DIVERSITY OF BACTERIA IN THE SEXUALLY SELECTED EPAULETTES OF THE LITTLE YELLOW-SHOULDERED BAT *Sturnira lilium* (CHIROPTERA: PHYLLOSTOMIDAE)

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SUMMARY

In bats, chemical signals are particularly important for communication. Although the scent from body fluids might be crucial to mating success, the presence of microbes in odor-producing structures might be indispensable because some substances must be metabolized by bacteria and experience biochemical changes before they acquire detectable odors and become meaningful signals. The goal of this study was to identify bacteria in sexually dimorphic shoulder glands ('epaulettes') of males of Sturnira lilium *and* S. bogotensis*, and determine whether some of these bacteria have been reported as present in sexually-selected male organs of other bat species. Identification of bacteria was attained through amplification and sequencing of their corresponding 16S rRNA genes. Fourty-two species of bacteria were identified in* S. lilium *male (n=3) and female (n=3) specimens and a* S. bogotensis *male. Males of* S. lilium *and* S. bogotensis *had 15 and 7 species of bacteria in epaulettes, respectively. Similarity between males and females, and between body parts in terms of their bacteriological profile was very low. Although there were common bacteria in epaulettes and backs,* Citrobacter freundii*,* Enterococcus faecalis*,* Exiguobacterium acetylicum *and* Flavobacterium mizutaii *were exclusively found in epaulettes of* S. lilium*. From the identified bacteria in epaulettes of males of* S. lilium*, four species (*Staphylococcus saprophyticus*,* S. sciuri*,* E. faecalis *and* Bacillus *cereus) have been found in sexually-selected male organs of other bat species. Common genera of bacteria in sexually selected male traits of bats are* Bacillus*,* Staphylococcus*,* Corynebacterium *and* Enterococcus*.*

Introduction

Chemical signals in bats are particularly important for communication (Bloss, 1999; Krutzsch, 2000; Dechmann and Safi, 2005), as for most mammals (Eisenberg and Kleiman, 1972; Blaustein, 1981; Andersson, 1994). Communication and social behavior of most bats is poorly known, making it difficult to determine the function of chemical signals and glandular scent organs. Like most other mammals, bats appear to make extensive use of chemical signals in a range of situations (Scully *et al*., 2000).

Odor production in bats is exceptionally diverse, and males have a more diverse and abundant repertoire of odors than females, mainly during the mating season (Quay, 1970; Eisenberg and Kleiman, 1972; Schmidt, 1985; Brooke and Decker, 1996; Krutzsch, 2000; Scully *et al*., 2000). Chemical signals are particularly important during attraction, individual recognition, and mate selection based on specific individual odor profiles (Blaustein, 1981; Höller and Schmidt, 1993; De Fanis and Jones, 1995; Voigt and von Helversen, 1999; Krutzsch, 2000; Bouchard, 2001; Safi and Kerth, 2003; Dechmann and Safi, 2005; Brennan and Kendrick, 2006). Several species of bats use secretions from glands and other odor-producing structures during courtship displays (Voigt and von Helversen, 1999; Krutzsch, 2000; Muñoz-Romo and Kunz, 2009; Muñoz-Romo *et al*., 2011). For example, males of the greater sac-winged bat, *Saccopteryx bilineata*, display courtship repertoires toward females using the scent produced in wing sacs. Males of *S. bilineata* combine different body fluids, store the mixture in wing sacs, and disperse the odor towards females during courtship flights (Voigt and von Helversen, 1999; Voigt, 2002).

While the scent from body fluids might be fundamental to mating success, the presence of microbes in wing sacs is indispensable to produce specific odor profiles to which females respond (Voigt *et al*., 2005). Although individual males on average carried two microbial strains in their wing sacs, and each male would have a unique microbiota, these authors estimated a minimum microbial richness of 40 for the whole population (Voigt *et al*., 2005). Males of *S. bilineata* had less diverse microbial communities than

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DIVERSIDAD DE BACTERIAS EN LAS CHARRETERAS DEL MURCIÉLAGO PEQUEÑO DE HOMBROS AMARILLOS, *Sturnira lilium* (CHIROPTERA: PHYLLOSTOMIDAE)
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RESUMEN

En los murciélagos, las señales químicas son particularmente importantes para comunicarse. Aunque el olor de los fluidos corporales puede ser importante para el éxito reproductivo, la presencia de microbios en estructuras productoras de olor puede ser indispensable porque algunas sustancias deben ser metabolizadas por bacterias y experimentar cambios bioquímicos, antes de adquirir olores que sean señales significativas. El objetivo de este estudio fue identificar bacterias en glándulas sexualmente dimórficas de los hombros ('charreteras') de machos de Sturnira lilium *y* S. bogotensis*, y determinar si alguna de estas bacterias ha sido reportada como presente en órganos masculinos sexualmente seleccionados de otras especies de murciélagos. La identificación fue lograda por amplificación y secuenciamiento de sus correspondientes genes rRNA16S. Se identificaron 42 especies de bacterias en machos (n=3) y hembras (n=3) de* S. lilium *y en un macho de* S. bogotensis*. En machos de* S. lilium *y* S. bogotensis *hubo 15 y 7 especies de bacterias en las charreteras, respectivamente. La similitud en términos de perfiles bacteriológicos fue muy baja entre machos y hembras, y entre partes del cuerpo. Aunque existen especies de bacterias comunes para charreteras y espaldas,* Citrobacter freundii, Enterococcus faecalis, Exiguobacterium acetylicum *y* Flavobacterium mizutaii *se encontraron exclusivamente en charreteras de* S. lilium*. De las bacterias identificadas en charreteras de machos de* S. lilium*, cuatro especies (*Staphylococcus saprophyticus*,* S. sciuri, E. faecalis *y* Bacillus cereus*) se han encontrado en órganos masculinos sexualmente seleccionados de otras especies de murciélagos. Géneros comunes de bacterias en atributos masculinos sexualmente seleccionados son* Bacillus*,* Staphylococcus*,* Corynebacterium *y* Enterococcus*.*

