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Comparative biogeography of *Chromobacterium* from the neotropics

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Abstract The genus *Chromobacterium* encompasses free-living Gram-negative bacteria. Until 2007, the genus consisted of only one species but six species are now recognized. *Chromobacterium violaceum* is the type species of the genus and is commonly found in soil and water in tropical and sub-tropical regions. We have investigated a collection of 111 isolates displaying violet pigmentation from undisturbed aquatic and soil environments from Brazilian Cerrado ecosystem. The 16S rRNA gene phylogeny revealed that all isolates were allocated in a monophyletic cluster inside the *Chromobacterium* genus and formed few clusters related most closely with *Chromobacterium piscinae*. The two sets of isolates from water and soil were analyzed by the repetitive extragenic palindromic (rep)-PCR genomic fingerprinting technique using a BOX-AR1 primer. The antimicrobial susceptibility and the different carbon sources utilized by these isolates were also investigated. Physiological profiles of the isolates generated by BIOLOG GN2 plates showed great versatility in the substrate utilization, much higher than the *C. violaceum* ATCC 12472. All isolates exhibited a high minimum inhibitory concentration (MIC) to ampicillin (MIC $>$ 512 μg/ml) and were inhibited by ciprofloxacin, tetracycline and mercury at the lowest concentration tested (MIC $<$ 2 μg/ml). Thirteen BOX-PCR band patterns were identified from 33 individual fingerprints. Eleven patterns provided evidence for endemic distributions. Antimicrobial susceptibility and BOX-PCR fingerprint clustering showed a clear distinction between *Chromobacterium* isolates from the water and soil. The results suggested that microenvironment barriers such as water and soil can play an important role in the periodic selection and diversification of *Chromobacterium* population ecotypes.

Keywords *Chromobacterium* · 16S rDNA · BOX-PCR · Antimicrobial susceptibility · BIOLOG · Cerrado · Neotropical

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Introduction

Bacteria are a very important component of Earth's biota. They exhibit a huge genetic and physiological diversity, and have essential roles in the biogeochemical cycling of energy and nutrients (DeLong 1997; Oren 2004). Over the past decades, their microbial diversity has attracted great attention from researchers. Several studies analyzing the 16S rRNA gene sequences have been reported (Freitas et al.

2008; Portillo and Gonzalez 2008; Lemke et al. 2009), but they can survey only a tiny fraction of the existing diversity. Nevertheless, they provided the basis for early studies of microbial biogeography.

For a long time, the knowledge about patterns, distribution and biogeography of microbial diversity has been neglected (Bowman and McCuaig 2003; Noguez et al. 2005). Because microbial biogeography is still poorly understood, it has been assumed that prokaryotes are cosmopolitan because of individuals' small size and crowded populations; consequently, they are potentially capable of dispersing everywhere (Nesbo et al. 2006). Microbial biogeography allows to explore spatial heterogeneity in macro and micro-scale (Vilas-Boas et al. 2002; Vogel et al. 2003; Horner-Devine et al. 2004). Some studies have demonstrated that, like macroorganisms, prokaryotic biogeography also reveals phylopatric pattern endemism in free-living microorganisms (Cho and Tiedje 2000; Fulthorpe et al. 1998; Pommier et al. 2007, Taylor et al. 2005).

We are studying in detail the *Chromobacterium* genus as part of an ongoing effort to evaluate whether free-living bacteria are mostly cosmopolitan or largely endemic and to analyze the distribution of bacterial taxa in undisturbed environments and microhabitats. This genus, of the family Neisseriaceae, was first proposed by Bergonzini (1881) while characterizing the *Chromobacterium violaceum* species bearing a typical violet pigment named violacein. Members of this species have often been isolated from various tropical and subtropical ecosystems, including soil and aquatic environments, and rarely cause infections in humans (Siqueira et al. 2005; Vijayan et al. 2009). They are free-living, aerobic, rod-shaped Gram-negative bacteria, and exhibit a great flexibility to survive in the most diverse environments (Creczynski-Pasa and Antonio 2004). The complete genome of *C. violaceum* has been sequenced and revealed a large biotechnological potential (Vasconcelos et al. 2003).

Six recognized species currently comprise the genus *Chromobacterium*, namely *C. violaceum* (Bergonzini 1881), *C. subtsugae* (Martin et al. 2007), *C. piscinae*, *C. pseudoviolaceum* (Kämpfer et al. 2009), *C. haemolyticum* (Han et al. 2008) and *C. aquaticum* (Young et al. 2008). Interestingly, the last two species do not exhibit the typical violacein pigment. Among other differentiating characteristics, the average index of similarity of the 16S rRNA gene sequences among the five new species and *C. violaceum* ATCC 12472 type species range from 96.1 to 99.8% (Han et al. 2008; Kämpfer et al. 2009; Martin et al. 2007, Young et al. 2008).

In the present study, we conduct a microbiogeographic survey through detailed genetic and phenotypic analyses of 111 violet *Chromobacterium* isolates from aquatic and soil environments in an undisturbed neotropical ecosystem. We have used molecular approaches through analysis of 16S rRNA gene sequences and BOX-PCR fingerprinting. In addition, we have investigated the adaptive potential of these free-living isolates by evaluating their antimicrobial susceptibility and use of different carbon sources.

Materials and methods

Study area

The Serra do Cipó National Park is located (19°–20° S; 43°–44° W, Fig. 1) in a protected area in the Cerrado (Brazilian savannah) biome, which is regarded as one of the most important biodiversity hotspots in the world with a high diversity of plant and animal species, many of them endemic (Myers et al. 2000). It has a humid subtropical climate with mild and rainy summers (annual rainfall ca.

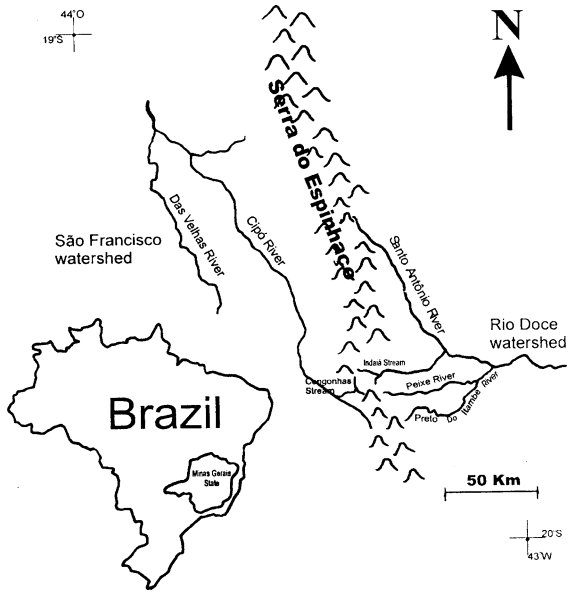

Fig. 1 Sampling site locations along the Indaiá Stream in the National Park of Serra do Cipó

1,500 mm, October–March) and dry winters from April to September (Galvão and Nimer 1965; Koppen 1936). Its soil is sandy oligotrophic and acid, with high levels of exchangeable aluminum and organic matter (Benites et al. 2005). The waters of this stream are of good quality (7 mg l^{-1} dissolved oxygen, 13 μS cm^{-1} electrical conductivity, 30 NTU turbidity, 14 μg l^{-1} total P and 234 μg l^{-1} total N) (Callisto et al. 2002; Lima-Bittencourt et al. 2007a).

