Phylogeny of symbiotic genes reveals symbiovars within legume-nodulating *Paraburkholderia* species

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ABSTRACT

Bacteria belonging to the genus *Paraburkholderia* are capable of establishing symbiotic relationships with plants belonging to the Fabaceae (=Leguminosae) family and fixing the atmospheric nitrogen in specialized structures in the roots called nodules, in a process known as biological nitrogen fixation (BNF). In the nodulation and BNF processes several bacterial symbiotic genes are involved, but the relations between symbiotic, core genes and host specificity are still poorly studied and understood in *Paraburkholderia*. In this study, eight strains of nodulating nitrogen-fixing *Paraburkholderia* isolated in Brazil, together with described species and other reference strains were used to infer the relatedness between core (16S rDNA, *recA*) and symbiotic (*nod*, *nif*, *fix*) genes. The diversity of genes involved in the nodulation (*nodAC*) and nitrogen fixation (*nifH*) abilities was investigated. Only two groups, one containing three *Paraburkholderia* species symbionts of *Mimosa*, and another one with *P. ribeironis* strains presented similar phylogenetic patterns in the analysis of core and symbiotic genes. In three other groups events of horizontal gene transfer of symbiotic genes were detected. *Paraburkholderia* strains with available genomes were used in the complementary analysis of *nifHDK* and *fixABC* and confirmed well-defined phylogenetic positions of symbiotic genes. In all analyses of *nod*, *nif* and *fix* genes the strains were distributed into five clades with high bootstrap support, allowing the proposal of five symbiovars in nodulating nitrogen-fixing *Paraburkholderia*, designated as mimosae, africana, tropicalis, atlantica and piptadeniae. Phylogenetic inferences within each symbiovar are discussed.

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Introduction

Diazotrophic symbiotic bacteria, generally called rhizobia, perform an important role in the nitrogen input into terrestrial systems, improving soil fertility and environmental sustainability. Rhizobia are capable of fixing atmospheric nitrogen (N_2) into available forms to plants, in a process known as biological nitrogen fixation (BNF), carried out by the enzymatic complex of nitrogenase [53]. The major contribution of the BNF process occurs in symbiotic associations with plants belonging to the Fabaceae (=Leguminosae) family, in specialized structures in the roots and occasionally in stems, called nodules [58].

Legume-rhizobia interactions initiate with a specific and coordinated molecular dialogue between the symbionts, in which the host plant exudates signals, mainly flavonoids, that will promote chemotaxis and the transcription of the regulator NodD protein in the compatible rhizobia, starting the expression of the nodulation genes that will lead to the synthesis and secretion of lipochitooligosaccharides (LCOs), called Nod factors (NF) [64,79]. When the host root hairs perceive the NFs, a series of morphological changes start that will lead to nodule organogenesis [57]

A common core structure of the NFs results from the expression of the nodulation *nodABC* genes [1,79]. NFs are composed of a chitin-like *N*-acetyl glucosamine oligosaccharide backbone with a fatty acyl chain at the non-reducing end in most rhizobia, but different species vary in their backbone length and in the size and saturation of the fatty acyl chain. The *nodA* gene product recognizes and transfers specific fatty acids to the chitin oligomers, whereas *nodC* is involved in the synthesis and length of the backbone of glucosamine oligosaccharide [35,80].

After nodule organogenesis, rhizobia differentiate into nitrogen-fixing bacteroids, and the synthesis of the nitrogenase complex takes place. The *nif* genes are responsible for the synthesis, processing and assembly of the nitrogenase complex. The nitrogenase complex is composed by the iron-protein (encoded by *nifH*) and the molybdenum-iron protein (encoded by *nifD* and *nifK* genes), and the operon *nifHDK* is regulated by the transcriptional activator *nifA* [43,80]. The *nifA* gene also controls the expression of other genes, such as the *fixABCX* [41]. The *nif* genes do not determine host specificity; nevertheless, their phylogenies have been largely studied, as they represent important markers of symbiotic plasmids/island, contributing to the distinction between symbiovar groups [69]. Similarly, some *fix* genes are critical for nitrogen fixation. The proteins FixA, FixB, FixC and FixX have been confirmed to be involved in the electron transfer pathway dedicated to the generation of reductants for the nitrogenase activity [27].

The term symbiovar (symbiotic variant) was suggested by Rogel [69], as a parallel term to pathovar in pathogenic bacteria, to distinguish rhizobial subgroups that are symbiotically distinct within one species. Therefore, the symbiovars are defined based on the symbiotic capabilities to nodulate the host plants, distinguished by differences in host range. However, due to rhizobia and legume promiscuity, the identification of a symbiovar has been based on the analysis of symbiotic genes [63,67].

Paraburkholderia (formerly *Burkholderia*) comprise many symbiotic species isolated mostly from South Africa and South America. Brazil is considered a center of diversity for legume-nodulating *Paraburkholderia*, especially in the Cerrado, Caatinga and Atlantic Forest biomes [4,14,15,25]. *Paraburkholderia* are preferred symbionts in Brazil for *Mimosa* [4,10,25,62] although their nodulation ability has also been reported in plants of the *Piptadenia* group [7] and *Calliandra* species [72]. Another legume-nodulating *Paraburkholderia* diversity center is the Fynbos biome in South Africa, where the genus is associated with endemic Papilionoid plants [19,20,21,28,33,39,49]. Interestingly, mimosoid-nodulating and papilionoid-nodulating *Paraburkholderia* differ considerably in the host range, since South African *Paraburkholderia* seem not to be able to nodulate *Mimosa* [28,50]. Studies conducted with the comparative phylogenetic analysis of South American and South African *Paraburkholderia* indicate different evolution patterns of symbiotic genes [7,18,55].

The phylogenetic analysis of symbiotic genes is necessary for a better understanding of host range, symbiovar definition and evolutionary processes. The objective of this study was to verify the correlation between core and symbiotic genes and also with the host specificity and geographic location of *Paraburkholderia* species. Based on the results obtained, we report and describe five symbiovars of nodulating *Paraburkholderia*.

Material and methods

Strains selection, DNA extraction, gene amplification and sequencing

Eight *Paraburkholderia* strains isolated from *Mimosa* spp. and one strain isolated from *Phaseolus vulgaris* were chosen for this study. The strains were selected from previous studies carried out by our group [60,61] and are listed in Table S1. All strains are deposited at the "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection # 1213, WDCM Collection #1054), in Londrina, State of Paraná, Brazil and in other culture collections. Stock cultures were maintained on modified-YMA medium [40] at 4 °C, and for long-term preservation strains were cryopreserved in modified-YM medium with 30% (v/v) glycerol at −80 °C and −150 °C, and lyophilized. The genomic DNA from these selected isolates was extracted using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's instructions.

The housekeeping genes 16S rRNA and *recA* were amplified as previously described [60]. For a better understanding of the evolutionary history of symbiotic genes, partial sequences of the nodulation genes *nodA* and *nodC* and the nitrogenase *nifH* gene were obtained. First, *nodA* and *nodC* genes were amplified according to the conditions described by Bontemps et al. [5] and Bournaud et al. [7], respectively, whereas the *nifH* gene amplification was performed with the primers and conditions described by Loiret et al. [52]. The PCR products were purified using the PureLink™ Quick PCR Purification Kit (Invitrogen™), following the manufacturer's instructions and sequenced using an ABI 3500xL (Applied Biosystems®). Sequences obtained in the study were submitted to the NCBI GenBank database and received the accession numbers from MT465888 to MT465925 (Table S2). In addition, we retrieved genomes and nucleotides sequences (*nifD*, *nifK*, *fixA*, *fixB*, *fixC*) of other nodulating species of *Paraburkholderia* from the GenBank and Joint Genome Portal (JGI/IMG/R) databases and the accession numbers are shown in the phylograms and/or in Table S2.

