



Characterization of the microbiome of the invasive Asian toad in Madagascar across the expansion range and comparison with a native co-occurring species

Bárbara Santos¹, Molly C. Bletz², Joana Sabino-Pinto³, Walter Cocca¹, Jean Francois Solofoniaina Fidy⁴, Karen LM Freeman⁴, Sven Kuenzel⁵, Serge Ndriantsoa⁶, Jean Noel⁴, Tsanta Rakotonanahary⁶, Miguel Vences³ and Angelica Crottini¹

¹ Cibio, Research Centre in Biodiversity and Genetic Resources, InBio, Universidade do Porto, Campus Agrário de Vairão, Rua Padre Armando Quintas, Portugal, Porto, Portugal

² Department of Biology, University of Massachusetts Boston, Boston, MA, USA

³ Zoological Institute, Braunschweig University of Technology, Mendelssohnstr. 4, Germany, Braunschweig, Germany

⁴ Madagascar Fauna and Flora Group, BP 442, 501 Toamasina, Madagascar, Toamasina, Madagascar

⁵ Max Planck Institute for Evolutionary Biology, August-Thienemann-Str. 2, Germany, Plön, Germany

⁶ Amphibian Survival Alliance c/o Durrell Wildlife Conservation Trust, Madagascar Programme, Lot II Y 49 J 12 Ampasanimalo, BP 8511 101 Antananarivo, Madagascar, Antananarivo, Madagascar

ABSTRACT

Biological invasions are on the rise, with each invader carrying a plethora of associated microbes. These microbes play important, yet poorly understood, ecological roles that can include assisting the hosts in colonization and adaptation processes or as possible pathogens. Understanding how these communities differ in an invasion scenario may help to understand the host's resilience and adaptability. The Asian common toad, *Duttaphrynus melanostictus* is an invasive amphibian, which has recently established in Madagascar and is expected to pose numerous threats to the native ecosystems. We characterized the skin and gut bacterial communities of *D. melanostictus* in Toamasina (Eastern Madagascar), and compared them to those of a co-occurring native frog species, *Ptychadena mascareniensis*, at three sites where the toad arrived in different years. Microbial composition did not vary among sites, showing that *D. melanostictus* keeps a stable community across its expansion but significant differences were observed between these two amphibians. Moreover, *D. melanostictus* had richer and more diverse communities and also harboured a high percentage of total unique taxa (skin: 80%; gut: 52%). These differences may reflect the combination of multiple host-associated factors including microhabitat selection, skin features and dietary preferences.

Submitted 7 October 2020

Accepted 7 May 2021

Published 28 June 2021

Corresponding author

Bárbara Santos,
barbarasantosbio@gmail.com

Academic editor

Lee Rollins

Additional Information and
Declarations can be found on
page 18

DOI 10.7717/peerj.11532

© Copyright

2021 Santos et al.

Distributed under

Creative Commons CC-BY 4.0

OPEN ACCESS

Subjects Biodiversity, Genetics, Microbiology, Molecular Biology

Keywords *Duttaphrynus melanostictus*, *Ptychadena mascareniensis*, Invasive species, Toamasina, Madagascar, 16s rRNA sequencing, Gut bacteria, Skin bacteria

INTRODUCTION

Biological invasions can cause dramatic biodiversity loss (*Enserink, 1999; Chornesky & Randall, 2003; Penk et al., 2016*), with climate change, habitat alterations and direct anthropogenic translocation being the main factors facilitating the worldwide spread of alien, invasive species (*Alpert, Bone & Holzapfel, 2000; Stachowicz et al., 2002; Walther et al., 2009; Crooks, Chang & Ruiz, 2011*). Although alien invasive species do not always have detrimental effects (*Schlaepfer, Sax & Olden, 2011*), their potential devastating effects can be stronger in fragile island ecosystems. Here, they often out-compete or predate on local species, interfering with trophic networks and ultimately altering natural ecosystem function and balance (*Lowe et al., 2000; Pitt, Vice & Pitzler, 2005*). Among amphibians, notable invasive species include the Cane toad, *Rhinella marina*, the Puerto Rican Coquí, *Eleutherodactylus coqui* and the American bullfrog, *Lithobates catesbeianus* (*Beard & Pitt, 2005; Shine, 2010; Snow & Witmer, 2010*) of which the former is especially notorious for its negative effects in its invasive range in Australia (*Shine, 2010*). Among the many impacts that invasive species can have on native ecosystems, the introduction and spread of pathogenic fungi and viruses is also emerging as an important factor that may contribute to the global amphibian population decline (*Miaud et al., 2016*).

Microbiome research with high-throughput DNA sequencing techniques has enabled a better understanding of how host-associated microbiomes vary across host species, age, sex and habitats, and how their composition and diversity is influenced by host related and habitat-dependent factors (*McKenzie et al., 2012; Bletz, Perl & Vences, 2017; Tiede et al., 2017*). Symbiotic microbial communities likely inhabit all multicellular organisms and play an important role in the ecology, physiology, behaviour and health of their hosts (*Dethlefsen, Mcfall-Ngai & Relman, 2007; Grice & Segre, 2012; Abdallah, Mijouin & Pichon, 2017; Lester et al., 2017*). The skin microbiome can influence host's ability to cope with environmental and habitat conditions and mediate immune responses (*Sanford & Gallo, 2013; Grice, 2014; Jani & Briggs, 2018; Rebollar et al., 2016; Xavier et al., 2019*), while gut-associated microbes can aid in food digestion, energy harvesting, development or immunity (*Turnbaugh et al., 2006; Heijitx et al., 2011; Tuddenham & Sears, 2015*). Microbiomes have been proposed to affect the host's capacity for colonization, adaptation, and boosting the immune system (*Rout et al., 2013; Gribben et al., 2017; Cheng et al., 2018*). For example, interactions between invasive plants and associated microbes were found to suppress the rhizosphere microbes and other beneficial symbionts in native plants (*Coats & Rumpho, 2014*); on the contrary, some fungal symbionts were found to increase survival of their insect host, an invasive ant species, when these were exposed to pathogens (*Konrad et al., 2014*). In amphibians, only a few recent studies have evaluated the microbial communities in invasive species (e.g., *Abarca et al., 2018; Christian et al., 2018; Kueneman et al., 2019*).

Madagascar is one of the most celebrated biodiversity hotspots (*Ganzhorn et al., 2001*), known not only for the high degree of endemism but also for the ongoing habitat loss. Amphibian diversity in Madagascar is exceptionally high (*Vieites et al., 2009; Perl et al., 2014; Brown et al., 2016*) and severely threatened by habitat loss and human exploitation (*Harper et al., 2007*). Invasive species and pathogens in Madagascar are emerging as a new

conservation concern since they may push native species further towards extinction (Bletz et al., 2015; Kull, Tassin & Carriere, 2015; Goodman et al., 2017). A naturalized population of the Asian common toad, *Duttaphrynus melanostictus*, was reported in Madagascar in 2014, and has since become a major conservation concern (Andreone et al., 2014; Crottini et al., 2014; Kolby, 2014). Originally from Asia, it is estimated to have been present in Madagascar since 2010, being first reported near the seaport city of Toamasina, on Madagascar's eastern coast in 2014 (Fig. 1; (Kolby, 2014)). *Duttaphrynus melanostictus* is believed to have arrived from Cambodia or Vietnam (Vences et al., 2017), possibly in shipping containers. At present, it occurs mainly in urban and rural landscapes with mixed *Eucalyptus* spp. forests, where native amphibian communities are highly impoverished. However, it is rapidly expanding (Licata et al., 2019; Licata et al., 2020) and it is feared that it may soon reach areas known to host richer amphibian communities such as Betampona Strict Nature Reserve and Parc Ivoloina (Rosa et al., 2012; Crottini et al., 2014).

Although with low incidence, predation of smaller herpetofauna has been observed in other invasive populations of this toad (Döring et al., 2017), but the major concern of this invasion is associated with toad toxicity and the devastating effects that this species might have on its predators. In fact, *D. melanostictus* is known to produce highly poisonous skin toxins that are likely to negatively affect the vast majority of potential native predators (Marshall et al., 2018). Skin secretions isolated from *D. melanostictus* individuals collected in its native range seem to contain potent antimicrobial agents and important pharmacological compounds (Garg et al., 2007) that may increase disease resistance, making this invasive amphibian species a particularly interesting candidate for microbiome studies in invasive scenarios. Due to its life history traits that promote the capacity to reach high abundances and high dispersal rate, *D. melanostictus* is considered to have high invasive potential (Reilly et al., 2017; Licata et al., 2019; Licata et al., 2020). High invasive potential has been observed in other toad species such as *R. marina*, where this capacity has been linked to reduced ecological pressures in invaded areas and a rapid physiological adaptation to new environments (Phillips et al., 2006).

Here, we provide the first assessment of skin and gut bacterial communities of the invasive *D. melanostictus* population after its recent introduction to Madagascar and we compare its microbiome with that of a co-occurring native frog species, the Mascarene ridged frog (*Ptychadena mascareniensis*), across its expansion range. We hypothesized that the invasive species will present richer and more diverse bacterial assemblages due to intrinsic physiological and ecological characteristics, but also due to the recent introduction to a new environment (the invaded area). We also expect that across sites the toad may have different bacterial assemblages, due to the different arrival time in the new environment, while the native species, due to the limited geographic scale and similar habitat type, may exhibit similar bacterial assemblages. We explore the correlation between the toad's microbial diversity and its high colonization and adaptation capacity, using as proxy measures of bacterial species richness, diversity and functional inference that may confer disease resistance or enhance wider diet range in different habitats.

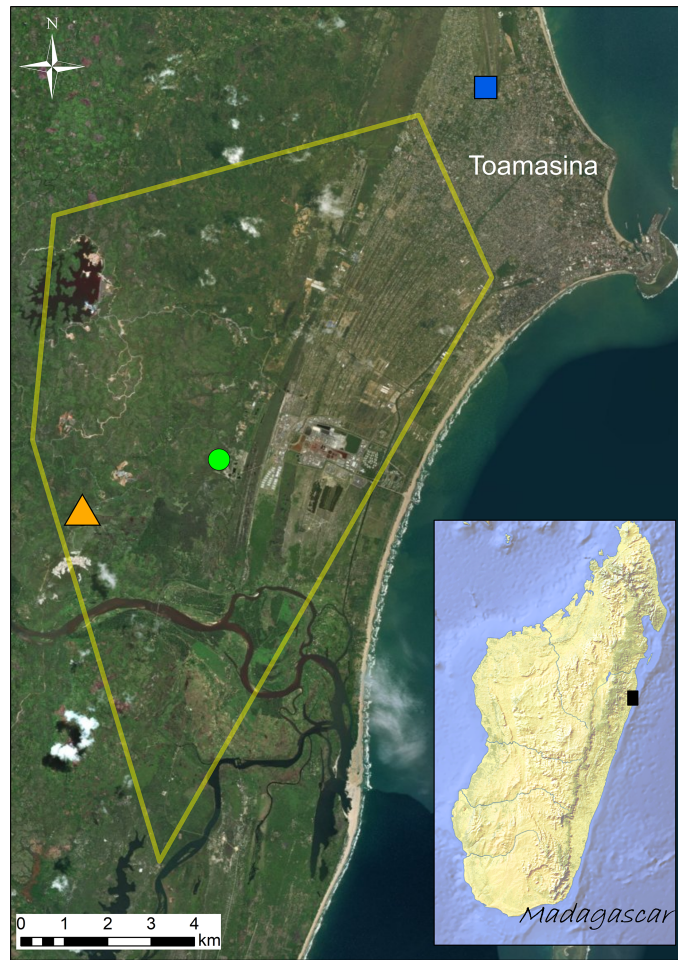


Figure 1 Distribution of the sampling sites (Site 1 = green circle; Site 2 = orange triangle; Site 3 = blue square) visited in September 2016. Known distribution area of the invasive population of *Duttaphrynus melanostictus* in Toamasina in late 2014 (yellow polygon; modified from Moore, Solofoniaina Fidy & Edmonds, 2015). The black square in the inset map shows the relative position of Toamasina in eastern Madagascar.

