Herpetological Conservation and Biology 20(1):57–66.
Submitted: 28 August 2024; Accepted: 24 January 2025; Published: 30 April 2025.

Skin Microbiota Composition of *Scinax x-signatus* (Anura: Hylidae) in Anthropized and Natural Areas in Northeast Brazil

Lara Valesca Mendonça da Costa Santos[1,3], Alcina Gabriela Maria Medeiros da Fonsêca Santos[1], Paula Mariana Salgueiro de Souza[2], Anna Carolina Soares Almeida[2], and Geraldo Jorge Barbosa de Moura[1]

[1]Laboratório de Estudos Herpetológicos e Paleoherpetológicos da Universidade Federal Rural de Pernambuco, Rua Dom Manuel de Medeiros, Dois Irmãos, Recife, Pernambuco 52171-900, Brazil
[2]Laboratório de Microbiologia Molecular, Departamento de Biologia, Universidade Federal Rural de Pernambuco, Rua Dom Manuel de Medeiros, Dois Irmãos, Recife, Pernambuco 52171-900, Brazil
[3]Corresponding author, e-mail: lara.santos@ufrpe.br

***Abstract.*—Some amphibian species adapted to urban ecosystems may have altered cryptic interactions with microorganisms. In anurans, the skin bacterial microbiota plays a fundamental role in host protection, yet little is understood about its composition in relation to habitat conditions. The Venezuelan Snouted Treefrog, *Scinax x-signatus*, occurs in both natural habitats, such as fragments of Atlantic Forest, and urbanized areas. We characterized the culturable bacterial microbiota from the skin of *S. x-signatus* by sequencing the 16S rRNA gene from the amplified polymerase chain reaction (PCR) product. We collected skin swab samples from 11 *S. x-signatus*, four in a natural area and seven in an urban area. We isolated bacteria using various rich and selective culture media and identified 20 bacterial isolates, seven from the natural area and 13 from the urban area. Composition of the families among the isolates was Enterobacteriaceae (54.6%; i.e., 7/20), Bacillaceae (18.2%; n = 2), and Moraxellaceae (18.2%; n = 2). *Enterobacter* was the most frequently occurring genus, and we found two human pathogens, *Escherichia coli* and *Klebsiella pneumoniae*, on the skin of treefrogs from the anthropized area. This initial report of culturable skin bacteria from *S. x-signatus* provides the first records of *E. coli* and *K. pneumoniae* in wild populations. We suggest that anurans like *S. x-signatus* be further investigated as potential sentinels for monitoring the presence of pathogenic bacteria, given their ecological role and proximity to human-modified environments.**

Key Words.—16s rRNA sequencing; amphibian conservation; anuran health; bacterial diversity; culturable bacteria; ecosystem monitoring; Enterobacteriaceae; frog-associated bacteria.

Introduction

Amphibians host diverse microbiomes, including bacteria on their skin, that play an essential role in maintaining their health through association with their innate immune system (Becker et al. 2015). As urban areas expand and overlap with natural habitats used by anurans, however, these animals, in particular, may be exposed to novel microbial communities (Bates et al. 2023). Indirect contact with humans, and their microorganisms, can occur when frogs are attracted to artificial waterbodies, such as water cisterns (García-Cardenete et al. 2014) and artificial light sources that attract their insect prey, (Dias et al., 2019). As demonstrated by Gibb et al. (2017) and Secondi et al. (2021), this interaction facilitates a bilateral transmission route for microorganisms and lineages with pathogenic potential (Cunningham et al. 2017; Cevallos et al. 2022). Thus, the co-occurrence of anurans and humans underscores the need to gain a deeper understanding of the microbial communities associated with anurans in different settings.

Despite the growing knowledge of amphibian microbial ecology, studies focusing on culturable bacteria in synanthropic species (species that live in close association with humans) remain scarce. The skin microbiota of amphibians commonly includes bacterial phyla such as Proteobacteria, Actinobacteria, and Bacteroidetes, and the composition of the assemblage has been shown to be influenced by factors that are intrinsic (e.g., species, sex; Brunetti et al. 2019) and extrinsic (e.g., habitat, temperature; Ross et al. 2019; Khalifa et al. 2021). Understanding how the extrinsic factor of proximity to urban

environments may impact the culturable bacterial microbiota of anurans is important for understanding the implications of human-animal interactions as well as the potential role of microbial communities in disease dynamics and ecosystem management (Chivian 2002; Woodhams et al. 2015; Bernardo-Cravo et al. 2020).

