A one-billion-year-old multicellular chlorophyte

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Abstract

Chlorophytes (which represent a clade within the Viridiplantae and a sister group of the Streptophyta) probably dominated marine export bioproductivity and played a key role in facilitating ecosystem complexity before the Mesozoic diversification of phototrophic eukaryotes such as diatoms, coccolithophorans, and dinoflagellates. Molecular clock and biomarker data indicate that chlorophytes diverged in the Mesoproterozoic or early Neoproterozoic, followed by their subsequent phylogenetic diversification, multicellular evolution, and ecological expansion in the late Neoproterozoic and Paleozoic. This model, however, has not been rigorously tested with paleontological data because of the scarcity of Proterozoic chlorophyte fossils. Here we report abundant millimeter-sized, multicellular, and morphologically differentiated macrofossils from ~1,000 Ma rocks. These fossils are described as Proterocladus antiquus new species and are interpreted as benthic siphonocladalean chlorophytes, suggesting that chlorophytes acquired macroscopic size, multicellularity, and cellular differentiation nearly a billion years ago, much earlier than previously thought.

The origin of photosynthetic eukaryotes represents a key evolutionary innovation that ultimately precipitated in major ecosystem-wide changes in Earth history. The Archaeplastida, which includes the Rhodophyta and Viridiplantae, have been the most ecologically successful photosynthetic eukaryotes today and in geological past. Rhodophytes donated their plastids to diatoms, coccolithophorans, and dinoflagellates, which have been the dominant contributors to marine export bioproduction since the Mesozoic1,2, whereas viridiplantae were likely the dominant export bioproducers in Paleozoic, Ediacaran, and Cryogenian oceans3,4. Whether viridiplantae were present before the Cryogenian Period and when they evolved multicellularity, however, is unclear. Some...
recent molecular clock analyses indicate that the Rhodophyta and Viridiplantae diverged in
the Paleoproterozoic–Mesoproterozoic Era\(^5-7\), crown-group Chlorophyta (which is a clade
within the Viridiplantae, a sister group of the Streptophyta, and includes prasinophytes
and the core Chlorophyta\(^8\)) diverged in the late Mesoproterozoic to early Neoproterozoic
eras\(^6,9-11\), but multicellular, siphonous, and siphonocladous chlorophytes evolved repeatedly
in the late Neoproterozoic and Paleozoic\(^10,12\). However, these molecular clock estimates
come with large uncertainties on the order of several hundred million years, particularly
for early divergence events within the Archaeplastida, Viridiplantae, and Chlorophyta\(^6,10\).
Furthermore, some of these molecular clocks give conflicting estimates; for example, the
divergence of crown-group Chlorophyta is estimated to have occurred at 0.466–0.792
Ga\(^11\) or 0.903–1.329 Ga\(^6\), and that of crown-group embryophytes in the Cambrian\(^13\) or
Ordovician Period\(^10\). These problems are in part related to the scarcity of reliable fossil
calibrations in the Proterozoic. Molecular clock studies had to choose between largely
ignoring Proterozoic fossils or calibrating clocks against putative Proterozoic archaeplastid
fossils, which are few and far between. For example, there are only a handful of Proterozoic
rhodophyte fossils even in the most optimistic view, including the \(-1.6\) Ga Ramathallus\(^14\)
(but see ref.\(^15\) that questions its rhodophyte or even archaeplastid interpretation), the
\(-1.05\) Ga Bangiomorpha\(^7\), and \(-0.6\) Ga florideophytes from the Doushantuo Formation\(^16\).
Similarly, Proterozoic chlorophytes are represented by only one plausible genus, the \(-0.72\)
Ma Proterocladus\(^17\), which is preserved as fragments and thus its phylogenetic interpretation
has been questioned due to the scanty morphological information\(^10,11,18\). The poor record
of Proterozoic archaeplastid fossils means large uncertainties in their stratigraphic ranges\(^19\),
hence limiting their values as fossil calibrations in molecular clock studies. Thus, it is
imperative to document Proterozoic archaeplastid fossils, particularly chlorophyte fossils,
not only to improve fossil calibrations so that molecular clocks are not entirely calibrated
on Phanerozoic fossils, but also to evaluate evolutionary models derived from molecular
clocks\(^11\) and fossil biomarkers\(^3,20-22\).

Here we report a multicellular fossil, *Proterocladus antiquus* new species, that occurs
in abundance in the ca. 1,000-Ma Nanfen Formation in North China (Extended Data
Fig. 1). Compared with previously reported fragments of *Proterocladus*\(^17\), the new
species offers a more complete suite of morphological features—including inferred
siphonocladous construction, multicellularity, and newly documented characters such as
cell differentiation, a multitude of branching, and a holdfast structure—that collectively
strengthen a phylogenetic position within the crown-group Chlorophyta. The new fossil
indicates that chlorophytes acquired multicellularity and cell differentiation no later than the
Tonian Period, and may have become phylogenetically diverse much earlier than predicted
by the molecular clock data\(^10\). Considering the abundant occurrence of *Proterocladus* in
the Nanfen Formation, chlorophytes may have played notable ecological and geobiological
roles, at least locally if not globally, prior to the Cryogenian Period when their biomarkers
became abundant\(^3\).

