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Change in Land Use Alters the Diversity and Composition of *Bradyrhizobium* Communities and Led to the Introduction of *Rhizobium etli* into the Tropical Rain Forest of Los Tuxtlas (Mexico)

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Abstract Nitrogen-fixing bacteria of the *Bradyrhizobium* genus are major symbionts of legume plants in American tropical forests, but little is known about the effects of deforestation and change in land use on their diversity and community structure. Forest clearing is followed by cropping of bean (*Phaseolus vulgaris*) and maize as intercropped plants in Los Tuxtlas tropical forest of Mexico. The identity of bean-nodulating rhizobia in this area is not known. Using promiscuous trap plants, bradyrhizobia were isolated from soil samples collected in Los Tuxtlas undisturbed forest, and in areas where forest was cleared and land was used as crop fields or as pastures, or where secondary forests were established. Rhizobia were also trapped by using bean plants. *Bradyrhizobium* strains were classified into genospecies by *dnaK* sequence analysis supported by *recA*, *glnII* and 16S-23S rDNA IGS loci analyses. A total of 29 genospecies were identified, 24 of which did not correspond to any described taxa. A reduction in *Bradyrhizobium* diversity was observed when forest was turned to crop fields or pastures. Diversity seemed to recover to primary forest levels in secondary forests that derived from abandoned crop fields or pastures. The shifts in diversity were not related to soil characteristics but seemingly to the density of nodulating legumes present at each land use system (LUS). *Bradyrhizobium* community composition in soils was dependent on land use; however, similarities were observed between crop fields and pastures but not among forest and secondary forest. Most *Bradyrhizobium* genospecies present in forest were not recovered or become rare in the other LUS. *Rhizobium etli* was found as the dominant bean-nodulating rhizobia present in crop fields and pastures, and evidence was found that this species was introduced in Los Tuxtlas forest.

Introduction

Soil bacteria known as rhizobia have an important role in maintaining soil fertility [1, 2]. These nitrogen-fixing bacteria associate with plants of the Fabaceae family (legumes), inducing the formation of nodules on roots where the bacteria reduce atmospheric N_2 and provide a substantial part of the plant nitrogen requirements. The Fabaceae is the third largest family of flowering plants and is well-represented in tropical forests [3]. Inventories of nodulating legume species are incomplete, but it has been shown that a significant proportion of the surveyed species in tropical forests are able to host rhizobia, especially in the Americas [4]. Rhizobia, due to the association with legumes, may contribute to a significant nitrogen input to the forest ecosystem.

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Species of rhizobia are distributed across several genera of α-Proteobacteria and of β-Proteobacteria [5–7]. Rhizobia belonging to the *Bradyrhizobium* genus are predominant legume symbionts in American tropical forests like the Barro Colorado island in Panama [8], the Rio Guabo forest of Costa Rica [9], the French Guiana fresh water swamp forest [10] and the Western Amazonian forest [11]. Los Tuxtlas is the rain forest located farthest north in the Americas, bordering the Gulf of Mexico and the Santa Martha volcano in the state of Veracruz, Mexico. Rhizobia from Los Tuxtlas have not been studied; however, due to floristic composition similarities with Neotropical forests of Panama and Costa Rica, it could be predicted that *Bradyrhizobium* will be as predominant as in other American tropical forests.

Los Tuxtlas forest has conserved areas and others significantly affected by deforestation [12]. After forest clearing, land is used mainly for agriculture, and a few years later when soil fertility declines, land is used for pastures or abandoned leading to the establishment of a secondary forest. Agriculture in Los Tuxtlas is characterized by traditional practices where maize and bean (*Phaseolus vulgaris*) are grown intercropped in a low-input system called "milpa" in small fields in contact with nearby forest [12]. Being legumes, beans associate with rhizobia. In Central Mexico, where beans originated and were domesticated, *Rhizobium etli* is their preferred symbiont, but when beans are introduced to other areas, local rhizobia can gain access to their nodules [13]. Little is known about which rhizobia nodulate beans in areas of Mexico such as Los Tuxtlas where this crop was introduced in pre-Hispanic times.

Tropical rain forests are rich in biological diversity not only of plants and animal species but also of microbes such as endophytic fungi [14] and bacteria [15]. Alterations in diversity and composition of whole soil bacterial communities after forest clearing and change in land use have been reported [16–22], but relatively few studies have focused on particular functional bacterial groups [23, 24] like rhizobia. It was the aim of this work to describe the diversity and composition of the symbiotic *Bradyrhizobium* community in the tropical rain forest of Los Tuxtlas, and in areas where forest has been cleared and the land was used for cropping or pastures, or where secondary forests had been established. Additionally, we investigated the identity of bean rhizobia in the different land use systems of Los Tuxtlas. This work was part of the "Conservation and Sustainable Management of Below-Ground Biodiversity" (CSM-BGBD) project funded by the Global Environment Facility (GEF) aimed at defining the impact of forest destruction on soil biota in several countries.