DIVERSIDADE DE BACTÉRIAS NAS DRAGONAS DO MORCEGO DE OMBROS AMARELOS, *Sturnira lilium* (CHIROPTERA: PHYLLOSTOMIDAE)
Nathaly González-Quiñonez, Gustavo Fermin e Mariana Muñoz-Romo

RESUMO

Nos morcegos, os sinais químicos são particularmente importantes para comunicar-se. Ainda que o cheiro dos fluidos corporais possa ser importante para o sucesso reprodutivo, a presença de micróbios nas estruturas produtoras de cheiro pode ser indispensável porque algumas substâncias devem ser metabolizadas por bactérias e experimentar mudanças bioquímicas, antes de adquirir cheiros que sejam sinais significativos. O objetivo de este estudo foi identificar bactérias em glândulas sexualmente dimórficas dos ombros ('dragonas') de machos de Sturnira lilium *e S.* bogotensis*, e determinar se alguma destas bactérias tem sido relatada como presente em órgãos masculinos sexualmente selecionados de outras espécies de morcegos. A identificação foi lograda por amplificação e sequenciamento de seus correspondentes gens rRNA16S. Identificaram-se 42 espécies de bactérias em machos (n=3) e fêmeas (n=3) de* S. lilium *e em um macho de* S. bogotensis*. Em machos de* S. lilium *e* S. bogotensis *hubo 15 e 7 espécies de bactérias nas dragonas, respectivamente. A similaridade em termos de perfis bacteriológicos foi muito baixa entre machos e fêmeas, e entre partes do corpo. Ainda que existam espécies de bactérias comuns nas dragonas e nas costas, Citrobacter freundii,* Enterococcus faecalis*,* Exiguobacterium acetylicum *e* Flavobacterium mizutaii *foram encontradas exclusivamente em dragonas de* S. lilium*. Das bactérias identificadas em dragonas de machos de* S. lilium*, quatro espécies (*Staphylococcus saprophyticus, S. sciuri, E. faecalis *y* Bacillus cereus*) tem sido encontradas em órgãos masculinos sexualmente selecionados de outras espécies de morcegos. Gêneros comuns de bactérias em atributos masculinos sexualmente selecionados são* Bacillus, Staphylococcus, Corynebacterium *e* Enterococcus.

females, but the microbe combinations of males might promote particular scents that allow distinguishing among individuals (Voigt *et al*., 2005). Sexually-selected male organs of other two species of bats have also been characterized in terms of their associated bacteria. Twelve species of bacteria were present in dorsal patches of the long-nosed bat, *Leptonycteris curasoae* (Nassar *et al*., 2009), and five in the inguinal pockets of the greater bulldog bat, *Noctilio leporinus* (Studier and Lavoie, 1984). Overall, from these studies, three species of bacteria (*Proteus mirabilis*, *Bacillus sphaericus*, and *Staphylococcus aureus*) were common between *S. bilineata*, *L. curasoae* and *N. leporinus.*

The strong, penetrating species-specific odors of many male mammals may result from some combination of glandular secretion, diet, and bacterial fermentation (Gorman and Trowbridge, 1989). Bats may feed on insects, fruits, nectar, vertebrates, and blood (Altringham, 1996); the metabolic transformation of these resources may produce different odors (Gorman and Trowbridge, 1989). Bacterial fermentation can be affected by the environment in which the bat roosts and by the odor-producing structure itself. The combination of roosting in warm, humid locations and the possession of specialized glandular scent organs provide ideal conditions for the proliferation of bacteria, which likely affects the odor of secretions (Scully *et al*., 2000).

Some chemical compounds found in glands and other odor-producing structures could be by-products of the microbiological breakdown of proteins, carbohydrates and cholesterol, due to the presence and activity of microorganisms, as has been reported for other odor-producing structures in bats (Dapson *et al*., 1977; Studier and Lavoie, 1984; Scully *et al*., 2000). Microbes are important in glands as some substances must first be metabolized by bacteria and experience biochemical changes before they acquire detectable odor characteristics and become meaningful

signals (Mykytowycz and Goodrich, 1974).

