FEMS Microbiology Ecology, 95, 2019, fiz099

doi: 10.1093/femsec/fiz099
Advance Access Publication Date: 27 June 2019
Research Article

RESEARCH ARTICLE

A leguminous species exploiting alpha- and beta-rhizobia for adaptation to ultramafic and volcano-sedimentary soils: an endemic *Acacia spirorbis* model from New Caledonia

Bryan Vincent[1,*], Farid Juillot[2], Emmanuel Fritsch[2], Agnieszka Klonowska[3], Noëmie Gerbert[1], Sarah Acherar[1], Cedric Grangeteau[1,†], Laure Hannibal[1], Antoine Galiana[4], Marc Ducousso[4] and Philippe Jourand[1]

[1]IRD, LSTM UMR040, TA A-82/J, Campus International de Baillarguet, 34398 Montpellier cedex 5, France, [2]IRD, IMPMC UMR206, 98848 Nouméa Cedex, New Caledonia, [3]IRD, Cirad, Univ. Montpellier, Interactions Plantes Microorganismes Environnement (IPME), 34394 Montpellier, France and [4]CIRAD, LSTM UMR082, TA A-82/J, Campus International de Baillarguet, 34398 Montpellier cedex 5, France

*Corresponding author: Université Catholique de Louvain - ELIM -Croix du Sud 2/L7.05.06 - 1348 Louvain-la-Neuve - Belgium. E-mail: bryan.vincent@uclouvain.be

One sentence summary: An endemic Acacia from New Caledonia establishes symbioses with diverse bacteria in order to survive soils harsh environments.

Editor: Paolina Garbeva

†Cedric Grangeteau, http://orcid.org/0000-0002-5286-632X

ABSTRACT

Acacia spirorbis subsp. *spirorbis* Labill. is a widespread tree legume endemic to New Caledonia that grows in ultramafic (UF) and volcano-sedimentary (VS) soils. The aim of this study was to assess the symbiotic promiscuity of *A. spirorbis* with nodulating and nitrogen-fixing rhizobia in harsh edaphic conditions. Forty bacterial strains were isolated from root nodules and characterized through (i) multilocus sequence analyses, (ii) symbiotic efficiency and (iii) tolerance to metals. Notably, 32.5% of the rhizobia belonged to the *Paraburkholderia* genus and were only found in UF soils. The remaining 67.5%, isolated from both UF and VS soils, belonged to the *Bradyrhizobium* genus. Strains of the *Paraburkholderia* genus showed significantly higher nitrogen-fixing capacities than those of *Bradyrhizobium* genus. Strains of the two genera isolated from UF soils showed high metal tolerance and the respective genes occurred in 50% of strains. This is the first report of both alpha- and beta-rhizobia strains associated to an *Acacia* species adapted to UF and VS soils. Our findings suggest that *A. spirorbis* is an adaptive plant that establishes symbioses with whatever rhizobia is present in the soil, thus enabling the colonization of contrasted ecosystems.

Keywords: *Bradyrhizobium*; *Paraburkholderia*; New Caledonia; *Acacia spirorbis*; nitrogen fixation; metal tolerance

Received: 11 April 2019; **Accepted:** 25 June 2019

INTRODUCTION

New Caledonia (NC) is a tropical archipelago located in the south-west Pacific, about 1200 km from the east coast of Australia. The geological history of NC is complex and as a result, many kinds of bedrocks shape the archipelago (Pelletier 2006; Bonvallot, Gay and Habert 2012). These bedrocks were altered by the climate, resulting in the formation of different soil types: acrisols, cambisol, leptosols and ferralsols. Thanks to this topographical and climatic diversity, the archipelago hosts noteworthy terrestrial biodiversity (Jaffré 1993; Bonvallot, Gay and Habert 2012). For instance, more than 3371 vascular plants species have been identified, of which 80% are endemic to NC (Morat *et al.* 2012). On the other hand, NC is affected by recurrent fires, extensive grazing, urbanization and mining (Jaffré, Bouchet and Veillon 1998; Jaffré, Munzinger and Lowry 2010; Losfeld *et al.* 2014; Ibanez *et al.* 2019). Given these threats, NC is considered a biodiversity hotspot (Myers *et al.* 2000; Marchese 2015).

Part of the terrestrial biodiversity has been shaped by the ultramafic (UF) environments that account for a third of the main island (Pillon *et al.* 2010; Isnard *et al.* 2016). The harsh properties of UF soils render plant development difficult: iron accounts for up to 50% of the total weight of the soil, and there are low concentrations of the main plant nutrients (nitrogen, phosphorus and potassium), a highly unbalanced calcium:magnesium ratio (up to 1:42) and high concentrations of metals such as cobalt, chromium, nickel and manganese (Proctor 2003; Kazakou *et al.* 2008). Nickel deposits are mined by several companies and play a key role in the NC economy today. In 2008, the mining industry accounted for 95% of NC exports and employed 6% of the labor force (Jaffré and L'Huillier 2010). On the other hand, mining companies are responsible for stripping over 20 000 ha of vegetation, which corresponds to 1.2% of the land territory (Jaffré and L'Huillier 2010). In accordance with local laws, the mining companies are responsible for rehabilitating the soil after extracting the nickel ore (Losfeld *et al.* 2014). Several Australian *Eucalyptus* and *Acacia* species have been introduced for the purpose of ecological restoration of post-mining soils, but none survived the harsh conditions of the UF soils (Sarrailh 2002). As a result, the use of endemic plant species that occur naturally in UF habitats was investigated.

One noteworthy candidate on the main island is the legume tree *Acacia spirorbis* subsp. *spirorbis* Labill., which naturally grows in UF and volcano-sedimentary (VS) environments. The presence of *A. spirorbis* in NC probably results from long-term dispersal from Australia (Richardson *et al.* 2011). Like many Australian *Acacia* species, *A. spirorbis* also displays invasive behavior in degraded ecosystems (Richardson, Le Roux and Wilson 2015). A phylogenetic study showed that *A. spirorbis* is endemic to the NC archipelago (Brown *et al.* 2012). This legume species is able to establish symbioses with both arbuscular and ectomycorrhizal fungi, as well as with nitrogen-fixing bacteria, in both UF and VS soils (Grangeteau *et al.* 2012; Ducousso *et al.* 2014; Houlès *et al.* 2018). The adaptation of *Acacia spirorbis* to such contrasted soils is unique and several authors have argued that this adaptation might be linked with its symbioses with microorganisms (Houlès *et al.* 2018; Vincent *et al.* 2018). For example, the ectomycorrhizal fungal communities, in which Basidiomycota account for 92% of the species, were structured by the soil type and abiotic parameters (Houlès *et al.* 2018). These authors thus concluded that the edaphic parameters of NC soils structure *A. spirorbis* ectomycorrhizal symbionts. A previous study by our team focused on the *in situ* nitrogen-fixing potential of *A. spirorbis* and reported that 80–100% of plant nitrogen was supplied by the nitrogen-fixing symbiosis throughout various UF and VS environments (Vincent *et al.* 2018).

However, little is known about the rhizobia that are symbiotically associated with *A. spirorbis*. The aims of the present study were thus to investigate (i) the diversity of the nitrogen-fixing bacteria symbiotically associated with *A. spirorbis* and (ii) the potential of these rhizobia to adapt to the harsh conditions of UF and VS soils. We addressed two specific questions: which rhizobia are naturally found in the nodules of *A. spirorbis* in UF and VS environments, and is there a relationship between the contrasted edaphic conditions and the characteristics of the rhizobia including taxonomy, nitrogen-fixing efficiency and heavy-metal tolerance? To answer these questions, *A. spirorbis* bacterial symbionts were isolated from root nodules naturally found in UF and VS environments. The molecular diversity of the bacterial strains was assessed with a multilocus analysis based on the 16S rRNA gene and on housekeeping genes including *dnaK*, *gyrB*, *atpD* and *recA*. The symbiotic efficiency of each strain was tested on *A. spirorbis* seedlings in a growth room. In addition, nitrogen-fixing markers were investigated (nodulation gene *nodA* and nitrogen-fixing gene *nifH*). The metal tolerance of rhizobia was also tested *in vitro*, and the presence of the nickel and cobalt resistance gene *cnrA* and nickel resistance gene *nreB* was investigated in parallel.

MATERIALS AND METHODS

Study sites and soil sampling

Natural populations of *A. spirorbis* were sampled at eight study sites distributed in UF and VS soils (Fig. 1, Table 1). Soil samples were collected as described in Vincent *et al.* 2018, from the top 30 cm layer, where nodules are assumed to find the best conditions for their development (Kessel and Roskoski 1981). Total concentrations of aluminum, calcium, chromium, cobalt, iron, magnesium, manganese, nickel, phosphorus, potassium, silicium, organic carbon and nitrogen in the soil were quantified as described in Vincent *et al.* 2018 (Table 2 and supplementary Figure S1 available online). Soils were identified using WRB Classification (http://www.fao.org/3/a-i3794e.pdf).

Isolation of bacterial strains

Five fresh nodules per plant were sampled from three *A. spirorbis* roots at each study site. All the trees sampled were mature and spaced at least 10 m away from each other, as described in Vincent *et al.* (2018). The nodules were kept in Falcon® tubes on ice and then placed in a cooler at 4°C. The nodules were individually washed with sterile ultrapure water, surface-sterilized in 70% ethanol and 3% calcium filtered hypochlorite, then crushed in 50 µL of sterile ultrapure water. The suspension was streaked on yeast mannitol agar plates (YMA; Vincent 1970) and incubated at 28°C for 48 h. The colonies growing in the first isolation plates were then streaked on second plates to obtain pure cultures. For long-term maintenance, bacterial strains were grown in YM broth and preserved in 25% glycerol at −80°C.

Molecular methods

For routine polymerase chain reaction (PCR) amplifications, bacterial DNA was obtained from cultures of pure bacterial isolates using a quick Proteinase K method, as described by Wilson (2001). PCR amplifications were performed with the GoTaq

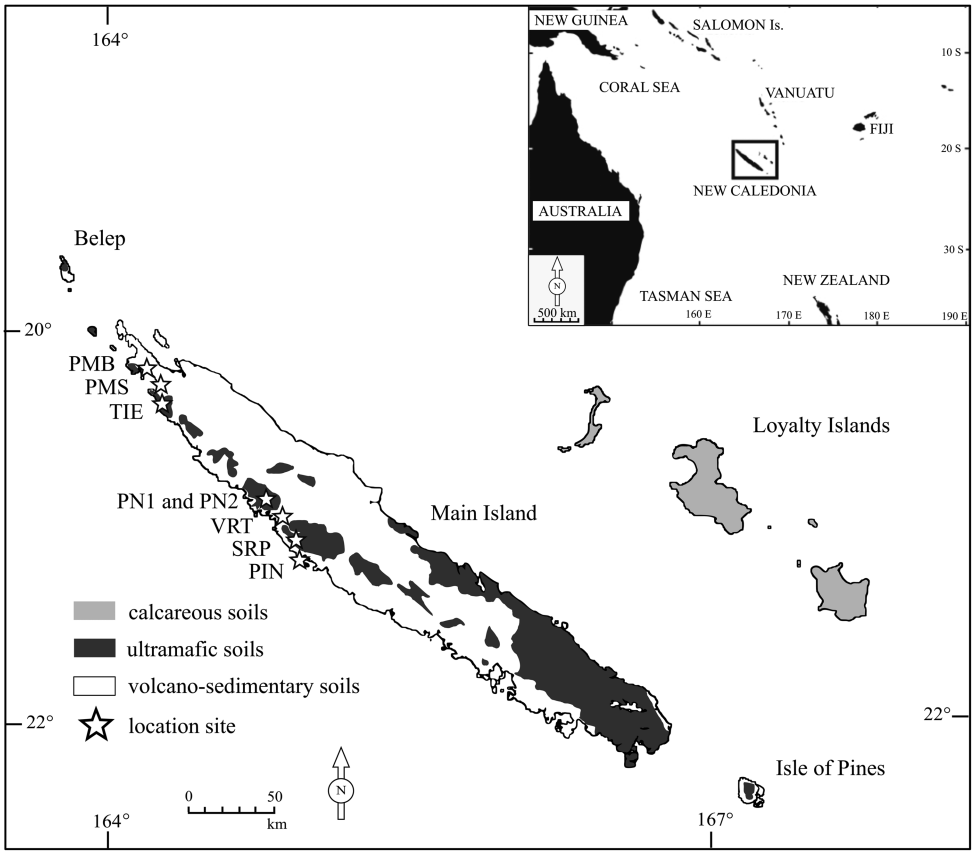

Figure 1. Map of New Caledonia showing the three main soils categories: calcareous (in grey), ultramafic (in dark grey) and volcano-sedimentary (in white). White stars show the location of the study sites. Legend of the study sites: PIN: Pindaï; PMB: Poum Malabou; PMS: Poum Silice; PN1: Pandanus 1; PN2: Pandanus 2; SRP: Serpent; VRT: Vertisol.