Methods

Study site and soil sampling

The study area (18°15′–18°26′ N and 94°44′–94°58′ W) was located in Sierra de Santa Marta (Veracruz, Mexico) within Los Tuxtlas Biosphere Reserve. The climate is warm and humid, with an average annual rainfall of 4900 mm. The soil is generally classified as an andisol, with alfisol located at some sites, and is generally acid (pH 4–6). Soil samples were obtained from four different land use systems (LUS): the original undisturbed primary forest, crop fields, pastures and secondary forests. Crop fields had intercropped maize and bean, with squash (*Cucurbita pepo*) and jicama (*Pachyrhizus erosus*) usually present. Pastures were dominated by the grasses *Paspalum conjugatum* or *Cynodon plectostachyus*. Secondary forests had an average age of 8 years. Different legumes species naturally occur in pastures and as weeds in crop fields. An unpublished inventory of legumes present at sampling points, kindly provided by E.B. López-Cano and G. Castillo-Campos, was used.

Twenty-four samples were taken from each LUS. Information about the proximity of sampling points within and between LUS is provided in Supplementary Table 1. At each sampling point, composite samples were obtained by mixing eight individual soil cores (20-cm depth) taken randomly within a 6-m radius circle. Collections were made in December 2003 and in January 2004, periods that do not correspond to the heavy rainy season (which ends at the end of October or beginning of November), but which have sporadic rains and residual humidity in the soil. Samples were maintained at room temperature and used as inocula within a few days after collection. The results of several physical and chemical analyses performed on soil samples were used as provided by the CSM-BGBD project (http://www3.inecol.edu.mx/csmbgbd/index.php/inicio). One-way ANOVA and Duncan's multiple range test were used to test for significant differences between the soil characteristics of the different LUS.

Bacteria isolation

Rhizobia were obtained from the soil samples using *Vigna unguiculata* (cowpea), *Macroptilium atropurpureum* (siratro) and *P. vulgaris* as trap plants. Cowpea and siratro were chosen because they are highly promiscuous hosts being able of forming symbioses with many different bradyrhizobia [25, 26] and some other genera of nodule bacteria [27]. *P. vulgaris* were used to investigate the identity of bean-nodulating rhizobia present in the same soil samples. All seeds were sterilized for 5 min in 70% ethanol and 15 min in 1.5% (w/v) sodium hypochlorite, rinsed five times with

sterile water and then germinated on plates with agar-water as previously described [26]. Seedlings were transplanted 2 days after germination and immediately inoculated with the appropriate fresh soil dilutions. Soil serial dilutions were prepared as follows: soil was suspended in sterile water (0.5 g per ml) from which 10^{-1} to 10^{-4} dilutions were prepared. Non-inoculated control plants were included.

Siratro plants were grown with Jensen plant medium [28] using vermiculite as substrate in glass tubes closed with a foam plug (to avoid contamination) only allowing the shoot to trespass. Cowpea and bean were grown in Fahraeus medium [29] in vermiculite in jars that were covered similarly. All plant manipulations were performed under axenic conditions, and all media, vermiculite and plant nutrients were sterilized. Plants were maintained in growth chambers at 28–30°C with a 12-h photoperiod.

Nodules were collected 21 (bean) or 30 days (cowpea and siratro) after inoculation. Non-inoculated control plants remained devoid of nodules. Plants did not show disease symptoms. Bacteria were recovered after nodule surface sterilization with 70% ethanol and then with 1.5% sodium hypochlorite for 3–5 min, followed by thorough rinsing with sterile water. Nodules were macerated on YEM medium with 25 mg/l bromothymol blue as a pH indicator [28]. Surface sterilization was checked by rubbing the nodules on YEM medium in plates before maceration. Plates were incubated at 30°C. Individual colonies were selected for further purification in YEM medium or peptone yeast extract (PY) medium [28]. The time of appearance of clear visible colonies was used to classify the isolates into slow-growing (≥5 days), intermediate-growing (4–5 days) or fast-growing (2–3 days) rhizobia. Purified isolates were named with numbers followed by a letter to indicate the isolation host (*p* for bean; *v* for cowpea; *m* for siratro) and maintained at −80°C in 20% glycerol.

DNA extraction, PCR amplification and sequencing

A loop full of a growing bacterial colony was suspended in 25 µl of sterile water, heated at 95°C for 10 min, centrifuged to pellet cell debris, and the supernatant containing the DNA was used as template for PCR reactions. When PCR reactions were not successful by this procedure single colonies were grown in 3 ml of liquid YEM or PY medium, and DNA was obtained using the Genomic Prep™ Cells and Tissue DNA isolation kit (Amersham Biosciences). 16S rRNA genes were amplified using primers fD1 and rD1 [30]. Primers and PCR conditions used for amplification of partial *dnaK*, *recA*, *glnII* and *rpoB* gene fragments, and the intergenic spacer (IGS) between the 16S and 23S rRNA genes, were as previously described [9, 13]. For the *dnaK* and 16S rRNA genes, one strand of the purified PCR products was sequenced with the respective forward primer using an automatic Applied Biosystems DNA-sequencer. Only sequences with a Phred score ≥25 were used. Both strands were sequenced for the remaining genes. In some cases, and only for bradyrhizobia, rep-PCR genomic fingerprints for groups of strains were generated using primers ERIC1R and ERIC2, or BOX A-1 as previously described [31]. Band patterns were visually analyzed, and strains differing in more than two bands were considered as members of different clone groups. Only one strain of each clone group was subjected to *dnaK* sequencing, and its classification was transferred to the remaining strains from the same group. PCR amplification with primers nodA-1, nodA-2 [32] and nifH2 [33] directed toward the 5′ end of *nodA*, 5′ end of *nodB* and 3′ end of *nifH*, respectively, was used to determine the arrangement of symbiotic genes in selected *R. etli* strains.

