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Nitrogen-fixing symbiosis inferred from stable isotope analysis of fossil tree rings from the Oligocene of Ethiopia

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ABSTRACT

The acquisition of reduced nitrogen (N) is essential for plant life, and plants have developed numerous strategies and symbioses with soil microorganisms to acquire this form of N. The evolutionary history of specific symbiotic relationships of plants with soil bacteria, however, lacks evidence from the fossil record confirming these mutualistic relationships. Here we use modern plants in the N-fixing clade of rosids to develop a geochemical method to assess the presence of symbiotic relationships with N-fixing soil bacteria via $\delta^{15}N$ values of tree rings. Application of this method to Oligocene tree rings confirms the symbiosis of certain arborescent legumes with N-fixing soil bacteria. The results suggest actinorhizal symbiosis for some Oligocene non-leguminous trees. The specific age, genera, and presence or absence of bacterial symbiosis of these fossil trees provide new information on genera that have maintained or lost the ability to form symbioses in the N-fixing clade. We envision that this approach, as applied to paleoecology, can lead to greater understanding of the response of plant symbioses under variations in atmospheric chemistry for N-limited ecosystems.

INTRODUCTION

Nitrogen is an essential nutrient for all forms of life. For plants, N is a limiting factor for growth (Vitousek et al., 2002). Competition for N results in unique strategies for nutrient uptake via symbiotic relationships of plants with soil microorganisms, such as the N-fixing clade of the rosids that maintain symbiosis with N-fixing bacteria (Terrer et al., 2016). The phylogeny of the N-fixing clade provides predictions of its evolutionary history, such as evolving from a yet-unknown common ancestor (Soltis et al., 1995; Werner et al., 2014). The origin of N-fixation is ancient, predating oxygenation of the atmosphere (Sprent and Raven, 1985; Stüeken et al., 2015); however, the fossil record of symbiotic relationships of N-fixing bacteria with plants is poorly understood because of the lack of fossil root anatomy of these interactions and the microorganisms (Taylor et al., 2015). While the origin of host plants of the N-fixing clade dates to the Late Cretaceous and Eocene (Schönenberger and von Balthazar, 2006; Sprent and James, 2007), the spectrum of affinities of N-fixing bacteria to host plants implies that speciation during the evolution of the host plant genera could result in losses or gains of this form of symbiosis (Wall, 2000). Thus, the origin, losses, and/or gains of this symbiosis along the evolutionary pathway of these plants are not clear from host plant phylogeny alone. To better constrain the evolutionary history of N-fixing symbiosis it is necessary to develop confirmation from the fossil record that such symbioses existed for certain fossil plants.

This study focuses on geochemical methods to detect the presence or absence of N-fixing symbiosis in fossil plants via two approaches: (1) demonstration that the geochemistry of modern, phylogenetically related, non–N-fixing plants and N-fixing plants can be distinguished based on N isotopes of wood, and (2) application of this approach to well-preserved leguminous and non-leguminous fossil woods from the Oligocene of Ethiopia. The nature of N preservation in the studied materials is assessed via solid-state ^{13}C nuclear magnetic resonance (NMR) and the preservation state of wood anatomy. N isotopes from peptide-like structures in a lignocellulose matrix are presented as the fossil archive of the N uptake strategies of these Oligocene trees. This wood anatomy and geochemistry method creates a framework to measure and interpret N isotope variations in fossil woods for the study of the evolution and paleoecology of N-fixation strategies of arborescent plants.

