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Research Article

Endophytic bacterial diversity in banana 'Prata Anã' (*Musa* spp.) roots

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Abstract

The genetic diversity of endophytic bacteria in banana 'Prata Anã' roots was characterized. Two hundred and one endophytic bacteria were isolated, 151 of which were classified as Gram-positive and 50 as Gram-negative. No hypersensitivity response was observed in any of the isolates. The rep-PCR technique generated different molecular profiles for each primer set (REP, ERIC and BOX). Fifty readable loci were obtained and all of the fragments were polymorphic. Amplified ribosomal DNA restriction analysis (ARDRA) of the isolates based on cleavage with four restriction enzymes yielded 45 polymorphic bands and no monomorphic bands. PCR amplified the *nifH* gene in 24 isolates. 16S rDNA sequencing of the 201 bacterial isolates yielded 102 high-quality sequences. Sequence analyses revealed that the isolates were distributed among ten bacterial genera (*Agrobacterium*, *Aneurinibacillus*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Lysinibacillus*, *Micrococcus*, *Paenibacillus*, *Rhizobium* and *Sporolactobacillus*) and included 15 species. The greatest number of isolates belonged to the genus *Bacillus*. The bacteria identified in this study may be involved in promoting growth, phosphate solubilization, biological control and nitrogen fixation in bananas.

Keywords: ARDRA, *Bacillus* sp., *nifH* gene, rep-PCR, 16S rDNA.

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Introduction

Bananas are important agricultural products in most tropical countries, with the world production estimated to be 90.7 million tons. Brazil is ranked fourth among banana producers, with a production of 7,116,808 tons and a harvested area of 510,825,000 hectares (FAO, 2010). Plants, including banana trees, are complex micro-ecosystems in which different niches are filled by a wide variety of microorganisms, including endophytes (Mia *et al.*, 2010). In recent years, there has been considerable interest in the study of endophytic microorganisms and the determination of their role in plants. Endosymbionts act as biological control agents in numerous diseases (Jie *et al.*, 2009), in the promotion of plant growth (Ryan *et al.*, 2008) and in the bioremediation of polluted areas (Germaine *et al.*, 2009). The use of these microorganisms is preferred compared to chemical fertilizers and pesticides because of their lower cost and their contribution to sustainable agriculture (Aung *et al.*, 2011).

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Various molecular techniques have been used to characterize endophytic bacteria, including repetitive extragenic palindromic sequence PCR (rep-PCR), which uses the conserved sequences of ERIC, REP and BOX, 16S rDNA amplification and restriction, and the cloning and sequencing of the amplified genes (Ryan *et al.*, 2008). Additionally, primers specific for the amplification of important bacterial genes, such as *nifH* (necessary for nitrogen fixation), or genes involved in the degradation of organic pollutants, have been used to study the potential participation of endophytic bacteria in important processes of the host plant (Ryan *et al.*, 2008).

For more than 60 years, bacteria have been known to co-exist with plants without causing any damage. The first reports of endophytic bacteria in banana trees were published in the 1990s and increased from 2000 onwards. However, few advances have been made in isolating and characterizing these endophytic bacteria and in understanding their diversity and functions in bananas. Some genera have been described as banana colonizers, including *Azospirillum amazonense*, *Azospirillum brasilense*, *Bacillus*, *Burkholderia cepacia*, *Burkholderia* spp., *Citrobacter* sp., *Enterobacter* spp., *Klebsiella* spp., *Klebsiella variicola*, *Ochrobactrum*, *Pantoea*, *Serratia* and *Staphylo-*

coccus epidermidis (Rosenblueth *et al.*, 2004; Thomas *et al.*, 2008; Ting *et al.*, 2008; Jie *et al.*, 2009).

The objective of this study was to isolate and identify banana endophytic bacteria and to assess their genetic diversity based on rep-PCR, ARDRA and partial 16S rDNA sequencing.

Material and Methods

Plant sampling and bacterial isolation

Roots from juvenile 'Prata Anã' plants were collected in four counties in Minas Gerais State and in one county in Bahia State, Brazil. The banana root fragments were immersed in 70% ethanol for 1 min and 4% sodium hypochlorite (NaClO) for 3 min and then washed three times in sterile, distilled water. The fragments were subsequently exposed to ultraviolet light in a flow chamber for 10 min and subjected to an ultrasound bath for 10 min. After two baths, the fragments were macerated and the suspensions were diluted 10 fold. A 0.1 mL aliquot was plated onto each of the following media: nutrient yeast dextrose agar (NYDA; 10 g dextrose, 5 g yeast extract, 3 g beef extract, 5 g peptone and 18 g agar), potato dextrose agar (PDA; 200 g potato starch, 20 g dextrose and 20 g agar) and tryptic soy agar (30 g TSA). The plates were incubated for 48 h at 25 ± 1 °C on a 12 h photoperiod. The different media described above were used for bacterial isolation and further culturing.

The bacterial isolates were characterized by Gram staining and, to assess pathogenicity, the isolates were inoculated into non-host plants, including pepper (*Capsicum annuum* Mill), tobacco (*Nicotiana tabacum* L.) and common bean (*Phaseolus vulgaris* L.) seedlings grown in a greenhouse. The infected plants were monitored for hypersensitivity responses as described by Romeiro (2001).

Total DNA extraction and 16S rDNA sequencing

For DNA extraction, each isolate was grown in liquid tryptic soy broth (TSB) for 24 h at 37 °C under constant mixing at 180 rpm. Bacterial genomic DNA was extracted with a DNA extraction kit (Qiagen, Valencia, CA, USA), according to the manufacturer's recommendations.

The isolates were identified by partial sequencing of the 16S region. Initially, the 16S region was amplified using primers 27 (5'-AGAGTTTGATC(AC)TGGCTCAG--3') and 1492R (5'-ACGG(CT)TACCTTGTTACGAC TT-3'). The reactions consisted of 2 µL of dNTPs (2.0 mM each), 2.5 µL of 10X buffer, 0.75 µL of 50 mM $MgCl_2$, 2.5 µL of each primer (5 mM), 0.3 µL of *Taq* polymerase (5 U/µL), 50 ng of template DNA and sterile Milli-Q water in a final volume of 25 µL. The amplification conditions consisted of an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 7 min. DNA was purified using a QIAquick gel extraction kit (Qiagen). Sample sequencing was done using an automated sequencer (ABI-PRISM 3100 Genetic Analyzer). The resulting sequences were compared with those present in the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov) by using the basic local alignment search tool (BLAST) software for nucleotides (Altschul *et al.*, 1997). The bacterial isolates were identified to the species level when similarity values varied between 98% and 100% and to the genus level when similarity values were < 98%.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper have been deposited in the GenBank nucleotide sequence database under accession numbers JQ979307-JQ979408.

