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Biogeografia e caracterização molecular e fisiológica de bactérias heterotróficas, com ênfase em *Chromobacterium*, dos biomas Mata Atlântica e Cerrado

CLÁUDIA IRACEMA LIMA BITTENCOURT

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BELO HORIZONTE

Maio - 2011

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Tese de Doutorado apresentado ao Programa de Pós-Graduação em Genética do Departamento de Biologia Geral do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Genética.

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"Resilience describes the speed with which a community returns to its former state after it has been perturbed and displaced from that state."

Ecology: from individuals to ecosystems / Michael Begon, Colin R. Townsend, John L. Harper.—4th Ed.

Dedico esta Tese ao meu pai, à minha mãe, à minha irmãzinha e ao meu marido, sem vocês nada disso teria sido possível.

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SUMÁRIO

LISTA DE FIGURAS

CAPÍTULO 4

LISTA DE TABELAS

LISTA DE ABREVIATURAS

µg	Micrograma
µl/µL	Microlitro
µm	Micrômetro
µM	Micromolar
µS	Microsiemens
abs	absorbance
Ak	Amicacina
Am	Ampicilina-ácido clavulânico
ampC	Gene de betalactamase
Ap	Ampicilina
ARDRA	Amplified rDNA restriction analysis
ATCC	American Type Culture Collection
AWCD	Average well-color development
BNGPC	Brazilian National Genome Project Consortium
BOX	BOX element of Streptococcus pneumoniae
bp	Base pair
C	Carbon
CaCl2	Cloreto de cálcio
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CCA	Canonical correspondence analysis
CFB	Grupo taxonômico Cytophaga-Flavobacteria-Bacteroidetes
CFU	Colony-forming unit
CLPP	Community level physiological profiles
Cm	Cloranfenicol
cm	Centímetro
CNPq	Conselho Nacioanal de Desenvolvimento Científico e Tecnológico.
CO2	Gás carbônico
Cp	Ciprofloxacina
d	Days
DNA	Ácido desoxirribonucléico
dNTP	Desoxinucleotídeo trifosfato
DO	Dissolved oxygen concentration
E	Equitabilidade
EDTA	Ácido etilenodiaminotetracético
ELISA	Enzyme linked immuno sorbent assay
FAPEMIG	Fundação de Amparo à Pesquisa do Estado de Minas Gerais
FeSO4	Sulfato ferroso
g	Grama
Gm	Gentamicina
GNS	Grupo taxonômico green nonsulfur
h	Hora
H’	Índice de diversidade Shannon-Weaver
H2O	Água

ha	Hectare
Hg	Bicloreto de mercúrio
ITS	Intergenic 16S-23S transcribed spacer
kb	Kilobase
KCl	Cloreto de potássio
Km	Canamicina
l/L	Litro
ln	Logarítimo natural
m	Metro
M	Molar
mg	Miligrama
MgSO4	Sulfato de magnésio
MH	Meio Mueller–Hinton
MIC	Minimum inhibitory concentration
MIC50	Concentração Inibitória Mínima (Minimum inhibitory concentration), na qual 50% dos isolados foram inibidos.
MIC90	Concentração Inibitória Mínima (Minimum inhibitory concentration), na qual 90% dos isolados foram inibidos.
min	Minuto
ml/mL	Mililitro
mM	Milimolar
mm	Milímetro
MnSO4	Sulfato de manganês II
N	Nitrogênio
NA	Nutrient agar
NaCl	Cloreto de sódio
NCCLS	National Committee for Clinical Laboratory Standards
ng	Nanograma
NH4	Amônio
NH4NO3	Nitrato de amônio
nm	Nanômetro
NO2	Nitrito
NO3	Nitrato
NTU	Nephlometric turbidity units
Nx	Ácido nalidíxico
ºC	Grau Celcius
OD	Optical density
ONU	Organização das Nações Unidas
OP	Divisões filogenéticas candidatas
ORF	Open reading frame
OTU	Operational taxonomic unit
P	Fósforo
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PERD	Parque Estadual do Rio Doce
pH	Potencial hidrogeniônico

PNSC	Parque Nacional da Serra do Cipó
PO4	Fosfato
PTYG	Meio formado por peptona, triptona, extrato de levedura e glicose
R	Riqueza
rDNA	Ácido desoxirribonucléico ribossômico
RDP	Ribosomal Database Project
rep-PCR	Repetitive extragenic palindromic PCR
RNA	Ácido ribonucléico
rRNA	Ácido ribonucléico ribossômico
s	Segundo
S	South (Sul)
Sm	Estreptomicina
TAE	Tampão Tris–acetate–EDTA
TBE	Tampão Tris borato de EDTA
Tc	Tetraciclina
tDNA	Ácido desoxirribonucléico transportador
TN	Total nitrogen
TP	Total phosphorus
tRNA	Ácido ribonucléico transportador
UPGMA	Unweighted Pair Group Method with Arithmatic Mean
UTO	Unidade taxonômica operacional
V	Volts
W	West (Oeste)
WS	Divisões filogenéticas candidatas

RESUMO

A Biogeografia, antes exclusiva de macrorganismos, estuda a distribuição espacial e temporal das espécies de seres vivos e tenta compreender os fatores que controlam sua abundância. Este estudo propôs investigar a diversidade e biogeografia de bactérias heterotróficas cultiváveis de duas unidades de conservação: Parque Nacional da Serra do Cipó (PNSC, Cerrado) e Parque Estadual do Rio Doce (PERD, Mata Atlântica), por meio de abordagem polifásica. Dos 936 isolados bacterianos estudados, 111 isolados exibiam pigmentação violeta e foram recuperados das águas do Córrego Indaiá (74) e solo do entorno (37), PNSC, enquanto 825 isolados foram recuperados de nove pontos dos gradientes eufótico (100%, 10% e 1% de penetração de luz) e horizontal do Lagoa Carioca (PERD), em junho e agosto de 2007. A filogenia do gene de rRNA 16S sugeriu que os 111 isolados foram relacionados com *Chromobacterium piscinae*. Todos apresentaram alta resistência à ampicilina e os perfis fisiológicos, gerados pelo BIOLOG GN2, mostraram grande versatilidade na utilização de substratos. Os agrupamentos por *fingerprint* BOX-PCR e de susceptibilidade a antimicrobianos mostraram clara separação entre os isolados de solo e de água, indicando uma forte endemicidade das populações. O perfil fisiológico, das 18 comunidades microbianas do lago Carioca, obtido com BIOLOG Ecoplates, revelou grande diversidade metabólica, tendo os dois agrupamentos encontrados delimitado as comunidades por sua origem temporal. Dentre os 825 isolados da lagoa, 673 deles produziram 360 UTOs (unidades taxonômicas operacionais) - ARDRA (análise de restrição do DNA ribossômico amplificado), das quais 313 foram únicas, indicando forte endemismo. Posteriormente, as UTOs-ARDRA foram identificadas pelo rDNA 16S e foram afiliadas a cinco filos, em ordem de abundância, Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes e Deinococcus-Thermus, representados por 39 gêneros. Teste de Mantel relacionou a composição das comunidades a poucos fatores abióticos (turbidez, N total e NO_2), principalmente nos períodos de tempo amostrados. Uma população de 31 isolados de *Chromobacterium*, gênero mais abundante, foi caracterizada e identificada pelo rDNA 16S como *C. haemolyticum*, sendo a primeira caracterização de isolados ambientais desta espécie. Os isolados exibiram heterogeneidade genética revelada pelo rDNA 16S e as análises de ITS e BOX-PCR.

Destaca-se, ainda, que durante o processo de purificação, algumas colônias peculiares (76/1196) albergavam dois a cinco isolados bacterianos, os quais foram denominados "isolados associados". Curiosamente, após a purificação alguns deles não sobreviveram. Das colônias abrigando os isolados múltiplos, aquelas com dois isolados associados predominaram, enquanto colônias abrigando quatro ou cinco isolados associados foram exclusivamente obtidos em 1% do gradiente eufótico. A identidade taxonômica dos 121 isolados associados revelou a presença dos mesmos cinco filos já encontrados, embora neste estudo os gêneros *Curtobacterium* e *Williamsia* tenham sido exclusivos.

ABSTRACT

Biogeography studies the space and temporary distribution of the species of living beings and tries to understand the factors that control their abundance. This study aimed to investigate the diversity and biogeography of cultivable heterotrophic bacteria from two Brazilian conservation units: Serra do Cipó National Park (PNSC, Cerrado) and Rio Doce State Park (PERD, Atlantic Rainforest), using a polyphasic approach. Of 936 bacterial isolates studied, 111 isolates exhibited violet pigmentation. These were isolated from the waters of Córrego Indaiá (74) and from the soil of its environs (37), PNSC. The remaining isolates (825) were retrieved from nine points of the euphotic and horizontal gradients (100%, 10% and 1% of light penetration) of the Carioca Lake (PERD), in June and August of 2007. Phylogeny of 16S rRNA gene suggested that the 111 violet isolates were related with *Chromobacterium piscinae*. All *C. piscinae* isolates presented high resistance to ampicillin, and their physiological profiles, generated by BIOLOG GN2, showed high versatility in the substrata use. The clusters obtained by BOX-PCR fingerprinting and by antimicrobial susceptibility showed clear separation among the isolates from soil and from water, indicating a strong endemism of the populations. The physiological profile of the 18 microbial communities from the Carioca Lake, obtained by BIOLOG Ecoplates, revealed high metabolic diversity, although it was possible to distinguish two groupings, which were delimited by their temporal origin. Among the 825 isolates from the lake, 673 of them produced 360 OTUs (operational taxonomic units) by ARDRA (Amplified Ribosomal DNA Restriction Analysis), of which 313 were unique, indicating strong endemism. Further, OTUs-ARDRA was identified for the 16S rDNA sequencing and was affiliated to five phyla, ordered by its abundance: Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Deinococcus-Thermus, represented by 39 genera. Mantel testing pointed the relationship between the communities composition and few abiotic factors (turbidity, total N, and NO2), especially when the comparison was done among the communities in July and August. A population of 31 isolates of *Chromobacterium*, the most abundant genus, was characterized and identified by 16S rDNA as *C. haemolyticum*. In our knowledge, this is the first characterization of environmental isolates from this species. These isolates exhibited

genetic heterogeneity revealed by the 16S rDNA and the analyses of ITS and BOX-PCR. It also stands out that, during the purification process, some peculiar colonies (76 from 1196) harbored two to five bacterial isolates, which were named “associated isolates”. Surprisingly, after the purification some of them lost their viability. Most of the colonies harboring multiple isolates presented two associated isolates. Interestingly, colonies harboring four or five associated isolates were obtained exclusively at 1% of the euphotic gradient. The taxonomic identity of the 121 associated isolates revealed the presence of the same five phyla already found, although in this study the *Curtobacterium* and *Williamsia* genera have been exclusive.

ESTRUTURA DA TESE

Esta tese é composta de um resumo, uma introdução geral, abordando os principais temas propostos no trabalho por meio de revisão da literatura, e objetivos gerais e específicos. Em seguida, quatro capítulos constituidos de um artigo publicado, "Comparative biogeography of *Chromobacterium* from the neotropics", e dois artigos submetidos, "A survey on cultivable heterotrophic bacteria inhabiting a thermally unstratified water column in Atlantic Forest Lake" e "Phylogeny of cultivable heterotrophic bacteria derived from mixed colonies"; e um artigo aceito para publicação "Characterization of a *Chromobacterium haemolyticum* population from a natural tropical lake". Finalmente, serão apresentadas uma conclusão geral e referências bibliográficas.

I) INTRODUÇÃO GERAL

Diversidade de Procariotos

A Organização das Nações Unidas (ONU) declarou 2010 como o Ano Internacional da Biodiversidade (http://www.cbd.int/2010/welcome/). No entanto, a biodiversidade começou a ser estudada cientificamente ainda no século XVIII quando Carl Linneaus desenvolveu o seu sistema taxonômico binomial, possibilitando a partir de então uma melhor catalogação de espécies. Até a década de 1980, o termo Diversidade Biológica foi utilizado para descrever a variedade de organismos vivos que, atualmente, é conhecida como Biodiversidade (Magurran, 2010). A ONU, na Convenção da Diversidade Biológica (1993), ampliou o conceito básico de Biodiversidade para "a variabilidade entre organismos vivos de todas as fontes, incluindo, ecossistemas terrestres e aquáticos, dentre outros, e a diversidade entre e dentro das espécies" (http://www.cbd.int/). Assim, uma característica universal de toda comunidade ecológica é apresentar a seguinte composição de espécies: algumas extremamente abundantes, algumas moderadamente comuns e a maioria em pequeno número. Deste modo, para avaliar a biodiversidade, as comunidades ecológicas são divididas em dois componentes: riqueza de espécies (número de diferentes espécies presentes em uma amostragem) e equitabilidade (proporção do número de indivíduos de cada espécie relativa ao total de indivíduos da comunidade). Estes dois componentes são importantes para se comparar duas comunidades, pois se elas apresentarem características semelhantes, a comunidade mais diversa será aquela com a maior equitabilidade (Magurran, 2005).

A vida na Terra é dividida em três Domínios: Eucarya, Bacteria e Archaea (Woese, 1987). Os três Domínios englobam, aproximadamente, 15 milhões de espécies, entretanto estima-se que existam, aproximadamente, 12 milhões de espécies só de procariotos (Domínios Bacteria e Archaea), tornando-os os seres mais abundantes do planeta (Guerrero et al, 2002). Além de estarem em grande número, eles também são os organismos mais diversos filogeneticamente e ecologicamente, representando, assim, a maior parte da diversidade genética da vida (Whitman et al, 1998). Consequentemente, esses microrganismos podem ser encontrados nos mais diferentes e inóspitos habitats, mostrando uma extensa adaptação fisiológica a diferentes condições ambientais

(Bowman & McCuaig, 2003; Freitas et al, 2008a; Portillo & Gonzalez, 2008; Lemke et al, 2009).

A história de vida na Terra evoluiu com o surgimento – aproximadamente há 3,8 bilhões de anos – de organismos procariotos, que habitavam uma Terra completamente diferente da atual, com habitats extremos, e diferentes do globo azul atualmente conhecido (Newman & Banfield, 2002). Assim, os procariotos foram e são fundamentais nos processos biogeoquímicos, sustentando a vida do Planeta. Devido à capacidade de adaptação e de utilização de fontes de energia inorgânica, as bactérias promoveram mudanças geoquímicas na atmosfera terrestre, favorecendo o surgimento de novos nichos ecológicos (DeLong, 1997; Oren, 2004).

Apesar dos procariotos estarem modificando a Terra há alguns bilhões de anos, o mundo microbiológico só começou a ser estudado no século XVII, com as observações de Antony van Leeuwenhoek. Entretanto, a Microbiologia só se desenvolveu a partir de dois eventos essenciais: Robert Koch, com o advento do cultivo de bactérias em meios de cultura, e Luis Pasteur, entre outros estudos, com o primeiro método de esterilização de substratos. Assim, começou-se a estudar bactérias de importância clínica e alimentar (Rossello-Mora & Amann, 2001). Estes espécimes foram, relativamente, fáceis de ser cultivados, pois os requerimentos necessários para o cultivo eram conhecidos. No entanto, atualmente, sabe-se que somente uma pequena fração de bactérias é cultivável por meio de protocolos padrões de cultivo (Connon & Giovannoni, 2002). Além disso, o curto período de tempo de estudo desses organismos, em relação aos eucariotos, e a dificuldade de diferenciá-los morfologicamente, os coloca em desvantagem numérica em relação ao que se conhece hoje em termos de diversidade das espécies. Atualmente, são conhecidas 10.504 espécies diferentes de procariotos (www.bacterio.cict.fr/) contra um milhão de plantas e animais (Staley & Gosink, 1999; Oren, 2004). Deve-se observar que com o desenvolvimento de novas técnicas moleculares e de cultivo este número de espécies pode aumentar imensamente. Entretanto, podem ocorrer grandes sinonímias nos bancos de dados (Konstantinidis et al, 2006).

Com o advento de técnicas de biologia molecular, a grande diversidade anteriormente não acessada pode ser estudada. As pesquisas baseadas principalmente nos genes de rRNA 16S trouxeram uma importante ferramenta para identificar os procariotos,

sem a necessidade de cultivo. Isso foi possível devido a algumas características intrínsecas desse gene. Essa molécula se mantém conservada, sendo estratégica para a síntese protéica da célula. Ela também é homóloga entre os organismos e fornecem informações suficientes para comparações filogenéticas (Olsen et al, 1986; Pontes et al, 2007). Porém, a limitação do uso do gene de rRNA 16S em distinguir bactérias no nível de espécie tem sido documentada. Uma alta similaridade (>99%) de sequências do gene de rRNA 16S tem sido encontrada para alguns organismos intimamente relacionados para os quais, entretanto, não há resolução no nível de espécie, como, por exemplo, membros do grupo *Bacillus cereus* (Ash et al. 1991; Fox et. al, 1992), *Enterobacter*, *Ochrobactrum* (Hoffmann & Roggenkamp 2003; Lebuhn et al., 2006) e *Chromobacterium* (Kämpfer et al., 2009). No entanto, essa ferramenta continua sendo a melhor estratégia para acessar massivamente a diversidade microbiana.

Métodos moleculares para acessar diversidade bacteriana

Muitos métodos de investigação de diversidade microbiana se baseiam na análise dos genes de rRNA 16S. Estes genes têm sido usados em abordagens independentes e dependentes de cultivo para o estudo de diversidade de procariotos. A forma mais rápida de se acessar a composição das comunidades de procariotos por meio dos genes de rRNA 16S é usar a amplificação por PCR (*Polymerase Chain Reaction*) e iniciadores universais (Bull et al, 2000). Os amplicons gerados podem ser usados, entre outros, para se construir bibliotecas de clones de genes de rRNA 16S ou serem digeridos com endonucleases de restrição para obtenção de *fingerprinting* (ARDRA – *amplified ribosomal DNA restriction analysis*; Rondon et al, 1999). A construção de bibliotecas de clones dos genes de rRNA 16S permitiu a descoberta de novos taxa. Entretanto, a análise dos clones do gene de rRNA 16S obtidos por sequenciamento é dispendiosa financeiramente e demanda muito tempo. Por isto, alguns pesquisadores têm preferido os métodos de *fingerprinting* de DNA (como ARDRA) para obter uma representação qualitativa da abundância de diferentes bactérias nas amostras ambientais (Logue et al, 2008).

Outras técnicas baseadas em PCR e que também correspondem a assinaturas genéticas são ITS-, tDNA- e rep-PCR. O espaçador transcrito interno entre os rRNAs 16S-23S (ITS – *internal transcribed spacer*) é uma região polimórfica que aparentemente

sofre menos pressão seletiva e apresenta por isso maior variabilidade que o gene de rRNA 16S. Além disso, normalmente um ou dois genes de tRNA estão presentes nesta região. Assim, a ITS-PCR gera *fingerprinting* que pode ser empregado para identificação bacteriana de amostras ambientais incluindo variações intra-específicas (Daffonchio et al., 1998; Louws et al., 1999; Bonizzi et al., 2006).

Um método de tipagem adicional é o *fingerprinting* de tDNA. A PCR dos genes de tDNA (tDNA-PCR) é baseada em suas sequências altamente conservadas (Welsh & McClelland, 1991). A técnica consiste na amplificação dos espaçadores entre dois genes de tRNA, usando os iniciadores consensos que se anelam nas extremidades desses genes. Os amplicons obtidos apresentam diferentes tamanhos, gerando perfis espécie-específicos (Daffonchio et al., 1998; Louws et al., 1999; Catry et al., 2004).

Perfis de sequências de elementos repetitivos genômicos (rep-PCR; *repetitive element sequence*-based PCR) têm sido usados em diversos organismos para inferir as relações filogenéticas entre isolados e comparação da diversidade genômica (Rademaker et al., 2000). As seqüências repetitivas, tipo BOX, que são dispersas pelo genoma, que é uma variedade de rep-PCR, têm sido frequentemente usadas para tipagens genômicas, principalmente por sua simplicidade e eficiência. Essa técnica tem melhor resolução para discriminar entre linhagens ou isolados bacterianos ambientais que outros métodos como, por exemplo, análises do gene de rRNA 16S (Brusetti et al., 2008).

Apesar da expansão das abordagens moleculares independentes de cultivo, a obtenção de culturas de microrganismos ainda é muito importante e permanece como um grande desafio para o estudo das comunidades microbianas. Deve-se salientar que o cultivo é necessário para que se possa conhecer a atividade metabólica e fisiológica dos microrganismos e sua participação em importantes processos ambientais. Biolog Ecoplate é um dos métodos mais usados para se medir o perfil fisiológico de uma comunidade microbiana . Este sistema fornece a caracterização da comunidade, acessando o potencial cultivável da mesma por meio da utilização de 31 fontes de carbono ecologicamente relevantes. Assim, os pesquisadores podem conhecer aspectos fisiológicos da comunidade como um todo (Garland, 1996; Foley et al, 2008).

Conceito de espécie

Intuitivamente, sabe-se que é imenso o mundo dos procariotos. Portanto, deve-se delimitar o conceito operacional de espécie, para quantificar e explorar esta diversidade, pois ela é a unidade básica para estudos de Biogeografia, Ecologia e Evolução (Sites Jr. & Marshall, 2004). Entretanto, é difícil de estabelecer um sistema de classificação adequado para procariotos, pois eles são morfologicamente indistintos. Além disso, os raros registros fósseis não são filogeneticamente informativos (Rossello-Mora & Amann, 2001). Ao contrário, dos eucariotos, não existe consenso para um conceito de espécie em procariotos. Devido à plasticidade apresentada pelos microrganismos, talvez um consenso do conceito de espécie realmente não se aplique a eles. Assim, os conceitos se tornam arbitrários e tendem a ser usados de acordo com a necessidade em uma determinada análise (Torsvik et al, 2002; Gevers et al, 2005). Portanto, como calcular a diversidade real se não existe um conceito universal de espécie bacteriana?

Um dos conceitos mais bem aceitos de espécie bacteriana é o filo-fenético: '*a espécie é uma categoria que circunscreve, preferencialmente, um grupo genotipicamente coerente de indivíduos (isolados ou linhagens) que compartilham um alto grau de similaridade em diversas características*' (Rossello-Mora & Amann, 2001). No entanto, esse conceito exige o conhecimento fenotípico e genotípico dos microrganismos, o quê é muito complicado atualmente, pois para acessar a grande diversidade microbiana de forma rápida é necessário usar métodos independentes de cultivo, o que dificulta o acesso às informações fenotípicas. Em conseqüência, é aceito, na comunidade científica, o uso do gene de rRNA 16S para a identificação de espécies já conhecidas (Glockner et al, 2000; van der Gast et al, 2005; Freitas et al, 2008). Portanto, essa importante ferramenta tem sido usada para quantificar a diversidade microbiana (Rappe & Giovannoni, 2003), mas ainda falta conhecer a real diversidade funcional, uma vez que a informação gerada pelas seqüências do gene de rRNA 16S só pode ser associada a um determinado táxon e mesmo assim com alguma dificuldade.

Biogeografia Microbiana

As atuais possibilidades de capturar a maioria dos microrganismos não cultiváveis abrem mais um campo de estudo para se entender a biodiversidade e a biogeografia, anteriormente exclusiva de macrorganismos. Essa ciência estuda a distribuição espacial e

temporal das espécies de seres vivos (Ward & Bora, 2006). Seus principais objetivos são registrar e compreender onde os organismos vivem, e os fatores que controlam sua abundância, ao longo de uma escala temporal. O principal ponto de partida para um estudo de biogeografia é determinar os padrões de distribuição de espécies, pois tais padrões podem ser analisados separadamente dos processos que os estabelecem (Myers & Giller, 1988a; Brown & Lomolino, 2006). Porém, este procedimento apresenta dificuldade para os botânicos, zoólogos, e, especialmente, para os microbiologistas.

A Biogeografia, ciência multidisciplinar, pode ser dividida em várias áreas, sendo as duas principais, Biogeografia Histórica e Ecológica. As duas tentam responder questões similares em escalas temporais diferentes. A Biogeografia Histórica se preocupa em reconstruir a origem, dispersão e extinção de táxons, levando em consideração eventos geológicos, como deriva continental e glaciação, para explicar os padrões de distribuição de espécies encontrados nos dias atuais. Além disso, possui duas importantes linhas de pesquisa: dispersão e vicariância. A dispersão considera como premissa o centro de origem do qual as espécies se dispersaram através de barreiras pré-existentes. Por outro lado, a vicariância leva em consideração eventos geográficos, constituindo os padrões de distribuição de espécies. Isso pode ser confirmado pela observação de táxons não relacionados, mas que apresentam os mesmos padrões de distribuição (Rosen, 1988). Por outro lado, a Biogeografia Ecológica estuda, principalmente, os táxons ainda não extintos. Para isso, procura entender a distribuição de espécies por meio de interações entre organismos e o meio ambiente. Essa ciência estuda as interações e padrões em uma escala de tempo atual ou ainda em um passado próximo. Além disso, também cabe a ela compreender padrões – diversos tipos de gradientes geográficos e temporais – e processos – extinção, especiação e dispersão – que moldam a distribuição de espécies (Brown, 1988; Myers & Giller, 1988b). A Biogeografia Ecológica contém uma área peculiar – Biogeografia Microbiana.

A Biogeografia Microbiana, ao contrário da Biogeografia que surgiu no século XIX, teve sua origem em 1934, com o cientista holandês Bass-Becking. Ele formulou uma máxima para descrever a distribuição de espécies bacterianas: “Tudo está em toda parte; o ambiente seleciona” (Bass-Becking, 1934 apud Martiny et al., 2006). No entanto, essa nova área do conhecimento ficou latente até os dias atuais, pois o acesso aos

microrganismos era difícil, sendo retomada com o advento da Biologia Molecular. Apesar do pouco conhecimento na área de Biogeografia Microbiana seu estudo possibilita o entendimento de processos importantes, uma vez que ao se conhecer a distribuição geográfica de uma determinada espécie é possível identificar seu papel ecológico e/ou se ela está ameaçada ou não de extinção (Staley & Gosink, 1999). Esse conhecimento não só favorece áreas como o controle epidemiológico de patógenos humanos, de animais e plantas, mas também a biorremediação (Ramette & Tiedje, 2007).

A hipótese de Bass-Becking considera que os microrganismos de vida livre seriam cosmopolitas, levando à conclusão de que habitats similares apresentariam a mesma distribuição de espécies, sendo que isso ocorreria por causa de uma fácil dispersão das mesmas entre as regiões geográficas da Terra. Durante muito tempo, pensou-se que os microrganismos eram ubíquos, ou seja, possuíssem uma distribuição cosmopolita. De certa forma, essa afirmação é verdadeira, uma vez que em biogeografia a escala de análise é muito importante. No nível de domínio, os procariotos possuem uma distribuição global. No entanto, no nível de gênero, alguns podem ser considerados cosmopolitas, como o *Bacillus*, mas o gênero *Synechococcus*, por exemplo, é exclusivo de habitats extremos (Papke et al, 2003; Ramette & Tiedje, 2007). Ainda, de acordo com a hipótese de Bass-Becking, todos os procariotos de vida livre deveriam ser capazes de dispersar a longas distâncias e chegar viáveis ao destino, qualquer que seja ele, e ainda conseguir colonizar o novo habitat. Essas suposições tornam-se verdadeiras, principalmente, para espécies capazes de gerar estruturas de proteção com as quais podem sobreviver com o metabolismo no nível basal. Essas estruturas (esporos e cistos) favorecem a dispersão, mas não necessariamente garantem a permanência do organismo no novo sítio (Staley & Gosink, 1999).

Os padrões de distribuição únicos de espécies na Terra são característicos dos seres vivos. Porém, esses padrões não se originam aleatoriamente, sendo moldados por processos ecológicos e evolutivos. Os principais processos envolvidos são: especiação, dispersão, extinção e interações ecológicas.

Especiação. Este é um processo muito importante para a Biogeografia, pois fornece novas espécies que podem ser dispersas para outros habitats. Assim, este processo gera e mantém a diversidade, promovendo a evolução de padrões

biogeográficos ao longo do tempo e espaço (Barton, 1988; Ramette & Tieje, 2007). Em procariotos, esse processo ocorre mais rápido do que em outros organismos, pois eles apresentam grande variabilidade genética que favorece a sua enorme adaptação ambiental, apesar de possuírem divisão basicamente clonal. A plasticidade do genoma dos procariotos é obtida por meio de ganho e perda de genes. Os mecanismos responsáveis são: mutações espontâneas e recombinação, incluindo a transferência gênica lateral – aquisição de fagos, plasmídios, transposons e integron-cassetes (Bailly et al, 2007).

Tendo como cenário essa variabilidade, as populações de uma mesma espécie podem evoluir e dar origem a outra espécie. Para isso, basta que as populações tornem-se suficientemente diferentes. Como o conceito de espécie em procariotos ainda não está unificado, a população que está se diferenciando pode ser considerada como pertencente a um novo ecótipo. O ecótipo é um variante da população da espécie original, que ainda não se diferenciou o suficiente para ser considerada como uma nova espécie. As espécies procarióticas podem ser consideradas como um conjunto clinal de ecótipos, pois, estes apresentam adaptações distintas ao mesmo habitat, sendo essa característica chamada tolerância ecológica (Myers & Giller, 1988b; Polz et al, 2006).

Para que haja diferenciação suficiente para ocorrer especiação não é necessária somente a existência de barreiras físicas impedindo a migração das populações, que é o fator responsável para a especiação alopátrica. Como os procariotos apresentam pequeno tamanho e grandes densidades populacionais a dispersão é facilitada, impedindo o isolamento. Assim, a especiação alopátrica não deve ser o principal mecanismo de especiação nesses organismos (Ramette & Tieje, 2007). Por outro lado, para microrganismos extremófilos, como arqueas hipertermófilas, a barreira se torna um processo fundamental para a divergência entre as populações (Whitaker et al, 2003). Outro exemplo de especiação por alopatria é a relação hospedeiro e simbionte, seja ele parasita ou comensal. Os simbiontes evoluem em paralelo com seus hospedeiros. Assim, populações de simbiontes tendem a divergir umas das outras (Papke & Ward, 2004). Em contrapartida, a especiação simpátrica parece ser o mecanismo mais provável para gerar divergência entre populações que coexistem, e isso é facilitado pela gradação adaptativa dos procariotos (Brown & Lomolino, 2006).

Dispersão. Este processo é definido pelo transporte, ativo ou passivo, de organismos ou populações de seu ponto de origem para uma nova área. No entanto, para esse movimento ser bem sucedido a nova população deve ser capaz de percorrer grandes percursos, sobreviver a situações pouco favoráveis e, por fim, colonizar o novo sítio. No processo de dispersão ativa os organismos devem ser capazes de deslocar por conta própria de uma região para outra. Os procariotos de vida livre apresentam flagelos, com os quais podem se deslocar alguns centímetros. No entanto, um deslocamento biogeograficamente significativo, por longas distâncias e através de barreiras geográficas, só terá êxito se mecanismos passivos de transporte estiverem envolvidos. Os mecanismos passivos são estocásticos, dependendo exclusivamente de vetores e de fatores climáticos (Ramette & Tieje, 2007). A dispersão é um processo estressante e nem todos os procariotos que iniciam a viagem conseguem chegar a seu destino, pois existem barreiras à sua dispersão. Elas podem ser físicas, devido à geografia, fisiológicas, se o microrganismo não está adaptado ao pH, à temperatura, à salinidade e ao suprimento de nutrientes, e ecológicas, devido a interações negativas com os organismos existentes no local de chegada. A não transposição dessas barreiras pode ser crucial para a existência de populações endêmicas de procariotos (Brown & Lomolino, 2006; Martiny et al, 2006).

Extinção. É um processo inevitável no qual espécies deixam de existir. Isso ocorre porque o ambiente está em constante mudança e a espécie não consegue se adaptar tão rápido quanto às alterações ambientais. Com isso, esse processo leva à diminuição da diversidade local. Porém, a extinção de uma espécie não significa que um determinado habitat ficará vazio. Aquela espécie pode ser substituída por outras, mantendo a diversidade local na mesma proporção ou aumentando-a (Brown & Lomolino, 2006). Ramette & Tieje (2007) sugerem que extinção em procariotos seja um evento raro, pois essas populações apresentam as principais características de táxons resistentes à extinção. Essas características são: tamanho individual diminuto, tempo de geração curto, alta densidade populacional, capacidade de dispersão e colonização. No entanto, pouco se sabe sobre esse processo em procariotos, pois o estudo ecológico de comunidades está no início. Extinção é fundamental na formação de padrões biogeográficos de diversidade, pois filtra populações ou espécies que deixaram de evoluir, permitindo que outras, mais adaptadas, continuem a existir. Geralmente, as espécies endêmicas sofrem mais com isso,

pois não apresentam amplitude geográfica nem capacidade de dispersão (Marshall, 1988; Martiny et al, 2006).