METHODS

Proxy Development

Genera (n = 9) from the N-fixing clade of the rosids, including tropical plants from Fabaceae (n = 7) and temperate plants from Betulaceae (n = 1) and Rosaceae (n = 1), were selected for N isotope analysis of tree rings to test whether N-fixing plants provide tree ring $\delta^{15}N$ values that are (1) diagnostic of N-fixing symbiosis and (2) distinct from trees without N-fixing symbiosis (Fig. 1A). Trees that maintain active N-fixing symbiosis (nodulating) are represented by four genera in Fabaceae and one genus of Betulaceae. Trees that do not maintain active N-fixing symbiosis (non-nodulating) are represented by three genera of Fabaceae and one genus of Rosaceae. For all plants, air-dried whole wood from tree rings was sampled along the circumference of stems at ~0.2-mm-wide increments, sequentially sampling in the radial direction; 25 mg of each sample was homogenized by mixing wood powders generated by subsampling a tree ring, and encapsulation in tin for N isotope analysis on an Elementar VisIon isotope ratio mass spectrometer in continuous-flow mode interfaced to a vario MICRO elemental analyzer at the University of California, Davis (USA; UC Davis) Stable Isotope Facility.

Geologic Setting

The fossil trees used in this study are from the Chilga Basin, Ethiopia; they are permineralized in quartz, contain well-preserved anatomical structure, and are late Oligocene in age (ca. 27 Ma; Kappelman et al., 2003; Jacobs et al., 2005). Fossil wood was collected at site CH107 in the Chilga district in the North Gondar Zone of the Amhara Regional State, Ethiopia (12.9167°N, 37.01°E). The most complete

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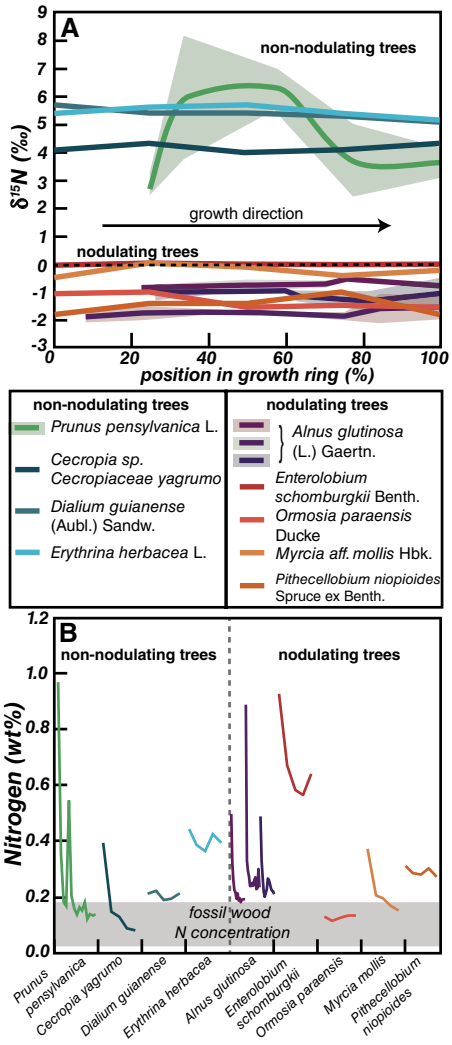

Figure 1. δ15N values and N concentrations for modern plants of the N-fixing clade. A: Average N isotope results of tree rings of temperate trees (*Alnus* and *Prunus*) and tropical trees (all other genera); relation of data to species is shown below. Samples were made along the direction of growth (indicated by arrow) for multiple rings (*Alnus* and *Prunus*) or single rings (all other genera). B: N concentrations for the studied tree rings, separated by non-nodulating trees versus nodulating trees. Gray shaded region defines the range of N concentrations of the studied fossil wood for comparison.

and well-dated section in this basin is located along the Guang River and is correlated to site CH107 (see Appendix DR1 in the GSA Data Repository[1] for details). Paleomagnetic reversal stratigraphy and radiometric geochronology indicate that the age of the fossiliferous horizons is between 28 and 27 Ma (Kappelman et al., 2003).