REP-PCR amplification

The isolates were compared using the genomic profiles obtained by rep-PCR (repetitive-PCR). The following primers were used: REP1R-I (5'-IIIICGICGICATCI GGC-3'), REP2-I (5'-ICGITTATCIGGCCTAC-3'), ERIC1R (5'-ATGTAAGCTCCTGGGGATTCA-3'), ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') and BOX 1AR (5'-CTCCGGCAAGGCGACGCTGAC-3') (Louws *et al.*, 1994). Each reaction contained 2.5 µL of 10X buffer, 0.7 µL of 50 mM $MgCl_2$, 2 µL of dNTPs (2.5 mM each), 1 µL of each primer (5 µM), 0.3 µL of *Taq* polymerase (5 U/µL), 3 µL of DNA (10 ng/µL) and sterile ultrapure (Milli-Q-treated) water in a total volume of 25 µL. The amplification conditions consisted of an initial denaturation at 95 °C for 7 min, followed by 30 cycles of 94 °C for 1 min, primer annealing for 1 min (53 °C for the BOX 1AR primer, 39 °C for the REP primer and 52 °C for ERIC), extension at 65 °C for 8 min and a final extension step (65 °C for 15 min). The PCR products were run on 1.5% agarose gels stained with ethidium bromide. The size of the amplified fragments was estimated with a 100-bp molecular weight DNA ladder.

ARDRA amplification

Endophytic bacterial DNA was amplified with the following primers: FGPS1490 5'-TGCGGCTGGATCAC CTCCTT-3' and FGPS132 5'-CCGGGTTTCCCCATTC GG-3'. The amplification reactions contained 0.8 µL of dNTPs (2.0 mM each), 2.5 µL of 10X buffer, 0.75 µL of 50 mM $MgCl_2$, 0.25 µL of each primer (5 mM), 0.2 µL of *Taq* polymerase (5 U/µL) and 50 ng of DNA in a final volume of 25 µL. The amplification conditions consisted of an initial denaturation step (95 °C for 3 min) followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, with a final extension at 72 °C for 3 min.

The amplification products were digested with the restriction enzymes *Hinf*I, *Msp*I, *Nde*I and *Rsa*I. Each enzy-

matic reaction contained 10 μL of PCR product, 2 μL of 10X enzyme-specific buffer, 2 μL of enzyme (5 U/reaction) and 18 μL of sterile Milli-Q water. The reactions were incubated for 16 h in a water bath at 37 °C. The fragments were analyzed by electrophoresis on 1.2% agarose gels in 1X TBE at 100 V for 3 h. The size of the amplified fragments was estimated with a 100-bp molecular weight DNA ladder.

nifH gene PCR amplification

DNA from root isolates was analyzed for the presence of the *nifH* gene using the universal primers 19f F (5'-GGAATTCTGTGACCTAAAGCTGA-3') and 407 R (5'-AGCATACATTGCCATCATTTCACC-3'). The amplification reaction mixtures contained 2.0 μL of dNTPs (2.0 mM each), 2.5 μL of 10X buffer, 1 μL of 50 mM $MgCl_2$, 0.5 μL of each primer (5 mM), 0.6 μL of *Taq* polymerase (5 U/μL) and 50 ng of DNA in a final volume of 25 μL. The amplification conditions consisted of denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 7 min. The amplification products were analyzed by electrophoresis on 1.2% agarose gels and fragments of ~270 bp were expected for the *nifH* gene. The size of the amplified fragments was estimated with a 100-bp molecular weight DNA ladder.

Statistical analyses

The ARDRA and rep-PCR results were analyzed cumulatively with R 2.13 software based on the coefficient of simple matching. Cluster analysis was done by the unweighted pair group method with arithmetic mean (UPGMA) using MEGA 5 software (Tamura *et al.*, 2011).

Results

Bacterial isolation and 16S rDNA sequencing

201 isolates of endophytic bacteria were obtained from the roots of 'Prata Anã' banana plants; 150 of these isolates were classified as Gram-positive and 51 as Gram-negative. There was no hypersensitivity response in tobacco (*N. tabacum* L.), bell pepper (*C. annuum* Mill) and common beans (*P. vulgaris* L.) five days after inoculation of the bacterial isolates.

Partial sequencing of the 16S rDNA from the 201 bacterial isolates yielded 102 high-quality sequences. Analysis of these sequences revealed that the isolates were from 15 species belonging to ten genera: *Agrobacterium*, *Aneurinibacillus*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Lysinibacillus*, *Micrococcus*, *Paenibacillus*, *Rhizobium* and *Sporolactobacillus* (Table 1).

The genus *Bacillus* was identified most frequently (87.3% of isolates), followed by the genus *Lysinibacillus*

Table 1 - Distribution of partially identified 16S rDNA sequences detected in endophytic isolates from 'Prata Anã' banana tree roots.

Isolates	E-value[1]	Identity[2]	Most closely related organism[3]	Gram test	*nifH* gene	GenBank accession no.[4]
EB-01	0.0	98%	*Bacillus pumilus*	+	-	HM006706.1
EB-04	0.0	98%	*Bacillus subtilis*	+	+	AY741264.1
EB-05	$7.e^{-119}$	99%	*Bacillus pumilus*	+	-	HQ218993.1
EB-07	0.0	98%	*Agrobacterium tumefaciens*	-	-	GU784794.1
EB-09	$1.e^{-116}$	98%	*Bacillus subtilis*	+	-	AY741264.1
EB-10	$1.e^{-136}$	98%	*Bacillus pumilus*	+	-	GQ917222.1
EB-11	$5.e^{-172}$	97%	*Bacillus* sp.	+	-	HQ218993.1
EB-12	$4.e^{-116}$	98%	*Bacillus pumilus*	+	-	GQ917222.1
EB-14	0.0	99%	*Bacillus pumilus*	+	-	HQ218993.1
EB-15	$4.e^{-127}$	98%	*Bacillus pumilus*	+	-	GQ917222.1
EB-16	$4.e^{-111}$	97%	*Bacillus* sp.	+	-	AJ550463.1
EB-17	$2.e^{-109}$	97%	*Bacillus* sp.	+	-	JF802184.1
EB-23	$3.e^{-179}$	98%	*Klebsiella pneumoniae*	-	+	JN201948.1
EB-24	0.0	98%	*Bacillus thuringiensis*	+	+	JF947357.1
EB-25	0.0	98%	*Bacillus cereus*	+	+	GU451184.1
EB-26	0.0	98%	*Bacillus methylotrophicus*	+	-	HM209756.1
EB-27	0.0	97%	*Bacillus* sp.	+	-	HQ256520.1
EB-28	$1.e^{-163}$	96%	*Paenibacillus* sp.	+	+	EF178460.1
EB-30	0.0	98%	*Bacillus axarquienses*	+	-	JF414764.1
EB-34	$1.e^{-131}$	98%	*Bacillus pumilus*	+	-	JN215511.1
EB-35	$2.e^{-88}$	94%	*Bacillus* sp.	+	-	GQ340516.1