Full-size DOI: 10.7717/peerj.11532/fig-1

METHODS

Sampling

The study species collected were the invasive Asian toad (*Duttaphrynus melanostictus*) and the native and co-occurring species Mascarene ridged frog (*Ptychadena mascareniensis*). Sampling was performed in the invaded area around Toamasina (eastern Madagascar) (Fig. 1) between September 20th and 24th, 2016. All sampling sites are highly anthropogenically transformed areas and the selection of sampling sites was based on the known distribution of *D. melanostictus* at the time (retrieved from Moore, Solofoniaina Fidy & Edmonds (2015) and based on field observations carried out by the staff of Madagascar Fauna and Flora Group). We aimed to analyse sites where the toad established in different years (Fig. 1): Site 1 (green circle, S1), point where the toad was likely introduced around

2010; Site 2 (orange triangle, S2), site where the toad was not found in early 2014 and detected only in late 2014; Site 3 (blue square, S3), site that was recently colonized at the time of sampling (September 2016). In each site we collected 16 individuals (eight males and eight females) of *D. melanostictus* and four individuals of *P. mascareniensis*. Each specimen was collected with new nitrile gloves, measured (snout-vent length and weight), and kept in individual sterile plastic bags until sampling. Each specimen was rinsed with sterile water to remove debris and transient microbes, and swabbed 10 times on the ventral side and five times on each thigh and foot using one sterile swab (MW113, Medical Wire Equipment & Co. Ltd., Corsham, United Kingdom). Swabs were air dried, placed in their individual tube and kept at ca. 4 °C during the expedition and during the export from Madagascar, and transferred to –20 °C upon their arrival in Europe.

To characterize the gut bacterial communities for both species, at each site we used a solution of Tricaine Methanesulfonate (MS-222, Sigma-Aldrich) to euthanize 4 individuals of *P. mascareniensis* and four (two males and two females) of the 16 individuals of *D. melanostictus*. After euthanasia the specimens were dissected and the gut (the entire intestine portion including gut contents) was removed and stored in RNA later. For each site, we pooled the dissected guts of the four *D. melanostictus* into one tube and the dissected guts of *P. mascareniensis* into another tube. The six tubes with the pooled gut samples per site and per species were kept in liquid nitrogen during fieldwork, transferred to cool conditions (ca. 4 °C) during the export from Madagascar, and stored at –80 °C upon their arrival to Europe.

DNA extraction, amplification and sequencing

Gut tissue samples from each tube were homogenized. DNA from swabs and gut tissue was extracted following a modified Qiagen DNeasy Blood & Tissue Kit protocol (Hilden, Germany) with an initial lysozyme incubation step at 37 °C to break up cell walls of Gram-positive bacteria. To enable comparison of our data with previously published studies on the microbiome of Malagasy amphibians we amplified the V4 region of the bacterial 16S rRNA gene using the following primer set: 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (*Caporaso et al., 2011*). Amplification of each sample was performed in duplicate in a volume of 12.5 µl including 0.2 µl of Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific, Waltham, Ma, USA), 0.25 µl of each primer (10 µM), 0.25 µl of dNTPs, 2.5 µl of buffer, 8.1 µl of H₂O and 1 µl of template DNA. The amplification protocol consisted of an initial denaturation step at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The two PCR products of each sample were pooled together in a total volume of 25 µl and visualized on 1% agarose gel. All samples were pooled together according to band brightness and the final pooled sample was run in a 1% agarose gel and purified with QIAQuick Gel Extraction Kit (Qiagen, Hilden, Germany). Samples were sequenced using paired-end 2 x 250 v2 chemistry on an Illumina MiSeq sequencing platform using a dual-index approach (*Kozich et al., 2013*). Raw sequences were deposited in NCBI under the following BioProject ID [PRJNA667830](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA667830).

Sequence processing

Sequences were processed in Quantitative Insights into Microbial Ecology (QIIME v1.9.1) (Caporaso *et al.*, 2010a). Due to the typical lower quality of reverse reads (Kwon *et al.*, 2013), only the forward reads were filtered under the following criteria: absence of Ns within the sequence, absence of barcode errors, and exclusion of reads containing three or more consecutive low-quality nucleotides. Sequences were clustered into sub-operational taxonomic units (sOTUs, hereafter called OTUs) following the deblur workflow (<https://github.com/biocore/deblur>) (Amir *et al.*, 2017). Sequences were trimmed to 150 bp and OTUs with less than 10 reads were excluded. The resulting OTUs were then assigned to a taxonomic group using the Greengenes 13.8 reference database (May 2013 release; <https://greengenes.lbl.gov/>). Non-bacterial taxa (e.g.: archaea, mitochondria and chloroplasts) were removed. All OTUs with less than 0.001% of the total reads of all analysed samples were excluded (Bokulich *et al.*, 2013). PyNAST (Caporaso *et al.*, 2010b) was used to align the OTU sequences and a phylogenetic tree was built with FastTree (Price, Dehal & Arkin, 2010). Data was organized into three datasets: Dataset A included only skin swabs from the two species; Dataset B included only skin swabs from males and females of *D. melanostictus*; and Dataset C included only gut samples from the two species (Table S1). Each dataset was rarefied to a specific number of reads per sample: Dataset A, B: 1,455; Dataset C: 1,867 (Table S1). Dataset A was additionally rarefied at 4,000 reads/sample to allow a better comparison with previous published works (e.g., Bletz *et al.*, 2017; Kueneman *et al.*, 2019). After filtering and rarefaction, the final Dataset A included a total of 37 samples (S1: 9 *D. melanostictus*, 4 *P. mascareniensis*; S2: 10 *D. melanostictus*, 3 *P. mascareniensis*; S3: 7 *D. melanostictus*, 4 *P. mascareniensis*) with 1,617 OTUs for the skin bacteria dataset. Dataset B included a total of 15 males (S1 =3, S2 =6, S3 =6) and 11 females (S1 =6, S2 =4, S3 =1) with the female samples from S3 being excluded from the analysis (Table S2). Dataset C included 3 pooled samples for each species (each containing the gut of 4 individuals per species per site) with 701 OTUs (Tables S1, S2).

Statistical analysis

Diversity indices and statistical analysis were performed using QIIME v1.9.1 and R v3.4.4 (R Core Team, 2016). Data was organized into three datasets. Dataset A included a total of 37 skin swabs from both species from all sites and was used to assess the effects of host and site on skin bacteria; Dataset B included 26 skin swabs from *D. melanostictus* from all sites and was used to assess the effect of sex on skin bacteria; Dataset C included six pooled (per species and per site) gut samples and was used to assess the effects of host species on the gut bacteria (Table S2 for more details).

Alpha diversity metrics were calculated to detect differences between host species, sexes and sites. Species richness was measured as number of observed OTUs (OTU Richness) and Chao1 diversity index; and diversity was measured using Shannon diversity index and Faith's phylogenetic distance (PD). Significant differences between alpha indices were assessed using ANOVA (aov, stats package, R Core Team, 2016). For Dataset A, we used a two-way ANOVA, with factors "species", "site" and their interaction; for Dataset B, we used a one-way ANOVA, with the factor "sex". In Dataset C, we used a non-parametric

Kruskal-Wallis Test (KW) using the variable “species” although the low sample size does not allow for a robust statistical analysis and values are indicative. Dissimilarity matrices were calculated using Weighted and Unweighted Unifrac distances (Lozupone & Knight, 2005) and visualized using a non-metric multidimensional scaling plot (NMDS, phyloseq package, McMurdie & Holmes, 2013). Differences in the bacterial community structure (Beta Diversity) were analysed with PERMANOVA (Adonis, vegan package, 999 permutations (Oksanen et al., 2016)) with species and site as predictor variables, including main effects and their interaction. When significant differences were observed, a test for homogeneity of groups dispersions was calculated using the function betadisper in vegan package in R (Oksanen et al., 2016). Community composition was visualized with bar plots including the most abundant taxa in each category (phylum, family and genus) after transforming the counts into relative abundances and grouping all other taxa with relative abundance lower than 1% (phylum from Dataset C), 5% (phylum from Datasets A and B), 15% (family and genus from Datasets A and B) and 10% (family and genus from Dataset C). An additional category “unidentified” represents the total relative abundance of taxa that were not identified at that taxonomic level. Total shared and unique OTUs for each species were represented as Venn diagrams for all groups using the collapsed biom tables retrieved from QIIME. Since no significant differences were found between sites, the subsequent analysis was performed with individuals from the three sites grouped together. Linear Discriminant Analysis Effect Size (LEfSe) method (LDA score > 3.0, $\alpha = 0.05$) (Segata et al., 2011) was used to determine OTUs responsible for the observed differences in the skin and gut communities between species (Datasets A and C) and sexes (Dataset B). We used PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013) to gain a better understanding of the possible functions of the symbiotic bacteria identified in the skin and in the gut. The OTUs were assigned to the Greengenes v13.5 database using the 97% similarity with the closed OTU-picking strategy, and a normalization of the copy numbers of each OTU was performed. Subsequently, the metagenome of each sample was predicted, and a functional categorization with respective abundances (following the Kyoto Encyclopedia of Genes and Genomes –KEGG –Orthology database) performed, using level 2 KEGG Orthologs (KO). Pathways with less than 10 counts were removed and abundances were rarefied. Both LEfSe and PICRUSt analysis were run on the Galaxy Web platform (<http://huttenhower.sph.harvard.edu/galaxy>). Significant differences between host species were assessed using the Kruskal-Wallis test (K-W) in QIIME. To better understand if the bacterial taxa could provide advantages regarding higher disease resistance in the toad, we mapped all skin bacterial OTUs (Datasets A and B) using a closed-reference OTU picking strategy, against the published database of antifungal amphibian skin bacterial isolates (Woodhams et al., 2015) - this database includes isolates that are likely able to inhibit or enhance the growth of the amphibian fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*). Taxa with a match of 97% were retrieved and the proportions of OTUs with putative *Bd*-inhibitory or *Bd*-enhancing properties were calculated.

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. Ministère de l'Environnement et du Développement Durable

provided the research permits for: collection, N°226/16/MEEF/SG/DGF/DSAP/SCB.Re of September 19th, 2016; transport, N°1679-16/MEEF/SG/DGF/DREEF.ATS/SREco and N°1680-16/MEEF/SG/DGF/DREEF.ATS/SREco of September 24th, 2016; and export, N°284N-EA10/MG16 of October 5th.