Phylogenetic analyses

The nucleotide sequences obtained in this study were assembled and corrected with the Bionumerics software (version 7.6). Multiple sequences alignments were performed with MUSCLE [26] and phylogenetic analyses were performed using MEGA 7 [44] with the maximum-likelihood (ML) algorithm.

For the 16S rRNA and the concatenated sequences of *fix* gene (*fixA*, *fixB*, *fixC*) phylogenies, the Tamura 3-parameter (T92) [76] model with gamma-distributed selection with invariant sites (G + I) was used. The *recA* and *nifH* phylogenies were performed with the T92 + G model. Phylogenies of single *nodC* and the concatenated sequences of *nodA* and *nodC* (*nodAC*) were built using T92 + I, while Kimura two-parameter + G [42] was used for the individual *nodA*. A phylogenetic tree of the concatenated *nifHDK* genes was conducted using Tamurei Nei + G model [76,77].

The statistical support for the trees was evaluated by bootstrap analysis with 1000 re-samplings [32]. Nucleotide identities (NI) were calculated using the Bioedit software (version 7.2.5) [37].

Results

Phylogenetic analysis of the 16S rRNA and recA genes

Aiming to compare core and symbiotic genes, first 16S rRNA (929 bp) (Fig. 1) and *recA* (414) (Fig. 2) phylograms were built. In the 16S rRNA phylogram, a large cluster included *Paraburkholderia* spp. strains JPY58, JPY604, STM604, JPY251, UYCP14C and CCGE1002 with *P. atlantica* strains. The phylogenies of the strains previously studied in the description of *P. franconis* (CNPSo 3191, 3200 and 3157T) and *P. atlantica* (CNPSo 3196, 3150 and 3155T) was confirmed [61]. *P. piptadeniae* STM7183T clustered together with *P. franconis* strains as the most closely related species. The remaining strains grouped together with *P. nodosa* CNPSo 1341, isolated from the Brazilian savanna (Cerrado) [15,16], and with *P. guarte-*
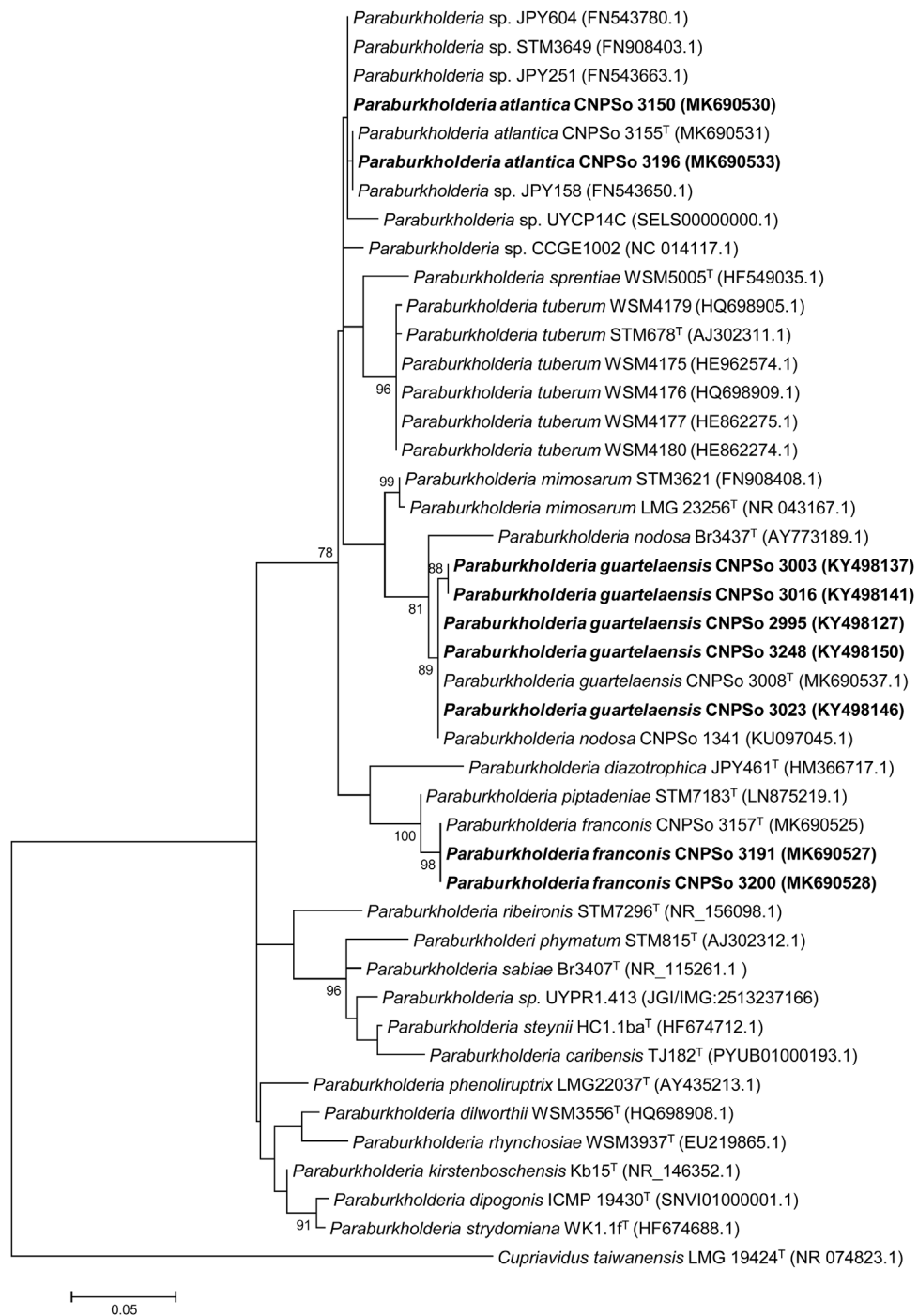
Fig. 1. Maximum likehood phylogenetic tree based on the 16S rRNA gene (929 bp) of nodulating *Paraburkholderia*. Accession numbers are indicated in parentheses and in Table S2. Strains under study are shown in boldface. Bootstrap values >70% are indicated at the nodes. *Cupriavidus taiwanensis* LMG 19424 was used as outgroup. Bar indicates five substitutions per 100 nucleotide positions.

laensis CNPSo 3008[T], in agreement with the study that indicated that the strains CNPSo 2995, CNPSo 3003, CNPSo 3016, CNPSo 3023 and CNPSo 3248 belong to this species [60]. Therefore, *P. nodosa* Br3437[T] is the closest neighbor to *P. guartelaensis*, followed by *P. mimosarum* PAS44[T] and *P. mimosarum* STM3621 (Fig. 1).

The phylogeny of the *recA* gene (Fig. 2) was in agreement with the 16S rRNA analysis (Fig. 1), indicating a consistent evolutionary pattern. It is worth mentioning that in both the 16S rRNA and *recA* phylograms, the strains classified as *P. tuberum*, JPY604, JPY158, STM3649, together with *Paraburkholderia* sp. CCGE1002 were more related to the newly described species *P. atlantica* than

with *P. tuberum* STM678[T], indicating that they should be reclassified.

