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ORIGINAL ARTICLE

Burkholderia species associated with legumes of Chiapas, Mexico, exhibit stress tolerance and growth in aromatic compounds

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KEYWORDS
Burkholderia;
Mimosa;
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Stress

Abstract Leguminous plants have received special interest for the diversity of β-proteobacteria in their nodules and are promising candidates for biotechnological applications. In this study, 15 bacterial strains were isolated from the nodules of the following legumes: *Indigofera thibaudiana*, *Mimosa diplotricha*, *Mimosa albida*, *Mimosa pigra*, and *Mimosa pudica*, collected in 9 areas of Chiapas, Mexico. The strains were grouped into four profiles of genomic fingerprints through BOX-PCR and identified based on their morphology, API 20NE biochemical tests, sequencing of the *16S rRNA*, *nifH* and *nodC* genes as bacteria of the *Burkholderia* genus, genetically related to *Burkholderia phenoliruptrix*, *Burkholderia phymatum*, *Burkholderia sabiae*, and *Burkholderia tuberum*. The *Burkholderia* strains were grown under stress conditions with 4% NaCl, 45 °C, and benzene presence at 0.1% as the sole carbon source. This is the first report on the isolation of these nodulating species of the *Burkholderia* genus in legumes in Mexico.

PALABRAS CLAVE
Burkholderia;
Mimosa;
Benceno;
Estrés

Especies de *Burkholderia* asociadas a leguminosas de Chiapas, México exhiben tolerancia a estrés y crecimiento en compuestos aromáticos

Resumen Las plantas leguminosas han recibido especial interés por la diversidad de β-proteobacteria que albergan en sus nódulos; algunas de estas bacterias son candidatas prometedoras para aplicaciones biotecnológicas. En el presente trabajo se aislaron 15 cepas

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bacterianas de los nódulos de las leguminosas *Indigofera thibaudiana, Mimosa diplotricha, Mimosa albida, Mimosa pigra* y *Mimosa púdica*, colectadas en 9 áreas de Chiapas, México. Las cepas fueron agrupadas en 4 perfiles de huellas genómicas por BOX-PCR e identificadas sobre la base de su morfología, pruebas bioquímicas API 20NE y secuenciación de los genes *16S ARNr*, *nifH* y *nodC* como bacterias del género *Burkholderia* relacionadas genéticamente con *Burkholderia phenoliruptrix, Burkholderia phymatum, Burkholderia sabiae* y *Burkholderia tuberum*. Las cepas de *Burkholderia* crecieron en condiciones de estrés con NaCl al 4%, a una temperatura de 45 °C y en presencia de benceno al 0,1% como única fuente de carbono. Este es el primer reporte del aislamiento de especies de *Burkholderia* nodulantes en leguminosas en México.

Introduction

Genetic diversity is reflected by the existence of multiple alleles in a population, it is a necessary requirement for individuals to evolve and adapt to new conditions, ensuring the preservation of the species over time. The information on the distribution of genetic diversity has been recognized as a useful tool for the efficient design of practices for the preservation of genetic heritage. *Burkholderia*, *Cupriavidus*, and *Rhizobium* genera of bacteria are promising candidates for biotechnological applications. Unfortunately, many of the species of the *Burkholderia* and *Cupriavidus* genera are associated with human infections, making their applications difficult[5,20]. New species of diazotrophic *Burkholderia* have been discovered, being phylogenetically distant from the *Burkholderia cepacia* complex (Bcc). Their environmental distribution and relevant features for agrobiotechnology applications are less known[9,33]. These genetically distinct species are grouped into a complex called non-pathogenic *Burkholderia*, which are atmospheric nitrogen fixers[28]. The presence of nitrogen-fixing *Burkholderia* species in the rhizosphere and rhizoplane of tomato plants grown in Mexico has revealed a high degree of diversity of diazotrophic *Burkholderia* including *Burkholderia unamae*, *Burkholderia xenovorans*, and *Burkholderia tropica*, two of which are undescribed species, and one is phylogenetically related to *Burkholderia kururiensis*[5,11]. Biological Nitrogen Fixation (BNF) or other bacterial activity that takes place in the inner tissues of plants suggests that products synthesized by bacteria may be released directly into the plant, influencing its metabolism, physiology, and development[21,26]. Until 2001, the bacteria involved in symbiotic nodules of legume plants were reported to be restricted to the α-proteobacteria genera (*Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, and *Azorhizobium*); however, Moulin et al.[18] reported that β-proteobacteria belonging to the *Burkholderia* genus form nodules on legumes in Africa and South America. Chen et al.[6] reported *Ralstonia taiwanensis* as a symbiont of *Mimosa pudica* in Taiwan; being this species later transferred to the *Cupriavidus* genus. Leguminous plants have received special interest in several countries because of their symbiotic diversity and adaptability to different soil types, which allows the microbiota associated with nodules to play an important role in nutrition and development[1,4,8,22]. However, there are few studies on the isolation of bacteria of the *Burkholderia* genus in legumes in Mexico. Therefore, the aim of this study is to identify *Burkholderia* nodulating wild legumes in Chiapas, Mexico.

