

1 **Metagenomic characterization of soil microbial communities in the Luquillo experimental**
2 **forest (Puerto Rico) and implications for nitrogen cycling**

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4 Smruthi Karthikeyan¹, Luis H. Orellana¹, Eric R. Johnston¹, Janet K. Hatt¹, Frank E. Löffler^{3,4},
5 Héctor L. Ayala-del-Río⁵, Grizelle González⁶, Konstantinos T Konstantinidis^{1,2*}

6

7 ¹ School of Civil and Environmental Engineering, Georgia Institute of Technology, Atlanta,
8 Georgia, USA

9 ² School of Biological Sciences, Georgia Institute of Technology, Atlanta, Georgia, USA

10 ³ Center for Environmental Biotechnology, Department of Microbiology, Department of Civil
11 and Environmental Engineering, Department of Biosystems Engineering and Soil Science,
12 University of Tennessee, Knoxville, Tennessee, USA

13 ⁴ Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA

14 ⁵ Department of Biology, University of Puerto Rico at Humacao, Puerto Rico, USA

15 ⁶ USDA Forest Service, International Institute of Tropical Forestry, Río Piedras, Puerto Rico,
16 USA

17

18 *Address correspondence to Konstantinos Konstantinidis, kostas@ce.gatech.edu

19 School of Civil & Environmental Engineering, Georgia Institute of Technology. 311 Ferst Drive,
20 ES&T Building, Room 3321, Atlanta, GA, 30332. Telephone: 404-639-4292

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23

24 **ABSTRACT**

25

26 The phylogenetic and functional diversity of microbial communities in tropical rainforests, and
27 how these differ from temperate communities remain poorly described but are directly related to
28 the increased fluxes of greenhouse gases such as nitrous oxide (N₂O) from the tropics. Towards
29 closing these knowledge gaps, we analyzed replicated shotgun metagenomes representing
30 distinct life zones from four locations in the Luquillo Experimental Forest (LEF), Puerto Rico.
31 These soils had a distinct microbial community composition and lower species diversity when
32 compared to temperate grasslands or agricultural soils. Unlike temperate soils, LEF soils showed
33 little stratification with depth in the first 0-30cm, with ~45% of community composition
34 differences explained solely by location. The relative abundances and nucleotide sequences of
35 N₂O reductases (*nosZ*) were highly similar between tropical forest and temperate soils. However,
36 respiratory NO reductase (*norB*) was 2-fold more abundant in the tropical soils, which might be
37 relatable to their greater N₂O emissions. Nitrogen fixation (*nifH*) also showed higher relative
38 abundance in rainforest compared to temperate soils (20% vs. 0.1-0.3% of bacterial genomes in
39 each soil type harbored the gene, respectively). Collectively, these results advance our
40 understanding of spatial diversity and metabolic repertoire of tropical rainforest soil
41 communities, and should facilitate future ecological modeling efforts.

42

43 **Importance:**

44 Tropical rainforests are the largest terrestrial sinks of atmospheric CO₂ and the largest natural
45 source of N₂O emissions, two critical greenhouse gases for the climate. The microbial
46 communities of rainforest soils that directly or indirectly, through affecting plant growth,

47 contribute to these fluxes remain poorly described by cultured-independent methods. To close
48 this knowledge gap, the present study applied shotgun metagenomics to samples selected from 3
49 distinct life zones within the Puerto Rico rainforest. The results advance our understanding of
50 microbial community diversity in rainforest soils and should facilitate future studies of natural or
51 manipulated perturbations of these critical ecosystems.

52

53 **INTRODUCTION**

54

55 Soil microbiomes are one of the most complex ecosystems owing to microenvironments
56 and steep physicochemical gradients, which can change on a micrometer or millimeter scale (1-
57 3). Temporal and spatial heterogeneity, demographic stochasticity, ecotype mixing, dispersion
58 and biotic interactions are the major drivers of soil microbial diversity in these ecosystems (4, 5).
59 The formation of such “metacommunities” coupled with biogeography and other edaphic factors
60 greatly influence the functional and taxonomic profile of a soil ecosystem at any given location
61 (6).

62 Tropical rainforests (“forests” hereafter) are characterized by humid and wet climate
63 patterns and account for a large portion of the world’s total forest cover (7). These forests have
64 high levels of primary productivity (~30% of the total global production) due to large amounts of
65 precipitation coupled with year-long warm temperatures and high levels of light (8).
66 Consequently, high levels of biodiversity are observed in these forest soils with unique microbial
67 genotypic signatures being exclusive to this habitat/location, along with only a few cosmopolitan
68 taxa that are shared with other (non-tropical forest) habitats (9, 10). Although tropical forest soils
69 are critical ecosystems that host a plethora of distinct ecological niches, little is known about the

70 metabolic potential of tropical soils, especially, across elevation and depth gradients. Describing
71 this metabolic diversity is important for studying and monitoring the microbial activities related
72 to greenhouse gas fluxes, namely, nitrous oxide (N₂O) and carbon dioxide (CO₂) from the
73 tropical soils (11).

74 Notably, tropical forests represent the largest terrestrial sinks of atmospheric CO₂ and the
75 largest natural source of N₂O emissions (12-15). Natural soils have been reported to contribute
76 over 43% of the total global N₂O emissions, with tropical ecosystems being the highest
77 contributors, having 2 to 4 times higher contributions compared to natural temperate ecosystems
78 (16-19). These soils are also responsible for about 70% of terrestrial nitrogen fixation, which
79 underlies, at least in part, their high rates of net primary productivity (11, 20).

80 Microbially-mediated nitrification and denitrification are the biotic processes contributing
81 the most to global N₂O soil emissions (60-70%) (19, 21, 22), although chemodenitrification, i.e.,
82 ferrous iron generated by ferric iron-reducing bacteria reacting with nitrite to produce N₂O
83 abiotically, is also likely high in iron-rich tropical soils (23). In soils, N₂O is biologically
84 produced as a result of incomplete nitrification, DNRA (dissimilatory nitrite reduction to
85 ammonium) or denitrification respiratory pathways (22, 24, 25). Respiratory nitric oxide
86 reductase (*nor*) is a key contributor to the microbial production of N₂O and is commonly
87 encoded in the genome of denitrifying bacteria as well as some ammonia-oxidizing organisms
88 (22, 26-30).

89 While both biotic and abiotic processes contribute to N₂O production, consumption of
90 N₂O is exclusively mediated by microbial N₂O reductase (NosZ) activity (31-34). Yet, whether
91 the denitrifying microorganisms in these soils differ from their counterparts in temperate soils
92 and, if their functional genes present in the community reflect the high nitrogen fluxes, remain

93 unanswered questions despite their apparent importance for better management and modeling of
94 tropical soil ecosystems. It has also been demonstrated that tropical forests have significantly
95 higher rates of nitrogen fixation (~70% of total terrestrial nitrogen fixation) compared to other
96 ecosystems, significantly affecting the nitrogen budgets in these ecosystems (3, 35-37). For
97 instance, higher rates of nitrogen fixation in soils have been linked to nitrous oxide emissions (N
98 loss) due to reduced N retention capacities (11, 38, 39). How these ecosystem rates translate to
99 the nitrogen-fixing microbial (sub)community diversity and gene potential remains unclear.

100 The Luquillo Experimental Forest (LEF), also known as the El Yunque National Forest in
101 Puerto Rico (PR), has been a long term ecological research (LTER) site since 1988. The site is
102 dedicated to the assessment of the effects of climate drivers on the biota and biogeochemistry.
103 The forest has been subjected to several disturbance regimes over the last few decades, mostly
104 natural and -to a smaller extent- anthropogenic such as tourism and experimental manipulations
105 (40, 41). This site encompasses distinct “life zones” characterized by sharp environmental
106 gradients even across small spatial scales (40, 42, 43). The broad life zones based on the
107 Holdridge classification system include the rain forest, wet forest, lower montane wet forest, and
108 lower montane rain forest. These life zones are distinguished by elevation, temperature and
109 rainfall patterns in addition to other edaphic factors (44-47). The elevation and rainfall patterns
110 also tend to influence oxygen availability, redox potential, nutrient uptake and organic
111 decomposition rates (44, 47, 48). The dynamic interplay of existing physicochemical gradients
112 and climatic factors gives rise to a complex mosaic of biodiversity patterns observed in this
113 forest (45). Hence, LEF represents an ideal environment to study tropical microbial community
114 diversity patterns and their impacts on carbon and nitrogen cycling. The four sampling sites of
115 this study were chosen to represent the distinct vegetation and life zones within the LEF.