Phylogeny of Paraburkholderia symbiotic genes

Phylogenetic analyses including all strains from this study and available sequences of other *Paraburkholderia* type and reference strains were performed with concatenated *nodAC* gene fragments (876 bp) (Fig. 3), and with partial sequences of the *nifH* gene (326 bp) (Fig. 4). In the *nodAC* phylogram, four different clusters were observed, all with 100% of bootstrap support, while *P. ribeiro-*
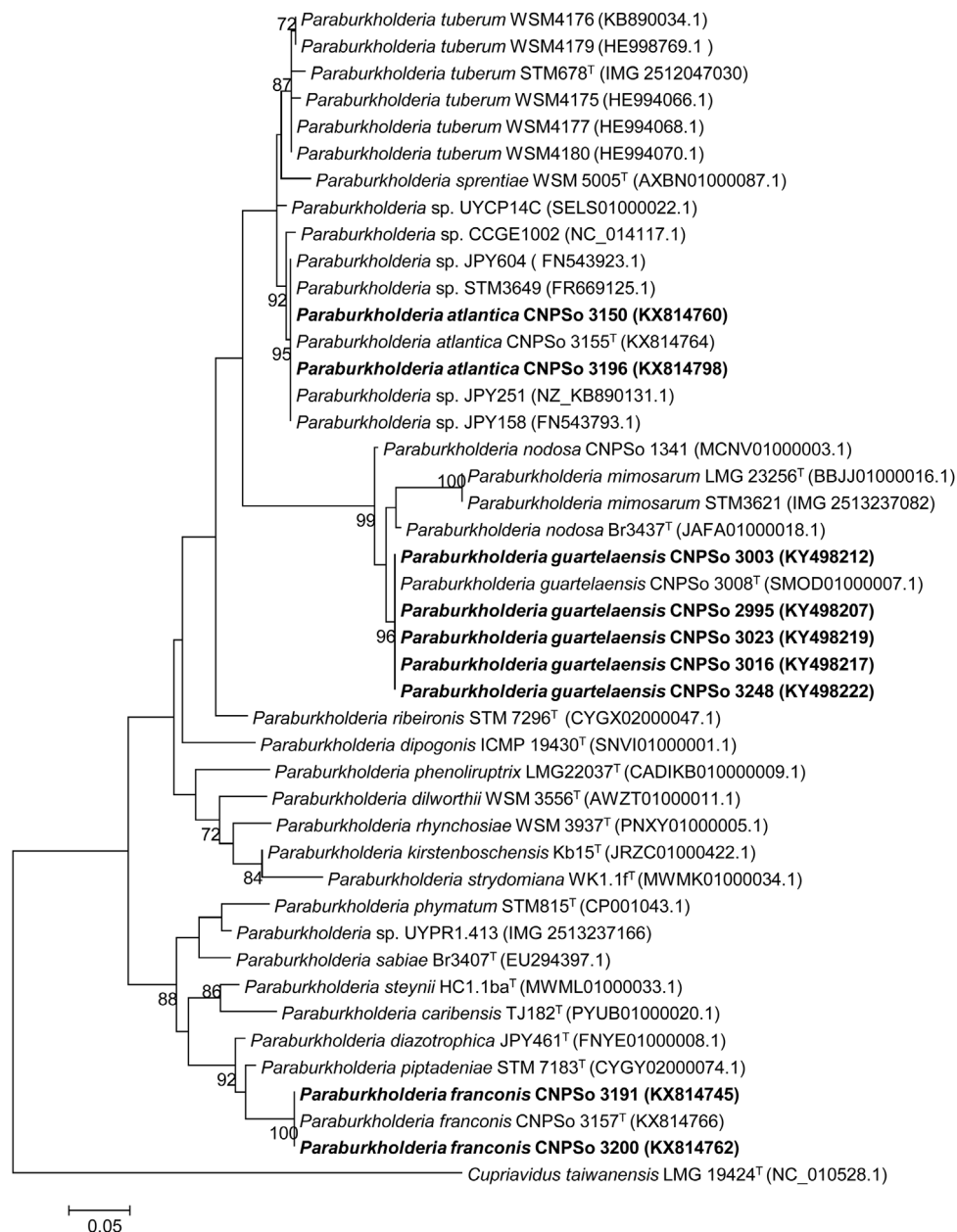
Fig. 2. Maximum-likelihood (ML) phylogeny based on parcial *recA* gene sequences (414 bp) showing the relationships between nodulating species of *Paraburkholderia*. Accession numbers are indicated in parentheses and in Table S2. Strains under study are shown in boldface. Bootstrap values >70% are indicated at the nodes. Scale bar indicates five substitutions per 100 nucleotide positions.

nis STM7296[T] and *Paraburkholderia* sp. UYPR1.413 showed unique positions (Fig. 3). Single phylogenies of *nodA* (257 bp) (Fig. S1) and *nodC* (442 bp) (Fig. S2) confirmed the phylogenetic positions of the strains. Unfortunately, the *nodC* sequence of *P. sabiae* Br3407[T] was not available in the databanks, therefore, we used the sequences of the strains *P. sabiae* STM7315 and *P sabiae* BRUESC861 in the *nodC* phylogeny (Fig. S2). The aligned sequences were shorter because of the inclusion of *P. sabia* STM 7315, with lower number of bp. Also, in the *nodC* phylogeny sequences of *P. ribeironis* STM7168 and STM7217 were used to verify the phylogenetic position of the *P. ribeironis* STM7296[T] and all strains grouped together with 99% of bootstrap support. In congruence with the *nodAC* phylogenetic analysis (Fig. 3), five distinct clusters were observed in the *nifH* phylogram, all with high bootstrap support (Fig. 4). Based on the *nodAC* and *nifH* phylogenies, we propose five symbiovar clusters for nitrogen-fixing symbiotic *Paraburkholderia*. Each symbiovar clus-

ter received the name of the first location or host plant of isolation. Other information as plant host, country of origin of the strains belonging to each symbiovar are shown in Table 1 and nucleotide identity information is presented in Table 2.

The first cluster of *nodAC* (Fig. 3) and *nifH* (Fig. 4) phylogenies composed the sv. mimosae (mi.mo'sae. N.L. gen. n. mimosae, first isolated from a plant of the genus *Mimosa*), grouping *P. mimosarum* strains STM3621 and LMG 23256[T], both isolated from Taiwan, all *P. guartelaensis* strains including CNPSo 3008[T], and *P. nodosa* strains Br3437[T] and CNPSo 1341, all isolated from Brazilian soils. Strains of sv. mimosae shared among them 92–100% and 94–100% of NI in the *nodAC* and *nifH* phylogenies, respectively (Table 2).