Table 1. Sampling sites: name, soil type, WRB classification, soil description, coordinates and elevation. Sites are grouped according to their soil type (ultramafic or volcano-sedimentary).

Site name	Soil type	Soil WRB classification	Soil description	Coordinates	Elevation
Pindaï	Ultramafic	Haplic ferralsol	Red-brown soil	S 21°19'55.45" E 164°58'19.24"	65 m
Pandanus 1	Ultramafic	Haplic cambisol	Brown gravely soil	S 21°01'33.36" E 164°46'59.12"	38 m
Pandanus 2	Ultramafic	Abruptic ferralsol	Brown clayey soil with pisolitic cover	S 21°01'58.8" E 164°46'40.97"	31 m
Tiébaghi	Ultramafic	Geric plinthosol	Red pisolitic soil	S 20°2819.20" E 164°15'35.16"	69 m
Poum Malabou	Volcano-sedimentary	Leptic cambisol	Brown gravel-rich soil	S 20°17'32.56" E 164°05'22.60"	48 m
Poum Silice	Volcano-sedimentary	Geric acrisol	Silicic bleached soil	S 20°13'38.5" E 164°04'44.52"	14 m
Serpent	Volcano-sedimentary	Haplic Cambisol	Thin silty grey-brown soil	S 21°10'27.74" E 164°52'40.47"	75 m
Vertisol	Volcano-sedimentary	Haplic Vertisol	Black clayey soil	S 21°07'59.75" E 164°54'55.49"	16 m

DNA polymerase kit (Promega, Madison, WI, USA) according to the manufacturer's instructions, using 0.625 unit of GoTaq and 20 μmol of each primer in 25 μL of reaction volume. A routine PCR protocol was applied to the DNA amplifications based on 35 cycles of 30 s of denaturation, 30 s of hybridization and 1 min of elongation. The full list of the primers and their respective melting temperatures (Tm) are available in supplementary Table S1 available online. Briefly, we performed PCR assays targeting the following genes: partial 16S rRNA, *dnaK*, *gyrB*, *atpD* and *recA* for the multilocus sequence analysis (MLSA), the genes *nodA* and *nifH* for the symbiotic characterization, and lastly, the genes *nreB* and *cnrA* for the metal tolerance approach. Each DNA resulting

Table 2. Physical-chemical properties of the soils at the eight study sites. Mean concentrations ($n = 3$) of each element are expressed in milligrams per kilogram of dry matter (mg kg^{-1} ± standard error). Sites are grouped according to their soil category.

Soil category	Study site	pH	C	N	C/N	P	K	Ca	Mg	Ca/Mg
Ultramafic	Pindaï	6.8 ± 0.1	4900 ± 1900	2175 ± 525	2.2 ± 0.4	141 ± 18	1151 ± 135	4635 ± 1135	9100 ± 1407	0.50 ± 0.05
	Pandanus 1	6.4 ± 0.1	3150 ± 350	1170 ± 20	2.7 ± 0.7	34 ± 15	73 ± 8	1380 ± 310	39 370 ± 14 215	0.037 ± 0.005
	Pandanus 2	5.9 ± 0.03	800 ± 100	380 ± 40	2.2 ± 0.5	54 ± 54	149 ± 58	150 ± 20	14 297 ± 4581	0.01 ± 0.002
	Tiébaghi	5.4 ± 0.1	1350 ± 250	470 ± 60	2.9 ± 0.2	129 ± 4	89 ± 15	225 ± 15	2107 ± 73	0.11 ± 0.01
Volcano-sedimentary	Poum Malabou	6.5 ± 0.2	2267 ± 896	1027 ± 226	2.1 ± 0.4	100 ± 16	6104 ± 2263	1020 ± 43	44 445 ± 3558	0.02 ± 0.003
	Poum Silice	4.2 ± 0.03	2800 ± 1667	387 ± 113	6.6 ± 2.1	27 ± 14	27 ± 19	183 ± 53	83 ± 23	2.2 ± 0.5
	Serpent	6.5 ± 0.02	3300 ± 3612	1427 ± 1243	1.8 ± 0.6	431 ± 131	4930 ± 1685	46 780 ± 8397	34 792 ± 4680	1.3 ± 0.9
	Vertisol	5.8 ± 0.5	4550 ± 2350	1695 ± 775	2.6 ± 0.2	162 ± 34	236 ± 69	6095 ± 285	8171 ± 497	0.75 ± 0.08

(continued)

Soil category	Study site	Al	Co	Cr	Fe	Mn	Ni	Si
Ultramafic	Pindaï	52 405 ± 2125	517 ± 109	19 965 ± 677	261 740 ± 11 460	4453 ± 371	7556 ± 2449	146 415 ± 3025
	Pandanus 1	17 860 ± 2640	595 ± 39	24 865 ± 4276	289 175 ± 38 305	5772 ± 937	8230 ± 1521	126 140 ± 28 770
	Pandanus 2	13 805 ± 2965	797 ± 404	31 598 ± 8648	327 700 ± 79 890	4783 ± 2272	3970 ± 688	110 525 ± 50 055
	Tiébaghi	45 875 ± 1865	753 ± 39	15 047 ± 1035	508 175 ± 2895	19 328 ± 2118	2650 ± 28	17 695 ± 445
Volcano-sedimentary	Poum Malabou	67 287 ± 9586	98 ± 11	1509 ± 116	61 920 ± 1971	818 ± 93	1792 ± 105	265 167 ± 14 164
	Poum Silice	250 ± 57	< 0.01	153 ± 74	103 ± 69	2 ± 0.8	31 ± 11	431 537 ± 8276
	Serpent	81 020 ± 2416	52 ± 3	359 ± 52	62 980 ± 1361	1178 ± 124	241 ± 59	209 353 ± 6289
	Vertisol	76 800 ± 2240	48 ± 2	344 ± 3	89 865 ± 1425	1008 ± 76	49 ± 2	226 270 ± 530

from PCR amplification was purified and sequenced as described in Moulin *et al.* (2001).

Phylogenetic analyses

Phylogenies were carried out using a MLSA, as previously reported by Bontemps *et al.* (2016). Briefly, each DNA consensus sequence was obtained with an alignment between forward and reverse sequences and manually edited on ChromasPro software, version 2.1.8. All DNA sequences were deposited in the DNA Data Bank of Japan (DDJB) for attribution of an accession number (supplementary Tables S2 and S3 available online). A set of sequences belonging to the same molecular marker were aligned using MultAlin (inra.fr/multalin/multalin.html) (Corpet 1988). Each phylogenetic analysis included a set of reference DNA sequences belonging to the same bacterial genus. Reference sequences used for molecular diversity for alpha- and beta-rhizobial references are listed in supplementary Tables S4 and S5 available online, respectively. Reference sequences of symbiotic markers *nodA* and *nifH* are listed in supplementary Tables S6 and S7 available online, respectively. Phylogenies were inferred using the maximum likelihood (ML) method using PhyML (Guindon *et al.* 2005). Bootstrap analyses were performed using 1000 replicates for ML using free phylogeny.fr software (http://www.phylogeny.fr/version2_cgi/index.cgi) (Dereeper *et al.* 2008, 2010). Concatenated phylogenetic trees were inferred from concatenated sequences obtained with the Genious software version 11.1.4 (Figs. 2 and 3). Concatemers were obtained by juxtaposing the DNA sequences of (i) the genes rRNA16S, *dnaK*, *atpD* and *recA* for alpha-rhizobia (Fig. 2) and (ii) the genes rRNA16S, *gyrB*, *atpD* and *recA* for beta-rhizobia (Fig. 3). Individual phylogenetic trees were obtained for each alpha- and beta-rhizobia gene (supplementary Figures S2–S9 available online). Leaf names and bootstrap values of the phylogenetic trees were edited with Inkscape software, version 0.92.3 (https://inkscape.org/fr/).

Plant nodulation and symbiotic efficiency tests

Plant nodulation and symbiotic efficiency tests were carried out according to Bontemps *et al.* (2016). Briefly, *A. spirorbis* seeds were surface sterilized for 10 min in 96% H_2SO_4, followed by five washes with sterile distilled water. Seeds were soaked overnight in sterilized water at 28°C and then placed on 0.8% water agar plates. Four days later, germinated seedlings were transferred in test tubes for *in vitro* nodulation tests. In each test tube, sterilized stiff paper was bent at the top and a 2-mm diameter hole was drilled. The seedling teguments were peeled off and the roots of the seedlings were passed through the hole in the paper. Test tubes were watered with Jensen medium (Jensen 1942). Seedlings were inoculated with bacterial strains grown in YM medium (Vincent 1970). The test tubes were closed with carded cotton plugs, then placed in a tropical plant chamber for 2 months (28°C, 70% humidity, day:night, 16:8 h). Five replicate test tubes were made for each bacterial strain tested. The symbiotic efficiency of each strain was measured from *in vitro* cultures after 2 months. The nitrogenase activity was assessed using the acetylene reduction assay as described by Hardy *et al.* (1968). After incubation in an oven for 48 h at 60°C, *A. spirorbis* seedling shoot and root dry biomass was weighed. All the nodules on each seedling were numbered and weighed. Raw values of nitrogen fixation are expressed in µmol of ethylene per hour and per sample (Hardy *et al.* 1968). In addition, the nitrogen-fixation efficiency is expressed in milligrams of nodule dry weight required to produce 100 mg of shoot dry weight (Jourand *et al.* 2005).