Materials and methods

Sample collection

Root nodules were collected from the following leguminous plants: *Indigofera thibaudiana*, *Mimosa diplotricha*, *Mimosa albida*, *Mimosa pigra*, and *M. pudica* in different geographical sites of Chiapas, Mexico. Soil pH was measured by adding 10 g of soil in 100 ml of distilled water while stirring for 30 min[2].

Bacterial strains and culture condition

The nodules collected were washed and immersed in 70% ethanol for 5 min, then they were disinfected with sodium hypochlorite at 25% for 15 min. The excess hypochlorite was removed by rinsing with sterile distilled water. Finally, the disinfected nodules were macerated and resuspended in a solution of $MgSO_4 \cdot 7H_2O$ at 10 mM[32]. Then, 100 μl of the suspension was inoculated into Burkholderia Azelaic citrullina (BAc) agar culture media and Yeast extract Mannitol Agar (YMA) culture media. The inoculated Petri dishes were incubated at 29 °C for 2 days and colonies with different morphology were then selected.

Gram staining

Gram-staining reaction was carried out by using a loopful of pure culture grown on Tryptone agar, which was then stained using the standard Gram staining procedure[27].

Box-Pcr

The BOX element (BOXA1) was amplified using the BOXA1R primer. Cycling conditions for BOX-PCR were as follows: 95 °C for 5 min and then 35 cycles of 95 °C for 1 min, 63 °C for 1 min and 72 °C for 3 min, and a final elongation cycle for 10 min at 72 °C. PCR and electrophoresis conditions were according to Estrada-de los Santos et al.[10]

16S rRNA, *nifH* and *nodC* gene sequencing

One strain of each group of the BOX-PCR profile was selected for its identification. The DNA extraction of the selected isolates was performed with the ZR Fungal/Bacterial DNA Miniprep™ kit. Subsequently, the *16S rRNA* gene was amplified by PCR using fD1 and rD1 oligonucleotides[31]. The *nifH* and *nodC* genes were amplified in the isolated strains by PCR using different oligonucleotides[3,15]. The PCR products from *16S rRNA*, *nifH* and nodC genes were cloned and the sequences were determined by the Institute of Biotechnology of the UNAM. The phylogenetic trees, based on *16S rRNA*, *nodC*, and *nifH* genes were constructed by the neighbor-joining method[14] using the Tamura-Nei model in Mega software version 6[29]. Multiple alignments of the sequences were performed using CLUSTALW software[30], based on 1400 nucleotide sites for the *16S rRNA* gene, 316 nucleotide sites for the *nifH* gene and 600 nucleotide sites for the *nodC* gene.

Biochemical characterization

The isolated strains were analyzed using the API 20NE microtest systematic gallery (bioMérieux) to identify their metabolic characteristics.

