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ESTRUCTURA GENETICA DE *Rhizobium etli* EN SAN MIGUEL ACUEXCOMAC, PUEBLA, MEXICO.

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RESUMEN

En esta tesis se realizó una análisis basado en electroforesis de isoenzimas para analizar la variación genética de *Rhizobium etli* bv. *phaseoli* de nódulos de *Phaseolus vulgaris* y *P. coccineus* . Los aislados se colectaron de seis parcelas manejadas tradicionalmente en San Miguel Acuexcomac, Puebla. Se encontró una diversidad genética alta (H= 0.531), y entre los 458 aislados se encontraron 126 genotipos diferentes. Se detectó un grado de diferenciación genética alto entre las parcelas y las localidades muestreadas. La mayor parte de la variabilidad genética se encontró entre los aislados que nodulan a cada planta. El análisis de grupos mostró dos linajes divergentes, separados a una distancia de 0.7. El análisis de desequilibrio de ligamiento realizado a los niveles jerárquicos mostró desequilibrio significativo. Sin embargo, cuando se realizó en análisis para los genotipos de los dos linajes genéticos se encontró equilibrio de ligamiento. Se propone una estructura genética reticulada y epidémica para *R. etli* en San Miguel, en la cual algunos genotipos aumentan en frecuencia para producir clonas epidémicas y el intercambio genético ocurre principalmente entre los genotipos dentro de cada linaje.

ABSTRACT

Although several studies have described the genetic structure of *Rhizobium* spp., little is known about the influence of traditional farming on the genetic structure of this nitrogen fixing bacteria. We conducted a MLEE based study to analyze genetic variation at 10 enzyme-encoding chromosomal loci in 482 *Rhizobium etli* bv. *phaseoli* isolates, sampled hierarchically from the root nodules of *Phaseolus vulgaris* and *P. coccineus* bean plants. The isolates were recovered from six traditionally managed agricultural plots in two localities in the State of Puebla, in Central Mexico. The total mean genetic diversity (H_e) for the six plots was 0.531. Among the 482 isolates collected, 126 distinctive multilocus genotypes (ETs) were obtained, where about half of the isolates are represented by 5 ETs. A significant degree of genetic differentiation among the six plots (G_{st}= 0.072) and between the two localities (G_{st}= 0.022) was detected. The main part of the observed variability (70%) was found within the isolates of the plants. The cluster analysis revealed two deeply diverging lineages, separated at a genetic distance of 0.7. When a multilocus linkage disequilibrium analysis was performed at the sampled hierarchical levels, we found significant linkage disequilibrium, but when the analysis was done for the genotypes within the two diverging lineages, we found evidences of recombination. We propose for *R. etli* bv. *phaseoli* a reticulated and epidemic genetic structure, in which few genotypes increase in frequency to produce epidemic clones, and genetic exchange occurs mainly among genotypes within each lineage.

INTRODUCCION

Con la agricultura moderna se ha simplificado la estructura del ambiente sobre grandes áreas, substituyendo la diversidad del ambiente natural por unas cuantas plantas cultivadas. Este proceso de simplificación alcanza su extremo en el caso de los monocultivos. El objetivo de la simplificación es aumentar la proporción de energía solar fijada que queda disponible para el humano. El resultado neto es un ecosistema artificial que requiere constante intervención humana para su operación. Aunque los agroecosistemas modernos han demostrado su capacidad de sostener grandes poblaciones de cultivos, hay considerable evidencia sobre la fragilidad de estos ecosistemas. La estrategia agrícola de estos sistemas es vista como el contrario de la secuencia sucesional en la naturaleza, se crean y mantienen estados sucesionales tempranos. A pesar de su alto rendimiento, tienen las desventajas de los ecosistemas inmaduros, en especial la falta de habilidad para llevar a cabo funciones de protección en términos de reciclaje de nutrientes, conservación de suelos y regulación de poblaciones (Altieri 1986). En los monocultivos se tiende a plantar variedades genéticamente uniformes. Un ejemplo de esto son las prácticas adoptadas a partir de la llamada "revolución verde", en las que se sembraron variedades únicas de alto rendimiento en lugar de las diferentes variedades tradicionales (Merrick 1990).

Existe gran variabilidad en el grado de diversificación, estabilidad, control humano, eficiencia energética y productividad entre los diferentes tipos de agroecosistemas. Para mantener niveles de producción altos, los ecosistemas modernos requieren considerablemente más control ambiental que los sistemas agrícolas tradicionales. Los sistemas modernos requieren del aporte de grandes cantidades de energía desde fuera del sistema para llevar a cabo el trabajo hecho por los procesos ecológicos en los sistemas menos perturbados. Aunque los monocultivos modernos pueden ser muy productivos, los policultivos tradicionales son más estables ecológicamente y eficientes económicamente, ya que requieren pocos insumos(Altieri 1986).

La estabilidad o resilencia del agrosistema tradicional así como su sustentabilidad (esta ha sido definida como la capacidad de utilizar un recurso renovable sin mermar su potencial de renovarse) está basada en la diversidad de los cultivares. Una de las ventajas de sembrar diversos genotipos de un cultivo es la oportunidad de explotar más eficientemente diferentes microhábitats y aumenta la estabilidad del rendimiento. Existe evidencia de que periódicamente ocurren ciclos de hibridización e introgresión entre los cultivos y sus contrapartes silvestres (Piñero y Eguiarte 1988), lo cual es una fuente de diversificación genética. Los genes derivados de la hibridización con parientes silvestres han contribuido a aumentar la adaptación local de las plantas cultivadas después de su introducción a ambientes nuevos (Merick 1990). En los sistemas tradicionales se fomenta la práctica de labranza mínima como metodología de cultivo. Los sistemas de mínima labranza reducen la entrada de materiales y energía requeridos y abaten la erosión del suelo. Además, como el resto de las prácticas agrícolas tradicionales, el gasto de tiempo, dinero y esfuerzo es mucho menor que en los sistemas modernos (Altieri 1986). La labranza mínima ha sido definida como una práctica agrícola que favorece la conservación del suelo (Altieri, 1986; Merick 1990). Ya que bajo este sistema, los residuos de la cosecha son dejados en la superficie del suelo, reduciendo la pérdida de humedad y proveyendo continuamente de sustrato para los microorganismos. Debido a que la labranza mínima aporta un ambiente más favorable para la biota del suelo se favorece la descomposición de la hojarasca. En ellos la entrada de materiales y la liberación de nutrientes es más gradual que en los agrosistemas donde hay roza-tumba-quema. Bajo estos agrosistemas, el crecimiento de las raíces se ve estimulado por el aumento de la actividad de los invertebrados terrestres como las lombrices (Altieri 1986).

El conocimiento de las asociaciones benéficas entre los componentes del sistema a través de los cultivos múltiples han creado sistemas que aprovechan mejor los recursos, mejoran la productividad global, amortiguan el efecto nocivo de las plagas y conservan el ecosistema. La producción se enfoca en la sustentabilidad del sistema

a largo plazo, más que enfatizar la maximización de la producción (Gliessman 1986).

Un sistema de cultivo múltiple muy estudiado es el policultivo tradicional de maíz, frijol y calabaza; también llamado sistema de milpa. La producción total de esta mezcla es mayor por unidad de área que la de los monocultivos respectivos (Gliessman 1990). La presencia de las plantas de calabaza ayudan a controlar las hierbas, ya que sus hojas anchas y horizontales bloquean la luz solar y la lluvia lixivia de sus hojas sustancias alelopáticas que inhibe el crecimiento desmedido de hierbas (Gilessman 1990). El rendimiento del maíz medido como toneladas de grano por hectárea, es 50% superior cuando se siembra con frijoles que en monocultivo. Los frijoles en policultivo con maíz nodulan más y fijan más nitrógeno, debido a que el maíz absorbe el nitrógeno disponible del suelo y aumenta la eficiencia de la nodulación al eliminar la inhibición por nitratos (Brockwell *et al.*, 1995). Se han observado ganancias netas de nitrógeno en el ecosistema cuando éstos cultivos se asocian, a pesar de la remoción de este elemento con la cosecha. Esto contribuye a la reducción de la dependencia en fertilizantes nitrogenados y provee una base estable para manejar los recursos dentro del sistema (Gilessman 1990).

El nitrógeno es uno de los componentes mas importantes para todos los seres vivos, ya que es elemento estructural de proteínas y ácidos nucleicos. En la biosfera el principal reservorio de nitrógeno se encuentra en la atmósfera como nitrógeno gaseoso (N_2). El cual es un gas inerte, por lo que el aporte de nitrógeno para los ecosistemas viene dado principalmente por el proceso de fijación biológica de nitrógeno. Los únicos organismos capaces de fijar nitrógeno son las bacterias. Estos microorganismos fijadores de nitrógeno son productores de amonio, el cual es incorporado por diversas vías a la cadena trófica, de tal forma que su aporte es de beneficio indirecto al hombre. Algunos de estos microorganismos tienen gran importancia ecológica, actuando como colonizadores pioneros en ambientes extremos y en ecosistemas pobres en nitrógeno (Sprent y Sprent 1990). En el grupo de las eubacterias fijadoras de nitrógeno existen algunas especies que se asocian con organismos eucariontes, como son la asociación entre las cianobacterias

fijadoras de nitrógeno con hongos para dar lugar a los líquenes, la asociación entre el actinomiceto *Frankia* y las plantas actinorrícicas, así como la asociación entre las bacterias del género *Rhizobium* y las plantas de la familia Leguminosae (Sprent y Sprent 1990). Debido al gran aporte alimenticio de las legumbres en la dieta de la humanidad y su importancia económica la asociación *Rhizobium*-leguminosa ha sido estudiada intensamente.

La estructura genética de las especies de *Rhizobium* ha sido estudiada desde hace más de una década. Los trabajos de Young (1985), Young *et al.* (1987) y Harrison *et al.*, (1989a, 1989b) fueron los primeros en utilizar la técnica de electroforesis de isoenzimas para detectar la variación genética en poblaciones naturales de *Rhizobium*. Algunos han abordado la estructura genética de *Rhizobium* utilizando colecciones de origen geográfico diverso (Piñero *et al.*, 1988; Eardly *et al.*, 1990; Martínez *et al.*, 1991). Otros han comparado la estructura genética de diferentes poblaciones en el espacio (Harrison *et al.*, 1989a; Souza *et al.*, 1994; Strain *et al.*, 1995; Hagen *et al.*, 1996) y en el tiempo (Souza *et al.*, 1994; Demezas *et al.*, 1995; Hagen *et al.*, 1996; Wilson *et al.*, 1998), así como la estructura en el suelo y en los nódulos (Young *et al.*, 1987; Segovia *et al.*, 1991; Bromfield *et al.*, 1995; Wilson *et al.*, 1998).

En todos estos estudios se ha hecho patente que los niveles de variación genética en *Rhizobium* son altos (H=0.426 - 0.689), aunque también se ha evidenciado que el número de genotipos es restringido (Young *et al.*, 1987; Harrison *et al.*, 1989a). Es decir, existe gran variación alélica, pero sus combinaciones exitosas están restringidas. En algunas especies se han encontrado genotipos cosmopolitas (Harrison *et al.*, 1989a; Eardly *et al.*, 1990; Martínez *et al.*, 1991), y en la mayoría de los estudios a nivel regional y local se han encontrado genotipos compartidos (Harrison *et al.*, 1989a; Young 1985; Strain *et al.*, 1995; Hagen *et al* .,1996). Aunque la frecuencia de estos genotipos diseminados suele variar no sólo en el espacio sino en el tiempo (Souza *et al.* 1994; Hagen *et al* 1996; Wilson *et al.* 1998). Generalmente, mientras más cercanas son las poblaciones, ya sea en el espacio o en el tiempo, comparten mayor proporción de genotipos (Strain *et al.* 1995; Hagen *et al.*, 1996). Sin

embargo, con frecuencia una alta proporción de los aislados locales representan genotipos únicos. Lo cual sugiere una estructura genética compuestas de genotipos cosmopolitas, que le dan cohesión a la especie, y genotipos locales producto de la diversificación ecológica. El hecho de encontrar genotipos cosmopolitas o al menos compartidos entre regiones geográficas diferentes, sugiere que existe una alta tasa de migración. Los mecanismos de dispersión de *Rhizobium* spp. no se conocen con precisión, sin embargo, existe evidencia de que el transporte de semillas puede ser un vector de dispersión importante (Pérez-Ramírez *et al.* en prensa).

En cuanto a las estimaciones de los niveles de intercambio genético dentro de las especies, los primeros trabajos indicaron una estructura genética subdividida en linajes clonales (Young 1985; Young *et al.*, 1987; Harrison 1989a, 1989b). El encontrar sólo a una pequeña fracción de las combinaciones genotípicas posibles, sugirió que éstas combinaciones representan genotipos coadaptados y que la recombinación es rara o que los genotipos recombinantes tiene menor adecuación (Young *et al.* 1985, 1987; Harrison *et al.*, 1989a, 1989b). Sin embargo estudios posteriores han encontrado que la fuente de desequilibrio de ligamiento tiene un componente de aislamiento genético más que geográfico (Eardly *et al.*, 1990; Strain *et al.*, 1995). En algunos trabajos se ha encontrado un nivel de variación genética muy alto para la especie y distancias genéticas elevadas cuando se consideran todos los genotipos. Sin embargo, la diversidad genética dentro de los grupos genéticos formados es menor, así como su distancia genética (Eardly *et al.*, 1990; Martínez *et al.*, 1991; Demezas *et al.*, 1995; Strain *et al.*, 1995). En algunos casos al analizar el desequilibrio de ligamiento dentro de los grupos genéticos se ha encontrado que las principales divisiones filogenéticas están en equilibrio de ligamiento (Eardly *et al.*, 1990; Demezas *et al.*, 1995) o que algún grupo genético es la fuente de desequilibrio para esta población (Strain *et al.*, 1995).

Por otra parte, Bromfield *et al.*, (1995) encontraron que los aislados de suelo y de nódulos compartían sólo la mitad de sus genotipos, además encontraron diferencias en la frecuencia de los genotipos compartidos, debido principalmente a que uno de los

genotipos predominó en los nódulos. Estos estudios indican que generalmente en los nódulos se encuentra una estructura de tipo epidémica, en la que unas cuantas clonas dominan la población de los nódulos. La explicación de este comportamiento epidémico en la población de los nódulos es multifactorial, e involucra aspectos como competencia intraespecífica por la nodulación, selección por parte del hospedero y efecto fundador de una fracción de la población (Brockwell *et al.*, 1995). Además el ambiente ecológico del sitio, como son el clima y el tipo de suelo, así como la presencia de plantas hospederas modifican las características de la interacción.

Por todo lo mencionado anteriormente, es importante conocer el impacto que ha tenido la agricultura sobre las poblaciones de *Rhizobium* . Esto es relevante, ya que en general las poblaciones de *Rhizobium* asociadas a plantas silvestres, presentan una estructura genética altamente dominante, con el caso extremo de una planta nodulada por un solo genotipo, mientras que las plantas cultivadas frecuentemente se encuentran noduladas por diferentes genotipos (Souza *et al.*, 1994). Lo que hace suponer que la especificidad por el hospedero y adaptación a las condiciones locales del suelo se ha ido perdiendo conforme el proceso de domesticación ha avanzado. Para observar plantas domesticadas que no hayan perdido su especificidad con sus simbiontes, es necesario estudiar a los sistemas tradicionales, ya que en ellos el ecosistema se ha mantenido estable por cientos de años, fomentando la coadaptación de linajes de frijol con su simbionte *R. etli*.

Todos los trabajos anteriores que abordan la estructura genética de *R. etli* han analizado cepas aisladas de campos de monocultivo de frijol (Souza *et al.*, 1994; Segovia *et al.*, 1991). Hasta el momento se desconoce cuál ha sido el efecto de la domesticación del frijol y de las diversas prácticas a agrícolas sobre la estructura genética de *Rhizobium* (Martínez-Romero y Caballero-Mellado 1996). El presente trabajo es parte de un proyecto encaminado a conocer el efecto que han tenido estas prácticas sobre la estructura genética de las poblaciones naturales de *R. etli* asociadas a plantas de frijol, en un gradiente de domesticación. En esta tesis se presentan los resultados del estudio de la estructura genética de *R. etli* asociado a plantas

de frijol cultivado bajo el sistema tradicional de milpa en San Miguel Acuexcomac, Puebla. El análisis detallado de los datos se presenta en el artículo titulado *"Reticulated and epidemic population genetic structure of Rhizobium etli bv. phaseoli in a traditionally managed locality in Mexico"*, el cual fue aceptado con correcciones para publicación en la revista Molecular Ecology. Adicionalmente, algunos de los resultados de San Miguel son comparados con los de otra comunidad en Puebla, Calpan, donde se practica el monocultivo de frijol; este trabajo se presenta en el artículo titulado *"Ethnomicrobiology: do agriculltural practices modify the population structure of the nitrogen fixing bacteria Rhizobium etli bv. phaseoli'*, el cual se encuentra en prensa en la revista Journal of Ethnobiology .

