UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE CIENCIAS

ANÁLISIS MOLECULAR DEL SIMBIOVAR MIMOSAE DE *Rhizobium etli*

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTOR EN CIENCIAS

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CIUDAD UNIVERSITARIA, CD. MX., 2021.
NOVIEMBRE

COORDINACIÓN DEL POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE CIENCIAS

OFICIO CPCB/1068/2021

ASUNTO: Oficio de Jurado

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Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 09 de noviembre de 2021

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POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

AGRADECIMIENTOS INSTITUCIONALES

Al posgrado en Ciencias Biológicas (PCB) de la Universidad Nacional Autónoma de México (UNAM), por darme la oportunidad de tener un desarrollo académico y profesional.

Al proyecto PAPIIT IN210021

Al CONACYT por el apoyo recibido en el posgrado.

A mi tutor principal: Dra. María Esperanza Martínez Romero por darme la oportunidad de desarrollar la tesis en su laboratorio.

A los miembros de mi comité tutor: Dr. Víctor Manuel González Zúñiga y el Dr. Enrique Merino Pérez por su apoyo académico.

DEDICATORIA

A

MARCO ANTONIO ROGEL LÓPEZ

Aline López López

A mis padres, la Sra. Ma. Isabel Hernández Hernández y el Sr. Estanislao Rogel Rebollar y a mis hermanos.

ÍNDICE **Página**

RESUMEN 6

ABSTRACT 7

INTRODUCCIÓN 8

¿Qué es un simbiovar? 9

Generalidades de los rizobios 10

Proceso de nodulación y FBN de los rizobios 11

Organización genómica de los rizobios 14

Mimosa afiinis, leguminosa endémica de Centro América 17

Justificación 18

Hipótesis y objetivos 19

ARTÍCULO REQUISITO 21

ARTÍCULOS CIENTÍFICOS PUBLICADOS 32

DISCUSIÓN GENERAL 56

CONCLUSIONES 65

REFERENCIAS BIBLIOGRÁFICAS 66

RESUMEN

Dentro de las especies de *Rhizobium* pueden existir diferentes simbiovares. *Rhizobium etli* posee el simbiovar (sv.) phaseoli y el sv. mimosae representados por las cepas CFN42 y Mim1 respectivamente; ambos sv. tienen la capacidad de nodular y fijar nitrógeno en plantas de *Phaseolus vulgaris*, sin embargo, éstos se diferencian por el rango de hospedero; mientras que el sv. phaseoli entabla una simbiosis efectiva solamente con *P. vulgaris*; el sv. mimosae tiene un rango de hospedero más amplio que le permite nodular efectivamente otras plantas como *Mimosa affinis* de donde fue aislado, *Leucaena leucocephala*, *Calliandra grandiflora* y *Acaciella angustissima*. El genoma del sv. phaseoli (CFN42) fue reportado por Victor Gonzalez y colaboradores en el 2006.

En este trabajo se secuenció el genoma del sv. mimosae (Mim1) utilizando diferentes plataformas de secuenciación masiva y una librería genómica de BAC´s (por sus siglas en inglés, "Bacterial Artificial Chromosome"). El genoma de la Mim1esta compuesto por un cromosoma de 4.8 Mb y 6 replicones extracromosomales que van de los 181 Kb a 1.08 Mb. En un análisis de genómica comparativa, se pudo observar que ambos sv. tienen sus cromosomas y algunos replicones extracromosomales muy conservados; sin embargo, sus plásmidos simbióticos poseen grandes diferencias tanto a nivel de secuencias como en contenido de genes, mientras que el plásmido simbiótico (pSim) del sv. phaseoli es de 371 kb, el pSim del sv. mimosae es de 615 kb.

El análisis genómico de los diferentes sv. de una misma especie de *Rhizobium*, nos ayudará a tratar de entender el papel que juegan los determinantes genéticos que les confieren las diferencias en cuanto al rango de hospedero.

ABSTRACT

Within *Rhizobium* species there may be different symbiovars. *Rhizobium etli* has the symbiovar (sv.) phaseoli and sv. mimosae represented by strains CFN42 and Mim1 respectively; both sv. have the ability to nodulate and fix nitrogen in *Phaseolus vulgaris* plants; however, they differ by host range; while sv. phaseoli establishes an effective simbiosis only with *P.vulgaris*; the sv. mimosae has a broader host range that allows it to effectively nodulate other plants such as *Mimosa affinis* from which it was isolated, *Leucaena leucocephala*, *Calliandra grandiflora* and *Acaciella angustissima*. The genome of sv. phaseoli was repored by Víctor González et al., in 2006.

In this work, the sv. mimosae (Mim1) genome was sequenced using different massive sequencing platforms and a genomic library of BAC´s ("Bacterial Artificial Crhomosome"). The Mim1 genome is composed of 4.8 Mb chromosome and 6 extrachromosomal replicons ranging from 181kb to 1.08 Mb. In a comparative genomics analysis, it was observed that both sv. have their chromosomes and some extrachromosomal replicons highly conserved; however, its symbiotic plasmids have great differences both at the level of sequences and in gene content, while the symbiotic plasmid (pSym) of sv. phaseoli is 371 kb, the pSym of sv. mimosae is 615 kb.

The genomic analysis of different sv. of the same *Rhizobium* species, it would help us to try to understand the role played by genetic determinants that the differences in host range confer on them.

INTRODUCCIÓN

Las bacterias fijadoras de nitrógeno juegan un papel importante en la asimilación del nitrógeno atmosférico. Aquellas que lo hacen en vida libre se encuentran distribuidas en diferentes grupos bacterianos por ejemplo: *Azospirillum brasilense*, *Burkholderia vietnamensis*, *Klebsiella variicola* y *Paenibacillus polymyxa* que pertenecen a las alfa (α), beta (β), gamma (γ) proteobacteria y Firmicutes respectivamente; estas bacterias han sido aisladas de diferentes plantas como la caña de azúcar, arroz, plátano, maíz, entre muchas otras (Tejera et al., 2005; Gillis et al 1995; Rosenblueth et al,. 2004; Von der Weid et al., 2000; Rosenblueth et al., 2006). Sin embargo, las bacterias que nodulan y llevan a cabo la fijación biológica de nitrógeno (FBN) en asociación con plantas que en su mayoría son leguminosas, pertenecen solo a las α y β-Proteobacterias y se les conoce como rizobios (Terpolilli et al., 2012); dentro de las β-Proteobacterias se han descrito 21 especies en el género *Paraburkholderia* y dos especies en el género *Cupriavidus*, capaces de nodular y fijar nitrógeno en plantas leguminosas (Velázques et al., 2017; Paulitsch et al., 2020; Steenkamp et al., 2015; Estrada de los Santos et al., 2014), pero dentro de las α-Proteobacterias en la actualidad ya se han descrito 16 géneros que son: *Agrobacterium*, *Allorhizobium*, *Aminobacter*, *Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Ciceribacter*, *Pararhizobium*, *Mesorhizobium*, *Methylobacterium*, *Microvirga*, *Ochrobactrum*, *Phyllobacterium*, *Rhizobium*, *Shinella* y *Ensifer* (Yuan T. et al., 2018), cada uno de éstos con diferentes especies que han sido descritas de acuerdo a sus propiedades de nodulación y con diferentes criterios moleculares, así como estudios polifásicos que se han establecido a través del tiempo (Graham P.H., et al. 1991; de Lajudie et al., 1994, 2019; Richter and Rosselló-Móra, 2009).

Rhizobium es el género que presenta el mayor número de especies (Shamseldin et al., 2017) y dentro de algunas de éstas, se han descrito diferentes variantes simbióticos o simbiovares.

¿Qué es un simbiovar?

El término simbiovar (sv.) fue propuesto en sustitución al biovar y como un término paralelo al patovar que se utiliza en las bacterias patógenas (Rogel et al. 2011). Los simbiovares pueden ser compartidos por diferentes especies, debido a la transferencia lateral de la información genética necesaria para que se lleve a cabo la simbiosis. En algunos géneros como *Bradyrhizobium* o *Mesorhizobium* ésta información se encuentra codificada como islas simbióticas en sus cromosomas, pero en el caso del género de *Rhizobium* se encuentra codificada en una estructura extracromosomal denominada plásmido simbiótico (pSim), por ejemplo en *R. gallicum* se encuentra el sv. *gallicum*, sv. *phaseoli* y sv. *orientale*, de éstos, se podría asumir que los simbiovares *phaseoli* y *orientale*, pudieron haberse adquirido por transferencia lateral de sus pSim (Rogel et. al., 2011), una lista más actualizada de los simbiovares de *Rhizobium* se encuentra en Velázques et al., 2017.

Los diferentes simbiovares pueden o no tener el mismo fondo genético, tal es el caso de *Rhizobium etli* sv. phaseoli que tiene la capacidad de nodular frijol (Segovia et al., 1993) y *R. etli* sv. mimosae, que además de nodular plantas de frijol, también tiene la capacidad de nodular plantas de *Mimosa affinis* de donde fue aislado (Wang et al., 1999), *Leucaena leucocephala*, *Calliandra grandiflora* y *Acaciella angustissima*. Ambos simbiovares comparten el 90 % de homología ADN-ADN demostrado con experimentos de hibridación, que nos habla de un mismo fondo genético pero con plásmidos simbióticos diferentes (Wang et al., 1999) que les pueden estar dando las diferencias en cuanto al rango de hospedero.

Generalidades de los rizobios

Los rizobios son bacterias de vida libre que tienen la capacidad de vivir en el suelo como saprofitos, además pueden adoptar un estilo de vida oligotrófica en la compleja comunidad microbiana del suelo (Poole et al., 2018). Cuando los rizobios posen la información genética necesaria para nodular y fijar nitrógeno, éstos pueden entablar una simbiosis con plantas leguminosas y se les conoce como simbiontes diazótrofos (Iyer et al., 2016). Se ha demostrado que coexisten poblaciones de *Rhizobium* simbióticos y no simbióticos en la rizósfera de leguminosas como el frijol, con proporciones que van de 1 a 40 respectivamente (Segovia et al., 1991), así como bacterias de *Bradyrhizobium* del suelo que no tienen la capacidad de nodular ni fijar nitrógeno (Jones et al., 2016). La mayoría de los rizobios tienen un estrecho rango de hospedero, sin embargo existen algunas excepciones como por ejemplo: *Sinorhizobium fredii* NGR234 que puede infectar 112 géneros de leguminosas (Pueppke and Broughton, 1999).

Para que se lleve a cabo la simbiosis bacteria-planta, los rizobios prenden toda una batería de genes involucrados en diferentes procesos; desde el pegado de las bacterias a la raíz, colonización de la rizósfera, nodulación y fijación de nitrógeno. Sin embargo, los genes más estudiados y caracterizados son los que están involucrados en el proceso de nodulación y FBN denominados genes *nod*, *nif* y *fix*. Para que los rizobios lleven a cabo la FBN en plantas leguminosas, las plantas desarrollan estructuras especializadas llamadas nódulos (Oldroyd et al., 2011). Una vez que se establece la simbiosis, las bacterias transforman el nitrógeno diatómico (N_2) en amonio (NH_3) (Burén and Rubio, 2018) y la planta le provee de fuentes de carbono derivadas de la fotosíntesis y todos los nutrientes necesarios que requiere la bacteria para su metabolismo (Udvardi and Poole, 2013).

Proceso de nodulación y FBN de los rizobios

La interacción planta-bacteria requiere de todo un mecanismo de señalización, en donde las plantas producen compuestos llamados flavonoides que son esenciales en la especificidad huésped-simbionte (Liu and Murray, 2016). Los flavonoides son censados por los rizobios, específicamente por la proteína NodD (Mulligan and Long, 1985) que actúa como regulador transcripcional de los genes *nodABC* (Chen et al., 2005), dichos genes son comunes entre todos los rizobios (Roche et al., 1996) y codifican para enzimas involucradas en la síntesis del esqueleto núcleo de metabolitos denominados factores de nodulación (factores Nod) (Roche et al., 1996; Kidaj et al., 2020). Los factores Nod son lipo-quitooligosacaridos hospedero-específicos (Oldroyd et al., 2011) producidos principalmente por los rizobios (Kidaj et al., 2020). Los genes *nodABC* son esenciales para que se lleve a cabo la simbiosis ya que mutaciones en estos genes resulta en un fenotipo Nod menos; es decir que la bacteria pierde la capacidad de producir factores Nod y por lo tanto no puede establecer la simbiosis (Kidaj et al., 2020). Además de los genes *nodABC*, se han reportado muchos otros genes *nod*, *nol* y *noe* cepa-específicos (Wang et al., 2018) que son considerados como los principales determinantes en la especificidad del hospedero (Andrews and Andrews, 2017); algunos de ellos están involucrados en modificaciones químicas de los factores Nod, por lo que éstos pueden presentar diferentes grupos funcionales que les da la especificidad por el hospedero, estas modificaciones pueden incluir acetilación, glicosilación, metilación y sulfatación (Andrews and Andrews, 2017). La figura 1 nos muestra un panorama general de como son los factores Nod y los genes que han sido caracterizados por su función en la generación y modificación del factor Nod, (Fig. 1).

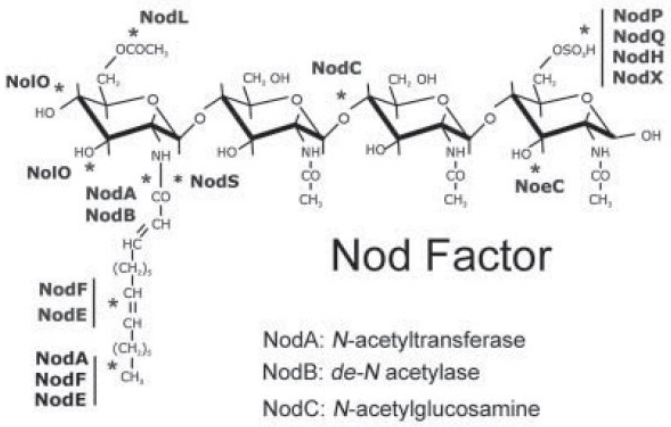

Fig. 1.- La cadena típica del factor Nod consiste de 4 o 5 residuos N-acetil-glucosamina ligados en la posición β-1-4. Los factores nod son sujetos de modificaciones químicas (la posición de los grupos que se agregan con frecuencia se indica con asteriscos) por la acción de los genes *nod* de los rizobios (en negritas). Los diferentes tipos de decoraciones dan como resultado una mezcla de factores Nod producidos por cada especie de rizobio. El producto de los genes *nodABC* participan en la síntesis de la cadena del factor Nod. (Figura extraída parcialmente de Via et al., 2016).

Cuando los factores Nod interactúan con las raíces de las plantas comienza la organogénesis del nódulo (Oldroyd et al., 2011). Los nódulos se clasifican como determinados o indeterminados en base a la actividad del meristemo (Li, et al., 2013). Los nódulos indeterminados mantienen el tejido meristemático, mientras que los nódulos determinados tienen un meristemo transitorio y dependerá de la planta hospedera el tipo de nódulo que se desarrolla (Andrews and Andrews, 2017). Cuando las raíces de las leguminosas detectan a los factores Nod, se produce un enroscamiento de los pelos radiculares en donde quedan atrapados los rizobios; posteriormente, se produce el hilo de infección a través del cual los rizobios llegan a las células epidérmicas para generar el nódulo (Wang et al., 2018). Conjuntamente a la organogénesis, los rizobios penetran al citoplasma de las células del nódulo y se transforman a bacteroides; que es una forma diferenciada de

los rizobios para fijar nitrógeno dentro de las células (Poole et al., 2018; Oldroy et al., 2011). La Fig. 2 nos muestra cómo se lleva a cabo el desarrollo de un nódulo indeterminado y las diferentes zonas que lo conforman una vez que ha alcanzado su madurez (Wang et al., 2018).

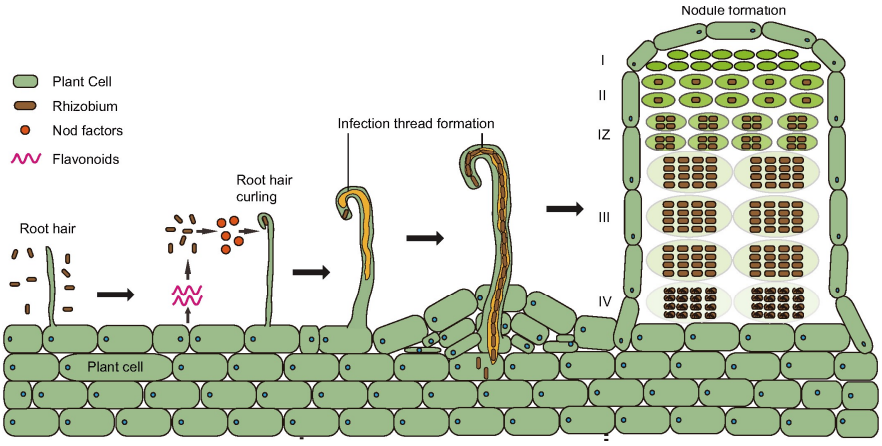

Fig. 2.- Proceso de infección y desarrollo del nódulo. Un nódulo indeterminado maduro contiene una zona meristemática (I), una zona de infección (II), una inter-zona (IZ), una zona de fijación de nitrógeno (III) y una zona de senescencia (IV) (Figura extraída parcialmente de Wang et al., 2018).

En el proceso de FBN, los genes *nif* codifican proteínas esenciales para este proceso, los cuales son compartidos por la mayoría de las bacterias fijadoras de nitrógeno (diazótrofos), pero en los rizobios (simbiontes diazótrofos) se requieren también los genes *fix* que codifican para proteínas específicas para dicho proceso (Poole et al., 2018). Los genes *nifHDK* representan los genes estructurales de la enzima nitrogenasa que es esencial en la FBN (Machado et al., 1996); además, en un análisis con 110 genomas secuenciados por completo se pudo comprobar que los genes *nifHDKEN* son genes universales en los microorganismos fijadores de nitrógeno (Raymond et al., 2004). El complejo enzimático de la nitrogenasa es la unidad central de FBN en todos los microorganismos diazotrófos

(Lindström and Mousavi, 2020). En la tabla 1 se muestran los genes *nod*, *nif* y *fix* más comunes en los rizobios.

Tabla 1. Lista de los genes *nod*, *nif* y *fix* más comunes en los rizobios (según Laranjo et al., 2014). (Tabla extraída de Lindström and Mousavi, 2020)

Genes	Function of gene product
Nodulation genes	
nodA	Acyltransferase
nodB	Chitooligosaccharide deacetylase
nodC	N-acetylglucosaminyltransferase
nodD	Transcriptional regulator of common *nod* genes
nodIJ	Nod factor transport
nodPQ	Synthesis of Nod factor substituents
nodX	Synthesis of Nod factor substituents
nofEF	Synthesis of Nod factor substituents
Other *nod* genes	Several functions in synthesis of Nod factors
nol genes	Several functions in synthesis of Nod factor substituents and secretion
noe genes	Synthesis of Nod factos substituents
Nitrogen fixation genes	
nifH	Dinitrogenase reductase (Fe protein)
nifD	α subunits of dinitrogenase (MoFe protein)
nifK	β subunits of dinitrogenase (MoFe protein)
nifA	Transcriptional regulator of the other *nif* genes
nifBEN	Biosynthesis of the Fe-Mo cofactor
fixABCX	Electron transport chain to nitrogenase
fixNOPQ	Cytochrome oxidase
fixLJ	Transcriptional regulators
fixK	Transcriptional regulator
fixGHIS	Copper uptake and metabolism
fdxN	Ferredoxin

Organización genómica de los rizobios

Para tratar de conocer y entender la organización genómica de las bacterias, se han desarrollado diferentes técnicas de biología molecular, con las cuales se puede determinar las moléculas de ADN de los genomas bacterianos, entre estas metodologías se encuentra la técnica de Eckhardt, mediante la cual se pueden visualizar los elementos de ADN extracromosomales denominados plásmidos (Eckhardt, T., 1978, Hynes et al., 1985), y en algunas bacterias como *Brucella* se pueden observar también sus cromosomas (Gándara et

al., 2001). Otra metodología que ha sido muy utilizada es la que se conoce como electroforesis de campos pulsados, con la cual se puede tener mayor certeza del tamaño real de un genoma bacteriano (Jumas-Bilak et al., 1998), pero sin duda, la secuenciación de sus genomas completos nos pone claramente de manifiesto su organización genómica.

Para obtener la secuencia genómica de los primeros rizobios, se utilizó la metodología denominada "shotgun" (por sus siglas en inglés), que consiste en la realización de librerías genómicas de alrededor de 3 a 8 kilobases para su posterior secuenciación. El primer reporte de la estructura genómica completa de un rizobio utilizando esta metodología fue en el año 2000, en donde se secuenció a *Mesorhizobium loti* cepa MAFF303099 (Kaneko et al., 2000), sin embargo, con las subsecuentes plataformas de secuenciación masiva de ADN como son Illumina, Ion torrent, 454 FLX Titanium, Solid, y recientemente PacBio y nonopore; en la actualidad se pueden secuenciar genomas bacterianos completos en muy poco tiempo y cada día a un menor costo.

Las nuevas tecnologías de secuenciación masiva han permitido conocer los genomas de bacterias representantes de casi todos los linajes bacterianos, aunque constantemente surgen nuevos grupos. A partir del año 2000 y hasta la fecha, se han reportado muchos genomas bacterianos que representan a casi todos los géneros de los rizobios, como por ejemplo: *Burkholderia phymatum* STM815 y *Cupriavidus taiwanensis* LMG19424 (Moulin et al., 2014; Amadou et al., 2008), que son las cepas tipo de los dos géneros descritos en las β-Proteobacterias, así como representantes de los géneros *Azorhizobium*, *Bradyrhizobium, Rhizobium* y *Sinorhizobium* de las α-Proteobacterias (Lee et al., 2008, Kaneko et al., 2002, González et al., 2006, Galibert et al., 2001), entre muchos otros.

Las bacterias en general pueden tener uno o más replicones de ADN, los cuales se pueden clasificar en cinco grupos que son: Cromosoma, cromosoma secundario, crómidos, megaplásmidos y plásmidos (diCenzo et al., 2017). De los genomas bacterianos analizados en la base de datos del NCBI (National Center for Biotechnology Information) hasta marzo del 2016, aproximadamente el 10% se encontraron divididos entre dos o más replicones; una arquitectura genómica conocida como genoma multipartita, esta organización se encuentra en muchos organismos importantes como los simbiontes vegetales (rizobios fijadores de nitrógeno) y los patógenos vegetales, animales y humanos (diCenzo et al., 2017).

Los rizobios simbiontes de leguminosas tienen diferentes arreglos genómicos, existen por ejemplo aquellos que solo tienen un cromosoma como la mayoría de los aislados que pertenecen al género *Bradyrhizobium* (Teamtisong et al., 2014), aunque se han reportado algunos aislados que también tienen plásmidos (Cytryn et al., 2008; Okazaki et al., 2015). Con respecto a los aislados del género *Rhizobium*, prácticamente todos los secuenciados tienen una organización genómica multipartita, los cuales tienen un cromosoma y pueden llegar a tener crómidos, megaplásmidos y plásmidos; además su organización es muy variable y prácticamente se da por aislado, aun cuando se trate de la misma especie.

R. etli sv. phaseoli CFN42 tiene una estructura multipartita, compuesta por 7 replicones que son: un cromosoma de 4.3 Mb, dos crómidos de 184 y 505 kb y 4 plásmidos de 194, 250, 371 y 642 Kb (González et al., 2006; Harrison et al., 2010). El psim de esta cepa está representado por el replicón de 371 Kb, sin embargo en la mayoría de las especies del género de *Rhizobium* los psim oscilan entre 400 a 600 kb.

Mimosa affinis, leguminosa endémica de Centro América

La familia de las leguminosas (Leguminosae o Fabaceae), se encuentra distribuida a nivel global y tiene una enorme importancia tanto ecológica como económica por el papel que desempeña tanto en los sistemas naturales como en los agrícolas (Sprent et al., 2017). Esta familia comprende alrededor de 19,500 especies en 751 géneros y se encuentra dividida en tres subfamilias: Caesalpinioideae, Papilionoideae y Mimosoideae (LPWG, 2013). Dentro de la subfamilia Mimosoideae se han definido cinco tribus: Mimozygantheae, Parkieae, Acacieae, Ingeae y Mimoseae.

El género *Mimosa* L. que pertenece a la tribu Mimoseae (Camargo et al., 2001), representa uno de los géneros más grandes dentro de la familia de las leguminosas con más de 500 especies distribuidas principalmente en el neotrópico (Simon et al., 2011). *Mimosa affinis* es una especie endémica de centro américa que se encuentra distribuida en diferentes países de centro américa como son Nicaragua, Guatemala, Belice y México. En México se encuentra en los estados de Sinaloa. Nayarit, Jalisco, Michoacan, Estado de México, Morelos, Guerrero, Oaxaca, Campeche, Yucatan y Quintana Roo (Camargo 2001).

