Spatially robust estimates of biological nitrogen (N) fixation imply substantial human alteration of the tropical N cycle

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Biological nitrogen fixation (BNF) is the largest natural source of exogenous nitrogen (N) to unmanaged ecosystems and also the primary baseline against which anthropogenic changes to the N cycle are measured. Rates of BNF in tropical rainforest are thought to be among the highest on Earth, but they are notoriously difficult to quantify and are based on little empirical data. We adapted a sampling strategy from community ecology to generate spatial estimates of symbiotic and free-living BNF in secondary and primary forest sites that span a typical range of tropical forest legume abundance. Although total BNF was higher in secondary than primary forest, overall rates were roughly five times lower than previous estimates for the tropical forest biome. We found strong correlations between symbiotic BNF and legume abundance, but we also show that spatially free-living BNF often exceeds symbiotic inputs. Our results suggest that BNF in tropical forest has been overestimated, and our data are consistent with a recent top-down estimate of global BNF that implied but did not measure low tropical BNF rates. Finally, comparing tropical BNF within the historical area of tropical rainforest with current anthropogenic N inputs indicates that humans have already at least doubled reactive N inputs to the tropical forest biome, a far greater change than previously thought. Because N inputs are increasing faster in the tropics than anywhere on Earth, both the proportion and the effects of human N enrichment are likely to grow in the future.

adaptive cluster sampling | free-living nitrogen fixation | nitrogen deposition | symbiotic nitrogen fixation

Over the last few decades, humans have dramatically altered the global nitrogen (N) cycle (1–3). Three main processes—Haber–Bosch fixation of atmospheric N_2, widespread cultivation of leguminous N-fixing crops, and incidental N fixation during fossil fuel combustion—collectively add more reactive N to the biosphere each year than all natural processes combined (2). Although human perturbation of the N cycle has brought substantial benefits to society (most notably, an increase in crop production) (4), it has also had a number of negative effects on both ecosystems (5, 6) and people (7).

Although humanity's large imprint on the global N cycle is clear, quantifying the extent of anthropogenic changes depends, in large part, on establishing baseline estimates of nonanthropogenic N inputs (1, 8, 9). Before recent human activities, biological N fixation (BNF) was the largest source of new N to the biosphere (9). Terrestrial BNF has been particularly challenging to quantify, because it displays high spatial and temporal heterogeneity at local scales, it arises from both symbiotic associations between bacteria and plants as well as free-living microorganisms (e.g., in leaf litter and soil) (10), and high atmospheric concentrations of N_2 make direct flux measurements unfeasible. Consequently, spatial estimates of BNF have always been highly uncertain (11), and global rate estimates have fallen precipitously in the last 15 y (from 100–290 to ∼44 Tg N y^{-1}) (9). This decline in BNF implies an increase in the relative magnitude of anthropogenic N inputs from 100–150% to 190–470% of BNF (9).

Historically, the largest anthropogenic changes to the N cycle have occurred in the northern temperate zone: first throughout the United States and western Europe and more recently, in China (12, 13). Large-scale estimates of BNF in natural ecosystems in these regions are consistently low (11), leading some to conclude that anthropogenic N inputs in the northern temperate zone exceed naturally occurring BNF and preindustrial atmospheric N deposition by an order of magnitude or more (1, 14). By contrast, the highest rates of naturally occurring BNF have been thought to occur in the evergreen lowland tropical rainforest biome (11), implying that, on a regional basis, human alteration of the tropical N cycle has been comparatively modest. However, in recent years, the tropics have seen some of the most dramatic increases in anthropogenic N inputs of any region on Earth—a trend that is likely to continue (2, 6, 13). Anthropogenic N inputs are increasing in tropical regions, primarily because of increasing fossil fuel combustion (13) and expanding high-N-input agriculture for both food and biofuels (6). These anthropogenic N inputs are having a measurable effect on tropical ecosystems (15). However, understanding and forecasting the effects of anthropogenic N depend, in part, on accurate estimates of BNF in lowland tropical rainforest.

Significance

Biological nitrogen fixation (BNF) is the largest natural source of new nitrogen (N) to terrestrial ecosystems. Tropical forest ecosystems are a putative global hotspot of BNF, but direct, spatially explicit measurements in the biome are virtually nonexistent. Nonetheless, robust estimates of tropical forest BNF are critical for understanding how these important ecosystems may respond to global change and assessing human perturbations to the N cycle. Here, we introduce a spatial sampling method to assess BNF and present evidence that tropical forest BNF is much lower than previously assumed. Our results imply that humans have roughly doubled N inputs to the tropical forest biome relative to N inputs through BNF.

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Unfortunately, the paradigm that the tropics have high rates of BNF is based on a paucity of evidence and several tenuous assumptions. For example, an early global synthesis of terrestrial BNF (11)—which included contributions from both symbiotic and free-living sources—included only one measured estimate of symbiotic BNF from tropical forest (16 kg N ha^{-1} y^{-1}) (16). That single estimate, scaled over thousands of square kilometers, represented the only direct evidence of high tropical BNF rates available at that time (Fig. 1). Subsequent modeled estimates (17) that indirectly estimated BNF have reinforced the notion that tropical BNF rates are high and dominated by the symbiotic form of fixation (Fig. 1). Such high estimates of symbiotic BNF are consistent with the large number of leguminous trees in tropical forest (18–20). However, many legume species do not form N-fixing nodules (21), and of those species that do, nodulation in individuals varies with soil nutrient status, N demand, and tree age (22). Several recent analyses (10, 22–24) indicate lower tropical forest BNF and suggest that symbiotic BNF may not be as important to total BNF as previously thought (Fig. 1), although few studies have simultaneously measured symbiotic and free-living BNF.

There is also a sound theoretical basis for questioning high estimates of BNF in tropical forest. Namely, high concentrations of soil N in the legume-rich tropics create something of a paradox. Although BNF could create N-rich conditions, the substantial energetic cost of BNF means—and some data show—that BNF should be suppressed under high N availability in primary forests (25). Because of high rates of net primary productivity and high N demand in secondary forests (26, 27), regenerating canopy gaps or abandoned agricultural land may have higher rates of BNF than late-successional forest ecosystems (26).

