Short Communication

# Elucidation of bacterial community structure on thin-spined porcupine (*Chaetomys subspinosus*) spines by denaturing gradient gel electrophoresis (DGGE) and sequencing

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**ABSTRACT.** Thin-spined porcupines (*Chaetomys subspinosus*) are threatened with extinction and are categorized as vulnerable. This is because of alteration to and loss of their habitat and possible hunting activities in their distribution area. Their spines constitute one of their defense mechanisms, which can be fomites for pathogens to humans. However, little is known about such pathogens. The present study aimed to detect bacteria on spines of *C. subspinosus,* from the Una Biological Reserve, South of Bahia, northeastern Brazil, by analyzing metagenomic DNA, isolating bacterial culture, using the denaturing gradient gel electrophoresis (DGGE) technique, and sequencing. Six anatomical

 

points were selected for withdrawing spine samples from an individual *C. subspinosus*. At all sample points, bacteria were detected by bacteriological culture and/or DGGE and sequencing of excised bands. When all samples were combined, standard PCR-DGGE analysis of bacteria present in the spines identified 15 distinct bands, thereby revealing a distinct bacterial community. The main pathogens identified through sequencing were *Bacillus cereus, B. thuringiensis, B. anthracis,* and *B. pumilus.* The present study demonstrated the isolation and identification of non-pathogenic and pathogenic bacteria on the spines of *C. subspinosus*.

**Key words:** Wild animal; Pathogenic bacteria; Microbial diversity; Host-pathogen interaction; Thin-spined porcupine; *Chaetomys subspinosus*

## INTRODUCTION

The thin-spined porcupine (*Chaetomys subspinosus*, Olfers, 1818) constitutes part of a group of rodents (order: Rodentia) from the suborder Hystricognatha and family Erethizontidae (Eisenberg and Redford, 1999). This monotypic species is the only representative of the subfamily Chaetomyinae. According to estimates, it originates from a single clade that diverged at least 10 million years before the diversification of the other species from Erethizontidae (Vilela et al., 2009). Therefore, there is great interest in the thin-spined porcupine, and it is referred to as one of the most distinct and important endemic mammals of the Brazilian Atlantic forest.

The thin-spined porcupine is nocturnal, solitary, and arboreal, with low activity levels and a herbivorous diet (Giné et al., 2010). These characteristics are common in other species of arboreal rodents (Eisenberg and Redford, 1999). The thin-spined porcupine also has hair that is modified into spines, similar to other species in this family. The spines are circular when viewed in a cross section, with few variations in color pattern. However, their shape, length, width, and tips enable them to be classified as follows: cranial, dorsal, ventral, and the base of the tail (Martinez et al., 2005).

The numbers of thin-spined porcupines are believed to decline dramatically owing to the reduction of and alteration to their natural habitat. In addition, hunting is possibly another factor that threatens this species. Large-scale hunting by rural populations is believed to occur throughout their distribution area. This has led to the classification of this species as “Vulnerable” on the official list of Brazilian Fauna Threatened with Extinction (Chiarello et al., 2008), and listing in the International Union for the Conservation of Nature and Natural Resources Red List of Threatened Species (Catzeflis et al., 2008).

Consumption of wild animal meat by local communities in Neotropical regions, such as the Atlantic forest of Brazil, is at a high level. This results in a high risk of pathogenic contamination in humans (Cullen et al., 2000). Disturbances in the ecosystem of the Atlantic forest could also bring humans in contact with fauna that are associated with pathogens. Some areas of the Atlantic rainforest are located within highly urbanized areas (Bradley and Altizer, 2007), thereby bringing wildlife and their associated pathogens into closer contact with domestic animals and humans. Thus, individuals who handle wildlife, such as the thin-spined porcupine can be at risk for infection, particularly if injured by the spines of *C. subspinosus*.

Little is known about the pathogens that exist in this species. We believe that such information can serve as a useful tool to curb hunting through education and awareness, and to warn against the risks posed by possible pathogens. In order to establish effective measures to

warn individuals against pathogenic risks of handling wildlife, it is necessary to adopt studies that emphasize quick and accurate identification of these pathogens.