Table 1 (cont.)

Isolates	E-value[1]	Identity[2]	Most closely related organism[3]	Gram test	*nifH* gene	GenBank accession no.[4]
EB-37	$2.e^{-146}$	96%	*Bacillus* sp.	+	-	JN215502.1
EB-38	$3.e^{-97}$	96%	*Bacillus* sp.	-	+	EU931559.1
EB-40	$7.e^{-99}$	97%	*Bacillus* sp.	+	+	GQ340516.1
EB-42	$2.e^{-89}$	96%	*Bacillus* sp.	+	-	JN082266.1
EB-44	$4.e^{-173}$	98%	*Bacillus amyloliquefaciens*	+	-	GU122948.1
EB-45	0.0	98%	*Lysinibacillus sp.*	+	+	JN215512.1
EB-46	0.0	99%	*Bacillus pumilus*	+	-	FJ236809.1
EB-47	$1.e^{-147}$	97%	*Bacillus* sp.	+	+	FJ611939.1
EB-48	0.0	98%	*Bacillus subtilis*	+	-	AY741264.1
EB-49	0.0	98%	*Bacillus licheniformis*	+	+	EU366371.1
EB-50	$3.e^{-108}$	96%	*Bacillus* sp.	+	+	HM769816.1
EB-51	0.0	98%	*Bacillus pumilus*	+	+	HQ218993.1
EB-52	$3.e^{-133}$	96%	*Bacillus* sp.	+	-	JF313264.1
EB-53	$7.e^{-85}$	92%	*Lysinibacillus*sp.	+	-	JN215512.1
EB-55	$3.e^{-108}$	98%	*Bacillus subtilis*	+	-	HQ334981.1
EB-56	$2.e^{-89}$	92%	*Bacillus* sp.	+	+	GU269573.1
EB-57	$3.e^{-102}$	96%	*Bacillus safensis*	+	-	JN092810.1
EB-58	$9.e^{-139}$	98%	*Bacillus pumilus*	+	-	JN082265.1
EB-60	$1.e^{-112}$	96%	*Lysinibacillus*sp.	+	-	JF906500.1
EB-62	$5.e^{-105}$	97%	*Bacillus* sp.	+	-	HQ334981.1
EB-63	$3.e^{-108}$	98%	*Bacillus pumilus*	+	-	GQ917222.1
EB-64	0.0	98%	*Bacillus pumilus*	+	+	JF271873.1
EB-65	$7.e^{-171}$	97%	*Bacillus* sp.	+	-	EU366378.1
EB-68	$2.e^{-136}$	98%	*Bacillus safensis*	+	-	JN092818.1
EB-69	$6.e^{-95}$	97%	*Bacillus* sp.	+	-	GQ34O516.1
EB-70	$5.e^{-167}$	96%	*Bacillus* sp.	+	-	GQ340516.1
EB-71	$2.e^{-166}$	97%	*Bacillus* sp.	+	+	HM461161.1
EB-73	$4.e^{-117}$	99%	*Bacillus pumilus*	+	-	GQ917222.1
EB-76	$7.e^{-130}$	97%	*Bacillus* sp.	+	-	FJ937920.1
EB-84	0.0	98%	*Bacillus subtilis*	+	-	HQ334981.1
EB-87	$8.e^{-155}$	98%	*Bacillus tequilensis*	+	+	HM770882.1
EB-88	$3.e^{-175}$	98%	*Bacillus flexus*	+	+	DQ870687.1
EB-89	$2.e^{-177}$	98%	*Bacillus subtilis*	+	-	HQ234331.1
EB-91	$6.e^{-100}$	97%	*Bacillus* sp.	+	-	JN092818.1
EB-98	$6.e^{-125}$	98%	*Micrococcus luteus*	+	-	FJ380958.1
EB-99	$2.e^{-136}$	96%	*Bacillus* sp.	+	-	AB301022.1
EB-101	$2.e^{-95}$	98%	*Bacillus pumilus*	+	-	JN082266.1
EB-107	0.0	99%	*Bacillus thuringiensis*	+	-	AM292316.1
EB-108	$2.e^{-84}$	95%	*Rhizobium* sp.	-	-	AY693664.1
EB-111	$8.e^{-114}$	99%	*Bacillus megaterium*	+	-	AM237398.1
EB-113	$9.e^{-120}$	92%	*Bacillus* sp.	+	-	JN208198.1
EB-117	$4.e^{-91}$	97%	*Bacillus* sp.	+	-	JN082257.1
EB-120	$6.e^{-131}$	97%	*Bacillus* sp.	+	-	AM921636.1
EB-124	$1.e^{-55}$	93%	*Bacillus* sp.	+	-	EU977719.1
EB-125	$4.e^{-137}$	98%	*Bacillus pumilus*	+	-	HQ858063.1
EB-126	0.0	98%	*Bacillus subtilis*	+	+	HM769817.1
EB-127	$2.e^{-89}$	97%	*Sporolactobacillus* sp.	+	+	D16282.1

Table 1 (cont.)

