RESEARCH ARTICLE

Biodiversity of *Mimosa pudica* rhizobial symbionts (*Cupriavidus taiwanensis*, *Rhizobium mesoamericanum*) in New Caledonia and their adaptation to heavy metal-rich soils

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Abstract

Rhizobia are soil bacteria able to develop a nitrogen-fixing symbiosis with legumes. They are taxonomically spread among the alpha and beta subclasses of the *Proteobacteria*. *Mimosa pudica*, a tropical invasive weed, has been found to have an affinity for beta-rhizobia, including species within the *Burkholderia* and *Cupriavidus* genera. In this study, we describe the diversity of *M. pudica* symbionts in the island of New Caledonia, which is characterized by soils with high heavy metal content, especially of Ni. By using a plant-trapping approach on four soils, we isolated 96 strains, the great majority of which belonged to the species *Cupriavidus taiwanensis* (16S rRNA and *recA* gene phylogenies). A few *Rhizobium* strains in the newly described species *Rhizobium mesoamericanum* were also isolated. The housekeeping and *nod* gene phylogenies supported the hypothesis of the arrival of the *C. taiwanensis* and *R. mesoamericanum* strains together with their host at the time of the introduction of *M. pudica* in New Caledonia (NC) for its use as a fodder. The *C. taiwanensis* strains exhibited various tolerances to Ni, Zn and Cr, suggesting their adaptation to the specific environments in NC. Specific metal tolerance marker genes were found in the genomes of these symbionts, and their origin was investigated by phylogenetic analyses.

Introduction

New Caledonia (NC), a tropical archipelago in the South-West Pacific, represents a remarkable environment for diversity studies. NC is considered as a hotspot of biodiversity, and part of its peculiarity comes from its specific and rich endemic flora adapted to the ultramafic soils, naturally rich in Mn, Fe, Ni, Cr and Co, that cover one-third of the main island (Pelletier, 2006; Pillon *et al.*, 2010). Moreover, owing to the only minor climatic fluctuations described for NC since its colonization by Australian flora 35–45 MYA (Chevillotte *et al.*, 2006), NC is considered as a refuge of 'relic' plant species. Despite it being an incontestably fascinating environment, only a few studies have been dedicated to the diversity of the New-Caledonian microflora and rhizosphere or to symbiotic microorganisms. These studies were generally concerned with tolerance to metal and the development of rehabilitation strategies for Ni mine spoils. Stoppel & Schlegel (1995) isolated and characterized several Ni-resistant strains: *Burkholderia* sp., *Hafnia alvei*, *Acinetobacter* sp., *Alcaligenes* sp., *Pseudomonas denitrificans* and *P. stutzeri*. This was followed by the study of Héry *et al.* (2003), who observed the emergence of bacteria related to *Ralstonia*/*Burkholderia* in samples of serpentine soils in response to Ni spiking. Finally, a recent study of symbionts of *Serianthes calycina*, a NC endemic legume plant, identified *Bradyrhizobium* strains characterized according to their Ni resistance, which is encoded by the *nre* and *cnr* operons (Chaintreuil *et al.*, 2007).

Mimosa pudica originates from America (Barneby, 1991; Simon *et al.*, 2011) and is now a pantropical weed. It was introduced to improve NC grassland at the end of the 19th century (Mac Kee, 1994; Gargominy *et al.*,

FEMS MICROBIOLOGY ECOLOGY

1996), but is now considered as a weed in NC, invading pastures and the dry forest. A risk assessment for *M. pudica* exists for the Pacific region (Pacific Island Ecosytem at Risk, PIER, http://www.hear.org/pier/). *Mimosa pudica*, as many *Mimosa* species (Chen *et al.*, 2003, 2005a, b; Bontemps *et al.*, 2010), interacts preferentially with rhizobia belonging to the beta-subclass of *Proteobacteria*, which are now termed beta-rhizobia (Moulin *et al.*, 2001; Gyaneshwar *et al.*, 2011). *Mimosa* spp. symbionts include several *Burkholderia* species, such as *B. phymatum* (Elliott *et al.*, 2007), *B. tuberum* (Vandamme *et al.*, 2002; Mishra *et al.*, 2012), *B. mimosarum* (Chen *et al.*, 2006), *B. nodosa* (Chen *et al.*, 2007), *B. sabiae* (Chen *et al.*, 2008a), *B. symbiotica* (Sheu *et al.*, 2011) and *B. diazotrophica* (Sheu *et al.*, 2012) and two species of *Cupriavidus*: *C. taiwanensis* (Chen *et al.*, 2001) and *Cupriavidus necator* (da Silva *et al.*, 2012; Taulé *et al.*, 2012). *Burkholderia* symbionts have been isolated from native and invasive *Mimosa* species across South Central and North America, Taiwan, China and Australia (Chen *et al.*, 2003, 2005a, b; Barrett & Parker, 2005, 2006; Liu *et al.*, 2007; Bontemps *et al.*, 2010; Mishra *et al.*, 2012). *Cupriavidus taiwanensis* was first isolated from invasive *Mimosa* species (*M. pudica*, *M. diplotricha* and *M. pigra*) in Taiwan (Chen *et al.*, 2001, 2003, 2005b), from *M. pudica* in India (Verma *et al.*, 2004) and then later from China (Liu *et al.*, 2011, 2012). It has also been isolated from *Mimosa* spp. in their native ranges in the New World, such as in Costa Rica (Barrett & Parker, 2006), Texas (USA) (Andam *et al.*, 2007) and French Guyana (Mishra *et al.*, 2012). Interestingly, no *Cupriavidus* isolates were found in a recent large biodiversity survey of native *Mimosa* symbionts in Brazil (Bontemps *et al.*, 2010; dos Reis *et al.*, 2010), which is the main centre of diversity of the large genus *Mimosa* (Simon *et al.*, 2011), thus emphasizing the current lack of clarity with regard to the origins of symbiotic *Cupriavidus*. A second symbiotic *Cupriavidus* species, *C. necator*, has been detected in South America. In Uruguay, *C. necator* strains were isolated from nodules of *Parapiptadenia rigida* (Taulé *et al.*, 2012), while in Brazil they were trapped from soils using *Leucaena leucocephala* and *Phaseolus vulgaris* (da Silva *et al.*, 2012).

Burkholderia is one of the most species-rich genera in the beta-Proteobacteria (> 50 species; Gyaneshwar *et al.*, 2011), and *Cupriavidus*, after several rearrangements and name changes (Alcaligenes-*Ralstonia*-*Wautersia*-*Cupriavidus*, Vandamme & Coenye, 2004), now includes 12 species. Bacteria belonging to the genera *Burkholderia* and *Cupriavidus* show extreme diversity in habitats and ecological lifestyles. They have been isolated from soil, water, plants, rhizosphere, insects (*Burkholderia*) and from infected humans (Tran Van *et al.*, 2000; Jeong *et al.*, 2003; Caballero-Mellado *et al.*, 2004; Reis *et al.*, 2004), illustrating their remarkable capacity for adaptation (Vial *et al.*, 2010). Within the *Cupriavidus* genus, several species seem particularly adapted to metal-rich environments (Mergeay *et al.*, 1985; Goris *et al.*, 2001; Sato *et al.*, 2006). The most well-known and studied strain is *C. metallidurans* CH34 that was isolated from metallurgical sediments in Belgium and represents the model bacterium for metal resistance studies (Mergeay *et al.*, 1985; Monchy *et al.*, 2007). *Cupriavidus eutrophus* H16, a metabolically versatile organism capable of subsisting in the absence of organic growth substrates on H(2) and CO(2) as its sole sources of energy and carbon (Pohlmann *et al.*, 2006), was recently shown to display high similarity with the genome of the *M. pudica* symbiont, *C. taiwanensis* LMG19424 (Amadou *et al.*, 2008).

The aim of this study was to investigate the biodiversity of rhizobial symbionts associated with *M. pudica* in the peculiar metal-rich soils of NC and their adaptations to this environment. The questions we addressed were as follows: first, what are the symbionts of *M. pudica* in NC? Are they native to NC or are they related to symbionts found in South America or Asia? Second, how did these symbionts adapt to heavy metals in the NC soils? To answer these questions, we isolated the *M. pudica* symbionts from New-Caledonian soils and analysed their taxonomic and symbiotic genetic diversity as well as their symbiotic performance with *M. pudica*. We also analysed the rhizobial adaptation to the NC environment with regard to their metal tolerance and the presence of genetic resistance determinants in their genome, in comparison with other model metal-resistant bacteria.