Most bats of the genera *Sturnira* are sexually dimorphic in the shoulder glands, commonly called 'epaulettes' in males (Figure 1). Males of *S. lilium* have dark hairs with a waxy secretion on their surface that displays a pleasant, sweet-smelling, spicy odor (Gannon *et al*., 1989; Scully *et al*., 2000). Although these structures are potentially involved in female attraction, courtship and mating (Gannon *et al*., 1989; Scully *et al*., 2000), whether specific bacteria associate with them and if they are able to contribute to odor profiles is currently unknown. An important step to fully understand the actual function of these structures in a sexual context is to characterize the bacteria associated with them. The goal of this study was to determine what bacteria might be present in the epaulettes of *S. lilium* and *S. bogotensis*, and whether some of these bacteria have been reported as present in sexually-selected male organs of other bat species. Finally, if bacteria are important in sexual signaling, as a first step, we would expect to find that the composition (presence or absence) of bacteria may differ between males and females, and between epaulettes and other body locations (i.e., backs or female shoulders). In this study, identification of bacteria was molecularly attained, in a rapid and accurate manner, by amplifying and sequencing the corresponding bacterial 16S rRNA genes.

Figure 1. Adult *Sturnira lilium* male showing a developed epaulette.

Materials and Methods

Species

The little yellow-shouldered bat *(Sturnira lilium)* is a small-sized (18-24g), neotropical, frugivorous species (Linares, 1998) that inhabits many different types of forest habitats, including mountainous forests, semi-deciduous tropical rainforests, and humid and semi-arid forests (Gannon *et al*., 1989). *S. lilium* is also found in tropical lowlands and open areas, such as fields or farmlands (Gannon *et al*., 1989). The little yellow-shouldered bat is found from northwestern Mexico (Sonora), southward through Central America into tropical and subtropical South America, to northern Argentina and Uruguay. This bat species also occurs in the Lesser Antilles north to Dominica, and on Trinidad (Gannon *et al*., 1989).

Study Site

Bats were captured at La Mucuy (08º36'49"N and 71º04'08"W), Mérida state, Venezuela, a low montane cloud forest at 1913masl, with an annual average temperature of 13-19ºC, and an annual precipitation of 1000-3000mm (Ataroff and Sarmiento, 2004).

Bat Sampling

All sampling protocols were performed following guidelines of the American Society of Mammalogists for capture, handling, and care of mammals (Sikes *et al*., 2011). Bats were captured using 12m long, 38mm mesh, 50 denier, four-shelf mist nets (Avinet, Dryden, New York, USA; Kunz *et al*. 2009) between 18:30 and 22:00. Once swab samples were taken (see below), each captured bat was individually placed into a clean cotton cloth bag and transported to a data collection station. Age of bats was estimated using the relative ossification of wing bones (Brunet-Rossini and Wilkinson, 2009), and sex and reproductive status were determined following standard criteria (Racey, 2009). All individuals were released at the site of capture immediately after measurements were recorded.

Sampling and collection of bacteria

During three field trips on February 2010, three males and three females of *S. lilium*, and a *S. bogotensis* male were captured and sampled. Swabs from the back and epaulettes (males), and from the back and shoulders (females) of each individual were directly streaked into Petri dishes containing Luria-Bertani agar. A contamination control plate consisted of a Petri dish kept opened the same time as required for the animal's sampling. Inoculated and control dishes were brought to the lab where they were incubated at a constant temperature of 37ºC and aerobic conditions.

Bacteria cultivation and purification

The dishes were incubated for 24-72h after collection, and then kept at 4ºC under aerobic conditions until use. Based on macro-morphological differences among colonies (size, color, elevation, border and shape) in every Petri dish, selected colonies were individually streaked again for further purification. Colonies were re-isolated in the same medium, and observed under the microscope after Gram staining to check for purity and Gram's reaction (Gerhardt *et al*. 1994). Five isolated, purified clones from each original colony were stored at -80ºC and used to streak a master Petri dish (one for every animal part per animal).

Colony PCR for the 16S rRNA gene

One day before the amplification of the *16S rRNA* gene by PCR, each individual colony was re-isolated as before and grown overnight at 37ºC. Fresh colonies were always used in all amplification protocols. Reaction mixtures for PCR amplification of the *16S rRNA* gene (Dekio *et al*., 2005) consisted of 10µl of the 1X GoTaq® Green master mix (Promega, Madison, WI) supplemented with universal primers 27F (5'AGAGTTTGAT CCT GGCTCAG3'; Lane, 1991) and 1492R (5'GGTTACCT TGTT ACGACTT3'; Turner *et al*., 1999). Once the reaction mixture was prepared, the colony to be tested was gently touched with a micropipette tip and washed in the reaction mixture tube. PCR amplification was attained according to the following program: an initial denaturation step at 95ºC for 10min, followed by 30 cycles of denaturation at 94ºC for 45sec, annealing at 51ºC for 45sec and extension at 72ºC for 90sec. A final extension step at 72ºC for 10min was also included (Batisson *et al*., 2009; Dekio *et al*., 2005). Amplification products quantity, quality and size were checked by agarose gel electrophoresis (Sambrook and Russell, 2001), and digitally recorded. Single, clear bands were salt and ethanol-precipitated and sent for sequencing without further purification, both strands, to the sequencing facility of the Instituto Venezolano de Investigaciones Científicas (IVIC).

Computational analysis

A contig for every sample was obtained, using reverse and forward sequences, with BioEdit (Hall, 1999), and the contig compared with equivalent sequences available in public databases (GenBank and Greengenes) using default parameters. A similarity of 98% or higher (Pei *et al*., 2010; Stackebrandt and Ebers, 2006) was used as the threshold value of success and identification if the query coverage was also higher or equal to 98%.