Isolates	E-value[1]	Identity[2]	Most closely related organism[3]	Gram test	*nifH* gene	GenBank accession no.[4]
EB-128	0.0	99%	*Bacillus pumilus*	+	-	EU379285.1
EB-129	1.e^{-137}	95%	*Bacillus* sp.	+	-	HM461228.1
EB-132	3.e^{-149}	98%	*Bacillus subtilis*	+	-	AY741264.1
EB-133	1.e^{-171}	98%	*Bacillus amyloliquefaciens*	+	+	AB301022.1
EB-134	6.e^{-74}	98%	*Bacillus amyloliquefaciens*	+	-	AB301022.1
EB-135	2.e^{-104}	98%	*Bacillus pumilus*	+	-	EU977790.1
EB-136	2.e^{-135}	98%	*Bacillus subtilis*	+	+	AB301012.1
EB-140	1.e^{-92}	94%	*Bacillus* sp.	+	-	GQ340516.1
EB-141	1.e^{-122}	96%	*Lysinibacillus* sp.	+	-	GU172164.1
EB-143	8.e^{-94}	96%	*Bacillus* sp.	+	-	JN092818.1
EB-144	0.0	92%	*Paenibacillus* sp.	+	+	EF178460.1
EB-145	0.0	94%	*Bacillus* sp.	+	-	JF896450.1
EB-146	0.0	96%	*Bacillus* sp.	+	-	HM461161.1
EB-147	0.0	99%	*Bacillus subtilis*	+	-	EU977724.1
EB-148	0.0	97%	*Aneurinibacillus* sp.	+	-	AB112723.1
EB-149	2.e^{-145}	97%	*Bacillus* sp.	+	-	EU977790.1
EB-150	6.e^{-121}	92%	*Bacillus* sp.	+	-	DQ915582.1
EB-151	2.e^{-157}	95%	*Bacillus* sp.	+	-	AM237389.1
EB-152	1.e^{-118}	95%	*Bacillus* sp.	+	-	JN082257.1
EB-153	1.e^{-127}	95%	*Bacillus* sp.	+	-	HM461228.1
EB-154	8.e^{-104}	98%	*Bacillus pumilus*	+	-	HQ334985.1
EB-157	1.e^{-122}	95%	*Bacillus* sp.	+	-	JN092818.1
EB-158	2.e^{-99}	97%	*Bacillus* sp.	+	-	AJ842964.1
EB-161	1.e^{-96}	96%	*Bacillus* sp.	+	-	HM461161.1
EB-162	3.e^{-107}	100%	*Bacillus pumilus*	+	-	GQ917222.1
EB-164	1.e^{-142}	96%	*Bacillus* sp.	+	-	AY484507.1
EB-169	5.e^{-106}	98%	*Bacillus pumilus*	+	+	FJ189791.1
EB-182	0.0	95%	*Bacillus* sp.	+	-	HG003422.1
EB-184	0.0	96%	*Bacillus* sp.	+	-	HQ218993.1
EB-187	2.e^{-94}	95%	*Bacillus* sp.	+	-	FM865689.1
EB-194	2.e^{-110}	93%	*Bacillus sp.*	-	+	FJ405377.1
EB-196	5.e^{-91}	95%	*Enterobacter* sp.	-	-	GQ260081.1
EB-199	1.e^{-92}	95%	*Bacillus* sp.	+	-	EF522800.1
EB-200	3.e^{-97}	99%	*Bacillus pumilus*	+	-	EU977790.1

[1]Probability of randomly finding the same alignment between two sequences.
[2]Percentage of sequence identity between the sequence of a banana isolate and a related organism.
[3]Organism with the partial 16S rDNA sequence most homologous to that of the banana isolate.
[4]Accession number of the related organism sequence.

(3.9% of isolates). Twelve *Bacillus* species were identified: *B. amyloliquefaciens*, *B. axarquiensis*, *B. cereus*, *B. flexus*, *B. megaterium*, *B. methylotrophicus*, *B. licheniformis*, *B. pumilus*, *B. safensis*, *B. subtilis*, *B. tequilensis* and *B. thuringiensis*, indicating intraspecific variability associated with banana 'Prata Anã' roots. *Bacillus pumilus* and *B. subtilis* predominated among the species identified in this study and represented 20.6% and 9.8% of the isolates identified, respectively (Table 1).

REP-PCR and ARDRA analysis

The rep-PCR technique generated different molecular profiles for each primer (REP, ERIC and BOX) separately. 50 readable loci were obtained and all of the fragments were polymorphic.

Nine major clusters were identified (Figure 1). Cluster I contained 34 isolates from three genera (*Paenibacillus*, *Bacillus* and *Lysinibacillus*), all belonging to the phylum Firmicutes. Despite the low dissimilarity among the isolates, this group showed high genetic diversity, with repre-

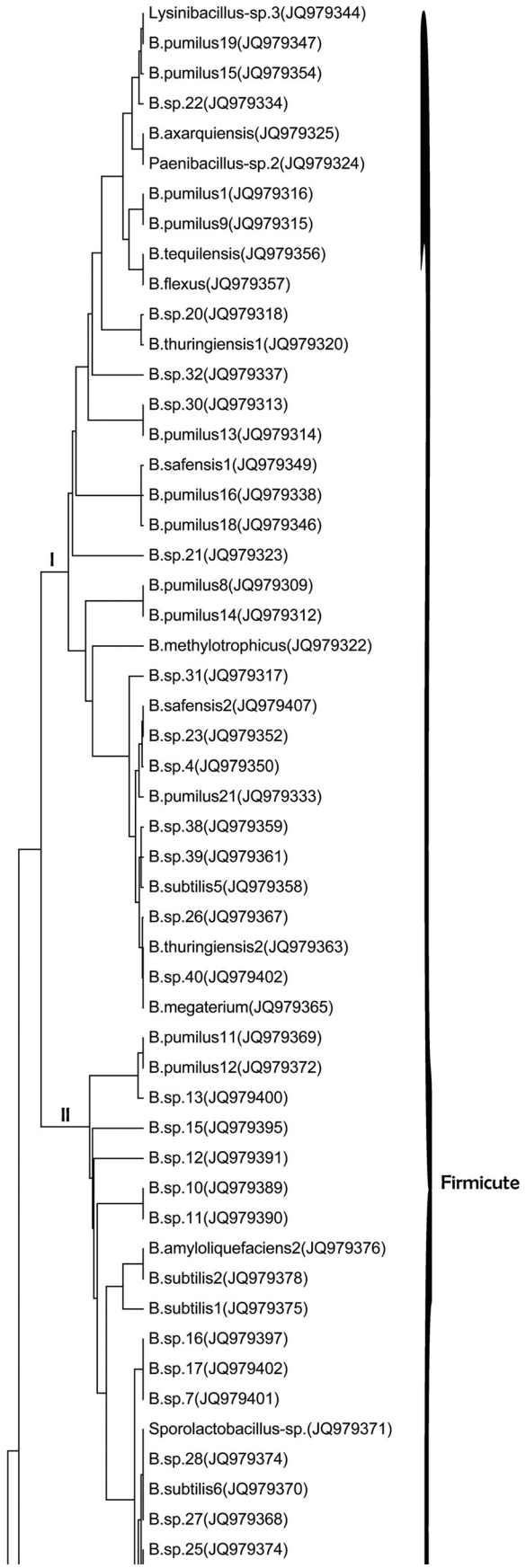

Figure 1 - Dissimilarity dendrogram based on rep-PCR amplicons of endophytic bacterial isolates from 'Prata Anã' banana roots.