Justificación

Los genes involucrados en el proceso de nodulación y FBN se encuentran codificados en plásmidos dentro de las cepas del género *Rhizobium*. Las variantes simbióticas (simbiovares) dependerán del tipo de pSim que posean. En *Rhizobium etli* se han descrito los simbiovares phaseoli y mimosae que nodulan preferentemente plantas de *Phaseolus vulgaris* y *Mimosa affinis* respectivamente. El sv. mimosae se diferencia del sv. phaseoli con base en su hospedero y algunos genes simbióticos como el *nifH* y la organización de los genes comunes *nodABC*. Sin embargo, existe mucha información en sus genomas que nos podrían ayudar a entender cuáles son los determinantes que les confieren las diferencias en cuanto al rango de hospedero. Por lo que la secuencia y análisis del genoma de la cepa Mim1 que representa al sv. mimosae, abona al conocimiento de lo que representan los simbiovares dentro de una especie bacteriana, además de darnos una idea más clara del porque se dan esas diferencias simbióticas dentro de una misma especie.

Hipótesis

Las diferencias genéticas de los simbiovares phaseoli y mimosae de *Rhizobium etli* determinan el rango de hospedero.

Objetivo principal

Analizar el genoma de *Rhizobium etli* sv. mimosae en comparación al del sv. phaseoli y definir los determinantes genéticos que le confieren el rango de hospedero.

Objetivos particulares

Secuenciar el genoma de *Rhizobium etli* simbiovar mimosae Mim1.

Determinar si *Phaseolus vulgaris* es capaz de atrapar al sv. mimosae en suelo.

ARTÍCULO REQUISITO

BMC Genomics

RESEARCH ARTICLE Open Access

Genomic basis of symbiovar mimosae in *Rhizobium etli*

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Abstract

Background: Symbiosis genes (*nod* and *nif*) involved in nodulation and nitrogen fixation in legumes are plasmid-borne in *Rhizobium*. Rhizobial symbiotic variants (symbiovars) with distinct host specificity would depend on the type of symbiosis plasmid. In *Rhizobium etli* or in *Rhizobium phaseoli,* symbiovar phaseoli strains have the capacity to form nodules in *Phaseolus vulgaris* while symbiovar mimosae confers a broad host range including different mimosa trees.

Results: We report on the genome of *R. etli* symbiovar mimosae strain Mim1 and its comparison to that from *R. etli* symbiovar phaseoli strain CFN42. Differences were found in plasmids especially in the symbiosis plasmid, not only in *nod* gene sequences but in *nod* gene content. Differences in Nod factors deduced from the presence of *nod* genes, in secretion systems or ACC-deaminase could help explain the distinct host specificity. Genes involved in *P. vulgaris* exudate uptake were not found in symbiovar mimosae but *hup* genes (involved in hydrogen uptake) were found. Plasmid pRetCFN42a was partially contained in Mim1 and a plasmid (pRetMim1c) was found only in Mim1. Chromids were well conserved.

Conclusions: The genomic differences between the two symbiovars, mimosae and phaseoli may explain different host specificity. With the genomic analysis presented, the term symbiovar is validated. Furthermore, our data support that the generalist symbiovar mimosae may be older than the specialist symbiovar phaseoli.

Keywords: Legume nodulation, Bacterial symbiosis, Nitrogen fixation, Host specificity

Background

Bacterial nitrogen fixation in legume nodules contributes to plant nutrition and allows plants to grow in nitrogen deficient soils. Genes for plant nodulation and nitrogen fixation are plasmid-borne in *Rhizobium* spp. (reviewed in [1]) and symbiovars define host specificity. There are over twenty different symbiovars reported not only in *Rhizobium* but also in *Bradyrhizobium* and in other genera of nodule forming bacteria [2-7]. The term symbiovar was proposed as a counterpart to the term pathovar in pathogenic bacteria [2]. A theoretical model proposes that a single species may exhibit alternative symbiovars depending on the presence of symbiotic plasmids or symbiotic islands [2]. The same symbiovar may be present in distinct species as a consequence of the lateral transfer of symbiotic plasmids or islands. Symbiotic genes and other genes associated with niche adaptation may have evolutionary histories independent of the evolution of the chromosomal genes [8]. Two symbiovars are recognized in *Rhizobium etli*: phaseoli (conferring the ability to nodulate *Phaseolus vulgaris*) and mimosae (involved in nodulating mimosas and *P. vulgaris*, [9]). Symbiovar mimosae strains were isolated from *Mimosa affinis* in Morelos and have a broad host range, including plants of *M. affinis, Leucaena leucocephala*, *Calliandra grandiflora, Acaciella angustissima* as well as *P. vulgaris* [9]. Symbiovar mimosae was originally distinguished from sv. phaseoli by the sequence of a few symbiotic genes and by the organization of *nif* and common *nod* genes. Multiple copies of *nifH* genes and a *nodA* gene separated from *nodBC* found in sv. phaseoli and not in sv. mimosae served as a molecular basis to distinguish these

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symbiovars [10,11]. Phylogenies indicated that symbiovar mimosae and phaseoli *nifH* genes were related [9] and similar to the corresponding gene in sv. gallicum [12]. Different origins of replication were found in sv. phaseoli and sv. mimosae symbiotic plasmids and both symbiotic plasmids were compatible [9]. It has been proposed that sv. mimosae is older than sv. phaseoli and that the phaseoli symbiotic plasmid was selected by *P. vulgaris* [13]. *P. vulgaris* is a recent species (probably two million years old [14]), while mimosas seem to be older.

Mimosas are distributed worldwide with Brazil and Mexico as main diversification sites. Mimosas in South America are nodulated by β-Proteobacteria like *Burkholderia* or other β-Proteobacteria [15-19] while mimosas in Mexico are only exceptionally nodulated by *Burkholderia* (unpublished). Mimosas from Mexico and Brazil are phylogentically separated [20]. Additionally abiotic conditions like pH and soil nitrogen content may account for their differences in symbionts [21]. Native mimosas in India are nodulated by sinorhizobia [22], that we have also found in some mimosa nodules in Mexico (unpublished).

Based on multilocus enzyme electrophoresis analysis, *M. affinis* isolate Mim1 was found to group closely to *R. etli* sv. phaseoli CFN 42 [9]. Other *M. affinis* isolates such as Mim2 were separated from CFN42 (Figure two in [9]) and thus Mim2 has been recently reassigned to *Rhizobium phaseoli* [1,23]. Therefore we recognize now that symbiovar mimosae exists in *R. phaseoli* as well as in *R. etli*. It is the aim of this work to define the genomic differences between two *R. etli* strains (CFN42 and Mim1) representing the symbiovars phaseoli and mimosa, respectively.

Methods

Strains and growth conditions

Rhizobium strains were grown overnight at 28ºC in PY medium [24] after recovery from glycerol stocks at -80ºC. Bacterial strains to be inoculated on plants were grown on solid PY media and resuspended in water to an OD_{600} of 0.5. For PCR or DNA isolation, bacteria were grown in liquid PY cultures [24].

Plasmid profiles

Plasmid profiles were visualized on agarose gels according to the protocol described by Hynes and McGregor [25]. Plasmid patterns from *R. etli* CFN42 or *R. leucaenae* CFN299 were used as references.

Plant nodulation assays

L. leucocephala seeds were treated with concentrated sulfuric acid for 15 min, rinsed with water and surface disinfected with sodium hypochlorite as described [26]; the same procedure was used to disinfect *P. vulgaris* seeds. Seeds were germinated in water-agar plates in the dark and transferred to flasks after 3 days. *L. leucocephala* plants were grown in vermiculite flasks with N free Fahraeus nutrient solution for 40 days and *P. vulgaris* plants in agar flasks with the same nutrient solution for 14 days.

Genome sequencing, assembly and annotation

Genomic DNA from *R. etli* Mim1 was sheared to produce two paired-end libraries for 454 pyrosequencing, one with 3 Kb inserts and the other with 8 Kb inserts. An additional 3 Kb library was sequenced only at one end. The total amount of reads were 512,236 paired reads and 112, 079 single reads. Library construction and sequencing was done at Mogene LC (St. Louis, MO, USA). Additionally two paired-end libraries were constructed, one with 200 bp inserts and the second with 2 Kb inserts. Both libraries were sequenced by Illumina at BGI (Beijing, China). A total amount of 16, 000, 000 short-paired readings (50–60 bases) were assembled. To improve the scaffolding a BAC library was constructed in pIndogo BAC-5™ vector by BIOS&T (Quebec, Canada) using fragments from a partial genomic DNA *Hind*III digestion. 105 BAC-ends were sequenced with ABI3730xl sequencer by Sanger method. Additionally, three BACs were completely sequenced with the same technology at the Center for Genomic Sciences (Cuernavaca, México). Two of these BACs were selected by hybridizing with *nifH* and other pSym probes; they embraced half of the pSym plasmid sequence. The third BAC was from the chromosome. Final assembly of the symbiotic plasmid was obtained using sequence reads from the three sources of information: BAC sequences, Illumina, and 454 pyrosequencing.

Different assembly strategies were used with the following programs: Newbler 2.5.3 (ROCHE), Velvet 1.1.06 [27], Sspace-Basic 2.0 [28], and Consed v23 [29]. ORFs were predicted with Glimmer 3 [30], and annotations were done in Artemis 12.0 graphic display [31] using previous annotations made for *R. etli* CFN42 [32] and comparing with the non-redundant data base of the Genbank [33], Interpro database [34], and IS database (http://www-is.biotoul.fr).

Sequence analysis

Average nucleotide identity (ANI) was calculated using the JSpecies software [35]. The DNA conservation between two genomes or replicons was estimated by obtaining an alignment with NUCmer [36], run with default parameters, and dividing the summed lengths of all aligned regions by the length of the genome or replicon and expressing the value obtained as a percentage. Common and specific protein families between *R. etli* CFN42 and Mim1 were detected using MCL as described [37].

Genomic islands

Alien Hunter v1.7 software [38] was used to analyze the chromosome sequence of *R. etli* sv. mimosae Mim1 and *R. etli* CFN42. The minimum region length for HT detection was 5 kbs. The score thresholds were 12.92 and 14.96 for Mim1 and CFN42, respectively.

Phylogenetic analysis

Alignments were performed with MUSCLE [39] and manually verified. Maximum likelihood trees were generated with PhyML [40] with tree node support evaluated by bootstrap analysis based on 1000 pseudoreplicate datasets. Phylogenetic relationships were also assessed by Bayesian inference using MrBayes 3.1 [41]. Analyses were initiated with random starting trees, run for 2,000,000 generations and three separate analyses were executed. Markov chains were sampled every 100 generations. We discarded 25% of trees as "burn in".

Genome accession numbers

Sequences and annotations were deposited in the Genbank database under accession numbers CP005950 (chromosome), CP005951 (pRetMim1a), CP005952 (pRetMim1b), CP005953 (pRetMim1c), CP005954 (pRetMim1d), CP005955 (pRetMim1e) and CP005956 (pRetMim1f).

Results and discussion

Genome of *R. etli* symbiovar mimosae strain Mim1

The final assembly of the *R. etli* sv. mimosae Mim1 genome rendered seven circular molecules: one chromosome and six plasmids at 150× coverage on average. The chromosome was 4.8 Mb in size while the plasmids ranged in size from 181 kb to 1.08 Mb (Figure 1). Average Nucleotide Identity (ANI) and the percentage of conserved DNA between Mim1 and CFN42 were 98.6% and 82.4% respectively on a whole genome analysis, confirming that both strains belong to the same species. Lower ANI (less than 90%) was found between Mim1 and strains of other species such as *R. phaseoli* and *R. leguminosarum*.

There were more than twice as many unique genes in Mim1 than in CFN42, mainly in plasmids. The respective chromosomes of each strain had around 260 unique genes. In chromosomes, 35 genomic islands were identified only in Mim1 and 17 only in CFN42. (Figure 2); genes found in Mim1 genomic islands are shown in Additional file 1: Table S1. Examples of unique genes found in Mim1 and not in CFN42 are those encoding cytochrome oxidases, some chaperonins, dipeptide transporters, lactate dehydrogenase, a PHB depolymerase, a ferritin–like protein, exopolysaccharide biosynthesis genes, a type VI secretion system and menaquinone biosynthesis as well as many hypothetical genes.

The conserved genome in *R. etli* strains Mim1 and CFN42 includes not only the chromosome but two extrachromosomal replicons (pRetMim1a-pRetCFN42b and pRetMim1d-pRetCFN42e) that have been designated as chromids in CFN42 [42] and one plasmid (pRetMim1b-pRetCFN42c) (Figure 3). Each of the chromids had less than 20 unique genes and the chromid pairs had an ANI around 99% (Table 1). The small replicons pReCFN42a and pRetMim1c were partially conserved and large genomic differences were found in the symbiotic plasmids (Figures 1 and 4, Table 1).

Mim1 and CFN42 chromosomes were syntenic, as were the chromid pairs pRetMim1a-pRetCFN42b and pRetMim1d-pRetCFN42e, and plasmids pRetMim1b-pRetCFN42c. In comparison to *R. leguminosarum* 3841, Mim1 plasmid

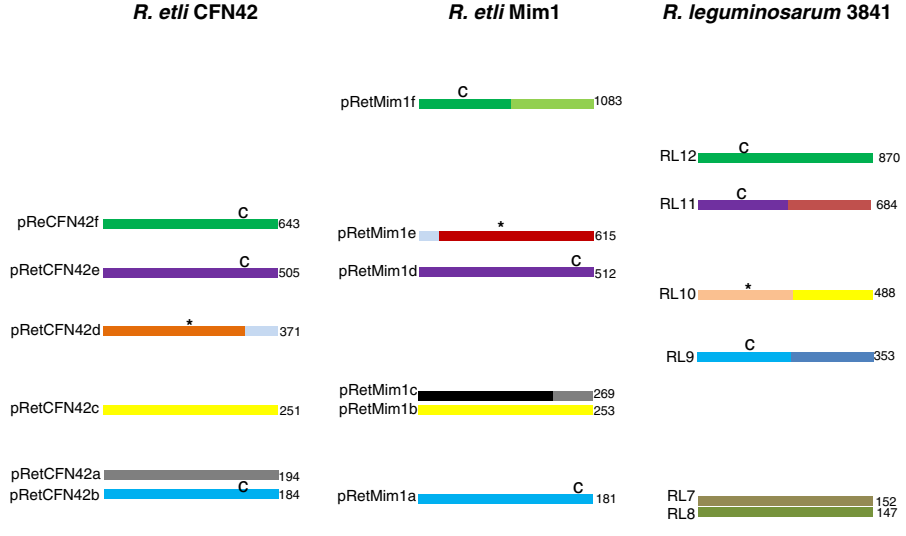

Figure 1 Schematic representation of plasmid patterns of *Rhizobium etli* symbiovars phaseoli and mimosae and *R. leguminosarum*. Equivalent replicons are indicated with the same color. *indicates the symbiotic plasmid. C indicates chromids.

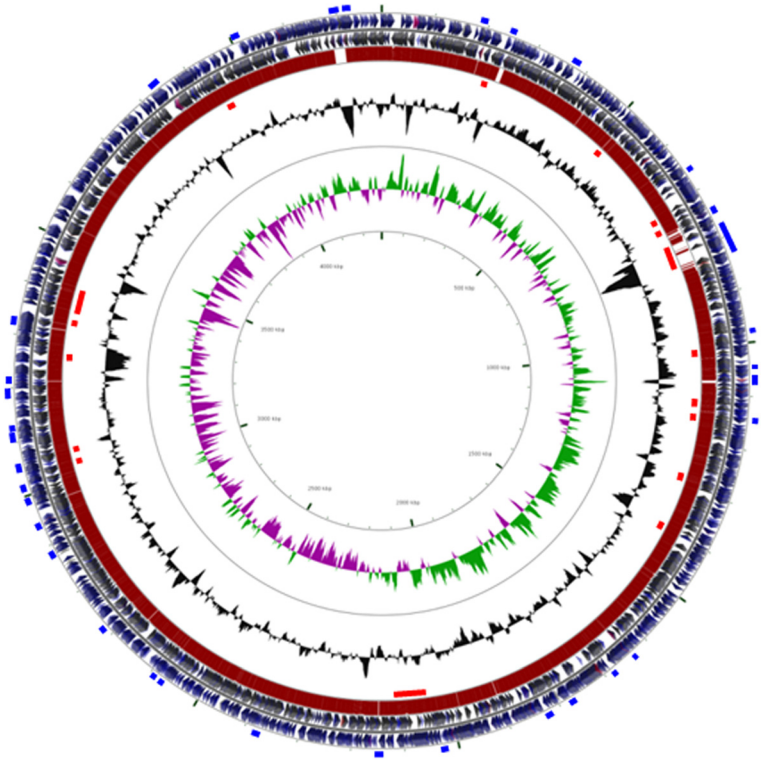

Figure 2 Representation of *R. etli* Mim1 chromosome. Circles from outermost to innermost indicate: genomic islands of Mim1 (in blue), ORFs in the leading strand, ORFs in the lagging strand, BLAST matches against CFN42 chromosome, genomic islands of CFN42 (in red), GC content, GC skew, coordinates.

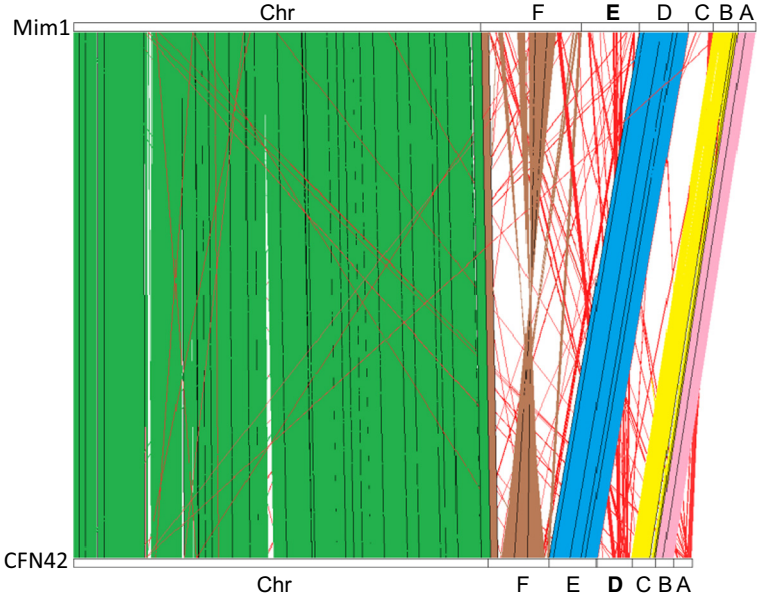

Figure 3 Graphic comparison of equivalences among the *R. etli* CFN42 and *R. etli* Mim1 genomes. Letters indicate the different extrachromosomal replicons found in both strains (see text). Letters in bold indicate the symbiotic plasmids.

Table 1 Average nucleotide identity (ANI) and conservation in percent between *R. etli* sv. mimosae Mim1 and *R. etli* sv. phaseoli CFN 42 or *R. leguminosarum* 3841 replicons

Mim1 replicons	ANI*/conservation[§] to the corresponding replicons in	
	R. etli CFN42	*R. leguminosarum* 3841
pRetMim1f	97.4/51.1 (pReCFN42f)	86.8/31.4 (pRL12)
pRetMim1e (pSym)	89.2/8.8 (ReCFN42d)	
pRetMim1d	98.9/97.5 (pReCFN42e)	88.2/61.2 (pRL11)
pRetMim1c	86.4/11.4 (pReCFN42a)	
pRetMim1b	99.1/92.5 (pReCFN42c)	87.2/55.7 (pRL10)
pRetMim1a	99.2/99.9 (pReCFN42b)	87.6/58.4 (pRL9)
RetMim1Ch	99.2/97.8 (ReCFN42Ch)	88.1/78.1 (RLChr)

*Average nucleotide identity was calculated using all portions of the replicons that could be aligned with the nucmer program. These regions included both genic and intergenic regions.
[§]Percentage of the Mim1 replicon involved in the ANI calculation is expressed here as the conservation value.

equivalences were similar to those reported previously for *R. etli* CFN42 [8]. ANI values were lower with *R. leguminosarum* 3841 that among the *R. etli* strains (Table 1).

All extrachromosomal replicons in Mim1 belong to the *repABC* plasmid family [43]. The protein products of the *repABC* operons of the replicon homologous pairs (pRetMim1a-pRetCFN42b, pRetMim1d-pRetCFN42e and pRetMim1b-pRetCFN42c) were almost identical with a sequence identity greater than 97.51%, strongly suggesting that members of each replicon pair belong to the same incompatibility group.

pRetCFN42f-pRetMim1f comparison

The largest extrachromosomal replicon in Mim1 (pRetMim1f) was only partially conserved in the putative chromid pRetCFN42f (Figures 1 and 4). pRetMim1f and pRetCFN42f possess two *repABC* operons: *repABC1* and *repABC2*. The sequence identity between the two *repABC* operons in Mim1 is low. The degree of sequence identities between the corresponding *repABC* genes of pRetCFN42f and pRetMim1f is large enough to suggest that both plasmids share the same incompatibility group and evolutionary origin. However only 51% of the pRetMim1f replicon

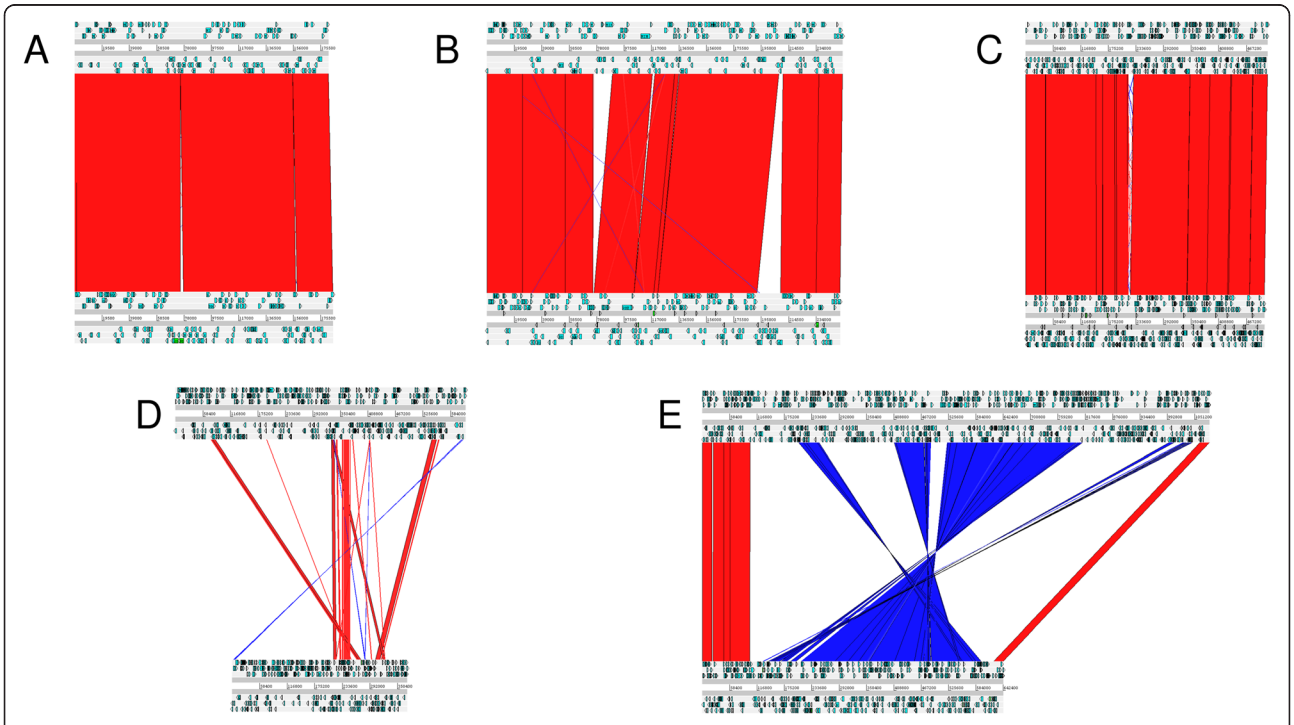

Figure 4 Graphic representation of the alignments of CFN42 (top)-Mim1 (bottom) replicons. A) pRetMim1a-pRetCFN42b, **B)** pRetMim1b-pRetCFN42c, **C)** pRetMim1d-pRetCFN42e, **D)** pRetMim1e-pRetCFN42d, **E)** pRetMim1f-pRetCFN42f. ORFs of each replicon are depicted with light blue arrows in their corresponding reading frame. Syntenic segments oriented in the same or opposite direction are joined by red and blue regions, respectively.

is conserved in CFN42 while 86% of pRetCFN42f was found in pRetMim1f (Figures 1 and 4).