The second cluster of sv. Africana (a.fri.ca'na. L. fem. adj. africana, first isolated from a legume in South Africa) grouped several strains isolated from South Africa, including *P. kirstenboschensis* KB15[T], *P. dilworthii* WSM3556[T], *P. rhynchosiae* WSM 3937[T], *P. spren-
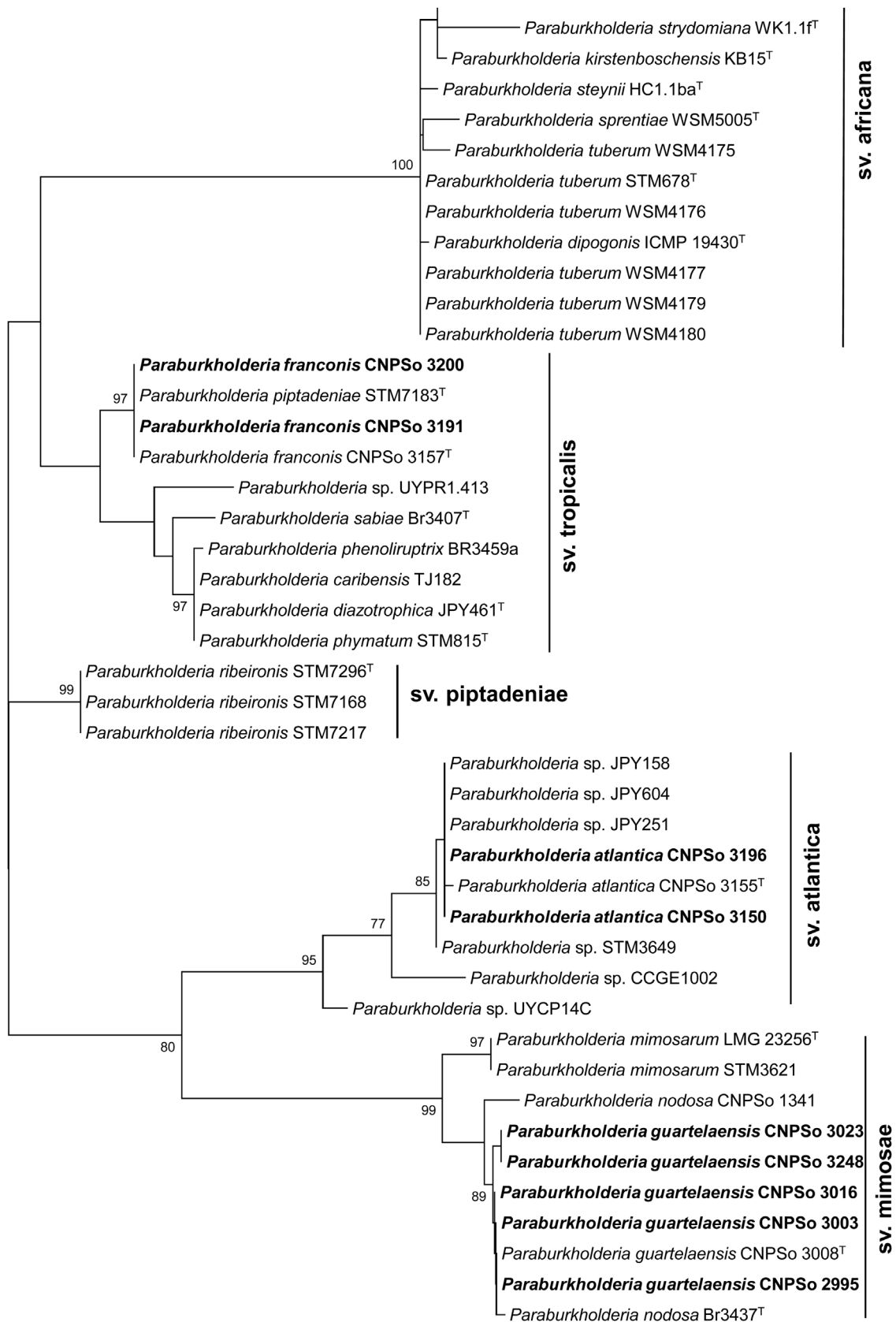
4
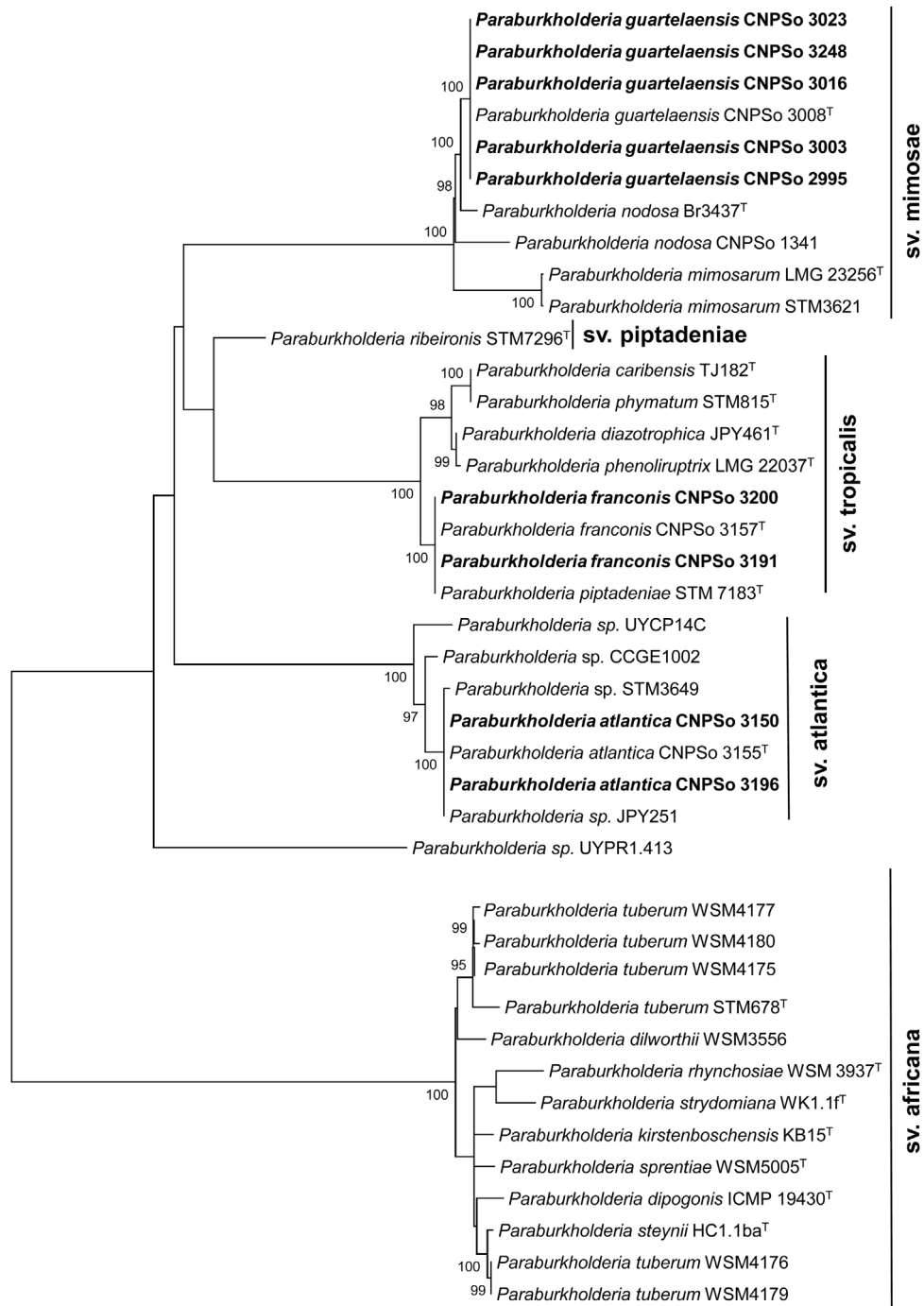
Fig. 3. Maximum-likelihood (ML) phylogeny based on the concatenated gene sequences (876 bp) of *nodA* (410 bp) + *nodC* (466 bp) showing the relationships between nodulating species of *Paraburkholderia*. Accession numbers are indicated in Table S2. Strains under study are shown in boldface. Bootstrap values >70% are indicated at the nodes. Scale bar indicates five substitutions per 100 nucleotide positions.

tiae WSM5005[T], *P. strydomiana* WK1.1ft[T], *P. steynii* HC1.1ba[T], and *P. tuberum* strains WSM4175, WSM4176, WSM4177, WSM4179, WSM4180 and STM678[T]. *P. dipogonis* ICMP 19430[T], isolated from New Zealand, was also included in this cluster (Figs. 3 and 4). The NI among *sv.* africana strains ranged from 93 to 100% in the *nodAC* phylogeny and from 95.3 to 100 % in the *nifH* phylogeny (Table 2).