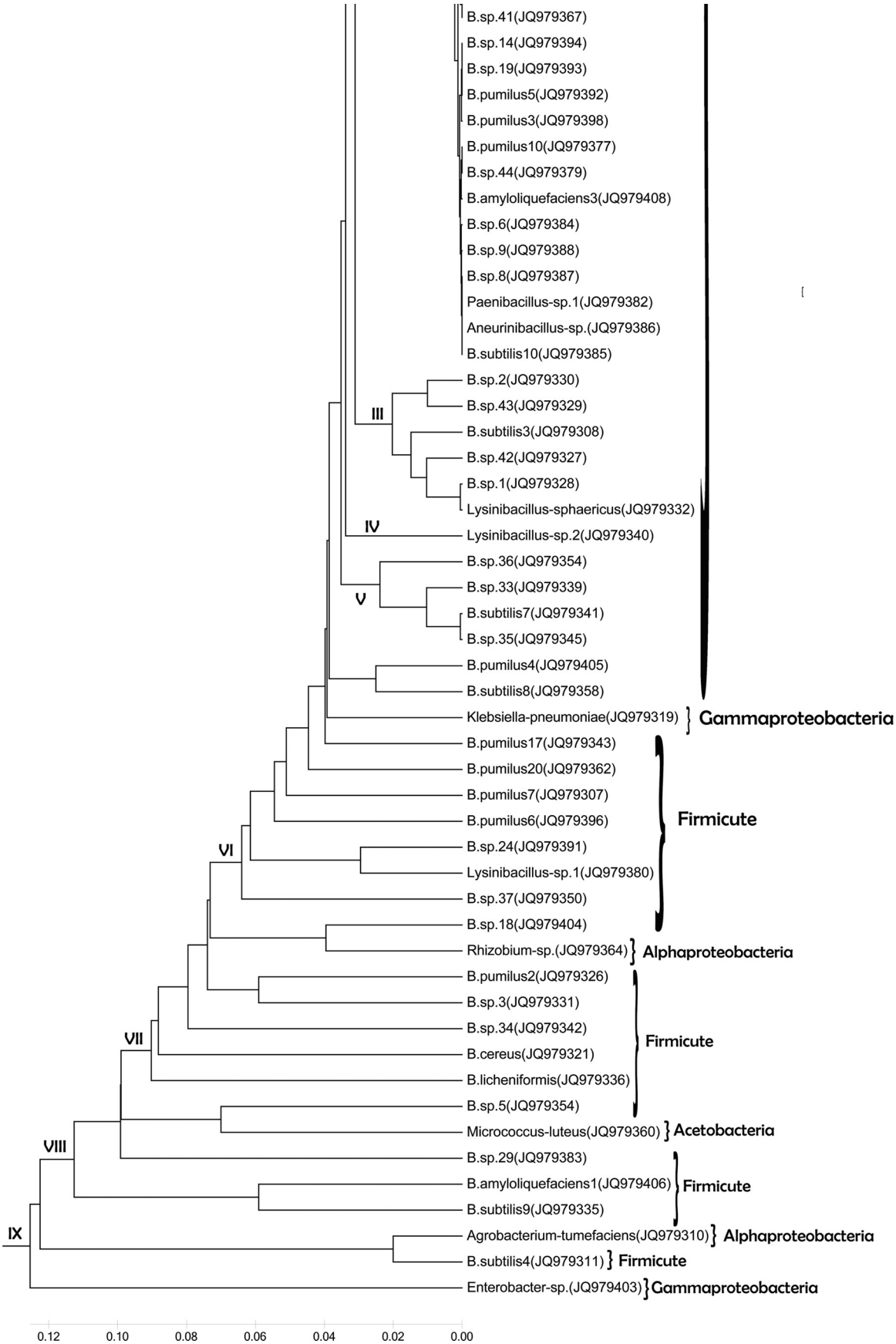

Figure 1 (cont.) - Dissimilarity dendrogram based on rep-PCR amplicons of endophytic bacterial isolates from 'Prata Anã' banana roots.

sentatives from eight species of *Bacillus*. In general, the groups were formed based on phylogenetic criteria. Groups I, II, III and V were formed exclusively by bacteria of the phylum Firmicutes while group IV consisted of a single representative of the genus *Lysinibacillus*. Group VI contained different bacterial genera, two of which (*Klebsiella* and *Rhizobium*) belonged to the phylum Proteobacteria. 75% of the isolates in group VIII belonged to the phylum Firmicutes, the exception being *Micrococcus luteus*, which belonged to the phylum Actinobacteria. Group IX contained three genera (*Agrobacterium tumefasciens*, *Enterobacter* sp. and *Bacillus subtilis*) that belonged to three classes (Alphaproteobacteria, Gammaproteobacteria and Bacilli, respectively).

ARDRA diversity analysis of the bacterial isolates, which compared the cleavage products from four restriction enzymes, yielded 45 polymorphic and non-monomorphic bands. The restriction enzyme *Rsa*I provided the most resolution by generating 13 bands, followed by *Msp*I with 12, *Hinf*I with 11 and *Nde*I with nine bands.

Phylogenetic analysis based on the ARDRA results showed that most (96%) of the isolates were affiliated with Firmicutes, including the dominant genus *Bacillus*. Figure 2 shows the phylogeny of *Bacillus* and *Lysinibacillus*, with the formation of eight groups. Groups III, V, VI, VII and VIII consisted exclusively of representatives belonging to *Bacillus*. The four representatives of *Lysinibacillus* formed three groups, with groups I and II containing one isolate each while group IV contained two isolates.

The phylum Proteobacteria accounted for 3.9% of all isolates. The only member of Actinobacteria identified was *Micrococcus luteus* (Figure 3). Figure 3 shows six groups in which groups II, V and VI were represented by a single bacterium each. There was no common criterion by which the bacteria were grouped. Group III contained two bacteria belonging to the phylum Firmicutes (*Paenibacillus* sp. and *Aneurinibacillus* sp.).