The Venezuelan Snouted Treefrog (*Scinax x-signatus,* Fig. 1), called tree frog and bathroom frog in Brazil, represents an interesting model for investigation due to its capacity for environmental adaptation and its association with human-modified environments. This species has thin, moist skin due to the secretion of mucous substances, which are crucial for the colonization of the cutaneous microbiome (Lin et al. 2023). It is nocturnal, small in size, and widely distributed across South America. It has been classified as Least Concern by the International Union for Conservation of Nature (IUCN; 2023) and the Brazilian Ministry of the Environment (MMA; Bastos et al. 2024). This species is frequently observed in urban fragments, forest edges, and residential areas (Pereira et al. 2016; Montezol et al. 2018), therefore, *S. x-signatus* is an excellent model species to study the microbiota of anurans in environments. The objective of this study was to compare the culturable bacterial microbiota associated with the skin of *S. x-signatus*, collected from two distinct areas (human impacted vs. natural) within an urban fragment of the Atlantic Forest. We wanted to highlight how the proximity of urbanization shapes the skin bacterial diversity and composition of anurans.

Figure 1. Individual Venezuelan Snouted Treefrog (*Scinax x-signatus*) on a bromeliad leaf in Control Area at Jardim Botânico do Recife, Pernambuco, Brazil. (Photographed by Ubiratã Ferreira-Souza).

Materials and Methods

Study site.—We collected bacteria from anurans at the Jardim Botânico do Recife (JBR), located in northeast Brazil (08°04'S, 34°59'W). The JBR spans 11.23 ha, with 60% consisting of fragments of the Atlantic Forest characterized as Ombrophilous Dense (De Oliveira *et al*., 2016). Thus, the JBR includes fragments of both native and secondary forest, with deactivated trails inaccessible to visitors and staff, which we designated as the Control Area (CA). In another sector, the JBR features facilities and areas designed for visitation, environmental education activities, and the reception of the public, which we categorized as the Anthropized Area (AA), located 700 m from the CA. We adopted this distance in our study based on movement estimates of < 700 m for anurans in the same genus and family (Bevier et al. 2008).

Microbiota collection.—We collected six male and five female adult *S. x-signatus* in December 2019, four from CA and seven from AA (Table 1). After capture, we washed individuals with 100 ml of sterile distilled water to remove particles and microorganisms associated with the environment. We aseptically swabbed the entire body of the animal: the ventrum, dorsum, head, and limbs (Lauer et al. 2007). We then released specimens at the respective sampling points.

After collection, we immediately inoculated the swabs into Luria Bertani (LB) broth and incubated overnight at 37º C. The following day, we sowed an aliquot of the samples on rich, selective and differential culture media (LB agar, Mueller-Hinton Agar, MacConkey Agar and Chromogenic Agar). The use of various specific media, ranging from low-nutrient, rich, selective, or differential media, has been standardly employed for cultivating microbiota that can be cultivated (Hughey et al. 2017; Assis et al. 2023). Different compositions and nutrient concentrations can favor bacteria with varying growth rates, allowing a broader range of microorganisms to be captured in culture media (Hughey et al. 2017). Based on the morphology and coloring of the colonies, we isolated around 20 distinct bacteria and stored them in Luria Bertani broth supplemented with 15% glycerol in an ultrafreezer (-80º C) for subsequent molecular identification.

Table 1. Collection points of amphibians within the Recife Botanical Garden, in northeast Brazil, categorized according to location within the garden and designation as either control or urban areas. Categories specify the location, description, number of animals collected (n), and coding attributed throughout the study. The symbols ♀ = females and ♂ = male

Collection site	Code	Description	Number of frogs (sex) / bacterial isolates per frog
Control area			
Orchidarium	A	An isolated orchidarium located along an abandoned trail, not intended for public access.	1(♀) / 2
Pond	B	Permanent water body located 700 m from the visitor areas of JBR	1(♀) / 1
Bromelial	C	A collection of terrestrial and epiphytic bromeliads located along an abandoned trail, with no intention for public access	1(♀) / 2
Leaf litter	D	Sites of intense deposition of organic plant material off abandoned trails.	1(♂) / 2
Anthropized area			
Library walls	E	Walls of libraries designated for environmental education for visiting public	1(♀)/ 2 1(♀) / 3 1(♂) / 1
Air conditioning unit	F	External portion of the air conditioning unit, exposed on the outside of the wall	1(♀) / 1
Bromeliad exhibit	G	A group of terrestrial bromeliads in a paved area of the JBR, intended for public visits and environmental education activities	1(♀) / 2
Bathroom	H	Public restroom with free access to visitors, characterized by an enclosed, humid environment with daily usage.	1(♀) / 2 1(♀) / 2
Total n			11