**Stratigraphic background**

The late Mesoproterozoic to Neoproterozoic sedimentary sequence in southern Liaoning
Province, North China, is well preserved with a maximum thickness of \(-12.7\) kilometers\(^23\).
The sequence comprises, in ascending order, the Yongning, Xihe, Wuhangshan, and Jinxian groups (see “Stratigraphy and sedimentary environment” in Supplementary Information; Extended Data Fig. 1). Briefly, the Nanfen Formation, which contains the Proterocladus material described in this paper, is conformably sandwiched between two sandstone units of the Xihe Group, the underlying Diaoyutai Formation and the overlying Qiaotou Formation. The Nanfen Formation is divided into three members. The lower member is dominated by dark grey and yellowish green silty shale and mudstone that preserves Proterocladus fossils; the middle member consists of thick-bedded greyish argillaceous limestone; and the upper member is mainly composed of grey, yellowish, and purple shale with thin-bedded sandstone interbeds. Although there are no reliable radiometric ages directly from the Nanfen Formation, the youngest population of detrital zircons from the underlying Diaoyutai Formation is dated at 1,056 ± 22 Ma, and a diabase sill emplaced in the overlying Qiaotou Formation gives a zircon SIMS U-Pb age of 947.8 ± 7.4 Ma. Hence, the depositional age of the Nanfen Formation is constrained between 1,056 Ma and 947.8 Ma, consistent with numerous other radiometric ages from the Xihe, Wuxingshan, and Jinxian groups (Extended Data Fig. 1; see also “Age constraints” in Supplementary Information). Considering that the fossiliferous horizon of Proterocladus is in the lower member of the Nanfen Formation, the first occurrence of Proterocladus is likely near the Mesoproterozoic–Neoproterozoic boundary, or ca. 1,000 Ma.

Results
Systematic Paleontology
Phylum Chlorophyta Pascher, 1914 (ref. 27)
Class Ulvophyceae Mattox and Stewart, 1984 (ref. 28)
Order Siphonocladales (Blackman & Tansley) Oltmanns, 1904 (ref. 29)
Genus Proterocladus Butterfield in Butterfield et al., 1994 (ref. 17), emended

*Type species.*—Proterocladus major Butterfield in Butterfield et al., 1994

*Emended diagnosis.*——Thallus consisting of multicellular, uniseriate, and branching filaments with intercellular septa. Filaments are typically constricted at septa. Branches typically emanate laterally from a cell in the central axis and subjacent to a septum. Lateral branches themselves can be septate. Maximally one branch per cell. Multiple orders of branches can occur, resulting in apical or upward growth. A sub-discoidal holdfast may be present. Cells typically elongate, thin-walled, mostly cylindrical, but globose, clavate, cyathiform, and doliform heteromorphic cells are occasionally present. Cell width gradually increases distally (or adapically). Apical cells round or capitate, sometimes bearing a narrow extension at the distal end.

*Remarks.*——Proterocladus was first erected by Butterfield based on fragmentary materials from the late Tonian Svanbergfjellet Formation in Svalbard. Abundant well-preserved specimens of Proterocladus from the Nanfen Formation reveal new diagnostic
features of the genus, including variations of cell shape and cell size, multiple orders of lateral branches, upward growth, apical extension, and sub-discoidal holdfast. Thus, the genus diagnosis is here emended to accommodate these features.

*Proterocladus* has been compared with extant siphonocladean *Cladophoropsis* because of their morphological similarity\(^1\). Indeed, both taxa are characterized by a distinctive branching pattern wherein a lateral branch emanates from the main axis subjacent to a septum. However, this branching pattern is not unique to *Cladophoropsis*; it is also present in other siphonocladeans, such as *Cladophora* and *Rhizoclonium*\(^2\),\(^3\). More importantly, although they all can have regular intercalary cell divisions and thus centripetal invagination\(^4\),\(^5\), some *Cladophoropsis* species can occasionally have segregative cell division and usually develop tenacular cells as an attachment structure\(^6\). However, no *Proterocladus* specimens show a sign of segregative cell division or tenacular cells (Supplementary Table 1). In this regard, *Proterocladus* is morphologically more similar to *Cladophora* (e.g., *C. herpestica*)\(^7\) and *Rhizoclonium* (e.g., *R. ramosum*)\(^8\) than to *Cladophoropsis*. Regardless, the extant Siphonocladeae provides the best interpretative analog for *Proterocladus*, suggesting that *Proterocladus* may be a member of the total-group Siphonocladeae. A more detailed discussion of the phylogenetic affinity of *Proterocladus* is presented in the discussion section.