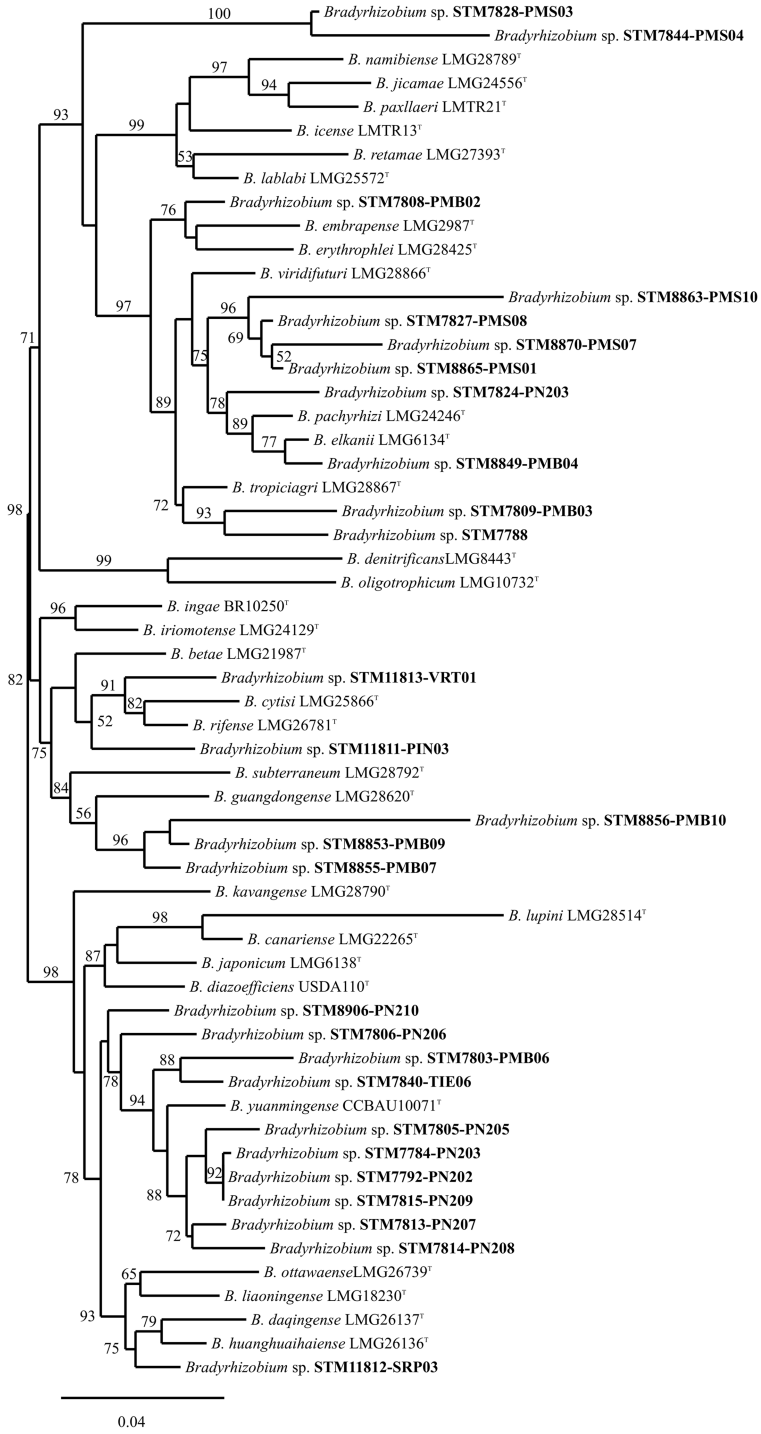

Figure 2 Phylogenetic tree of *Acacia spirorbis* alpha-rhizobial symbionts and related species. The tree was inferred from concatenated sequences of the genes 16S rRNA, *dnaK*, *atpD* and *recA*. The number at the node represents the bootstraps percentage of 1000 replicates (shown only when > 50%). Strains isolated in this study (New Caledonia) are in bold. The scale bar indicates the number of substitutions per site.

Metal tolerance tests

The tolerance of bacterial isolates to aluminium, cobalt, chromium, iron, manganese and nickel was assessed through the half maximal inhibitory concentration (IC_{50}) according to Maier (2015). Bacterial strains were grown in 96-well microplates filled with 200 μL of YM medium per well (Vincent 1970). Each metal was supplemented as follows: for Al^{3+}, $Al(NO_3)_3$ was added with final concentrations of Al^{3+} of 0.1, 0.5, 1, 5, 10 and 39 mM; for Co^{2+}, $CoCl_2$ was added with final concentrations of Co^{2+} of 0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 50 mM; for Cr^{6+}, $K_2Cr_2O_7$ was added with final concentrations of Cr^{6+} of 0.02, 0.1, 0.2, 1, 2, 10, 20 and 100 mM; for Ni^{2+}, $NiCl_2$ was added with final concentrations of Ni^{2+} of 0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 50 mM; for Mn^{2+}, $MnCl_2$ was added with final concentrations of Mn^{2+} of 0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 50 mM; and for Fe^{3+}, $FeCl_3$ was added with

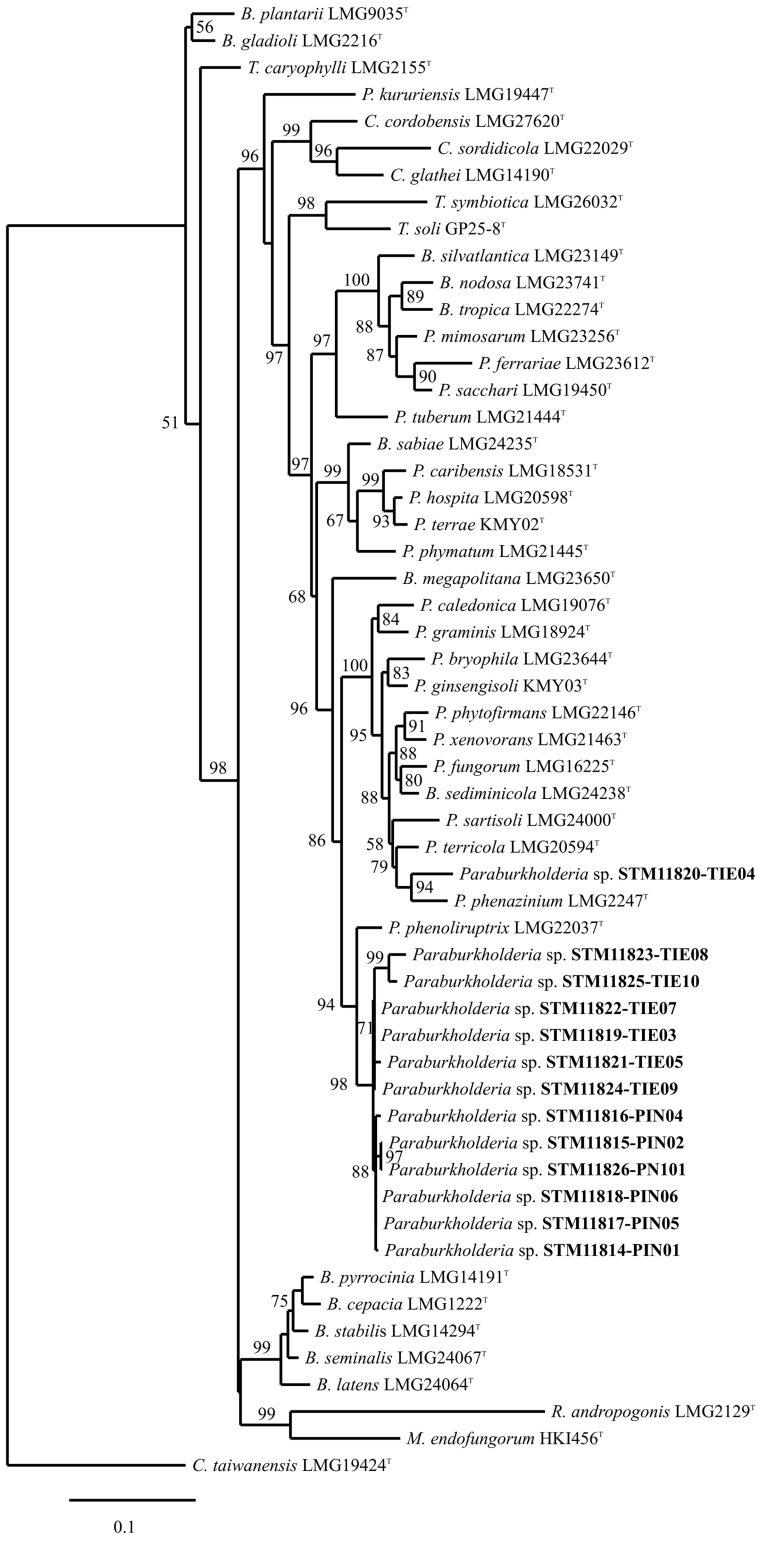

Figure 3. Phylogenetic tree of *Acacia spirorbis* beta-rhizobial symbionts and related species. The tree was inferred from concatenated sequences of the genes 16S rRNA, *gyrB*, *atpD* and *recA*. The numbers at the node represents the bootstraps percentage of 1000 replicates (shown only when >50%). Strains isolated in this study (New Caledonia) are in bold. The scale bar indicates the number of substitutions per site.

final concentrations of Fe^{3+} of 0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 50 mM. Cultures of rhizobia were calibrated at optical density (OD) measured at $\lambda = 600$ nm ($OD_{\lambda 600nm}$) = 1 using an Infinite M200 Microplate Spectrophotometer (TECAN, Männedorf, Switzerland) and incubated for up to 6 days at 28°C under agitation. Bacterial growth was monitored by $OD_{\lambda 600nm}$ after 0, 18, 24, 41 and 48 h post-inoculation. Bacterial strains in YM medium (Vincent 1970) without supplemented metal were used as controls.

Statistical analyses

Statistical analyses were performed with R software, using the 'RVAideMemoire' package, version 0.9–54, available at the following link: http://CRAN.R-project.org/package=RVAideMemoire, and the package 'agricolae', version 1.2–3, available at http://CRAN.R-project.org/package=agricolae. When the data did not follow a normal distribution, non-parametric tests were used. Correlations between numerical variables were assessed using a non-parametric Spearman's rank correlation test. Kruskal–Wallis tests were used to evaluate the effect of a factor on a given numerical parameter. When a factor was found to have a significant effect ($P < 0.05$) on a given parameter, pairwise Wilcoxon rank sum tests were applied at a confidence level of 0.05 (Houlès *et al.* 2018; Vincent *et al.* 2018). Principal component analyses (PCA) were performed to analyze the distribution of the rhizobial symbiotic efficiency and heavy metal tolerance according to the bacterial genus, the soil type (UF or VS) and the combination of genus and soil type. In the following, the term 'strains sampled in UF or VS' is an abbreviation for rhizobial strains isolated from nodules grown in UF or VS soils.

Nucleotide sequence accession numbers

Partial DNA sequences have been deposited in the DDJB database (supplementary Tables S2 and S3 available online). Accession numbers for *Bradyrhizobium* DNA sequences are: LC383474 to LC383500 for the 16S rRNA gene; LC383501 to LC383527 for the *atpD* gene; LC383555 to LC383581 for the *dnaK* gene; LC383528 to LC383554 for the *recA* gene; LC383656 to LC383682 for the *nodA* gene; and LC383683 to LC383709 for the *nifH* gene (supplementary Table S2 available online). Accession numbers for *Paraburkholderia* DNA sequences are: LC383582 to LC383594 for the 16S rRNA gene; LC383595 to LC383607 for the *atpD* gene; LC383608 to LC383619 for the *gyrB* gene; LC383620 to LC383632 for the *recA* gene; and LC416540 for the *nifH* gene (supplementary Table S3 available online).

