Response of Free-Living Nitrogen-Fixing Microorganisms to Land Use Change in the Amazon Rainforest

Babur S. Mirza,[a] **Chotima Potisap,**[a,b] **Klaus Nüsslein,**[c] **Brendan J. M. Bohannan,**[d] **Jorge L. M. Rodrigues**[a]

Department of Biology, University of Texas, Arlington, Texas, USA[a]; Department of Biochemistry, Khon Kaen University, Khon Kaen, Thailand[b]; Department of Microbiology, University of Massachusetts, Amherst, Massachusetts, USA[c]; Institute of Ecology and Evolution, University of Oregon, Eugene, Oregon, USA[d]

The Amazon rainforest, the largest equatorial forest in the world, is being cleared for pasture and agricultural use at alarming rates. Tropical deforestation is known to cause alterations in microbial communities at taxonomic and phylogenetic levels, but it is unclear whether microbial functional groups are altered. We asked whether free-living nitrogen-fixing microorganisms (diazotrophs) respond to deforestation in the Amazon rainforest, using analysis of the marker gene *nifH*. Clone libraries were generated from soil samples collected from a primary forest, a 5-year-old pasture originally converted from primary forest, and a secondary forest established after pasture abandonment. Although diazotroph richness did not significantly change among the three plots, diazotroph community composition was altered with forest-to-pasture conversion, and phylogenetic similarity was higher among pasture communities than among those in forests. There was also 10-fold increase in *nifH* gene abundance following conversion from primary forest to pasture. Three environmental factors were associated with the observed changes: soil acidity, total N concentration, and C/N ratio. Our results suggest a partial restoration to initial levels of abundance and community structure of diazotrophs following pasture abandonment, with primary and secondary forests sharing similar communities. We postulate that the response of diazotrophs to land use change is a direct consequence of changes in plant communities, particularly the higher N demand of pasture plant communities for supporting aboveground plant growth.

T he Amazon rainforest is the largest equatorial forest in the world and acts as one of the major carbon dioxide (CO_2) sinks, absorbing 0.4 Pg C · year^{-1} (1 Pg = 10^{15} g) of this greenhouse gas annually [1, 2]. To absorb this enormous amount of CO_2, a significant input of nitrogen (N) is required. This can occur through the decomposition of the organic material, atmospheric deposition of N, and biological N_2 fixation. In terrestrial ecosystems, 97% of the natural N input comes from biological N_2 fixation [3] performed by *Bacteria* and *Archaea* [4, 5]. The majority of these nitrogen-fixing microorganisms (diazotrophs) are present in a free-living state, and their contribution to the N budget in tropical ecosystems can be from 12.2 to 36.1 kg · ha^{-1} · year^{-1} [6].

The composition and abundance of the free-living diazotrophic community in soil is directly related to its N_2 fixation rate [7] and can be altered by a number of factors, including land management practices [7–10], soil N content [11–14], C and P availability [15–18], soil texture [19], pH and clay content [20, 21], season [22], and presence of different plant species [23, 24]. Despite the importance of free-living diazotrophic communities to ecosystem processes, our knowledge of their diversity, abundance, and community composition in tropical forests remains very limited.

The Amazon rainforest continues to be threatened by deforestation at alarming rates, with 70% of the conversion associated with pasture formation. Primary forests, which can contain up to 179 unique tree species ha^{-1} [25, 26], are replaced with one or two fast-growing grasses (primarily *Urochloa brizantha* and *Panicum maximum*). This shift in plant community composition is likely to impact ecosystems through microbially mediated processes. For example, the processes of mineralization, nitrification, and denitrification have been reported to decrease in response to the conversion of Amazon forest to pasture [27–29]. Similarly, methane consumption has been reported to decrease in response to deforestation [30].

The impact of deforestation on the bacterial diversity of Amazon rainforest soils has been limited to a few studies, all of which have either used phylogenetic markers (i.e., the 16S rRNA gene) [31–34] or the functional gene marker *pmoA* (a genetic marker for methane-oxidizing bacteria) [30]. These studies reported significant differences in bacterial community compositions in forest and pasture soils. However, it remains virtually unknown how other microbial groups responsible for specific functional processes respond to deforestation. In addition, it is also unclear to what extent altered functional diversity can recover after the reestablishment of forest following pasture abandonment.