Thus, the objective of this study was to detect bacteria present in the spines of *C. subspinosus* from the Una Biological Reserve, South of Bahia, northeastern Brazil, by means of analyzing metagenomic DNA and isolating bacterial cultures by denaturing gradient gel electrophoresis (DGGE) and sequencing.

## MATERIAL AND METHODS

### Collection of biological samples

In July 2013, the biological samples (spines) were obtained from an adult thin-spined porcupine captured in the Una Biological Reserve, Brazil. The animal was sedated following the method set out by Giné et al. (2010). Following deep anesthesia, six anatomical locations were selected for withdrawal of spine samples: the head region (frontal), the dorsal region (cervical, thoracic, lumbar, and tail base), and lateral region. The samples were placed in sterile vials and transferred to the Laboratory of Agroindustrial Microbiology and the Laboratory of Microbial Biotechnology at Universidade Estadual de Santa Cruz (UESC). All procedures were performed with legal approval and consent from the Brazilian Federal Authority (ICMBio, license Nos.: 25184-1; 23468-2; and 27021-1) and the Committee of Ethics for the Use of Animals - UESC (protocol No. 024/2013).

### Cultivation and isolation of bacteria

Three spines from each of the five regions (frontal, cervical, thoracic, lumbar, and tail base) were placed on a Petri dish containing Tryptic soy agar (TSA) culture medium (5 g peptone, 15 g tryptone, 5 g NaCl, 15 g agar, and 1000 mL distilled water). These samples (spines) were distributed over the surface of the medium with a Drigalski spatula.

In order to obtain pure cultures, the plates were kept in a biochemical oxygen demand incubator at 26°C, thereby allowing the growth of isolated colonies. After 48 h, individual colonies showing distinct morphological features were selected, and transferred with a platinum spatula to Petri dishes with TSA medium. Molecular tests were performed following bacterial isolation.

### Total metagenomic DNA extraction from spines and bacterial colonies

Metagenomic DNA was extracted from spines of the lateral area and from isolated bacterial colonies according to procedures modified from Ferreira and Grattapaglia (1998). Approximately 200 mg of each sample (spines or bacterial colonies) were crushed in liquid nitrogen. The crushed tissue was exposed to 700 µL extraction buffer (2% cetyltrimethylammonium bromide, 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA) and incubated for 30 min at 65°C, followed by two extractions: the first with 450 µL chloroform:isoamyl alcohol (24:1) and the second with 450 µL buffered phenol. After 5 min centrifugation at 12,000 rpm, the DNA was precipitated with 500 µL isopropanol at -20°C for 60 min. The precipitate was centrifuged at 12,000 rpm for 5 min and successively washed with 70 and 100% ethanol. After drying, the DNA was resuspended in 50 µL Tris-EDTA buffer and treated with 2 µL RNAse for 30 min at 37°C.

## PCR-DGGE

PCR was performed using a region of about 1500 bp from the gene that encodes the 16S rRNA, which was amplified using the primers F27 (5'-AGAGTTTGATCGGCTCAG-3') and R1525 (5'-AAGGAGGTGTCCARCC-3') (Lane, 1991). The amplification cycle consisted of an initial 5 min denaturation step at 94°C, followed by 35 1-min cycles at 94°C, 2 min at 55°C, 1 min at 72°C, and a final 5-min extension step at 72°C. The product from the first reaction (2 µL) was used for the second reaction. In the second step, a region of about 360 bp was amplified using the primers F984 (5'-CCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3') and R1378 (5'-CGGTGTGTACAAGGCCCGGGAACG-3') (Heuer et al., 1997). The underlined sequence in F984 indicates the GC clamp required for DGGE (Sheffield et al., 1989).

The amplification cycle consisted of an initial 3-min denaturation step at 94°C, followed by 30 1-min cycles at 94°C, 1 min at 60°C, 1 min at 72°C, and a final 15-min extension step at 72°C. The amplification reactions had a final volume of 25 µL containing a mixture of 60 mM Tris-HCl, pH 9.0, 0.2 mM of each dNTP, 10 pmol of each primer, 2.5 mM $MgCl_2$, and 1 U Taq DNA Polymerase. In order to view PCR products, 5 µL of the suspension was subjected to electrophoresis on 1% agarose gel in 1X Tris/Borate/EDTA buffer, and then stained with ethidium bromide and examined under UV light.