Cluster analysis (Joining method, tree clustering; single

amalgamation (linkage) rule; 1-Pearson r distance) was used to identify groups of individuals that shared similar bacteria, using Statistica 6.0 (1998, Statsoft). Additionally, the Sørensen index was used to compare the similarity of epaulettes and shoulders in terms of their associated bacteria; it varies between 1 (maximum similarity) and 0 (no similarity) (Molles 2009).

Results

Overall, 89 isolated and purified bacterial samples yielded data on the presence of 42 different species in *S. lilium* males (n=3) and females (n=3), and a *S. bogotensis* male, including samples from epaulettes and backs (males), and shoulders and backs (females) of every individual sampled. Thirty-three bacteria were identified to species, and only nine to genus (Table I). A list of all samples whose sequences were sent to the GenBank public database is presented (Table II). Considered together, bacteria were represented by 80.9% bacilli and 19.1% cocci; 76.4 % were $Gram^+$ and 23.6% $Gram^-$.

Fifteen species of bacteria were found in epaulettes (average 5 ±3; n=3) and 12 in backs (average 4 ±1; n=3) of *S. lilium* males, whereas females had 18 species of bacteria in shoulders (average 6 ±1; n=3) and 15 in backs (average 5 ±1; n=3). Moreover, 10 species of bacteria were identified in the *S. bogotensis* male: 7 in his epaulettes, and 6 in his back.

When comparing body parts in terms of their associated bacteria it was found that all body parts of males and females of *S. lilium* tend to have a unique set of bacteria, as indicated by the very low Sørensen indices (Table III), corresponding to low similarity in all cases. Despite differences between males and females, and between body parts in terms of bacteria, epaulettes of males tend to be more similar between them than to any other body region, considering all sampled animals together (see A in Figure 2). On the other hand, samples from *S. bogotensis* (B in Figure 2) differed from the bacteria found in all three males of *S. lilium* (B in Figure 2). In fact, both the epaulette and the back of *S. bogotensis* shared common bacteria, separating them from the remaining samples. Four species (*Citrobacter freundii, Enterococcus faecalis, Exiguobacterium acetylicum*, and *Flavobacterium mizutaii*) were exclusively found in epaulettes of *S. lilium*, whereas two species (*Lysinibacillus sphaericus* and *Microbacterium lacticum*) were exclusively found in epaulettes of *S. bogotensis*.

All species fall into four phyla (Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria; Table I) of the 24 recognized for Eubacteria. Actinobacteria were found only on the back or epaulettes

TABLE I
SUMMARY OF THE BACTERIA (42 SPECIES, 89 SAMPLES) FOUND ON THE SKIN OF *Sturnira lilium* AND *S. bogotensis* SAMPLED AT LA MUCUY, MÉRIDA, VENEZUELA

Phylum (A,B)*	Class (A,B)	Order (A,B)	Family (A,B)	Species (B) b, present in *S. bogotensis*; i: present in *S. lilium*
Actinobacteria (6,7)	Actinobacteria (6,7)	Actinomycetales (6,7)	Corynebacteriaceae (1,2)	*Corynebacterium variabile* (2,i)
			Microcobacteriaceae (4,4)	*Curtobacterium citreum* (1,b), *Curtobacterium* sp. 1 (1,i), *Leucobacter aridicollis* (1,i), *Microbacterium lacticum* (1,b)
			Micrococcaceae (1,1)	*Arthrobacter agilis* (1,i)
Bacteroidetes (2,2)	Flavobacteria (2,2)	Flavobacteriales (2,2)	Flavobacteriaceae (2,2)	*Flavobacterium mizutaii* (1,i), *Myroides odoratus* (1,i)
Firmicutes (24,61)	Bacilli (24,61)	Bacillales (23,60)	Bacillaceae (14,35)	*Bacillus cereus* (11,i), *B. flexus* (1,i), *B. megaterium* (5,bi), *B. mycoides* (1,i), *B. pseudomycoides* (2,i), *B. pumilus* (2,b), *B. subtilis* (1,i), *B. thuringiensis* (2,i), *B. weihenstephanensis* (4,bi), *Lysinibacillus fusiformis* (1,i), *L. sphaericus* (1,b), *Bacillus* sp.1 (2,i), *Bacillus* sp. 2 (1,i), *Bacillus* sp. 3 (1,i)
			Planococcaceae (2,4)	*Caryophanon* sp. 1 (3,bi), *Solibacillus silvestris* (1,i)
			Staphylococcaceae (4,12)	*Staphyloccocus hominis* (1,i), *S. saprophyticus* (6,i), *S. sciuri* (4,i), *Staphyloccocus* sp. 1 (1,i)
			XII *Incertae sedis* (3,9)	*Exiguobacterium acetylicum* (1,i), *E. indicum* (1,i), *E. sibiricum* (7,bi)
		Lactobacillales (1,1)	Enterococcaceae (1,1)	*Enterococcus faecalis* (1,i)
Proteobacteria (10,19)	Betaproteobacteria (1,1)	Burkholderiales (1,1)	Comamonadaceae (1,1)	*Lampropedia hyalina* (1,i)
	Gammaproteobacteria (9,18)	Pseudomonadales (3,12)	Moraxellaceae (3,12)	*Acinetobacter lwoffii* (9,bi), *Acinetobacter* sp. 1 (2,bi), *Acinetobacter* sp. 2 (1,i)
		Enterobacteriales (6,6)	Enterobacteriaceae (6,6)	*Enterobacter hormaechei* (1,i), *Escherichia coli* (1,i), *Citrobacter freundii* (1,i), *C. koseri* (1,i), *Citrobacter* sp. 1 (1,i), *Shigella sonnei* (1,i)

