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Molecular identification and phylogenetic analysis of *Bothrops insularis* bacterial and fungal microbiota

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ABSTRACT

Bothrops insularis, known as the golden lancehead snake, has its natural habitat restricted to Queimada Grande Island on the southern coast of Brazil. This culture-dependent study aimed to identify microorganisms obtained from the mouth, eyes, and cloaca of this species. Swabs from 20 snakes were collected for fungal and bacterial isolation. DNA was extracted from all samples, and identification was performed by amplifying the ITS1-5.8S-ITS2 regions and the 16S rDNA gene, respectively. All strains were identified and deposited in the GenBank nucleotide database. MEGA v6.0 software was utilized to construct phylogenetic trees. In total, 100 strains were isolated and characterized, from which 42 fungi were distributed into 23 species and 58 bacteria into 13 species. The genus *Fusarium* was predominant since 11 strains and probably a new species was isolated from this fungus. *Pseudomonas aeruginosa* and *Enterococcus faecalis* were the predominant groups of aerobic bacteria isolated. Phylogenetic analyses between bacterial and fungal sequences suggest a similarity between the microorganisms found on the island and on the continent. These findings may be attributed to anthropic actions resulting from both expeditions to the island and actions of migratory birds, which are the main sources of food for snakes.

Introduction

The snake *Bothrops insularis* may have originated from *Bothrops jararaca* from the Brazilian continent through isolation of ancestral populations, a phenomenon known as allopatric speciation. According to Marques, Martins, and Sazima (2002), two populations separated by a geographical barrier may be differentiated over time and become distinct species. The current population of *B. insularis* is small, estimated at only 2000 animals, which has been declining in recent years (Martins, Sawaya, and Marques 2008). The biggest threats are illegal removal by biopiracy interested in venom pharmacological potential and profitability for the international pet market. Other potential threats are fires, infectious and parasitic diseases, introduction of exotic species, and food shortage (Bataus and Reis, 2011; Marques, Martins, and Sazima 2002; Martins, Sawaya, and Marques 2008). Therefore, this snake has been included in the Red List of The International Union for Conservation of Nature as critically endangered (http://www.iucnredlist.org/).

B. insularis, first described in 1921 by Afrânio do Amaral (Marques, Martins, and Sazima 2002), is endemic to Queimada Grande Island, located 33 km of the coast of São Paulo state, Brazil, (Machado, Drummond, and Paglia 2008) and its habitat is restricted to this island (Machado, Drummond, and Paglia 2008). Since there were no small terrestrial mammals on the island, this snake needed to adapt to a new diet during a

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L.N.B., R.S.F. Jr., and L.D.S. participated and designed the study, study planning, performed data analysis, and wrote the manuscript. P.L.M., H.G.G., J.L.C., T.F., and L.C.B. performed data analyses. S.M.G.B., E.B., A.F. Jr., and B.B. collected the data and critically reviewed the manuscript. All authors read and approved the final version of the manuscript.

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diurnal cycle in an arboreal habitat to enable survival. This behavioral change was accompanied by modifications of the venom, which became more effective at killing birds (Zelanis, Travaglia-Cardoso, and Furtado 2008).

Although the species *B. insularis* and its habitat have been studied for years, data are still scarce. The biochemical and biological activities of the venom and its fractions, population dynamics, breeding in captivity, as well as proteomics of venoms and antivenoms have been previously described (Della-Casa et al. 2011; Gonçalves-Machado et al. 2016; Guimarães et al. 2014; Silva et al. 2015; Valente et al. 2009). However, no apparent investigators have thus far explored the bacterial and fungal microbiota of this species. These findings may reveal the influence of microbiota on the health of the snakes, birth and mortality rates, and role as a secondary etiological agent in infections resulting from snakebites (Costello et al. 2010; Ferreira Junior et al. 2009; Fonseca et al. 2009; Paula Neto et al. 2005). The aim of this study was to characterize microbiota in *B. insularis* snakes using molecular identification of aerobic bacteria and fungi isolated from mouth, eyes, and cloaca using a culture-dependent approach.

Methods

Island, snakes, and swab collection

Queimada Grande Island (24° 29′ 8.5″ S, 46° 40′ 31.22″ W), the only natural habitat of *B. insularis*, is located about 18 nautical miles, or 35 km off the coast of São Paulo State, Brazil (Figure 1). Queimada Grande Island is approximately 430,000 m^2, with an irregular topography and maximum altitude of 206 m. According to Marques, Kasperoviczus, and Almeida-Santos (2013), the climate is subtropical with two seasons: rainy and hot from October to March and dry and cold from April to September. The island is uninhabited and may be reached only by environmental analysts and scientists authorized by the Brazilian government.

Twenty healthy *B. insularis* snakes of both sexes were captured on the island before clinical and physical examinations. After microchipping, snakes were placed in a transparent plastic box (40 × 30 × 40 cm) filled with CO_2. Exposure time was directly proportional to the size of the animal (3–5 min), and animals completely recovered in the next 5 min. Swabs were collected from the oral, ophthalmic, and cloacal cavities, the material identified and placed in appropriate boxes for further processing.

