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Rhizobium etli and *Rhizobium gallicum* Nodulate Common Bean (*Phaseolus vulgaris*) in a Traditionally Managed Milpa Plot in Mexico: Population Genetics and Biogeographic Implications

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The stability of the genetic structure of rhizobial populations nodulating *Phaseolus vulgaris* cultivated in a traditionally managed milpa plot in Mexico was studied over three consecutive years. The set of molecular markers analyzed (including partial *rrs*, *glnII*, *nifH*, and *nodB* sequences), along with host range experiments, placed the isolates examined in *Rhizobium etli* bv. phaseoli and *Rhizobium gallicum* bv. gallicum. Cluster analysis of multilocus enzyme electrophoresis and plasmid profile data separated the two species and identified numerically dominant clones within each of them. Population genetic analyses showed that there was high genetic differentiation between the two species and that there was low intrapopulation differentiation of the species over the 3 years. The results of linkage disequilibrium analyses are consistent with an epidemic genetic structure for both species, with frequent genetic exchange taking place within conspecific populations but not between the *R. etli* and *R. gallicum* populations. A subsample of isolates was selected and used for 16S ribosomal DNA PCR-restriction fragment length polymorphism analysis, *nifH* copy number determination, and host range experiments. Plasmid profiles and *nifH* hybridization patterns also revealed the occurrence of lateral plasmid transfer among distinct multilocus genotypes within species but not between species. Both species were recovered from nodules of the same plants, indicating that mechanisms other than host, spatial, or temporal isolation may account for the genetic barrier between the species. The biogeographic implications of finding an *R. gallicum* bv. gallicum population nodulating common bean in America are discussed.

Rhizobia are soil bacteria that are capable of inducing the formation of nitrogen-fixing nodules on the roots or stems of particular legume host plants (51). Rhizobial species seem to have coevolved with their hosts at their centers of diversification (30). Common bean (*Phaseolus vulgaris*) originated in America; this plant was exported to the rest of the world starting in the early 16th century, and it is currently an important crop worldwide (17, 18). At least five species have been reported to nodulate common bean. *Rhizobium etli* bv. phaseoli is the predominant *P. vulgaris*-nodulating species in Mexico, Colombia, and Argentina (1, 11, 42). *R. etli* bv. phaseoli is found in regions where common bean has been introduced, such as Spain, France, Austria, Senegal, Gambia, and Tunisia (10, 21, 24, 33, 34, 44). However, in these countries other *Rhizobium* species also nodulate this legume. *Rhizobium leguminosarum* bv. phaseoli is commonly found in Europe, and it has also been reported to be present in Tunisia and Colombia (11, 21, 33, 34). *Rhizobium tropici* is present in acid soils of South America (31) and has been found in France (2) and several African countries (4, 10). *Rhizobium giardinii* has been found only in European and Tunisian soils (3, 21, 34). *Rhizobium gallicum* has been found nodulating beans in Europe (3, 21) and Tunisia (33, 34), and one Mexican strain (strain FL27) has been identified as a member of this species (44).

In *Rhizobium* a large proportion of the genome is composed of plasmids (16), which contribute significantly to the ecological fitness and symbiotic performance of rhizobial strains (6, 8, 16). The symbiotic genes for nodulation (*nod*) and nitrogen fixation (*nif*, *fix*) are located on the symbiotic plasmid (pSym). The pSym plasmids of biovar phaseoli strains that nodulate *P. vulgaris* have multiple copies of the nitrogenase reductase gene (*nifH*) and confer a restricted host range (28). In contrast, *R. tropici* and *R. gallicum* bv. gallicum pSym plasmids carry a single *nifH* copy and confer a broader host range that includes *Leucaena* spp. (3, 9). Segovia and collaborators (41) suggested that *R. leguminosarum* bv. phaseoli is the result of pSym transfer from *R. etli* bv. phaseoli in historic times. The *R. gallicum* and *R. giardinii* bv. phaseoli strains that nodulate *P. vulgaris* harbor pSym plasmids similar to those found in *R. leguminosarum* bv. phaseoli and *R. etli* bv. phaseoli, which led Amarger and colleagues (3) to propose that the acquisition of *R. etli* bv. phaseoli pSym took place via *R. leguminosarum* strains. The presence of viable *R. etli* bv. phaseoli strains on the testa of *P. vulgaris* seeds has been demonstrated (36). This finding could explain the geographical spread of *R. etli* bv. phaseoli and provides a scenario for the lateral transfer of pSym among indigenous *Rhizobium* species in historic and recent times along with the introduction of bean crops worldwide.

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Besides the strong evidence for interspecies pSym transfer, population level analyses of *Rhizobium* species have also revealed the existence of lateral transfer of pSym within species in agricultural fields and pastures (25, 27, 40, 60). However, Wernegreen and colleagues (57) found a limited pattern of *pSym* transfer (48) in *R. leguminosarum* bv. trifolii populations associated with native *Trifolium* species growing in mountain meadows of California.

The effects of agricultural practices and plant host domestication on the genetic structure of *Rhizobium* populations have scarcely been addressed (30, 47). The domestication of wild *P. vulgaris* plants began around 4,000 years ago in Mesoamerica and the Andean region of South America (22). Beans were probably codomesticated with maize in Mesoamerica, since these two crops are grown in association in a traditional agrosystem called milpa (29). The milpa system is a prehispanic cultivation method, in which beans are intercropped with maize and squash, together with diverse other plant species that are locally used for medicinal and nutritional purposes (47). This cultural practice promotes bean nitrogen fixation, and its advantages have been recognized (5, 29). In a previous study we compared the genetic structures of *R. etli* bv. phaseoli strains associated with beans under different degrees of domestication (47). We found that the bacteria associated with milpa beans had a genetic structure intermediate between the genetic structures of the bacteria isolated from wild beans and monocultured beans (47). In a more recent study, we analyzed the genetic structure of a *Rhizobium* population obtained from nodules of *P. vulgaris* and *Phaseolus coccineus* plants from milpa plots in San Miguel, Puebla, Mexico, with the aim of understanding its spatial variation (45). To do this, six plots were sampled in a single year, and a hierarchical analysis of multilocus enzyme electrophoresis (MLEE) data revealed (i) the coexistence of two distantly related genetic groups, designated genetic divisions I and III, and (ii) the existence of numerically dominant genotypes within each division; in addition, linkage disequilibrium analyses indicated that recombination is frequent within each genetic division but not between the divisions, which led us to propose a reticulated and epidemic genetic structure (32, 45). In the present study, *P. vulgaris* plants from one of the previously studied milpa plots were sampled over three consecutive years to determine (i) the temporal stability of the population genetic structure, (ii) the structure and dynamics of the plasmidic compartment, and (iii) the taxonomic affiliations of the genetic groups identified.