Wood Anatomy

Slides of silicified wood were prepared by standard thin section techniques. Images and measurements were taken on an Olympus BX40 microscope with a Diagnostic Instruments SPOT Insight digital camera. Anatomical descriptions follow the International Association of Wood Anatomists (IAWA list of microscopic features for hardwood identification; Wheeler et al., 1989). Images and descriptive data on modern woods were accessed using the InsideWood database and the references therein (http://insidewood.lib.ncsu.edu/; Wheeler, 2011). Identification was to genus of an extant nearest living relative, and no attempt was made to describe new fossil genera (Appendix DR2).

Fossil Wood

The mineralogy of the samples was assessed via X-ray diffraction of powdered samples (Appendix DR3). Fossil wood was microsampled from the tangential plane along the direction of growth by sequentially grinding away 0.3 mm parallel increments of wood with a diamond-coated bit on a rotary drill. The powdered sample was homogenized via mixing and 20–25 mg was loaded into cleaned tin capsules for stable isotope analysis. (For data reduction and normalization, see Appendix DR3.) Replicate analyses at the University of Wisconsin-Milwaukee (UWM) and UC Davis indicate analytical precision ($\delta^{15}N$) of 0.2‰ and 0.1‰ (1 standard deviation), respectively. Accuracy ($\delta^{15}N$) is better than 0.4‰ and 0.6‰ for UWM and UC Davis, respectively. Stable isotope compositions of C and N in fossil wood are reported in the conventional δ (‰) notation. N isotope data from samples with <0.01 wt% N were discarded because they yield insufficient N_2 peaks for precise isotope analysis. Thermal diagenetic effects were evaluated with pyrolysis experiments that were carried out on sample splits, with one split being a control. Matching areas of both the control and pyrolized materials were microsampled sequentially, and homogenized powders were analyzed for C and N isotopes (Appendix DR3).

Solid-State ^{13}C NMR

Solid-state ^{13}C NMR spectroscopy can provide chemical structure information on organic matter that cannot be extracted from samples dissolved in solvents. Several ^{13}C NMR methods were applied to the analysis of organic matter in fossil wood (see Appendix DR4 for details). Cross-polarization with magic angle spinning (CPMAS) NMR provides a sensitive semi-quantitative measurement of carbon functional groups. Direct polarization MAS NMR spectra have lower sensitivity, but are quantitative in the absence of paramagnetic metals (e.g., Fe and Mn). Therefore, we used a dilute (10 wt%) hydrofluoric acid solution to remove metals and silicates prior to ^{13}C NMR analyses. The information from ^{13}C NMR includes the relative abundance of the functional groups composed of C-C, C-N, C-O, and C-H bonds, as well as calculated molecular structural dimensions.

RESULTS

Nitrogen Isotopes of Modern Tree Rings

Concentrations of N in modern tree rings range from 1% to 0.1% for a given specimen; the highest concentrations are in the vascular cambium and ring boundaries. Nitrogen concentrations exponentially decrease from ring boundaries to the interior of a ring (Fig. 1B). No clear distinction between nodulating or non-nodulating trees and N concentration is observed.

For non-nodulating trees (Fig. 1A), the mean intra-annual $\delta^{15}N$ values are 4.7‰ (±1.8‰, n = 30). Intra-annual $\delta^{15}N$ trends are either invariant or exhibit an increase in $\delta^{15}N$ values near the middle of a ring. Nodulating trees have mean intra-annual $\delta^{15}N$ values of –1.0‰ (±0.5‰, n = 62). Tropical nodulating trees have intra-annual $\delta^{15}N$ variation ranging from invariant to displaying an increase in $\delta^{15}N$ in the middle of a ring. The temperate-latitude nodulating tree displays invariant $\delta^{15}N$ values.

Fossil Wood

A total of 36 collected fossil wood specimens from site CH107 contain 16 wood genera (see Appendix DR2). Of the specimens identified, seven genera with identifiable growth intervals and/or well-preserved wood anatomy permineralized in quartz are used in this study (Tables DR1 and DR2). The specimens represent the leguminous and non-leguminous wood diversity from the CH107 site to test whether N isotopic composition would differ significantly among specific genera.