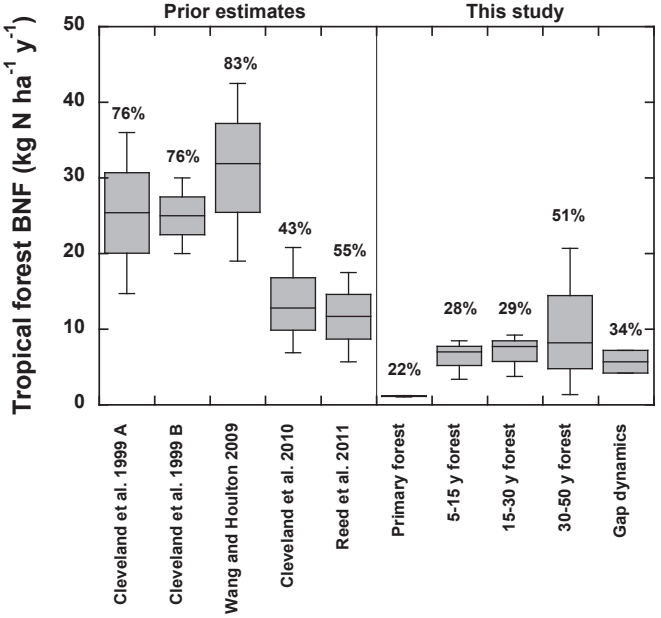

Fig. 1. Previous estimates of BNF in tropical rainforest and BNF measured in this study. Percentages indicate the proportion of total BNF from symbiotic BNF. Cleveland et al. 1999 A (11) is a literature database-derived estimate of tropical forest BNF; Cleveland et al. 1999 B (11) is a modeled estimate of BNF based on the correlation between net primary productivity (NPP) and BNF derived with remotely sensed NPP and evergreen broadleaved forest (EBF) land cover classification. Central estimates and variance for Cleveland et al., 1999 A (11) and Reed et al. 2011 (10) represent the low, central, and high data-based estimates of BNF assuming 5%, 15%, and 15% legume cover, respectively. Central estimates and variance for Wang and Houlton 2009 (17) represent the modeled mean and SD of BNF predicted for the EBF biome. Central estimates and variance for Cleveland et al. 2010 (23) represent the low, central, and high estimates of symbiotic BNF plus free-living BNF or modeled BNF plus free-living BNF. Central estimates and variance for BNF in the four forest ages measured here (primary, 5–15 y, 15–30 y, and 30–50 y) represent means ± 1 SD (n = 3). Our estimate of BNF in a dynamic primary forest (gap dynamics) lacks SD, because it consisted of only two measurements: low and high estimates of forest turnover times equal to 150 and 75 y, respectively.

Resolving the uncertainty in the tropical (and global) N cycle requires that we overcome the enduring challenge of quantifying BNF in any ecosystem. How do we estimate large-scale rates of a process that displays extreme spatial heterogeneity at local scales? Whether using acetylene reduction assays, ^{15}N tracer incubations, or the ^{15}N natural abundance method, most past approaches to empirically estimate symbiotic BNF have relied on spatial extrapolations of BNF rates measured at the level of individual trees. Typically, such extrapolations are based on legume abundance (e.g., percent cover) and make species- or genera-level assumptions about nodulation status of putative N fixers. Here, we applied a method commonly used by community ecologists to measure rare species abundances—stratified adaptive cluster sampling (SACS) (28)—to measure symbiotic BNF. This approach could be used in any ecosystem, and in contrast to other methods, SACS generates unbiased estimates of mean symbiotic BNF (independent of legume abundance) and can more robustly capture the irregular distribution of nodules on the landscape. We simultaneously measured symbiotic and free-living BNF multiple times over the course of 1 y to generate spatially explicit rates of BNF inputs in primary and secondary (5–50 y old) lowland tropical forest in Costa Rica and then used the understanding gained from those estimates to revisit estimates of BNF and anthropogenic N inputs in the tropical forest biome.

Results and Discussion

Taken together, our data suggest far lower rates of total BNF in a region of mixed primary and secondary tropical forest than have been previously reported (Fig. 1). The mean rate of total BNF that we measured in primary forest was only 1.2 kg N ha^{-1} y^{-1}, 10–20 times lower than previously published empirical (11.7 kg N ha^{-1} y^{-1}) (10) or modeled rates (25.4–31.9 kg N ha^{-1} y^{-1}) (Fig. 1) (11, 17). Secondary forest had higher total BNF than primary forest (6.2–14.4 kg N ha^{-1} y^{-1}), and rates increased with age in the three successional forest age classes (although with substantial intraage variability) (Fig. 1). However, the primary forest BNF estimate does not explicitly account for the important role of frequent small-scale disturbances that create canopy gaps in primary forest. Gaps promote species turnover and thereby contribute to small-scale BNF variability in primary forest. Although the primary forest sites that we studied showed no signs of recent disturbance and lacked large canopy gaps, data from other Costa Rican rainforests suggest tree turnover times that average between 75 and 150 y (29). Assuming that secondary forest BNF approximately represents BNF in disturbed forest patches (a reasonable assumption based on a recent analysis in nearby Panama) (22), we suggest a time-integrated mean estimate for BNF of 5.7 kg N ha^{-1} y^{-1} for primary forest in this region (Fig. 1, gap dynamics). Although substantially higher than our estimate of BNF in undisturbed primary forest alone, this estimate is still low relative to previous estimates (Fig. 1).

Symbiotic BNF, which is typically assumed to be the dominant source of BNF in tropical forests, accounted for only 20–50% of total BNF in our study depending on forest age (Fig. 1). Therefore, free-living BNF may represent an equal, if not greater, source of new N to both primary and secondary tropical forests than symbiotic BNF. On a per-mass basis, symbiotic nodules have the highest rates of BNF measured in nature (30), whereas mass-based rates of free-living BNF tend to be much lower (10). However, free-living BNF is much more consistent across the landscape than symbiotic BNF. Thus, N inputs through

Table 1. Legume (*Fabaceae*) species abundance in five primary forest sites [Korup, Luqillo, Yasuni, Pasoh, and Barro Colorado Island (BCI)] that contribute to the Center for Tropical Forest Science plot network (34) as well as the primary forest (1°) and secondary forest (2°) measured in this study near the Piro Biological Station in southwest Costa Rica

	Site						
Fabaceae abundance and basal area	Korup, Cameroon	Luqillo, Puerto Rico	Yasuni, Ecuador	Pasoh, Malaysia	BCI, Panama	Piro 1°, Costa Rica*	Piro 2°, Costa Rica*
Family rank	3	6	1	2	2	8 ± 2	5 ± 2
Basal area (m^2 ha^{-1})	2.9	0.8	2.2	2.6	3.2	1.0 ± 0.5	9.1 ± 3.3
Basal area (%)	9.0	6.5	14.9	8.5	9.9	0.7 ± 0.39	10.3 ± 3.0
Trees (ha^{-1})	387.6	36.0	377.2	—	343.7	67.3 ± 20.7	371.1 ± 95.6
Trees (%)	5.9	2.7	13.0	3.3	7.5	8.2 ± 3.9	39.9 ± 9.9
Total tree species (ha^{-1}; all families)	38	6	108	—	37	90 ± 6	58 ± 6