MATERIALS AND METHODS

Description of the sampling site and procedure. San Miguel Acuexcomac is a village with a semiarid climate (annual rainfall, 600 mm) and an alkaline soil (pH 8.4) and is located in the state of Puebla, Mexico (45). This area has a long history of bean cultivation that extends for centuries and has never been inoculated with rhizobial strains (47). Agricultural plots are traditionally managed as typical milpas, in which bean, maize, and squash are cultivated together. Low levels of fertilizer, minimal tillage, and hand weeding practices are used. The main *P. vulgaris* variety grown at this site is the climbing landrace called mantequilla. The germplasm is actively maintained by the indigenous community, and each year seeds from the previous crop are sown (47). One plot (plot B of Silva et al. [45]) was sampled in three consecutive years (1994, 1995, and 1996). *Rhizobium* isolates were obtained in the field from root nodules of *P. vulgaris* plants. One isolate was obtained from each nodule, as previously described (45). All isolates were tested for growth on plates coated with PY medium (per liter, 5 g of peptone, 3 g of yeast extract, and 1 g of calcium chloride) supplemented with nalidixic acid (60 μg/ml) and on Luria-Bertani plates. Isolates were deposited in the collection of the Instituto de Ecología, Universidad Nacional Autónoma de México.

MLEE. Cell lysates of the isolates were obtained as previously described (45), and electrophoresis was performed on cellulose acetate membranes (20, 45). The following six enzymes were assessed: isocitrate dehydrogenase (EC 1.1.1.42), peptidase (EC 3.4.13), phosphoglucomutase (EC 5.4.2.2), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), xanthine dehydrogenase (EC 1.1.1.204), and malate dehydrogenase (EC 1.1.1.37). Isocitrate dehydrogenase, peptidase, and phosphoglucomutase each exhibited one band of activity, glucose-6-phosphate dehydrogenase and xanthine dehydrogenase each exhibited two bands, and malate dehydrogenase exhibited three bands, which yielded a total of 10 loci for analysis.

Genetic diversity and cluster analysis. Distinctive mobility variants of each enzyme, numbered in order of decreasing anodal mobility, were considered alleles at the corresponding locus (43). In the case of enzymes that produced more than one band, each band was considered a locus. The absence of enzyme activity was scored as a null allele and was treated as an ordinary allele. The combined allele profiles were defined as multilocus genotypes (electrophoretic types [ETs]). Based on allele frequencies for ETs, the genetic diversity for an enzyme locus (h) was calculated as follows: $h = (1 - \Sigma x_i^2)[n/(n - 1)]$, where x_i is the frequency of the ith allele and n is the number of ETs (43). The total mean genetic diversity (H) is the arithmetic mean of h values for all loci and represents the proportion of loci at which two randomly chosen genotypes can be expected to differ. To compute the H values, we used the program ETDIV, version 2.2 (58).

The genetic distance between each pair of different ETs was estimated by determining the mean character differences, and a similarity matrix was constructed by using the PAUP* program (53); the data were clustered by the unweighted pair group method with arithmetic averages (UPGMA) (46).

Genetic differentiation. To estimate the relative genetic differentiation (G_{st}), we used Nei's equation, $G_{st} = (H_t - H_s)/H_t$ (49), where H_t is the expected diversity in an equivalent randomly mating total population and H_s is the average diversity of the subpopulations. To compute the G_{st} values, we used the program ETDIV, version 2.2 (58). The indices range from 0, if there is no genetic differentiation at a given level, to 1, if there is maximal genetic differentiation (35). To test if the G_{st} values were significantly different from 0, we performed a chi-square test of independence as follows: $\chi^2 = nG_{st}(a - 1)$, where n is the number of individuals and a is the total number of alleles. The degrees of freedom are $(k - 1)(a - 1)$, where k is the number of subdivisions. The degrees of freedom and χ^2 values were summed across loci, and significance was examined at a P value of <0.05 (19, 59).

Linkage disequilibrium analyses. To determine the extent to which populations exhibited nonrandom associations of alleles between loci, we used a multilocus index based on the distribution of allelic mismatches between pairs of isolates for all loci. The ratio of the variance in mismatches observed in a population (V_o) to the expected variance of the corresponding population at linkage equilibrium (random association of alleles) (V_e) provides a measure of linkage disequilibrium. If there is no linkage disequilibrium, V_o/V_e is 1. The significance of the difference between V_o and V_e was calculated by using a Monte Carlo procedure with 1,000 iterations, which was carried out with the LDV program (50).

Visualization and cluster analysis of plasmid profiles. The plasmid contents of the isolates were visualized by using the Eckhardt procedure (12). Plasmid mobilities were determined in 0.7% agarose gels by using plasmids of *R. etli* bv. phaseoli CFN42 and *Sinorhizobium meliloti* 1021 as molecular size references. A plasmid profile similarity matrix was constructed by using the PAUP* program (53), and data were clustered by using the UPGMA algorithm (46).

PFGE of plasmids and *pSym* determination. Pulsed-field gel electrophoresis (PFGE) was used to obtain accurate size estimates for plasmids from selected isolates and to identify the *pSym* plasmids. Intact genomic DNA was prepared in 0.8% agarose plugs and subjected to PFGE in a contour-clamped homogeneous electric field apparatus (CHEF-DRII; Bio-Rad) by following the manufacturer's instructions. Electrophoresis was carried out at 13°C with a constant voltage of 4.5 V cm^{-1} by using a two-block program as follows: block 1, 30-s initial switch, 60-s final switch, and 8-h run time; and block 2, 50-s initial switch, 100-s final switch, and 28-h run time. *Saccharomyces cerevisiae* chromosomes (Bio-Rad) were used as molecular size markers. Gels were stained with ethidium bromide, photographed, and transferred to nylon filters. Membrane hybridization with a *nifH* probe was performed as described below.

Analysis of *nifH* gene organization. Genomic DNA from 34 isolates was digested with endonuclease *Bam*HI, subjected to 1% agarose gel electrophoresis, stained with ethidium bromide, photographed, and transferred to nylon filters.

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An internal *nifH* fragment (~450 bp) from strain CFN42 was amplified with primers o1 and o3 (37), as described below. This fragment was labeled with digoxigenin-dUTP by using random primers, and detection was performed with anti-digoxigenin-alkaline phosphatase Fab fragments by using the chemiluminescence system and following the instructions of the manufacturer (Roche). Membrane hybridization and washing were performed under high-stringency conditions (65°C, 0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 sodium citrate]). Some membranes were also hybridized with a lambda probe to obtain accurate estimates of the hybridization signal sizes on gels normalized with *Hin*dIII-digested lambda DNA.