Saline and thermal stress tests

The strains identified as *Burkholderia* were grown in culture media containing 2 and 4% NaCl. For the thermal stress test, the strains were incubated at temperatures of 37 and 45 °C for 4 days[7].

Aromatic compound growth

The strains identified as *Burkholderia* were grown in salts-ammonium-aromatic compound (SAAC) culture media containing phenol and benzene at 0.1% as sole carbon source[5].

Nucleotide sequence accession numbers

The obtained 1400-bp portion of the *16S rRNA* gene sequences in this study was deposited in GenBank under accession numbers KY569371, KY569372, KY569373 and KY569374. The *nifH* sequences were deposited under accession numbers KY574497, KY574498, KY574499 and KY574500, and the *nodC* sequences under accession numbers KY574501, KY574502 and KY574503.

Results

Isolation

All the plants examined had nodules on their roots, and fifteen strains were obtained; isolates were selected from the highest tenfold serial dilutions using the predominance of the morphological colony types (Table 1). The strains were characterized as gram- negative, oxidase-negative, and catalase-positive bacilli.

Box-PCR

The genetic diversity of the isolates was further analyzed by BOX-PCR, amplification products yielded complex genomic fingerprints consisting of fragments ranging in size from 100 to 1000 bp. A binary matrix was constructed with the BOXA1R profiles of the analyzed strains; they were divided into 4 groups based on the UPGMA method using Jaccard's coefficient with a cut of 70% (Fig. 1).

Sequencing of the 16S Ribosomal RNA

The sequences obtained from each of the strains were compared against the GenBank nucleotide database. The four strains were genetically related to the *Burkholderia* genus. The analysis placed YP50.2 (KY569371) strain close to *Burkholderia phymatum* STM815 (99%, NR074668), BP52.3 (KY569374) strain to *Burkholderia sabiae* (99%, NR043180), while the BP15.1 (KY569373) and BP10.4 (KY569372) strains were placed close to *Burkholderia phenoliruptrix* (99%, AY435213). The genetic relatedness of the isolated species strains with reported nodulating *Burkholderia* spp. can be seen in the phylogenetic tree constructed using the neighbor-joining method[23], based on 1400 nucleotides of the 16S ribosomal gene sequences according to the distance matrix developed by Jukes and Cantor[14] (Fig. 2).

Table 1 Isolated strains from different leguminous plants in the state of Chiapas, Mexico

Code strains	Leguminous plant	Locality	Soil pH
BP10.4, YP8.7, BP6.2, BP10.3, YP11.1, YP7.5, YP8.1	*Mimosa pigra*	Tapachula	5.3–8.7
BP20.1, BP15.1, BP20.2, YP13.2	*Mimosa pigra, Mimosa diplotricha*	Mazatán	5.4–7.4
BP52.3, YP50.2	*Mimosa albida, Indigofera thibaudiana*	Tonalá	7.0–7.8
BP25.2	*Mimosa pudica*	Huehuetán	5.2–7.1
BP34.1	*Indigofera thibaudiana*	Huixtla	7.5–8.2