Type VI secretion system genes were only found in the megaplasmid pRetMim1f and the conserved *impB* component is phylogenetically related to the corresponding genes in *Rhizobium* sp. KIM5 (corresponding to PEL1 lineage, [44]) and *R. leguminosarum* strain 3841. Mim1 pRetMim1f has a duplicated citrate synthase gene as in sv. tropici symbiotic plasmids. The plasmid duplicated citrate synthase gene in sv. tropici is required for eliciting a normal number of nodules and is regulated differently than the copy in the chromosome [45,46]. A plasmid borne citrate synthase was not found in CFN42. Phylogenetic analysis of citrate synthase genes showed that the Mim1 chromosomal gene product is identical to that of CFN42, while the gene in pRetMim1f has a novel sequence only distantly related to a plasmid copy of *R. gallicum* R602 (55% identity).

Genes such as *raiI* and *raiR* were found in the conserved region of pRetCFN42f and pRetMim1f. RaiI produces homoserine lactones and RaiR is the transcriptional regulator. The *rai* system in *R. phaseoli* sv. phaseoli CNPAF 512 affects nodule number but not nitrogen fixation in *P. vulgaris* [47]. This system also controls growth in *R. phaseoli* [48] and if this is the case in *R. etli*, this would explain its conservation in both symbiovars.

A transcriptomic study compared the genome expression of *R. phaseoli* Ch24-10 in maize and *P. vulgaris* rhizospheres [49]. Over 50% of the extrachromosomal genes highly expressed in *P. vulgaris* but not in maize roots were found in a Ch24-10 replicon equivalent to pRetCFN42f. It seems that genes in this replicon are involved in plant specific interactions.

Symbiosis plasmid gene comparison

Large differences were observed between the symbiotic plasmids of CFN42 and Mim1 (Figure 4). Around 10% and 15% of the symbiotic plasmids of Mim1 and CFN42 had conserved syntenic regions with an average nucleotide identity of 89.2% (Table 1).

Differences in symbiosis genes in CFN42 and Mim1 genomes are shown in Table 2. The Nod factor from *R. etli* sv. phaseoli strain CFN42 is a pentamer of *N*-acetylglucosamine with an acetyl fucose at the reducing end and methyl and carbamoyl groups at the non reducing end [50]. The heterologous expression in *Azorhizobium caulinodans* of Nod modification genes showed that fucosylated Nod factors were the most suitable to induce *P. vulgaris* nodulation [51]. In symbiovar mimosae no genes related to Nod factor fucosylation (*nodZ*) were observed (Table 2), in their place, *nodHPQ* genes that modify the Nod factor with sulfate, were found. Such genes are present in sv. tropici strains that are also *Leucaena* symbionts [10]. Like *R. etli* strain CFN42, Mim1 may produce nodulation factors bearing carbamoyl groups at their non reducing end residues but the position of these decorations must differ because their pSyms encoded distinct carbamoyl transferases, NolO in CFN42 and NodU in Mim1. Carbamoylation at the C-6 position introduced by NodU maybe promotes *Leucaena* nodulation [52,53]. Both Mim1 and CFN42 symbiotic plasmids carry *nodS* involved in methylation at the non reducing end, a decoration that is essential for bean and *Leucaena* nodulation in *R. tropici* CIAT 899 [52]. A *nodO* homologue, 70% identical to that of *Rhizobium* sp. BR816, was found only in the sv. mimosae pSym. It has been shown that heterologous expression of *nodO* can improve nodulation of *L. leucocephala* by different rhizobia and can even extend the host range [54,55].

Table 2 Relevant symbiotic plasmid differences between *R. etli* sv. mimosae Mim1 and *R. etli* sv. phaseoli CFN 42

Gene*	symbiovar	
	mimosae	phaseoli
nodHPQ	+	-
nodZ	-	+
nolL	-	+
nodO	+	-
nolO	-	+
nodU	+	-
fixKL	+	-
fxkR	+	-
acdS	+	-
teu	-	+
hup-hyp	+	-

*Functions of each gene are explained in the text.

Mim1 *nod* gene phylogenies are congruent, resembling the corresponding genes from sv. *giardinii* (not shown) while Mim1 *nifH* genes resemble those from sv. phaseoli. Different NodDs in phaseoli and mimosae symbiovars may reflect their affinities for the different flavonoids exuded by the different host plants. Mim1 *nodH* gene (encoding the sulfotransferase involved in the synthesis of the Nod factor) resembles the corresponding gene in *Rhizobium* sp. IE4771 that represents a novel genomic lineage related to *R. etli* and *R. phaseoli* [1,44].

acdS gene coding ACC-deaminase was found in the symbiotic plasmid of Mim1 but not in CFN42. ACC-deaminase decreases the amount of ACC that is a precursor of ethylene that may diminish nodule number. A heterologous ACC-deaminase in *Rhizobium* sp TAL 1145 enhanced nodulation in *Leucaena* [56].

In CFN42, the *fixGHIS-fixNOQP* genes required for biosynthesis of the symbiotic terminal oxidase are present in the pSym and also in pRetCFN42f [57]. The regulatory genes *fixK* and *fixL* are adjacent to this reiteration in pRetCFN42f while a *fixK* pseudogene is found in the pSym [58]. In Mim1, we found that the symbiotic terminal oxidase genes are also reiterated in pRetMim1f but, in contrast to

CFN42, the sv mimosae pSym carried complete *fixK* and *fixL* genes. The *fixGHIS-fixNOQP-fixKL* region shared by pSym and pRetMim1f plasmids in Mim1 is 95% identical while the reiterated regions in CFN42 are only 87% identical. The recently described *fxkR* gene in pRetCFN42f [59], coding for a response regulator that acts in conjunction with FixL and FixK, is present in the sv. mimosae pSym. There is a reiteration of this gene in pRetMim1f as well.

Genes involved in *P. vulgaris* exudate uptake (*teu* genes, [60]) are found in the symbiotic plasmids in symbiovar phaseoli strains *R. etli* CFN42 and *R. phaseoli* CIAT652 but they were not found in the Mim1 genome. *Rhizobium* mutants in *teu* genes had reduced nodulation competitiveness in *P. vulgaris* [60].

The symbiosis plasmid of Mim1 has genes for a type III secretion system (T3SS) that are more closely related to those found in *Mesorhizobium* and *Sinorhizobium* strains than to those encoded in the CFN42 pSym (not shown). This difference may contribute to the disparate host ranges displayed by sv. phaseoli and mimosae strains considering the function of rhizobial T3SS in specificity [61].

A cluster of *hup-hyp* genes encoding the components of an uptake hydrogenase (Hup) was found in the pSym of Mim1 but not in CFN42. The Mim1 products showed high identities (>70%) to their counterparts coded in *R. leguminosarum* and *R. tropici* [10,62]. *R. tropici* CIAT 899 lacks several *hup* genes and displays a Hup minus phenotype. In Mim1, all genes except *hupE* are present. HupE is an uptake transporter for nickel [63], a metal required for Hup function. Since another nickel transporter is encoded elsewhere in Mim1 symbiotic plasmid, the Hup system could be functional.

Mim1 symbiotic plasmid has a *repABC* origin of replication as well as a *repC* gene that are not phylogenetically related to the *R. etli* CFN42 corresponding genes. pRetMim1e *repC* resembles those from *R. gallicum* and *Rhizobium* sp. sv. giardinii IE4771 (corresponding to PEL1 lineage). Mim1 *repABC* from the pSym resembles the corresponding genes in *R. endophyticum* CCGE502 but an extensive plasmid conservation was not observed. CCGE502 is Nod$^-$ and does not have a symbiotic plasmid [64].

The analysis of insertion sequences in *R. etli* CFN42 suggested that the symbiotic plasmid did not significantly share IS sequences with the rest of the genome [65]. This was interpreted as evidence that the pSym was a recent acquisition in this bacterium. The analysis of IS sequences in Mim1 indicated that the symbiotic plasmid had the largest number of IS sequences, some of them shared with the chromosome, pRetMim1f and pRetMim1b, this may perhaps indicate that this symbiotic plasmid has an older history with the *R. etli* genomic background than the phaseoli plasmid. Mim1 has a large number of IS66 that are common in rhizobia.

Genome similarities were found among the different Mim1 replicons. pRetMim1e (pSym) has similar sequences to pRetMim1c and the same is observed among pRetMim1e (pSym) and pRetMim1f (Figure 5). The similarities of Mim1 symbiotic plasmid and the putative chromid pRetMim1f could support that symbiovar mimosae symbiotic plasmid is ancestral in *R. etli*. In contrast the phaseoli symbiotic plasmid was found dissimilar to the rest of the genomic background in *R. etli*, except to pReCFN42a [32,66].

In *R. etli* CFN42, the symbiotic plasmid and pRetCFN42a have common sequences such as *tra* and *vir* genes and repeated sequences that mediate the natural cointegration of both plasmids for the conjugative transfer of CFN42 pSym [67]. In contrast to pReCFN42a, pRetMim1c from Mim1 does not seem to participate in the transfer of the Mim1 symbiotic plasmid, which we have been unable to transfer to other bacterial hosts (Marco A. Rogel, unpublished observations).

Plant-interaction genes not in the symbiosis plasmid

Some genes involved in plant interactions were found conserved in both symbiovars, such as those encoding Rmr extrusion pumps that may be involved in eliminating plant produced phytoalexins. *R. etli* mutants in these genes had reduced nodulation [68]. Those genes are encoded in chromid pRetCFN42b in CFN42 and in the corresponding replicon pRetMim1a. *rmrA* gene had 97% identity and *rmrB* and *rmrR* genes 98% in CFN42 and Mim1 genomes. Homologous genes were found being expressed in different plant rhizospheres [1,49,69].

Even though sv. mimosae strains are capable of forming nodules in *Leucaena*, we did not find genes resembling *mid* or *pyd* genes involved in the catabolism of the toxic

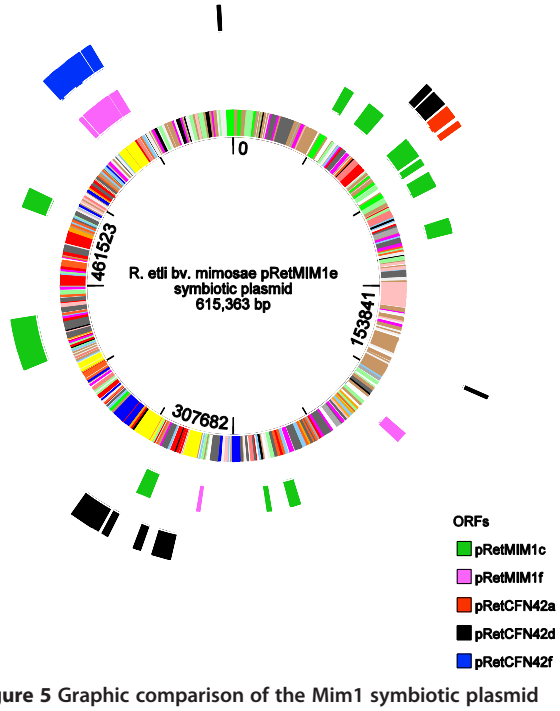

Figure 5 Graphic comparison of the Mim1 symbiotic plasmid pRetMim1e compared to other rhizobial replicons.

amino acid derivative mimosine found in *Leucaena* plants. Such genes were described from a *Rhizobium* sp. strain TAL 1145 (related to *R. tropici*) that was isolated from *Leucaena* plants [70]. *Mimosa pigra* has a much lower level of mimosine that *Leucaena* plants [71] and data for other mimosa species is not available.

Symbiovar phaseoli is prevalent in different *Phaseolus vulgaris* nodule bacteria

R. etli sv. mimosae strains have a broader host range than symbiovar phaseoli strains. In particular, *L. leucocephala* plants served as a host to distinguish symbiovar mimosae strains. Thirty-six *P. vulgaris* nodulating bacteria (with *R. etli*-like 16S rRNA gene sequences) obtained from the rain forest of Los Tuxtlas in Mexico corresponded to symbiovar phaseoli on the basis of *nodA* gene organization [10] and for being unable to nodulate *Leucaena* plants. We found that some strains that were previously classified as *R. etli* such as CIAT652, Ch24-10, CNPAF512, 8C-3 and Brasil5 now assigned to *R. phaseoli* [1], as well as others like Kim5, GR56 and CIAT 894 now assigned to recently named novel lineages [44] corresponded to sv. phaseoli when we analyzed their genomes. Symbiotic plasmids are highly conserved in symbiovar phaseoli [66] perhaps from being recently evolved [13]. Considering that the majority of *P. vulgaris* nodule isolates tested corresponded to sv. phaseoli we may conclude that this symbiovar is better adapted to its host, thus phaseoli seems to be a specialist symbiovar having a narrow range not including mimosa plants. The phaseoli symbiovar is found in several *Rhizobium* species or lineages (*R. gallicum, R. giardinii, R, phaseoli, R. etli* and *Rhizobium* sp. corresponding to PEL1 lineage). The widespread of this symbiovar may be in relation to its host historic worldwide distribution and to the transferability of the symbiotic plasmid, seemingly an epidemic plasmid. Besides having the phaseoli symbiovar, the *Rhizobium* species mentioned above have additional generalist symbiovars: gallicum or giardinii or mimosae Having alternative symbiovars with different host ranges may be advantageous in rhizobia, as it expands their legume niches and allows them to avoid the specialist-generalist dilemma.

Conclusions

The term symbiovar is validated with genomic analyses that show that a common genomic background may harbor different symbiotic plasmids determining host specificity. However, besides differences in the symbiotic plasmids there were differences in other ERs and in the chromosomes in the two strains analyzed, CFN42 and Mim1. In Mim1, Nod factors with sulfate modifications, secretion systems or ACC-deaminase may help explain the extended host range of symbiovar mimosae. In CFN42, *teu* genes that participate in exudate uptake [60] and genes involved in Nod factor fucosylation (*nodZ*) may contribute to *P. vulgaris* host specialization. The discussion that mimosae is older than phaseoli may apply to gallicum and giardinii, thus we propose that gallicum and giardinii are older than phaseoli.

Additional file

Additional file 1: Table S1. Genes found in different genomic islands exclusive of Mim1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EMR initiated and designed the study. MAR performed experiments. VG, DR, MAC contributed reagents and analyzed data. EOO, PB, RIS, JMR, JCM, LL performed data analysis. EMR, EOO, MAR wrote the paper. All authors read and approved the manuscript.

Acknowledgements

To PAPIIT 205412, CONACyT CB 131499 and CONACyT 154453 for financial support. To Michael Dunn for reading the paper. MA Rogel was a Ph.D. student at the Biological Sciences program in UNAM Mexico and supported by a PASPA fellowship.

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Received: 2 September 2013 Accepted: 1 July 2014
Published: 8 July 2014

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doi:10.1186/1471-2164-15-575
Cite this article as: Rogel *et al.*: Genomic basis of symbiovar mimosae in *Rhizobium etli*. *BMC Genomics* 2014 **15**:575.

ARTÍCULOS CIENTÍFICOS PUBLICADOS

Systematic and Applied Microbiology 34 (2011) 96–104

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Minireview

Symbiovars in rhizobia reflect bacterial adaptation to legumes

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ARTICLE INFO

Article history:
Received 14 September 2010

Keywords:
Legumes
Symbiosis
Nitrogen fixation
Rhizobium diversity

ABSTRACT

Legume specificity is encoded in rhizobial genetic elements that may be transferred among species and genera. Dissemination (by lateral transfer) of gene assemblies dictating host range accounts for the existence of the same biological variant (biovar) in distinct microbiological species. Different alternative biovars may exist in a single species expanding their adaptation to different niches (legume nodules). A review of all reported biovars is presented. Instead of the term biovar, symbiotic variant (symbiovar) is proposed as a parallel term to pathovar in pathogenic bacteria. Symbiovars should be determined based on the symbiotic capabilities in plant hosts, distinguished by the differences in host range and supported by symbiotic gene sequence information.

Introduction

Nitrogen fixing bacteria in legume nodules collectively designated as rhizobia have been known since 1888, reviewed in [58]. They were the first biofertilizers produced and allow savings of millions of dollars in chemical fertilizers [23] that may contaminate soil and water. Interest in these bacteria is increasing as plants to produce biofuels may profit from bacterial nitrogen fixation to attain a sustainable process. Nodule formation culminating in nitrogen fixation has been well studied and different symbiosis genes such as *nod*, *nif* and *fix* genes are known. Different host specificities may be determined by the symbiotic gene content. Excellent reviews have been published on the molecular basis of nodulation and on Nod factors, the modified lipochitooligosaccharides that induce nodule formation [10,11,16,27,43,56]. Nod factors, type 3 secretion systems and other rhizobial functions are needed to establish symbioses with legumes [43]. The *nod* gene similarity found in some cases in *Rhizobium*, *Agrobacterium* and *Sinorhizobium* species evidences the mobilization of symbiosis genes between these genera, reviewed in [41].

Biovars (biological variants) have been described in diverse bacterial species and reveal the different biochemical and enzymatic characteristics within a species. A biovar represents a group of bacterial strains distinguishable from other strains of the same species on the basis of physiological or biochemical characters. Biovars were formerly known as biotypes. In rhizobia biovars have been used to distinguish symbiotically distinct subgroups within a single rhizobial species. Biovars can be shared by different species due to the lateral transfer of symbiotic information. Biovars were first described in *Rhizobium leguminosarum* in a taxonomical revision of rhizobial species [28]. Since then biovars have been identified in other *Rhizobium* species, in *Ensifer* (*Sinorhizobium*), *Mesorhizobium* and *Bradyrhizobium* (Fig. 1A, Table 1, Supplementary Fig. 1) [2,5,35,45,59,62,66,76,77,79] but not so far in beta-rhizobia. Lateral transfer of *nod* genes from alpha rhizobia to *Burkholderia* and from these bacteria to *Ralstonia* seems to account for the existence of nodulating species in these genera [1,3,8,47].

Biovars in *Rhizobium*

Biovars viciae (nodulating pea), trifolii (nodulating clover) and phaseoli (nodulating *Phaseolus vulgaris* beans) were all ascribed to *R. leguminosarum* [28] considering that there was a single bacterial species (a common chromosomal background) that could alternatively contain symbiotic plasmids with different specificities. In a multilocus enzyme electrophoresis study (though with few metabolic enzymes), there were electrophoretic types common in all biovars supporting their belonging to a single taxon [82]. These results were confirmed by RFLP (restriction fragment length polymorphism) in hybridization assays using chromosomal probes showing the same pattern types in isolates from different host species [30]. Although there was a taxonomy revision of *R. leguminosarum* in 1984, officially the species *R. phaseoli* and *R. trifolii* were never rejected and according to taxonomy rules these species were still valid. When the *R. phaseoli* type strain (ATCC 14482) was characterized by DNA–DNA hybridization and sequencing of 16S rRNA, *recA* and *atpD* genes it was found that it was different from all described species and clearly did not correspond to *R. leguminosarum* bv. phaseoli [60]. The biovar of the *R. phaseoli* type strain (ATCC 14482) is bv. phaseoli as it nodulates *P. vulgaris* bean and has

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doi:10.1016/j.syapm.2010.11.015

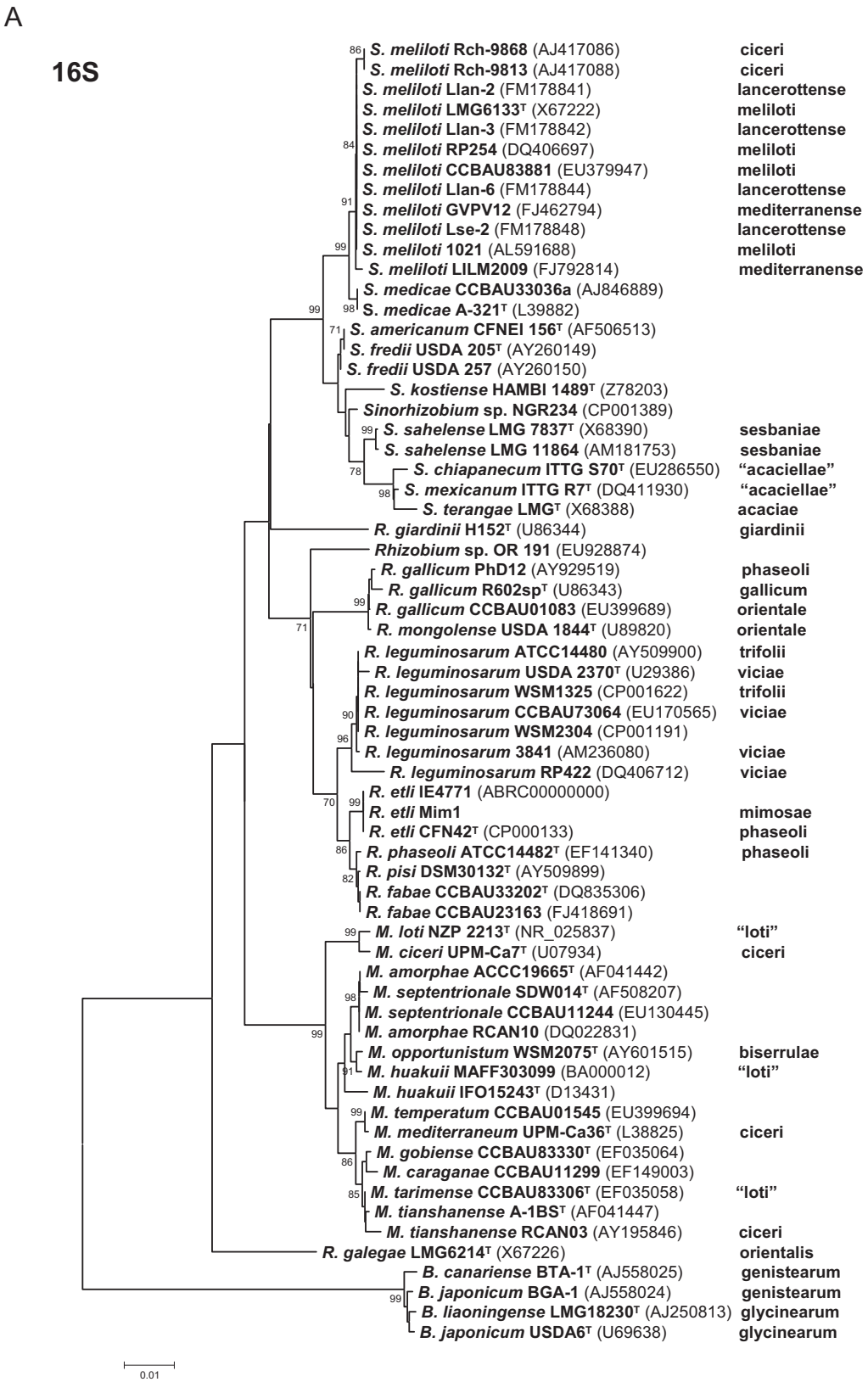

Fig. 1. Maximum likelihood phylogenies (A) 16S gene tree of rhizobial species with assigned biovars. Type strains are indicated with a superscript T. Biovars are indicated in the right column, (B) *nodC* gene tree, (C) *nifH* gene tree. Biovars names are indicated with brackets.

a *nodC* gene sequence as that found in bv. phaseoli [19]. Additionally when the *R. leguminosarum* type strain was similarly analyzed, there were some surprises: not all type strains from different collections were the same. DSM 30132 supposedly corresponding to *R. leguminosarum* type strain was different from other *R. leguminosarum* isolates and from the synonymous USDA 2370 type strain. Therefore DSM 30132 strain was assigned to a novel species *R. pisi* nodulating pea and other legumes [60] and its biovar is viciae

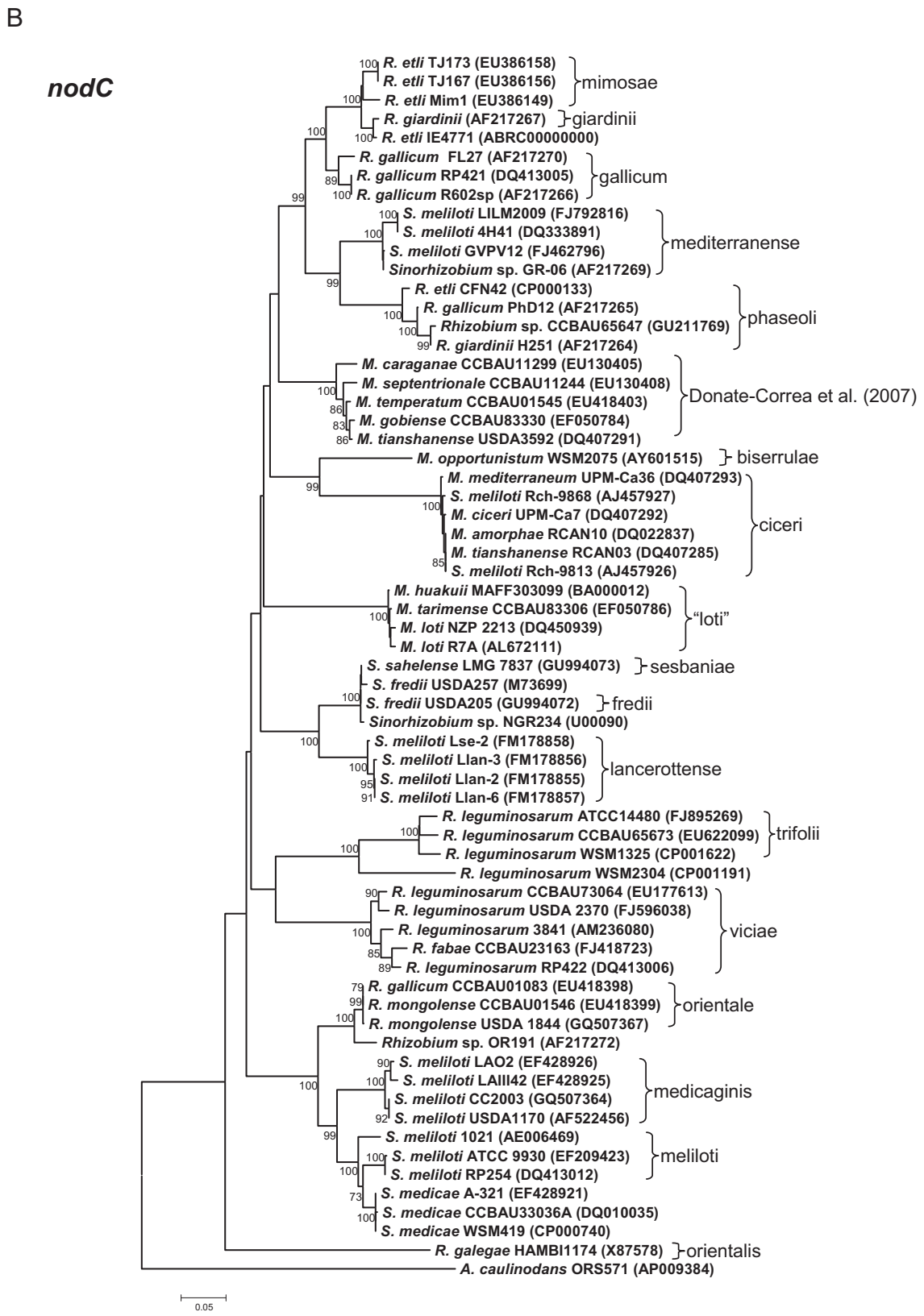

Fig. 1. (*Continued*)

(Velazquez, personal communication). This is also the biovar in the closely related *R. fabae* that was obtained from *Vicia faba* nodules in China and is capable of nodulating pea [71] with *nodC* genes similar to those found in bv. viciae (Fig. 1B), and related *nodA* genes as well (our own unpublished data).