*Proterocladus* is superficially similar to *Aimonema* Hermann in Hermann and Podkovyrov, 2010 (ref.\(^9\)), an articulated form of *Palaeovaucheria* Hermann, 1981 (ref.\(^10\)), in having a branching thallus and clavate terminal cells. However, *Proterocladus* is distinguished from *Aimonema* and *Palaeovaucheria* in its apical or upward branching pattern and discoidal holdfast, whereas *Aimonema* has a reticulate thallus similar to extant nematode-trapping fungi\(^11\). Additionally, filamentous algal fragments from the Middle Ordovician Winneshiek Shale are similar to *Proterocladus* in having a *Cladophora*-style branching system\(^12\). However, their cells are much larger than those of *Proterocladus* (90–380 μm vs. 6–35 μm in cell width), although the Winneshiek fossils may represent a younger record of siphonocladean chlorophytes\(^13\).

The organic-walled microfossil *Jacutianema* is morphologically similar to *Proterocladus* in having side branches adjacent to one end of the mother cell and possible siphonous/siphonocladean construction\(^14\). Given that *Jacutianema* co-occurs with *Proterocladus* in the Svanbergfjellet Formation of Svalbard, an interesting hypothesis is that *Jacutianema* may represent the akinete of *Proterocladus*, and this hypothesis needs to be investigated further by a detailed restudy of the Svalbard material. Additionally, branching thalli from the ca. 1.078 Ma Nonesuch Formation (fig. 2L, M of ref.\(^15\)) are morphologically similar to fragmented specimens of *Proterocladus* in the Nanfen Formation (e.g., Fig. 2b, d), suggesting that they may represent broken pieces of *Proterocladus*. However, more completely preserved specimens from the Nonesuch Formation are needed in order to confirm their taxonomic identification as *Proterocladus*.

**Occurrence.**——*Proterocladus* has been recovered from latest Mesoproterozoic to Tonian successions, including the late Tonian Svanbergfjellet Formation in Svalbard\(^16\), the late Tonian Khastakh Formation in Siberia\(^17\), the latest Mesoproterozoic to early Tonian Nanfen
Formation in North China, and possibly the latest Mesoproterozoic Nonesuch Formation in North America.\textsuperscript{37}

*Proterocladus antiquus* new species

2018 *Proterocladus* sp.; Xiao and Tang\textsuperscript{39}, fig. 3B.

**Holotype.** —— VPIGM-4762 in Fig. 2g, repositored at Virginia Polytechnic Institute Geoscience Museum.

**Paratype.** —— VPIGM-4799 in Fig. 1l, repositored at Virginia Polytechnic Institute Geoscience Museum.

**Diagnosis.** —— A species of *Proterocladus* characterized by a differentiated sub-discoidal holdfast, morphologically distinct akinetes, multiple orders of lateral branches constructing an upward-growing thallus, and an apical extension. Cells defined by robust septa and associated constrictions. Cell shape and size are variable in a thallus.

**Etymology.** —— Species epithet derived from Latin, *antiquus*, referring to the Proterozoic age of the species.

**Material.** —— 1,028 specimens from the lower Nanfen Formation in North China.

**Occurrence.** —— *Proterocladus antiquus* has been recovered from the latest Mesoproterozoic to early Tonian Nanfen Formation in North China.

**Description.** —— Well-preserved specimens of *P. antiquus* consist of a branching thallus and a holdfast (Fig. 1a). The thallus, 0.3–3.3 mm and 0.1–2.4 mm in maximum height and width respectively, consists of uniseriate filaments that branch sparsely or profusely (Fig. 1; Extended Data Fig. 2). Branches are laterally and asymmetrically inserted, and are alternately or unilaterally arranged along the main axis (Fig. 1; Extended Data Fig. 2). The main axis and branches tend to widen distally (Fig. 1a, b, f, h, i). Lateral branches grow apically or upward and away from the insertion point (Fig. 1a, h–k). Multiple orders of lateral branches can occur (Extended Data Fig. 3a), leading to complex thallus with numerous branches and aggregates (Extended Data Fig. 3b–d). The rarely preserved holdfast is lobate or sub-discoidal in shape and 53–57 μm in maximum dimension (Fig. 1a, l; Extended Data Fig. 4).