RESULTS

Dataset A - Comparison of the skin microbiome of *Duttaphrynus melanostictus* and *Ptychadena mascareniensis* across the expansion range

Host species had a significant effect on alpha diversity indices of the cutaneous microbiome (Figs. 2A–2D): *D. melanostictus* showed significantly higher values for OTU richness (ANOVA, $F = 33.15$, $p < 0.001$), phylogenetic diversity (ANOVA, $F = 40.66$, $p < 0.001$), Chao1 diversity (ANOVA, $F = 29.64$, $p < 0.001$) and Shannon diversity (ANOVA, $F = 7.289$, $p = 0.006$) compared to the native *P. mascareniensis*. Site did not have an effect on alpha diversity (ANOVA, OTUs: $F = 0.505$, $p = 0.61$; PD: $F = 1.830$, $p = 0.180$; Chao1: $F = 1.274$, $p = 0.30$; Shannon: $F = 1.074$, $p = 0.36$, SM Fig. S1A), and the interaction between species and site was not statistically significant (ANOVA, OTUs: $F = 0.729$, $p = 0.49$; PD: $F = 1.078$, $p = 0.35$; Chao1: $F = 0.334$, $p = 0.72$; Shannon: $F = 0.783$, $p = 0.47$). However, a trend was observed with *D. melanostictus* showing greater values in all alpha indices across sites while *P. mascareniensis* showed an irregular pattern.

Beta diversity significantly differed between host species when measured by both weighted Unifrac (Fig. 2E, PERMANOVA: Pseudo- $F_{(1,36)} = 4.896$, $R^2 = 0.118$, $p = 0.002$) and unweighted Unifrac metrics (Fig. 2F, PERMANOVA: Pseudo- $F_{(1,36)} = 6.565$, $R^2 = 0.156$, $p = 0.001$); but did not differ across sites (Figs. 2E–2F, PERMANOVA: weighted Pseudo- $F_{(2,36)} = 1.138$, $R^2 = 0.055$, $p = 0.32$; unweighted Pseudo- $F_{(2,36)} = 1.036$, $R^2 = 0.049$, $p = 0.36$). Similarly, the interaction of species and site did not affect beta diversity (Figs. 2E–2F, PERMANOVA: weighted Pseudo- $F_{(2,36)} = 1.664$, $R^2 = 0.080$, $p = 0.06$; unweighted Pseudo- $F_{(2,36)} = 1.217$, $R^2 = 0.058$, $p = 0.15$). Analysis of dispersion indicated no significant differences between species (Weighted: $F_{(1,35)} = 0.863$, $p = 0.37$; Unweighted: $F_{(1,35)} = 1.996$, $p = 0.17$) or sites (Weighted: $F_{(2,34)} = 0.0649$, $p = 0.94$; Unweighted: $F_{(2,34)} = 0.943$, $p = 0.40$).

The skin bacterial communities from the two species were mainly composed of the same phyla (Actinobacteria, Bacteroidetes and Proteobacteria) but with several differences in relative abundances at lower taxonomic levels (family and genus; Figs. 2G–2H). The *D. melanostictus* skin community had higher abundances of the families Alteromonadaceae, Comamonadaceae, Moraxellaceae and Sphingobacteriaceae while the *P. mascareniensis* skin community had higher abundances of Enterobacteriaceae, Moraxellaceae (only at Site 3), Pseudomonadaceae and Xanthomonadaceae. Notably, *P. mascareniensis* had a higher abundance of bacteria of the genus *Pseudomonas* while *D. melanostictus* had *Cellvibrio* as the most abundant genus. Across sites, the differences observed between host species were concordant. Within species, the patterns varied: *D. melanostictus* skin bacterial communities were more stable across sites and *P. mascareniensis* showed more variability in taxonomic

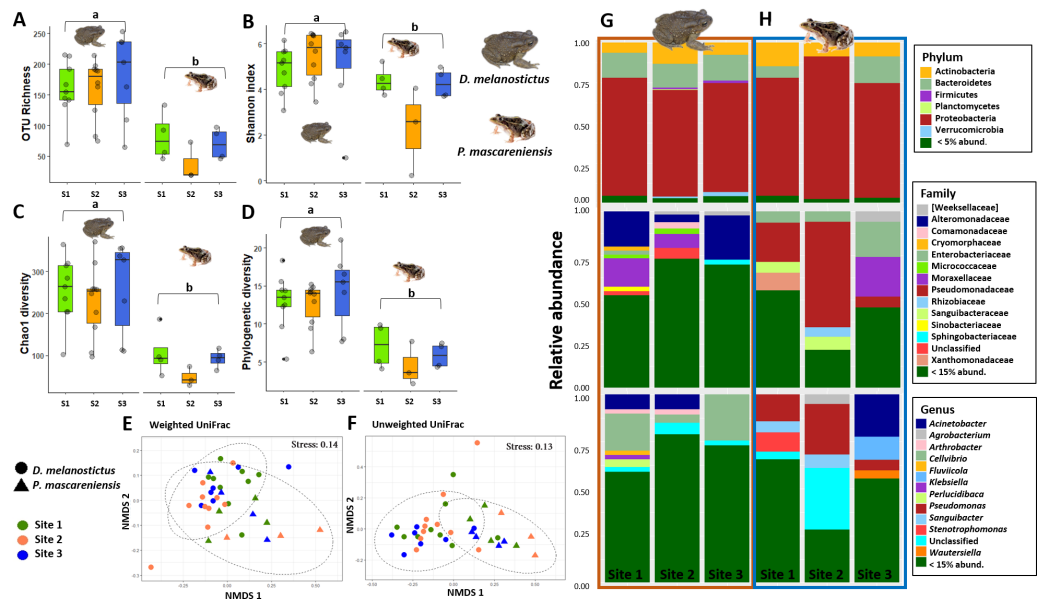


Figure 2 Skin bacterial diversity and composition of *Duttaphrynus melanostictus* and *Ptychadena mascareniensis* across sites. Alpha diversity: (A) OTU Richness. (B) Shannon index. (C) Chao1 diversity. (D) Phylogenetic diversity. Different letters (a, b) indicate significant different groups; Beta diversity: (E) Non-metric multidimensional scaling (NMDS) ordination of Weighted Unifrac Distances, (F) Non-metric multidimensional scaling (NMDS) ordination of Unweighted Unifrac Distances; Abundance plots: Composition of the skin bacterial communities including the most abundant taxa from each taxonomic level: phylum (top), family (middle) and genus (bottom) in (G) *D. melanostictus* and (H) *P. mascareniensis* across the three sites. Photo credit: Angelica Crottini, Javier Lobon-Rovira.

Full-size [DOI: 10.7717/peerj.11532/fig-2](https://doi.org/10.7717/peerj.11532/fig-2)

abundance (Figs. 2G–2H). LEfSe analysis revealed 39 taxa that were differently abundant in the two host species including 13 taxa that exhibited higher relative abundance in *P. mascareniensis* and 26 in *D. melanostictus* (Fig. 3). Specifically, *P. mascareniensis* only had differently abundant taxa from the phylum Proteobacteria and only one from the phylum Firmicutes, while *D. melanostictus* was characterized by significant differential abundance of taxa from the Actinobacteria, Proteobacteria, Bacteroidetes and Verrucomicrobia phyla. In *P. mascareniensis*, all differentially abundant taxa were included within the class Gammaproteobacteria with the exception of one Alphaproteobacteria taxon. In the case of *D. melanostictus*, differentially abundant bacteria belonged to several classes and families within different phyla (Fig. 3A).

In total, *D. melanostictus* had more than 1,000 unique OTUs (equivalent to 80% of total number of OTUs) and shared only 238 (15%) with *P. mascareniensis*, while the latter had only 5% unique OTUs (Fig. S1A). The percentage of shared OTUs between the species was similar at sites 1 and 3 and lower at site 2 (Fig. S1C). This lower percentage was coupled with higher number of unique OTUs found in the toad (Fig. S1C). Individuals of *D. melanostictus* across sites shared between 30–40% of OTUs, while the percentage of unique OTUs found at each site was around 20% (Fig. S1B); individuals from sites 1 and 2 shared more OTUs than in comparison with the number of shared OTUs between each of the first two sites with site 3 (Fig. S1B). *P. mascareniensis* had similar trends, with individuals

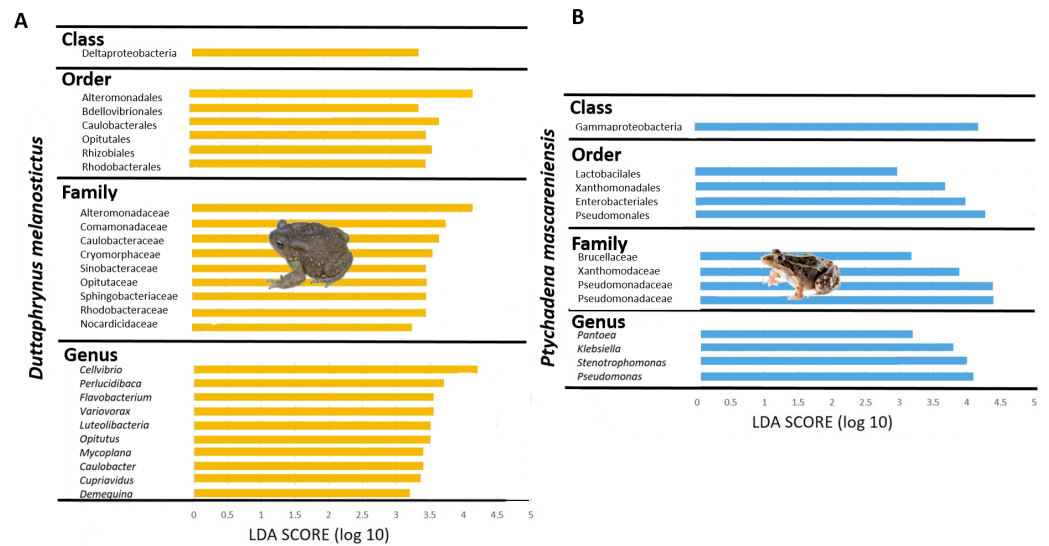


Figure 3 Differently abundant skin taxa occurring in *Duttaphrynus melanostictus* (yellow bars) and *Ptychadena mascareniensis* (blue bars). –LDA scores of detected OTUs in LEfSe analysis. Photo credit: Angelica Crottini, Javier Lobon-Rovira.

Full-size DOI: 10.7717/peerj.11532/fig-3

sharing a higher number of OTUs compared with the unique OTUs found at each site. However, the percentage of OTUs found at all three sites was only 9% (Fig. S1B).