Materials and methods

Soil sampling and analyses

Four sites (S1–S4) were chosen for this study corresponding to different soil conditions where *M. pudica* was observed to grow (Table 1). Site S1 is located in the Northern part of NC, at the bottom of the Koniambo Massif on a basaltic weathered substrate. Site S1 is approximately 300 km away from the three sites located in the Southern part of the island, that are at a distance of 8–16 km from each other. Site S2 is located on the coastal side West of Noumea and is composed mainly of coral calcareous materials (Cote Blanche), site S3 is located 16 km East of Noumea, near the abandoned mining site of Mont Dore on fine saprolithic colluvial deposit, and site S4 is located 5 km North of Noumea (Ko We Kara) on burnt nickel slag. S4 is a sterile substrate that could be considered as an anthropogeomorphic soil material according to FAO (World reference

Table 1. Characteristics of soils used for rhizobial trapping

Soil	Site characteristics and flora*	GPS coordinates	Alt.†	Soil parameters‡					Total metal ions§					Bioavailable metal ions¶			
				Gr.	pH	OM	Corg	Ntot	P	Ni	Cr	Co	Zn	Ni	Cr	Co	Zn
S1	Koniambo, North NC, basaltic weathered soil, mine roadside, *M. pudica*	21°00′52.88″S 164°44′27.96″E	196	Sandy loam	8.2	7.1	4.1	0.2	0.04	1562	1106	132	111	3.5	0.03	0.13	0.4
S2	Cote Blanche, South NC, coral calcareous soil, sea coast, *M. pudica*	22°18′14.59″S 166°27′30.00″E	0	Sandy loam	6.6	8.3	4.8	0.7	0.03	22.5	63	11	69	0.15	0.01	0.06	0.6
S3	Mont Dore, South NC, saprolitic colluvial soil, *C. collina*, *A. spirorbis*, *M. pudica*	22°15′16.51″S 166°36′44.27″E	112	Loamy sand	6.8	8.1	4.7	0.2	< 0.01	10177	15652	690	388	3.2	5.1	0.5	0.6
S4	Ko We Kara, South NC, open area, burnt nickel slag, *M. pudica*	22°13′53.08″S 166°27′28.80″E	11	ASM sand	9.2	2.8	1.6	0.1	0.05	1090	6860	57	170	0.4	0.2	0.03	0.5

*C. *collina*, *Casaurina collina*; *A. spirorbis*, *Acacia spirorbis*; *M. pudica*, *Mimosa pudica*.

†Alt., site altitude (m).

‡Gr, granulometry-soil texture according to particle size, ASM, anthropogeomorphic soil material.

§pH, soil pH (in water); OM, organic matter (g kg^{-1}); Corg, organic carbon content (g kg^{-1}); Ntot, total nitrogen content (g kg^{-1}); P, phosphate content Olsen (g kg^{-1}).

¶Total metal ions, total metal quantity (mg kg^{-1}); Bioavailable metal ions, metal quantity extracted with DPTA (mg kg^{-1}).

base for soil resources). For each site (represented by a surface area of 4–10 m^2), five soil samples (200 g) were randomly collected from under *M. pudica* plants and then mixed together for physico-chemical analysis and for isolation of *M. pudica*-compatible rhizobia. Soil granulometric analysis was performed by water dispersion and sedimentation, and the mineralogy of samples was established by X-ray diffraction. To measure total quantity of Fe, Mn, Cu, Zn, Ni, Cr, Co, Pb and Cd in the soils, samples were analysed as described previously (Perrier *et al.*, 2004). To estimate the availability of metals, diethylene triamine pentaacetic acid (DTPA) extraction was performed according to the Lindsay and Norvell method (Lindsay & Norvell, 1978). Elements were measured by ICP-AES (CIRAD Montpellier).

Isolation and maintenance of bacterial strains

Rhizobia were trapped from soil samples using *M. pudica* as a trap-host (*M. pudica* seeds were purchased from B&T World Seeds, Paguignan, France). Soil suspensions were prepared by mixing 5 g of soil with 50 mL of sterile water and then vortexing thoroughly. Three-day-old germinated *M. pudica* seeds (see later) were then inoculated with 1 mL of the soil dilution in a Jensen Gibson tube (Vincent, 1970). Fifteen tube replicates were prepared for each soil sample (with 10 replicates without soil added as negative control)and incubated for 4 weeks in a tropical chamber (28 °C, 55% humidity, day/night 16 h/8 h). Five nodules per plant were randomly harvested for bacterial isolation. Nodules were thoroughly washed in running tap water, surface sterilized with 3% calcium hypochlorite (filtered) for 5 min and rinsed six times in sterile water. The nodules were individually crushed in 50 μL of sterile water, and the suspension was streaked onto yeast mannitol agar plates (YMA, Vincent, 1970) and incubated at 28 °C for 48 h. Bacterial isolates were subcultured twice and checked for nodulation of *M. pudica* using Jensen Gibson's tubes in similar growth condition as described for trapping, except 1 mL of exponential bacterial culture was used for inoculation. For long-term maintenance, bacterial strains were grown in YM broth and preserved in 25% glycerol at −80 °C.

Plant nodulation and symbiotic efficiency tests

Mimosa pudica seeds were surface sterilized in 96% H_2SO_4 and 3% calcium hypochlorite (15 min each treatment, followed by 5–6 washes with sterile distilled water), soaked overnight in sterilized water and transferred for overnight germination at 37 °C on 0.8% water agar plates. Seedlings were then transferred to Gibson tubes for trapping or nodulation tests. Gibson tubes were kept

in a tropical plant chamber (28 °C, 55% humidity, day/night 8 h/16 h) for 3 weeks.

Symbiotic efficiency was assessed in plants grown in pots (15 replicates) containing a mixture of sand and vermiculite (50/50) and supplemented with nitrogen-free nutrient solution, except for an initial single application of 0.5 mM KNO_3. Dry weight of shoots, roots and nodules, and nodule number were measured at 30 days post-inoculation in a tropical greenhouse.

Acetylene reduction assays (ARA) were performed at 4 weeks after inoculation on excised roots, as described by Miché *et al.* (2010).

Molecular methods

For routine PCR amplifications, bacterial DNA was obtained from isolates using a quick Proteinase K method as described by Wilson (1987). PCR amplifications were performed with GoTaq DNA Polymerase (Promega) according to the manufacturer's instructions and using 0.625 unit of GoTaq and 20 μmol of each primer in 25 μL of volume reaction. A routine PCR protocol was applied to the DNA amplification based on 35 cycles with 30 s of denaturation, 30 s of hybridization and 1 min of elongation steps. The hybridization temperature was adjusted according to the primers used (Table 2).

A nearly full-length 16S rRNA gene was amplified with primers FGPS6 and FGPS1509 (Normand *et al.*, 1992) and sequenced as previously described in Moulin *et al.* (2001). PCR-restriction fragment length polymorphism (RFLP) analysis was performed using 10 μL of 16S rRNA gene PCR amplification products obtained with CfoI, HinfI and MspI restriction enzymes. Analysis of digestion products by agarose gel electrophoresis was performed as previously described (Laguerre *et al.*, 1994). PCR amplification of *recA* fragments of 800 bp were obtained for beta-rhizobia strains using primers recABurk1F and recABurk1R, and fragments of 600 bp were obtained for alpha-rhizobia using primers recA-6-F and TS2recAR (Table 2). *nodA* PCR amplification (800 bp) and sequencing were performed with primers nodABurkF and nodABurkR for both alpha- and beta-rhizobia. *nifH* amplification (400 bp), and sequencing for beta-rhizobia were performed with primers nifH-R and for alpha-rhizobia using primers nifHF and nifHI. Finally, amplification of the metal resistance determinants *nreB* (800 bp fragment) and *ncc*/*cnrA* (1100 bp, protein N-terminal fragment) was performed as described by Chaintreuil *et al.* (2007). REP-PCR fingerprints of genomic DNA were produced according to a protocol for BOX-PCR from Kaschuk *et al.* (2006), except that the primer annealing temperature was reduced to 40 °C, and the primers REP1R-I and REP2-I were used.

Phylogenetic analyses

Sequences were aligned using ClustalX (Thompson *et al.*, 2002) and manually edited and corrected with Genedoc (Nicholas & Nicholas, 1997). Phylogenies were inferred by distance on mega4 (Tamura *et al.*, 2011) or by maximum likelihood (ML) methods using PhyML (Guindon *et al.*, 2005) or Paup4. Bootstrap analyses were performed

Table 2. Primers used for PCR and sequencing

Gene	Primer name	Sequence	Reference
16S rRNA gene	FGPS6	GGAGAGTTAGATCTTGGCTCAG	Normand *et al.* (1992)
	FGPS1509	AAGGAGGGGATCCAGCCGCA	
recA	recABurk1F	GATCGARAAGCAGTTCGGCAA	Payne *et al.* (2005)
	recABurk1R	TTGTCCTTGCCCTGRCCGAT	
	recA-6-F	GTAGAGGAYAAATCGGTGGA	Modified from Gaunt *et al.* (2001)
	TS2recAR	CGGATCTGGTTGATGAAGATCACC	Stepkowski *et al.* (2005)
nodA	nodABurkF	CRGTGGARGGTBYGYTGGGA	This study
	nodABurkR	TCAYARCTCDGGBCCGTTBCG	
nifH	nifH-F	AARGGNGGNATYGGHAARTC	Chen *et al.* (2003)
	nifH-R	GCRTAVAKNGCCATCATYTC	
	nifHF	TACGGNAARGGSGGNATCGGCAA	Laguerre et al. (2001)
	nifHI	AGCATGTCYTCSAGYTCNTCCA	
nreB	*nre*B-425F	CCTTCACGCCGACTTTCCAG	Chaintreuil *et al.* (2007)
	*nre*B-1179R	CGGATAGGTAATCAGCCAGCA	
cnrA	*cnrA*-F	AACAAGCAGGTSCAGATCAAC	Chaintreuil *et al.* (2007)
	cnrA-R	TGATCAGGCCGAAGTCSAGCG	
REP sequence*	REP1R-I	IIIICGICGICATCIGGC	Versalovic *et al.* (1991)
	REP2-I	ICGICTTATCIGGCCTAC	

*Repetitive Extragenic Palindromic [REP] elements.

using 1000 replicates for distance and 100 replicates for ML using the phylogeny.fr software package (BIONJ, PHYML; Dereeper *et al.*, 2008).