Phylogenetic analysis

Sequences from reference rhizobial strains retrieved from the GenBank database and those obtained in this study were compared. *Bradyrhizobium japonicum* DNA homology groups I (from here on *B. japonicum*) and Ia [34] were treated as separate species as they did not appear as a monophyletic group in most phylogenies. Sequences were aligned using CLUSTAL W [35] and edited with BioEdit [36]. The lengths of the analyzed alignments after gap removal were (in nt): 567, 405, 569, 658 and 582 for *dnaK*, *recA*, *glnII*, 16S rRNA and *rpoB*, respectively. For identical sequences only one was used in the phylogenetic analyses. Gene phylogenies were generated by the minimum evolution (ME) and maximum likelihood (ML) methods using MEGA [37] and PhyML [38], respectively. ME shows a significant improvement over the more commonly used neighbor joining (NJ) method in terms of topological accuracy [39]. The best model of sequence evolution for each gene was selected with JModelTest using the Akaike information criterion [40]. ME and ML phylograms showed differences in topology but were largely congruent in species-level clade delineation, thus only ME phylogenies were used in further analyses. As recommended by Willems et al. [41], the IGS phylogeny was inferred by the NJ method with a matrix of uncorrected distances calculated without penalizing gaps using the *p*-distance and pairwise-deletion options of MEGA. The length of the analyzed IGS sequences ranged from 746 to 1057 bp.

Classification of the strains into (geno)species

The *dnaK* and 16S rRNA gene sequences were used as the main criteria for the classification of bradyrhizobia, and the fast-growing or intermediate-growing rhizobia, respectively. The *dnaK* gene was chosen instead of the 16S rRNA gene for bradyrhizobia due to the very low sequence variability of the latter gene in this taxon [42] and to the availability of *dnaK* sequences from Neotropical bradyrhizobia at the beginning of this work. An initial classification of the *dnaK* sequences was performed by delineating operational taxonomic units (OTUs) using average clustering at a distance of 0.014 with MOTHUR [43] based on the report of Rivas et al. [44] that bradyrhizobial *dnaK* sequences sharing ≥98.6% similarity belong to the same species. Forty-two OTUs including bradyrhizobial sequences were obtained. Most reference strains of the same species were clustered into the same OTU; however, *B. japonicum* strains were split into two OTUs, and *B. elkanii* and *B. pachyrhizi* strains were joined into a single one. Rivas et al. [44] pointed out that, irrespective of sequence similarity cut-off levels, a multilocus sequence analysis can be used to recognize species as they formed separate clades in most gene tree topologies. Thus, phylogenies of three additional phylogenetic markers (*recA*, *glnII* and IGS) were obtained for selected strains representing the diversity within each *dnaK*-defined OTU. Thirty-one OTUs were recovered as separate clades in all gene phylograms as expected for OTUs representing individual species. Four pairs of closely related OTUs were detected. Each pair consistently clustered in at least three out of the four phylograms and share ≥98.4% *dnaK* similarity. Although this similarity is slightly lower than the 98.6% threshold, it is still above the maximum 97.9% inter-species similarity reported by Rivas et al. [44], thus each pair was considered a single OTU. The two OTUs including *B. japonicum* strains together with another OTU were merged as they clustered in all phylogenies despite showing a minimum sequence similarity of 97.5%. *B. elkanii* and *B. pachyrhizi* reference strains shared 98.5–99.2% *dnaK* sequence similarity but formed separate clades in all phylogenies and thus were considered as separate OTUs. For the fast-growing and intermediate-growing rhizobia, the use of the 16S rRNA gene allowed a clear delineation of most OTUs at a 99.5% similarity level [45]. Nevertheless, to resolve species affiliation sequencing and analysis of the *rpoB* gene was necessary for some OTUs. Some of the OTUs identified here were equated with named species while the remaining were referred to as genospecies to be in line with nomenclature previously used in rhizobial taxonomy. The genospecies were named TUXTLAS followed by a number (e.g. TUXTLAS-12).

Diversity analyses

Rarefaction curves and Good's coverage estimator [46] were used to evaluate how well the bradyrhizobial diversity was sampled. Total richness was predicted with the non-parametric estimator S_{Chao1} [47, 48]. The reciprocal of Simpson's index (1/D) and the exponential of Shannon's index (e^{H}) were used as measures of diversity [49]. Equitability was evaluated using the Pielou's evenness index [49]. The community compositions of the different LUS were compared using the incidence-based similarity index of Jaccard, and the abundance-based Bray–Curtis and Chao's modified Jaccard-type indices [49, 50]. All diversity estimates as well as the similarity indices were calculated with EstimateS (http://viceroy.eeb.uconn.edu/EstimateS) or SPADE (http://chao.stat.nthu.edu.tw/indexE.html) software. Statistical differences were evaluated using the *t*-test [51]. Pearson product correlation coefficients were calculated to explore the relationships between *Bradyrhizobium* diversity and soil characteristics or legume density.