Interações ecológicas. Com o advento do isolamento microbiano, no século XIX, por Robert Koch, é possível estudar os microrganismos em um ambiente controlado, com poucas variáveis, o que não acontece quando eles estão em sua comunidade ecológica original, interagindo com outros organismos vivos (Shapiro & Dworkin, 1997; Rudi et al., 2007; Little et al., 2008). Em um ecossistema, indivíduos de uma espécie não existem isoladamente, mas podem participar de interações simbióticas tão dependentes que os membros do consórcio não podem ser isolados como culturas puras, pois eles não sobrevivem separadamente. Essas interações ecológicas influenciam diretamente a estrutura da comunidade – composição da comunidade leva em consideração as espécies presentes em um habitat e o número de indivíduos de cada população. As interações podem ser intra ou interespecífica. As interações interespecíficas são de três tipos: protagonistas (mutualismo), benignas (comensalismo) e antagonistas (competição, predação e parasitismo). O mutualismo é uma interação, na qual ambos os organismos envolvidos se beneficiam. O comensalismo é similar ao mutualismo, mas apenas um dos organismos se beneficia, enquanto o outro não perde nem ganha nada com a associação (Little et al, 2008). Na competição, na predação e no parasitismo, por outro lado, uma das partes sempre está em desvantagem. A competição pode ser intra ou interespecífica. A competição é a disputa entre dois organismos pelos mesmos recursos ou espaço. A predação é o consumo de um organismo por outro. No entanto, essa interação, é aparentemente rara entre os procariotos, mas é a sua principal causa de morte por micro-eucariotos. A última das interações antagonistas, o parasitismo, ocorre quando um organismo explora parte de outro indivíduo, sem levá-lo à morte imediatamente. Geralmente, a proporção dessas interações que ocorrem em uma comunidade microbiana é responsável pela sua função no ecossistema (Schoener, 1988; Little et al, 2008).

Esses quatro processos moldam a distribuição de espécies ao longo do Globo Terrestre, sendo que o balanço entre especiação e extinção é fundamental para o estabelecimento de diversidade. Por outro lado, a estrutura da comunidade é determinada, basicamente, pela proporção entre competidores e predadores, sendo que os predadores podem fazer o controle de populações de grandes densidades. Em procariotos, no entanto,

esses processos ainda necessitam de esclarecimentos. O aumento dos estudos na área de Biogeografia Microbiana pode levar à expansão desses conhecimentos.

Ecossistemas Aquáticos de Água Doce

As variáveis ambientais e o tipo de ambiente influenciam a composição das comunidades de microrganismos, mas ainda não está claro como ocorre essa influência. Diferentes ambientes, como os aquáticos e terrestres, suportam quantidades distintas de táxons procarióticos, sendo que o aquático apresenta menor número que o solo (Horner-Devine et al, 2004). Assim, conhecer as características ambientais onde habitam as comunidades em estudo pode ajudar a elucidar a estrutura das comunidades de microrganismos.

A Terra é composta de dois tipos de águas continentais, sistemas lacustres (correspondendo a 3% da superfície terrestre) e fluviais. A maioria dos lagos naturais e profundos é encontrada no hemisfério norte. Essa predominância de lagos no hemisfério norte é devido aos períodos de glaciação que ocorreram no Pleistoceno. No Brasil, ao contrário da situação encontrada no hemisfério norte, ocorre a predominância de rios ao invés de lagos. No Brasil, as águas continentais são formadas principalmente por bacias hidrográficas e, assim, a atividade geológica do sistema hídrico é responsável pela formação da maioria dos lagos brasileiros. Portanto, estes lagos são pequenos e pouco profundos, não ultrapassando 20 m de profundidade (Esteves, 1998).

Em Minas Gerais, as bacias do Rio São Francisco e do Rio Doce abrigam, respectivamente, duas importantes áreas de conservação: o Parque Nacional da Serra do Cipó (PNSC) e o Parque Estadual do Rio Doce. O PNSC (19-20ºS; 43-44ºW; FIG. 1) situa-se no centro de Minas Gerais, possuindo o papel de divisor de águas dos rios São Francisco e Doce. Apresentando 33.800 hectares, abriga uma vegetação composta de Cerrado nas altitudes inferiores a 1.000 m, campos rupestres nas partes mais altas e mata ciliar nos vales mais úmidos e ao longo dos rios (Galdean et al., 2000). A flora e a fauna do PNSC são de extraordinária diversidade, apresentando também alto grau de endemismo. Além disso, o PNSC ainda acolhe algumas espécies ameaçadas de extinção como o lobo-guará, tamanduá-bandeira, veado campeiro e onça parda, entre outros

(http://www.portalsaofrancisco.com.br/alfa/meio-ambiente-parques-nacionais-brasileiros/parque-nacional-da-serra-do-cipo.php).

FIG. 1 – Mapa do Parque Nacional da Serra do Cipó.
FONTE: Galdean et al., 2000 (modificado).

O Parque Estadual do Rio Doce (PERD), que está localizado no trecho médio do Vale do Rio Doce, MG, faz limite com os rios Doce, ao leste e Piracicaba, ao norte (FIG. 2). O PERD constitui o maior fragmento de Mata Atlântica de Minas Gerais, sendo o seu sistema hídrico formado por cerca de 50 lagoas, correspondentes a 6% de sua área (Andrade et al, 1997). O clima da região é o tropical úmido, no qual os meses de chuva se estendem de outubro a março, sendo de 1500 mm a precipitação anual média de chuva nesses meses. Uma precipitação menor (1000 mm) ocorre no mês de julho, estação de seca (Tundisi, 1997a). O PERD como o PNSC também contém muitos organismos como a capivara, o macaco-prego e a anta, mas também, mas também abriga animais ameaçados de extinção, como a onça- pintada e o muriqui, o maior primata das Americas.

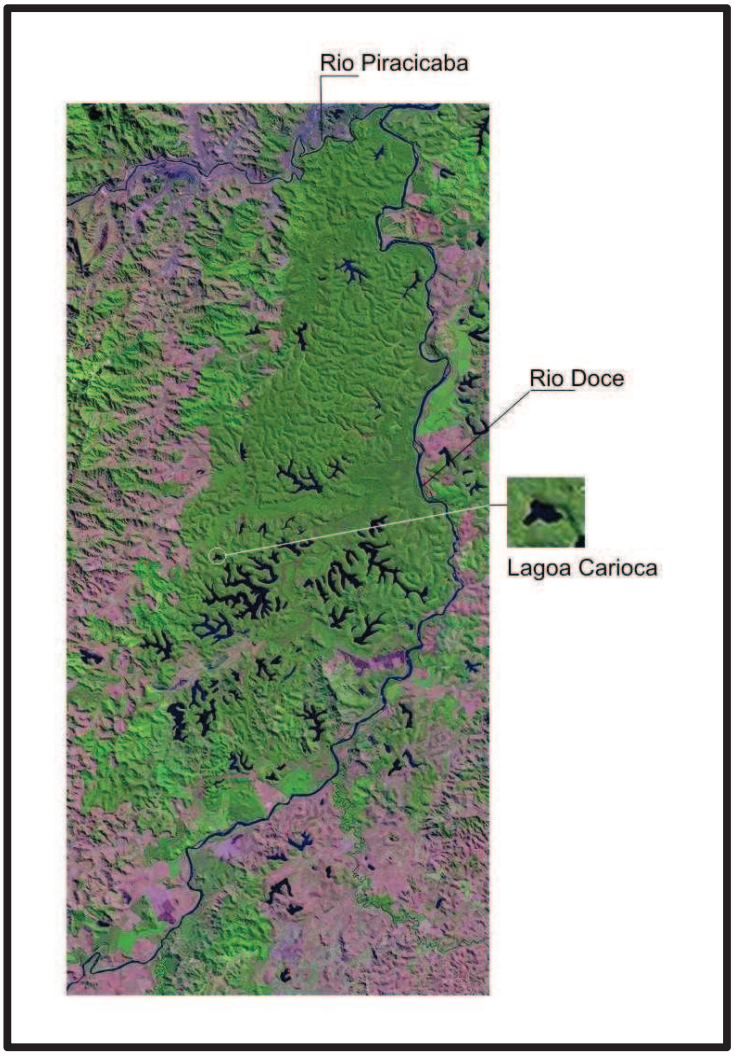

FIG. 2 – Imagem do Parque Estadual do Rio Doce, pelo satélite Landsat (Miranda & Coutinho, 2004).

A lagoa Carioca é relativamente pequena, apresentando apenas 14,3 ha, sendo considerada rasa (11,8 m de profundidade) e moderadamente eutrofizada. Além disso, não possui afluentes, nem efluentes. Por ser pequena e pouco profunda, apresenta boa circulação de nutrientes, sendo classificada como monomíticos quentes (Barbosa, 1981; Henry et al, 1997). Essa lagoa teve sua origem, provavelmente, pela atividade dos rios Doce e Piracicaba no Pleitoceno, sendo considerada como um lagoa de barragem (Esteves, 1998).

Em um ecossistema lacustre a penetração de luz na coluna exerce um papel fundamental, pois a base da cadeia alimentar se inicia pela fotossíntese. A incidência de

luz no verão é muito mais intensa que no inverno. Curiosamente, a produção primária dessa lagoa no inverno é maior que no verão. Isso ocorre porque, no verão, a luz que incide no espelho da água tem baixa reflexão, fazendo com que a alta energia que acompanha a penetração da luz iniba a fotossíntese, oxidando, por exemplo, o fotossistema presente no fitoplâncton (Esteves, 1998). Por outro lado, no inverno a intensidade da luz que penetra no corpo de água é de menor intensidade, fazendo com que sua energia seja quase que totalmente aproveitada pelas algas microscópicas, associando-se a isso uma melhor distribuição de nutrientes, e favorecendo assim um aumento do número de organismos em todos os níveis da cadeia alimentar (Barbosa, 1981; Esteves, 1998).

Outro papel importante da luz em um corpo de água é a estratificação da coluna, no período chuvoso. A estratificação corresponde a camadas de águas, com densidades diferentes, na coluna. Isso ocorre porque variáveis climatológicas, ventos, precipitação, radiação e temperatura, tornam a coluna de água mais estável termicamente. Esse processo está associado ao aquecimento mais rápido da superfície do lago, com baixa força do vento, que impede a circulação de água nas regiões mais profundas do lago. Isso gera como conseqüência a distribuição irregular de nutrientes e compartimentalização de organismos na coluna de água (Barbosa & Tundisi, 1989; Tundisi, 1997b; Marques et al, 1999).

As comunidades dos seres vivos de água doce são compostas pelos seguintes grupos de organismos: bento, nécton e plâncton. Os bentos, residentes dos sedimentos dos lagos, são também o tipo de comunidade mais freqüente em rios. Os representantes desta comunidade variam de produtores primários a consumidores. Os néctons – os peixes são os representantes mais característicos– e os plânctons são habitantes das regiões pelágicas dos lagos. Essas duas comunidades diferem, principalmente, pela capacidade que os néctons possuem de nadar livremente, , enquanto os plânctons são levados horizontalmente pelas correntes aquáticas podendo, entretanto,realizarem migrações verticais. Os plânctons ocorrem com memor freqüência em rios, principalmente, em regiões com menos correnteza (Esteves, 1998; Lampert & Sommer, 2007).

Os plânctons podem ser divididos em: fitoplânctons, zooplânctons e bacterioplâncton. A comunidade fitoplanctônica participa do nível trófico como produtores primários e é composta de algas e de Cyanobacteria. O zooplâncton é composto por protozoários, micro-crustáceos, larvas de insetos e de peixes. Esta comunidade participa da cadeia alimentar como consumidores (herbívoros, zooplanctívoros e bacterívoros). Os bacterioplânctons são os organismos que estão presentes na maioria dos níveis tróficos. Podem ser encontrados como produtores primários fotolitoautotróficos, decompositores, parasitas e outros (Lampert & Sommer, 2007). Estudos de inferência filogenética baseados em sequências do gene de rRNA 16S de ecossistema de água doce revelaram que os principais filos que caracterizam a comunidade bacterioplantônica são Proteobacteria (Alfaproteobacteria, Betaproteobacteria e Gamaproteobacteria – sendo bastante abundantes em sistemas de água doce – e), Actinobacteria, Bacteroidetes (Grupo CFB - Cytophaga-Flavobacteria-Bacteroidetes) e Verrucomicrobia. Outros filos estão presentes, mas não são dominantes, como Chloroflexi (*Green nonsulfur*) e Candidatus (OP e WS) e outros taxa que podem ser detectados em ecossistemas de água doce, como Planctomycetes e Deltaproteobacteria. (FIG. 3; Zwart et al, 2002; Logue et al 2008).

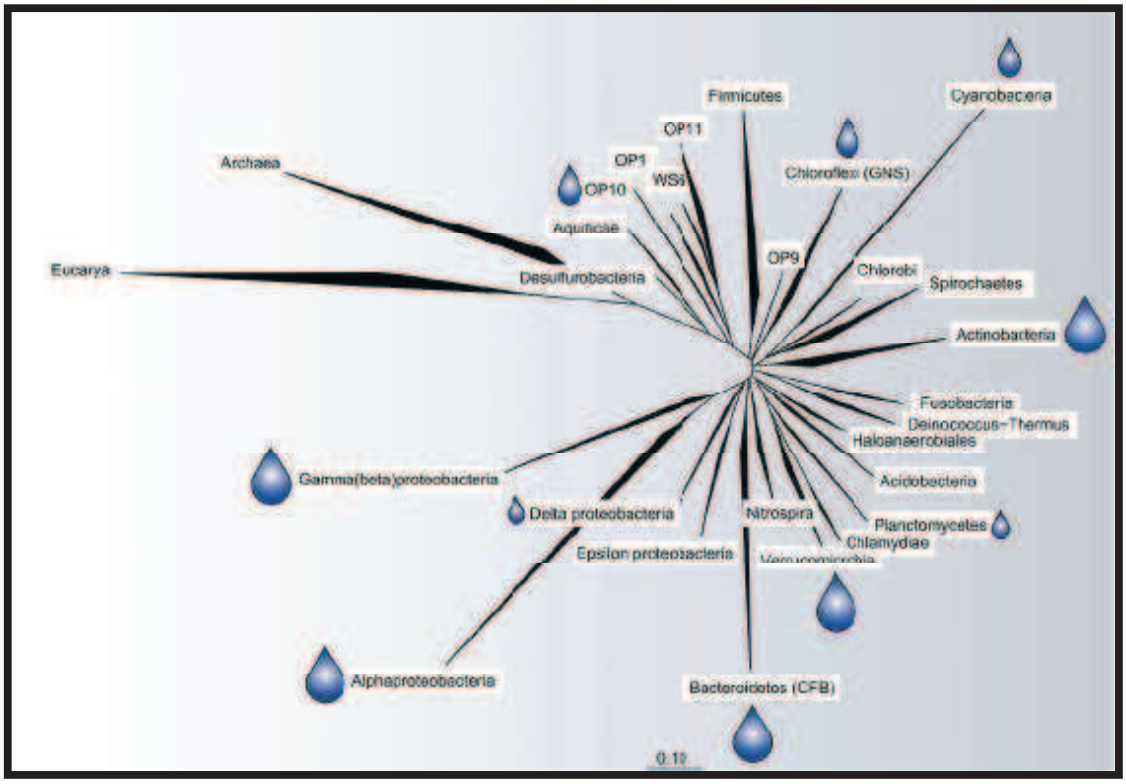

FIG. 3 – Inferência filogenética baseada em sequências do gene de rRNA 16S, mostrando os principais táxons bacterianos presentes em ecossistemas de água. Gotas grandes indicam grupos típicos e freqüentemente dominantes de bactérias de água doce; gotas de tamanhos intermediários representam grupos bacterianos não freqüentemente dominantes e gotas pequenas indicam outros grupos que podem ser observados em ecossistemas de água doce. Abreviações: CFB, grupo cytophaga-flavobacteria-bacteroidetes; GNS, green nonsulfur; OP e WS, divisões filogenéticas candidatas. (Logue et al 2008).

Gênero Chromobacterium

O gênero *Chromobacterium* consiste em seis espécies: *C. violaceum* (Bergonzini, 1881), *C. subtsugae* (Martin et al. 2007a), *C. aquaticum* (Young et al. 2008), *C. haemolyticum* (Han et al. 2008), *C. pseudoviolaceum* e *C. piscinae* (Kämpfer et al. 2009, FIG. 4). Este gênero pertence à família Neisseriaceae e à classe Betaproteobacteria. Membros deste gênero são bastonetes Gram-negativos, móveis, a temperatura de crescimento varia de 25 a 32ºC, com tempo de crescimento de 24h a 48h (Tabela 1). Com exceção de *C. haemolyticum* que foi recuperada de uma secreção clínica, as outras cinco espécies foram isoladas de ecossistemas naturais. No Brasil, a ocorrência desse gênero tem sido documentada no Rio Negro, Amazônia (Dall'Agnol et al, 2008), e também em

rios e solo do Parque Nacional da Serra do Cipó, MG (Lima-Bittencourt, 2007, Lima-Bittencourt, 2011) e no Parque Estadual do Rio Doce, MG (Lima-Bittencourt et al, 2011 *unpublished*).

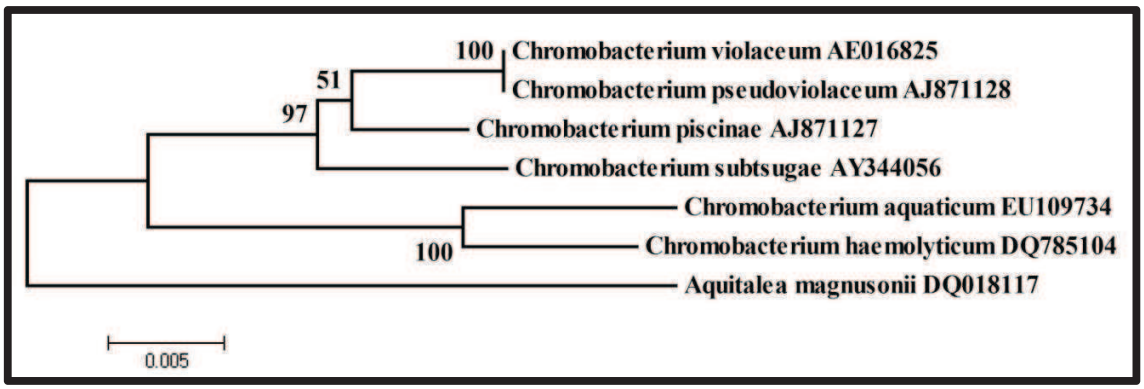

FIG. 4 – Análise filogenética baseada em sequências de rRNA 16S depositadas no Genbank com o método de *minimum evolution* (1000 repetições de *Bootstrap*).

Tabela 1 – Características fisiológicas de todas as linhagens de *Chromobacterium*.

Características	***C. violaceum***	***C. subtsugae***	***C. aquaticum***	***C. haemolyticum***	***C. pseudoviolaceum***	***C. piscinae***
Cor da colônia	violeta	violeta	não violeta	não violeta	violeta	violeta
Fonte de isolamento	água/solo	solo	água de fonte	escarro	amostras ambientais	amostras ambientais
Crescimento:						
4ºC	-	+	ND	ND	ND	ND
25ºC	+	-	ND	ND	ND	ND
2% NaCl	-	+	ND	ND	ND	ND
3% NaCl	-	-	+	ND	ND	ND
Agar sangue	sem hemólise	ND	ND	β-hemólise	ND	ND
Utilização como única fonte de carbono:						
Sacarose	+	-	ND	ND	ND	ND
Citrato	+	-	ND	+	ND	ND
Acetato	-	+	ND	ND	ND	ND
Redução de nitrato a nitrito	-	+	ND	ND	ND	ND
Hidrólise de esculina	+	-	+	ND	ND	ND
Assimilação de:						
L-manose	-	+	ND	-	ND	ND
Ácido capríco	-	+	ND	ND	ND	ND
D-manitol	-	-	-	+	-	-
i-inositol	-	-	-	+	-	-
Sorbitol	-	-	-	+	-	-
Putrecina	-	-	+	+	-	-
Cis-aconitato	-	+	+	-	+	-
Propionato	-	+	+	-	+	-
DL-3-hidroxibutirato	+	-	-	-	+	-
Oxiglutarato	-	-	+	+	-	-
L-Aspartato	-	+	+	+	+	+
L-leucina	-	-	+	-	-	-
L-prolina	-	+	+	+	+	+
Catalase	+	+	-	+ (fraca)	ND	ND
Oxidase	-	ND	ND	+	ND	ND
Fermentação de ribose	+	-	-	ND	ND	ND
Fermentação de glicose	+	ND	ND	+ (fraca)	ND	ND
Produção de indol	-	-	-	-	ND	ND
Gelatinase	+	-	+	ND	ND	ND
Formação de ácido:						
D-manose	+	-	-	-	+	+

Chromobacterium violaceum foi descoberta em 1880 por Bergonzini (Bergonzini, 1881). Ela é a espécie mais antiga do gênero e melhor estudada, permanecendo por um longo tempo como a única espécie do gênero, sendo considerada a espécie tipo, *C. violaceum* ATCC 12472, sendo isolada em sistemas de água doce da Malásia. *C. violaceum* é constituída por bastonetes com espessura variando de 0,6-0,9 μm e comprimento de 1,5-3,5 μm. Possuem mobilidade, apresentando um único flagelo polar e flagelos laterais ou subpolares mais longos. Produzem colônias, em meio sólido, cremosas e violetas, enquanto ocorre formação de um halo violeta na superfície do caldo e, em alguns casos, podem colorir o meio totalmente. São bactérias anaeróbicas facultativas. (Sivendra, et al., 1975; Holt & Krieg, 1984). *C. violaceum* também é naturalmente resistente às penicilinas, possuindo em seu genoma o gene de resistência *ampC* (Fantinatti-Garboggini et al., 2004). Essa espécie ocorre em água doce e solo de vários ecossistemas de regiões tropicais e subtropicais. É considerada como um microrganismo saprófito, não patogênico para humanos, podendo, no entanto, tornar-se um patógeno oportunista (Durán & Menck, 2001). As infecções por *C. violaceum* são geralmente associadas com pessoas imunocomprometidas, entretanto existem relatos de infecção, no Brasil, que ocorreu em pessoas saudáveis (Martinez et al., 2000; Dias et al, 2005). *C. violaceum* pode infectar outros mamíferos, como porcos, primatas em geral e gado (Zins et al., 2001).

Ela desperta grande interesse biotecnológico, devido ao seu amplo potencial para uso industrial, farmacêutico e ecológico (Carepo et al., 2004). Dentre os vários produtos biológicos interessantes destacam-se: a violaceína, polihidroxialcanoatos, cianeto de hidrogênio, antibióticos e quitinase. Essas características peculiares de *C. violaceum* foram algumas das razões para que ela fosse selecionada para ter seu genoma seqüenciado pela Rede Nacional de Seqüenciamento do Projeto Genoma Brasileiro, patrocinado pelo CNPq. O seu genoma é formado por um cromossomo circular com 4.751.080 pares de bases, com 89% de regiões codificadoras. Destas ORFs, aproximadamente 61% codificam proteínas conhecidas. As outras 39% são proteínas hipotéticas, que são divididas em proteínas conservadas e exclusivas de *C. violaceum*. O genoma apresenta 98 genes de tRNA, que são capazes de transcrever transportadores para os 20 aminoácidos. Os genes de rRNA estão agrupados em oito operons, com seqüências idênticas (BNGPC, 2003).

A segunda espécie a ser descrita foi *C. subtsugae*, em 2007, sendo isolada do solo de Maryland, EUA. Essa espécie apresenta colônias pigmentadas de violeta com

crescimento ocorrendo de 24h a 48h, sendo constituída por bastonetes de 0,7 x 2,4 µm. O crescimento ideal ocorre nas temperaturas de 25ºC a 28ºC (Martin et al, 2007a). Essa espécie pode ser usada como controle biológico do besouro da batata do Colorado, entre outros insetos (Martin et al, 2004; Martin et al, 2007b).

Chromobacterium aquaticum foi isolada de amostras de água da fonte da montanha de Yang-Ming, condado de Taipei, Taiwan, é constituída por bastonetes com espessura variando de 0,3-0,5 µm e comprimento de 1,5-2 µm. Possuem mobilidade, apresentando um único flagelo polar e são aeróbias. Produzem colônias, em meio sólido, cremosas e não pigmentadas de violeta. O crescimento ótimo ocorre em 48h a 32ºC (Young et al. 2008). *C. haemolyticum* também não apresenta colônias pigmentadas, sua característica mais notável é a produção de β-hemolisinas em agar sangue de carneiro. Além disso, em meio líquido, lisa eritrócitos humanos e seu crescimento ótimo ocorre a 37ºC por 24h. Essa espécie tem uma grande importância clínica, tendo sido recuperada em ambiente hospitalar. Essa espécie apresenta a menos similaridade em relação ao gene de rRNA 16S (96,1%) quando comparada com a espécie tipo (*C. violaceum*; Han et al, 2008).

Chromobacterium pseudoviolaceum é a espécie que apresenta maior similaridade em relação ao gene de rRNA 16S (99,8%) quando comparada com a espécie tipo (*C. violaceum*). As suas células são constituídas por bastonetes móveis, com espessura variando de 0,3-0,5 µm e comprimento de 1,5-2 µm. Produzem colônias cremosas e violetas. Além disso, são aeróbias, apresentando crescimento máximo a 32ºC quando incubadas por 48h. *C. piscinae* é a segunda espécie filogeneticamente mais próxima a *C. violaceum*, além disso, apresenta características similares à *C. pseudoviolaceum* (Kämpfer et al. 2009).

As espécies *C. aquaticum*, *C. haemolitycum*, *C. pseudoviolaceum* e *C. piscinae* ainda não possuem estudos mostrando seus potenciais biotecnológicos, como se observa em relação à *C. violaceum* e *C. subtsugae*. No entanto, estudos de diversidade genética de populações naturais das espécies *C. piscinae* (Lima-Bittencourt et al, 2010) e *C. haemolyticum* (Lima-Bittencourt et al, 2011 *unpublished*) têm sido relatados, mas novos estudos devem ser realizados para melhor conhecimento desse gênero.

II) OBJETIVOS

Objetivo Geral

Estudar a biogeografia, diversidade e estrutura das comunidades bacterianas cultiváveis e sua associação com parâmetros abióticos, espacialmente e/ou temporalmente, do Lago Carioca –Parque Estadual do Rio Doce, PERD–, e de água do Córrego Indaiá e solo adjacente do Parque Nacional da Serra do Cipó (PNSC).

Objetivos Específicos

a) Caracterizar fatores físicos e químicos nos três pontos da coluna de água do Lago Carioca.
b) Isolar bactérias heterotróficas em gradientes eufótico (1%, 10% e 100% de luz) – vertical – e horizontal da zona limnética do Lago Carioca.
c) Isolar bactérias, a partir de colônias violetas, de água do Rio Indaiá e solo adjacente do Parque Nacional da Serra do Cipó.
d) Pesquisar a diversidade funcional, usando BIOLOG GN2 e BIOLOG Ecoplate, em *Chromobacterium* (PNSC) e comunidades microbianas (PERD), respectivamente.
e) Testar a susceptibilidade de isolados de *Chromobacterium* a diversos antimicrobianos.
f) Avaliar por Box-PCR a diversidade inter e intra-específica de *Chromobacterium*.
g) Amplificar, por PCR, o gene de rRNA 16S das bactérias isoladas do PERD e PNSC.
h) Pesquisar os perfis de diversidade, obtidos pela técnica molecular ARDRA, das comunidades bacterianas do Lago Carioca.
i) Analisar as sequências dos genes de rRNA 16S e identificar taxonomicamente os isolados bacterianos do PERD e PNSC.
j) Avaliar e analisar a associação dos fatores abióticos, fontes de carbono e dados de sequências de rDNA 16S nos padrões biogeográficos das comunidades bacterianas do PERD, usando os seguintes métodos estatísticos: PCA, CCA e Mantel

III) CAPÍTULOS

CAPÍTULO 1 – Comparative biogeography of *Chromobacterium* from the neotropics

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ORIGINAL PAPER

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 microscale (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),

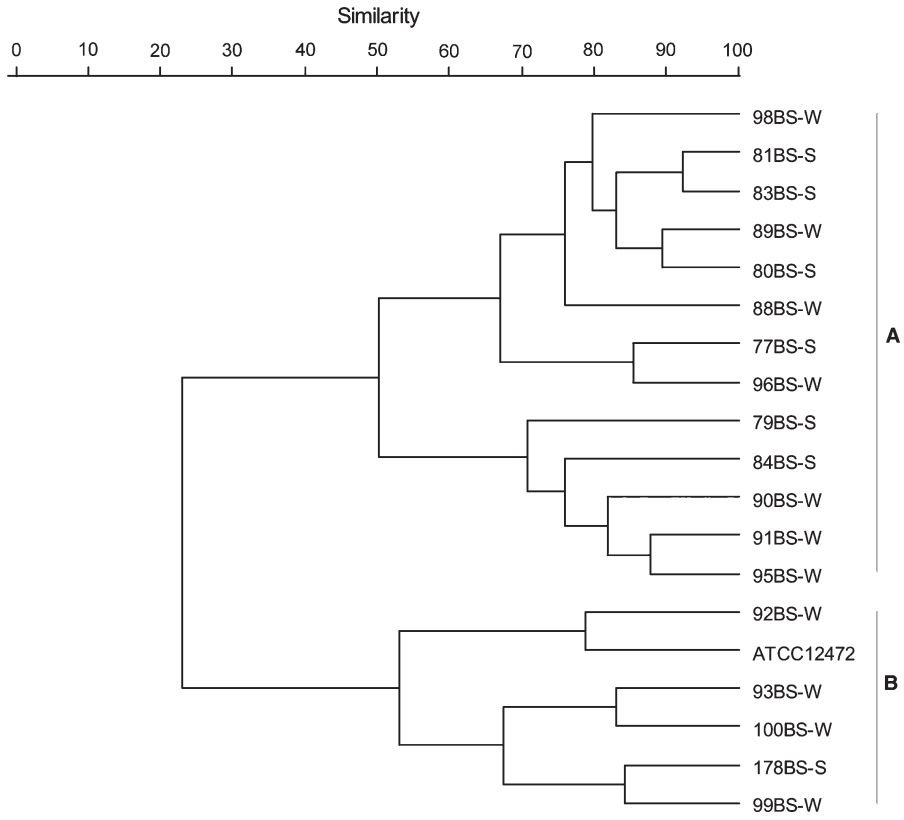

Fig. 2 Cluster analysis of *Chromobacterium* sp. isolates and of *C. violaceum* ATCC 12472 according to BIOLOG GN2 microplates profiles. A distance matrix of simple similarity coefficients was clustered with the UPGMA algorithm. *W* isolates recovered from water; *S* isolates recovered from soil

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 $ml^{-1)}$

			Environments			
			Water		Soil	
Antimicrobials	Range	Type species	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 *Chomobacterium* 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,

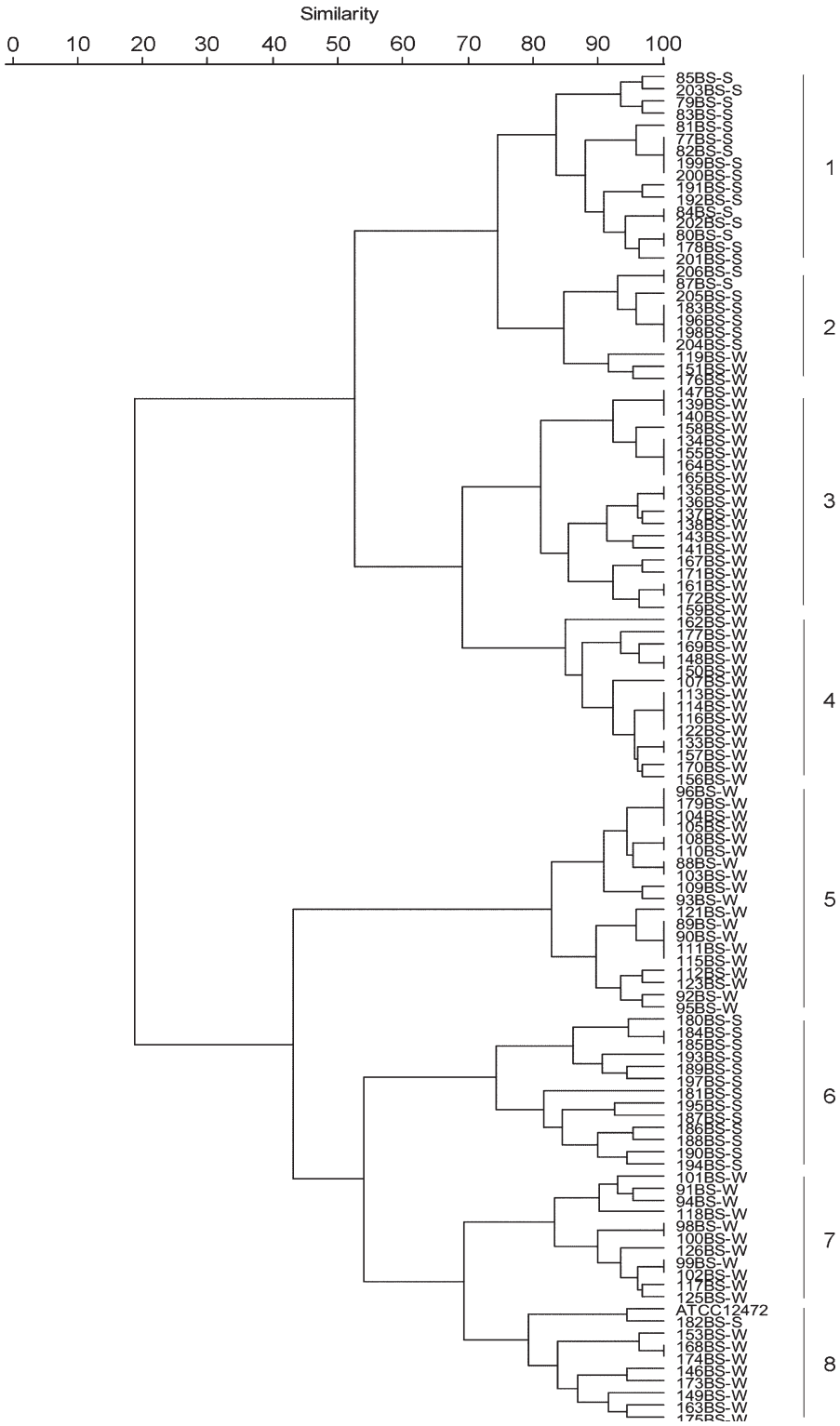

Fig. 3 Cluster analysis of *Chromobacterium* sp. isolates and of *C. violaceum* ATCC 12472 according to antimicrobial susceptibility profiles. A distance matrix of simple similarity coefficients was clustered with the UPGMA algorithm