In this study, we sought to address the above gap in knowledge by studying the free-living diazotrophic community, a functional group of microorganisms that makes a significant contribution to the N pool in tropical systems [6, 35]. The objectives of the present study were (i) to assess the diazotrophic community composition and abundance of the Amazon rainforest and determine its response to land use change from primary forest to pasture, (ii) to evaluate the extent of recovery for diazotrophic communities following pasture abandonment (and the growth of secondary forest), (iii) to determine whether diazotrophic community composition and abundance are linked to the changes in soil physiochemical characteristics, and (iv) to identify indicator diazotrophic taxa associated with changes in land use in tropical forest ecosystems.

MATERIALS AND METHODS

Soil collection and DNA extraction. Soil samples were collected from Fazenda Nova Vida (10°10′18.71″S, 62°47′15.67″W), situated in the state of Rondônia, Brazil, in 2009. The state of Rondônia was selected for sampling because it was one of the three states (Rondônia, Mato Grosso, and Pará) which accounted for more than 85% of all Amazon deforestation from 1996 to 2005, converting an average of 16,600 km^2 year^{-1} of forest to pasture land (36). Samples were collected from a primary forest, a pasture established in 2004, and a secondary forest established after pasture abandonment in 1999. All plots share common boundaries within a 2-km distance. A 100-m^2 transect was placed in each plot, and nested transects of 10, 1, 0.1, and 0.01 m^2 were established from the same initial point as previously described by Rodrigues et al. (34). A total of five soil samples per plot were collected: three on the transect with the cardinal direction north (1, 10, and 100 m) and one each (100 m) on the transects from the directions northeast and east.

After removal of the litter layer, soils were taken with a 5-cm-diameter corer to a 10-cm depth, kept on ice, and transported to the laboratory, where they were stored at −80°C until used for DNA extraction. Soil cores were individually sieved with a 2-mm mesh and subdivided for total soil carbon and nitrogen determination with the Auto Analyzer LECO TruSpec CN at the Centro de Energia Nuclear na Agricultura, University of Sao Paulo, Brazil, and elemental concentrations were analyzed at the Laboratorio de Fertilidade do Solo, Department of Soil Sciences, University of Sao Paulo, as described previously (34). The details of the sampling sites and soil physiochemical characteristics are presented in Table S1 in the supplemental material.

DNA extraction and PCR amplification. Total genomic DNA was extracted from soil using the Power Lyzer PowerSoil DNA isolation kit (MO BIO, Carlsbad, CA) according to the manufacturer's instructions. Soil DNA was quantified, diluted to a concentration of 25 ng/μl, and used to amplify the *nifH* gene with primers PolF (TGCGAYCCSAARGCBGACTC) and PolR (ATSGCCATCATYTCRCCGGA) (37). The reaction volume of 50 μl contained 0.2 mM each deoxynucleoside triphosphate, 0.4 μM each primer, 1× PCR buffer, 2 mM MgCl₂, 50 ng of template DNA, and 2 U of *Taq* DNA polymerase (Gene Script, Piscataway, NJ). The PCR conditions were 2 min of denaturation, followed by 35 rounds of temperature cycling (95°C for 30 s, 59°C for 30 s, and 72°C for 45 s), and a final extension at 72°C for 7 min. Aliquots (5 μl) of the amplified products were visualized on ethidium bromide-stained 1% agarose gels. Along with polymerase (Pol) primers, three other primer sets—Ueda19F-Ueda470R (15), PicenoF/R (38), and Z-primer (39)—were tested for *nifH* amplification (40). Only the Pol primer set resulted in successful amplification for all 15 samples, and hence it was used for the subsequent analyses.

Cloning and DNA sequencing. Amplified products were purified with the Ultra Clean 15 DNA purification kit (MO BIO, Carlsbad, CA), ligated into the TOPO TA pcr2.1 vector, and transformed into *Escherichia coli* One Shot TOP10 competent cells (TOPO TA cloning kit; MO BIO, Carlsbad, CA), as recommended by the manufacturer. Transformants were grown in Luria-Bertani medium supplemented with kanamycin (50 ng/μl) and analyzed for the presence of an insert of approximately 362 bp by PCR amplification with primers M13F (GTAAAACGACGGCCAG) and M13R (CAGGAAACAGCTATGAC). Amplified fragments were treated with ExoSAP-IT (USB, Cleveland, OH) and sequenced with the M13F primer. A total of 825 *nifH* gene clones (55 clones per sample) were sequenced with the 3130XL genetic analyzer (Applied Biosystems, Foster City, CA) at the University of Texas—Arlington Genomics Core Facility.

Sequence analyses and OTU assignment. A total of 825 sequences of the *nifH* gene were first aligned using CLUSTAL_X (41) and trimmed to 362 bp using Sequencher 4.2.2 (Gene Codes Corp., Ann Arbor, MI), and a final alignment was performed with MUSCLE (42). All sequences were compared to those in NCBI databases for confirmation as *nifH* gene sequences and deposited in GenBank.