RESULTS

Soil properties at the study sites

The soil properties of the eight study sites are listed in Table 2. As revealed by PCA (supplementary Figure S1 available online), the eight study sites can be grouped in two contrasted soil types: UF and VS soils, based on their chemical composition (Table 2). The UF soils from the Pindaï, Pandanus 1, Pandanus 2 and Tiébaghi study sites contained significantly higher concentrations of metallic elements, notably iron, than the VS soils from Poum Malabou, Poum Silice, Serpent and Vertisol sites (Table 2). Concentrations of cobalt ranged from 517 ± 109 to 797 ± 404 mg kg^{-1} in UF soils and from nil (<0.01 mg kg^{-1}) to 98 ± 11 mg kg^{-1} in VS soils (Table 2). Chromium concentrations ranged from 15 047 ± 1035 to 31 598 ± 8648 mg kg^{-1} in UF soils and from 344 ± 3 to 1509 ± 116 mg kg^{-1} in VS soils (Table 2). Iron concentrations ranged from 289 175 ± 38 305 to 508 175 ± 2895 mg kg^{-1} in UF soils and from 103 ± 69 to 89 865 ± 1425 mg kg^{-1} in VS soils (Table 2). Manganese concentrations ranged from 4453 ± 371 to 19 238 ± 2118 mg kg^{-1} in UF soils and from 2 ± 0.8 to 1178 ± 124 mg kg^{-1} in VS soils (Table 2). Nickel concentrations ranged from 2650 ± 28 to 8230 ± 1521 mg kg^{-1} in UF soils and from 31 ± 11 to 1792 ± 105 mg kg^{-1} in VS soils (Table 2). Silicon concentrations differed between VS and UF, ranging from 209 353 ± 6289 to 431 537 ± 8276 mg kg^{-1} in VS soils and from 17 695 ± 445 to 146 415 ± 3025 mg kg^{-1} in UF soils (Table 2).

Molecular characterization of the bacterial strains

A total of 40 bacterial strains were isolated from the root nodules of *A. spirorbis* (24 strains from UF soils and 16 strains from VS soils). The MLSA based on concatenated sequences of four genetic markers confirmed that all the strains we isolated belonged either to the genus *Bradyrhizobium* and *Paraburkholderia* (Figs 2 and 3). This result is confirmed by independent phylogenetic analyses based on the following genetic markers: 16S rRNA, *dnaK*, *atpD* and *recA* for *Bradyrhizobium* sp. strains (supplementary Figures S2, S3, S4 and S5 available online, respectively) and the genetic markers 16S rRNA, *gyrB*, *atpD* and *recA* for *Paraburkholderia* sp. strains (supplementary Figures S6, S7, S8 and S9 available online, respectively). Among the 40 strains, 67.5% belonged to the genus *Bradyrhizobium* and 32.5% to the genus *Paraburkholderia*. For the *Bradyrhizobium* sp. strains, a phylogenetic tree was constructed by MLSA, based on the concatenate sequences of the 16S rRNA gene (LC383474 to LC383500), the *atpD* gene (LC383501 to LC383527), the *dnaK* gene (LC383555 to LC383581) and the *recA* gene (LC383528 to LC383554) (Fig. 4). The MLSA showed that the strains of *Bradyrhizobium* sp. clustered in three putative major groups (Fig. 2). Group 1 contained five strains from two study sites (PMS and PN2; see Fig. 1 for site abbreviations) and formed a cluster next to *Bradyrhizobium pachyrhizi* and *Bradyrhizobium elkanii*. Group 2 contained three strains from PMB, next to *Bradyrhizobium guangdongense*. Group 3 contained 10 strains from three study sites (PMB, PN2 and TIE) and formed a cluster next to *Bradyrhizobium yuanmingense*. The nine remaining strains of *Bradyrhizobium* sp. isolated were grouped in two clusters of two strains and the last five strains were individual lineages scattered inside the phylogeny (Fig. 2).

A phylogenetic tree was constructed for the *Paraburkholderia* sp. strains by MLSA, based on the concatenated sequences of the 16S rRNA gene (LC383582 to LC383594), the *atpD* gene (LC383608 to LC383619), the *gyrB* gene (LC383608 to LC383619) and the *recA* gene (LC383620 to LC383632). The MLSA (Fig. 3) showed that, except for one strain, the strains of *Paraburkholderia* sp. clustered in one homogeneous putative group. A total of 12 strains of *Paraburkholderia* sp. isolated from *A. spirorbis* nodules formed a cluster next to *Paraburkholderia phenoliruptix*, while the strain STM11820-TIE04 was located close to *Paraburkholderia phenazinium* (Fig. 3).

All the *Paraburkholderia* sp. strains were only found in UF soils, whereas 41% and 59% of *Bradyrhizobium* sp. strains were isolated from UF and VS soils, respectively. Regarding *Bradyrhizobium* sp. strains, no correlations were found between the clusters and the soil type.

Symbiotic efficiency of the bacterial strains

The *in vitro* experiments conducted to test the symbiotic efficiency of the bacterial strains showed that all *Bradyrhizobium* sp.

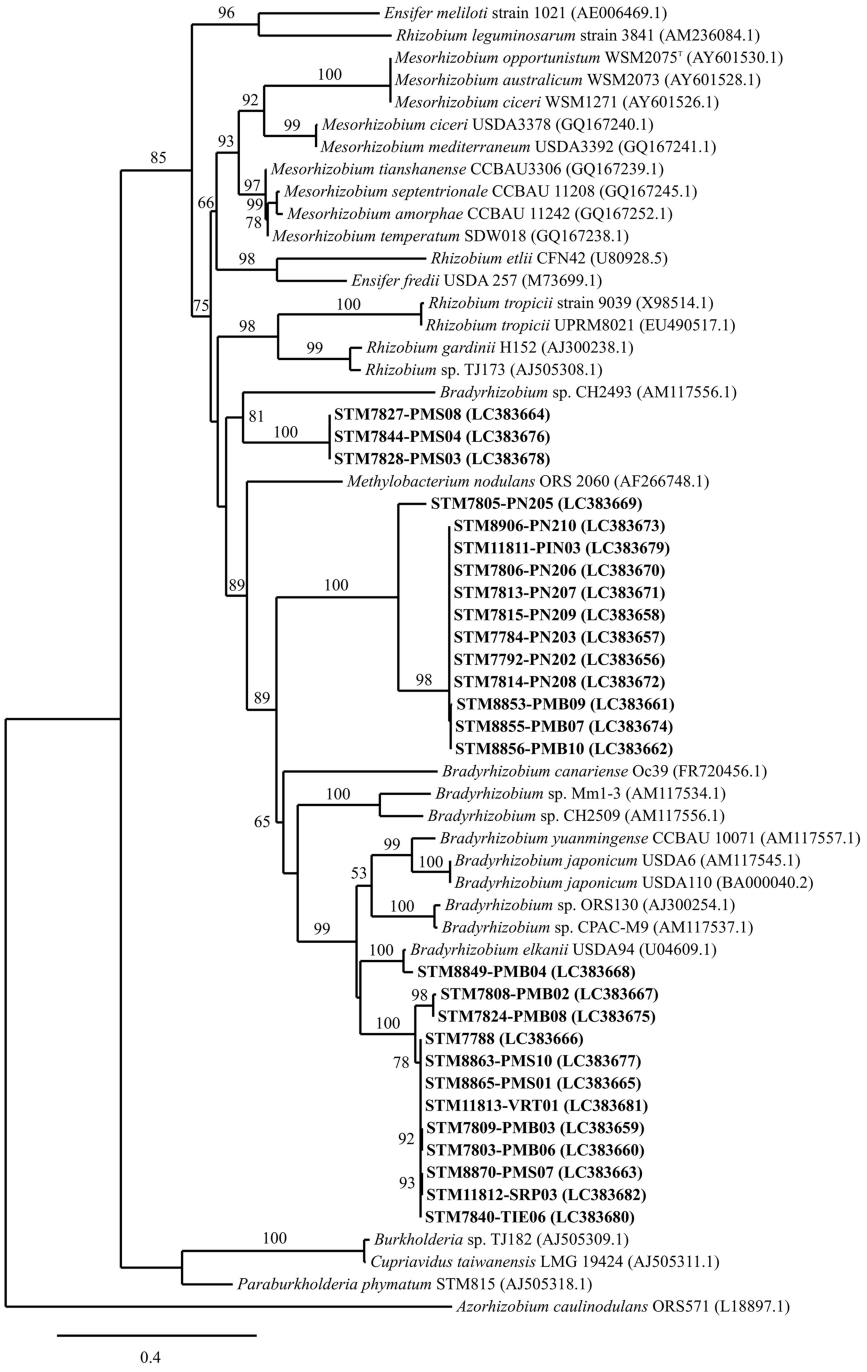

Figure 4. Rooted phylogenetic tree of *Acacia spirorbis* alpha-rhizobial symbionts and related species. The tree was inferred from sequences of the gene *nodA*. The numbers at the node represents bootstraps percentage of 1000 replicates (shown only when >50%). Strains isolated in this study (New Caledonia) are in bold. The scale bar indicates the number of substitutions per site.

and *Paraburkholderia* sp. strains described in the present study were able to form efficient nitrogen-fixing nodules on *A. spirorbis* seedlings (supplementary Tables S8 and S9 available online).

The symbiotic phenotypes (i.e. the number of nodules, biomass of nodules and symbiotic efficiency) of *A. spirorbis* seedlings grown *in vitro* differed significantly ($P < 0.05$) depending on the bacterial genus inoculated. The inoculation of *Bradyrhizobium* sp. strains induced significantly more nodules and total nodule biomass per plant (8.3 ± 3.9 nodules per plant with a mean weight of 3.9 ± 1.5 mg) than *Paraburkholderia* sp. strains (3.1 ± 1.6 nodules per plant with a mean weight of 1.9 ± 0.9 mg). The *Paraburkholderia* sp. strains showed a significantly higher symbiotic efficiency (with only 5.9 ± 2.1 mg dry weight of nodule required to form 100 mg of shoot dry weight) than *Bradyrhizobium* sp. strains (with 13.6 ± 6.9 mg nodule dry weight required to form 100 mg of shoot dry weight). However, no significant difference was found in overall nitrogen fixation in *Bradyrhizobium* sp. and *Paraburkholderia* sp. strains, with averages of 0.13 ± 0.07 and 0.14 ± 0.08 μmol of ethylene per plant per hour, respectively. Moreover, the difference in the symbiotic

Table 3. Means ± standard deviation of the data collected from the nodulation test on *A. spirorbis* seedlings. The nitrogenase activity estimated by the acetylene reduction assay is expressed in μmol of ethylene formed per hour and per seedling. Dry weights of the shoots (shoot dw), roots (roots dw) and nodules (nodule dw) are expressed in mg per plant. The number of nodules corresponds to the mean number of nodules on each seedling. The efficiency of nitrogen fixation corresponds to the dry biomass of nodules required to grow 100 mg of *A. spirorbis* shoot. The data are grouped according to (i) the genus of the rhizobia inoculated, all soil types combined, (ii) the soil type, with both rhizobia genera combined, and (iii) the genus and soil type combined. For each numerical parameter, means were compared with Kruskal–Wallis tests followed by Wilcoxon tests. The superscript letters 'a', 'b' and 'c' correspond to different mean levels significantly different between each other.