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

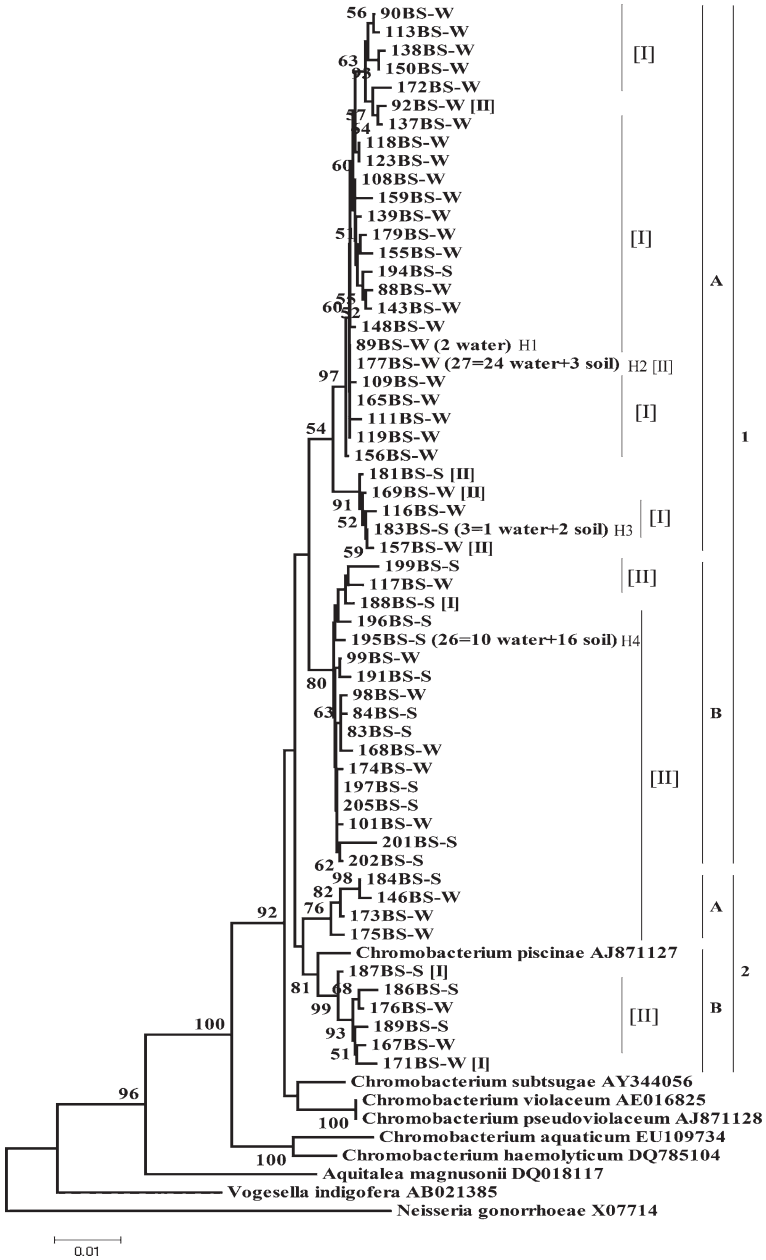

Fig. 4 Phylogenetic tree based on 16S rRNA gene sequences of all *Chromobacterium* sp. isolates from water and soil. One thousand bootstrap resamplings were used to evaluate the robustness of the inferred trees. *Numbers* in *parentheses* represent the number of the isolates exhibiting the same haplotype.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

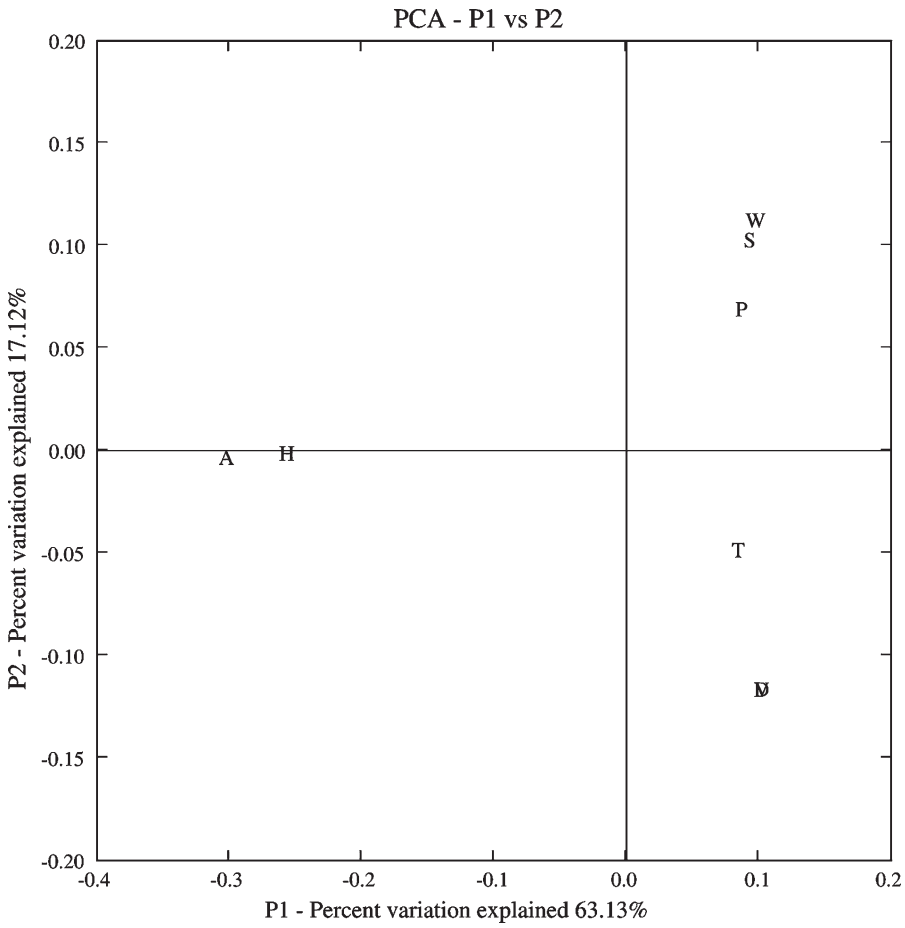

Fig. 5 Principal component analysis ordination plot for the 16S rRNA gene. The percent of variation explained by each principal component is indicated on the axis labels. Environments are represented by the following letters: Water W, Soil S. *Chromobacterium* species are represented by the following 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

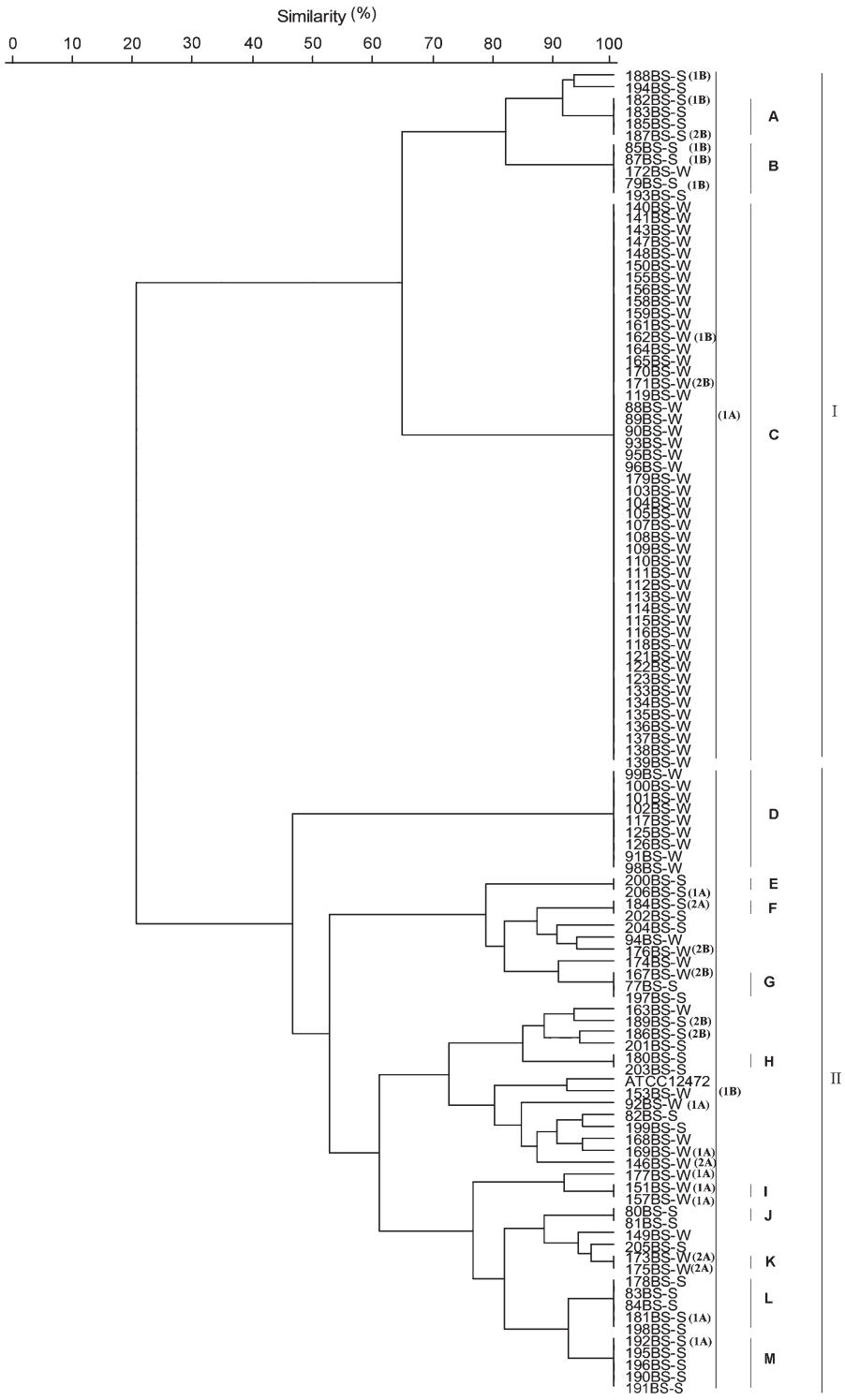

Fig. 6 Cluster analysis of *Chromobacterium* sp. isolates and of *C. violaceum* ATCC 12472 according to BOX-PCR fingerprinting. A distance matrix of simple similarity coefficients was clustered with the UPGMA algorithm. *Letters A–M* correspond to the 13 patterns. A and B correspond to 16S rRNA phylogenetic 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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CAPÍTULO 2 – A survey on cultivable heterotrophic bacteria inhabiting a thermally unstratified water column in Atlantic Forest Lake

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A survey on cultivable heterotrophic bacteria inhabiting a thermally unstratified water column in an Atlantic Rainforest lake

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Running title: A survey on cultivable lake heterotrophic bacteria

Abstract

Temporal and/or spatial molecular and physiological investigations of the diversity and distribution of cultivable heterotrophic bacteria from lake water have not been extensively studied, despite the key role of these organisms in biogeochemical cycles and their influence on water quality. Eighteen heterotrophic bacteria communities distributed over time and space in the water column of Carioca Lake were analyzed to characterize their composition. A polyphasic approach was used, including 16S rDNA restriction analysis (ARDRA), 16S rRNA gene sequence analysis, BIOLOG Ecoplates and statistical methods. Great diversity was observed in the physiological profiles. Dendrogram analysis of the BIOLOG Ecoplates divided the communities into two clusters based on their temporal origin. The 673 isolates obtained yielded 360 ARDRA OTUs, 326 of them unique. There were few overlapping ARDRA OTUs in temporal and spatial scales, indicating strong endemism. A subset of representative isolates for each ARDRA OTU was identified by 16S rRNA gene fragment sequencing and categorized into five phyla, Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Deinococcus-Thermus, represented by 39 genera. Rarefaction analysis of the genera richness of the isolates suggested that the total cultivable diversity by our method was almost saturated (13 of 18 communities). Mantel testing revealed that the biogeographical patterns of the communities were influenced by abiotic factors (turbidity, total N and NO_2) on a temporal scale.

Introduction

Microorganisms in the soil, marine and in freshwater ecosystems are recognized as fundamental regulators of biogeochemical cycles that play a key role in the cycling of nutrients and energy on our planet and are likely necessary for evolution. In recent years, several studies were performed to describe bacterial diversity and community changes in various ecosystems. Soil is often described as one of the most diverse environments on Earth, harboring vast species-level diversity. In contrast, the diversity in freshwater is orders of magnitude less than in marine and soil environments (41). Freshwater lake ecosystems have been investigated less frequently than have marine environments. Most freshwater bacteria fall into one of the following classes: alpha- beta- and gamma-Proteobacteria, the Cytophaga-Flavobacterium-Bacteroides group, Cyanobacteria, Actinobacteria, Verrucomicrobia, green non-sulfur bacteria and candidate division OP10 (53). Moreover, studies of the composition of bacterial communities from lentic environments have mainly used culture-independent approaches, which do not allow the characterization of heterotrophic bacteria in the environment (4).

In 1944, the Parque Estadual do Rio Doce (PERD; Fig. 1) was established as an Atlantic Rainforest conservation unity in Brazil. The PERD makes up the largest residual area of the Atlantic Rainforest biome (36,000 ha of forests) and provides locations for long-term ecological research investigation. Its water system occupies 9.8% of the total area (6). Many ecological studies have focused mainly on macro-organisms, microcrustaceans and cyanobacteria (e.g. 7, 8, 37). The PERD contains diverse organisms such as capybara, capuchin monkey and agouti as well as endangered species, such as jaguar (onça pintada) and woolly spider monkey (muriqui), which is the largest primate in the Americas. The PERD also contains a remarkable lake system that is composed of forty natural lakes, including Carioca Lake, which is a shallow lake (11.8 m maximum depth) with a small surface area (14.1 ha) (6). Its water circulates only from June to August (24), and in this period the water column is thermally unstratified.

Currently, little is known about the ecology, physiology and community composition of cultivable bacteria from lakes. Therefore, in the present study, we used the cultivation of heterotrophic bacteria coupled with techniques based on 16S rRNA gene sequencing, the BIOLOG EcoPlate and statistical methods to evaluate the taxonomic diversity and composition of cultivable bacterial communities in the water

column of Carioca Lake. The relationship between microbial community function as assessed by community level physiological profiles (CLPP) and abiotic variables was also investigated.

Materials and Methods

Sampling and bacterial isolation

Water samples (500 ml) in three replicates from three points 100 m distant from each other across a euphotic gradient from a limnetic zone (Carioca Lake) were taken with Van Dorn bottles. Collections took place in June and August 2007, which is the dry season. Samples were taken from the water column at different degrees of light penetration (100%, 10% and 1%) as determined by Secchi disk. To assess water conditions, selected physical and chemical variables were measured at three points in the euphotic gradient. Water temperature, pH, turbidity and dissolved oxygen concentration (DO) were measured *in situ* with a multiprobe (Horiba, model U-22). Concentrations of total nitrogen (TN), total phosphorus (TP), ammonium nitrogen (NH_4), nitrite nitrogen (NO_2), nitrate nitrogen (NO_3), soluble reactive phosphorus (PO_4) and chlorophyll *a* were measured as previously described (19, 32, 36).

Bacteria were isolated by plating 100 µl of a water sample directly on PTYG agar plates (0.5% peptone, 0.5% tryptone, 0.5% yeast extract, 1.0% glucose, 0.06% $MgSO_4$, 0.006% $CaCl_2$, 1.5% agar), which were then incubated at 28°C for up to seven days. The PTYG was chosen to allow the overall growth of culturable aquatic bacteria with different metabolic demands. The isolates were further streaked onto the same medium and checked for purity prior to molecular and phenotypic analyses. Subsequently, the isolates were stored in glycerol at -70°C. The isolates in this study were named according to each point of the limnetic zone (A, B and C), specific euphotic gradient (1%, 10% and 100% of light penetration) and month of collection (J, June and A, August) from which they were retrieved, in that order (e.g., LimA-1-J, LimB-10-A and LimC-100-J).

Microbial metabolic diversity

Community-level physiological profiles (CLPP) were measured using a BIOLOG 96-well Ecoplate (BIOLOG Inc., USA). This system is composed of 3 x 31 single carbon sources and a water blank well; each well also contains tetrazolium redox dye. The

microplate detection system monitors the reduction of the dye at the beginning of microbial respiration, meaning that the carbon sources have been used by the microbial community. This process causes the tetrazolium dye to change to a purple color, which can be measured by the absorbance of light at 590 nm. One hundred twenty µl of sample water was inoculated into each well of the EcoPlate and incubated at 28ºC in the dark. Color development was measured at OD_{590} every 24 h for 5 d using an ELISA plate reader (BIO-RAD Model 3550 Microplate Reader). The absorbance in the water blank was subtracted from the absorbance readings of all other wells. Negative optical density values were set to zero. Plate readings at 72 h of incubation were used for the assessment of bacterial functional diversity and statistical analyses because it represented the optimal range of optical density readings (0.3–1.0 abs).

Ecoplate data analysis

Since raw OD_{590} values were corrected the microbial activity for each microplate, expressed as the average well-color development (AWCD), was calculated as follows: $AWCD = \sum OD_i/31$ where OD_i is the optical density value from each well. Richness (the number of oxidized C substrates) and the Shannon–Weaver index (the richness and evenness of response) were calculated using an OD of 0.25 as the threshold for positive response (17). Shannon's diversity index was calculated as $H' = -\sum p_i (\ln p_i)$ where p_i is the ratio of the activity on each substrate (OD_i) to the sum of activities on all substrates ($\sum OD_i$). Shannon's evenness was calculated as $E = H'/\ln R$, where H' is Shannon's diversity and R is substrate richness.

Statistical analysis

According to Legendre and Legendre (29), ordered data do not have to be transformed before they are analyzed numerically. Thus, the Ecoplate data were normalized and transformed logarithmically before multivariate analyses were applied. The 72 h absorbance values were normalized by the AWCD, as recommended by Garland (17):

$$\overline{OD_k} = \frac{OD_k}{\frac{\sum OD_i}{31}}$$

where $\overline{OD_k}$ represents the normalization data of well k, OD_k is the absorbance reading of well k and the denominator in this equation represents the AWCD.

This transformation is likely to normalize skewed data (29). A natural logarithmic transformation was used in this study:

$OD' = \ln(\overline{OD}_k + 1)$

where OD' represents the value of the transformed data.

Two types of multivariate analyses were used to study the relationship between microbial community function (assessed by CLPP) and abiotic variables: principal component analysis (PCA) and canonical correspondence analysis (CCA) using CANOCO for windows version 4.51 (Biometrics, Plant Research International). PCA was applied to reduce the dimensionality of the CLPP and abiotic data.

The Shannon–Weaver index, Shannon's evenness and rarefaction curve were determined using PAST software (23). The Shannon–Weaver index and Shannon's evenness were calculated for ARDRA OTUs and sequence data. The rarefaction curve was estimated for communities' sequence data. Bacterial isolate sequence coverage was calculated using the equation C= [1-(n_i/N)] x 100, where n_i represents the number of genera represented by a bacterial isolate sequence and N represents the total number of sequences in the community.

DNA extraction and 16S rRNA gene amplification

Genomic DNA of the isolates was purified as described previously (45). The complete 16S rRNA gene was amplified by touchdown PCR according to Pontes et al. (44), using the conserved primer set PA (5'-TCCTGGCTCAGATTGAACGC-3') (modified from 27) and U2 (5'-ATCGGYTACCTTGTTACGACTTC-3') (35).

Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA was performed to analyze bacterial diversity and to compare the composition of the communities from each point sampled. 16S rDNA was amplified with the PA and U2 primers and then digested separately with two restriction enzymes (*Afl*III and *Alu*I; New England Biolabs) that recognize sequences of six nucleotides, according to the supplier's recommendations. Restriction fragments were separated on 2.5% agarose gels in Tris–acetate–EDTA (TAE) buffer. After electrophoresis at 75 V for 2.5 h, the gels were observed and photographed. The restriction fingerprints were analyzed using BioNumerics version 6.0 software (Applied Maths, St. Martens-Latem, Belgium). Digitized gel images were converted and normalized using the 1 Kb Plus DNA Ladder (Invitrogen). A band-matching algorithm (band-matching tolerance of 1.0%) was used

to calculate pairwise similarity matrices with the Dice coefficient. Cluster analyses of similarity matrices were performed by UPGMA (unweighted pair group method using arithmetic averages). For each ARDRA pattern, the 16S rRNA gene of one to three isolates was sequenced, thus minimizing the number of sequence reactions that had to be performed.

Sequencing and phylogenetic analysis

The partial 16S rRNA gene sequence was obtained using the primers 8F (5'-GAGTTTGATYMTGGCTCAG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') (28). Sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and an ABI 3130 sequencer (Applied Biosystems) according to the manufacturers' instructions. The 16S rRNA gene sequences were analyzed, checked for quality, aligned, and edited to produce a consensus using Phred v.0.20425 (15), Phrap v.0.990319 (22) and Consed 12.0 (21) software. To determine the approximate phylogenetic relationships of our 673 isolates, the 16S rRNA gene consensus sequences were compared against sequences in GenBank using BLASTN and against sequences in the Ribosomal Database Project (RDP) using the Classifier search. Phylogenetic relationships were inferred by MEGA 4 (48) using the Minimum Evolution method, and the evolutionary distances were computed using the Maximum Composite Likelihood method. The robustness of the phylogenetic tree topology was evaluated with 1,000 replicates of bootstrap analyses. The nucleotide sequences generated were deposited in the GenBank database with accession numbers xxx to xxx.

Bacterial community analysis

The Unifrac metric method (http://bmf.colorado.edu/unifrac) was used to compare bacterial communities from each point of the limnetic zone with those from each euphotic gradient using phylogenetic information (33). The phylogenetic data were used to compare bacterial communities, testing statistical differences among all samples, with UPGMA and PCA. The Cluster Environments function of Unifrac was used to determine which environments in the tree had similar bacterial communities, and the PCA function was used to find the most important axes along which samples vary. Jackknifing was used to support UPGMA clustering results, and significance tests were also performed as previously described (34).

The simple Mantel test was performed using PAST software (23). This program calculated whether biotic similarity correlated with environmental factors and whether this correlation was statistically significant. The value of r^2 was the correlation value, and positive or negative values reflected the type of relationship between the two matrices, while *p* was the probability associated with r^2. Values of *p* were significant if they were <0.05; values that were >0.05 indicated that the null hypothesis (distances in the biotic matrix were independent of the distances in the environmental factors matrix) applied.

Results

Environmental characterization

The physicochemical and biological parameters of the water column are presented in Table 1. There was little variability in temperature along the euphotic gradient during the sampling periods, indicative of a thermally unstratified water column. The June collection showed lightly acidic water, with pH values decreasing with depth, whereas in August the pH remained close to neutrality and was constant with depth. Turbidity was ≤7 nephelometric turbidity units (NTU) in August, and no vertical variation existed in June. Carioca Lake was deficient in dissolved oxygen at the bottom of the water column in both sampling periods when compared to top of the water column and was especially pronounced in August. Total phosphorous and nitrogen concentrations increased with depth in both sampling periods. According to the model of Salas and Martino (1991), establishing the trophic state of tropical lakes with total phosphorus scales, the Carioca Lake was mesotrophic during the sampling periods. The highest primary productivity, determined by chlorophyll *a* concentration, was observed at the layer receiving 1% light penetration, not in the surface layer.

The first two axes of the PCA accounted for 98.8% of the total variance. Temperature, pH, nitrite nitrogen (NO_2) and dissolved oxygen (DO) were positively correlated with the first axis (95.7%). Chlorophyll *a*, depth, total phosphorus and nitrate nitrogen (NO_3) were positively correlated with the second axis (3.1%). The August communities from horizontal and euphotic gradients, which showed the highest concentration of phosphorus and pH levels, were the farthest from the origin (Fig. 2).

Microbial community level physiological characteristics

To better evaluate the community level physiological profiles (CLPP), BIOLOG EcoPlates were used to analyze four parameters: substrate richness (R), average well-

color development (AWCD), Shannon-Weaver index (*H'*) and evenness (E). Significant differences ($p<0.05$) were found in R, AWCD and *H'* between the sampling periods. In contrast, R, AWCD and *H'* were not significantly different ($p>0.05$) at each point of the horizontal and vertical gradients of the limnetic zone in June and August (Table 2).

The highest functional diversity among the 18 microbial communities was detected in the vertical gradient (LimB-10) in August, which utilized 30 C sources. In contrast, the LimB-10 sample in June, which was negative for 12 substrates, was the least responsive in EcoPlate substrate utilization. 2-hydroxy benzoic acid was the single C source that was not utilized by any microbial community from the two sampled periods. Moreover, D-xylose, γ-hydroxybutyric acid and putrescine were not utilized by any microbial community sampled in June, whereas the other C sources were used differentially. Cluster analysis of the data from the Ecoplate (Fig. 3) grouped the microbial communities into two principal clusters, and the highest similarity among the microbial communities occurred in August. There was a clear separation of the sampled periods and no observable pattern in the groupings with respect to horizontal and vertical gradients.

In addition to analyzing individual substrates, CLPP data were subdivided into six substrate categories (9, 52): carboxylic acids (pyruvic acid methyl ester, D-glucosaminic acid, D-galactonic acid γ-lactone, D-galacturonic acid, 2-hydroxy benzoic acid, 4-hydroxy benzoic acid, γ-hydroxybutyric acid, itaconic acid, α-ketobutyric acid and D-malic acid); complex carbon sources (Tween 40, Tween 80, α-cyclodextrin and glycogen); carbohydrates (D-cellobiose, α-D-lactose, β-methyl-D-glucoside, D-xylose, i-erythritol, D-mannitol and N-acetyl-D-glucosamine); phosphate-carbon (glucose-1-phosphate and D,L-α-glycerol phosphate); amino acids (L-arginine, L-asparagine, L-phenylalanine, L-serine, L-threonine and glycyl-L-glutamic acid) and amines (phenylethylamine and putrescine). Significant differences ($p<0.05$) in the average utilization of specific substrate categories were observed between the horizontal and vertical gradients. The EcoPlate substrates that induced the highest responses by the microbial communities were complex carbon sources followed by amino acids, amines, carbohydrates, phosphate-carbon, and carboxylic acids from LimB-10-J, LimB-100-A, LimC-100-A, LimA-100-J, LimB-10-A and LimC-100-J, respectively.

The results of the PCA performed on the CLPP of the different aquatic microbial communities obtained after 72 h of incubation are shown in Fig 4. Principal component 1 accounted for 25.9% of total variability and was mainly associated to C sources L-

asparagine, N-acetyl D-glucosamine, pyruvic acid methyl ester, Tween 80, Tween 40, β-methyl-D-glucoside and D-cellobiose. Principal component 2 accounted for 18.8% of total variability and was mainly associated with L-serine, L-arginine and D-Galactonic Acid γ-Lactone. LimB-10-J, which showed the lowest substrate richness (R=19) and Shannon-Weaver index (*H'*=2.6 and E=0.88) values, was the farthest from the origin in the PCA.

Two types of multivariate analysis were used to study the relationship between microbial community function (assessed by CLPP) and abiotic variables: PCA and CCA. PCA was applied to reduce the dimensionality of the CLPP and abiotic data. Thus, only ten C sources and eight abiotic variables were analyzed by canonical correspondence, which showed no correlation between CLPP and abiotic variables. The eigenvalues were low for the two first ordination axes (0.011 and 0.007, respectively), explaining 16.5 and 26.6% of the CLPP variance and 44.8 and 72.3% of the cumulative variance of the relationship CLPP versus abiotic variables, respectively. Correlations of CLPP versus abiotic variables at the first two axes were moderately significant (0.70 and 0.58, respectively). In addition, CLPP and abiotic variables were not correlated to axes 1 and 2 ($F = 1.97$; $p>0.01$; Fig. 5) according to Monte Carlo's permutation test.

Recovery of isolates

Results from direct cultivation of bacteria from horizontal and euphotic gradients of the limnetic zone on PTYG agar showed low isolate recovery, ranging from 1.2×10^2 CFU ml^{-1} (June) to 3.8×10^2 CFU ml^{-1} (August). After purification of the colonies, a total of 673 randomly selected bacterial isolates (LimA 122 and 128, LimB 109 and 113, and LimC 82 and 119 in June and August, respectively, Table 3) were identified by 16S rRNA gene fragment sequencing.

ARDRA OTU distribution and biogeographic analysis

The ARDRA data from all examined isolates obtained with two restriction endonucleases (*Afl*III and *Alu*I) were combined into a single restriction pattern for each isolate. The number of restriction sites for *Afl*III was from one to eight and for *Alu*I from one to nine (data not shown). The analysis of combined restriction patterns revealed 360 distinct restriction patterns. Operational taxonomic units (OTUs) were defined as unique ARDRA patterns. The ARDRA patterns were primarily composed of single-isolate OTUs (252 of 360 OTUs). The geographic ARDRA OTU distribution

showed that 313 of them were unique, whereas the remaining ARDRA OTUs (47) were scattered throughout the euphotic gradient (Table 3). Moreover, the temporal ARDRA OTU distribution showed that only 13 of them were detected in both collection months (Table 3).

To measure the diversity of the ARDRA OTUs found in the bacterial communities, the Shannon-Weaver diversity index was calculated (Table 3). All bacterial communities were significantly different ($p < 0.05$) at each point of the horizontal and vertical gradients of the limnetic zone. In addition, only three communities (LimA-10-J, LimB-1-J and LimB-1-A) showed evenness ≥ 0.90.

The simple Mantel test was performed for biogeographical analysis. The results showed that the depth in the water column was not a significant factor in influencing bacterial community composition ($p > 0.05$). Few abiotic factors appeared to significantly influence the bacterial community composition, with the exception of LimB (Table 4). The abiotic factors mainly influenced the temporal biogeographic patterns. Turbidity and total N were significant factors in influencing LimA and LimC bacterial community composition ($p < 0.05$), whereas the C source was significant only for the LimC community. All r^2 values were positive, showing a direct influence of abiotic factors on the composition of bacterial communities. When all abiotic factors, C sources and ARDRA OTU data sets from June samples were combined (nine communities), light penetration, pH, temperature, electrical conductivity, NH_4 and chlorophyll *a* were the significant factors ($p < 0.05$). In contrast, no significant correlations between C sources, abiotic factors, ARDRA OTUs and biogeographic patterns were obtained from samples collected in August. Moreover, when the 18 bacterial communities were examined, turbidity, total N and NO_2 mainly influenced the temporal biogeographic patterns ($p < 0.05$, Table 4).

