Symbiotic skin bacteria as a source for sex-specific scents in frogs

Andrés E. Brunetti,a,1 Mariana L. Lyra,b Weilan G. P. Melo,b Laura E. Andrade,a Pablo Palacios-Rodríguez,c Bárbara M. Prado,a Célio F. B. Haddad,a Mônica T. Pupo,a and Norberto P. Lopesa,1

Centro de Aquicultura, Instituto de Biociências, Universidade Estadual Paulista, 13506-900 Rio Claro, SP, Brazil; and Departamento de Ciências Biológicas, Universidad de los Andes, AA4976 Bogotá DC, Colombia

Amphibians are known to possess a wide variety of compounds stored in their skin glands. While significant progress has been made in understanding the chemical diversity and biological relevance of alkaloids, amines, steroids, and peptides, most aspects of the odorous secretions are completely unknown. In this study, we examined sexual variations in the volatile profile from the skin of the odorous secretions are completely unknown. In this study, we examined sexual variations in the volatile profile from the skin of the tree frog Boana prasina and combined culture and culture-independent methods to investigate if microorganisms might be a source of these compounds. We found that sesquiterpenes, thioethers, and methoxypyrazines are major contributors to the observed sex differences. We also observed that each sex has a distinct profile of methoxypyrazines, and that the chemical origin of these compounds can be traced to a Pseudomonas sp. strain isolated from the frog’s skin. This symbiotic bacterium was present in almost all individuals examined from different sites and was maintained in captive conditions, supporting its significance as the source of methoxypyrazines in these frogs. Our results highlight the potential relevance of bacteria as a source of chemical signals in amphibians and contribute to increasing our understanding of the role that symbiotic associations have in animals.

Chemical signaling is regarded as the most ancient mode of communication (1). Because all living organisms emit, detect, and respond to chemical cues, there is an enormous number and diversity of potential chemical interactions within and between species across all kingdoms, from bacteria and archaea to plants and animals (1–3). Several vertebrate species are known to produce volatile substances that convey information related to species and kin recognition, as well as recognition and assessment in sexual interactions, thus mediating social and sexual behavior (4). Like other substances used in communication, volatiles come from four main sources: (i) de novo synthesis, generally in specific secretory glands; (ii) metabolic by-products released with excreted material; (iii) environmental sequestration; and (iv) the products of microbial symbionts (2).

In particular, the “fermentation hypothesis” proposed in the 1970s states that symbiotic bacteria in mammals metabolize proteins and lipids occurring in the scent glands, producing volatile compounds used by their host to communicate (5). Recent advances in various “Omic” technologies (6) have enabled scientists to examine this hypothesis in different mammal species (7–9). However, this hypothesis is not supported by empirical evidence in other vertebrate groups (8), which hinders its generalization, as well as the examination of the coevolution of host–microbe interactions.

In anuran amphibians (frogs and toads), communication has traditionally been assumed to rely almost exclusively on acoustic signals, whereas other sensory modalities were considered minor subjects (10). However, these assumptions have been recently challenged, as we now know that several families use visual signals (10), which are often accompanied by acoustic signals in multimodal (multisensory) displays (11, 12). In addition, behavioral, morphological, and chemical evidence suggest that chemically mediated interactions could actually be much more common and phylogenetically widespread in anuran amphibians than what had traditionally been thought (13–16). In particular, the skin glands distributed along the body in hundreds of species secrete volatile compounds with characteristic odors (17–19). The few studies that have investigated these volatile secretions suggest that compounds likely come from two different sources: de novo synthesis by anuran amphibians and environmental sequestration (20, 21). Alternatively, just as they occur in mammals, volatile compounds may originate from interaction with the rich microbiota that inhabit amphibian skin (22, 23). Unraveling this interaction could help to solve diverse ecological questions, like the impact of symbiotic bacteria in individual recognition and mate choice (8, 24).