Reticulated and epidemic population genetic structure of *Rhizobium etli* biovar *phaseoli* in a traditionally managed locality in Mexico

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Running title: *Rhizobium etli* genetic structure

Abstract

We conducted a multilocus enzyme electrophoresis based study to assess the genetic structure of the nitrogen fixing bacteria *Rhizobium etli* bv. *phaseoli*. We analyzed the genetic variation at 10 enzyme-encoding chromosomal loci of 482 isolates from root nodules of *Phaseolus vulgaris* and *P. coccineus* bean plants. The isolates were recovered from six traditionally managed agricultural plots in two localities in the State of Puebla, in Central Mexico. The total mean genetic diversity (H_e) for the six plots was 0.531. Among the 482 isolates collected, 126 distinctive multilocus genotypes (ETs) were obtained, where about half of the isolates are represented by 5 widespread ETs. A significant degree of genetic differentiation among the six plots (G_{st}= 0.072) and between the two localities (G_{st}= 0.022) was detected. The main part of the observed variability (70%) was found among the isolates of the plants. The cluster analysis revealed two deeply diverging lineages, separated at a genetic distance of 0.7. When a multilocus linkage disequilibrium analysis was performed at different hierarchical levels, we found significant linkage disequilibrium, but when the analysis was done for the genotypes within the two diverging lineages, we found evidence of recombination. We propose for *R. etli* bv. *phaseoli* a reticulated and epidemic genetic structure, in which few genotypes increase in frequency to produce numerically dominant clones, and genetic exchange occurs mainly among genotypes within each lineage.

Introduction

Most studies on the interaction between *Rhizobium* and plants have a strong emphasis on controlled environments and monocultural systems, where influences of other plants are few, due to agricultural management practices (Brockwell *et al.* 1995). Although some studies have been done in the sites of origin and diversification of the symbionts (Eardly *et al.* 1990; Demezas *et al.* 1991; Segovia, *et al.* 1991; Souza *et al.* 1992; 1994), none of them had studied the genetic structure of rhizobia in a traditionally managed agricultural system. This is an interesting question, since the traditionally managed plots are complex plant communities, where interactions among different plant species, at the aerial and root level, are the rule (Gliessman 1986; 1990; Alcorn 1990; Souza *et al.* in press).

In Mexico, the annual bean *Phaseolus vulgaris* and the related perennial species *P. coccineus*, are normally nodulated by strains of the nitrogen-fixing bacteria *Rhizobium etli* bv. *phaseoli* (Segovia *et al.* 1993; Souza *et al.* 1994). *P. vulgaris* and *P. coccineus* bean landraces are traditionally cultivated in an agricultural system known as milpa (Altieri & Merrick 1987; Alcorn 1990). In this system, beans are intercropped with corn and squash, as well as with diverse plant species that are locally used for medicinal and nutritional purposes (Altieri & Merrick 1987; Alcorn 1990; Souza *et al.* in press). The milpa system is an ancient way of bean culture, and probably it is the original ecosystem in which the beans were domesticated by the inhabitants of Mesoamerica (Bush 1986; Altieri & Merrick 1987).

Several studies on the genetic structure of rhizobia associated with beans have been done using collections of isolates from different geographical origins (Piñero *et al.* 1988; Geniaux *et al.* 1993; Laguerre *et al.* 1993). Piñero *et al.*(1988) analyzed *Phaseolus* isolates from various geographic sources, mainly in Mexico, and they described a high genetic diversity and a strong linkage disequilibrium. A number of those Mexican isolates originally classified as *R. leguminosarum* bv. *phaseoli* were reclassified as *R.*

etli bv. *phaseoli*, and also significant multilocus linkage disequilibrium was detected when only *R. etli* bv. *phaseoli* ETs were considered (Souza *et al.* 1992). But as pointed out by Maynard Smith *et al.* (1993), linkage disequilibrium may arise in samples in which strains or populations are geographically isolated. Furthermore, linkage disequilibrium may be minimal in some local populations of bacterial species such as *Bacillus subtilis* (Istock *et al.* 1992), *Bradyrhizobium* sp. (Bottomley *et al.* 1994), *Burkhoderia cepacia* (Wise *et al.* 1995) and *Pseudomonas* (Haubold & Rainey, 1996). In *R. etli*, some studies at a local scale have found lower levels of linkage disequilibrium than the one reported by Piñero *et al.* (1988) (Souza *et al.* 1992; 1994; Gordon 1995), suggesting that recombination occurs locally, and that the previous evidences of clonality were the product of geographical isolation. The reproductive isolation among genetically distant isolates can also be source of linkage disequilibrium (Maynard Smith *et al.* 1993). Evidence of this has been observed in *R. meliloti* from wild *Medicago* in the Mediterranean basin and from cultivated worldwide *Medicago* (Eardly *et al.* 1990). This population structure is described by Maynard Smith *et al.* (1993) as reticulated, "in which recombination does not occur between isolates from the major branches, but frequent recombination occurs between isolates within each major branch". An epidemic structure could be another source of linkage disequilibrium in bacteria (Maynard Smith *et al.* 1993). In these populations, recombination occurs but, occasionally, a highly successful genotype arises and increases in frequency to produce an epidemic clone. Maynard Smith *et al.* (1993) reported an epidemic genetic structure for clinical isolates of *Neisseria meningitidis* collected worldwide.

In the present study we analyzed the genetic structure of native *R. etli* bv. *phaseoli* populations in a traditionally managed locality in Puebla, Mexico. This study was designed with a sampling scheme at different hierarchical levels, and the MLEE data were examined with the purpose of elucidating the importance of the different evolutionary forces in shaping the population genetic structure of *R. etli* bv. *phaseoli* in a complex agricultural system.

Materials and methods

Description of the site and sampling procedure

San Miguel Acuexcomac is a small village located in the state of Puebla, in Central Mexico (98°05'W, 18° 50'N), at 2,100 m above sea level, ca. 36 km from Puebla city. We studied the nitrogen fixing bacteria *Rhizobium etli* bv. *phaseoli* associated with two bean landraces species: *Phaseolus vulgaris* (common bean, "mantequilla" variety) and *P. coccineus* (red runner or ayocote bean) in six traditionally managed agricultural plots. In order to study the geographic differentiation among local populations, the sampled plots were distributed in two localities, one herein called "town", that includes the cultivated plots that are near the village houses, and the other called "field", which includes plots that are ca. 2 km apart from the village (Figure 1). The soil conditions are different between these localities. Town soils have a sandy clay loam texture and higher content of total nitrogen (139.0 vs. 117.2 ppm), nitrates (1.9 vs. 0.9 ppm), organic matter (2.9 vs. 1.9 %) and pH (8.4 vs. 8.0) than field soils. In contrast, field soils have a clay texture and higher content of magnesium (85.0 vs. 17.5 meq/100g) and calcium (431.7 vs. 185.0 meq/100g) than town soils (Souza *et al.* in press; unpublished data).

In each locality, three milpa plots were sampled, plots A, B and C are town plots, and D, E, and F are field plots (Figure 1). The mean distance among the plots of each locality was about 100 m, and the mean distance between the town and field localities was about 2 km. In each plot, 10 plants were randomly chosen (including *P. coccineus* plants when present) at a mean distance ca. 5 m, and fifteen nodules were sampled per plant. The nodules were washed with 15 % sodium hypochlorite, rinsed twice with sterile water, squashed onto Petri dishes with peptone yeast extract medium (PY) and incubated for 2 days at 30 °C (Souza *et al.* 1994). A single colony was restreaked in a new PY Petri dish and incubated for 2 days. In order to obtain pure *R. etli* bv. *phaseoli* isolates, a single colony was restreaked onto PY medium added with nalidixic acid (20 µg/ml)

and incubated for 2 days. As described by Segovia (1993), all the *R. etli* isolates are resistant to nalidixic acid. Each isolate so obtained was kept at -80°C in glycerol peptone liquid medium (UL) (Souza *et al.* 1994). We eliminated from the analysis the plants in which less than 5 isolates could be recovered, to insure a statistically more adequate sample size. Sampling scheme and number of recovered isolates are shown in Table 1.

Multilocus enzyme electrophoresis

Frozen isolates were streaked onto PY plates and incubated for 2 days at 30 °C and then grown in 50 ml PY for a day at 30 °C. Cultures were then centrifuged at 6,000 rpm for 5 minutes, the supernatant was eliminated and the pellets were resuspended in 1 ml of buffer Tris-HCl pH 8. To break bacterial cell walls, 0.1 ml of lysozyme (7.5 mg/ml) were added; the resulting suspension was frozen at -80 °C twice for 15 minutes and centrifuged at 12,000 rpm for 3 minutes. The supernatant, which contains the protein lysate, was distributed into three 0.5 ml plastic tubes and stored at -80 °C.

Electrophoresis were done on acetate cellulose membranes following the procedures recommended by Hebert and Beaton (1993). Six enzymes were assessed: isocitrate dehydrogenase (IDH, EC 1.1.1.42), peptidase (PEP, EC 3.4.13), phosphoglucomutase (PGM, EC 5.4.2.2), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), xanthine dehydrogenase (XDH, EC 1.1.1.204) and malate dehydrogenase (MDH, EC 1.1.1.37). For all of them, the buffer system used was Tris Glycine (TG) pH 8.5 (Hebert and Beaton, 1993). The enzymes IDH, PEP and PGM showed one band of activity, the G6PDH and XDH two bands, and the MDH three bands, yielding a total of 10 analyzed loci.

Genetic diversity

Distinctive mobility variants of each enzyme, numbered in order of decreasing anodal mobility, were considered alleles at the corresponding locus (Selander *et al.* 1986). In the case of enzymes with more than one band, each one was equated with a locus. The

absence of enzyme activity was scored as a null allele and was treated as an ordinary allele. The allele profiles (electrophoretic types or ETs) for the 10 loci analyzed were equated with multilocus genotypes (Selander *et al.* 1986).

Based on allele frequencies for ETs, genetic diversity for an enzyme locus was calculated as $h=(1-\sum x_i^2)[n/(n-1)]$, where x_i is the frequency of the ith allele and n is the number of ETs (Selander *et al.* 1986). The mean genetic diversity per locus (H_e) 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_e values we used the program ETDIV version 2.2 (Whittam 1990). To compare the allelic frequencies in a given locus, we used the Workman and Niswander (1970) chi-square test of heterogeneity.

Genetic differentiation

To estimate the relative genetic differentiation at different levels, we used various modified indices related to Nei's $G_{st}=(H_t-H_s)/H_t$ (Souza *et al.* 1994), where H_t is the expected diversity in an equivalent random mating total population and H_s is the average diversity of the subpopulations. In the case of plot populations, and for total *P. vulgaris* and *P. coccineus* populations, H_s is the average genetic diversity of the plants. In the case of town, field and total populations, H_s is the average genetic diversity of the plots. To compute the G_{st} values we used the program ETDIV version 2.2 (Whittam 1990).

These indices range from 0, if there is no genetic differentiation at a given level, to 1 if there is maximal genetic differentiation (Nei 1987). To test if the G_{st} indices were significant different from 0, we performed a chi-square test of independence as $\chi^2=nG_{st}(a-1)$, where n is the number of individuals and a is the total number of alleles. Degrees of freedom are $(k-1)(a-1)$, where k is the number of subdivisions. Degrees of freedom and χ^2

values were summed across loci and significance was examined at $p<0.05$ (Workman & Niswander 1970; Hagen & Hamrick 1996b).

The amount of gene flow among populations was calculated by substituting Nei's G_{st} for F_{st} in Wright's island model of gene flow (Wright 1951), that describes the effective number of migrants per generation as $Nm=(1-G_{st})/2G_{st}\alpha$, in which $\alpha=(n/(n-1))^2$, where n is the number of populations (Crow & Aoki 1984).

Also, we calculated the average Nei's genetic identities (I) for all pairs of plots based on the allele frequencies for the ETs (Nei 1987).

We performed a variance component estimation using both isolates and ETs. In order to get estimates of the proportion of the variability explained at each sampling level we performed a three level nested ANOVA (Weir & Cockerham 1984; Weir 1996) with the levels: plots, plants within plots, and isolates within plants. These analysis consider the isolates within plants as the error of the ANOVA.

Linkage disequilibrium

To determine the extent to which populations exhibit non-random associations of alleles between loci, we used a multilocus index based on the distribution of allelic mismatches between pairs of isolates over all loci. An allelic mismatch frequency distribution is calculated by comparing each isolate to every other isolate for a total of $n(n-1)/2$ pairwise comparisons, where n is the number of isolates in the population. An equation of the variance of the distribution has been derived by Brown *et al.* (1980) and the procedures have been described in detail elsewhere (Souza *et al.* 1992; Leung *et al.* 1994). The ratio of the variance in mismatches observed in a population (V_O) to the expected variance in a corresponding population at linkage equilibrium (random association of alleles) (V_e), provides a measure of multilocus linkage disequilibrium. If there is no linkage disequilibrium $V_O/V_e= 1$. The significance of the difference between V_O and V_e was calculated using

a Monte Carlo procedure with 1,000 iterations, done with the LDV program (Souza *et al.* 1992).

Cluster analysis

The genetic distance between each pair of different multilocus genotypes (ETs) was estimated as the proportion of loci with different alleles (mismatches). Clustering from a matrix of pairwise mismatched distances was performed by the UPGMA method (unweighted pair-group method with arithmetic mean) (Sokal and Michener 1958). The analysis was done using the program ETCLUS version 2.1 (Whittam 1990). For this analysis we included four reference strains, one of *R. meliloti* (strain 1021, kindly given by Frans de Bruijn, Center for Microbial Ecology, Michigan State University) and three *R. etli* bv. *phaseoli* (strains CFN42, TAL182 and CIAT895, kindly provided by David Romero, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México).

Results

Genetic diversity

Levels of genetic variation of *R. etli* bv. *phaseoli* in San Miguel were high (Table 2) and displayed a high degree of genotype dominance, meaning that among the ETs identified, some of them were represented by many isolates. Among the 482 isolates collected from six plots and 48 plants (Table 1), only 126 distinctive multilocus genotypes (ETs) were found (Table 2). The total mean genetic diversity (H_e) for the six plots was 0.531. All the loci examined were polymorphic, and the mean number of alleles per locus was 4.2.

The plots showed a genetic diversity H_e ranging from 0.327 to 0.549 (Table 2; plot F and E, respectively). We found that the mean genetic diversity for the town plots (H_e= 0.523) was higher than for the field plots (H_e= 0.481). In Table 2, it can be observed that the mean number of alleles and genetic diversity were higher for the plots in which *P. coccineus* plants were sampled, than for the plots with only *P. vulgaris*. These results suggest that the *P. coccineus* plants nodulate with a wider range of *R. etli* bv. *phaseoli* genotypes. This is also reflected in the *P. coccineus* ET/isolates ratio (0.48), that is twice the *P. vulgaris* ET/isolates ratio (0.24).

Of the 126 ETs found from the 485 isolates, 85 were represented by only one isolate, comprising 67.5% of the ETs and 17.6% of the isolates. Of the 41 ETs represented by more than one isolate, ETs 7, 9, 11, 13 and 27 (with 26, 42, 70, 34 and 59 isolates, respectively) were shared by all the plots, comprising about half (48%) of the total number of isolates. A chi-square test showed that the frequencies of these five widespread ETs varied significantly among the six plots (χ^2_{20}= 78.2, p<0.005), and for all the pairwise combinations between town and field plots (AD χ^2_4= 19, AE χ^2_4= 22.2, AF χ^2_4= 17.2, BD χ^2_4=2 3.2, BE χ^2_4= 28, BF χ^2_4= 21.7, CD χ^2_4= 19.3 CE χ^2_4= 24.6 CF χ^2_4= 19.7; p<0.005). In contrast, the differences in the frequencies of these widespread ETs among the town plots and among the field plots were not significant. This suggests that the

differences are due to the local characteristics of the plots, but are averaged (and lost) when considering the localities.