The thallus of *P. antiquus* comprises multiple cells that are defined by complete septa (Figs. 1a; 2a–d). A constriction typically occurs at a septum, such that cell boundaries can also be recognized by constrictions when septa are poorly preserved (Figs. 1a, i, k; 2c). Cells are 14–510 μm (average = 123 μm; s.d. = 0.45; n = 321) and 6–49 μm (average = 25 μm; s.d. = 0.12; n = 321) in length and width, respectively, with a length/width ratio 0.9–26.9. They are mostly thin-walled and cylindrical in shape (Figs. 1a, i; 2a–d), but heteromorphic cells are also observed occasionally (Fig. 1a), including globose (Fig. 1j, k; Extended Data Fig. 5a–c), clavate (Figs. 1j; 2e; Extended Data Fig. 5d–f), doliform (Fig. 2f–h; Extended Data Fig. 5g–k), and cyathiform cells (Figs. 1c, g, i; 2a, i, j; Extended Data Fig. 5l). These
heteromorphic cells are also distinctive in their greater maximum cell width and nearly opaque cell-wall (Figs. 1b, j; 2g, h, j), suggesting thicker or more recalcitrant cell walls. Some cells appear to have a minute lateral pore (Figs. 1a; 2e; Extended Data Fig. 5m–o). Lateral branches are always developed subjacent to a septum, either freely communicating with the parent cell (Figs. 1a, i–l; 2b; Extended Data Figs. 5g, l; 6) or separated from the mother cell by a septum at the branching point (Figs. 1a, j, l; 2b, f). Some terminal cells have a distinct narrow apical extension (Figs. 1a, e, i; 2a, j, k; Extended Data Fig. 6d), possibly representing apical cell division, which is supported by the development of septa in presumably more mature apical extensions (Extended Data Figs. 6e; 7).

**Remarks.**——The type species *Proterocladus major*, along with two other species of *Proterocladus*—*P. minor* and *P. hermannae*, was erected based on fragmentary specimens from the late Tonian Svanbergfjellet Formation in Svalbard. The new species *P. antiquus* is distinguished from other species of *Proterocladus* in the presence of a holdfast, akinetes, multiple orders of lateral branches that grow upward, and an apical extension. However, given that the type species *P. major* was erected on the basis of fragmentary specimens, it is possible that *P. antiquus* and *P. major* are synonymous as some fragments of *P. antiquus* appear identical to *P. major*.

*Proterocladus major*, *P. minor*, and *P. hermannae* were distinguished by their cell size, the prominence of constrictions, and the frequency of septa. However, our investigation on a large collection of well-preserved specimens of *P. antiquus* in the Nanfen Formation shows that cell width can vary gradually from the base to the top of the thallus (e.g. Fig. 1b, f, i, j). Thus, cell size alone is probably not a reliable criterion to differentiate species of *Proterocladus*, particularly if they are preserved as fragments. The frequency of intercellular septa and constrictions is also variable, as indicated by the highly variable cell length measurements. Thus, if *P. antiquus* and *P. major* are synonymous, it is possible that all four known species of *Proterocladus* are synonymous. This possibility needs to be evaluated by a restudy of the Svanbergfjellet material and the discovery of more completely preserved specimens of *Proterocladus* from the Svanbergfjellet Formation. At the present, to preserve taxonomic objectivity and to highlight the morphological complexity as revealed by the well-preserved Nanfen material, we choose to place the Nanfen material in *Proterocladus antiquus* new species. In addition, the *Proterocladus* specimen reported in ref. is here regarded as *P. antiquus* given that it was recovered from the same horizon of *P. antiquus* reported in this study.

**Discussion**

The well-preserved specimens of *P. antiquus*, which is broadly similar to and could even be synonymous with the type species *P. major* from Svalbard (see “Systematic paleontology”), reveal a suite of features that were not preserved in the fragmentary material of *P. major* but can assist in the morphological reconstruction as well as ecological and phylogenetic interpretations of *Proterocladus*. Specifically, the more opaque and larger-sized heteromorphic cells may represent specialized akinetes that form in unfavorable conditions, typically with a larger cell size and a thicker cell wall enclosing condensed cytoplasm (Fig. 3a, b). The lateral pore (Figs. 1a; 2e; Extended Data Fig. 5m–o) is identical to lateral...
openings of reproductive cells (e.g., gametangia and sporangia) where gametes and spores are released through such openings (Fig. 3c; fig. 10 of ref. 30). The swollen apical cell with a narrow apical extension (Figs. 1e, i; 2g, j, k; Extended Data Fig. 6d) indicates that thallus growth was mainly achieved by apical growth, an interpretation supported by the subsequent development of septa in the apical extension at maturation (Extended Data Figs. 6e; 7). In addition, the presence of a holdfast and evidence for apical growth suggest that Proterocladus had an erect epibenthic habit. The dense branching pattern and the formation of aggregates suggest that Proterocladus likely formed tufts, which may have facilitated its colonization on the ocean floor (Fig. 4). This is also consistent with the massive preservation of Proterocladus in the Nanfen mudstone (Extended Data Figs. 8, 9). More importantly, the co-occurrence of extremely large cells (up to several hundred μm long; e.g., Fig. 2g) and small cells (dozens of μm long; e.g., Fig. 2g) in the same thallus indicates that the larger cells were likely coenocytic and thus Proterocladus was a siphonocladous (i.e., coenocytic and multicellular) organism17. Therefore, well-preserved Nanfen specimens indicate that Proterocladus was an erect epibenthic multicellular organism with filamentous branches, a unique branching pattern with asymmetrical lateral branches arising subjacent to septa, a differentiated holdfast, differentiated heteromorphic akinete-like cells, reproductive cells as inferred from the presence of lateral pores, and an inferred siphonocladous construction (Fig. 1a).