A: number of species, B: number of samples.

of both bat species, while Bacteroidetes were found only in the back or epaulettes of two different *S. lilium* individuals. On the other hand, Proteobacteria were found in the three body parts of both bat species considered together, but those belonging to the family Enterobacteriaceae were present only in *S. lilium* females, except for one *C. freundii* collected from the epaulette of a *S. lilium* male. On the contrary, the ubiquitous *Acinetobacter* (Moraxellaceae) species were found in several individuals regardless of bat species, body part or sex. Finally, the most numerous group of bacteria analyzed here, the Firmicutes (22 different species from 61 samples), are represented by five different families: Enterococcaceae with one species (one epaulette sample) and Staphylococcaceae with four species (12 samples from any body part) only from *S. lilium*, and Planococcaceae with two different species (*Caryophanon* sp. and *S. silvestris*) and Bacillaceae (with 12 species represented by 35 samples) from both bat species. Interestingly, *Bacillus cereus*, the most commonly found bacteria of all analyzed here (11 samples), was only found on the skin of *S. lilium*, regardless of body part or sex of the individual. A *Bacillus* species exclusive of *S. bogotensis* was represented by *B. pumillus* (2 samples), while those present in both bat species included *B. megaterium* (five samples) and *B. weihenstephanensis* (four samples). The rest of the bacteria (eight species, 13 samples) belonging to the Bacillaceae family were found only in *S. lilium*. Bacteria belonging to the XXII *Incertae sedis* family (Bacillales) included two different species of *Exiguobacterium* only present in *S. lilium*, besides *E. sibiricum*, found only in males of both bat species. *Arthrobacter luteolus* and *Bacillus niacini* were the only species found in the plates used as control.

TABLE II
GENBANK ACCESSION NUMBERS FOR THE RNA 16S GENE SEQUENCES OF THE BACTERIA ISOLATED FROM DIFFERENT BODY PARTS OF MALE AND FEMALE *Sturnira lilium* AND *S. bogotensis* COLLECTED AT LA MUCUY (MÉRIDA, VENEZUELA)