Results

Characteristics and classification of bradyrhizobia

A total of 585 and 184 isolates were obtained with cowpea and siratro, respectively. As expected, most isolates were slow-growing bacteria with colonies appearing after 5 days of incubation and showing alkaline or neutral reaction in YEM medium, typical characteristics of *Bradyrhizobium* [52]. The number of nodules (and therefore isolates) obtained from different samples of the same LUS was very variable precluding a sample-based analysis. Hence, all isolates from each LUS were grouped and analyzed as a single, composite sample. Randomly chosen isolates obtained from each LUS were classified into genospecies by *dnaK* gene sequence analysis (Fig. 1), additionally supported and refined by *recA*, *glnII* and IGS sequence analyses (Supplementary Figs. 1–3). Although the trap plants used are highly promiscuous, they may have different affinities for some bradyrhizobial groups. To prevent this potential bias, for each LUS equal or approximately equal number of isolates was chosen from each plant species. From the 233 isolates analyzed, a total of 29 genospecies were identified, 13 trapped with both hosts, seven only with cowpea and nine only with siratro. Only one case of lateral gene transfer was detected involving genospecies TUXTLAS-23 that received a *dnaK* allele from an unidentified Rhizobiales bacterium but that have *Bradyrhizobium*-affiliated sequences in the remaining loci. The genospecies assignment, isolation host and LUS origin of all analyzed strains are provided in Supplementary


F C P SF
TUXTLAS-1
TUXTLAS-3
TUXTLAS-2
B. yuanmingense
TUXTLAS-6
TUXTLAS-4
B. liaoningense
TUXTLAS-5
B. japonicum Ia
B. canariense
TUXTLAS-7
B. japonicum
TUXTLAS-8
TUXTLAS-9
TUXTLAS-14
TUXTLAS-16
TUXTLAS-15
B. betae
TUXTLAS-13
TUXTLAS-12
TUXTLAS-10
TUXTLAS-30
TUXTLAS-11
TUXTLAS-22
TUXTLAS-19
TUXTLAS-20
TUXTLAS-21
TUXTLAS-31
TUXTLAS-18
B. elkanii
TUXTLAS-17
TUXTLAS-29
B. jicamae
TUXTLAS-29b
Bradyrhizobium genosp. VIII
TUXTLAS-32
R. palustris
TUXTLAS-23
0.01

◀ **Figure 1** Minimum evolution *dnaK* gene phylogeny of the *Bradyrhizobium* genospecies found in Los Tuxtlas and other bradyrhizobial species. The total number of identical sequences represented by each terminal branch is indicated within *parentheses*. Bootstraps support values higher than 70% are shown. Distribution of each genospecies in the different land use systems is indicated at the *right* (*F* primary forest, *C* crop fields, *P* pastures, *SF* secondary forest). Species not found in Los Tuxtlas are shown in *smaller lettering*

Table 2. The distinctiveness of all genospecies named in this study in relationship to taxa not included in Fig. 1 is depicted in Supplementary Figs. 1–3.

We did not have access to seeds of the majority of native legumes from Los Tuxtlas. Nevertheless, a limited number of *Inga* sp. seeds could be obtained and were inoculated with eight strains representing six genospecies. *Inga* is the legume genus with more species in Los Tuxtlas rain forest and has been reported to be nodulated by *Bradyrhizobium* [53]. Seven out of the eight tested strains were able to induce effective nodulation on the inoculated plants. These results suggested that the *Bradyrhizobium* strains isolated from the trap plants used here are the common symbionts of the native legumes in Los Tuxtlas. More evidence for that assumption was given by the fact that we recovered three genospecies that have been reported to nodulate legumes in Central American tropical forests.

Bradyrhizobium diversity in relation to LUS

Rarefaction curves indicated that the *Bradyrhizobium* communities of each LUS were not completely sampled (Supplementary Fig. 4). Nevertheless, sample coverage was high for all LUS (>79%) indicating that the majority of the bradyrhizobial diversity accessible with the used trap plants was obtained (Table 1). The number of observed genospecies in forest, crop fields, pastures and secondary forest were 10, 16, 16 and 15, respectively. Several measures were used to describe and compare the bradyrhizobial diversity of the different LUS. To account for differences in sample effort, all measures from crop fields and pastures were rarefied to forest sample size. Predicted genospecies richness by the S_{Chao} estimator indicated that secondary forests harbored the highest number of genospecies followed by pastures and crop fields, and the forest (Table 1). Nevertheless, these differences in richness between LUS were not significant. The Simpson index indicated a decrease in *Bradyrhizobium* diversity when the forest was turned to crop fields or pastures and that the secondary forest harbored a diversity equivalent to the primary forest (Table 1). Both forest types had highest Shannon index values than crop fields and pastures, but only for pastures and secondary forests this difference was significant (Table 1). The discrepancies observed between both indices may be explained by how they treat abundant species. In contrast to the Shannon index, the Simpson measure gives more weight to abundant species and thus is more sensitive to the dominance patterns exhibited by communities with low diversity. Accordingly, the two LUS revealed as having the lowest diversity by this index showed the lowest equitability, or alternatively the highest dominance (Table 1).