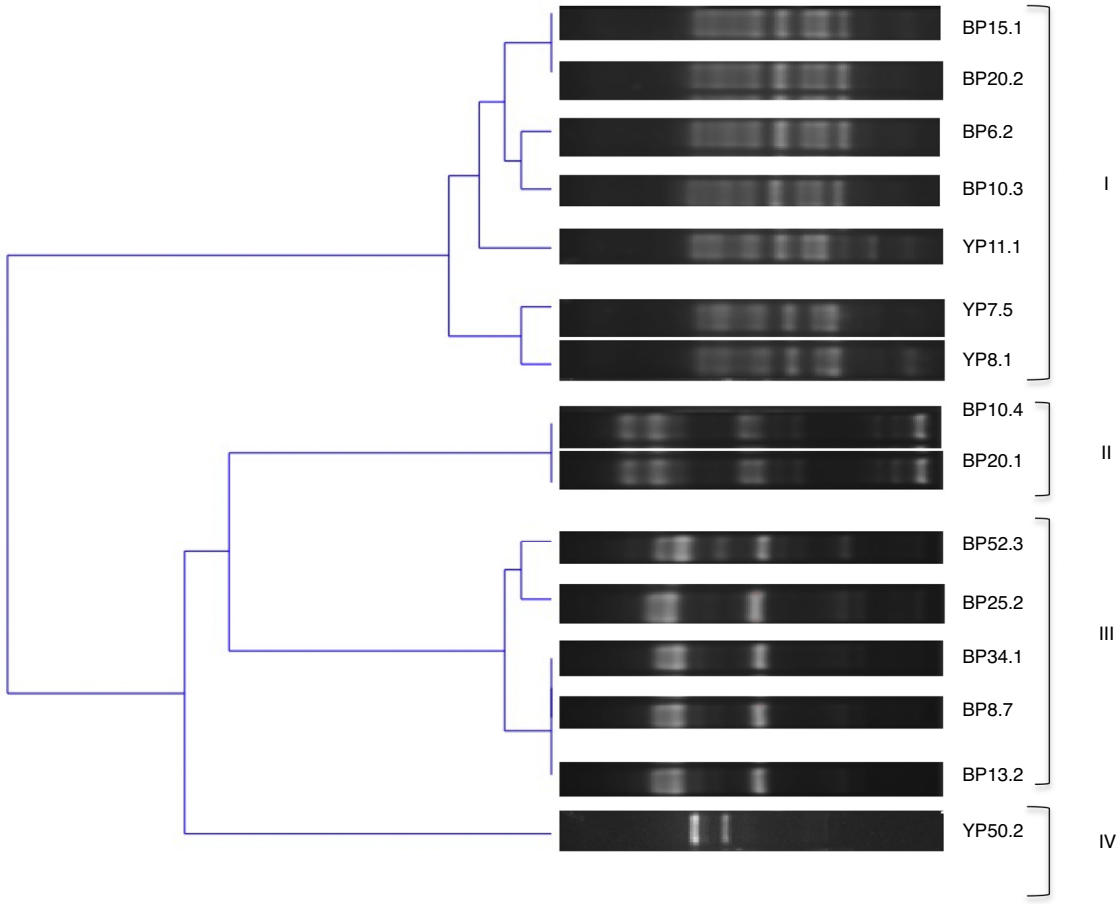

Figure 1 Dendrogram obtained from BOX-PCR profiles using BOXAR1 oligonucleotide of bacteria isolated from nodules of leguminous plants.

Analysis of genes *nifH* and *nod*C

Four strains of *Burkholderia* spp. were chosen for the sequencing of their *nifH* and *nodC* genes based upon their different lineages; partial *nifH* gene sequences encoding dinitrogenase reductase, a key enzyme in N_2 fixation, were determined, and the phylogenies of the obtained sequences were compared with the *nifH* sequences in the databases (Fig. 3). The analysis placed BP52.3 (KY574498), YP50.2 (KY574500) and BP10.4 (KY574497) strains close to *B. phymatum* STM815 (with 90–97% similarity, NR074668) and BP15.1 (KY574499) strain close to *Burkholderia* sp. STM (93%, FN544053). The phylogenetic analysis of the partial *nodC* gene sequences of BP52.3 (KY574502) and YP50.2 (KY574503) strains placed them close to *B. phymatum* STM815 (99%, NR074668) and the BP10.4 strain to *B. phenoliruptrix* (99%, AY435213) (Fig. 4). The genetic relationship of the isolated *Burkholderia* species with nodulating *Burkholderia* spp. strains described above can be seen in the phylogenetic tree constructed using the neighbor-joining method[23], based on 316 nucleotides of the *nifH* gene sequences and 600 nucleotides of the *nodC* gene, according to the distance matrix proposed by Jukes and Cantor[14].

Biochemical characterization

The four selected strains exhibited different metabolic characteristics in the use of carbon sources (Table 2).