GenBank	Bat species	Location	Sex	Isolate	Bacteria species
JF935051	*S. bogotensis*	Back	Male	CtST10.2	*Acinetobacter lwoffii*
JF935052	*S. bogotensis*	Back	Male	CtST10.3	*Acinetobacter lwoffii*
JF935053	*S. bogotensis*	Back	Male	CtST10.4	*Curtobacterium citreum*
JF935054	*S. bogotensis*	Back	Male	CtST10.5	*Bacillus weihenstephanensis*
JF935055	*S. bogotensis*	Back	Male	CtST10.6	*Bacillus weihenstephanensis*
JF935056	*S. bogotensis*	Back	Male	CtST10.7	*Exiguobacterium sibiricum*
JF935057	*S. bogotensis*	Back	Male	CtST10.8	*Exiguobacterium sibiricum*
JF935058	*S. bogotensis*	Back	Male	CtST10.9	*Bacillus pumilus*
JF935059	*S. bogotensis*	Back	Male	CtST10.10	*Bacillus megaterium*
JF935060	*S. bogotensis*	Epaulettes	Male	ChST10.1	*Bacillus weihenstephanensis*
JF935061	*S. bogotensis*	Epaulettes	Male	ChST10.2	*Exiguobacterium sibiricum*
JF935062	*S. bogotensis*	Epaulettes	Male	ChST10.4	*Acinetobacter lwoffii*
JF935063	*S. bogotensis*	Epaulettes	Male	ChST10.5	*Exiguobacterium sibiricum*
JF935064	*S. bogotensis*	Epaulettes	Male	ChST10.6	*Caryophanon* sp.
JF935065	*S. bogotensis*	Epaulettes	Male	ChST10.7	*Lysinibacillus sphaericus*
JF935066	*S. bogotensis*	Epaulettes	Male	ChST10.9	*Microbacterium lacticum*
JF935067	*S. bogotensis*	Epaulettes	Male	ChST10.10	*Acinetobacter lwoffii*
JF935068	*S. bogotensis*	Epaulettes	Male	ChST10.12	*Acinetobacter lwoffii*
JF935069	*S. bogotensis*	Epaulettes	Male	ChST10.13	*Acinetobacter lwoffii*
JF935070	*S. bogotensis*	Epaulettes	Male	ChST10.15	*Acinetobacter* sp.
JF935071	*S. lilium*	Back	Female	CtST1.1	*Bacillus thuringiensis*
JF935072	*S. lilium*	Back	Female	CtST1.2	*Citrobacter koseri*
JF935073	*S. lilium*	Back	Female	CtST1.3	*Bacillus* sp.
JF935074	*S. lilium*	Back	Female	CtST1.4	*Bacillus cereus*
JF935075	*S. lilium*	Back	Female	CtST1.5	*Exiguobacterium indicum*
JF935076	*S. lilium*	Back	Female	CtST1.6	*Bacillus cereus*
JF935077	*S. lilium*	Back	Female	CtST1.7	*Bacillus cereus*
JF935078	*S. lilium*	Back	Female	CtST1.8	*Bacillus* sp.
JF935079	*S. lilium*	Back	Female	CtST3.1	*Caryophanon* sp.
JF935080	*S. lilium*	Back	Female	CtST3.3	*Curtobacterium* sp.
JF935081	*S. lilium*	Back	Female	CtST3.4	*Solibacillus silvestris*
JF935082	*S. lilium*	Back	Female	CtST3.5	*Bacillus megaterium*
JF935083	*S. lilium*	Back	Female	CtST3.6	*Bacillus cereus*
JF935084	*S. lilium*	Back	Female	CtST3.7	*Bacillus megaterium*
JF935085	*S. lilium*	Back	Female	CtST9.1	*Citrobacter* sp.
JF935086	*S. lilium*	Back	Female	CtST9.2	*Bacillus cereus*
JF935087	*S. lilium*	Back	Female	CtST9.3	*Acinetobacter* sp.
JF935088	*S. lilium*	Back	Female	CtST9.4	*Bacillus flexus*
JF935089	*S. lilium*	Shoulders	Female	ChST1.1	*Bacillus pseudomycoides*
JF935090	*S. lilium*	Shoulders	Female	ChST1.2	*Bacillus cereus*
JF935091	*S. lilium*	Shoulders	Female	ChST1.3	*Staphylococcus saprophyticus*
JF935092	*S. lilium*	Shoulders	Female	ChST1.4	*Bacillus weihenstephanensis*
JF935093	*S. lilium*	Shoulders	Female	ChST1.5	*Staphylococcus hominis*
JF935094	*S. lilium*	Shoulders	Female	ChST1.6	*Lysinibacillus fusiformis*
JF935095	*S. lilium*	Shoulders	Female	ChST1.7	*Bacillus pumilus*
JF935096	*S. lilium*	Shoulders	Female	ChST3.1	*Bacillus cereus*
JF935097	*S. lilium*	Shoulders	Female	ChST3.2	*Bacillus* sp.
JF935098	*S. lilium*	Shoulders	Female	ChST3.3	*Bacillus subtilis*
JF935099	*S. lilium*	Shoulders	Female	ChST3.5	*Bacillus megaterium*
JF935100	*S. lilium*	Shoulders	Female	ChST3.6	*Caryophanon* sp.
JF935101	*S. lilium*	Shoulders	Female	ChST9.1	*Escherichia coli*
JF935102	*S. lilium*	Shoulders	Female	ChST9.2	*Enterobacter hormaechei*
JF935103	*S. lilium*	Shoulders	Female	ChST9.3	*Shigella sonnei*
JF935104	*S. lilium*	Shoulders	Female	ChST9.4	*Staphylococcus* sp.
JF935105	*S. lilium*	Shoulders	Female	ChST9.5	*Exiguobacterium sibiricum*
JF935106	*S. lilium*	Shoulders	Female	ChST9.7	*Acinetobacter lwoffii*
JF935107	*S. lilium*	Shoulders	Female	ChST4.2	*Bacillus mycoides*
JF935108	*S. lilium*	Back	Male	CtST7.1	*Bacillus cereus*
JF935109	*S. lilium*	Back	Male	CtST7.2	*Staphylococcus sciuri*
JF935110	*S. lilium*	Back	Male	CtST7.3	*Staphylococcus sciuri*
JF935111	*S. lilium*	Back	Male	CtST7.4	*Acinetobacter lwoffii*
JF935112	*S. lilium*	Back	Male	CtST7.5	*Corynebacterium variabile*
JF935113	*S. lilium*	Back	Male	CtST7.6	*Staphylococcus saprophyticus*
JF935114	*S. lilium*	Back	Male	CtST7.7	*Staphylococcus saprophyticus*
JF935115	*S. lilium*	Back	Male	CtST7.8	*Leucobacter aridicollis*
JF935116	*S. lilium*	Back	Male	CtST2.1	*Bacillus thuringiensis*
JF935117	*S. lilium*	Back	Male	CtST2.3	*Bacillus pseudomycoides*
JF935118	*S. lilium*	Back	Male	CtST2.4	*Exiguobacterium sibiricum*

It continues in following page.