Aligned *nifH* gene sequences were used to create a distance matrix followed by clustering into operational taxonomic units (OTU) at 99, 97,

95, 90, and 80% DNA identity using the software Mothur (43). Rarefaction curves were generated using the same software.

DNA translation and OPU assignment. In order to increase the taxonomic resolution of the *nifH* sequences (44) and associate their presence with a specific land use, DNA sequences were translated into deduced amino acid sequences and aligned using the functional gene pipeline (Fungene) of the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu) (45). Aligned protein sequences were used to generate the distance matrix and clustered into operational protein units (OPU) at 99, 97, 95, 90, and 80% protein similarity using Mothur as described above.

OPU representing more than five sequences were selected for construction of an amino acid-based maximum likelihood (ML) phylogenetic tree using the PHYML 3.0 software (46) with settings previously described elsewhere (47). The software MEGA (version 4) was used to collapse sequences at a 97% amino acid similarity level (48).

Phylogenetic diversity. Phylogenetic diversity of diazotrophic communities was compared among different land use systems using the unweighted UniFrac algorithm (49). A DNA-based ML phylogenetic tree along with the branch length matrix was used as the input file and a principal coordinate analysis (PCoA) was performed using the Fast UniFrac matrix (http://bmf2.colorado.edu/fastunifrac/) (50).

Diazotrophic community structure. To assess changes in diazotrophic community composition across different land use systems, an OTU-based DNA similarity data matrix (at 99, 97, 95, 90, and 80% identity) was square root transformed and analyzed using Primer 6 (version 6.1.11) computer software (Primer-E Ltd., Plymouth, United Kingdom) with the permutational multivariate analysis of variance (PerMANOVA) add-on package (51). Differences in the diazotrophic communities were evaluated through total species richness, Margalef's richness index, and the Chao1 estimator (52). Changes in OTU composition among communities were evaluated with the Sørensen index of similarity calculated from presence/absence data (53).

Patterns of the diazotrophic community structure were visualized by nonmetric multidimensional scaling (NMDS) of the Bray-Curtis similarity matrix. Statistical significance of differences among the data sets was analyzed by PerMANOVA (54), followed by a calculation of a pseudo-*F* statistic and comparison of the total variance explained by sample identities to that explained by random permutations. Treatments were compared to 9,999 permutations.

Associations between the diazotrophic species composition data matrix and the environmental data matrix (soil moisture, N, C, C/N, pH, P, S, K$^+$, Ca^{2+}, Mg^{2+}, Al^{3+}, H$^+$, CEC, V, and Al + H [potential acidity]) were examined using the "BEST" procedure in Primer (51). All environmental data were log transformed and standardized prior to the BEST analysis.

Indicator species analysis. Translated amino acid-based data (OPU 97% amino acid similarity) was also assessed with PerMANOVA. In order to identify the indicator species and the relative contribution of each OPU to the differences in groups observed by PerMANOVA, we performed a similarity percentage (SIMPER) analysis (51).

qPCR. For quantitative PCR (qPCR), reactions were performed in triplicate for each biological sample and carried out in a 20-μl volume containing 10 μl of iTaq Fast SYBR green super mix with ROX (Bio-Rad, Inc., Hercules, CA), 100 nM the same primers used for cloning, PolF and PolR, and 10 ng of DNA. Amplification was performed with the Applied Biosystems 7300 real-time PCR system under the following conditions: 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 59°C for 1 min, and extension at 72°C for 1 min. PCR-grade water (no template) was used as a negative control. The specificity of the qPCR products was confirmed by melting curve analysis and gel-based post-PCR analysis. Standard curves (10^0 to 10^7 *nifH* copies per reaction) were generated with plasmid DNA containing a partial fragment of the *nifH* gene from *Herbaspirillum seropedicae* ATCC 35892. The qPCR efficiency (*E*) was calculate according to the equation $E = 10^{[-1/\text{slope}]}$. The real-time efficiency of *nifH* was 1.80 ± 0.01, and the R^2 of the standard
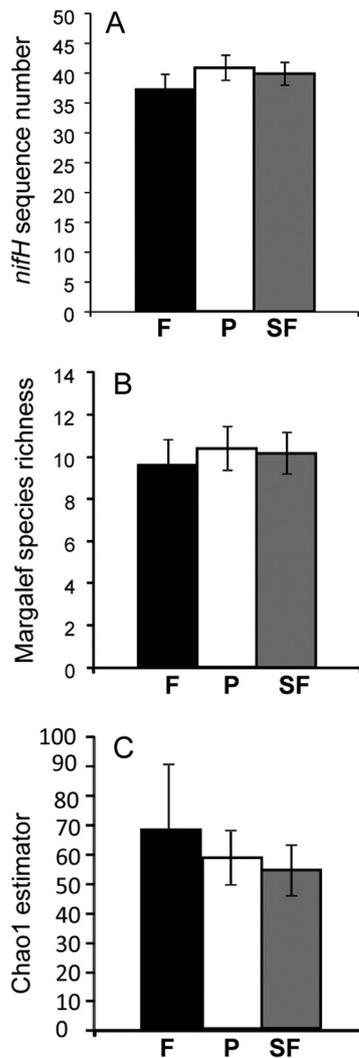
FIG 1 *nifH* gene diversity in response to land use change in the Amazon. (A) Number of unique genes; (B) Margalef species richness; (C) Chao1 estimate. Land use treatments are follows: F, primary forest (black bars); P, pasture (white bars); SF, secondary forest (gray bars). Error bars represent standard errors.