Abiotic stress and aromatic compound growth

The strain *Burkholderia* spp. BP15.1, which is closely related to *B. phenoliruptrix*, grew in culture media containing 2 and 4% NaCl, and also at temperatures of 37 °C and 45 °C respectively. Nonetheless, the other 6 strains belonging to the same group did not exhibit the same behavior in the stress tests, which is contrary to the degradation of benzene (Table 3). The strain *Burkholderia* spp. YP50.2, which is closely related to *Burkholderia caribensis*, did not grow in high salinity and temperature conditions; however, it did use benzene as a carbon source for growth, while The strain *Burkholderia* sp. BP52.3, which is closely related to *B. sabiae*, did not grow under stress conditions at 45 °C; it only used benzene as a carbon source, while *Burkholderia* spp. BP10.4 metabolically behaved much like *Burkholderia* spp. BP52.3 (Table 3).

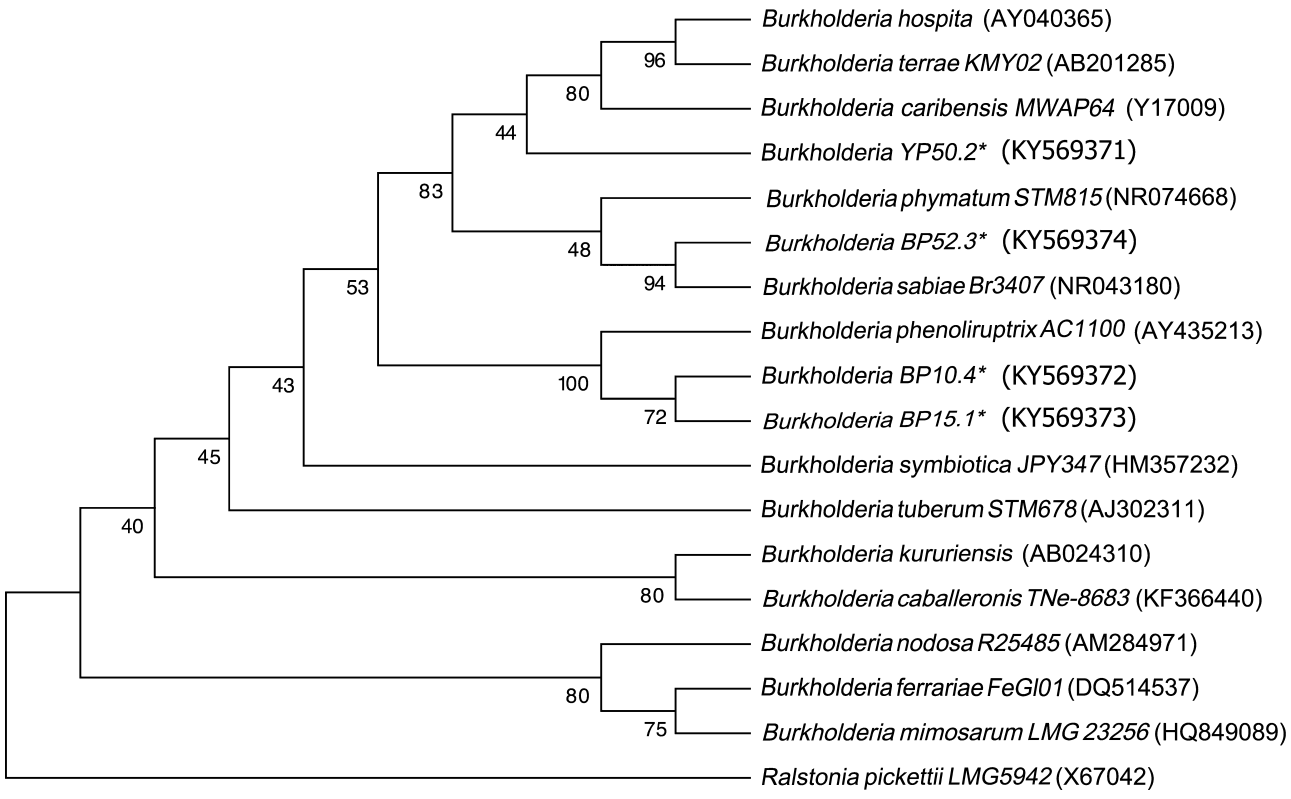

Figure 2 Phylogenetic tree based on *16S rRNA* gene sequences, showing the relatedness among the nodulating *Burkholderia* species. The bar represents 1 nucleotide substitution per 100 nucleotides. Nodal robustness of the tree was assessed using 1000 bootstrap replicates. The NCBI GenBank accession number