To address some of these questions, we used the South American tree frog Boana prasina as a biological model (SI Appendix, Fig. S1). This species has a prolonged reproductive period with males displaying a rich vocal repertoire in different social contexts (25, 26) and, like in other members of the B. pulchella group, emits a strong and characteristic smell (19, 21). In a prior study, we found that the volatile secretion in two other species of this group is formed by a blend of 35–42 compounds from nine different chemical classes. Although no functional study has yet been conducted, the variety of components of the secretion suggests that they may be linked to


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Data deposition: The sequences reported in this paper have been deposited in the Sequence Read Archive, www.ncbi.nlm.nih.gov/sra (Bioproject ID PRJNA498895). The sequences reported in this paper have been deposited in Genbank, https://www.ncbi.nlm.nih.gov/genbank/ (accession nos. MK100851–MK100897).

1To whom correspondence may be addressed. Email: andresbrunetti@gmail.com or npeoples@fcfrp.usp.br.

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different functions, including defense and reproduction (21). Here, we report sex-specific odor profiles in *B. prasina*, identify the chemical classes that account for such differences, and examine through culture and culture-independent methods whether the skin microbiota could be the source of these sex-specific components. The results presented here have implications for the role of the skin microbiome in amphibians and highlight the relevance of biosynthesis on chemical communication in less-studied vertebrates.

**Results**

**Diversity of Volatile Components in the Skin of *B. prasina***. Solid-phase microextraction (SPME) and gas chromatography/mass spectrometry (GC/MS) analyses revealed that the volatile skin secretion of adult males and females of *B. prasina* is a multi-component blend of 60–80 compounds including alcohols, aldehydes, alkenes, ethers, ketones, methoxy pyrazines (MOPs), terpenes [hemiterpenes, monoterpenes, and sesquiterpenes (SQTs)], and thioethers (TOEs) (Fig. 1, Table 1, SI Appendix, Fig. S2, and Dataset S1 A–E). Our results show that regardless of the sex, the mean relative abundance of hemiterpenes, ketones, and alcohols combined make a large contribution (49.9–78.3%) to the overall volatile composition in the species (Table 1). Hemiterpenes and ketones were relatively unaffected by the sampling procedures (i.e., in vivo or skin sampling), whereas alcohols were only abundant when using skin sampling (Table 1).

**Sex Differences in the Composition of the Volatile Secretion**. Principal component analysis (PCA) revealed that SQTs, TOEs, and MOPs contributed the most to the observed variations between males and females (Fig. 2 A and B). The created linear discriminant analysis (LDA) function shows that frogs can be discriminated by sex based on their volatile profile using one dimension (Kruskal-Wallis test: DF = 1, P = 0.001; Fig. 2C). Of these three compound classes, MOPs were, on average, more abundant in females than males, regardless of the sampling procedure [likelihood ratio tests (LRTs): interaction; sex: $\chi^2 = 1.202; P = 0.2729$; sex: $\chi^2 = 12.778; P = 0.0004$; Table 1]. However, relative abundance of SQTs were higher in males than in females, but only in in vivo sampling [LRTs: interaction $\chi^2 = 23.817; P < 0.0001$; sex(in vivo sampling): $\chi^2 = 22.933; P < 0.0001$; sex(skin sampling): $\chi^2 = 1.317; P = 0.2511$; Table 1]. Similarly, the relative abundance of TOEs was higher in males than in females only in in vivo sampling [LRTs: interaction $\chi^2 = 10.618; P = 0.0011$; sex(in vivo sampling): $\chi^2 = 10.9505; P = 0.0009$; sex(skin sampling): $\chi^2 = 2.0051; P = 0.1568$; Table 1]. When considering the chemical composition within SQTs and TOEs, we noticed that only one compound contributed mainly to the total abundance in each of these two classes, namely, dihydroadulene II and (2E)-4-(methylsulfanyl)-2-pentene, respectively (Dataset S1A).