Molecular analysis.—We obtained the DNA used in the molecular analyses by osmotic lysis from a solution of a 10 µL calibrated loop with bacterial culture diluted in 200 µl of sterile Milli-Q water. After the procedure, we used 3µL of this suspension to amplify the 16S rRNA gene by polymerase chain reaction (PCR). For each reaction, we used 5 µl of buffer, 2.5 µl of MgCl2, 0.5 µl of dNTP and 1 µl of each primer 27F (GAGTTTGATCCTGGGGCTCAG) and 1093R (GTTGCGCTCGTTGCGGAACT; Lane 1991). We obtained 31 isolates, but we removed 11 from the set as they did not amplify during PCR, or the DNA quantity was insufficient for high-quality sequencing. The remaining 20 isolates were viable for sequencing and subsequent analyses.

We purified the amplified samples with phenol-chloroform, quantified by spectrophotometry on NanoDrop® 2000 (Thermo Scientific, Wilmington, Delaware, USA) and subsequently sequenced on the ABI3500 Genetic Analyser (Applied Biosystems, Foster City, California, USA). We analyzed the electropherograms in BioEdit 7.2 (https://thalljiscience.github.io/) to clean up the sequences and then analyzed them in the National Centre for Biotechnology Information (NCBI) database to search for homologies, applying a threshold for query cover, percentage identity, or e-value as the basis (Appendix Table).

Results

The 20 isolates from the skin of *S. x-signatus* included 11 bacterial species belonging to the families Enterobacteriaceae, Bacillaceae, and Moraxellaceae (Table 2). Enterobacteriaceae and Bacillaceae were present in both areas, whereas Moraxellaceae was the least frequent family. Enterobacteriaceae was the most representative family overall. Bacillaceae was the second most predominant family in both areas, whereas Moraxellaceae was exclusively found in the AA (Table 2).

Three bacteria were common to both sample areas: *Enterobacter hormechei*, *Enterobacter cloacae*, and *Bacillus cereus*. These three species represent the entire diversity sampled in the natural sites, with *Enterobacter hormaechei* being the most abundant. Enterobacteriaceae exhibited greater diversity in the AA, with eight additional Enterobacteriaceae

Table 2. The absolute and relative frequencies of bacterial families, genera, and species collected from the skin of Venezuelan Snouted Treefrogs (*Scinax x-signatus*) in the control and anthropized areas within the Recife Botanical Garden, northeast Brazil. Sampling location codes are A = orchidarium; B = pond; C = bromeliads; D = leaf litter; E = library; F = air condition unit (outdoor); G = bromeliad at visitor facility; H = bathroom.

Family / Species	Collection point	Absolute frequency per area	Relative frequency per area (%)
Control area (n = 4 animals)			
Bacillaceae		1	14.29
Bacillus cereus	A	1	14.29
Enterobacteriaceae		6	85.71
Enterobacter cloacae	C, D	2	28.57
Enterobacter hormaechei	A, B, C, D	4	57.14
Total		7	100
Anthropized area (n = 7 animals)			
Bacillaceae		2	15.38
Bacillus cereus	E	1	7.71
Exiguobacterium acetilium	H	1	7.71
Enterobacteriaceae		7	53.85
Enterobacter cloacae	G	1	
Enterobacter hormaechei	E, H	3	23.07
Enterobacter sp.	H	1	7.71
Escherichia coli	H	1	7.71
Klebsiella pneumoniae	F	1	7.71
Pantoea dispersa	E	1	7.71
Serratia marcescens	E	1	7.71
Moraxellaceae		2	15.38
Acinetobacter junii	E	1	7.71
Acinetobacter pittii	G	1	7.71
Total		13	100

species occurring exclusively in this area. Among them, we identified *Escherichia coli* and *Klebsiella pneumoniae* at sampling points frequently used by humans, specifically the buildings called the Library and Bathroom, respectively. Additionally, we identified the Moraxellaceae family, exclusive to the AA, represented by the species *Acinetobacter junii* and *A. pittii* (Table 2).