## DGGE

The procedure for electrophoresis and for preparation of the gel was adapted from Muyzer et al. (1993). The amplified products in the second PCR were separated by polyacrylamide gel electrophoresis at 8% (37.5:1 acrylamide:bisacrylamide) prepared with a 20-50% linear gradient of denaturant solution (a 100% denaturant solution contains 40% formamide and 7 M urea). The PCR products (5 µL) were applied and subjected to electrophoresis at 70 V for 15 min, followed by 200 V for 4 h. The electrophoretic run was performed in 1X Tris-acetate-EDTA buffer at a constant temperature of 60°C.

The gel was subsequently stained with 0.1% silver nitrate and digitized using a Scanner (HP Officejet Pro J5780). The profiles were analyzed and a binary data matrix was created from the gel showing the presence or absence of bands. This matrix was used to analyze species richness, considering the total number of bands detected in each DGGE profile. Dice similarity dendrograms and Venn diagrams were generated using the PAST (Palentological Statistics) software (Version 2.17c) (Hammer et al., 2001).

## DGGE band sequencing

Based on the patterns obtained, the bands of interest were excised from DGGE gel using sterile scalpel blades and eluted in 50 µL ultrapure sterile water (Milli-Q, Millipore Corporation, Billerica, MA, USA). The diffused DNA was used as a template in the PCR with primers F984 and R1378 without the GC clamp, according to the protocol described by Heuer et al. (1997). The samples were subjected to sequencing and the sequences obtained were compared to sequences from the Ribosomal Database Project II version 10 (http://rdp.cme.msu.edu/) and from GenBank at the National Center for Biotechnology Information (NCBI), using the nucleotide blast program from the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

## RESULTS

Bacteria were present at all six sample points of the *C. subspinosus* specimen indicated in Figure 1. When all samples were combined, standard PCR-DGGE band analysis of bacteria present in the spines, identified 15 distinct bands. A mean of 5.7 distinct bands were observed among the sampled points (Figure 2).

There proved to be a distinct bacterial community on the spines of the specimen. Of the bands present at point 1 (metagenomic DNA), none were common to all samples, but 50% of bands present in this sample were present in other sampled points. Band patterns were used to estimate the structure of the bacterial community in the *C. subspinosus* specimen. This estimate was made by means of a cluster analysis of the observed DGGE bands for each sample (Figure 2). The dendrogram that was generated based on the 16S rDNA DGGE profile (bacteria), distinguished points 3 and 4 as one external group that shared similarity. Points 1 and 6 were also revealed to have a closely related bacterial community.

The Venn diagram in Figure 3 shows a grouping of bands that can be shared between sampling points, or between points that are unique to the sample. There were a greater number of bands shared between points 1 and 6 in the bacterial DGGE. These two sampling points also shared two exclusive groups, not present at other points, thereby demonstrating similarity in the distribution of bacterial species. DGGE bacterial profiles suggest greater species richness at points 1 and 6. By excising the most intense bands generated by DGGE of each sampled point, and subsequent sequencing, detected bacterial species are recorded in Table 1.