*Means ± 1 SE.

widespread (but low) rates of free-living BNF may exceed N inputs through isolated (but high) rates of symbiotic BNF. Recent syntheses have made this argument (Fig. 1) (10, 23), but until now, direct evidence has been lacking. We note that, although soil and litter are likely the dominant sources of free-living BNF in tropical forest (10), if we included N inputs from unmeasured sources such as decaying wood (31), canopy epiphytes (32), or termites (33), the relative contribution of free-living BNF would almost certainly increase. Surprisingly, the proportion of the three sources of BNF that we measured (soil, litter, and symbiotic) was similar between primary and secondary forests (Fig. S1), raising questions about the biophysical and/or biogeochemical factors that regulate symbiotic and free-living BNF across forest age classes (10). However, different BNF rates between primary and secondary forest occurred, despite similar soil N and P availability among the sites (Table S1).

We measured low rates of symbiotic BNF in primary tropical forest, despite the presence of legumes in all our sites and an abundance of legumes in secondary forest sites. In fact, both legume abundance and legume basal area in our sites were similar to those in multiple well-studied tropical forests around the world (Table 1 and Fig. S2) (34). Therefore, we suggest that differences between our direct spatial measurement of symbiotic BNF and previous estimates reflect the ability of the SACS method to provide a spatially unbiased estimate of nodule biomass within a site and to generate more robust spatial estimates of symbiotic BNF.

Accurately scaling symbiotic BNF from nodules to ecosystems has been a major impediment to generating spatial estimates of symbiotic BNF in tropical forest. Scaling approaches that rely on legume abundance assume that legumes are actively fixing and/or that symbiotic BNF is correlated with tree size. However, studies using N isotopes have shown that only 36–53% of

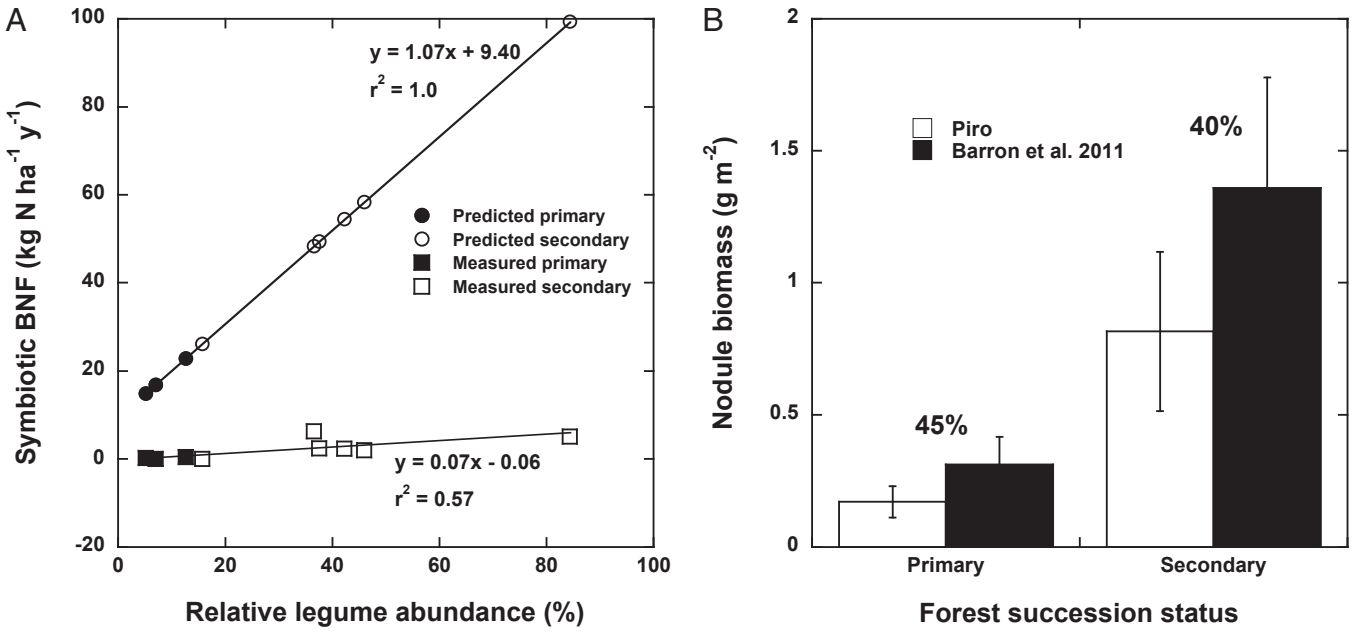

Fig. 2. Comparisons between the SACS approach used in this study and existing methods of spatially extrapolating BNF and nodule biomass. (*A*) Based on legume abundance in the plots that we used, the regression equation used by Cleveland et al. (11) predicts much higher BNF in both primary and secondary forests than we actually measured. In primary forests, the regression approach predicts a symbiotic BNF flux of 14–23 kg N ha^{-1} y^{-1}; we measured a flux of 0.1–0.5 kg N ha^{-1} y^{-1}. (*B*) Using maximum likelihood estimates of nodule biomass and legume abundance, Barron et al. (22) estimated nodule biomass, on a spatial basis, in both primary and secondary forests in Panama. Using the SACS design, we measured 45% less nodule biomass in primary forests near the Piro Biological Station and 40% less nodule biomass in secondary forests near the Piro Biological Station than estimated by Barron et al. (22) in Panama.

legumes are actively fixing in the Amazon (35, 36), and legume tree size and nodule biomass were only weakly correlated in a Panamanian rainforest (22). By comparing our estimates of BNF using the SACS method with two other methods that have scaled BNF based on legume abundance, we show that the SACS method was the proximate cause of the difference between the existing BNF estimates and our lower measurement of BNF. We used legume relative abundance in our plots (Table 1) to estimate BNF with the same regression equation that was used by Cleveland et al. (11). Compared with our measured BNF, the legume abundance-based regression predicted much higher rates of BNF in our primary forest sites (14–22 kg N ha^{-1} y^{-1}) (Fig. 2)—BNF rates were similar to the rates that generated the biome-level estimate in the work by Cleveland et al. (11). Because legumes were more abundant in secondary forest sites, the regression approach predicted secondary forest BNF rates as high as 100 kg N ha^{-1} y^{-1}, a value strikingly higher than the measured values (Fig. 2). Furthermore, our estimate of nodule biomass was 40% lower in primary forest and 45% lower in secondary forest than a legume-based maximum likelihood scaling approach used in Panama (22) that predicted much lower rates of symbiotic BNF than had been reported in the literature (16) (Fig. 2). Because the SACS method incorporated both isolated areas, where nodules are abundant, and extensive areas, where nodules are absent, we argue that it represents a significant advance in the measurement of symbiotic BNF in tropical forest.