The third cluster, of *sv.* tropicalis (tro.pi.ca'lis. N.L. adj. tropicalis, first isolated from a legume in French Guiana, in the tropics), was named after *P. phymatum* STM815[T], isolated from *Machaerium lunatum* in French Guiana [78] and included *P. caribensis* TJ182 isolated in Taiwan. All other strains of this group were iso-

lated in Brazil, and included *P. diazotrophica* JPY461[T], *P. franconis* strains CNPSo 3157[T], CNPSo 3191 and CNPSo 3200, *P. piptadeniae* STM 7183[T], *P. phenoliruptrix* BR3459a[T] and *P. sabiae* Br3407[T] (Figs. 3 and 4). The NI among the strains ranged from 95.8 to 100% and from 95.3 to 100% in the *nodAC* and *nifH* phylogenies, respectively (Table 2).

The fourth symbiovar, *sv.* atlantica (at.lan'ti.ca. L. fem. adj. atlantica, first isolated, from the Atlantic Forest) was named after *P. atlantica* CNPSo 3155[T] and clustered *P. atlantica* strains CNPSo 3150, CNPSo 3196, JPY251, JPY158 and JPY604, isolated in Brazil and *Paraburkholderia* sp. CCGE1002 isolated in Mexico.

Fig. 4. Maximum-likelihood (ML) phylogeny based on partial nitrogen fixing *nifH* gene sequences (326 bp) showing the relationships between nodulating species of *Paraburkholderia*. Accession numbers are indicated in Table S2. Strains under study are shown in boldface. Bootstrap values >70% are indicated at the nodes. Scale bar indicates five substitutions per 100 nucleotide positions.

Table 1
Symbiovar clusters of nodulating Paraburkholderia.

Symbiovar cluster	Strain	Host	Country of origin	Also nodulate	References
sv. mimosae	*P. guartelaensis* CNPSo 3008[T] *P. guartelaensis* CNPSo 2995 *P. guartelaensis* CNPSo 3003 *P. guartelaensis* CNPSo 3016 *P. guartelaensis* CNPSo 3023 *P. guartelaensis* CNPSo 3248	*Mimosa gymnas*	Brazil	*Mimosa pudica, Phaseolus vulgaris, Macroptilium atropurpureum*	Paulitsch et al. [60]
	Paraburkholderia nodosa DSM 1341[T]	*Mimosa scabrella*	Brazil	Mimosa spp/*Piptadenia, Parapiptadenia, Anadenanthera* and *Phaseolus vulgaris*	Chen et al. [10]; Bontemps et al. [4]; Bournaud et al. [7]; Dall'agnol et al. [14]
	Paraburkholderia nodosa CNPSo 1341	*Phaseolus vulgaris*	Brazil	ND	Dall'agnol et al. [15]
	Paraburkholderia mimosarum LMG 23256[T]	*Mimosa pigra*	Taiwan	*Mimosa* spp., *Phaseolus vulgaris, Vigna unguiculata, Macroptilium atropurpureum*	Chen et al. [11,12]; Bontemps et al. [4]; Liu et al. [51]; Gehlot et al. [34]; Lardi et al. [46]
	Paraburkholderia mimosarum STM3621	*Mimosa pudica*	French Guiana	ND	Mishra et al. [54]
sv. africana	*Paraburkholderia tuberum* STM678[T]	*Aspalathus carnosa*	South Africa	*Cyclopia* spp., *Lebeckia ambigua, Macroptilium, Atropurpureum, Crotalarieae, Hypocalypteae, Indigofereae,* and *Podalyrieae*	Moulin et al. [56]; Taulé et al. [78]; Elliot et al. [28]; Howieson et al. [39]; Lemaire et al. [47]; De Meyer et al. [22];
	Paraburkholderia kirstenboschensis KB15[T]	*Virgilia oroboides*	South Africa	*Hypocalyptus sophoroides, Hypocalyptus oxalidifolius*	Beukes et al. [3]; Steenkamp et al. [74]
	Paraburkholderia dilworthii WSM3556[T]	*Lebeckia ambigua*	South Africa	*Lebeckia sepiaria*	Howieson et al.[39]; De Meyer et al. [21]
	Paraburkholderia dipogonis ICMP 19430[T]	*Dipogon lignosus*	New Zealand	ND	Liu et al. [50]; Sheu et al. [70]
	Paraburkholderia rhynchosiae WSM 3937[T]	*Rhynchosia ferulifolia*	South African Western Cape	ND	De Meyer et al. [20]
	Paraburkholderia sprentiae WSM5005[T]	*Lebeckia ambigua*	South Africa	ND	De Meyer et al. [19]
	Paraburkholderia strydomiana WK1.1f[T] *Paraburkholderia steynii* HC1.1ba[T]	*Hypocalyptus sophoroides*	South Africa	*Macroptilium atropurpureum, Vigna unguiculata*	Beukes et al. [2,3]
	Paraburkholderia tuberum WSM4175			ND	De Meyer et al. [22]
	Paraburkholderia tuberum WSM4176	*Lebeckia ambigua*	South Africa	ND	De Meyer et al. [23]
	Paraburkholderia tuberum WSM4177			*Lebeckia sepiaria*	Howieson et al. [39]; De Meyer et al. [22]
	Paraburkholderia tuberum WSM4179 *Paraburkholderia tuberum* WSM4180			ND	De Meyer et al. [22]
sv. tropicalis	*Paraburkholderia caribensis* TJ182	*Mimosa diplotricha*	Taiwan	*Dalbergia louveli*	Taulé et al. [78], Chen et al. [13]; Rasolomampianina et al. [66]
	Paraburkholderia diazotrophica JPY461[T]	*Mimosa candollei*	Brazil	Mimosa spp., *Anadenanthera, Parapiptadenia blanchetti, Piptadenia viridiflora, P. gonoacantha,Calliandra* spp.	Sheu et al. [71]; Bontemps et al. [4]; Mishra et al. [54]; Bournaud et al. [7]; Silva et al. [72]
	Paraburkholderia franconis CNPSo 3157[T] *Paraburkholderia franconis* CNPSo 3191 *Paraburkholderia franconis* CNPSo 3200	*Mimosa pudica*	Brazil	*Phaseolus vulgaris*	Paulitsch et al. [61]
	Paraburkholderia piptadeniae STM 7183[T]	*Piptadenia gonoacantha*	Brazil	*Mimosa pudica*	Bournaud et al. [8]
	Paraburkholderia phenoliruptrix BR3459a	*Mimosa flocculosa*	Brazil	*Mimosa pudica, Macroptilium atropurpureum, Piptadenia gonoacantha, Microlobius foetidus Anadenanthera peregrina, Parapiptadenia rígida, Pityrocarpa monoliformis*	Zuleta et al. [81]; Bournaud et al. [7]

Table 1 (*Continued*)