Continuation Table II

GenBank	Bat species	Location	Sex	Isolate	Bacteria species
JF935119	*S. lilium*	Back	Male	CtST2.5	*Arthrobacter agilis*
JF935120	*S. lilium*	Back	Male	CtST8.1	*Staphylococcus sciuri*
JF935121	*S. lilium*	Back	Male	CtST8.13	*Lampropedia hyalina*
JF935122	*S. lilium*	Back	Male	CtST8.14	*Myroides odoratus*
JF935123	*S. lilium*	Epaulettes	Male	ChST7.1	*Exiguobacterium acetylicum*
JF935124	*S. lilium*	Epaulettes	Male	ChST7.2	*Bacillus cereus*
JF935125	*S. lilium*	Epaulettes	Male	ChST7.3	*Staphylococcus saprophyticus*
JF935126	*S. lilium*	Epaulettes	Male	ChST7.4	*Flavobacterium mizutaii*
JF935127	*S. lilium*	Epaulettes	Male	ChST7.5	*Corynebacterium variabile*
JF935128	*S. lilium*	Epaulettes	Male	ChST7.6	*Acinetobacter* sp.
JF935129	*S. lilium*	Epaulettes	Male	ChST7.7	*Enterococcus faecalis*
JF935130	*S. lilium*	Epaulettes	Male	ChST7.8	*Staphylococcus sciuri*
JF935131	*S. lilium*	Epaulettes	Male	ChST2.1	*Bacillus megaterium*
JF935132	*S. lilium*	Epaulettes	Male	ChST2.2	*Citrobacter freundii*
JF935133	*S. lilium*	Epaulettes	Male	ChST2.3	*Bacillus cereus*
JF935134	*S. lilium*	Epaulettes	Male	ChST2.4	*Bacillus* sp.
JF935135	*S. lilium*	Epaulettes	Male	ChST2.5	*Bacillus cereus*
JF935136	*S. lilium*	Epaulettes	Male	ChST2.6	*Staphylococcus saprophyticus*
JF935137	*S. lilium*	Epaulettes	Male	ChST2.71	*Acinetobacter lwoffii*
JF935138	*S. lilium*	Epaulettes	Male	ChST2.72	*Exiguobacterium sibiricum*
JF935139	*S. lilium*	Epaulettes	Male	ChST8.3	*Staphylococcus saprophyticus*

TABLE III
SØRENSEN INDEX VALUES FROM COMPARISON BETWEEN MALES AND FEMALES, AND DIFFERENT BODY REGIONS OF *Sturnira lilium* BASED ON THEIR ASSOCIATED BACTERIA

Sex or body region compared	Sørensen Index
Males vs. females (all body parts)	0.318
Epaulettes vs. males' backs	0.435
Shoulders vs. females' backs	0.267
Epaulettes vs. Shoulders	0.167
Males' back vs. Females' back	0.207

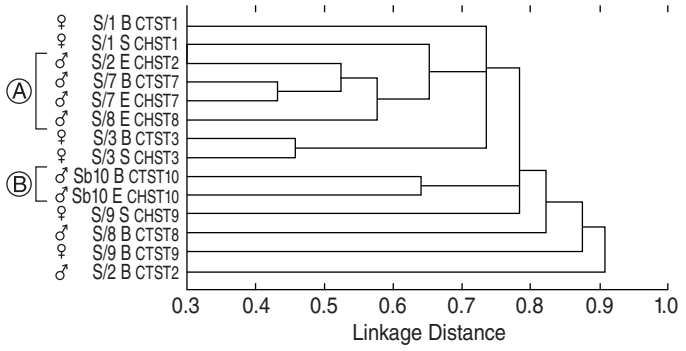

Figure 2. Dendrogram from cluster analysis showing epaulettes (E) of males of *S. lilium* (*Sl*) forming the group A based on common bacteria. Samples of *S. bogotensis* (*Sb*) (epaulette (E) and back (B)) also separate in group B from the remaining samples of males and females of *S. lilium*. Note that samples from female shoulders (S) and backs (B) do not form isolated groups. See methods for details.

Discussion

As an initial attempt to characterize the bacterial flora present in epaulettes, shoulders, and backs of *S. lilium* and *S. bogotensis*, a molecular approach based on the amplification and sequencing of the *16S rRNA* bacterial gene was used in this study, taking as a cutoff value of identification more than 98% similarity between our sequences and those reported at public databases. Overall, 42 bacteria species were identified, most of them bacilli and Gram$^+$.

Although few individual bats were sampled, and this limits the discussion about variability of microbial composition across sexes and species, these bats provided an important list of bacteria sampled from epaulettes, shoulders, and backs. Thus, this allows us to postulate possible bacteria associated to those body regions.

It is important to keep in mind that culturing bacteria in Petri dishes containing the Luria-Bertani agar used here represents a selective method where only some bacteria will grow. The newer, culture-independent next generation sequencing methods would provide orders of magnitude more bacterial taxa from this kind of sampling, simply because most bacteria do not grow in culture (Mardis, 2008; Metzker, 2010; Bariuso *et al*., 2011; Rastogi and Sani, 2011). Presumably, many rare or uncultivable bacterial taxa present on the bats were not detected in our study. Thus, more samples from individuals, and a less selective culture method would provide a more complete list of bacteria, and this would allow to provide a more appropriate interpretation of the results in the context of the sexual signaling concept.

The fact that epaulettes of males were more similar between them than to any other body region would suggest that epaulettes would share distinguishing species that could make them unique, which is presumably important in the context of chemical communication. The fact that the sample from a male back (CtSt7) remained together with the sample of the epaulette (ChSt7) of the same individual of *S. lilium* could be related to grooming (males might 'contaminate' their own backs while grooming the whole body). Although we had only one sample from *S. bogotensis*, it is noteworthy that his epaulette (ChSt10) and his back (CtSt10) remained together. It remains to be determined whether each species of *Sturnira* distinguishes from one another in terms of their bacteriological profile.