A large number of isolates from *P. vulgaris* bean nodules in Spain corresponded to *R. leguminosarum* bv. phaseoli, the most frequently isolated species from *P. vulgaris* bean in that region [19]. A new revision of *Trifolium* nodulating strains based on the sequence of some genes and DNA–DNA hybridization showed that they should

C

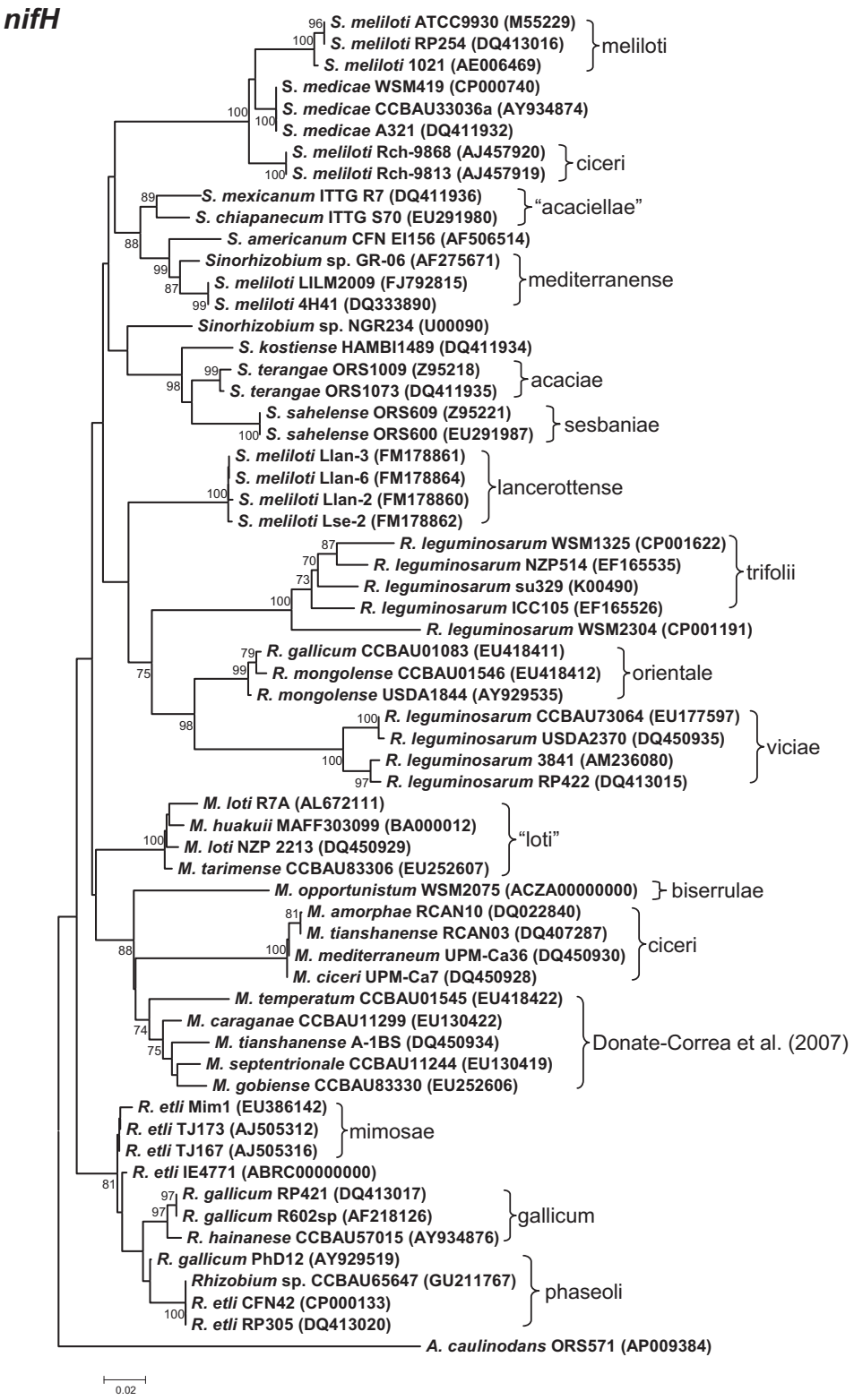

Fig. 1. (*Continued*).

be considered *R. leguminosarum* [60] and their biovar is bv. trifolii [60]. Comparisons of the gene sequences from the genomes of *R. leguminosarum* bv. viciae strain 3841 [83] isolated from pea in fields in England [26] and *R. leguminosarum* bv. trifolii strain WSM1325 (isolated from an annual clover, *Trifolium* sp. in Greece, R. Yates, PhD thesis, Murdoch University, 2008) shows their belonging to a single species, their different specificities (*Trifolium* versus *Pisum*) strongly supports the concept of biovars. However *R. leguminosarum* bv. trifolii strains WSM1325 and WSM2304 (isolated from the perennial *Trifolium polymorphum* in Uruguay [61]) do not

Table 1
Biovars in different rhizobial species and host legumes.

Biovar	Rhizobial species	Legume host	References
acaciae	*S. terangae*	*Acacia*	[37]
	S. sahelense	*Acacia*	[22]
	S. meliloti	*Acacia tortilis*	[5]
acaciellae	*S. chiapanecum*	*Acaciella angustissima*	This work
	S. mexicanum	*Acaciella angustissima*	This work
biserrulae	*M. opportunistum*	*Biserrula pelecinus*	[50]
ciceri	*M. amorphae*	*Cicer arietinum*	[63]
	M. tianshanense	*Cicer arietinum*	[63]
	M. ciceri	*Cicer arietinum*	[50]
	M. mediterraneum	*Cicer arietinum*	[51]
	S. meliloti	*Cicer arietinum*	[38]
gallicum	*R. gallicum*	*Phaseolus vulgaris*, *Leucaena leucocephala*	[2]
	R. giardinii	*Phaseolus vulgaris*, *Leucaena leucocephala*	[2]
genistearum	*B. japonicum*	*Genisteae*, *Loteae*	[77]
giardinii	*R. giardinii*	*Phaseolus vulgaris*, *Leucaena leucocephala*	[2]
glycinearum	*B. japonicum*	*Glycine*	[77]
lancerottense	*S. meliloti*	*Lotus lancerottense*	[34]
medicaginis	*S. meliloti*	*Medicago laciniata*	[76]
mediterranense	*S. fredii*	*Phaseolus vulgaris*	[45]
	S. meliloti	*Phaseolus vulgaris*	[45]
meliloti	*S. meliloti*	*Medicago sativa*, *Medicago truncatula*	[76]
mimosae	*R. etli*	*Phaseolus vulgaris*, *L. leucocephala*, *Mimosa affinis*	[79]
officinalis	*R. galegae*	*Galega officinalis*	[59]
orientalis	*R. galegae*	*Galega orientalis*	[58]
orientale	*R. mongolense*, *Rhizobium* spp.	*Medicago ruthenica*, *Phaseolus vulgaris*	[66]
phaseoli	*R. gallicum*	*Phaseolus vulgaris*	[2]
	R. giardini	*Phaseolus vulgaris*	[2]
	R. leguminosarum	*Phaseolus vulgaris*	[28]
	R. etli	*Phaseolus vulgaris*	[64]
	R. phaseoli	*Phaseolus vulgaris*	[60]
sesbaniae	*S. terangae*	*Sesbania*	[37]
	S. sahelense	*Sesbania*	[37]
	Agrobacterium sp.	*Sesbania*	[12]
trifolii	*R. leguminosarum*	*Trifolium*	[28]
viciae	*R. leguminosarum*	*Vicia sativa*	[28]
	R. fabae	*Vicia faba*	[71]
	R. pisi	*Vicia sativa*	[60]

have the same specificity and nodulate and fix nitrogen in different *Trifolium* species from distinct geographical origin [81]. In theory they should not correspond to the same biovar. Only WSM 1325 corresponds to bv. trifolii, then WSM 2304 should deserve a distinct biovar. In agreement *nodD* genes (that recognize plant flavonoids and regulate the expression of other *nod* genes) were more divergent than chromosomal genes and *nolR* was found only in WSM1325 and not in WSM2304; probably they produce different Nod factors [81, Yates, PhD thesis, Murdoch University, 2008].

Diversity of *R. leguminosarum* bv. viciae was determined by the plant [14,24,49]. Furthermore, not all *R. leguminosarum* bv. viciae strains exhibited the same host range when tested with different legumes [49] suggesting that biovars may be more complex than we think; probably subtle genetic differences may have specificity effects. However the species designation of such isolates should be revised considering the novel related species recently described.

Rhizobium gallicum bv. gallicum and bv. phaseoli were found in *P. vulgaris* bean nodules in Europe [2]. Later *R. gallicum* bv. gallicum was also found in *Phaseolus coccineus* and in few *P. vulgaris* nodules in Mexico [65] and in Tunisia [44]. *R. gallicum* was reported from other legumes such as *Oxytropis* and *Onobrychis* in Canada [32] and in other sites, reviewed in [65]. Biovars gallicum and phaseoli have different *nod* gene sequences and host ranges, biovar gallicum has a broad host range including *Leucaena* while bv. phaseoli strains do not form nodules in this host.

Biovar gallicum and biovar giardinii *nod* genes are related to those found in *Mimosa* nodulating bacteria including bv. mimosae from *R. etli* and also *R. tropici* [42]. *R. giardinii* bv. giardinii and bv. phaseoli were found as well in *P. vulgaris* bean nodules and were distinguished by their different specificities [2], they seem to be less efficient for nitrogen fixation in *P. vulgaris* bean than other rhizobia.

Biovar phaseoli is found in *R. etli*, *R. phaseoli*, *R. leguminosarum*, *R. gallicum* and in *R. giardinii* (Supplementary Fig. 1) with conserved *nodC* and *nifH* genes [31]. The phaseoli symbiotic plasmid of *R. etli* strains is well conserved in nucleotide sequences as well as in gene content, as shown in a recent comparative genomic study [20]. It remains to be established if the symbiotic plasmid is also conserved in the other species harboring bv. phaseoli.

In addition to bv. phaseoli, a new biovar was described in *R. etli*, biovar mimosae, for *Mimosa* nodulating bacteria, [79], that is considered the ancestral biovar in *R. etli*. Our recent analysis of the genome of *R. etli* bv. mimosae Mim1 showed that there is an extensive identity to *R. etli* bv. phaseoli strains CFN42 and CIAT 652 in the chromosome and in some of the plasmids (supporting their belonging to the same species) but not in the symbiotic plasmids. The conserved plasmids but not the symbiotic plasmid corresponded to the recently defined chromids [21]. Differences in the overall gene content of the bv. phaseoli and bv. mimosae symbiotic plasmids were observed in addition to differences in the *nod* and *nif* gene sequences. Genes involved in the biosynthesis of sulfated Nod factors were found in bv. mimosae but not in bv. phaseoli (Rogel et al. unpublished). *R. etli* strain IE4771 that was considered as corresponding to bv. mimosae [66] seems to correspond to bv. giardinii (Fig. 1B). Although bv. giardinii and bv. mimosae are closely related in *nodC* gene phylogenies (Fig. 1B), the genomic comparison of the respective symbiotic plasmids in Mim1 and IE4771 showed that they are significantly different (unpublished) suggesting different evolutionary histories. If *nodB* and *nifH* gene sequences are indica-

tive of overall differences in symbiotic plasmids, then there seems to be some heterogeneity inside bv. phaseoli [65,66]. Different alleles of *nodC* genes are known as well [19]. Additionally different Nod factors have been reported among *R. etli* bv. phaseoli strains [55] as well as differences in the regulation of nitrogen fixation, reviewed in [40]. A characteristic of biovar phaseoli is the multiple (three) copies of the *nif* operon [57] (bv. gallicum carries a single *nifH* copy, and bv. mimosae 2 *nifH* gene copies). A *nodA* gene separated from the common *nod* operon was observed in bv. phaseoli strains [74] but such *nod* gene organization is not observed in *R. etli* bv. mimosae. Different *nifH* gene hybridization patterns exist in bv. phaseoli strains [39]. Although *nifH* genes do not determine host specificity, they represent characteristic markers of symbiotic plasmids and they clearly allow the distinction and grouping of biovars (Fig. 1C).

Rhizobium mongolense was isolated from *Medicago ruthenica* from Mongolia [73]. It was argued that *R. mongolense* may be considered as *R. gallicum sensu lato* [66] and as synonymous with *R. gallicum* (E. Velázquez, personal communication). Interestingly *R. mongolense nodB* genes resemble the respective genes from sinorhizobia that nodulate *Medicago* and biovar orientale was proposed [66]. In contrast *R. mongolense nifH* genes resembled *R. leguminosarum* bv. viciae. In spite of the similarities in *nodB* genes, the existence of biovar orientale in both *R. mongolense* and in *R. yanglingense* [66] is doubtful due to the differences in legume specificities.

Rhizobium galegae strains nodulate the legumes *Galega orientalis* and *G. officinalis* from the Caucasus and biovars orientalis and officinalis were proposed in relation to host nodulation and differences in sequences of *nod* genes were related to the legume species [59]. Later the bacteria from the two distinct biovars were found to correspond to different groups in AFLP analysis and it was suggested that *R. galegae* was diverging into subspecies perhaps driven by host specificity [70]. Consequently no evidence of recombination was detected between the biovars [4].

R. huautlense, a related species to *R. galegae*, that forms nodules in *Sesbania* has *nodA* genes similar to sinorhizobia biovar sesbaniae [62], meaning that there are peculiar genetic determinants for *Sesbania* nodulation [36]; however, symbiosis genes in *Azorhizobium* and in mesorhizobia nodulating *Sesbania* are not related. Interestingly *nodA* and *nifH* genes in *Agrobacterium* strain IRBG74 nodulating *Sesbania* are similar to those from bv. sesbaniae of *Sinorhizobium* [12] and probably the biovar in IRBG74 is bv. sesbaniae. *R. huautlense* and *Mesorhizobium plurifarium* from *Sesbania* in South America did not equally nodulate distinct *Sesbania* species and other legumes and it was suggested that different biovars may exist in both species [78].

Biovars in *Sinorhizobium*

Sinorhizobium sahelense (corrected name of *S. saheli*) and *S. terangae* were isolated in Africa from *Acacia* and *Sesbania* trees and were found to be closely related in 16S rRNA and *nifH* gene phylogenies. Biovars sesbaniae and acaciae were described in both *Sinorhizobium sahelense* and *S. terangae* [7]. Different Nod factors are produced by the different biovars [35,36]. *nodA* genes of the biovar acaciae from the different species *S. terangae*, *S. sahelense*, and *S. arboris* are similar [62].

S. meliloti is the best studied rhizobial species in regard to the molecular mechanisms involved in plant nodulation. It is well known for its capacity to nodulate alfalfa (*Medicago sativa*) plants but also forms nodules in *Trigonella* and *Melilotus*. Novel biovars, bv. acaciae [5], bv. medicaginis [76] and bv. mediterranense [45] besides bv. meliloti and probably bv. ciceri [38] were recognized in *Sinorhizobium meliloti* (Table 1, Figs. 1 and Supplementary Fig. 1). *S. meliloti* bv. acaciae was obtained from *Acacia tortilis* nodules and produces a Nod factor similar to that produced by *S. terangae* bv. acaciae and by other rhizobia and mesorhizobia nodulating acacias [5]. *S. meliloti* strains that effectively nodulated *P. vulgaris* beans corresponded to bv. mediterranense, their *nodC* and *nifH* genes were not related to those of bv. meliloti nor to bv. phaseoli but were more closely related to those of Mediterranean *Sinorhizobium fredii* strains nodulating *Phaseolus vulgaris*; they are salt tolerant.

Isolates from *Medicago laciniata* that also nodulate *M. sauvagei* but not *M. truncatula* were classified as *S. meliloti* by chromosomal characteristics such as 16S rRNA genes and DNA–DNA hybridization but different host range, sequence of *nodA* and RFLP patterns of *nifDK* genes justified its designation as a novel biovar, medicaginis.

Additionally, another biovar, lancerottense, has been reported in *S. meliloti* with distinct symbiotic genotypes and effectively nodulating *Lotus lancerottensis* [34]. These isolates did not nodulate *Medicago* and this was the first time that *S. meliloti* was described as symbiont of *Lotus*; the isolates seem to be the preferred symbionts of *L. lancerottensis*. The *Lotus* isolates were tolerant to salinity and alkaline conditions [34].

nodC genes from bv. medicaginis and bv. meliloti are related (Fig. 1B). It is interesting to note that bv. mediterranense *nodC* gene cluster is related to the cluster bv. phaseoli and those genes from bv. lancerottense are more similar to those from *S. fredii* USDA 257 and NGR234. This similarity was not observed with *nifH* genes: the *nifH* phylogeny showed no close relationship between bv. mediterranense and bv. phaseoli (Fig. 1C), nor between *S. fredii* and *S. meliloti* bv. lancerottense [34]. *nif* and *nod* gene phylogenies are not congruent in some cases (Fig. 1A and B) meaning that recombination has had a role in the evolution of biovars. Genetic rearrangements have been observed in *R. etli* biovar phaseoli [18]. In addition to recombination, gene loss and gain may be responsible for generating particular gene assemblies that eventually determine biovars. Horizontal gene transfer and recombination drive the diversity of sinorhizobia associated with *Medicago* [6]. Although NGR234 clusters with bv. fredii by *nodC* gene sequences (Fig. 1), its remarkably broader host range and its lack of soybean nodulation would place NGR234 out of biovar fredii.

Biovar mediterranense was designated in *S. fredii* for strains with specificity for *Leucaena leucocephala* and *P. vulgaris* and unable to nodulate soybean [45]. This biovar was also identified in *S. meliloti* as described above. It was argued that bv. mediterranense was not the bv. in *Sinorhizobium mexicanum* or *S. chiapanecum* in spite of the similarities in *nod* gene sequences because bv. mediterranense strains did not efficiently nodulate *Acaciella angustissima*, the original host for *S. mexicanum* and *S. chiapanecum* [62]. *S. mexicanum* or *S. chiapanecum* have the same host specificity (for *Acaciella*) and very similar symbiosis genes, their corresponding biovar should be named bv. acaciellae.

Biovars in *Mesorhizobium*

Mesorhizobium amorphae biovar ciceri and *M. tianshanense* biovar ciceri nodulate chickpea [63], whereas the originally described species do not nodulate chickpea. *M. amorphae* nodulates *Amorpha fruticosa* [80] and *M. tianshanense* nodulates various legumes native to arid China [9], thus the different biovar determines alternative specificity. *M. ciceri* [52] and *M. mediterraneum* [51] were described as species nodulating chickpeas (*Cicer arietinum*). In *M. ciceri* two biovars were described, biserrulae and ciceri distinguished by different *nodA* and *nifH* gene sequences and by host specificities [50]. Isolates corresponding to bv. biserrulae do not nodulate chickpea and those from bv. ciceri do not nodulate *Biserrula pelecinus*. Do chickpea nodulating bacteria share symbiosis genes? A conserved *nodC* gene [33] was found in all species nodulating chickpea (Fig. 1B).

Both *Mesorhizobium huakuii* and *M. huakuii* bv. renge [48] form nodules in milkvetch (*Astragalus sinicus*) that is used as green manure in rice crops. By nodulating the same host, bv. renge does not meet the criterium to be considered as a different *M. huakuii* biovar. Differences in 16S rRNA gene sequences and sensitivity to bacteriophages led to the proposal of subspecies in *M. huakuii*. Strains corresponding to bv. renge were found to belong to one of the subspecies further supporting their misclassification as bv. renge [53].

The *Mesorhizobium* strain MAFF 303099 formerly considered as *Mesorhizobium loti* has been classified as belonging to *M. huakuii* [72]. A biovar loti having the capacity to nodulate *Lotus* seems to be contained in different mesorhizobial species [34].

Different *Mesorhizobium* species that nodulate the shrub *Anagyris latifolia* have similar symbiosis genes (Fig. 1B and C). All these species probably share a single novel biovar in mesorhizobia [15].

Biovars in *Bradyrhizobium*

Biovar genistearum was found in *B. canariense* and in *B. japonicum* meaning that both species shared the capacity to nodulate genistoid plants, brooms [77]. Biovar glycinearum was described in *B. japonicum* and *B. liaoningense* strains that nodulate soybean. It is conceivable that symbiotic islands could be exchanged among related *Bradyrhizobium* species as has been described in *Mesorhizobium* strains [68,69]. In *Bradyrhizobium* genomic islands have been identified [25] but up to now symbiotic plasmids have not been found although other plasmids exist in this genus [13].

Is the term biovar adequate to define symbiotic capabilities in rhizobia?

In *Agrobacterium* three biovars were recognized for a long time. They now correspond to different species [54] and even to different genera [67,75]. The term biovar as used in *Agrobacterium* and in other bacteria has not the same connotation in rhizobia. The term biovar in *Rhizobium* as reviewed here has been used to refer to the symbiotic capabilities and it would be more adequate or appropriate to use the term symbiotic variant (abbreviated symbiovar) as a parallel term to pathovar in pathogenic bacteria. A revision to the International Standards for naming pathovars of phytopathogenic bacteria [17] was published in 1991. Pathovars are defined "on the basis of distinctive pathogenicity to one or more plant hosts." "Usually pathovars are distinguished in terms of proved differences in host range. Clear differences in symptomatology on the same plant species can warrant different pathovar designations." Similarly symbiovars should be defined on the basis of the symbiotic capabilities in plant hosts distinguished by the differences in host range. If different plant effects (symptomatology in the case of pathogens) would be taken into account, then the efficiency in nitrogen fixation should also be evaluated and considered. As symbiotic gene sequences are commonly analyzed in rhizobial studies then the proposal of a biovar should be additionally supported with sequence data of symbiosis genes. Gene sequence data would be particularly useful when dealing with promiscuous hosts. Symbiovars would reflect a successful assembly of genes (some maybe yet unknown) that provide suitable host specificity. A symbiovar is determined by a symbiotic plasmid or island but may be conditioned as well by other replicons (chromosome or plasmids) carrying symbiotic determinants. A particular symbiovar may be maintained in different diverging bacteria lineages (it seems that preferentially in related species) by lateral transfer of symbiotic information.