Table 1 Diversity measures of the four land use systems (LUS) rarefied to forest sample size

Diversity measure	LUS			
	Forest	Crop fields	Pastures	Secondary forest
Good's coverage estimate (%)	92.7	85.4	82.9	78.0
Genospecies richness (S_{obs})[a]	10	11	12	15
S_{Chao1} richness estimate[b]	12 (2.6) a	20 (8.7) a	23 (9.8) a	27 (10.8) a
Simpson's index (1/D)[b]	6.72 (0.21) a	4.85 (0.78) b	3.35 (0.59) b	6.78 (0.28) a
Exponential Shannon (e^{H})[b]	7.30 (0.83) ab	6.36 (0.9) ab	5.37 (0.88) b	9 (1.4) a
Pielou's evenness index (E_H)	0.86	0.77	0.67	0.81

Values were estimated using *Bradyrhizobium* genospecies as operational taxonomic units

[a] The number of observed genospecies was 16 for the complete crop fields and pastures samples

[b] Standard errors are indicated within parentheses. Values followed by different letters are significantly different at the 5% level

Influence of soil characteristics and legume density on bradyrhizobial diversity

No significant differences were detected in soil carbon and micronutrients content between LUS (data not shown). As discussed by Hughes et al. [54], the capacity of these soils to sequester large quantities of organic matter and their relatively high cation exchange capacity may explain these observations. Soil characteristics found to be significantly different between LUS were pH, density, porosity, nitrogen and phosphorus content (Table 2). Forest soils were the least dense and more porous, and the richest in nitrogen and phosphorus content. All LUS had acids soils, but crop fields were the least acid. Soil characteristics did not seem to influence *Bradyrhizobium* diversity as no significant correlations ($P>0.05$) were found between any soil parameter and diversity indices.

Legume species found at each LUS are shown in Supplementary Table 3. Given that not all legumes are able to nodulate or to host *Bradyrhizobium*, species were classified in three groups according to published data [55–57]: non-nodulating species, nodulating species, and spe-

Table 2 Soil characteristics of the different land use systems (LUS)

Parameter	LUS			
	Forest	Crop fields	Pastures	Secondary forest
Soil characteristic[a]				
pH	4.86 b[b]	5.18 ac	4.88 b	4.94 bc
Bulk density (g/cm^{-3})	0.53 c	0.79 a	0.77 a	0.69 b
Porosity (%)	75.9 a	67.7 b	65.9 b	71.3 a
P_{Bray} (ppm)	3.67 a	1.54 b	1.38 b	1.66 b
N (NO_3) (ppm)	60.3 a	28.7 b	32.4 b	39.1 b
N (NH_4) (ppm)	21.8 a	15.5 b	17.6 ab	15.7 b

[a] No significant differences were found in organic matter, C, Na, K, Mg and Ca contents, humidity and cation exchange capacity between the different LUS

[b] Values in the same row followed by the same letter were not significantly different ($P>0.05$) by the Duncan's multiple range test

cies showing preferences for nodulation with *Bradyrhizobium*. Excluding legumes sown in crop fields, total legume density was higher in forest and secondary forest when compared to pastures and crop fields (Table 3). A high proportion of non-nodulating legume species were found in forests and secondary forests (Supplementary Table 3). Similar number of non-nodulating species can be found in floristic inventories reported for this region [58, 59]. Nevertheless, when only nodulating or *Bradyrhizobium*-specific legume species were considered, forest and secondary forests still had the highest legume densities (Table 3). Interestingly, significant positive correlations were found between *Bradyrhizobium* diversity, as measured by the Shannon index, and the density of nodulating ($P=0.012$) and *Bradyrhizobium*-specific legumes ($P=0.002$). Albeit not significant, high correlation coefficient values ($r\geq0.89$) were also obtained between legume density and the Simpson diversity index.

Community composition variation

The *Bradyrhizobium* community composition changed considerably when the forest was replaced with crop fields, pastures or secondary forest as judged by the low Jaccard and Bray–Curtis similarity index values obtained between forest and the other LUS (Table 4). The modified Jaccard index adjusted to account for unseen species [50] also revealed differences between forest and other LUS. Crops and pastures, the two LUS suffering from the highest human intervention, were those showing the highest similarity in community composition (Table 4). Remarkably, seven out of the 10 genospecies recovered from forests became less abundant or were no longer recovered in all other LUS (Fig. 2).

The relatively high abundance of certain genospecies seemed to be characteristic of each LUS (Fig. 2), and they may constitute marker species. Genospecies TUXTLAS-14 and TUXTLAS-19 were of the primary forest, while TUXTLAS-5 and TUXTLAS-6 were of crop fields. Secondary forests showed high abundances of *B. japonicum* and genospecies TUXTLAS-11. In pastures, *Bradyrhizobium* genosp. TUXTLAS-17 was the dominant bradyrhizobial group, the abundance of this group also increased in pastures, crop field and secondary forest when compared to the forest.

A high number of novel *Bradyrhizobium* genospecies were found in Los Tuxtlas

B. japonicum was recovered from the secondary forest and also from two *Inga* nodules we collected during soil sampling (Fig. 1). Based on all the analyzed loci, genospecies TUXTLAS-17 seemed to be equivalent to *Bradyrhizobium* "genotype J" [60] described from strains nodulating native legumes in Central American tropical forests [8], and both groups most likely correspond to the recently described *B. pachyrhizi* species isolated from *P. erosus* nodules in Costa Rica and Honduras [61]. Remarkably, the remaining 27 genospecies found in Los Tuxtlas were not affiliated with any of the currently described bradyrhizobial species.