	Genus		Soil type		Genus and soil type combined		
	Paraburkholderia	*Bradyrhizobium*	UF	VS	BK-UF	BR-UF	BR-VS
Nitrogenase activity	0.14 ± 0.09[a]	0.13 ± 0.07[a]	0.11 ± 0.08[a]	0.17 ± 0.04[a]	0.14 ± 0.09[a]	0.08 ± 0.07[b]	0.17 ± 0.04[a]
Shoot dw	33.4 ± 5.6[a]	30.2 ± 7.9[a]	29.8 ± 6.4[a]	33.4 ± 8.3[a]	33.4 ± 5.6[a]	25.7 ± 4.3[b]	33.4 ± 8.3[a]
Root dw	19.8 ± 3.6[a]	20.1 ± 3.5[a]	19.5 ± 3.1[a]	20.8 ± 3.9[a]	19.8 ± 3.6[a]	19.1 ± 2.4[a]	20.8 ± 3.9[a]
Nodule dw	2.0 ± 0.8[b]	3.8 ± 1.6[a]	3.0 ± 1.7[a]	3.5 ± 1.4[a]	2.0 ± 0.8[b]	4.2 ± 1.7[a]	3.5 ± 1.4[a]
Nodule nb	3.2 ± 1.5[b]	8.0 ± 4.1[a]	5.8 ± 4.1[a]	7.5 ± 3.9[a]	3.2 ± 1.5[b]	8.8 ± 4.3[a]	7.5 ± 3.9[a]
Efficiency	5.9 ± 2.1[b]	13.6 ± 6.9[a]	10.9 ± 7.6[b]	11.3 ± 5.6[a]	5.9 ± 2.1[c]	16.9 ± 7.4[a]	11.3 ± 5.6[b]

UF: ultramafic soils; VS: volcano-sedimentary soils; BK-UF: *Paraburkholderia* strains sampled in ultramafic soils; BR-UF: *Bradyrhizobium* strains sampled in ultramafic soils; BR-VS: *Bradyrhizobium* strains sampled in volcano-sedimentary soils.

phenotypes measured between *Bradyrhizobium* or *Paraburkholderia* sp. strains had no significant impact on the dry biomass of *A. spirorbis* seedlings, with (i) shoot dry weights of 30.6 ± 7.9 and 32.6 ± 6.2 mg, respectively, and (ii) root dry weights of 20.3 ± 3.5 and 19.6 ± 3.5 mg, respectively.

Regarding the relationship between symbiotic efficiency and the soil type from which the strains were isolated (Table 3), our data clearly showed that *Paraburkholderia* sp. strains from UF soils were significantly more efficient than *Bradyrhizobium* sp. strains that were also isolated from UF soils. However, the *Bradyrhizobium* sp. strains isolated from VS and UF soils produced a similar number of nodules and similar nodule biomass (7.5 ± 3.9 nodules weighing 3.5 ± 1.4 mg for *Bradyrhizobium* sp. strains from VS soils and 8.8 ± 4.3 nodules weighing 4.2 ± 1.7 mg for *Bradyrhizobium* sp. strains from UF; Table 3). Despite these similarities, the nitrogen fixation and efficiency of *Bradyrhizobium* sp. strains isolated from VS soils differed significantly from those isolated from UF soils (with, respectively, 0.17 ± 0.04 and 0.08 ± 0.07 μmol of ethylene formed per hour and per seedling and 11.3 ± 5.6 and 16.9 ± 7.4 mg of nodules required to form 100 mg of shoot; Table 3). Moreover, the shoot dry biomass of *A. spirorbis* seedlings inoculated by *Bradyrhizobium* sp. strains from VS soils (33.4 ± 8.3 mg; Table 3) weighed significantly more than *Bradyrhizobium* sp. strains from UF soils (25.7 ± 4.3 mg; Table 3).

An overview of these data is presented in a PCA (supplementary Figure S10 available online), completed by a correlation matrix (supplementary Table S10 available online).

Molecular symbiotic characterization of the bacterial strains

Molecular data on the *nodA* gene found in *Bradyrhizobium* sp. are reported in Fig. 4. The *Bradyrhizobium* sp. strains isolated in this study represent three major groups of the *nodA* gene (Fig. 4). Group 1 of *nodA* contained three strains from VS soils and clustered next to *Bradyrhizobium* sp., strain CH2493 (Fig. 4). Group 2 of the *nodA* gene contained a total of 12 strains distributed between UF soils (75%) and VS soils (25%) and clustered between *Methylobacterium nodulans* and *Bradyrhizobium canariense* (Fig. 4). Group 3 of the *nodA* gene contained a total of 12 strains distributed between UF soils (17%) and VS soils (83%) and clustered next to *B. elkanii* (Fig. 4). In spite of several amplifications we did not manage to amplify the expected *nodA* fragment as it corresponded to a gene fragment coding for peptide-methionine (S)-S-oxide reductase.

Molecular data concerning the *nifH* gene found in *Bradyrhizobium* sp. strains are reported in Fig. 5. Two major putative groups were identified. Group 1 contained 10 strains from three study sites (PMB, PMS and TIE) and formed a cluster next to *B. elkanii* (Fig. 5). Group 2 contained 17 strains from five study sites (PMB, PMS, PN2, SRP and VRT) and formed a cluster next to *Bradyrhizobium ganzhouense* (Fig. 5).

Concerning *Paraburkholderia* sp. strains, the *nifH* nucleotidic sequence number LC416540 of the strain STM11825-TIE10 matched the *nifH* sequence from *Paraburkholderia phymatum*.

Metal tolerance of *A. spirorbis* bacterial symbionts

Metal tolerance of *Bradyrhizobium* sp. and *Paraburkholderia* sp. strains were assessed in relation to metal contents found in the UF and VS soils: aluminum, cobalt, chromium, iron, manganese and nickel. Data are summarized in Table 4 (means of IC_{50} are expressed in mM or μM ± standard deviation) and presented in relation to three factors: (i) the rhizobial genus, (ii) the soil type and (iii) the combination of the genus and the soil type. An overview of the data with a PCA is given in supplementary Figure S11 available online.

The *Bradyrhizobium* sp. strains presented significantly higher ($P < 0.05$) IC_{50} values for aluminium, chromium, cobalt and iron than the *Paraburkholderia* sp. strains (Table 4). More precisely, mean IC_{50} values in the *Paraburkholderia* sp. differed significantly from those in *Bradyrhizobium* sp. strains with, respectively: 110 and 367 μm of chromium; 564 and 254 μM of cobalt; 2123 and 2482 μM of iron; and 12 and 21.8 mM of aluminium (Table 4). Metal tolerances were similar in strains sampled in UF and VS soil, except for chromium and iron, where strains from VS soils tolerated higher concentrations of chromium and iron than strains from UF soils (Table 4). The IC_{50} values in *Bradyrhizobium* sp. strains isolated from UF and VS soils varied with the metal (Table 4).

The correlation matrix of IC_{50} values of metals in supplementary Table S11 available online shows that IC_{50} values of aluminum, cobalt, nickel and manganese were positively correlated ($P < 0.05$ and $r > 0$).

The occurrence of two metal resistance genes (*cnrA* and *nreB*) are listed in Table 5. It is worth noting that in the strains, either

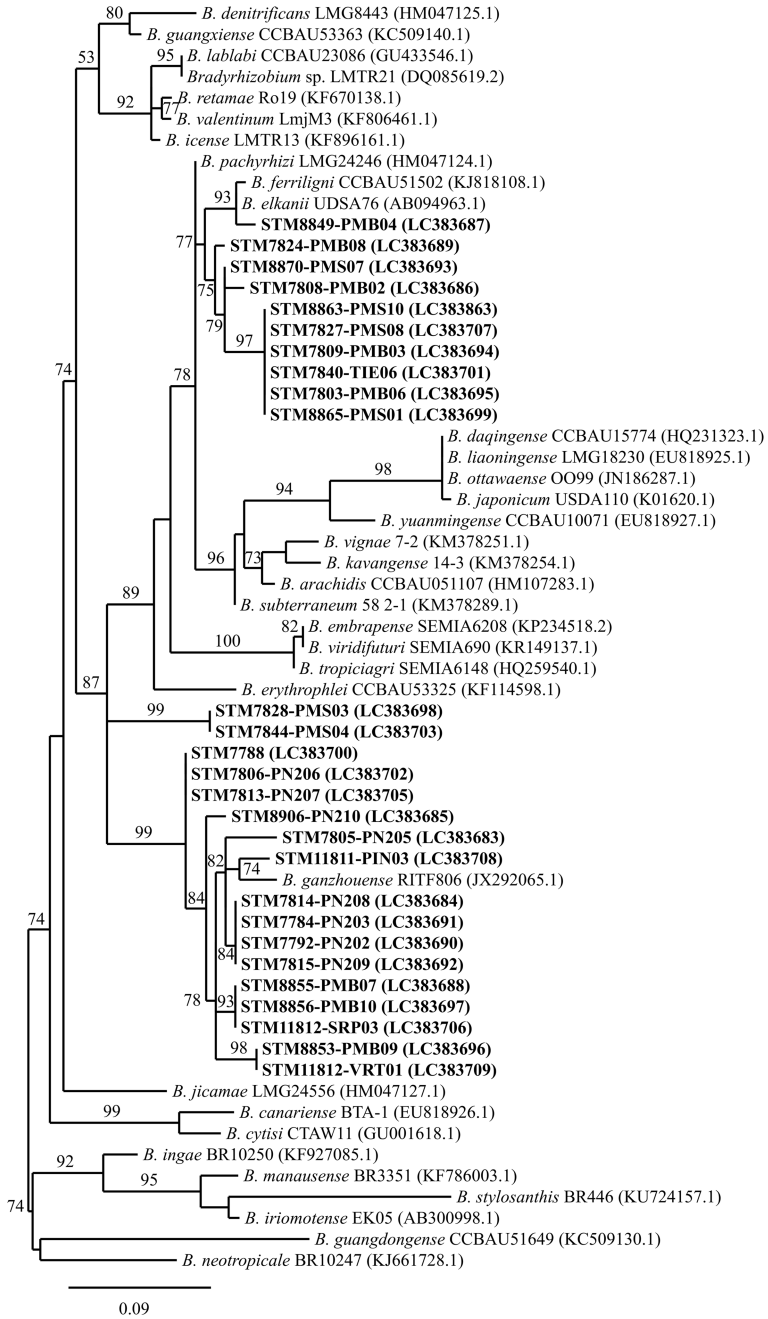

Figure 5. Unrooted phylogenetic tree of *Acacia spirorbis* alpha-rhizobial symbionts and related species. The tree was inferred from sequences of the gene *nifH*. The number at the node represents the bootstraps percentage of 1000 replicates (shown only when >50%). Strains isolated in this study (New Caledonia) are in bold. The scale bar indicates the number of substitutions per site.

both genes occurred simultaneously or were absent (Table 5). The *cnrA* and *nreB* genes were detected on average three times more frequently in *Paraburkholderia* sp. strains than in *Bradyrhizobium* sp. strains. The *cnrA* gene was present in 77% of the *Paraburkholderia* sp. strains versus 22% of the *Bradyrhizobium* sp. strains (Table 5). Similarly, the *nreB* gene was present in 69% of the *Paraburkholderia* sp. strains versus 19% in *Bradyrhizobium* sp. strains (Table 5). The strains from UF soils had twice as many *cnrA* and *nreB* genes than strains from VS soils (Table 5). Despite these differences, the occurrence of metal resistance genes *cnrA* and *nreB* was similar in *Bradyrhizobium* sp. strains from UF and VS soils (Table 5). Among BR-UF strains, the genes *cnrA* and *nreB* were detected in 18% of the strains, while the *cnrA* and *nreB* genes of the BR-VS strains was detected in 25% and 19% of the strains, respectively (Table 5). No statistical correlations were found between the IC_{50} values and the occurrence of *cnrA* and *nreB* genes in the strains presented in this study.