116 Previous studies in the LEF, and similar forest regions, have mostly focused on the
117 effects of redox dynamics, litter decomposition, nitrogen (N) and other nutrient fertilization on
118 microbial community activity through enzyme assays. Few studies have examined microbial
119 diversity patterns across an elevation gradient and were only based on low-resolution techniques
120 such as terminal restriction fragment length polymorphism analysis (14, 49-53). Furthermore,
121 studies linking marker-gene abundances (related to nitrogen cycling) with *in-situ* flux
122 measurements showed very high N₂O fluxes in the forest soils (54). However, the *nosZ* primers
123 targeted only the typical (Clade I) clades, thereby introducing a primer bias, which can be
124 circumvented by employing metagenomic analyses.

125 With recent developments in next generation DNA sequencing and associated
126 bioinformatics binning algorithms, near-complete metagenome-assembled genomes (MAGs) can
127 been recovered without cultivation (55, 56), opening new windows into studying soil microbial
128 communities. Here, shotgun metagenomes originating from soils from the four different
129 locations/life zones and three different depths in the LEF were analyzed to describe the microbial
130 community diversity, biogeographical patterns, and metabolic potential differences across
131 samples. Furthermore, the metagenomic data obtained from these soils were also compared to
132 similar data from temperate grasslands in Oklahoma (OK) (57) and agricultural soils from
133 Illinois (IL), USA (56) obtained previously by our team. By analyzing near-complete MAGs, we
134 show that the most abundant microbial population (based on number of reads recruited) at each
135 of the sampling locations represent sequence-discrete populations, similar to those observed in
136 other habitats (58). Using such sequence-discrete populations as the fundamental unit of
137 microbial communities, we subsequently assess the population distribution at high resolution

138 across the sampling sites (biogeography) and the gene content they encoded, with a focus on
139 nitrogen metabolism.

140

141 **RESULTS**

142

143 **Diversity of forest microbial communities**

144 The LEF soil communities were compared to those of intensively studied ecosystems,
145 namely the Oklahoma temperate grassland (OK) (57, 59) and Illinois agricultural soils (IL) (56),
146 which were previously characterized with similar shotgun metagenomics approaches. Shotgun
147 metagenomic sequencing recovered a total of 370 million reads across the 4 sites (Suppl. Table
148 S2). Nonpareil 2.0 (60) was used to estimate sequence coverage, i.e., what fraction of the total
149 extracted community DNA was sequenced. Nonpareil analysis of community diversity (Suppl.
150 Fig. S1) showed that the agricultural Urbana (IL) site had the highest diversity of all the soils
151 compared (NP diversity 24.02; note that NP values are given in log scale), and consequently, the
152 lowest sequence coverage at (only) 37.23%. El Verde and Pico del Este (20-30cm) were the least
153 diverse or most completely sequenced with 87.1% and 73.4% coverage respectively (NP
154 diversity of 19.6 and 20.6 respectively or about 2-3 orders of magnitude less diverse). Overall,
155 OK and IL soils appear to be more diverse than the PR soils by about two orders of magnitude,
156 on average, with an average Nonpareil value of 22.75 ± 0.37 . Nearly complete coverage for El
157 Verde and Pico del Este (20-30cm samples) would require 2.402×10^9 bp and 8.735×10^9 bp,
158 respectively, and, for the same level of coverage, the more complex communities in Urbana (IL)
159 would require a substantially higher sequencing effort of 1.282×10^{12} bp. The OK soils had an
160 estimated sequencing depth of $2.063 \times 10^{11} \pm 1.436 \times 10^{11}$ bp.

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Community composition variation across the forest sites based on 16S rRNA gene sequences.

The number of total 16S-rRNA gene-based OTUs (Operational Taxonomic Unit) observed in each metagenome as well as the Chao1 estimate of total OTUs present reflected the degree of undersampling at each site (Suppl. Fig. S1 and S2), and were also consistent with the Nonpareil coverage estimates (Fig. 1). When Puerto Rico tropical soils (PR) were compared with the agricultural and grassland soils from the United States at the phylum level, *Proteobacteria*, *Acidobacteria* and *Actinobacteria* were the most abundant taxa across all ecosystems. However, in the forest soils, a few highly abundant OTUs dominated the entire soil community whereas in the OK and IL soils, OTUs were more evenly distributed (Suppl. Fig. S2), consistent with the Nonpareil diversity results. Only 1.28% of the total detected OTUs (out of a total 8019, non-singleton OTUs) were shared among all PR samples, while 49.95% of OTUs were exclusive to a particular sampling site in PR, reflecting partly the under-sampling of the extant diversity by sequencing. Only 0.37% of the OTUs (out of a total 13760, non-singleton OTUs) were shared among all the sites across all 3 ecosystems, all of which were assignable to *Alphaproteobacteria*, *Acidobacteria*, *Verrucomicrobia* and *Actinobacteria*.

Further, applying four additional DNA extraction methods on a selected subset of our samples, including two manual phenol chloroform-based methods that are often advantageous for iron rich soils like those in tropical forest, revealed similar levels of diversity, more or less (Suppl.Fig. S3). Hence, the diversity patterns reported here are robust and independent of the DNA method used.

184 **Factors driving community diversity in the forest soils: Multidimensional scaling analysis**
185 **of beta diversity**

186 The PCoA (Principal Coordinate Analysis) plots, constructed based on the MASH
187 distances among whole metagenomes, showed a clustering pattern that was primarily governed
188 by site/location. Accordingly, site explained 45.22% of the total diversity (Fig. 2B). The non-
189 metric multidimensional scaling (NMDS) analysis of the data revealed only site, pH and soil
190 moisture to be statistically significant physicochemical parameters in explaining the observed
191 community diversity (Fig. 2C, Suppl. Table S3). ANOSIM values also indicated site to be a
192 more important factor than depth, with a P value of 0.001 and 0.94, respectively. Based on the
193 distance-based redundancy analysis (dbRDA), site was the most significant factor, even when the
194 interplay between site and sampling depth was accounted for (Suppl. Table S4). Table 1 shows
195 the partitioning of the variance between the proportion that is explained by constrained axes (i.e.,
196 environmental variables measured) and the porportion explained by unconstrained axes (i.e.,
197 variance not explained by environmental variables measured). The total variance explained by all
198 (measured) environmental variables was 80.2% (Table. 1), which is remarkably high for a soil
199 ecosystem (61).

200

201 **Major N cycling pathways**

202 Genes encoding proteins involved in denitrification and nitrogen fixation were the most
203 abundant nitrogen (N) cycling pathway genes detected at different sites. Overall, the forest soils
204 harbored about a 2-3-fold higher abundance of denitrification genes, i.e., *narG*, *nirK*, and *norB*
205 catalyzing the reduction of nitrate, nitrite, and nitric oxide, respectively, compared to the
206 grassland and agricultural soils (Fig. 2A). For instance, the *norB* gene abundance was found to

207 be at the highest abundance among the denitrification genes, with ~37% (SD 9.5%) of the
208 genomes in the PR soils predicted to contain a *norB* gene, compared to ~17% (SD 4%) and
209 ~14% (SD 1.3%) at IL and OK, respectively. Similarly, *narG* showed a 3-fold higher abundance
210 in the PR soils compared to IL and OK soils (Fig.2B). While denitrification gene abundances
211 appeared higher in the tropical soils, the relative abundance of *nosZ* gene, i.e., 11.6% (SD 3%) of
212 the total genomes across the four locations in the LEF were predicted to encode *nosZ*, similar to
213 *nosZ* relative abundance in IL and OK soils, i.e., 11.75% (SD 5%) and 11.08% (SD 3%),
214 respectively (not statistically significant at $p=0.05$). Similar to *nosZ*, DNRA gene abundances
215 (namely, *nrfA*) was similar across all sites studied herein (9%, SD 1.9%).