Differences in one or few symbiosis genes may have specificity effects as has been clearly shown in *B. japonicum* [29,46]. The genetic basis of host specificity needs to be further studied in plants and in rhizobia and will provide a better understanding of symbiovars that will also derive from genomic studies. An extensive analysis of bradyrhizobial specificity and *nodA* genes in relation to the presence of other different *nod* genes that modify Nod factors has been published [46].

Acknowledgements

To PAPIIT grant IN200904 from UNAM, to Julio Martinez for technical support and Michael Dunn for review. We are grateful to Encarna Veláquez and Alvaro Peix for valuable comments. M.A. Rogel was a Ph.D. student in the Ciencias Biológicas program in UNAM and received fellowships from CONACyT. To the reviewers for their careful revision.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2010.11.015.

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Research

Endemic *Mimosa* species from Mexico prefer alphaproteobacterial rhizobial symbionts

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Received: *9 February 2015*
Accepted: *15 June 2015*

New Phytologist (2016) **209:** 319–333
doi: 10.1111/nph.13573

Key words: *Burkholderia*, *Ensifer*, legume nodulation, *Mimosa*, nitrogen (N) fixation, *Rhizobium*.

Summary

- The legume genus *Mimosa* has > 500 species, with two major centres of diversity, Brazil (*c.* 350 spp.) and Mexico (*c.* 100 spp.). In Brazil most species are nodulated by *Burkholderia*. Here we asked whether this is also true of native and endemic Mexican species.
- We have tested this apparent affinity for betaproteobacteria by examining the symbionts of native and endemic species of *Mimosa* in Mexico, especially from the central highlands where *Mimosa* spp. have diversified. Nodules were tested for betaproteobacteria using *in situ* immunolocalization. Rhizobia isolated from the nodules were genetically characterized and tested for their ability to nodulate *Mimosa* spp.
- Immunological analysis of 25 host taxa suggested that most (including all the highland endemics) were not nodulated by betaproteobacteria. Phylogenetic analyses of *16S* rRNA, *recA*, *nodA*, *nodC* and *nifH* genes from 87 strains isolated from 20 taxa confirmed that the endemic Mexican *Mimosa* species favoured alphaproteobacteria in the genera *Rhizobium* and *Ensifer*: this was confirmed by nodulation tests.
- Host phylogeny, geographic isolation and coevolution with symbionts derived from very different soils have potentially contributed to the striking difference in the choice of symbiotic partners by Mexican and Brazilian *Mimosa* species.

Introduction

Bacteria called 'rhizobia' form nodules on the roots of many legumes (Fabaceae) (Graham, 2008; Sprent, 2009) and are recognised as the main contributors of biologically-fixed nitrogen to undisturbed terrestrial ecosystems (Cleveland *et al.*, 1999). Until early this century, known rhizobia were confined to a few genera in the order Rhizobiales of the class Alphaproteobacteria (Graham, 2008), but it is now known that some legumes may also form effective nodules with Betaproteobacteria in the genera *Burkholderia* and *Cupriavidus* (Gyaneshwar *et al.*, 2011). Most studies so far have been carried out on the genus *Mimosa* (tribe Mimoseae, subfamily Mimosoideae). Approximately 500 species are native to the tropical and subtropical New World, but there are two Old World centres in Madagascar/East Africa (*c.* 30 spp.) and Asia (6 spp.) (Simon *et al.*, 2011). Species vary in habit from tall trees and shrubs to vines and herbs. They are found in a wide variety of habitats from wet to dry, growing on many different soils, including those that are very low in nutrients and organic matter, and low in pH. *Mimosa* is particularly abundant and diverse in the Cerrado and Caatinga biomes of Brazil, where there are many endemics, particularly at elevations above 1000 m a.s.l. (Barneby, 1991; Simon & Proença, 2000; Simon *et al.*, 2011). Almost all of the > 100 species that have been examined have been found to be nodulated, and thus it appears that nodulation is a generic character (Chen *et al.*, 2005a; dos Reis Junior *et al.*, 2010; Gehlot *et al.*, 2013; Lammel *et al.*, 2013). In terms of their symbionts, most work has been on widespread and/or invasive species. Mainly betarhizobial strains, particularly in the species *C. taiwanensis*, *B. mimosarum* and *B. phymatum*, have been isolated from the three major invasive *Mimosa* weed species (*M. diplotricha*, *M. pigra* and *M. pudica*) in many Southeast Asian tropical regions, such as Taiwan (Chen *et al.*, 2001, 2005b), India (Gehlot *et al.*, 2013), northern Australia (Parker *et al.*, 2007), Papua-New Guinea (Elliott *et al.*, 2007, 2009), southern China

(Liu *et al.*, 2012), the Philippines (Andrus *et al.*, 2012) and New Caledonia (Klonowska *et al.*, 2012).

Although we have learnt much about the symbionts of these three aggressive invasive species, are they representative of the vast majority of *Mimosa* species, most of which are highland endemics with a highly restricted range and distribution (Simon & Proença, 2000; Simon *et al.*, 2011)? In order to address this, Bontemps *et al.* (2010) and dos Reis Junior *et al.* (2010) examined the symbionts of *Mimosa* spp. native to the largest centres of radiation – the Cerrado and Caatinga biomes in central Brazil (together containing *c.* 250 spp.). They found that almost all of the 70 (mostly endemic, but also some widespread) species examined were exclusively nodulated by *Burkholderia*. Regardless of their degree of endemism, all the *Mimosa* species nodulated with *Burkholderia* strains that were genetically similar to each other, but the widespread ones were also capable of nodulating with other symbiont types, such as promiscuous strains of *Burkholderia* and *C. taiwanensis* (dos Reis Junior *et al.*, 2010). This suggests not only that the environment in which they have evolved is of great importance for the selection of *Mimosa* rhizobial symbionts, but also that their restriction to very particular localities has meant that the endemic Brazilian species have become very specialized in their selection of symbionts, whereas the widespread ones have remained capable of nodulating with a more diverse range of rhizobia (Elliott *et al.*, 2009; Bontemps *et al.*, 2010; Melkonian *et al.*, 2014).

The general aim of the present study was to investigate further the relationships between rhizobial symbionts and their *Mimosa* hosts, but in this case in the second largest centre of radiation of the genus, Mexico, which houses *c.* 100 species (Barneby, 1991; Grether *et al.*, 1996; Simon *et al.*, 2011). As in Brazil, native Mexican *Mimosa* species are a mixture of widespread and endemic species, with many of the latter residing in the central highlands/altiplano at altitudes above 1000 m a.s.l. (Supporting Information Fig. S1) (Martínez-Bernal & Grether, 2006; Grether *et al.*, 2007; Martínez-Bernal *et al.*, 2008). The widespread Mexican species are also found throughout the tropical New World, including Brazil, but the central Mexican endemics, which are closely related to each other, are confined to particular clades that are quite distant from those containing, for example, the central Brazilian endemics (Simon *et al.*, 2011). It is possible that these Mexican endemics have selected different rhizobial symbionts as a result of their geographic and taxonomic separation from the Brazilian endemics, and their subsequent evolution in a different environment (e.g. within neutral–alkaline rather than acid soils). Indeed, one of the few earlier studies conducted on the symbionts of Mexican *Mimosa* showed that a common Mesoamerican species, *M. affinis* (Grether, 2001), was nodulated by *Rhizobium etli* sv mimosae, a close relative of symbionts of common bean (*Phaseolus vulgaris* L.) (Wang *et al.*, 1999), although a more recent study has shown that the Mexican native species *M. occidentalis* was nodulated by *Burkholderia* (Ormeño-Orrillo *et al.*, 2012).

The present study had the following specific aims: to study nodulation of *Mimosa* species in the central and western Mexican highlands/altiplano; to isolate rhizobia from *Mimosa* nodules collected from plants growing in their native environments and/or grown in soil collected from their rhizospheres, and genetically characterize the rhizobial isolates by comparing sequences of some of their 'housekeeping' and symbiosis-essential genes with those in the databases; and to perform cross-inoculation studies to determine the symbiotic preferences and host range of representative Mexican *Mimosa* isolates.

Materials and Methods

Sampling of nodules, seeds and soils

Species of *Mimosa* were sampled from various locations in central and western Mexico in September 2007 and in October 2008 (Tables 1, 2, S1; Figs S1, S2). Strain CCGE1002 was isolated in 2006 from a field in Nayarit in western Mexico (Ormeño-Orrillo *et al.*, 2012) (Tables 1, S1). Voucher specimens were taken for all species and deposited in the herbarium at UAM-Iztapalapa (UAMIZ), Mexico City, and the locations from where they were sampled can be seen using Google Earth© (Notes S1). As many of the species are rare and nodule harvesting is destructive, we minimised the number of plants taken. Seeds were collected if present. In both expeditions, nodules (if present) were collected and preserved in silica gel for later bacterial isolation. Some nodules (3–4 per plant) were also cut in half to determine if they were potentially active and effective by the appearance of a pink colouration due to the presence of leghaemoglobin (Lb), and these were then placed into vials containing 2.5% glutaraldehyde in 50 mM phosphate buffer (pH 7.5) for microscopical analysis. Soil was also taken for rhizobial 'trapping' experiments using seedlings of the species that was originally found in that soil. Soil characteristics are listed in Table S2. The trapping experiments were conducted at CCG, UNAM, Cuernavaca, Mor., Mexico. Seeds of *Mimosa* spp. were germinated according to Elliott *et al.* (2007), and were placed in the appropriate rhizosphere soil in small pots (300 ml). Seeds of Mexican species that did not have soil particular to them were rooted in a mixture of all the soils. Nodules were sampled 3 months after the seeds were sown, and treated as for field-collected nodules.

Microscopy and *in situ* detection of microsymbionts

Pink nodules collected in the field or from trap experiments were prepared and sectioned for light microscopy to determine general nodule structure, and then were further analysed by *in situ* immunogold labelling plus silver-enhancement (IGL-SE) using antibodies raised against *Burkholderia phymatum* STM815^T and *Cupriavidus taiwanensis* LMG19424^T according to dos Reis Junior *et al.* (2010).

Bacterial strains, DNA extraction and amplification

Rhizobia were isolated from *Mimosa* nodules according to Bontemps *et al.* (2010). Bacteria from glycerol stocks were grown at 28°C for 3 d on TY medium (Beringer, 1974); a single colony for each sample was then transferred to 5 ml of liquid TY medium and grown at 28°C in a shaking incubator for 3 d. As

Table 1 Nodulation of *Mimosa* species collected in central and western Mexico in 2006 and 2007

Nodule sample (Herbarium voucher no.)	Species (E, endemic to Mexico; R, restricted to Mexico & Central America; W, widespread in the Americas)	State and location/vegetation type from where nodules were collected (more details are given in Supporting Information Table S3 and can be viewed on GoogleEarth; Notes S1)	Elevation (m a.s.l.)	Rhizobial isolates obtained (JPY) and their generic identification via sequencing of their *16S* rRNA and *recA* genes (*16S–recA* 'clusters' in parentheses)
UoD 189	*M. affinis* B.L. Rob. (R)	Mor., Cuernavaca – Tepotzlán. Roadside.	1418	None isolated
UoD 191	*M. albida* H. & B. ex. Willd. var. *albida* (W)	Mor., Cuernavaca – Tepotzlán. Roadside.	1418	None isolated
UoD 199	*M. albida* H. & B. ex. Willd. var. *strigosa* (Willd.) B.L. Rob. (W)	Mor., Cuernavaca – Tepotzlan. Roadside.	2019	*Rhizobium* **(5) 1075^{Mp}**
UoD 200	*M. albida* H. & B. ex. Willd. (W)	Mor., Cuernavaca. Roadside.	1907	None isolated
UoD 201	*M. affinis* B.L. Rob. (R)	Pue., Tepexco. Pasture.	1242	None isolated
UoD 207	*M. tricephala* Schltdl. & Cham. var. *tricephala* (E)	Pue., Izucar de Matamoros. Roadside.	1372	*Rhizobium* **(3) 820$^{**, Mp}$** **(6) 810^{Mp},** 811^{nt}
UoD 208	*M. benthamii* J.F. Macbr. var. *malacocarpa* (B.L. Rob.) J.F. Macbr.* (E)	Pue., Izucar de Matamoros. Pasture.	1273	None isolated
UoD 210	*M. mollis* Benth.* (E)	Pue. Izucar – Cuatla. Roadside.	1453	None isolated
UoD 211	*M. lactiflua* Delile ex Benth.* (E)	Pue. Izucar – Cuatla. Roadside.	1288	None isolated
UoD 212	*M. tricephala* Schltdl. & Cham. var. *tricephala** (E)	Pue., Izucar de Matamoros. Roadside.	1323	*Ensifer* **(2) 851$^{**, Mp}$, 996^{Mp}, 998^{Mp}**
UoD 222	*M. albida* H. & B. ex. Willd. var. *strigosa* (Willd.) B.L. Rob. (W)	Mor., Xochicalco. Pasture.	1368	*Rhizobium* **(6) 773$^{**, Mp}$**
UoD 223	*M. depauperata* Benth.* (E)	Qro., Tequisquiapan. Roadside.	1900	None isolated
UoD 224	*M. lacerata* Rose* (E)	Qro., Cadereyta. Roadside.	2174	None isolated
UoD 230	*M. depauperata* Benth. (E)	Qro., Toliman. Roadside.	1735	None isolated
UoD 232	*M. aculeaticarpa* Ortega (E)	Gto., Ranch Santa Ines. Roadside.	2192	None isolated
UoD 233	*M. monancistra* Benth.* (E)	Gto., San Miguel de Allende. Roadside.	1939	*Rhizobium* **(6) 826$^{**, Mp}$**
UoD 236	*M. albida* H. & B. ex. Willd. var. *albida* (W)	Gto., Campuzana. Roadside.	2141	*Rhizobium* **(5) 880^{Mp}, 888^{Mp+}**
UoD 239	*M. tequilana* S. Watson* (E)	Jal., Tequila. Roadside.	1174	*Rhizobium* **(4) 934^{Mp},** 947^{nt} **(6) 936, 940^{Mp}** 924^{nt}, 926^{nt}, 946^{nt}
UoD 244^{Bp}, 246	*M. skinneri* Benth. var. *skinneri* (W)	Jal., Tequila – Tepic. Roadside.	1203	*Burkholderia* **(1) 807^{Mp+}** *Rhizobium* **(4) 794^{Mp},** 785^{nt}, 792^{nt} **(5) 877^{Mp+}** **(6) 783^{Mp+},** 740^{nt}
UoD 245^{Bp}	*M. somnians* H. & B. ex. Willd. (W)	Jal., Tequila – Tepic. Roadside.	1203	*Burkholderia* **(1) 681^{Mp+}, 690^{Mp+},** 682^{nt}, 687^{nt}, 694^{nt}, 697^{nt}, 802^{nt}, 804^{nt}
UoD 247	*M. diplotricha* C. Wright ex. Sauvalle var. *diplotricha* (W)	Jal., Tequila – Tepic. Roadside.	1203	None isolated
MFS821	*M. occidentalis* Britton & Rose (R)	Nay., Tepic. Roadside.	716	*Burkholderia* **(1) 655 (CCGE1002)$^{Mp+}$**

New reports of nodulation are indicated by an asterisk after the species, and effective nodulation was confirmed by microscopical examination of the nodules in each case. The *in situ* reaction of the symbionts in the nodules to antibodies against *Burkholderia phymatum* STM815 (Bp) and *Cupriavidus taiwanensis* LMG19424 (Ct) was found to be negative for all samples except for those marked ^{Bp}. Strains isolated from the nodules are also listed, and unless marked otherwise each strain was tested positive for its ability to nodulate *M. affinis* (^{nt}, not tested; **, no nodulation). Strains marked ^{Mp} were also tested for their ability to nodulate *M. pudica*, and those marked $^{Mp+}$ nodulated it. Bold indicates that strains have been tested for nodulation on *M. affinis*.

> 700 isolates were obtained from the nodules, it was necessary to reduce these to a more manageable number for detailed analysis. Potential rhizobia were selected visually according to their colony morphology on yeast mannitol broth (YMB) + Congo Red agar plates (Vincent, 1970); most of the isolates from individual nodules appeared to be very similar, and so only one or two were selected for further analysis. DNA extractions were carried out according to Chomczynski & Sacchi (1987). Amplifications were performed with GoTaq® (Promega) according to the manufacturer's instructions using the primers shown in Table S3. DNA was amplified using a standard temperature profile with an initial DNA denaturation step at 95°C for 5 min followed by 30 cycles

Table 2 Nodulation of *Mimosa* species in rhizobial trapping experiments using soil collected in central and western Mexico in 2007 and 2008

Nodule sample (Herbarium voucher no.)	Species tested (E, endemic to Mexico; R, restricted to Mexico & Central America; W, widespread in the Americas)	State and location/vegetation type from where soil was collected (more details are given in Table S3 and can be viewed on GoogleEarth; Notes S1)	Elevation (m a.s.l.)	Rhizobial isolates obtained (JPY) and their generic identification via sequencing of their *16S* rRNA and *recA* genes (*16S–recA* clusters in parentheses)
na	*M. biuncifera* Benth. (R)	Mixture of all soils collected in Pue., Qro., Jal. & Mor.	–	*Ensifer* **(2) 1210** *Rhizobium* **(5)1206** **(6) 1209**
na	*M. borealis* A. Gray (R)#	Mixture of all soils collected in Pue., Qro., Jal. & Mor.	–	*Ensifer* **(2) 1220^{Mp}, 1226^{Mp}, 1228, 1229** *Rhizobium* **(6) 1225**
na	*M. dysocarpa* Benth. (R)	Mixture of all soils collected in Pue., Qro., Jal. & Mor.	–	*Ensifer* **(2) 1260** *Rhizobium* **(5) 1252^{Mp}, 1263**
na	*M. orthocarpa* Spruce ex. Benth. (W)	Mixture of all soils collected in Pue., Qro., Jal. & Mor.	–	No nodules
na	*M. robusta* R. Grether* (E)	Mixture of all soils collected in Pue., Qro., Jal. & Mor.	–	*Rhizobium* **(5) 1283** **(6) 1269**
UoD 215, 216	*M. luisana* Brandg. (E)	Pue., Tehuacan. Pasture.	1632	*Ensifer* **(2) 1111, 1123, 1165**
UoD 217	*M. polyantha* Benth. (E)	Pue., Tehuacan. Pasture.	1144	*Ensifer* **(2) 1114, 1118, 1132****
UoD 219	*M. luisana* Brandg. (E)	Mor., Xochicalco. Pasture.	1282	*Ensifer* **(2) 1088^{Mp}, 1091^{Mp}**
UoD 224	*M. lacerata* Rose (E)	Qro., Cadereyta. Roadside.	2174	*Ensifer* **(2) 1139^{Mp}**
UoD 227	*M. similis* Britton & Rose* (E)	Qro., Cadereyta. Roadside.	1545	*Ensifer* **(2) 1142**
UoD 239	*M. tequilana* S. Watson (E)	Jal., Tequila. Roadside.	1174	*Rhizobium* **(4) 1153** **(5) 1145^{Mp}, 1152^{Mp}** **(6) 1151, 1154**
UoD 325	*M. polyantha* Benth. (E)	Mor., Sierra de Huautla. Roadside.	1021	*Rhizobium* **(6) 1198^{Mp}, 1201, 1202**
UoD 326	*M. goldmanii* B.L. Rob.* (E)	Mor., Sierra de Huautla. Roadside.	1021	*Rhizobium* **(6) 1300, 1301, 1321, 1322, 1323**
UoD 328	*M. albida* H. & B. ex. Willd. (W)	Mor., Sierra de Huautla. Roadside.	1148	*Ensifer* **(2) 1168**** *Rhizobium* **(6) 1166, 1170, 1171, 1172, 1385, 1388, 1389, 1390**
UoD 333	*M. albida* H. & B. ex. Willd. (W)	Mor., Sierra de Huautla. Roadside.	1060	*Rhizobium* **(5) 1403, 1404, 1405**
UoD 335	M. benthamii J.F. Macbr. (E)	Mor., Sierra de Huautla. Pasture.	1234	*Rhizobium* **(6) 1359, 1363**, 1367**
UoD 336	*Mimosa* sp. X* (E)	Mor., Sierra de Huautla. Roadside.	1043	*Ensifer* **(2) 1431, 1432** *Rhizobium* **(5) 1429**

R#, restricted to southern USA. New reports of nodulation are indicated by an asterisk after the species, and effective nodulation was confirmed by microscopical examination of the nodules in each case. The *in situ* reaction of the symbionts in the nodules to antibodies against *Burkholderia phymatum* STM815 (Bp) and *Cupriavidus taiwanensis* LMG19424 (Ct) was found to be negative for all samples. Strains isolated from the nodules are also listed, and unless marked otherwise each strain was tested positive for its ability to nodulate *M. affinis* (**, no nodulation). Strains marked ^{Mp} were also tested for their ability to nodulate *M. pudica*, and those marked $^{Mp+}$ nodulated it.

for the *16S* rRNA and *recA* genes or 40 cycles for *nifH, nodA* and *nodC* consisting of 30 s at 95°C, 30 s of primer annealing and 30 s of DNA amplification (or 1 min 30 s for *16S* rRNA) at 72°C. Annealing temperatures were 63°C for *recA* and *nifH*, 50°C for *nodA* and *nodC* and 56°C for *16S* rRNA. Amplifications were finished with a final extension step at 72°C for 7 min.

For *Burkholderia* isolates, PCR of these genes was performed as above, but with modifications according to Bontemps *et al.* (2010).

Restriction fragment length polymorphism analysis and sequencing

PCR-amplified *16S* rRNA, *recA* and *nodC* genes were digested with the restriction enzymes HinfI and MspI in order to classify the isolates into groups (Laguerre *et al.*, 1994; Chen *et al.*, 2003, 2005a,b). Five microlitres of each PCR product was incubated with 5 units of enzyme and the appropriate buffers at 37°C for a minimum of 3 h. The digestion products were separated on a 2% gel for 2.5 h at 80 V and visually compared. In order to better establish their taxonomic position, profiles of *16S* rRNA and *recA* were combined, and the isolates were considered to be similar when profiles were identical for both genes with both enzymes. The efficiency of the restriction fragment length polymorphism (RFLP) grouping was then checked by sequencing several isolates from each group. The PCR-amplified products were sequenced in both directions by Macrogen Inc. (Seoul, Korea) or by the sequencing service at the James Hutton Institute, Dundee (UK). The sequences were aligned with the MAFFT software (Katoh *et al.*, 2009) and their quality checked with BioEdit (Hall, 1999). Accession numbers are given in Table S1.

Phylogenetic and statistical analyses

Nucleotide alignments and phylogenetic trees were constructed and edited with Mega6 (Tamura *et al.*, 2013) using a maximum-likelihood (ML) method based on a GTR + G + I model. Support for the tree branches was estimated with 100 bootstrap replicates and all positions with < 80% site coverage were eliminated for the *16S* rRNA, *recA* and *nifH* genes, whereas all positions containing gaps and missing data were eliminated for the *nodA* and *nodC* genes. A total of 1074 positions were used for the concatenated *16S* rRNA-*recA* phylogenetic tree, 737 for the *16S* rRNA, 337 for the *recA*, 285 for the *nodA*, 424 for the *nodC* and 479 for the *nifH* phylogenetic trees. Canonical discriminant analysis (CDA) was applied to assess the plant host and ecological preferences of the different rhizobial genera (*Burkholderia*, *Ensifer* and *Rhizobium*) according to different qualitative (plant clade, site, plant-status) and quantitative (elevation) variables with XLSTAT. The distribution of the genera was summarized by their centroids. The plant status was used and defined according to the plant distribution within the Americas (W, widespread in the Americas; R, restricted to certain parts of Central and North America; E, Endemic to Mexico). The different plant clades are those defined in the *Mimosa* phylogeny of Simon *et al.* (2011). The locations refer to the sampling locations that can be found together with their elevations in Tables 1, 2 and S1.