FIG 2 Nonmetric multidimensional scaling representation of the *nifH* gene sequence composition based on 97% DNA identity for three different land use systems in the Amazon: black triangles, primary forest samples (F); white circles, pasture samples (P); gray squares, secondary forest samples (SF). (A) Bray-Curtis similarity index; (B) Sørensen similarity index. The two-dimensional stress for each panel was 0.11.

curve was 0.99. The detection limit of the qPCR assay was ca. 100 copies/μl. Differences in the abundance of the *nifH* gene were assessed by single-factor ANOVA using the software package R, version 2.9.2 (www.R-project.org).

Nucleotide sequence accession numbers. All sequences confirmed as *nifH* gene sequences have been deposited in GenBank under accession no. JX865783 through JX866606.

RESULTS

OTU assignment and univariate analyses. A total of 825 *nifH* gene sequences were clustered based on different percent identity values (99, 97, 95, 90, and 80%), which resulted in 632, 466, 367, 262, and 80 OTU, respectively. We present the results below based on 97% identity, as different cutoff values yielded similar community compositional patterns for the same treatment plot.

The total number of unique *nifH* sequences, the Chao1 estimate, and the value for the Margalef's species richness index were not significantly different between forest and pasture sites (Fig. 1).
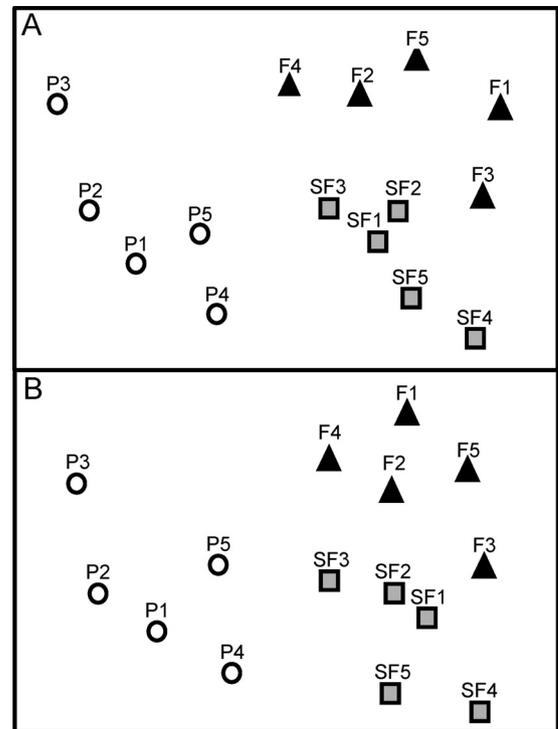
Rarefaction curve analysis indicates that the number of OTU did not reach an asymptote, which suggests that additional sequencing effort could capture more diversity (see Fig. S1 in the supplemental material).