216

217 **Predominant NosZ clades are shared among soil ecosystems**

218 Placing *nosZ*-encoding reads to a reference *nosZ* phylogenetic tree revealed that atypical
219 clades (clade II *nosZ*), affiliated predominantly with *Opitutus*, *Anaeromyxobacter* and other
220 closely related genera, dominated the *nosZ* gene pool in the tropical forests (Figs. 3, Suppl.
221 Figs.S4-S7). In contrast, a very small fraction of reads (<10% of total *nosZ* reads) were recruited
222 to typical *nosZ* clades (or clade I). Members belonging to the clade II *nosZ* dominated the *nosZ*
223 gene pool in OK and IL soils as well, with IL agricultural soils showing the greatest *nosZ*
224 sequence diversity among the three regions. Notably, *O. terrae*-affiliated sequences represented
225 the most abundant sub-clade (*nosZ* OTUs/sub-clades were defined at the 95% nucleotide
226 sequence identity level) in all regions. Furthermore, most of the *O. terrae*-affiliated reads in the
227 forest soil dataset appeared to be assigned to a single sub-clade, while their counterparts in the
228 OK and IL soils appeared to be more evenly distributed among several closely related *nosZ* sub-
229 clades, i.e., showing higher sequence diversity (Fig. 3, Suppl. Figs. S4-S7). *O. terrae* (strain

230 DSM 11246/PB90-1) *nosZ* reads at >95% identity made up between 20% and 60% of the total
231 *nosZ* reads recovered from the El Verde site and, together with the second most abundant sub-
232 clade from *Anaeromyxobacter* sp., contributed over 30% of the total *nosZ* reads across all four
233 PR locations (Fig. 5). Despite the significant taxonomic diversity observed in these soils (Suppl
234 Fig. S2), the soils from PR shared several abundant *nosZ* gene sequences/sub-clades at >95
235 nucleotide identity with soils in OK and IL (Fig. 3). Furthermore, in order to compare the
236 predominant *nosZ* clades across the samples shown here, a new phylogenetic reference tree was
237 constructed based on almost full length sequences obtained from the assemblies/MAGs obtained
238 from the metagenomes studied here (namely PR,OK,IL). The short-reads identified as *nosZ* from
239 the PR soils were placed on this tree and show that the majority of these reads are recruited by
240 the *nosZ* sequences obtained from these assemblies/MAGs, indicating that the *nosZ* sequences
241 across these ecosystems studies here are similar (Suppl. Fig. S8)

242

243 **Nitrogen fixation potential**

244 The nitrogen fixation genes (mainly *nifH*) were present at a much lower abundance in the
245 lower altitude forest samples. For instance, only ~1-3% of all genomes in the lower altitude
246 samples were predicted to encode *nifH* compared to a ~20% of the genomes in the higher
247 elevation samples (Pico del Este) (Fig. 2A), and almost none of the reads from IL and OK
248 metagenomes appeared to encode *nifH* (<0.1%). Therefore, nitrogen fixation gene abundance
249 patterns indicated a much stronger selection for nitrogen fixation in the tropical forest relative to
250 temperate agricultural or natural prairie soils, especially at higher elevations. Furthermore, no
251 ammonia oxidizing genes (*amoA*) were detected in any of the soils except for Urbana soils (IL),
252 which had a history of fertilizer (N) input.

253

254 **Recovery of metagenome-assembled genomes (MAGs) representative of each site**

255 In order to test the effect of biogeography (i.e., limits to dispersion) of taxa across the
256 elevation gradient sampled, the distribution of abundant MAGs recovered from each PR
257 sampling site (assembly and MAG statistics provided in Suppl. Table S6) were assessed across
258 the sites using read-recruitment plots (62). Taxonomic assignment using the Microbial Genomes
259 Atlas (63) revealed that the most abundant MAG at site El Verde (lowest elevation), representing
260 4.39% of the total metagenome, and was affiliated with an unclassified *Verrucomicrobia*. The
261 second most abundant (1.8% of total) was likely a member of the genus *Ca. Koribacter*
262 (*Acidobacteria*) followed by an unclassified member of *Acidobacteria* (1.45% of total). The
263 *Verrucomicrobium* MAG was found at an abundance of 1.03% of the total population at Sabana,
264 and at 0.07% and 0.03% in Palm Nido and Pico del Este (highest elevation), respectively.
265 Uneven coverage across the length of the reference sequence and nucleotide sequence identities
266 were observed in the recruitment of short-reads from Palm Nido and Pico del Este as well as
267 with all OK datasets, indicating that the related populations in the latter samples were divergent
268 from the reference MAG (Suppl. Fig. S10). Therefore, at least this abundant low-elevation
269 *Verrucomicrobial* population did not appear to be widespread in the other samples analyzed here
270 (Suppl. Fig. S10). Similarly, the other abundant MAGs from other sites in the forest soils were
271 unique to the corresponding sites (elevation) from which they were recovered. Almost all MAGs
272 used in the analyses were assignable to a novel family, if not higher taxonomic rank, according
273 to MiGA analysis (when compared to 11,566 classified isolate genomes available in the NCBI
274 prokaryotic genome database), underscoring the large unexplored diversity harbored by the PR
275 tropical rainforest soils. The sequence diversity/complexity as well as sequencing depth limited
276 large-scale recovery of high-quality MAGS.

277

278 **Functional gene content of the MAGs**

279 The genome sequences of the most abundant MAGs from each location (n=6) were
280 analyzed in more detail to assess the functions they encoded, especially with respect to N cycling
281 pathways (Fig. 4). MAGs from Pico del Este (highest elevation) showed a high abundance of N
282 metabolism related genes compared to MAGs from other sites (Fig. 4). Most notably, genes
283 related to nitrogen fixation were found only in the Pico del Este MAG, which was consistent
284 with the short read analysis datasets showing greater relative abundance of *nifH* at this site.
285 Nitrification (ammonia oxidation related genes) gene clusters were not detected in any of the
286 recovered MAGs. *norB* and *nosZ* genes were found in three out of the six abundant MAGs
287 analyzed. The most abundant El Verde MAG, most closely related to *O. terrae* (AAI = 40 %),
288 possessed a *nosZ* gene, which was congruent with the *nosZ* phylogeny described above (i.e.,
289 ~60% of the *nosZ*-encoded reads from El Verde had a closest match to *O. terrae nosZ*
290 sequences).

291

292 **DISCUSSION**

293

294 The present study reported the taxonomic and gene content diversity of the poorly
295 characterized tropical rainforest soils by using whole-community, shotgun metagenomic
296 sequencing of samples from the El Yunque forest, Puerto Rico. The recovered near-complete
297 MAGs represented several abundant and widespread organisms within this ecosystem that could
298 serve as model organisms for future studies. Furthermore, since the Luquillo Experimental Forest
299 (LEF) within El Yunque is subjected to varying natural as well as experimental (e.g., warming,

300 phosphorus fertilization) perturbations, our study could also provide a baseline for these
301 perturbations and future soil microbial studies at LEF. Our results revealed that the LEF soils
302 harbor distinct microbial communities at sites with distinct elevation from sea-level. In contrast,
303 and unlike several other soil ecosystems, sampling depth did not have a substantial impact on
304 structuring community diversity, revealing no depth stratification in the LEF soils, at least for the
305 depths sampled here (5-30cm). This could be due to the lack of distinct soil horizons within the
306 first 30cm of the sampling sites, and indicates that the soil formation and/or physicochemical
307 properties in these ecosystems could differ markedly from those in their temperate counterparts
308 (44).

309 A recent study examining the dominant bacterial phylotypes across the globe found that
310 the predominant phylotypes were widespread across ecosystems. The only exception to this
311 pattern was the forest tropical soils which harbor distinct phylotypes (10). Consistent with these
312 conclusions, the MAGs recovered from each LEF site represented at least novel species and
313 genera, further underlining the under-tapped microbial diversity harbored by tropical forest soils.
314 Currently, the environmental factors driving these diversity patterns remain poorly understood
315 for tropical forest soils (10), but our study provided several new insights into this issue.