Nodulation tests

Seeds were not available for most of the *Mimosa* species to test for their ability to nodulate with their potentially symbiotic isolates, so *M. affinis* was chosen as a 'model' host, as it is widespread in Mexico and Central America, is herbaceous and fast growing, and has an ability to nodulate with a wide range of rhizobial types, both Alpha and Beta (Wang *et al.*, 1999; Elliott *et al.*, 2007, 2009). Out of the 87 strains used in the phylogenetic analysis, 74 strains from 17 *Mimosa* species, as well as reference type strains, were tested on *M. affinis*. Similar tests were also conducted on the pan-tropical species *M. pudica* with 28 strains (all of which were also tested on *M. affinis*), as this species has been used in several studies from South America as a model host for *Mimosa* symbionts, particularly Betaproteobacterial ones (Chen *et al.*, 2005a; Bontemps *et al.*, 2010; Mishra *et al.*, 2012). The *M. affinis* and *M. pudica* plants were grown hydroponically in a sterile solution of Jensen's medium (quarter strength), and they were inoculated according to Elliott *et al.* (2009). The plants were harvested 6 wk after inoculation and were scored for the presence of nodules. *Rhizobium etli* sv mimosae strain Mim-1 served as a positive control with *M. affinis*, and *C. taiwanensis* LMG19424 with *M. pudica*. Cross-inoculation tests were performed with selected isolates on various *Mimosa* spp. native to Mexico and/or to South America, as well as on common bean cv Negro Jamapa. The seeds were sourced and germinated according to Elliott *et al.* (2007), and the tests were performed under sterile conditions in glass tubes (70 ml volume) that were quarter-filled with an autoclaved mixture of vermiculite and perlite, and fed with Jensen's N-free medium. The plants were inoculated according to Elliott *et al.* (2009). The mimosas were harvested at 6–8 wk and the beans at 3 wk after inoculation, when they were scored for the green colour of their aerial parts and the presence of pink nodules, which are indications of effective nitrogen fixation. Nodules were also taken for microscopical analysis.

Additional nodulation tests were performed at CCG-UNAM, Cuernavaca on a range of *Mimosa* spp. inoculated with *R. etli* sv mimosae Mim-1, which was originally isolated from *M. affinis* by Wang *et al.* (1999). In this case the plants were rooted in agar made with Jensen's medium inside enclosed tubes according to Chen *et al.* (2003), and were harvested at 8 wk after inoculation. Uninoculated plants served as controls in all the experiments.

Results

Nodules on endemic Mexican *Mimosa* spp. do not contain betaproteobacteria

In 2007, nodules were obtained directly from 21 separate *Mimosa* plants in the field, representing 15 separate taxa (Table 1; Fig. S2). Eight of the endemic species (*M. benthamii*, *M. biuncifera*, *M. depauperata*, *M. lactiflua*, *M. monancistra*, *M. mollis*, *M. tequilana* and *M. tricephala*) are new reports of nodulation (Table 1; Fig. S3). Nodules that were prepared and sectioned for microscopy had a structure typical of *Mimosa* nodules and were effective in appearance (see Fig. S3 for representative examples). None of the nodules on the endemic and Central American species reacted with the specific betaproteobacterial antibodies using IGL-SE, but nodules from the widespread species *M. skinneri* and *M. somnians* reacted with the

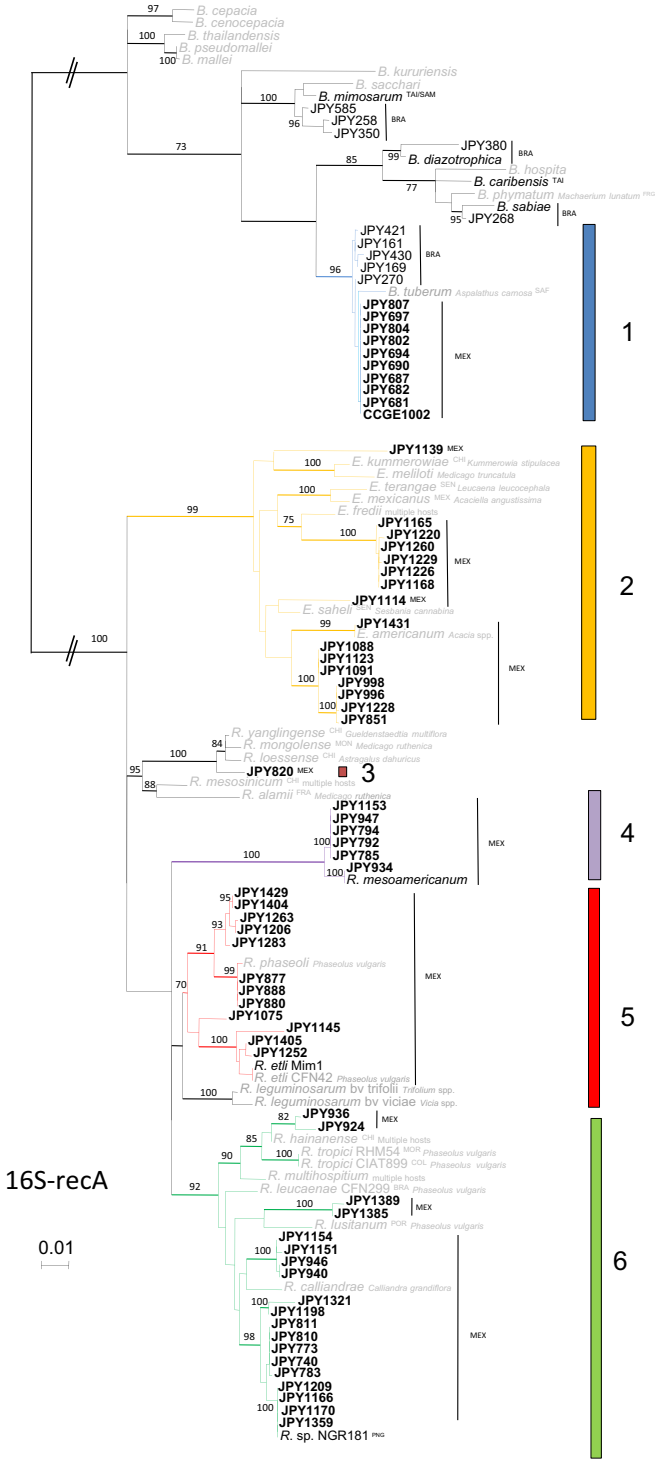


Fig. 1 Phylogenetic relationships of strains isolated from *Mimosa* nodules in this study, and reference strains, based on a 1074 nucleotide 16S rRNA-*recA* concatenated sequence. The tree was built using a maximum-likelihood method and heavy lines indicate branches supported by bootstrap values > 70% (100 replicates). The scale represents mutations per nucleotide. *Mimosa* symbionts are in black, and those isolated in this study are in bold. Taxa in grey are nonsymbiotic bacteria or symbionts of other hosts that are indicated beside the strain name. Full sequence identifiers, accession numbers and strain numbers can be found in Supporting Information Fig. S3 and Table S3. Coloured bars indicate clusters 1–6 that are discussed in the main text. When known, geographical origins of the strains are indicated as follows: BRA, Brazil; CHI, China; COL, Colombia; FRA, France; FRG, French Guiana; MEX, Mexico; MON, Mongolia; MOR, Morocco; PNG, Papua New Guinea; POR, Portugal; SAM, South America; SAF, South Africa; SEN, Senegal; TAI, Taiwan. B, *Burkholderia*; E, *Ensifer*; R, *Rhizobium*.

B. phymatum STM815 antibody (Table 1). Nodules were also harvested from *Mimosa* spp. grown as 'trap plants' in rhizosphere soils, as well as from four Mexican *Mimosa* species grown in a mixture of all the rhizosphere soils. A fifth species, *M. orthocarpa*, did not form any nodules (Table 2). In total, these trap experiments yielded nodules on a further 15 taxa, including 10 that were different from those of the field samplings. There were new reports of nodulation by the endemic species *M. goldmanii*, *M. robusta*, *M. similis* and by another endemic species from Sierra de Huautla, which is still awaiting a formal description (*Mimosa* sp. X; Fig. S2f). Sections of all of the nodules from the trap experiments showed that the nodules were effective (Fig. S3), but none reacted with either antibody using IGL-SE (Table 2).

Endemic Mexican *Mimosa* spp. are specifically associated with alphaproteobacteria

After genetic analyses and nodulation tests, the survey of Mexican *Mimosa* symbiont (MMS) diversity resulted in 87 isolates from single nodules from 26 plants in 18 locations. These represented potential symbionts of 19 *Mimosa* taxa (17 species), 33 of which came from nodules collected from eight *Mimosa* taxa in the field, and 54 from 13 *Mimosa* taxa grown in the trap experiments (Tables 1, 2, S1). According to their *16S* rRNA and *recA* sequences, isolates from the field-sampled nodules were classified in both Alphaproteobacteria (*Rhizobium*, *Ensifer*) and Betaproteobacteria (*Burkholderia*), with the latter being almost confined to the widespread species *M. somnians*, although another widespread species *M. skinneri* also yielded some *Burkholderia* isolates amongst its largely alphaproteobacterial microbiota. All isolates from the trap experiments were alphaproteobacteria, and belonged to either *Rhizobium* or *Ensifer*.

The taxonomic positions of the 87 isolates were assessed by a phylogenetic tree based on concatenated *16S* rRNA and *recA* sequences (Fig. 1). According to bootstrap values and reference strain positions, five clusters and a single-strain lineage (JPY820) were defined in the concatenated tree and in the *16S* rRNA and *recA* trees (Fig. S4a,b). Nine closely related isolates (Cluster 1) belonged to *Burkholderia*; these were isolated from the widespread species *M. skinneri* and *M. somnians* and they grouped with *B. tuberum* strains isolated from Brazilian *Mimosa* spp. (JPY161–JPY430; Bontemps *et al.*, 2010) and from *M. occidentalis* (CCGE1002). Cluster 2 encompassed *Ensifer* isolates from 10 *Mimosa* species. The remaining Clusters (3–6) belonged to the genus *Rhizobium*. Cluster 3 grouped one isolate (JPY820 from *M. tricephala*) with the reference species *R. loessense*, *R. mongolense* and *R. yanglingense*, Cluster 4 was closely related to *R. mesoamericanum*, Cluster 5 grouped with reference strains already known to nodulate *Mimosa* or *P. vulgaris*, and Cluster 6 grouped with the *R. tropici* (CIAT899^T),

R. leucaenae (CFN299^T) and *R. calliandrae* (CCGE524^T) type strains, as well as the Papua New Guinea *Mimosa* strain NGR181 (Elliott *et al.*, 2009). In addition, Cluster 6 also contained JPY491 (Fig. S4a), one of only two *Rhizobium* strains that were isolated from central Brazil by Bontemps *et al.* (2010), both from the widespread species *M. xanthocentra*.

Mexican *Mimosa* symbionts have diverse, but specific, stable and ancient nodulation genes

The symbiotic phenotype was confirmed for all 87 isolates at the molecular level by amplification and sequencing of the symbiosis-related genes, *nodA*, *nodC* and *nifH*, and/or by nodulation tests (Tables 1, 2, S1). Both *nodA* and *nodC* were sequenced from 45 strains, *nodA* from 10 strains and *nodC* from a further 35 strains; *nifH* was sequenced from 34 strains. Phylogenetic trees were constructed for all three genes (Fig. 2). Similar clusters as for *16S* rRNA/*recA* were generally observed, that is Cluster 1 (*Burkholderia*), Cluster 2 (*Ensifer*) and Clusters 3–6 (*Rhizobium*). Clusters 2–6 also frequently encompassed common bean symbionts and, more rarely, other mimosoid symbionts that are also capable of nodulating *P. vulgaris*. The clusters of MMS strains defined in the 16S/*recA* phylogeny were also seen in the phylogenies of the symbiosis-related genes, and groupings within the major clusters 2, 5 and 6 were largely conserved. However, there were four strains that had *nodA* sequences typical of Cluster 6 rather than of their own cluster (Fig 2): the Cluster 4 *Rhizobium* strains JPY1153 (from *M. tequilana*) and JPY785 (from *M. skinneri*), the Cluster 5 *Rhizobium* strain JPY1429 (from *Mimosa* sp. X), and the Cluster 2 *Ensifer* strain JPY1168 (from *M. albida*). JPY1153 also had a Cluster 6 *nodC* sequence, whereas the *nifH* sequence of JPY1168 was in Cluster 2. It thus seems that these four strains may be the recipients of *nod* genes, but not necessarily *nif* genes, from donors in Cluster 6. Further evidence for horizontal transfer of nodulation genes is that JPY996 and JPY998 (both from *M. tricephala*) formed a separate lineage for *nodA* (but not *nodC*) from the other *Ensifer* strains in Cluster 2; these *nodA* sequences are more closely related to those of the *Rhizobium* strains in Cluster 3, which also contains a *M. tricephala* symbiont (JPY820).

Relationship between rhizobial type, plant host and location

On the one hand, there are no obvious differences between MMS from field-collected nodules and those obtained from trap plants. Soils in which the plants were growing were relatively similar, at least in pH (Table S1), which is often the major determinant of bacterial diversity in the soil, including that of *Burkholderia* (Stopnisek *et al.*, 2014). On the other hand, 18 sites were sampled across 600 km (Fig. 3) and there are evident geographic trends: symbiotic *Burkholderia* strains were isolated only in the west (from the widespread species *M. skinneri* and *M. somnians* and the Mexican–Central American-restricted species *M. occidentalis*), whereas *Ensifer* strains were most prominent in the centre and the east of Mexico (Fig. 3a). The results of a CDA test indicated that the different genera were, indeed, not randomly distributed (Fig. 3b). One of two main factors explaining this distribution was the sampling location (Fig. 3c). The other factor was the plant phylogeny based upon the *Mimosa* clades defined by Simon *et al.* (2011), and presented in selected form in Fig. 4. In Mexico, there were preferential associations of: *Burkholderia* with widespread *Mimosa* spp. from clades L, M and N; *Rhizobium* with *Mimosa* spp. from clades R, T and V, where the studied species have a more restricted geographical range (except for *M. albida* and *M. skinneri*); and *Ensifer* with *Mimosa* spp. from clade B that are mainly endemic to Mexico (Figs 3c, 4).

Endemic Mexican *Mimosa* species have a preference for nodulating with alphaproteobacteria

Mimosa affinis was nodulated effectively by the four *Burkholderia* strains (CCGE1002, JPY681, JPY690, JPY807) and all but six of the 70 alphaproteobacterial strains tested (as well as *R. etli* sv mimosae Mim7-4) (Tables 1, 2, S1). Those that did not nodulate *M. affinis* included the single strain in Cluster 3 (JPY820), and some strains from Clusters 2 and 6. By contrast, *M. pudica* was only nodulated (partially) effectively by the *Burkholderia* strains, whereas *Rhizobium* strains JPY783 (Cluster 6) and JPY877 (Cluster 5) from *M. skinneri* and JPY888 (Cluster 5) from *M. albida* formed ineffective nodules. The other *Rhizobium* and *Ensifer* strains did not nodulate this host (Tables 1, 2, S1). Of the reference strains, *R. etli* CFN42^T, *R. leucaenae* CFN299^T and *R. tropici* CIAT899^T nodulated *M. affinis* ineffectively and failed to nodulate *M. pudica* (Table S1). No uninoculated control plants nodulated.

Cross-inoculation tests (Table 3; Fig. 5) showed that all species had the capacity to nodulate effectively with most *Rhizobium* strains, but there were also differences in host range between *Mimosa* species: those in the southern USA–Mexican Clade B (*M. borealis*, *M. dysocarpa*, *M. luisana* and *M. polyantha*) were less capable of nodulating effectively (or at all) with betarhizobia. This contrasts with the four species in Clade T (including the Mexican endemic *M. tequilana*,) and *M. orthocarpa* in Clade M, which were all capable of nodulating effectively with *B. phymatum* STM815 (Table 3, this study; Elliott *et al.*, 2007; dos Reis Junior *et al.*, 2010). Finally, all of the alphaproteobacterial strains tested, JPY1220 (Cluster 2), JPY820 (Cluster 3), JPY934 (Cluster 4), Mim-1 (Cluster 5) and JPY940 and 1198 (both Cluster 6) nodulated *P. vulgaris* effectively (Table S1), but *Burkholderia* sp. CCGE1002 (Cluster 1) only formed occasional ineffective nodules on this host.

Discussion

Betaproteobacteria are not the usual symbionts of Mexican *Mimosa* spp.

In this study, we have confirmed nodulation of the Mexican *Mimosa* species studied by Elliott *et al.* (2007), but have also presented evidence that several other species are capable of nodulation, with new reports of nodulation for 12 Mexican endemics.

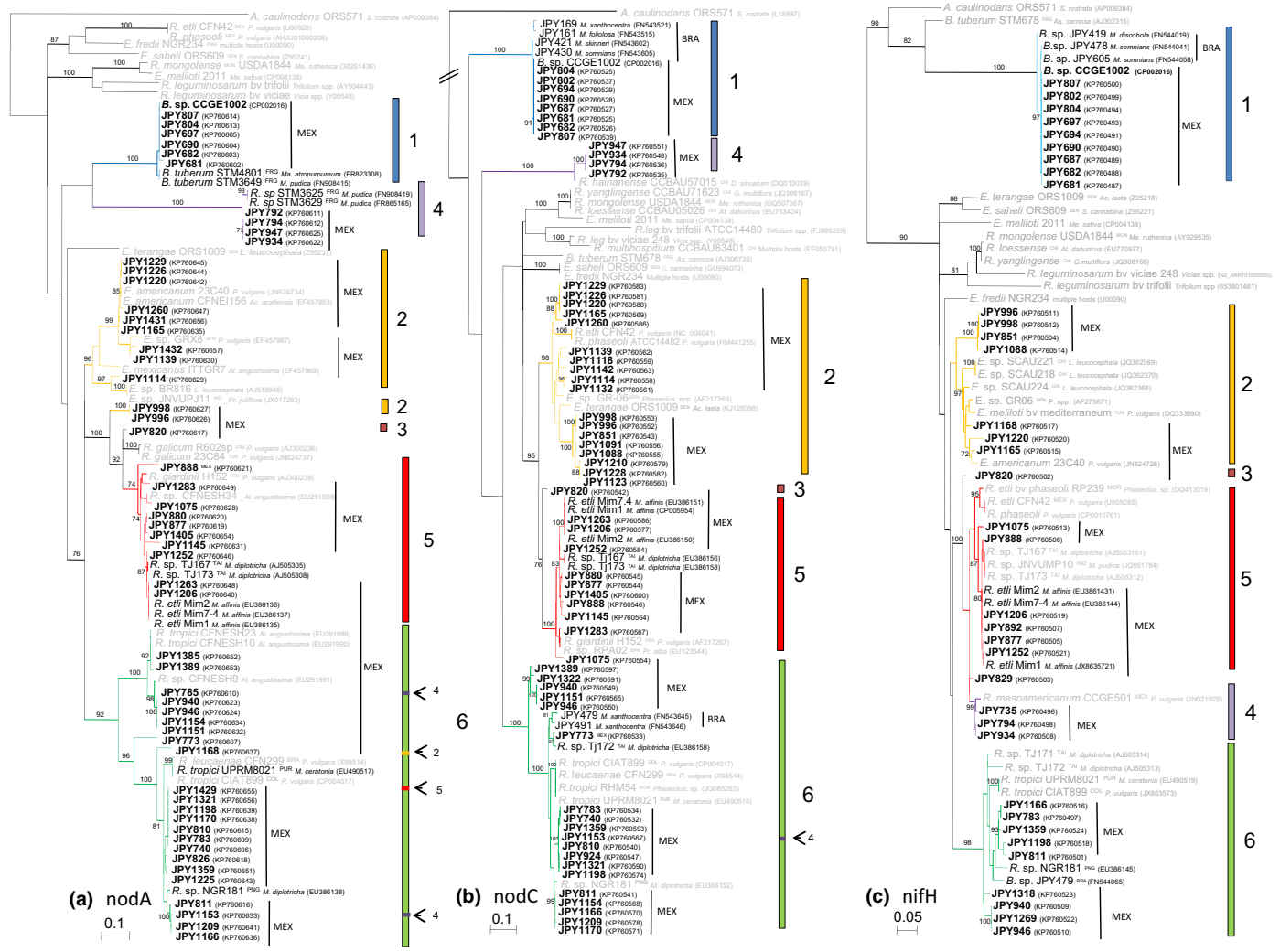
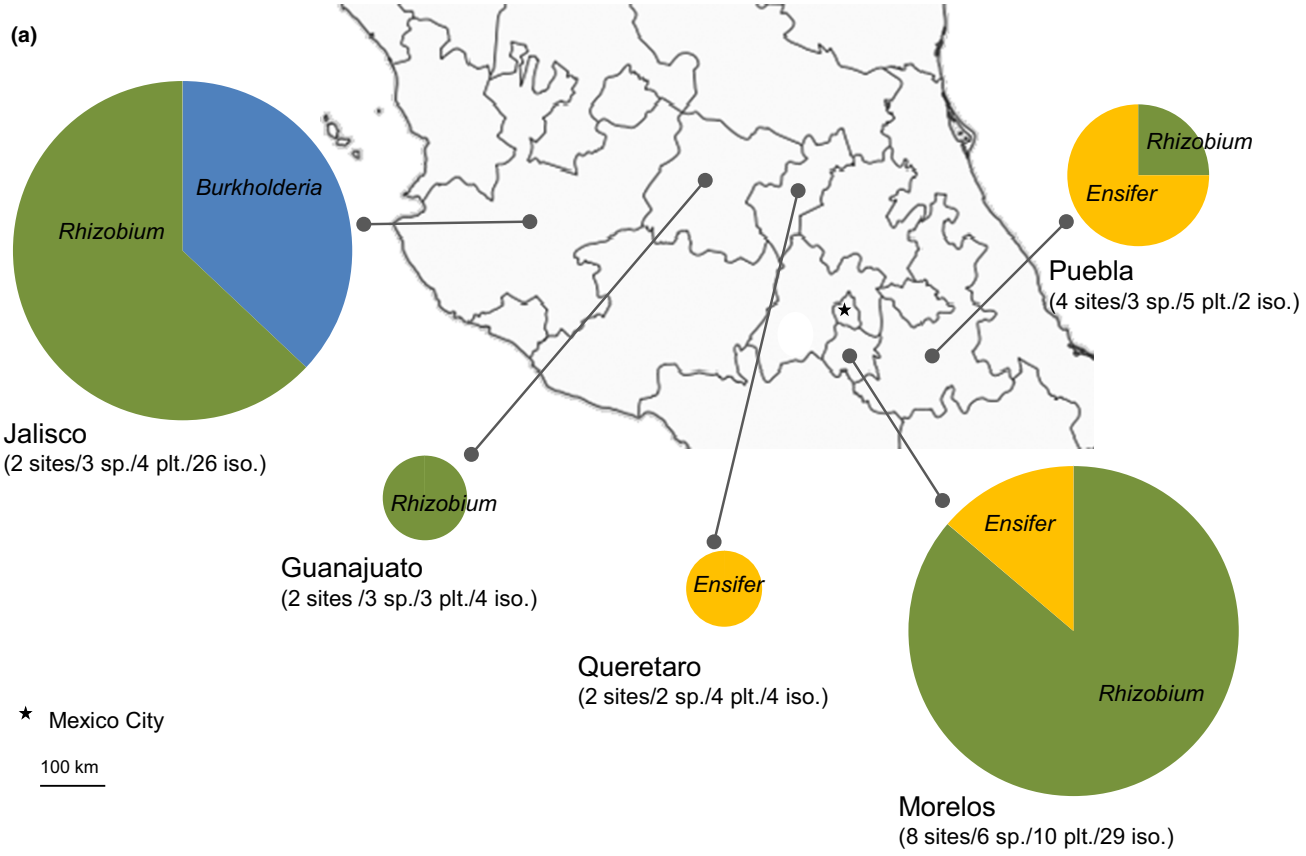
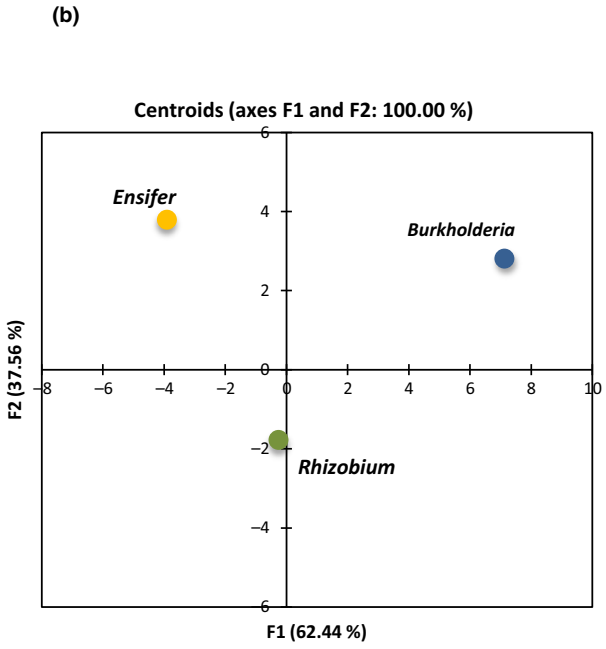


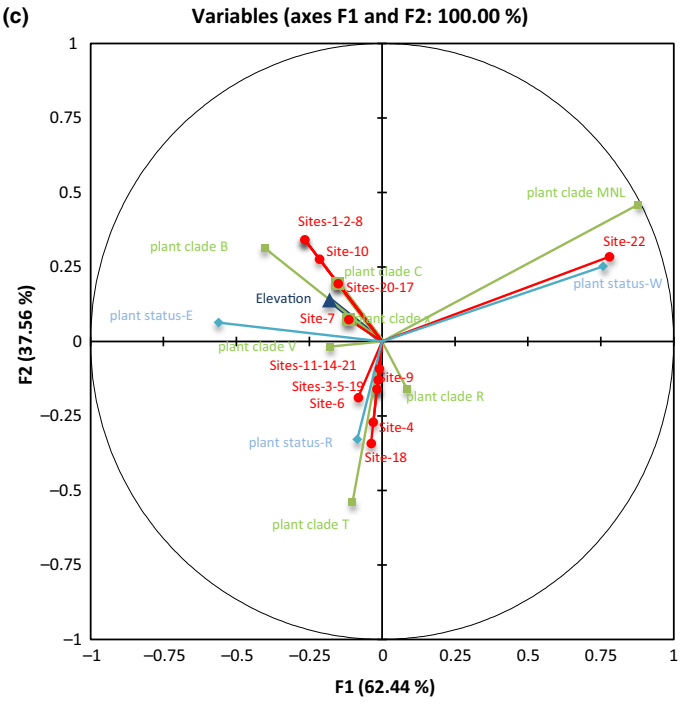
Fig. 2 Phylogenies of symbiosis-related genes in strains isolated from *Mimosa* nodules in this study and reference strains. (a) *nodA*, (b) *nodC* and (c) *nifH*. The trees were built using a maximum-likelihood method with a 285-nt alignment for *nodA*, a 424-nt alignment for *nodC* and a 479-nt alignment for *nifH*. Heavy lines indicate branches supported by bootstrap values > 70% (100 replicates). The scale represents mutations per nucleotide. *Mimosa* symbionts are in black and those isolated in this study are in bold. Symbionts of other hosts are in grey, and their hosts are indicated. For previously published sequences, the host plant is indicated, and the sequence accession number in parentheses. Coloured bars indicate clusters defined in the *16S* rRNA-*recA* phylogeny from Fig. 1. Arrows indicate potential horizontal transfer of symbiosis genes between clusters. When known, geographical origins of isolates are indicated as follows: BRA, Brazil; COL, Colombia; IND, India; MEX, Mexico; MON, Mongolia; MOR, Morocco; FRA, France; FRG, French Guiana; PNG, Papua New Guinea; PUR, Puerto Rico; SEN, Senegal; SPN, Spain; TAI, Taiwan; TUN, Tunisia. A, *Azorhizobium*; B, *Burkholderia*; E, *Ensifer*; R, *Rhizobium*; Ac, *Acacia*; Al, *Acaciella*; As, *Aspalathus*; At, *Astragalus*; G, *Gueldenstaedtia*; L, *Leucaena*; M, *Mimosa*; Ma, *Macroptillium*; Me, *Medicago*; P, *Phaseolus*; Pr, *Prosopis*; S, *Sesbania*.