Plant nodulation tests and acetylene reduction assay. Seeds of *P. vulgaris* cv. Negro Jamapa, *Macroptilium atropurpureum*, and *Leucaena leucocephala* cv. Peruvian were surface sterilized with sodium hypochlorite (54). Pregerminated seeds were placed in flasks filled with vermiculite, watered with an N-free plant nutrient solution (13), and inoculated with each of the 34 selected isolates. Plants were maintained in a growth chamber at 28°C with a photoperiod of 15 h. After 4 weeks, the numbers of nodules were counted, and nitrogen fixation was measured by the acetylene reduction assay (54).

PCR-restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene. The 16S rRNA genes of 34 isolates were PCR amplified by using primers fD1 and rD1 (55). The amplification products were digested with endonucleases *Sau*3AI, *Msp*I, and *Pst*I and visualized in agarose gels as described elsewhere (23). Type and reference strains of the five recognized species that nodulate common bean (*R. etli* bv. phaseoli CFN42, *R. tropici* CIAT899 and CFN299, *R. leguminosarum* bv. phaseoli USDA2671, *R. gallicum* bv. gallicum R602sp and FL27, and *R. giardinii* H152) were included for comparison.

PCR amplification and nucleotide sequencing. Two isolates were selected for partial DNA sequencing of two chromosomal genes, *rrs* coding for 16S rRNA and *glnII* coding for glutamine synthetase, and two pSym genes, *nifH* and *nodB*, coding for the dinitrogenase reductase and *N*-acetylglucosamine deacetylase, respectively. For PCR amplification, a reaction mixture (50 μl) containing 1× PCR buffer (Gibco BRL), 1.5 mM $MgCl_2$, each deoxynucleotide triphosphate at a concentration of 200 μM, each primer at a concentration of 0.2 μM, and 2 U of *Taq* polymerase was used. The following temperature profile was used for all amplifications: 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at the appropriate annealing temperature, and 1 min of extension at 72°C and a final extension cycle consisting of 72°C for 5 min. The annealing temperatures for the different primer pairs are indicated below. *rrs* gene fragments were amplified with primers PF2 (TACTGTCGATCTGGAGTATG) and PR1 (ATTGTAGC ACGTGTGTAGCC) (annealing temperature, 60°C); *glnII* gene fragments were amplified with primers GSF1B and GSR2seq (56) (annealing temperature, 70°C); *nifH* sequences were amplified with primers o1 and o3 (37) (annealing temperature, 58°C); and almost complete *nodB* genes were amplified with primers nodB3F (56) and nodCRR (GAGACGGCGRCRRTGCTGGTTG) (annealing temperature, 65°C). The same primers were used for sequencing reactions with ABI Prism Big Dye chemistry, and the products were analyzed with an ABI377 sequencer (ABI, Foster City, Calif.).

Nucleotide sequence accession numbers. The sequences obtained in this study have been deposited in the GenBank sequence database under accession numbers AF529011 to AF529022.

RESULTS

Plant sampling and bacterial isolation. In 1994, 1995, and 1996 six, seven, and eight plants were sampled and 30, 38, and 58 isolates were obtained, respectively (Table 1). Over the 3 years a total of 21 plants were sampled and 126 isolates were obtained. All isolates grew on PY plates supplemented with nalidixic acid (60 μg/ml) but were unable to grow on Luria-Bertani plates. A total of 108 isolates formed gummy colonies on PY plates (classified as *R. etli* [see below]), and the remaining 18 isolates exhibited a rough colony appearance (classified as *R. gallicum* [see below]). Ten of the 21 plants sampled harbored *R. etli* isolates exclusively, whereas 11 plants harbored both *R. etli* and *R. gallicum* isolates.

Genetic diversity, genetic differentiation, and cluster analysis. The MLEE survey yielded 43 different multilocus genotypes for the 126 isolates. Cluster analysis of the MLEE data revealed two genetic divisions separated at a mean genetic distance of 0.7 (Fig. 1), corresponding to genetic divisions I and III described previously (45). On the basis of the nucleotide sequence, plasmid profiling, *nifH* copy number, and host range analyses described below, the 18 division I isolates were classified as *R. gallicum* bv. gallicum, and the 108 division III isolates were confirmed to be *R. etli* bv. phaseoli. The *R. gallicum* and *R. etli* isolates had similar mean genetic diversities (Table 2), and both populations displayed ET dominance, since a few ETs were represented by many isolates (Table 1 and Fig. 1). For the *R. gallicum* population, ET4 was recovered at the highest frequency and ET4 isolates comprised 33% of the *R. gallicum* isolates, although it was not found in the 1996 samples (Table 1). In the *R. etli* population, ET1, ET2, and ET3 (with 38, 18, and 15 isolates, respectively) were the most abundant ETs, were found in all 3 years and comprised 66% of the *R. etli* isolates (Table 1 and Fig. 1). A chi-square test showed that the frequencies of these three ETs did not vary significantly over the 3 years ($\chi^2 = 7.14$; df = 4; $P = 0.129$), suggesting that there was temporally stable genotype dominance in the population. These dominant *R. etli* genotypes grouped in a tight cluster, as shown in Fig. 1 (mean genetic distance, <0.2), indicating that they form a clonal complex (14).

The level of genetic differentiation between the *R. etli* and *R. gallicum* populations was high and significant ($G_{st} = 0.285$; $P < 0.001$), whereas the levels of intrapopulation genetic differentiation over the 3 years for *R. etli* ($G_{st} = 0.073$; $P = 0.243$) and *R. gallicum* ($G_{st} = 0.045$; $P = 0.999$) were not significant (Table 2). Furthermore, of the 37 alleles found in the 126 isolates, 8 were detected exclusively in the *R. gallicum* population, while 12 were unique to the *R. etli* population, and the 17 shared alleles were found at disparate frequencies in the two species (the MLEE data set is available from the corresponding author upon request). These results indicate that the two species do not share the main part of their alleles and that within the species the levels of genetic variability remained constant during the 3 years sampled.

Linkage disequilibrium analyses. A hierarchical linkage disequilibrium analysis was performed with the MLEE data set to estimate the extents of genetic exchange within and between the *R. etli* and *R. gallicum* populations. As shown in Table 2, both populations showed linkage equilibrium when only ETs were included in the analysis. When all isolates of each species were considered, the populations appeared to be in linkage disequilibrium, due to the presence of numerically dominant ETs (epidemic clones [32]). These results revealed an epidemic genetic structure for both species, in which the frequency of a few dominant genotypes increases to produce epidemic clones, but frequent genetic exchange occurs among the members of the population. Linkage disequilibrium was detected when the analysis was performed with either all 126 isolates or 43 ETs (Table 2), indicating that the extent of genetic exchange between *R. etli* and *R. gallicum* is negligible, if there is any exchange at all.