Three genospecies from Los Tuxtlas seemed to be equivalent to taxa not formally described as species but reported elsewhere. Interestingly, those taxa are composed of strains isolated from wild legumes occurring in tropical forests or from cultivated crops growing in subtropical acid soils. Based on *dnaK*, *recA* and IGS sequences, genospecies TUXTLAS-10 is equivalent to the "common BCI *Bradyrhizobium* lineage" found to be widespread in tropical

Table 3 Legume density at the different land use systems (LUS)

Legume density[a]	LUS			
	Forest	Crop fields	Pastures	Secondary forest
All species	2.22	0.88	0.45	2.39
Nodulating species	1	0.88	0.45	1.5
Bradyrhizobium specific species	0.87	0.69	0.45	1.17

[a] Individuals per sampling point. Beans and *P. erosus* sown in crop fields were not counted

Table 4 Number of shared *Bradyrhizobium* genospecies and community similarity indices between the land use systems (LUS)

LUS pair		Number of shared genospecies	Similarity index		
			Jaccard	Bray–Curtis	$Jaccard_{abund}$
Forest	Crop fields	6	0.30	0.25	0.41
Forest	Pastures	8	0.44	0.41	0.67
Forest	Secondary forest	5	0.25	0.29	0.50
Secondary forest	Crop fields	8	0.35	0.37	0.61
Secondary forest	Pastures	6	0.24	0.35	0.51
Crop fields	Pastures	11	0.52	0.63	0.81

forests of Costa Rica and Panama [62]. Sequences of *recA* and *glnII* indicate that strain SEMIA 6434, isolated from *Inga* sp. and recommended as commercial inoculant in Brazil, belongs to genospecies TUXTLAS-10 [63]. By *dnaK* and *recA* sequences, strain Cp5-3 isolated from *Centrosema pubescens* in Panama belongs to genospecies TUXTLAS-14. Strain Cp5.3 has been previously assigned to "genotype K" based on polymorphisms in the ribosomal RNA region [60]. By *recA* and *glnII* sequences, strain CB756 isolated from *Macrotyloma africanum* in Zimbabwe and the Australian strain SEMIA 6077 from *Stylosanthes* sp. [63] belong to genospecies TUXTLAS-6. Strain CB756 has been previously referred to as the sole member of bradyrhizobial "lineage SA-4" [64].

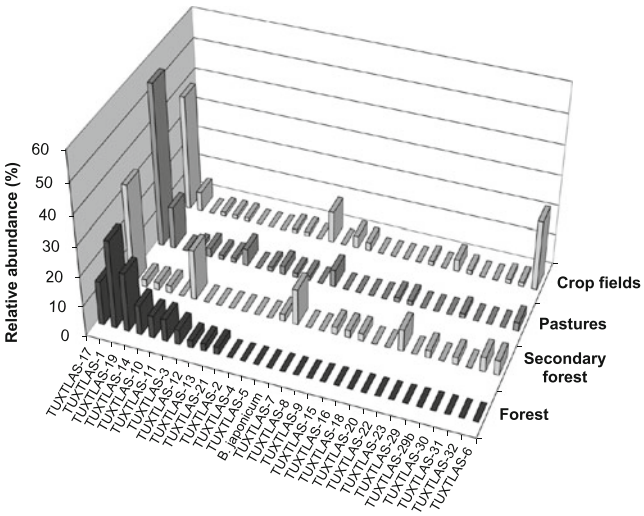

Figure 2 Relative abundance of the *Bradyrhizobium* genospecies in each land use system

Identity of bean rhizobia in Los Tuxtlas

A total of 1153 isolates were obtained using *P. vulgaris* as a trap plant (63, 463, 354 and 273 from forest, crop fields, pastures and secondary forest, respectively). Random samples corresponding to approximately 7–12% of the isolates from each LUS were characterized. The 83 bean rhizobia were classified into eight genospecies by 16S rRNA gene analysis (Fig. 3, Supplementary Table 4) additionally supported in some cases with *rpoB* gene sequence analysis (Supplementary Fig. 5). Species nodulating bean in Los Tuxtlas included *R. etli*, *R. leucaenae* (former *R. tropici* type A [45]), *R. hainanense*, *R. mesoamericanum*, *Burkholderia tuberum*, and the two novel *Rhizobium* genospecies TUXTLAS-27 and TUXTLAS-28 (Fig. 3). *R. etli* was the predominant species in samples from crop fields and pastures (70 and 76% of the analyzed isolates), while *R. leucaenae* strains represented the majority (50%) of strains in samples from the secondary forest where only 25% were assigned to *R. etli*.