**Males and Females Exhibit a Distinct Profile of MOPs**. In addition to the higher percentage of total MOPs in females, we also detected differences within the four MOPs (MOP 1: 2-isopropyl-3-methoxy pyrazine, MOP 2: 3-isopropyl-2-methoxy-5-methylpyrazine, MOP 3: 2-sec-butyl-3-methoxy pyrazine, and MOP 4: 3-sec-butyl-2-methoxy-5-methylpyrazine) occurring in both males and females (Figs. 3 and 4 and Dataset S1 F-H). We found that MOP 3 was the major MOP in males and was poorly represented in females (Fig. 3). The relative abundance of this compound was much higher in males than in females, regardless of the sampling procedure [LRTs: interaction $\chi^2 = 0.1219; P = 0.7277$; sex: $\chi^2 = 49.4548; P < 0.0001$], with no significant differences found between sampling procedures (LRTs: sampling procedure $\chi^2 = 1.077; P = 0.3$; Fig. 3]. In contrast, MOP 4 was the major MOP in females. The relative abundance of this compound was much higher in females than in males in both sampling procedures (Fig. 3), but the difference between sexes was larger in vivo in skin samples than in skin samples [LRTs: interaction $\chi^2 = 5.3977; P = 0.0202$; sex(in vivo sampling): $\chi^2 = 25.5523; P < 0.0001$; sex(skin sampling): $\chi^2 = 33.1275; P < 0.0001$].

MOP 1 and MOP 2 were less represented in the total abundances of MOPs in both sexes (0–12.8%). MOP 1 showed higher percentages in males than in females [absent in in vivo females; sex (skin sampling): $\chi^2 = 21.766; P < 0.0001$; Fig. 3], whereas the relative abundance of MOP 2 was higher in females (LRTs: interaction $\chi^2 = 0.0181; P = 0.8929$; sex: $\chi^2 = 9.8289; P = 0.0017$; Fig. 3). This latter compound showed a higher relative abundance in skin samples than in those obtained in vivo sampling (LRTs: sampling procedure $\chi^2 = 4.621; P = 0.0316$; Fig. 3).

**A Skin-Associated Pseudomonas Produces the Same MOPs Found in Frogs**. Of the three components responsible for sex differences, TOEs and MOPs are compounds typically produced by microorganisms. To explore whether this is the case in *B. prasina*, we isolated,
showed lower and similar abundances (MOP 3 = 2.6 ± 5.0%, MOP 4 = 2.7 ± 2.1%).

The MOP-Producing Pseudomonas Occurs in All Specimens from Different Sites Surveyed and Is Maintained in Captive Conditions.

The general structure of skin bacterial communities varies among sites and lengths of time in captivity (Fig. 5 A and B, SI Appendix, Fig. S3, and Dataset S3 C and D). Despite this variation, more than half of the relative abundance of operational taxonomic units (OTUs) observed in the skin bacterial communities of four sites (60–63%) and in five different captivity times (56–66%) can be explained by members of seven and nine genera from the orders Enterobacterales, Pseudomonadales, Methylobacteriales, Oceanospirillales, Actinomycetales, Bacillales, Alteromonadales, and Burkholderiales, respectively (Dataset S3 E–H).

Among them, the genera Klebsiella (Enterobacteriales) and Pseudomonas (Pseudomonadales) were the two most prominent OTUs shared by all sites (relative abundance 7–30%) and were also abundant in all captive frogs (relative abundance 4–35%; Fig. 5 A and B). We found that OTU richness is not very different among the sites surveyed (mean number of observed OTUs site A: 115 ± 31, site B: 113 ± 17, site C: 161 ± 20, and site D: 143 ± 24; Dataset S3C). We also found that the OTU richness was larger at the beginning of the captivity, decreasing in subsequent periods (mean no. of OTUs 1-d: 163 ± 26, 3-d: 145 ± 35, 10-d: 100 ± 9, 2-wk: 104 ± 41, 8-mo: 155 ± 34; Dataset S3D).