Diversity and cluster analysis of plasmid profiles. Eleven plasmid size classes were identified among the 18 *R. gallicum* isolates; the sizes ranged from ~50 to >1,500 kb and represented seven distinct profiles (Table 1 and Fig. 1). *R. gallicum* isolates harbored two to four plasmids (average, 3.1 plasmids), and all isolates contained a >1,500-kb megaplasmid, which was

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TABLE 1. Distribution of ETs and plasmid profiles among 18 *R. gallicum* and 108 *R. etli* isolates from nodules of *P. vulgaris* plants over three consecutive years[a]

ET	Sampling year			Total no. of isolates
	1994	1995	1996	
R. gallicum				
ET4	**P13** (2)[b]	**P12** (2), P21, P22		6
ET8			**P4,** P31	2
ET12	**P4**			1
ET15		**P4**		1
ET16		**P12**		1
ET19		**P14**		1
ET26		**P4**		1
ET29			**P4**	1
ET30			**P4**	1
ET38			**P4**	1
ET39			**P14**	1
ET40			**P4**	1
R. etli				
ET1	**P1** (2), **P2** (5), **P5** (2), **P6, P11,** P19	**P1** (4), **P2**	**P1** (9), **P2** (2), **P3, P5** (2), **P6** (3), **P8, P11,** P26, P27	38
ET2	**P3, P9, P10,** P17, P18, P20	**P1, P3** (2), **P7, P9, P10,** P23	**P1, P16,** P25, P29, P32	18
ET3	**P2** (4), **P3** (3)	**P3** (3)	**P1, P2** (2), **P3, P15**	15
ET5	**P9**		P9, **P10**	3
ET6			**P1** (3)	3
ET7		**P1** (2), **P2**		3
ET9			**P1,** P30	2
ET10			**P1, P8**	2
ET11	**P2**	**P3**		2
ET13		**P3**		1
ET14		**P11**		1
ET17		**P2**		1
ET18		**P7**		1
ET20		**P3**		1
ET21		**P5**		1
ET22		**P16**		1
ET23		**P15**		1
ET24		**P7**		1
ET25		**P7**		1
ET27		P24		1
ET28			**P1**	1
ET31			**P3**	1
ET32			**P5**	1
ET33			P28	1
ET34			**P2**	1
ET35			**P8**	1
ET36			**P1**	1
ET37			**P3**	1
ET41			**P1**	1
ET42			**P8**	1
ET43			P33	1

[a] A total of 126 isolates were obtained (30 isolates in 1994, 38 isolates in 1995, and 58 isolates in 1996).
[b] Plasmid profiles found in multiple isolates are indicated by boldface type, and the number of isolates is given in parentheses when there was more than one isolate.

absent in the *R. etli* population (Table 3). Profile P4 was the dominant profile; it was observed in 44% of the isolates (Table 1 and Fig. 1) and was indistinguishable from the profile of the type strain of *R. gallicum* bv. gallicum R602sp (Table 3). Fifteen plasmid size classes were detected among the population of 108 *R. etli* isolates, and the plasmid sizes ranged from ~125 to ~700 kb. Twenty-six plasmid profiles were found, consisting of two to five plasmids (average, 3.5 plasmids). All of the *R. etli* profiles included the ~700-kb plasmid, which was not found in the *R. gallicum* population (Table 3). The *R. etli* population displayed a high degree of plasmid profile dominance, with many isolates sharing a few plasmid profiles. Profiles P1, P2, and P3 were found in 57% of the *R. etli* isolates (Table 1 and Fig. 1); these profiles were the predominant profiles and were recovered in all 3 years. A chi-square test indicated that the frequencies of these three profiles varied significantly over the 3 years ($\chi^2 = 19.32$; df = 4; $P < 0.001$). Profile P1 was the most abundant profile in 1996, P2 was the most abundant profile in 1994, and P3 was the most abundant profile in 1995. These apparent temporal changes in plasmid profile dominance in the *R. etli* population could have been due simply to the small sample size or could reflect actual changes in the plasmid combinations selected in the crop seasons (for example, due to fluctuations in environmental conditions).

Figure 1 shows the separation of *R. gallicum* and *R. etli* populations on the basis of both MLEE and plasmid profiles.

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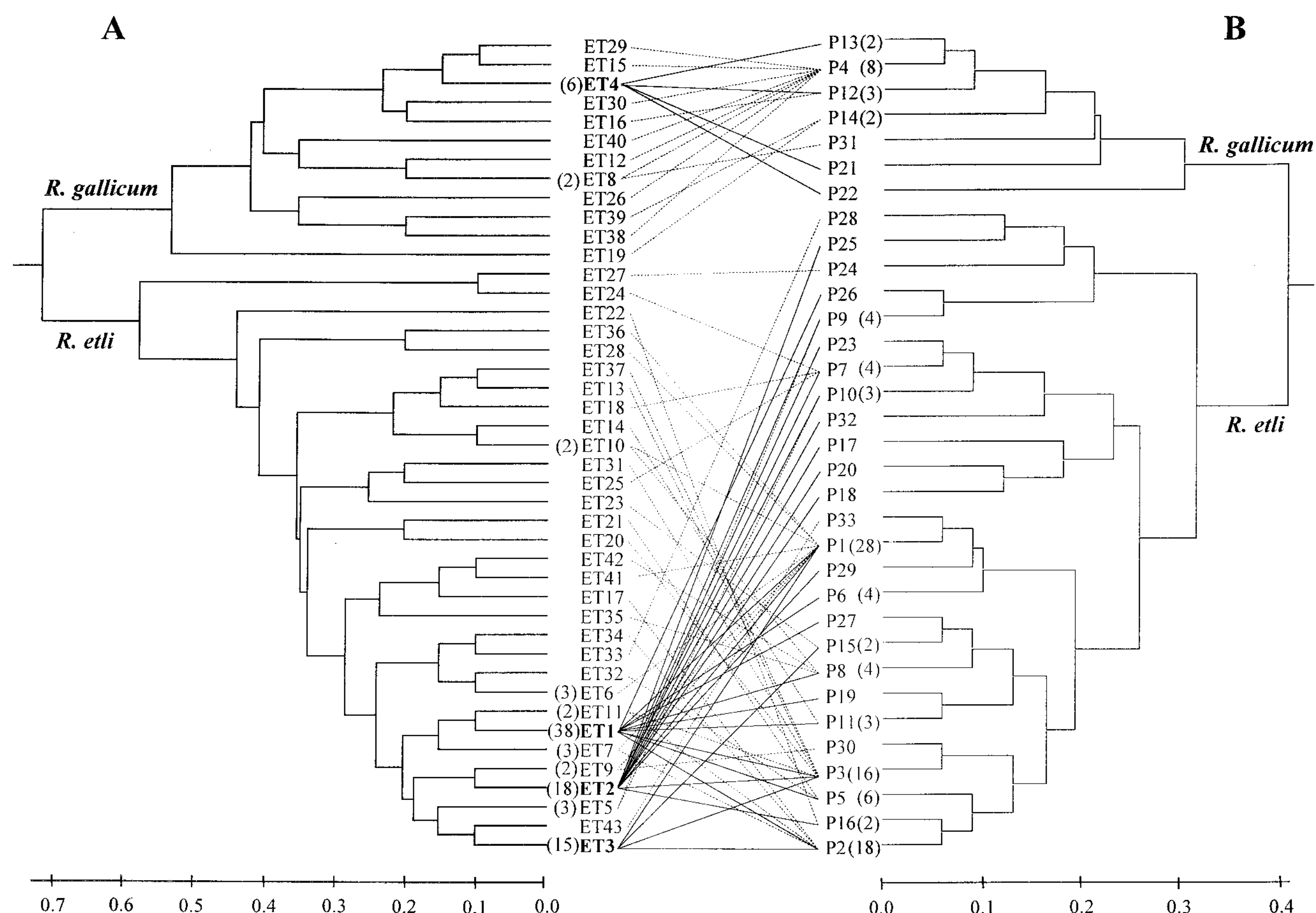