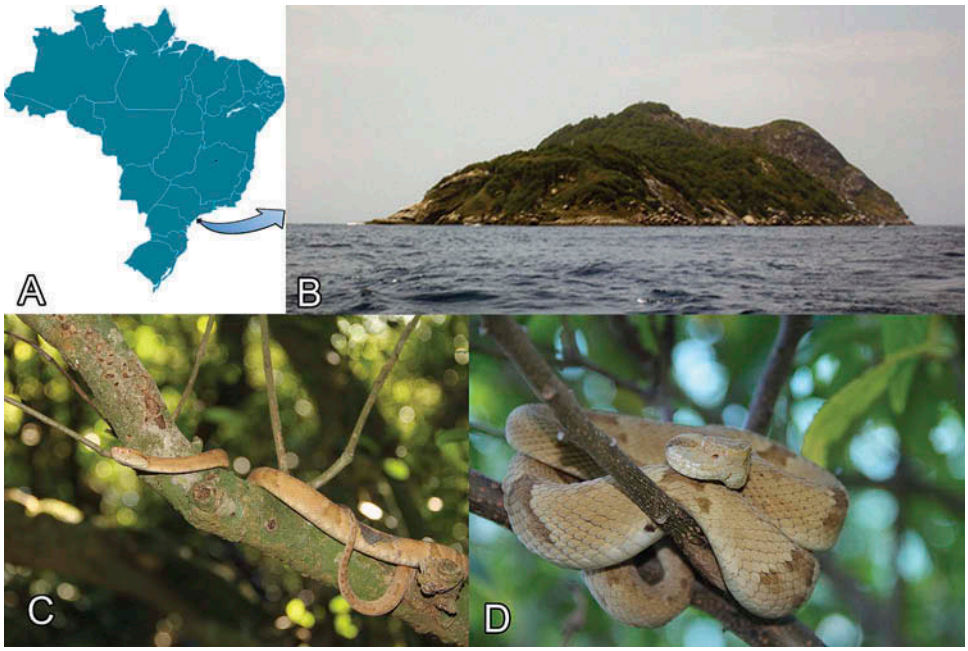

Figure 1. (A) Brazil map*; (B) Queimada Grande Island coast view; (C, D) Arboricle behavior of *Bothrops insularis* snakes. (*Image elaborated by Adobe Photoshop v.13.0.1.3. http://www.adobe.com/br/products/photoshop.html).

Strains

The collected samples were plated on Sabouraud Dextrose agar plus chlorphenicol (50 µg/mL) for fungal isolation, and MacConkey agar and blood agar for bacterial isolation. Media were incubated at 27°C for fungi or in aerobiosis at 37°C for bacteria. After primary isolation of microbial strains, subcultures were carried out in new plaques until pure cultures of the isolates were reached. Fungi visualization was obtained by staining with cotton-blue lactophenol and/or microculture technique (Riddell 1950). Bacterial morphology was visualized by Gram staining, and conventional biochemical identification tests were performed (Koneman and Allen, 2008).

DNA extraction

The fungi/yeast genomic DNA isolation® kit (Norgen Biotek Corporation, Throld, Canada) was employed in order to obtain yeast DNA and filamentous fungi DNA. Bacterial DNA was extracted by using the Illustra blood genomic Prep Mini Spin® kit (GE Healthcare Life Sciences, Buckinghamshire, UK).

PCR amplifications

For fungi identification, the ITS1-5.8S-ITS2 region was amplified using the primers ITS4 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS5 (5′-TCCTCCGCTTATTGATATTGC-3′) described by White et al. (1990). In order to differentiate the species of the genus *Fusarium*, partial amplification of gene for the elongation factor 1α (EF-1α) was utilized with primers EF1 (5′-ATGGG TAAGGAAGACAAGAC-3’) and EF2 (5′-GGAAGTACCAGTGATCTGTT-3′) (Wang et al. 2011). The 16S rDNA gene was used for bacterial identification, and for this purpose, a pair of primers were designed from bacterial sequences deposited in the public databases. The primers employed were BG1 (5′-AGACTC CTACGGGAGGCAGC-3′) and BG2 (5′-GGA CTACCAGGGTATCTAATCC-3′). PCR was performed on a Veriti Thermocycler® thermocycler (Applied Biosystems, Foster City, CA, USA).

For the reactions, 12.5 µl DNA polymerase enzyme (Go Taq® G2 Colorless Master Mix, GE Healthcare Life Sciences, Madison, USA), 3 µl DNA, 10 µM of each primer and water nuclease free up to 25 µl were used. The thermal cycling conditions were 95°C/5 min followed by 35 cycles of 94°C/1 min, 55°C/1 min, and 72°C/1 min (ITS region), and 94°C/4 min followed by 35 cycles of 94°C/40 s, 55°C/40 s, and 72°C/90 s (EF region). For bacterial amplification, the conditions were 95°C/4 min followed by 35 cycles of 95°C/1 min, 60°C/1 min, and 72°C/30 s (16S region). For all amplifications, final extension was performed at 72°C/10 min. The amplification of the products was verified by electrophoresis using a 1.5% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, USA). The PCR products were detected as a single band of approximately 500 bp for ITS1-5.8s-ITS2 and 16S rDNA and approximately 700 bp for EF-1α.