Metal tolerance tests

Tolerance of bacterial strains to Ni, Cr, Co, Zn and Cd was determined based on the presence or absence of bacterial growth in 96-well microplates filled with 200 μL per well of YM medium supplemented with $NiSO_4$ (3, 7, 15, 30 mM), K_2CrO_4 (0.1, 0.33, 0.83, 1.6, 3.3, 6.6 mM), $CoCl_2$ (2, 3, 5, 10 mM), $ZnSO_4$ (0.5, 3, 5, 7, 10, 15 mM) and $CdCl_2$ (0.1, 0.5, 3 mM). The microplates were inoculated with bacterial cultures calibrated at $OD_{600\ nm} = 0.12$ and incubated under agitation at 28 °C up to 6 days. The bacterial growth was followed by spectophotometry at $OD_{600\ nm}$ using an Infinite M200 microplate spectrophotomer (TECAN). Strains were considered as tolerant when their culture $OD_{600\ nm}$ was $\geq$ 0.3 (and non-tolerant when $OD_{600\ nm}$ did not increase). To confirm the presence or absence of metal tolerance in NC isolates, three reference strains were included: *C. metallidurans* CH34, a highly resistant bacterium to heavy metal (Mergeay *et al.*, 2003) serving as a positive control, *C. taiwanensis* LMG19424^T that is the type strain of the species and *Escherichia coli* S17-1 (Simon *et al.*, 1983) as a negative control.

Statistical analysis

Difference in the distribution of the genotypes across the soils was tested by Chi-square test of row (soils) × column (genotypes) independency from the contingency table using the STATXACT software.

Nucleotide sequence accession numbers

Partial sequences have been deposited in the GenBank/EMBL database under accession numbers FN908229 to FN9082346 for the 16S rRNA gene; HE687278, HE687279, HE687280 and FN908247 to FN908253 for *rec*A; FN908240 to FN908244 for *nod*A; FN908238 to FN908239 for *nifH* gene, FN908237 for *ncc*-like, FN908245 to FN908246 for *nre*B, FN908235 to FN908236 for *czc*A-like.

Results

Characteristics of New Caledonian soils hosting *M. pudica*

The four selected locations represented diverse geological conditions: basaltic weathered substrate, coral calcareous materials, saprolitic colluvial deposit and burnt nickel slag. All four soil samples were characterized by a low quantity of organic matter (2.8–8.3 g kg^{-1}), total nitrogen (0.1–0.7 g kg^{-1}), and by a deficiency in phosphorous (P_{Olsen} 0.01–0.05 g kg^{-1}). These characteristics were especially pronounced for the anthropogenic soil S4 (Table 1). Samples S1 and S4 were characterized by basic pH (8.2 and 9.2, respectively), while S2 and S3 were almost neutral (pH 6.6 and 6.8, respectively). The two soil samples S1 and S2 collected in the vicinity of metal-rich ultramafic rocks (Koniambo and Mont Dore, respectively) and the soil sample from mine spoils (S4 Ko We Kara) were characterized by high contents of putatively toxic heavy metals, such as Ni (1562–10 177 mg kg^{-1}) and Cr (1106–15 652 mg kg^{-1}). A high concentration of Co was observed in soil S1 (690 mg kg^{-1}). The results of metal extraction with DTPA showed the presence of available Ni in soil S1 (3.47 mg kg^{-1}, respectively), and Ni and Cr in soil S3 (3.19 and 5.1 mg kg^{-1}, respectively). It should be noted that total contents of Ni and Cr for soils S1 and S3 were comparable with quantities described for ultramafic soils (Perrier *et al.*, 2006), while concentrations of these DTPA-extractable metals were approximately a 100-fold lower than in ultramafic soils. The principal component analysis (PCA) with regard to soil physico-chemical characteristics confirmed the pronounced differences between the four soils (Supporting Information, Fig. S1). The four soils were well separated by F1 and F2 first components or by F1 and F3. The main soil characteristics contributing to these components were pH, sand content (higher for soil S4) and bioavailable content of Cr and Co (higher for soil S3) as well as Ni and Zn, clay and total nitrogen content (for soil S2).

Trapping and molecular characterization of New Caledonian *M. pudica* symbionts diversity

Nineteen to 31 isolates per soil were trapped from nodules of *M. pudica* and were able to re-nodulate this host plant, leading to a collection of 96 confirmed *M. pudica* symbionts from NC (Table S2).

Three 16S rRNA gene haplotypes were identified according to their RFLP profiles (AAA, AAB and DCC; Table 3). The nearly full 16S rRNA gene of one representative strain of each haplotype (STM6077, STM6117 and STM6155 for AAA, AAB and DCC, respectively) was sequenced. BLASTN analyses showed that 16S rRNA gene haplotype AAA (71% of isolates) and AAB (25% of isolates) shared 98% nucleotide identity (11 nt were different out of 1365 nt). Both haplotypes showed 99% nucleotide identity with LMG19424, the type strain of *C. taiwanensis*, indicating that these strains might belong to this species. The other closest hits were those of

Table 3. Distribution of *Cupriavidus* and *Rhizobium* genotypes in four soils of New Caledonia

			No of isolates in soils‡					
Genotypes*	16S RFLP haplotype†	REP-PCR pattern	S1 (31)	S2 (25)	S3 (21)	S4 (19)	Total (96)	Representative strains
Ct I	AAA	1			7	19	26	STM6162
Ct II	AAA	2	18	1			19	STM6077
Ct III	AAA	3	13		2		15	STM6070
Ct IV	AAA	4			8		8	STM6133
Ct V	AAB	5		24			24	STM6117
Rsp VI	DCC	6			4		4	STM6155

*Genotypes were categorized based on combined data of 16S rRNA gene haplotypes and REP-PCR patterns; Ct, *C. taiwanensis*; Rsp, *Rhizobium* sp.

†16S rRNA gene haplotypes were defined by combination of MspI, HinfI and CfoI restrictions enzymes patterns by PCR-RFLP; numbers between brackets indicates the number of isolates per site.

‡S1, S2, S3 and S4 refer to the soil samples listed in Table 1 and Table S1.

C. eutrophus H16 (98%) and *C. metallidurans* CH34 (94% nucleotide identity). The third haplotype DCC (4% of isolates) fell into the genus *Rhizobium* (100% nucleotide identity with *Rhizobium mesoamericanum* CCGE501 and 99% identity with *Rhizobium* sp. tpud 22.2).

As the majority of isolates belonged to the genus *Cupriavidus*, we assessed the intraspecific diversity of the rhizobial collection by REP-PCR fingerprinting (Table 3). The most abundant 16S rRNA gene haplotype (AAA) was subdivided into four REP-PCR genomic profiles, the two other 16S rRNA gene haplotypes being each associated with only one specific REP-PCR genomic profile. The combination of 16S rRNA gene haplotypes and REP-PCR profiles allowed for the differentiation of six rhizobial genotypes, five within *Cupriavidus* (I to V) and one representing the *Rhizobium* isolates (VI). Four of the defined genotypes was each represented by 16–27% of the isolates. The minority genotypes (IV and VI) together represented the remaining 13% of the total isolates.

Phylogeny of 16S rRNA and *recA* genes

16S rRNA gene and *recA* sequences were obtained for representative strains of each NC rhizobial genotype, and phylogenies for both genes were performed. Each *C. taiwanensis* genotype had a unique sequence with substitutions varying from 5 to 14 nucleotides over 1394 bp for 16S rRNA gene and from 5 to 10 nucleotides over 529 bp for *recA* sequences. As represented in Fig. 1a and b, as the relative positioning of sequences in the *C. taiwanensis* phylogeny were not strongly supported by bootstrap values, it is difficult to deduce the exact geographical origin of NC isolates. It can be observed, however, that in the first instance the *C. taiwanensis* genotypes formed distinct lineages. Secondly, in both phylogenies, all the genotypes I-V are closer to *C. taiwanensis* LMG19425 than to *C. taiwanensis* LMG19424 (strains which have been shown to share 98% nucleotide identity and 71% of genome identity by DNA-DNA hybridization; Chen *et al.*, 2001).