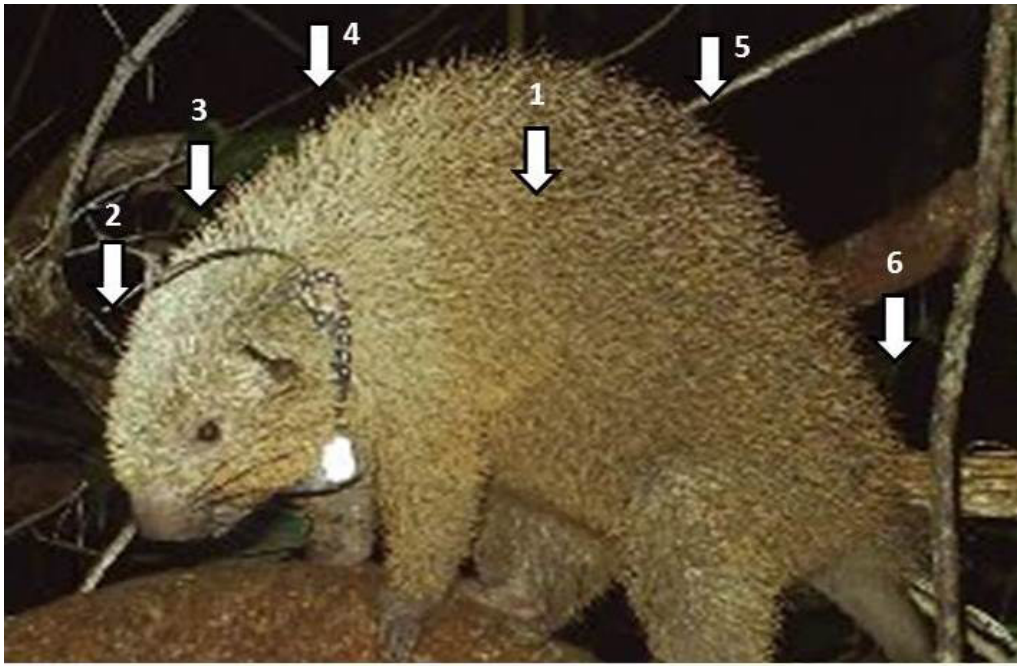


**Figure 1.** Sample points along the *Chaetomys subspinosus* specimen from the Una Biological Reserve, Brazil. Point 1: spine removed for metagenomic DNA extraction; point 2: head region (frontal); point 3: cervical region; point 4: thoracic region; point 5: lumbar region; point 6: base of the tail.

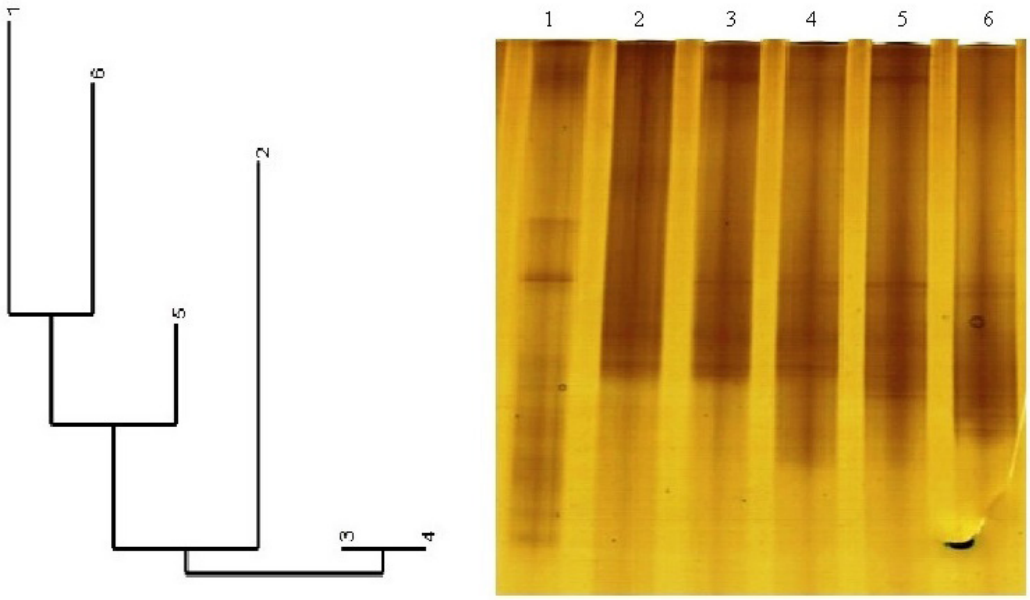


**Figure 2.** Dice dendrogram generated from denaturing gradient gel electrophoresis of amplified fragments with 16S primers.