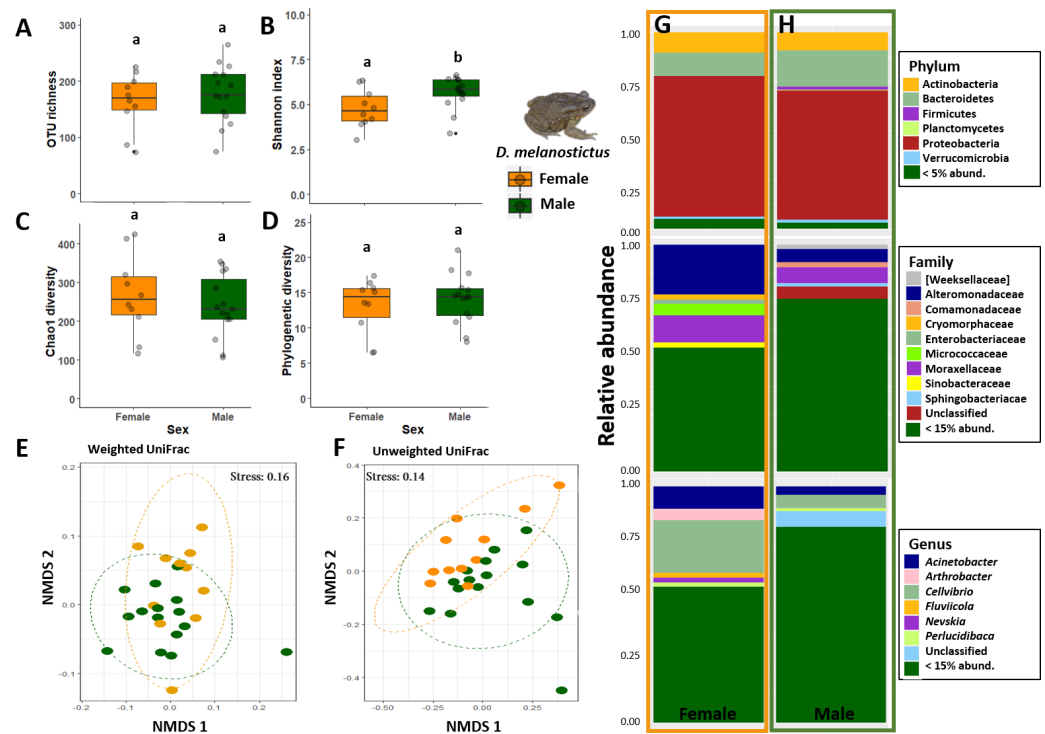
A total of 39 KEGG pathways (Level 2) were predicted for the two amphibians' skin microbiomes, of which 18 exhibited significantly different relative abundance between species (Table S3). From these, *D. melanostictus* had 11 enriched functional groups including cell growth and death, transport and catabolism, biosynthesis of secondary metabolites, energy and lipid metabolism, xenobiotics biodegradation and environmental adaptation. *P. mascareniensis* had seven enriched functional groups including membrane transport, infectious diseases, cellular processes and signalling, among others.

The two amphibians had significant differences in the proportion of OTUs with putatively *Bd*-inhibitory capacities but not of *Bd*-enhancing skin OTUs (K-W: $\chi^2 = 11.5$, $p < 0.001$ and $\chi^2 = 3.10$, $p = 0.078$ respectively) with *P. mascareniensis* carrying higher proportions of putative *Bd*-inhibitory OTUs (Fig. S3A).

Dataset B - Comparison of the skin bacterial community of males and females of *Duttaphrynus melanostictus*

In terms of alpha diversity, Shannon was the only metric that was significantly different between the sexes (ANOVA: $p = 0.04$, Figs. 4A–4D). Beta diversity showed that sex was significant when assessing weighted Unifrac distances (PERMANOVA: Sex, Pseudo- $F = 2.35$, $R^2 = 0.09$, $p = 0.02$, Fig. 4E) but not with the Unweighted Unifrac distances (PERMANOVA: Sex, $p > 0.05$, Fig. 4F). Analysis of dispersion indicated a significant difference between sexes (Weighted Unifrac, $F_{(1,24)} = 4.8089$, $p = 0.032$).

Males had higher abundances of Sphingobacteriaceae and a high rate of unidentified taxa when compared with females (Figs. 4G–4H). At the genus level, sex seemed to influence the



Full-size [DOI: 10.7717/peerj.11532/fig-4](https://doi.org/10.7717/peerj.11532/fig-4)

abundance level of the most common taxa (*Arthrobacter*, *Cellvibrio*, *Devosia*) but without a clear pattern. Once again, males had a higher abundance of unidentified genus than females (Figs. 4G–4H). However, LefSe analysis indicated that there were no differently abundant taxa.

Males had double the number of unique OTUs compared to females when samples from the three sites were grouped (Fig. S2). A total of five predicted KEGG pathways (Level 2) were more abundant in males and four in females (Table S4). Among these, females exhibited significantly higher abundances of functional groups associated with Immune System Diseases while males had higher abundances of functional groups associated to other diseases and associated to cellular processes (e.g., Transport and Catabolism) (Table S4).

Comparing the skin bacterial communities with the antifungal database showed marginal differences with males exhibiting a slightly higher proportion of putative *Bd*-inhibitory skin OTUs (K-W: $\chi^2 = 3.59$, $p = 0.058$). Both sexes exhibited similar proportions of putative *Bd*-enhancing skin OTUs (K-W: $\chi^2 = 0.12$, $p = 0.74$) (Fig. S3B).

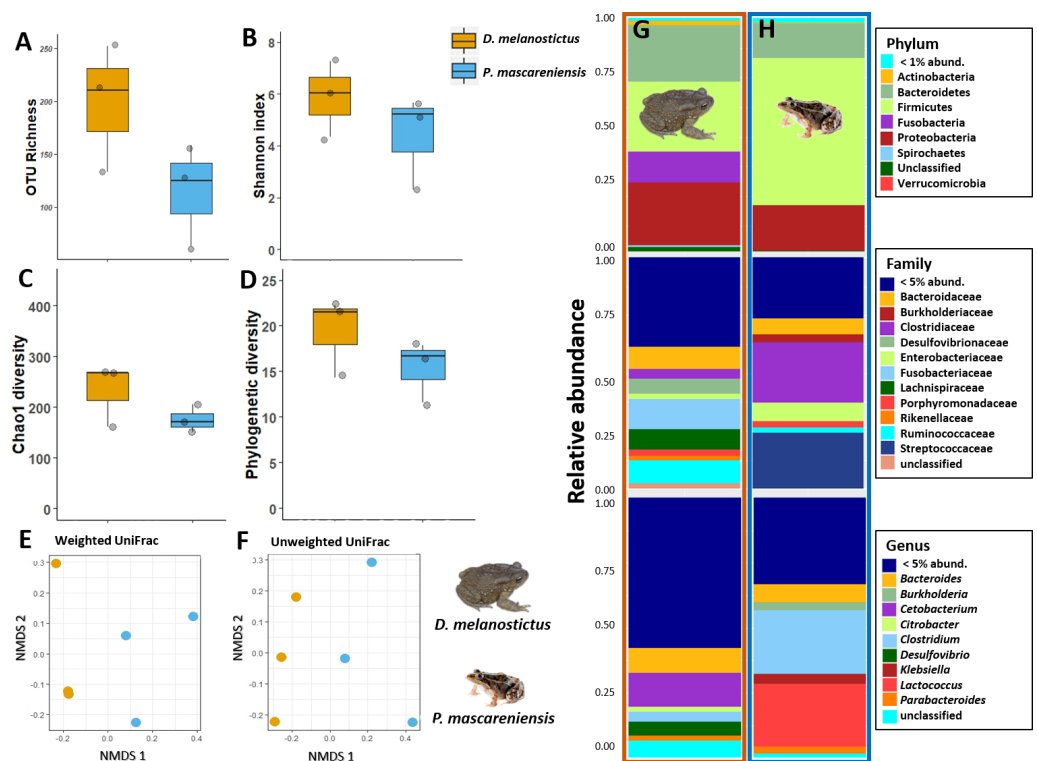


Figure 5 Gut bacterial diversity and composition of *Duttaphrynus melanostictus* (yellow) and *Ptychocheilus mascareniensis* (blue). Alpha diversity: (A) OTU Richness. (B) Shannon index. (C) Chao1 diversity. (D) Phylogenetic diversity. No significant differences; Beta diversity: (E) Non-metric multidimensional scaling (NMDS) ordination of Weighted Unifrac Distances, (F) Non-metric multidimensional scaling (NMDS) ordination of Unweighted Unifrac Distances; Abundance plots: Composition of the skin bacterial communities including the most abundant taxa from each taxonomic level phylum (top), family (middle) and genus (bottom) in (G) *D. melanostictus* and (H) *P. mascareniensis*. Photo credit: Angelica Crottini, Javier Lobon-Rovira.

Full-size DOI: 10.7717/peerj.11532/fig-5

Dataset C - Comparison of gut bacterial communities of *Duttaphrynus melanostictus* and *Ptychocheilus mascareniensis*

In dataset C, only one pooled sample (with four individuals each) per site and species was obtained, thus all the statistics were performed to compare only the effect of the host species (Figs. 5A–5D). The complete plots with separated sites are available in supplementary material (Figs. S4A–S4D). The gut communities did not present significant differences in alpha diversity between host species for any of the indices (KW, OTUs: $\chi^2 = 2.33$, $df = 1$, $p = 0.13$; Chao1: $\chi^2 = 1.19$, $df = 1$, $p = 0.28$; PD: $\chi^2 = 1.19$, $df = 1$, $p = 0.28$; Shannon: $\chi^2 = 1.19$, $df = 1$, $p = 0.28$) although a trend for an increase in bacterial richness and diversity was observed in *D. melanostictus* (Figs. 5A–5D; SM Fig. S4A).

No significant differences in gut community composition were found between the two species using both weighted Unifrac (PERMANOVA: Pseudo- $F_{1,5} = 1.66$; $R^2 = 0.3$, $p = 0.30$) and unweighted Unifrac (PERMANOVA: Pseudo- $F_{1,5} = 1.75$; $R^2 = 0.3$, $p = 0.10$) distances (Figs. 5E–5F, Fig. S4B).

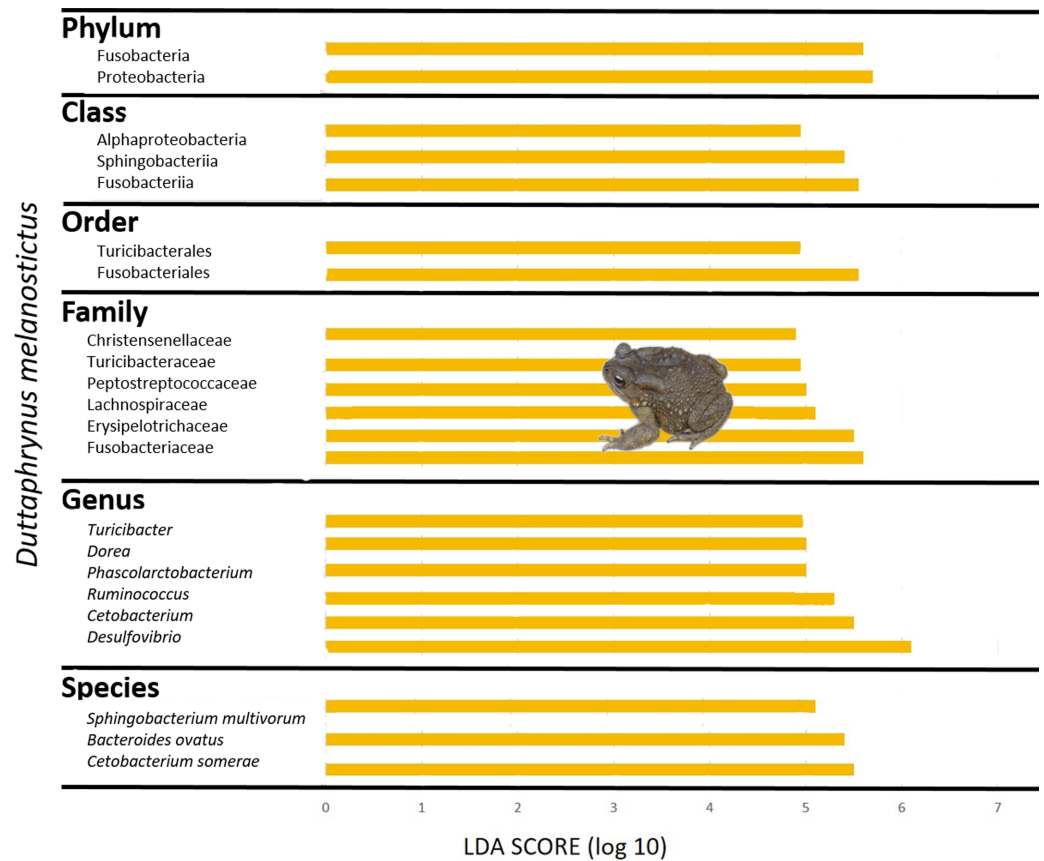


Figure 6 Differently abundant gut taxa occurring in *Duttaphrynus melanostictus* with LDA score. No differently abundant gut taxa were identified in *Ptychadena mascareniensis*. Photo credit: Angelica Crot-tini.