In contrast to other LUS, only two samples from forest did promote nodulation of bean plants, and all *R. etli* strains came from one of those samples collected in a forest patch located in close vicinity to a crop field. Aiming at gaining a further insight into the identity of bean rhizobia in the forest, we analyzed all fast-growing and intermediate-growing forest isolates ($n=13$) trapped with cowpea and siratro. *Rhizobium* genospecies TUXTLAS-24, *R. etli* and *R. mesoamericanum* (one, two and eight isolates, respec-

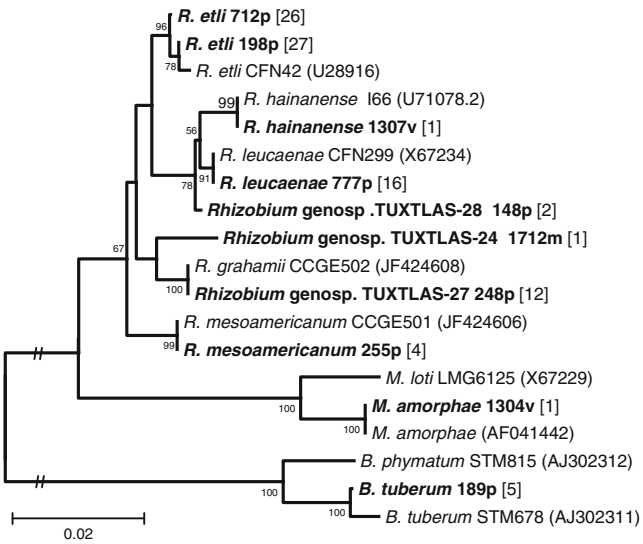

Figure 3 Minimum evolution 16S rRNA gene phylogeny of the bean-nodulating rhizobial species found in Los Tuxtlas (shown in *bold*). Only one strain is shown in each terminal branch, and the total number of strains having identical sequences is indicated within *brackets*. Sequence accession numbers are shown within *parentheses*. Bootstraps support values higher than 70% are shown. *R Rhizobium*, *M Mesorhizobium*, *B Burkholderia*

tively) were obtained with siratro, while two cowpea isolates were ascribed to *R. mesoamericanum* and *Mesorhizobium amorphae* (Fig. 3). The two *R. etli* strains trapped with siratro came from the same forest sample from which *R. etli* were obtained using bean traps. The low numbers of *R. etli* obtained from all forest soils sampled indicate that this species is not normally present in the forest.

Symbiotic genotypes of *R. etli* from Los Tuxtlas

Two symbiotic varieties (symbiovars, sv.) have been described in *R. etli* strains depending on their host preference. Strains specific for beans belong to sv. phaseoli while those adapted to nodulate *Mimosa* plants correspond to sv. mimosae [65]. Since *Mimosa pudica* and *M. albida* have been reported in Los Tuxtlas [66], we sought to determine the symbiotic genotype of *R. etli* from this region. A random sample of 15, 10 and three strains from crop fields, pastures and secondary forest, respectively, and all strains from forest were assayed taking advantage of the different arrangement of the nodulation (*nod*) and nitrogen fixation (*nif*) genes in the symbiotic plasmids from each symbiovar. The *nodA* gene in sv. mimosae strains is contiguous to *nodBC* (Rogel-Hernández, unpublished) while in sv. phaseoli this gene is separated from *nodBC* but adjacent to *nifH* [67]. PCR with primer pair nodA-1/nodA-2 is expected to amplify a 660-bp fragment in sv. mimosae and no product in sv. phaseoli, while primer pair nodA-1/nifH2 should amplify a ~3.5-kb product in sv. phaseoli and no product in the other symbiovar. *R. etli* sv. phaseoli CFN42 and *R. etli* sv. mimosae Mim1 were used as controls. Interestingly, all 36 *R. etli* strains assayed showed the gene arrangement corresponding to sv. phaseoli (data not shown). *nodA* gene sequencing and phylogenetic analysis confirmed that these strains belong to sv. phaseoli (Supplementary Fig. 6).

Discussion

For whole soil bacterial communities, increases in diversity have been reported after forest clearing and change in land use to pastures [18] and crop fields [16]. In contrast, when the forest in Los Tuxtlas was cleared and the land used for cropping or pastures, the bradyrhizobial diversity decreased. Changes in soil properties after deforestation, such as decrease in C, N and micronutrient content; decrease in porosity and increase in density; and changes in pH have been reported in other studies [15, 22]. Several of those changes were also observed in Los Tuxtlas. Nevertheless, none of the recorded changes in soil properties seemed to explain the diversity loss. Possibly, other factors like different land management practices used in agriculture and pastures are affecting rhizobial diversity. For example, some tillage systems [68], nitrogen fertilization at levels normally recommended for agriculture in Mexico [69] and bovine slurry depositions to soil [70] are known to negatively affect rhizobial diversity. Additionally, agrochemicals like fungicides that are normally applied to seeds or herbicides used in crops and pastures diminish rhizobial survival and colonization of plants [71–73].

Changes in vegetation cover associated with land use modification have a large impact on soil microbial communities [17, 22] and likely explain better the shifts in diversity and composition of these communities than soil characteristics [74]. Legumes are predicted to have a large impact on soil rhizobial communities. Indeed, we found that bradyrhizobial diversity in Los Tuxtlas was highly correlated with the density of legumes able to nodulate with *Bradyrhizobium*. Crop fields have a large density of legumes because of the presence of bean crops; however, the density of legumes with specificities for *Bradyrhizobium* in crop fields and pastures is lower than in the undisturbed forest. The secondary forests, as rich in bradyrhizobial diversity as the primary forest, showed also a high legume density. Others have also reported a high density of legumes in the secondary forests of Los Tuxtlas [75, 76]. Secondary forests have a high rate of aboveground biomass accumulation [54] that may be limited by N losses during previous land use as crop fields or pastures [77]. Interestingly, in the rain forest of Central Amazonia, biological nitrogen fixation (BNF) by the legume–rhizobia association is higher in the secondary regrowth than in the mature forest [78]. It is conceivable that a high legume density may have promoted an increase in bradyrhizobial diversity, which, in turn, may aid in sustaining the high BNF required for secondary forests growth.