Genetic differentiation

The genetic differentiation indices (G_{ST}) are shown in Table 2. The genetic differentiation of the six plots in relation to the total genetic diversity found in San Miguel was significantly different from 0 (G_{ST}= 0.072; Table 2), indicating that a 7.2% of the variability in allele frequencies is attributable to differences among plots. The differences between the two localities accounted for a smaller, but a significant, fraction of the observed variability (G_{ST}= 0.022± 0.007 SE, $p<0.001$; not shown in Table 2). We also found a significant degree of differentiation among town (G_{ST}= 0.062) and field (G_{ST}= 0.046) plots (Table 2).

At the plant level, none of the mean genetic differentiation values were significant; the G_{ST} ranged from 0.015 to 0.135 (plot D and plot C, respectively; Table 2), indicating considerable variation. When the plants of the two bean species were analyzed separately, we found that the bacteria associated to *P. coccineus* plants showed a higher, but non significant, degree of differentiation than the *P. vulgaris* plants (Table 2).

From the G_{ST} related values, the migration parameter *Nm* was estimated. All the *Nm* at the plot level were relatively high and similar (Table 2). We also found that the number of effective migrants per generation between town and field localities was high (*Nm*= 5.1; not shown in table).

In contrast, the migration parameter at the plant level varied considerably (Table 2). The lowest value was found for the plants from plot C (*Nm*= 2.5) while the highest value was found for the plants from plot D (*Nm*= 24.1). The migration parameter for the total *P. vulgaris* sample was more than five-fold higher than the parameter for the total *P. coccineus* sample (Table 2), suggesting a lower degree of migration among *P. coccineus* plants compared with *P. vulgaris* plants.

The pairwise genetic identities (I) and percentage of shared ETs are shown in Table 3. Despite heterogeneity in allele frequencies among the six plots, the pairwise genetic identity values were very high (mean I= 0.914), with the exception of most comparisons with plot A. This supports our earlier analysis indicating a low degree of differentiation among plots. Moreover, because the identity values were not higher between pairs of plots of the same locality than the pairs among localities (Table 3), we found no evidence of isolation by distance between the two localities. These findings are reinforced by the results of the percentage of shared ETs (Table 3).

The three level nested ANOVA showed that, in the case of isolates, 70.1% of the variability was due to differences among isolates within plants, while less variability can be explained by differences at higher levels: 13.1% is due to differences among plants and a slightly higher proportion (16.8%) to differences among plots. Similar results were obtained when the analysis was done using only ETs. The genetic component of the variance at the isolates level was about 88.3%, the variance due to differences among plants was 5.3%, and finally, the variance due to differences among plots was 6.4%. These results indicate that the main proportion of the variability is found within plants level, rather than at higher local geographic scales.

Linkage disequilibrium at hierarchical levels

Table 4 summarizes the results of the linkage disequilibrium analysis. In general, the V_O/V_e ratios were lower when only the ETs were analyzed, while the mean number of mismatches increased. The V_O/V_e ratio for the 482 isolates was 3.93, while it dropped to 2.27 if done for the 126 ETs; both values are high and significantly different from 1 ($p<0.001$; Table 4), indicating non random association of alleles.

The V_O/V_e ratio for isolates within plots ranged from 1.71 to 4.58 (Table 4; plots F and C, respectively), and when only ETs were taken into account, the value ranged from 1.46 to 3.23 (Table 4; plots D and C, respectively).

The isolates V_O/V_e ratio was higher for the town plots than for the field plots, and higher for *P. coccineus* than for *P. vulgaris* (Table 4). Plots in which both bean species were sampled had higher V_O/V_e ratios and mean number of mismatches than plots in which only *P. vulgaris* was sampled (Table 4); suggesting again that the *P. coccineus* plants nodulate with a wider range of *R. etli* bv. *phaseoli* genotypes than *P. vulgaris*.

In all the cases, the V_O/V_e ratio was significantly larger than 1, indicating significant linkage disequilibrium at all the analyzed hierarchical levels. These results show that *R. etli* bv. *phaseoli* have a clonal population structure at the different spatial and host levels.

Cluster Analysis

The genetic relatedness of the 126 ETs are depicted in a UPGMA dendrogram (Figure 2). The first division of the dendrogram is at 0.9 genetic distance (measured as the proportion of mismatches) separating the *R. etli* bv. *phaseoli* isolates from the *R. meliloti* strain. The *R. etli* bv. *phaseoli* cluster can be divided at a genetic distance of 0.7 in three clusters (Figure 2). Cluster I contains 95 isolates of this study, cluster II contains only the *R. etli* bv. *phaseoli* reference strains, while cluster III contains 387 isolates of this study.

The composition of clusters I and III are explained in Table 5. Cluster I is composed of 19.7% of the total isolates, mainly from the town plots and the *P. coccineus* plants. 53.9% of the *P. coccineus* isolates were within this cluster, while only 10.3% of *P. vulgaris* isolates were represented herein. More than half of the isolates in this cluster belong to plot A (62.1%; Table 5). Even though this cluster is not the largest one, it contains a high proportion of unique ETs (of the 48 ETs in this cluster, 40 were unique), representing 83.3% of the cluster ETs and 47% of the unique ETs in the total sample. This clustering reflects the finding that the genotypes from plot A and from *P. coccineus* plants are the most distinct populations.

Cluster III comprises the biggest part of both the total isolates (80.3%) and the total ETs (61.9%). Almost all the field isolates and the *P. vulgaris* isolates are included here (Table 5). This cluster had a more homogeneous plot distribution than cluster I, since most of the isolates of each plot were found herein, with the exception of plot A (Table 5). Of the 78 ETs in this cluster, only 45 were unique, representing about half (57.7%) of the ETs in the cluster. The five common and widespread ETs were found herein, grouped in two smaller subclusters, named subclusters III.1 and III.2 (Figure 2).

Subcluster III.1 contains the widespread ETs 11, 7 and 27 (with 70, 26 and 59 isolates, respectively), and the unique ET 22 (Figure 2), comprising 32.4% of the total isolates. Subcluster III.2 contains the widespread ETs 13 and 9 (with 34 and 42 isolates, respectively), ET 75 with two isolates, and the unique ET 81 (Figure 2), comprising 16.4% of the total population. The genetic distance within each subcluster was 0.1; ETs within subcluster III.1 had a mismatch in the XDH2 locus, and ETs within subcluster III.2 had an allelic difference in the PGM locus. The separation between these subclusters was due to the presence of different fixed alleles in the G6PDH2 locus, so the number of mismatches between the ETs of these subclusters ranged from 1 to 3, depending on the XDH2 and PGM alleles. These results suggests that these abundant and widespread genotypes are closely related, and could be the result of the diversification of an original successful genotype.

We performed genetic diversity and linkage disequilibrium analysis for both the genotypes and the isolates within clusters I and III (Table 6). Cluster I showed a higher genetic diversity, mean number of mismatches, and V_O/V_e ratios than cluster III, both for ETs and isolates (Table 6). The isolates V_O/V_e ratios for both clusters showed significant linkage disequilibrium, but the ETs V_O/V_e ratios were not significantly different from 1 (Table 6), showing random allele association between loci (Maynard Smith *et al.* 1993). These results indicate that frequent mixis occurs among the genotypes within the two main genetic divisions.

Discussion

Bacteria that nodulate beans in San Miguel are very diverse, globally and within each population. The level of genetic variation found for the total population (*H*= 0.531) is similar to that reported for other *Rhizobium* species (e.g. *H*= 0.493 for *R. leguminosarum* bv. *viciae*, Gordon *et al.* 1995; *H*= 0.426 for *R. leguminosarum* bv. *trifolii*, Hagen and Hamrick 1996a) and for others *R. etli* bv. *phaseoli* populations associated with cultivated *P. vulgaris* (*H*= 0.499 in 1987 and *H*= 0.407 in 1988, Souza *et al.* 1994; *H*= 0.513, Segovia *et al.* 1991).

Recently, Sullivan *et al.* (1995) showed that genetic diversity in *Rhizobium* can be generated by recombination events between local bacteria and an inoculant strain. The authors propose that these new nodulating strains may account for the rapid diversification of rhizobia in the field. In the case of *R. etli* populations, Segovia *et al.* (1991) found a proportion of at least 1 symbiotic strain to 40 nonsymbiotic strains in the soil, and they suggest the participation of these nonsymbiotic populations in the generation of new symbiotic strains with different adaptive traits. In San Miguel, we found a high proportion of genotypes represented by only one isolate (67.3% of the ETs or 17.6% of the isolates), which could be persisting rare genotypes or the outcome of mutation and/or recombination events within the *R. etli* genetic pool. Nevertheless, these unique genotypes may be outcompeted by other genotypes preferentially selected by the host plants. Among the preferred strains are the five widespread and genetically related genotypes that account for almost half of the isolates. These few, very successful genotypes, may be adapted to the global environmental conditions of the site to survive during the drought season, and selected by a wide range of the bean landraces genotypes. However, competitive differences among genotypes under specific ecological conditions could account for their differential abundance between the two localities, probably due to differences in soil composition. For example, ET 1 with 22 isolates, was the third most abundant among town plots, but this ET was not represented in the field plots. It is interesting that this ET belongs to cluster I as most of the genotypes from town plots. We found a slightly higher

average genetic diversity in town plots than in field plots, perhaps indicating that town environmental conditions allow the development of a wider range of *R. etli* bv. *phaseoli* genotypes. A high content of soil organic matter has been correlated with the maintenance of higher rhizobial populations, and maybe higher genetic diversity (E. Martínez-Romero, personal communication). The association between isolate distribution and plot of origin suggests an ecotypic structure, with clones adapted to the local conditions (Maynard Smith 1991; Haubold & Rainey, 1996).

The G_{ST} related values were significant at the higher hierarchical levels (total and locality), although not significantly different from 0 within each plot or at the bean species level. These results indicate that a high proportion of the genetic diversity is found within individual plants. On the basis of the high ET/isolates ratio, mean genetic diversity, G_{ST} value, mean number of mismatches, and *Vo/Ve* ratio, we proposed that the *P. coccineus* plants nodulate with a wider range of *R. etli* bv. *phaseoli* genotypes than the *P. vulgaris* plants. The plot pairwise genetic identity values were very high, indicating that most alleles are shared among the different plots. Identity values and percentage of shared ETs, indicate that plot A is the most differentiated population. An explanation for the different genotypes found in plot A, could be the differences in soil conditions, as a steeper inclination of this terrain can result in higher erosion and lower water retention.

The variance component estimation over the total genetic diversity showed that the main part of the differences were found among the isolates that nodulate the plants, whereas only a small fraction of the variability was explained by differences among plots or plants. This points out that the founder effect of the isolates that nodulate a plant and the genotype-specific selection of the host are important processes that are shaping the local population structure of this symbiotic nitrogen fixing bacteria. This finding is in agreement with the results observed for *R. trifolii* by Hagen & Hamrick (1996a). They suggest that the gene flow acts at a larger scale, while founder effect is important at local scales. Our data supports a high degree of gene flow among plots and localities, but

also among most plants. This may reflect the soil movement due to tillage practices, in contrast with the non cultivated roadside *R. trifolii* populations analyzed by Hagen & Hamrick (1996a). All *Nm* values obtained for the sampled populations are higher than 1, and most were higher than 4, suggesting that the sampled plots may experience sufficient gene flow to prevent substantial genetic divergence and may belong to a single evolutionary unit (Wright 1951).

The total *R. etli* bv. *phaseoli* sampled population is in linkage disequilibrium, indicating clonality. But as pointed out by Maynard Smith *et al.* (1993), linkage disequilibrium may arise by geographic or ecological isolation. In the present study, the linkage disequilibrium analysis of the ETs within the two main genetic clusters, showed evidence of linkage equilibrium, indicating frequent mixis among genetically related genotypes. Since genotypes of both clusters were present in all the sampled plots, these results could explain why significant linkage disequilibrium was found at all the hierarchical levels analyzed. This, together with the high gene flow detected in the population, suggests that geographic separation may not be an important source of linkage disequilibrium. Further suggesting that the linkage disequilibrium found in the sampled populations of *R. etli* bv. *phaseoli* in San Miguel was due to the coexistence of two main genetic lineages sexually isolated.

The fact that for the global San Miguel population we found linkage disequilibrium, but linkage equilibrium for the genotypes within the two main genetic clusters, suggests a reticulate genetic structure (Maynard Smith *et al.* 1993). On the other hand, the fact that the clusters linkage disequilibrium analysis performed with isolates showed disequilibrium, and when performed only with ETs showed linkage equilibrium, points towards an epidemic genetic structure (Maynard Smith *et al.* 1993). These two features brought together point towards a reticulated-epidemic population genetic structure (Figure 3).

Evidence of reticulate genetic structure has been reported for *R. meliloti* and *R. tropici* (Eardly *et al.* 1990; Martínez-Romero *et al.* 1991). As pointed out by Gordon *et al.* (1995) and Lenski (1993),

conclusions derived from the analysis of subgroups will be much stronger when there is independent evidence that the subgroups are biological meaningful entities. In the case of our *R. etli* bv. *phaseoli* isolates, it would be interesting to have additional information that would support the existence of these lineages and establish if these genetic divisions are part of the *R. etli* genetic pool or if they are different species.

This study strongly suggests that in San Miguel the local conditions have shaped a complex genetic structure. Within the *R. etli* genetic pool, mutation and recombination within the two lineages can generate diversity. On one hand, we found ecotypes locally adapted, and on the other, we observed widespread genotypes adapted to the global conditions. The selection imposed by the bean landraces coupled with the founder effect of the different ecotypes, produces an epidemic structure in the nodules, while gene flow prevents genetic divergence within the lineages and maintains the populations as a single evolutionary unit.

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Table 1. Sampling scheme and number of recovered isolates of *R. etli* asssociated with *Phaseolus vulgaris* and *P. coccineus* plants recovered from six plots in two localities (town and field) in San Miguel, Puebla, Mexico.

Plot	No. of Plants	No. of P. v.[¢]	No. of P. c.[£]	No. of Isolates	No. of P. v.[§]	No. of P. c.[ß]
A	10	5	5	94	43	51
B	7	7	0	69	69	0
C	9	8	1	96	85	11
Town	26	20	6	259	197	62
D	7	7	0	77	77	0
E	7	3	4	72	30	42
F	8	8	0	74	74	0
Field	22	18	4	223	181	42
Total	48	38	10	482	378	104

[¢]Number of *Phaseolus vulgaris* plants; [£]Number of *P. coccineus* plants; [§]Number of *R. etli* isolates recovered from *P. vulgaris*; [ß]Number of *R. etli* isolates recovered from *P. coccineus*.

Table 2. Genetic diversity and genetic differentiation estimates at different hierarchical levels for the 10 loci surveyed in *R. etli* associated with *P. vulgaris* and *P. coccineus* plants in two localities (town and field) in San Miguel, Mexico.

Data set	No. of isolates	No. of ETs	No. of alleles[£]	H_e [§]	G_{st}[ß]	Nm[∂]
A	94	46	3.3	0.521 (0.069)	0.068 (0.020)[a)]	5.6
B	69	18	2.7	0.346 (0.068)	0.040 (0.014)[a)]	8.8
C	96	33	3.4	0.473 (0.065)	0.135 (0.033)[a)]	2.5
D	77	27	2.6	0.336 (0.083)	0.015 (0.005)[a)]	24.1
E	72	33	3.5	0.549 (0.054)	0.066 (0.015)[a)]	5.2
F	74	30	2.9	0.327 (0.077)	0.090 (0.035)[a)]	3.9
Town	259	75	3.7	0.523 (0.066)	0.062*(0.015)[b)]	3.4
Field	223	69	3.9	0.481 (0.060)	0.046*(0.019)[b)]	4.6
P. v.[+]	378	91	3.7	0.501 (0.066)	0.045 (0.010)[a)]	10.1
P. c.[‡]	104	50	4.0	0.548 (0.067)	0.173 (0.039)[a)]	1.9
Total	482	126	4.2	0.531 (0.066)	0.072*(0.017)[b)]	4.5

[+]*Phaseolus vulgaris*; [‡]*P. coccineus*; [£]Mean number of alleles; [§]Mean genetic diversity, values in parenthesis represent ± SE; [ß]Genetic differentiation, values in parenthesis represent ± SE; * G_{st} values significanly different from 0 at $p<0.05$; a) Genetic diferenciation at the plant level; b) Genetic differentiation at the plot level; [∂]Efective number of migrants per generation.