Species identification

Amplicons were purified with the ExoProstar® enzyme (GE Healthcare Life Sciences, Buckinghamshire, UK) and sequenced in 3500® Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The sequences obtained were analyzed in the MEGA v6.0 software and compared with GenBank databases using *Blastn* tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) from the database of the National Center for Biotechnology Information. A % identity greater than 98% of the reference sequence was considered when compared with the sequence submitted.

Phylogenetic analysis

Two phylogenetic analyzes were performed also utilizing the MEGA® v6.0 software, one with the ITS1-5.8S-ITS2 sequences while the other one with 16S rDNA sequences. The alignment was carried out using Clustal W, and the evolutionary history was inferred using the maximum likelihood method (Harris and Stocker, 1998) based on Kimura's two-parameter model (Kimura 1980) with 1000 bootstrap replicates. *Pythium insidiosum* (access number AB971190.1) and *Halorubrum lacusprofundi* (access number NR028244.1) were employed as outgroup sequences for ITS and 16S rDNA regions, respectively.

Nucleotide sequence access numbers

The sequences of the isolates identified in this study were deposited in the GenBank nucleotide database under the access codes KX538818 to KX538820 and KY490646 to KY490684 (for the ITS region); KY273925, KY273926, and KY490592 to KY490645 (for the 16S rDNA region).

Ethics approval

All procedures performed involving snake specimens were conducted in accordance with the ethical standards of the Institutional and/or National Research Committee. This investigation was approved by the Ethics Committee on Animal Use of Botucatu Medical School (no. 1187/2016 – CEUA) in Botucatu, Sao Paulo, Brazil. The permission for researchers to enter Queimada Grande Island and collection of research material was authorized by the Chico Mendes Institute for Biodiversity Conservation, Brazil (SISBIO no. 41307-4).

Results

In view of the difficulty to identify bacterial strains by traditional biochemical tests, identification of environmental isolates was conducted through partial gene sequencing. The universal primers initially employed were not satisfactory; therefore, a pair of primers (BG1/BG2) from the alignment of different sequences deposited in the databases needed to be designed and amplified with the 16S rDNA gene. The selection of strains was based upon the diversity found in the samples. In contrast, all fungal strains amplified the ITS1-5.8S-ITS2 region using universal fungal primers. The identification of the nucleotide sequences of these environmental isolates in the database provided 99% or 100% identity to fungi and bacteria.

One hundred environmental strains were present in the culture plates. Among these strains, 42 were identified as fungi, with 3 (7.2%) yeasts and 39 (92.8%) filamentous fungi. Regarding bacterial strains, 58 were identified as bacteria, with15 (25.9%) Gram-positive and 43 (74.1%) Gram-negative.

Among the most frequent filamentous fungi were *Fusarium* spp. with 12 isolates (28.6%), *Penicillium* spp. with 6 isolates (14.3%), and *Cladosporium* spp. with 5 isolates (12%) (Table 1). The identification of the bacterial strains isolated from *B. insularis* (Table 2) showed that among the Gram-negative bacteria *Pseudomonas* spp. was the most abundant with 18 isolates (31%), followed by *Serratia* spp. With 10 isolates (17.2%) and *Klebsiella* spp. with 5 isolates (8.6%). Regarding Gram-positive bacteria, a single strain of *Micrococcus* sp. was detected, while the rest were identified as *Enterococcus faecalis* (93.3%).

The greatest source of fungal isolation was obtained in the eyes followed by cloaca and then mouth. For bacterial strains, the greatest source was mouth, cloaca, and eyes, respectively (supplementary material, Tables S1 and S2).

Phylogenetic trees of fungi (ITS region) and bacteria (16S rDNA gene) are shown in Figures 2 and 3, respectively. The bootstrap values demonstrate the quality of the analyses.

Diversity was observed among the species of fungi of Ascomycota and Basidiomycota phyla with isolates from eleven families (Nectriaceae, Clavicipitaceae, Hypocreaceae, Cordycipitaceae, Ophiocordycipitaceae, Trichocomaceae, Davidiellaceae, Didymellaceae, Periconiaceae, Saccharomycetaceae, and Sporidiobolaceae). Therefore, the pseudo-fungus *P. insidiosum* (*Oomycota*) was selected as outgroup because of distance between species.

It was clearly noted that bacterial microbiota of *B. insularis* is composed predominantly of Gram-negative isolates from Proteobacteria phylum, Enterobacteriaceae, Xanthomonadaceae, and Pseudomonadaceae families. As for Gram-positive, the majority of isolates were of the Firmicutes phylum and a strain of the Actinobacteria phylum. In this case, the archaeobacterium *Halorubrum lacusprofundi* was used as outgroup.

Discussion

Filamentous fungi are often isolated from snakes, and although most fungi make up the normal microbiota of reptiles, some were reported as an etiological agent of mycoses. The ones most commonly found are the

Table 1. Identification of Fungal Species Isolated from *Bothrops insularis* by DNA Sequencing of the ITS Region.