Significant changes in species composition were also observed after forest clearing. Some *Bradyrhizobium* genospecies seemed to disappear or became very rare while others increased their abundances, resulting in a general decrease in community evenness. Either the new soil conditions or vegetation may differentially affect the bradyrhizobial genospecies inducing the observed compositional changes. Communities from crop fields and pastures were the more similar sharing the highest number of genospecies. Soils from those LUS appear to be different by being denser and less porous than soils from forest and secondary forest. These modifications affect soil microniches were bacteria reside promoting bacterial predation by protozoa [79] or anoxic conditions under which only some genospecies may thrive. Vegetation may also affect the structure of bacterial communities by stimulating the growth of species than can catabolize its root exudates.

Legumes, by hosting selectively some rhizobia and not others, can have a large impact on the rhizobial community [69, 80]. In Los Tuxtlas, *B. pachyrhizi* (=genospecies TUXTLAS-17) became the dominant species in pastures, crop fields and secondary forest. Interestingly, legumes able to host *B. pachyrhizi* occur in relatively high density in pastures and crop fields. *Desmodium* spp. plants grow naturally in pastures and as weeds in crop fields, and the tuberous legume jicama (*P. erosus*) is intermittently sown in crop fields. The dominance of *B. pachyrhizi* in secondary forests may be a residual effect of previous land use as pastures or crop fields, maintained by the occurrence of some *Desmodium* species in the secondary regrowth.

Maize and bean have been intercropped in traditional agriculture in Mexico for around 7000 years. This kind of agriculture was introduced long ago, and it is still practiced in Los Tuxtlas. *R. etli* is the most common symbiont of beans in Central Mexico [13] and a natural maize endophyte that has been found to colonize the maize rhizosphere in large numbers [81, 82]. We found that *R. etli* was the dominant bean rhizobia in crop fields and pastures. Cropping of maize and bean surely explains this prevalence in agricultural fields. Since *R. etli* associates with maize, it also may be able to colonize other members of the Poaceae family like the grasses that are predominant plants in pastures. It has been reported that *R. etli* is able to colonize other plants besides beans and maize [83].

Beans can nodulate with a wide range of rhizobia [84], so it was not unexpected to find other rhizobial genospecies within their nodules. *R. leucaenae* was already known as a bean symbiont [45], and *R. hainanense* and *B. tuberum* strains have been reported as being able to induce bean nodulation [85, 86]. *Rhizobium* genospecies TUXTLAS-27 was closely related to *R. grahamii* that together with *R. mesoamericanum* have been reported as low competitors for bean nodulation [87]. *Rhizobium* genospecies TUXTLAS-28 and *R. alamii* are here reported as novel bean symbionts.

As discussed for *Bradyrhizobium* genosp. TUXTLAS-17, the presence of *R. etli* in secondary forests may be a residual effect of previous land use as crop fields. However, in contrast to crop fields and pastures, *R. leucaenae* became the dominant bean rhizobia in secondary forests. It is a common practice in Los Tuxtlas to use live fences to delineate pasture plots and crop fields. The legume tree *Gliricidia sepium* is one of the two most commonly used plants in these fences [88]. These trees commonly act as secondary forest regeneration nuclei after the land is abandoned [75]. We have previously found that *R. leucaenae* (previously named *R. tropici* type A [45]) is the major symbiont of *G. sepium* in the state of Veracruz where Los Tuxtlas is located [89]. It seems likely that the use of *G. sepium* in live fences is inducing local soil enrichment of *R. leucaenae* that is subsequently reflected in the abundance of this species in the secondary forest.

Unexpectedly, we did not find *R. etli* sv. mimosae in Los Tuxtlas, despite the presence of two *Mimosa* species. Preliminary inoculation experiments suggest that *M. pudica*, one of the species found in this region, is not able to host *R. etli* sv. mimosae strains (Rogel-Hernández, unpublished results). The sole presence of sv. phaseoli coupled to the absence of *R. etli* in the primary forest strongly indicates that this species is not native to Los Tuxtlas. As beans were introduced to this region it seems probable that *R. etli* sv. phaseoli was introduced as well. Bean seeds carry *R. etli* bv. phaseoli on their coats [13], and this may account as a mechanism for bacterial introduction at the same time as the seeds spread to novel geographical regions.

This study underscored that destruction of tropical forest may have a large impact in the diversity of the *Bradyrhizobium* legume symbionts, a functionally important group of soil bacteria. The fact that the bradyrhizobial community composition of the secondary forest soil showed little similarity with that of the original forest soil may have implications for programs aimed at restoring the original forest vegetation. Some legumes may have specific rhizobial requirements for nodulation and may not be successfully established if their symbionts were lost, thus requiring inoculation.

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