FIG. 1. Dendrograms showing the genetic relatedness among chromosomal and plasmidic genotypes of *R. etli* and *R. gallicum* and the chromosome-plasmid profile combinations. (A) Genetic relatedness of the multilocus genotypes based on 10 isoenzymatic loci. The ET designations are indicated. (B) Genetic relatedness of the plasmid profiles based on the presence or absence of the different plasmid size classes. The plasmid profile designations are indicated. The number of isolates for each multiple ET or plasmid profile is given in parentheses. The genetic distance between each pair of ETs or plasmid profiles was estimated by determining mean character differences, and data were clustered by the UPGMA. The four most abundant ETs and their plasmid profile combinations are indicated by boldface type and solid lines; the other combinations are indicated by dashed lines.

It is noteworthy that within species isolates belonging to the same ET could have different plasmid profiles and, conversely, that a particular plasmid profile could be found in isolates with different ETs, suggesting that plasmid transfer occurs within conspecific populations but not between species, as graphically shown in Fig. 1.

Based on the MLEE and plasmid profile data, 34 isolates were selected for further molecular analyses; this selection included 10 isolates from the *R. gallicum* population and 24 isolates from the *R. etli* population. This selection included isolates from dominant ETs and plasmid profiles, as well as isolates displaying unique ETs or plasmid profiles (Table 3).

TABLE 2. Genetic diversity, genetic differentiation, and linkage disequilibrium estimates for *R. gallicum* and *R. etli* populations associated with *P. vulgaris* plants in San Miguel Acuexcomac, Mexico

Population	No. of isolates	No. of ETs	Mean genetic diversity	Mean no. of alleles	G_{st}	Isolates			ETs		
						Mean no. of mismatches	V_o/V_e[a]	P[b]	Mean no. of mismatches	V_o/V_e[a]	P[b]
R. gallicum	18	12	0.388 (0.086)[c]	2.5	0.045	3.3	1.66	<0.001	3.9	0.96	NS[d]
R. etli	108	31	0.351 (0.059)	2.9	0.073	1.9	1.72	<0.001	3.5	1.06	NS
Total	126	43	0.501 (0.060)	3.7	0.285[e]	3.3	3.90	<0.001	5.0	2.18	<0.001

[a] Observed variance/expected variance of the mismatch distribution.
[b] Probability of rejecting by chance the null hypothesis that $V_o = V_e$.
[c] The values in parentheses are standard errors.
[d] NS, not significant.
[e] G_{st} is significantly different from 0.

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TABLE 3. Multilocus genotypes (ETs), plasmid profiles, 16S ribosomal DNA RFLP patterns, *nifH* hybridization patterns, and estimated sizes of plasmids and *nifH* hybridization bands for 34 selected *P. vulgaris* isolates and reference strains

Strain[a]	ET	16S ribosomal DNA RFLP pattern[b]	Plasmid		*nifH*	
			Profile	Sizes (kb)[c]	Pattern	Size(s) (kb)
R. gallicum isolates						
IE992	ET4	AAB	P13	>1,500, **550,** 350, 250	C1	8.3
IE2703	ET4	AAB	P22	>1,500, 650, 125, 50	C1	8.3
IE4868	ET40	ABA	P4	>1,500, **550,** 250	C1	8.3
IE4845	ET38	AAA	P4	>1,500, 550, 250	C1	8.3
IE2729	ET15	AAB	P4	>1,500, 550, 250	C2	9.5
IE988	ET12	AAA	P4	>1,500, 550, 250	C2	9.5
IE4770	ET29	AAB	P4	>1,500, 550, 250	C1	8.3
IE4872	ET8	AAA	P31	>1,500, **550,** 180, 125	C1	8.3
IE2735	ET16	ABA	P12	>1,500, 550	C1	8.3
IE2751	ET19	ABA	P14	>1,500, **525,** 250	C3	12.0
R. etli isolates						
IE4810	ET6	BAB	P1	700, 550, 450	A1	4.6, 12.4, 16.6
IE4813	ET32	BAB	P5	700, 450, **390,** 125	B1	5.6, 9.8
IE4794	ET31	BAB	P3	700, 450, **390,** 250	A2	2.6, 12.4, 16.6
IE4803	ET5	BAB	P9	700, **500,** 425	A1	4.6, 12.4, 16.6
IE4815	ET33	BAB	P28	700, **425,** 350, 270	A2	2.6, 12.4, 16.6
IE4876	ET43	BAB	P33	700, 550, 450, 225	A1	4.6, 12.4, 16.6
IE954	ET1	BAB	P1	700, **550,** 450	A1	4.6, 12.4, 16.6
IE4777	ET1	BAB	P1	700, 550, 450	A1	4.6, 12.4, 16.6
IE963	ET1	BAB	P2	700, 450, **390**	B1	5.6, 9.8
IE2730	ET1	BAB	P2	700, 450, 390	B1	5.6, 9.8
IE4804	ET1	BAB	P3	700, 450, **390,** 250	A2	2.6, 12.4, 16.6
IE4877	ET1	BAB	P5	700, 450, 390, 125	B1	5.6, 9.8
IE994	ET1	BAB	P5	700, 450, 390, 125	B1	5.6, 9.8
IE1006	ET1	BAB	P6	700, **550,** 450, 125	A1	4.6, 12.4, 16.6
IE1009	ET1	BAB	P11	700, **450**	A1	4.6, 12.4, 16.6
IE2737	ET2	BAB	P1	700, **550,** 450	A1	4.6, 12.4, 16.6
IE4795	ET2	BAB	P1	700, 550, 450	A1	4.6, 12.4, 16.6
IE950	ET2	BAB	P3	700, 450, **390**, 250	A2	2.6, 12.4, 16.6
IE2704	ET2	BAB	P3	700, 450, **390**, 250	A2	2.6, 12.4, 16.6
IE1004	ET2	BAB	P10	700, 450, **350**, 300, 250	A2	2.6, 12.4, 16.6
IE4874	ET3	BAB	P1	700, 550, **450**	A1	4.6, 12.4, 16.6
IE951	ET3	BAB	P2	700, 450, **390**	B2	4, 5.6, 9.8
IE4837	ET3	BAB	P2	700, 450, 390	B2	4, 5.6, 9.8
IE4771	ET3	BAB	P3	700, 450, **390**, 250	B1	5.6, 9.8
Reference strains						
CFN42	ET44	BAB	P34	650, 510, **390**, 270, 180, 150	B2	4, 5.6, 9.8
FL27	ND[d]	AAB	P35	>1,500, 600, 390	C1	8.3
R602sp	ND	AAA	P4	>1,500, 550, 250	C1	8.3