Within the genus Pseudomonas, the haplotype network showed that although some OTUs were exclusively found in one site (Fig. 5C; e.g., P. umongensis, haplotype 34) or only under captive conditions (Fig. 5D; e.g., P. stutzeri; haplotypes 32 and 33), four strains occurred in most of the samples and were common to all categories independent of the geographical origin of the frogs and captivity times (Fig. 5 C and D). These OTUs correspond to the MOP-producing Pseudomonas (haplotypes 11 and 12, Pseudomonas sp. (occurring also in environmental samples; haplotypes 17 and 18), P. veronii (haplotypes 35 and 36), and P. fragi (haplotypes 26 and 27). When specifically examining the MOP-producing Pseudomonas, we found that this bacterium was present in most field samples from all of the sites (n = 10 of 11 males, n = 1 female) and in samples from all of the different captivity times (n = 6 males) (Fig. 5). The mean relative abundance of this bacterium in male samples collected in the field was 1.7% (CI95% 0.6–2.8; Dataset S3A), and although we were only able to analyze one female sample, its relative abundance (2.4%; Dataset S3A) was similar to that observed in the males. We also detected variations in the abundance of the MOP-producing Pseudomonas during the time in captivity, which was particularly evident in the first 3 d immediately after a frog’s collection (1 d = 8.8 ± 4.3%, 3 d = 0.4 ± 0.2%; Dataset S3B).

Fig. 2. Differences in the skin volatile profile of females and males of the tree frog B. prasina. (A) Biplot of PCA performed on relative abundance of volatile compounds. Centroids of specimens’ dispersion are depicted as a big circle for females and a triangle for males. (B) Relative contribution of each compound class to the first four principal components obtained in the PCA analysis. The absolute value of each contribution is depicted according to the size of the circle, whereas blue and red colors show positive and negative contributions, respectively. (C) Discriminant function (=Sex Volatile Profile). Confidence intervals and the medians obtained from Bayesian inferences are depicted as color boxes and horizontal lines, respectively. The shape around the rectangles represents the complete data distribution in each group.

Fig. 3. MOPs identified in females and males of B. prasina. Graphical representation (Left) and table with mean, SEM, and P values (Right) of the relative abundance of each MOP (abundance of MOP/overall abundance of MOPs, where # = 1, 2, 3, or 4 ± SEM). All MOPs have significant sexual differences.

Supplementary information: Dataset S1, Dataset S2, Dataset S3, SI Appendix.
production of frog TOEs by unidentified bacteria; or (iv) the synthesis of TOEs by the frogs.

MOPs presented marked sex differences in B. prasina and were found to be major constituents of a skin symbiotic Pseudomonas sp. Pyrazines are widely distributed in nature, possess intense odors, and are some of the major volatile compounds produced by bacteria (29, 30). They likewise mediate communication in different organisms such as bacteria (31), insects (32, 33), and mammals (34, 35). Particularly, MOPs have been described as mate attractants and aposematic signals in insects (32, 36). However, within vertebrates, they had only been reported in two other species of Boana (21). The biosynthesis of pyrazines is still an open debate with different pathways proposed (33, 37). Specifically, animals are not known to synthesize MOPs, but instead, their biosynthetic pathways have been described in two species of Pseudomonas, namely, P. perolens (38) and P. taetrolens (39), and in plants (40). In this scenario, it seems likely that symbiotic bacteria are the source of MOPs in frogs, whereas in insects, they could be derived either from symbiotic bacteria or, especially for phytophagous species, sequestered from plants.

Two biosynthetic pathways have been suggested for MOPs, and current evidence indicates that either pathway is possible (38-41). Both involve the condensation of one amino acid (e.g., leucine, isoleucine, and valine) with either another amino acid (e.g., glycine) or glyoxal. Following either pathway, the synthetic origin of MOPs reported here (Fig. 4) could be traced to valine (MOPs 1 and 2), isoleucine (MOPs 3, 4, and 8), alanine (MOP 5), and leucine (MOPs 6 and 7). Since our results showed that the same MOPs found in B. prasina are produced by the symbiotic Pseudomonas sp.,

Fig. 4. (A) MOPs found in males and females of B. prasina (1-4) and in the skin-associated Pseudomonas sp. (1-8) (G) GCMS total ion current chromatogram showing production of MOPs 1-8 (Left) by Pseudomonas sp. cultivated on Mueller–Hinton agar medium (Right). Their relative abundances are shown in Dataset S2 D–F.