The SACS approach estimated nodule biomass independently of legume abundance, thereby providing the opportunity to correlate nodule biomass and legume abundance at the ecosystem scale. There has been a surprising lack of empirical evidence to validate the intuitive positive relationships among legume abundance, nodule biomass, and symbiotic BNF rates. For instance, ter Steege et al. (18) suggested that, across the Amazon basin, legume abundance and nodule biomass were negatively correlated, implying that legume abundance may not predict symbiotic BNF rates. Here, we observed strong positive correlations among the basal area of putatively N-fixing legumes (21), nodule biomass, and symbiotic BNF rates (Fig. S3). We argue that these relationships emerged because we measured BNF across a secondary successional chronosequence where the number and size of legumes varied. Both the shift in legume abundance through succession and the strength of these correlations provide empirical evidence for emerging theory and models that suggest that symbiotic BNF is up- and down-regulated by facultative mechanisms (25) or species replacement (37) to meet N demand. The correlations also provide compelling evidence that symbiotic BNF may be a function of legume basal area during succession and suggest a promising avenue for future work attempting to scale symbiotic BNF rates from legume basal area estimates.

Although our estimates of BNF were collected in one set of sites, two lines of evidence suggest that the low rates of symbiotic BNF that we observed might be common in lowland tropical forest. First, as mentioned above, legume abundance and basal area were similar between our sites and other tropical forest sites around the world (Table 1 and Fig. S2) (34), implying that the low rates of BNF that we observed were not because of a lack of legumes. Second, a reanalysis of a recent lower global BNF estimate that used a top-down ^{15}N isotope model approach (9) also implied lower rates of tropical forest BNF. Vitousek et al. (9) estimated that preindustrial global BNF was 58 Tg N y^{-1} (equal to 5.3 kg N ha^{-1} y^{-1} on vegetated land), much less than previous estimates of 128–195 Tg N y^{-1} (8, 11, 17). Because tropical forest is believed to contribute a disproportionate amount to global BNF (11), we argue that fixation in this biome cannot remain high if global BNF is one-half or one-third the value suggested by previous estimates. We show this point by constraining two existing global models of BNF (11, 17) with our empirically derived estimate of 5.7 kg N ha^{-1} y^{-1} in tropical forest, while holding BNF constant in all other biomes. With tropical forest BNF constrained, global BNF estimated by these models was reduced to the range predicted by the top-down isotope-based estimate (Fig. 3). Although we do not suggest that our empirically derived estimate of BNF represents the value for all tropical forests, it is noteworthy that our measured rate is much easier to reconcile with 58 Tg N y^{-1} global BNF than previous estimates (e.g., 25–30 kg N ha^{-1} y^{-1}) (Fig. 1). We also note that it is the first, to our knowledge, field-based estimate of BNF that supports, by direct measurement, the top-down ^{15}N isotope-based model estimate of BNF (9). Although some areas with exceptionally high rates of BNF undoubtedly exist in tropical forest, these high rates of BNF may either be transient (Fig. 1) (26) or lack sufficient spatial extent to generate high rates of BNF throughout the tropical rainforest biome.

If, as multiple lines of evidence suggest, low rates of BNF are common in tropical forest, this finding would fundamentally alter our understanding of both the tropical N cycle and the impact that humans have had on it. In undisturbed tropical forest, where relatively high soil N availability is common (38–42), lower rates of BNF are consistent with theoretical tradeoffs in the energetics of nutrient acquisition (37, 43–45). Although high BNF has been invoked as a driver of those N-rich conditions, the maintenance of such high rates in the face of N abundance has always been paradoxical (25). The data that we present here support recent evidence suggesting that the majority of BNF-derived N that enters tropical forest may do so episodically (i.e., during periods of forest regeneration), when N demand and biomass accrual are high (26). Subsequent development of N-rich conditions may, therefore, result more from shifting patterns in nutrient limitation and forest growth strategies than from chronically high rates of BNF.

Low tropical forest BNF rates also imply that recent human activity has enriched the tropical N cycle far more than previously thought. Anthropogenically derived N deposition inputs in tropical regions are already similar to (or exceed) the rates of BNF that we report here (15, 46). In areas deforested for agriculture, massive per-area increases in N inputs are common because of fertilizer application and/or the growth of N-fixing crops—most notably, soy (47). Deforestation is often the result of economically expedient (and politically supported) agricultural extensification (*sensu lato*, ref. 48), after which exhausted agricultural areas are abandoned to secondary succession.

To provide context for the importance of BNF in evaluating the overall anthropogenic change in human vs. natural N inputs to the tropical forest biome, we compiled biome-wide atmospheric N deposition estimates (49), inorganic and organic fertilizer use data (50), and an estimate of agricultural N fixation based on crop area and yield statistics (51) within the historical tropical rainforest biome (i.e., the tropical rainforest biome in the absence of human activity) (52). Most strikingly, this analysis shows the importance of accurate BNF estimates in considering human changes to the tropical N cycle. In particular, anthropogenic N sources only represented 31% of total nonanthropogenic N inputs using prior assumptions of high forest BNF (Fig. 4). Using the lower BNF value indicated by our field study, we suggest that humans have increased the amount of reactive N entering the tropical rainforest biome by 134% (Fig. 4). We note that our estimate of anthropogenic N flux does not include organic fertilizer, like manure, which recycles both endogenous and exogenous N within ecosystems (assuming that most manure is not a net import from other biomes). However, in Fig. 4, we depict biome-level organic fertilizer inputs to illustrate that manure application in the tropics rivals the biome-wide forest BNF value derived from our estimate and that manure is an important alternative to inorganic fertilizer manufactured by the Haber–Bosch process (Fig. 4). The values for both agricultural BNF and