DISCUSSION

The broad symbiotic promiscuity of *A. spirorbis*

In the present study, we observed that natural populations of *A. spirorbis* from both UF and VS soils could establish nitrogen-fixing symbioses with both alpha- and beta-rhizobia,

Table 4. Nickel (Ni), chromium (Cr), cobalt (Co), iron (Fe), manganese (Mn) and aluminum (Al) IC_{50} values (means ± standard deviation) in the rhizobial strains isolated from *A. spirorbis* nodules. Results are presented according to three factors: (i) the rhizobial genus, (ii) the soil type and (iii) genus and soil type combined. For each numerical parameter, means were compared using Kruskal–Wallis tests followed by Wilcoxon tests. The superscript letters 'a', 'b' and 'c' correspond to different mean levels significantly different between each other.

IC_{50}	Genus		Soil type		Genus and soil type combined		
	Paraburkholderia	*Bradyrhizobium*	UF	VS	BK-UF	BR-UF	BR-VS
Ni (μM)	449 ± 471[a]	502 ± 640[a]	569 ± 715[a]	358 ± 281[a]	449 ± 471[b]	711 ± 904[a]	358 ± 281[b]
Cr (μM)	110 ± 107[b]	367 ± 495[a]	108 ± 100[b]	468 ± 546[a]	110 ± 107[b]	100 ± 52[b]	468 ± 546[a]
Co (μM)	564 ± 1065[b]	254 ± 141[a]	419 ± 802[a]	259 ± 163[a]	564 ± 1065[b]	247 ± 101[a,b]	259 ± 163[a]
Fe (μM)	2123 ± 499[b]	2482 ± 789[a]	2165 ± 536[b]	2533 ± 832[a]	2123 ± 499[b]	2347 ± 642[a,b]	2533 ± 832[a]
Mn (mM)	17.3 ± 15.0[a]	23.6 ± 15.9[a]	20.4 ± 15.3[a]	23.1 ± 16.5[a]	17.3 ± 15.0[a]	24.2 ± 15.0[a]	23.1 ± 16.5[a]
Al (mM)	12.0 ± 4.01[b]	21.8 ± 11.1[a]	18.7 ± 10.7[a]	18.5 ± 10.0[a]	12.0 ± 4.01[c]	26.7 ± 10.7[a]	18.5 ± 10.1[b]

UF: ultramafic soils; VS: volcano-sedimentary soils; BK-UF: *Paraburkholderia* strains sampled in ultramafic soils; BR-UF: *Bradyrhizobium* strains sampled in ultramafic soils; BR-VS: *Bradyrhizobium* strains sampled in volcano-sedimentary soils.

Table 5. Occurrence of *cnrA* and *nreB* metal resistance genes among the 40 rhizobial strains (expressed as a percentage) isolated from *A. spirorbis* nodules collected in ultramafic (UF) and volcano-sedimentary (VS) soils. Results are presented according to: (i) the rhizobial genus, all soil types combined, (ii) the soil type with both rhizobia genera combined and (iii) rhizobial genus and soil type combined.

	Genus		Soil type		Genus and soil type combined		
	Paraburkholderia	*Bradyrhizobium*	UF	VS	BK-UF	BR-UF	BR-VS
Occurrence of *cnrA* (%)	77	22	50	25	77	18	25
Occurrence of *nreB* (%)	69	19	46	19	69	18	19

UF: ultramafic soils; VS: volcano-sedimentary soils; BK-UF: *Paraburkholderia* strains sampled in ultramafic soils; BR-UF: *Bradyrhizobium* strains sampled in ultramafic soils; BR-VS: *Bradyrhizobium* strains sampled in volcano-sedimentary soils.

i.e. *Bradyrhizobium* sp. and *Paraburkholderia* sp., on UF and VS soils. Symbiotic promiscuity varies among *Acacia* species: on one hand, *A. saligna*, *A. salicina* and *A. stenophylla* are known to harbor broad symbiotic promiscuity with several alpha-rhizobia including the genera *Rhizobium*, *Bradyrhizobium*, *Ensifer* and *Mesorhizobium* (Boukhatem *et al.* 2012). On the other hand, Australian *Acacia* species like *A. dealbata*, *A. mearnsii* and *A. melanoxylon* showed a narrower diversity of symbionts, mainly *Bradyrhizobium* species (Lafay and Burdon 2001). The *Bradyrhizobium* sp. symbionts found in *A. spirorbis* nodules revealed high diversity as they showed similarities with nine diverse *Bradyrhizobium* species (Fig. 2). Ten *Bradyrhizobium* sp. strains clustered with *B. yuanmingense* isolated from *Lespedeza* sp. (Yao *et al.* 2002). Other *Bradyrhizobium* sp. strains formed clusters including one to five strains, with multiple *Bradyrhizobium* species (i.e. *B. cytisi*, *B. elkanii*, *B. embrapense*, *B. guangdongense*, *B. huanghuaihaiense*, *B. pachyrhizi* and *B. tropiciagri*), isolated from a variety of hosts (i.e. *Arachis hypogea*, *Cytisus villosus*, *Desmodium heterocarpon*, *Glycine max*, *Neonotonia wightii* and *Pachyrhizus erosus*), as described by several authors (Kuykendall *et al.* 1992; Ramírez-Bahena *et al.* 2009; Chahboune *et al.* 2011; Zhang *et al.* 2012; Delamuta *et al.* 2015; Li *et al.* 2015). Lastly, two strains (STM7828-PMS03 and STM7844-PMS04) clustered far from known *Bradyrhizobium* species, indicating a putative undescribed species of *Bradyrhizobium* (Fig. 2).

It is noteworthy that the symbiotic promiscuity of *A. spirorbis* shares a common pattern with Australian acacias. On their native soil, Australian acacias establish nitrogen-fixing symbioses mostly with *Bradyrhizobium* species, notably with *Bradyrhizobium japonicum* and *B. elkanii*, while African and American acacia species *sensus lato*, the majority of which are now grouped in the *Vachellia* and *Senegalia* genera (LPWG 2017), are mainly associated with strains of *Rhizobium*, *Ensifer* and *Mesorhizobium* (Lafay and Burdon 2001; Leary *et al.* 2006; Leroux *et al.* 2009). Outside Australasia, introduced *Acacia* spp. such as *A. auriculiformis* Benth., *A. dealbata* Link, *A. longifolia* (Andrews) Willd., *A. mangium* Willd. and *A. mearnsii* De Wild. are still mainly associated with *Bradyrhizobium* species (Joubert 2003; Weir *et al.* 2004; Le Roux *et al.* 2009). The symbiotic promiscuity of *A. spirorbis* involving alpha-rhizobia is in agreement with these studies, thus supporting the hypothesis that *A. spirorbis* subsp. *spirorbis* originates from Australia (Richardson *et al.* 2011).

In addition to the presence of *Bradyrhizobium* sp., *A. spirorbis* symbiotic promiscuity includes two putative species of *Paraburkholderia* sp. from the beta-rhizobia. Multilocus analysis revealed that *A. spirorbis* beta-rhizobial symbionts clustered in (i) one homogenous group next to *P. phenoliruptix* and (ii) a putative second group represented by one strain next to *P. phenazinium* (Fig. 3). The molecular data of *Paraburkholderia* and *Bradyrhizobia* could lead to a new species and need further investigations (e.g. DNA–DNA hybridization analyses, and physiological and biochemical tests), as described by other authors (e.g. De Meyer *et al.* 2014 and Guentas *et al.* 2016). The beta-rhizobia/leguminous symbiosis was first described by Moulin *et al.* (2001) as being widespread in nature, and included *Burkholderia sensu lato* and *Cupriavidus* (formerly *Ralstonia*) species (Chen *et al.* 2003). Recent studies showed that species of *Burkholderia sensus lato* included human, animal and plant pathogenic species, as well as plant beneficial and environmental species. Therefore, five distinct lineages were recently introduced: *Burkholderia sensus stricto*, *Caballeronia*, *Paraburkholderia rhizoxinica*, *Paraburkholderia* and *Robbsia* (Beukes *et al.* 2017; Lopes-Santos *et al.* 2017). *Paraburkholderia* species correspond to plant-associated strains, including free-living diazotrophs and symbiotic rhizobia (Sawana, Adeolu and Gupta 2014). *Paraburkholderia* species were found to be the major symbionts of nitrogen-fixing symbioses among *Mimosa* spp. (47 different species tested in Bontemps *et al.* 2010). Moreover, *Paraburkholderia* symbiotic promiscuity involves leguminous plants such

as *Anadenanthera* spp., *Cyclopia* spp., *Dalbergia* spp., *Lebeckia ambigua*, *Macroptilium atropurpureum*, *Microlobius foetidus*, *Parapiptadenia* spp., *Phaseolus vulgaris*, *Piptadenia* spp., *Podalyria canescens* and *Pseudopiptadenia* spp. (Rasolomampianina *et al.* 2005; Elliott *et al.* 2007; Bournaud *et al.* 2013; De Meyer *et al.* 2014).

So far, only a few studies have reported that a given leguminous species is able to form symbioses with alpha- and beta-rhizobia. One study in Brazil showed that *Mimosa* species including *Mimosa acutistipula* Benth., *Mimosa kalunga* M. F. Simon & C. E. Hughes, *Mimosa radula* Benth. and *Mimosa xanthocentra* Mart. nodulated with alpha-rhizobial (*Rhizobium*) and beta-rhizobial (*Paraburkholderia*) strains (dos Reis *et al.* 2010). Another recent study argued that *Vachellia karroo* (Hayne) Banfi & Galasso (formerly *Acacia karroo*) from southern Africa is also able to establish symbioses with both alpha- and beta-rhizobia (Beukes *et al.* 2018). However, the nitrogen-fixing ability of these beta-rhizobial strains was not confirmed by nodulation tests in this latter study. Caution is warranted because beta-rhizobia are known to occur naturally as endophytic bacteria inside nodules, notably in *Mimosa pudica* L., *G. max* (L.) Merr. and *Vachellia seyal* (Delille) P.J.H. Hurter Mabberley's (formerly *Acacia seyal*) (Pandey, Kang and Maheshwari 2005; Diouf *et al.* 2007; Li *et al.* 2008). Hence, our study may be one of the first to report that an endemic *Acacia* species establishes symbioses with both alpha- and beta-rhizobial and to provide evidence for the nitrogen-fixing ability of each strain characterized.

Exploiting alpha- and beta-rhizobia for adaptation to contrasted soils

The results of our study show that in NC, *A. spirorbis* naturally establish nitrogen-fixing symbioses with local rhizobia in both UF and VS soils. The symbiotic promiscuity of *A. spirorbis* is structured by the type of soil, and *Paraburkholderia* sp. strains were only found in nodules sampled in UF soils, whereas *Bradyrhizobium* sp. strains were isolated from both UF and VS soils. Molecular data showed that the metal resistance gene *cnrA* and the *nreB* gene were more frequent in strains sampled from UF soil than those sampled from VS soil (Table 5).