Strain	% identification	Identification	Accession number	Final identification
BIF 1	99	*Cladosporium* sp. SQU-QU11	KU945953.1	*Cladosporium* sp.
BIF 2	100	*Cladosporium* sp.	LN834425.1	*Cladosporium* sp.
BIF 3	100	*Aspergillus flavus*	KU296252.1	*Aspergillus flavus*
BIF 4	99	*Fusarium circinatum*	HQ631016.1	*Fusarium circinatum*
BIF 5	99	*Fusarium subglutinans*	HQ631016.1	*Fusarium subglutinans*
BIF 6	100	*Yamadazyma phyllophila*	NR19421.1	*Yamadazyma phyllophila*
BIF 7	100	*Fusarium solani* CBS 118931	JX435204.1	*Fusarium solani*
BIF 8	100	*Aschersonia* sp. XL-D20	EF488395.1	*Aschersonia* sp.
BIF 9	100	*Fusarium solani* isolate Z162	KP265359.1	*Fusarium solani*
BIF 10	100	*Penicillium paxilli* strain CBS 547.77	JN617709.1	*Penicillium paxilli*
BIF 11	100	*Fusarium solani*	JN637954.1	*Fusarium solani*
BIF 12	100	*Fusarium solani* strain IHEM 22464	KP132232.1	*Fusarium solani*
BIF 13	100	*Fusarium subglutinans*	KP979602.1	*Fusarium subglutinans*
BIF 14	100	*Penicillium commune* strain A531CE	KT264644.1	*Penicillium commune*
BIF 15	100	*Penicillium commune* strain 108A	KT264644.1	*Penicillium commune*
BIF 16	100	*Penicillium commune* strain 108A	KT264644.1	*Penicillium commune*
BIF 17	100	*Penicillium commune* strain 108A	KT264644.1	*Penicillium commune*
BIF 18	99	*Acremonium variecolor*	HE608647.1	*Acremonium variecolor*
BIF 19	100	*Epicoccum sorghinum* strain CBS 179.80	FJ427067.1	*Epicoccum sorghinum*
BIF 20	100	*Cladosporium* sp. HNC13-10	KT959282.1	*Cladosporium* sp.
BIF 21	100	*Paecilomyces lilacinus* NRRL22958	GU980033.1	*Purpureocillium lilacinum*
BIF 22	100	*Purpureocillium lilacinum* INTR-2	FJ765024.1	*Purpureocillium lilacinum*
BIF 23	99	*Nectria* sp. P79-320	KJ439166.1	*Nectria* sp.
BIF 24	99	*Nectria* sp. P79-320	KJ439166.1	*Nectria* sp.
BIF 25	99	*Fusarium pseudocircinatum*	KP979602.1	*Fusarium pseudocircinatum*
BIF 26	99	*Fusarium pseudocircinatum*	KM655517.1	*Fusarium pseudocircinatum*
BIF 27	100	*Fusarium oxysporum* strain CBS 140424	KT794176.1	*Fusarium oxysporum*
BIF 28	100	*Fusarium* sp. LK-2016	KU182491.1	*Fusarium* sp.
BIF 29	99	*Rhodotorula mucilaginosa* strain UCDFST 13–478	KU609508.1	*Rhodotorula mucilaginosa*
BIF 30	100	*Trichoderma harzianum* voucher Th.12	KJ010951.1	*Trichoderma harzianum*
BIF 31	100	*Fusarium oxysporum* strain CBS 140424	KT794176.1	*Fusarium oxysporum*
BIF 32	100	*Penicillium paxilli* strain CBS 547.77	JN617709.1	*Penicillium paxilli*
BIF 33	100	*Trichoderma harzianum* voucher Th.12	KJ010951.1	*Trichoderma harzianum*
BIF 34	100	*Candida orthopsilosis* strain YCH159	KM982962.1	*Candida orthopsilosis*
BIF 35	100	*Paecilomyces lilacinus* strain NRRL22958	GU980033.1	*Purpureocillium lilacinum*
BIF 36	100	*Trichoderma harzianum* voucher Th.12	KJ010951.1	*Trichoderma harzianum*
BIF 37	100	*Cladosporium* sp. HNC13-10	KT959282.1	*Cladosporium* sp.
BIF 38	100	*Beauveria bassiana* isolate NBAII-Bb-69	JQ434752.1	*Beauveria bassiana*
BIF 39	100	*Paecilomyces lilacinus* strain NRRL22958	GU980033.1	*Purpureocillium lilacinum*
BIF 40	99	*Periconia* sp. 2501 FrLB	LN997731.1	*Periconia* sp.
BIF 41	100	*Cladosporium oxysporum*	KU945954.1	*Cladosporium oxysporum*
BIF 42	100	*Trichoderma atroviride* culture-collection CCTCC:AV429	KT588284.1	*Trichoderma atroviride*

BIF, *Bothrops insularis* fungi.

species of *Aspergillus* spp., *Penicillium* spp., *Acremonium* spp., *Cladosporium* spp., *Mucor* spp., and dermatophytes (Bohuski et al. 2015; Dehghani et al. 2016; Freire et al. 2015). Among the fungi identified in *B. insularis* snakes, the most prevalent were ascomycetes *Trichoderma* spp., *Purpureocillium* spp., *Cladosporium* spp., *Penicillium* spp., *Nectria* spp., and *Fusarium* spp., which represented 78.6% of the total strains. *Acremonium* spp. and *Aspergillus* spp. were also identified having one representative each.