Community composition and phylogenetic diversity. The diazotrophic community composition across different land uses was assessed by multivariate analyses of the *nifH* OTU. There was a distinct clustering of samples by land use with the Bray-Curtis index (Fig. 2A), and subsequent PerMANOVA analysis confirmed that the observed differences were statistically significant [$F_{(2, 12)} = 1.72$, $P = 0.001$]. Pairwise comparisons of diazotrophic communities between land uses indicated that they were significantly different ($P < 0.05$). To test whether these differences were associated with changes in composition only (as opposed to changes in relative abundance and composition), we calculated the Sørensen index using an OTU data set transformed to a presence/absence matrix. The NMDS ordination of the Sørensen index also showed a distinct clustering of the diazotrophic community across the three sites (Fig. 2B). These groups were significantly different from each other [$F_{(2, 12)} = 1.81$, $P = 0.001$], which was subsequently confirmed with pairwise *t* test comparisons. These results were consistent regardless of the selected percent similarity cutoff value for the OTU (i.e., OTU_{95}, OTU_{90}, or OTU_{80}) (see Fig. S2 in the supplemental material), although differences in community composition became nonsignificant between primary and secondary forests at higher taxonomic levels (see Fig. S2).
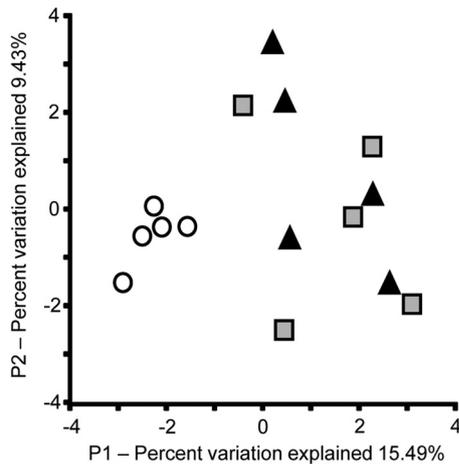
FIG 3 Principal coordinate analysis plot of phylogenetic similarities among *nifH* gene sequences from three different land use systems in the Amazon: black triangles, primary forest samples; white circles, pasture samples; gray squares, secondary forest samples. Plots were generated using unweighted UniFrac distances from a maximum likelihood-based analysis.

Diazotrophic communities from pastures were different from those recovered from forests. A principal coordinate analysis of Fast UniFrac distances resulted in separation between the pasture and forest sites (Fig. 3). Furthermore, the relatively tight clustering of all five replicates from pastures suggests that the diazotrophic communities within pastures were much more closely related than those in forest (Fig. 3).

Environmental attributes. Despite significant differences in the plant communities, all three different land use systems had similar values for 15 soil physicochemical characteristics (see Table S1 in the supplemental material). The total soil N concentration was slightly higher at the forest and secondary forest sites (140 mg/kg soil) than in the pasture (100 mg/kg soil), but these differences were not statistically significant. To test whether there was an association between the diazotrophic community and environmental factors, an individual regression analysis was performed between the Bray-Curtis similarity results and each environmental parameter. Only three factors—total soil N, C/N ratio, and Al + H (potential acidity)—showed a significant association with the diazotrophic community ($P < 0.001$, $R^2 = 0.367$).

OPU analyses. We tested whether the observed differences were maintained at the protein level and performed a phylogenetic analysis with translated amino acid sequences (44). We selected OPU comprising a minimum of 5 sequences, and a representative from each group was used for identification. The numbers of OPU were 19, 14, 2, and 1, for similarities of 99, 97, 95, and 90%, respectively. We used the 97% similarity cutoff value because it represented the majority of the sequences (85%; $n = 691$) observed in this study. The 14 different OPU groups used for constructing a maximum-likelihood phylogenetic tree (Fig. 4) yielded two distinct NifH clusters (I and III), corresponding to the two previously recognized NifH groups (55). No sequences belonging to clusters II, IV, and V were observed. The majority of the sequences (84%) belonged to NifH cluster I, and within this cluster, OPU1 and OPU5 (identified as belonging to *Proteobacteria*) were detected as predominant groups (90%) among the total

number of sequences (see Table S2 in the supplemental material). These OPU varied between the pasture and the two forest sites. For example, OPU1 (most closely related to the *Deltaproteobacteria*) was more commonly found at the pasture site (53% of the total sequences in that cluster were from pasture), whereas OPU5 (most closely related to *Alpha*- and *Betaproteobacteria*) was predominantly composed of sequences from primary and secondary forest sites (38% and 42%, respectively). Four OPU were only detected at the primary and secondary forest sites (OPU56, OPU4, OPU63, and OPU51; most closely related to *Paenibacillus*, *Nostoc*, and *Firmicutes*). NifH cluster III was also diverse and was represented by seven OPU. Three of these (OPU2, OPU9, and OPU72) were only observed at the primary and secondary forest sites. OPU7 and OPU9 (related to archaeal species in the genera *Methanocella* and *Methanosphaerula*) also varied between the forest and pasture sites. Three OPU (OPU2, OPU16, and OPU19) were most closely related to uncultured bacteria, suggesting that these groups could be novel diazotrophic taxa (see Table S2 in the supplemental material).