316 In particular, sites El Verde and Sabana (lowest elevation sites) had similar community
317 structure and diversity compared to the two higher-elevation sampling sites with certain MAGs
318 being present at both sites but not in any of the other (higher-elevation) sites examined. This is
319 presumably attributable to both sites having similar climate and vegetation patterns (i.e.,
320 Tabonuco forest). On the other hand, Pico del Este was the highest elevation site and experiences
321 almost continuous cloud cover as well as horizontal precipitation. The unique topology of Pico
322 del Este was reflected in distinct and deeply novel MAGs and gene content, which differed

323 markedly from the other three sampling sites within the LEF (PCoA plots, Fig. 2B). The high
324 water content of the Pico del Este soils gives rise to a unique ecosystem dominated by epiphytes
325 (e.g., moss) (64). The epiphytic community has presumably significant impacts on nutrient (e.g.,
326 nitrogen) cycling (65), and influences the water input to the soil, thereby shaping a unique
327 habitat/niche for the soil microbes. Free-living microbes have been shown to be one of the
328 highest contributors to biological N fixation in these forests with high rates of nitrogenase
329 activity associated with the presence of moss/epiphytes (53, 66). Consistent with these previous
330 results and interpretations, the Pico del Este showed an extremely high potential for nitrogen
331 fixation, i.e., it was estimated that 1/5 of the total bacterial genomes sampled possessed genes for
332 N fixation, which is at least 10 times greater than any other site evaluated herein. Accordingly,
333 we found that site (location) alone explained about half (45%) of the beta diversity differences
334 observed among the four sampling sites, which reached ~80% when a few physicochemical
335 parameters namely pH and moisture were also included in the analyses (Fig. 2B, Table 1). This
336 is a remarkably high fraction of beta diversity explained by measured parameters for a soil
337 ecosystem (61) and likely reflected that location and the physical properties that characterized
338 different locations within LEF structured diversity much stronger than in other soil ecosystems.
339 Tropical forests have also been shown to have significantly higher rates of nitrogen fixation
340 compared to other ecosystems, which can exceed the N retention capacity of the soil resulting in
341 large N loss as N₂O (67). The findings reported here on denitrification gene abundances were
342 generally consistent with these previous observations as well.

343 Links between soil community structure and nitrogen cycling can help close the
344 knowledge gaps on how the forest ecosystems impact the release and mitigation of certain highly
345 potent greenhouse gases such as N₂O. The gene abundances observed here, e.g., more than two-

346 fold higher abundance of *norB* (associated with NO reduction to N₂O) and similar *nosZ* (N₂O
347 consumption) abundances in tropical soils relative to temperate soils were consistent with higher
348 N₂O emissions observed previously from the tropics. Further, in acidic soils such as the tropical
349 forest soils evaluated in this study, lack of N limitation can suppress complete denitrification,
350 thereby leading to higher N₂O release compared to other soil ecosystems (35). These
351 interpretations were consistent with our observation that the PR soils harbored a relatively high
352 abundance of respiratory (related to denitrification) *norB* genes as well. Previous studies have
353 also suggested that most denitrifying bacterial genomes possess the genes required to reduce
354 nitrate to nitrous oxide but do not possess the gene responsible for the last step i.e., N₂O
355 reduction to N₂, leading to the release of N₂O gas (Braker and Tiedje, 2003; Richardson et al.,
356 2009; Giles et al., 2012; (22, 26-29), consistent with the findings of our study.

357 It has been established that tropical forest soils are the single highest contributor of
358 natural N₂O emissions. While several abiotic and microbial processes can contribute to soil N₂O,
359 N₂O consumption is an exclusively microbial process, catalyzed by the enzyme product of the
360 *nosZ* genes (34). Based on the assessment of the *nosZ* gene phylogeny, it appears that almost all
361 of the *nosZ* genes from the tropical forest soils studied here belong to a previously overlooked
362 Clade II or atypical *nosZ* genes (32, 34, 68). This clade consists mainly of non-denitrifying, and
363 secondary denitrifying N₂O reducers. Despite the unique phylogenetic diversity harbored by
364 tropical soils in general, the *nosZ* gene sequence diversity appears to be shared between
365 temperate and agricultural soils (Fig 4). These findings imply strong selection pressure for
366 conservation of nitrous oxide reductase sequences across tropical and temperate soil ecosystems
367 that are not apparently applicable to other N-cycling genes and pathways, which warrants further
368 attention in the future.

369 Integration of functional (e.g., gene expression) data with *in-situ* rate measurements will
370 provide a more complete picture of the composition and functioning in tropical forest soils. The
371 identification of certain biomarker genes such as *nosZ* sequences in our study could facilitate
372 future investigations on biogeochemical N-cycling and greenhouse gas emissions. For instance,
373 the assembled MAGs and gene sequences provided here could be useful for the design of
374 specific PCR assays for assessing transcript levels (activity), allowing potential linking of carbon
375 dioxide, methane, nitrogen, SOM, etc. turnover to the activity of individual populations. It would
376 also be interesting to assess how the findings reported here for the LEF apply (or not) to other
377 tropical forests especially because our study is based on a relative small sample size. While the
378 diversity in the Puerto Rico soils appears to be lower than that in temperate grassland and
379 agricultural soils, and different DNA extraction methods, including phenol-chloroform- and kit-
380 based, provided for similar results (Fig. S3), it is important to note that DNA of the temperate
381 soil samples was extracted using different methods (OK soils were extracted using the PowerSoil
382 kit). Therefore, it would be important to confirm these preliminary findings by using the exact
383 same DNA extraction and sequencing procedures in all soils. Despite the sample size, however,
384 our results showed differences along the elevation gradient sampled at the LEF that are
385 independent of DNA extraction (Suppl.Fig. S3) or sequencing methods, and consistent with our
386 metadata (Fig. 2), and previous process rate measurements. As the gradients at the LEF also
387 provide a natural setting to interpret the potential ramifications of climate change scenarios such
388 as altered participation patterns, the DNA sequences provided here could facilitate future
389 manipulation experiments with an emphasis on understanding and predicting the effects of
390 climate change on microbial community dynamics along the elevation gradient.
391

392 MATERIALS AND METHODS

393

394 Sampling sites

395 Soil samples were collected on February 2016 from four locations/sites across the LEF (18.3' N,
396 65.80' W). The four sites namely, Sabana, El Verde field station, Palm Nido and Pico del Este,
397 each located at different elevations from the mean sea level, i.e., 265, 434, 634 and 953 m,
398 respectively, were chosen due to their unique landscape and rainfall patterns, thereby creating
399 distinct ecological niches (Fig. 2A).

400 The El Yunque forest is categorized into four distinct vegetation zones namely, the
401 Tabonuco, Palo Colorado, Sierra Palm and Dwarf/Elfin forests. Site Sabana and El Verde, which
402 are located at the lowest elevation among the four sites within the LEF, fall under the Tabonuco
403 forest category in terms of vegetation, dominated by the tree species *Dacryodes excelsa* (native
404 to Puerto Rico). They are characterized by canopy cover and low light intensities at the ground
405 level which account for the sparsely vegetated forest floor. However, these sites still harbor the
406 richest flora of all sites (69). Palm Nido is characterized by unstable, wetter soils, steeper slopes
407 and the vegetation is dominated by the Sierra Palm (*Prestoea montana*). The site at the highest
408 elevation, Pico del Este (dwarf forest ecosystem or “elfin woodlands”) is characterized by higher
409 winds, lower temperatures and the vegetation is enveloped by clouds (41, 70) and its main
410 vegetation is comprised of moss and epiphytes. Furthermore, highly acidic soil and continuously
411 water-saturated soils deficient in oxygen are some major characteristics of this ecosystem with
412 most mineral inputs for plants become dissolved in the rain and fog.

413 Three adjacent soil profiles were taken from each of the four LEF sites (4 sites
414 encompassing 3 lifezones, Palo Colorado was not sampled). For each profile, individual soil

415 cores were taken at each depth (0-5cm, 5-20cm, 20-30cm) using a 3-cm diameter x 15-cm length
416 soil corer (AMS Inc, Idaho) that was decontaminated between samplings by washing with 70%
417 ethanol. Soil samples were stored in sterile Whirl-pak bags and kept on ice during transport and
418 until storage at -80° C. The three cores at each sampling depth were pooled together for
419 community DNA extraction, producing a total of twelve samples across the four sites.