Phylogenetic analysis of the 16S rRNA gene and *recA* sequences placed genotype VI (STM6155) in a well-defined clade including isolates from a newly described species, *R. mesoamericanum* (strains CCGE501 and CCGE503, 100% identity over 1346 bp for 16S rRNA gene), which was isolated from *Phaseolus vulgaris* nodules in Mexico (López-López *et al.*, 2011) (Fig. 1a). In this clade were also placed *Rhizobium* sp. tpud. 40a and tpud22.2 that were isolated from nodules of *M. pudica* in Costa Rica (Barrett & Parker, 2006) and which showed 69% of DNA-DNA homology with *R. mesoamericanum* CCGE 501, as well as *Rhizobium* sp. STM3625 from *M. pudica* in French Guiana (Mishra *et al.*, 2012). Our *Rhizobium* isolates were thus considered as belonging to *R. mesoamericanum*. This group was clearly separated from the *Rhizobium etli* and *Rhizobium tropici* clades that contain strains isolated from the *Mimosa* species *M. diplotricha*/*M. invisa*, *M. affinis* and *M. ceratonia*.

Phylogenetic analyses of *nodA* and *nifH* symbiotic genes

Phylogenies of *nodA* and *nifH* gene fragments of representative strains of each rhizobial genotype were built to investigate the origin and evolutionary relationships of symbiotic genes within the NC collection. Sequences for the two gene fragments (*nodA* 530 bp and *nifH* 350 bp) were strictly identical for all the *Cupriavidus* genotypes isolated in NC. Sequences of both genes formed a highly supported clade (97% bootstrap – BP) together with several *C. taiwanensis* strains from various countries (Taiwan, Costa Rica and Papua New Guinea; Fig. 2a and b), with the closest one being that of strain NGR193 from Papua New Guinea.

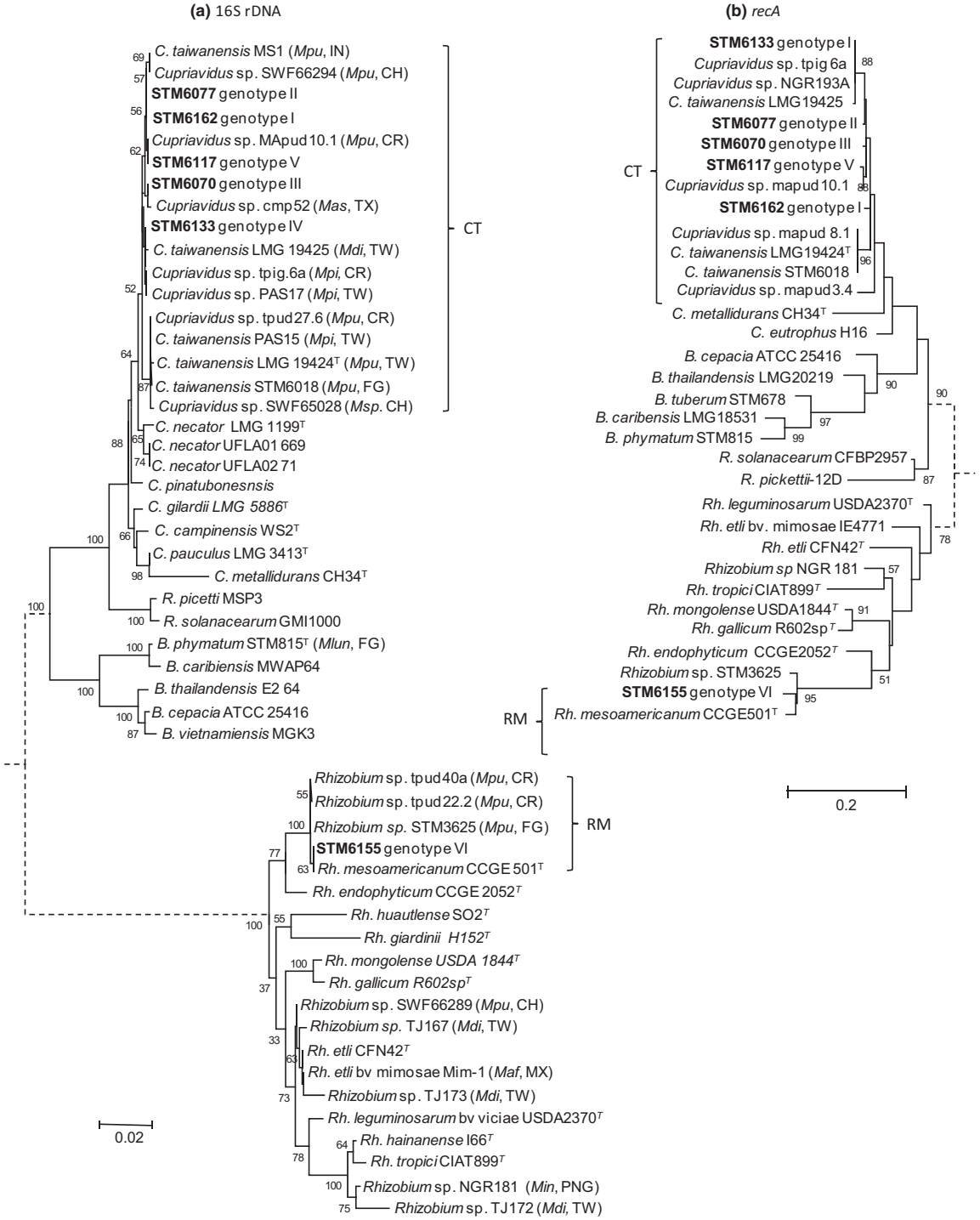

Fig. 1. 16S rRNA gene (a) and *recA* (b) phylogenetic trees of *Mimosa* symbionts and related species (among alpha- and beta-Proteobacteria). Both trees were built by neighbour-joining from a matrix corrected by the Kimura-2 parameter method. Numbers at nodes are boostrap% from 1000 replicates (shown only when > 50%). Broken tree lines indicate that branch length is not informative (upper branch trees were reduced to fit to page). Both trees were rooted with *Shewanella oneidensis* MR1 (gamma-Proteobacteria). Strains isolates from this study (New Caledonia) are in bold. Information about plant host and geographical origin are only given for *Mimosa* symbionts. Scale bar indicates numbers of subsitutions per site. Mpu, *M. pudica*; Mdi, *M. diplotricha*; Mpi, *M. pigra*; Maf, *M. affinis*; Msp, *Mimosa* sp.; Mlu, *Macchaerium lunatum*; IN, India; CH, China; CR, Costa Rica; FG, French Guyana; TW, Taiwan; TX, Texas; PNG, Papua New Guinea; PN, Panama; MX, Mexico. Accession numbers of sequences included in the trees are given in Table S2.