For fungi identification in a database, attention needs to be paid to different species (anamorph and teleomorph) since the sequences submitted represent varying points of view and differing historical periods (Garcia Garces et al. 2016). An example of this is *Paecilomyces lilacinus*, currently designated as *Purpureocillium lilacinum*, which was isolated from three (15%) *B. insularis* snakes. *P. lilacinus* is an entomopathogenic fungus that commonly infects various species of reptiles (Schmidt 2015). Bohuski et al. (2015) previously isolated this fungal species from the skin of snakes closely related to *Ophidiomyces ophiodiicola* species normally found in the skin of these animals.

The ITS1-5.8S-ITS2 region sequencing identified a wide variety of fungi because it has the most clearly defined barcode gap between inter- and intraspecific variation (Schoch et al. 2012). However, out of the 12

Table 2. Identification of Bacterial Species Isolated from *Bothrops insularis* through Partial Sequencing of the 16S rDNA Gene.

Strain	% identification	Identification	Accession number	Final identification
BIB 1	100	*Pseudomonas fulva*	AM161143.1	*Pseudomonas fulva*
BIB 2	100	*Serratia marcescens* strain CSE_5	KX027341.1	*Serratia marcescens*
BIB 3	100	*Klebsiella oxytoca* strain ATCC 13182	NR_041749.1	*Klebsiella oxytoca*
BIB 4	100	*Klebsiella oxytoca* strain ATCC 13182	NR_041749.1	*Klebsiella oxytoca*
BIB 5	100	*Stenotrophomonas maltophilia* strain LEM12	KU180334.1	*Stenotrophomonas maltophilia*
BIB 6	100	*Citrobacter* sp. K36	KX156768.1	*Citrobacter* sp.
BIB 7	100	*Pseudomonas aeruginosa* strain NBAII AFP-4	HM439965.1	*Pseudomonas aeruginosa*
BIB 8	100	*Morganella morganii* strain ATCC 8076	FJ971868.1	*Morganella morganii*
BIB 9	100	*Klebsiella oxytoca* strain ATCC 43863	KC155255.1	*Klebsiella oxytoca*
BIB 10	100	*Enterococcus faecalis* strain 081513	KU353623.1	*Enterococcus faecalis*
BIB 11	100	*Klebsiella pneumoniae* strain SRP2	KR092085.1	*Klebsiella pneumoniae*
BIB 12	99	*Serratia* sp. F1390	JQ417649.1	*Serratia* sp.
BIB 13	100	*Enterococcus faecalis* strain K530	KT005522.1	*Enterococcus faecalis*
BIB 14	100	*Enterococcus faecalis* strain K530	KT005522.1	*Enterococcus faecalis*
BIB 15	100	*Enterococcus faecalis* strain K530	KT005522.1	*Enterococcus faecalis*
BIB 16	100	*Stenotrophomonas maltophilia*	LN890169.1	*Stenotrophomonas maltophilia*
BIB 17	99	*Ochrobactrum* sp./*Brucella* sp.	N.A.	*Ochrobactrum* sp./*Brucella* sp.
BIB 18	100	*Enterococcus faecalis* strain K530	KT005522.1	*Enterococcus faecalis*
BIB 19	100	*Pseudomonas aeruginosa* strain ATCC 27853	CP015117.1	*Pseudomonas aeruginosa*
BIB 20	100	*Pseudomonas aeruginosa* strain ATCC 27853	CP015117.1	*Pseudomonas aeruginosa*
BIB 21	99	*Serratia marcescens* strain P4565	KT215407.1	*Serratia marcescens*
BIB 22	100	*Enterococcus faecalis* strain K530	KT005522.1	*Enterococcus faecalis*
BIB 23	100	*Enterococcus faecalis* strain K530	KT005522.1	*Enterococcus faecalis*
BIB 24	100	*Enterococcus faecalis* strain K530	KT005522.1	*Enterococcus faecalis*
BIB 25	100	*Citrobacter* sp. K36	KX156768.1	*Citrobacter* sp.
BIB 26	100	*Enterococcus faecalis* strain K530	KT005522.1	*Enterococcus faecalis*
BIB 27	100	*Enterococcus faecalis* strain K530	KT005522.1	*Enterococcus faecalis*
BIB 28	100	*Morganella morganii* strain ATCC 8076	FJ971868.1	*Morganella morganii*
BIB 29	100	*Micrococcus* sp. M7	KX131225.1	*Micrococcus* sp.
BIB 30	100	*Enterococcus faecalis* strain K530	KT005522.1	*Enterococcus faecalis*
BIB 31	99	*Enterococcus faecalis* strain K530	KT005522.1	*Enterococcus faecalis*
BIB 32	100	*Pseudomonas aeruginosa* strain ATCC 27853	CP015117.1	*Pseudomonas aeruginosa*
BIB 33	99	*Pseudomonas aeruginosa* strain ATCC 27853	CP015117.1	*Pseudomonas aeruginosa*
BIB 34	100	*Pseudomonas aeruginosa* strain ATCC 27853	CP015117.1	*Pseudomonas aeruginosa*
BIB 35	99	*Stenotrophomonas maltophilia* strain F3-1–27	KX350012.1	*Stenotrophomonas maltophilia*
BIB 36	100	*Pseudomonas aeruginosa* strain ATCC 27853	CP015117.1	*Pseudomonas aeruginosa*
BIB 37	100	*Pseudomonas aeruginosa* strain NBAII AFP-4	HM439965.1	*Pseudomonas aeruginosa*
BIB 38	100	*Pseudomonas aeruginosa* strain ATCC 27853	CP015117.1	*Pseudomonas aeruginosa*
BIB 39	100	*Pseudomonas* sp. Rhizorgl	KT318820.1	*Pseudomonas* sp.
BIB 40	99	*Klebsiella oxytoca* strain ATCC 13182	NR_041749.1	*Klebsiella oxytoca*
BIB 41	100	*Pseudomonas aeruginosa* strain ATCC 27853	CP015117.1	*Pseudomonas aeruginosa*
BIB 42	100	*Pseudomonas aeruginosa* strain ATCC 27853	CP015117.1	*Pseudomonas aeruginosa*
BIB 43	100	*Stenotrophomonas maltophilia* strain ATCC 19861	NR_040804.1	*Stenotrophomonas maltophilia*
BIB 44	100	*Serratia marcescens* strain P4565	KT215407.1	*Serratia marcescens*
BIB 45	99	*Serratia marcescens* strain P4565	KT215407.1	*Serratia marcescens*
BIB 46	100	*Serratia marcescens* strain REDSIVNE-ABHI001	KR866113.1	*Serratia marcescens*
BIB 47	100	*Serratia marcescens* strain P4565	KT215407.1	*Serratia marcescens*
BIB 48	99	*Serratia marcescens* strain CSMB14A	KM091719.1	*Serratia marcescens*
BIB 49	100	*Serratia marcescens* strain T6-02	GU294130.1	*Serratia marcescens*
BIB 50	99	*Serratia* sp. DZ	HQ437667.1	*Serratia* sp.
BIB 51	100	*Pseudomonas aeruginosa* strain ATCC 27853	CP015117.1	*Pseudomonas aeruginosa*
BIB 52	100	*Enterococcus faecalis* strain K530	KT005522.1	*Enterococcus faecalis*
BIB 53	100	*Enterococcus faecalis* strain K530	KT005522.1	*Enterococcus faecalis*
BIB 54	100	*Pseudomonas aeruginosa* strain ATCC 27853	CP015117.1	*Pseudomonas aeruginosa*
BIB 55	100	*Pseudomonas aeruginosa* strain NBAII AFP-4	HM439965.1	*Pseudomonas aeruginosa*
BIB 56	99	*Ochrobactrum* sp./*Brucella* sp.	N.A.	*Ochrobactrum* sp./*Brucella* sp.
BIB 57	100	*Pseudomonas aeruginosa* strain ATCC 27853	CP015117.1	*Pseudomonas aeruginosa*
BIB 58	100	*Pseudomonas aeruginosa* strain NBAII AFP-4	HM439965.1	*Pseudomonas aeruginosa*