420 Soil pH was determined using an automated LabFit AS-3000 pH Analyzer, and soil
421 extractable P, K, Ca, Mg, Mn, and Zn were extracted using the Mehlich-1 method and measured
422 using an inductively coupled plasma spectrograph at the University of Georgia Agricultural and
423 Environmental Services Laboratories (Athens, GA, USA). Soil extractable P using this method is
424 interpreted as the bioavailable fraction of P. NH₄-N and NO₃-N were measured by first
425 extracting them from soil samples with 0.1 N KCl, followed by the colorimetric phenate method
426 for NH₄⁺ and the cadmium reduction method NO₃. The physicochemical conditions at the sites
427 during the time of sampling are provided in Supplementary Table (S1).

428

429 **Community DNA extraction and sequencing**

430 Total DNA from soil was extracted using the FastDNA SPIN KIT (MP Biomedicals, Solon, OH)
431 following manufacturer's procedure with the following modifications (71). Soils were air dried
432 under aseptic conditions followed by grinding employing a mortar and pestle. Cells were lysed
433 by bead beating and DNA was eluted in 50 µl of sterile H₂O. DNA sequencing libraries were
434 prepared using the Illumina Nextera XT DNA library prep kit according to manufacturer's
435 instructions except the protocol was terminated after isolation of cleaned double stranded
436 libraries. Library concentrations were determined by fluorescent quantification using a Qubit HS
437 DNA kit and Qubit 2.0 fluorometer (ThermoFisher Scientific), and samples were run on a High

438 Sensitivity DNA chip using the Bioanalyzer 2100 instrument (Agilent) to determine library insert
439 sizes. An equimolar pool of the sequencing libraries was sequenced on an Illumina HiSeq 2500
440 instrument (located in the School of Biological Sciences, Georgia Institute of Technology) using
441 the HiSeq Rapid PE Cluster Kit v2 and HiSeq Rapid SBS Kit v2 (Illumina) for 300 cycles (2 x
442 150 bp paired end). Adapter trimming and demultiplexing of sequenced samples was carried out
443 by the HiSeq instrument. In total, 12 metagenomic datasets were generated (3 per site for the
444 three depths), and statistic details on each dataset are provided in Supplementary Table S2.

445 In order to test for any DNA extraction biases of the kit used above, especially for the
446 high iron/clay content that characterizes tropical forest soils and is known to affect the extraction
447 step, four additional DNA extraction methods were performed in parallel on a small subset of
448 samples collected in 2018 from the same sites (6 samples per extraction method for 5 extraction
449 methods covering the 4 sites). The methods included two manual (as opposed to kit-based)
450 phenol-chloroform based methods (72, 73) as well as two other kit-based methods namely;
451 DNeasy PowerSoil and DNeasy PowerSoil Pro (Qiagen Inc.). For this evaluation, the soils were
452 first homogenized and subsequently in five subsamples to use with each method (including the
453 FastDNA SPIN KIT-based method mentioned above). The libraries were constructed and
454 sequenced the same way as described above for the FastDNA SPIN KIT method.

455 All metagenomic datasets were deposited in the European Nucleotide Archive (ENA) under
456 project PRJEB26500. Additional data is available at <http://enve-omics.ce.gatech.edu/data/prsoils>.

457

458 **Bioinformatics analysis of metagenomic reads and MAGs**

459 The paired end reads were trimmed and quality checked using the SolexaQA (74) package with a
460 cutoff of $Q > 20$ ($\geq 99\%$ accuracy per base-position) and a minimum trimmed length of 50 bp.

461 *i) Assembly and population genome binning*: Co-assembly of the short reads from the same
462 location was performed using IDBA-UD (75) and only resulting contigs longer than 500 bp in
463 length were used for downstream analysis (e.g. functional annotation and MyTaxa
464 classification). Genes were predicted on the co-assembled contigs using MetaGeneMark (76) and
465 the predicted protein-coding regions were searched against the NCBI All Genome database using
466 Blastp (77). Since the assembly of individual datasets resulted mostly in short contigs (data not
467 shown), the contigs from the co-assembly (combining metagenomes from the three sampling
468 depths, for each site) were used for population genome binning. Contigs longer than 1Kbp were
469 binned using MaxBin (78) to recover individual MAGs (default settings). The resulting bins
470 were quality checked for contamination and completeness using CheckM (79), and were further
471 evaluated for their intra-population diversity and sequence discreteness using fragment
472 recruitment analysis scripts as part of the Enveomics collection (62) essentially as previously
473 described (80).

474 *ii) Functional annotation of MAGs*: Genes were predicted for each MAG using MetaGeneMark
475 and the predicted protein-coding regions were searched against the curated Swiss-Prot (81)
476 protein database using Blastp (77). Matches with a bitscore higher than 60 or amino acid identity
477 higher than 40% were used in subsequent analysis. The Swiss-Prot database identifiers were
478 mapped to their corresponding metabolic function based on the hierarchical classification
479 subsystems of the SEED subsystem category (Level 1) (82). The relative abundance of genes
480 mapping to each function was calculated based on the number of predicted genes from each
481 MAG assigned to the function (for read-based assessment, see below). Relative abundance data
482 were plotted in R using the “superheat” package (<https://arxiv.org/abs/1512.01524>). Individual
483 biomarker genes for each step of the nitrogen cycling pathway were manually verified by

484 visually checking the alignment of the identified sequences by the pipeline outlined above
485 against verified reference sequences.

486 *iii) Functional annotation of short reads*: Protein-coding sequences present in short reads were
487 predicted using FragGeneScan (83) using the 1% Illumina error model. The predicted genes were
488 then searched against the Swiss-Prot database using Blastp (best match). Low quality matches
489 (bitscore < 60) were excluded, and relative abundance of genes mapping to each function was
490 determined as described in the previous section.

491

492 **Community diversity estimation**

493 *i) Nonpareil*: Nonpareil (60) was used to estimate sequence coverage, i.e., what fraction of the
494 total extracted community DNA was sequenced and predict the sequencing effort required to
495 achieve "nearly complete coverage"($\geq 95\%$). The default parameters in Nonpareil were used for
496 all datasets. Only one of the two paired reads (forward) for each dataset was used to avoid
497 dependency of the paired reads, which can bias Nonpareil estimates (60).

498 *ii) MASH and multidimensional scaling*: MASH, a tool employing the MinHash dimensionality
499 reduction technique to compare sample-to-sample sequence composition based on k-mers (84),
500 was used to compute pairwise distances between whole metagenomic datasets and construct the
501 distance matrix to be used in multidimensional scaling. Pairwise MASH distances between the
502 metagenomic datasets were computed from the size-reduced sketches (default parameters).

503 PCoA (Principal coordinate analysis) and NMDS (Non-metric multidimensional scaling) were
504 employed to visualize the distance matrix and evaluate the physicochemical parameters driving
505 community diversity, respectively. Furthermore, dbRDA (distance based redundancy analysis),

506 was used to obtain a finer resolution on the observed compositional variation. All of the above
507 startistical analysis were performed using the vegan package in R (85), with default settings.
508 *iii) 16S rRNA gene fragments recovered from shotgun metagenomes:* 16S ribosomal rRNA (16S)
509 gene fragments were extracted from the metagenomic datasets using Parallel-META (86). 16S-
510 carrying reads were classified taxonomically using the GreenGenes database.
511 Recovered 16S fragments were clustered ('closed-reference OTU picking' strategy using
512 UCLUST (87)) and taxonomically classified based on their best match in the GreenGenes
513 database (88) at an ID \geq 97% in QIIME (89, 90). The relative abundance of the OTUs were
514 calculated based on the number of reads assigned to each OTU. Community composition was
515 assessed based on OTU taxonomic assignments at the genus and the phylum ranks and was
516 compared between the sites based on the relative abundance of OTUs at each site.