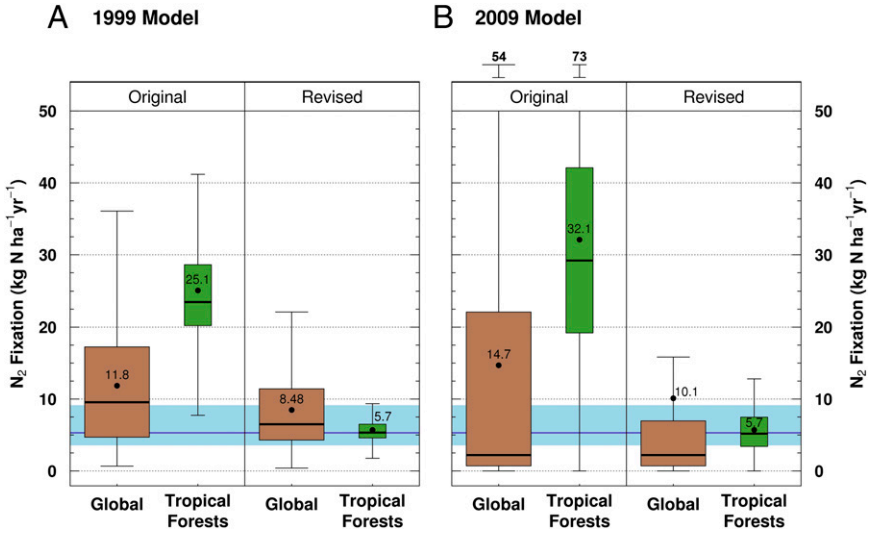

Fig. 3. Revising global BNF models with our gap-based estimate of BNF in tropical forests (5.7 kg N ha^{-1} y^{-1}) places existing global estimates in the range of BNF measured by a top-down isotope model of global BNF (9) (dark blue horizontal line, mean; shaded blue horizontal area, high and low estimates). (*A*) By revising tropical BNF in the original 1999 model (11), we reduced global BNF from 11.8 to 8.5 kg N ha^{-1} y^{-1}. (*B*) By revising tropical BNF in the original 2009 model (17), we reduced global BNF from 14.7 to 10.1 kg N ha^{-1} y^{-1}. Dots represent means, with values noted. Black horizontal bars within box plots represent the median values, box plots represent 1 SEM, and whiskers represent the total range of estimates. Global mean BNF estimates using these two models remain substantially higher than the median value because of high legume-derived estimates of BNF in tropical savannas.

fertilizer use are undoubtedly conservative: although they represent compilations of the currently available data (circa 2000), they do not integrate the fact that, over the past 15 y, both overall N fertilizer use and the extent of soy cultivation (an important source of agricultural BNF) have expanded dramatically in tropical regions (6, 53).

Regardless of the extent to which human activities have actually perturbed the tropical N cycle, there is no doubt that, as the proportion of anthropogenic N inputs increases relative to natural inputs, the region will continue to see significant ecological and socioeconomic impacts. In temperate regions, anthropogenic N inputs have contributed to shifts in species composition, even when below so-called critical loads—thresholds below which inputs of N are supposedly safe for ecosystems (54). Given that tropical regions contain much of the biological diversity on Earth (55), many tropical species may be vulnerable to the unintended, indirect effects of increasing anthropogenic N. Similarly, as seen in much of the temperate zone, increased anthropogenic N inputs could have positive effects on human health, but without effective management, they could also have profound negative effects (7). Low rates of BNF in tropical regions add urgency to growing calls to manage increasing N inputs in tropical biomes and require local, regional, and international policy instruments that, for the most part, are not yet in place (6).

Methods

We measured BNF in 12 0.5-ha plots (50 × 100 m) on the Osa Peninsula, Costa Rica near the Piro Biological Station (8°24′ N, 83°20′ W; 87 m above sea level) (56). *SI Methods*, sections 1 and 2 provide additional site description. We measured symbiotic, soil, and litter N fixation four times in 2012 and 2013, capturing all seasonal precipitation variation that the sites experience—January, May, July, and October. To measure symbiotic BNF, we measured nodule biomass during the early wet season at each site using SACS (28). *SI Methods*, section 2 and Fig. S4 give additional details. In total, we searched for nodules in ∼1,500 5.5-cm-wide × 10-cm-deep cores. We measured BNF rates on excised nodules, soil, and litter using the acetylene reduction assay. *SI Methods*, sections 3 and 4 give additional details. We aggregated the 5- to 15-, 15- to 30-, and 30- to 50-y forest age classes into the category of secondary forest. We measured primary forest turnover (gap dynamics) using two scenarios—low turnover (150 y) and high turnover (75 y) based on estimates in ref. 29. *SI Methods*, section 5 details measurement of BNF in each scenario. We measured the relationship between nodule biomass, symbiotic BNF, and basal area of putative N-fixing legumes using linear regression in the statistical package R (57). We constrained two existing spatially explicit global N_2 fixation models (11, 17) by our empirically derived estimate of tropical N_2 fixation and compared both previous and constrained models with the top-down global BNF measured in ref. 9. *SI Methods*, section 6 gives additional details on constraining the models. We quantified the extent of anthropogenic impacts on the tropical N cycle by comparing preindustrial N

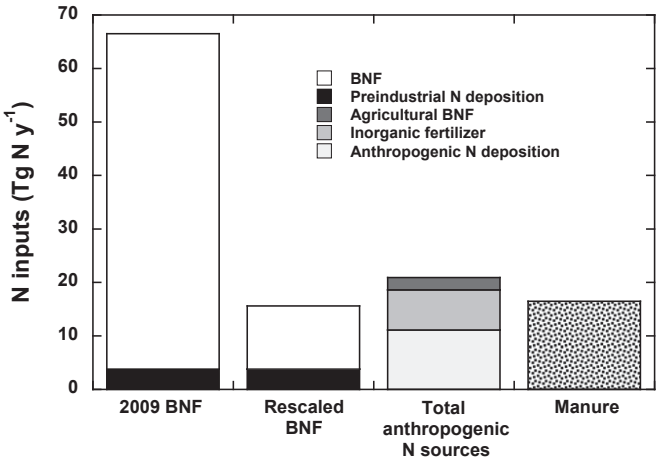

Fig. 4. The rate of BNF in tropical rainforest generated using the SACS approach is much lower than previous estimates, and it implies that the human perturbation to the tropical N cycle is approximately four times greater than previous N fixation estimates would suggest. We estimated BNF using an existing (2009) model (17) that generated high estimates of BNF, and then, we estimated lower rates of BNF by downscaling the 2009 model (17) to our mean BNF estimate of 5.7 kg N ha^{-1} y^{-1}. Anthropogenic N deposition was calculated as the difference between total tropical N deposition (49) and preindustrial N deposition (58). Manure was not treated as an anthropogenic N input, because we assumed that it is regionally produced and that it represents a recycling of previous anthropogenic or naturally fixed N. However, manure is a major source of N to tropical ecosystems nearly equivalent to all other anthropogenic N sources combined.

inputs (BNF and N deposition) with recent estimates of anthropogenic N inputs (postindustrial N deposition, agricultural BNF, and agricultural fertilization) across total historical tropical forest area. *SI Methods*, section 7 gives additional details.