Full-size DOI: 10.7717/peerj.11532/fig-6

The gut community of both species was dominated by three phyla (Bacteroidetes, Proteobacteria and Firmicutes) (Figs. 5G–5H; Fig. S4C), with differences in the relative abundances at lower taxonomic levels. *D. melanostictus* also exhibited high relative abundance of Fusobacteria. At the family level, *D. melanostictus* showed a more diverse gut community in terms of relative abundance including 10 families almost equally abundant (Figs. 5G–5H), while *P. mascareniensis* had 7 families of high relative abundances, and among these, Clostridiaceae and Streptococcaceae were the most abundant. At the genus level, the gut community of *D. melanostictus* exhibited high abundance of *Bacteroidetes* and *Cetobacterium* while *P. mascareniensis* gut community was dominated by higher abundances of *Clostridium* and *Lactococcus*. Across sites, the gut communities of both amphibian species also exhibited significant differences in terms of relative abundances of several taxa (Fig. S4C). With LefSe analysis, a total of 22 taxa were identified as being significantly more abundant in the gut of *D. melanostictus*, including members of the three phyla (Bacteroidetes, Firmicutes, Proteobacteria) while no taxa were enriched in *P. mascareniensis* (Fig. 6).

D. melanostictus had 365 unique OTUs (52%), while *P. mascareniensis* had 256 (37%) and only 11% of the bacterial OTUs (corresponding to a total of 80 OTUs) was shared (Fig. S5).

From the gut communities of the two species a total of 39 KEGG pathways (Level 2) were predicted, with both amphibians exhibiting the same functional groups. Significant differences in the abundance levels of these pathways were detected (K-W, p -value < 0.05) (Table S5). *D. melanostictus* had four enriched functional groups: Biosynthesis of Secondary Metabolites, Energy Metabolism, Endocrine System and Information Processing –Folding, Sorting and Degradation, while *P. mascareniensis* had only one enriched functional group associated with membrane transport (Table S5).

DISCUSSION

Our study provides the first characterization of the skin and gut microbiomes of the Asian common toad *Duttaphrynus melanostictus* in its invasive range in Madagascar occurring in a highly human impacted area, and includes a comparison with the co-occurring native species *Ptychadena mascareniensis*. To our knowledge, only five recent studies characterized the microbiome of an invasive amphibian: [Christian et al. \(2018\)](#) found that *Rhinella marina* had the poorest and most dissimilar skin bacterial community in comparison with native amphibians in Australia; [Abarca et al. \(2018\)](#) found, for the same species, higher skin bacterial diversity in individuals from the invaded range compared to the native range; while, [Kueneman et al. \(2019\)](#) found that on a global scale the skin microbiome of *Lithobates catesbeianus* was more similar to that of the native amphibians than to itself in different parts of its invasive range. Two more recent studies focused on the gut microbiome of invasive species. The first one characterized the microbiome of *R. marina* while comparing the bacterial community across gut sections and found that sex influenced the gut microbiota and that cloacal swabs can be a good proxy to study intestinal microbes ([Zhou et al., 2020](#)). The second study found that the gut microbiome of the invasive guttural toad (*Sclerophrys gutturalis*) exhibited greater microbial diversity and functional flexibility when compared with bacterial communities from the native populations ([Wagener, Mohanty & Measey, 2020](#)). We investigated a very recent invasion and aimed at characterizing the microbiome of *D. melanostictus* across its expansion range and how it differs from the microbiome of a native species from across three sites with similar levels of human impact. Skin bacterial communities were strongly correlated with host species, with *D. melanostictus* showing higher richness and diversity. To a lesser extent, sex also influenced these communities. However, no significant differences in the skin bacterial composition were observed between sampling sites for both species, which might be related to the small geographic area and the overall similar habitat of the three sampling sites (all sampling sites were urban areas). It also indicates that the skin microbiome of *D. melanostictus* individuals has likely been stable across its early expansion.

Skin bacteria diversity differs between the invasive toad *D. melanostictus* and the native frog *P. mascareniensis* but not across sites

The skin bacterial community of the two amphibian species differed in terms of richness, diversity, community structure and functional inferences. *D. melanostictus* hosted a richer community than the co-occurring native species, harboured many unique OTUs (80%) and average richness values similar to those found for other terrestrial species of amphibians in Madagascar (Table S6; Bletz et al., 2017; Kueneman et al., 2019). On the other hand, the native species *P. mascareniensis* showed lower values of bacterial richness than the average values found in previous studies comparing terrestrial or aquatic species from Madagascar (Table S6; Bletz et al., 2017; Kueneman et al., 2019). However, previous studies mostly included terrestrial amphibians (including *P. mascareniensis*) from multiple habitats (Bletz et al., 2017), while our study included only urban sites around Toamasina. This habitat is characterized by high anthropogenic pressures, such as the presence of cattle, human waste, no natural vegetation cover and poor availability of clean water bodies, that can potentially have impoverished the environmental bacterial pool and consequently reduced the richness of the bacterial communities in *P. mascareniensis* (Becker et al., 2017; Jiménez et al., 2020) but this should be further investigated in future assessments.

Notably, *D. melanostictus* showed a dramatically higher percentage of unique OTUs (80%) compared to the native species, which may be related with the toad skin characteristics and therefore different skin microenvironment, and other host ecological factors. The percentage of shared bacterial taxa (15%) between our two species is low compared with that observed in previous works comparing different species (25–70%), or between aquatic and terrestrial ecomorphs or different life stages (Rebollar et al., 2016; Bletz et al., 2017; Kueneman et al., 2014). Although not significant, we observed some variation in alpha diversity levels in the skin microbiota of *D. melanostictus* in the three analysed sites. This could be linked to different environmental bacteria colonizing the skin in each site. This was accompanied by higher percentage of shared OTUs with *P. mascareniensis* which can also support the hypothesis that the toad is being colonized by new environmental bacteria. To explain differences in bacterial composition between the two target species, skin texture may play a major role. Tubercles in *D. melanostictus* skin may provide alternative microniches for the bacteria compared to the smoother skin of *P. mascareniensis*. Moreover, the skin of amphibians has been suggested to select and filter for specific bacteria from the surrounding environment due to the secretion of skin compounds that may block colonization by some taxa and favour others, and this selection could differ among host species (Flechas et al., 2019; Walke et al., 2014). Terrestrial amphibian species (such as *D. melanostictus*) are expected to have richer skin communities than aquatic or arboreal amphibians (Bletz et al., 2017; De Assis, Barreto & Navas, 2017; Kueneman et al., 2019; Walke et al., 2014) partly because the soil usually harbours a richer bacterial pool than aquatic systems and the habitat is known to greatly influence amphibians' skin communities. Although both species were found in the highly anthropized areas in Toamasina, *P. mascareniensis* were mostly found in the grass and often

close to small water-bodies, while individuals of *D. melanostictus* were conspicuous within villages, sometimes near domestic animals or anthropogenic waste.

So far, only one recent study attempted to assess the role of bacterial communities in the adaptation of amphibians to novel habitats by studying the gut communities in invasive and native populations (Wagener, Mohanty & Measey, 2020). In other systems, more diverse microbiomes have been linked to higher host fitness, such as, for instance, pathogen resistance in wheat (Matos, Kerkhof & Garland, 2005), or defence against chemical compounds in beetles (Cheng et al., 2018). In amphibians, richer microbiomes have been linked to a higher resistance to pathogens (Becker & Harris, 2010; Harrison et al., 2017) and a richer microbiome could conceivably aid in the colonization of novel habitats (Wagener, Mohanty & Measey, 2020). Bacterial taxa associated with disease resistance were among the most abundant groups in both hosts but with specific differences in taxa identity and abundances. *P. mascareniensis* seems to carry a more diverse bacterial community with antifungal properties while the toad carried more OTUs from the Comamonadaceae family that contains taxa used in probiotic assays (Becker et al., 2015). *D. melanostictus* also had very low abundance of *Pseudomonas*, a genus that is ubiquitous in the environment (soil, water), plants and other organisms and is linked to resistance to pathogens like *Bd* (Becker et al., 2015). *Pseudomonas* was highly prevalent in *P. mascareniensis* and is usually abundant in amphibians from tropical regions (Bletz et al., 2017). Notably, the low abundance of *Pseudomonas* found in *D. melanostictus* agrees with the pattern found in invasive populations of *R. marina*: lower in invasive populations compared with native ones (Abarca et al., 2018), and lower in comparison with co-occurring native amphibians from Australia (Christian et al., 2018) and further analysis of this similarity should be applied.

The functional redundancy here observed was congruent with previous studies (Bletz et al., 2016; Huang et al., 2018), demonstrating that different microbiome assemblages from different hosts can succeed in the same environment and are probably more associated with host identity. From the host's perspective this is crucial since it means that it maintains functional stable microbial community despite carrying different bacterial assemblages. The functional category of xenobiotics biodegradation and metabolism that was enriched in *D. melanostictus* could be related with a high capacity to cope with environmental alteration and anthropogenic stress which would be the case in Toamasina, and therefore higher adaptability or resilience to highly impacted or new habitats (Claus, Guillou & Ellero-Simatos, 2016). A future comparison with individuals occurring in less human impacted areas could provide more insights regarding the functional capacity of the microbiome in relation to the host habitat.

In a recent study with *R. marina* collected near its invasion front and where chytridiomycosis is absent, it was observed that individuals had lower *Bd*-inhibitory bacteria when compared with areas where *Bd* was present, highlighting the hypothesis that these bacteria are selected when the pathogen is present (Weitzman et al., 2019). In the species analysed here, the lower proportion of bacteria with putative *Bd*-inhibiting functions in *D. melanostictus* (in comparison with *P. mascareniensis*) may be related with its occurrence in Toamasina where *Bd* has not yet been detected (Bletz et al., 2015). However,

it is important to note that the antifungal database include taxa isolated from amphibian species from Africa, America and Australia with no representatives from Asia, which can partially be responsible for the high proportion of taxa found in the native *P. mascareniensis* (Woodhams et al., 2015). Moreover, this classification is based on 97% similarity to bacteria that can inhibit *Bd* which does not necessarily mean that these bacteria actually have this function.

Moreover, *D. melanostictus* has been showed to have a high *Bd* prevalence (43%) in its native areas in India (Thorpe et al., 2018), which may be linked to a low prevalence of *Bd*-inhibitory bacteria, although it has not been tested there. A screen of the microbiome diversity of *D. melanostictus* from its native areas, and where *Bd* has been detected, could give new insights about microbiome patterns.

