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Morpho-Biochemical characterization of cutaneous bacterial isolates of three endemic frogs from Mindanao Island, Philippines

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Abstract

We characterized through morphological features and some biochemical tests bacterial isolates from the skin of three endemic frog species: *Kalophyrnus sinensis* (Peters, 1867), *Limnonectes magnus* (Stejneger, 1910), and *Megophrys stejnegeri* (Taylor, 1920) from Mt. Andapon Barangay Campawan, in Baganga, Davao Oriental, Philippines. The bacterial isolates were acquired through skin swabs from five representative adult individuals per species, grown in select solid media, and subjected to various standard biochemical tests. Nine bacterial isolates were obtained: *Citrobacter* sp., *Salmonella* sp., *Pseudomonas* sp., *Enterobacter* sp., *Micrococcus* sp., *Proteus* sp., *Staphylococcus* sp., *Staphylococcus aureus*, and *Diplococcus* sp. Eight of these isolates were found in *Megophrys stejnegeri* (Taylor, 1920). Many of the bacterial isolates obtained were associated with soil. *Citrobacter* sp. was the common bacterial isolate found in all the frog species on both dorsal and ventral sides. The presence of bacterial isolates on these frogs maybe suggestive of a mutualistic relationship. Further studies maybe done to decipher role of these bacterial isolates and to validate if these are the only microorganism thriving on the skin of these Philippine endemic frogs.

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Introduction

The Philippine tropical forest is home to several endemic frog species with increasing frequency of new species discovered almost every year (Brown, De Layola, Lorenzo, and Diesmos, 2015; Mcleod *et al*, 2011; Supsup, Guinto, Redoblado, and Gomez, 2017). Majority of the studies conducted are inventories focused on identifying what species are present in different habitat types and deciphering elevational distribution (Sanguilla *et al*, 2016; Diesmos, Brown, and Gee, 2003; Siler *et al*, 2012). Data on ecology on these endemic frog species remains depauparate and limited reports are published pertinent to microbial species assciated with frog species (Diesmos, Brown, and Gee, 2003; Ates and Delima, 2008; Warquez, Mondejar, and Demayo, 2013; Dacalus *et al*, 2017).

Ecological interactions such as microbial associations in frogs were previously reported (Conlon, 2011;, McKenzie *et al*, 2012). Microbial symbionts especially those found in the cutaneous region has been identified to have substantial significant influence in the capability of frogs to combat pathogens. Skin microbiome play as positive inducer for release of antimicrobial peptides in frog's skin of some anuran species (Mangoni *et al*, 2001; Becker *et al*, 2014). Moreover species-specific resistance to pathogens such as fungi maybe influenced by host-specific bacteria (Mangoni *et al*, 2001). Bacterial taxa in frog's skin may also act as barrier against pathogens, thereby forming a potential safetynet for the frog host (Sanguilla *et al*, 2016).

Despite increasing number of literature reports on the seemingly important role of bacterial symbionts of the frog's skin, we found only one report on bacterial taxa associated with Philippine frog species (Eda, Diesmos, Vredenburg, and Chan, 2015). Thus this study provides baseline information on bacterial isolates associated with the skin of three Philippine endemic frogs sampled from Mindanao and additional information on cutaneous bacterial isolates from frogs of the Philippines