Analysis of the *nif*H gene

PCR amplification of the *nifH* gene was detected in 24 of the 102 bacterial isolates; 79% of the isolates belonged to the genus *Bacillus*. Seven species of *Bacillus* were *nifH*-positive, including: *B. amyloliquefaciens*, *B. cereus*, *B. flexus*, *B. licheniformis*, *B. pumilus*, *B. subtilis* and *B. tequilenses*. The other isolates belonged to the genera *Klebsiella*, *Lysinibacillus*, *Paenibacillus* and *Sporolactobacillus* (Table 1).

DISCUSSION

Studies of plants and endophytic bacteria have demonstrated the importance of such interactions for plant adaptation to diverse ecosystems and for enhancing soil health and quality. However, little is known about the colonizing species, the relationship between these bacteria and banana trees, and the possible benefits of this interaction.

Of the 201 bacterial isolates collected in this study, 75% were classified as Gram-positive. In contrast, Thomas *et al.* (2008), in a study of endophytic bacteria isolated from banana shoot tip cultures during the first passage *in vitro* encountered more Gram-negative organisms (75%). Together, these findings agree with other reports regarding the marked diversity of Gram-negative and Gram-positive endophytic bacteria in banana (Habiba *et al.*, 2002; Ganen *et al.*, 2009).

The lack of a hypersensitivity reaction for the 201 bacterial isolates suggested the absence of phytopathogenic bacteria. The species *A. tumefaciens* was identified here but did not elicit a hypersensitivity response in non-host plants, although it behaved as an endophytic bacterium in banana trees. According to Kobayashi and Palumbo (2000), some

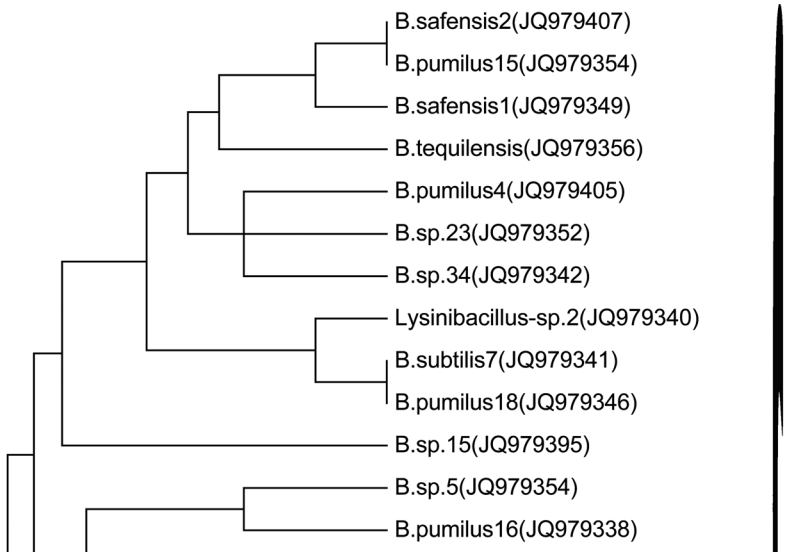

Figure 2 - Dendrogram based on genetic dissimilarity matrix values of *Bacillus* and *Lysinibacillus* isolates analyzed by PCR-ARDRA.