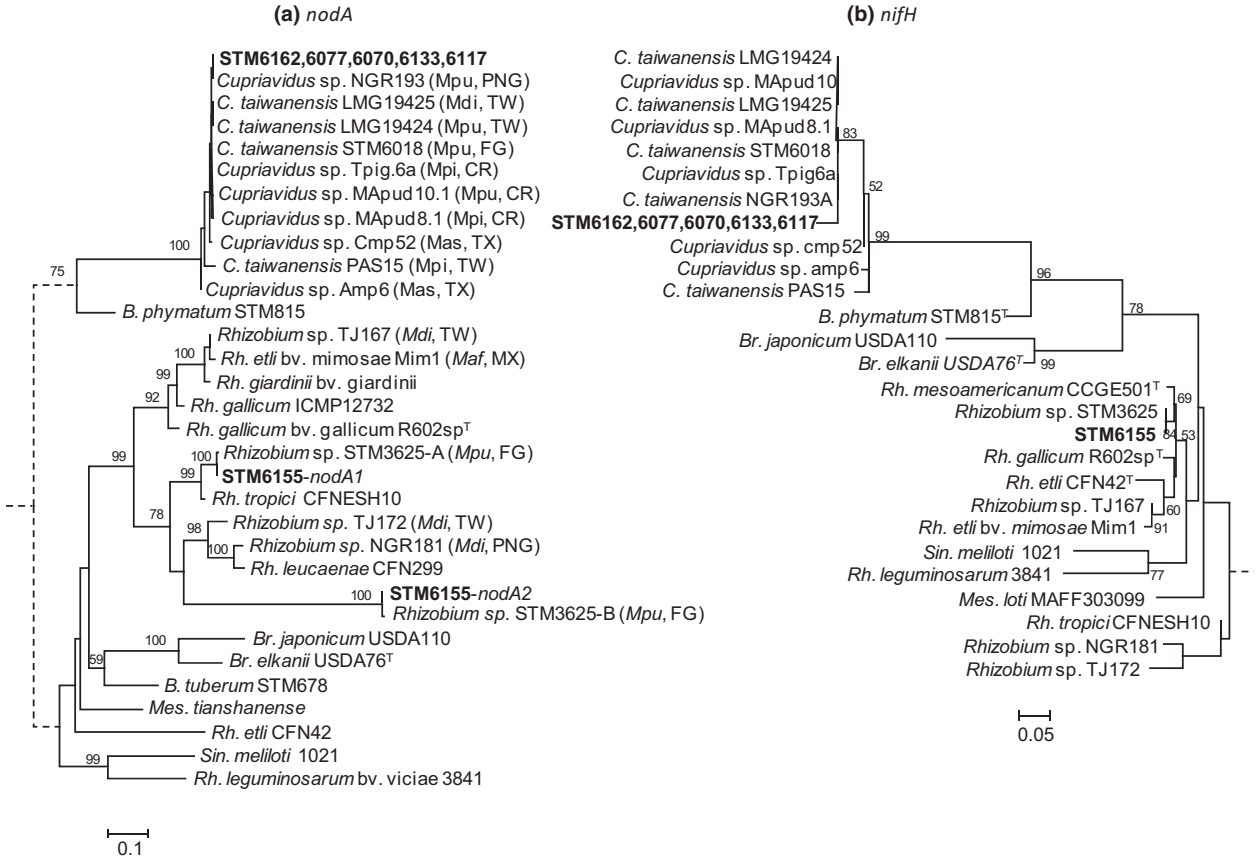

Fig. 2. *nodA* (a) and *nifH* (b) phylogenetic trees of *Mimosa* symbionts and related species (among alpha- and beta-Proteobacteria). Both trees were built by maximum likelihood at Phylogeny.fr (PhyML). Numbers at nodes are boostrap% from 100 replicates (shown only when > 50%). Broken tree lines indicate that branch length is not informative (upper branch trees were reduced to fit to page). *nodA* trees was rooted with *Azorhizobium caulinodans* ORS571 (alpha-Proteobacteria, L18897), while *nifH* tree was rooted with *Klebsiella pneumonia* (gamma-Proteobacteria). Strains isolates from this study (New Caledonia) are in bold. Information about plant host and geographical origin are only given for *Mimosa* symbionts. Scale bar indicates numbers of subsitutions per site. Abbreviations are the same as those given in Fig. 1 legend. Accession numbers of sequences included in the trees are given in Table S2.

Interestingly, analysis of the *nodA* gene sequence from the NC *R. mesoamericanum* strain STM 6155 (genotype VI) showed the presence of two different copies of *nodA* gene sharing 66% of identity at the nucleotide level, and each being nearly identical to the *nod*A1 and *nod*A2 genes of *Rhizobium* sp. STM3625 (99% nucleotide identity), a strain isolated from *M. pudica* in French Guiana (Mishra *et al.*, 2012). The *nodA1* genes of STM6155 and STM3625 formed a clade together with *R. tropici* strains isolated from nodules of *Acaciella angustissima* from Mexico (Rincón-Rosales *et al.*, 2009). The *nodA2* genes formed a new branch, clearly separated from other known sequences (Fig. 2a), at the base of a clade (but with a very low bootstrap support) grouping several *Rhizobium* spp. strains isolated from diverse *Mimosa* species, such as NGR181, TJ172 and *Rhizobium leuaenae* CFN299 (a *P. vulgaris* symbiont from Mexico, Debellé *et al.*, 1996). The *nifH* gene phylogeny showed that the NC *Rhizobium* sp. *nifH* sequence was again localized in a well-defined clade together with *R. mesoamericanum* (López-López *et al.*, 2011) and *Rhizobium* sp. STM3625 (Mishra *et al.*, 2012).

Geographical distribution of genotypes

The distribution of species and REP-PCR genotypes in soils is presented in Table 3. All soils hosted *C. taiwanensis* but *R. mesoamericanum* was found only in soil S3. Indeed, the highest diversity of genotypes was found in soil S3, which hosted three *Cupriavidus* and the *Rhizobium* genotypes. Soil sample S1 showed nearly equal abundances of genotypes II and III. The lowest diversity was observed in soil S2, where genotype V dominated (96%) over genotype II, and in soil S4, where only genotype I was detected. These results were confirmed by calculating the Simpson's Index of Diversity (Brower & Zar, 1984) which showed significant variation among soils in

the order S3 > S1 > S2 = S4 (1/D = 3.45, 2.03, 1.08 and 1, respectively). Concerning the differences between the populations, we calculated Variance of Simpson's Diversity Index for all soils (S^2 = 0.0005, 0.0041, 0.00117 and 0 for S1, S2, S3 and S4, respectively) and compared the variance with a *t*-test. A site-dependent distribution was observed and confirmed statistically by a Chi-square test. No single genotype was present in all sites. The genotypes IV, V and VI were each detected in only one soil, genotype II was abundant in soil S1 and infrequent in soil S2, and genotype I was much more frequent in soil S4 than in soil S3.

Metal tolerance of New-Caledonian *M. pudica* symbionts

Bacterial isolates were tested for their tolerance to several heavy metals detected in three of four soil analyses: Ni, Co and Cr. Cd and Zn were added to the tests because the main known Ni and Co resistance mechanisms confer simultaneous resistance to these two metals (Grass *et al.*, 2000). The detailed results are presented in supplementary data Table S2 and are resumed in Fig. 3. Ni tolerance was observed for 16% of NC isolates that showed resistance to a higher Ni concentration (15 mM) than that of the positive control *C. metallidurans* CH34 (7 mM). No other isolates from the NC collection, nor *C. taiwanensis* LMG19424, could grow in the presence of Ni (even at levels as low as 3 mM in YM medium). The Ni-tolerant NC symbionts belonged mostly to genotype III (14 out of 15 isolates), and a few to genotype II (two of 19 isolates). Surprisingly, these Ni-tolerant isolates originated mainly from soil S1 (88% of isolates) which is characterized by only slightly higher DTPA-extractable Ni content and a lower total Ni concentration than soil S3. Two levels of Zn tolerance were observed. Eighty per cent of the isolates tolerated the presence of 10–15 mM Zn; these Zn-tolerant isolates belonged to genotypes I, II (with one exception), IV and V and were found in all soils. Three isolates tolerated 5–7 mM Zn that was a lower concentration than that tolerated by *C. taiwanensis* LMG19424 (10 mM) and *C. metallidurans* CH34 (15 mM), but higher than that tolerated by the negative control *E. coli* (3 mM). The remaining isolates (16.6%) that grew only in the presence of 3 mM Zn were considered as non-tolerant. Tolerance to Cr and Cd that was comparable with that of the reference strain, *C. metallidurans* CH34, was detected in 28% and 24% of the NC isolates for Cr and Cd, respectively. The Cr- and Cd-tolerant isolates were distributed within almost all genotypes and soils, with the exception of genotype III (which showed no Cr tolerance) and VI (which showed no Cd tolerance). None of the isolates obtained in this study showed tolerance to the presence of Co.

Phylogenetic analyses of Ni resistance determinants

Nickel tolerance seemed to be restricted to a specific soil and to two specific genotypes (II and III). We further investigated this phenotype by looking for the presence of previously described Ni resistance determinants (*ncc/cnrA*- and *nrb*-type genes) and analysing the sequence polymorphism of putative gene fragments. Thirty-one isolates were analysed: 12 of 16 Ni-tolerant isolates, all reference strains, and 14 randomly chosen non-tolerant isolates. Results showed a nearly total correlation between Ni tolerance and the PCR amplification of gene fragments from both *ncc/cnrA* and *nreB* (Table S1). Two non-resistant isolates showed the presence of one of the two markers. To characterize the *ncc/cnrA* fragments, the PCR products from six isolates (STM6070, STM6073, STM6081, STM6085, STM6094 and STM6160) were sent for sequencing. Obtained sequencing results showed the presence of two paralogs in the PCR products. The two paralogs were subcloned for strain STM6160 and sequenced separately. Both sequences showed 67.6% of identity at amino acid level and were closely related to the CzcA and Ncc/CnrA protein family which is involved in the transport of transition metals (Tseng *et al.*, 1999). Phylogenetic analysis (performed on amino acid sequences) separated these two sequences into two

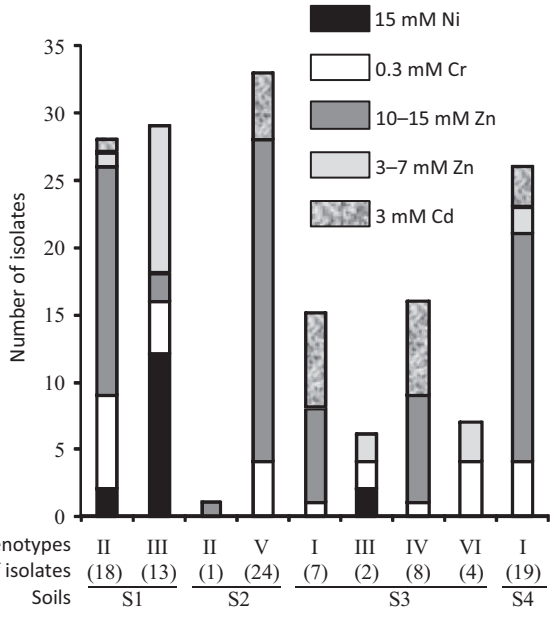

Fig. 3. Distribution of metal tolerance according to the genotype and the soil sample. To simplify the graphic presentation for each genotype, the number of tolerant isolates was presented independently for each metal tolerance, but several isolates displayed double tolerance (see also Table S1).