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Supporting Information

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SI Methods

(1) Plot Description. Mean annual temperature at the Piro Biological Station is 26 °C, and mean annual precipitation is 3,450 mm (1). The area experiences a climate that can be divided into three seasons: a short dry season between late December and April, an early wet season between April and September, and a late wet season between September and December. The 12 sites used in this study consisted of four age classes: 5–15 y, 15–30 y, 30–50 y, and primary forest. Each age class had three replicates ($n = 3$). Slopes were <10% at all 12 sites. Each plot was subject to a census for tree size and species identification (or genus if species identification was impossible) in 2010–2011.

(2) Estimating Nodule Biomass Using Stratified Adaptive Cluster Sampling. The stratified adaptive cluster sampling (SACS) design generates an unbiased estimate of the mean nodule biomass per site (2). Beginning with an initial sample, it adaptively adds more samples from the adjacent neighborhood of the original sample if nodules are present, and the neighborhood grows until no additional samples have nodules present. To use the SACS approach, we first identified a 10 × 70-m strip within each plot, which we partitioned into seven 10 × 10-m strata that consist of $N_c = 9{,}604$ possible cores each (Fig. S4). In each stratum, we sampled 10 cores (n_c), one at each 1 m along a transect on the central axis of each stratum that is perpendicular to the long axis of the total area T. If nodules were present in any of 10 sampled cores along the central transect, we expanded our search at each point that nodules were initially present until no nodules were present on all sides of the network or we were physically obstructed from digging the hole (e.g., by a tree) (Fig. S4). As described by Thompson (2), we defined the mass of nodules in a core (w_{ci}), weighted by the number of networks in the sample, as

$$w_{ci} = \frac{\Sigma M_n}{N_n},$$

where M_n is the mass of the nodules in the network and N_n is the number of holes containing nodules in the network. Because our networks never crossed strata, we then estimated mean nodule biomass ($\hat{\mu}$) (2) using the equation

$$\hat{\mu} = \frac{1}{T}\sum_{c=1}^{L}\frac{N_c}{n_c}\sum_{i=1}^{n_c} w_{ci}.$$

We calculated the average mass of nodules per area (M_A; in grams meter^{-2}) by multiplying $\hat{\mu}$ by the area sampled with each core.

(3) Acetylene Reduction Assay with Nodules. In a Panamanian tropical forest, it was shown that nodule biomass does not change substantially seasonally (including in a dry season) (3); thus, we measured nodule biomass one time (during the early wet season) but assessed variability of biological nitrogen fixation (BNF) rates (nodule activity) four times throughout the year. When we measured nodule biomass using the SACS approach, we used the acetylene reduction assay (ARA) on nodules excavated from the SACS sampling; in those samplings and subsequent samplings, we used between 6 and 10 samples of ARA per site, and each ARA sample had one to five nodules (depending on nodule size) in each sample. We performed the ARA using the following protocol. After excavating nodules and gently separating them from attached roots, we incubated them in a 50-mL clear acrylic tube for 1 h with a 10% acetylene atmosphere. After incubation, 14-mL headspace samples were removed from tubes with a sampling syringe, placed in 10-mL Vacutainers (Becton-Dickinson), and returned to the United States for analysis by GC using a Shimadzu GC-2014 equipped with a flame ionization detector. We accounted for the ethylene produced from soil and litter without acetylene exposure, the ethylene produced from tubes and Vacutainers, and the concentration of ethylene within our acetylene as well as the ethylene lost because of photodegredation during transport. To convert acetylene reduction rates into BNF rates, we measured the uptake of ^{15}N-labeled N_2 in nodules to generate a site-specific ethylene:N conversion ratio of 2.8:1 for symbiotic BNF. Although the ARA method requires the excision of nodulated roots from the host plant, we are unable to assess the artifact of this sampling method on actual rates of BNF but instead, sought to minimize these artifacts by using a short (1 h) incubation—an approach that is frequently used (3, 4).

(4) ARA with Soil and Litter. We measured free-living soil BNF in intact cores taken from the top 2 cm of the mineral soil but converted these rates to 10-cm estimates, because BNF is relatively constant in the top 10 cm of a soil profile (5). Leaf litter was collected by hand and placed in acrylic tubes for incubation, and area-based estimates of leaf litter BNF were estimated by collecting all of the litter within several 0.09-m^2 quadrants at each site and each sampling time, allowing for mass-to-area conversions. Both soil and litter samples were exposed to 10% acetylene atmosphere for 18 h. After incubation, 14-mL headspace samples were removed from tubes with a sampling syringe, placed in 10-mL Vacutainers (Becton-Dickinson), and returned to the United States for analysis by GC using a Shimadzu GC-2014 equipped with a flame ionization detector. We accounted for the ethylene produced from soil and litter without acetylene exposure, the ethylene produced from tubes and Vacutainers, and the concentration of ethylene within our acetylene as well as the ethylene lost because of photodegredation during transport. To convert acetylene reduction rates into BNF rates, we applied the theoretical conversion ratio of 3:1 (moles ethylene:moles N) to free-living BNF (5).

(5) Estimating BNF in Primary Forests with Disturbance. To estimate BNF in the low- and high-turnover scenarios, we assumed that mean total BNF from each age class was constant and multiplied total BNF by the number of years in each category (and allowed the 5- to 15-y category to apply to the first 5 y of forest development). Primary forest rates were applied to forest ages greater than 50 y.