Gut bacteria show no differentiation between the two species

In gut bacterial communities some patterns were similar to skin communities. For example, *D. melanostictus* individuals hosted a bacterial gut community characterized by more unique OTUs and higher richness values (although not statistically significant). Although both species are generalist feeders (Döring et al., 2017; Fatroandrianjafinonjasolomiovazo et al., 2011), *P. mascareniensis* feeds mainly on arthropods while the diet of *D. melanostictus* includes other invertebrates and occasionally also small vertebrates (e.g., worm snakes) (Hahn, 1976; O'Shea et al., 2013). A larger body size probably allows the consumption of larger and more diverse prey whereas the microhabitat type (soil, water, leaves) may also hold different invertebrate groups influencing the potential prey availability for the two species. A richer bacterial community has been related with richer diets probably aiding the host in the digestion and metabolization of different items (Tiede et al., 2017; Wagener, Mohanty & Measey, 2020). The dominant bacterial phyla identified in the guts of the two amphibian species were similar to other studies (Fig. 5C) (Chang et al., 2016; Huang et al., 2018), which might be explained by the stable gut environment across species (compared for instance to the external environment). The relative abundance of taxa, however, varied between the two species probably associated with gut physiology, host diet and habitat conditions although the low sample size and the fact that the samples were pooled prevents us from obtaining robust comparisons (Ley et al., 2008; Tiede et al., 2017; Zhang et al., 2010). Members of the phyla Firmicutes (mainly belonging to the class Clostridia) are linked to fermentation of carbohydrates and found to be common in terrestrial animals, thus its high occurrence in *P. mascareniensis* was expected. *D. melanostictus* has a longer gut, and the lower oxygen availability associated with this environment (in addition to the host's generalist diet), might explain the dominance of Bacteroidetes (Döring et al., 2017; Nelson et al., 2013). Bacteroidetes can also assist in metabolizing different energy sources (Flint et al., 2012). The higher proportion of members of the family Desulfovibrionaceae in *D. melanostictus* should be further studied since the group includes taxa that can be opportunist pathogens and produce endotoxins (Zhang et al., 2010). The overall absence of significant differences between the gut communities of these two species could also be the result of the low sample size used for this dataset, even if each pool included a mix of gut samples from 4 individuals. Besides the lack of significance in alpha and beta diversity,

it is worth noting that we observed differences in the relative abundance levels, number of unique OTUs and functional inference, but further investigation is needed in this respect.

CONCLUSIONS

The expansion of the Asian common toad *Duttaphrynus melanostictus* in Madagascar is ongoing and comparing the recently introduced populations across its invaded range and subsequent expansion into different habitat types could help understand how microbiome changes through the process of invasion in a contemporary scenario. Our study shows that the skin microbiome of *D. melanostictus* is richer and more diverse than the skin microbiome of the native species, and this diversity is probably associated with the toad's intrinsic physiological and ecological traits (e.g., the toad's skin microenvironment), or can be linked to the colonization of new areas, with the toad being less likely to be selecting for any specific taxa. Expanding this study to other native amphibian species and other habitats (as shown in [Licata et al., 2020](#), the toad is currently occupying urban, rural/agricultural, palm-oil plantation and *savoka* (degraded forest and mixed scrubland) habitats) is needed to further understand these differences. Additional data from its natural range in Asia and from other invasive populations could help to better characterize the degree of variation between native and invasive populations. For Madagascar, we encourage the development of new studies aimed at characterizing skin secretions and the antifungal properties of the skin microbiome of *D. melanostictus*. Similarly, we think that it will be beneficial to further investigate the connection between the toad's diet and its gut microbiome (composition and functional roles), especially in this invasion scenario where the host may have to adapt fast as it will expand to different areas in Madagascar (colonization of different habitats).

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

Portuguese National Funds through FCT (Foundation for Science and Technology) supported the Investigador FCT (IF/00209/2014) and the 2020.00823. CEECIND contracts for Angelica Crottini and the doctoral fellowships for Bárbara Santos (PB/BD/106055/2015) and Walter Cocca (SFRH/BD/102495/2014). This work was also supported by National Funds through FCT/MCTES under the UIDB/50027/2020 funding. There was no additional external funding received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:

The Portuguese National Funds through FCT (Foundation for Science and Technology). Investigador FCT (IF/00209/2014) 2020.00823. CEECIND: PB/BD/106055/2015, SFRH/BD/102495/2014.

National Funds through FCT/MCTES under the UIDB/50027/2020.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Bárbara Santos conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Molly Bletz and Joana Sabino-Pinto analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Walter Cocca, Jean Francois Solofoniaina Fidy, Serge Ndriantsoa, Jean Noel and Tsanta Rakotonanahary performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Karen L.M. Freeman conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Sven Kuenzel and Miguel Vences analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Angelica Crottini conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

Ministère de l'Environnement et du Développement Durable approved all the research permits:

Collection: No 226/16/MEEF/SG/DGF/DSAP/SCB.Re of September 19th, 2016

Transport: No 1679-16/MEEF/SG/DGF/DREEF.ATS/SREco and No 1680-16/MEEF/SG/DGF/DREEF.ATS/SREco of September 24th, 2016

Export: No 284N-EA10/MG16 of October 5th.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

Ministère de l'Environnement et du Développement Durable provided the research permit (N° 226/16/MEEF/SG/DGF/DSAP/SCB.Re of September 19th, 2016).

Data Availability

The following information was supplied regarding data availability:

Data are available at NCBI: BioProject ID [PRJNA667830](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA667830).

The raw sequences and metadata are available at Zenodo: Bárbara Santos. (2021). PeerJ49550. [http://doi.org/10.5281/zenodo.4518953](https://doi.org/10.5281/zenodo.4518953).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.11532#supplemental-information>.

REFERENCES

- Abarca JG, Zuniga I, Ortiz-Morales G, Lugo A, Viquez-Cervilla M, Rodriguez-Hernandez N, Vázquez-Sánchez F, Murillo-Cruz C, Torres-Rivera EA, Pinto-Tomás AA, Godoy-Vitorino F. 2018. Characterization of the skin microbiota of the cane toad *Rhinella cf. marina* in Puerto Rico and Costa Rica. *Frontiers in Microbiology* 8(2624):1–13 DOI 10.3389/fmicb.2017.02624.
- Abdallah F, Mijouin L, Pichon C. 2017. Skin immune landscape: inside and outside the organism. *Mediators of Inflammation* 5095293:1–17 DOI 10.1155/2017/5095293.
- Alpert P, Bone E, Holzapfel C. 2000. Invasiveness, invasibility and the role of environmental stress in the spread of non-native plants. *Perspectives in Plant Ecology, Evolution and Systematics* 3(1):52–66 DOI 10.1078/1433-8319-00004.
- Amir A, McDonald D, Navas-Molina JA, Kopylova E, Morton JT, Xu ZZ, Kightley EP, Thompson LR, Hyde ER, Gonzalez A, Knight R. 2017. Deblur rapidly resolves single-nucleotide community sequence patterns. *American Society for Microbiology* 2(2):1–7 DOI 10.1128/mSystems.00191-16.
- Andreone F, Rabibisoa N, Randrianantoandro C, Crottini A, Edmonds D, Kraus F, Lewis JP, Moore M, Rabemananjara FCE, Rabemanantsoa JC, Vences M. 2014. Risk review is under way for invasive toad. *Nature* 512:253 DOI 10.1038/512253c.
- Beard KH, Pitt WC. 2005. Potential consequences of the coqui frog invasion in Hawaii. *Diversity and Distributions* 11(5):427–433 DOI 10.1111/j.1366-9516.2005.00178.x.
- Becker MH, Harris RN. 2010. Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLOS ONE* 5(6):1–6 DOI 10.1371/journal.pone.0010957.
- Becker CG, Longo AV, Haddad CFB, Zamudio KR. 2017. Land cover and forest connectivity alter the interactions among host, pathogen and skin microbiome. *Proceedings of the Royal Society B: Biological Sciences* 284:20170582 DOI 10.1098/rspb.2017.0582.
- Becker MH, Walke JB, Murrill L, Woodhams DC, Reinert LK, Rollins-Smith LA, Burzynski EA, Umile TP, Minbiole KPC, Belden LK. 2015. Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis*. *Molecular Ecology* 24(7):1628–1641 DOI 10.1111/mec.13135.
- Bletz MC, Archer H, Harris RN, McKenzie VJ, Rabemananjara FC, Rakotoarison A, Vences M. 2017. Host ecology rather than host phylogeny drives amphibian skin microbial community structure in the biodiversity hotspot of Madagascar. *Frontiers in Microbiology* 8(1530):1–14 DOI 10.3389/fmicb.2017.01530.
- Bletz MC, Goedbloed DJ, Sanchez E, Reinhardt T, Tebbe CC, Bhujju S, Geffers R, Jarek M, Vences M, Steinfartz S. 2016. Amphibian gut microbiota shifts differentially in community structure but converges in habitat-specific predicted functions. *Nature Communications* 7(13699):1–12 DOI 10.1038/ncomms13699.
- Bletz MC, Perl RGB, Vences M. 2017. Skin microbiota differs drastically between co-occurring frogs and newts. *Royal Society Open Science* 4(4):170107 DOI 10.1098/rsos.170107.

- Bletz MC, Rosa GM, Andreone F, Courtois EA, Schmeller DS, Rabibisoa NH, Rabemananjara FCE, Raharivololoniaina L, Vences M, Weldon C, Edmonds D, Raxworthy CJ, Harris RN, Fisher MC, Crottini A. 2015. Widespread presence of the pathogenic fungus *Batrachochytrium dendrobatidis* in wild amphibian communities in Madagascar. *Scientific Reports* 5(8633):1–10 DOI 10.1038/srep08633.
- Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA, Caporaso JG. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature Methods* 10(1):57–59 DOI 10.1038/nmeth.2276.
- Brown JL, Sillero N, Glaw F, Bora P, Vieites DR, Vences M. 2016. Spatial biodiversity patterns of Madagascar's amphibians and reptiles. *PLOS ONE* 11(1):1–26 DOI 10.1371/journal.pone.0144076.
- Caporaso JG, Bittinger K, Bushman FD, SeSantis TZ, Andersen GL, Knight R. 2010b. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26(2):266–267 DOI 10.1093/bioinformatics/btp636.
- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010a. QIIME allows analysis of high-throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. *Nature Publishing Group* 7(5):335–336 DOI 10.1038/nmeth0510-335.
- Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America* 108:4516–4522 DOI 10.1073/pnas.1000080107.
- Chang CW, Huang BH, Lin SM, Huang CL, Liao PC. 2016. Changes of diet and dominant intestinal microbes in farmland frogs. *BMC Microbiology* 16(1):1–13 DOI 10.1186/s12866-016-0660-4.
- Cheng C, Wickham JD, Chen L, Xu D, Lu M, Sun J. 2018. Bacterial microbiota protects an invasive bark beetle from a pine defensive compound. *Microbiome* 6(1):132 DOI 10.1186/s40168-018-0518-0.
- Chornesky EA, Randall JM. 2003. The threat of invasive alien species to biological diversity: setting a future course. *Annals of the Missouri Botanical Garden* 90(1):67–76 DOI 10.2307/3298527.
- Christian K, Weitzman C, Rose A, Kaestli M, Gibb K. 2018. Ecological patterns in the skin microbiota of frogs from tropical Australia. *Ecology and Evolution* 8(21):10510–10519 DOI 10.1002/ece3.4518.
- Claus SP, Guillou H, Ellero-Simatós S. 2016. The gut microbiota: a major player in the toxicity of environmental pollutants? *Npj Biofilms and Microbiomes* 2(16003):1–12 DOI 10.1038/npjbiofilms.2016.3.
- Coats VC, Rumpho ME. 2014. The rhizosphere microbiota of plant invaders: an overview of recent advances in the microbiomics of invasive plants. *Frontiers in Microbiology* 5(368):1–10 DOI 10.3389/fmicb.2014.00368.