A pairwise comparative analysis among sites using translated sequences (grouped at 97% similarity) indicated that primary and secondary forests were not significantly different ($t = 0.74$, $P = 0.761$). However, the diazotrophic communities in both secondary and primary forests were significantly different from those observed in the pasture [PerMANOVA; $F_{(2, 12)} = 3.80$, $P = 0.006$]. We performed an indicator species analysis (SIMPER) to identify OPU associated with different land uses. Three OPU (OPU1, OPU5, and OPU16) contributed to 32.68% of the total dissimilarity detected between the primary forest and pasture. OPU1 and OPU16 were predominantly detected in pasture samples, whereas OPU5 was dominant in primary forest samples. Likewise, three OPU (OPU1, OPU5, and OPU51) were found to be important for discrimination of secondary forest and pasture sites, contributing to 42.09% of the total dissimilarity.

***nifH* gene abundance.** The number of copies of the *nifH* gene differed significantly at each site ($P < 0.05$), with pasture samples containing the highest values (mean of 2.2×10^5 copies g^{-1} dry soil), while the primary forest had the smallest number of copies of the *nifH* gene (2.1×10^4 copies g^{-1} dry soil) (Fig. 5; see Fig. S3 in the supplemental material). The secondary forest contained an intermediate number of the copies of the *nifH* gene (5.0×10^4 copies g^{-1} dry soil).

DISCUSSION

The *nifH* gene, encoding the reductase subunit of nitrogenase, has been widely used as a genetic marker to study the diversity and abundance of N$_2$-fixing microorganisms (55–57). Previous studies, based on the 16S rRNA gene, reported increases in richness and α diversity following deforestation and pasture establishment in the Amazon (33, 34). We observed a different response to deforestation for diazotrophic communities than that reported for the total bacterial community (31–34). In our study, while richness and diversity of diazotrophic communities remained similar among the three land uses (Fig. 1), the community composition and the *nifH* gene abundance were drastically altered with land use (Fig. 2A and B). To our knowledge, this work is the first to report that microbial communities associated with a particular ecosystem function, biological nitrogen fixation, are altered with Amazon deforestation.
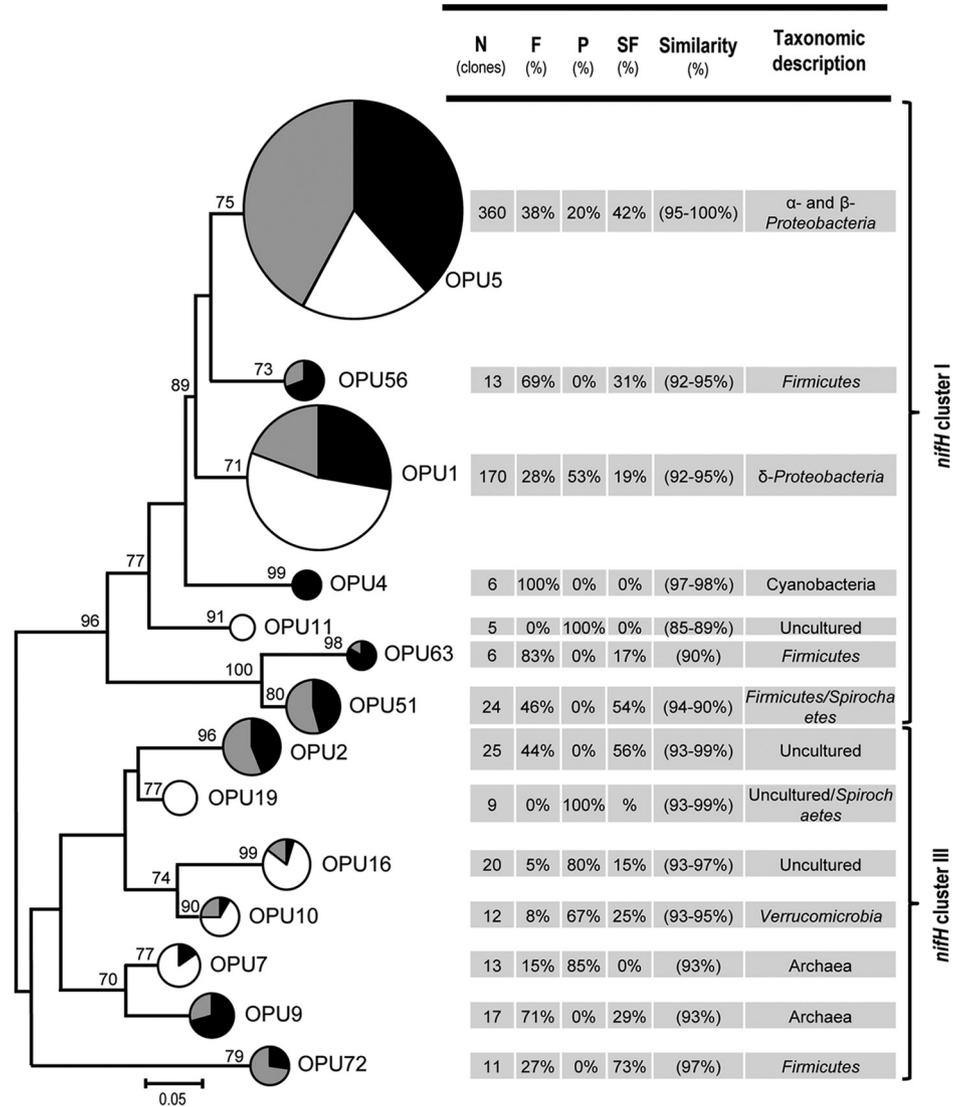
We postulate that the changes in composition and abun-