[a] The underlined isolates were used for determination of *rrs*, *glnII*, *nifH*, and *nodB* gene sequences.
[b] Each letter refers to a restriction pattern obtained with the enzymes *Sau*3AI, *Msp*I, and *Pst*I. Restriction sites for each enzyme were mapped on the *rrs* sequence of *R. gallicum* R602sp (accession number U86343), which had an AAA pattern. The type B pattern for *Sau*3AI contained additional restriction sites at nucleotides 501, 505, and 561. The *Msp*I type B pattern lacked the site at position 951. For *Pst*I digestion, the type B pattern had an additional restriction site at nucleotide 926.
[c] *pSym* plasmids are indicated by boldface type.
[d] ND, not determined.

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PFGE and pSym identification. PFGE was used to obtain accurate plasmid size estimates for the 34 isolates selected for molecular analyses, and the values are shown in Table 3. These values were used to estimate the plasmid sizes for the whole sample of 126 isolates, based on Eckhardt plasmid profiles.

The pSym plasmids of 20 isolates (4 *R. gallicum* and 16 *R. etli* isolates) were identified by Southern analysis of plasmid profiles resolved by PFGE by using a *nifH* probe and are indicated in Table 3. Most *R. gallicum* isolates harbored 550-kb pSym plasmids; the single exception was isolate IE2751, which harbored a 525-kb pSym. The sizes of the pSym plasmids of *R. etli* isolates ranged from 350 to 550 kb. One-half of the *R. etli* isolates contained a 390-kb pSym, like the *R. etli* type strain CFN42. In general, pSym plasmids of the same size were associated with identical or similar plasmid profiles; for example, isolates with profiles P2 and P3 harbored 390-kb pSym plasmids. The only exception was isolate IE4874, which contained a 450-kb pSym, whereas the other two isolates with profile P1 contained 550-kb pSym plasmids (Table 3).

Determination of *nifH* copy number by Southern hybridization. For the 34 isolates analyzed, six different hybridization patterns were recorded (Table 3). All *R. gallicum* isolates exhibited a single hybridization signal, and most of them shared an ~8.3-kb hybridizing band (pattern C1) with *R. gallicum* bv. gallicum strains R602sp and FL27 (Table 3), supporting classification of our isolates as members of *R. gallicum* bv. gallicum. All *R. etli* isolates contained multiple (two or three) *nifH* copies, and two groups of related patterns were observed. Patterns A1 and A2 had two bands with sizes of ~12.4 and ~16.6 kb, while patterns B1 and B2 had two bands with sizes

TABLE 4. Average numbers of nodules formed by 10 *R. gallicum* and 24 *R. etli* isolates on three leguminous hosts[a]

Organism	No. of nodules formed on:		
	P. vulgaris	*M. atropurpureum*	*L. leucocephala*
R. gallicum	12.6 ± 3.1 (F)[b]	15.1 ± 1.2 (F)	5.3 ± 1.1 (F)
R. etli	36.6 ± 3.4 (F)	4.5 ± 0.75	0.0

[a] See Table 3.
[b] Average ± standard error. (F), nitrogen fixation (acetylene reduction was detected in nodules).

of ~5.6 and ~9.8 kb; the latter pattern was identical to that displayed by type strain *R. etli* CFN42 (Table 3).

Within the *R. gallicum* and *R. etli* populations, *nifH* hybridization patterns could be found scattered throughout the different branches of the MLEE and plasmid profile dendrograms, further supporting the conclusion derived from the comparative cluster analyses of MLEE and plasmid profile data that lateral transfer of plasmids occurs within each species but not between the species. However, in some instances an association between plasmid profile and *nifH* pattern within species was observed. For example, all the *R. etli* isolates that had a P1 plasmid profile displayed a type A1 *nifH* hybridization pattern, as summarized in Table 3. Notable associations between pSym size and *nifH* hybridization pattern were also found. For example, *R. etli* isolates with the A1 *nifH* pattern harbored 450- to 550-kb pSym plasmids, the A2 *nifH* patterns were associated with 390- to 425-kb pSym plasmids, and the B1 and B2 *nifH* patterns were strictly associated with 390-kb pSym plasmids (Table 3). *R. gallicum* isolates with 550-kb pSym plasmids yielded only the C1 *nifH* pattern, while a 525-kb pSym was associated with the distinct C3 *nifH* pattern. A 550-kb pSym was detected in the *R. etli* and *R. gallicum* populations, but the *nifH* hybridization patterns revealed that these plasmids were distinct.

Plant nodulation tests and symbiotic effectiveness. All 34 selected isolates (Table 3) nodulated and fixed nitrogen with *P. vulgaris* plants (Table 4). However, *R. gallicum* isolates induced only one-third of the nodules induced by *R. etli* isolates. All *R. gallicum* isolates formed effective nodules on *M. atropurpureum*, whereas *R. etli* isolates induced less than one-third of the nodules induced by *R. gallicum* isolates on this host and were ineffective (Table 4). All *R. gallicum* isolates nodulated and fixed nitrogen on *L. leucocephala* plants, further supporting classification of these isolates as *R. gallicum* bv. *gallicum*, while all *R. etli* isolates were unable to nodulate this host (Table 4).