Table 3. Nei's genetic identities (*I*) and percentage of shared ETs between pairs of plots[Ø].

Plot	A	B	C	D	E	F	Average*
A		0.811	0.918	0.804	0.891	0.791	0.843
B	10.9		0.955	0.984	0.911	0.978	0.928
C	13.9	21.6		0.934	0.954	0.936	0.939
D	8.2	20.0	13.3		0.922	0.990	0.927
E	7.6	13.7	10.6	13.3		0.929	0.921
F	7.9	16.7	15.9	19.3	12.7		0.925
Average*	9.7	16.6	15.1	14.8	11.6	14.5	

[Ø]Above the diagonal are the genetic identities and below the diagonal are the percentage of shared ETs; * Average over all the comparisons for each plot.

Table 4. Linkage disequilibrium parameters at different hierarchical levels at the 10 loci surveyed for *R. etli* associated with *P. vulgaris* and *P. coccineus* in two localities (town and field) in San Miguel, Mexico.

Data set	Isolates		ETs		P[†]
	mism[π]	*Vo/Ve*[∂]	mism	*Vo/Ve*	<0.001
A	4.7	3.97	5.2	2.25	<0.001
B	1.9	3.13	3.5	2.71	<0.001
C	3.2	4.58	4.7	3.23	<0.001
D	2.3	1.76	3.4	1.46	<0.001
E	4.1	4.23	5.6	3.25	<0.001
F	2.5	1.71	3.3	1.76	<0.001
Town	4.2	4.60	5.2	2.44	<0.001
Field	3.1	3.16	4.8	2.51	<0.001
P. v.[+]	3.1	3.36	5.0	2.30	<0.001
P. c.[‡]	5.0	4.10	5.5	2.91	<0.001
Total	3.8	3.93	5.4	2.27	<0.001

[+]*Phaseolus vulgaris*; [‡]*P. coccineus*; [π]Mean number of mismatches; [∂]Observed variance / expected variance of the mismatch distribution; [†]Probability of rejecting by chance alone the null hypothesis that Vo = Ve, for both isolates and ETs.

Table 5. Distribution of *R. etli* isolates belonging to clusters I and III, as defined in Figure 2.

Data set	Cluster I			Cluster III		
	N∞	%Pop£	%Clu¢	N	%Pop	%Clu
A	59	62.8	62.1	35	37.2	9.0
B	3	4.3	3.2	66	95.7	17.1
C	15	15.6	15.8	81	84.4	20.9
D	2	2.6	2.1	76	98.7	19.6
E	15	20.8	15.8	56	77.8	14.5
F	1	1.4	1.1	73	98.6	18.9
Town	77	29.7	81.1	182	70.3	47.0
Field	18	8.1	18.9	205	91.9	53.0
P.v.+	39	10.3	41.0	339	89.7	87.6
P.c.‡	56	53.8	59.0	48	46.2	12.4
Total	95	19.7	100.0	387	80.3	100.0

+*Phaseolus vulgaris*; ‡*P. coccineus*; ∞Number of isolates in the correponding population; £Percentage of the corresponding population; ¢Percentage of the cluster.

Table 6. Genetic diversity and multilocus linkage disequilibrium estimates for R. etli within clusters I and II, as defined in Figure 2.

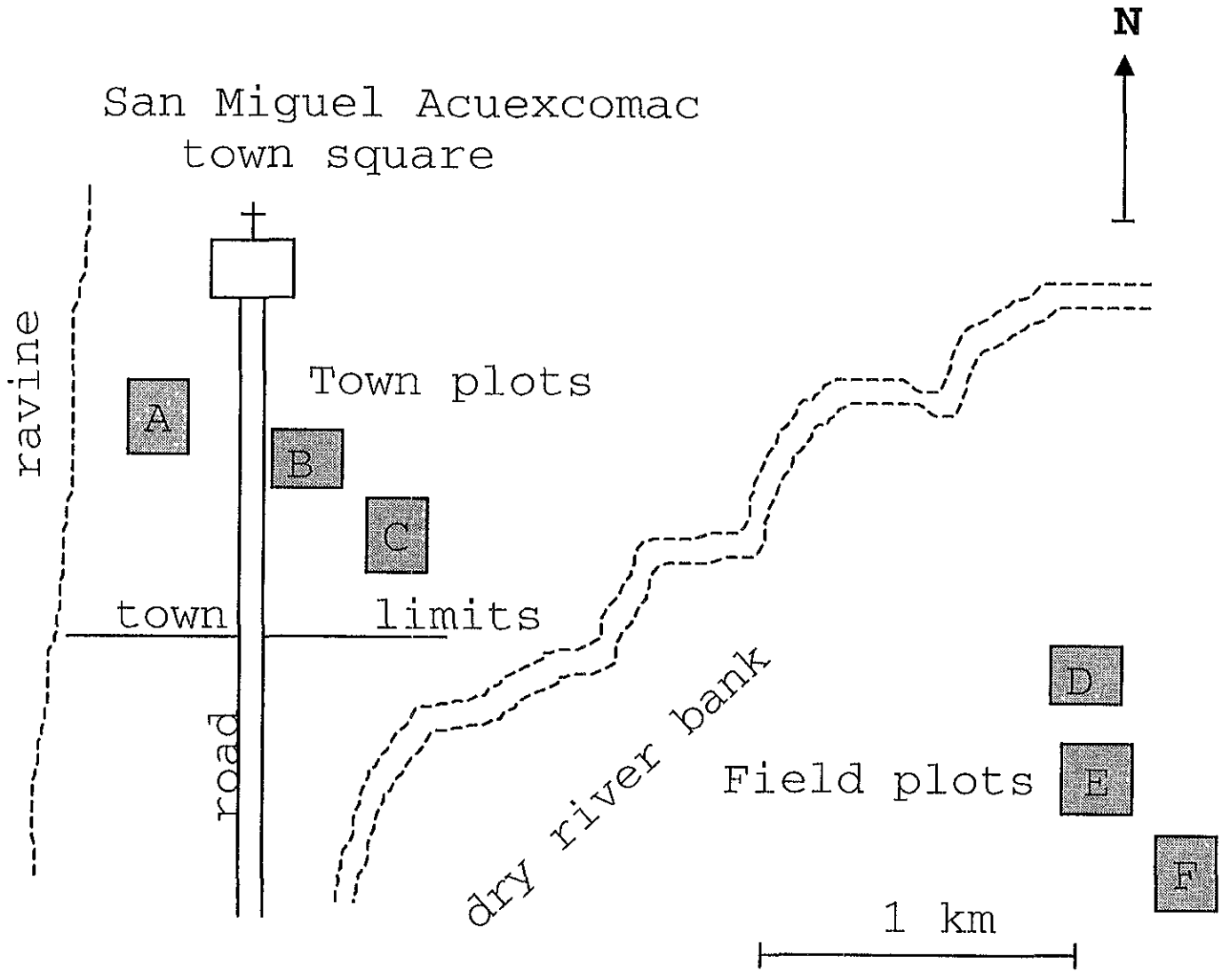
Cluster	No. isoð	No. ETs	No. allef	H§	Isolates mismπ	Vo/Veð	P†	ETs mism	Vo/Veð	P
I	95	48	3.4	0.400 (0.082)	2.8	1.87	<0.001	4.1	1.15	N. S.
III	387	78	3.6	0.347 (0.067)	2.0	1.18	<0.001	3.4	0.75	N. S.
Total	482	126	4.2	0.531 (0.066)	3.8	3.93	<0.001	5.4	2.27	<0.001

ðNumber of isolates; £Mean number of alleles; §Mean genetic diversity, values in parenthesis represent _ SE; πMean number of mismatches; ðObserved variance/expected variance of the mismatch distribution; †Probability of rejecting by chance alone the null hypothesis that Vo=Ve.

Figure 1.- Schematic representation of the six plots in both field and town localities. Letters refer to plot designation along the text.

Figure 2.- Genetic relatedness among 126 ETs of R. etli bv. phaseoli based on 10 polymorphic loci, generated by distance method UPGMA. The genetic distances were estimated as the proportion of allelic mismatches. Only genetic distances above 0.2 are shown. The terminal codes represents the ET number and in parenthesis the number of isolates for multiples ETs. The R. etli bv. phaseoli clusters I, II and III are indicated. The bold lines and black arrows refer to subclusters III.1 and III.2.

Figure 3.- Representation of the reticulated-epidemic genetic structure proposed for R. etli. The structure is basically reticulated, where recombination occurs between the members of the two main lineages, but is restricted among them. Occasionally a successful genotype increase in frequency to produce epidemic clones.


N
San Miguel Acuexcomac
town square
ravine
Town plots
A
B
C
town
limits
road
dry river bank
D
Field plots
E
F
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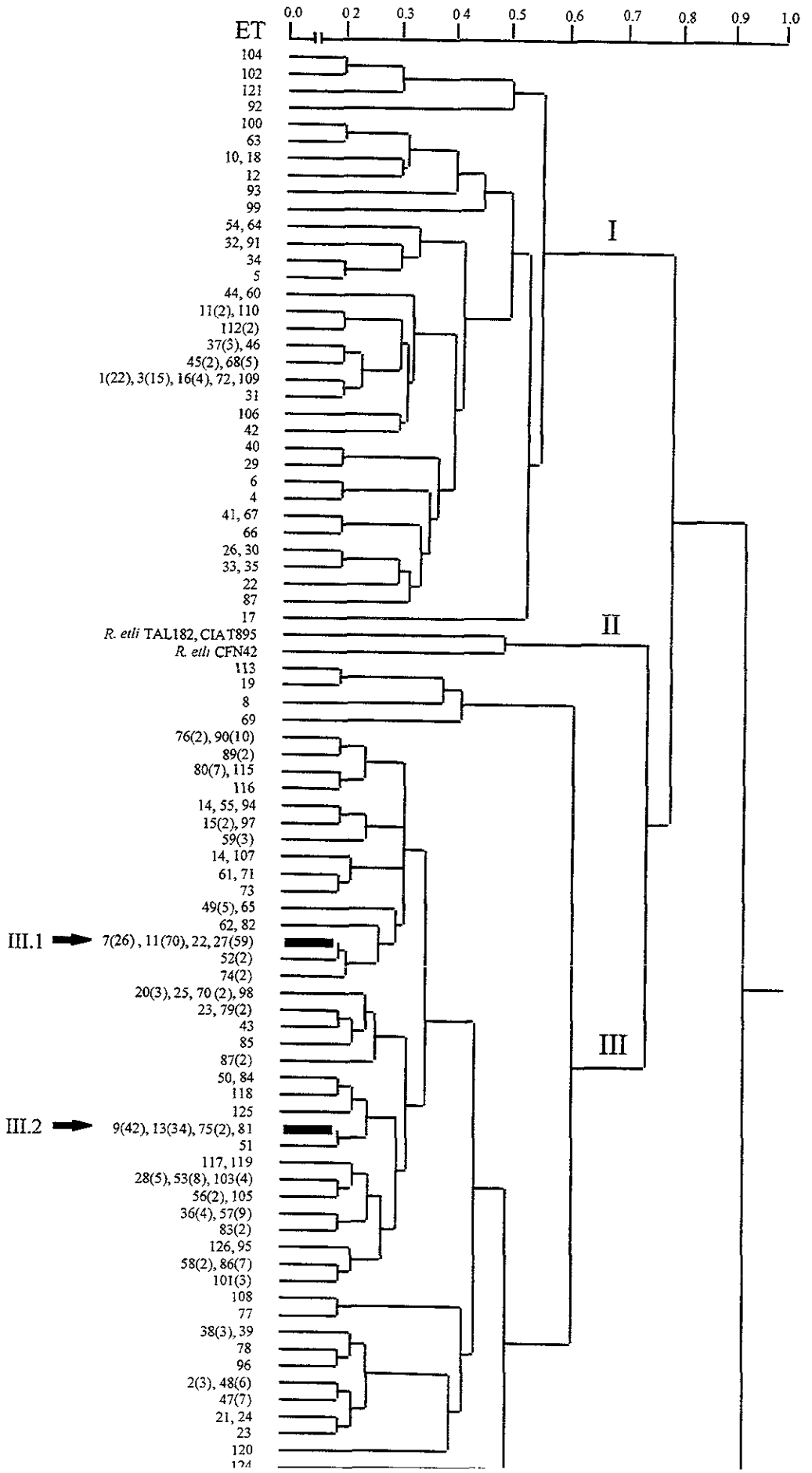
ET
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0.2
0.3
0.4
0.5
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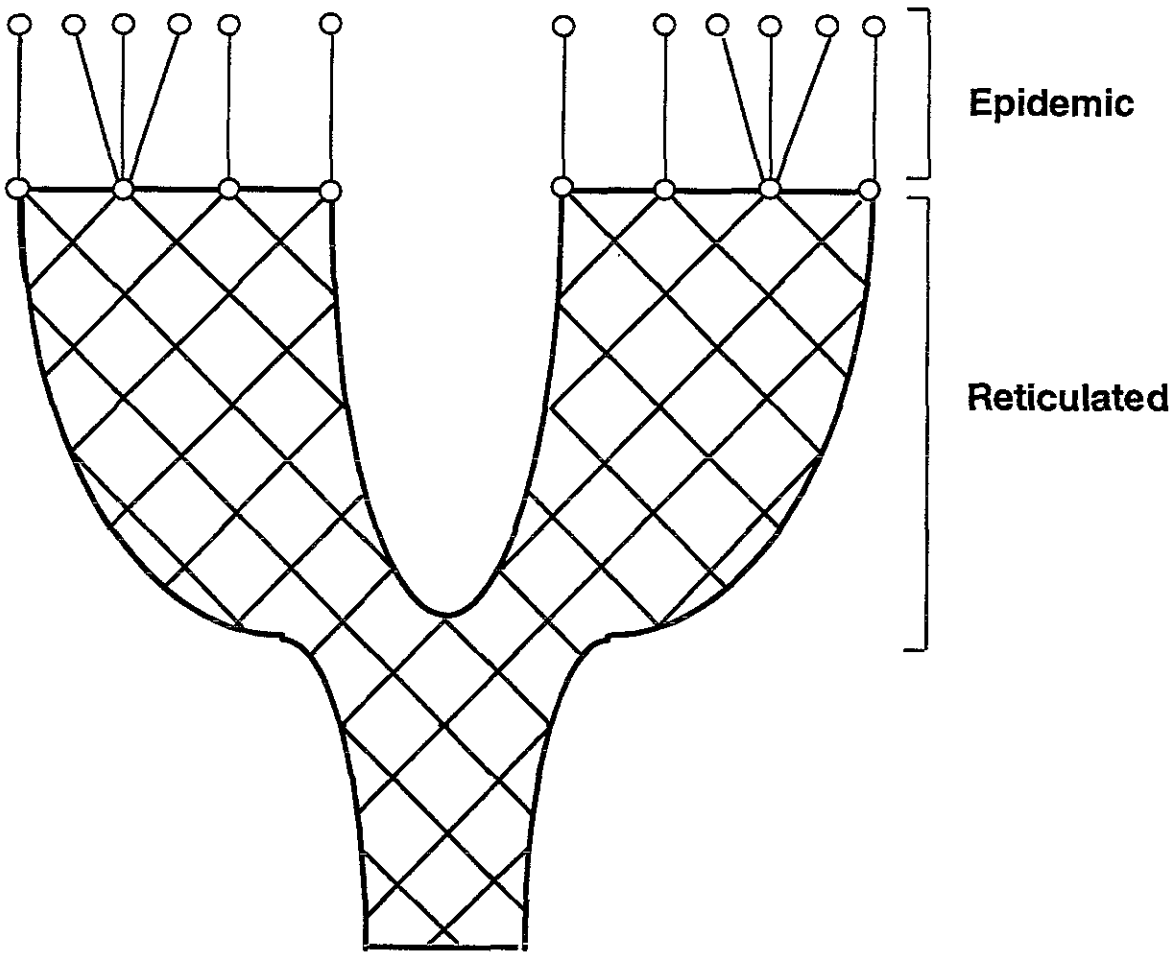
0.9
1.0
104
102
121
92
100
63
10, 18
12
93
99
54, 64
32, 91
34
5
44, 60
11(2), 110
112(2)
37(3), 46
45(2), 68(5)
1(22), 3(15), 16(4), 72, 109
31
106
42
40
29
6
4
41, 67
66
26, 30
33, 35
22
87
17
R. etli TAL182, CIAT895
R. etli CFN42
113
19
8
69
76(2), 90(10)
89(2)
80(7), 115
116
14, 55, 94
15(2), 97
59(3)
14, 107
61, 71
73
49(5), 65
62, 82
III.1
7(26), 11(70), 22, 27(59)
52(2)
74(2)
20(3), 25, 70 (2), 98
23, 79(2)
43
85
87(2)
50, 84
118
125
III.2
9(42), 13(34), 75(2), 81
51
117, 119
28(5), 53(8), 103(4)
56(2), 105
36(4), 57(9)
83(2)
126, 95
58(2), 86(7)
101(3)
108
77
38(3), 39
78
96
2(3), 48(6)
47(7)
21, 24
23
120
I
II
III


Epidemic
Reticulated

ETHNOMICROBIOLOGY: DO AGRICULTURAL PRACTICES MODIFY THE POPULATION STRUCTURE OF THE NITROGEN FIXING BACTERIA RHIZOBIUM ETLI BIOVAR PHASEOLI?