(6) Constraining Global Models of BNF to Low Tropical BNF. To constrain two existing spatially explicit global N_2 fixation models (6, 7), we used the following equation:

$$mBNF_{adj} = \sum_{i=0}^{n_{trop}}\left[\frac{\overline{eBNF}}{\overline{mBNF}} \times mBNF_i\right],$$

where $mBNF_i$ represents spatially explicit modeled N_2 fixation rates, $\overline{mBNF}$ represents the mean of modeled tropical N_2 fixation, $\overline{eBNF}$ represents our gap-filled estimate of mean tropical N_2 fixation (5.7 kg N ha^{-1} y^{-1}), and $mBNF_{adj}$ represents total tropical N_2 fixation constrained by our empirically derived estimate. Modeled tropical N_2 fixation was derived from (i) the central

N_2 fixation empirical model based on net primary productivity (6) applied to moderate resolution imaging spectroradiometer net primary productivity data (8) ($\overline{mBNF}$ equaled 25.1 kg N ha^{-1} y^{-1}) and (*ii*) the Carnegie Ames Stanford Approach biogeochemical process model (7) ($\overline{mBNF}$ equaled 32.1 kg N ha^{-1} y^{-1}). Tropical forest pixels (n_{trop}) were defined using the University of Maryland evergreen broadleaf forest land cover classification (9).

(7) Quantifying the Extent of Anthropogenic Impacts on the Tropical N Cycle. Historical tropical forest extent (i.e., tropical forest extent in the absence of human activity) was calculated by combining the tropical evergreen forest and tropical deciduous forest land cover classifications within the global potential vegetation data product from ref. 10. Preindustrial N deposition was estimated from ref. 11, whereas natural rates of N fixation were derived from ref. 7. Rescaled total natural tropical N inputs were estimated by applying our updated estimate of mean tropical forest N fixation across the total historical tropical forest area. Total anthropogenic N inputs were defined to include postindustrial N deposition (calculated as the difference between total N deposition and preindustrial N deposition) (12), agricultural N fertilization (13), and agricultural N fixation (14) inputs. We also highlight the scale of N inputs from manure additions (13), although they are not truly additional anthropogenic inputs, because most manure is locally and naturally derived and thus, represents within-biome N cycling. Postindustrial N deposition was estimated for total historical tropical forest areas using the EDGAR 4.2 emission database (15) generated with a global 3D chemistry transport model for the year 2008, the most recent year available. Total N fertilization and N inputs from manure application occurring within historically tropical forest areas were estimated from a fertilization and manure application database generated from year 2000 agricultural statistics data (13). Total agricultural N fixation was estimated by combining year 2000 crop area and yield data for all Food and Agricultural Organization-recognized crop types (14) with known crop-specific rates of symbiotic and free-living N fixation (16, 17).

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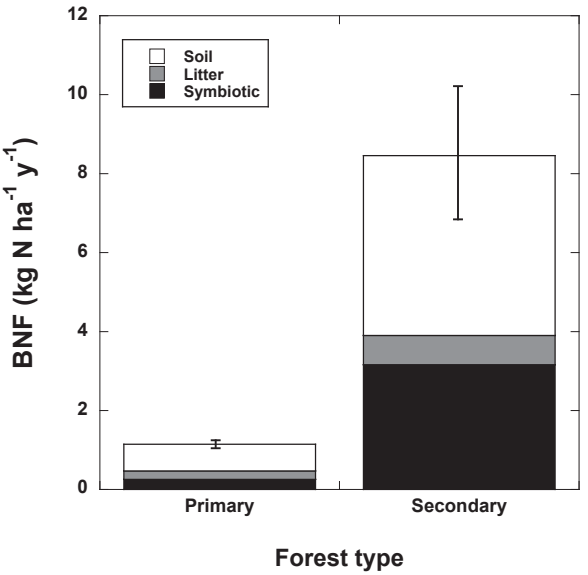

Fig. S1. Total annual BNF inputs from soil, litter, and symbiotic pools in primary and secondary forest on the Osa Peninsula, Costa Rica. In this study, we define free-living BNF as the sum of soil and litter BNF. The BNF rate in secondary forest was calculated as the mean of symbiotic and free-living BNF rates in each of the three secondary forest ages that we measured: 5–15, 15–30, and 30–50 y. Error bars depict ±1 SE of mean total N_2 fixation in primary ($n = 3$) and secondary ($n = 9$) forest.

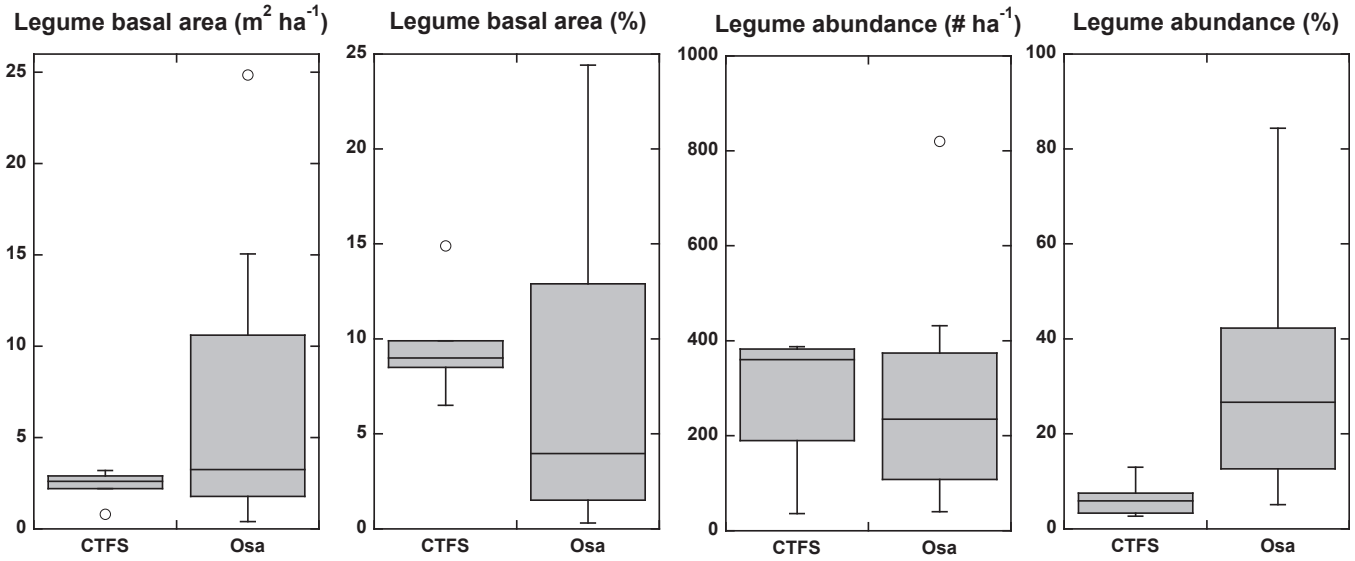

Fig. S2. Distribution of legume basal area and legume abundance (both in absolute values and percent) in 5 Center for Tropical Forest Science (CTFS) plots located around the world (Table 1) and 12 secondary and primary forest plots on the Osa Peninsula, Costa Rica.