517

518 **Identification of N cycling genes using ROcker**

519 ROcker (91) was employed for a precise identification and quantification of *nosZ* (encoding
520 nitrous oxide reductase), *norB* (encoding respiratory nitric oxide reductase, cytochrome bc
521 complex associated), *nirK* (encoding nitrite reductase), *narG* (encoding nitrate reductase), *nrfA*
522 (encoding nitrite reductase, DNRA related) *amoA* (encoding ammonia monooxygenase) and *nifH*
523 (encoding nitrogenase) encoding metagenomic reads ([http://enve-](http://enve-omics.ce.gatech.edu/rocker/models)
524 [omics.ce.gatech.edu/rocker/models](http://enve-omics.ce.gatech.edu/rocker/models)). Briefly, the short-read nucleotide sequences were searched
525 (using Blastx) against a training set for each abovementioned protein; training sets were
526 manually curated to encompass experimentally verified reference sequences as suggested
527 previously (91). The resulting matching sequences were then filtered using the ROcker compiled
528 model (model for 150bp-long reads for PR and OK soils and 100 bp model for IL soils). Protein

529 abundances (based on the number of reads assigned to the protein) were normalized by
530 calculating genome equivalents. For the latter, the ROcker-filtered read counts were normalized
531 by the median length of the sequences of each protein reference, and the corresponding genome
532 equivalents were calculated as the ratio of NosZ (or another protein of interest) read counts to the
533 RNA polymerase subunit B (*rpoB*), a universal single copy marker, read counts.

534

535 *NosZ phylogenetic analysis*

536 The NosZ reference protein sequences were aligned were aligned using CLUSTAL Omega (92)
537 and a maximum likelihood reference tree was created using RAxML v 8.0.19 (93) with a general
538 time reversible model option, gamma parameter optimization and '-f a' algorithm. The ROcker
539 identified NosZ-encoding reads were extracted from all datasets, translated into protein
540 sequences using FragGeneScan, and then added to the reference alignment using Mafft (94). The
541 reads were placed in the phylogenetic tree using RAxML EPA algorithm and visualized using
542 iTOL (95).

543

544 **Intra-population diversity assessment based on recovered MAGs**

545 The taxonomic affiliation of individual contig sequences of a MAG was evaluated based on
546 MyTaxa, a homology based classification tool (96). The MiGA (Microbial Genomes Atlas,
547 www.microbial-genomes.org) webserver was used for the taxonomic classification of the whole
548 MAG using the ANI/AAI concept. To assess intra-population diversity and sequence
549 discreteness, each target population MAG was searched against all the reads from each location
550 by Blastn (only contigs longer than 2Kbp were used). Fragment recruitment plots were
551 constructed based on the Blastn matches (threshold values: nucleotide identity $\geq 75\%$ and

552 alignment length ≥ 80 bp) using the Enveomics collection of scripts (62). The evenness of
553 coverage and sequence diversity of the reads across the length of the reference genome sequence
554 were used to evaluate the presence and discreteness of the population in the chosen dataset.

555

556

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564 their help in soil sampling.

565

566

567 **TABLES:**

568 **Table 1: Proportion of total microbial community diversity explained by measured soil**
569 **environmental factors.**

	Inertia	Proportion	Rank
Total	0.1092	1	
Constrained	0.0876	0.8021	6
Unconstrained	0.02161	0.1978	5

Site, sampling depth, pH, total nitrogen, total carbon, moisture data were considered in the analysis

570

571 **FIGURE LEGENDS**

572

573 **Fig. 1: Sampling location map and microbial community diversity among the study sites. A.**

574 Map of the four sampling sites within the Luquillo Experimental Forest (LEF). B. Principal co-

575 ordinate analysis (PCoA) plots based on MASH distances, colored by sampling site, C.

576 Nonmetric multidimensional scaling (NMDS) plot with the soil physicochemical parameters

577 incorporated. The arrow lengths are proportional to the strength of the correlations obtained

578 between measured soil physicochemical parameters and each ordination axis.

579

580 **Fig. 2: Abundance of N cycling genes and their distribution across soil ecosystems. A.**

581 Abundance of hallmark genes for denitrification, DNRA and nitrogen fixation pathways,

582 represented as genome equivalents (% of total bacterial genomes sampled that carry the gene) in

583 the metagenomes studied (see Figure key). B. Frequency of genomes carrying the respective

584 denitrifying gene across the three ecosystems studied. Genes denoted by the same letter are not
585 statistically significantly different between ecosystems (ANOVA Tukey test). Statistical
586 significance reported at $p < 0.05$. Note that nitrification genes were not detected in any of the
587 Puerto Rico sites.

588

589 **Fig. 3: Phylogenetic diversity of *nosZ*-encoding sequences recovered in each soil ecosystem.**

590 *nosZ* sequences were identified by the ROCKER pipeline and placed in a reference *nosZ*
591 phylogeny as described in the Materials and Methods section. The radii of the pie charts are
592 proportional to the number of reads assigned to each sub-clade and the colors represent the
593 sampling sites from each ecosystem (see Figure key). Sub-clades highlighted in grey indicate the
594 most abundant sub-clades across all three ecosystems whereas the ones highlighted in blue were
595 abundant only in agricultural soils (IL). A. *nosZ* reads from every sampling site recruiting to
596 atypical (Clade II) clades. B. *nosZ* reads recruiting to typical (Clade I) clades. Inset shows the
597 most abundant sub-clade (*Opitutus terrae*) from panel A and its distribution across all sites. Note
598 that in all three ecosystems most of the reads recruit to atypical sub-clades. Suppl. Fig. S7 shows
599 the distribution of the reads among the most abundant sub-clades in detail.

600

601 **Fig. 4: Functions encoded by the recovered population MAGs.** Heatmap showing the relative

602 abundance of genes encoding the major metabolic functions (Level 1 of the SEED subsystem

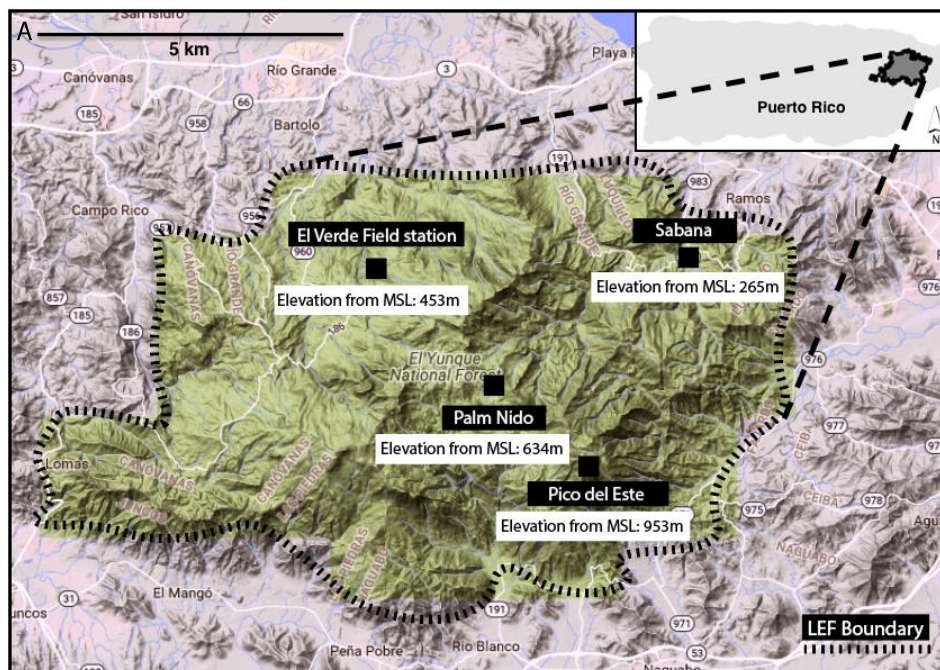
603 category) for each MAG recovered from the four sites in Puerto Rico. The taxonomic

604 classification of each MAG based on MiGA is shown on the bottom left. The symbols at the