for each strain type tested is shown in parentheses. Phylogenetic relationship of the *16S rRNA* gene sequence of isolates of *Burkholderia phenoliruptrix*, *Burkholderia caribensis* and *Burkholderia sabiae* in leguminous plants.

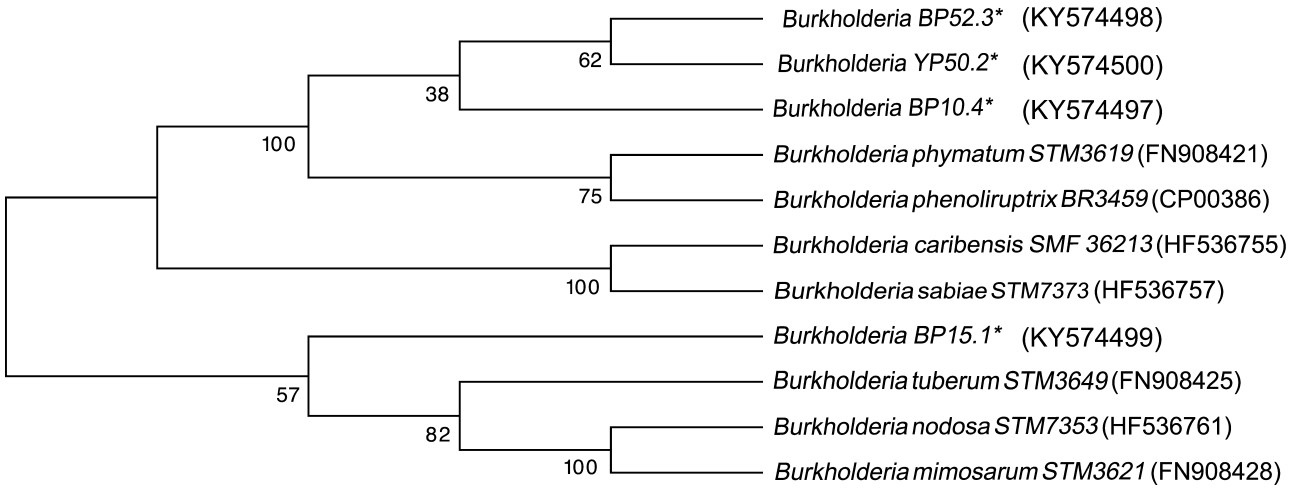

Figure 3 Phylogenetic tree based on *nifH* gene sequences, which shows *Burkholderia* species associated with leguminous plants.

Discussion

The main objective of this work was to explore the presence of *Burkholderia* species in Southeastern Mexico. The strains isolated from nodules of *I. thibaudiana*, *M. diplotricha*, *M. albida*, *M. pigra*, and *M. pudica* plants, in the state of Chiapas, Mexico, were selected based on their morphological characteristics. The 15 strains were grouped into 4 genomic fingerprint profiles obtained by BOX-PCR. One strain of each profile was selected for sequencing. The *16S rRNA* gene sequences obtained were analyzed in the GenBank nucleotide database; all 4 strains were genetically related to species of the *Burkholderia* genus. Strain YP50.2 (KY569371), which is genetically related to the *B. caribensis* species, was isolated in the YMA culture medium from *I. thibaudiana* nodules collected in the municipality of Tonala.