Sampling and bacterial isolation

The samples were collected in the Indaiá stream and from soil of the adjacent area about 100 m from the stream, situated in high-altitude fields (1,450 m). Samples were collected in summer (rainy season), and taken in triplicate from water and soil at a depth of 15 and 10 cm, respectively. The samples were then pooled into a single water or soil sample. Soil was sampled at three random spots located 10 m from each other. Aliquots of 0.1 ml of undiluted sampled water were plated directly on 25%-strength nutrient agar (NA, Difco Laboratories). Soil suspensions (5 g) were homogenized with 100 ml of $MgSO_4$ sterilized buffer (0.1 M), and the mixture was shaken at 25°C for 24 h. Next, they were serially diluted and plated on 25%-strength NA. The plates were incubated at 25°C for up to 7 days. Only colonies producing violet pigments were selected and purified by restreaking on 25%-strength NA. To certify that the violet isolates were *Chromobacterium*, they were incubated at 4°C, 15 and 37°C (Logan and Moss 1992) prior to molecular and phenotypic analyses. *C. violaceum* ATCC 12472 was included as the type species in all analyses.

Phenotypic analysis

The ability of the isolates to utilize 95 different compounds was tested using a BIOLOG GN2 MicroPlate (BIOLOG Inc., USA) according to the manufacturer's instructions. Each 96-well plate consists of 95 sole carbon sources and one water blank together with a tetrazolium redox dye. Formation of purple coloring, measured by optical density (OD) at 590 nm, takes place when microbial respiration reduces the dye. Dilutions of the cultures were prepared up to 10^{-2} in sterile saline, and 120 μl of these dilutions were inoculated into a BIOLOG GN2 MicroPlate and incubated in the dark at 28°C for 24 h.

The minimum inhibition concentration (MIC) was determined by the agar dilution method performed in Mueller–Hinton medium (MH; Difco Laboratories). Antimicrobial susceptibilities to ampicillin (Ap), amoxicillin-clavulanic acid (Am), tetracycline (Tc), chloramphenicol (Cm), nalidixic acid (Nx), amikacin (Ak), gentamicin (Gm), kanamycin (Km), streptomycin (Sm), ciprofloxacin (Cp) and the heavy metal mercury bichloride (Hg) were tested. All antimicrobials were obtained from Sigma Chemical Co., and mercury was obtained from Merck Co.

The isolates were tested for chitinase enzyme production. Chitinase activity and the colloidal chitin preparation were performed using the modified method of Kang et al. (1999). The bacterial isolates were spotted onto SM-chitin agar plates (1.5% glucose; 0.5% NH_4NO_3; 0.5% $MgSO_4 \cdot 7H_2O$; 0.05% KCl; $FeSO_4 \cdot 7H_2O$ 0.001%; $MnSO_4 \cdot H_2O$ 0.001% plus 10% of Luria–Bertani broth and 0.2% colloidal chitin).

DNA extraction and 16S ribosomal RNA gene amplification

Total genomic DNA of each isolate was extracted from the bacterial cultures as described elsewhere (Sambrook and Russel 2001). The complete 16S rRNA gene was amplified by PCR using the primers PA 5′-TCCTGGCTCAGATTGAACGC-3′ (Kuske et al. 1997) and U2 5′-ATCGGYTACCTTGTTACGACTTC-3′ (Lu et al. 2000). Polymerase chain reaction mixtures (20 μl) consisted of 0.4 mM of each dNTP, 0.5 μM of each primer, 1 unit of *Taq* DNA polymerase (Phoneutria, Brazil), and 40 ng of bacterial DNA. The thermal cycling conditions consisted of one cycle at 95°C for 10 min followed by 30 cycles of 30 s of denaturation at 95°C, 40 s of annealing at 48°C, and 2 min of extension at 72°C, and a final extension step of 15 min at 72°C.

Sequencing and phylogenetic analysis

The 16S rRNA gene sequencing was made using the primers PA, U2 and E926R (5′-CCGTCAATTCC TTTGAGTTT-3′). Sequencing reactions were performed using standard protocols with DYEnamic ET dye terminator kit (GE Healthcare) and the MegaBACE 1000 capillary sequencer (GE Healthcare). Each sequence in forward and reverse directions was

repeated at least three times for every bacterial isolate. The 16S rRNA gene sequences were base called, checked for quality, aligned and analyzed using Phred v.0.20425 (Ewing and Green 1998), Phrap v.0.990319 (Gordon et al. 2001) and Consed 12.0 (Gordon et al. 1998) software. Phylogenetic analysis was inferred by MEGA 3 software (Kumar et al. 2004) using the neighbor-joining method to calculate trees from Kimura 2P distances. One thousand bootstrap resamplings were used to evaluate the robustness of the inferred trees. Subsequently, a test was carried out to detect differences between isolates from distinct environments using the UniFrac statistics software that performed principal component analyses. Additional 16S rRNA gene sequences of *C. violaceum* (AE016825), *C. subtsuga* (AY344056), *C. haemolyticum* (DQ785104), *C. aquaticum* (EU109734), *C. piscinae* (AJ871127), *C. pseudoviolaceum* (AJ871128), *Aquitaleae magnusonii* (DQ018117), *Vogesella indigofera* (AB021385) and *Neisseria gonorrhoeae* (X07714) were obtained from the GenBank database. *A. magnusonii*, *V. indigofera* and *N. gonorrhoeae* were used as outgroup. The nucleotide sequences generated were deposited in the GenBank database with accession numbers GU216165 to GU216221.

Rep-PCR genomic fingerprinting

The repetitive and conservative chromosomal DNA regions were amplified with the BOX-A1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′) (Koeuth et al. 1995). Polymerase chain reaction mixtures (20 µl) consisted of 0.2 mM of each dNTP, 0.4 µM of the BOX-A1R primer, 1 unit of *Taq* DNA polymerase (Phoneutria, Brazil), and 60 ng of bacterial DNA. The thermal cycling conditions consisted of one cycle at 94°C for 5 min followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 52°C, and 3 min of extension at 72°C, and a final extension step of 10 min at 72°C. Products were separated by electrophoresis in 2.5% agarose and 1 X TBE (100 mM Tris–HCl, 90 mM boric acid, 1 mM Na_2EDTA, pH 8.0) running buffer for 3.5 h at 65 V, and visualized by staining with ethidium bromide (0.5 mg ml^{-1}). The generated fingerprints were compared visually. The reproducibility of the fingerprint profiles was assessed in at least three separate experiments.