N.A., not applicable; BIB, *Bothrops insularis* bacteria.

fungal isolates of the genus *Fusarium*, 7 did not present species-level definition with amplification of ITS region. Thus, EF-1α gene amplification was also performed, resulting in identification of five different species (*Fusarium subglutinans, Fusarium pseudocircinatum, Fusarium circinatum, Fusarium oxysporum e F. solani*). Due to the high genetic diversity of *Fusarium*, the EF-1α gene has been a useful phylogenetic marker that frequently provides discrimination at the species level. This gene seems to be present as a single copy in *Fusarium* allowing more quantitative comparisons among species (Geiser et al. 2004; Karlsson et al. 2016). Even using the EF region, a strain still remained unclassified (Figure 2), suggesting that this may constitute a new species of *Fusarium* detected in Queimada Grande Island. This uninhabited Island has peculiar environmental characteristics that enable discovery of new microbial species.

Different species of *Fusarium* have already been isolated from snakes and other reptiles. *F. solani, Fusarium moniliforme*, and *F. oxysporum* were associated with abscesses, pneumonia, and opportunistic pathogens (Dadone et al. 2010; Frelier, Sigler, and Nelson 1985; Williams et al. 2012). In general, these are considered agents of secondary infections (Barber et al. 2016).

The predominance of filamentous fungi was observed in relation to yeasts in *B. insularis*. It is estimated that yeasts comprise approximately 1% of fungal population (Kurtzman and Piškur 2006) and, to some extent, this finding reflects a low %. The yeast fungus *Rhodotorula mucilaginosa* was the only representative of the phylum Basidiomycota isolated from *B. insularis*. The genus *Rhodotorula* occurs naturally in air and soil and may be isolated from human skin, feces, and food. Although most species are not pathogenic, *R. mucilaginosa* was considered an emerging opportunistic pathogen (Capoor et al. 2014; Hazen 1995).

O. ophiodiicola and *Chrysosporium* anamorph of *Nannizziopsis vriesii* are among the agents that produce fungal diseases in snakes. These fungi, which have been isolated from several species, produce serious skin infections, and may be even fatal (Bertelsen et al. 2005; Eatwell 2010; Lorch et al. 2016; McBride et al. 2015; Rzadkowska et al. 2016). Based upon lesions suggestive of mycosis found in *Bothrops* and *Crotalus* snakes scales in captivity, *Trichosporon asahii* was also isolated (Campagner et al. 2012). However, none of these species was identified in *B. insularis*. It is of interest that most of the fungal isolates were found in the eyes. One might postulate that dissemination of filamentous fungi occurs because of intensive winds on the island and their tree-dwelling behavior.