Symbiovar cluster	Strain	Host	Country of origin	Also nodulate	References
	Parabukholderia phymatum STM815[T]	*Machaerium lunatum*	French Guiana	Mimosa spp., *Phaseolus vulgaris, Parapitadenia. Pterosperma, Cyclopia* spp., *Virgilia oroboides,*	Taulé et al. [78]; Elliott et al. [29]; dos Reis et al. [25]; Talbi et al. [75]; Mishra et al. [54]; Liu et al. [51]; Bournaud et al. [7]
	Paraburkholderia sabiae Br3407[T]	*Mimosa caesalpiniifolia*	Brazil	Pitadeniae, Parapiptadeniae, Anadenanthera	Chen et al. [9,11]; Taulé et al. [77]; Bournaud et al. [7]; Silva et al. [72]
sv. atlantica	*Paraburkholderia atlantica* CNPSo 3155[T] *Paraburkholderia atlantica* CNPSo 3150	*Mimosa pudica*	Brazil	*Phaseolus vulgaris, Macroptilium atropurpureum*	Paulitsch et al. [61]
	Paraburkholderia atlantica CNPSo 3196	*Phaseolus vulgaris*		*Mimosa pudica, Macroptilium atropurpureum*	
	Paraburkholderia sp. CCGE1002	*Mimosa occidentalis*	Mexico	ND	Ormeño-Orrillo et al. [59]
	Paraburkholderia sp. JPY251	*Mimosa velloziana*	Brazil	ND	Gyaneshwar et al. [36]
	Paraburkholderia sp. UYCP14C	*Calliandra parvifolia*	Uruguay	ND	Langleib et al. [45]
	Paraburkholderia tuberum JPY158	*Mimosa pigra*	Brazil	ND	Bontemps et al. [4]
	Paraburkholderia tuberum JPY604	*M. xanthocentra var. xanthocentra*			
	Paraburkholderia tuberum STM3649	*Mimosa pudica*	French Guiana	ND	Mishra et al. [54]
sv. piptadeniae	*Paraburkholderia ribeironis* STM7296[T]	*Piptadenia gonoacantha*	Brazil	*Mimosa pudica*	Bournaud et al. [8]
	Paraburkholderia sp. UYPR1.413	*Parapiptadenia rigida*	Uruguay	ND	Tamura et al. [77]

The other two strains within this symbiovar were isolated from neighbor countries, *Paraburkholderia* sp. UYCP14C (Uruguay) and *Paraburkholderia* sp. STM3649 (French Guiana) (Figs. 3 and 4). The NI in the *nodAC* and *nifH* ranged from 95.5 to 100% and from 94.7 to 100% respectively (Table 2).

The fifth and last symbiovar, sv. piptadeniae (pip.ta.de'ni.ae. N.L. gen. n. piptadeniae, first isolated from a plant of the genus *Piptadenia*) included only *P. ribeironis* species, isolated from soils of the Brazilian Atlantic Forest. *P. ribeironis* STM7296[T] presented a unique position in the *nodAC* phylogeny (Fig. 3), and the phylogenetic position was confirmed with two other *P. ribeironis* strains, STM7168 and STM7217 in the analysis of *nifH* (Fig. 4), as well as on the single *nodC* phylogeny (Fig. S2). The NIs among the strains in the *nodAC*, *nodC* and *nifH* were of 100% (Table 2).

Paraburkholderia sp. strain UYPR1.413 presented unique positions in the *nodAC* (Fig. 3) and *nifH* (Fig. 4) phylograms, not grouping with any of the proposed symbiovars.

To confirm the phylogenetic position of the symbiovars, phylogenetic analyses of the concatenated sequences of *nifHDK* (3065 bp) (Fig. S3) and *fixABC* (2986 bp) (Fig. S4) genes were performed, including only nodulating *Paraburkholderia* strains with available genomes. All strains maintained the same clusters observed in the *nodAC* (Fig. 3) and *nifH* (Fig. 4) phylogenies. The exception was *Paraburkholderia* sp. strain UYPR1.413, that clustered with the sv. caribensis in the *nifHDK* phylogeny but confirmed a unique position in the *fixABC* phylogram, not allowing a clear classification of the symbiotic genes.

Discussion

Phylogenetic studies using core and symbiotic genes suggest that nodulating *Paraburkholderia* can be split into two major groups: (i) strains from Central and South America that nodulate *Mimosa* and other members of the Mimosoideae, also called Mimosoid-nodulating *Paraburkholderia* [7,8,36,60,73], and (ii) South African strains that nodulate papilionoid species native to the Cape Floristic Region (CFR) [18,33,47,49], also called Papilionoid-nodulating *Paraburkholderia* (Fig. 5).

Rogel et al. [69] suggested that one symbiovar represents a subset of strains, at the intra- or interspecific levels, differentiated on the bases of symbiotic abilities concerning the host plants and the variability of the symbiotic genes. Since this definition, as the phylogenies of *nodA* and *nodC* genes are intimately associated with host specificity, they have been used for symbiovar definition, with an emphasis on *nodC* [35,63,68,69,79].

Mishra et al. [54] were the first to suggest that symbiotic *Paraburkholderia* could present different symbiovars. In their study, the sv. mimosae comprised the American strains considered as *P. tuberum* and the sv. papilionoid comprised South African strains such as *P. tuberum* STM678[T]. However, our results have clearly shown that symbiotic genes of nodulating *Paraburkholderia* are far more complex and diverse than previously thought, and the results do not agree with the two symbiovars proposed by Mishra et al. [54]. Our results indicate that the previously suggested "sv. mimosae" should be split into four different and well-defined symbiovars that are now proposed.

Based on the analysis of nodulation and nitrogen-fixation genes, we were able to identify five well-defined symbiovars in nodulating *Paraburkholderia*. Interestingly, the results confirm previous analyses of core and symbiotic genes by De Meyer et al. [18] and Estrada de los Santos et al. [31]. Based on our analysis of NI values (Table 2), we were also able to suggest a 92% threshold for symbiovar delimitation in the analysis of *nodA*, *nodC*, *nodAC*, *nifH*, *nifHDK* and *fixABC* genes of *Paraburkholderia*. This cut-off value has also been suggested for symbiotic genes of alpha-rhizobia [6,24].

The strains belonging to the sv. mimosae presented the same phylogenetic pattern in the analyses of core and symbiotic genes. Despite grouping few strains of a unique species, *P. ribeironis*, indications are that core and symbiotic genes are also congruent in sv. piptadeniae. However, many strains belonging to the three other symbiovars (sv. africana, sv. tropicalis and sv. atantica) showed different phylogenetic positions in the comparison of core and symbiotic genes phylogenies. This finding supports the hypothe-

Table 2
Nucleotide identity (NI, %) of core and symbiotic genes of nodulating *Paraburkholderia* belonging to four different symbiovars. Length of aligned regions were as follows: 16S rRNA (929 bp), recA (414 bp), nodAC (876 bp), nifH (326 bp), nifHDK (3065 bp) and fixABC (2986 bp).

16S rDNA

between:	sv. mimosae	sv. africana	sv. tropicalis	sv. atlantica	sv. piptadeniae
sv. mimosae	97.4–100	95.5–98.1	95.3–98.1	96.6–98.8	96.1–96.3
sv. africana	95.5–98.1	96–100	95.4 –98.9	96.4–99.1	96.2–97.1
sv. tropicalis	95.3–98.1	95.4–98.9	96.1–100	96–98.3	96–97
sv. atlantica	96.6–98.8	96.4–99.1	96–98.3	96.1–100	96.1–96.6
sv. piptadeniae	96.1–96.3	96.2–97.1	96–97	96.1–96.6	100

recA

between:	sv mimosarum	sv. africana	sv. tropicalis	sv. atlantica	sv. piptadeniae
sv. mimosae	95.4–100	85.7–92.7	86.9–92.9	89.8–92.9	89.3–91.3
sv. africana	85.7–92.7	89.3–100	87.9–98.5	89.8–99	91.3–95.1
sv. tropicalis	86.9–92.9	87.9–98.5	89.3–100	89.1–99.7	89.6–92.5
sv. atlantica	89.8–92.9	89.8–99	89.1–99.7	98.5–100	94.6–95.1
sv. piptadeniae	89.3–91.3	91.3–95.1	89.6–92.5	94.6–95.1	100

nodAC

between:	sv. mimosae	sv. africana	sv. tropicalis	sv. atlantica	sv. piptadeniae
sv. mimosae	92.4–100	69.8–71.6	76.9–78.8	78.3–80	81.6–83.7
sv. africana	69.8–71.6	93–100	71.9–73.6	71.4–73.1	72.6–73.9
sv. tropicalis	76.9–78.8	71.9–73.6	95.8–100	77.6 –78.6	84.4–86.2
sv. atlantica	78.3–80	71.4–73.1	77.6–78.6	95.5–100	83.3–83.7
sv. piptadeniae	81.6–83.7	72.6–73.9	84.4–86.2	83.3–83.7	100