Discussion

Hundreds of amphibian species from different lineages worldwide are known to secrete strong and characteristic smells from their skin, but the biological function and origin of their components remain largely unknown (17, 19). Using a multidisciplinary approach, we examined sex differences in the volatile profile of the tree frog B. prasina and explored whether the compounds responsible for these differences have a bacterial origin. Consistent with previous descriptions in two related species of the B. pulchella Group (21), our results showed that the skin secretion of males and females of B. prasina is rich in volatile chemicals, including at least 10 different compound classes. Our results also showed that the volatile profile of this species varies with sex and, most notably, that some of the compounds responsible for the sex variation are derived from symbiotic bacteria.

The greater abundance of SQTs and TOEs in males than in females may be associated with functions such as female attraction, male-male competition, and aggression (see below in this section). Among terrestrial vertebrates, these compound classes are known to mediate sexual behavior in mammals (27, 28). With regard to their origin, SQTs are likely obtained from environmental sources. A previous study has demonstrated that SQTs in the skin of the Australian tree frog Ranoidea caerulea (20) can be sequestered from its diet, thus suggesting that the diet of males and females of B. prasina might differ in the content of SQTs. Because sulfur derivatives are widespread across bacterial taxa (29, 30), it is plausible that a frog's skin bacteria may be involved in the synthesis of TOEs. Although we have identified TOEs in the bacterial isolates, none of them was identical to the TOEs found in the frogs. These results can be explained by different (albeit not mutually exclusive) possibilities, including: (i) the bacterial production of frog TOEs only under certain substrate conditions; (ii) the sequestration of TOEs from the bacteria and subsequent metabolization by the frogs; (iii) the
we can hypothesize that the characteristic sex profile of MOPs in frogs might be a consequence of distinct microenvironmental and/or metabolic conditions in males and females. This hypothesis is supported by experimental evidence showing that the production of volatile compounds in microorganisms is strongly influenced by environmental variables like nutrient availability and metabolic interactions with the host (33, 42). Furthermore, the variation in the MOP profile of *Pseudomonas* sp. in culture mediums and in the frogs themselves may be due to strong differences in both environments, among them, a high content of nutrients without competition in cultures vs. limited nutrient supply with several competitors in the frogs (42). Similar reasoning applies to the volatile profile of other bacteria isolated from *B. prasina*.

Our study suggests that the environment may affect the structure of the bacterial community and the composition of the *Pseudomonas*. However, it also reveals a similar bacterial composition across samples. In particular, it showed that frogs from all sites and times under captive conditions shared four *Pseudomonas* lineages, which might constitute part of the core microbiome of *B. prasina*. One of them is the MOP-producing *Pseudomonas* sp., whereas the others are symbiotic bacteria that might affect different aspects of their host’s biology. It has been proposed that the particular composition of amphibian skin, such as the presence of several classes of bio-molecules, acts as a host filtering mechanism, thus creating a unique skin-associated bacterial community (23, 43, 44). Subsequently, natural selection may act on the hosts and their associated microbiota. Indeed, mammalian species would have a strong selection for gland microenvironments favorable to odor-producing bacteria (7). Lastly, the volatile compounds emitted by the MOP-producing *Pseudomonas* sp. in the frog’s skin can act as infochemicals mediating interactions with other members of the bacterial community, and reciprocally, the bacterial community may have effects on the production of MOPs. Because our experimental design was not planned to evaluate sex differences in the bacterial community, future studies are crucial to examine the potential effects of other skin microorganisms in the MOP profile of males and females of *B. prasina*.