Discussion

We identified the bacterial families Enterobacteriaceae, Bacillaceae, and Moraxellaceae on the skin of *S. x-signatu*s in the course of our study, with Enterobacteriaceae being the most prevalent. Our findings are consistent with those of Proença et al. (2021), who also reported the prevalence of enterobacteria on the skin of anurans in areas with and without environmental impacts, such as metal pollution. Although these families were present in both areas, the frogs from the human-impacted area exhibited a greater diversity of enterobacteria, with the first reports of *Klebsiella pneumoniae* and *Escherichia coli* on the skin of wild populations.

The presence of *Enterobacter hormaechei*, *E. cloacae*, and *Bacillus cereus* in frogs from both study areas aligns with previous studies on anuran skin microbiota (Ienes-Lima et al. 2023; Assis et al. 2016; Martin et al. 2020; Proença et al. 2021). These bacteria are generally regarded as ubiquitous species, widely distributed across diverse microenvironments, including soil, water, vegetation, and anthropogenic structures (Murray et al. 1998). This ubiquity may explain their presence at all four sampling sites within the control area of our study.

Similarly, the occurrence of *Bacillus cereus*, a member of the Bacillaceae family, in both study areas can be attributed to its ubiquity and ability to form endospores, structures that allow the microorganism to survive for extended periods in adverse environments

(Mandic-Mulec et al. 2015). Although the number of isolates in our study is relatively small, our results are also consistent with previous studies that have suggested that *Bacillus cereus* and other *Bacillus* species may constitute the symbiotic skin microbiota of ranids, bufonids, and hylids (Bletz et al. 2017). Therefore, this may indicate that Enterobacteriaceae and Bacillaceae not only exhibit higher adaptability to environmental challenges (Gonzales-Siles and Sjöling, 2016) but can also colonize the skin of anurans under similar conditions.

The records of bacterial species that are restricted to the anthropized area offer valuable insights. Of particular interest is the identification of *Pantoea dispersa* and *Serratia marcescens*, which may have implications for amphibian skin defense, particularly in the context of defending against *Batrachochytrium dendrobatidis* (Bd), the pathogen responsible for chytridiomycosis in amphibians (Ienes-Lima et al. 2023). Additionally, *Serratia marcescens* has been associated with multidrug resistance (MDR) in the Brazilian endemic Admirable Redbelly Toad (*Melanophryniscus admirabilis*) populations inhabiting anthropized environments (Ienes-Lima et al. 2023). In light of these findings, we posit that the greater bacterial diversity observed in frogs from the anthropized area may be associated with both the higher number of isolates obtained in the anthropized area and the environmental complexity of the sampling sites.

One of the key findings in our study is the first report of *Escherichia coli* and *Klebsiella pneumoniae* on the skin of wild anuran populations. While these bacteria are well known to inhabit the gastrointestinal tract of endothermic animals (Schierack et al. 2009), their presence on anuran skin raises questions about their role in the microbiota of *S. x-signatus*. These opportunistic pathogens are often associated with human and animal infections (Rogers et al. 2011). In anurans, only one study with *K. pneumoniae* has been documented as a producer of carbapenemases (MDR-KP), causing infections with clinical symptoms such as spasms, hyperemia, and inflammation in farmed American Bullfrogs (*Rana catesbeiana*) in China (Lin et al. 2023). Moreover, multidrug-resistant (MDR) strains of enterobacteria were recorded in Brazilian endemic species of anurans found in anthropized environments (Ienes-Lima et al. 2023). Our study suggests that these bacteria could be transmitted to *S. x-signatus* through direct or indirect contact with human feces or surfaces contaminated by humans in the human-impacted sites. As *S. x-signatus* is commonly found in urban vegetation and decorative bodies of water (Pereira et al. 2016), we suggest that these bacteria were introduced to the specimens through human activities.

We highlight the significant potential of anurans to act as sentinels for monitoring the extent of spread of MDR bacteria in the environment (Goulas et al. 2020). Most anuran species, such as the hylids *S. x-signatus*, live at the interface between aquatic and terrestrial habitats to prevent skin desiccation, complete their life cycle, and achieve reproductive success (Vági and Székely, 2023). Thus, anurans are exposed to both human and wildlife pathogens, and the presence of rivers and surface water bodies connecting urban to less degraded neighboring areas can facilitate the spread of pathogens (Fernandes et al. 2016; Wyres and Holt, 2018). Our findings suggest that *S. x-signatus* may carry and spread resistant bacteria, which requires further research to document.