Clustering analysis

For cluster analysis, the data were converted into a binary matrix, where the digit 1 represents the presence of a phenotypic character or DNA band and the digit 0 represents its absence. The similarity matrix was generated with Euclidean distances, which were used to build a tree with the unweighted pair group mean averages (UPGMA) algorithm. Analysis of data was performed using the software PAST (Hammer et al. 2001).

Results

Phenotypic characterization

In total, 111 isolates exhibiting violet pigmentation retrieved from the water (74) and soil (37) were recovered on 25%-strength NA and analyzed phenotypically and molecularly. All isolates were unable to grow at 7 or 15°C. A subset of these isolates was analyzed using chitinase qualitative screening (67 from water and 9 from soil), and the majority of the isolates (82%) were chitinase producers.

A subset of 18 isolates (11 from water and 7 from soil) were analyzed for their metabolic capacity with BIOLOG GN2 plates (Table 1). The population from water isolates was able to utilize all the 95 carbon substrates, whereas the population from soil isolates used 92 carbon substrates. The substrates α-Keto valeric acid, sebacic acid and phenylethylamine were not consumed by soil *Chromobacterium* isolates. The *C. violaceum* ATCC 12472 type species had the lowest metabolic versatility of all isolates and grew only on 43 substrates. Only nine substrates, glycogen, turanose, pyruvic acid methyl ester, L-alanylglycine, L-asparagine, L-glutamic acid, glycil-L glutamic acid, L-serine and D, L α-glycerol phosphate, were degraded for all isolates tested, including *C. violaceum* ATCC 12472. In contrast, seven substrates, α-D-glucose, D-manose, D-trehalose, β-hydroxybutyric acid, D, L-lactic acid, L-alanine and inosine, were exclusively used by the 18 *Chromobacterium* isolates but not the type species (Table 1). The dendrogram generated from the clustering analysis of richness is shown in Fig. 2. All the isolates and the type species exhibited unique metabolic combinations, showing evidence of a great diversity of carbon utilization. The 92BS-W

Table 1 Phenotypic characteristics of 18 *Chromobacterium* sp. isolates

Biochemical characteristics	Percentage of positive bacterial isolates		
		Environments	
	Type species	Soil (7)	Water (11)
α-Cyclodextrin	+	28.6	36.4
Dextrin	+	100.0	90.9
Glycogen	+	100.0	100.0
Tween 40	+	85.7	90.9
Tween 80	+	42.9	81.8
N-acetyl-D-glucosamine	+	100.0	90.9
L-arabinose	+	100.0	72.7
D-arabitol	+	85.7	72.7
D-cellobiose	+	100.0	90.9
D-fructose	+	100.0	90.9
α-D-glucose	+	100.0	100.0
D-mannose	+	100.0	100.0
D-psicose	+	85.7	72.7
Sucrose	+	100.0	81.8
D-trehalose	+	100.0	100.0
Turanose	+	100.0	100.0
Pyruvic acid methyl ester	+	100.0	100.0
Cis-aconitic acid	+	100.0	63.6
D-gluconic acid	+	100.0	72.7
β-Hydroxybutyric acid	+	100.0	100.0
D, L-lactic acid	+	100.0	100.0
Succinic acid	+	100.0	90.9
Bromosuccinic acid	+	100.0	81.8
L-alaninamide	+	85.7	90.9
D-alanine	+	100.0	90.9
L-alanine	+	100.0	100.0
L-alanylglycine	+	100.0	100.0
L-asparagine	+	100.0	100.0
L-aspartic acid	+	100.0	90.9
L-glutamic acid	+	100.0	100.0
Glycyl-L aspartic acid	+	85.7	100.0
Glycyl-L glutamic acid	+	100.0	100.0
L-histidine	+	85.7	90.9
L-leucine	+	42.9	36.4
L-ornithine	+	100.0	72.7
L-phenylalanine	+	85.7	72.7
L-proline	+	100.0	90.9
L-serine	+	100.0	100.0
L-threonine	+	85.7	81.8
Urocanic acid	+	100.0	90.9
Inosine	+	100.0	100.0
Thymidine	+	71.4	90.9
D, L-α-glycerol phosphate	+	100.0	100.0
D-glucose-6-phosphate	+	100.0	90.9
α-Keto valeric acid	−	0.0	18.2
Phenyethylamine	−	0.0	18.2
Sebacic acid	−	0.0	18.2
N-acetyl-D galactosamine	−	100.0	90.9
Adonitol	−	85.7	63.6
i-Erythritol	−	57.1	45.5
L-fucose	−	85.7	63.6
D-galactose	−	100.0	81.8
Gentiobiose	−	100.0	72.7
m-Inositol	−	85.7	90.9
α-D-lactose	−	100.0	63.6
Lactulose	−	71.4	63.6
Maltose	−	100.0	72.7
D-mannitol	−	85.7	90.9
D-melibiose	−	85.7	63.6
β-Methyl-D-glucoside	−	85.7	72.7
D-raffinose	−	100.0	54.5
L-rhamnose	−	57.1	45.5
D-sorbitol	−	100.0	81.8
Xylitol	−	71.4	72.7
Succinic acid mono-methyl-ester	−	100.0	81.8
Acetic acid	−	28.6	27.3
Citric acid	−	100.0	90.9
Formic acid	−	85.7	72.7
D-galactonic acid lactone	−	85.7	63.6
D-galacturonic acid	−	100.0	81.8
D-glucosaminic acid	−	71.4	63.6
D-glucuronic acid	−	100.0	90.9
α-Hydroxybutyric acid	−	85.7	63.6
γ-Hydroxybutyric acid	−	57.1	45.5
p-Hydroxy phenylacetic acid	−	57.1	45.5
Itaconic acid	−	14.3	18.2
α-Keto butyric acid	−	42.9	18.2
α-Keto glutaric acid	−	85.7	90.9
Malonic acid	−	42.9	72.7

Table 1 continued

Biochemical characteristics	Percentage of positive bacterial isolates		
		Environments	
	Type species	Soil (7)	Water (11)
Propionic acid	–	28.6	27.3
Quinic Acid	–	71.4	63.6
D-saccharic acid	–	85.7	63.6
Succinamic acid	–	42.9	45.5
Glucuronamide	–	85.7	8.2
Hydroxy-L proline	–	100.0	63.6
L-pyroglutamic acid	–	71.4	72.7
D-serine	–	85.7	54.5
D, L-carnitine	–	28.6	63.6
γ-Amino butyric acid	–	85.7	72.7
Uridine	–	85.7	90.9
Putrescine	–	85.7	72.7
2-Aminoethanol	–	42.9	27.3
2,3-Butanediol	–	14.3	27.3
Glycerol	–	85.7	90.9
α-D-glucose-1-phosphate	–	85.7	90.9

isolate showed the closest physiological profile to the type species.