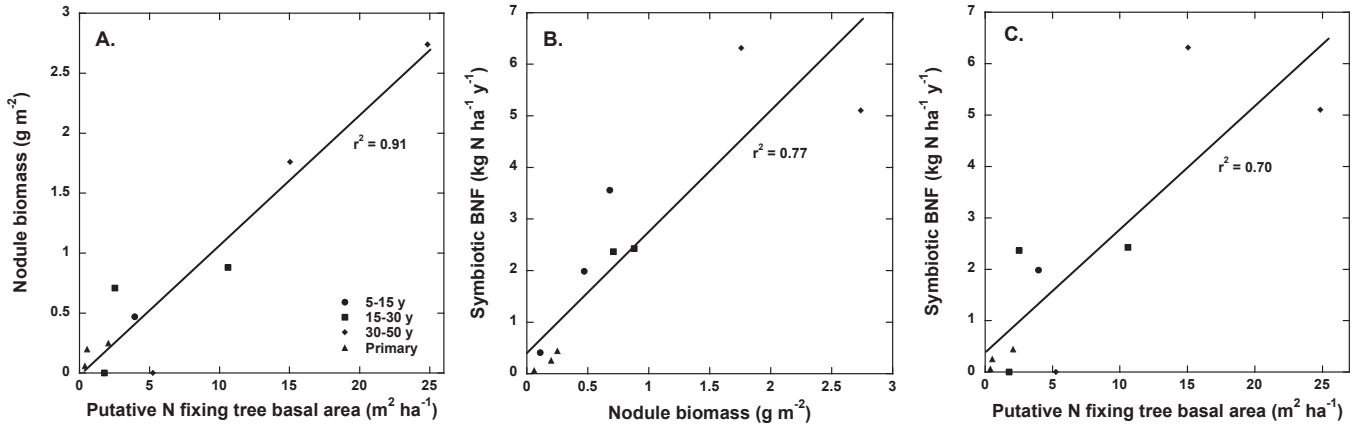

Fig. S3. Relationships between (*A*) nodule biomass and the basal area of legume species putatively capable of forming nodules and fixing N, (*B*) symbiotic BNF (on a spatial basis) and nodule biomass, and (*C*) symbiotic BNF rates (on a spatial basis) and the basal area of legume species putatively capable of forming nodules and fixing N. All relationships are significant ($P < 0.05$), and the strength of each correlation is described by r^2 values within each panel.

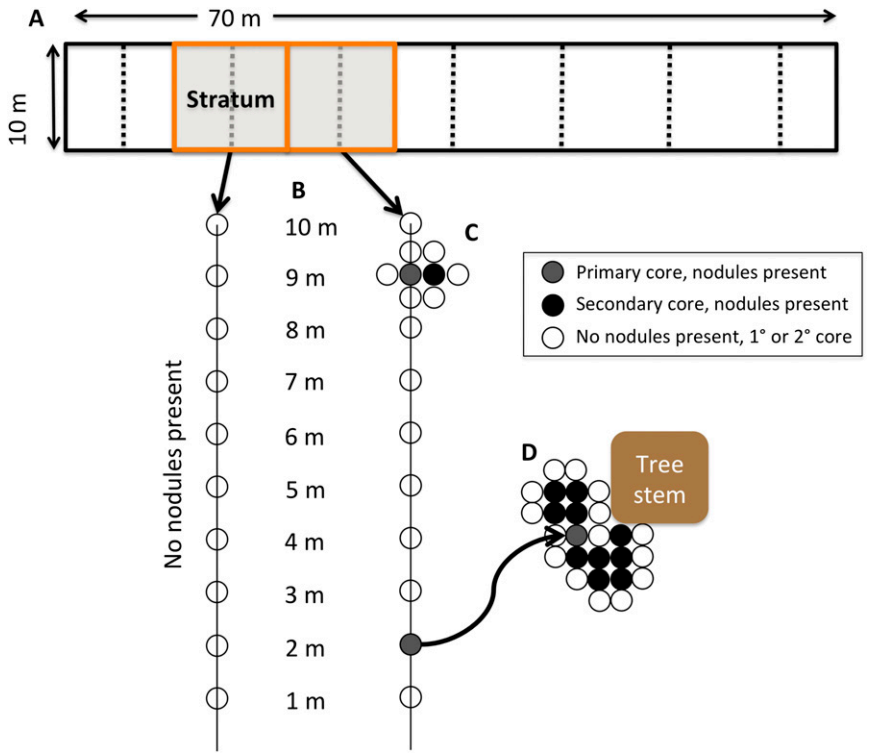

Fig. S4. The SACS experimental design. (*A*) First, we identified a 10 × 70-m sample area. (*B* and *C*) Second, we sampled 10 cores along the central transect of each stratum. If nodules were present in 1 or more of 10 primary transect cores, (*C*) we expanded the sampling design to include all samples in the neighborhood of the original core that had nodules present and stopped expanding the neighborhood when no more adjacent samples had nodules present or (*D*) we were physically prevented from sampling. The clusters of nodules presented in *C* and *D* represent actual patterns of nodules that we found on the landscape.

Table S1. Soil total and inorganic N, soil total and extractable P, and soil pH in the surface soil (0–10 cm) among the sites occupying the four forest age classes of the Piro chronosequence

	Forest age classification			
Soil parameters	5–15 y	15–30 y	30–15 y	Primary
Total N (%)	0.37	0.36 (0.05)	0.44 (0.12)	0.39 (0.04)
Inorganic N (mg kg^{-1})	2.57 (4.31)	3.31 (6.40)	3.69 (3.82)	4.13 (2.72)
Total P (mg kg^{-1})	499	522 (275.7)	489 (277.8)	665 (110.5)
Extractable P (mg kg^{-1})	0.94 (0.84)	3.90 (5.22)	2.54 (3.08)	1.56 (2.61)
pH	5.4	5.2 (0.42)	5.4 (0.42)	5.4 (0.21)

Inorganic N was calculated as the sum of ammonium and nitrate; extractable P was measured using dilute ammonium fluoride extraction. pH was measured in 1:1 soil:water slurry. Inorganic N and extractable P concentrations represent the average of the four seasonal sampling events. Values are mean ± 1 SD (n = 3 per age class).