The presence of Moraxellaceae, specifically by the species *Acinetobacter junii* and *Acinetobacter pitii* only in urban sites represents a different finding. Although these species were previously considered environmental microorganisms (Atrouni et al. 2016), their detection exclusively in the human-impacted area suggests they may also be part of the human-associated microbiota and could be opportunistic pathogens to other vertebrates, such as fishes, causing septicemic diseases (Malick et al. 2020). This highlights the potential for amphibians living in human environments to serve as reservoirs for human-associated bacteria, contributing to the spread of opportunistic pathogens in both wildlife and human populations. For example, the establishment of pathogenic agents on frog skin, such as the bacterium *Aeromonas hydrophila* and the fungus *Batrachochytrium dendrobatidis*, can result in fatal and highly transmissible infections (Khalifa et al. 2021; Jiménez et al. 2020; Ruthsatz et al. 2020).

In consideration of bacterial diversity, high diversity is typically associated with complex environments in relatively unfragmented and connected ecosystems (Hibbing et al. 2010; Walters and Martiny, 2020). Our study indicates that the elevated bacterial diversity observed in the frogs from areas with people can be attributed to two primary factors. First, the greater number of *S. x-signatus* specimens collected in the human-impacted area relative to the control area may have increased the probability of detecting additional bacterial species. We collected seven frogs in the area with people, resulting in eight additional bacterial

species compared to the control area. Secondly, the environmental complexity of this site, resulting from human activities, may have constituted a significant driver of bacterial colonization and dispersion. This phenomenon has also been documented by Ramey and Ahlstrom (2020), who demonstrated that human activities are a significant source of environmental fragmentation, contributing to the creation of complex microenvironments that facilitate the dispersion of microorganisms to synanthropic fauna. The adaptation of *S. x-signatus* individuals to modified environments and the spatial heterogeneity caused by human habitats may have contributed to the presence of human-associated bacterial species on the skin of these treefrogs.

It is important to acknowledge the limitations of this study. The relatively modest sample size and the subsequent loss of some samples due to the lack of 16S gene amplification, the inferior quality of certain isolates, and the laboratory access restrictions encountered during the ongoing COVID-19 pandemic have affected the representativeness of the results. Without next-generation sequencing techniques, our understanding of bacterial diversity in this study was limited to species of bacteria that could be cultivated. The focus on only 20 isolates per area may not fully capture the bacterial community associated with this species. Furthermore, the restricted sample size precluded an evaluation of potential biological factors that may influence the microbiota, including sex-related differences or specific environmental conditions beyond the anthropization gradient.

Notwithstanding these limitations, our findings offer valuable insights into the characterization of the culturable bacterial microbiota of *S. x-signatus*. Furthermore, we highlight the necessity for future studies to consider the potential of animals with a biphasic life cycle, such as anuran amphibians, as indicators of MDR strain dissemination in the environment. It is imperative to identify this indicative potential, particularly in endemic species, to facilitate effective ecosystem management and enhance our understanding of biotic interactions.

Acknowledgments.—We express our gratitude to the Jardim Botânico do Recife and the Sistema de Autorização e Informação em Biodiversidade (SisBio) for granting permission for collections in the fragment. We also extend thanks to the Universidade Federal Rural de Pernambuco (UFRPE) and the Plataforma Tecnológica de Genômica e Expressão Gênica do Centro de Ciências Biológicas-CCB/UFPE for their partnership in the proposed analyses. Special thanks to Ubiratã Ferreira Souza, Carla Rodrigues Ribas, and Lucas Tavares for their insightful help and consideration in the analysis and manuscript writing. Additionally, we note that this article was partially produced during the PPGBIO00047 - Tópicos Especiais em Biodiversidade IX course of the Biodiversity Postgraduate Program at the Universidade Federal Rural de Pernambuco. We also thank the Sistema de Autorização e Informação em Biodiversidade (SISBio) for the approval of this study (2019/80088).

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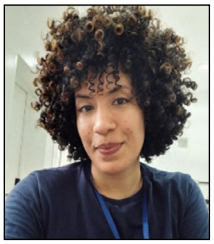
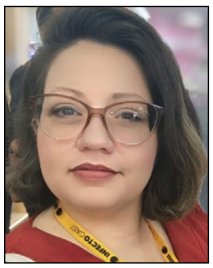
Appendix Table. Identification of sequences through BLAST analysis on NCBI, showing the scientific names of the identified organisms, with query cover, E-value, and percentage identity (Percentage ID) obtained for each sequence.