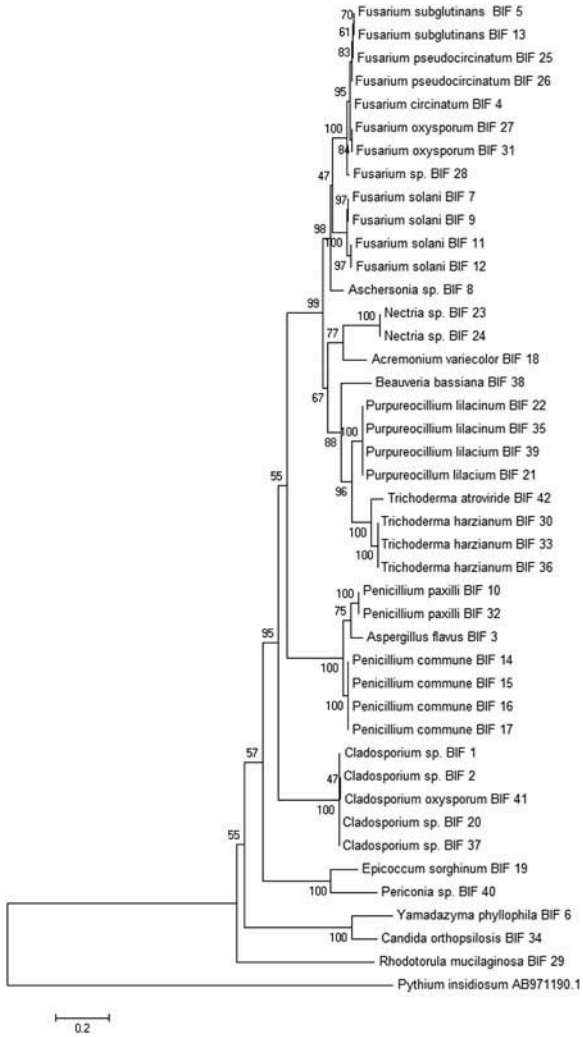

Figure 2. The evolutionary history using ITS1-5.8S-ITS2 region sequences was inferred by using the maximum likelihood method (Tamura et al. 2013) based on the Kimura two-parameter model (Kimura 1980). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (White et al. 1990).

Initially, traditional biochemical tests were used to identify bacterial strains; however, many results were inconclusive (data not shown). In agreement with Drancourt et al. (2000) and Torsvik et al. (1998)

described the difficulty in identifying environmental isolates through phenotypic tests. 16S rDNA gene sequencing is considered a reliable method for identifying a variety of phenotypically non-identifiable bacterial isolates (Drancourt et al. 2000). The sequences of these two strains, which were isolated but not identified (BIB 17 and BIB 56), were similar; however, it was not possible to differentiate between *Ochrobactrum* and *Brucella* when comparing these sequences to sequences deposited in the GenBank nucleotide database using the BLAST algorithm. Scholz et al. (2008) reported that species of *Ochrobactrum anthropi, Ochrobactrum intermedium*, and *Brucella* spp. are phenotypically and genetically related pathogens whose identification and differentiation were difficult to be carried out.

Gram-negative bacteria were predominant with 32.7% belonging to the family Enterobacteriaceae (*Citrobacter* sp., *Klebsiella pneumoniae* and *Klebsiella oxytoca, Morganella morganii*, and *Serratia marcescens* and *Serratia* sp.). Enterobacteria are one of the most frequent bacterial groups found in snakes, including those of the genus *Bothrops*, isolated from the cloaca and mouth (Campagner et al. 2012; Ferreira Junior et al. 2009, 2010). The examination of intestinal microbiota of *B. jararaca* performed by Bastos et al. (2008) also verified the prevalence of enterobacteria, whose most frequent isolates were *Salmonella* (27.3%), *Citrobacter* (26.0%), and *Escherichia* (12.3%).

Bacterial infections are common complications after snakebites, and the pathogens isolated from these lesions have been associated with oral microbiota (Jorge and Ribeiro 1997; Jorge et al. 1994). It is believed that oral microbiota reflect fecal microbiota of its ingested preys. However, oral microbiota characterization of reptiles using culture-independent high-throughput sequencing found few potential pathogens at low frequencies. Another interesting finding is that microbiota of the oral cavity exhibited little resemblance to fecal microbiota of the prey, which does not support the hypothesis that the oral cavity of these animals acts as a reservoir of pathogenic agents (Zancolli et al. 2015).

The most common bacterium in *B. insularis* was *Pseudomonas* (31%), with *Pseudomonas aeruginosa* the predominant species in this group (88.8%). This species is an opportunistic pathogen considered to be one of the main agents of nosocomial infection. This Gram-negative bacillus is widely distributed in the environment and detected in feces of humans and animals (Lavenir et al. 2008). *P. aeruginosa* was also reported to constitute a part of normal microbiota of healthy captive snakes, which spread easily among animals (Colinon et al., 2010).