separate branches (Fig. 4a). One of the sequences grouped with proteins containing the double insertion (2 × 10–13 aa) in a periplasmic loop, as suggested by TMHMM prediction (Sonnhammer *et al.*, 1998). It was closely related to the NccA protein from *C. metallidurans* CH34 with 92% of identity, and subsequently named *nccA*-like. The second sequence was localized on the opposite branch of the phylogenetic tree, together with the CzcA proteins of *C. metallidurans* and *C. taiwanensis* (Fig. 4a); it was therefore named *czcA*-like, even though it was not closely related to any members of this clade (67.9% and 70% of amino acid identity to CzcA from pMOL30 of *C. metallidurans* and of *C. taiwanensis*, respectively). Both sequences were distinct from the CnrA fragment (obtained with the same primers) of *Bradyrhizobium* sp. STM2460 (51% of identity), and the symbiont of *S. calycina* isolated from serpentine soils in NC (Chaintreuil *et al.*, 2007). Concerning the *nreB* gene fragment, the phylogenetic analysis (Fig. 4b) grouped its amino acid sequence in a clade together with those from *C. metallidurans* 31A (previous *Achromobacter xylosoxidans*, Grass *et al.*, 2001) (85% of amino acid identity) and *C. metallidurans* CH34 (78% of amino acid identity). Again, it was distant from the clade formed by sequences of NreB proteins of *Bradyrhizobium* STM2464 and STM2457 (64% of identity).

Symbiotic effectiveness of *C. taiwanensis* STM6070 and *R. mesoamericanum* STM6155

Two strains, representing the alpha-rhizobia (*R. mesoamericanum* STM6155) and the beta-rhizobia (the nickel-tolerant strain *C. taiwanensis* STM6070 – genotype III) detected in our study, were compared for their symbiotic efficiency on *M. pudica*. The reference strain *B. phymatum* STM815 was used as a positive control as it is known to form a highly effective symbiosis with *M. pudica* (Elliott *et al.*, 2007). As shown in Table 4 the inoculation of *M. pudica* with *C. taiwanensis* STM6070 led to a plant biomass comparable with that obtained with *B. phymatum*

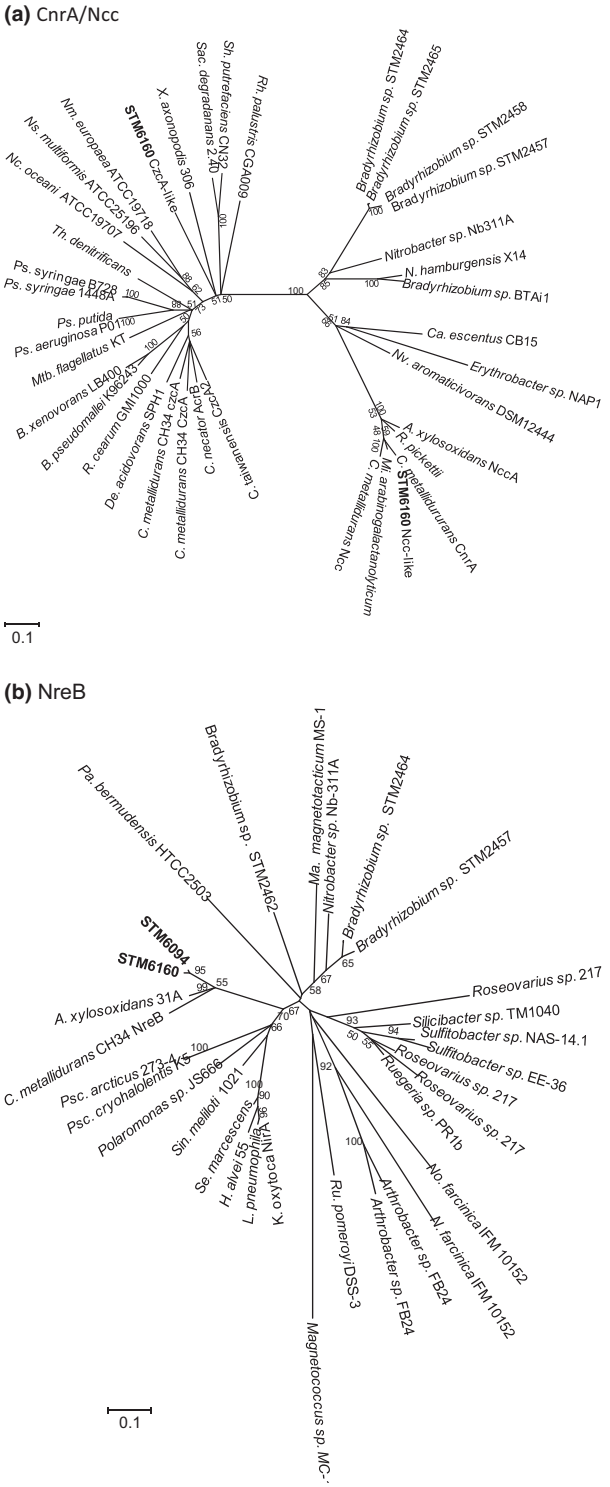

Fig. 4. Maximum likelihood phylogenies of metal resistance determinants based on amino acid alignments of (a) NccA/CnrA (nickel–cobalt–cadmium/cobalt–nickel resistance) with the close homologue CzcA (cobalt–zinc–cadmium resistance) and (b) NreB (nickel resistance). Numbers at nodes are boostrap% from 100 replicates (shown only when > 50%). Scale bar indicates numbers of substitutions per site. New sequences from this study are indicated by bold type. A., *Achromobacter*; B., *Burkholderia*; Ca., *Caulobacter*; C., *Cupriavidus*, De., *Delftia*; H., *Hafnia*; K., *Klebsiella*; L., *Legionella*; Ma., *Magnetospirillum*; Mtb., *Methylobacillus*; Mb., *Microbacterium*; Nm., *Nitrosomonas*; Ns., *Nitrosospira*; Nc., *Nitrosococcus*; No., *Nocardia*; Nv., *Novosphingobium*; Pa., *Parvularcula*; Ps., *Pseudomonas*; Psc., *Psychobacter*; Rh., *Rhodopseudomonas*; Ru., *Ruegeria*; Sac., *Saccharophagus*; Se., *Serratia*; Sh., *Shewanella*; Sin., *Ensifer* (*Sinorhizobium*); Th., *Thiobacillus*; X. *Xanthomonas*. Accession numbers of sequences included in the trees are given in Table S2.

Table 4. Effect of the rhizobial strain on *Mimosa pudica* plant growth and nodule traits 4 weeks after inoculation

Rhizobial strain	No of nodules per plant	DW (mg per plant) of			Nodule DW (mg per nodule)
		Shoots	Roots	Nodules	
C. taiwanensis STM6070	25.5 ab	78.22 a	34.37 a	4.28 ab	0.17
R. mesoamericanum STM6155	15.9 a	41.79 b	14.47 b	11.88 a	0.59
B. phymatum STM815	14.2 a	64.84 ab	27.31 a	2.61 b	0.2
Non-inoculated	–	45.71 b	26.83 a	–	–

DW, dry weight.
Mean values (n = 15) followed by the same letter within a column are not significantly different ($P \leq 0.05$).

STM815. In contrast, the biomass of *M. pudica* plants nodulated by *R. mesoamericanum* STM6155 was almost twofold lower and was not statistically different from the uninoculated control. During this procedure, we observed differences in nodule morphology between *R. mesoamericanum* STM6155 and *C. taiwanensis* STM6070 which was also reflected in their mean nodule weights (Table 4, Fig. S2) and in the proportion of the total plant dry weight that was taken up by nodules (DW). Indeed, the nodule DW induced by *R. mesoamericanum* STM6155 was far higher than that obtained with either STM6070 and STM815, and it represented 13.9% of plant total DW, while the nodules formed by *B. phymatum* STM815 and *C. taiwanensis* STM6070 represented only 2.5% and 3.7% of total plant DW, respectively. Interestingly, the capacity to fix N_2 was confirmed for both NC isolates (*C. taiwanensis* STM6070 and *R. mesoamericanum* STM6155) by the presence of leghaemoglobin and by the detection of nitrogenase activity (via ARA; data not shown).