The morphological reconstruction described above are useful in phylogenetic inference of Proterocladus (see Supplementary Table 1). Specifically, its branching style, differentiated cells, the presence of a holdfast, inferred siphonocladous construction, and the lack of a common outer sheath place Proterocladus in the kingdom of Eukarya. Some stigonematalean cyanobacteria, such as Nostochopsis and Thalpophila, can develop uniseriate trichomes with differentiated akinetes, true branches, and apical growth41. However, stigonematalean trichomes are always surrounded by a robust outer sheath, which has reasonably good preservation potential42 but is not present in Proterocladus.

More importantly, no stigonematalean cyanobacteria are known to develop siphonocladous construction. To the best of our knowledge, no bacteria or archaeabacteria are known to have the combination of features present in Proterocladus (Supplementary Table 1). Although coenocyte or syncytium exists in many groups of eukaryotes43, a filamentous siphonocladous construction is characteristic of only a handful of extant eukaryote groups, including some filamentous fungi (e.g., Neurospora44), xanthophytes (e.g., Vaucheria in reproductive stage45), rhodophytes (e.g., Griffithsia46), and chlorophyte (e.g., Rhizoclonium30). The vegetative structure of multinucleate fungi, septate hyphae, could be morphologically similar to Proterocladus in having large cells and lateral branches that sometimes adjacent to a septum44,47. However, fungal septa are very different from the complete septa of Proterocladus in that they are perforated47. More importantly, septate hyphae usually consist of cells with a relatively uniform size and tend to form a complex network of branching structure (i.e., mycelium), the hyphae themselves do not differentiate into a filamentous holdfast, and fungal cells can fuse to form loops of various shapes44,48. In addition, the reproductive organs of multinucleate fungi (such as sporangium, zygospore, ascus, conidia47) are also markedly different from the reproductive
cells observed in *Proterocladus*. Therefore, given its morphological differences from extant fungi, *Proterocladus* is unlikely a fungus.

The xanthophycean alga *Vaucheria* is morphologically similar to *Proterocladus* in developing apical extensions\(^\text{49}\). However, vegetative thallus of *Vaucheria* is siphonous (i.e., coenocytic but unicellular). Septation in *Vaucheria* only occurs in the reproductive stage at the apical end of filaments where akinetes or zoospores are produced as either detached individuals or loose chains with constricted connections in a filament sheath\(^\text{45}\). These features are conspicuously different from those of *Proterocladus*, where septation occurs intercalary along the entire filament.

Some uniseriate filamentous rhodophytes, such as *Griffithsia*\(^\text{50}\), can develop siphonoclados construction, but their intercellular septa are usually characterized by pit plugs which are different from the complete septa of *Proterocladus*\(^\text{17}\) (Fig. 2a–c; Extended Data Fig. 6a, b). More importantly, the unique branching pattern of *Proterocladus*, characterized by lateral branches originating subjacent to a septum, is distinct from the dichotomous or trichotomous branching pattern of siphonoclados rhodophytes. Thus, *Griffithsia* is not a morphological analog of *Proterocladus*.

To the best of our knowledge, modern siphonocladean chlorophytes provide the most appropriate morphological analog of *Proterocladus*. Among uniseriate filamentous chlorophytes, siphonoclados construction is most common in the class Ulvophyceae, particularly in the order Siphonocladales (= Cladophorales and is the preferred name for this group of chlorophytes according to ref.\(^\text{50}\)). Importantly, the initiation of lateral branches as outpocketing structures always subjacent to a septum is a key feature among extant siphonocladaleans such as *Cladophora* and *Rhizoclonium*\(^\text{30,31}\) (Fig. 3d). Indeed, in addition to siphonoclados construction and the unique branching pattern, *Proterocladus* also shares with *Cladophora* and *Rhizoclonium* a number of other morphological features, including a holdfast and an epibenthic habit, intercalary cell division with centripetal invagination as indicated by constrictions at septa, as well as differentiated cells with lateral pores likely representing sexually reproductive cells\(^\text{51}\) (Supplementary Table 1). Thus, among all morphological analogs discussed above, *Proterocladus* compares best with siphonocladaleans, and their morphological similarities are suggestive of a phylogenetic relationship. Of course, we cannot rule out the possibility that *Proterocladus* may represent an extinct group of siphonoclados eukaryotes that independently evolved a siphonocladean-style branching pattern, but the Occam’s razor leads us to hypothesize that *Proterocladus* is a possible siphonocladean chlorophyte.