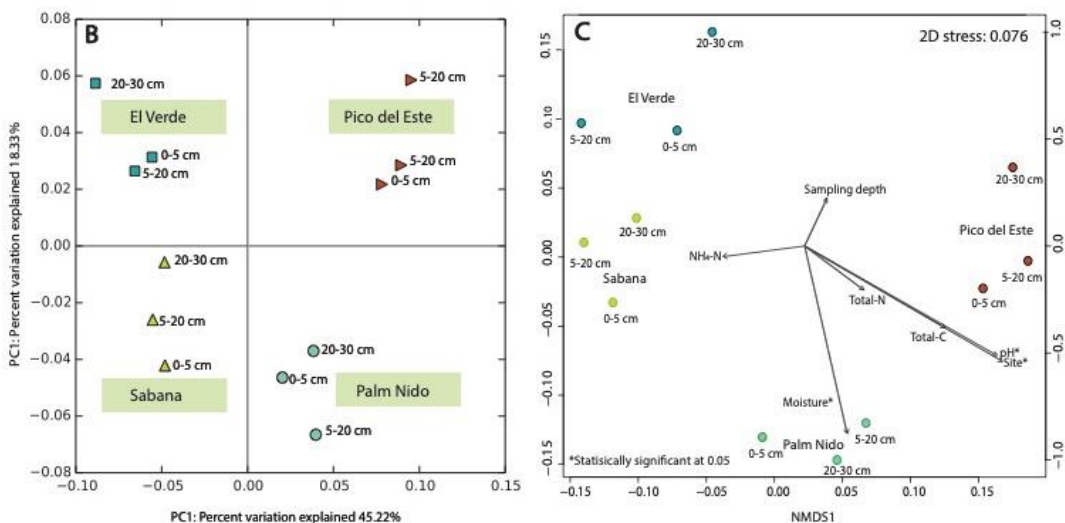
605 bottom of the heatmap denote the presence (or absence) of specific N-cycling genes, namely

606 denitrification and nitrogen fixation. No genes involved in nitrification were detected in any of
607 the bins.

608 **FIGURES**
609



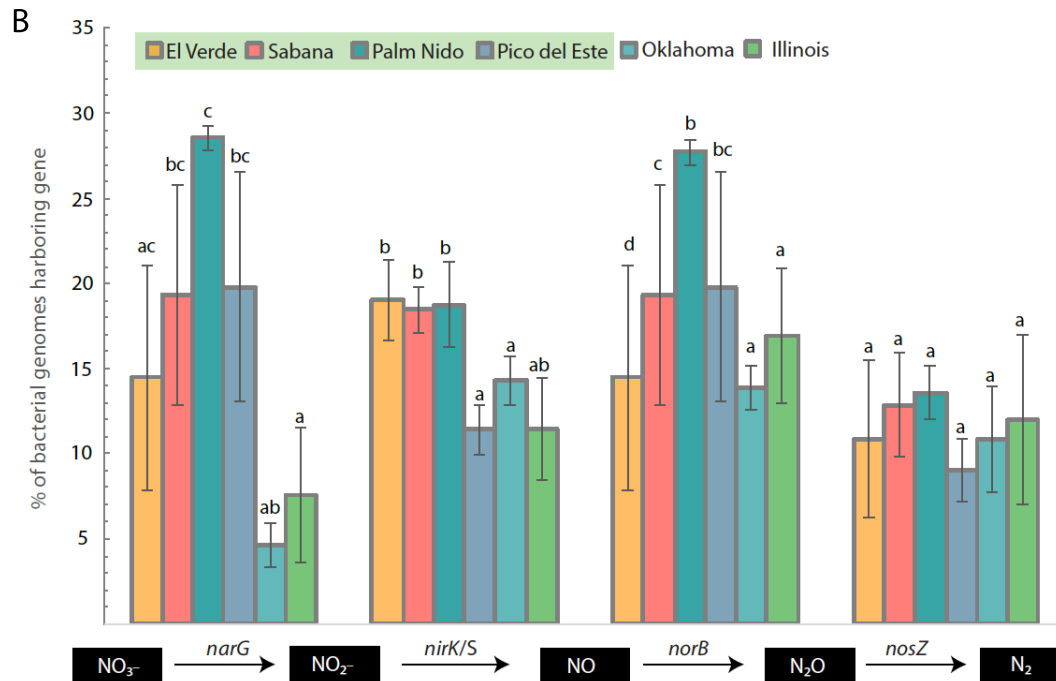
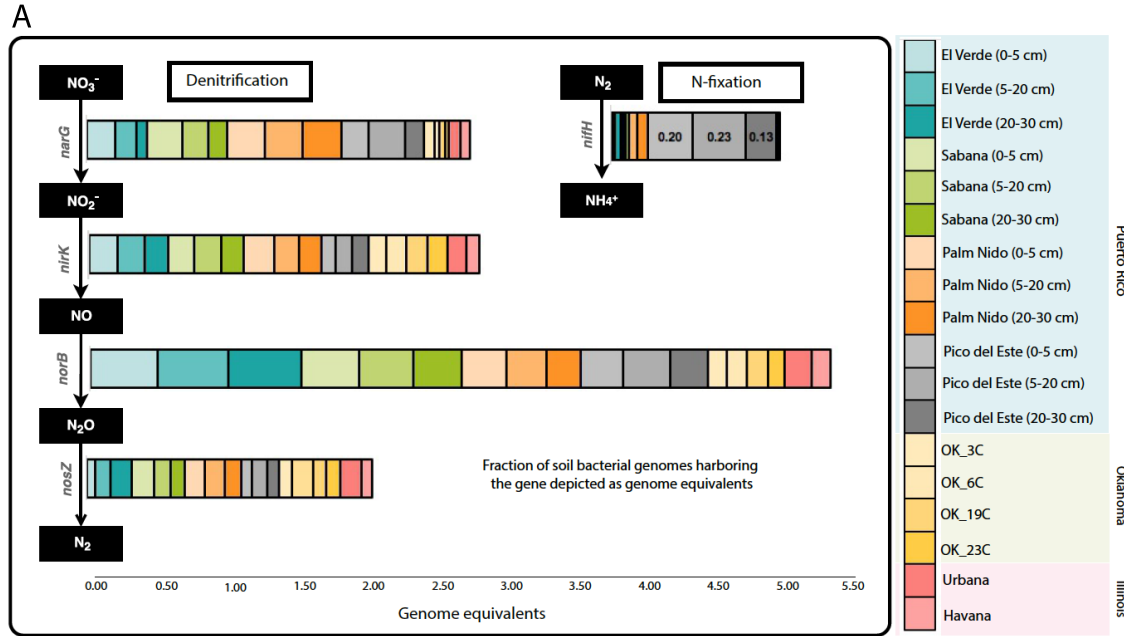
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Figure 1: Sampling location map and microbial community diversity among the study sites.