Isolate identity based on 16S rRNA gene sequence

All sequences (557), corresponding to 454 bp from the variable V2 to V4 regions of 16S rDNA, were categorized into five phyla: Proteobacteria (61.8%, includes Alpha-, Beta- and Gamma-proteobacteria), Firmicutes (18.6%), Actinobacteria (10.2%), Bacteroidetes (8.5%) and Deinococcus-Thermus (0.9%, Fig. 6). Proteobacteria sequences were from Gamma-proteobacteria (53%), Beta-proteobacteria (43.2%) and Alpha-proteobacteria (3.8%). A total of 183 sequences were from Gamma-proteobacteria, including 15 genera: *Aeromonas* (19.1% and 25.7% of the isolates in

June and August, respectively); *Pseudomonas* (10.9% and 6%); *Acinetobacter* (6% and 9.3%); *Enterobacter* (9.3% and 0.5%); *Serratia* (2.2% and 0%)*; Morganella, Stenotrophomonas, Plesiomonas* (1.6% and 0% each)*; Cronobacter*/*Kluyvera* and *Enhydrobacter* (1.1% and 0% each)*; Rahnella* and *Shewanella* (0% and 1.1% each)*; Cedeceae* and *Citrobacter* (0% and 0.6% each) and *Klebsiella* (0.6% and 0%). Beta-proteobacteria sequences were found to belong to *Chromobacterium* (58.4% and 28.8%)*, Aquitalea* (6% and 3.4%), *Janthinobacterium* (0% and 2.1%) and *Herbaspirillum* (1.3% and 0%). Thirteen sequences belonged to three genera of Alpha-proteobacteria: *Brevundimonas* (30.8% and 30.7%), *Sphingomonas* (7.7% and 15.4%) and *Paracoccus* (15.4% and 0%).

The taxonomic assignment of 104 bacterial isolates to the Firmicutes phylum revealed that they belonged to six genera. The majority of the isolates were *Staphylococcus* (41.3 and 5.8% of the isolates in June and August, respectively), *Bacillus* (26.9% and 13.5%), *Paenebacillus* (1.9% and 3.9%), *Brevibacillus* (1% and 3.8%), *Macrococcus* (1% and 0%) and *Exiguobacterium* (0% and 1%).

Actinobacteria (57 bacterial isolates) consisted of four families, Micrococcaceae, Microbacteriaceae, Intrasporangiaceae and Brevibacteriaceae, including eight genera, namely *Arthrobacter* (22.8% and 33.3% of the isolates in June and August, respectively), *Microbacterium* (8.8% and 5.2%), *Janibacter* (7% and 1.8%), *Micrococcus* (3.5% and 3.5%), *Kocuria* (1.8% and 3.5%)*, Brevibacterium* and *Rothia* (3.5% and 0% each) and *Streptomyces* (0% and 1.8%).

Bacteroidetes and Deinococcus-Thermus were less represented with few genera in each phylum. The isolates associated with Bacteroidetes were represented by the genera *Chryseobacterium* and *Elizabethkingiamiricola*, and *Deinococcus* represented the Deinococcus-Thermus phylum.

To measure the diversity of the genera assemblages, the Shannon-Weaver diversity index was calculated using the 16S rRNA gene sequences. The bacterial communities were significantly different ($p < 0.05$) at each point of the horizontal and vertical gradients of the limnetic zone. However, significant differences ($p > 0.05$) were not found between the sampling periods. The values of the Shannon-Weaver diversity index obtained for the 18 communities showed that the community from LimB-1-A had the highest diversity ($H' = 2.35$) among the communities, whereas the community from LimC-100-A had the lowest Shannon-Weaver index ($H' = 1.27$). In addition, all communities presented evenness ≤ 0.70 (Fig. 7A and 7B).

Rarefaction curves were used to determine whether the sampling was enough to evaluate genera diversity with some level of confidence. Rarefaction curves were obtained by plotting the genera richness observed against the number of isolates analyzed for each of the 18 communities (Fig. 7A and 7B). A decrease in the rate of genera detection was observed in the bacterial communities from August, except for in LimC-1-A (coverage = 64%; Fig. 7B). In June, a decrease was observed in five of the nine bacterial communities, indicating that the majority of the diversity was detected. This result was further supported by calculating the coverage of the communities (≥ 62%; Fig. 7A).

Temporal and spatial dynamics of bacterial communities

The distribution and abundance of the five bacterial phyla, Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and Deinococcus-Thermus (Fig. 8), differed in all bacterial communities from June and August ($p < 0.01$). Proteobacteria was consistently found in all communities and in both collection months. In contrast, Bacteroidetes was only detected in LimA-100-J in eight of the nine communities from August. Members of Firmicutes and Actinobacteria were also cosmopolites. Deinococcus-Thermus was distributed only at three points at ≤ 10% of light penetration (Fig. 8). Consequently, the distribution of the bacterial genera also differed considerably among the 18 bacterial communities (Fig. 9). Bacterial communities from June had a higher number of genera (31) than the bacterial communities from August (27), and the bacterial communities from LimB-1-A and LimA-10-J showed the highest phylogenetic heterogeneity, with 14 and 15 genera, respectively. Among the 39 genera, *Chromobacterium* was the most common genus, missing only from LimA-10-A. Other common genera were: *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Arthrobacter* and *Staphylococcus*. Moreover, some genera were detected in only one of the collection months: *Paracoccus*, *Herbaspirillum*, *Stenotrophomonas*, *Enhydrobacter*, *Morganella*, *Cronobacter*/*Kluyvera*, *Plesiomonas*, *Klebsiella*, *Serratia*, *Janibacter*, *Brevibacterium*, *Rothia* and *Macrococcus* in June (Fig. 9A); and *Janthinobacterium*, *Cedeceae*, *Citrobacter*, *Rahnella*, *Shewanella*, *Streptomyces*, *Exiguobacterium* and *Elizabethkingiamiricola* in August (Fig. 9B). Likewise, some genera from each sampling period were restricted to a specific euphotic gradient point, predominately at 1% and 10% of light penetration. Interestingly, *Sphingomonas* (LimB-100-J) and

Brevibacillus (LimB-1-A) were restricted to the same horizontal and vertical gradient points in both months (Fig. 9).

To compare the composition of bacterial communities and specific features of the ecosystems, we used the UniFrac metric analysis. This analysis revealed three main clusters of related communities. Cluster 1 matches the August collection period, except for LimC-1-A, which is located in cluster 3. Clusters 2 and 3 combined the isolates from June (Fig. 10). The communities were grouped with low similarities, showing the difference of genera composition. The robustness of the inferred UniFrac tree topology to the presence of specific isolates represented was confirmed by jackknife analysis ($p < 0.01$). Principal components analyses also suggested that there were significant differences among communities ($p < 0.01$, Fig. 11).

Discussion

One of the primary goals of biogeography is to comprehend biodiversity patterns, their distribution and the processes that interfere with these patterns over space and time. Ecological biogeography provides a more comprehensive understanding of the specific role of an organism in its natural habitat and the influence of physical and biotic environments on community composition. These processes are particularly difficult to study in microbial biogeography. Hence, the majority of microbial biogeography studies have searched for biodiversity patterns and community composition. The simplest and most widely accepted way to measure the α diversity of aquatic bacterial communities is to use values of species richness and evenness (e.g., the Shannon-Weaver index). Diversity statistics also favor the comparison among microbial communities (β diversity).

The present study aimed to assess the composition of bacterial communities and their spatial and temporal distribution relative to environmental variables. A total of 18 water samples were retrieved from different points in the euphotic gradient (1%, 10% and 100% light penetration) at three limnetic points of the mesotrophic Carioca Lake in June and August. In general, abiotic parameters and chlorophyll *a* were spatially and temporally variable in the water columns, as expected (43, 50).

BIOLOG Ecoplate has been recognized as a useful tool to study the functional diversity and to compare bacterial communities (46). Community level physiological profiles (CLPP) reflect the potential of the bacterial community to respond to different C sources. According to Charazenc et al (2010), complex C sources allow the best

growth of bacterial communities because these compounds can produce more energy. In the present study, CLPPs revealed differences in the functional diversity of the microbial communities between June and August. These communities greatly metabolized complex C sources (Tween 80, Tween 40, α-cyclodextrin and glycogen). Moreover, all categories of C sources were metabolized, possibly indicating that these bacterial communities are generalist species. In general, the 18 bacterial communities studied here showed high AWCD and R values. It is interesting to note that functional Shannon-Weaver diversity value (*H*'=2.60-3.33) found in this study is similar to that found by other groups (16, 20, 30, 47). The past studies were done on soil, which shows more diversity than freshwater (49). Species evenness values were similar in 17 communities (0.93 to 0.98), indicating how equal the community was numerically. The LimB-10-J was the unique uneven community (0.88); however, this community had similar functional activity to communities from June. A possible explanation could be that even though the communities have a different phylogenetic composition, they could have functional similarities allowing them to metabolize the assayed carbon sources at a similar rate (3). This functional redundancy is very important for the stability of the communities, making them resilient and able to survive environmental changes (13).

ARDRA is an effective technique to examine the composition of bacterial communities over space and time and to reduce the number of sequences requiring further analysis (14). The ARDRA were distinct for the most isolates, showing 313 unique OTUs (Table 3). Thus, strong endemicity was seen in all bacterial communities. Additionally, distinct ARDRA OTUs were found representing the same genus. Dang and Lovell (14), working with 16S rRNA gene clone libraries, found similar results. Therefore, this approach reveals the number of different 16S rRNA genes retrieved from a sampling site, but not the number of different varieties of organisms in the sample. Moreover, these genetic variants could represent ecotypes, reflecting physiological adaptations to their habitats (10, 40). Differences in the compositions of bacterial communities, especially in relation to the composition of ecotypes and the number of isolates, were also found in other environments (1). In addition, the Shannon-Weaver diversity value obtained by this approach (*H'*=2.60-3.47) was similar to that obtained by CLPP analysis. However, the species evenness obtained by the ARDRA OTUs revealed uneven bacterial communities, in contrast to functional evenness obtained by BIOLOG EcoPlates. This may be explained by fact that although BIOLOG EcoPlates is a culture-based method, it is possible to obtain data from almost the entire

bacterial community, including uncultivable microorganisms (2). This is interesting because the most general law of community composition states that communities are composed of many rare and few abundant species (38).

The biogeographical patterns of bacterial communities in the water column varied over time and space. Statistical analysis showed that the composition of LimA and LimC communities were significantly influenced by a few abiotic variables, but no correlation was observed in LimB communities. These data suggest that these heterotrophic bacterial communities are regulated by other environmental factors. Therefore, the statistical analysis suggests that the temporal scale has a biogeographical effect on the local spatial scale. Previous studies also could not associate bacterial communities with abiotic variables (5, 12). However, they related the composition of the community to the hydrodynamic composition of aquatic systems.

Despite the fact that the cultivation technique is restrictive in recovering bacterial isolates from environmental samples (49), the use of PTYG medium was effective in recovering bacteria with different metabolic demands, totaling 39 genera belonging to five phyla. These data were supported by the coverage values (52% to 84%), showing that the diversity found has a level of confidence. Previous studies using a culture-independent approach with clone libraries from water samples obtained coverage values in a similar range and lower than those found in the present study (11, 39, 42). Moreover, in this study, it was observed that the community species evenness values were low. Lower evenness, when compared to evenness values for soil communities, is due to a moderate number of bacterial groups appearing to dominate the freshwater environment (53).

The comparative analysis of 557 freshwater lake bacterial 16S rDNA sequences revealed that the most of them (422 of 557 sequences) were closely related to sequences originating from freshwater environments. These data suggest that the isolates are indigenous to freshwater. The remaining sequences were retrieved from other environments. The 39 bacterial genera to which the isolates were assigned based on partial sequence analysis of 16S rRNA genes belonged to five bacterial phyla: Proteobacteria (especially Gamma-proteobacteria), Firmicutes, Actinobacteria, Deinococcus-Thermus and Bacteroidetes. Among these phyla, Proteobacteria (Gamma-, Beta- and Alpha-proteobacteria), Actinobacteria and Bacteriodetes are considered typical freshwater bacteria (53). The two other phyla (Firmicutes and Deinococcus-Thermus) are also found in freshwater environments, although at a lower frequency

(31). The phyla Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes represent diverse phylotypes, ecological niches and trophic capabilities (18, 26, 51). Although these phyla were relatively common in the heterotrophic bacterial communities, differences were observed in the temporal and spatial distribution and in the abundance in the horizontal and euphotic gradients. An interesting result from our study was the presence of the *Deinococcus* genus, whose members are resistant to ionizing radiation (25).

In conclusion, our results provide evidence of complex and diverse bacterial communities in a tropical freshwater lake, which seem well adapted to different nutrients provided by the lake and litter therein. Shifts in the composition of the communities were spatially and temporally evident, possibly being caused by a few abiotic factors (turbidity, total N and NO2), although other environmental factors may be involved on the spatial scale. Despite the low number of isolates, the diversity of the 16S rRNA gene sequences and the huge occurrence of unique ARDRA OTUs were surprising, indicating a strong endemicity. The members of the Proteobacteria, Bacteroidetes and Actinobacteria phyla dominate in broad assessments of typical freshwater bacteria diversity in non-cultivable and cultivable heterotrophic bacteria. Deinococcus-Thermus can also be considered a typical freshwater bacteria, at least in tropical lakes.

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Table1. Environmental parameters obtained in the water column from Carioca Lake in 2007.

Environmental parameters	Sample period					
	June			August		
Light penetration	100%	10%	1%	100%	10%	1%
pH	7.3	6.3	5.6	7.4	7.7	7.3
Temperature (ºC)	23	22.4	22	23.6	23	21.6
Turbidity (NTU)	7	7	7	2	2	7
Dissolved oxygen (mg.l-1)	9.1	8.7	7.3	8.4	8.3	6.8
P total (µg/l)	18.1	19.1	25.6	25.4	26.8	34.6
PO_4 (µg/l)	1.2	5.9	2.2	1.9	ND	3.9
N total (µg/l)	370.9	354.7	404.5	201.4	221.6	365.6
NH_4 (µg/l)	121.9	114.6	112.7	43.1	41.6	22.8
NO_3 (µg/l)	32.2	38.5	32.2	30.1	47.6	43.5
NO2	1.7	2.1	1.6	1.6	2.3	1.4
Chlorophyll *a*	55.1	58.8	62	25.7	32.6	96.6

Table 2. Carbon source utilization by bacterial communities from Carioca Lake and average well-color development (AWCD), richness (R), Shannon–Weaver index (H') and evenness (E) calculated on carbon substrate used in BIOLOG EcoPlate.

Carbon Sources	**Sites sampled**																	
	June									August								
	Lim A			Lim B			Lim C			Lim A			Lim B			Lim C		
	100%	10%	1%	100%	10%	1%	100%	10%	1%	100%	10%	1%	100%	10%	1%	100%	10%	1%
Pyruvic Acid Methyl Ester	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween 40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween 80	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ciclodextrin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycogen	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Cellobiose	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
α-D-Lactose	+	-	-	-	-	-	+	-	-	+	+	-	-	+	+	-	+	+
β-Methyl-D-Glucoside	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-
I-Erythritol	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-Acetyl-D-Glucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucosaminic Acid	+	+	+	-	+	+	+	+	+	+	-	+	+	+	-	-	+	+
Glucose-1-Phosphate	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
D,L-α-Glycerol Phosphate	+	-	+	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+
D-Galactonic Acid γ-Lactone	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galacturonic Acid	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
2-Hydroxy Benzoic Acid	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4-Hydroxy Benzoic Acid	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
γ-Hydroxybutyric Acid	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	+	+
Itaconic Acid	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
α-Ketobutyric Acid	-	-	-	-	+	+	+	-	-	-	+	+	+	+	+	+	+	+
D-Malic Acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
L-Arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Asparagine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LPhenylalanine	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Serine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Threonine	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
Glycyl-LGlutamic Acid	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Phenylethylamine	-	+	+	-	-	+	-	+	-	+	+	+	+	+	-	+	+	+
Putrescine	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
R	23	20	24	20	19	24	24	23	24	28	29	28	25	30	26	26	28	28
AWCD	0.7	0.3	0.7	0.5	0.4	0.6	0.7	0.6	0.7	1.0	1.1	1.0	0.8	1.1	0.9	1.0	1.0	1.0
H'	2.97	2.92	3.05	2.86	2.60	2.96	2.98	2.97	3.03	3.18	3.28	3.17	3.07	3.33	3.11	3.15	3.18	3.19
E	0.95	0.97	0.96	0.95	0.88	0.93	0.94	0.95	0.95	0.96	0.97	0.95	0.95	0.98	0.95	0.97	0.96	0.96

Table 3 - Spatial distribution of ARDRA OTUs and statistical diversity of communities of bacterial isolates.

Euphotic and Horizontal Gradients	No. of isolates	No. of unique ARDRA OTUs	ARDRA OTUs repeated between:		*H'*	E
			Euphotic and Horizontal Gradients	Month of collection		
LimA-100-J	36	18	1	1	2.76	0.77
LimA-10-J	37	22	6	2	3.41	0.94
LimA-1-J	49	35	1	0	3.47	0.89
LimB-100-J	44	26	0	2	3.28	0.87
LimB-10-J	38	20	0	3	3.27	0.89
LimB-1-J	27	19	1	0	3.08	0.95
LimC-100-J	29	13	1	2	2.6	0.77
LimC-10-J	22	13	2	2	2.73	0.87
LimC-1-J	31	17	0	1	2.99	0.88
LimA-100-A	36	12	7	0	2.66	0.74
LimA-10-A	49	16	1	0	2.69	0.70
LimA-1-A	43	22	2	0	3.15	0.85
LimB-100-A	40	13	6	0	2.84	0.88
LimB-10-A	35	7	1	0	2.48	0.74
LimB-1-A	38	22	1	0	3.28	0.90
LimC-100-A	39	16	2	0	2.89	0.80
LimC-10-A	47	11	2	0	2.79	0.72
LimC-1-A	33	11	0	0	2.89	0.83
Total	**673**	**313**	**34**	**13**		

H' = Shannon-Weaver index; E = Shannon's evenness.

Table 4 - Simple Mantel test for the bacterial communities of this study.

Factors	LimA						LimB						LimC						LimA+LimB+LimC					
	June (J)		August (A)		J+A		June		August		J+A		June		August		J+A		June		August		J+A	
	r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*
Light penetration	0.62	0.17	-0.86	0.83	-0.13	0.65	0.91	0.32	-0.99	0.84	-0.11	0.67	0.99	0.16	-0.99	1	0.09	0.31	**0.41**	**0.01**	-0.25	0.97	0.01	0.43
Depth	0.84	0.33	0.42	0.49	-0.10	0.62	-0.33	0.68	0.05	0.50	-0.20	0.69	0.19	0.51	-0.04	0.66	-0.19	0.69	0.11	0.24	-0.16	0.87	-0.09	0.74
pH	0.97	0.17	0.41	0.51	0.03	0.38	0.46	0.49	0.72	0.33	-0.27	0.74	0.85	0.16	0.8	0.17	-0.015	0.61	**0.29**	**0.02**	0.26	0.09	-0.11	0.78
Temperature	0.96	0.16	0.49	0.51	0.09	0.39	0.50	0.50	0.14	0.50	-0.34	0.88	0.87	0.17	0.005	0.67	-0.02	0.51	**0.29**	**0.02**	-0.15	0.83	-0.05	0.63
Turbidity	0.001	0.68	0.82	0.33	**0.78**	**0.03**	-0.002	0.17	0.55	0.34	0.20	0.24	0.002	1.00	0.43	0.66	**0.65**	**0.03**	-0.002	0.90	-0.06	0.59	**0.33**	**0.004**
Dissolved oxygen	0.68	0.34	0.78	0.33	-0.04	0.49	-0.55	0.83	0.50	0.50	0.05	0.44	-0.05	0.51	0.38	0.66	-0.16	0.69	0.03	0.42	-0.08	0.61	-0.09	0.74
Electrical condutivity	0.55	0.33	0.74	0.33	-0.03	0.39	0,94	0.17	0.44	0.50	-0.05	0.58	0.98	0.33	0.32	0.66	-0.21	0.68	**0.42**	**0.02**	-0.09	0.64	-0.09	0.66
Total P	0.58	0.32	0.71	0.34	-0.09	0.56	-0.66	0.84	0.40	0.50	0.31	0.21	-0.19	0.49	0.27	0.67	-0.21	0.70	-0.02	0.52	-0.10	0.67	-0.06	0.64
PO_4	-0.95	0.84	-0.99	1.00	-0.33	0.91	0.08	0.49	-0.86	1.00	0.16	0.26	-0.44	0.68	-0.79	0.83	-0.06	0.58	-0.17	0.83	-0.07	0.65	-0.04	0.64
Total N	-0.03	0.67	0.74	0.33	**0.72**	**0.04**	-0.98	1.00	0.43	0.50	0.005	0.48	-0.74	0.83	0.31	0.68	**0.58**	**0.03**	-0.20	0.92	-0.10	0.64	**0.25**	**0.02**
NO_3	-0.99	1.00	-0.99	1.00	0.23	0.18	-0.19	0.67	-0.97	1.00	0.08	0.41	-0.66	1	-0.93	0.83	0.36	0.09	-0.29	0.92	-0.15	0.81	**0,25**	**0.01**
NH_4	0.74	0.17	0.78	0.33	0.26	0.13	0.83	0.34	0.50	0.50	0.18	0.14	0.99	0.17	0.37	0.66	0.13	0.25	**0.38**	**0.01**	-0.08	0.60	0.05	0.18
Chlorophyll *a*	0.99	0.17	0.76	0.33	0.41	0.17	0.31	0.50	0.46	0.49	-0.02	0.52	0.75	0.16	0.34	0.68	0.22	0.27	**0.26**	**0.04**	-0.09	0.61	0.11	0.23
C Source	-0.50	0.84	-0.99	1.00	-0.09	0.58	0.002	0.51	0.82	0.18	0.50	0.1	0.92	0.35	-0.02	0.49	**0.52**	**0.03**	-0.07	0.53	-0.23	0.78	-0.07	0.65

r^2 is the correlation value; positive or negative values reflect the type of relationship between the two matrices, while *P* is the probability associated with r^2. *P* values are significant if *P* is <0.05 (boldface).

Figure legends

Figure 1. Rio Doce State Park and the sampling site location, Carioca Lake.

Figure 2. Correlation of the abiotic parameters biplot with the first two axes of principal component analysis (PCA) and distributions of communities sampled in June and August. The parameter abbreviations are Total N, total nitrogen; NH4, ammonium nitrogen; NO3, nitrate nitrogen; NO2, nitrite nitrogen; Turb, turbidity; Total P, total phosphorus; PO4, soluble reative phosphorus; Chl a, chlorophyll *a*; Temp, temperature; DO, dissolved oxygen. The community abbreviations are
1, LimA-100-J; 2, LimA-10-J; 3, LimA-1-J; 4, LimB-100-J; 5, LimB-10-J; 6, LimB-1-J; 7, LimC-100-J; 8, LimC-10-J; 9, LimC-1-J; 10, LimA-100-A; 11, LimA-10-A; 12, LimA-1-A; 13, LimB-100-A; 14, LimB-10-A; 15, LimB-1-A; 16, LimC-100-A; 17, LimC-10-A and 18, LimC-1-A.

Figure 3. UPGMA cluster analysis of microbial communities based on metabolic diversity obtained through BIOLOG EcoPlates after incubation at 28ºC for 72 h.

Figure 4. Correlation of the BIOLOG EcoPlates CLPP biplot with the first two axes of principal component analysis (PCA) and distributions of communities sampled in June and August. The C source abbreviations are AcPiv, Pyruvic Acid Methyl Ester; T40, Tween 40; T80, Tween 80; CiDes, α-Cyclodextrin; Gly , Glycogen; Dcell, D-Cellobiose; Dlac, α-D-Lactose; MetGlu, β-Methyl-D-Glucoside; Dxyl, D-Xylose; Eryt, i-Erythritol; Man, D-Mannitol; AceGluc, N-Acetyl-D-Glucosamine; AcGluc, D-glucosaminic Acid; GluFos, Glucose-1-Phosphate; GliFos, D,L-α-Glycerol Phosphate; AcLac, D-Galactonic Acid γ-Lactone; AcGal, D-Galacturonic Acid; HidBen, 4-Hydroxy Benzoic Acid; AcBut, γ-Hydroxybutyric Acid; AcIta, Itaconic Acid; AcAlfBut, α-Ketobutyric Acid; AcMal, D-Malic Acid; Arg, L-Arginine; Asp, L-Asparagine; Fen, LPhenylalanine; Ser, L-Serine; Teo, L-Threonine; AcGlut, Glycyl-Lglutamic Acid; PAmina, Phenylethylamine; Put, Putrescine. The community abbreviations are 1, LimA-100-J; 2, LimA-10-J; 3, LimA-1-J; 4, LimB-100-J; 5, LimB-10-J; 6, LimB-1-J; 7, LimC-100-J; 8, LimC-10-J; 9, LimC-1-J; 10, LimA-100-A; 11, LimA-10-A; 12, LimA-1-A; 13, LimB-100-A; 14, LimB-10-A; 15, LimB-1-A; 16, LimC-100-A; 17, LimC-10-A and 18, LimC-1-A.

Figure 5. Triplot of Ecoplate CLPP, environmental variables and distributions of communities sampled in June and August along the first two axes produced by the Canonical correspondence analyses (CCA). △, C sources. ○, microbial communities. AcPiv, Pyruvic Acid Methyl Ester; T40, Tween 40; T80, Tween 80; Dcell, D-Cellobiose; MetGlu, β-Methyl-D-Glucoside; AceGluc, N-Acetyl-D-Glucosamine; AcLac, D-Galactonic Acid γ-Lactone; Arg, L-Arginine; Asp, L-Asparagine; Ser, L-Serine; NO3, nitrate nitrogen; NO2, nitrite nitrogen; Total P, total phosphorus; Chl a, chlorophyll *a*; Temp, temperature; DO, dissolved oxygen. The communities abbreviations are 1, LimA-100-J; 2, LimA-10-J; 3, LimA-1-J; 4, LimB-100-J; 5, LimB-10-J; 6, LimB-1-J; 7, LimC-100-J; 8, LimC-10-J; 9, LimC-1-J; 10, LimA-100-A; 11, LimA-10-A; 12, LimA-1-A; 13, LimB-100-A; 14, LimB-10-A; 15, LimB-1-A; 16, LimC-100-A; 17, LimC-10-A and 18, LimC-1-A.

Figure 6. Compressed tree of the evolutionary relationships of phyla. The evolutionary history was inferred using the Minimum Evolution method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange algorithm. The neighbor-joining algorithm was used to generate the initial tree.

Figure 7. Rarefaction curves for each bacterial community based on the 16S rRNA gene sequences. H', Shannon-Weaver index; E, Shannon's evenness; C, coverage. 7A- bacterial communities from June; 7B- bacterial communities from August.

Figure 8. Relative abundance of phyla in Carioca Lake based on sequence analysis of the 16S rRNA gene.

Figure 9. Relative abundance of genera in Carioca Lake based on sequence analyses of the 16S rRNA gene. 9A- bacterial communities from June; 9B- bacterial communities from August.

Figure 10. UPGMA dendrogram, generated by UniFrac, of isolates from 18 bacterial communities from Carioca Lake.

Figure 11. Principal components analysis ordination plot for the 16S rRNA gene. The percent of variation explained by each principal component is indicated on the axis labels.

Figure 1

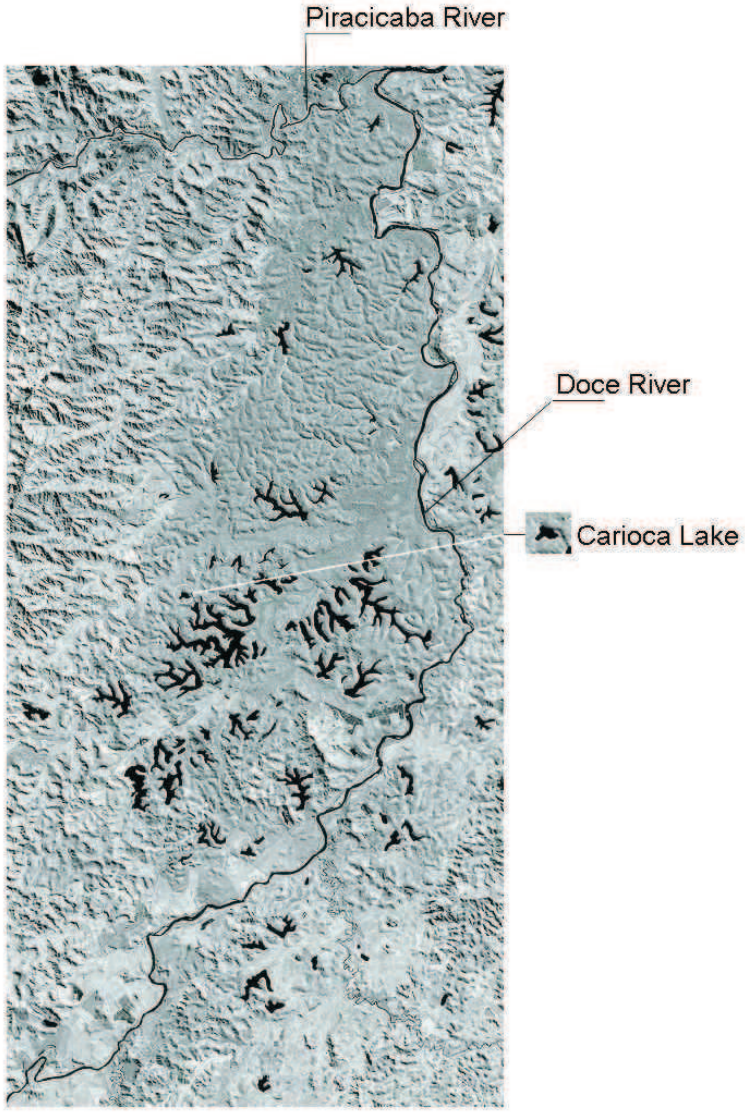


Figure 2

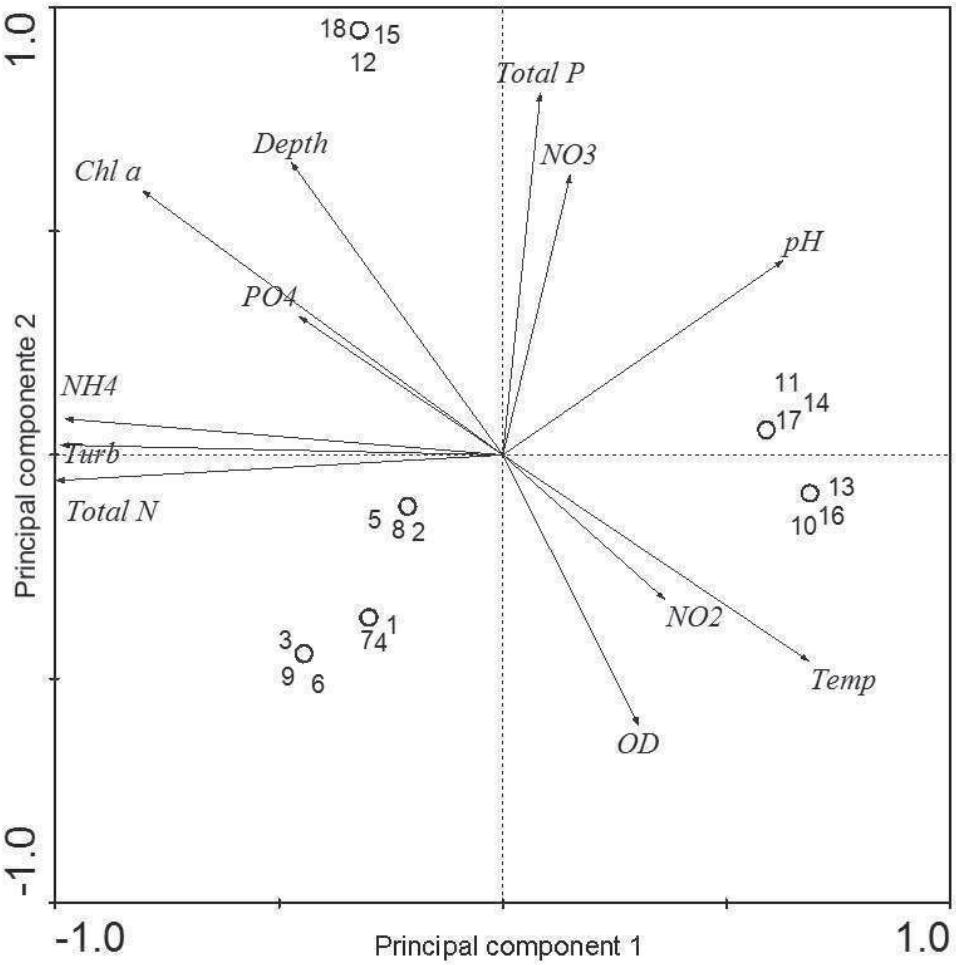

1.0
-1.0
-1.0
1.0
Principal componente 2
Principal component 1
18
15
12
Total P
Depth
NO3
Chl a
pH
PO4
NH4
11
14
17
Turb
13
Total N
10
16
5
8
2
NO2
3
1
7
4
9
6
Temp
OD