If our interpretation is correct, then *Proterocladus antiquus* from the ca. one-billion-year-old Nanfen Formation represents one of the earliest known multicellular chlorophytes. Chlorophyte fossils are key to test various molecular clock estimates of the origin of primary plastids and the crown-group Archaeoplastida, which range from ca. 1,900 Ma\(^{\text{6}}\) to 900 Ma\(^{\text{52}}\); the divergence of crown-group Viridiplantae and Chlorophyta, which probably occurred in the Mesoproterozoic–Tonian\(^{\text{6,9,10}}\); and the internal divergences within the Chlorophyta, which were proposed to have occurred in the late Neoproterozoic and Paleozoic\(^{\text{10,12}}\). If the ca. 1,047 Ma fossil *Bangiomorpha* is accepted as a rhodophyte\(^{\text{51}}\),...
the divergence between the Rhodophyta and Viridiplantae must have occurred no later than the late Mesoproterozoic. But the Proterozoic fossil record of the Viridiplantae and particularly the Chlorophyta is sparse and controversial at best. Various Proterozoic fossils have been interpreted as potential chlorophytes, including some Paleoproterozoic leiosphere acritarchs, Tonian macrofossils such as *Chuaria, Longfengshania, Protoarenicola, Pararenicola,* and *Purinia,* and the late Tonian colonial microfossil *Palaearustrum.* However, the morphological simplicity of these taxa means that diagnostic chlorophyte features are few and subject to evolutionary convergence. The interpretation of *Proterocladus* as a chlorophyte and specifically as a siphonocladalean has also been questioned, largely on the basis of its morphological simplicity. Compared with previously described specimens of *Proterocladus,* the new material reported here offers additional phenotypic features—including a differentiated holdfast, akinetes, siphonocladous organization, and distinct branching pattern—that strengthen a morphological comparison and suggest a phylogenetic affinity with siphonocladalean chlorophytes (Supplementary Table 1). If this phylogenetic interpretation is confirmed, *Proterocladus* provides a minimum age calibration for the origin of photosynthetic eukaryotes, the divergence between the Rhodophyta and Viridiplantae, the internal divergences within the Chlorophyta, and even the Ulvophyceae. Thus, *Proterocladus* suggests that the Chlorophyta may have diverged nearly a billion years ago, consistent with some molecular clock analyses but not others.

The abundant occurrence of *Proterocladus* in the Nanfen Formation indicates that chlorophytes may have played important ecological and geobiological roles at least locally. It has been postulated that the pre-Cryogenian oceans were stratified in redox condition due to the lack of metazoans and the dominance of cyanobacterial phytoplankton as primary producers. This postulation is mostly grounded on the earliest known chlorophyte and sponge biomarkers, which suggest that chlorophytes and filter-feeding metazoans diversified in the Cryogenian Period. However, the abundant occurrence of chlorophytes such as *Proterocladus* in the ca. one-billion-year-old Nanfen Formation and other Mesoproterozoic–Tonian rocks, including the late Tonian Svanbergfjellet Formation in Svalbard, the late Tonian Khastakh Formation in Siberia, and possibly the latest Mesoproterozoic Nonesuch Formation in North America (see “Systematic paleontology”), indicates that macroscopic chlorophytes may have had more than a local impact on the Tonian ecosystem. Benthic macroscopic algae such as *Proterocladus* are expected to contribute markedly to local bioproduction and organic carbon burial in coastal environments, to foster a myriad of ecological habits through the formation and ecological engineering of algal turfs (e.g., Fig. 4; Extended Data Figs. 3d; 8), and to facilitate the ecological complexity and diversification of eukaryotes in Tonian oceans. Therefore, together with other Tonian evolutionary innovations, such as nitrogen fixing heterocystous cyanobacteria, biomineralization, eukaryovorous predation, emergence of fungi, and perhaps the origin of animals, the rise of multicellular chlorophytes such as *Proterocladus* may have had a transformative impact on oceanic redox structures and ecosystem complexity. The ecological and geobiological roles of Tonian chlorophytes can be further tested by more focused search for stigmastane, a possible chlorophyte biomarker, in abundantly
fossiliferous units such as the Nanfen Formation. Currently available biomarker data indicate that the transition from bacterium- to eukaryote-dominated marine primary producers occurred sometime between 1,100 Ma$^{21}$ and 780–729 Ma$^{20,22}$, and the 1,047 Ma red algal fossil *Bangiomorpha* and the ~1,000 Ma green algal fossil *Proterocladus* reported here are consistent with this scenario.