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ABSTRACT.- We analyzed how agricultural practices affect the levels of genetic variation and the genetic structure of beans (Phaseolus vulgaris and P. coccineus) and of their associated Rhizobium etli bacteria, and the role of agriculture in the maintenance of this genetic diversity. Two contrasting communities in the state of Puebla, Central Mexico, were selected: San Miguel, a Nahuatl community where traditional agricultural work is done almost exclusively by women, and Calpan, a Mestizo community where men cultivate crops using modern techniques. The results were compared with previous research from Morelos, also in Central Mexico. We found that San Miguel has maintained its agricultural tradition for generations. In recent years, women have played an important role not only in preserving this tradition but also in the conservation of the biological diversity in their plots. However, in Calpan the local varieties of beans have been substituted by commercial varieties and the women are losing their traditions and their contact with the land. In general terms, the genetic diversity of R. etli associated with cultivated beans (both P. vulgaris and P. coccineus), is high in all the communities studied, while it is lower for the rhizobia associated with wild beans. The population structure of Rhizobium etli is different in the communities studied: the most fertile and intensively managed plots are similar, while the least managed plots resemble the site of wild P. vulgaris. This research indicates that agricultural practices, and local environmental conditions affect the genetic structure of both crops and their associated bacteria.

RESUMEN.- En este artículo analizamos cómo las prácticas agrícolas afectan los niveles de variación genética y la estructura genética de los frijoles (*Phaseolus vulgaris* y *P. coccineus*) y de sus bacterias

asociadas, *Rhizobium etli*, y el papel de los sistemas agrícolas en el mantenimiento de esta diversidad genética. Se seleccionaron para trabajar dos comunidades contrastantes en el estado de Puebla, en el centro de México: San Miguel, una comunidad náhuatl donde las actividades agrícolas tradicionales se llevan a cabo de manera casi exclusiva por mujeres, y Calpan, una comunidad mestiza donde los hombres cultivan sus parcelas usando técnicas modernas. Los resultados se comparan con investigaciones previas realizadas en Morelos, también en el centro de México. Encontramos que en San Miguel se han mantenido las tradiciones agrícolas por generaciones. En años recientes, las mujeres han jugado un papel importante no solo preservando estas tradiciones, sino también la diversidad biológica en sus parcelas. Por otra parte, en Calpan las variedades locales de frijol han sido substituidas por variedades comerciales y las mujeres han perdido en buena parte las tradiciones y el contacto con la tierra. En términos generales, la diversidad genética de *R. etli* asociado a frijol cultivado (tanto en *P. vulgaris* como en *P. coccineus*) es alta en todas las comunidades estudiadas, mientras que para los rhizobia asociados a frijoles silvestres es menor. La estructura poblacional de *Rhizobium etli* es diferente en las distintas comunidades estudiadas, siendo las parcelas más fértiles y cultivadas las más parecidas entre sí, mientras que las parcelas menos manejadas se parecen más bien al sitio de *P. vulgaris* silvestre. Este trabajo indica que las prácticas agrícolas, la influencia de las mujeres y las condiciones ambientales locales afectan la estructura genética de ambos, los cultivos de frijol y sus bacterias asociadas.

INTRODUCTION

Mexico is a country with an enormous cultural and biological richness (Mittermeier, 1988; Flores-Villela and Gerez, 1994). Nevertheless this diversity is being lost at an unprecedented rate because of increasing demographic, economic and technological pressures. In order to preserve both the cultural and biological richness of crops, it is necessary to understand the relationship between the human management of these species and their genetic diversity. The process of domestication is an ideal system to understand the influence of humans over biological diversity (Doebley, 1989). Usually, the domestication process erodes the natural genetic diversity of the organism under domestication (Doebley, 1989; Escalante et al., 1994). Nevertheless, relatively high levels of genetic variation can be maintained as landraces by indigenous cultures (Kaplan, 1981; Brush, 1986; Altieri and Merrick, 1987; Doebley, 1989). In addition, it has also been suggested that rural women hold an important role in the preservation of genetic diversity by their use of traditional knowledge of agricultural practices as well as by the use of seeds with "old" genotypes (Brush, 1986; Bain, 1993).

The common bean, *Phaseolus vulgaris*, is an ancient crop species. Domesticates appear in the archeological record 7,000 yr. BP. in both Mesoamerica and South America (Gentry, 1969; Kaplan, 1981; Delgado et al., 1988). In Mexico, the common bean and the related cultivated perennial species *P. coccineus*, are normally nodulated by strains of the nitrogen-fixing bacteria, *Rhizobium etli* (Segovia et al., 1993; Souza et al., 1994).

Beans, as most legumes, allow certain strains of *Rhizobium* to penetrate in their roots and subsequently to develop nodules where the nitrogen fixation occurs. The nodule structure and the transformation of the atmospheric nitrogen to ammonia is an active process mediated by both the plant and the bacteria in the nodules, representing one of the most clear examples of symbiosis (Long, 1989). Nevertheless, nitrogen fixation efficiency, number of nodules and their size depends on the correct molecular signals between both symbiotic partners (Long, 1989; Souza, 1990). Traditionally, the

evolutionary biology of both, the plant host and the bacteria, had been studied as two separate entities without considering human influence on their genetic diversity. Although there is evidence that man has contributed to spread rhizobia around the world by moving seeds, crops and soil from one continent to the other (Martínez-Romero and Caballero Mellado, 1996).

Piñero and Eguiarte (1988) and Escalante et al. (1994) have previously reported population genetics of wild and cultivated P. vulgaris and P. coccineus from Central Mexico. *Phaseolus coccineus* from Central Mexico has a high genetic diversity (measured as *H*, the mean expected heterozygosity in Hardy Weinberg equilibrium, range 0.18-0.27) and intermediate outcrossing rates (*t* range 0.59 to 0.69, Escalante et al., 1994). In this species, the domestication process has neither eroded the levels of genetic variation nor changed the mating system (Escalante et al., 1994). *Phaseolus vulgaris*, in contrast, is highly inbred (almost entirely self-pollinated), and has very low levels of genetic variation (*H*= 0.041; Escalante et al., 1994).

The population genetics of *Rhizobium* sp. have been studied by several authors (Piñero et al., 1988; Demezaz et al., 1991; Segovia et al., 1991; Souza et al., 1992, 1994; Eardly et al., 1990, 1995; Strain et al., 1995), who have found high levels of genetic diversity in *Rhizobium* associated with cultivated legumes (*H* ranges from 0.46 to 0.69). Souza et al. (1994) described similar results in the rhizobia associated with cultivated beans of Morelos in Central Mexico (*H*= 0.41). However, the rhizobia associated with wild *P. vulgaris* in Morelos presented a much lower genetic diversity (*H*= 0.11).

The main objectives of this research were to analyze how agricultural practices affect the genetic diversity not only of crops, but also of the bacteria associated with them, and explore the role of women in the conservation and management of the genetic diversity of the beans and their rhizobia.

In order to achieve these objectives, two contrasting communities in the central Mexican state of Puebla were selected: San Miguel Acuexcomac, a Nahuatl community where traditional agricultural

work is done almost exclusively by women, and San Andrés Calpan, a Mestizo community where men cultivate using modern techniques. The results were compared with those of previous research from Tepoztlan and Santiago Tepetlapa, Morelos (also in Central Mexico) (Souza, 1990; Souza et al. 1994). In both communities we studied the role of women in agriculture from the ethnobiological point of view, and the population genetics of both the host plants (*Phaseolus vulgaris* and *P. coccineus*) and the nitrogen fixing bacteria *Rhizobium etli*.

This is the first study of this kind, where the interaction between the bean and the nitrogen fixing bacteria *Rhizobium etli* is analyzed in different agrosystems with the purpose of understanding the effect of bean and soil management on the bacteria.

METHODS

Study Sites.- The sites where this research was carried out are near Mexico city, in the highlands of Central Mexico, their general characteristics are described in Table 1. We chose these communities because one (San Miguel) presented the lowest registered bean production of the state of Puebla, while Calpan has one of the highest yields in the state (Table 2). (Calpan, INEGI, 1994, M. Colunga and D. Piñero unpublished data).

a.- San Miguel Acuexcomac, Puebla. (hereafter San Miguel). This is a traditionally indigenous (Nahuatl) community, that has been marginal rural land since pre-Columbian times, when it was called "the site of the ants" (Acuexcomac). Agriculture is mainly a polyculture system: corn, beans, squash, chile and different herbs. At present, mainly women cultivate the land in San Miguel, since most of the men are working in Los Angeles, CA (Niehe, 1988; V. Souza personal obs.). The soil has low levels of soluble nitrogen (nitrates), and a moderate alkalinity (Figure 1); rainfall is unpredictable (Niehe, 1988).

b.- San Andrés Calpan, Puebla (hereafter Calpan). This is a traditional Mestizo community, with a suburban socioeconomic structure (Table 1). Here, the capitalization process and the input of technology have allowed an increase of their agricultural productivity (Zamora Rodriguez, 1989) but at a high cultural and biological price: traditions as well as germplasm are being lost. In Calpan, the land is cultivated mostly by men with the help of the family (V. Souza pers. obs.). The soil has more soluble nitrogen and a more balanced pH than San Miguel, making it more fertile (Figure 1); Calpan has a more predictable rain pattern and a more temperate climate than San Miguel because of the shadow of the Popocatepetl volcano (Zamora Rodríguez, 1989).

c.- Santiago Tepetlapa, Morelos. (hereafter Santiago). Santiago is a small community with a long history of bean cultivation since pre-Columbian times (Souza, 1990; Souza et al., 1992, 1994). Although the culture is Nahuatl, the language is being lost in the younger generations (Table 1). The rain pattern in this site is predictable and abundant in the summer (García, 1988). Santiago has changed a lot

in the last 10 years, since it is very close to the village of Tepoztlán (an expensive resort for the people of Mexico City). The rural traditions are being lost since most of the agricultural sites have been sold to build country villas (V. Souza personal observations). In Santiago all the plots are being cultivated mostly by men without the help of women. This is due to the fact that women are working mostly as housekeepers in the villas or at home. The soil in Santiago is acid and very rich in nitrates (Figure 1) due to the input of chemical fertilizers.

d.-Tepoztlan, Morelos (hereafter wild site). In this site we found wild populations of *P. vulgaris* and *P. coccineus* (Souza, 1990; Souza et al., 1992, 1994). This is a very disturbed oak forest that is heavily grazed by cows and horses. It is located 3.7 km northwest of the town of Tepoztlán (99° 07'W, 19° 00'N; 1,850 msnm), and 7 km north of the town of Santiago, in all the region the rain pattern is predictable and abundant in summer. The soil in this site is the poorest in Calcium but richer in organic mater (Figure 1).

Ethnography.- We made 8 visits to the communities of Calpan and San Miguel Acuexcomac, Puebla. We interviewed the women, taping the conversations, having first obtained permission from them to do so. Although a questionnaire was prepared, the interviews were made in an informal and casual manner that brought into conversation previously prepared questions. Subsequently, the questions and answers were analyzed in order to obtain general response patterns, to asses the way in which the women from these communities perceive their relationship with the crops they tend and with their surrounding natural resources. The interviews took place mainly in homes, and a couple of times we approached the women while they were doing their shopping, so those persons accompanying them (usually their children or friends) participated too. We interviewed 13 women in Calpan and 12 in San Miguel. In both places we looked for women of different ages. A canonical discriminant analysis (Cuadras, 1981) was performed using the variables that explained most of the variance among sites.

Sampling procedures for the beans and the rhizobia.- San Miguel and Calpan were sampled during 1994 (Table 2). The approximate number of active nodules present in each plant was counted and ten active

nodules were collected from each plant. A leaf sample of each sampled plant was collected and stored at -80°C, in order to determine the genetic structure of the plants for all plots. A soil sample from each plot was also taken. 100 randomly selected seeds from each plot, where measured and weighted and their color was scored. The productivity per plant was determined *in situ* by hand harvesting 10 randomly selected plants. The aerial part of the plant was stored in a large plastic bag taking care of getting all the loose leaves. The seeds from each plant where stored in a separated bag. In the laboratory the plants and the seeds where dried in a stove at 350°C. The dry weight of the plants and the seeds from each site was compared using a t student test (Sokal and Rohlf, 1981).

Bacterial isolates.- The nodules were washed with 10% sodium hypo chloride and rinsed twice with sterile water. Nodules were smashed in Petri dishes with Peptone Yeast Extract medium (PY) and grown for two days at 30 °C. One strain was isolated and grown again in a new Petri dish. This procedure was repeated twice to obtain a pure strain or clone. Each strain so obtained was kept at -80_°C in UL (Glicerol-Peptone minimum media, Souza, 1990; Souza et al., 1994). We isolated 351 strains for this analysis: 229 from San Miguel and 122 from Calpan, from three randomly selected plots of each site.

Electrophoresis procedures.- a.- Bean electrophoresis: We analyzed the genetic diversity of both species of beans in the studied sites using isoenzyme electrophoresis in cellulose acetate membranes (Hebert and Beaton, 1993). Since most of the studied loci in *P. vulgaris* are monomorphic (Escalante et al., 1994), we were able to analyze only three polymorphic loci: malic enzyme (ME, EC 1.1.1.40), isocitrate dehydrogenase (IDH, EC 1.1.1.42) and 6-phosphoglucose dehydrogenase (6-PGDH, EC 1.1.1.49). For *P. coccineus*, four polymorphic loci were analyzed (ME, IDH, 6-PGDH and malate dehydrogenase (MDH, EC 1.1.1.37) as an extra enzyme). The genetic variation was estimated as the average expected heterozygosity in Hardy-Weinberg equilibrium, *H* (Hedrick, 1983; Escalante et al., 1994) that ranges from 0, if there is no genetic variation, to a theoretical maximum of 1, if there is an infinite number of alleles, each with the same allelic frequency.

b.-Bacteria electrophoresis: Before each electrophoresis the strains to be analyzed were grown in solid PY and two days later transferred to liquid PY (50 ml). After two days, the cultures were centrifuged at 6000 rpm during 5 minutes, to obtain the cell pellet. The supernatant was eliminated and the pellet re-suspended in 1 ml of Tris HCl pH8 buffer. Addition of 0.1 ml of lisozyme (0.075 mg/ml) ensured lysis of the bacterial walls and the resulting suspension was frozen twice at -80°C for 15 minutes. It was then centrifuged at 12,000 rpm during 5 minutes. The supernatant containing the protein lysate was distributed in three 1.5 ml plastic tubes and stored at -80°C. The strains from Morelos were analyzed using isoenzyme electrophoresis in starch as described by Selander et al. (1986) and Souza et al. (1994). For the strains from Puebla, electrophoresis was performed in membranes of acetate cellulose (Hebert and Beaton, 1993). Seven polymorphic enzymes were used: isocitrate dehydrogenase (IDH, EC 1.1.1.42), peptidase (PEP, EC 3.4.11), phosphoglucomutase (PGM, EC 5.4.2.2), glucose 6-phosphate dehydrogenase (G-6PDH, EC 1.1.1.49), xanthine dehydrogenase (XDH, EC 1.1.1.204), malate dehydrogenase (MDH, EC 1.1.1.37), and the malic enzime (ME, EC 1.1.1.40).