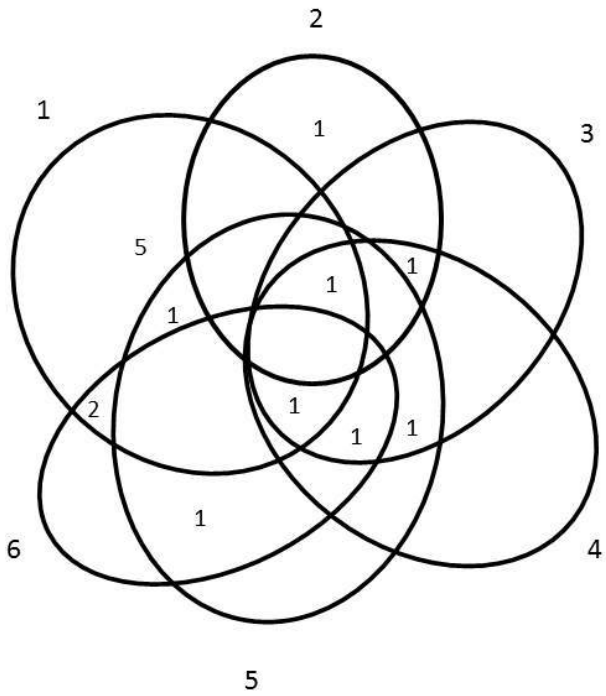


**Figure 3.** Venn diagram showing the richness of bands detected by DGGE profiles at different sampling points.

**Table 1.** Bacterial detection at each sampled point of the *Chaetomys subspinosus* specimen, through sequencing of the most intense bands from denaturing gradient gel electrophoresis (DGGE).

| Sequence with the highest-scoring segment pairs | Max score | Ident; e-value | GenBank accession No. |
|---|---|---|---|
| Point 1. Metagenomic DNA | | | |
| *Bacillus pumilus* strain JUN-15 16S ribosomal RNA gene, partial sequence | 279 | 82%; 3e-71 | KF228933.1 |
| *Bacillus* sp PPB16 16S ribosomal RNA gene, partial sequence | 275 | 81%; 4e-70 | HM771669.1 |
| *Bacillus funiculus* strain WRA-6 16S ribosomal RNA gene, partial sequence | 275 | 81%; 4e-70 | EF636899.1 |
| *Bacillus cereus* strain MSCS33 16S ribosomal RNA gene, partial sequence | 269 | 81%; 2e-68 | KJ882426.1 |
| Point 2. Head region | | | |
| *Bacillus weihenstephanensis* strain BPLa23 16S ribosomal RNA gene, partial sequence | 345 | 83%; 3e-91 | KF387717.1 |
| *Bacillus cereus* strain p20_D01 16S ribosomal RNA gene, partial sequence | 345 | 83%; 3e-91 | JN236260.1 |
| *Bacillus thuringiensis* serovar finitimus gene for 16S rRNA, partial sequence | 343 | 83%; 9e-91 | AB617473.1 |
| *Bacillus anthracis* strain A1a 16S ribosomal RNA gene, partial sequence | 342 | 83%; 3e-90 | KJ808579.1 |
| Point 3. Cervical region | | | |
| *Bacillus thuringiensis* strain b24sy 16S ribosomal RNA gene, partial sequence | 695 | 99%; 0.0 | KC574674.1 |
| *Bacillus* sp B56 16S ribosomal RNA gene, partial sequence | 693 | 99%; 0.0 | GU321100.1 |
| *Bacillus cereus* 16S rRNA gene, isolate AB1A1 | 693 | 99%; 0.0 | AM062679.1 |
| *Bacillus* sp 7S7 16S ribosomal RNA gene, partial sequence | 691 | 99%; 0.0 | KM374750.1 |
| Point 4. Thoracic region | | | |
| *Bacillus thuringiensis* strain KNUC2103 16S ribosomal RNA gene, partial sequence | 682 | 98%; 0.0 | JN084031.1 |
| *Bacillus* sp Pc119 16S ribosomal RNA gene, partial sequence | 682 | 98%; 0.0 | EU333134.1 |
| *Bacillus thuringiensis* strain b24sy 16S ribosomal RNA gene, partial sequence | 681 | 98%; 0.0 | KC574674.1 |
| *Bacillus cereus* strain BGZ-8 16S ribosomal RNA gene, partial sequence | 679 | 98%; 0.0 | KM378612.1 |
| Point 5. The lumbar region | | | |
| *Bacillus cereus* strain DCB1 16S ribosomal RNA gene, partial sequence | 646 | 96%; 0.0 | JN650544.1 |
| *Bacillus* sp 7S7 16S ribosomal RNA gene, partial sequence | 645 | 96%; 0.0 | KM374750.1 |
| *Bacillus cereus* strain BGZ-8 16S ribosomal RNA gene, partial sequence | 645 | 96%; 0.0 | KM378612.1 |
| *Bacillus cereus* strain MSSRF Q10 16S ribosomal RNA gene, partial sequence | 645 | 96%; 0.0 | KM596819.1 |
| Point 6. The base of the tail | | | |
| *Bacillus pumilus* strain JUN-15 16S ribosomal RNA gene | 279 | 82%; 3e-71 | KF228933.1 |
| *Bacillus* sp PPB16 16S ribosomal RNA gene, partial sequence | 275 | 81%; 4e-70 | HM771669.1 |
| *Bacillus funiculus* strain WRA-6 16S ribosomal RNA gene, partial sequence | 275 | 81%; 4e-70 | EF636899.1 |
| *Bacillus* sp S2-3 16S ribosomal RNA gene, partial sequence | 271 | 81%; 5e-69 | FJ217159.1 |