Fig. 3 (a) Distribution of the bacterial genera found in association with *Mimosa* spp. in each state sampled in Mexico. The area of each circle is proportional to the number of isolates. For each state, the number of sampled sites, the number of sampled *Mimosa* species (sp.), the number of individual plants (plt.), and the number of bacterial isolates (iso.) are indicated. Coloured circles represent the proportion of each genus among the isolates: blue, *Burkholderia*; green, *Rhizobium*; yellow, *Ensifer*. Only confirmed nodulating strains were included in the analysis. Map source: http://en.wikipedia.org/wiki/Template:Location_map_Mexico. (b) Canonical discriminant analysis (CDA) of the distribution of the different bacterial clusters according to qualitative (plant clade, site, plant-status) and quantitative (elevation) variables. The sample distribution for each bacterial genus associated with *Mimosa* in Mexico is summarized by their centroids. The bacterial clusters correspond to those in Fig. 1: *Burkholderia* are in Cluster 1, *Ensifer* in Cluster 2 and *Rhizobium* in Clusters 4–6. Cluster 3, a single strain, was omitted from the analysis. The first axis explains 62.44% of the variation in bacterial cluster distribution and showed a strong difference between *Ensifer* and *Burkholderia* distribution. The second axis explains 37.56% of this variation and also showed a differentiation between *Rhizobium* distribution and those of the two other genera. (c) Correlation circle of the variables on the first factorial plane (F1 × F2) of the CDA. The different plant clades are those defined in the *Mimosa* phylogeny (Fig. 4). Plant status: W, widespread in the Americas; R, restricted to certain parts of Central or North America; E, Endemic to Mexico. The locations and elevations can be found in Tables 1, 2, S3. The distribution of the genus *Burkholderia* appeared to be mainly associated with widespread *Mimosa* found in location 22 and those species that belonged to the Clades L, M and N (see Simon *et al.* (2011) and Fig. 4 (this study)), whereas *Ensifer* distribution was linked more with *Mimosa* clade B (mostly Mexican endemics) in locations 1, 2, 8 and 10. The most explanatory variable for *Rhizobium* distribution appeared to be the *Mimosa* clade T, especially the widespread species *M. albida*.

Betarhizobia were not detected by *in situ* hybridization in nodules from the Mexican native or endemic *Mimosa* species in Clades B, T and V. This is in contrast to the study of Brazilian native and endemic *Mimosa* spp. by dos Reis Junior *et al.* (2010), in which nodules from 67 out of 70 species reacted with the *Burkholderia phymatum* STM815 antibody. It should be noted, though, that nodules from the two species that were common to both studies, *M. skinneri* and *M. somnians*, reacted to this antibody in both Mexico (this study) and Brazil (dos Reis Junior *et al.*, 2010). It is also noteworthy that in neither study were any nodules sampled







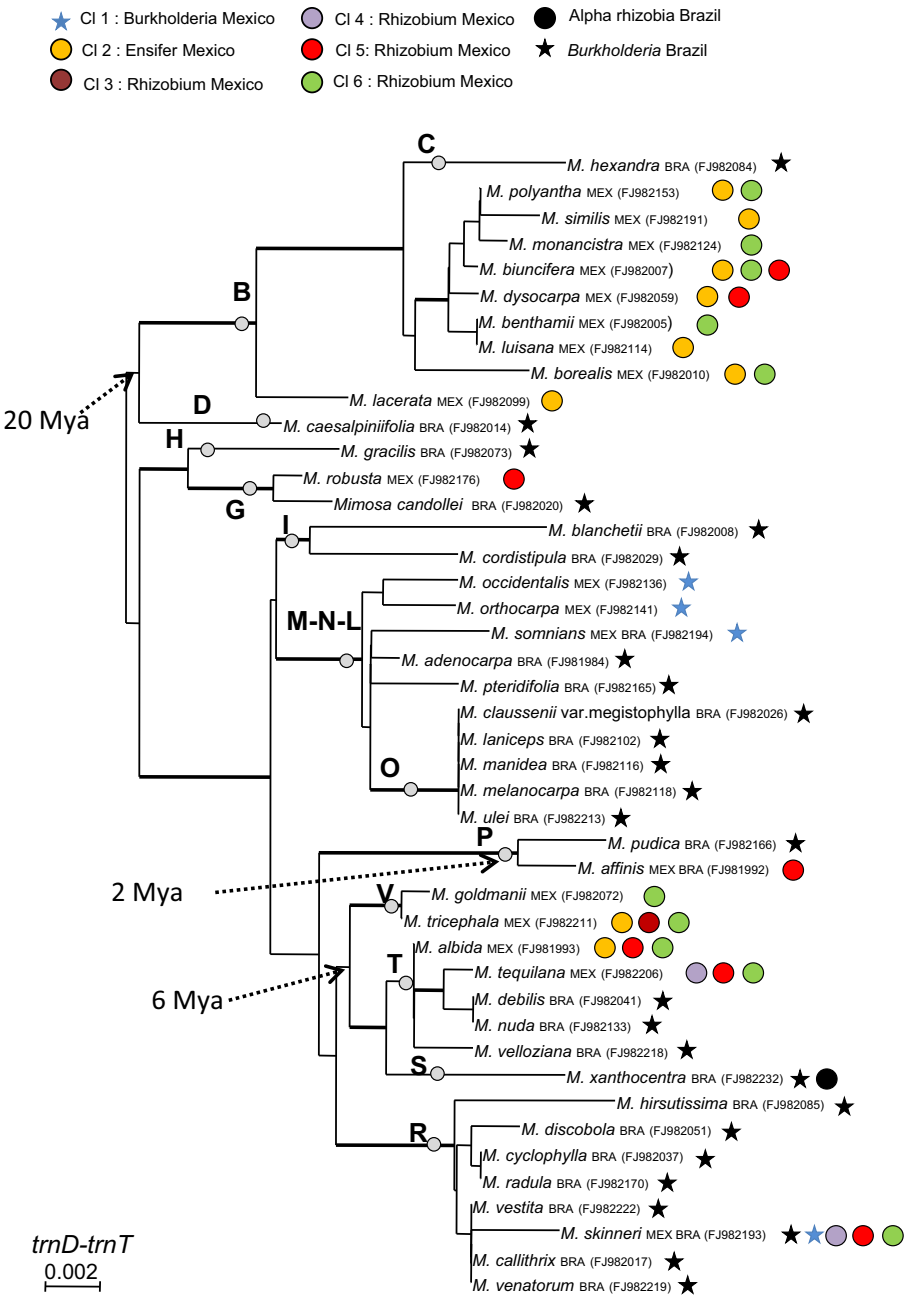


Fig. 4 Phylogeny of *Mimosa* based on DNA sequences of the *trnD-trnT* noncoding plastid locus. Only *Mimosa* species sampled from this study in Mexico, those in Brazil from Bontemps *et al.* (2010), the study of Elliott *et al.* (2007), and the nodulation test species *M. pudica* and *M. affinis* were used to build the tree. The tree was built using the distance (BioNJ) method. Bootstrap values > 75% are indicated with heavy lines (1000 replicates). Letters on branches indicate well-supported *Mimosa* clades defined by Simon *et al.* (2011). The origin of the sampled *Mimosa* species is indicated (MEX, Mexico; BRA, Brazil; MEX BRA, sampled in both locations). Taxonomic groups of associated symbionts are indicated with circles for alpharhizobia and stars for *Burkholderia*. Symbiont data for Brazilian *Mimosa* species are from Bontemps *et al.* (2010), dos Reis Junior *et al.* (2010) and Elliott *et al.* (2007), and those for *M. affinis* from Elliott *et al.* (2009). For Mexican isolates, the associated coloured circle corresponds to the taxonomic clusters defined in Fig. 1. Arrows indicate the ages of nodes as estimated by Simon *et al.* (2011). M, *Mimosa*; Cl, Cluster. Ma, Myr ago.

that reacted with the *Cupriavidus taiwanensis* LMG19424 antibody, even though *C. taiwanensis* is widespread in invasive *Mimosa* spp. in the tropics (see the Introduction section). Furthermore, Elliott *et al.* (2007) found that the Mexican *Mimosa* species in their study, which included some of the Clade B endemics that we also sampled, were not nodulated effectively (and in some cases not at all) by either *B. phymatum* STM815 or *C. taiwanensis* LMG19424.

The *Burkholderia* isolates were confined to two *Mimosa* species, *M. skinneri* and *M. somnians*, nodules from which also reacted positively with the antibody against *B. phymatum* STM815. Both species are widespread in the Neotropics, and were previously reported to be nodulated by *Burkholderia* in Brazil (Elliott *et al.*, 2007; Bontemps *et al.*, 2010; dos Reis Junior *et al.*, 2010). The *Burkholderia* strains from *M. skinneri* and *M. somnians* in Mexico were all very closely related to the sequenced strain CCGE1002 (Ormeño-Orrillo *et al.*, 2012), which was originally isolated from *M. occidentalis*, a Mexican–Central American-restricted species. These Mexican burkholderias in Cluster 1 are most closely related to *B. tuberum* sv mimosae, a species/symbiovar (Rogel *et al.*, 2011) that has been widely isolated from *Mimosa* spp. in South America (Bontemps *et al.*, 2010; Mishra *et al.*, 2012); this is species complex 6, as defined by Bontemps *et al.* (2010). The low diversity of the Mexican *Burkholderia* strains contrasts with the very diverse *Burkholderia* lineages found in the South American studies of Bontemps *et al.* (2010), Mishra *et al.* (2012) and Lammel *et al.* (2013), but the sample is very small, from just two collection sites and three *Mimosa* species, because *Burkholderia* was

Table 3 Cross-inoculation tests with Mexican *Mimosa* rhizobia and *Mimosa* spp. from various locations and clades (Simon *et al.*, 2011)

Mimosa sp. (Clade; Simon *et al.*, 2011)	*Ensifer* sp. JPY1220 (Cluster 2)	*Rhizobium* sp. JPY934 (Cluster 4)	*Rhizobium* sp. JPY1198 (Cluster 6)	*Rhizobium* sp. JPY940 (Cluster 6)	*Rhizobium etli* sv mimosae Mim-1 (Cluster 5)	*Burkholderia* sp. CCGE1002 (Cluster 1)	*B. phymatum* STM815^T	*C. taiwanensis* LMG19424^T
***M. borealis* (B)**	E	E	E	E	N	N	N[a]	N[a]
***M. dysocarpa* (B)**	E	E	E	E	N	N	N[a]	N[a]
***M. biuncifera* (B)**	nt	nt	nt	E	N	N	N[a]	N[a]
***M. luisana* (B)**	E	–	E	E	N	–	–[a]	–[a]
***M. polyantha* (B)**	–	–	–	E	N	N	N[a]	–[a]
***M. tequilana* (T)**	–	E	N	E	N	N	E	N
M. albida (T)	N	E	E	N	N	N	E[a]	N
M. velloziana (T)	nt	N	nt	–	nt	N	E[a]	N[a]
M. debilis (T)	nt	nt	nt	–	nt	nt	E[a]	N[a]
M. pudica (P)	–	–	–	–	N[b]	N	E[a]	E[a]
***M. affinis* (P)**	E	E	E	E	E	N	E[a]	N
M. orthocarpa (M)	nt	nt	nt	nt	nt	N	E	N

North and Central American spp.	
Widespread and Brazilian spp.	
Effective nodulation	E
Ineffective nodulation	N
No nodulation	–
Not tested	nt

Plants (two to four replicates per species/strain combination) were grown in sterile glass tubes and rooted in sterile vermiculite/perlite (1 : 1) for 6–8 wk after inoculation.
[a]Results obtained by Elliott *et al.* (2007) or dos Reis Junior *et al.* (2010).
[b]Results obtained by Elliott *et al.* (2009).

so uncommon at the sample locations. A dedicated search, particularly of nonendemic *Mimosa* species in the *M. occidentalis*/*M. orthocarpa* clade M of Simon *et al.* (2011), would no doubt reveal greater diversity.

Many species of *Rhizobium* and *Ensifer* are symbionts of endemic Mexican *Mimosa* species

The *Mimosa*-nodulating alphaproteobacteria were divided into five distinct *16S* rRNA-*recA* clusters: one *Ensifer* and four *Rhizobium*. Some individual clusters encompassed several reference species, so they can be regarded as species complexes. There was substantial sequence diversity among the MMS within each cluster, indicating their affiliation to more than one species. *Ensifer* (Cluster 2) has not previously been reported as a *Mimosa* symbiont in the Neotropics, but has been isolated from other mimosoid legumes in central Mexico, such as *E. americanum* from *Acacia* (*s.l.*) spp. (Toledo *et al.*, 2003) and *E. mexicanum* and *E. chiapanecum* from *Acaciella angustissima* (Lloret *et al.*, 2007; Rincón-Rosales *et al.*, 2009). Three *Ensifer* (*Sinorhizobium*) isolates were reported from the USA–Mexican native *M. strigillosa* in Texas (Andam *et al.*, 2007). In addition, *E. mexicanum* strains were isolated from nodules on *M. himalayana*, an Indian species that was used for trap experiments in Brazilian Cerrado soils by Gehlot *et al.* (2013). The *Ensifer* symbionts isolated in the present study fall into at least five species-level clades (Fig. 1), including some that are not closely related to any described species. The three main *Rhizobium* clusters (4, 5 and 6) contained *R. mesoamericanum*, *R. etli*/*R. phaseoli* and *R. tropici*/*R. leucaenae*/*R. calliandrae*, respectively, plus strains not yet given a formal species designation; these species all contain strains already known to nodulate *Mimosa* spp. and/or other mimosoids, such as *Leucaena* and *Calliandra*, but also to nodulate the promiscuous papilionoid legume *P. vulgaris* (Wang *et al.*, 1999; Zurdo-Piñeiro *et al.*, 2004; Elliott *et al.*, 2009; Klonowska *et al.*, 2012; López-López *et al.*, 2012; Mishra *et al.*, 2012; Ribeiro *et al.*, 2012; Rincón-Rosales *et al.*, 2013; Melkonian *et al.*, 2014). The present study has greatly extended the sampling of these clusters to include other closely related strains and possibly new *Mimosa*-nodulating species. Indeed, the type strains of *R. etli*, *R. tropici* and *R. leucaenae*, which are efficient nodulators of *P. vulgaris*, are also capable of nodulating *M. affinis* (albeit ineffectively), and *Mimosa* strains from each of the five Alphaproteobacterial clusters (2–6) can nodulate *P. vulgaris* effectively (this study).

The *nodA* and *nodC* phylogenies of the *Ensifer* and *Rhizobium* symbionts are largely congruent with those of the *16S* rRNA and *recA* genes, indicating that horizontal gene transfer (HGT) has not been common. The depth of the branches in the trees indicates that the common ancestor of these sets of symbiosis genes was ancient. The Mexican *Mimosa* rhizobia situation is, therefore, quite similar to that observed with the *Burkholderia* symbionts of the Brazilian *Mimosa* endemics, as the latter also exhibited very

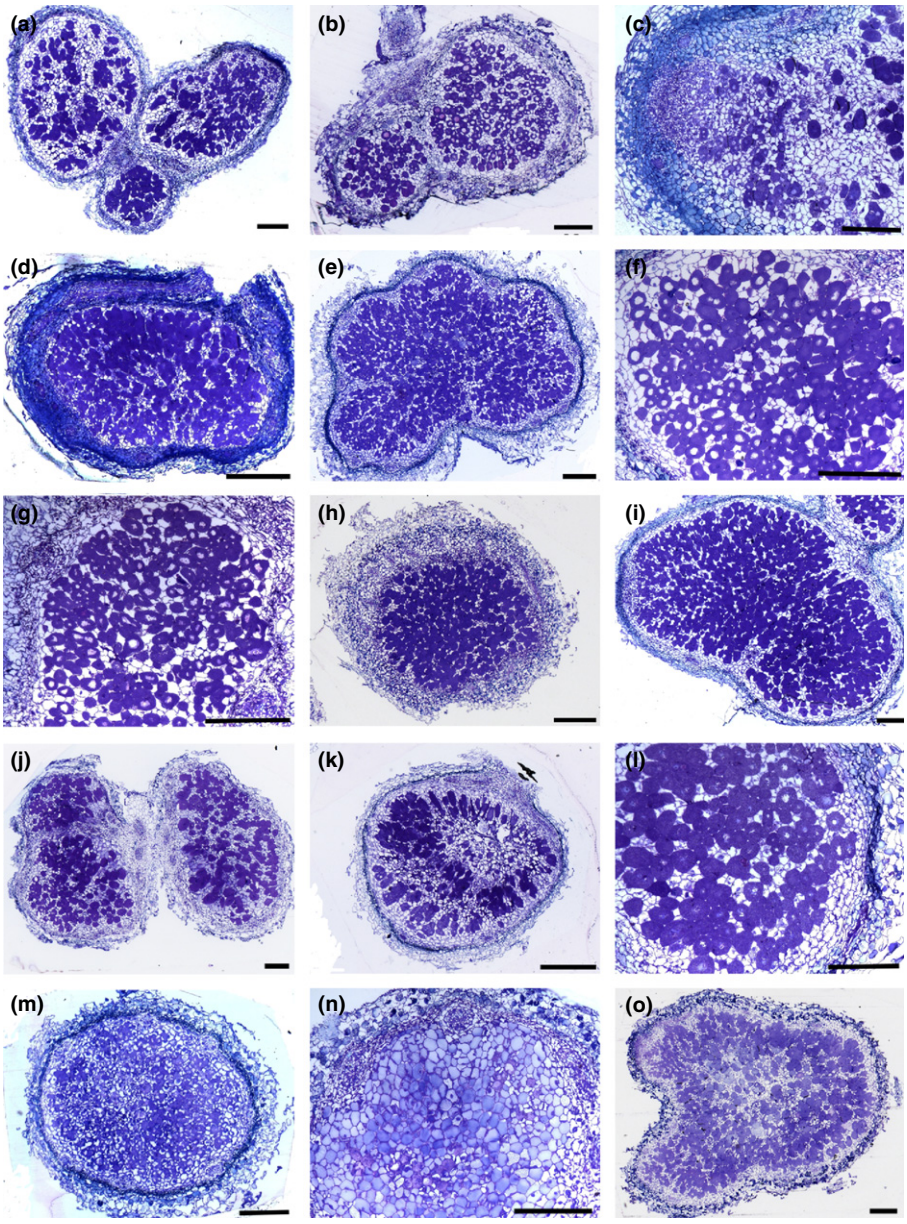


Fig. 5 Light microscopy of semi-thin (1-μm-thick) sections of Mexican *Mimosa* nodules from cross-inoculation experiments. All the nodules were effective, except for those indicated. *Ensifer* sp. JPY1220+ (a) *M. borealis*, (b) *M. luisana* and (c) *M. albida*; *Rhizobium* sp. JPY934+ (d) *M. albida* (ineffective); *Rhizobium* sp. JPY940+ (e) *M. borealis*, (f) *M. dysocarpa*, (g) *M. polyantha* and (h) *M. tequilana*; *Rhizobium* sp. JPY1198+ (i) *M. borealis* and (j) *M. luisana*; *R. etli* sv mimosae Mim-1+ (k) *M. borealis* (ineffective) and (l) *M. aculeaticarpa*; *Burkholderia* sp. CCGE1002+ (m) *M. borealis* (ineffective) and (*n*) *M. polyantha* (ineffective); (o) *B. phymatum* STM815+ *M. orthocarpa*. Bars, 200 μm.

little HGT, and the phylogenies of their housekeeping genes were closely aligned with those of the symbiotic loci (Bontemps *et al.*, 2010). Nevertheless, our phylogenetic studies also showed that some *P. vulgaris* symbionts have genes closely related to those of the MMS, supporting a possible symbiotic overlap which we have confirmed for selected MMS by nodulation tests. It is now clear that the diversity of rhizobia able to nodulate *Mimosa* is much greater than previously thought, but that this diversity is only found in certain rhizobial species and nodulation gene clades, indicating that *Mimosa* nodulation requires some degree of specificity, the basis of which is still unknown.

Mexican *Mimosa* species prefer Alphaproteobacterial symbionts

The widespread Mesoamerican species, *M. affinis*, was found to be a good 'common' host for nodulation tests, as it could nodulate with most of the MMS. Interestingly, very few MMS strains could form nodules on the widespread and pan-tropical species *M. pudica*, whereas this has been a useful common host for testing the symbionts of South American *Mimosa* spp., which are mainly betarhizobia (Bontemps *et al.*, 2010; Mishra *et al.*, 2012). This is not so surprising, in fact, as *M. pudica* has shown only a slight ability to nodulate with alpharhizobia, and the nodules formed are often ineffective or partially effective (Barrett & Parker, 2006; Elliott *et al.*, 2009; Gehlot *et al.*, 2013; Melkonian *et al.*, 2014). Cross-inoculation tests have confirmed that the native southern USA and Mexican *Mimosa* spp. prefer alpha- to betarhizobia, but also that the degree of preference depends on the clade. None of the closely related species in the southern USA–Mexican Clade B could nodulate effectively with promiscuous *Burkholderia* strains (Elliott *et al.*, 2007; this study), but those in the 'mixed' Clade T could. The latter includes *M. albida*, which is a very common species in Central America and Mexico, and *M. tequilana*, which

is endemic to the Tequila municipality in Jalisco; both could nodulate effectively with *B. phymatum* STM815, but in the field they appear to be nodulated exclusively by alpharhizobia. The other species in this clade, such as *M. debilis* and *M. velloziana* are nodulated by *Burkholderia* in Brazil (Bontemps *et al.*, 2010; dos Reis Junior *et al.*, 2010), and cannot nodulate with the promiscuous *Mimosa*-nodulating *Rhizobium* strain JPY940 (this study); in this respect they differ from their cousins *M. albida* and *M. tequilana* in Mexico, which have adapted to nodulate with the local alpharhizobial MMS. A similar situation was recently reported for a native Indian *Mimosa* species (*M. himalayana*), which is related to Brazilian species (Simon *et al.*, 2011); although it nodulates with 'local' *Ensifer* spp. in the field, it can still nodulate with *Burkholderia* (Gehlot *et al.*, 2013).