Scientific name	Query cover	E-value	Percentage ID
Enterobacter hormaechei	100%	0.0	100%
Bacillus cereus	100%	0.0	100%
Enterobacter hormaechei	100%	0.0	100%
Enterobacter hormaechei	100%	0.0	100%
Enterobacter cloacae	100%	0.0	100%
Enterobacter hormaechei	100%	0.0	97.99%
Enterobacter hormaechei	100%	0.0	100%
Acinetobacter junii	100%	0.0	99.78%
Pantoea dispersa	100%	0.0	100%
Enterobacter hormaechei	100%	0.0	100%
Enterobacter hormaechei	100%	0.0	100%
Klebsiella pneumoniae	99%	0.0	91.52%
Enterobacter cloacae	100%	0.0	99.88%
Enterobacter cloacae	100%	0.0	99.86%
Escherichia coli	100%	0.0	100%
Exiguobacterium acetylicum	100%	0.0	99.76%
Bacillus cereus	100%	0.0	99.14%
Enterobacter sp.	100%	0.0	100%
Bacillus cereus	100%	0.0	99.73%
Acinetobacter pitii	100%	0.0	100%



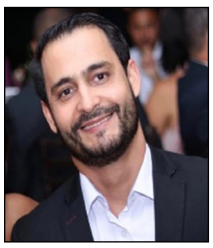
Lara V. M. Costa Santos is a Ph.D. candidate in the Biodiversity Graduate Program at the Federal Rural University of Pernambuco (UFRPE). She holds a B.Sc. in Biological Sciences and an M.Sc. in Ethnobiology and Nature Conservation. Lara is a member of the Herpetological and Paleoherpetological Studies Lab and Molecular Microbiology Lab at UFRPE, and her research focuses on environmental microbiology and amphibian conservation, specializing in amphibian marking, microbiological and molecular techniques, and applied ecology. Her work emphasizes anuran microbiology and conservation. (Photographed by Lara V.M. da Costa Santos).

Alcina G. M. M. F. Santos is a Ph.D. candidate in Animal Biosciences at the Federal Rural University of Pernambuco (UFRPE). She holds a B.Sc. in Biological Sciences (2017) and an M.Sc. in Ecology (2020), both from UFRPE. Alcina interned at the Herpetological and Paleoherpetological Collection and the Cavouco Group, both at UFPE. She is a member of the Herpetological and Paleoherpetological Studies Lab and Molecular Microbiology Lab and is a collaborator on projects at the Catholic University of Salvador, Brazil, and at UFRPE, specializing in wildlife marking, parasitology, and environmental DNA. (Photographed by Alcina G.M.M.F. Santos).



Paula M. S. Souza earned a Ph.D. from the Postgraduate Programme in Applied Cellular and Molecular Biology at the University of Pernambuco, Brazil. She has experience in molecular genetics and applied microbiology, and her work focuses on the phenotypic and genotypic characterization, regulation, and surveillance of Gram-negative bacteria that cause healthcare-associated infections and their antibiotic resistance mechanisms. Paula is currently a Postdoctoral Researcher at the Federal Rural University of Pernambuco, Brazil, investigating the potential of bacteriophages to combat antibiotic-resistant human and animal pathogens. (Photographed by Paula M.S. Souza).

Anna C. S. Almeida is an Assistant Professor in the Genetics, Department of Biology, at the Federal Rural University of Pernambuco, Brazil (UFRPE). She holds a B.Sc. in Biological Sciences (UFRPE), an M.Sc. in Cellular and Molecular Biology (University of Pernambuco), and a Ph.D. in Genetics (UFPE). Anna is a permanent faculty member of the Graduate Program in Cellular and Molecular Biology at the Institute of Biological Sciences, University of Pernambuco, and coordinates the Molecular Microbiology Laboratory at UFRPE. Her research focuses on pathogen identification, resistance mechanisms, and environmental impact remediation. (Photographed by Anna C.S. Almeida).



Geraldo J. B. Moura is a Professor and Researcher, a National Council for Scientific and Technological Development 1D Research Productivity Fellow. He holds a Postdoctoral degree in Behavior from the Center for the Study of Biodiversity at the University of Porto, Portugal, a Ph.D. in Biological Sciences from the Federal University of Paraíba, João Pessoa, Brazil, and an M.Sc. in Paleontology from the Federal University of Pernambuco/University of Buenos Aires, Brazil specializing in Human Anatomy (Federal University of Pernambuco) and Zoology (Federal Rural University of Pernambuco). Geraldo also is the Director of Environmental Monitoring at the State Environmental Agency of Pernambuco. (Photographed by Letícia Costa Moura).