Isotope Geochemistry

Concentrations of N in fossil wood range from 0.12% to 0.01%, with trends of increased concentration at inferred ring boundaries. Trends in $\delta^{15}N$ values of the specimens, which average all isotope values for specific percentages of a growth interval, indicate three types: (1) $\delta^{15}N$ values during the inferred growth interval that are consistently close to 0‰ (*Detarium*, *Magnistipula*, Fig. 2A); (2) relatively invariant $\delta^{15}N$ values (*Antiaris*, *Tetrapleura*, *Trilepisium*, Fig. 2B); and (3) fluctuating $\delta^{15}N$ trends by >1‰ (*Entandrophragma*, *Trilepisium*, Fig. 2B).

Organic Geochemistry

The ^{13}C CPMAS NMR spectrum of organic matter preserved in a fossil *Carapa* specimen, which was selected for analysis as a

[1]GSA Data Repository item 2017223, Appendix DR1 (map of collection site), Appendix DR2 (wood anatomy, characters, and figures), Appendix DR3 (geochemistry: XRD, pyrolysis experiment, stable isotope analysis, and data set), Appendix DR4 (NMR methods), is available online at http://www.geosociety.org/datarepository/2017/, or on request from editing@geosociety.org.

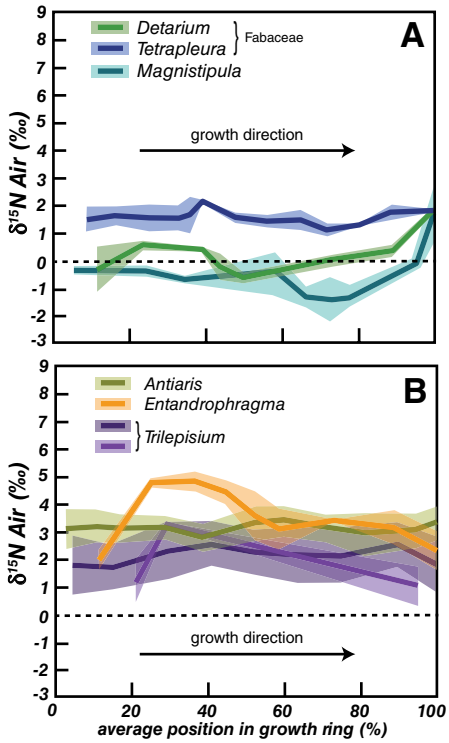

Figure 2. $\delta^{15}N$ values of growth intervals. Results are reported as averages (bold line) of growth ring position and standard deviation (shaded regions), and 2-point weighted average of one growth interval for *Magnistipula*. A: Average N isotope results for growth rings of *Tetrapleura*, *Detarium*, and *Magnistipula*. B: Results for *Trilepisium*, *Entandrophragma*, and *Antiaris*.

representative of the range of diagenetic alteration of the studied wood, is dominated by aromatic and phenolic (aromatic ether) C, as indicated by the predominance of the signals near 130 ppm and 150 ppm, respectively (Fig. 3). The dipolar-dephasing spectrum indicates that aromatic structures have bridging alkyl side chains of 3–4 carbon atoms on average (see Table DR4-1). This is very consistent with the structure of the lignin polymer in wood. The spectra

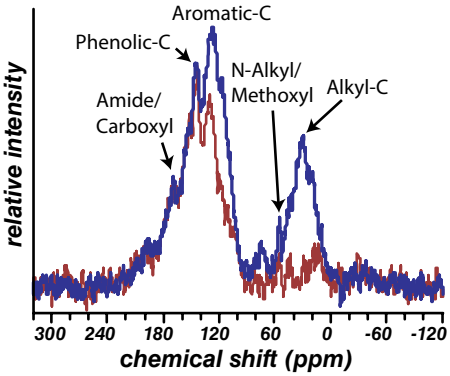

Figure 3. Cross-polarization ^{13}C nuclear magnetic resonance (blue) with dipolar-dephasing (red) spectra of organic matter in fossil *Carapa* specimen.

are consistent with those from lignin pyrolysis experiments conducted at temperatures below 350 °C on laboratory time scales (Sharma et al., 2004). Analysis of the N-containing functional groups indicates that amine (N-H) and amide (N-C = O) nitrogen are both likely to be present.