UF soils are characterized by low concentrations of the major elements (nitrogen, phosphorus and potassium), a strongly unbalanced calcium:magnesium ratio and high concentrations of iron oxides with heavy metals, notably cobalt, chromium, manganese and nickel (Brooks 1988). Growth conditions in UF soils are reasonably harsh for plant development, and consequently promote plant endemism and speciation (Isnard *et al.* 2016). Several authors have reported that plant speciation towards heavy metals found in UF soils also involves rhizobia, in particular with *Paraburkholderia* sp. and *Bradyrhizobium* sp. (Chaintreuil *et al.* 2007; Klonowska *et al.* 2012; Gonin *et al.* 2013). One study from Gonin *et al.* (2013) in NC showed that the rhizosphere of naturally occurring *Costularia* spp. (Cyperceae) in UF soils is dominated by *Paraburkholderia* sp. (28%), followed distantly by *Bradyrhizobium* sp. (9%). This study of *Costularia* spp.-associated rhizobacteria led to the description of two novel rhizospheric *Paraburkholderia* sp. (formerly *Burkholderia* sp.) that have adapted to UF conditions. Another study done in NC by Klonowska *et al.* (2012) described the biodiversity of *M. pudica* rhizobial symbionts (*Cupriavidus taiwanensis*, *Rhizobium mesoamericanum*) and their adaptation to UF soils. Moreover, one study reported the presence of *Bradyrhizobium* sp. symbionts on *Serianthes calycina* Benth., an endemic species (Caesalpinieae) from NC growing on both UF and nickel-free soils (Chaintreuil *et al.* 2007). All rhizobial symbionts isolated from nodules of *S. calycina* were reported to closely resemble *B. elkanii* (Chaintreuil *et al.* 2007). *Serianthes calycina* rhizobial symbionts from UF soils were more tolerant to nickel and cobalt toxicity than strains from nickel-free soils, thanks to the occurrence of metal resistance genes such as the *cnrA* and the *nreB* genes (Chaintreuil *et al.* 2007). The present study also shows that the *cnrA* and *nreB* genes harbored by *A. spirorbis* symbionts are more frequent in rhizobia from UF soils (Table 5), in agreement with the results of the previously performed study (Chaintreuil *et al.* 2007). Taken together, these observations suggest that the *cnrA* and *nreB* genes found in rhizobia associated with *A. spirorbis* are involved in the adaptation of the symbiont to the extreme soil properties found in UF soils, enabling the legume to establish symbioses in this harsh environment.

In vitro experiments conducted to test the metal tolerance of *A. spirorbis* symbionts revealed no significant correlation between the presence of metal resistance genes (*nre* or *cnr*) and the values of IC_{50}. This suggests that other mechanisms may be involved in bacterial tolerance to metals, such as exclusion, active removal, biosorption, precipitation or bioaccumulation in external and intracellular spaces, transport mechanisms and/or chelation, all such mechanisms being driven by genes other than the *nre* or *cnr* gene families (Haferburg and Kothe 2007).

In VS soils, the rhizobial symbionts of *A. spirorbis* belonged to *Bradyrhizobium* sp. These strains were also tolerant to metals but at lower levels than those observed in *Bradyrhizobium* sp. strains isolated from UF soils. These results may be related to the presence of metals in VS soils at lower concentrations that those found in UF soils (Table 2), suggesting that when present in VS soils, *Bradyrhizobium* sp. have adapted to the specific edaphic conditions of these soils. These results are in agreement with those reported for Australian *Acacia* species that occur naturally on heavy metal-free acidic soil (Ashwath *et al.* 1995) and establish symbioses with *Bradyrhizobium* species, mainly *B. japonicum* and *B. elkanii* (Lafay and Burdon 2001; Leary *et al.* 2006). A previous study reported that *Bradyrhizobium* bacteria are adapted to acidic soil and more tolerant than other rhizobia-nodulating *Acacia* species (Marsudi, Glenn and Dilworth 1999). Therefore, the fact that *A. spirorbis* is able to establish nitrogen-fixing symbioses with *Bradyrhizobium* sp. adapted to both VS or UF edaphic conditions confirms the capacity of this legume to develop symbiotic interactions with any local rhizobia present in soils and, in this way, to colonize other environments. Lastly, it is worth noting that the diversity of symbionts associated with *A. spirorbis* in natural conditions reported in the present study has no impact on its nitrogen-fixing capacity (Vincent *et al.* 2018). These results suggest that the symbiotic promiscuity of *A. spirorbis* with local rhizobia adapted to edaphic conditions and their optimal nitrogen-fixing rates in UF and VS soils may be two of many keys to the successful adaptation of *A. spirorbis* to contrasted environments. In that perspective, it would be interesting for future research to address the question of the possible synchronous activity of both alpha- and beta-rhizobia, either through an *in vitro* experiment of seedlings co-incubation and time-dependent RNA or protein analysis, or through a similar approach on the original habitat using a meta-transcriptomic approach.

CONCLUSIONS

Our study is one of the first to report (i) symbionts of an endemic *Acacia* species within its natural ecosystems with the presence

in nodules of both alpha- and beta- rhizobia, genera *Bradyrhizobium* and *Paraburkholderia*, (ii) evidences of their nitrogen-fixing capacity and (iii) that their taxonomic and symbiotic efficiency are adapted to edaphic conditions, whereby *Paraburkholderia* sp. symbionts are mainly found in UF soils and the symbiotic efficiency of *Bradyrhizobium* sp. strains differed between UF and VS soils.

Such an establishment of nitrogen-fixing symbioses by *A. spirorbis* with both *Bradyrhizobium* and *Paraburkholderia* species may be involved with its adaptation to UF and VS soils. On one hand, rhizobial taxonomy, symbiotic efficiency and metal tolerance are structured by the soil properties (Tables 3 and 4). On the other hand, a legume tree can interact with a limited number of rhizobial species (Peix *et al.* 2015). Taken together, these findings suggest that the more a legume tree can interact and establish symbioses with rhizobial symbionts, the better the plant can adapt to contrasted soils and colonize different ecosystems. This hypothesis has already been confirmed for *A. spirorbis*, as we previously reported that the biological nitrogen fixation of this tree legume in natural ecosystems reaches optimal rates, regardless of the soil type (Vincent *et al.* 2018). Moreover, this rhizobial broad symbiotic promiscuity is complementary to the one observed in ectomycorrhizal fungi symbiosis in *A. spirorbis* (Houlès *et al.* 2018), which could be one of the many keys to the successful adaptation of *A. spirorbis* to contrasted soils.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

ACKNOWLEDGEMENTS

This work was supported by (i) the French National Agency for Research (ANR) financial support through the BIOADAPT Program 2012 (grant number ANR-12-ADAP-0017 ADASPIR) and (ii) the project CNRT-BIOINDIC. Part of this work was conducted in the ISO 9002 certified Laboratoire des Moyens Analytiques (LAMA) of the IRD Center in Noumea (New Caledonia). We are grateful to S. Mouah, an MSc student, who undertook the initial work of strain isolation.

Conflicts of interest. None declared.

REFERENCES

Ashwath N, Dart PJ, Edwards DG *et al.* Tolerance of Australian tropical and subtropical Acacias to acid soil. *Plant Soil* 1995;**171**:83–7.

Beukes CW, Palmer M, Manyaka P *et al.* Genome data provides high support for generic boundaries in *Burkholderia sensu lato*. *Front Microbiol* 2017;**8**:1154, DOI: 10.3389/fmicb.2017.01154.

Beukes CW, Boshoff F, Phalane FL *et al.* Alpha- and beta-rhizobia nodulate *Vachellia karroo* in South Africa. *S Afr J Bot* 2018;**115**:279–80.

Bontemps C, Elliott GN, Simon MF *et al.* *Burkholderia* species are ancient symbionts of legumes. *Mol Ecol* 2010;**19**:44–52.

Bontemps C, Rogel MA, Wiechmann A *et al.* Endemic *Mimosa* species from Mexico prefer alphaproteobacterial rhizobial symbionts. *New Phytol* 2016;**209**:319–33.

Bonvallot J, Gay J-C, Habert E. *Atlas de la Nouvelle-Calédonie*. IRD Editions. Marseille: Institut de Recherche pour le Développement, 2012 .

Boukhatem ZF, Domergue O, Bekki A *et al.* Symbiotic characterization and diversity of rhizobia associated with native and introduced acacias in arid and semi-arid regions in Algeria. *FEMS Microbiol Ecol* 2012;**80**:534–47.

Bournaud C, de Faria SM, dos Santos JMF *et al.* *Burkholderia* species are the most common and preferred nodulating symbionts of the Piptadenia group (tribe Mimoseae). *PLoS One* 2013;**8**:e63478.

Brooks R. Serpentine and its vegetation. A multidisciplinary approach. *Brittonia* 1988;**40**:268.

Brown GK, Murphy DJ, Kidman J *et al.* Phylogenetic connections of phyllodinous species of *Acacia* outside Australia are explained by geological history and human-mediated dispersal. *Aust Syst Bot* 2012;**25**:390–403.

Chahboune R, Carro L, Peix A *et al.* *Bradyrhizobium cytisi* sp. nov., isolated from effective nodules of *Cytisus villosus*. *Int J Syst Evol Microbiol* 2011;**61**:2922–7.

Chaintreuil C, Rigault F, Moulin L *et al.* Nickel resistance determinants in *Bradyrhizobium* strains from nodules of the endemic New Caledonia legume *Serianthes calycina*. *Appl Environ Microbiol* 2007;**73**:8018–22.

Chen W-M, Moulin L, Bontemps C *et al.* Legume symbiotic nitrogen fixation by beta-proteobacteria is widespread in nature. *J Bacteriol* 2003;**185**:7266–72.

Corpet F. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* 1988;**16**:10881–90.

Delamuta JRM, Ribeiro RA, Ormeño-Orrillo E *et al.* *Bradyrhizobium tropiciagri* sp. nov. and *Bradyrhizobium embrapense* sp. nov., nitrogen-fixing symbionts of tropical forage legumes. *Int J Syst Evol Microbiol* 2015;**65**:4424–33.

De Meyer SE, Cnockaert M, Ardley JK *et al.* *Burkholderia dilworthii* sp. nov., isolated from *Lebeckia ambigua* root nodules. *Int J Syst Evol Microbiol* 2014;**64**:1090–5.

Dereeper A, Guignon V, Blanc G *et al.* Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* 2008;**36**:W465–469.

Dereeper A, Audic S, Claverie J-M *et al.* BLAST-EXPLORER helps you building datasets for phylogenetic analysis. *BMC Evol Biol* 2010;**10**:8.

Diouf D, Samba-Mbaye R, Lesueur D *et al.* Genetic diversity of *Acacia seyal* Del. rhizobial populations indigenous to senegalese soils in relation to salinity and pH of the sampling sites. *Microb Ecol* 2007;**54**:553–66.

dos Reis FB, Simon MF, Gross E *et al.* Nodulation and nitrogen fixation by *Mimosa* spp. in the Cerrado and Caatinga biomes of Brazil. *New Phytol* 2010;**186**:934–46.