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	N (clones)	F (%)	P (%)	SF (%)	Similarity (%)	Taxonomic description	
OPU5	360	38%	20%	42%	(95-100%)	α- and β-Proteobacteria	*nifH* cluster I
OPU56	13	69%	0%	31%	(92-95%)	Firmicutes	
OPU1	170	28%	53%	19%	(92-95%)	δ-Proteobacteria	
OPU4	6	100%	0%	0%	(97-98%)	Cyanobacteria	
OPU11	5	0%	100%	0%	(85-89%)	Uncultured	
OPU63	6	83%	0%	17%	(90%)	Firmicutes	
OPU51	24	46%	0%	54%	(94-90%)	Firmicutes/Spirochaetes	
OPU2	25	44%	0%	56%	(93-99%)	Uncultured	*nifH* cluster III
OPU19	9	0%	100%	%	(93-99%)	Uncultured/Spirochaetes	
OPU16	20	5%	80%	15%	(93-97%)	Uncultured	
OPU10	12	8%	67%	25%	(93-95%)	Verrucomicrobia	
OPU7	13	15%	85%	0%	(93%)	Archaea	
OPU9	17	71%	0%	29%	(93%)	Archaea	
OPU72	11	27%	0%	73%	(97%)	Firmicutes	

Bootstrap values at nodes: 75, 89, 73, 71, 77, 99, 96, 91, 98, 100, 80, 96, 77, 74, 99, 90, 70, 77, 79

Scale bar: 0.05

FIG 4 Maximum likelihood phylogenetic tree based on translated amino acid sequences of the *nifH* gene from three different land use systems in the Amazon rainforest: black, primary forest (F); white, pasture (P); gray, secondary forest (SF). Only operational protein units (OPU) representing ≥5 sequences were used. The size of the circle is proportionate to the number of sequences within an OPU. Numbers at the nodes reflect bootstrap support values above 70% with branches within clusters being collapsed. The percent contribution of sequences from different land uses and percent similarities of translated *nifH* gene sequences to closely related NifH protein sequences are provided.
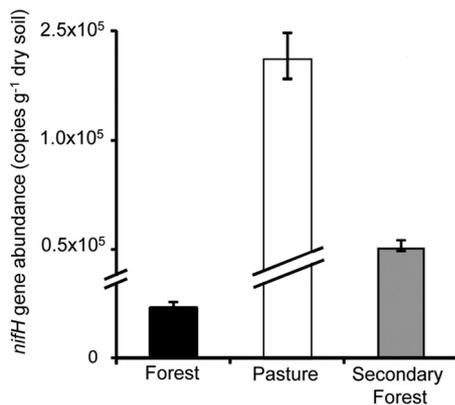

FIG 5 Copy numbers of the *nifH* gene per gram of dry soil determined for three different land use systems in the Amazon. Each bar represents the average of the 15 replicates (5 biological replicates × 3 technical replicates).

dance of diazotroph communities that we observed are a direct consequence of the plant composition observed in each land use. An increase in plant diversity is often associated with higher rates of N mineralization carried out by microorganisms and a greater net N supply (58, 59). Consistent with this idea, higher rates of N mineralization were observed in forests compared to pastures at our field site (28). Consequently, the forest demand for N is mainly filled (>80%) by the recycling of above- and belowground litter (60), with N_2 fixers providing for the remaining N requirements. In contrast, pastures are constantly N depleted due to grazing of aboveground biomass and rely heavily on free-living diazotrophs to fulfill the N requirements for supporting plant growth. The grass species *Urochloa brizantha* and *Panicum maximum* obtain up to 41% of their N through biological nitrogen fixation by allocating large quantities of easily degradable carbon sources as root exudates