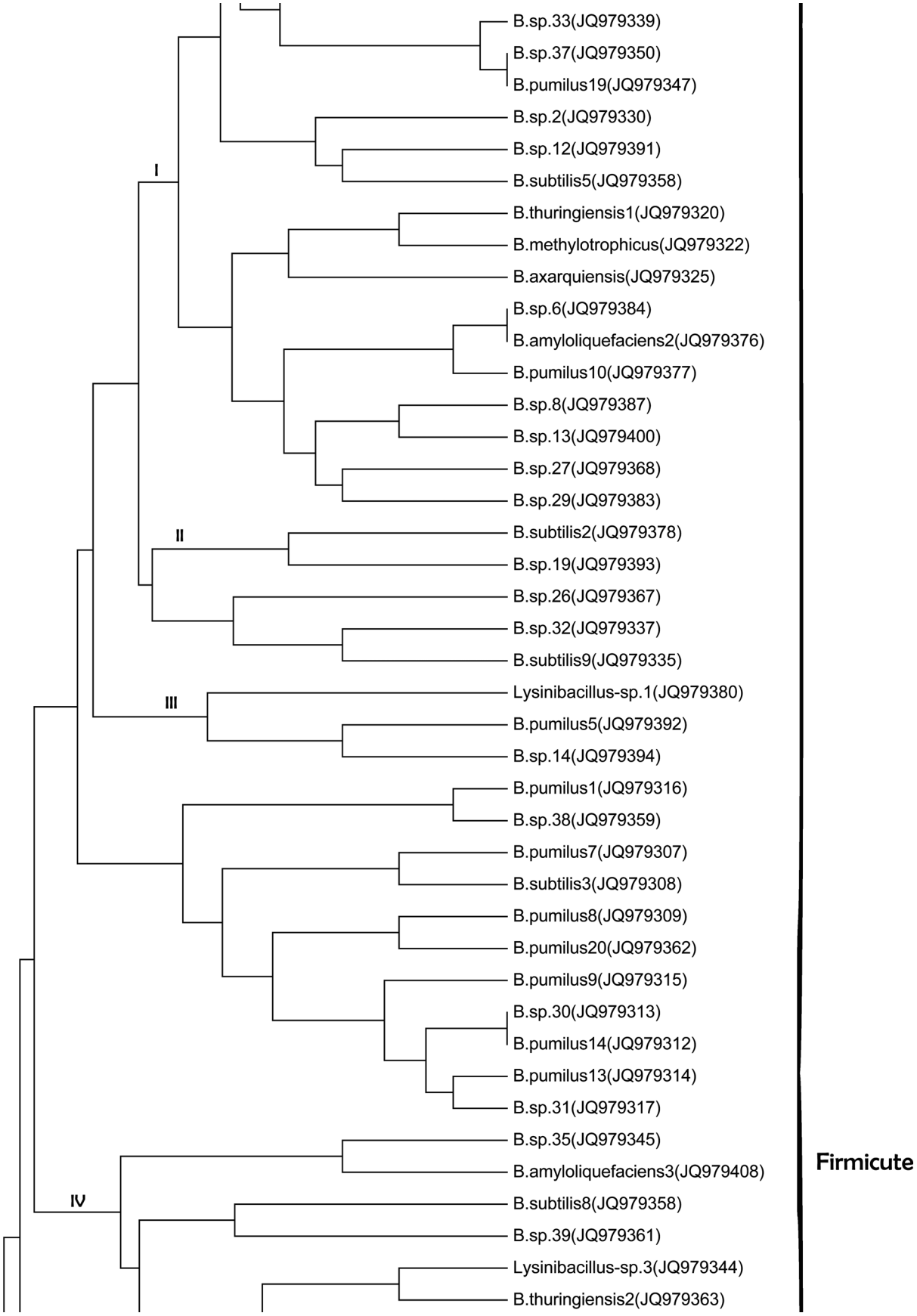

Figure 2 (cont.) - Dendrogram based on genetic dissimilarity matrix values of *Bacillus* and *Lysinibacillus* isolates analyzed by PCR-ARDRA.

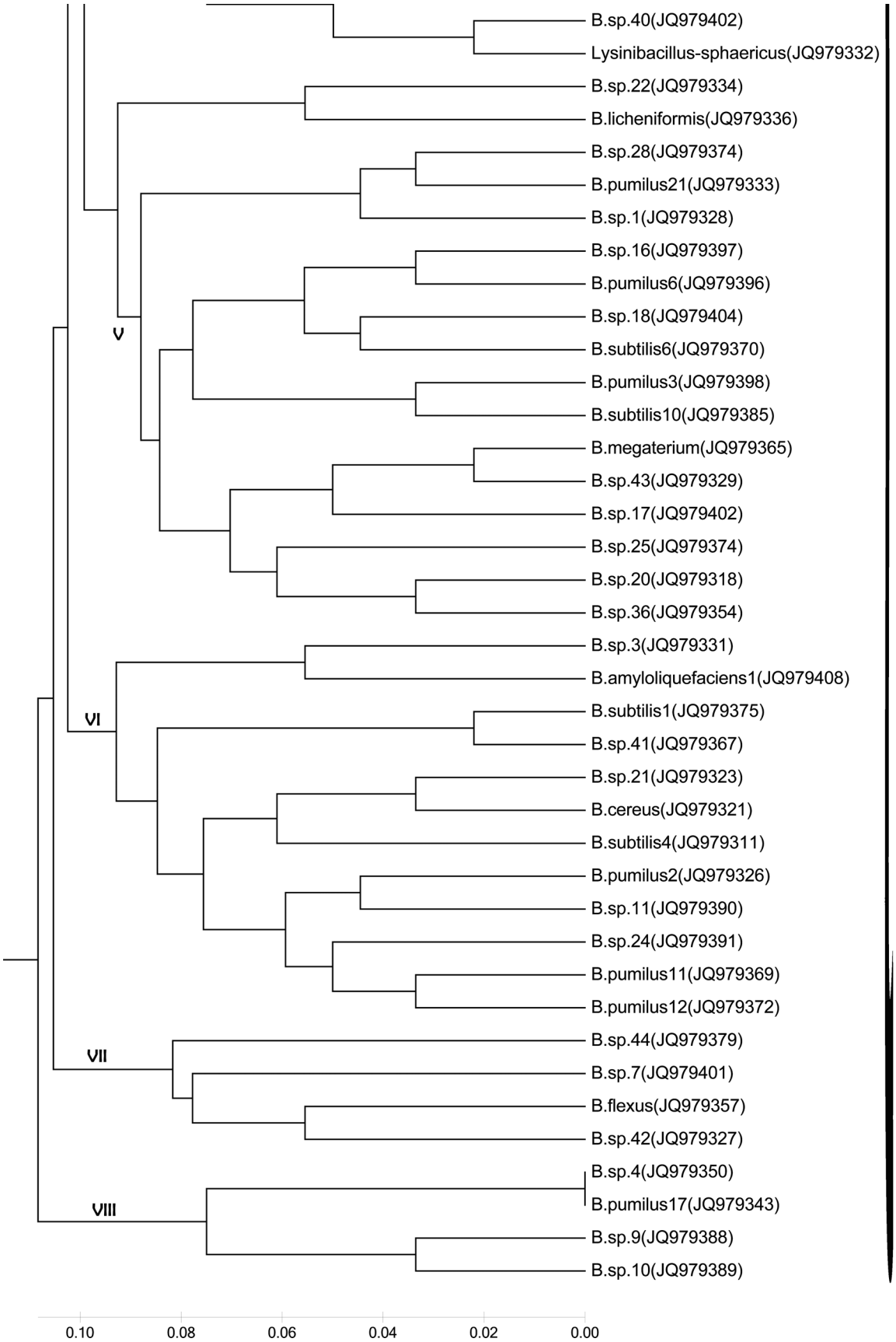

Figure 2 (cont.) - Dendrogram based on genetic dissimilarity matrix values of *Bacillus* and *Lysinibacillus* isolates analyzed by PCR-ARDRA.

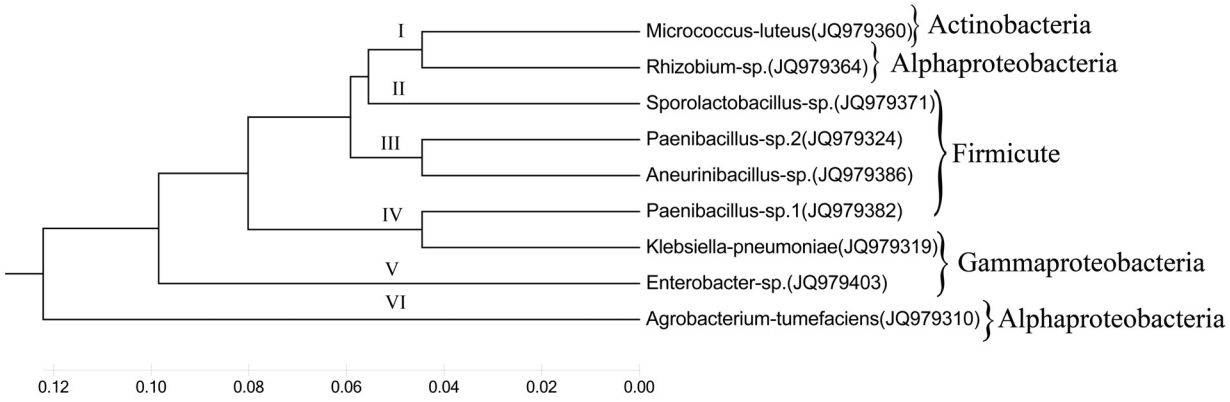

Figure 3 - PCR-ARDRA dendrogram of genetic dissimilarity showing the phylogenetic relationships among nine isolates from banana 'Prata Anã' roots.

phytobacteria genera are typical endophytic bacteria without causing any symptoms of illness in the associated plant. The host and pathogen developmental stage, environmental changes and host defense responses (Schulz and Boyle, 2005), as well as genetic, structural and physiological factors can determine whether a microorganism is endophytic or phytopathogenic (Andreote *et al.*, 2008).