PCR-RFLP analysis of the 16S rRNA gene. Almost complete 16S rRNA genes (~1.5 kb) for 34 isolates were PCR amplified and digested with endonucleases *Sau*3AI, *Msp*I, and *Pst*I. Four different composite restriction patterns were found (Table 3). *R. gallicum* isolates displayed three different patterns (AAA, AAB, and ABA); pattern AAB was identical to the pattern of *R. gallicum* FL27, while pattern AAA was identical the pattern of *R. gallicum* R602sp. All *R. etli* isolates displayed the same restriction pattern (BAB), which was identical to the pattern of *R. etli* CFN42 (Table 3). The restriction sites for each enzyme were mapped on the *rrs* sequence of *R. gallicum* R602sp (accession number U86343) and are indicated in Table 3. The patterns displayed by other *Rhizobium* reference species were different from those of our isolates (data not shown).

Nucleotide sequence analyses. Partial sequences of two chromosomally encoded genes (*rrs* and *glnII*) and two pSym-encoded genes (*nifH* and *nodB*) were determined for *R. gallicum* isolate IE988 (ET12 in Table 1 and Fig. 1) and *R. etli* isolate IE2730 (ET1 in Table 1 and Fig. 1) and, when the sequences were not available in public sequence databases, also for *R. gallicum* strains R602sp and FL27.

The 558-bp *rrs* sequence segment of isolate IE988 was very similar to that of strain R602sp (Table 5). However, the level of similarity with the R602sp and FL27 sequences reported by Sessitsch et al. (44) was lower, due to the presence of several insertions which are absent in the R602sp sequence reported by Amarger et. al. (3) and in our IE988 sequence. The *rrs* sequence of isolate IE2730 differed only at two nucleotides from the *rrs* sequence of *R. etli* CFN42 (Table 5).

A 540-bp *glnII* sequence segment was obtained for isolates IE988 and IE2730 and for strains R602sp and FL27. The highest levels of similarity were found between isolate IE988 and strains FL27 and R602sp, while isolate IE2730 showed the highest level of similarity with *R. etli* CFN42 (Table 5).

The 389-bp *nifH* sequence segment obtained for isolate IE988 was most similar to the *nifH* sequence segments of strains R602sp and FL27, while the *nifH* sequence of isolate IE2730 was identical to the sequence of the three paralogous *nifH* copies of *R. etli* CFN42 (Table 5).

An almost complete *nodB* sequence segment (571 bp) was obtained for isolates IE988 and IE2730 and for strains R602sp and FL27. The *nodB* sequence segment of IE988 was very similar to the *nodB* sequence segments of strains R602sp and FL27, while the *nodB* sequence of IE2730 was identical to the *nodB* sequence of *R. etli* CFN42 (Table 5).

Taken together, these results strongly support the taxonomic placement of isolate IE988 in *R. gallicum* bv. gallicum and the taxonomic placement of isolate IE2730 in *R. etli* bv. phaseoli.

DISCUSSION

In this study we analyzed the genetic structure of rhizobial populations nodulating *P. vulgaris* plants growing in a traditionally managed milpa plot in San Miguel Acuexcomac, Mexico, over three consecutive years. This site was selected for our study because of its long history of bean cultivation that extends back to pre-Columbian times (47). In a previous study of the genetic structure of rhizobial populations associated with beans grown in six milpa plots at this site, low levels of genetic differentiation among the plots (G_{st} = 0.072) were found, and five dominant ETs were recovered from all six plots, indicating that the population structure was stable at the spatial scale analyzed (45). Based on these findings, we decided to sample a single representative plot to study the temporal stability of the genetic structure, as well as to gain insight into the structure and dynamics of the plasmidic compartment, an issue that has not been addressed previously.

The three dominant *R. etli* ETs identified in the present work correspond to those found in the previous study (45), indicating that the genetic composition of the populations at this site is stable both in terms of space (six plots sampled in a single year [45]) and in terms of time (one plot sampled over 3

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years [this study]). Furthermore, the two distinct lineages that were consistently recovered over the 3 years sampled correspond to the previously described genetic divisions I and III (45). Linkage disequilibrium analyses confirmed that recombination takes place within but not between these lineages. Importantly, the set of molecular markers analyzed in this study, along with the host range experiments, unambiguously placed our division I population in *R. gallicum* bv. gallicum and the division III population in *R. etli* bv. phaseoli. Therefore, the two lineages correspond to two distinct species and are not compartments within an *R. etli* population, as suggested previously (45). As a consequence, we concluded that the genetic structure of the *R. etli* and *R. gallicum* populations at this site is epidemic but not reticulated, as defined by Maynard Smith and colleagues (32).

Strong evidence that there is genetic exchange within but not between the *R. etli* and *R. gallicum* populations was also provided by the analysis of the plasmid compartment. Both the plasmid profiles and the *nifH* hybridization patterns provided evidence that there is plasmid transfer among distinct multilocus genotypes within each species (Fig. 1 and Table 3). The panmictic plasmid transfer pattern (48) found for both species could have been the result of the selective pressure imposed by agricultural practices, as suggested by Wernegreen and colleagues (57), or may have been an intrinsic feature of these *Rhizobium* species. The evidence that recombination took place also in the chromosomal compartment, as demonstrated by the linkage disequilibrium analyses, favors the latter possibility. Taken together, these results indicate that genetic exchange is an important source of variation and cohesion in the ecology and evolution of the two species, although it is not great enough to prevent the emergence of epidemic clones that are recovered from nodules at a high frequency. This finding may be interpreted as evidence that there is strong selective pressure imposed by the host, which favors the maintenance of particular chromosome-plasmid associations. It will be interesting to test whether the epidemic clones are particularly competitive for nodulation or if they are simply numerically dominant clones in the soil. It is important that both species nodulate the same individual plants, indicating that mechanisms other than host, spatial, or temporal isolation may account for the genetic barrier between them.