The degree of resistance in the two populations is shown as the MIC for 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates (Table 2). Analysis of the MIC revealed that there was a wide range in the inhibitory concentration of a particular antimicrobial agent between the populations. All isolates exhibited high MIC to ampicillin and were inhibited by ciprofloxacin, tetracycline and mercury at the lowest concentration tested. A wide variability of MIC was clearly revealed by 80 distinguishable profiles (Fig. 3). Fifty-three of the 111 isolates exhibited a unique profile for a combination of the antimicrobials tested. No isolate showed the same MIC profile as the type species. The dendrogram based on MIC revealed a clear separation between water and soil isolates and exhibited nine distinct clusters with a similarity cut-off of about 80% (Fig. 3). Clusters 3, 4, 5, 7 and 8 assembled isolates exclusively from water, with an exception of one soil isolate (182BS). The remaining clusters comprised only soil isolates, with the exception of three water isolates (119BS, 151BS and 176BS),

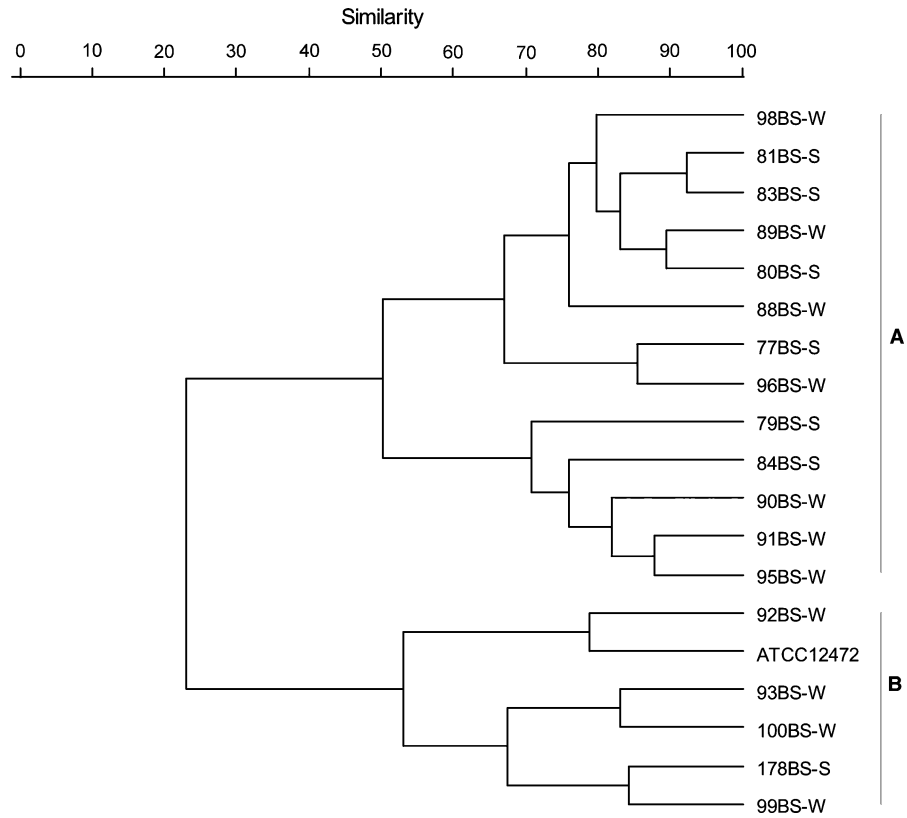

Fig. 2 Cluster analysis of *Chromobacterium* sp. isolates and of *C. violaceum* ATCC 12472 according to BIOLOG GN2 microplates profiles. A distance matrix of simple similarity coefficients was clustered with the UPGMA algorithm. *W* isolates recovered from water; *S* isolates recovered from soil

Table 2 Minimum inhibitory concentration (MIC) at which 50 and 90% of water and soil *Chromobacterium* sp. isolates in the overall population are inhibited (μg ml^{-1})

Antimicrobials	Range	Type species	Environments			
			Water		Soil	
			MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Ap	2–1024	1024	256	>1024	>1024	>1024
Am	2–1024	256	16	1024	512	1024
Cp	2–128	<2	<2	<2	<2	<2
Ak	2–128	<2	<2	>128	8	16
Gm	2–128	<2	<2	64	<2	4
Km	2–128	4	4	16	4	4
Sm	2–128	16	8	>128	<2	16
Cm	2–128	32	<2	16	4	8
Nx	2–128	<2	<2	32	<2	<2
Tc	2–128	<2	<2	<2	<2	<2
Hg	2–16	8	<2	<2	<2	<2

which grouped with the soil isolates (cluster 2). The type species grouped with one soil isolate (182BS) and eight water isolates in cluster 8.

Isolate identity based on 16S rRNA gene sequence

The almost complete 16S rDNA sequences used for phylogenetic analyses were 1,245 nucleotides long and spanned the V2 to V8 variable regions corresponding to *C. violaceum* ATCC 12472. There was a high level of 16S rRNA gene sequence similarity between all the *Chromobacterium* species (Table 3). The sequences of the 111 isolates exhibited 97.8% similarity to the 16S rRNA gene sequence of *C. violaceum* ATCC 12472, whereas 16S rDNA sequences presented 99% similarity among water and soil isolates. The interspecies similarity was greatest with *C. piscinae* (98.3%) and least with *C. haemolyticum* (96.6%). Fifty-eight of these isolates (37 and 21 from water and soil, respectively) had identical 16S rRNA gene sequences and were represented in the phylogenetic tree as four haplotypes. The phylogenetic tree based on these sequences confirmed their assignment to *Chromobacterium* and their phylogenetic proximity to *C. piscinae* (Fig. 4). Moreover, the resulting tree revealed two main clusters containing 111 isolates and six *Chromobacterium* species and confirmed that the isolates did not group well according to their origin.

To compare the association between genetic similarity and specific features of the two microenvironments, we have applied the UniFrac metric analysis. The UniFrac and principal component analyses showed that there were no significant environmental differences ($P > 0.01$; Fig. 5), confirming the absence of a clear separation according to the 16S rRNA gene phylogenetic tree (Fig. 4).