Figure 3

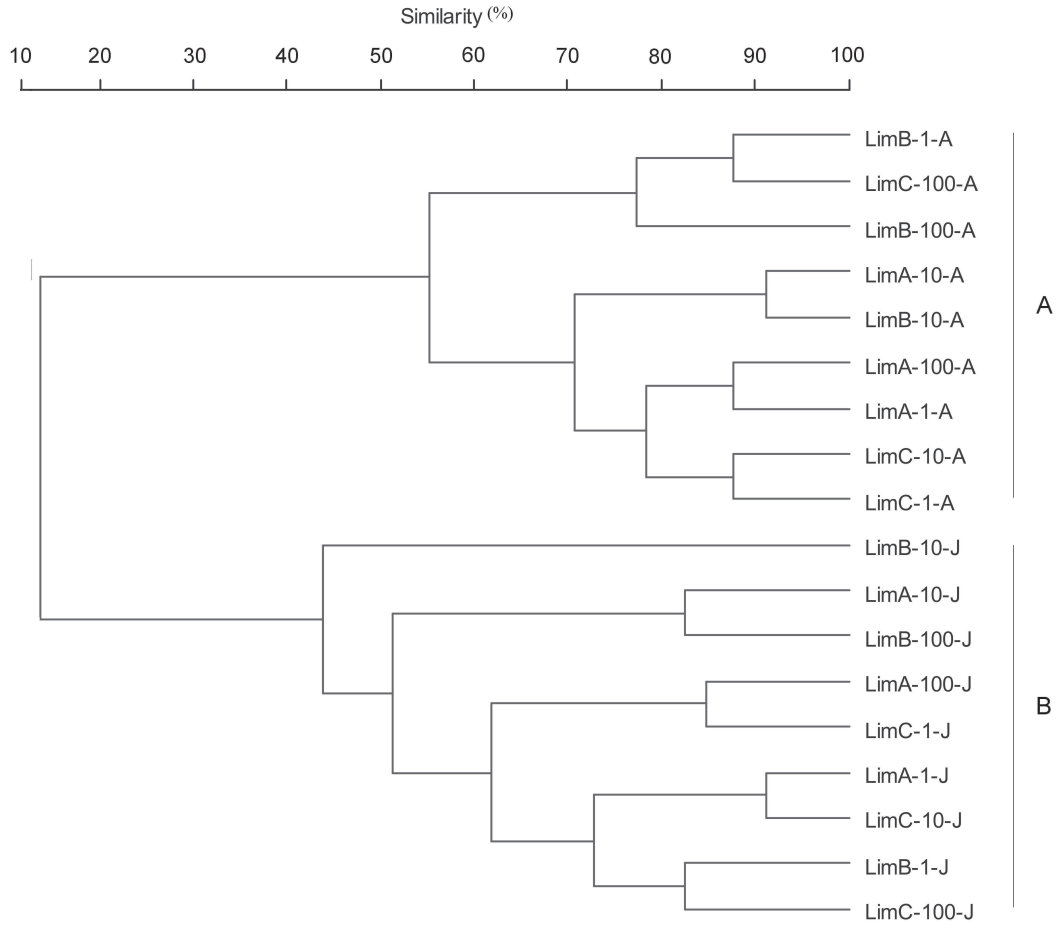

Similarity (%)
10
20
30
40
50
60
70
80
90
100
LimB-1-A
LimC-100-A
LimB-100-A
LimA-10-A
LimB-10-A
LimA-100-A
LimA-1-A
LimC-10-A
LimC-1-A
LimB-10-J
LimA-10-J
LimB-100-J
LimA-100-J
LimC-1-J
LimA-1-J
LimC-10-J
LimB-1-J
LimC-100-J
A
B

Figure 4

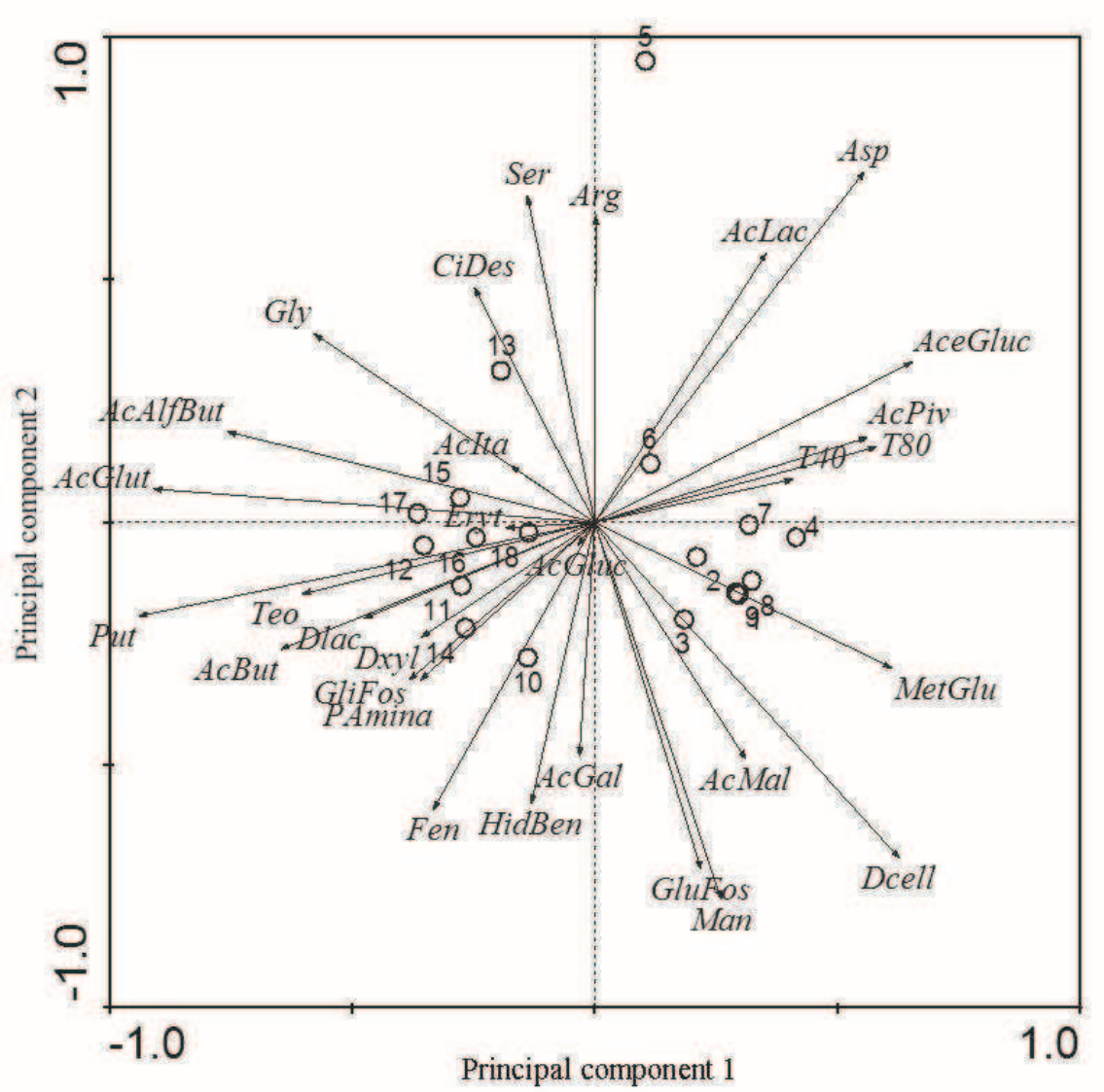

1.0
-1.0
-1.0
1.0
Principal component 2
Principal component 1
Asp
Ser
Arg
AcLac
CiDes
Gly
AceGluc
AcPiv
T80
T40
AcAlfBut
AcIta
AcGlut
Ervt
AcGluc
Put
Teo
Dlac
Dxyl
AcBut
GliFos
PAmina
MetGlu
AcGal
AcMal
Fen
HidBen
GluFos
Man
Dcell
5
13
6
15
17
7
4
12
16
18
2
11
9
8
3
14
10

Figure 5

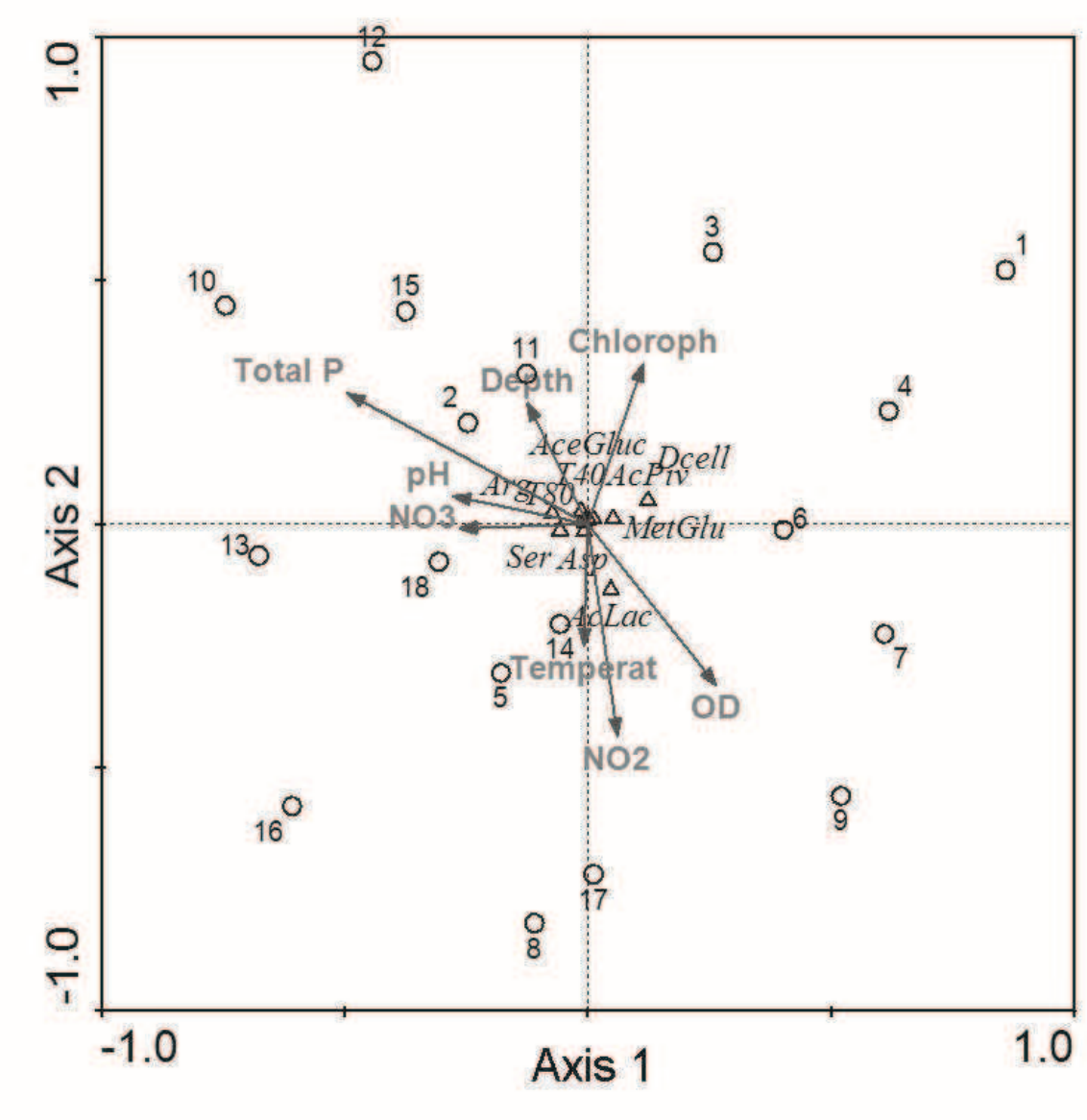

1.0
-1.0
Axis 2
-1.0
Axis 1
1.0
12
3
1
10
15
11
Chloroph
Total P
Depth
2
4
AceGluc
Dcell
T40AcPiv
pH
ArgTS0
NO3
MetGlu
6
13
Ser Asp
18
AcLac
14
7
Temperat
5
OD
NO2
16
9
17
8

Figure 6

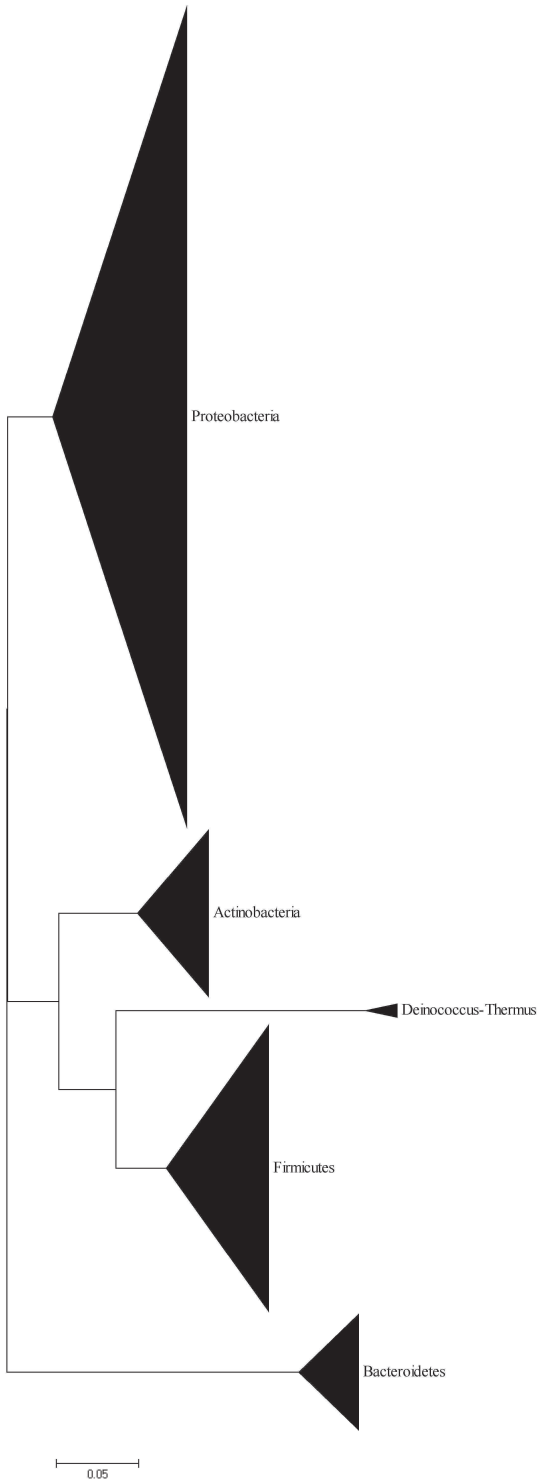

Proteobacteria
Actinobacteria
Deinococcus-Thermus
Firmicutes
Bacteroidetes
0.05

Figure 7

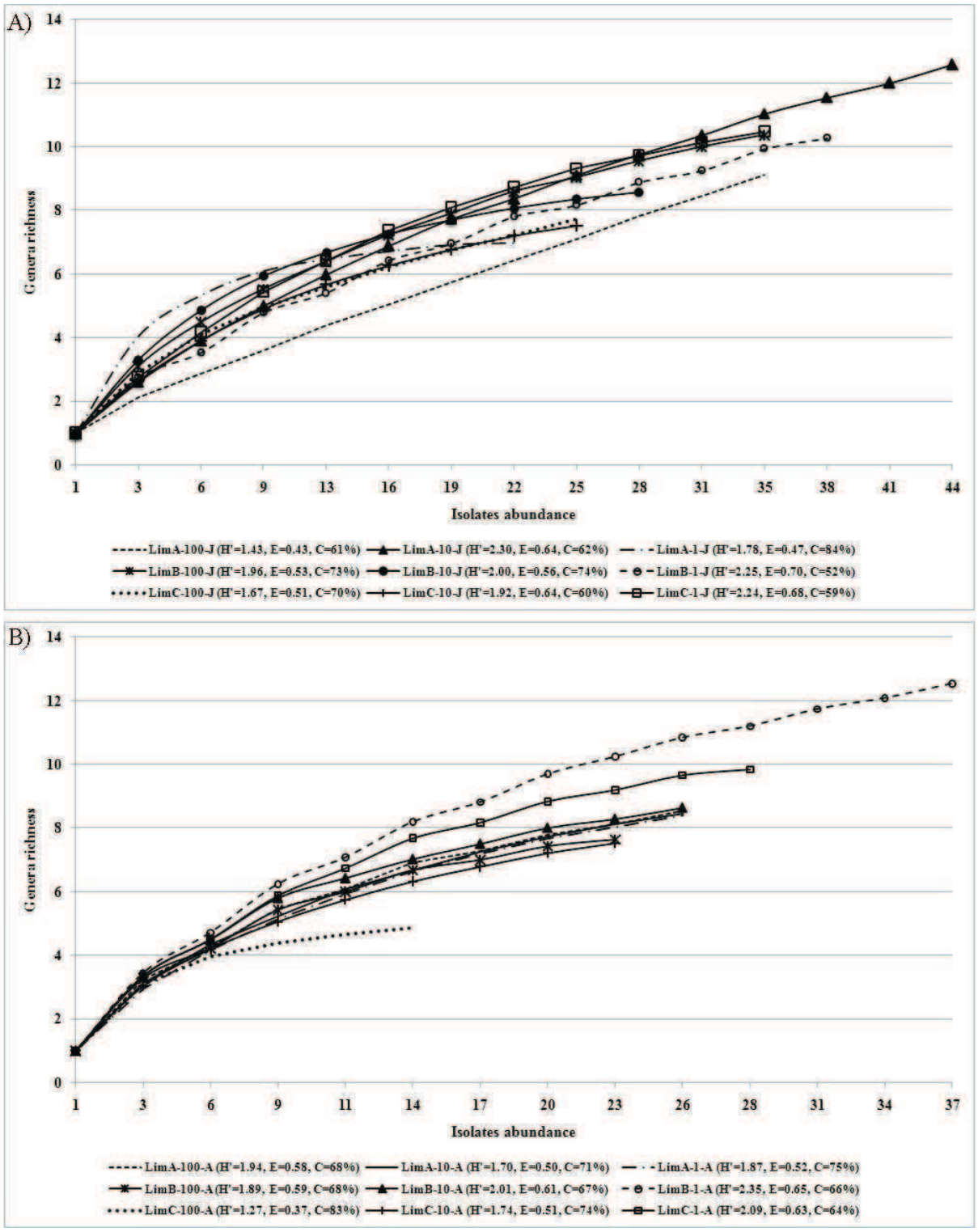

A)
Genera richness
Isolates abundance
LimA-100-J (H'=1.43, E=0.43, C=61%)
LimA-10-J (H'=2.30, E=0.64, C=62%)
LimA-1-J (H'=1.78, E=0.47, C=84%)
LimB-100-J (H'=1.96, E=0.53, C=73%)
LimB-10-J (H'=2.00, E=0.56, C=74%)
LimB-1-J (H'=2.25, E=0.70, C=52%)
LimC-100-J (H'=1.67, E=0.51, C=70%)
LimC-10-J (H'=1.92, E=0.64, C=60%)
LimC-1-J (H'=2.24, E=0.68, C=59%)
B)
Genera richness
Isolates abundance
LimA-100-A (H'=1.94, E=0.58, C=68%)
LimA-10-A (H'=1.70, E=0.50, C=71%)
LimA-1-A (H'=1.87, E=0.52, C=75%)
LimB-100-A (H'=1.89, E=0.59, C=68%)
LimB-10-A (H'=2.01, E=0.61, C=67%)
LimB-1-A (H'=2.35, E=0.65, C=66%)
LimC-100-A (H'=1.27, E=0.37, C=83%)
LimC-10-A (H'=1.74, E=0.51, C=74%)
LimC-1-A (H'=2.09, E=0.63, C=64%)

Figure 8

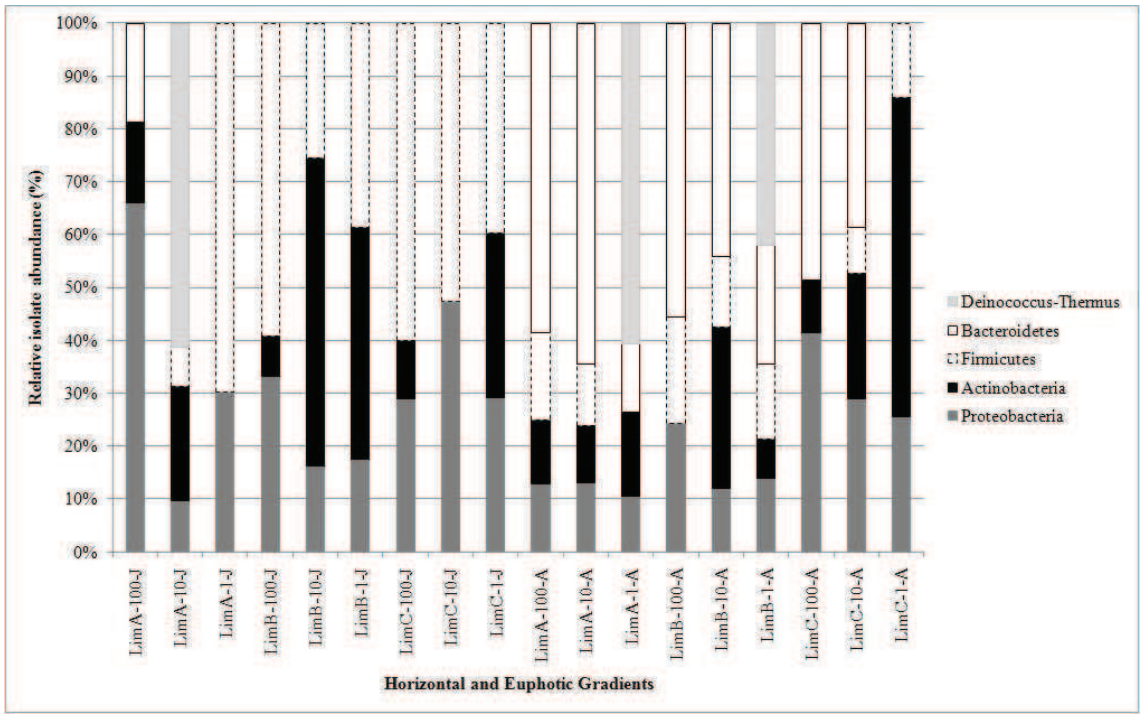

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative isolate abundance (%)
LimA-100-J
LimA-10-J
LimA-1-J
LimB-100-J
LimB-10-J
LimB-1-J
LimC-100-J
LimC-10-J
LimC-1-J
LimA-100-A
LimA-10-A
LimA-1-A
LimB-100-A
LimB-10-A
LimB-1-A
LimC-100-A
LimC-10-A
LimC-1-A
Horizontal and Euphotic Gradients
Deinococcus-Thermus
Bacteroidetes
Firmicutes
Actinobacteria
Proteobacteria

Figure 9

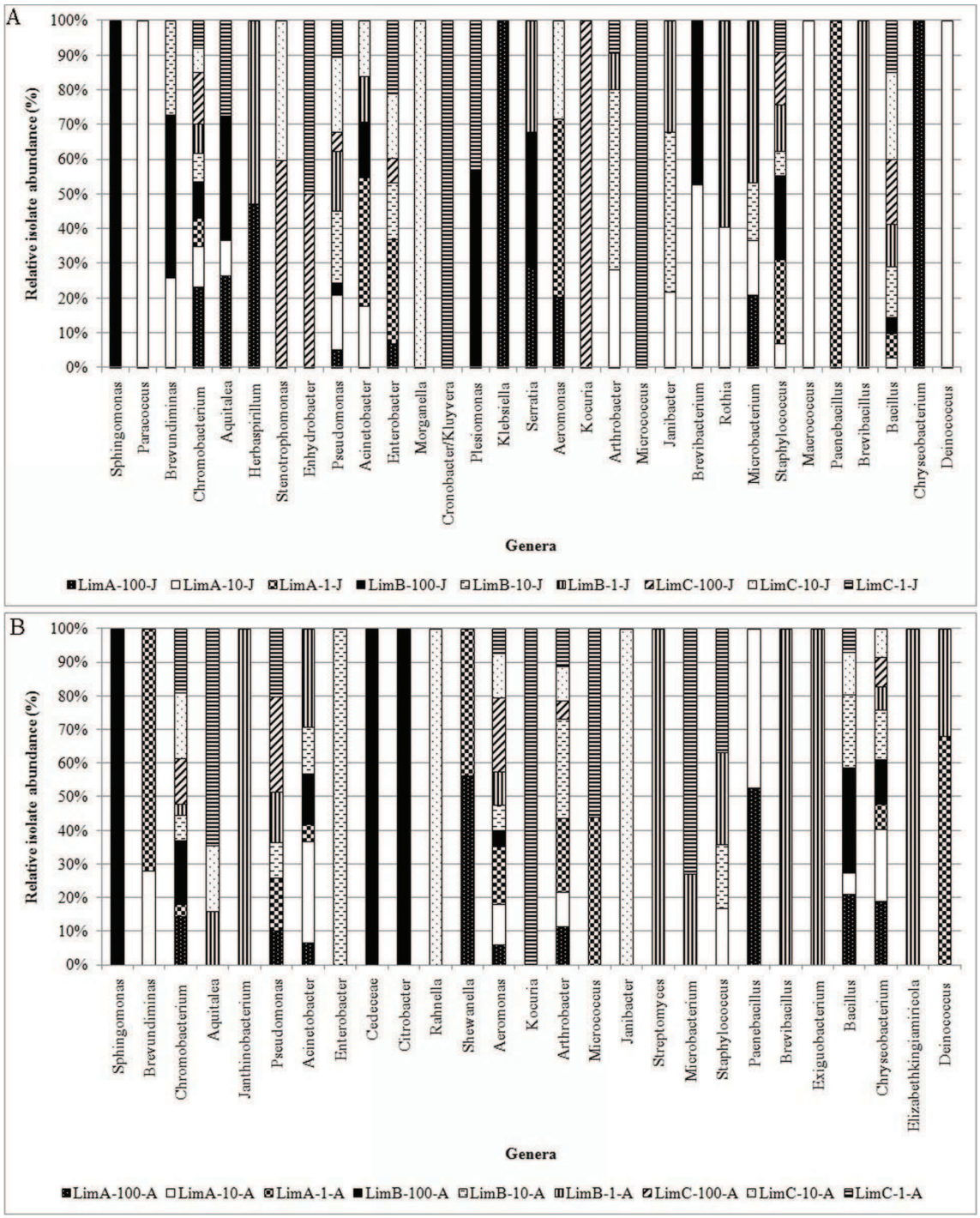

A
Relative isolate abundance (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Sphingomonas
Paracoccus
Brevundimonas
Chromobacterium
Aquitalea
Herbaspirillum
Stenotrophomonas
Enhydrobacter
Pseudomonas
Acinetobacter
Enterobacter
Morganella
Cronobacter/Kluyvera
Plesiomonas
Klebsiella
Serratia
Aeromonas
Kocuria
Arthrobacter
Micrococcus
Janibacter
Brevibacterium
Rothia
Microbacterium
Staphylococcus
Macrococcus
Paenebacillus
Brevibacillus
Bacillus
Chryseobacterium
Deinococcus
Genera
LimA-100-J
LimA-10-J
LimA-1-J
LimB-100-J
LimB-10-J
LimB-1-J
LimC-100-J
LimC-10-J
LimC-1-J
B
Relative isolate abundance (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Sphingomonas
Brevundimonas
Chromobacterium
Aquitalea
Janthinobacterium
Pseudomonas
Acinetobacter
Enterobacter
Cedeceae
Citrobacter
Rahnella
Shewanella
Aeromonas
Kocuria
Arthrobacter
Micrococcus
Janibacter
Streptomyces
Microbacterium
Staphylococcus
Paenebacillus
Brevibacillus
Exiguobacterium
Bacillus
Chryseobacterium
Elizabethkingia miricola
Deinococcus
Genera
LimA-100-A
LimA-10-A
LimA-1-A
LimB-100-A
LimB-10-A
LimB-1-A
LimC-100-A
LimC-10-A
LimC-1-A

Figure 10

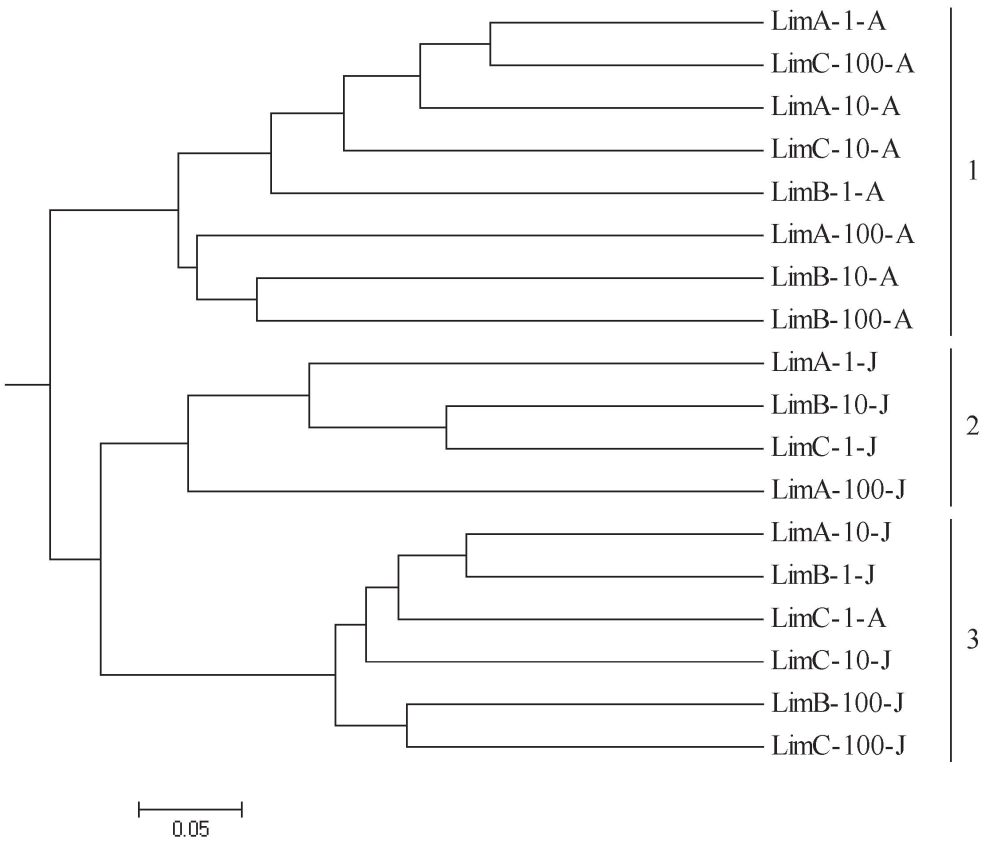
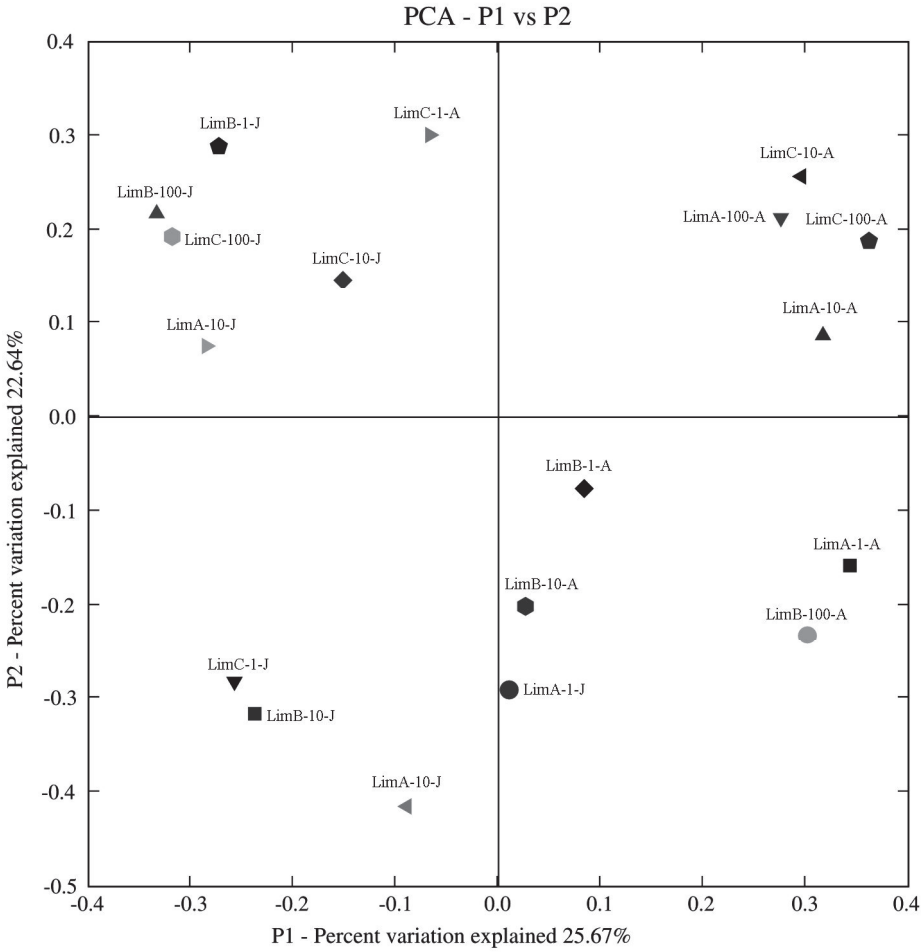

LimA-1-A
LimC-100-A
LimA-10-A
LimC-10-A
LimB-1-A
LimA-100-A
LimB-10-A
LimB-100-A
LimA-1-J
LimB-10-J
LimC-1-J
LimA-100-J
LimA-10-J
LimB-1-J
LimC-1-A
LimC-10-J
LimB-100-J
LimC-100-J
1
2
3
0.05

Figure 11


PCA - P1 vs P2
P2 - Percent variation explained 22.64%
P1 - Percent variation explained 25.67%
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.4
-0.3
-0.2
-0.1
0.0
0.1
0.2
0.3
0.4
LimB-1-J
LimC-1-A
LimC-10-A
LimB-100-J
LimA-100-A
LimC-100-A
LimC-100-J
LimC-10-J
LimA-10-A
LimA-10-J
LimB-1-A
LimA-1-A
LimB-10-A
LimB-100-A
LimC-1-J
LimA-1-J
LimB-10-J
LimA-10-J

CAPÍTULO 3 – Phylogeny of cultivable heterotrophic bacteria derived from mixed colonies

Artigo submetido à revista Archives of Microbiology

Phylogeny of cultivable heterotrophic bacteria derived from mixed colonies

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Abstract

In nature, bacteria interact with each other in ways that do not occur in pure laboratory cultures. However, when bacteria are purified from environmental samples, the resulting colonies occasionally harbor diverse bacterial isolates, which we have termed "associated isolates". Bacteria were obtained from a freshwater lake along euphotic gradients (100%, 10% and 1% light penetration). Surprisingly, 76 of the 1196 resulting colonies harbored between 2 and 5 associated isolates (for a total of 168 associated isolates), and 121 of these associated isolates were recovered as pure cultures. A portion of the isolates (47/168) was unviable after re-streaking, suggesting an inability to survive and reproduce in the absence of their associates. Partial sequencing of 16S rDNA revealed that these isolates were affiliated with *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Deinococcus*-*Thermus*, and represented 26 genera. Colonies harboring associated isolates and those that harbored the largest number of isolates were primarily sampled from the 1% euphotic gradient. Significant differences in the distribution of associated isolates along the euphotic gradient suggest that environmental factors might be driving these associations.