- Crooks JA, Chang AL, Ruiz GM. 2011.** Aquatic pollution increases the relative success of invasive species. *Biological Invasions* **13**(1):165–176 DOI [10.1007/s10530-010-9799-3](https://doi.org/10.1007/s10530-010-9799-3).
- Crottini A, Andreone F, Edmonds D, Hansen CM, Lewis JP, Rabemanantsoa JC, Moore M, Kraus F, Vences M, Rabemananjara F, Randrianantoandro C. 2014.** A new challenge for amphibian conservation in Madagascar: the invasion of *Duttaphrynus melanostictus* in Toamasina Province. *FrogLog* **22**(3):46–47.
- De Assis AB, Barreto CC, Navas CA. 2017.** Skin microbiota in frogs from the Brazilian Atlantic Forest: Species, forest type, and potential against pathogens. *PLOS ONE* **12**(7):e0179628 DOI [10.1371/journal.pone.0179628](https://doi.org/10.1371/journal.pone.0179628).
- Dethlefsen L, Mcfall-Ngai M, Relman DA. 2007.** An ecological and evolutionary perspective on human –microbe mutualism and disease. *Nature* **449**(7164):811–818 DOI [10.1038/nature06245](https://doi.org/10.1038/nature06245).
- Döring B, Mecke S, Kieckbusch M, O’Shea M, Kaiser H. 2017.** Food spectrum analysis of the Asian toad, *Duttaphrynus melanostictus* (Schneider, 1799) (Anura: Bufonidae), from Timor Island, Wallacea. *Journal of Natural History* **51**(11–12):1–17 DOI [10.1080/00222933.2017.1293182](https://doi.org/10.1080/00222933.2017.1293182).
- Enserink M. 1999.** Predicting invasions: biological invaders sweep in. *Science* **285**(5435):1834–1836 DOI [10.1126/science.285.5435.1834](https://doi.org/10.1126/science.285.5435.1834).
- Fatroandrianjafinonjasolomiovazo TNL, Rasiamampionona NR, Vieites DR, Vences M. 2011.** Diet of the Mascarene grass frog, *Ptychadena mascareniensis*, in Madagascar. *Malagasy Nature* **5**:68–74.
- Flechas SV, Acosta-González A, Escobar LA, Kueneman JG, Sánchez-Quitian ZA, Parra-Giraldo CM, Rollins-Smith LA, Reinert LK, Vredenburg VT, Amézquita A, Woodhams DC. 2019.** Microbiota and skin defense peptides may facilitate coexistence of two sympatric Andean frog species with a lethal pathogen. *The ISME Journal* **13**:361–373 DOI [10.1038/s41396-018-0284-9](https://doi.org/10.1038/s41396-018-0284-9).
- Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. 2012.** Microbial degradation of complex carbohydrates in the gut. *Gut Microbes* **3**(4):289–306 DOI [10.4161/gmic.19897](https://doi.org/10.4161/gmic.19897).
- Ganzhorn JU, Lowry PP, Schatz GE, Sommer S. 2001.** The biodiversity of Madagascar: one of the world’s hottest hotspots on its way out. *Oryx* **35**(4):346–348 DOI [10.1046/j.1365-3008.2001.00201.x](https://doi.org/10.1046/j.1365-3008.2001.00201.x).
- Garg AD, Kanitkar D, Hippargi R, Gandhare AN. 2007.** Antimicrobial activity of skin secretions isolated from Indian toad, *Bufo melanostictus* Schneider 1799. *Nature Precedings* **2007**:1–8 DOI [10.1038/npre.2007.1204.1](https://doi.org/10.1038/npre.2007.1204.1).
- Goodman SM, Raselimanana AP, Andrianiaina HA, Gauthier N, Ravaoianahary F, Sylvestre M, Raherilalao M. 2017.** The distribution and ecology of invasive alien vertebrate species in the greater Toamasina region, central eastern Madagascar. *Malagasy Nature* **12**:95–109.
- Gribben PE, Nielsen S, Seymour JR, Bradley DJ, West MN, Thomas T. 2017.** Microbial communities in marine sediments modify success of an invasive macrophyte. *Scientific Reports* **7**(9845):1–8 DOI [10.1038/s41598-017-10231-2](https://doi.org/10.1038/s41598-017-10231-2).

- Grice E. 2014. The skin microbiome: potential for novel diagnostic and therapeutic approaches to cutaneous disease. *Seminars in Cutaneous Medicine and Surgery* 33(2):98–103 DOI 10.12788/j.sder.0087.
- Grice EA, Segre JA. 2012. The human microbiome: our second genome. *Annual Review of Genomics and Human Genetics* 13(1):151–170 DOI 10.1146/annurev-genom-090711-163814.
- Hahn DE. 1976. Worm snakes in the diet of a toad, *Bufo melanostictus*. *Herpetological Review* 7(4):167.
- Harper GJ, Steininger MK, Tucker CJ, Juhn D, Hawkins F. 2007. Fifty years of deforestation and forest fragmentation in Madagascar. *Environmental Conservation* 34(4):325–333 DOI 10.1017/S0376892907004262.
- Harrison XA, Price SJ, Hopkins K, Leung WTM, Sergeant C, Garner T. 2017. Host microbiome richness predicts resistance to disturbance by pathogenic infection in a vertebrate host. *BioRxiv*. DOI 10.1101/158428.
- Heijitx RD, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, Hibberd ML, Forssberg H, Pettersson S. 2011. Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences of the United States of America* 108(7):1–6 DOI 10.1073/pnas.1010529108.
- Huang B-H, Chang C, Huang C, Gao J, Liao P-C. 2018. Composition and functional specialists of the gut microbiota of frogs reflect habitat differences and agricultural activity. *Frontiers in Microbiology* 8:1–14 DOI 10.3389/fmicb.2017.02670.
- Jani AJ, Briggs CJ. 2018. Host and aquatic environment shape the amphibian skin microbiome but effects on downstream resistance to the pathogen *Batrachochytrium dendrobatidis* are variable. *Frontiers in Microbiology* 9(487):1–17 DOI 10.3389/fmicb.2018.00487.
- Jiménez RR, Alvarado G, Sandoval J, Sommer S. 2020. Habitat disturbances influences the skin microbiome of a rediscovered neotropical-montane frog. *BMC Microbiology* 20:292 DOI 10.1186/s12866-020-01979-1.
- Kolby JE. 2014. Stop Madagascar's toad invasion now. *Nature* 509:563 DOI 10.1038/509563a.
- Konrad M, Grasse AV, Tragust S, Cremer S. 2014. Anti-pathogen protection versus survival costs mediated by an ectosymbiont in an ant host. *Proceedings of the Royal Society B: Biological Sciences* 282:20141976 DOI 10.1098/rspb.2014.1976.
- Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Applied and Environmental Microbiology* 79(17):5112–5120 DOI 10.1128/AEM.01043-13.
- Kueneman JG, Bletz MC, McKenzie VJ, Becker CG, Joseph MB, Abarca JG, Archer H, Arellano AL, Bataille A, Becker M, Belden LK, Crottini A, Geffers R, Haddad CFB, Harris RN, Holden WM, Hughey M, Jarek M, Kearns PJ, Kerby JL, Kielgast J, Kurabayashi A, Longo AV, Loudon A, Medina D, Nuñez JJ, Perl RGB, Pinto-Tomás A, Rabemananjara FCE, Rebolgar EA, Rodríguez A, Rollins-Smith L, Stevenson R, Tebbe CC, Asensio GV, Waldman B, Walke JB, Whitfield SM, Zamudio KR, Chaves IB, Woodhams DC, Vences M. 2019. Community richness of

- amphibian skin bacteria correlates with bioclimate at the global scale. *Nature Ecology & Evolution* 3:381–389 DOI 10.1038/s41559-019-0798-1.
- Kueneman JG, Parfrey LW, Woodhams DC, Archer HM, Knight R, McKenzie V. 2014.** The amphibian skin-associated microbiome across species, space and life history stages. *Molecular Ecology* 23(6):1238–1250 DOI 10.1111/mec.12510.
- Kull C, Tassin J, Carriere S. 2015.** Approaching invasive species in Madagascar. *Madagascar Conservation & Development* 9(2):60–70 DOI 10.4314/mcd.v9i2.2.
- Kwon S, Park S, Lee B, Yoon S. 2013.** In-depth analysis of interrelation between quality scores and real errors in illumina reads. In: *2013 35th annual international conference of the IEEE engineering in medicine and biology society (EMBC)*. 635–638 DOI 10.1109/EMBC.2013.6609580.
- Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepille DE, Thurber RLV, Knight R, Beiko RG, Huttenhower C. 2013.** Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology* 31(9):814–821 DOI 10.1038/nbt.2676.
- Lester PJ, Sébastien A, Suarez AV, Barbieri RF, Gruber MAM. 2017.** Symbiotic bacterial communities in ants are modified by invasion pathway bottlenecks and alter host behavior. *Ecology* 98(3):861–874 DOI 10.1002/ecy.1714.
- Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI. 2008.** Evolution of mammals and their gut microbes. *Science* 320(5883):1647–1651 DOI 10.1126/science.1155725.
- Licata F, Andreone F, Freeman K, Rabesihanaka S, Robsomanitrاندrasana E, Reardon J, Crottini A. 2020.** The Asian Toad (*Duttaphrynus melanostictus*) in Madagascar: a report of an ongoing invasion. In: Angelici FM, Rossi L, eds. *Problematic wildlife II, new conservation and management challenges in the human-wildlife interactions*. Berlin: Springer, 617–638.
- Licata F, Ficetola GF, Freeman K, Mahasoah RH, Ravololonarivo V, Solofo Niaina Fidy JF, Koto-Jean AB, Nahavitatsara ER, Andreaone F, Crottini A. 2019.** Abundance, distribution and spread of the invasive Asian toad *Duttaphrynus melanostictus* in eastern Madagascar. *Biological Invasions* 21(5):1615–1626 DOI 10.1007/s10530-019-01920-2.
- Lowe S, Browne M, Boudjelas S, De Poorter M. 2000.** 100 of the World's Worst Invasive Alien Species. A selection from the Global Invasive Species Database. Published by The Invasive Species Specialist Group (ISSG) a specialist group of the Species Survival Commission (SSC) of the World Conservation Union.
- Lozupone C, Knight R. 2005.** UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology* 71(12):8228–8235 DOI 10.1128/AEM.71.12.8228.
- Marshall BM, Casewell NR, Vences M, Glaw F, Andreone F, Rakotoarison A, Zancolli G, Woog F, Wüster W. 2018.** Vulnerability of Malagasy predators to the toxins of an introduced toad. *Current Biology* 28(11):R654–R655 DOI 10.1016/j.cub.2018.04.024.