**Methods**

**Fossil collection and extraction.**

*Proterocladus* specimens are abundant in the lower Nanfen Formation (Extended Data Fig. 8). They are preserved as carbonaceous compressions on the bedding surface (Extended Data Fig. 9a–d). They are typically concentrated in thin and relatively dark-colored fossiliferous layers, which contrast with the intervening light-colored layers with sparse occurrence of fossils (e.g. Extended Data Fig. 9e–i). Totally 1,028 specimens of *Proterocladus*, including 301 well-preserved specimens discovered on bedding surfaces and 727 specimens extracted from the rock matrix using the HF acid maceration technique$^{71}$, were collected from mudstone/shale of the basal to lower Nanfen Formation.

**Optical and electron microscopic analyses.**

Extracted specimens were examined using an Olympus CX41 biomicroscope. Well-preserved specimens on bedding surfaces were examined using an Olympus SZX7 stereomicroscope. Both microscopes were connected with an Infinity 1 camera, which was used to photograph the fossils. Selected specimens were further analyzed using backscattered electron scanning electron microscopy (BSEM), energy dispersive X-ray spectroscopy (EDS), and EDS element mapping at the Virginia Tech Institute of Critical Technology and Applied Science Nanoscale Characterization and Fabrication Laboratory. These tests were conducted on a FEI QUANTA 600FEG environmental scanning electron microscope with a pole piece backscattered electron solid-state detector, a secondary electron Everhart-Thornley detector, and a Bruker EDX with a silicon drifted detector. BSEM specimens were coated with a ~20 nm conductive gold-palladium layer. The operating voltage in BSEM and EDS modes was 20 kV in high-vacuum condition.

**Data availability**

All specimens illustrated in this paper are reposited and available at Virginia Polytechnic Institute Geoscience Museum (Blacksburg, Virginia, USA; museum catalog numbers VPIGM-4749 to 4794 and VPIGM-4799).
Extended Data

Extended Data Fig. 1. Geological map and stratigraphic column of Proterozoic successions in southern Liaoning Province, North China.

Question mark in stratigraphic column denotes poor age constraint on the Dalinzi Formation, which could be either Neoproterozoic or Cambrian in age. Stars in geological map and stratigraphic column mark sample locality (near Shileicun, 39°35.6566’N, 121°35.8379’E) and sample horizon, respectively. Ca = Cambrian, Pa = Paleoproterozoic, Fm = Formation, CLZ = Changlingzi, NGL = Nanguanling, GJZ = Ganjingzi, YCZ = Yingchengzi, SSLT = Shisanlitai, MJT = Majiatun, CJT = Cuijiatun, XMC = Xingmincun, GT = Getun. Radiometric ages (924 ± 5 Ma and 947.8 ± 7.4 Ma) of diabase sills emplaced in the Cuijiatun and Qiaotou formations are from ref. 22 and ref. 25; detrital zircon ages (<924 ± 25 Ma and <1056 ± 22 Ma) are from ref. 24. See ref. 25 for a compilation of radiometric ages from Neoproterozoic successions in North China. Geological map drawn by authors based on ref. 22 with permission, and stratocolumn drawn by authors.
Extended Data Fig. 2. *Proterocladus antiquus* new species on bedding surface, showing lateral branches.

a–f, VPIGM-4763, VPIGM-4764, VPIGM-4765, VPIGM-4766, VPIGM-4767, and VPIGM-4768, respectively. All photos taken by authors.
Extended Data Fig. 3. *P. antiquus* preserved on bedding surface, showing multiple orders of lateral branches (a) and aggregates of thalli (b–d).

a–d, VPIGM-4769, VPIGM-4770, VPIGM-4771, and VPIGM-4772, respectively. All photos taken by authors.
Extended Data Fig. 4. Thallus of *P. antiquus* with a sub-discoid holdfast preserved on bedding surface. 

**b** is a close-up view of labeled black frame in **a**. VPIGM-4773. All photos taken by authors.
Extended Data Fig. 5. Heteromorphic cells (a–l) and reproductive cells (m–o) of *P. antiquus*. a–l, Filaments with globose (yellow arrowheads), clavate (black arrowheads), doliform (cyan arrowheads), and cyathiform (blue arrowhead) heteromorphic cells. b, k are magnifications of labeled frames in a and j, respectively. VPIGM-4774, VPIGM-4775, VPIGM-4776, VPIGM-4777, VPIGM-4778, VPIGM-4779, VPIGM-4780, VPIGM-4781, VPIGM-4782, and VPIGM-4783, respectively. m–o, Inferred reproductive cells with minute lateral pores (blue arrows), possibly representing openings through which reproductive gametes or zoospores were released. VPIGM-4784, VPIGM-4785, and VPIGM-4786.
respectively. Specimens in a and j were photographed on bedding surface, and all other specimens were extracted from the rock matrix using HF acid maceration technique. All scale bars equal 100 μm unless otherwise specified. All photos taken by authors.