Keywords: associated bacteria, intra-colony, diversity, freshwater, 16S rDNA

Introduction

In the 1880s, Robert Koch and his co-workers established the field of bacteriology with the development of laboratory techniques for isolating organisms in pure culture, and these methods remain essential for studies in microbiology. By isolating microorganisms from their communities, it is possible to focus on their behavior in biologically simple environments such as Petri dishes and test tubes, while ignoring the complex network of interactions that occur in their natural environments. Indeed, microbial communities are dynamic consortia, interacting with other microorganisms and other forms of life (Shapiro and Dworkin 1997; Rudi et al. 2007; Little et al. 2008).

In the natural environment, individual organisms do not exist in isolation. Rather, many microorganisms live in close symbiotic associations, forming consortia whose members depend on one other for existence and therefore cannot be isolated in pure culture. Interactions between microorganisms can be negative (competition, predation and parasitism) or positive (commensalism and mutualism), and these interactions may be related to the environment in which these microorganisms live (Little et al. 2008). Another interaction that occurs between microorganisms in the environment is syntrophy, which is the combination of metabolic processes of two organisms, usually through of the transfer of electrons facilitating a metabolism that is thermodynamically unfavorable. There are many reported cases of microorganisms using metabolites, such as precursors of certain vitamins or amino acids excreted by another organism, to save energy, even if the microorganism can synthesize these compounds (Schink 2002; Ueda and Beppu 2007).

While it is known that bacterial communities contain many different organisms that may all grow together in culture, pure cultures are primarily used to study the physiological and genetic features of a specific type of bacteria. As part of an ongoing effort to investigate bacterial taxa from natural environments, we have used re-streaking from an isolated colony as a strategy for obtaining pure cultures. However, over the years we have noticed that ascertaining the purity of a culture is not always easy, and it is often necessary to go through several stages of re-streaking to obtain pure cultures. Moreover, in many cases, when the physical separation of associated isolates is accomplished, one or all of them does not survive, suggesting their inability to grow in pure culture. Therefore, we performed a detailed molecular and phylogenetic

characterization of isolates derived from a single original colony. These bacteria were collected from a natural freshwater lake in a tropical region.

Methods

Study area and sampling site

Carioca Lake is a natural body of water situated in the middle of the Rio Doce Basin of Brazil. It is located in a Conservation Unit (Parque Estadual do Rio Doce, PERD, 19º29'24''–19º48'18''S and 42º28'18''–42º38'30''W) that is the largest remnant of Atlantic Forest in the state of Minas Gerais. Carioca Lake is mesotrophic, round, shallow (11.8 m of maximum depth) and relatively small, with an area of 14.1 hectares (Bortoluzzi et al. 2004, Bezerra-Neto et al. 2010).

Sampling and bacterial isolation

Water samples (500 mL) across a euphotic gradient in the limnetic (Lim) zone (Carioca Lake) were taken with Van Dorn bottles. Collections took place in June and August 2007, which is the dry season. Samplings were conducted in the water column at different degrees of light penetration (100%, 10% and 1%), as determined by Secchi disk. To assess water conditions, selected physical and chemical variables were measured at three points in the euphotic gradient. Water temperature, pH and dissolved oxygen concentration (DO) were measured *in situ* with a multiprobe (Horiba, model U-22) (Mackereth et al. 1978). Concentrations of total nitrogen (TN), total phosphorus (TP), ammonium nitrogen, nitrite nitrogen, nitrate nitrogen, and soluble reactive phosphorus were measured as previously described (Golterman et al. 1978; Mackereth et al. 1978).

Bacteria were isolated by plating 100 µL of water sample directly on PTYG agar plates (Brown and Balkwill 2009), which were then incubated at 28°C for up to seven days. The PTYG was chosen to allow an overall growth of the culturable aquatic bacteria with different metabolic demands. The resulting colonies were re-suspended in saline, vortexed and repeatedly streaked on the same medium to accomplish their purification. Vortexing was applied to separate randomly-stuck cells before each streaking. Isolates derived from a single original colony that harbored isolates with visually different colony morphologies (size, shape, surface, color, texture and elevation) were named "associated isolates" and were chosen for subsequent molecular analysis. The isolates in this study were named according to the specific euphotic

gradient from which they were retrieved (Lim1, Lim10 and Lim100). Moreover, to facilitate the recognition of associations, isolates derived from a single colony were designated by the same number followed by different letters, e.g., Lim-1-01A and Lim-1-01B.

Statistical analyses were performed with STATISTICA data analysis software, version 7 (www.statsoft.com; StatSoft, Inc., 2004). The Pearson's correlation coefficient was used to test differences between associated isolates and environmental variables. A p value ≤ 0.05 was considered to be statistically significant.

DNA extraction and 16S rRNA gene amplification

Genomic DNA of the isolates was purified as described previously by Dramsi et al. (1995). The complete 16S rRNA gene was amplified by touchdown PCR according to Pontes et al. (2009), using the conserved primer set PA (5'-TCCTGGCTCAGATTGAACGC-3',), modified from Kuske et al. (1997) and U2 (5'-ATCGGYTACCTTGTTACGACTTC-3'), described by Lu et al. (2000).

Amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA was performed to minimize the sequencing of isolates. The 16S rDNA was amplified with primers PA and U2 and then digested separately (according to the supplier's recommendations) with two restriction enzymes (*Nla*IV and *Afl*III; New England Biolabs) that recognize sequences of six nucleotides. Digested DNA was separated in 2% agarose gels in Tris–acetate–EDTA (TAE) buffer. After electrophoresis at 75 V for 2.5 h, the gels were recorded and photographed. The data obtained from ARDRA were converted into binary matrix and the patterns were grouped by UPGMA (unweighted pair group method with arithmetic mean). One to three isolates per ARDRA pattern were subsequently sequenced.

Genomic fingerprinting

Repetitive extragenic palindromic (rep)-PCR genomic fingerprinting was performed using the $(GTG)_5$ primer and $(GTG)_5$-PCR amplification cycling conditions described by Freitas et al. (2008). Products were separated by electrophoresis in 2% agarose gels in 1X TAE buffer for 3 h at 75 V and visualized by staining with ethidium bromide (0.5 mg/mL). Fingerprints were analyzed using BioNumerics version 6.0 software (Applied Maths, St. Martens-Latem, Belgium). Digitized gel images were converted and

normalized using a 1 Kb Plus DNA Ladder (Invitrogen). Similarity between sets of fingerprint patterns was calculated using the pairwise Pearson's product-moment correlation coefficient (r-value; these values are often represented by % similarity where an r-value of 1 is equivalent to 100%). This approach compares the entire densitometric curves of the fingerprints (Hane et al. 1993). Cluster analysis of pairwise similarity values was performed using the UPGMA algorithm.

Sequencing and phylogenetic analysis

The partial 16S rRNA gene sequence was obtained using the primers PA and E926R (5'-CCGICIATTIITTTIAGTTT-3') (Watanabe et al. 2001). Sequencing reactions were performed with a DYEnamic ET Dye Terminator Kit (GE Healthcare) and a MegaBACE 1000 capillary sequencer (GE Healthcare) according to the manufacturers' instructions. The 16S rRNA gene sequences were analyzed, checked for quality, aligned, and edited to produce a consensus using Phred v.0.20425 (Ewing and Green 1998), Phrap v.0.990319 (http://www.phrap.org.) and Consed 12.0 (Gordon et al. 1998) software. To determine the approximate phylogenetic affiliations of our 121 isolates, the 16S rRNA gene consensus sequences were aligned to sequences in GenBank using BLASTN and to sequences in the Ribosomal Database Project (RDP) using Classifier search. Phylogenetic relationships were inferred by MEGA 4 (Tamura et al. 2007) using the neighbor-joining method (Saitou and Nei 1987) and Kimura's 2-P model of sequence evolution. The robustness of the phylogenetic tree topology was evaluated with 1,000 replicates of bootstrap analysis. The nucleotide sequences generated were deposited in the GenBank database with accession numbers HQ234363 to HQ234483.
The haplotypic affiliation of each rRNA 16S gene sequence was inferred by DNAsp v. 5.0 software (http://www.ub.edu/dnasp/). Using the haplotypes generated by DNAsp, median-joining networks (Bandelt et al. 1999) were constructed by NETWORK software (www.fluxus-engineering.com) to investigate phylogenetic relationships and the distribution of haplotypes throughout the euphotic gradient.

Bacterial community analysis

The UniFrac metric method (http://bmf.colorado.edu/unifrac) was used to compare bacterial communities from each euphotic gradient using phylogenetic information (Lozupone et al. 2006). The phylogenetic data were used to compare bacterial communities and statistical differences were tested among all samples by using

UPGMA and principal component analysis (PCA). The cluster environments function of UniFrac was used to determine which environments in the tree had similar bacterial communities, and PCA was used to find the most important axes along which the samples varied. Jackknifing was used to support UPGMA clustering results, and significance tests were also performed, as previously described (Lozupone and Knight 2005).

Results

Abiotic features of the water column

To relate the associated isolates to physical and chemical features of the water column, measurements of temperature, pH, DO and several other chemical variables were made at different points of the euphotic gradient (Table 1). In both samplings, the water column exhibited isothermal conditions. In August, the pH was close to neutral. The maximum difference in pH between points at 100% and 1% in the euphotic gradient was 1.7 in June, while in August this difference was only 0.1. In June, the maximum DO concentration was 9.1 mg/L (100% euphotic gradient) and the difference in DO concentration in the sampling points of the euphotic gradient was 1.8, compared to 1.6 on the later sampling date.

Inorganic phosphorus and nitrogen are limiting nutrients in aquatic environments. In Carioca Lake, the nitrogen and phosphorus ratio was greater than nine. According to Salas and Martino (1991), this ratio indicates that phosphorus was the most limiting nutrient. Additionally, according to the Salas and Martino (1991) model, the lake was classified as mesotrophic for both months.

Associated isolates

The colony forming unit (CFU) counts on PTYG plates indicated that there were 10^3 bacteria per mL of water. No statistically significant difference in CFUs was detected throughout the euphotic gradient in both samplings ($P>0.05$). A total of 1196 colonies, uniformly distributed throughout the euphotic gradient, was screened to obtain pure cultures. Seventy-six of 1196 colonies harbored between two and five isolates with visually different colony morphologies, and a total of 168 isolates was obtained from these colonies. Two or more distinct morphologically isolates derived from a single colony were designated as associated isolates. Forty-seven (derived from 21 colonies)

of 168 isolates were unable to grow in a second subculture, leaving 121 isolates for molecular and phylogenetic characterization. Thirty-seven of these also failed to grow later. Most of the isolates that were unable to grow were retrieved from the 1% euphotic gradient (Fig. S1).

Of the colonies harboring multiple isolates, those with two associated isolates predominated (Fig. S2). Interestingly, colonies harboring two or three associated isolates were scattered across the euphotic gradient, while colonies harboring four or five associated isolates were exclusively from the 1% euphotic gradient.

Identity of associated isolates based on 16S rRNA gene sequence

To avoid sequencing several isolates with identical 16S rDNA sequences, amplicons were digested separately with two restriction endonucleases (*Nla*IV and *Afl*III) and grouped into different ARDRA patterns. A total of 88 distinct patterns was generated, of which 73 were unique (found only once in this study). We then sequenced the 16S rRNA gene fragments (490 bp) of these isolates to determine their identities. Phylogenetic analyses of these sequences revealed that the isolates represented a wide diversity of both gram-positive and gram-negative heterotrophic bacteria. Representatives of five phyla were identified: *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Deinococcus-Thermus* (Figs. S3-S5).

Within these phyla, we identified a variety of genera (Table 2). Phylogenetic analysis of the isolates revealed a predominance of *γ-Proteobacteria* (39.1%) belonging to nine genera: *Enterobacter* (13.3%), *Aeromonas* (10%), *Acinetobacter* (6.6%), *Brevundimonas* (3.3%), *Pseudomonas* (3.3%), *Rahnella* (2.5%), *Moraxella* (2.5%), *Burkholderia* (1.6%) and *Providencia* (0.83%). A great diversity was also found in *Firmicutes*, with representatives of the following genera: *Staphylococcus* (15%), *Bacillus* (9.1%), *Micrococcus* (3.3%), *Paenibacillus* (1.6%), *Exiguobacterium* (1.6%), *Kocuria* (1.6%), *Microbacterium* (0.83%), and *Brevibacillus* (0.83%). The other isolates were represented by the genera *Chromobacterium* (5.8%), *Herbaspirillum* (1.6%) and *Aquitalea* (0.83%) *(β-Proteobacteria*); *Chryseobacterium* (1.6%) (*Bacteroidetes*); and *Arthrobacter* (9.1%), *Janibacter* (0.83%), *Curtobacterium* (0.83%) and *Williamsia* (0.83%) (*Actinobacteria*). The genera *Aquitalea*, *Providencia*, *Brevibacillus*, *Deinococcus*, *Microbacterium*, *Janibacter*, *Curtobacterium* and *Williamsia* were each represented by only one isolate. Of the 26 identified genera, *Staphylococcus, Enterobacter* and *Aeromonas* constituted 38.3% of the isolates.

Of the 55 colonies studied, 42 exhibited unique combinations of associations. Associations that were detected more than once were between members of the same genus (*Enterobacter*, Lim1-12 and Lim1-13; *Arthrobacter*, Lim1-22 and Lim10-51; *Chromobacterium*, Lim1-09 and Lim10-39; *Staphylococcus* Lim1-14, Lim1-45 and Lim1-46) and members of different genera (*Staphylococcus* and *Moraxella*, Lim1-19 and Lim100-24) (Table 2).

Eleven of the 26 genera identified were found associated with the same and different genera (Table 2): *Staphylococcus*, *Brevundimonas Aeromonas*, *Arthrobacter*, *Bacillus*, *Chromobacterium*, *Enterobacter*, *Herbaspirillum*, *Acinetobacter*, *Pseudomonas* and *Paenibacillus alvei* (the only organism found in association only with itself). We also found associations between three different genera that belonged to different phyla (Lim1-06, Lim1-31 and Lim10-49; Table 2). *Proteobacteria* and *Proteobacteria* (17/55) was the most common association, followed by *Proteobacteria* and *Firmicutes* (12/55). Associations between different phyla (*Deinococcus-Thermus* and *Actinobacteria*, *Bacteroidetes* and *Firmicutes*, *Actinobacteria* and *Firmicutes*) were also found.

Comparison of bacterial genera along the euphotic gradient

A total of 26 genera were represented in the associated isolates. The distribution of bacterial genera varied considerably across the euphotic gradient. Samples from the 1% euphotic gradient revealed the highest phylogenetic heterogeneity, with 21 genera identified. The following genera were restricted to only one part of the euphotic gradient: *Providencia*, *Micrococcus*, *Herbaspirillum*, *Aquitalea*, *Pseudomonas*, *Rahnella*, *Paenibacillus* (1% euphotic gradient); *Kocuria*, *Deinococcus*, *Burkholderia*, *Curtobacterium* and *Williamsia* 10% euphotic gradient); and *Microbacterium* and *Brevibacillus* (100% euphotic gradient). In contrast, the genera *Arthrobacter*, *Staphylococcus*, *Bacillus*, and *Rahnella* were found scattered throughout the euphotic gradient.

To compare the phylogenetic compositions of the communities, we used UniFrac to statistically compare sequence sets representative of each community. We used the cluster environments function to compare the communities from each part of the euphotic gradient. Samples were clustered using UPGMA. The communities from each part of the gradient were on a completely different branch, and the 1% euphotic gradient community was more similar to the 100% euphotic gradient community than to the 10% euphotic gradient community (Fig. 1). The robustness of this result was

confirmed by jackknife analysis ($p < 0.001$). Bacterial communities retrieved from the tree points of the euphotic gradient were significantly different, as shown by UniFrac significance tests ($p \leq 0.05$). In PCA analysis, the first principal component separated the 1% and 100% euphotic gradient communities from the bacterial community at 10% (Fig. 2). The highest similarity was between the 1% and 100% euphotic gradient communities.

Haplotypic distribution of associated isolates

To explore the distribution of the associated isolates, we constructed a haplotypic network based on media joining. Each unique combination of nucleotides was considered a distinct haplotype (Table S1). The media-joining network (Fig. 3) illustrates the phylogenetic relationships among the 47 haplotypes and their distribution throughout the euphotic gradient. The haplotypes were distributed into the groups comprising the phyla *Proteobacteria* (22), *Firmicutes* (13), *Actinobacteria* (9), *Bacteroidetes* (2) and *Deinococcus-Thermus* (1). The network contained five clusters. Cluster 1 grouped the phylum *Firmicutes*. Nine haplotypes were unique to a specific euphotic gradient (5, 1 and 3 haplotypes in the 1%, 10% and 100% euphotic gradients, respectively). The remaining four haplotypes (H4, H9, H13 and H21) contained isolates from two (1 % and 10%; 1% and 100%) or three euphotic gradients. There was a larger number of isolates from the 1% euphotic gradient. Cluster 2 assembled haplotypes belonging to the phylum *Actinobacteria*. Seven of the nine haplotypes were exclusive to a single euphotic gradient. Haplotypes H34, H41, H42 and H47 were exclusive to the 10% euphotic gradient, whereas haplotypes H11 and H16 were unique to the 1% gradient and haplotype H23 was unique to the 100% gradient. Two haplotypes occurred in more than one euphotic gradient. Cluster 3 (haplotype H43) belonged to the phylum *Deinococcus-Thermus* and had only one isolate from the 100% euphotic gradient. Cluster 4, which consisted of the phylum *Proteobacteria*, had the large number of haplotypes. Fifteen of the 22 haplotypes were unique to the 1% and 10% euphotic gradients (12 and 3 haplotypes, respectively). There was no haplotype exclusive to the 100% euphotic gradient in this cluster. Cluster 5 assembled haplotypes belonging to the phylum *Bacteroidetes* and was the single phylum that no had representative haplotype from the 100% euphotic gradient. The dominant haplotype, H12, accounted for 93% (13/14) of all *Enterobacter* isolates and appeared in the 1% and 10% euphotic gradients. Most of the haplotypes (72.3%) revealed endemicity to a specific euphotic gradient.

Detection of genomic variability between associated isolates

We used interspersed repetitive sequence PCR to discern genetic relationships among isolates derived from the same original colony. Fingerprints generated by $(GTG)_5$-PCR were composed of 1 to 14 bands of varying sizes (300 bp to 5000 bp) and intensities. Fifty-five dendrograms (46 with two associated isolates, 7 with three associated isolates and 2 with four associated isolates) were generated from $(GTG)_5$ fingerprinting. Overall, the dendrograms revealed that no isolate presented 100% similarity with its associated isolates (Fig. S6).

Discussion

Previous studies have documented the existence of interactions among microorganisms in the natural environment, in which at least one of the participants is dependent on another for survival and reproduction, i.e. mandatory association (Ueda and Beppu 2007; Litle et al. 2008). In the present study, the purification of the bacterial isolates revealed several associations, some of them likely mandatory. The non-viability of some isolates after physical separation could be an indication of their physiological inability to survive and reproduce in the absence of certain associations. This dependence was observed in 27 of 55 colonies harboring associated isolates. In contrast, isolates that were able to grow in pure cultures after physical separation were likely involved in facultative interactions with mutual benefits. According to Schink (2002), such associations can facilitate the production of energy through the exchange of metabolites, allowing organisms to save energy and survive in a hostile environment. It is also possible that the failure of some associated isolates (47/168) to grow in pure culture could be due to a depletion of their endogenous reserves or even to an absence of minimum levels of certain metabolites in the PTYG medium.

Most of the colonies harboring associated isolates and those that harbored the largest number of isolates were derived from the 1% euphotic gradient. This result can be explained by the fact that light is essential in water environments. In a situation when light penetration levels are ideal, phytoplankton produces glucose from CO_2 by photosynthesis and releases their excess exudates (dissolved organic matter), is the cornerstone of the food web. These exudates are then used by heterotrophic bacteria for the maintenance of cell components. By contrast, heterotrophic bacteria liberate orthophosphate from organic phosphate, creating a symbiotic cycle (Pomeroy et al.

2007; Pringault et al. 2009). Alternatively, at 1% light penetration, the cycle is disturbed. The phytoplankton no longer produces exudates because the rate of photosynthesis is low, and they consume everything they produce. Accordingly, those bacteria that live in a microbial loop interact with other bacteria as a strategy to seek other ways to get and exchange energy. This hypothesis is supported by a negative correlation ($p< 0.05$) between DO concentration and the largest number of isolates derived from a single colony.

Analysis of the V2 to V4 variable regions of the 16S rRNA gene allowed us to make an accurate phylogenetic assignment of the associated isolates at the genus level. The phylogenetic tree created from 16S rRNA gene sequences from associated isolates included several bacterial taxonomic groups. Five phyla were identified: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Deinococcus-Thermus* and *Proteobacteria*. This result is in agreement with previous studies of bacterial diversity in freshwater, which have reported the occurrence of these phyla (Hiorns et al. 1997; Berg et al. 2009). Moreover, the dominance of *Proteobacteria* and the relatively small numbers of *Bacteroidetes* and *Deinococcus-Thermus* have also been reported in previous studies (Pontes et al. 2009; Lemke et al. 2009). *Enterobacter*, *Moraxella Staphylococcus* and *Acinetobacter* have also been detected in lentic environments (Berg et al. 2009, Lemke et al. 2009).

In the present study, we selected isolates on the basis of colony morphotype in order to include distinct taxa derived from a single colony; however, this selection method may have imposed some bias because a single bacterial species can exhibit several distinct colony morphologies, and morphologically indistinguishable colonies can contain taxonomically different bacteria. Indeed, phylogenetic analysis of bacterial 16S rRNA gene fragments demonstrated that the re-streaking technique is an effective tool for separating taxa. Nevertheless, analysis of 16S rRNA gene sequences also indicated that some of the isolates, although representing different colony morphotypes, were phylogenetically related. For these reasons, the associated isolates in this study may represent a subset of a larger natural bacterial consortium. The fact that some isolates with identical 16S rRNA gene sequences were found in associations suggests that they might benefit from interactions with organisms with different physiological capacities and ecological niches. In addition, it should be pointed out that some isolates affiliated with the same species, although unable to grow after physical separation, were represented in our culture collection.

To best explore the genetic relationships between associated isolates, we performed $(GTG)_5$-PCR fingerprinting. This genomic fingerprinting technique has been successfully used in several studies on the separation of non-clonal strains and can reveal intraspecies polymorphisms (Tindall et al. 2010). The results obtained from genomic fingerprinting revealed that associated isolates exhibiting identical 16S rDNA sequences exhibited genomic variability, indicating that these isolates are non-clonal. Therefore, the results indicate that the purification treatment was suitable to separate randomly-stuck cells before streaking and to identify truly associated cells.

In conclusion, we have identified diverse taxa, including distinct phyla, among associated isolates derived from mixed colonies. Our genomic fingerprinting analysis indicates that phylogenetically distant bacteria coexist in natural environmental associations. In addition, environmental parameters appear have important effects on the distribution and number of associations. From an ecological perspective, these associations could have a physiological basis, with compounds produced by one member being useful for the other. Thus, the data obtained in the present study are very likely of ecological relevance, and now the challenge is to understand how these bacteria coexist.

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Table1 - Environmental parameters obtained in the water column from Carioca Lake 2007

Parameters	Sampling month					
	June			August		
Light penetration	100%	10%	1%	100%	10%	1%
pH	7.3	6.3	5.6	7.4	7.7	7.3
Temperature (ºC)	23	22.4	22	23.6	23	21.6
DO (mg/l)	9.1	8.7	7.3	8.4	8.3	6.8
TP (µg/L)	18.06	19.09	25.64	25.38	26.76	34.62
PO_4 (µg/L)	1.22	5.87	2.20	1.87	-	3.93
TN (µg/L)	370.90	354.70	404.50	201.35	221.55	365.55
NH_4 (µg/L)	121.85	114.55	112.65	43.11	41.56	122.75
NO_3 (µg/L)	32.23	38.48	32.17	28.54	45.31	42.14
NO_2 (µg/L)	1.71	2.10	1.62	1.58	2.28	1.38

Table 2. Affiliation of associated isolates from each original colony.

Colonies	Affiliation of the associate isolates and accession number in GenBank			
Lim1-01	*Arthrobacter woluwensis* AB244483.1 (99%)	*Acinetobacter* sp. GQ202271.1 (99%)		
Lim1-02	*Arthrobacter woluwensis* AB244483.1 (99%)†	*Aeromonas jandaei* *X74678.1* (99%)†		
Lim1-03	*Staphylococcus epidermidis* *FJ976549.1* (100%)	*Staphylococcus epidermidis* *FJ976549.1* (100%)		
Lim1-04	*Aeromonas* sp. FJ494898.1 (99%)	*Aeromonas jandaei* *X74678.1* (99%)		
Lim1-05	*Aeromonas jandaei* *X74678.1* (100%)	*Enterobacter* sp. GQ247734.1 (99%)	*Aeromonas* sp. FJ494898.1 (96%)†	
Lim1-06	*Aeromonas jandaei* FJ940821.1 (100%)	*Aeromonas popoffii* DQ133182.1 (100%)†	*Staphylococcus* sp. FJ957450.1 (99%)†	*Chromobacterium* sp. AB426118.1 (99%)
Lim1-07	*Aeromonas jandaei* *FJ940821.1* (99%)	*Providencia rettgeri* EU587107.1 (99%)†		
Lim1-08	*Bacillus cereus* GQ344805.1 (99%)	*Bacillus cereus* GQ344805.1 (100%)		
Lim1-09	*Chromobacterium* sp. EU244725.1 (99%)	*Chromobacterium* sp. AB426118.1 (100%)		
Lim1-10	*Micrococcus* sp. GU367133.1 (100%)	*Bacillus* sp. DQ985283.1 (99%)		
Lim1-11	*Enterobacter cloacae* EF185910.1 (99%)	*Enterobacter* sp. GQ247734.1 (99%)		
Lim1-12	*Enterobacter cloacae* EF185900.1 (99%)	*Enterobacter cloacae* EF185900.1 (99%)		
Lim1-13	*Enterobacter cloacae* EF185907.1 (99%)	*Enterobacter cloacae* EF185900.1 (99%)	*Chromobacterium sp.* AY117572.1 (96%)	
Lim1-14	*Staphylococcus sp.* GQ179690.1 (99%)	*Staphylococcus* sp. EF469678.1 (100%)		
Lim1-15	*Herbaspirillum* sp. AF364861.1 (100%)	*Staphylococcus epidermidis* *FJ976549.1* (100%)†	*Herbaspirillum sp.* AF364861.1 (100%)	
Lim1-16	*Janibacter melonis* FJ811878.1 (100%)†	*Pseudomonas stutzeri* *EF587985.1* (99%)		
Lim1-17	*Bacillus macauensis* AY373018.1 (99%)	*Bacillus weihenstephanensis* FN433021.1 (99%)		
Lim1-18	*Bacillus cereus* EU857430.1 (99%)†	*Aquitalea magnusonii* EU548073.1 (99%)		
Lim1-19	*Staphylococcus epidermidis* FJ976549.1 (99%)	*Moraxella osloensis* GQ284472.1 (99%)		
Lim1-20	*Enterobacter cloacae* EF185896.1 (99%)	*Exiguobacterium* sp. EU182850.1 (99%)†	*Enterobacter cloacae* EF185900.1 (99%)	
Lim1-21	*Enterobacter cloacae* EF185910.1 (99%)†	*Enterobacter* sp. GQ247734.1 (99%)		
Lim10-22	*Arthrobacter woluwensis* AB244483.1 (99%)	*Arthrobacter woluwensis* AB244483.1 (99%)		
Lim10-23	*Chromobacterium* sp. AB426118.1 (100%)	*Arthrobacter woluwensis* AB244483.1 (100%)†		
Lim100-24	*Staphylococcus epidermidis* FJ976549.1 (99%)	*Moraxella osloensis* GQ284472.1 (100%)		
Lim100-25	*Staphylococcus* sp. AM988975.1 (99%)	*Microbacterium sp.* AB461113.1 (99%)		
Lim100-26	*Acinetobacter* sp. FJ389742.1 (99%)†	*Acinetobacter* sp. FJ389742.1 (100%)†		
Lim100-27	*Rahnella sp.* EU826664.1 (97%)	*Exiguobacterium* sp. EU182850.1 (99%)		
Lim100-28	*Staphylococcus* sp. GQ179690.1 (100%)†	*Chryseobacterium jejuense* EF591303.1 (98%)		

†Bacteria unable to grow after subculture
Number in parenthesis represents percent identity

Table 2. Continued

Colonies	Affiliation of the associate isolates and accession number in GenBank			
Lim1-29	*Bacillus pumilus* EF528273.1 (96%)	*Aeromonas jandaei* FJ940821.1 (100%)		
Lim1-30	*Acinetobacter sp.* EU260218.1 (99%)†	*Acinetobacter* sp. EU260218.1 (99%)†	*Moraxella osloensis* AF005190.1 (99%)†	*Micrococcus luteus* GQ369519.1 (99%)†
Lim1-31	*Bacillus cereus* GQ844975.1 (99%)†	*Arthrobacter woluwensis* AB244483.1 (100%)†	*Staphylococcus epidermidis* FJ976549.1 (99%)†	
Lim10-32	*Aeromonas popoffii* DQ133177.1 (100%)	*Aeromonas* sp. AM989270.1 (99%)†		
Lim100-33	*Arthrobacter woluwensis* AB244483.1 (99%)	*Bacillus* sp. FJ977607.1 (99%)†		
Lim1-34	*Enterobacter* sp. GQ247734.1 (99%)	*Arthrobacter woluwensis* AB244483.1 (100%)		
Lim1-35	*Pseudomonas aeruginosa* GQ342301.1 (99%)	*Pseudomonas aeruginosa* GQ339107.1 (100%)		
Lim1-36	*Brevundimonas diminuta* FJ843099.1 (99%)	*Brevundimonas diminuta* FJ843099.1 (99%)	*Brevundimonas diminuta* FJ843099.1 (99%)	
Lim10-37	*Arthrobacter woluwensis* AB244483.1 (99%)†	*Kocuria* sp. GQ391989.1 (100%)†		
Lim10-38	*Staphylococcus capprae* Y12593.1 (99%)†	*Rahnella* sp. FJ210846.1 (97%)		
Lim10-39	*Chromobacterium* sp. AB426118.1 (100%)	*Chromobacterium sp.* AB426118.1 (99%)		
Lim10-40	*Micrococcus luteus* GQ369519.1 (99%)†	*Bacillus cereus* GQ344805.1 (100%)		
Lim10-41	*Micrococcus luteus* FJ440960.1 (99%)†	*Staphylococcus conii* DQ870684.1 (99%)		
Lim100-42	*Brevibacillus agri* EF368355.1 (100%)	*Enterobacter* sp. GQ247734.1 (99%)		
Lim1-43	*Acinetobacter* sp. GQ178045.1 (99%)†	*Rahnella sp.* FJ210846.1 (97%)		
Lim1-44	*Paenibacillus alvei* AB377108.1 (99%)†	*Paenibacillus alvei* AB377108.1 (99%)†		
Lim1-45	*Staphylococcus* sp. GQ179690.1 (99%)	*Staphylococcus* sp. GQ179690.1 (99%)		
Lim1-46	*Staphylococcus* sp. GQ179690.1 (99%)	*Staphylococcus* sp. GQ179690.1 (99%)		
Lim1-47	*Bacillus cereus* GQ199742.1 (100%)	*Enterobacter* sp. GQ871449.1 (100%)		
Lim1-48	*Staphylococcus* sp. GQ179690.1 (99%)	*Acinetobacter* sp. FJ389742.1 (99%)		
Lim10-49	*Brevundimonas* sp. EU876670.1 (100%)	*Kocuria* sp. GQ391989.1 (100%)	*Burkholderia cepacia* FJ907187.1 (97%)	
Lim10-50	*Deinococcus* sp. AM988958.1 (99%)†	*Curtobacterium* sp. AJ784400.1 (98%)		
Lim10-51	*Arthrobacter woluwensis* AB244483.1 (99%)	*Arthrobacter woluwensis* AB244483.1 (99%)†		
Lim100-52	*Enterobacter* sp. FJ611880.1 (99%)	*Enterobacter* sp. GQ247734.1 (99%)		
Lim1-53	*Aeromonas popoffii* DQ133182.1 (100%)†	*Pseudomonas* sp. AB461692.1 (99%)†		
Lim1-54	*Acinetobacter* sp. FN435916.1 (99%)†	*Burkholderia cepacia* FJ907187.1 (99%)		
Lim1-55	*Chryseobacterium* sp. AM982789.1 (98%)†	*Williamsia* sp. AB498612.1 (99%)		

†Bacteria unable to grow after subculture
Number in parenthesis represents percent identity

Figure legends

Figure 1. UPGMA cluster environmental analysis, generated by UniFrac, of isolates from the 1%, 10% and 100% euphotic gradients.