- Matos A, Kerkhof L, Garland J. 2005.** Effects of microbial community diversity on the survival of *Pseudomonas aeruginosa* in the wheat rhizosphere. *Microbial Ecology* 49(2):257–264 DOI 10.1007/s00248-004-0179-3.
- McKenzie VJ, Bowers RM, Fierer N, Knight R, Lauber CL. 2012.** Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. *The ISME Journal* 6:588–596 DOI 10.1038/ismej.2011.129.
- McMurdie PJ, Holmes S. 2013.** phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLOS ONE* 8(4):e61217 DOI 10.1371/journal.pone.0061217.
- Miaud C, Pozet F, Gaundin NCG, Mantel A, Pasmans F, Labrut S. 2016.** *Ranavirus* causes mass die-offs of the Alpine amphibians in the southwestern Alps (France). *Journal of Wildlife Diseases* 52(2):242–252 DOI 10.7589/2015-05-113.
- Moore M, Solofoniaina Fidy JF, Edmonds D. 2015.** The new toad in town: distribution of the Asian toad, *Duttaphrynus melanostictus*, in the Toamasina area of eastern Madagascar. *Tropical Conservation Science* 8(2):440–455 DOI 10.1177/194008291500800210.
- Nelson TM, Rogers TL, Carlini AR, Brown MV. 2013.** Diet and phylogeny shape the gut microbiota of Antarctic seals: a comparison of wild and captive animals. *Environmental Microbiology* 15(4):1132–1145 DOI 10.1111/1462-2920.12022.
- Oksanen J, Blanchet F, Friendly M, Kindt R, Legendre P, McGlenn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H. 2016.** vegan: community ecology package. R package version 2.4-5.. Available at <https://cran.r-project.org/web/packages/vegan/>.
- O'Shea M, Kathriner A, Mecke S, Sanchez C, Kaiser H. 2013.** Fantastic Voyage: a live blindsnake (*Ramphotyphlops braminus*) journeys through the gastrointestinal system of a toad (*Duttaphrynus melanostictus*). *Herpetological Notes* 6:467–470.
- Penk MR, Jeschke JM, Minchin D, Donohue I. 2016.** Warming can enhance invasion success through asymmetries in energetic performance. *Journal of Animal Ecology* 85(2):419–426 DOI 10.1111/1365-2656.12480.
- Perl RGB, Vences M, Wollenberg KC, Sonet G, Glaw F, Nagy ZT. 2014.** DNA barcoding Madagascar's amphibian fauna. *Amphibia-Reptilia* 35(2):197–206 DOI 10.1163/15685381-00002942.
- Phillips BL, Brown GP, Webb JK, Shine R. 2006.** Invasion and the evolution of speed in toads. *Nature* 439(7078):803.
- Pitt W, Vice D, Pitzler M. 2005.** Challenges of invasive reptiles and amphibians. In: *Proceedings of the 11th wildlife damage management conference*. 112–119.
- Price MN, Dehal PS, Arkin AP. 2010.** FastTree 2 –approximately maximum-likelihood trees for large alignments. *PLOS ONE* 5(3):e9490 DOI 10.1371/journal.pone.0009490.
- R Core Team. 2016.** R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Available at <https://www.R-project.org/>.
- Rebollar EA, Hughey MC, Medina D, Harris RN, Ibáñez R, Belden LK. 2016.** Skin bacterial diversity of Panamanian frogs is associated with host susceptibility

- and presence of *Batrachochytrium dendrobatidis*. *ISME Journal* **10**:1682–1695 DOI [10.1038/ismej.2015.234](https://doi.org/10.1038/ismej.2015.234).
- Reilly SB, Wogan GO, Stubbs AL, Arida E, Iskandar DT, McGuire JA. 2017. Toxic toad invasion of Wallacea: a biodiversity hotspot characterized by extraordinary endemism. *Global Change Biology* **23**(12):5029–5031 DOI [10.1111/ijlh.12426](https://doi.org/10.1111/ijlh.12426).
- Rosa GM, Andreone F, Crottini A, Hauswaldt JS, Noël J, Rabibisoa NH, Randriambahiniarime MO, Rebelo R, Raxworthy CJ. 2012. The amphibians of the relict Betampona low-elevation rainforest, eastern Madagascar: an application of the integrative taxonomy approach to biodiversity assessments. *Biodiversity and Conservation* **21**(6):1531–1559 DOI [10.1007/s10531-012-0262](https://doi.org/10.1007/s10531-012-0262).
- Rout ME, Chrzanowski TH, Westlie TK, DeLuca TH, Callaway RM, Holben WE. 2013. Bacterial endophytes enhance competition by invasive plants. *American Journal of Botany* **100**(9):1726–1737 DOI [10.3732/ajb.1200577](https://doi.org/10.3732/ajb.1200577).
- Sanford JA, Gallo RL. 2013. Functions of the skin microbiota in health and disease. *Seminars in Immunology* **25**(5):370–377 DOI [10.1016/j.smim.2013.09.005](https://doi.org/10.1016/j.smim.2013.09.005).
- Schlaepfer MA, Sax DF, Olden JD. 2011. The potential conservation value of non-native species. *Conservation Biology* **25**(3):428–437 DOI [10.1111/j.1523-1739.2010.01646.x](https://doi.org/10.1111/j.1523-1739.2010.01646.x).
- Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. 2011. Metagenomic biomarker discovery and explanation. *Genome Biology* **12**(6):R60 DOI [10.1186/gb-2011-12-6-r60](https://doi.org/10.1186/gb-2011-12-6-r60).
- Shine R. 2010. The ecological impact of invasive cane toads (*Bufo marinus*) in Australia. *The Quarterly Review of Biology* **85**(3):253–291 DOI [10.1086/655116](https://doi.org/10.1086/655116).
- Snow NP, Witmer GW. 2010. American bullfrogs as invasive species: a review of the introduction, subsequent problems, management options, and future directions. *USDA National Wildlife Research Center - Staff Publications* **1288**:86–89.
- Stachowicz JJ, Terwin JR, Whitlatch RB, Osman RW. 2002. Linking climate change and biological invasions: Ocean warming facilitates nonindigenous species invasions. *Proceedings of the National Academy of Sciences of the United States of America* **99**(24):15497–15500 DOI [10.1073/pnas.242437499](https://doi.org/10.1073/pnas.242437499).
- Thorpe CJ, Lewis TR, Fisher MC, Wierzbicki CJ, Kulkarni S, Pryce D, Davies L, Watve A, Knight ME. 2018. Climate structuring of *Batrachochytrium dendrobatidis* infection in the threatened amphibians of the northern western Ghats, India. *Royal Society Open Science* **5**(6):180211 DOI [10.1098/rsos.180211](https://doi.org/10.1098/rsos.180211).
- Tiede J, Scherber C, Mutschler J, McMahon KD, Gratton C. 2017. Gut microbiomes of mobile predators vary with landscape context and species identity. *Ecology and Evolution* **7**(20):8545–8557 DOI [10.1002/ece3.3390](https://doi.org/10.1002/ece3.3390).
- Tuddenham S, Sears CL. 2015. The intestinal microbiome and health. *Current Opinion in Infection Diseases* **28**(5):464–470 DOI [10.1097/QCO.000000000000196](https://doi.org/10.1097/QCO.000000000000196).
- Turnbaugh P, Ley R, Mahowald M, Magrini V, Mardis E, Gordon J. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**(7122):1027–1031 DOI [10.1038/nature05414](https://doi.org/10.1038/nature05414).
- Vences M, Brown JL, Lathrop A, Rosa GM, Cameron A, Crottini A, Dolch R, Edmonds D, Freeman KLM, Glaw F, Grismer LL, Litvinchuk S, Milne MG, Moore M, Solofo

- JF, Noël J, Nguyen TQ, Ohler A, Randrianantoandro C, Raselimanana AP, van Leeuwen P, Wogan GOU, Ziegler T, Andreone F, Murphy RW. 2017. Tracing a toad invasion: lack of mitochondrial DNA variation, haplotype origins, and potential distribution of introduced *Duttaphrynus melanostictus* in Madagascar. *Amphibia-Reptilia* 38(2):197–207 DOI 10.1163/15685381-00003104.
- Vieites DR, Wollenberg KC, Andreone F, Kohler J, Glaw F, Vences M. 2009. Vast underestimation of Madagascar's biodiversity evidenced by an integrative amphibian inventory. *Proceedings of the National Academy of Sciences of the United States of America* 106(20):8267–8272 DOI 10.1073/pnas.0810821106.
- Wagener C, Mohanty NP, Measey J. 2020. The gut microbiome facilitates ecological adaptation in an invasive vertebrate. *BioRxiv* DOI 10.1101/2020.12.10.418954.
- Walke JB, Becker MH, Loftus SC, House LL, Cormier G, Jensen RV, Belden LK. 2014. Amphibian skin may select for rare environmental microbes. *The ISME Journal* 8(11):2207–2217 DOI 10.1038/ismej.2014.77.
- Walther G-R, Roques A, Hulme PE, Sykes MT, Pyšek P, Kühn I, Zobel M, Bacher S, Botta-Dukát Z, Bugmann H, Czúcz B, Dauber J, Hickler T, Jarosík V, Kenis M, Klotz S, Minchin D, Moora M, Nentwig W, Ott J, Panov VE, Reineking B, Robinet C, Semchenko V, Solarz W, Thuiller W, Vilà M, Vohland K, Settele J. 2009. Alien species in a warmer world: risks and opportunities. *Trends in Ecology & Evolution* 24(12):686–693 DOI 10.1016/j.tree.2009.06.008.
- Weitzman CL, Kaestli M, Gibb K, Brown GP, Shine R, Christian K. 2019. Disease exposure and antifungal bacteria on skin of invasive Cane toads, Australia. *Emerging Infectious Diseases* 25(9):1770–1771 DOI 10.3201/eid2509.190386.
- Woodhams DC, Alford RA, Antwis RE, Archer H, Becker MH, Belden LK, Bell CA, Bletz M, Daskin JH, Davis LR, Flechas SV, Lauer A, Gonzalez A, Harris RN, Holden WM, Hughey MC, Ibáñez R, Knight R, Kueneman J, Rabemananjara F, Reinert LK, Rollins-Smith LA, Roman-Rodríguez F, Shaw SD, Walke JB, Mckenzie VJ. 2015. Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens. *Ecology* 96(2):595–595 DOI 10.1890/14-1837.1.
- Xavier R, Mazzei R, Pérez-Losada M, Rosado D, Santos JL, Veríssimo A, Soares MC. 2019. A risky business? Habitat and social behavior impact skin and gut microbiomes in Caribbean cleaning gobies. *Frontiers in Microbiology* 10:1–9 DOI 10.3389/fmicb.2019.00716.
- Zhang C, Zhang M, Wang S, Han R, Cao Y, Hua W, Mao Y, Zhang X, Pang X, Wei C, Zhao G, Chen Y, Zhao L. 2010. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. *The ISME Journal* 4(2):232–241 DOI 10.1038/ismej.2009.112.
- Zhou J, Nelson TM, Lopez CR, Sarma RR, Zhou SJ, Rollins LA. 2020. A comparison of nonlethal sampling methods for amphibian gut microbiome analyses. *Molecular Ecological Resources* 20:844–855 DOI 10.1111/1755-0998.13139.