Alpharhizobia have been isolated from *Mimosa* in previous studies, but have been only a minor part of the symbiont diversity, and they are often ineffective or non-nodulating (Barrett & Parker, 2006; Elliott *et al.*, 2009; Mishra *et al.*, 2012; Melkonian *et al.*, 2014). In central Mexico, however, they are clearly the major part of the rhizobial diversity associated with the genus, at least as it is represented by the 25 species in the present study, and this apparent preference of Mexican species for alpharhizobial symbionts is in almost complete contrast to Brazilian species, where all but two of the 143 symbionts isolated from 47 *Mimosa* spp. were *Burkholderia* (Bontemps *et al.*, 2010). Mexico is second only to Brazil as a centre of diversity of the large and important genus *Mimosa*, and geographical separation of these two diversification centres has most likely affected the type of symbiont selected by the *Mimosa* spp. in each. Differences in soils, such as fertility (e.g. N content) and pH, are important factors in governing how and why *Mimosa* spp. select particular symbionts (Elliott *et al.*, 2009; Liu *et al.*, 2012; Mishra *et al.*, 2012; Gehlot *et al.*, 2013). For example, under N-limited conditions, invasive *Mimosa* spp. overwhelmingly prefer to nodulate with *Burkholderia* rather than *Cupriavidus* or *Rhizobium*, (Elliott *et al.*, 2009; Melkonian *et al.*, 2014), but the predominance of *Burkholderia* can be overcome at higher N concentrations, which demonstrates that soil N-content is an important factor in *Mimosa* symbiont selection (Elliott *et al.*, 2009). Furthermore, soils in central Brazil are generally acid (many are less than pH 5.0; dos Reis Junior *et al.*, 2010), which would favour the acid-tolerant genus *Burkholderia* (Garau *et al.*, 2009; Stopnisek *et al.*, 2014), whereas those from central Mexico are either weakly acidic, neutral or slightly alkaline (Camargo-Ricalde *et al.*, 2010; this study), which would favour most species of *Rhizobium*, and also *Cupriavidus* (Klonowska *et al.*, 2012; Liu *et al.*, 2012; Mishra *et al.*, 2012; Gehlot *et al.*, 2013). Further studies using soils and seeds from both Brazil and Mexico could help to determine if (and what) soil characteristics are factors in the selection of symbionts by *Mimosa* spp. endemic to Brazil and Mexico.

Concluding remarks

Independent evolution following geographic separation of *Mimosa* lineages may help to explain symbiont preferences in the various clades. A possible scenario is that after the ancestors of the main Mexican and Brazilian lineages became separated, their descendants coevolved with the 'local' rhizobial microflora inhabiting the mainly highland soils in which the genus speciated: *Burkholderia* in the case of Brazil and *Rhizobium*/*Ensifer* in the case of Mexico. These old plant lineages, particularly those rich in endemic habitat-specific species, have had time to become increasingly specialized to a particular group of symbionts, and so have eventually lost (or possibly never had) an ability to associate with other types of bacteria. For example, *Burkholderia* is not a symbiont of Clade B, which diverged *c.* 20 Myr ago (Ma), soon after the emergence of the genus *c.* 28 Ma (Simon *et al.*, 2011). However, there have been several subsequent divergences between Mexican and Brazilian *Mimosa* lineages, and many are quite recent (1–6 Ma; Simon *et al.*, 2011). These younger lineages, such as those in the 'mixed' Clade T which diverged 2–6 Ma, have also adopted alpharhizobial symbionts in Mexico, but have retained their ability to nodulate with the *Burkholderia* symbionts of their South American cousins. If the closely related Indian species *M. hamata* and *M. himalayana* are an appropriate example (Gehlot *et al.*, 2013), it might be expected that the ability to nodulate with multiple symbiont types will eventually be lost in the more endemic Mexican species in this clade, such as *M. tequilana*, but retained in the widespread species *M. albida*. Another example is *M. affinis*, the Mesoamerican 'sister' to the widespread *M. pudica*: in the short time since they diverged (*c.* 2 Ma) *M. affinis* has developed a much stronger affinity for Alphaproteobacteria than its Betaprotobacteria-loving sister (Elliott *et al.*, 2009; Melkonian *et al.*, 2014; this study). In summary, although plant symbiotic preference can evolve relatively rapidly following the colonization of a new area/continent (e.g. a shift from beta to alpha preference in Mexican mimosas that recently diverged from South American lineages), there appears to be a trend towards symbiotic specialization, particularly in endemic plant lineages confined to specific habitats.

Acknowledgements

We thank the Millenium Seedbank, Kew, UK for supplying the seeds of several of the *Mimosa* spp. used in this study, Colin Hughes and Pete Iannetta for discussions, and Ivonne Toledo for technical help. This work was funded by the Natural Environment Research Council (NE/B505046/1 and NE/B505038/1) and the Carnegie Trust for the Universities of Scotland. E.M-R. was funded by grant IN207615 from UNAM.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Notes S1 Sampling locations with *Mimosa* herbarium voucher numbers in KML format for visualization in Google Earth.

Fig. S1 Mexican *Mimosa* species growing *in situ*.

Fig. S2 Light microscopy of semi-thin sections of Mexican *Mimosa* nodules collected in the field.

Fig. S3 Phylogenetic relationships of strains isolated from *Mimosa* nodules in this study and reference strains based on 737 bp 16S rRNA and 337 bp *recA* sequences.

Table S1 Plant voucher and bacterial accession numbers, together with location details of Mexican *Mimosa* spp.

Table S2 Characteristics of rhizosphere soil from *Mimosa* species collected in Mexico

Table S3 Primers (Oligonucleotides) which were used for PCR and sequencing

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DISCUSIÓN GENERAL

El genoma de *Rhizobium etli* simbiovar mimosae Mim1

El ensamble del genoma de *R. etli* sv. mimosae Mim1 generó 7 replicones circulares: un cromosoma y seis replicones extracromosomales con una cobertura de 150X. El cromosoma tuvo un tamaño de 4.8 megabases (Mb), mientras que los replicones extracromosomales oscilaron en un rango de 181 kilobases (Kb) a 1.08 Mb. La cantidad y tamaño de los replicones extracromosomales se pudo corroborar mediante la técnica de Eckhardt (Eckhardt, 1978; Hynes et al., 1985) (Fig. 3); aunque en la actualidad, con el avance tecnológico de las plataformas de secuenciación y el desarrollo de programas para su ensamble, se pueden obtener ensambles precisos de los genomas bacterianos.

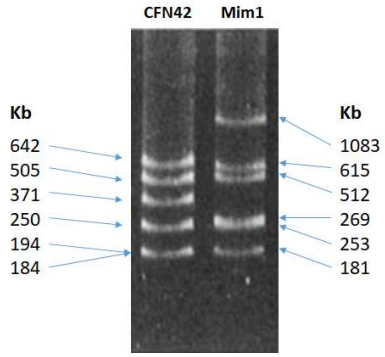

Fig. 3 Perfil de los replicones extracromosomales de los simbiovares phaseoli (CFN42) y mimosae (Mim1). Los tamaños de los replicones expresados en Kb se obtuvieron de los ensambles genómicos (González et al., 2006; Rogel et al., 2014).

En un análisis del genoma completo, el porcentaje de Identidad Nucleotídica Promedio (ANI por sus siglas en ingles) y el porcentaje del ADN conservado entre la Mim1 y la CFN42 fue de 98.6% y 82.4% respectivamente, lo que confirma que ambas cepas

pertenecen a la misma especie. Se encontraron más del doble de genes únicos en la Mim1 que en la CFN42, principalmente en plásmidos, lo que se puede explicar por la diferencia en los tamaños de sus plásmidos. En cada uno de sus cromosomas, se encontraron alrededor de 260 genes únicos respectivamente y 35 islas genómicas en la Mim1, mientas que en la CFN42 solo se encontraron 17. Ejemplos de genes únicos que se encuentran en la Mim1 y no en la CFN42, son los que codifican para citocromo oxidasas, algunas chaperoninas, transportadores de dipéptidos, lactato deshidrogenasa, una PHB depolimerasa, una proteína similar a la ferritina, genes de biosíntesis de exopolisacáridos, un sistema de secreción tipo VI y genes para la biosíntesis de menaquinona, así como muchos otros genes hipotéticos.

El genoma conservado entre los dos simbiovares Mim1 y CFN42, no solo incluye el cromosoma sino dos replicones extracromosomales (pRetMim1a-pRetCFN42b y pRetMim1d-pRetCFN42e) que han sido designados como crómidos en la CFN42 (Harrison et al., 2010) y un plásmido (pRetMim1b-pRetCFN42c); todos ellos conservan la misma sintenia. Cada uno de los crómidos tuvo menos de 20 genes únicos y un ANI alrededor del 99%. Los replicones pRetMim1c y pRetCFN42a están parcialmente conservados y entre sus plásmidos simbióticos se encontraron grandes diferencias.

Todos los replicones extracromosomales de la Mim1 pertenecen a la familia de plásmidos *repABC* (Ceballos et al., 2008). Las proteínas que codifican los operones *repABC* de los tres pares de replicones homólogos entre ambas cepas tuvieron un porcentaje de identidad por arriba del 97.51%, sugiriendo fuertemente que los miembros de cada par de replicones pertenecen al mismo grupo de incompatibilidad.

Comparación de los plásmidos pRetMim1f y pRetCFN42f

El replicón más grande de la cepa Mim1 (pRetMim1f) está parcialmente conservado con el supuesto crómido de la CFN42 (pRetCFN42f); ambos replicones tienen dos operones *repABC* (*repABC*1 y *repABC*2) pero la identidad entre los dos operones dentro de la misma cepa (Mim1 o CFN42) es muy baja; sin embargo, el alto grado de identidad de los *repABC* correspondientes entre ambas cepas, sugiere que ambos plásmidos comparten el mismo grupo de incompatibilidad y origen evolutivo. Solo el 51% del replicón pRetMim1f es conservado en la CFN42, mientras que el 86% del pRetCFN42f fue encontrado en el pRetMim1f.

Los genes del sistema de secreción tipo VI fueron encontrados solo en el megaplásmido de la Mim1 (pRetMim1f); este sistema de secreción permite que las bacterias trasladen proteínas efectoras a otras bacterias o células eucariotas (Salinero-Lanzarote et al., 2019). Se demostró que mutantes en el sistema de secreción tipo VI tienen disminuida la nodulación, lo que indica que este sistema tiene un rol positivo en la simbiosis (Salinero-Lanzarote et al., 2019), aunque no todos los rizobios lo poseen.

Una duplicación del gene de citrato sintasa, fue encontrado en el pRetMim1f de la Mim1 al igual que en el pSim del sv. tropici. Este gene es requerido por el sv. tropici para desarrollar un numero normal de nódulos, además de que es regulado diferencialmente al gene cromosomal (Pardo et al., 1994; Hernández-Lucas et al., 1995). El gene de citrato sintasa del pRetMim1f de la Mim1 presenta una secuencia nueva con apenas el 55% de identidad con la copia plasmídica del mismo gene de la cepa de *R. gallicum* R602. Recientemente una comparación genómica de las islas de simbiosis de distintas especies de *Bradyrhizobium* aisladas de *Phaseolus lunatus* mostró que entre los genes conservados se encontraba el que codifica para citrato sintasa (datos no publicados).

Los genes *raiI* y *raiR* se encontraron en las regiones conservadas de los pRetMim1f y pRetCFN42f de ambos simbiovares. RaiI produce homoserine lactonas y RaiR es el regulador transcripcional. El sistema *rai* en *R. phaseoli* sv. phaseoli CNPAF512 afecta el número de nódulos pero no la fijación de nitrógeno (Rosemeyer et al., 1998), además este sistema controla el crecimiento en *R. phaseoli* (Daniels et al., 2002), esto podría explicar su conservación en ambos simbiovares.

En un análisis transcriptómico de *R. phaseoli* Ch24-10 en la rizosfera de frijol y maíz, se observó que más del 50% de los genes de un replicón extracromosomal de la Ch24-10 equivalente al pRetMim1f se expresaban en el frijol pero no en el maíz (López-Guerrero et al., 2012), lo que sugiere que los genes de este replicón en la Mim1 están involucrados específicamente en la interacción con la planta.

Comparación de plásmidos simbióticos

Alrededor del 10 al 15% de los plásmidos simbióticos de la Mim1 y la CFN42 tienen regiones sinténicas conservadas con un ANI del 89.2%. Las diferencias en cuanto a los genes simbióticos entre la CFN42 y la Mim1 se encuentran en la tabla 2.

Tabla2.- Diferencias relevantes en sus plásmidos simbióticos entre *R. etli* sv. mimosae Mim1 y *R. etli* sv. phaseoli CFN42, (Tabla extraída de Rogel et al., 2014).

Gene*	symbiovar	
	mimosae	phaseoli
nodHPQ	+	-
nodZ	-	+
nolL	-	+
nodO	+	-
nolO	-	+
nodU	+	-
fixKL	+	-
fxkR	+	-
acdS	+	-
teu	-	+
hup-hyp	+	-

*Las funciones de cada uno de los genes son explicadas en el texto.

El factor Nod de *R. etli* sv. phaseoli CFN42 es un pentámero de *N*-acetilglucosamina con una acetil fucosa en el extremo reductor y grupos metilo y carbamoilo en el extremo no reductor (Poupot et al., 1995). El gene *nodZ* involucrado en la fucosilación del factor Nod estuvo ausente en la Mim1; sin embargo, en un estudio de expresión heteróloga en *Azorhizobium caulinodans* se demostró que los factores Nod fucosilados fueron los más adecuados para inducir la nodulación en *P. vulgaris*, pero al parecer no es un determinante ya que *P. vulgaris* al ser una planta promiscua reconoce un amplio espectro de factores Nod (Laeremans et al., 1999). Los genes *nodHPQ* que modifican al factor Nod con un sulfato estuvieron presentes en la Mim1, dichos genes se encuentran presentes en cepas del sv. tropici que son también simbiontes de *Leucaena* (Ormeño-Orrillo et al., 2012). Los genes que codifican para carbamoil transferesas y que modifican al factor Nod se encontraron en ambos simbiovares Mim1 y CFN42, pero la posición de los grupos carbamoil en los factores Nod podría diferir dado que son genes diferentes, *nolO* en la CFN42 y *nodU* en la Mim1. La

carbamoilación en la posición C-6 introducida por NodU podría promover la nodulación de *Leucaena* (Waelkens et al., 1995; Perret et al., 2000). Tanto la Mim1 como la CFN42 tienen *nodS* en el psim, involucrado en la metilación del extremo no reductor; una decoración en el factor Nod que es esencial para la nodulación de frijol y *Leucaena* en *R. tropici* CIAT899 (Waelkens et al., 1995). Los estudios de expresión heteróloga del gene *nodO* en diferentes rizobios, mostraron que puede mejorar la nodulación en *L. leucocephala*, además de extender el rango de hospedero (van Rhijn et al., 1996; Vlassak et al., 1998), dicho gene se encontró solamente en la Mim1 lo que podría ayudar a entender el amplio rango de hospedero de esta cepa. Diferentes NodDs en los simbiovares mimosae y phaseoli podrían reflejar sus afinidades por diferentes flavonoides exudados por las diferentes plantas hospederas.

El gene *acdS* que codifica para la ACC-deaminasa se encontró solamente en el psim de la Mim1 y no en la CFN42. La ACC-deaminasa decrementa la cantidad de ACC que es el precursor del etileno, el cual puede disminuir el número de nódulos. Una ACC-deaminasa heteróloga en *Rhizobium* sp. TAL1145 mejoró la nodulación en *Leucaena* (Tittabutr et al., 2008).

Los genes *fixGHIS-fixNOQP* requeridos para la biosíntesis de una oxidase simbiótica terminal, están presentes en los plásmidos simbióticos de ambos simbiovares Mim1 y CFN42; además, están reiterados en sus plásmidos pRetMim1f y pRetCFN42f respectivamente. Los genes *fixK* y *fixL* que regulan a los genes *fixGHIS-fixNOQP* se encuentran adyacentes a estos genes en la Mim1, sin embargo en la CFN42 solo se encontró un pseudogene *fixK* en su psim. El porcentaje de identidad de los genes reiterados *fixGHIS-fixNOQP* en la Mim1 es mayor que en la CFN42 con un 95 y 87% respectivamente.

Los genes *teu* involucrados en la captación de exudados de *P. vulgaris* (Rosenblueth et al., 1998) no se encontraron en la Mim1, mientras que en la CFN42 y *R. phaseoli* CIAT652

sí; mutantes en estos genes reducen la competitividad de nodulación en *P. vulgaris* (Rosenblueth et al., 1998).

Los genes del sistema de secreción tipo III (T3SS por sus siglas en ingles) en los plásmidos simbióticos de ambos simbiovares Mim1 y CFN42 son muy distantes; estas diferencias podrían contribuir a la disparidad en el rango de hospedero, si se considera la función del T3SS en la especificidad de los rizobios (Fauvart et al., 2008).

Un grupo de genes *hup-hyp* que codifican para los componentes de una hydrogenasa de captura (Hup) se encontró en el psim de la Mim1 pero no en la CFN42; su identidad fue por arriba del 70% con cepas de *R. leguminosarum* y *R. tropici* (Baginsky et al., 2002). *R. tropici* CIAT899 carece de varios genes *hup* y muestra un fenotipo Hup menos. En la Mim1 se encuentran prácticamente todos los genes *hup*, menos el *hupE* que codifica para un transportador de níquel, un metal requerido para la función Hup (Brito et al., 2010), sin embargo existen otros genes que codifican para transportadores de níquel en el psim de la Mim1, por lo que el sistema Hup podría ser funcional. Los genes del origen de replicación *repABC* de los plásmidos simbióticos de ambos simbiovares, no están filogenéticamente relacionados.

El análisis de secuencias de inserción (IS por sus siglas en ingles) en *R. etli* CFN42 sugirió que el plásmidos simbiótico no compartió significativamente secuencias IS con el resto del genoma, esto se interpretó como evidencia de una reciente adquisición en esta cepa (Lozano et al., 2010). El análisis de IS en la Mim1 indicó que el psim tiene un amplio número de secuencias de inserción, algunas de ellas compartidas con el cromosoma, pRetMim1f y pRetMim1b, esto podría indicar que el psim del sv. mimosae tiene una historia más antigua en el fondo genético de *R. etli* que el de phaseoli.

Se encontraron algunas similitudes entre los diferentes replicones de la Mim1; el pRetMim1e (psim) tiene secuencias similares al pRetMim1f y al pRetMim1c, estas similitudes del psim de la Mim1 con el supuesto crómido pRetMim1f, podrían apoyar la hipótesis de que el psim del sv. mimosae es ancestral en *R. etli*. En contraste con el psim de la CFN42 que solo comparte algunas secuencias con el pRetCFN42a (González et al., 2010).

Los genes de interacción con la planta no se encuentran en el plásmido simbiótico

Algunos genes involucrados en la interacción con la planta se encontraron conservados en ambos simbiovares, tales como los que codifican para bombas de extrusión Rmr que podrían estar involucradas en la eliminación de fitoalexinas producidas por la planta. Las mutantes en estos genes redujeron la nodulación en la CFN42 (González-Pasayo, et al., 2000). Estos genes están codificados en el crómido pRetCFN42b de la CFN42 y en el replicón correspondiente de la Mim1 (pRetMim1a), con porcentajes de identidad del 97% para *rmrA* y 98% para *rmrB* y *rmrR* de ambos simbiovares (CFN42 y Mim1). Se reportó que genes homólogos se expresan en la rizósfera de diferentes plantas (Ramachandran et al., 2011).

Aun cuando el sv. mimosae es capaz de nodular a *Leucaena*, no se encontraron los genes *mid* o *pyd* que están involucrados en el catabolismo del aminoácido tóxico mimosina que se encuentra en estas plantas. Estos genes fueron descritos en *Rhizobium* sp. TAL1145 (relacionado a *R. tropici*) que fue aislado de plantas de *Leucaena* (Borthakur y Soedarjo, 1999).

El simbiovar phaseoli prevalece en los nódulos de diferentes bacterias que nodulan plantas de *Phaseolus vulgaris*

R. etli sv. mimosae tiene un rango de hospedero más amplio que el sv. phaseoli. En particular las plantas de *L. leucocephala* sirven como planta hospedera para distinguir al simbiovar mimosae. Treinta y seis cepas aisladas de nódulos de *P. vulgaris* (que se agruparon como *R. etli* por secuencia del 16S rRNA) obtenidas del bosque lluvioso los Tuxtlas en Veracruz-México, correspondieron al sv. phaseoli con base en la organización de sus genes *nodA* (Ormeño-Orrillo et al., 2012) y por su incapacidad de nodular plantas de *Leucaena*. Diferentes bacterias que están clasificadas como *R. phaseoli* (CIAT652, Ch24-10, CNPAF512, 8C-3 y Brasil5) (López-Guerrero et al., 2012) y otras que han sido descritas como nuevos linajes (Kim5, GR56 y CIAT894) (Ribeiro et al., 2013), correspondieron al sv. phaseoli en base al análisis de sus genomas. Considerando que la mayoría de los aislados de nódulos de *P. vulgaris* correspondieron al sv. phaseoli, podemos concluir que este simbiovar está mejor adaptado a su hospedero; por lo tanto, phaseoli parece ser un simbiovar especializado con un rango estrecho de hospedero que no incluye a las plantas de mimosa. El sv. phaseoli se encuentra en varias especies de *Rhizobium* o linajes como *R. gallicum*, *R. giardinii*, *R. phaseoli*, *R. etli* y *Rhizobium* sp. que corresponden al linaje PEL1 (phaseoli-etli-leguminosarum) (Ribeiro et al., 2013). La distribución de este simbiovar puede estar en relación a la distribución histórica de su hospedero en el mundo y la transferibilidad del psim que pareciera ser un plásmido epidémico. Además de tener el sv. phaseoli, las especies de *Rhizobium* mencionadas anteriormente, tienen simbiovares adicionales más generalistas, como gallicum, giardinii o mimosae. Teniendo alternativas simbiovares con diferentes rangos de hospedero, puede ser una ventaja para los rizobios, que les puede ampliar sus nichos de leguminosas y evitar el dilema de ser especialista-generalista.

CONCLUSIONES

El término simbiovar es validado con el análisis genómico que mostró que en un fondo genómico común, se pueden albergar diferentes plásmidos simbióticos que determinan la especificidad por el hospedero. Sin embargo, además de las diferencias en los plásmidos simbióticos, hubo también diferencias en sus cromosomas y en otros replicones extracromosomales de las dos cepas analizadas CFN42 y Mim1. En la Mim1, los factores Nod con modificaciones de sulfato, sistemas de secreción o ACC-deaminasa podrían ayudar a explicar el amplio rango de hospedero del simbiovar mimosae. En la CFN42, los genes *teu* que participan en la captación de exudados y genes involucrados en la fucosilación del factor Nod (*NodZ*), podrían contribuir a la especialización del hospedero. La discusión de que el sv. mimosae es más antiguo que el phaseoli, podría aplicar también para gallicum y giardinii, por lo que proponemos que gallicum y giardinii son más antiguos que phaseoli.

La secuencia del genoma completo del sv. mimosae Mim1 nos mostró que tiene una estructura multipartita representada por un cromosoma y 6 replicones extracromosomales, que por comparación con el sv. phaseoli CFN42 dos ellos podrían ser crómidos. También se encontró que el pRetMim1c es un plásmido único en la Mim1.

Los datos obtenidos nos muestran que el sv. mimosae generalista podría ser más antiguo que el sv. phaseoli especialista.

Dado que el sv. mimosae tiene la capacidad de nodular tanto a plantas de *Phaseolus vulgaris* como de *Leucaena leucocephala*, se pudo corroborar que los nódulos son una característica propia de la planta ya que las primeras producen nódulos determinados, mientras que las segundas nódulos indeterminados.

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