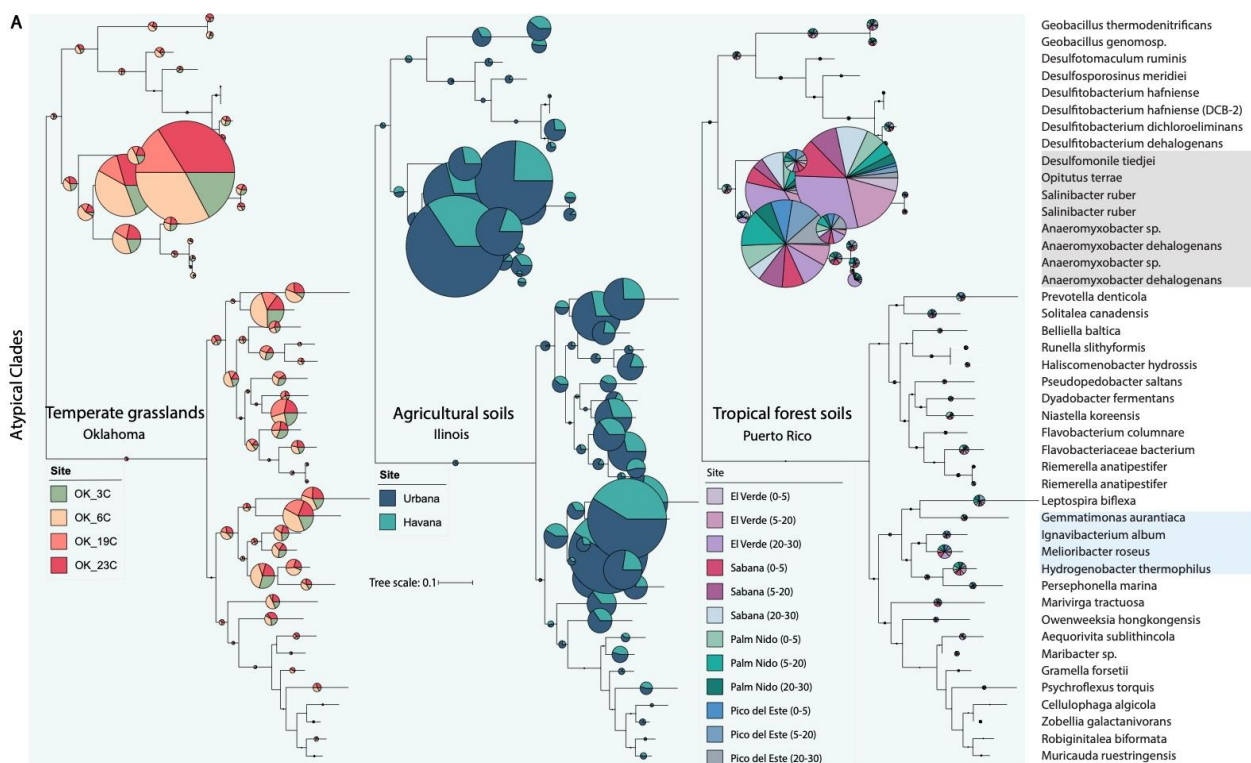
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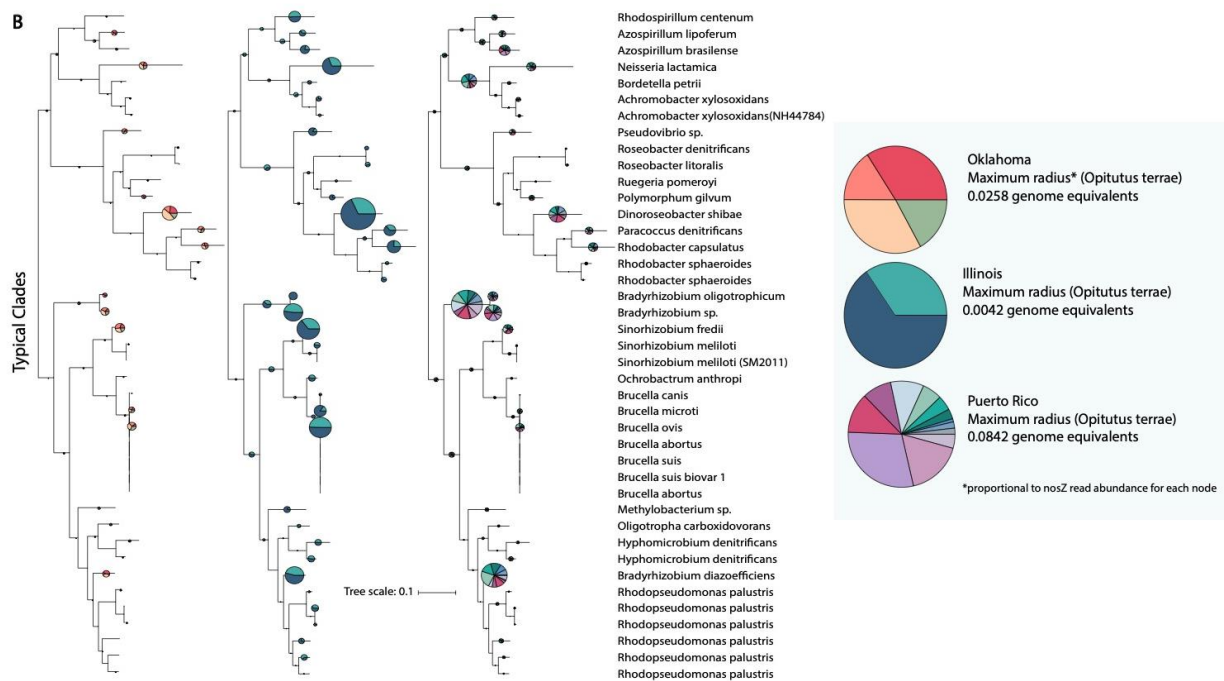
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Figure 2: Abundance of N cycling genes and their distribution across soil ecosystems.

625



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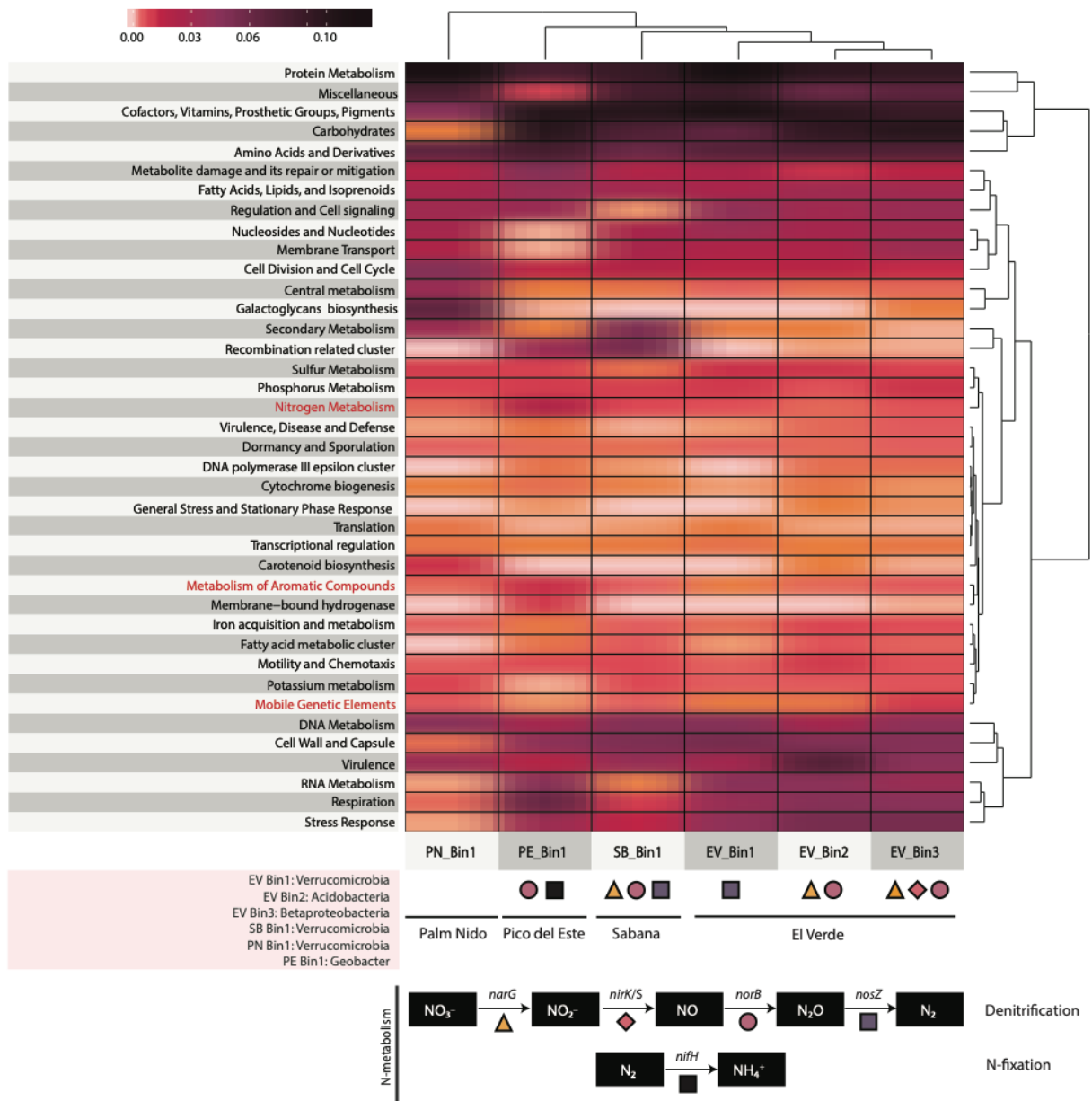
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630

Figure 3: Phylogenetic diversity of *nosZ*-encoding sequences recovered in each soil ecosystem.

631



632

633

Figure 4: Functions encoded by the recovered population MAGs.

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