Discussion

Cupriavidus taiwanensis is the predominant species nodulating *M. pudica* in NC

New Caledonia represents a particular environment originating from its geomorphological history and geographical isolation. The aim of this work was to study the diversity and adaptation of symbionts of *M. pudica*, an invasive tropical weed introduced to this island over a hundred years ago. During the prospecting campaign for sampling sites, we observed that *M. pudica* could be often found in disturbed environments where the native flora was absent and never in well established environments. The diversity analysis of four sites chosen for examination shows that the main symbiotic partner of *M. pudica* was *C. taiwanensis* (96% of obtained isolates), represented by five different genotypes. The presence of *R. mesoamericanum* (4% of total isolates) was observed in only one site. No symbiotic *Burkholderia* could be detected. The isolation method being a plant-trapping approach, we wondered if the specificity of the *M. pudica* variety used for trapping could not have biased the symbiont diversity by selecting preferentially *Cupriavidus* towards *Burkholderia*. For example, it was reported that *M. pigra* seems to associate preferentially with *Burkholderia* in comparison with *M. pudica* (Chen *et al.*, 2005a, b; Barrett & Parker, 2006). However, in a recent study, performed on soils of French Guiana, an identical trapping approach with the same *M. pudica* seeds allowed us to trap diverse *Burkholderia* species, as well as *Cupriavidus* and *Rhizobium* species, showing this strategy and the variety of *M. pudica* used does not preferentially select *Cupriavidus* strains over *Burkholderia* (Mishra *et al.*, 2012). Apart from *M. pudica*, only one other species of *Mimosa* has been observed in NC, *M. diplotricha*, another pantropical invasive weed (Simon *et al.*, 2011) which is known to be nodulated by *C. taiwanensis* in Taiwan (Chen *et al.*, 2003), but also by both *Cupriavidus* and *Burkholderia* in China (Liu *et al.*, 2011). In the case of *M. pigra*, this weed is not present in NC (V. Blanfort, CIRAD, personal communication).

Diversity studies worldwide on symbionts of *Mimosa* spp. presents an emerging picture of *C. taiwanensis* being symbiotically restricted to pantropical invasive *Mimosa* species, such as *M. pudica*, *M. diplotricha* and *M. pigra* (Chen *et al.*, 2001, 2003, 2005b; Verma *et al.*, 2004; Elliott *et al.*, 2007; Gyaneshwar *et al.*, 2011), which contrasts with *Burkholderia* species which are the dominant symbionts of *Mimosa* spp in their native ranges, including the main centre of diversification in Central Brazil (Chen *et al.*, 2005a; Bontemps *et al.*, 2010; dos Reis *et al.*, 2010). The relative scarcity of *C. taiwanensis* in the native ranges of *Mimosa* spp. is illustrated by a study in French Guiana by Mishra *et al.* (2012), where *M. pudica* was found to associate mainly with *Burkholderia* spp., and *C. taiwanensis* was found in only one of the eight sites sampled (in which it constituted 85% of the isolates from that particular soil). Similarly, in Costa Rica, *C. taiwanensis* represented from 8% to 69% of the rhizobial population of native *M. pudica* together with *Burkholderia* spp. and *Rhizobium* sp. (Barrett & Parker, 2006). Interestingly, though, in Taiwan *C. taiwanensis* was shown to be the dominant symbiont (98.6%) for introduced *M. pudica* (Chen *et al.*, 2003) in spite of the proven (and dominant) presence of

Burkholderia spp. obtained from nodules of *M. pigra* (Chen *et al.*, 2005b).

In NC, we encountered again the situation of *C. taiwanensis* dominating as an invasive *Mimosa* symbiont and wondered, therefore, about the factors in this part of the world that might be influencing the supremacy of *C. taiwanensis* over other potential symbionts of *M. pudica*. The absence of any *Burkholderia* symbiotic strains in our NC sampling could result from (1) the restricted number of sampled locations, (2) a very low proportion of symbiotic *Burkholderia* populations or their poor competitiveness for nodulation and/or (3) a negative selective pressure of the NC environment acting against *Burkholderia* symbionts. From our results, we can conclude that if there were any symbiotic *Burkholderia*, given that it has a high competitive ability over *C. taiwanensis* in soils with low amounts of inorganic nitrogen (Elliott *et al.*, 2009), such as those which we sampled in NC, *Burkholderia* populations must be very low in comparison with *C. taiwanensis*.

Local environmental factors may have an impact on bacterial diversity (Barrett & Parker, 2006). One of the important soil characteristics that seems to influence the *Burkholderia*/*Cupriavidus* ratio is the soil pH and inorganic nitrogen content (Elliott *et al.*, 2009). Previous reports have shown that acidic soils could be more conducive to the survival of *Burkholderia* symbionts (Garau *et al.*, 2009; dos Reis *et al.*, 2010; Mishra *et al.*, 2012); the soils analysed in the present study exhibited neutral to basic pH and so might be more favourable for *Cupriavidus* and *Rhizobium*. In the case of two NC soils, S1 and S4, pH levels higher than eight were measured, but for the other two soils, S2 and S3, pH was just under 7, which is more similar to the pH measured for the *M. pudica* rhizosphere soils in French Guiana where the presence of *Burkholderia* spp. was recorded (Mishra *et al.*, 2012). Other soil characteristics that might be limiting the growth of *Burkholderia* spp. are soil structure, organic matter, carbon, nitrogen and phosphorus (Ashworth & Alloway, 2004; Antoniadis *et al.*, 2008; Garau *et al.*, 2009). Indeed, the four soils are constituted in large part of sand and are poor in organic matter, as well as in phosphorus content. The genus *Cupriavidus* includes several species (*C. metallidurans* CH34, *C. eutrophus* H16, *C. pinatubonensis* and *C. laharis*) known to be facultative chemolithotrophs that are adapted well to extremely poor mineral environments (Pohlmann *et al.*, 2006; Sato *et al.*, 2006). The largest chromosome in *C. taiwanensis* (CHR1) harbours 62% of the genes that it shares with *C. metallidurans* CH34, *C. pinatubonensis* JMP134 and *C. eutrophus* H16, and it shows an even higher orthology with *C. eutrophus* H16 (Amadou *et al.*, 2008; Janssen *et al.*, 2010). These data suggest that even if *C. taiwanensis* has a reduction in metabolic properties in comparison with other *Cupriavidus* spp., such as autholithotrophy, it could still profit from the presence of its ancestral genetic heritage. For example, there are several predicted transport proteins that are conserved in *C. taiwanensis* LMG19424 genome in comparison with those from *C. metallidurans* CH34, *C. pinatubonensis* JMP134 and *C. eutropfus* H16 (Janssen *et al.*, 2010).

Adaptation of *C. taiwanensis* to NC soils

The apparently dominant symbiont, *C. taiwanensis*, was represented by five genotypes (as visualized by REP-PCR). These results revealed a relatively low diversity of rhizobia, and the soil-dependent distribution of the *C. taiwanensis* genotypes that could have resulted from specific soil conditions. Given the high metal content of NC soils, it was thus expected that the presence of particular metals might influence the presence and/or distribution of rhizobia. ACP analysis suggested that the presence of Cr and Co could play a role in the distribution of genotypes, but, surprisingly, the NC isolates did not display any tolerance to Co. In the case of Cr, however, tolerance was spread among the genotypes. Moreover, it was found in isolates from all the soils and was not particularly attributed to isolates from soil S3, which is the soil that contained the highest bioavailable Co content. The second metal that was shown to be potentially important for the distribution of genotypes between soils was Ni (especially for S1 and S3), and in this case, we could observe a relation between the metal resistance pattern and the soil characteristics. It was shown that isolates belonging to genotype III (14 out of 15 isolates), which were characteristic of site S1, displayed a tolerance to high concentrations of Ni (15 mM). Such tolerance was not detected in the *C. taiwanensis* LMG19424 reference strain. Nor was high Ni tolerance observed among 200 *C. taiwanensis* isolates from a Taiwanese collection, as the most resistant isolate, TJ208, could not grow in concentrations of Ni higher than 7 mM (in comparison with a reference strain, *C. metallidurans* CH34; Chen *et al.*, 2008b). Our results thus suggest that *Cupriavidus* strains from genotype III have adapted to the presence of Ni in NC soils. New-Caledonian Ni-tolerant isolates appeared to harbour *ncc*-like and *nreB*-like genes which showed higher amino acid sequence identity with Ni resistance determinants from beta-Proteobacteria (Fig. 4), especially to a strain isolated from serpentine soils (Abou-Shanab *et al.*, 2007), than to determinants from alpha-rhizobia (*Bradyrhizobium* STM2464 and STM2463) that were isolated from nodules of *S. calycina* in NC ultramafic soils (Chaintreuil *et al.*, 2007). We can thus speculate that the Ni tolerance of the NC Genotype III *C. taiwanensis*