DISCUSSION

Diagenetic Alteration

To understand the effects of diagenesis on the isotope patterns we used three evaluation methods: (1) thermal effects via pyrolysis experiments (Appendix DR3); (2) compositional effects via ^{13}C NMR and wood morphology; and (3) systematic versus unsystematic isotope variation via comparison of inter-ring isotope patterns in specimens with defined growth intervals.

Pyrolysis experiments indicate that C isotope fractionation is systematic following thermal alteration, and that intra-ring isotope patterns are preserved in spite of a positive shift in $\delta^{13}C$ values (Figs. DR3 and DR3). The N content, however, was reduced below nominal detection limits in pyrolized material, resulting in a lack of intra-ring patterns, and large variance in the likely erroneous $\delta^{15}N$ values. Thus, high-temperature alteration (>350 °C) will likely mitigate the use of N isotopes in this manner on fossil woods.

NMR results indicate that carbohydrate-like (60–90 ppm) signals, methoxyl (45–60 ppm) signals, and aromatic bridgehead C (≤20%) have low relative abundances, which are indicative of early-stage lignin diagenesis (Filley et al., 2002) and low thermal maturity, similar to coal with the rank of lignite (Solum et al., 1989). All specimens preserve fine morphological and structural elements such as pitting, vessel elements, and parenchyma bands, indicating that open-system diagenetic alteration was insignificant, because these features would have been destroyed in the process (Hatcher and Clifford, 1997).

C and N isotopes do not display covariation with sample mass that would be indicative of diagenetic alteration as seen in pyrolysis experiments. Repetition of isotopic patterns within and between specimens suggests that diagenetic effects from organic matter decomposition (NMR), isotope exchange, and thermal alteration (pyrolysis experiments) were systematic in these samples, resulting in low variance of mean intra-ring delta values. C and N abundance is relatively consistent for a given specimen, displaying similar reductions in C and N from assumed precursor values of 45 wt% (C) and 0.2 wt% (N) to the measured values that range from ~2 wt% (C) and ~0.04 wt% (N).

Nitrogen Isotopes in Tree Rings

Modern tree ring N isotope data indicate that mean tree ring $\delta^{15}N$ values are distinct when phylogenetically related non–N-fixing plants are compared to tree rings of N-fixing plants (Fig. 1A). Non-nodulating plants displays $\delta^{15}N$ values >+2‰ in repeatable patterns, indicating a predominant supply of soil N. Nodulating plants, however, display mean tree ring $\delta^{15}N$ values consistent within the range of N-fixing biomass of –0.8‰ ± 1.3‰ (Steele et al., 1983), indicating sufficient nitrogenase activity in the root nodules to supply fixed N, as a dominant source of N, to these plants. Overlaps in $\delta^{15}N$ values occur in nodulating and non-nodulating plants, and may be linked with the abundance of soil-derived N versus microbial fixed N for nodulating plants, or pioneer species colonizing sites previously occupied by N-fixing plants (Craine et al., 2009). The use of multiple consecutive rings for a given tree, modern or fossil, is useful in distinguishing growth-dependent trends in $\delta^{15}N$ values related to changes in N source (Poulson et al., 1995) or yearly translocation of N within a tree (Hart and Classen, 2003). The analysis of multiple rings from a fossil specimen can provide quantification for time-dependent variations in $\delta^{15}N$ values that are unrelated to the source of N uptake (Fig. 2B), which could confound interpretations of plant-microbe symbiosis.