Measurement of Bacterial Diversity.- From allele frequencies, the genetic diversity for an enzyme locus was estimated again as the expected virtual (since the bacteria are haploid) heterozygosity in Hardy-Weinberg equilibrium (H), and was estimated as $H = (1-\Sigma x_i^2)[n/(n-1)]$, where x_i is the frequency of the i-th allele and n is the number of genotypes (or electrotypes, ET) (Selander et al., 1986; Souza et al., 1994). The average genetic diversities were calculated in a hierarchical way considering the diversity of the rhizobia within each plant, within each plot, from each site and the total diversity using the ETDIV program for bacterial population genetics (Whittham, 1990).

Genetic diversity was also estimated by the number of electrophoretic morphs (ET's)/ the number of strains analyzed. The higher value (ET /strain=1) is obtained when all the isolates are genetically different, and the lowest (1/n strains) is obtained when all the isolates are identical. This estimate is obviously more

sensitive to the number of strains collected and the number of loci used in the analysis, but it reflects information on the degree of diversity and clonality of the population (Souza et al., 1994).

Genetic Differentiation of the Bacteria.- We used three modified indices related to the G_{st} index to estimate the genetic differentiation at three hierarchical levels (Souza et al., 1994):

a.- plant level: $G_{pp} = (H_{plot} - H_{plants})/H_{plot}$

b.- plot level: $G_{ps} = (H_{site} - H_{plots})/H_{site}$

c.- site level: $G_{st} = (H_{total} - H_{sites})/H_{total}$

where H_{plants} is the average genetic diversity of *R. etli* within a plant, H_{plot} is the average genetic diversity within a plot, H_{site} is the average genetic diversity within a community and H_{total} is the average genetic diversity of the total sample in Puebla. These indices range from 0, if there is no genetic differentiation at that level (this can happen if all units at that level have exactly the same alleles with the same frequencies), to 1, if there is maximum genetic differentiation (meaning that the compared units share no alleles) (Souza et al., 1994).

RESULTS

Ethnographic Studies.- From the data shown in Table 3, it is clear that more women in San Miguel than in Calpan participate and understand the agricultural practices, conservation, identification and selection of seeds as well as the weather patterns. Since there is no fruit cultivation in San Miguel, the women of Calpan are more knowledgeable in this issue. Women from San Miguel also posses more knowledge of the management of the local vegetation (collection of medicinal and edible plants, and woody plants for fuel, Table 3). A Canonical analysis of discriminants showed that there is a significative difference ($X^2 = 42.23$ $p < 0.001$) among the way women from both communities growth their crops, manage their soil and use the local vegetation.

In San Miguel there is an almost complete absence of men due to migration to the USA in an effort to increase their low income and improve their extremely poor living. Women see themselves as mere "helpers" of their absent husbands, even though it is them who are in charge of the agricultural and commercial activities. Because of the enormous work pressure on these women and because of the lack of control by men, the community in general can be compared to a great extended family in the way the daily activities are organized. Traditional agriculture in San Miguel can be seen as an expression of indigenous culture, maintained by the older women's teachings.

Calpan has a more "traditional" family structure in the sense that most men are present during the year and women's activities are limited to that which the husband or father "allows them" to do (Table 3). In Calpan there has been a gradual loss of agricultural traditions: "Because working in the fields is not profitable any more, people are leaving; young people study but cannot find jobs so they just stay around, doing nothing". On the other hand, the seed stock of the community (germplasm) has suffered pressure from the market and local varieties have been substituted by commercial varieties which "although they do not taste as good, sell better".

Bean morphology and diversity.- The seeds is San Miguel are larger and heavier than beans from Calpan (Table 2, $t=15.67$ $p<0.001$, $t=$

18.72 $p<0.001$ respectively), and show a high diversity both in color (6 colors in SM vs. 4 colors in Calpan) and size (variation coefficient 17.21 in San Miguel vs. 10.21 in Calpan, Table 2) and weight (variation coefficient 51.80 in San Miguel vs. 23.69 in Calpan, Table 2). The criollo varieties from San Miguel are also as rich in protein as those from Calpan (an average of 3.26% total nitrogen in Calpan and 3.32 % in San Miguel, analyses done at the Instituto Nacional de Nutrición). Besides, the yield per plant (dry weight of the seeds/dry weight of the plant) is significantly larger in San Miguel than in Calpan (Table 2, $t=6.9$ $p<0.001$).

Cultivated and wild *P. vulgaris* have very low levels of genetic variation (*H* range from 0.016 to 0.025, Figure 2), with slightly higher levels for the wild *P. vulgaris* and the cultivated population from San Miguel ($H=0.025$ and 0.023, respectively). Low levels of genetic variation for cultivated *P. vulgaris* have been described previously by Escalante et al. (1994). However, the slightly higher genetic diversity in San Miguel may be due to a more diverse stock of local seeds. The germplasm in San Miguel is actively and carefully maintained by each family since the criteria to harvest, store and select seeds is more complex than in Calpan (Table 3) where the seed supply is changing constantly due to the input of government agencies.

Phaseolus coccineus populations have substantially higher levels of genetic diversity than the common bean (Figure 2, range $H = 0.24$-0.368). The cultivated population of *P. coccineus* in San Miguel has lower variation levels than *P. coccineus* from other localities in Central Mexico ($H = 0.24$), which may be due to the fact that the weather and soil in San Miguel are not part of the ecological range of this species, which occurs naturally in temperate oak forest with cooler climate and higher humidity (Escalante et al., 1994).

Genetic Structure of Rhizobium etli.- a.-Genetic diversity of Rhizobia and beans: Both the *H* and the ET/strains indices and their relationships with the variation levels of the host plants are shown in Figure 2. In terms of *H*, rhizobia associated with wild *P. vulgaris* and wild *P. coccineus* have lower levels of genetic variation (*H* range = 0.118 - 0.335), while the rhizobia associated with both cultivated

P. vulgaris and *P. coccineus* have higher levels of genetic variability *H*, ranging from 0.407 to 0.542 (Figure 2).

The ET/strains index indicates, again, that the wild *P. vulgaris* associated rhizobia have the lowest levels of genetic variation while the second lowest are the cultivated *P. vulgaris* population and the highest levels of genetic variation occur in the rhizobia associated with *P. coccineus*, regardless of being wild or cultivated.

When we compare the levels of genetic variation of the host plants with their associated bacteria (Figure 2), in *P. vulgaris*, there is a negative relationship between plant and bacterial genetic variation (Figure 2). This is explained, at least in part, by the dominance patterns discussed below.

b) Distribution of the strains and degree of dominance: In Figure 3 we show the distribution of strains with the same ET for four contrasting populations of rhizobia. We detect a gradient of dominance, which appears to depend on the degree of agricultural management. In Calpan, the most technified site, the community of *R. etli* presents the lowest dominance, with many ET's represented by just one or few strains in each. Both communities with lower levels of agricultural technology, Santiago Tepetlapa and San Miguel, have intermediate dominance levels, with most ET's being unique or with few strains, but some ET's represented by several strains. The wild *P. vulgaris* site is the extreme of the gradient, with a strong dominance, as most of the collected strains belong to a single ET.

c) Genetic differentiation at different hierarchical levels. The genetic levels of differentiation are described by the G_{st} analogs at the different levels, shown in Table 4. In Calpan, the G_{pp} indicates there is very little genetic differentiation of the bacterial isolates among plants in a given plot (G_{pp} range 0.009-0.052), and thus we found in each plant most of the rhizobia variation described for the entire plot. In contrast, in San Miguel the G_{pp} was substantially larger, ranging from 0.379 to 0.555, indicating a large degree of genetic differentiation of the bacterial isolates among plants. This means that each plant had a lower proportion of the rhizobia genetic variation found in the whole plot. This pattern was also found in the cultivated rhizobia from Santiago, (G_{pp}=0.58) and

in the wild *P. vulgaris* (Gpp=0.53) where most of the variation is found between plants.

At a higher level, differentiating plots within a site (G_{ps}), in both communities in Puebla each plot had most of the genetic variation present in that site (Calpan G_{ps}= 0.101, San Miguel G_{ps}= 0.142), meaning that there was little bacterial genetic differentiation among the plots in a given site. In contrast, in Santiago the two cultivated plots were quite different (G_{ps}=0.67) and this was even more dramatic in the wild bean plots (G_{ps}=0.97).

At the site level within each state, the G_{st} indicates that each site in Puebla represented most of the genetic variation, and very little genetic differentiation was found between sites (Gst=0.09), while the sites sampled in 1987 in Morelos were quite different (Gst =0.55). On the other hand, we have not found a single shared strain between the two sites in Puebla, although *P. coccineus* and *P. vulgaris* in San Miguel shared 5 ETs. In Morelos, very few strains were shared among neighboring sites (Souza et al., 1994). This may be due in part to the lack of mobility of this bacteria, but also to a lack of adaptation to new environments.

DISCUSSION

We can summarize the main findings of our research as follows: 1) San Miguel is a Nahuatl community that has maintained its agricultural tradition and its rich and diverse bean germplasm for generations. In recent years, women have played an important role in preserving this tradition. In Calpan, on the other hand, the seed stock of the community has suffered an increasing pressure from the market: local varieties of beans have been substituted by commercial varieties that are less diverse and smaller that the beans in San Miguel.

2) The genetic diversity of *R. etli* associated with cultivated beans, both *P. vulgaris* and *P. coccineus*, is high in all the studied communities, while it is lower for the *Rhizobium* associated to wild beans.

3) The population structure of *Rhizobium etli* is different in all studied populations. The most fertile and intensively managed plots are similar, while the least managed plots resemble the wild site of *P. vulgaris*; the genetic structure of *Rhizobium* depends on the agricultural practices, bean genetic diversity and soil conditions.

The people, the beans and the environment.- One of the objectives of this research was to define the role women play in these communities as managers of their natural resources and germplasm, and therefore, to assess their effect on the conservation and management of the genetic diversity of beans and the nitrogen-fixing bacteria *Rhizobium etli*.

Women in San Miguel like heterogeneity in their crops, as was expressed by a woman: "me gusta que mi canasta esté pinta" ("I like a mottled basket"), meaning she liked her corn and bean seeds of many colors and sizes (e.g. tortillas in San Miguel are brownish due to a mixture of grains of different colors). In the interviews we observed, that women in San Miguel take more variables into account when the select their seeds for the next cycle. This selection for heterogeneity is confirmed by the morphological and genetic analysis of the beans, where the maintenance of a mixture of genotypes is evident. The beans in San Miguel are also morphologically larger and

heavier than the commercial seeds from Calpan. The higher diversity of the beans found in San Miguel has been observed elsewhere in several of the ancestral crop varieties known as landraces (Brush, 1986; Altieri and Merrick, 1987; Martin and Adams, 1987). While genetic diversity is directly related to ancient agricultural traditions and may be explained as a response to a complex and competitive environment, sometimes it is also associated with low productivity (Jennings and Cock, 1978). This is only partially true for San Miguel, where the bean productivity per hectare is lower than in Calpan and it is one of the lowest in the state of Puebla (INEGI, 1994), but they have a significantly higher yield per plant. This paradox is due to the fact than in San Miguel they use beans varieties with undetermined (vine) growth that get so big that they can crush the corn plant that is their support. While in Calpan the beans have a determinated growth (bush) and do not need any support so they can grow in a monoculture. The women in San Miguel choose only certain number of corn plants to support their beans and they do not have more than 50-100 plants per hectare, while in Calpan the density is very high (close to 15,000 per hectare).

The bean plants in San Miguel also may be better symbionts with rhizobia than the beans in Calpan, as indicated by the number of active nodules (red nodules are evidence for active nitrogen fixation) per plant that in San Miguel was 229, while in Calpan it was on average of only 122. However, this has to be proved in the greenhouse by controlled inoculation of different seeds.

Besides the rich bean germplasm, the botanical research shows that regardless of the region's semi-arid climate and eroded soils, San Miguel has a higher plant diversity inside the parcels than Calpan, which is explained in part by the Nahuatl agricultural practices that promotes the growth of weeds that are used as plant resources and by the active presence of healers in their community. While most women in San Miguel collect plants and fuel wood from their plots and immediate surroundings, in Calpan, women buy fuel and medicinal plants from the market and when they collect plants, this is never done in their plot. Furthermore, due to the family structure, the women in Calpan are more detached of the agricultural

practices and known less of the weather and the soil management than the women in San Miguel.

Agricultural techniques.- The agricultural traditions in both sites are different. Plots in San Miguel are seldom hand weeded and fertilized. This may cause a certain nutrient competition among the various plants growing simultaneously in the plots, by reducing the amount of resources available to the bean plant. Bean plants from Calpan, where fields are managed intensively and fertilized, obtain more nutrients. Nevertheless, peasants in San Miguel obtain other benefits from the presence of a tolerable level of weeds in their fields (Datta and Banerjee, 1978). In San Miguel, 11-21 species of weeds are found in the parcels, most of them are used for medicinal and culinary purposes, while in Calpan only 6-8 species are found in association with the beans. Weed communities may also increase biological insect pest control (Altieri et al., 1977) and enhance organic matter accumulation and soil conservation (Chacon and Gliessman, 1982). The better symbiotic relationship among beans and rhizobia in San Miguel could be due to both the differences in beans varieties and to the absence of agrochemicals, since large amounts of ammonia and nitrates in the soil inhibits the nodulation process (Long, 1989).

Even though the beans in San Miguel are much more diverse morphologically than the beans in Calpan, the bean genetic structure is similar in the 4 sampled sites. These results suggest that the morphological diversity could be due to the expression few genes of that are not scored in the Multiloci Electrophoresis. In that case, even if the mating system of the species reduces heterozigocity (Escalante et al., 1994) the selection criteria of seeds in San Miguel may be favoring morphological diversity by selecting diverse bean lines that coexist in a cultivar. The genetic erosion of the commercial varieties is common all over the world in the sites of domestication. The replacement of the local landraces with modern varieties usually implies the loss of all or most of the old cultivars and of their genetic diversity. Although productivity can increase through this substitution, an increase in management costs

and a reliance on purchased inputs, like fertilizers and seeds is common (Brush, 1986; Altieri and Merrick, 1987).

Rhizobium genetic structures and diversity.- Agricultural practices have indirectly changed the genetic structure of the nitrogen fixing bacteria *Rhizobium etli*. The bacteria associated with cultivated plants have higher levels of genetic variation than the bacteria associated with wild plants. This may seem a paradoxical result, as it is well known that most domesticated organisms have lower levels of genetic variation than their wild relatives (Doebley, 1989). This may be due solely to the agricultural practices, e.g., in the cultivated plots there are more bacteria than in the wild sites, and thus the plants can sample from a larger pool of genotypes, or it may be due to the domestication process that has indirectly changed the plant specificity for bacteria and thus their root can be colonized by a genetically wider pool of bacteria than the wild plants. We suspect that the correct explanation is a change in specificity (see also Souza et al., 1994). Future experiments regarding specificity of both beans and rhizobia will help to sustent this hypothesis.

The abundance of some well adapted ETs (dominance) suggests both adaptation to the local soil conditions and adaptation to the local bean varieties (Souza, 1990). This hypothesis is reinforced by the number of nodules per plant and the ratio of strains per ET (Table 2). In San Miguel, the community with minimum agricultural practices, nodulation was high, but apparently the environmental pressures reduced the genetic diversity (measured as the ratio of ET's/strains) and increased the differentiation from plant to plant within a plot. In Calpan, where the soil conditions are good and the technified agriculture may increase the movement of strains within a plot, the ratio of ET's/strain is higher, suggesting high genetic diversity but a lower nodulation efficiency than in San Miguel. This could be due to a wider host range of the cultivated varieties and also to the lower adaptation of some strains of *R. etli* to the commercial lines of common bean. In Santiago the dominance and low nodulation efficiency may be explained by the low calcium concentration (Lodeiro et al., 1995) found in this site. In Santiago there is also an apparent low migration of strains from other sites (Souza et al.,

1994), which reduces the genetic diversity within each site and increases the genetic differentiation. These results suggest that the genetic structure of *Rhizobium* depends not only on the number of different strains found in one site but also on the biology of the host plant and on the agronomic practices of each community.