strains could have been acquired from other Ni-resistant beta-Proteobacteria present in NC, such as *Alcaligenes* spp. (Stoppel & Schlegel, 1995) or the other bacterial populations closely related to the *Ralstonia*/*Oxalobacter*/*Cupriavidus* group (*C. necator* and *C. pauculus*) that have been reported in NC ultramafic soils by Héry *et al.* (2003). These indigenous species represent potential reservoirs of heavy metal tolerance genes and could be the origins of gene transfer to symbiotic *Cupriavidus* species, such transfer being facilitated by the close phylogenetic position of these species. Considering that the metal tolerance genes are often localized on chromosomal islands or on small plasmids, it is intriguing that we did not observe any multiple metal tolerance phenotypes among the NC isolates (Ni/Co/Zn, Ni/Co/Zn/Cd/Cu), as described for other *beta-* and gamma-Proteobacteria, such as *C. metallidurans* CH34 (Monchy *et al.*, 2007) and *C. metallidurans* 31A (previously *Achromobacter xyloxidans* 31A, Stoppel & Schlegel, 1995; Goris *et al.*, 2001). While tolerance to 15 mM Zn was quite commonly observed in all soils, the Ni-tolerant isolates could not grow in Zn concentrations higher than 7 mM. Moreover, the lack of Co tolerance was observed even in the Ni-resistant isolates that were shown to harbour the *cnrA* gene which has been described as conferring Co/Ni resistance to bacteria (Grass *et al.*, 2000). Ni tolerance was not observed in the other genotypes (except Genotype II with two isolates of 19 that were Ni-tolerant) present in soils S1 and S3, which were characterized by the presence of bioavailable Ni. This observation raises questions about the survival strategies of symbionts in soil S1 given the presence in it of several heavy metals. One explanation could be the fact that one soil sample is constituted of at least five subsamples collected in a 5 m radius around the site where the *M. pudica* plants were growing and thus could contain a mixture of samples that are more or less protected from the impacts of metal toxicity. On the other hand, the persistence of different micro-environments within the same soil sample was postulated even in soils that were characterized by a high metal content, and these might explain the presence of heterogeneous metal tolerance within a bacterial population from a particular site.

Cupriavidus taiwanensis and *R. mesoamericanum* symbionts were probably introduced to NC together with *M. pudica* seeds

Another result from this study is the low symbiont diversity found at the species and genotypic levels compared with previous studies, thus raising questions about the origin of these symbionts. Genetic differences in *C. taiwanensis* were observed at the level of sequences of 16S rRNA gene (5–15 nucleotide substitutions among 1394 bp in pairwise comparison) and *recA* (5–10 nucleotide substitutions among 529 bp) (Fig. 1). The existence of different lineages of *C. taiwanensis* has also been shown for isolates from Taiwan (Chen *et al.*, 2003) and Costa Rica (Barrett & Parker, 2006). Among 73 isolates of *C. taiwanensis* obtained from *M. pudica* in Taiwan seven PFGE, four 16S rRNA gene RFLP and four *nod*A RFLP patterns were described. In contrast, we observed that the NC *C. taiwanensis* genotypes are characterized by identical *nodA* (530 bp), *nodC* (600 bp, data not shown) and *nifH* (350 bp) gene fragments.

Cupriavidus taiwanensis LMG19424 harbours on its pSym the most compact (35-kb) symbiotic island (*nod*, *fix* and *nif* genes) identified so far in any rhizobial species (Amadou *et al.*, 2008). The most probable hypothesis that could explain the observed conservation of *nod*A, *nod*C and *nif*H gene fragments among our different *C. taiwanensis* genotypes, and their close phylogenetic relationship with other worldwide *C. taiwanensis* is the introduction of several *Cupriavidus* genotypes in NC together with *M. pudica* seeds and the transfer and maintenance of the symbiotic island from the best symbiotically adapted lineage under plant host selection. Seed-borne rhizobia have already been described in the literature and are suspected to be responsible for the spread of rhizobial populations (Pérez-Ramirez *et al.*, 1998; Stepkowski *et al.*, 2005).

The presence of *R. mesoamericanum* strains in the nodules of *M. pudica* in NC supports the hypothesis of the introduction of symbionts with their *Mimosa* hosts. The presence of these isolates does not seem to be anecdotal as *R. mesoamericanum* was found in *M. pudica* nodules in Costa Rica and in French Guiana (Barrett & Parker, 2006; Mishra *et al.*, 2012), as well as a symbiont of *P. vulgaris*, cowpea (*Vigna unguiculata*) and siratro in Mexico (López-López *et al.*, 2011). This species could thus have been brought to NC together with *M. pudica* seeds. Taken together, these data suggest that *M. pudica* might have been imported for reason of distance or commercial agreements from China or Taiwan where *C. taiwanensis* was described as a symbiont in *M. pudica* nodules (Chen *et al.*, 2003; Liu *et al.*, 2011) with a very low level of *Burkholderia* spp (1.4%) in Taiwan. However, no *Rhizobium* sp. isolates close to *R. mesoamericanum* were detected in these geographic areas. On the other hand, *Mimosa* plants could have been imported directly from the New World, particularly from Central America, where the presence of three symbiotic genera (*Burkholderia*, *Cupriavidus* and *R. mesoamericanum*) were detected in *M. pudica* (Barrett & Parker, 2006). Finally, genetical analyses on *M. pudica* plants from NC in comparison with those in Asia and in the New World may also help

unravel the origin of introduced *Mimosa* species and their symbionts.

Symbiotic effectiveness

The NC *Rhizobium* isolates were found to be inefficient symbionts of *M. pudica*. These results corroborate the observations of Barrett & Parker (2006) for *Rhizobium* sp. strains tpud. 40a and tpud22.2 collected in Costa Rica, which are closely related to the NC strains and could belong to the same species. Assuming that the symbionts were introduced into NC along with their *M. pudica* host an obvious question concerns their survival strategy in their new environment. A partial answer could come from the analysis of the collection sites. The NC *Rhizobium* isolates were obtained only from one soil collected in a site characterized by the presence of unidentified grasses, *Casuarina collina*, which is known for its nitrogen-fixing symbiosis with *Frankia* (Navarro *et al.*, 1997), and *Acacia spirorbis*, a nodule-forming legume species (M. Ducousso, personal communication). *Acacia* belong to the Mimosoideae, the same subfamily as *Mimosa*, and a recent study showed that, like *Mimosa*, *Acacia* species from Australia could be nodulated by alpha- and beta-rhizobia (Hoque *et al.*, 2010). Moreover, a recently described new species, *R. mesoamericanum*, was isolated from nodules of *P. vulgaris* (López-López *et al.*, 2011) and *Rhizobium* sp. STM3625 that was isolated from *M. pudica* nodules in French Guiana could belong to this species (Mishra *et al.*, 2012). The symbiotic efficiency test performed with NC *C. taiwanensis* and *R. mesoamericanum* isolates on native *A. spirorbis* (seeds from NC) and commercial *P. vulgaris* seeds showed that only the *R. mesoamericanum* isolate was able to nodulate both plants (results not shown). The accumulated data and our results raise questions about the original host of the NC *R. mesoamericanum* strain, the eventual adaptative strategy towards invasive *M. pudica* and the role in this adaptation of the second *nodA* gene. Experiments to obtain *nodA1* and *nodA2* deletion mutants are currently under way to better understand this atypical rhizobial feature. The NC *Cupriavidus* and *Rhizobium* isolates are also candidate strains in the GEBA-RNB sequencing project (Root Nodulating Bacteria) at the Joint Genome Institute (USA) to further investigate the adaptation of symbionts to NC environment in comparison with the type strain LMG19424 and another *M. pudica* symbiont *Rhizobium* sp. STM3625 that was obtained from French Guiana (Mishra *et al.*, 2012).

Conclusion

We investigated the diversity of symbionts of *M. pudica* that were introduced into NC at the end of the 19th century. The majority of symbionts belonged to the beta-rhizobia and were identified as belonging to *C. taiwanensis*, a species that was first discovered in Taiwan. These symbionts were probably introduced to the island together with *Mimosa* plants at the time that seeds of this plant were first introduced there. The prevalence of *Cupriavidus* might be due in part to its adaptation to NC soils. An enlarged study concerning the *Mimosa* symbionts in Asia and Oceania is needed to reveal the origin of *M. pudica* symbionts in NC, as apart from Taiwan and some isolates from the Philippines, Papua New Guinea and India, and a larger study already begun in China, little is known about them. To investigate the origins of *C. taiwanensis* and the distribution more representative collections should be obtained from India, Australia and Polynesia in parallel with comparisons of soil characteristics and the occurrence of adjacent legume populations.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Principal component analysis of soil parameters and bioavailable metal ions content.

Fig. S2. Nodules of *R. mesoamericanum* STM6155 (a, c, e and g) and *C. taiwanensis* STM6070 (b, d and f) obtained on *M. pudica* (a–f) and on *P. vulgaris* plants (g).

Table S1. Genotypic and metal tolerance characteristics of isolates and reference strains used in this study.

Table S2. Listing of isolates and reference strains used in this study.

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