BOX-PCR genomic fingerprinting analysis

BOX-PCR fingerprinting generated profiles of 2–13 bands, ranging in size from approximately 200 bp to 4 kb. Moreover, BOX-PCR fingerprinting provided a higher resolution and showed that the haplotypes H2, H3 and H4 (Fig. 4) exhibited genetically divergent isolates (5, 2 and 17, respectively). In contrast with the 16S rRNA gene tree, the BOX-PCR dendrogram (Fig. 6) showed a clear separation between water and soil isolates, which was consistent with the antimicrobial susceptibility results. There was a larger cluster including 48 water isolates. The presence of many clusters is compatible with the prevalence of unique fingerprinting patterns among the isolates. BOX-PCR fingerprints were analyzed using a 100% similarity cut-off to discern distinct band patterns. Thus, 33 fingerprints for the 111 isolates were divided into 13 BOX-PCR patterns (A–M), each represented by between 2 to 48 isolates. Pattern C was numerically dominant in the aquatic environment, assembling 48 isolates. Interestingly, patterns restricted to a single sampling site were common; 11 of these patterns are potentially endemic. By contrast,

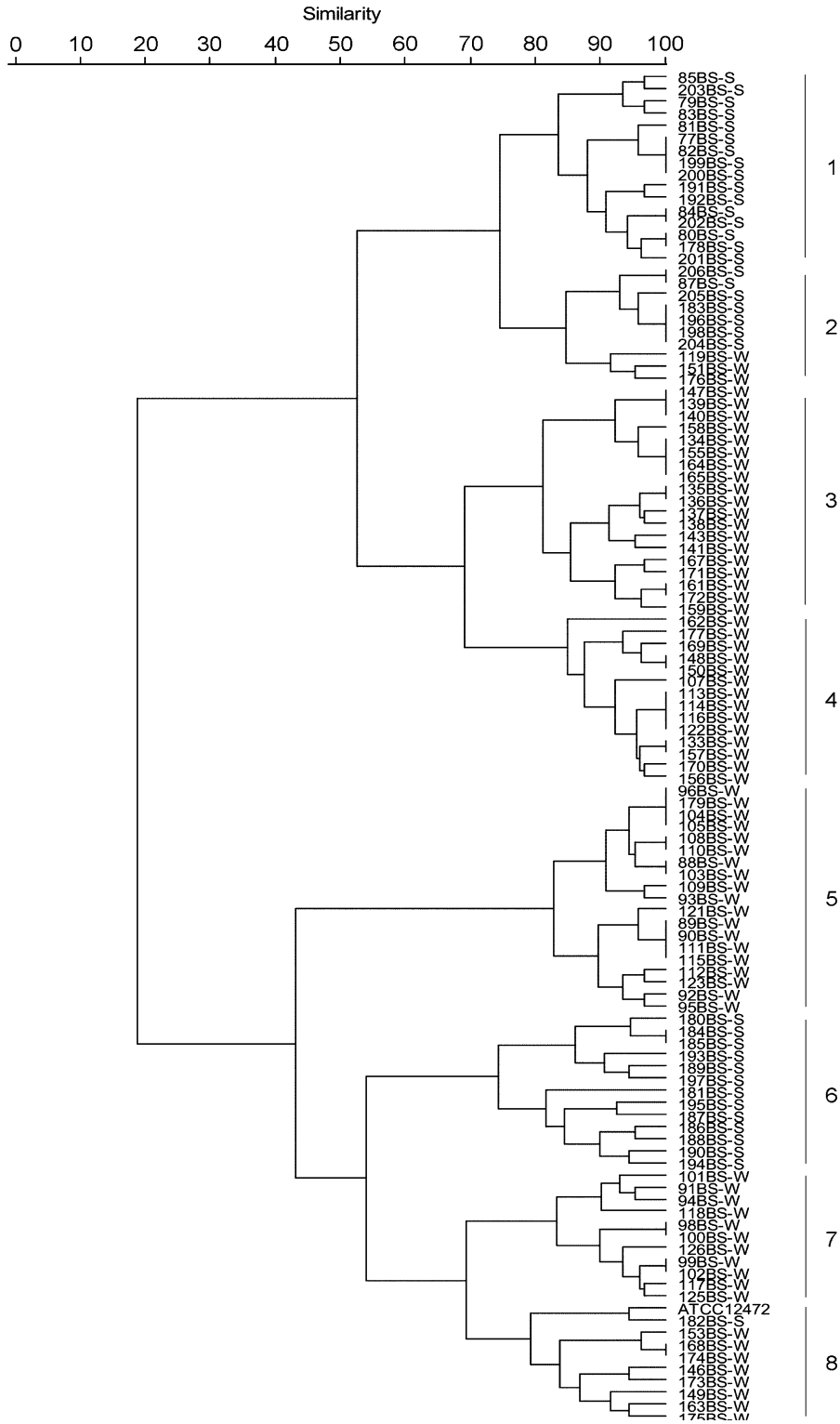

Fig. 3 Cluster analysis of *Chromobacterium* sp. isolates and of *C. violaceum* ATCC 12472 according to antimicrobial susceptibility profiles. A distance matrix of simple similarity coefficients was clustered with the UPGMA algorithm

Table 3 Average similarity of 16S rRNA sequences among individual isolates and *Chromobacterium* species

Isolates	Average similarity of 16S rRNA sequences (%)					
	C. piscinae	*C. subtsuga*	*C. violaceum*	*C. pseudoviolaceum*	*C. haemolyticum*	*C. aquaticum*
88BS-W	97.7	98.1	97.6	97.6	96.3	95.7
89BS-W	98.1	98.4	98.0	98.0	96.6	96.1
90BS-W	97.9	98.1	97.6	97.6	96.3	95.7
92BS-W	97.9	97.9	97.5	97.5	96.1	95.6
98BS-W	98.7	97.7	97.9	97.9	97.1	96.4
99BS-W	99.0	97.8	98.0	98.0	97.2	96.5
101BS-W	98.8	97.8	98.0	98.0	97.2	96.5
108BS-W	98.0	98.3	97.9	97.9	96.5	96.0
109BS-W	98.0	98.3	97.9	97.9	96.5	96.0
111BS-W	97.9	98.2	97.8	97.8	96.4	95.9
113BS-W	97.8	98.0	97.6	97.6	96.2	95.7
116BS-W	98.4	98.2	98.6	98.6	96.1	95.7
117BS-W	98.5	97.3	97.5	97.5	96.7	96.0
118BS-W	97.9	98.2	97.8	97.8	96.4	95.9
119BS-W	98.1	98.4	98.0	98.0	96.6	96.1
123BS-W	97.9	98.2	97.8	97.8	96.4	95.9
137BS-W	97.9	97.9	97.5	97.5	96.1	95.6
138BS-W	97.5	97.8	97.4	97.4	96.0	95.5
139BS-W	97.9	98.2	97.8	97.8	96.4	95.9
143BS-W	97.7	98.1	97.6	97.6	96.3	95.7
146BS-W	98.3	98.6	97.6	97.6	96.6	96.2
148BS-W	98.0	98.3	97.9	97.9	96.5	96.0
150BS-W	97.6	97.9	97.5	97.5	96.1	95.6
155BS-W	97.7	98.1	97.6	97.6	96.3	95.7
156BS-W	98.2	98.3	98.0	98.0	96.7	96.2
157BS-W	98.3	98.2	98.7	98.7	96.2	95.7
159BS-W	97.7	98.1	97.6	97.6	96.3	95.7
165BS-W	98.1	98.4	98.0	98.0	96.6	96.1
167BS-W	98.7	97.9	97.7	97.7	96.1	95.4
168BS-W	98.7	97.6	97.8	97.8	97.0	96.4
169BS-W	98.5	98.3	98.7	98.7	96.4	95.9
171BS-W	98.7	97.8	97.6	97.6	96.0	95.3
172BS-W	97.6	97.8	97.4	97.4	96.0	95.5
173BS-W	98.5	99.1	97.5	97.5	96.8	96.3
174BS-W	98.9	98.0	98.0	98.0	97.2	96.5
175BS-W	98.5	98.8	97.5	97.5	96.8	96.3
176BS-W	98.8	98.1	97.7	97.7	96.3	95.6
177BS-W	98.1	98.4	98.0	98.0	96.6	96.1
179BS-W	97.8	98.2	97.7	97.7	96.4	95.8
189BS-W	98.7	97.8	97.6	97.6	96.1	95.4
83BS-S	98.8	97.8	98.0	98.0	97.2	96.5
84BS-S	98.7	97.7	97.9	97.9	97.1	96.4
181BS-S	98.4	98.3	98.7	98.7	96.3	95.8