Primers - Sequence - 16S rDNA target - (*Escherichia coli* numbering): F984 (5'-GCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3') - Bacteria (968-984) and R1378 (5'-CGGTGTGTACAAGGCCCGGGAACG-3') - Bacteria (1378-1401).

## DISCUSSION

The present study provides evidence that spines of *C. subspinosus* are colonized by bacteria that may be pathogenic to humans, domestic, and wild animals. Case studies in medical literature indicate that pathogenic bacteria can be introduced through the skin, following injury by the animal's spines, and can cause serious infections (Azizi et al., 2014).

Sampling points 1 and 6 showed the largest number of bands in DGGE, indicating greater bacterial diversity. The spines analyzed from these anatomical regions are not capable of breaking the skin, but seem to be the only vehicles of specific bacteria. However, flies (*Chrysomya megacephala* and *Musca domestica*) and cockroaches (*Periplaneta americana* and *Blattela germanica*) are also mechanical vectors of *Bacillus* sp that can cause infections in humans (Chaiwong et al., 2014; Isaac et al., 2014).

Pathogenic bacteria, such as *Bacillus cereus* and *Bacillus anthracis*, identified at sampling points 2 and 3, showed comparatively lower diversity; however, spines from these points are capable of puncturing the epidermis. Black Pakistan cobras (*Naja naja karachiensis*) also carry and can transmit *Bacillus* sp through bite injuries and wound contamination (Iqbal et al., 2014).

The evidence from the present study suggests that the spines of *C. subspinosus* harbor a variety of pathogenic bacteria that are much more harmful than the pain inflicted by the physical

injury. Several bacteria have defensive functions associated with the penetration of porcupine spines (Bisharat et al., 1999).

Among the pathogens identified in the present study, there were *B. cereus*, which is an opportunistic pathogen that causes food poisoning and provokes diarrheal or emetic conditions (Ivanova et al., 2003), and *B. thuringiensis,* which is a well-known insect pathogen (Aronson and Shai, 2001). In addition, *Bacillus anthracis* is an etiologic agent of anthrax, causing acute disease in animals and humans (Jensen et al., 2003). *Bacillus pumilus* rarely infects humans, but when it does, it is associated with skin infections related to animal contamination (Tena et al., 2007; Azizi et al., 2014).

The present study indicates the pathogenic potential of bacteria isolated and identified from *C. subspinosus* spines. These spines serve a defensive function by inflicting physical injuries, but harm can also be caused by opportunistic pathogenic bacteria introduced via the injuries.

## Conflicts of interest

The authors declare no conflict of interest.

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