Figure 2. Principal component analysis ordination plot for 16S rRNA gene sequences. The percent variation explained by each principal component is indicated on the axis labels.

Figure 3. Media-joining network showing the haplotype distribution into groups according to phyla. Circle size represents the number of isolates present in each haplotype, and circle color represents the location in the euphotic gradient.

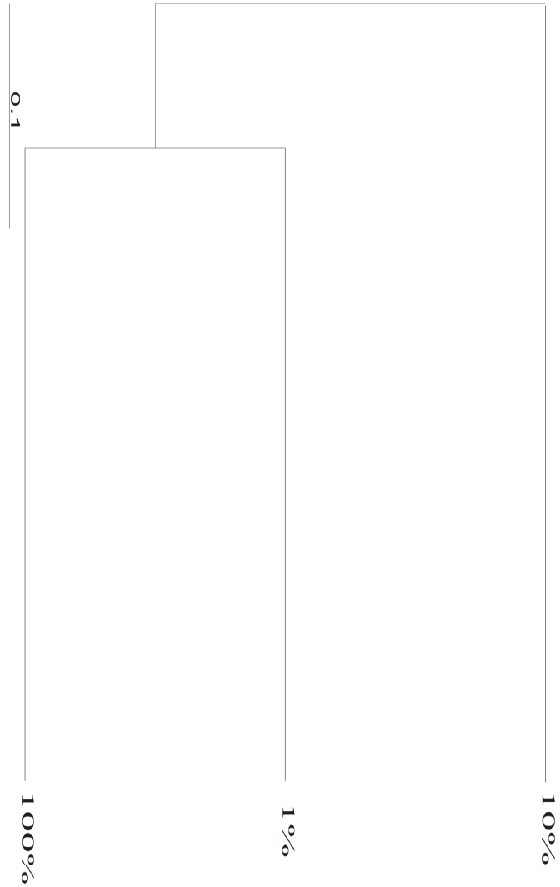

Fig.1 – Costa2011

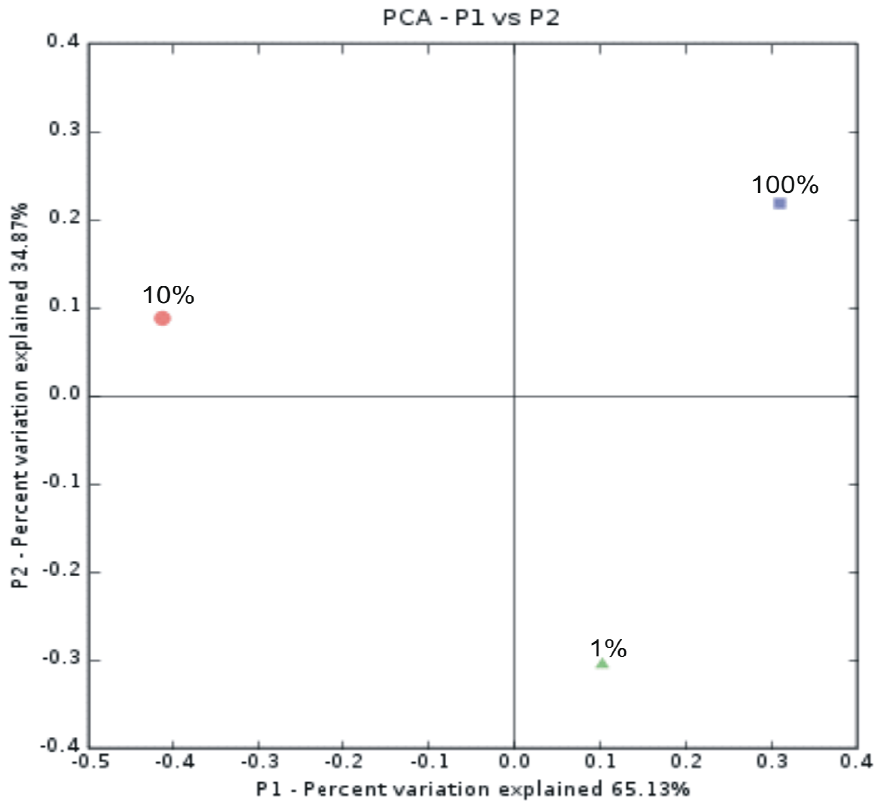

Fig.2 – Costa2011

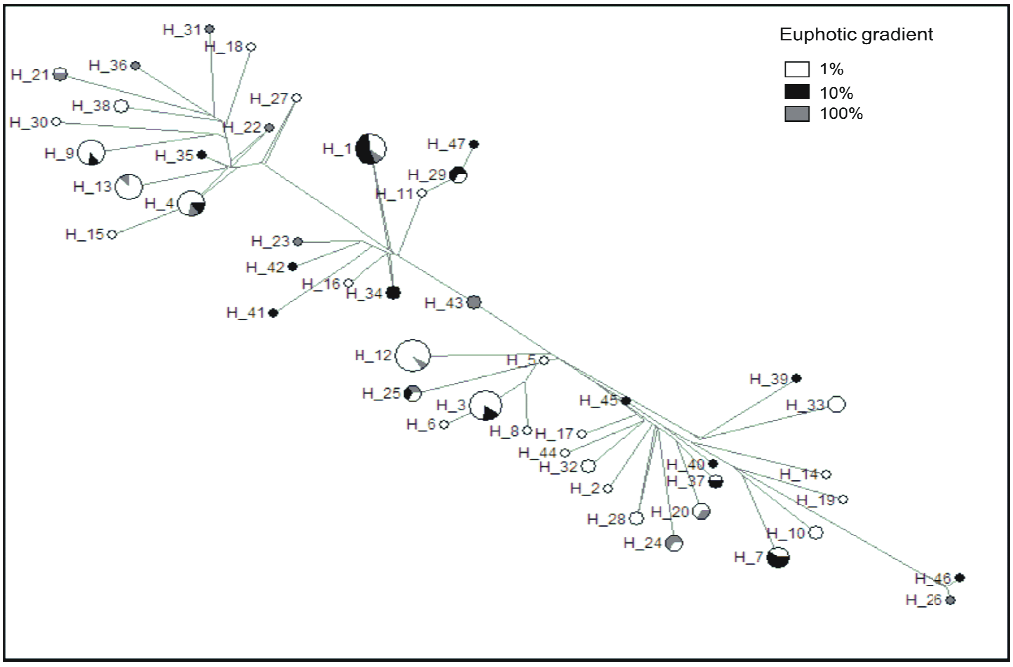

Fig.3 – Costa2011

Supplemental Table S1. Origin and phylogenetic affiliation of haplotypes

Haplotypes	Origin of haplotypes *	Phylogenetic affiliation
H 1	Lim1(4), Lim10(6), Lim100 (1)	*Arthrobacter*
H 2	Lim1(1)	*Acinetobacter*
H 3	Lim1(9), Lim10(2)	*Aeromonas*
H 4	Lim1(5), Lim10(1), Lim100 (1)	*Staphylococcus*
H 5	Lim1(1)	*Enterobacter*
H 6	Lim1(1)	*Aeromonas*
H 7	Lim1(2), Lim10(3)	*Chromobacterium*
H 8	Lim1(1)	*Providencia*
H 9	Lim1(6), Lim10(1)	*Bacillus*
H 10	Lim1(2)	*Chromobacterium*
H 11	Lim1(1)	*Micrococcus*
H 12	Lim1 (12), Lim100(1)	*Enterobacter*
H 13	Lim1(7), Lim100(1)	*Staphylococcus*
H 14	Lim1(1)	*Herbaspirillum*
H 15	Lim1(1)	*Staphylococcus*
H 16	Lim1(1)	*Janibacter*
H 17	Lim1(1)	*Pseudomonas*
H 18	Lim1(1)	*Bacillus*
H 19	Lim1(1)	*Aquitalea*
H 20	Lim1(2), Lim100 (1)	*Moraxella*
H 21	Lim1(1), Lim100 (1)	*Exiguobacterium*
H 22	Lim100(1)	*Staphylococcus*
H 23	Lim100(1)	*Microbacterium*
H 24	Lim1(1), Lim100(2)	*Acinetobacter*

*Number in parenthesis corresponds to number of isolates found in each part of the euphotic gradient.

Supplemental Table S1. Continued.

Haplotypes	Origin of haplotypes	Phylogenetic affiliation
H 25	Lim1(1), Lim10(1), Lim100(1)	*Rahnella*
H 26	Lim100(1)	*Chryseobacterium*
H 27	Lim1(1)	*Bacillus*
H 28	Lim1(2)	*Acinetobacter*
H 29	Lim1(1), Lim10(2)	*Micrococcus*
H 30	Lim1(1)	*Bacillus*
H 31	Lim100(1)	*Bacillus*
H 32	Lim1(2)	*Pseudomonas*
H 33	Lim1(3)	*Brevundimonas*
H 34	Lim10(2)	*Kocuria*
H 35	Lim10 (1)	*Staphylococcus*
H 36	Lim100 (1)	*Brevibacillus*
H 37	Lim1(2)	*Acinetobacter*
H 38	Lim1(2)	*Paenibacillus*
H 39	Lim10 (1)	*Brevundimonas*
H 40	Lim10 (1)	*Burkholderia*
H 41	Lim10 (1)	*Deinococcus*
H 42	Lim10 (1)	*Curtobacterium*
H 43	Lim100 (2)	*Enterobacter*
H 44	Lim1 (1)	*Pseudomonas*
H 45	Lim1(1)	*Burkholderia*
H 46	Lim1 (1)	*Chryseobacterium*
H 47	Lim1 (1)	*Williamsia*

* Number in parenthesis corresponds to number of isolates found in each point of the euphotic gradient.

Supplemental material legends

Supplemental Figure 1. Distribution of the number of associated isolates died, identified and unidentified, throughout the euphotic gradient.

Supplemental Figure 2. Distribution of the number of original colonies that harbored associated isolates.

Supplemental Figure 3a and 3b. Phylogenetic tree of isolates from the 1% euphotic gradient based on 16S rRNA gene sequences. One thousand bootstrap resamplings were used to evaluate the robustness of the inferred trees. *Halobacterium salinarium* was used as an outgroup.

Supplemental Figure 4. Phylogenetic tree of isolates from the 10% euphotic gradient based on 16S rRNA gene sequences. One thousand bootstrap resamplings were used to evaluate the robustness of the inferred trees. *Halobacterium salinarium* was used as an outgroup.

Supplemental Figure 5. Phylogenetic tree of isolates from the 100% euphotic gradient based on 16S rRNA gene sequences. One thousand bootstrap resamplings were used to evaluate the robustness of the inferred trees. *Halobacterium salinarium* was used as an outgroup.

Supplemental Figure 6. Dendrogram showing the genetic relatedness among associated isolates as determined by $(GTG)_5$-PCR fingerprint analysis. Similarity (%) between patterns was calculated using the Pearson coefficient, and data were sorted by UPGMA clustering. Colony Lim1-12, which harbored associated isolates *Enterobacter cloacae*.

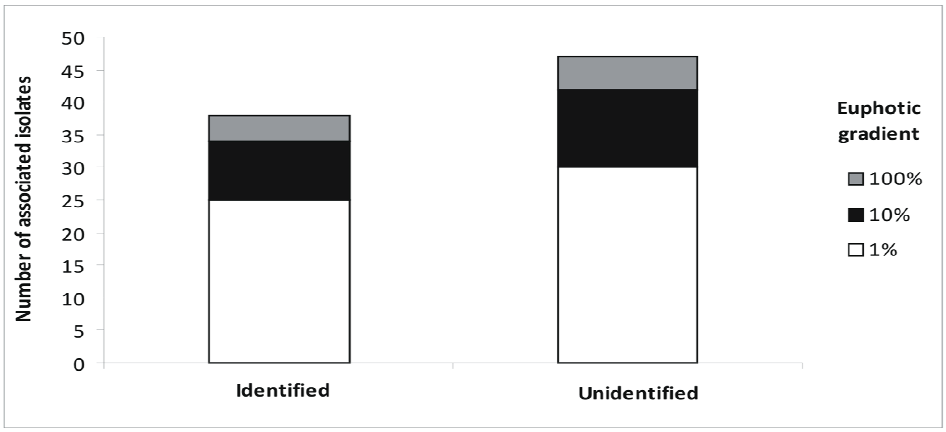

Fig.S1 – Costa2011

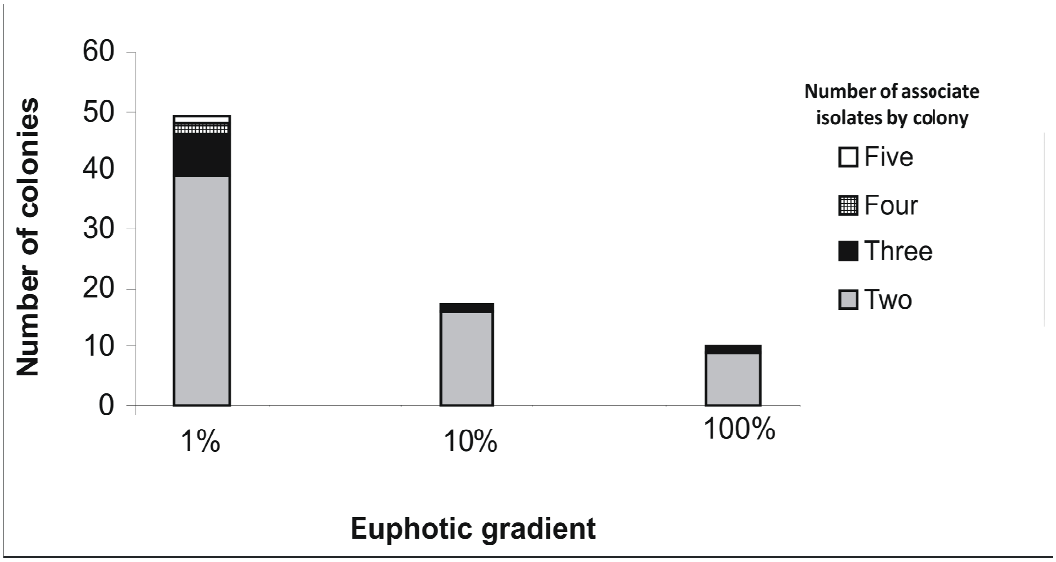

Fig.S2 – Costa2011

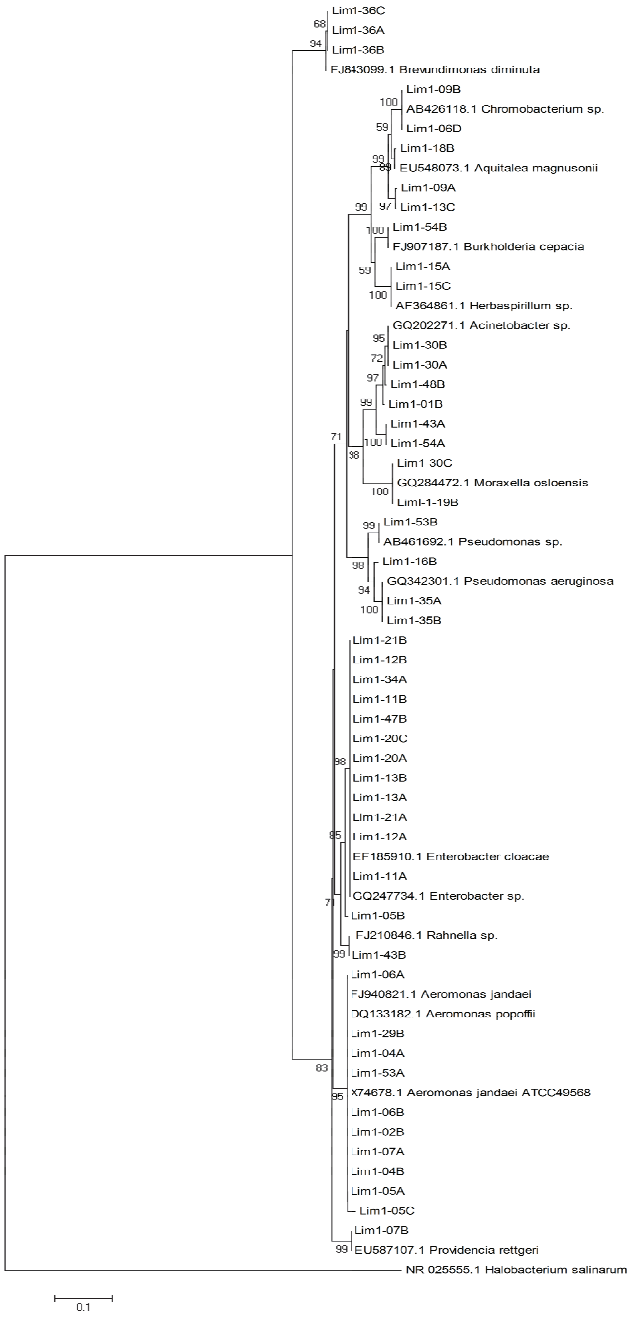

Fig.S3A - Costa2011

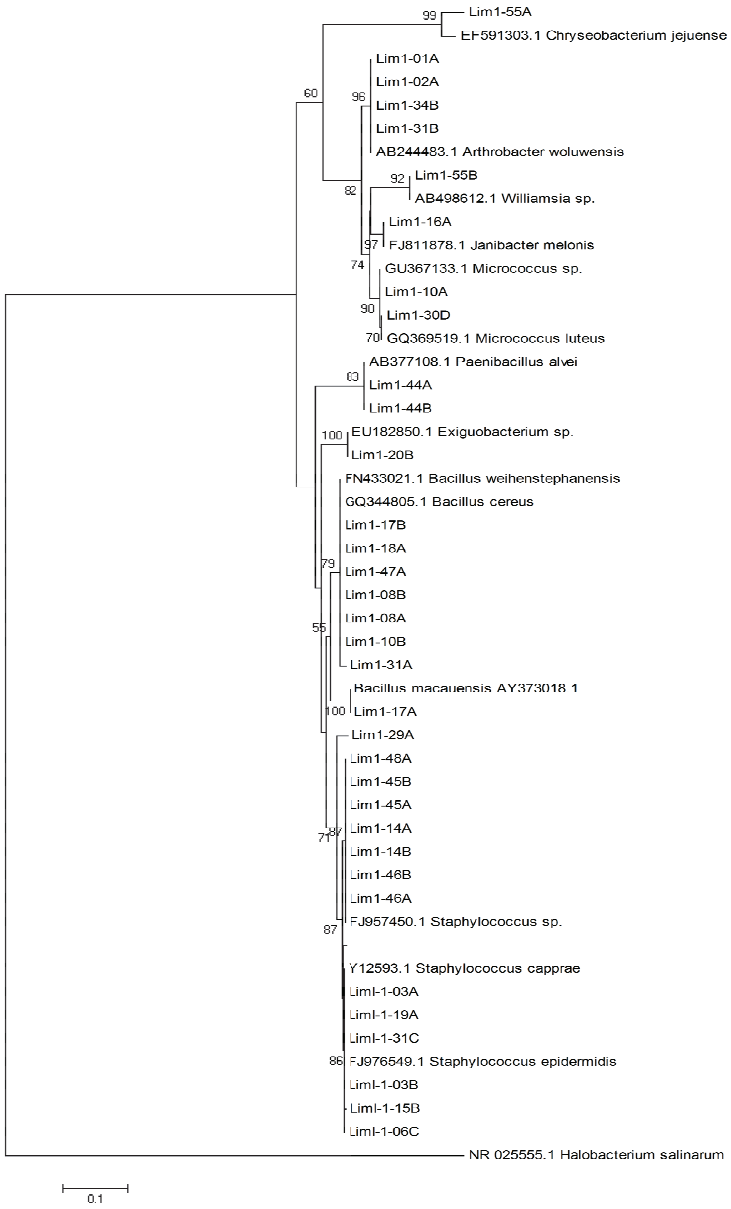

Fig.S3B – Costa2011

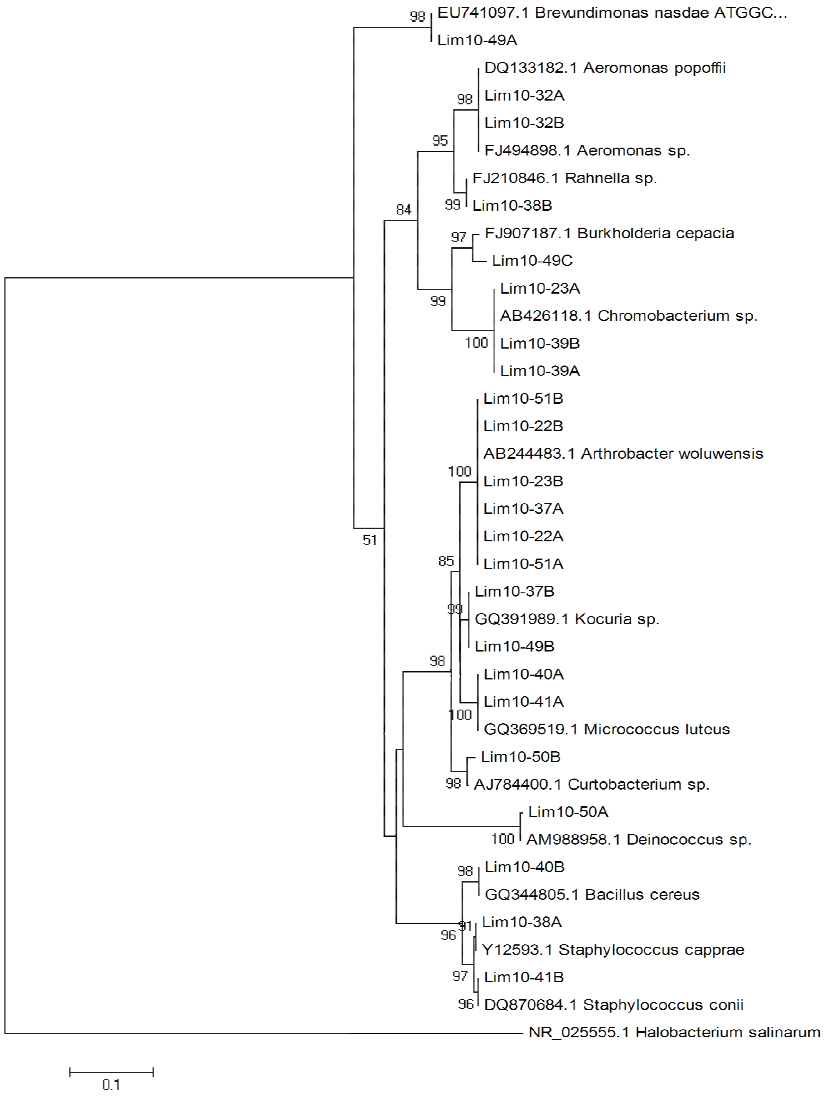

Fig.S4 – Costa2011

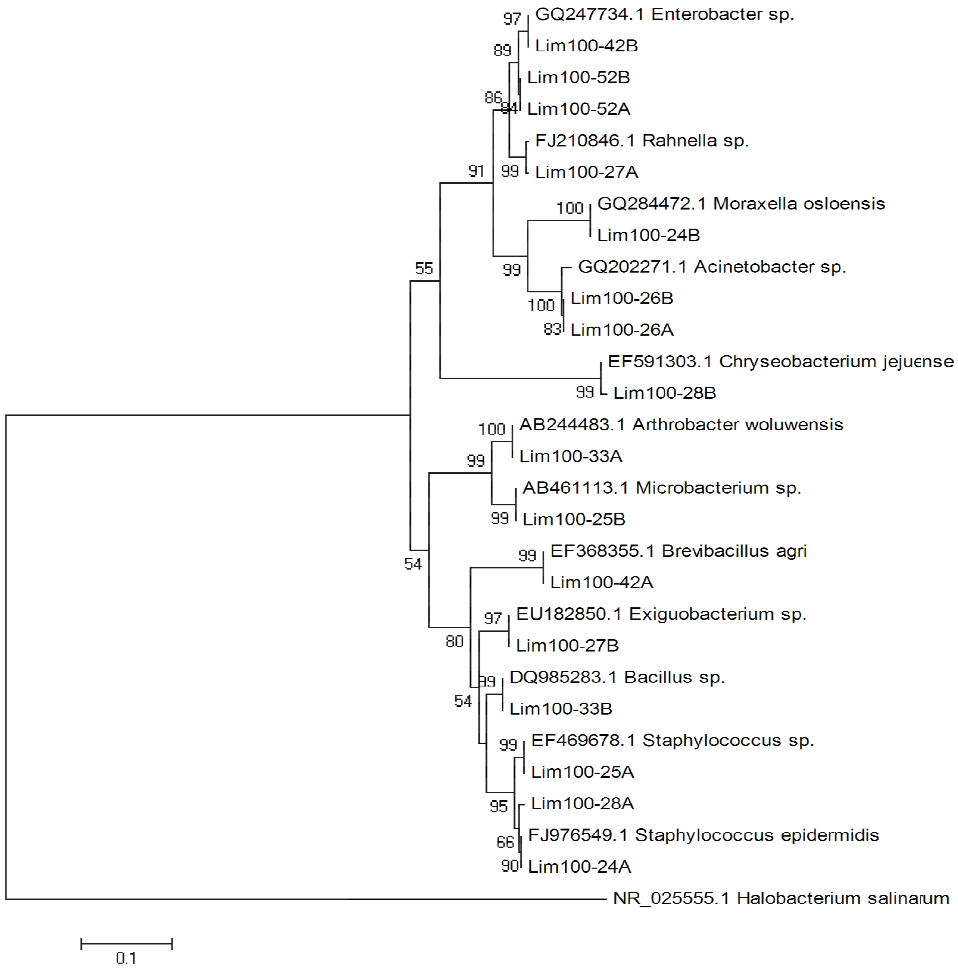

Fig.S5 – Costa2011

Fig.S6 – Costa2011

CAPÍTULO 4 – Characterization of a *Chromobacterium haemolyticum* population from a natural tropical lake

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ORIGINAL ARTICLE

Characterization of a *Chromobacterium haemolyticum* population from a natural tropical lake

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Keywords
BOX-PCR, *Chromobacterium*, freshwater lake, ITS-PCR, tDNA-PCR.

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Abstract

Aim: To study genetic diversity of *Chromobacterium haemolyticum* isolates recovered from a natural tropical lake.
Methods and Results: A set of 31 isolates were recovered from a bacterial freshwater community by conventional plating methods and subjected to genetic and phenotypic characterization. The 16S ribosomal RNA (rRNA) gene phylogeny revealed that the isolates were related most closely with *C. haemolyticum*. In addition to the molecular data, our isolates exhibited strong β-haemolytic activity, were nonviolacein producers and utilized i-inositol, D-mannitol and D-sorbitol in contrast with the other known chromobacteria. Evaluation of the genetic diversity in the 16S rRNA gene, tRNA intergenic spacers (tDNA) and 16S-23S internal transcribed spacers (ITS) unveiled different levels of genetic heterogeneity in the population, which were also observed with repetitive extragenic palindromic (rep)-PCR genomic fingerprinting using the BOX-AR1 primer. tDNA- and ITS-PCR analyses were partially congruent with the 16S rRNA gene phylogeny. The isolates exhibited high resistance to β-lactamic antibiotics.
Conclusion: The population genetic heterogeneity was revealed by 16S rRNA gene sequence, ITS and BOX-PCR analysis.
Significance and Impact of the Study: This study provides for the first time an insight into the genetic diversity of phylogenetically close isolates to *C. haemolyticum* species.

Introduction

The genus *Chromobacterium* consists of six recognized species: *Chromobacterium violaceum* (Bergonzini 1881), *C. subtsugae* (Martin *et al.* 2007), *C. aquaticum* (Young *et al.* 2008), *C. haemolyticum* (Han *et al.* 2008), *C. pseudoviolaceum* and *C. piscinae* (Kämpfer *et al.* 2009). The complete genome of *C. violaceum* has been sequenced, which revealed a potential role in biotechnological applications (Vasconcelos *et al.* 2003). *Chromobacterium violaceum* is the type species of the genus and is commonly found in soil and water of tropical and subtropical regions. This organism produces a violet pigment, violacein; however, *C. aquaticum* and *C. haemolyticum* do not produce violacein. *C. haemolyticum* has a remarkable ability to lyse human and sheep erythrocytes. Interestingly, it was the only isolate that was not recovered from environmental samples, but from a clinical sputum culture (Han *et al.* 2008). Its closest phylogenetic relative is *C. aquaticum* with 98·1% similarity to the 16S rRNA gene. Moreover, some exclusive features of *C. haemolyticum* MD0585^T that allow the differentiation among *Chromobacterium* validly described species are its strong haemolytic activity on sheep blood agar culture, and the utilization of i-inositol, D-mannitol and D-sorbitol (Han *et al.* 2008; Kämpfer *et al.* 2009).

Several micro-organisms have been reclassified with the use of modern molecular biology techniques. For other previously unidentified species, identification was made possible based on the determination of their molecular signatures. Classifying bacteria according to the 16S rRNA gene sequence has been popular for at least the last decade among taxonomists. It allows for rapid and reliable

identification of bacterial isolates, can be used to investigate the population structure and is suitable for phylogeny studies (Ward 2006). Thus, the introduction of 16S rRNA gene analysis changed bacterial taxonomy and systematics, improving the classification of many groups and allowing more confidence in the reconstruction of their natural history (Cohan 2002; Staley 2006). Analyses of tDNA-, internal transcribed spacers (ITS)- and rep-PCR fingerprints have been successfully used to better discriminate closely related bacterial species and can reveal intraspecies polymorphisms (Louws *et al.* 1999; Lanoot *et al.* 2004).

Chromobacterium haemolyticum has been recovered from a clinical sputum culture; nevertheless, no information is yet available on the genotypic or phenotypic characteristics of this species from natural populations and its diversity in the environment. In this study, we sought to gain insight into the genetic and physiological diversity and the phylogeny of isolates related most closely with *C. haemolyticum* recovered from a natural lake of a Conservation Unity.

Materials and methods

Sampling site

Carioca Lake is a natural body of water situated in the middle of the Rio Doce basin of Brazil. It is located in a Conservation Unity (Parque Estadual do Rio Doce, PERD, 19°29′24″-19°48′18″S and 42°28′18″-42°38′30″W, Fig. S1), which is the largest remnant of the Atlantic Forest in the state of Minas Gerais. The region climate is tropical humid, and the rainy season extends from October to March, with average annual rain precipitation during these months of 1500 mm the lesser precipitation volume (1000 mm) occurs in July, dry season (Tundisi 1997). Carioca Lake is round, shallow (11·8 m of maximum depth) and relatively small with an area of 14·1 hectares (Bezerra-Neto *et al.* 2010).

Bacterial isolation

As part of an ongoing effort to investigate bacterial taxa in undisturbed environments, we have primarily identified bacterial communities by their 16S rRNA gene sequence.