Table 3 continued

Isolates	Average similarity of 16S rRNA sequences (%)					
	C. piscinae	*C. subtsuga*	*C. violaceum*	*C. pseudoviolaceum*	*C. haemolyticum*	*C. aquaticum*
183BS-S	98.3	98.4	98.7	98.7	96.4	95.7
184BS-S	98.5	98.8	97.8	97.8	96.8	96.4
186BS-S	98.7	97.7	97.5	97.5	96.1	95.4
187BS-S	99.2	97.9	97.5	97.5	96.3	95.7
188BS-S	98.5	97.5	97.6	97.6	96.9	96.2
191BS-S	99.0	97.6	97.8	97.8	97.0	96.4
194BS-S	97.7	98.1	97.6	97.6	96.3	95.7
195BS-S	98.7	97.7	97.9	97.9	97.1	96.4
196BS-S	98.7	97.6	97.8	97.8	97.0	96.4
197BS-S	98.9	97.9	98.1	98.1	97.3	96.6
199BS-S	98.2	97.1	97.3	97.3	96.5	95.8
201BS-S	98.3	97.3	97.5	97.5	96.7	96.0
202BS-S	98.8	97.8	98.0	98.0	97.2	96.5
205BS-S	98.9	97.9	98.1	98.1	97.3	96.6
Total average similarity	98.3	98.0	97.8	97.8	96.6	96.0

two patterns (B and G) collected in both environments appear to be widespread among these isolates. Moreover, BOX-PCR fingerprinting suggested significantly greater diversity in the soil isolates. The fingerprint from one water isolate was clustered with the type species in the UPGMA tree with a similarity of 92%.

Discussion

The present investigation aimed at assessing the spatial distribution of *Chromobacterium* isolates, and it can provide a more comprehensive picture of the ecology, evolution and population structure of *Chromobacterium* isolates. The screening using 4, 15 and 37°C of incubation in this investigation was reliable in distinguishing the *Chromobacterium* genus from other genera that also produce the violet pigment, such as *Janthinobacterium* and *Iodobacter* (Logan and Moss 1992). The identification was further confirmed by amplifying and sequencing the16S rRNA genes from all isolates (111). Thirty-five isolates were unable to grow in a second subculture, clearly indicating the difficulties of keeping these environmental bacteria long term. Hence, only 76 isolates were assayed for chitinase production. In contrast to the type species, 14 isolates were not chitinase producers. This can be explained due to bacterial response to environmental conditions and their different capability in regulating gene expression (Brückner and Titgemeyer 2002; Peng and Shimizu 2003) or even through variation in genome sizes that can occur within the same species (Medini et al. 2005).

Polyphasic approaches by 16S rRNA gene sequencing, rep-PCR genomic fingerprinting with the BOX primer, biochemical characterization and antimicrobial susceptibility testing were used as tools to assess genetic relationships and the level of physiological difference that might be discernible between the geographical locations of *Chromobacterium* isolates. The biochemical and antimicrobial susceptibility data revealed physiologically distinct bacterial populations even when some of the isolates exhibited identical haplotypes. It should be pointed out that the violacein has a wide reading spectrum (431–699 nm) (Logan and Moss 1992), and, consequently, could interfere with sample readings which were done at 590 nm. Therefore, this limitation restricted the number of isolates analyzed to 18, which were those *Chromobacterium* isolates that did not longer produce the violacein pigment. However, the number of the isolates analyzed was clearly sufficient to reveal the physiological heterogeneity. The presence of high inter- and intra-population

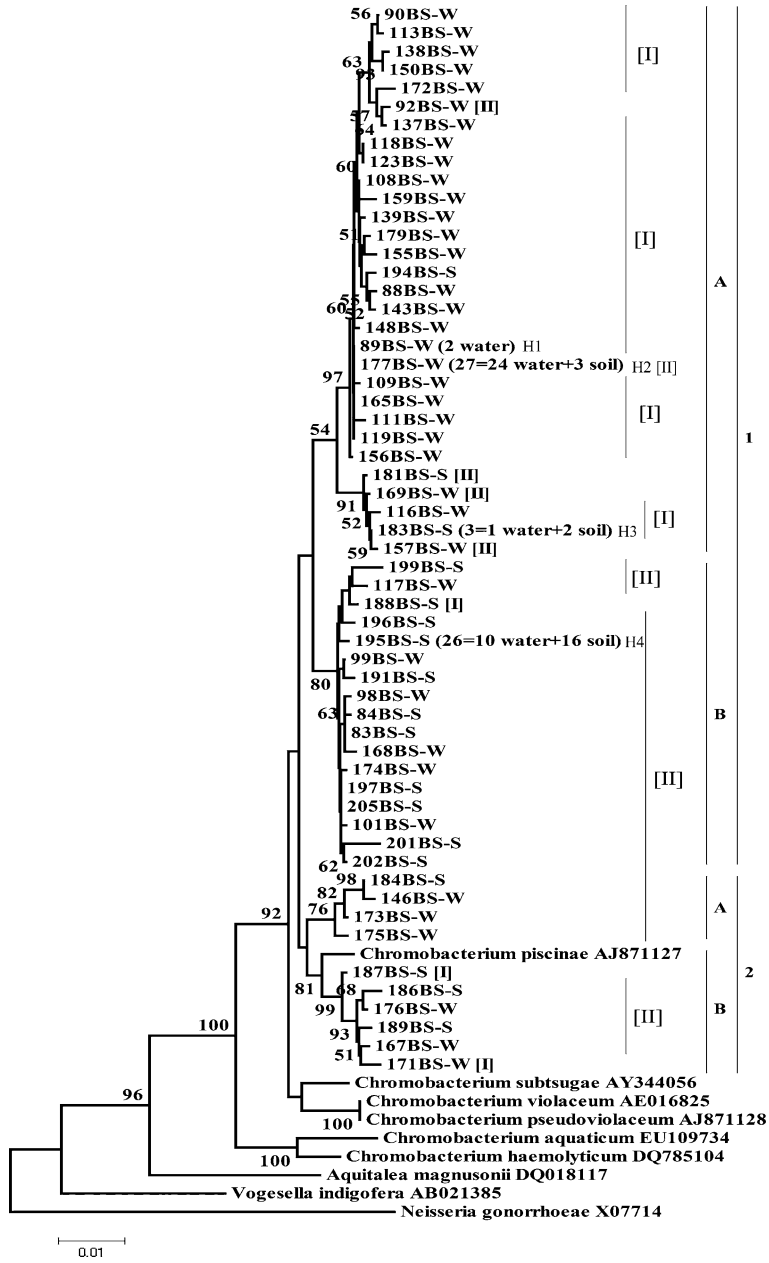

Fig. 4 Phylogenetic tree based on 16S rRNA gene sequences of all *Chromobacterium* sp. isolates from water and soil. One thousand bootstrap resamplings were used to evaluate the robustness of the inferred trees. *Numbers* in *parentheses* represent the number of the isolates exhibiting the same haplotype. *Numbers* in *brackets* correspond to BOX-PCR fingerprinting dendrogram clusters