The NMR data collected from the fossil *Carapa* specimen suggest that N in the fossil wood is found in amine and amide functional groups, resembling peptide-like structures (Fig. 3). The presence of these functional groups, absence of thermal and/or microbial degradation products, and presence of a lignocellulose matrix suggest that N is in a well-preserved state, of compositional form analogous to N in modern wood (Werner and Schmidt, 2002). Therefore, the N isotope data are interpreted as Oligocene archives of the source of N uptake by these plants.

Leguminous plants (Fabaceae), which can have symbiosis with the rhizobia class of N-fixing soil bacteria, composed a major plant functional type of Oligocene ecosystems in the study area (Pan et al., 2010). Two of the studied trees are arborescent legumes, *Tetrapleura* and *Detarium* (Fig. 2A). As members of Fabaceae, it is possible that these genera maintained rhizobia symbiosis, in which case their $\delta^{15}N$ values would be –0.8‰ ± 1.3‰ (Steele et al., 1983). The $\delta^{15}N$ values tend to be near 0‰ during wood growth for *Detarium* (a legume) (Fig. 2A). *Magnistipula* also displays $\delta^{15}N$ values near 0‰. It is not reported that *Magnistipula* (Chrysobalanaceae) is actinorhizal in modern ecosystems; however, the $\delta^{15}N$ data from the studied specimen strongly suggest some form of N-fixing symbiosis for this tree in the Oligocene as compared to extant actinorhizal trees (Fig. 2A). The *Tetrapleura* specimen, as a member of Fabaceae, displays $\delta^{15}N$ values >+1‰, inconsistent with the hypothesized 0‰ $\delta^{15}N$ expected for trees with rhizobia symbiosis. *Tetrapleura*, however, is known to be a non–nodule-forming tree and

therefore may not have symbiosis with rhizobia (Sprent, 2005), perhaps explaining the $\delta^{15}N$ values >+1‰ for this specimen. Except for *Magnistipula*, non-leguminous trees maintain more positive $\delta^{15}N$ values (+2‰ to +5‰) during the growing season (Fig. 2B), with invariant trends over the growth interval or with more negative $\delta^{15}N$ values at ring boundaries. Non-nodulating trees display elevated $\delta^{15}N$ values, consistent with the ranges of $\delta^{15}N$ values of other non–N-fixing plants. For trees with $\delta^{15}N$ values inconsistent with N-fixing symbionts, more refined paleoecological inferences could be made by comparing relationships of these trees with other microorganisms (e.g., mycorrhizae), fauna, and soil types.

CONCLUSIONS

We show here that high-resolution sampling of tree rings for stable N isotopes offers insight into N uptake strategies through established symbiotic relationships utilized by woody plants. Application of this approach to Oligocene fossil wood reveals rhizobia symbiosis with some leguminous trees, and likely actinorhizal symbiosis of a non-legume tree. The intra-ring $\delta^{15}N$ values from this study fall into two classes of persistent trends: (1) elevated $\delta^{15}N$ values during the growth interval that are possibly related to a larger component of soil-derived N, with differences in the $\delta^{15}N$ values possibly related to rates of N cycling, or differences in the sources of N from the soil; and (2) $\delta^{15}N$ values persistently near 0‰ consistent with N-fixer symbiosis. Actinobacteria and rhizobia fix N from the atmosphere in modern ecosystems, resulting in $\delta^{15}N$ values near 0‰ for the plant-available NH_4^+. This study provides the platform to screen fossil wood broadly for potential N-fixing strategies, perhaps even in plants that lost the capacity for N-fixing symbioses during the evolutionary history of the N-fixing clade of the rosids. The methods developed here also define an experimental framework for future studies of the adaptation to N limitation in ancient ecosystems.

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