Ethnomicrobiology, an emerging field.- The process of plant domestication, as well as introduction of crops to novel environments, may have an impact on both the introduced and the native rhizobia (Souza, 1990). The extent of this effect has not been evaluated (Martínez-Romero and Caballero Mellado, 1996). In this study we observed that rhizobia performance is much better in San Miguel than in Calpan. In San Miguel the interaction between *R. etli* and the local races of beans seems to be very efficient, which could be due to seeds selection, cultivar management and to the adaptation of the *R. etli*-bean interaction towards alkaline soils and unpredictable rains. In Calpan the introduction of nitrogen via fertilizers and the use of novel and homogeneous bean strains are changing the genetic structure of the native rhizobia and their interaction with beans. This could also be happening to other microbes associated to crops and most important, it could be happening to pathogens.

The direct and indirect effects of traditional human activities on the microbiota are overlooked aspects of ethnobiology, but potentially important. We suggest that the interdisciplinary study of the biological, ecological and cultural bases of both direct and indirect interactions between microbes and humans as well as their relationships in time could be studied by the ethnomicrobiology.

Is Biotechnology the solution? The results from this study present a grim panorama for biotechnology. The introduction of genetically engineered strains to Mexico is an impossible task due to the enormous genetic diversity of *Rhizobium* in soil. Besides, cultivation techniques and diverse soil conditions are very diverse and the interactions involved among beans-people-soil-rhizobia are very complicated. Thus, to improve the bean production in the studied

sites with the inoculation of *Rhizobium etli*, we suggest the following:

1) In places like San Miguel where there are few well adapted strains, the odds that a laboratory designed bacteria will survive and fix nitrogen are very low. The strategy would be to choose local isolates that are abundant, survive for several years, are well adapted to their bean varieties, and have good nitrogen fixing abilities.

2) Santiago and Calpan have several genotypes that can be selected, but the task is harder because the diversity is high and therefore individual genotypes are difficult to locate. We have to sample more years to have a notion of the best genotypes to select. In the long term, we will try to choose generalist strains, inoculate them on an average plot using the average commercial bean and assess them on different sites for survival, abundance and good nitrogen fixing abilities. If we do get improved strains, these will be explored extensively by rural people before incorporating them into the productive system.

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TABLE 1.- General characteristics of the studied communities in Puebla and Morelos, Mexico.

Site Characteristics	Calpan, Puebla	Santiago, Morelos[b]	San Miguel, Puebla[c]
Climate[a]	temperate	subtropical	semi-arid
Altitude[a]	2,500 mt	1,500 mt	2,100 mt
Coordinates[a]	99º30´W 19º05´N	99º05´W 18º59´N	98º 05´W 18º50´N
Vegetation[a]	oak and pine forest	tropical deciduous forest	scrubland
Rainfall[a]	800-1,000mm predictable	1,000 mm predictable	500-600mm unpredictable
Soil management	tractor, hand weeding	hand weeding	"labranza mínima" little weeding
Use of agrochemicals	fertilizer pesticide	fertilizer pesticide	none
Soil structure and fertility	sandy clay loam sandy loam fertile	sandy clay loam fertile	clay sandy clay loam eroded
Soil pH	6.2	5.5	8.3
Culture	suburban Mestiza	rural Mestiza-Nahualt	rural Nahuatl
Family	male dominant	male dominant	female based[d]
Cultivars in same plot	bean plots separated by a row of fruit trees	beans	many bean varieties, corn, squash and associated herbs
Average number of plant spp.	7 species per plot	not studied	16 species per plot

[a] Zamora Rodríguez, 1989. Inegi, 1994. [b] Souza 1990, Souza et al., 1994. [c] Niehe, 1988

TABLE 2.- Sampling scheme, characteristics of the beans, number of strains and electrotypes (ET's) of *Rhizobium etli* obtained from each site.

Site characteristics	Calpan Puebla	Santiago, Morelos	San Miguel, Puebla
Sampled plots (year)	3 (1994)	1 (1987) 1 (1988)	3(1994)
Sampled plant/plot	10	100 (1987)[a] 32 (1988)	10
Bean productivity (INEGI,1994)	high (2.5 ton/ha)	medium (1 ton/ha)	subsistence (ranges from 1 kg to 0.5 ton./plot)
Average dry weight seed /plant(± s.d)	0.68 ± 0.13	---	0.890 ± 0.01
Average bean size (cm ± s.d)	1.23 ± 0.12	1.02 ± 0.10	1.42 ± 0.24
Average bean weight (gr ± s.d)	0.38 + 0.09	0.32 + 0.05	0.59 + 0.31
Different colors of bean seeds	4	1	6
Bean variety	"amarillo" and "mantequilla" *P. vulgaris* wild *P. coccineus*	"negro jamapa" *P. vulgaris*	landraces of *P. vulgaris*; cultivated *P. coccineus*
Average active nodules/plant	34.5	14	48
Num. strains isolated from 10 random plants	122	270	229
ET/stains	0.508	0.352	0.393

[a] From 99 plants only 1 nodule was extracted, while from 1 plant all the nodules (52) were extracted, see Souza et al. (1994).

TABLE 3.- Ethnographic studies on women from both communities in Puebla. Percentage of interviewed women that participate in each activity. The number of interviews in San Miguel=12, in Calpan=13.

Activity	San Miguel %	Calpan %
Bean cultivation	91.7	7.7
Weeding	91.7	53.3
Separation of seed by size	100.0	76.9
Separation of seed by color	33.3	7.7
Separation of seed by aspect	41.7	30.8
Corn cultivation	66.7	0.0
Believes in moon's influence	41.7	0.0
Collects fuelwood	66.7	30.8
Buys fuelwood	33.3	84.6
Collects plants	83.3	46.2
Tends animals	66.7	46.2
Knowledge of rain patterns	75.0	30.8
Knowledge of chemical fertilizers	91.7	61.1
Uses chemical fertilizers	58.3	0.0
Knowledge of natural fertilizers	25.5	30.8
Grows fruits/flowers in their home gardens	100.0	100.0
Palm weaving	16.7	0.0
Is a healer	8.3	0.0
Collects fruits	0.0	46.2
Grows fruit trees	0.0	23.1

TABLE 4.- Relative genetic differentiation in *Rhizobium etli* in Puebla and Morelos.

Site	N strains[a]	*H R. etli*[b]	G_{pp}[c]	G_{ps}[d]
CALPAN, PUEBLA	123	0.545		0.101
plot 1	54	0.463	0.052	
plot 2	33	0.466	0.014	
plot 3	36	0.540	0.009	
SANTIAGO, MORELOS (1988)	190	0.407	0.58	0.67
SN.MIGUEL PUEBLA	229	0.508		0.142
plot 10	95	0.498	0.379	
plot 11	74	0.440	0.555	
plot 12	60	0.371	0.482	
WILD *P. vulgaris* MORELOS	33	0.216	0.53	0.97
TOTAL PUEBLA	351	0.579	G_{st} =	0.09
TOTAL MORELOS	223	0.487	G_{st} =	0.55

[a]: Number of strains in each spatial scale

[b]: *H R. etli*: A measure of the genetic variation in *Rhizobium*, the virtual expected heterozygosity (Souza et al., 1994).

[c]: Relative genetic differentiation among plants within a plot (G_{pp}= *H* plot- *H* plant/ *H* plot).

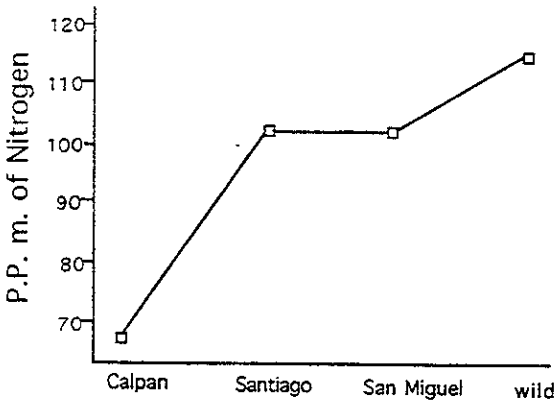
[d]: Relative genetic differentiation among plots within a site (G_{ps}= *H* site - *H* plot / *H* site)

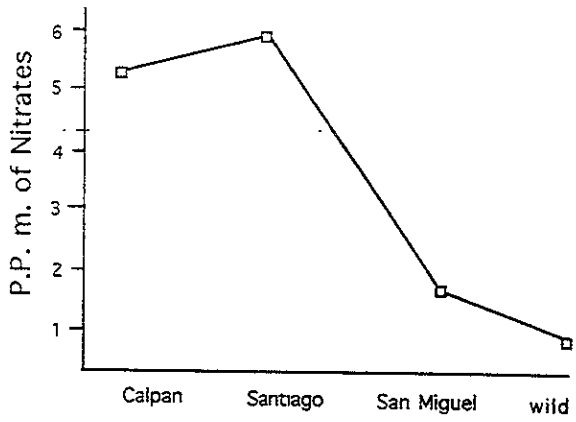
[e] Relative genetic differentiation among sites within each state (G_{st}= *H* total - *H* site / *H* total)

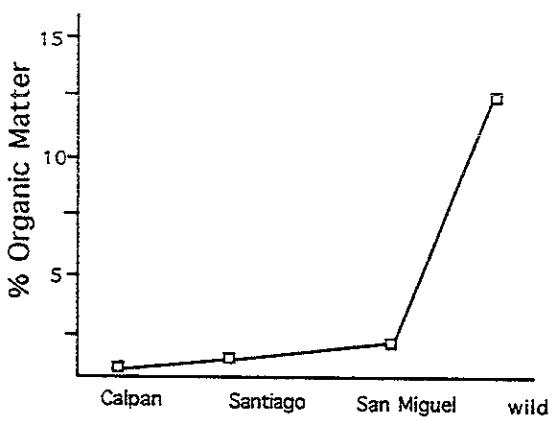
Figure 1.- Concentration of total nitrogen, soluble nitrates, organic matter, pH and Calcium in the soil of the studied sites.

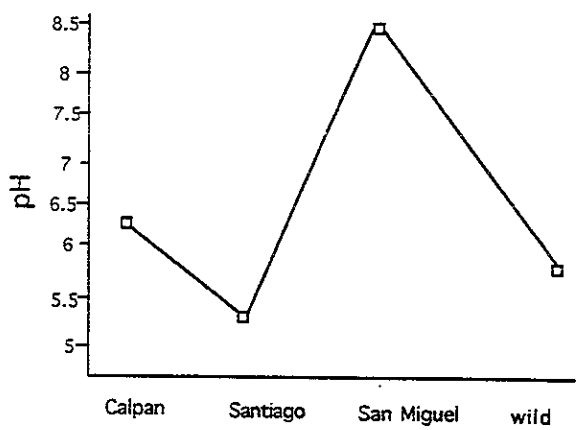
Figure 2.- Genetic diversity of beans and associated rhizobia in four sites in Mexico. The first column shows the genetic diversity of *Phaseolus vulgaris* and the genetic variation of their associated *R. etli* ; the second column shows the genetic diversity of *P. coccineus* and their associated *R. etli* (see text). The rhizobia associated with *P. coccineus* in Calpan, Puebla and in Santiago, Morelos are not represented in the figure due to the small sample size. Open squares show the data of 1994, filled squares show the data from Souza et al., 1994. [a] is for the 1987 studies and [b] is for the 1988 studies. The bars show standard errors.

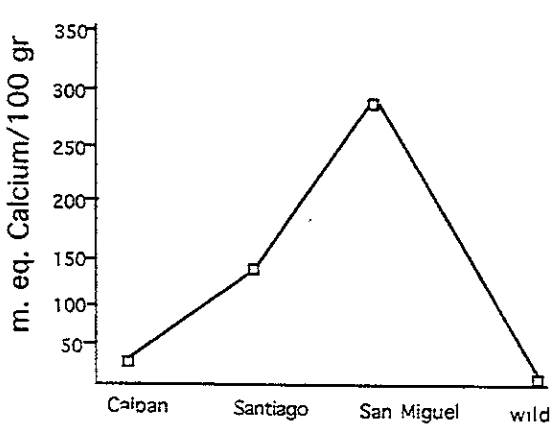
Figure 3.- Distribution of the frequencies of strains with the same genotype (Et) in the studied sites.