Our results were consistent with other studies in which reported bacteria from skin of bats are also commonly found in other animals. Gram-positive bacilli found in this study are widespread in nature and easily found in soil, water, sand, pasteurized milk, cow feces, food and clinical specimens, animals and animal products, and skin of human and animals. Many of them can cause foodborne disease, or are associated with urinary infections in humans (for examples see Gilbert and Kramer, 1987; Funke *et al*., 2005). The diversity of bacteria found in this study would indicate that despite the common bacteria found between body regions of bats, each individual has a combination of specific bacteria as has been shown in other animals, including humans (Gao *et al*., 2007; Fierer *et al*., 2008; Grice *et al*., 2008).

From the identified bacteria in epaulettes of males of *S. lilium*, two species (*Staphylococcus saprophyticus* and *Enterococcus faecalis*)

have been found in the sexually selected wing sacs of *S. bilineata* (Voigt *et al*., 2005). Moreover, *Bacillus cereus* and *Staphylococcus sciuri* have been reported in both the sexually-selected wing sacs of males of *S. bilineata* (Voigt *et al*., 2005), and the dorsal patches of males of *Leptonycteris curasoae* (Nassar *et al*., 2009). The microbiota of sexually-selected organs is likely influenced by an individual's major histocompatibility complex, and thus, microbial products may play important roles during mate-choice recognition (Wyatt, 2003; Voigt *et al*., 2005). For example, important compounds for communication found in the interdigital secretions of the bontebok (*Damaliscus dorcas dorcas*) and the blesbok (*D. dorcas phillipsi*), could be by-products of *Bacillus brevis* (Burger *et al*., 1999). Studier and Lavoie (1984) suggested that microbes in inguinal pockets of the greater bulldog bat (*Noctilio leporinus*) and lesser bulldog bat (*N. albiventris*) were involved in odor production, being *Staphylococcus aureus* responsible for the intense odor of at least *N. leporinus* (Studier and Lavoie, 1984). Dapson *et al*. (1977) suggested something similar for the big brown bat (*Eptesicus fuscus*) and two free-tailed bat species (*Molossus bondae* and *Tadarida brasiliensis*). In *S. bilineata*, volatile compounds such as indole derivatives and aminoacetophenones are likely of microbial origin (Caspers *et al*., 2009). In fact, aminoacetophenones are recognized as a male-specific substance present in sexually-selected organs of adult males of *S. bilineata* (Caspers *et al*., 2011) and *L. curasoae* (Muñoz-Romo *et al*., 2012), and presumably involved in female attraction.

Results from Studier and Lavoie (1984), Voigt *et al*. (2005), Nassar *et al*. (2009) and this study would indicate that important genera of bacteria in sexually-selected organs of male bats are *Bacillus* and *Staphylococcus* (present in four species), *Corynebacterium* and *Enterococcus* (present in three species), and *Proteus*, *Acinetobacter*, and *Exiguobacterium* (present in two species) (Figure 3). These genera, and particularly *Bacillus* and *Staphylococcus*, should be the focus of studies on bacterially-mediated chemical communication in bats and other mammals. As Voigt *et al*. (2005) stated, microbes may be more involved in olfactory communication, especially in female choice, than we expected, and in the evolution of morphological and behavioral adaptations.

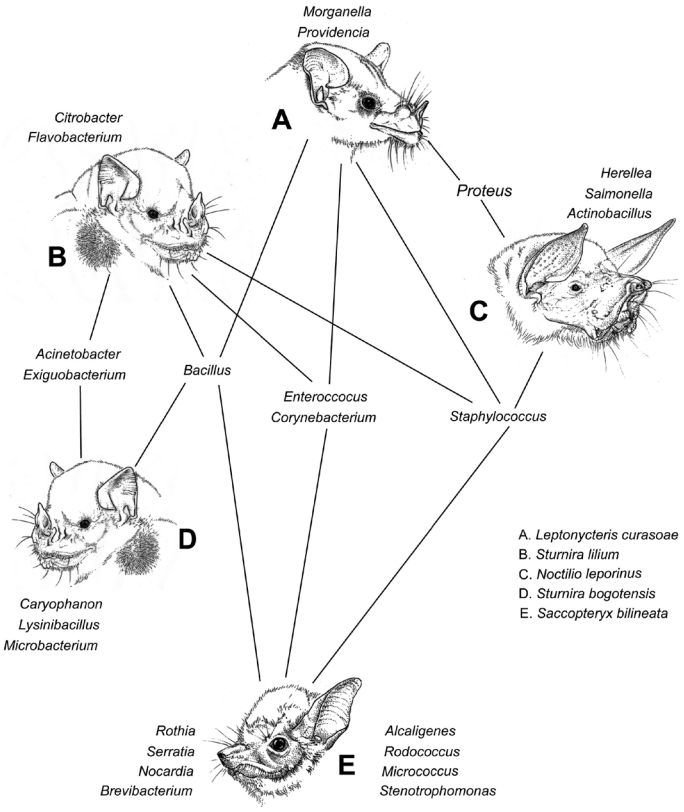

Figure 3. Common genera of bacteria in sexually-selected male organs of five species of bats: epaulettes of *S. lilium* and *S. bogotensis* (n=3 and n=1, respectively; this study), inguinal pockets of *N. leporinus* ("several" individuals; Studier and Lavoie 1984), wing sacs of *S. bilineata* (n=22; Voigt *et al*. 2005), and dorsal patches of *L. curasoae* (n=8; Nassar *et al*. 2009). Lines indicate common species of bacteria between bats. Other genera of bacteria found in sexually-selected organs of these bats are listed by a side of each bat species.

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