nifH

between:	sv. mimosae	sv. africana	sv. tropicalis	sv. atlantica	sv. piptadeniae
sv. mimosae	94.7–100	81.9–83.7	83.7–87.4	86.1–88.3	86.1–87.4
sv. africana	81.9–83.7	95.3–100	85.8–88.6	81- 84.9	85.2–86.8
sv. tropicalis	83.7–87.4	85.8–88.6	95.3–100	85.2–89.2	92–94.4
sv. atlantica	86.1–88.3	81–84.9	85.2–89.2	94.7–100	86.8–87.1
sv. piptadeniae	86.1–87.4	85.2–86.8	92–94.4	86.8–87.1	100

nifHDK

between:	sv. mimosae	sv. africana	sv. tropicalis	sv. atlantica	sv. piptadeniae
sv. mimosae	94.4–99.9	78.1–80.1	85.4–86	83–84.3	86.3–86.5
sv. africana	78.1–80.1	95.9–99.4	82.4–83.2	78.9–80.4	82.2–83.4
sv. tropicalis	85.4–86	82.4–83.2	96.6–100	83.9–85.5	90.9–91.4
sv. atlantica	83–84.3	78.9–80.4	83.9–85.5	92.8–99.9	86–87
sv. piptadeniae	86.3–86.5	82.2–83.4	90.9–91.4	86–87	100

fixABC

between:	sv. mimosae	sv. africana	sv. tropicalis	sv. atlantica	sv. piptadeniae
sv. mimosae	92.9–98.6	75.8–77.3	80.4–81.5	78.8–80.2	82.5–83.6
sv. africana	75.8–77.3	92–99.4	79.2–80.8	77–78.4	80.2–81.8
sv. tropicalis	80.4–81.5	79.2–80.8	94.1–100	81.8–83	88.1–89.6
sv. atlantica	78.8–80.2	77–78.4	81.8–83	94.7–99.9	84.7–85.5
sv. piptadeniae	82.5–83.6	80.2–81.8	88.1–89.6	84.7–85.5	100

sis that horizontal gene transfer of symbiotic genes occurred after the divergence of *Paraburkholderia* lineages [18]. The greater division in clades of Mimosa-nodulating and Papilionoid-nodulating *Paraburkholderia* is clear in the symbiotic gene phylogenies (*nodAC*, *nifH*, *nifHDK* and *fixABC*) (Figs. 3 and 4, S3 and S4). Strains belonging to the sv. mimosae are considered as *Mimosa*-nodulating *Paraburkholderia*. This correlation between core (16S rRNA, *recA*) and symbiotic genes (specially *nodC*) phylogenies has been reported in most *Mimosa*-nodulating *Paraburkholderia*, suggesting an ancestral acquisition of those genes, with rare events of horizontal gene transfer [4,7,18,60,61].

It is important to highlight that, in both 16S rRNA and *recA* phylogenies, some strains classified as *P. tuberum*, including JPY604 and JPY158 isolated from *Mimosa xanthocentra* and *M. pigra*, respectively, in Brazil [4], *Paraburkholderia* sp. STM3649 isolated from *M. pudica* in French Guiana [54], and *Paraburkholderia* sp. CCGE1002

isolated from *M. occidentalis* in México [59] are more phylogenetic related to the newly described *P. atlantica* species [61] than with *P. tuberum* STM678[T] [78], and should be reclassified. Furthermore, *P. atlantica* CNPSo 3155[T] was isolated from the Brazilian Atlantic forest, while *P. tuberum* STM678[T] was isolated from South Africa, an additional endorsement that *Paraburkholderia* sp. JPY604, JPY158 and STM3649 are more closely related to *P. atlantica* species. As discussed before our results also do not agree with the suggestion [55] that *P. tuberum* species can include both the previously called "sv. mimosae" and "sv. papilionoideae". We clearly show that Center-South American and South African strains differ drastically regarding their symbiotic genes, demonstrating a different source of acquisition of these genes.

The third symbiovar tropicalis was named after *P. phymatum* STM815[T], isolated in French Guiana, in the tropics. This strain was first isolated from *Machaerium lunatum* but failed to re-nodulate
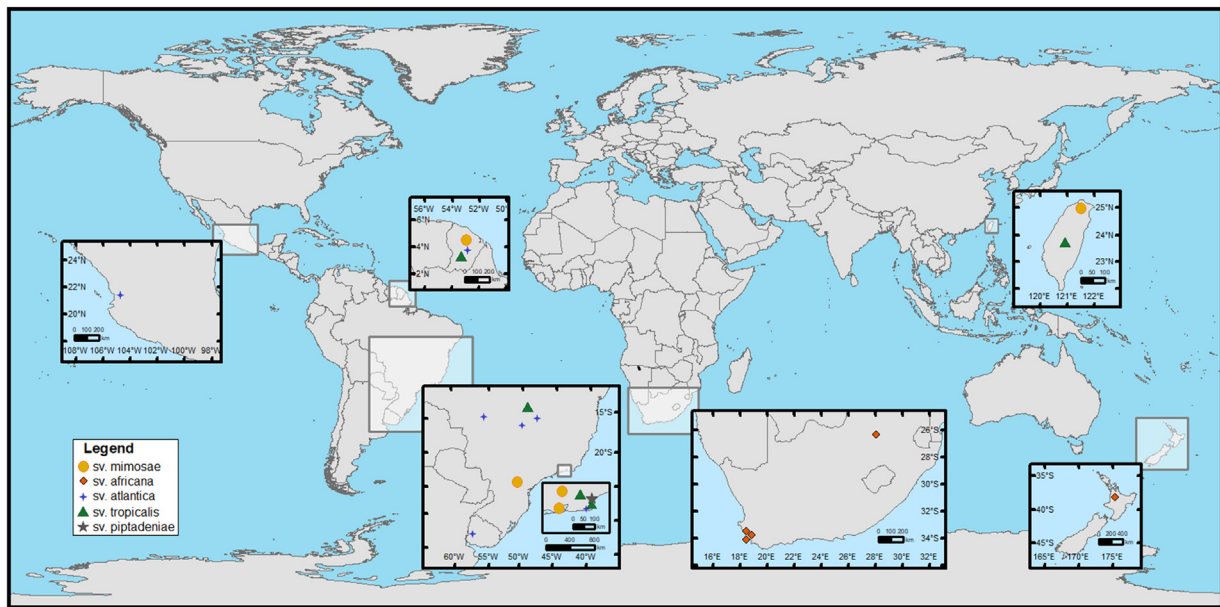
Fig. 5. World distribution of symbiovars of *Paraburkholderia*.

its original host or any *Machaerium* species [29]; however, it has the remarkable ability to nodulate many species of *Mimosa, Parapiptadenia, Pterosperma, Cyclopia* spp., *Virgilia oroboide* and *Phaseolus vulgaris* [7,25,28,29,49,51,54,75]. In our results, *P. phymatum* STM815[T] and *P. caribensis* TJ182 presented almost identical patterns in all symbiotic genes, as also observed by Estrada-de los Santos et al. [31], implying that they share a close evolutionary history of symbiotic gene acquisition and that both are probably Mimosa-nodulating *Paraburkholderia*. Still in the sv. tropicalis, *P. sabiae* and *P. phenoliruptrix* are reported to carry *nodC* variants. In the study conducted by Bournaud et al. [7], *P. sabiae* strains showed two different *nodC* genes, exhibiting different nodulation phenotypes, indicating that the variability in the *nodC* affects both conditions of host specificity and effectiveness of nodules [7]. Recently, *P. sabiae* strains were also isolated from *Calliandra* spp. in Brazil, but all strains showed high similarity in the *nodC* gene [72]. As the *nodC* sequence of the type strain of *P. sabiae* is not available, we used two other strains. *P. sabiae* STM7315 came from the study of Bournaud et al. [7], representing the most frequent *nodC* variant (*nodC3*), while the other strain BRUESC861 was isolated by Silva et al. [72], and both shared high similarity (Fig. S2).