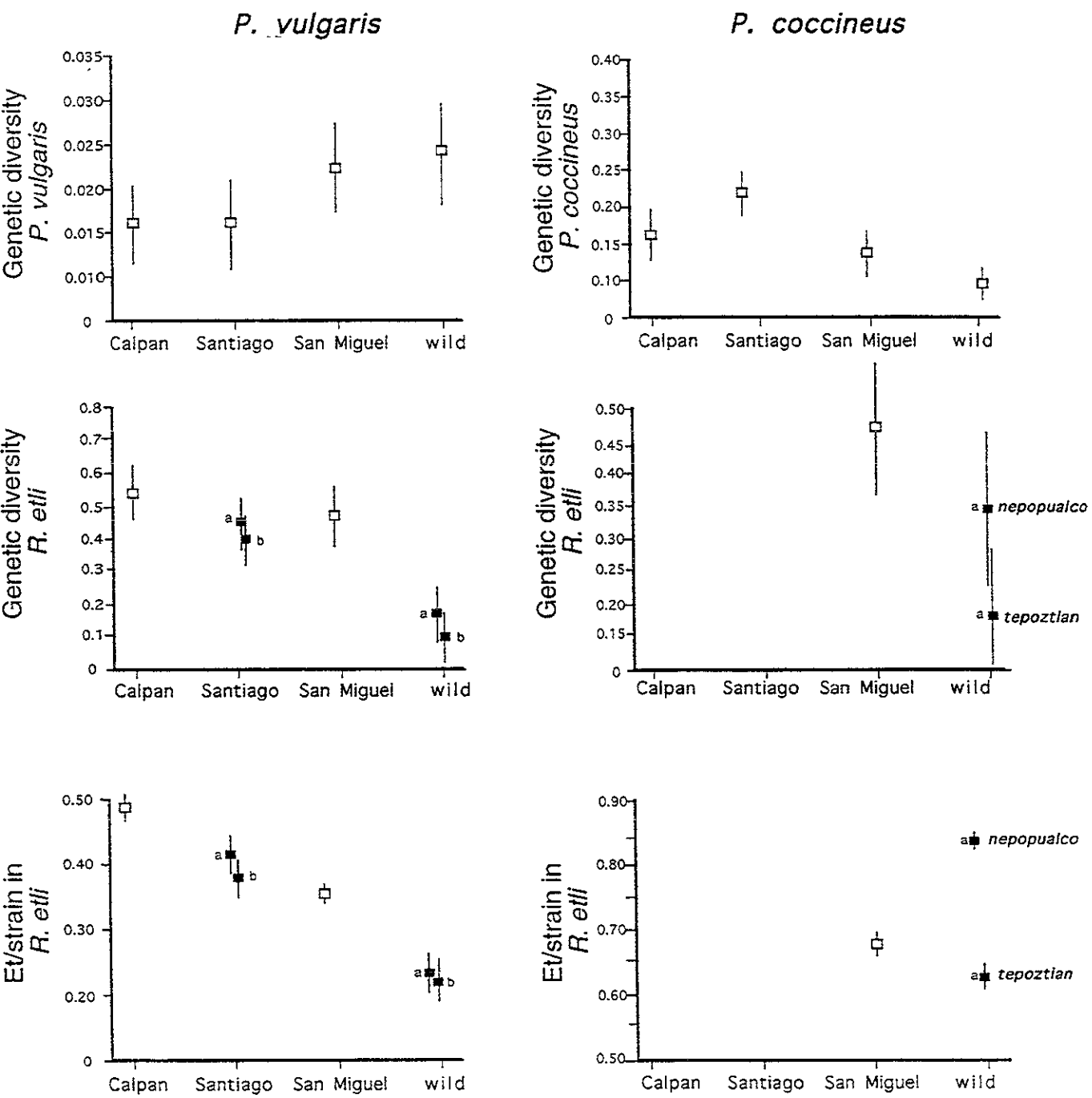


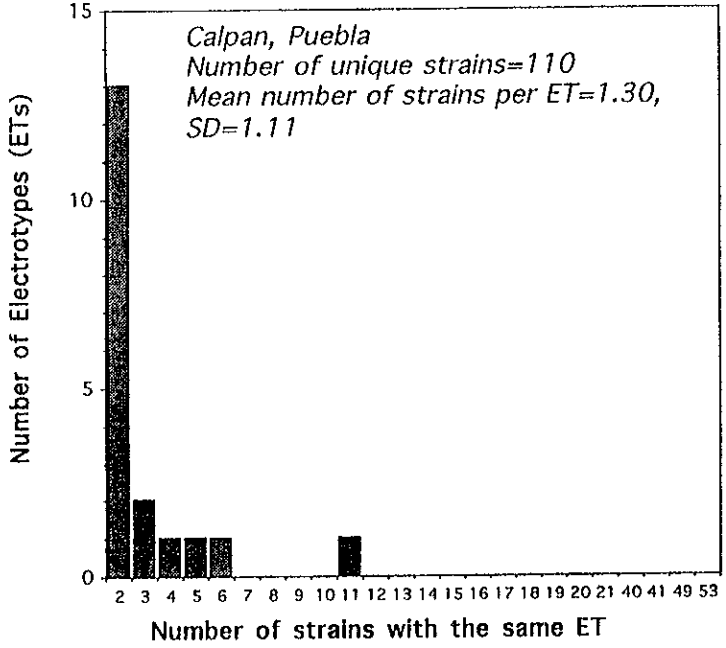


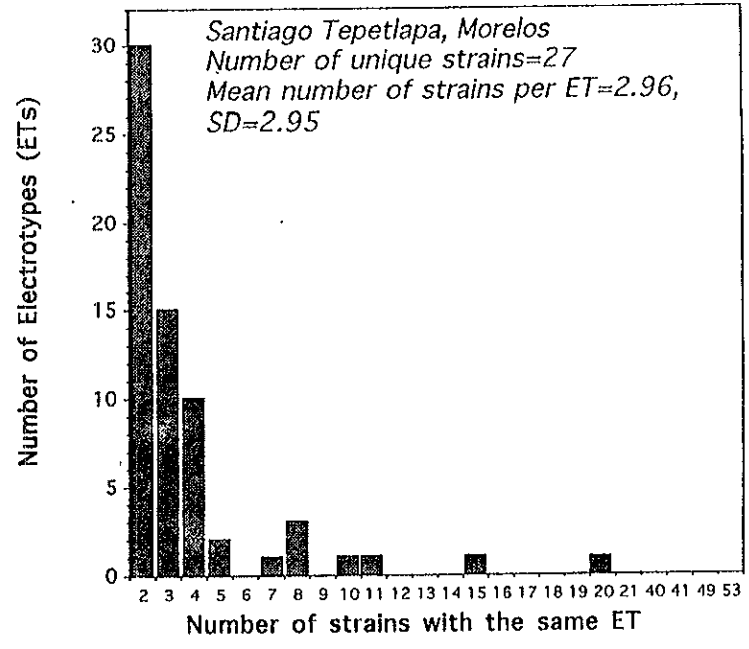


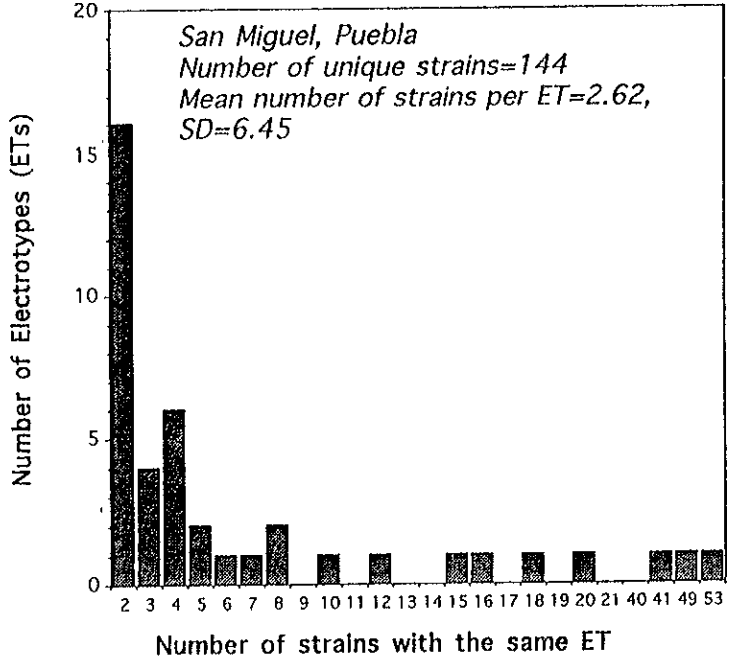


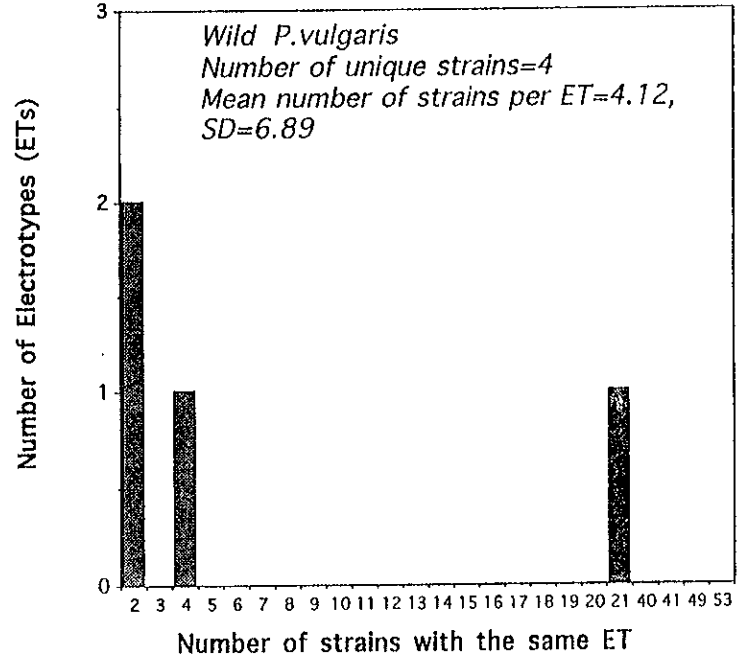



P. vulgaris
P. coccineus
Genetic diversity
P. vulgaris
Genetic diversity
P. coccineus
Genetic diversity
R. etli
Et/strain in
R. etli
Calpan
Santiago
San Miguel
wild
nepopualco
tepoztlan


Calpan, Puebla
Number of unique strains=110
Mean number of strains per ET=1.30,
SD=1.11
Number of Electrotypes (ETs)
Number of strains with the same ET


Santiago Tepetlapa, Morelos
Number of unique strains=27
Mean number of strains per ET=2.96,
SD=2.95
Number of Electrotypes (ETs)
Number of strains with the same ET


San Miguel, Puebla
Number of unique strains=144
Mean number of strains per ET=2.62,
SD=6.45
Number of Electrotypes (ETs)
Number of strains with the same ET


Wild P.vulgaris
Number of unique strains=4
Mean number of strains per ET=4.12,
SD=6.89
Number of Electrotypes (ETs)
Number of strains with the same ET

CONCLUSIONES Y PERSPECTIVAS

Los valores de diversidad genética de *R. etli* en San Miguel encontrados en este trabajo son similares a los reportados para otras especies de *Rhizobium*, (Piñero *et al.*, 1988; Harrison *et al.*, 1989a; Eardly *et al.*, 1990; Martínez *et al.*, 1991; Souza *et al.*, 1994; Demezas *et al.*, 1995; Strain *et al.*, 1995; Hagen *et al.*, 1996; Wilson *et al.*, 1998) así como para otras especies bacterianas del suelo (Martínez-Romero y Caballero-Mellado, 1996). Mientras que en Calpan se encontró una diversidad genética un poco más alta que la de San Miguel.

Sin embargo, aunque la diversidad genética sea similar a la encontrada en otros estudios; la estructura genética de *R. etli* en San Miguel es diferente. En las milpas de este sitio se observó una alta dominancia ecológica para *R. etli*. Esta estructura poblacional sugiere una estructura fuertemente epidémica, similar a la observada en los nódulos de *R. meliloti*, donde se encuentra esta misma estructura donde unas cuantas clonas dominan la población de los nódulos (Bromfield *et al.*, 1995). Este tipo de estructura genética también se ha reportado para bacterias patógenas como *Neisseria meningitidis* (Maynard Smith *et al.* 1993) donde algunas cepas o clonas son capaces de causar epidemias. En el caso de las poblaciones de San Miguel, esta estructura epidémica sugiere por una parte que existe un intenso proceso de selección sobre las bacterias que van a formar los nódulos. Por otra parte, es posible que este sea la estructura original de las poblaciones nativas de *Rhizobium*, ya que en los frijoles silvestres de Morelos (Souza et al., 1994) se observó una fuerte dominancia similar a la encontrada en San Miguel, lo cual apoya la idea de que la diferencia entre los ecosistemas naturales y los agrícolas depende en gran media de la intensidad el manejo que se les dé (Altieri 1986). Estas similitudes con el ecosistema natural se pueden deber a que este es el primer estudio que se realiza en un cultivo tradicional de frijol donde la labranza mínima provee un ambiente más favorable para la biota del suelo y hojarasca que el monocultivo. Esto posiblemente sea debido a que la entrada y la liberación de nutrientes es gradual tanto en la milpa como en los

ecosistemas naturales. Asimismo, se ha demostrado que en estos agrosistemas el crecimiento de las raíces se ve estimulado por el aumento de la actividad de los invertebrados terrestres (Altieri 1986).

Por otra parte, aunque los agroecosistemas modernos han demostrado su capacidad de sostener grandes poblaciones de cultivos, hay considerable evidencia sobre la falta de resilencia de esos ecosistemas. Esta fragilidad fue constatada en la estructura genética de *R. etli* en Calpan donde se observó una estructura genética diversa y azarosa; donde no parece haber adaptación local ni al suelo ni a cierto tipo de frijoles. Esta estructura se refleja en el hecho de que a pesar de la cercanía geográfica entre parcelas, el número de genotipos compartidos entre ellas es muy bajo, tanto en el tiempo como el espacio (Bouchet, 1996; Valera 1997); mientras que en San Miguel los genotipos dominantes son estables tanto en el espacio como en el tiempo (Silva *et al.*, en preparación). Esto podría deberse en gran medida al manejo agrícola al que están sometidos los frijoles monocultivados, ya que, a pesar de su alto rendimiento, estos agrosistemas tienen las desventajas de los ecosistemas inmaduros, donde es patente la falta de habilidad para llevar a cabo funciones de protección en términos de reciclaje de nutrientes, conservación de suelos y regulación de poblaciones (Altieri 1986), por lo que es necesaria la aplicación de agroquímicos. Los agroquímicos pueden estar influyendo negativamente sobre el proceso de infección y nodulación (Brockwell *et al.*, 1995). Por otro lado, en Calpan no se favorece la coevolución entre el frijol y su simbionte ya que las variedades de frijol que se siembran varía de año en año de acuerdo a las necesidades del mercado.

Tanto en San Miguel como en Calpan existe desequilibrio de ligamiento significativo para las poblaciones de *R. etli*. Sin embargo, este desequilibrio es diferente en cada sitio; ya que en San Miguel existe una estructura reticulada, donde es frecuente la recombinación dentro de grupos genéticos similares e infrecuente entre grupos genéticos distantes. Esta recombinación asortativa puede favorecer el incremento de la diversidad genética dentro del grupo; pero evita que se pierdan genotipos que hacen de este conjunto de

cepas emparentadas, un grupo adaptado ecológicamente, tanto a ciertas condiciones ambientales como a ciertos genotipos y especies de frijol. Esto se refleja en el hecho de que todos los genotipos dominantes pertenezcan a un grupo genéticamente relacionado. Por otra parte, en Calpan la probabilidad de recombinación entre grupos genéticos disimiles es alta, reflejándose esto en la poca estabilidad genética de las cepas y en la alta diversidad (Bouchet, 1996).

En estudios anteriores se ha sugerido la participación de las poblaciones no simbióticas de *Rhizobium* en la generación de diversidad de las poblaciones simbióticas (Segovia *et al.* 1991; Sullivan *et al.* 1995). Los resultados del presente estudio se basan en el análisis de una muestra de *R. etli* simbiótico, aislados de nódulos, pero además sería interesante conocer los patrones de variación en el suelo. Con estos datos podríamos saber si los niveles de diversidad y la estructura genética de *Rhizobium* son similares en sus diferentes nichos ecológicos. Sería también interesante conocer si en Calpan la recombinación genética entre cepas incapaces de nodular genera una poza génica amplia de cepas que pueden adquirir por transferencia horizontal el plásmido simbiótico. De esta manera las cepas de *R. etli* pueden ser capaces de nodular, aunque sea de forma poco eficiente, con un rango muy amplio de frijoles. En contraste con Calpan, en San Miguel, donde los linajes de frijol criollo parecen ser menos promiscuos, suponemos que la poza génica de las cepas de *Rhizobium* capaces de nodular, se ve purgada constantemente por la selección natural, ya que solo aquellos genotipos que cumplen con una serie de condiciones particulares pueden sobrevivir en las duras condiciones del San Miguel (suelo alcalino (pH= 8.27) y baja precipitación (600 mm/año)) y competir con aquellos genotipos bien adaptados a estos frijoles.

La baja diferenciación genética encontrada en San Miguel indica que existe suficiente flujo génico como para prevenir la divergencia genética de estas bacterias. Este es un resultado importante, ya que los mecanismos y el alcance de dispersión de *Rhizobium* no se conocen (Martinez-Romero y Caballero Mellado 1996). Los resultados encontrados en San Miguel indican que a una escala geográfica local existe un tamaño efectivo de migrantes elevado. Todos los valores del

número efectivo de migrantes por generación (*Nm*) son mayores que 4. Con un *Nm* mayor o igual a 4 se considera que existe suficiente migración como para prevenir la divergencia genética de las poblaciones. En Calpan la diferenciación genética es mayor (Bouchet 1996) que en San Miguel, lo cual es producto de la baja estabilidad genética de *R. etli* en este sitio. Sin embargo, sería recomendable utilizar otros métodos poblacionales indirectos (como es la presencia de alelos privados (Slatkin, 1985)), así como tratar de hacer estimaciones directas de la migración de estas bacterias en su hábitat natural. Esta última estimación podría hacerse liberando cepas con algún marcador genético fácil de detectar y seguir en el campo.

La existencia de dos grupos genéticos principales en San Miguel es un resultado que se ha encontrado al analizar otras especies de *Rhizobium* (Eardly *et al.*, 1990; Martínez *et al.*, 1991; Demezas *et al.*, 1995; Strain *et al.*, 1995). Para demostrar que estos grupos genéticos son entidades biológicas con sentido y determinar su posición taxonómica, se podrían utilizar otras técnicas moleculares. Para tener evidencia de una barrera reproductiva entre estos dos grupos genéticos sería necesario contar con información adicional. Encontrar plásmidos o patrones de plásmidos exclusivos de cada grupo, podría ser evidencia de trasferencia horizontal dentro de los grupos pero no entre ellos. Encontrar bacteriófagos exclusivos a cada grupo aportaría evidencia de vectores de intercambio genético dentro de los grupos. Si al liberar cepas marcadas de alguno de los dos grupos se encontrara que existe trasferencia lateral del marcador a cepas del mismo grupo, pero no del otro grupo, apoyaría la idea de transferencia lateral dentro de los grupos y aislamiento reproductivo entre ellos.

Es importante abordar la problemática de los agroecosistemas, tomando en cuenta el manejo que se les dé y las consecuencias que esto tiene sobre las interacciones bióticas y abióticas generadas dentro del ecosistema. Por lo tanto, un objetivo importante de la agroecología podría ser diseñar agroecosistemas estables con una productividad similar a la de los ecosistemas naturales, permitiendo esto alcanzar la autosuficiencia y sustentabilidad de la producción

agrícola. Un ejemplo de estos agrosistemas es el sistema de milpa, donde las interacciones entre *R. etli* y *Phaseolus* sp. parecen mantener relaciones similares a las observadas entre *Rhizobium* y las leguminosas silvestres.

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