(61). A combination of C-rich and N-depleted conditions in pastures may provide a competitive advantage for free-living diazotrophs. Therefore, we sought to identify soil physicochemical variables associated with differences in *nifH* gene sequences. Three soil variables, namely, N, C/N ratio, and potential acidity (Al + H), showed significant association values ($P < 0.001$) with land use. The decrease in soil N concentration and increase in C/N ratio are suggested drivers of environmental selection for N_2 fixers (19). The third variable, potential acidity, is related to pH, a well-established factor affecting the diversity of microbial communities (33, 62). In agreement with the above results, we observed a dramatic shift in *nifH* gene abundances with land use change. Future *in situ* estimates of biological nitrogen fixation (BNF) in the Amazon rainforest will help to improve nutrient flux models for this terrestrial ecosystem.

We investigated the possibility of diazotrophic community recovery after pasture abandonment and establishment of a secondary forest. Retrieved *nifH* sequences from both primary and secondary forests showed high levels of similarity at taxonomic and phylogenetic levels (Fig. 2 and 3). We also detected a decrease in *nifH* gene abundance in secondary forest samples relative to pasture samples, although gene abundance was still higher in secondary forests than in primary forests. Together, these results suggest that recovery of diazotrophic communities may be possible following pasture abandonment. This has important implications for restoration of degraded areas since 30 to 50% of cleared land in the Amazon is under secondary forest succession (63). At the phylogenetic level, the return of *nifH* gene sequences similar to those observed in primary forest suggests compositional restoration within 10 years or less after abandonment. At the functional level, the decrease in copy numbers of the *nifH* gene implies a less N-limited environment as secondary forests mature. We recognize that fewer gene copies may not translate into lower BNF rates and remain cautious about establishing a direct link; however, there is strong evidence suggesting that as plant diversity increases, the total N accumulated and recycled through the forest alleviates the need for external N input (64). A botanical survey at our research site showed that shrubs and woody plant species comprised 37% of the secondary forest plant community after 2 years of pasture abandonment (65), which may help to explain the resilience of the free-living diazotrophic community.

We performed an indicator species analysis to assess operational protein units contributing to the compositional differences we observed among land use types. The two distinct clusters observed in our study are typically found in soils: while cluster I is represented by aerobic N_2 fixers, cluster III consists of anaerobic diazotrophs from the domains *Bacteria* and *Archaea* (55). Most of the sequences were related to *Proteobacteria* (64.2%), the most abundant group in terrestrial ecosystems (57), and sequences in this group contributed greatly to the observed differences between the pasture and the two forest sites. For example, OPU5, the largest group of *nifH* sequences we detected, is related to *Alpha-* and *Betaproteobacteria*, and we found it predominantly in primary (38%) and secondary (42%) forests relative to pastures (20%). On the other hand, the proportion of the community comprised of OPU1 (related to the *Deltaproteobacteria*) increased from 28% to 53% with forest-to-pasture conversion (Fig. 4). Many OPU were observed only in forest samples, suggesting an impact on diazotrophic diversity with ecosystem conversion. The effects of the

loss of particular functional groups in tropical forests are not known, nor have they been extensively evaluated in other forest ecosystems, but they do raise concerns about the long-term stability of the biogeochemical process.

Noteworthy was the presence of sequences associated with *Archaea*. Our results indicate a clear shift, with OPU9 present only in forest sites, while 85% of sequences binned as OPU7 came from the pasture sample. OPU9 is related to *Methanoregula* and *Methanosphaerula*, two genera isolated from soils with pH below 5.5, while OPU7 belongs to *Methanocella*, a genus with representatives obtained from soils with pH 6.5 to 7.0 (Fig. 4). Despite the fact that the ability of methanogenic *Archaea* to fix N_2 has long been known (66, 67) and interconnections between the C and N biogeochemical cycles have been well established, how the processes of methanogenesis and BNF are related in nature remains virtually unknown. Because Fazenda Nova Vida pastures have been characterized as methane sources (68), one would expect that archaeal biological N_2 fixation plays an important role in the Amazon rainforest under threat of deforestation.

Conclusions. Land use changes in the Amazon rainforest result in a series of ecosystem alterations that include an increase in belowground productivity (32) and a more open N cycle (69), two ecological controls known to influence free-living diazotrophic communities (64). Our study found that diazotrophic communities in tropical terrestrial ecosystems can indeed respond to land use change, and we documented a shift in their community composition and abundance following forest-to-pasture conversion. Understanding the causes and consequences of these alterations may lead to refined models that incorporate molecular-level dynamics into ecosystem-level processes.

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