Ducousso M, Galiana A, Houlès A *et al.* *Acacia spirorbis* a model plant to study the contribution of symbiotic root microorganisms in plant adaptation to soil constraints: news and prospects, Acacia 2014 Conference "Sustaining the Future of Acacia Plantation Forestry" held in Hue, Vietnam. 2014.

Elliott GN, Chen W-M, Bontemps C *et al.* Nodulation of *Cyclopia* spp. (Leguminosae, Papilionoideae) by *Burkholderia tuberum*. *Ann Bot* 2007;**100**:1403–11.

Gonin M, Gensous S, Lagrange A *et al.* Rhizosphere bacteria of *Costularia* spp. from ultramafic soils in New Caledonia: diversity, tolerance to extreme edaphic conditions, and role in plant growth and mineral nutrition. *Can J Microbiol* 2013;**59**:164–74.

Grangeteau C, Ducousso M, Fritsch E *et al.* Diversity of nitrogen fixing bacteria associated to the New Caledonian ubiquitous

tree *Acacia spirorbis*, EGU General Assembly 2012, held 22-27 April. 2012, Vienna, Austria, 4892.

Guentas L, Gensous S, Cavaloc Y *et al*. *Burkholderia novacaledonica* sp. nov. and *B. ultramafica* sp. nov. isolated from roots of *Costularia* spp. pioneer plants of ultramafic soils in New Caledonia. *Syst Appl Microbiol* 2016;**39**:151–9.

Guindon S, Lethiec F, Duroux P *et al*. PHYML Online–a web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Res* 2005;**33**:W557–9.

Haferburg G, Kothe E. Microbes and metals: interactions in the environment. *J Basic Microbiol* 2007;**47**:453–67.

Hardy RWF, Holsten RD, Jackson EK *et al*. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation 1. *Plant Physiol* 1968;**43**:1185–207.

Houlès A, Vincent B, David M *et al*. Ectomycorrhizal communities associated with the legume *Acacia spirorbis* growing on contrasted edaphic constraints in New Caledonia. *Microb Ecol* 2018;**76**:964–75.

Ibanez T, Birnbaum P, Gâteblé G *et al*. Twenty years after Jaffré et al. (1998), is the system of protected areas now adequate in New Caledonia? *Biodivers Conserv* 2019;**28**:245–54.

Isnard S, L'Huillier L, Rigault F *et al*. How did the ultramafic soils shape the flora of the New Caledonian hotspot? *Plant Soil* 2016:**403**:1–24.

Jaffré T. The relationship between ecological diversity and floristic diversity in New Caledonia. *Biodivers Lett* 1993;**1**:82–7.

Jaffré T, Bouchet P, Veillon JM. Threatened plants of New Caledonia: is the system of protected areas adequate? *Biodivers Conserv* 1998;**7**:109–35.

Jaffré T, L'Huillier L. La végétation des roches ultramafiques ou terrains miniers. In: L'Huillier L, Jaffré T, Wulff A (eds). *Mines et environnement en Nouvelle Calédonie : les milieux sur substrats ultramafiques et leur restauration*. Païta: IAC, 2010, 45–103.

Jaffré T, Munzinger J, Lowry PP. Threats to the conifer species found on New Caledonia's ultramafic massifs and proposals for urgently needed measures to improve their protection. *Biodivers Conserv* 2010;**19**:1485–502.

Jensen HL. Nitrogen fixation in leguminous plants. I. General characters of root-nodule bacteria isolated from species of *Medicago* and *Trifolium* in Australia. *Proc Linn Soc N S W* 1942;**67**:98–108.

Joubert C. Rhizobia associated with Australian *Acacia* species (*Acacia mearnsii*, *Acacia dealbata* and *Acacia decurrens*) in South Africa as determined by sodium dodecyl-sulphate polyacrylamide gel electrophoresis. Thesis report, University of Pretoria, South Africa, 2003.

Jourand P, Renier A, Rapior S *et al*. Role of methylotrophy during symbiosis between *Methylobacterium nodulans* and *Crotalaria podocarpa*. *Mol Plant Microbe Interact* 2005;**18**:1061–8.

Kazakou E, Dimitrakopoulos PG, Baker AJM *et al*. Hypotheses, mechanisms and trade-offs of tolerance and adaptation to serpentine soils: from species to ecosystem level. *Biol Rev* 2008;**83**:495–508.

Kessel C van, Roskoski JP. Nodulation and N_2 fixation by *Inga jinicuil*, a woody legume in coffee plantations. *Plant Soil* 1981;**59**:207–15.

Klonowska A, Chaintreuil C, Tisseyre P *et al*. Biodiversity of *Mimosa pudica* rhizobial symbionts (*Cupriavidus taiwanensis*, *Rhizobium mesoamericanum*) in New Caledonia and their adaptation to heavy metal-rich soils. *FEMS Microbiol Ecol* 2012;**81**:1–18.

Kuykendall LD, Saxena B, Devine TE *et al*. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can J Microbiol* 1992;**38**:501–5.

Lafay B, Burdon JJ. Small-subunit rRNA genotyping of Rhizobia nodulating Australian *Acacia* spp. *Appl Environ Microbiol* 2001;**67**:396–402.

Leary JK, Singleton PW, Scowcroft PG *et al*. Symbiotic diversity in the cosmopolitan genus *Acacia*. *Symbiosis* 2006;**4**: 107–17.

Le Roux C, Tentchev D, Prin Y *et al*. Bradyrhizobia nodulating the *Acacia mangium* × *A. auriculiformis* interspecific hybrid are specific and differ from those associated with both parental species. *Appl Environ Microbiol* 2009;**75**:7752–9.

Li JH, Wang ET, Chen WF *et al*. Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China. *Soil Biol Biochem* 2008;**40**:238–46.

Li YH, Wang R, Zhang XX *et al*. *Bradyrhizobium guangdongense* sp. nov. and *Bradyrhizobium guangxiense* sp. nov., isolated from effective nodules of peanut. *Int J Syst Evol Microbiol* 2015;**65**:4655–61.

Lopes-Santos L, Castro DBA, Ferreira-Tonin M *et al*. Reassessment of the taxonomic position of *Burkholderia andropogonis* and description of *Robbsia andropogonis* gen. nov., comb. nov. *Antonie Van Leeuwenhoek* 2017;**110**:727–36.

Losfeld G, L'Huillier L, Fogliani B *et al*. Mining in New Caledonia: environmental stakes and restoration opportunities. *Environ Sci Pollut Res* 2014;**22**:5592–607.

LPWG. The Legume Phylogeny Working Group. A new subfamily classification of the Leguminosae based on a taxonomically comprehensive phylogeny. *Taxon*2017;**66**:44–77.

Maier RM. Bacterial growth. In: Pepper IL, Gerba CP, Gentry TJ (eds). *Environmental Microbiology*. Elsevier, 2015, 728.

Marchese C. Biodiversity hotspots: A shortcut for a more complicated concept. *Glob Ecol Conserv* 2015;**3**:297–309.

Marsudi NDS, Glenn AR, Dilworth MJ. Identification and characterization of fast- and slow-growing root nodule bacteria from South-Western Australian soils able to nodulate *Acacia saligna*. *Soil Biol Biochem* 1999;**31**:1229–38.

Morat P, Jaffré T, Tronchet F *et al*. The taxonomic reference base Florical and characteristics of the native vascular flora of New Caledonia. *Adansonia* 2012;**34**:179–221.

Moulin L, Munive A, Dreyfus B *et al*. Nodulation of legumes by members of the beta-subclass of Proteobacteria. *Nature* 2001;**411**:948–50.

Myers N, Mittermeier RA, Mittermeier CG *et al*. Biodiversity hotspots for conservation priorities. *Nature* 2000;**403**:853–8.

Pandey P, Kang SC, Maheshwari DK. Isolation of endophytic plant growth promoting *Burkholderia* sp. MSSP from root nodules of *Mimosa pudica*. *Curr Sci* 2005;**89**:177–80.

Peix A, Ramírez-Bahena MH, Velázquez E *et al*. Bacterial associations with legumes. *Crit Rev Plant Sci* 2015;**34**:17–42.

Pelletier B. Geology of the New Caledonia region and its implications for the study of the New Caledonian biodiversity. Payri C, Richer de Forges B, Colin F (eds). *Compendium of Marine Species from New Caledonia:* 2nd edn. Nouméa: IRD, 2006, 19–32.

Pillon Y, Munzinger J, Amir H *et al*. Ultramafic soils and species sorting in the flora of New Caledonia. *J Ecol* 2010;**98**: 1108–16.

Proctor J. Vegetation and soil and plant chemistry on ultramafic rocks in the tropical Far East. *Perspect Plant Ecol Syst* 2003;**6**:105–24.

Ramírez-Bahena MH, Peix A, Rivas R *et al*. *Bradyrhizobium pachyrhizi* sp. nov. and *Bradyrhizobium jicamae* sp. nov., isolated from effective nodules of *Pachyrhizus erosus*. *Int J Syst Evol Microbiol* 2009;**59**:1929–34.

Rasolomampianina R, Bailly X, Fetiarison R *et al*. Nitrogen-fixing nodules from rose wood legume trees (*Dalbergia* spp.) endemic to Madagascar host seven different genera belonging to alpha- and beta-Proteobacteria. *Mol Ecol* 2005;**14**:4135–46.

Richardson DM, Carruthers J, Hui C *et al*. Human-mediated introductions of Australian acacias – a global experiment in biogeography. *Divers Distrib* 2011;**17**:771–87.

Richardson DM, Le Roux JJ, Wilson JRU. Australian acacias as invasive species: lessons to be learnt from regions with long planting histories. *South Forests* 2015;**77**:31–9.

Sarrailh JM. La revégétalisation des exploitations minières: l'exemple de la Nouvelle-Calédonie. *Bois et Forêts des Tropiques* 2002; **272**:21–31.

Sawana A, Adeolu M, Gupta RS. Molecular signatures and phylogenomic analysis of the genus *Burkholderia*: proposal for division of this genus into the emended genus *Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. nov. harboring environmental species. *Front Genet* 2014;**5**:429.

Vincent B, Jourand P, Juillot F *et al*. Biological *in situ* nitrogen fixation by an *Acacia* species reaches optimal rates on extremely contrasted soils. *Eur J Soil Biol* 2018;**86**:52–62.

Vincent JM. *A Manual for the Practical Study of Root-Nodule Bacteria*. Oxford: Published for the International Biological Programme by Blackwell Scientific, 1970, 440.

Weir BS, Turner SJ, Silvester WB *et al*. Unexpectedly diverse *Mesorhizobium* strains and *Rhizobium leguminosarum* nodulate native legume genera of New Zealand, while introduced legume weeds are nodulated by *Bradyrhizobium* species. *Appl Environ Microbiol* 2004;**70**:5980–7.

Wilson K. Preparation of genomic DNA from bacteria. *Curr Protoc Mol Biol* 2001;**56**:241–5.

Yao ZY, Kan FL, Wang ET *et al*. Characterization of rhizobia that nodulate legume species of the genus *Lespedeza* and description of *Bradyrhizobium yuanmingense* sp. nov. *Int J Syst Evol Microbiol* 2002;**52**:2219–30.

Zhang YM, Tian CF, Sui XH *et al*. Robust markers reflecting phylogeny and taxonomy of Rhizobia. *PLoS One* 2012;**7**: e44936.