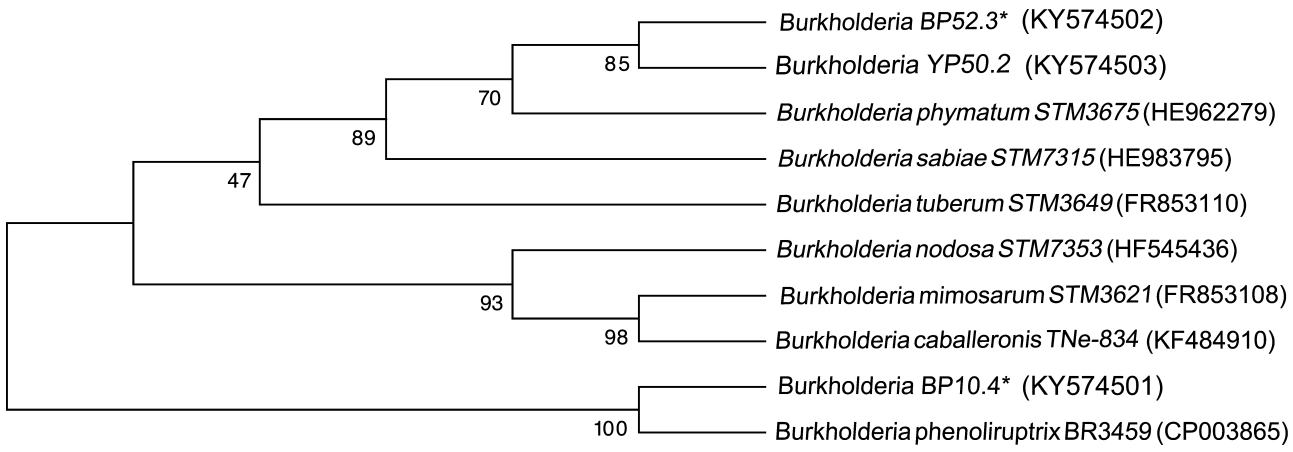

Figure 4 Phylogenetic tree based on *nodC* gene sequences, which shows *Burkholderia* species associated with leguminous plants.

Table 2 Biochemical characteristics of Burkholderia isolates from leguminose of state of Chiapas, Mexico

Characteristics	BP10.4 (*Mimosa pigra*)	BP52.3 (*Mimosa albida*)	BP15.1 (*Mimosa pigra*)	YP50.2 (*Indigofera thibaudiana*)
Nitrate	+	+	+	+
Tryptophane	–	–	–	–
Glucose	–	+	–	–
Arginine	–	+	–	–
Urea	–	–	–	–
Esculine Ferric citrate	–	+	–	+
Gelatin	–	–	–	+
p-nitrophenyl-β-D-galactopyranoside β	+	+	+	+
Asimilation of				
D-Glucose	+	+	+	+
L-Arabinose	–	+	–	+
D-Mannose	+	+	+	+
D-Manitol	+	+	+	+
N-acetyl-glucosamine	+	+	+	+
D-Maltose	–	+	–	+
K-Gluconate	+	+	+	+
Capric acid	+	–	–	–
Adipic acid	–	–	+	–
Malic acid	+	+	+	+
Trisodium citrate	+	+	+	+
Phenylacetic acid	+	+	+	–
Oxidase test	–	–	–	–

Strain BP52.3 (KY569374), isolated in BAc culture medium from the plant *M. albida* collected in the town of Tonala has a genetic relationship with *B. sabiae*. Strain BP15.1 (KY569373), which was isolated in BAc culture medium from *M. pigra* nodules collected in the municipality of Mazatan and BP10.4 (KY569372) strain also isolated from *M. pigra* in the municipality of Tapachula have a genetic relationship with *B. phenoliruptrix*. In the analysis of the sequences of the *nifH* genes of *Burkholderia* YP50.2 (KY574500), BP52.3 (KY574498), and BP10.4 (KY574498) strains, a genetic relationship was found with *B. phymatum* and *B. phenoliruptrix*, while strain BP15.1 (KY574499) was related to *B. sabiae* and *Burkholderia tuberum* (Fig. 3). The same genetic relationship was observed in the sequence analysis of the *nodC* genes; *Burkholderia* BP52.3 (KY574502) and YP50.2 (KY574503) strains are related with *B. phymatum* and *B. phenoliruptrix*, while the BP10.4 (KY574501) strain has a genetic relationship with *B. sabaie* and *B. tuberum* (Fig. 4).