The 34 strains selected for molecular and host range analyses revealed that the subsample of 10 *R. gallicum* isolates constitutes a diverse lineage that displays three of the four 16S PCR-RFLP patterns detected, three different *nifH* hybridization patterns, and two pSym sizes (Table 3). In addition to *P. vulgaris*, these isolates effectively nodulated the other two hosts tested (Table 4). On the other hand, the 24 *R. etli* isolates effectively nodulated only *P. vulgaris*. All of the isolates displayed the same 16S PCR-RFLPs, but they harbored six pSym size classes and displayed four *nifH* hybridization patterns. The comparison of plasmid profiles, *nifH* hybridization patterns, and pSym sizes revealed the complex and dynamic structure of the plasmidic compartment within species. Generally, pSym plasmids that were the same size and had identical *nifH* hybridization patterns were associated with identical plasmid profiles. However, there were some exceptions that might indicate the existence of genetic rearrangements which affect the plasmidic compartment. These rearrangements can operate

TABLE 5. Numbers of pairwise nucleotide differences and percentages of similarity for aligned *rrs, glnII, nifH,* and *nodB* gene segments of the *R. gallicum* IE988 and *R. etli* IE2730 isolates compared with reference strains

Strain	No. of nucleotide differences (% of similarity)												
	rrs gene				*glnII* gene			*nifH* gene			*nodB* gene		
	R. gallicum R602sp (U86343)[a]	*R. gallicum* R602sp (AF008130)[a]	*R. gallicum* FL27 (AF008129)[a]	*R. etli* CFN42 (U28916)[a]	*R. gallicum* R602sp (AF529015)[b]	*R. gallicum* FL27 (AF529016)[b]	*R. etli* CFN42 (AF169585)[b]	*R. gallicum* R602sp (AF218126)[a]	*R. gallicum* FL27 (M55226)[a]	*R. etli* CFN42 (M15942)[a]	*R. gallicum* R602sp (AF529022)[b]	*R. gallicum* FL27 (AF529021)[b]	*R. etli* CFN42 (M58626)[a]
IE988[c]	5 (99.1)[e]	15 (97.3)	15 (97.3)	11 (98.0)	10 (98.1)	7 (98.7)	68 (86.4)	5 (98.7)	10 (97.1)	13 (96.7)	4 (99.3)	6 (98.9)	109 (80.9)
IE2730[d]	12 (97.8)	20 (96.4)	18 (96.8)	2 (99.6)	73 (86.4)	70 (87.0)	25 (96.0)	15 (97.2)	14 (96.1)	0 (100)	108 (81.1)	109 (80.9)	0 (100)

[a] The number in parentheses is the accession number of the sequences retrieved from GenBank.
[b] The number in parentheses is the accession number of the sequence generated in this study.
[c] The accession numbers for *rrs, glnII, nifH,* and *nodB* gene segments of *R. gallicum* IE988 are AF529011, AF529013, AF529017, and AF529019, respectively.
[d] The accession numbers for *rrs, glnII, nifH,* and *nodB* gene segments of *R. etli* IE2730 are AF529012, AF529014, AF529018, and AF529020, respectively.
[e] Pairwise nucleotide difference, including gaps (percentage of similarity).

within particular strains, as extensively documented for CFN42 and NGR234 (7, 15, 38, 39), and may be coupled with lateral transfer within populations, as proposed in the present study.

Only 14% of all of the isolates examined in this study are *R. gallicum* isolates. This is in good agreement with the results of a previous study (45), in which it was reported that 10% of the isolates obtained from *P. vulgaris* corresponded to genetic division I (*R. gallicum*). In that study, however, *R. gallicum* isolates were recovered from *P. coccineus* nodules at nearly the same frequency (54%) as *R. etli* isolates. These results indicate that *R. gallicum* and *R. etli* nodulate both hosts, although the latter species is clearly more competitive for *P. vulgaris* nodulation. Thus, although *R. gallicum* was originally isolated from *P. vulgaris* nodules in France (3), this plant may not be its primary host. This possibility is further supported by the report that *R. gallicum* strains were isolated in Canada from *Onobrychis viciifolia* and *Oxytropis riparia* (tribe Galegae), suggesting that not all potential hosts for this species have been identified yet (26).

Besides its broad host range, *R. gallicum* has a wide geographic distribution, which raises a question about its biogeography. *R. gallicum* populations have been isolated from bean nodules in several European countries and Tunisia (3, 21, 33), and only one American *R. gallicum* bean isolate (strain FL27) has been reported previously (44). The present study provides the first report of an *R. gallicum* population nodulating beans in America, and it is the first population genetic analysis for the species that was performed. Sessitsch and colleagues (44) reported that the European *R. gallicum* strains can be distinguished from the Mexican FL27 strain by an RFLP resulting from a single nucleotide difference in the 16S rRNA gene, which created a *Pst*I restriction site that was absent in all the French and Austrian isolates which they analyzed but was present in the Mexican strain FL27. However, this restriction site was absent in 60% of our *R. gallicum* isolates (Table 3) and hence cannot be used to distinguish European *R. gallicum* strains from American *R. gallicum* strains. Furthermore, the plasmid profiles and nucleotide sequences of chromosomal and plasmidic genes of the Mexican population were very similar to those of *R. gallicum* type strain R602sp (in some instances even more similar than they were to those of Mexican strain FL27). These observations suggest that if *R. gallicum* was imported from America to Europe, as previously suggested (44), the lineages that gave rise to the European isolates remain in America and that the divergence between intercontinental populations is less pronounced than the divergence suggested by Sessitsch et al. (44). The presence of *R. etli* and *R. gallicum* in Tunisian soils recently cropped with beans and the absence of these organisms in fields which had not been cultivated with beans (33) further support the hypothesis that *P. vulgaris* microsymbionts were introduced by being carried along with bean seeds (36). Interestingly, *R. gallicum* bv. phaseoli was not present in the isolates from San Miguel Acuexcomac, even though *R. etli* bv. phaseoli and *R. gallicum* bv. gallicum may have coexisted at this site for centuries. One possible explanation for this is that *R. gallicum* bv. phaseoli may be outcompeted by *R. etli* bv. phaseoli and *R. gallicum* bv. gallicum strains. An alternative explanation is that since *R. leguminosarum* has not been found in Mexico, the hypothesized bridge for the conjugal transfer of pSym from *R. etli* bv. phaseoli to *R. gallicum* is missing (3). Perhaps the introduction of common beans along with their seed-borne symbionts to other continents exerted selective pressure and provided the ecological opportunity for lateral transfer of symbiotic information to resident *Rhizobium* populations, as in the case of the symbiotic island acquired by saprophytic *Mesorhizobium* spp. native soil populations in New Zealand (52).

In conclusion, our data are consistent with the biogeographical hypothesis of an American origin of the *R. gallicum* lineage (44), a view that is also consistent with the report of Canadian *R. gallicum* isolates obtained from *O. viciifolia* and *O. riparia* (26). Alternatively, however, *R. gallicum* could have a wide geographical distribution and a long evolutionary history of adaptation to different environments and leguminous hosts. Further research is clearly needed to examine these alternative hypotheses. We are currently analyzing the sequences of several chromosomal and plasmidic genes of *R. gallicum* isolates from different continents and hosts in order to elucidate the phylogeographic origin and dispersal pathways of this species.

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