microdiversity in *Chromobacterium* isolates suggests the existence of physiological adaptations to a new ecological niche. From an ecological perspective, the presence of these different members in the same habitat can be understood only if they occupy distinct ecological niches. It is interesting to note that, although the isolates were collected in environments with different nutrient availability (Benites et al. 2005; Callisto et al. 2002; Lima-Bittencourt et al. 2007a), the samplings were performed on the same day in the rainy season. The topology of the region could facilitate the flow of nutrients from the soil to the watercourse, therefore harmonizing the nutrient load in both environments (www.ibama.gov.br). Moreover, it is detected the changing of the Indaiá stream water pH from 6.1 (dry season) (Callisto et al. 2002; Lima-Bittencourt et al. 2007a) to 4.5 (rainy season) close to the soil pH 4.0 (Benites et al. 2005). It is well known that bacterial populations in their natural environments suffer periodic selection and

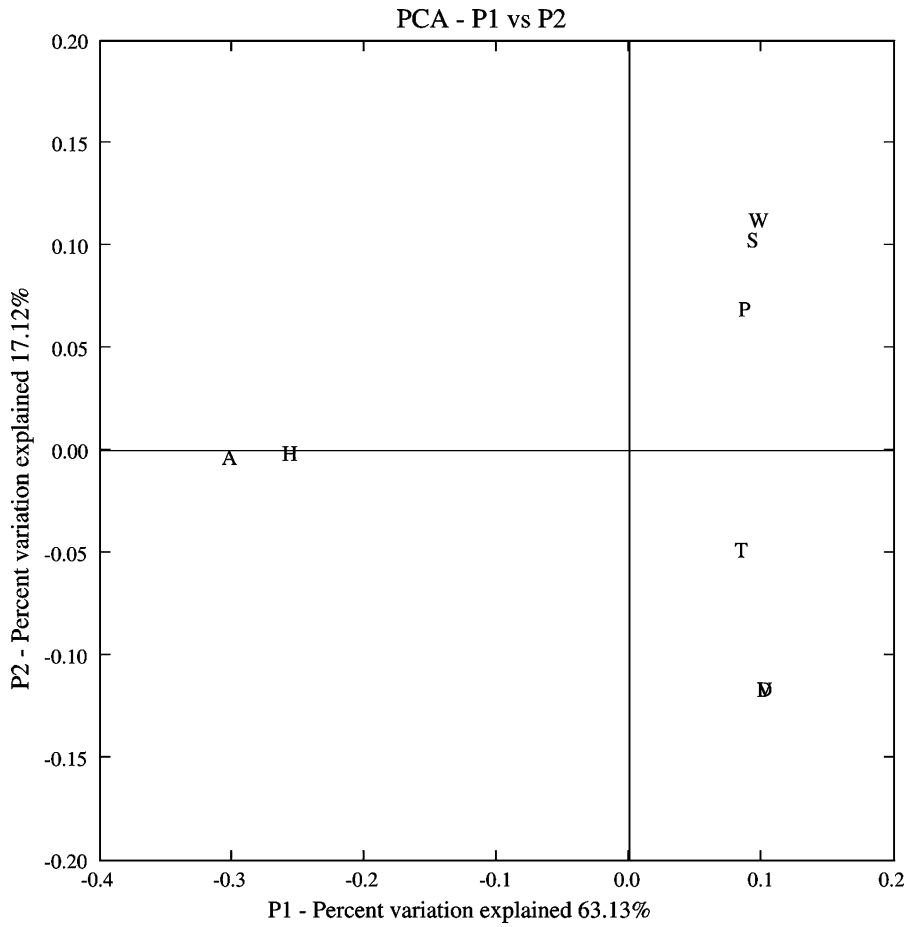

Fig. 5 Principal component analysis ordination plot for the 16S rRNA gene. The percent of variation explained by each principal component is indicated on the axis labels. Environments are represented by the following letters: Water W, Soil S. *Chromobacterium* species are represented by the following letters: *C. piscinae* P, *C. aquaticus* A, *C. haemolyticum* H, *C. subtsugae* T, *C. violaceum* V and *C. pseudoviolaceum* D

that individuals with fitter variants grow to be numerically dominant (Levin 1981). In this study, we suggest that individuals with higher fitness brought from soil to water by the rain colonized the aquatic environment. This is further supported by the fact that lower genetic diversity was found in water isolates, which are derived from a fewer more adapted individuals to the new environment.

Surprisingly, antimicrobial susceptibility testing proved to be as discriminatory in achieving the separation of the *Chromobacterium* isolates between the environments as the genomic fingerprinting technique BOX-PCR. This approach is simple, low-cost and easy to perform. Therefore, it appears to be a suitable method for assessment of genetic variability and distribution of the *Chromobacterium* isolates populations and their adaptation to local environments. To our knowledge, this is the first time that the antimicrobial susceptibility testing was used successfully in distinguishing *Chromobacterium* populations inhabiting different but nearby environments, opening the possibility of studying bacterial biogeography on a microgeographic scale. The sensitivity of the technique has been shown to be greatly influenced by the particular environments studied therein. The soil harbors the great majority of antibiotic producers and it maintains conditions so that they are retained, especially by their chemical combination (D'Costa et al. 2007). Concomitantly, the presence of humic acids in soil and water creates a feedback loop, making a strong association between antibiotic and soil and water particles, thus delaying the biodegradation (Baquero et al. 2008). This information could explain the difference in selective pressure that antibiotics exert on bacteria from soil and water.