Bournaud et al. [7] also isolated *P. phenoliruptrix* strains and were the first to report nodulation in the *Piptadeniae* group. The *nodC* variants within this species were also observed and many strains harbor *nodC3*, as some of the *P. sabiae* strains, suggesting horizontal gene transfer between the species [7]. A comparative genomic analysis revealed that several *Mimosa*-nodulating *Paraburkholderia* present a unique copy of nodulation genes, whereas those of the papilionoid-nodulating clade present two copies of *nodC* and *nodB* genes, of different sizes, and located at a considerable distance in the chromosome [18]. Interestingly, the shorter *nodC* copy may also play a role in host range.

The sv. africana clustered South African CFR (Cape Floristic Region) strains isolated from nodules of native and endemic Papilionoid legumes, except for *P. dipogonis* ICMP 19430[T], isolated from nodules of *Dipogon lignosus* in New Zealand (Fig. 5). It is worth mentioning that both *P. tuberum* STM678[T] and *P. dipogonis* ICMP 19430[T] failed to nodulate mimosoids [28,50]. This reflects the highly divergent *nod* genes (and probably Nod factors) of the papilionoid and mimosa nodulating *Paraburkholderia*.

Finally, the sv. piptadeniae is for now represented only by one species, *P. ribeironis,* exhibiting the same phylogenetic pattern in all phylograms. *P. ribeironis* STM7296[T] was isolated from the same geographic location and same plant host as *P. piptadeniae* STM 7183[T] but presented different phylogenetic positions in the symbiotic genes analysis.

The *nodAC*, *nifH* and *nifHDK* (Figs. 3 and 4, S3) phylogenies revealed that the *nodABC* and *nifHDK* of the svs. mimosae, tropicalis, atlantica and piptadenia have probably been acquired simultaneously, given the monophyletic character [18]. Oppositely, *nod* and *nif* genes from papilionoid-nodulating *Paraburkholderia* belonging to sv. africana appear to have a different origin. Previous comparative genomic analysis [18,31] revealed that South-African strains have their symbiotic genes in chromosomal islands, while in strains from Central and South-American they are plasmid-borne. In the Papilionoid-nodulating strains, *nod* and *nif* genes are very closely related to alpha-rhizobia, particularly *Methylobacterium nodulans* and *Bradyrhizobium*, from which those genes have been probably acquired [18,31]. Altogether, this suggests a different evolutionary origin of these genes, similar to the observed in the sv. africana. Differences in nodulation genes should affect *Paraburkholderia* host range, explaining why South-African and American strains fail in nodulating each other hosts [36,47].

The phylogram of *fixABC* (Fig. S4) indicates the same evolutionary pattern of *nod* and *nif* phylogenies, reinforcing the proposed symbiovar clusters. Results obtained by De Meyer et al. [18] suggest that *fix* genes were obtained from a free-living diazotrophic ancestor in all beta-rhizobia, which could explain the ability of *P. phymatum* and *P. tuberum* to fix nitrogen *ex planta* [18,29]. However, in the study by De Meyer et al. [18] several differences in *nod*, *nif* and *fix* genes were detected between *Mimosa*-nodulating and *Papilionoid Paraburkholderia*, with an emphasis on *nifA*, indicating different evolutionary stories. Other phylogenetic studies with symbiotic *Paraburkholderia* are necessary to truly clarify the origin of symbiotic genes.

Differences in the phylogenetic position of core and symbiotic genes are also reported in alpha-rhizobia. For example, a study conducted with symbionts of *Phaseolus dumosus* shows that *Rhizobium* strains belonging to the sv. tropici present different position in the 16S rRNA and MLSA phylogenies. However, they share a very

similar symbiotic plasmid, apparently indicating horizontal gene transfer [65].

In the sv. mimosae, *P. mimosarum* LMG 23256[T] was isolated from the invasive *M. pigra* in Taiwan [12]. Interestingly, *M. pigra* is originally from tropical America, but is now widespread throughout the tropics (e.g. Australia, Africa, Southeast Asia, Florida) [38]. *M. pigra* was possibly introduced in these countries together with its symbionts, explaining the phylogenetic pattern with the other strains of the sv. mimosae. Furthermore, *P. mimosarum* STM3621 was isolated from French Guiana soils using *M. pudica* as trap plant [54], reaffirming that *P. mimosarum* strains have a South American origin.

Strains belonging to the sv. tropicalis, *P. piptadeniae* STM 7183[T] and *P. franconis* strains CNPSo 3157[T], CNPSo 3191 and CNPSo 3200 are phylogenetic related and they have all been isolated from the Brazilian Atlantic Forest biome. Bournaud et al. [8] isolated *P. piptadeniae* using *Piptadenia gonoacantha* as trap plant and reported differences in the rhizobia diversity between site samples, indicating that soil conditions influence the survival and biogeography location of the symbionts, modulating rhizobia diversity [7].

Finally, *Paraburkholderia* sp. UYPR1.413 isolated from *Parapiptadenia rigida* in Uruguay did not group with any of the proposed symbiovars, indicating that others symbiovars should soon be described.

Altogether, our results confirm Brazil as a major center of nodulating nitrogen-fixing *Paraburkholderia* diversity [4,7,14,62,72], as well as South Africa [3,18,39,48] (Fig. 5), with potential influence of biogeographical [7,25,17,72] and abiotic [7,30,33,51,54] conditions, and of co-evolution with the host plants, that resulted in differences in the evolution of the symbiotic genes, clearly defining symbiovars. The delineation of symbiovars and other bacterial ecotypes contributes to the understanding of functional diversity and evolutionary ecology. We also highlight the importance of studies conducted with native and endemic species of legumes and their *Paraburkholderia* symbionts, as they are key to define the evolutionary relationships among the symbiotic partners.

The following new sequences have been deposited in GenBank database:

nodA of *P. guartelaensis* CNPSo 2995 (MT465921), *P. guartelaensis* CNPSo 3003 (MT465922), *P. guartelaensis* CNPSo 3016 (MT465923), *P. guartelaensis* CNPSo 3023 (MT465924), *P. guartelaensis* CNPSo 3248 (MT465925), *P. atlantica* CNPSo 3150 (MT465909), *P. atlantica* CNPSo 3196 (MT465910), *P. franconis* CNPSo 3191 (MT465907) and *P. franconis* CNPSo 3200 (MT465908).

nifH of *P. guartelaensis* CNPSo 2995 (MT465902), *P. guartelaensis* CNPSo 3003 (MT465903), *P. guartelaensis* CNPSo 3016 (MT465904), *P. guartelaensis* CNPSo 3023 (MT465905), *P. guartelaensis* CNPSo 3248 (MT465906), *P. atlantica* CNPSo 3150 (MT465890), *P. atlantica* CNPSo 3196 (MT465891), *P. franconis* CNPSo 3191 (MT465888) and *P. franconis* CNPSo 3200 (MT465889).

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Ethics approval and consent to participate

The authors declare no ethical conflicts; authors declare that they have consented to participate in the manuscript and publish it.

Competing interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.syapm.2020.126151.

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