In this study, the sample was collected in the dry season during June 2007. Water samples, in triplicate, from the limnetic zone were collected with disinfected Van Dorn bottle, transferred to sterilized 500-ml glass bottle and subsequently analysed in the laboratory. The water was collected at a depth of 3 m, which corresponds to penetrance of 1% light determined by a Secchi disc. Water temperature, pH and dissolved oxygen concentration (DO) were measured *in situ* with a multiprobe (Horiba, model U-22) (Mackereth *et al.* 1978). Concentrations of total nitrogen (TN), total phosphorus (TP), ammonium nitrogen (NH_4), nitrite nitrogen (NO_2), nitrate nitrogen (NO_3) and soluble reactive phosphorus (PO_4) were measured as previously described (Golterman *et al.* 1978; Mackereth *et al.* 1978).

Aliquots of 0·1 ml of undiluted sample were plated directly on PTYG agar (0·5% peptone, 0·5% tryptone, 0·5% yeast extract, 1·0% glucose, 0·06% $MgSO_4$, 0·006% $CaCl_2$, 1·5% agar) and incubated at 28°C for up to 7 days. The bacterial isolates were purified by restreaking on the same medium. The *C. violaceum* ATCC 12472^T type species was included as a reference strain in all analyses.

DNA extraction and 16S ribosomal RNA gene amplification

Total genomic DNA was extracted from each isolate as described elsewhere (Sambrook and Russel 2001). The 16S rRNA gene was PCR-amplified using the primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGHTACCTTGTTACGACTT-3′; Martin-Laurent *et al.* 2001). PCR mixtures (20 μl total volume) consisted of 0·4 mmol l^{-1} of each dNTP, 0·5 μmol l^{-1} of each primer, 0·5 units of Taq DNA polymerase (Fermentas, Belo Horizonte, MG, BR) and 40 ng of bacterial DNA. The thermal cycling conditions consisted of a period at 95°C for 10 min followed by 30 cycles of 30 s at 95°C (denaturation), 40 s at 48°C (annealing) and 2 min at 72°C (extension). The final extension step was 15 min at 72°C.

Sequencing and phylogenetic analysis

Sequencing reactions were performed using standard protocols of the DYEnamic ET dye terminator kit (GE Healthcare, Piscataway, NJ) and the MegaBACE 1000 capillary sequencer (GE Healthcare). Forward and reverse sequencing reactions were repeated at least three times for every bacterial isolate. The 16S rRNA gene sequences were checked for quality, aligned and analysed using Phred ver. 0.20425 (Ewing and Green 1998), Phrap ver. 0.990319 (Gordon *et al.* 2001) and Consed 12.0 (Gordon *et al.* 1998) software. Phylogenetic analysis was inferred by Mega 4 software (Tamura *et al.* 2007) using the minimum evolution method to calculate trees from Kimura 2P distances. One thousand bootstrap resamplings were used to evaluate the robustness of the inferred trees. The DnaSP ver. 5 software was used for haplotype analysis (Librado and Rozas 2009). Additional 16S rRNA gene sequences of *C. violaceum* (AE016825 and AY117553), *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 outgroups. Nucleotide sequences that were generated were deposited in the GenBank database with accession numbers GU997701 to GU997731.

tDNA-PCR, ITS-PCR and BOX-PCR DNA fingerprinting

The primers and the amplification conditions for the tDNA-, ITS- and BOX-PCR were in accordance with Freitas *et al.* (2008a). Products were separated by electrophoresis in 2·5% agarose and 1X TBE (100 mmol l^{-1} Tris–HCl, 90 mmol l^{-1} boric acid, 1 mmol l^{-1} Na_2EDTA, pH 8·0) running buffer for 3·5 h at 65 V. Gels were visualized by staining with ethidium bromide (0·5 mg ml^{-1}). The fingerprints were analysed using BioNumerics version 6.0 software (Applied Maths, St Martens-Latem, Belgium). Digitized gel images were converted and normalized using the 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA). The ITS- and BOX-PCR patterns were analysed separately. Similarity between sets of fingerprint patterns was calculated using the pair-wise Pearson's product–moment correlation coefficient (*r*-value; these values are often represented by % similarity where an *r*-value of 1 is equivalent to 100%). This approach compares the whole densitometric curves of the fingerprints (Hane *et al.* 1993). Cluster analysis of the pair-wise similarity values was performed using the UPGMA algorithm. The reproducibility of the fingerprint patterns was assessed in at least three separate experiments.

Phenotypic analysis

Nutrient agar medium with 5% sheep blood was used for haemolysis testing; a clear zone around the colony indicates β-haemolysis. The physiological characterization was made as described by Kämpfer *et al.* (1991). The minimum inhibitory concentration (MIC) was determined by the agar dilution method in Mueller–Hinton medium (MH; Difco Laboratories, Franklin Lakes, NJ). Several antimicrobial agents were tested as representatives of important classes: ampicillin, amoxicillin-clavulanic acid, tetracycline, chloramphenicol, amikacin, gentamicin and ciprofloxacin. All antimicrobials were obtained from Sigma Chemical Co., and mercury was obtained from Merck Co. The data were interpreted according to MIC breakpoints, as recommended by the Clinical and Laboratory Standards Institute (2005) for *Pseudomonas aeruginosa* and other non-Enterobacteriaceae.

Results

Abiotic features

In the sampled period, the water column was thermally unstratified (temperature average 22°C) and slightly acid water (pH 5·6). The dissolved oxygen at 1% of light penetration was 7·3 mg l^{-1}. Inorganic phosphorus and nitrogen are limiting nutrients in aquatic environments. In Carioca Lake, the phosphorus was the most limiting nutrient (TP = 25·64 μg l^{-1}, PO_4 = 2·2 μg l^{-1}), whereas the nitrogen presented higher values (TN = 404·5 μg l^{-1}, NO_2 = 1·62 μg l^{-1}, NO_3 = 32·17 μg l^{-1}, NH_4 = 112·65 μg l^{-1}). According to the Salas and Martino (1991) model, the lake was classified as mesotrophic.

Isolation and isolate identity based on 16S rRNA gene sequence

The colony-forming unit (CFU) counts on PTYG plates indicated that there were 10^3 bacteria per ml of water. In this study, the 16S rRNA gene sequences used for phylogenetic analysis were approximately 551 nucleotides long and spanned the V2 to V5 variable regions corresponding to the *C. violaceum* ATCC 12472T 16S rRNA gene. The 16S rRNA gene sequences of the 31 isolates were 99·6% similar to *C. haemolyticum* MDA0585 (GenBank accession no. DQ785104). For other species of this genus, similarity ranged from 96 to 98·5% (Fig. 1). This phylogenetic assignment was strongly supported by the tree topology generated by the minimum evolution method (Fig. 1). The phylogenetic tree formed from the almost complete 16S rRNA gene sequences (1237 bp) of six isolates showed monophyletic relationships among the isolates and *C. haemolyticum* (Fig. S2). Moreover, these isolates were 99·7% similar to *C. haemolyticum*. Of these isolates, 28 were distributed into four haplotypes (H1, H3, H5 and H7) with identical 16S rRNA gene sequences. The haplotypes H2, H4 and H6 (CA2·42, CA2·45 and CA2·35, respectively) were found only once in this study. The haplotypic diversity indicated by the DnaSP software (Librado and Rozas 2009) was 0.64.

tDNA-, ITS- and BOX-PCR fingerprinting analyses

To evaluate whether the genetic heterogeneity observed in the isolates through 16S rRNA gene sequence analyses was consistent in other conserved genomic regions, we performed tDNA-, ITS- and BOX-PCR fingerprinting. The results of tDNA-PCR fingerprinting of the analysed isolates were identical (data not shown), and the same banding pattern with amplicon lengths

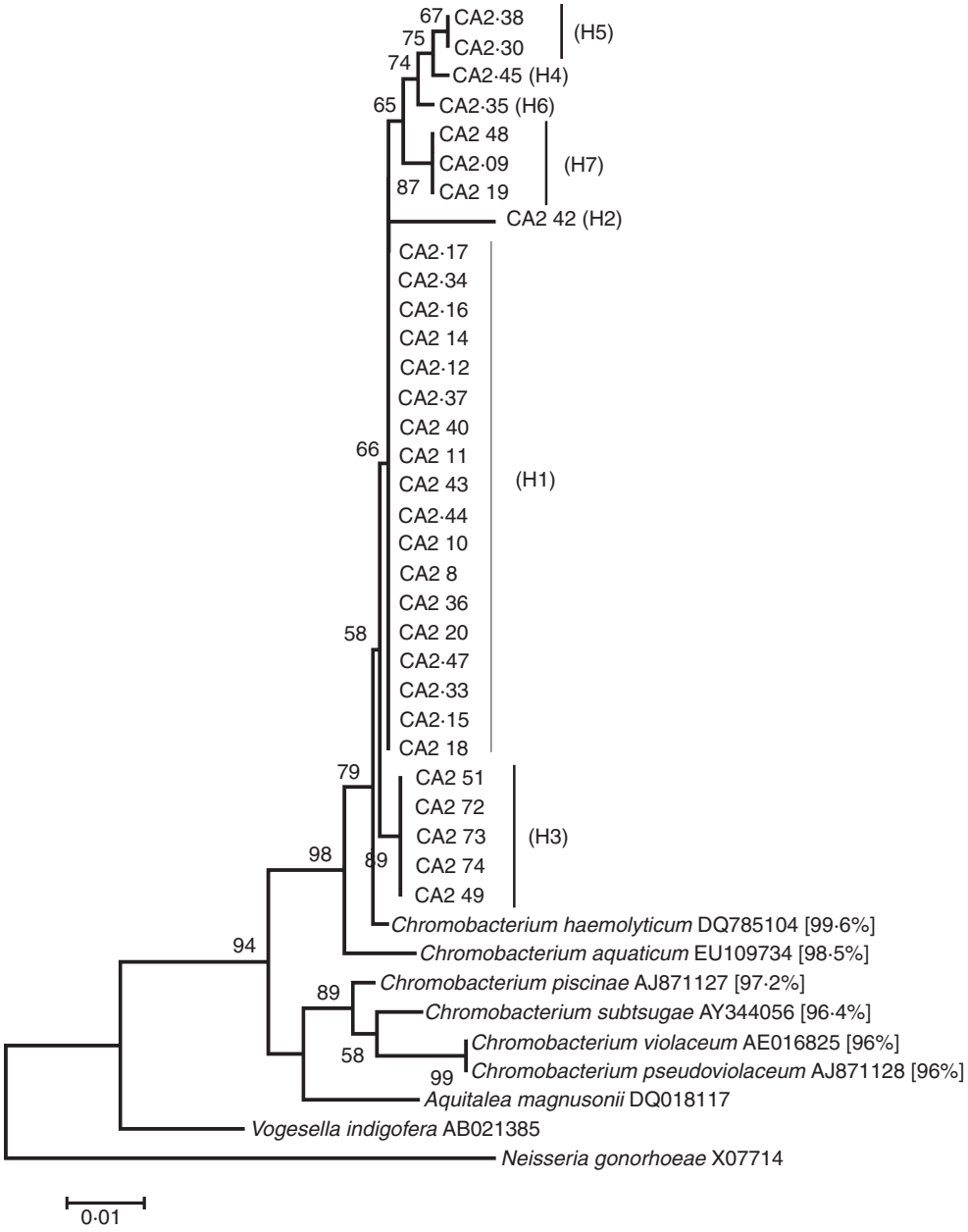

Figure 1 Phylogenetic tree based on 16S rRNA gene sequences showing *Chromobacterium haemolyticum* isolates and other *Chromobacterium* species. One thousand bootstrap resamplings were used to evaluate the robustness of the inferred trees. The haplotypes are represented in parentheses. Numbers in brackets correspond to the average similarity values between isolates and *Chromobacterium* species. *Aquitaleae magnusonii* (DQ018117), *Vogesella indigofera* (AB021385) and *Neisseria gonorrhoeae* (X07714) were used as outgroups.

ranging from 100 to about 1000 bp was observed. However, this pattern differed from that obtained for the *C. violaceum*.

For a better resolution in ITS-PCR fingerprinting cluster analysis, a similarity value (r) of 0·99 was employed resulting in 17 distinct patterns with the number of bands ranging from 3 to 10 (Fig. 2). ITS-PCR amplifications were negative for three isolates (CA2·36, CA2·49 and CA2·73) in three independent experiments. Within each cluster, the isolates generally exhibited a high degree of similarity (Fig. 2).

In addition to the genetic approaches described earlier, the intraspecific diversity of the isolates was analysed by BOX-PCR genomic fingerprinting. BOX-PCR for the 31 isolates yielded genomic fingerprints consisting of 1–11 amplified bands of varying intensity. The simi-

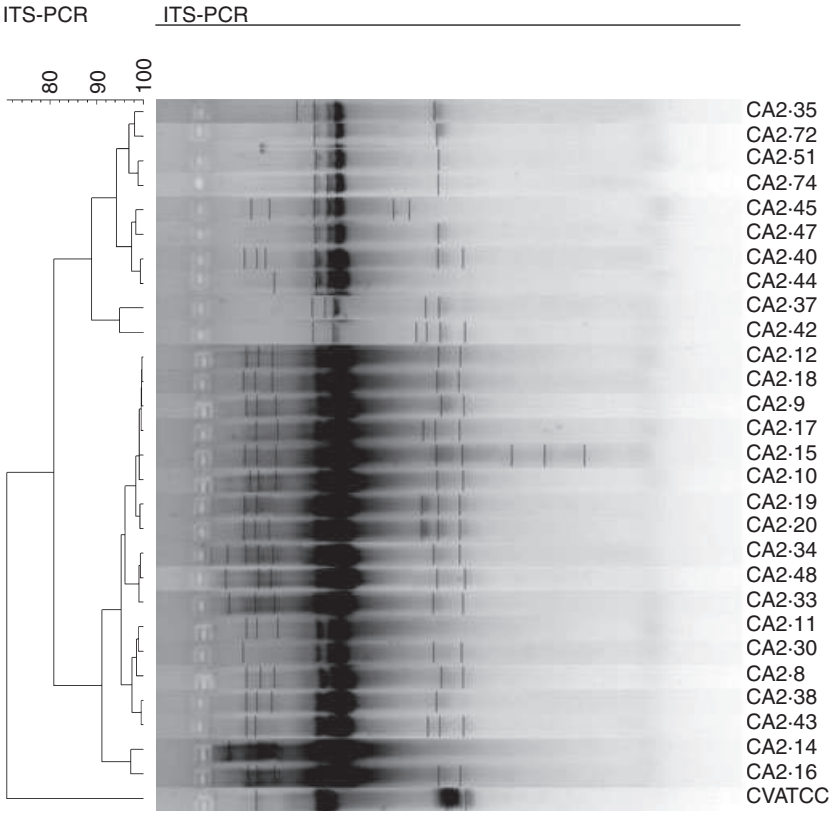

Figure 2 Dendrogram showing the genetic relatedness among *Chromobacterium haemolyticum* isolates and *C. violaceum* ATCC12472^T based on the ITS-PCR fingerprint. Similarity (%) between patterns was calculated using the Pearson coefficient. The data were sorted with the UPGMA clustering method.

larity value (r) for BOX-PCR fingerprinting analysis was 0·9, and this generated 15 patterns. Cluster analysis revealed three distinct clusters (Fig. 3). The first cluster was partially congruent with haplotype 1 as detected by the 16S rRNA gene sequence analysis. The type species presented unique patterns in these three fingerprinting analyses.

Phenotypic characterization

In the haemolytic assay, a clear zone around the colony revealed that the isolates presented β-haemolytic activity on sheep blood agar culture (Fig. S3). The biochemical tests revealed that the isolates were positive for i-inositol, D-mannitol, D-sorbitol, citrate, glucose fermentation and catalase, whereas were negative for mannose and indole production. The isolates were characterized for their antimicrobial resistance phenotype. The isolates were resistant to ampicillin (100%), amoxicillin-clavulanic acid (100%), tetracycline (100%), chloramphenicol (29%), amikacin (100%) and gentamicin (16%) and sensitive to ciprofloxacin (100%). All isolates exhibited the highest MIC to ampicillin, amoxicillin-clavulanic acid (1024 μg ml^{-1}) and were inhibited by ciprofloxacin at the lowest concentrations tested (2 μg ml^{-1}).

Discussion

Sequence analysis of 16S rRNA genes was effective in identifying the phylogenetic affiliation of our isolates in *C. haemolyticum* and revealed genetic heterogeneity among the isolates as shown by the haplotypic diversity. This species was detected in low density (0·3 CFU ml^{-1}) in the Carioca Lake. In addition to the molecular data, our isolates exhibited strong β-haemolytic activity, are nonpigmented and utilize i-inositol, D-mannitol and D-sorbitol in contrast with the other known chromobacteria.

Although the tDNA and ITS regions are more variable than the 16S rRNA gene, they are still considered to be highly conserved genomic regions. PCR-generated polymorphic bands usually provide resolution at the desired taxonomic level allowing the discrimination between two species by producing species-specific patterns (Louws

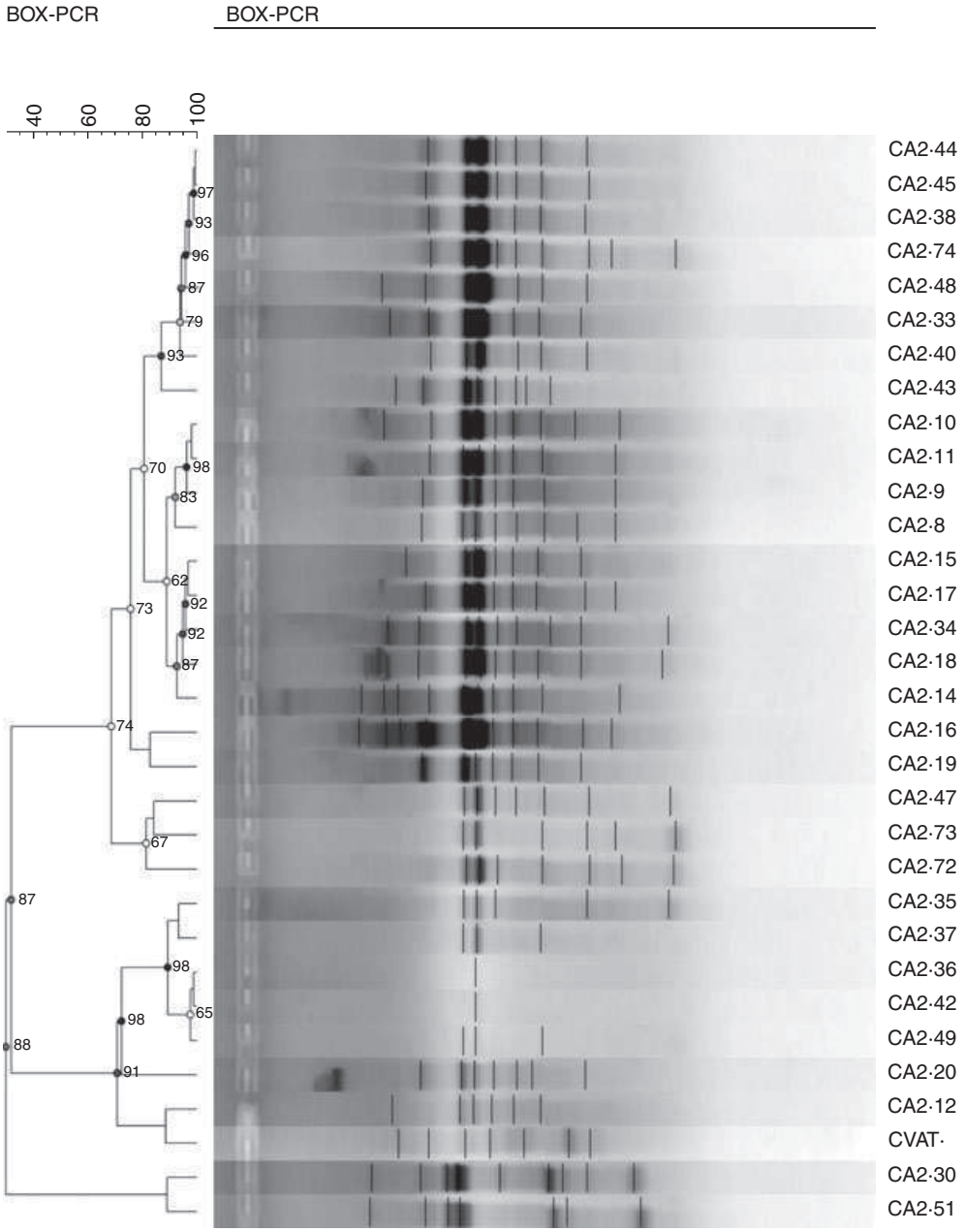

Figure 3 Dendrogram showing the genetic relatedness among *Chromobacterium haemolyticum* isolates and *C. violaceum* ATCC12472^T as determined by BOX-PCR fingerprint analysis. Similarity (%) between patterns was calculated using the Pearson coefficient. The data were sorted with the UPGMA clustering method.

et al. 1999; Bonizzi *et al.* 2007). Thus, tDNA- and ITS-PCR have been successfully employed for bacterial identification, determination of intraspecies variation and characterization of environmental samples. tDNA-PCR revealed a single pattern within the *Chromobacterium* sp. population. By contrast, the ITS-PCR revealed intragenomic diversity and was also efficient in differentiating *C. violaceum* from isolates.

The use of BOX-PCR for analysing bacterial genomes has proven to be a reliable fingerprinting tool for studying microbial diversity, ecology and evolution (Ishii and Sadowsky 2009). We found that isolates with the same partial 16S rRNA gene sequence often had quite dissimilar BOX-PCR patterns. Therefore, the BOX-PCR analysis was effective in detecting genetic diversity. On the other hand, a correlation between the results of BOX-PCR and

16S rRNA analysis was not detected; the BOX-PCR analysis grouped *C. violaceum* with the isolates. Our data are in agreement with studies carried out on a variety of different bacterial genera and species, which have revealed that the correlation with the classification based on the 16S rRNA sequences are not always identical (Hungria *et al.* 2005; Freitas *et al.* 2008b).

Because antimicrobial resistance is recognized as a worldwide clinical problem, and because the *C. haemolyticum* type strain was recovered from clinical sputum culture, the antimicrobial susceptibility of a natural *C. haemolyticum* population was investigated. The isolates in our study exhibited high resistance to β-lactamic. Similar results were reported by Lima-Bittencourt *et al.* (2007a, 2011) in environmental *Chromobacterium* species isolates. Moreover, other studies have shown that resistance to antimicrobials, particularly of the β-lactam class, is common in environmental isolates from undisturbed environments (Ash *et al.* 2002; Lima-Bittencourt *et al.* 2007b; Pontes *et al.* 2009). It is interesting to note that the genome of the type species contains genes related to β-lactam- and multidrug-resistance (Fantinatti-Garboggini *et al.* 2004) and that both species (*C. violaceum* and *C. haemolyticum*) are phylogenetically close.

The results of this study provide the first insights into the genetic diversity and distribution of this bacterium and contribute to its microbial ecology.

Emended description of *Chromobacterium haemolyticum*

The description is based on that provided by Han *et al.* (2008), with the following amendments. It occurs in undisturbed natural lake (Atlantic Rain Forest, Brazil). Colonies on PTYG agar are grey, flat and present irregular borders. In addition, the isolates showed to be positive for arginine dihydrolase, gelatinase, and negative for β-galactosidase, lysine decarboxylase, ornithine decarboxylase, H_2S production, urease, tryptophan deaminase, acetoin production, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. The isolates are resistant to ampicillin (1024 μg ml^{-1}), amoxicillin-clavulanic acid (1024 μg ml^{-1}), tetracycline (128 μg ml^{-1}), chloramphenicol (≥32 μg ml^{-1}), amikacin (≥64 μg ml^{-1}) and gentamicin (16 μg ml^{-1}) and exhibit a genotypic heterogeneity revealed by 16S rRNA gene sequences, ITS- and BOX-PCR analysis. The type strain is *C. haemolyticum* MD0585^T.

Acknowledgements

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Supporting Information

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

Figure S1 Rio Doce State Park and the sampling site location, Carioca Lake.

Figure S2 Phylogenetic tree based on 16S rRNA gene sequences (1237 bp) showing *Chromobacterium haemolyticum* isolates and other *Chromobacterium* species. One thousand bootstrap resamplings were used to evaluate the robustness of the inferred trees. *Aquitaleae magnusonii* (DQ018117), *Vogesella indigofera* (AB021385) and *Neisseria gonorrhoeae* (X07714) were used as outgroups.

Figure S3 Isolate with marked haemolysis on sheep blood agar after 24 h culture.

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Supporting Information

Figure S1

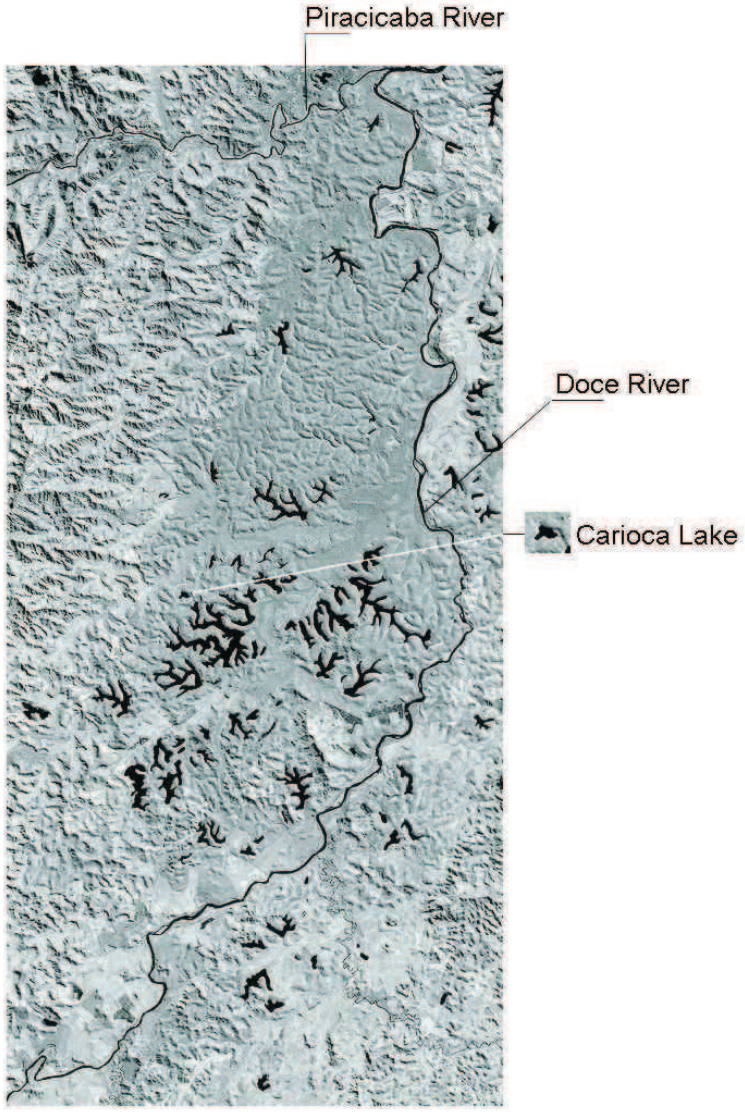
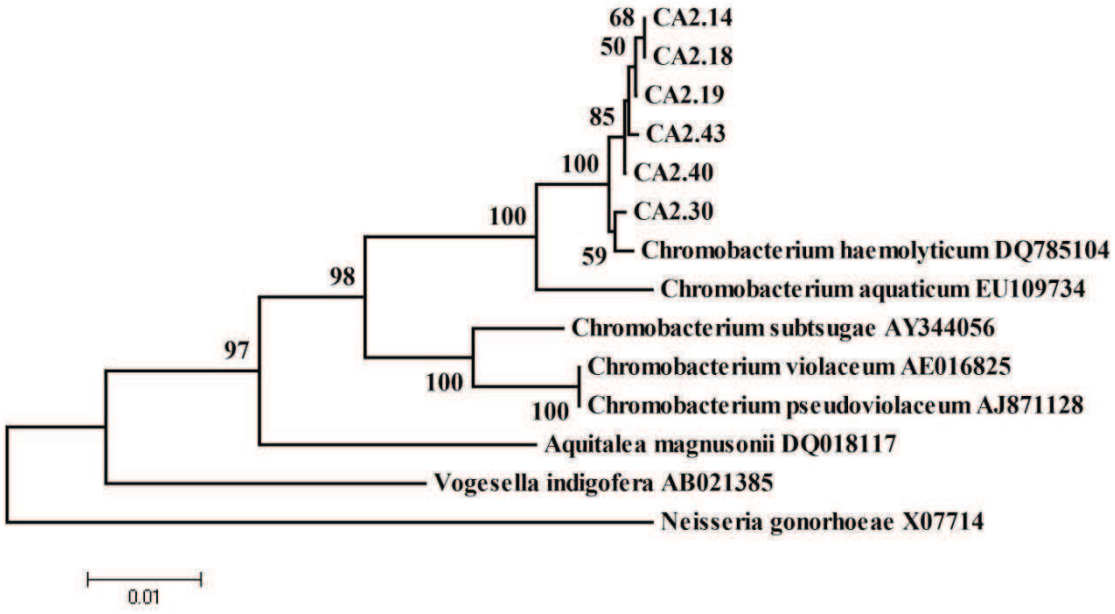


Figure S2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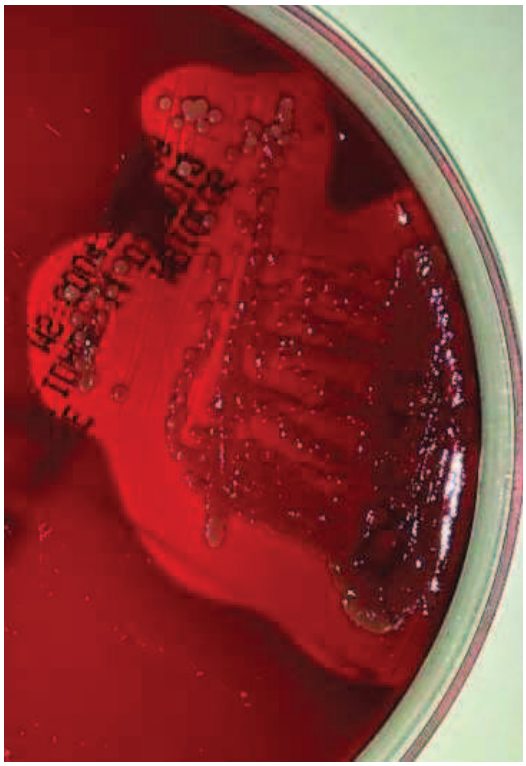
CA2.14
50
CA2.18
CA2.19
85
CA2.43
100
CA2.40
100
CA2.30
59
Chromobacterium haemolyticum DQ785104
98
Chromobacterium aquaticum EU109734
Chromobacterium subtsugae AY344056
97
Chromobacterium violaceum AE016825
100
100
Chromobacterium pseudoviolaceum AJ871128
Aquitalea magnusonii DQ018117
Vogesella indigofera AB021385
Neisseria gonorhoeae X07714
0.01

Figure S3



IV) CONCLUSÃO GERAL

Este foi o primeiro estudo, ao nosso conhecimento, abrangendo Biogeografia e biodiversidade de comunidades bacterianas heterotróficas cultiváveis, presentes no córrego Indaiá e solo do entorno, do Parque Nacional da Serra do Cipó (PNSC), e na lagoa Carioca do Parque Estadual do Rio Doce (PERD). Os resultados evidenciaram que a as variações temporais influenciam biogeograficamente as composições das comunidades. Isso pôde ser evidenciado pelas mudanças na estrutura da diversidade das 18 comunidades bacterianas nos gradientes eufótico e horizontal, nos períodos amostrados. Dentre as 360 UTOs-ARDRA, 313 foram únicas, indicando um alto grau de endemismo dentro dessas comunidades. Dos cinco filos, três filos (Proteobacteria, Actinobacteria e Bacteroidetes) encontrados eram típicos de ecossistemas de água doce, sendo Proteobacteria o filo mais abundante. Em relação ao total (121) das bactérias associadas suas sequências de rDNA 16S foram afiliadas nos mesmos cinco filos (Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes e Deinococcus-Thermus) encontrados nas 18 comunidades bacterianas da lagoa Carioca. O gênero mais frequente e comospolita na lagoa Carioca foi *Chromobacterium.* Surpreendentemente, todos isolados deste gênero pertenciam à espécie *C. haemolyticum* e apresentaram grande variabilidade genotípica e fenotípica. Em contraste, no PNSC, todos os isolados bacterianos apresentando coloração violeta pertenciam à espécie *C. piscinae*. Duas populações desta espécie, provenientes do ecossistema terrestre e aquático, foram estudadas, e apresentaram características que indicam suas diferentes origens geográficas. Apesar do que está sendo indicado pelos fatores abióticos e interações microbianas, não se sabe, ainda, quais variáveis ecológicas realmente influenciam a estrutura e composição das comunidades bacterianas, principalmente porque a maioria dos fatores é interdependente. Em resumo, há necessidade de um maior número de amostras e de isolados para se determinar a real ubiquidade e distribuição de grupos procarióticos no plancton.

V) REFERÊNCIAS

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