In the sequence analysis of the *16S rRNA*, *nifH* and *nodC* genes of *Burkholderia* spp. strains, it can be observed that

Table 3 *Burkholderia* strains growth in stress and aromatic compounds

BOX-PCR profile (n)	Heat stress		Salt stress		Growth phenol	Growth benzene
	37 °C	45 °C	2%	4%		
Burkholderia caribensis						
YP50.2 (IV)	–	–	–	–	–	+
Burkholderia phenoliruptrix						
BP10.4 (II)	+	–	–	–	–	+
BP20.1	+	–	+	+	–	+
Burkholderia phenoliruptrix						
BP15.1 (I)	+	–	–	–	–	+
BP20.2	+	–	+	+	–	+
BP6.2	+	–	+	+	–	+
BP10.3	+	–	–	–	–	+
YP11.1	+	+	+	+	–	–
YP7.5	+	–	+	+	–	+
YP8.1	–	–	–	–	–	–
Burkholderia sabiae						
BP52.3 (III)	+	–	–	–	–	+
BP25.2	+	–	–	–	–	+
BP34.1	+	–	–	–	–	+
YP8.7	+	–	+	+	–	–
YP13.2	+	+	+	–	–	+

there is a genetic relationship with the nodulating species already described; however, it can also be observed that in the *nifH* and *nodC* gene sequences the strains isolated from leguminous plants might be new species of *Burkholderia*. *Burkholderia* spp. strains have the capacity to metabolize different carbon sources, which is a metabolic characteristic of the genus (Table 2). Therefore, to confirm whether they are new species, the gene sequencing will be done on the *atpD*, *recA*, and *rpoB* genes, as well as the protein profiles and DNA hybridization tests with species that have greater genetic relationship to confirm that these strains belong to new *Burkholderia* species. The description of few nodulating *Burkholderia* species was done in Mexico; among these species is *Burkholderia caballeronis* which was isolated from the rhizosphere of the tomato plant, which is not a legume, and *Burkholderia* spp. CCGE1002, isolated from nodules of *Mimosa occidentalis*[17,19]. It is also important to note that although *B. phenoliruptrix* was isolated from nodules of *Mimosa flocculosa* in Brazil, this is the first report of this species isolated from nodules of *M. pigra* in Mexico[3,6,12,13,24,25]. In Mexico, it is the first report of these *Burkholderia* species genetically related to *B. phymatum*, *B. phenoliruptrix*, *B. sabiae*, *B. caballeronis*, and *B. tuberum*.

The *Burkholderia* spp. strains were subjected to various stress conditions, they grew in the presence of benzene at 0.1% as the only carbon source, and also at 2 and 4% NaCl and temperatures of 37 and 45 °C. Most *Burkholderia* spp. strains were tolerant to heat and salinity stress conditions, and also had the ability to grow in 0.1% benzene. This ability of tolerance to saline and thermal stress has been evaluated in strains of rhizobia[7]. Lopez et al.[16] evaluated the ability of *Rhizobium tropici* to degrade polycyclic aromatic hydrocarbons. Caballero-Mellado et al.[5] reported on nitrogen-fixing strains *B. unamae* and *B. xenovorans* growing in the presence of phenol, benzene, and biphenyl. Furthermore, species of the *B. cepacia* complex, such as *B. cepacia* G4, degrade toluene. This work contributes to confirming the ability of the members of nodulating- *Burkholderia* species to degrade some xenobiotic compounds.

In conclusion, members of the *Burkholderiaceae* family, particularly of the genus *Burkholderia*, were identified in Mexico. This genus was found in nodules of leguminous plants growing in Chiapas State. However, the presence of *Burkholderia* seems to be limited, as only a few strains were identified among the isolates analyzed.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

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