Extended Data Fig. 6. Cell branching pattern and apical extensions in extracted specimens of P. antiquus.

a–c, Fragmented filaments with unilateral (a, c) and alternate branches (two lower lateral branches in b). VPIGM-4787, VPIGM-4788, and VPIGM-4789, respectively. d–e, Branching filaments with an inflated apical cell subtending a narrower apical extension

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Extended Data Fig. 7. Branching thallus of *P. antiquus* with a cell (in black frame) that has a distinct constriction at base (blue arrowhead).

The branching pattern is superficially similar to H-shaped branching in the early vascular plant *Zosterophyllum*. b is a magnification of white box in a, showing the basal constriction of the cell that initially may represent an apical extension that subsequently develops septa and branches at maturation. VPIGM-4792. All photos taken by authors.
Extended Data Fig. 8. Dense population of fragmented *P. antiquus* specimens preserved on bedding surface.

VPIGM-4793. All photos taken by authors.
Extended Data Fig. 9. Taphonomy of *P. antiquus* preserved in the Nanfen mudstone.

*a–b*. A partially exposed specimen. *b* is a backscattered electron scanning electron microscopy (BSEM) photograph of the same specimen in *a*. VPIGM-4794.  
*c*, Energy dispersive X-ray spectroscopy (EDS) point analysis at the blue spot in *b*, showing the presence of carbon in the fossil specimen.  
*d*, EDS elemental maps of labeled box in *b*, showing the enrichment in C and deficiency in O, Al, and Si in fossil relative to matrix.  
*e–g*, Nanfen mudstone fractured obliquely relative to bedding plane, showing darker-colored fossil layers and lighter-colored background layers. *f* and *g* are magnifications of labeled
boxes in e. h–i, Polished slab cut perpendicular to bedding surface, showing darker-colored fossil layers and lighter-colored background layers. i is a close-up view of labeled box in h with blue arrowheads denoting fragmented fossils in a fossil layer. All photos taken by authors.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1 | Gross morphology of *Proterocladus antiquus* new species from the Nanfen Formation.

a. Morphological reconstruction and terminology. b–h. Slender thalli preserved on bedding surfaces. c, e, and g are magnifications of white frames in b, d, and f, respectively, showing cyathiform heteromorphic cells (in c and g) and apical extension (in e). VPIGM-4749, VPIGM-4750, VPIGM-4751, and VPIGM-4752, respectively. i–l. Branching thalli extracted from rock matrix using HF acid maceration technique. A close-up view is provided for the holdfast in the black frame in l. VPIGM-4753, VPIGM-4754, VPIGM-4755, and VPIGM-4799 (paratype), respectively. Blue arrowheads in a, c, g, and i: cyathiform
heteromorphic cell; purple arrowheads in a and e: apical extension; black arrowheads in a and j: clavate cell; yellow arrowheads in a, j, and k: globose heteromorphic cell; black arrows in a, j, and l: septum and constriction. Scale bars equal 200 μm unless otherwise specified. All photos taken by authors.
Figure 2 | Cellular structures of Proterocladus antiquus new species.

a–f, Fragmentary specimens extracted from rock matrix using HF acid maceration technique. VPIGM-4756, VPIGM-4757, VPIGM-4758, VPIGM-4759, VPIGM-4760, and VPIGM-4761, respectively. g–k, Well-preserved thallus on bedding surface. VPIGM-4762 (holotype). h–k are magnifications of labeled frames in g. Black and blue arrows denote robust septa and lateral pore, respectively. Cyan, blue, and purple arrowheads denote morphologically differentiated doliform heteromorphic cells, cyathiform heteromorphic cells,
and narrow apical extensions, respectively. Scale bars equal 100 μm unless otherwise specified. All photos taken by authors.
Figure 3. | Extant Siphonocladales of the genera Cladophora and Rhizoclonium for comparison with Proterocladus.

a, General morphology of a branching thallus of Cladophora, showing elongate cells and unique lateral branching system. Compare with Fig. 1b, d. b, Doliform akinetes (cyan arrowheads) of Rhizoclonium under stressed conditions in contrast to the cylindrical vegetative cells (white arrowhead). Compare with Fig. 2f. c, Reproductive cells of Rhizoclonium with lateral pore (white arrows) after the liberation of gametes or zoospores. Compare with Fig. 2e. d, Lateral branches (blue arrowheads) of Cladophora arising from mother cell subjacent to septa and remaining cytoplasmic contact with mother cell. Compare with Fig. 2a. a adapted from ref.68 under a Creative Commons License, b from ref.69 with permission, c from ref.70 under a Creative Commons License, and d from ref.31 with permission.
Figure 4.
An artist’s reconstruction of *Proterocladus antiquus*. Artwork by Dinghua Yang.