Sequence analyses using BLASTn revealed high bacterial diversity in the roots of 'Prata Anã' banana plants. Many bacterial genera, such as *Bacillus* and other species identified here have also been identified in other studies of endophytic bacteria. The genera *Bacillus*, *Enterobacter* and *Pantoea* have been identified as endophytes in several plants, such as citrus, sugar cane and soybean (Medrano and Bell, 2007; Magnani *et al.*, 2010).

Bacillus was the most frequently encountered genus in this study. The *Bacillus* species identified here (*B. cereus*, *B. subtilis*, *B. megaterium* and *B. pumilus*) have been described as endophytic species (Elvira-Recuenco and Van Vuurde, 2000; Araújo *et al.*, 2002) and have also been isolated from contaminated banana explants in tissue culture (Odutayo *et al.*, 2007; Jie *et al.*, 2009). *Bacillus* bacteria are classified as growth promoters (characterized by the production of auxins and gibberellins by *B. pumilus*), nitrogen fixers and phosphate solubilizers (Forchetti *et al.*, 2007).

Lysinibacillus was the second most frequently identified genus. Species belonging to this genus have been associated with ginseng plants and the isolates evaluated were positive for most of the plant growth promoters (Vendan *et al.*, 2010). Despite the predominance of *Bacillus* isolates, species of *Paenibacillus* were also identified and are of considerable biotechnological potential. *Paenibacillus* species not only have the capacity to produce plant growth hormones (auxins and cytokinins), enzymes (chitinases, amylases and proteases) and antibiotics but also can solubilize organic phosphates (Coelho *et al.*, 2009).

Only one representative each of *Enterobacter* and *Rhizobium* was found to be associated with banana roots. *Enterobacter* species are endophytes in *Citrus* spp., soybean and banana (Araujo *et al.*, 2002; Kuklinsky-Sobral *et al.*, 2004; Thomas *et al.*, 2008; Jie *et al.*, 2009). *Enterobacter* species can fix nitrogen and solubilize phosphate (Asis Jr and Adachi, 2003; Dalton *et al.*, 2004), thereby increasing plant growth and productivity.

Rhizobium species have been widely used as biofertilizers for various legume species. In banana, a few reports have described *Rhizobium* spp. associated with *Musa* spp. A study by Martinez *et al.* (2003) reported significant increases in stem and leave fresh weight in bananas after the inoculation of some *Rhizobium* isolates.

Although *A. tumefaciens* is described as phytopathogenic this species has been reported as an endophytic bacterium that is asymptomatically associated with the roots of *Triticum aestivum* L. (Sharma *et al.*, 2005), the stems of *Rosa grandiflora* (Martí *et al.*, 1999) and the roots and stems of *Crotalaria pudica*, *Crotalaria pallida* and *Mimosa pudica* (Wang *et al.*, 2008). Wang *et al.* (2008) also observed that *A. tumefaciens* was able to thrive in nodules formed by *Sinorhizobium meliloti* in *Melilotus dentatus*. According to Llop *et al.* (2009), the asymptomatic association of *A. tumefaciens* with host plants may reflect a loss of pathogenicity. To our knowledge, this is the first report on identify *A. tumefaciens* living as an endophyte in *Musa* spp. This association suggests that these isolates may be present in the soil and enter the plant through secondary roots.

The BOX primer yielded the greatest number of polymorphic bands and had the best resolving power among the bacterial isolates. According to Van Berkum (1999), BOX regions are associated with a high degree of polymorphisms and consequently participate in adaptive evolution by mediating the interactions of microorganisms with harsh or adverse environments.

The ARDRA technique was efficient in identifying variation among bacterial isolates evaluated by polymorphisms and estimates of genetic distance. The variations observed among bacteria are based on the generation of different band profiles that reveal the diversity among and within bacterial groups and group individuals of similar genotypes (Kuklinsky-Sobral *et al.*, 2004). Assumpção *et al.* (2009) stated that the diversity and structure of microbial

communities varies with the sample size because the probability of finding rare species increases with increasing sample size.

The detection of the *nifH* gene indicated that 23.5% of the endophytic bacteria analyzed could act as nitrogen fixers. Most of these nitrogen fixers belonged to the genus *Bacillus*, which has already been described as potential nitrogen fixers (Raymond *et al.*, 2004). The *nifH* gene was also present in the genus *Rhizobium* and *K. pneumoniae.* The presence of this gene is strong evidence of the nitrogen-fixing ability in bacteria because this gene encodes the Fe-nitrogenase subunit of the nitrogenase complex. The *nifH* gene has therefore become a useful marker for studying the diversity of endophytic bacteria with the potential to fix N_2 in independent culture studies (Izquierdo and Nüsslein, 2006).

The distribution of phylogenetic groups showed a predominance of members of Firmicutes (96%). In contrast, Jie *et al.* (2009), in a study of the re-introduction of naturally-occurring endophytes into tissue culture banana plantlets, noted a high proportion of members of the phylum Proteobacteria (87.7%). Both studies demonstrated the potential effect of cultivars, climatic conditions and soil on the genetic diversity of endophytic bacteria in bananas.

The isolation, identification and screening of endophytic bacteria as plant growth regulators, as well as their ability to increase plant nutrient absorption and stimulate the development of resistance towards abiotic and biotic stress, are essential leads for establishing their applications in agriculture, especially the cultivation of bananas (Cao *et al.*, 2004; Jaizme-Vega *et al.*, 2004; Jie *et al.*, 2009).

The marked genetic diversity observed here and in other studies of endophytic bacteria in banana represents an emerging trend in biotechnology. However, further studies on the molecular and biochemical mechanisms of growth promotion and on the usefulness of artificial inoculation must be done in order to meet the expectations of large-scale banana producers.

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