Although chemical communication in anurans may be favored in species that lack acoustic signals, most species in which communication by chemical signals was suggested based on morphological (10, 15, 16), chemical (13, 14, 45), and/or behavioral (13, 14, 45) evidence also vocalize. Our results, along with a vocalization analysis and field behavioral observation (assessed for *B. prasina* (23, 26)), indicate that the reproductive behavior of this species may include a combination of chemical and acoustical signals. Examples of these signal’s interactions in anurans could be found in: (i) female recognition by satellite and vocalizing males (45), (ii) male recognition and assessment by females (45), (iii) female mate assessment, and (iv) recognition of competing males. Because behavioral studies in amphibians have focused almost exclusively on the acoustic components, our study points out the need to address the role of chemical signaling within a more comprehensive context that includes signals from different sensory modalities.

As previously seen in insects and mammals (6–8), our analyses support the concept that symbiotic bacteria are involved in the production of frog’s volatile compounds, which might act as chemical signals in distinct social interactions. Similar cases may also occur in other amphibians. For instance, macrodiles, which are polyketyte natural products typically produced by microorganisms, have also been reported functioning as sexual pheromones in mantellid frogs (13). In mammals, the host–symbiont interaction leading to the emission of particular odors is called fermentation hypothesis, because the compounds are known products of bacterial fermentation (5). However, to include other microbial metabolites that may participate in host chemical communication, a broader terminology is needed. Our results illustrate that studies on the ecological role of host–microbiome associations in amphibians are in their early infancy and delineate some crucial questions to increase our understanding of this interaction.

**Materials and Methods**

Full experimental details including citations are provided in the SI Appendix.

**Specimen Collecting and Handling.** Adults of the hyliid tree frog *B. prasina* were collected at night from four sites in Brazil: Nova Friburgo, Rio de Janeiro (site A: 22°16′55″ S, 42°26′25″ W; site B: 22°16′55″ S, 42°26′25″ W; site C: 22°16′55″ S, 42°26′25″ W; site D: 22°16′55″ S, 42°26′25″ W). Males were distinguished from females based on the presence of pigmented vocal sacs. Individuals were transported to the laboratory and were kept in glass terraria and provided with dechlorinated tap water. Various different plant species and rocks that serve as refuges were added. Frogs were kept at 22–26 °C, with a 14.10 h (light/dark) period, and fed with crickets.

**Bacterial Cultures of Skin Microbiota.** Isolation of microorganisms was performed through swabbing the dorsal skin from two groups of specimens. In the first group, two males and one female were collected in site A and swabbed in the field. Each swab was suspended in 5 mL of TSB medium (Trypticase Soy Broth-BD) and kept at room temperature for 96 h. Then, in the laboratory, a 100-μL aliquot of each tube was transferred to agar plates with TSA (Trypticase Soy Agar-BD) or ISP2 (International Streptomycetes Project) medium. In the second group, six males were collected in site B and swabbed in the laboratory, 2 d (three individuals) or 2 wk (three individuals) after collection of the specimens. The swabs were directly scrubbed onto ISP2 media plates. All media were supplemented with antifungal agents (nystatin: 0.04 g/L; cycloheximide: 0.05 g/L), and plates were incubated in biochemical oxygen demand (BOD) at 28 °C for 3 d. Different bacterial colonies were identified according to distinct morphotypes and were preserved in a liquid ISP2 medium with 30% glycerol at −80 °C for posterior identification by DNA sequencing and volatile analysis.

**Volatile Surveys of Frogs and Bacteria.** In vivo sampling and skin sampling procedures were employed for volatile surveys of frogs as described (21). Fourteen males and three females were examined for in vivo sampling, and six males and four females were examined for skin sampling. For volatile surveys, the bacteria culture or bacteria culture plus a streptomycete system was grown in two lidless Petri dishes laid in opposing positions and sealed with two layers of thin plastic film (Parafilm, Bemis NA) was used. After cultivating the bacteria for 72 h, the headspace was sampled by inserting a SPME into this system. All volatile analyses were undertaken on a Shimadzu GC coupled to a Mass Spectrometer Detector (GC/MS QP2010 Plus). Data analyses were performed using the Shimadzu GCMS solution software Version 2.53 SUJ, which includes the NIST21 and NIST107 Mass Spectral Libraries.