Previously designated as *Pseudomonas maltophilia* and subsequently *Xanthomonas maltophilia*, the bacterium *Stenotrophomonas maltophilia* is a commensal species that was reported to act as an infectious agent in reptiles (Hejnar et al. 2007). In this study, the presence of *S. maltophilia* was detected in the mouth of 20% of the snakes examined. Using pulsed-field gel electrophoresis, Hejnar et al. (2007) demonstrated the presence of *S. maltophilia* in the mouth of 30% of the snakes studied. As with the change in classification of the genus *Stenotrophomonas*, the phylogenetic tree (Figure 3) places this group apart from the *Pseudomonas* sequences displaying evolutionary distance between these strains.

Gram-negative bacteria are the most common pathogens although aerobic bacteria and pathogenic fungi may also be important components of diseases in reptiles. On the other hand, most Gram-positive bacteria are not considered pathogenic for reptiles and are part of the existing microbiota. Some Gram-positive bacteria may produce diseases particularly in immunocompromised animals (Paré et al. 2006).

Among the Gram-positive bacteria, a strain of *Micrococcus* sp. (usually non-pathogenic) and 14 strains of *E. faecalis* were isolated from the mouth and cloaca of the snakes (85.7% of the strains of this species). Jho et al. (2011) verified the presence of *E. faecalis* in the cloacal cavity of *Python molurus*. This bacterium is part of normal microbiota of the gastrointestinal tract belonging to the phylum Firmicutes. Costello et al. (2010) assessed the remodeling of the postprandial intestinal microbiota in Burmese python snakes and noted that Bacteroidetes and Firmicutes are the two numerically dominant phyla. Between 12 h and 3 d after feeding, Firmicutes gradually surpassed the number of Bacteroidetes in relation to the fasting state, and an increase occurred in the diversity of the species in the intestine of fed animals.

Finally, the composition of microbiota of a snake is influenced by several factors including species, origin (Shek et al. 2009), feeding and fasting patterns (Costello et al. 2010), type of food, health, and human manipulation. In addition, the methodology

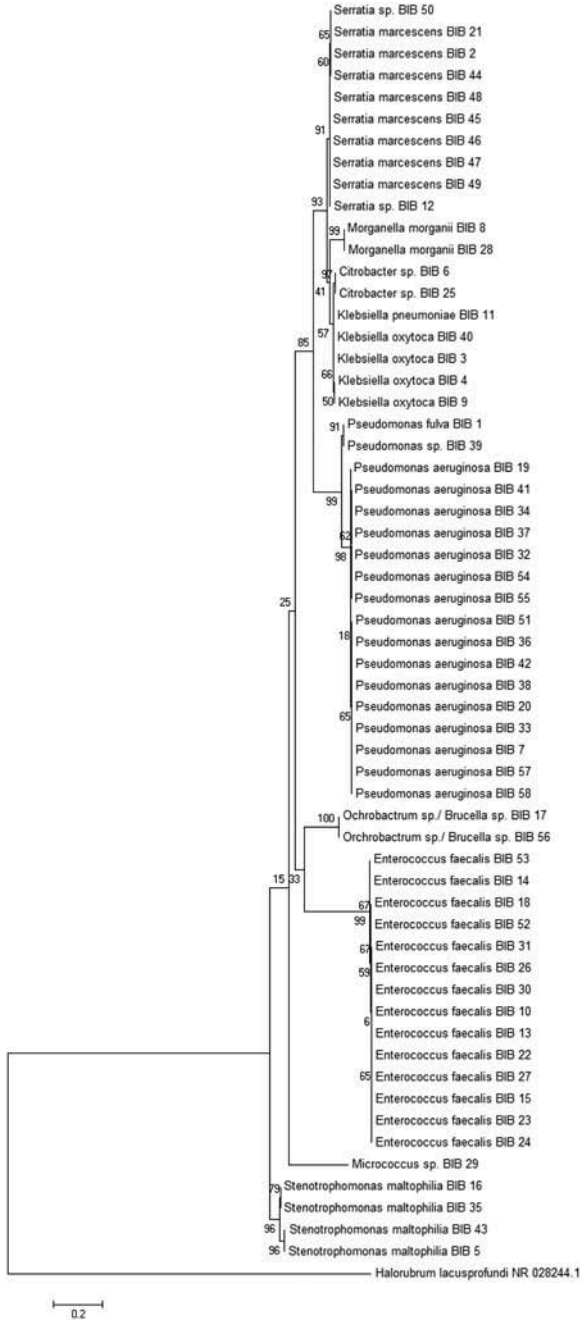

Figure 3. The evolutionary history using 16S rDNA gene sequences was inferred by using the maximum likelihood method (Tamura et al. 2013) based on the Kimura two-parameter model (Kimura 1980). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 (White et al. 1990).

used for collecting and cultivating the material may also influence the results. Our data showed an overview of the diversity of bacterial and fungal microorganisms associated with *B. insularis* in a wild and isolated environment.

Conclusions

The strains isolated in this study reflect microbial diversity existing in Queimada Grande Island as some of these microorganisms are certainly components of the transient microbiota of the snakes examined. The isolation of potentially pathogenic species does not imply that there is a disease in these animals or that they are able to carry pathogens. Despite its isolation for hundreds of centuries (Marques, Martins, and Sazima 2002), Queimada Grande Island snake microbiota did not differ from those on the continent, which demonstrates bacterial balance in healthy animals. New studies need to be conducted in order to understand the complex relationship among microbiota, health, and conservation of this unique island snake species in the world.

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