Since the advent of molecular biology techniques, bacterial taxonomy has employed molecular approaches for the identification of bacteria. The

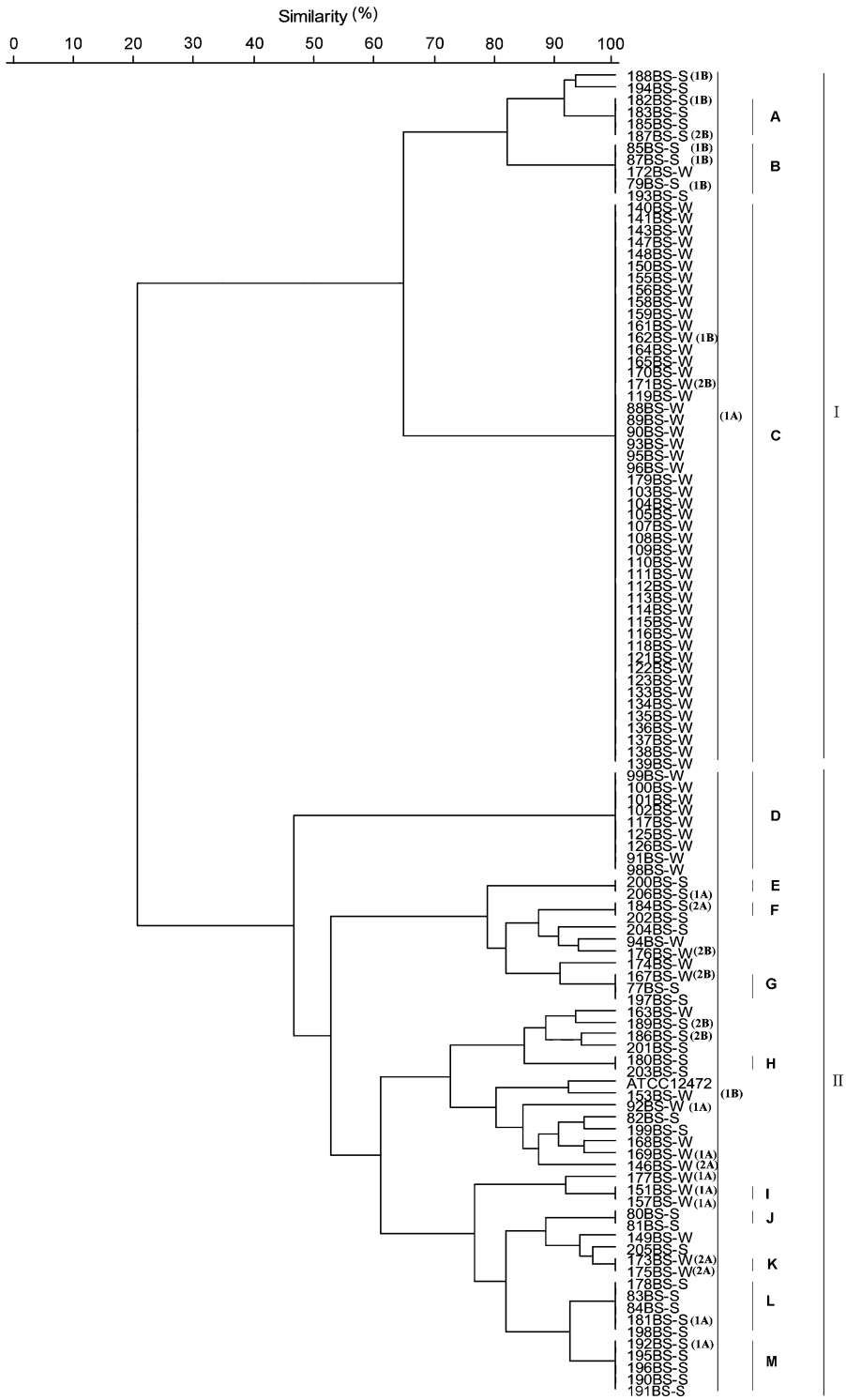

Fig. 6 Cluster analysis of *Chromobacterium* sp. isolates and of *C. violaceum* ATCC 12472 according to BOX-PCR fingerprinting. A distance matrix of simple similarity coefficients was clustered with the UPGMA algorithm. *Letters A–M* correspond to the 13 patterns. A and B correspond to 16S rRNA phylogenetic tree clusters

16S rRNA gene sequence has been frequently used as a molecular marker, turning this sequence into a bacterial molecular signature. Sequence similarity of ≥97% was proposed by Stackebrandt and Goebel (1994) for bacterial species delineation. Nevertheless, the 16S rRNA gene is considered a neutral marker

used to distinguish bacteria at the species level according to their evolutionary history from common ancestry, albeit with some limitations (Oren 2004). For example, *Sporosarcina globispora* and *S. psychrophila* have 100% identity in 16S rRNA gene sequence (Fox et al. 1992). A high similarity of 16S rRNA sequences has also been described in *Chromobacterium* species. In the present study, the phylogenetic tree topology revealed that the isolates were closely related to *C. piscinae* and exhibited a very high genetic diversity. Considering that many *Chromobacterium* isolates were phenotypically diverse, reflecting adaptations to their local habitats, the different phenotypic isolates within sub-clusters shown in the 16S rRNA phylogenetic tree may represent bacterial ecotypes and novel species (Cohan 2002; Achtman and Wagner 2008). Other *Chromobacterium* populations from Cerrado were also found to be phenotypically and phylogenetically diverse according to our previous work (Lima-Bittencourt et al. 2007b). Despite being an effective tool to survey the taxonomy in bacteria, the 16S rRNA gene cannot differentiate the environmental origin of the isolates, as periodical selection is constantly acting on individual bacterial isolates.

The application of BOX-PCR to generate fingerprints of bacterial populations present in the natural environment represents an important tool in studies of bacterial diversity. It is well suited to distinguish between genotypically related strains of a broad range of bacterial species, detecting the intraspecific diversity (Brusetti et al. 2008). The 16S rRNA gene sequences clusters for *Chromobacterium* isolates diversity were further subdivided by the high genetic diversity obtained for BOX-PCR, including those that had identical 16S rRNA gene sequences. One likely explanation is that the repetitive sequences detected by BOX-PCR have had time to accumulate divergence at rapidly evolving loci, but not at the 16S rRNA loci. Overall, the BOX-PCR groupings were similar to the topology of the 16S rRNA gene tree, with the exception of some isolates (Figs. 4 and 6), which is likely due to convergent evolution of bands in the BOX-PCR. In accordance with Rademaker et al. (2000), this molecular tool is a powerful strain and species discriminator; however, our results indicate that due to its higher variability, convergent patterns can appear. Despite this, BOX-PCR has proven a good correlation with the phenotypic analysis of *Chromobacterium* isolates, and can be used to ascertain geographically close, but environmentally different, isolates.

This is the first study with a large set of *Chromobacterium* isolates most closely related to *C. piscinae* in the neotropical region, and our data support the findings of Cho and Tiedje (2000) that there is a high degree of endemicity in bacterial populations. Nevertheless, it should be pointed out that the antimicrobial susceptibility testing and rep-PCR used here were more suitable techniques, allowing us to assess patterns of the adaptively correlated genetic diversity. This diversity brings an endemic character to the two populations, indicating that ecological barriers could play an important role in the periodic selection and rapid diversification of ecotypes in *Chromobacterium* populations from water and soil. A future perspective is the detailed biochemical and physiological study of the bacterial isolates, particularly their enzymes, in biotechnologically relevant processes.

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