**Statistical Analyses of Volatile Compounds in Frogs.** For statistical analysis, we used the relative abundances for all compounds. This abundance was calculated as the ratio of the area of each compound over the area of all compounds combined. The area of each chemical class was determined as the sum of all compounds within that class. Multivariate analyses approaches (PCA and LDA) were used to assess differences and females and males were discriminated based on their volatile profile and to identify whether any of the compound classes is most likely to be associated with one sex rather than the other.

Generalized least squares (GLS) models were used to test differences between sexes in those compound classes identified by PCA (i.e., SQTs, TOEs, and MOPs). Two fixed factors (sex and sampling procedure) and the two-way interaction between those factors were tested using a model selection approach that compares nested models with a likelihood ratio test, as suggested by Zuur et al. (46). For each of these three compound classes, we used models that accounted for the observed heterogeneity of variance. In addition, we analyzed differences between sexes in relation to the relative abundance of each of the four MOPs identified in the frogs using GLS models. All analyses were conducted with the help of the software R-studio. See SI Appendix for description of the models.

**Identification of Bacterial Cultures and Dorsal Skin Bacterial Community Assessment.** An aliquot of 100 μL of each isolated bacterium was transferred to a 1.5-mL microcentrifuge tube with ISP2 medium. After 24–36 h, the cells were centrifuged for 1 min at 10,000 × g and genomic DNA extraction was conducted using the ammonium acetate precipitation method (47). Bacterial 16S rDNA was amplified and sequenced using the bacterial 16S rDNA primers 27F and 1492R (48). Amplified fragments were purified and sent to Macrogen Inc. for sequencing. The sequences were quality verified and trimmed using Geneious V.6 (49), and the preliminary identification of each bacterium was performed using the online BLASTN 2.7.0 (50) and the 16S ribosomal RNA (Bacteria and Archaea) Database. New sequences were submitted to GenBank (Dataset S2A and BioProject ID PRJNA498895).
For the skin bacterial community profiling, we followed procedures described in Bietz et al. (51) (see Supplement for a full description of methods). Since we were interested in exploring the ecological role of the isolated MOP-producing *Pseudomonas* sp. strain in amphibian skin, we used the filtered OTU table (not rarefied) to verify the presence of the *Pseudomonas* sp. strain in each sample. We also explored *Pseudomonas* diversity by constructing a haplotype network with the OTU sequences classified by qiime. This approach allowed us to access the geographical and temporal diversity and similarities of the *Pseudomonas* strains in amphibian skin and to take into account possible population variation of the isolated MOP-producing *Pseudomonas* sp. strain. For the haplotype network, we used Haploviewer (www.cibiv.at/~greg/haploviewer) and a maximum parsimony tree was inferred using the Phylog 3.695 package (S2) with the DNA Pars extension. Sequences that differed in only one pair basis were considered a variation within the same strain.

**Animal Use and Care.** Collection permits were issued by the Instituto Chico Mendes de Conservação da Biodiversidade/Biodiversity Information and Authorization System (SISBIO)/National System of Genetic Resource Management and Associated Traditional Knowledge (SiGen) (Permits 45180-8, 50071-1, 50077-2, A1FC13). Experimental procedures were performed according to the regulations specified by the Conselho Nacional de Controle Animal and Ministério da Ciência, Tecnologia e Inovação, Brazil and were approved by the Ethics Committee on Animal Use (Comitê de Utilização de Animais of Universidade Estadual Paulista (UNESP/Ribeirão Preto) and the Pharmacological Sciences of Ribeirão Preto-CEPA (N7/1.7.1047.60.0). Voucher specimens are housed in the herpetological collection of C.F.B.H., Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil (praisia: CFBH 41014–41033).

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