Materials and methods

Isolation of cutaneous bacteria

Five individuals each of *Kalophrynus sinensis*, *Limnonectes magnus,* and *Megophrys stejnegeri* were collected from a forest interior of Mt. Andapon, Sitio Casunugan, Barangay Campawan, Baganga, Davao Oriental, Mindanao, Philippines. Andapon Mountains form part of the Mt. Mayo-Tagub Kampalili Complex, one of the identified major Key Biodiversity Area of the country. Adult frog individuals were handpicked from the site and transported through native bamboo basket to the site were swabbing was done. The handlers wore nitrile gloves to prevent transfer of microbes from the skin of the handlers to the frogs' skin. Prior to swabbing, frog individuals were washed with sterile water twice for about 20 seconds on both dorsal and ventral sides to remove transient bacterial species (Woodhams *et al*, 2007). Fresh nitrile gloves were used to handle the frogs prior to swabbing. Each frog individual was swabbed twice: dorsal and ventral sides, using separate fresh, sterile swabs, 20 times each side in order to get ample inoculum from the tip of the snout to the webbings of the hindfoot's digits. Swabs for each side and individuals per species were directly inoculated in separate tubes in freshly prepared nutrient broth in the field (Rahman, Somsiri, Tajima, and Ezura, 2004). Frog individuals were released immediately after swabbing. Tubes were incubated at 25^0C for 24 hours. Subsequently, one ml inoculum from each turbid tube were diluted serially, three-fold and poured on freshly prepared Trypticase soy agar, nutrient agar, Mannitol-salt agar, and MacConkey agar plates. Pure cultures of each of the colonies were obtained and utilized for morphological and biochemical characterizations.

Morpho-Biochemical characterization of bacterial isolates

Bacterial isolates obtained from plates with solid medium were initially characterized based on coloration of the colony/colony forming unit, shape of margins of the colony, pattern of growth (after 24 hours), capacity to ferment lactose, and Gram staining reactions. Moreover biochemical tests were performed for each isolate that have grown in McConkey and Mannitol-Salt agar plates. The biochemical tests performed included: triple sugar iron agar (TSI), Lysine iron agar (LSI), urease, citrase, coagulase, sulfide and indole formation and motility following standard procedures.

Results and discussion

Bacterial isolates obtained

Nine bacterial isolates were obtained and morpho-biochemically characterized from the swabs of *Kalophrynus sinensis* (Peters, 1867), *Limnonectes magnus* (Stejneger, 1910), and *Megophrys stejnegeri* (Taylor, 1920) (Table 1). Eighty eight percent (88%) of the bacterial isolates were obtained from *M. stejnegeri* while the least number of bacterial isolates was obtained from *K. sinensis*. Majority of these bacterial isolates are reported to be thriving in soil (Tortora, Funke, and Case, 2013). The presence then of these bacterial isolates in the skin of the frogs examined is expected since these frogs are ground dwelling species (International Union for the Conservation of Nature, 2004; Inger, 1954).

The variation in the number of isolates obtained per frog species maybe attributed to the surface area available for the bacterial isolates to colonize. Among the three species, *K. sinensis* has the least wide dorsum and ventral surface (International Union for the Conservation of Nature, 2004; Inger, 1954) area which could explain why it has the least number of bacterial isolates. Although *L. magnus*, attributed to its stocky body (International Union for the Conservation of Nature, 2004; Siler McVay, Diesmos, and Brown, 2009), provides a wider area for bacterial species colonization, the number of isolates obtained was way less compared to that of *M. stejnegeri*. One reason for such maybe the presence of pronounced ridges on the dorsum of *L. magnus* compared to the lesser ridges and tubercles of *M. stejengeri* dorsum (International Union for the Conservation of Nature, 2004; Siler McVay, Diesmos, and Brown, 2009). The presence of the ridges may have influenced the inoculum obtained through swabbing as ridges may limit areas that can be reached by the tip of the sterile swab and such could possibly hamper access to other bacterial isolates residing on areas proximal to the ridges.

Table 1. Morpho-biochemical characters of the bacterial isolates from three endemic frogs sampled from Mindanao, Philippines.

Frog Species	Putative Id of bacterial isolate	Morphological characteristics		Biochemical Tests Results								
		Color	Shape	GSR	LF	TSI	LIA	Urease	Citrase	Coagulase	Sulfide	Indole
	Citrobacter sp. (D, V)	White	*Bacilli Mucoid Motile*	-	NLF, LF	A, A +, +	-, +	+	+		+	+, -
	Salmonella sp. (D)	White	*Bacilli*	-	NLF	K, A +, -	+, -	+	+		+, -	+
	Pseudomonas sp. (V)	colorless	*mucoid*	-	NLF	K, K +,-	-,+	+	+		-	-
L. magnus	*Citrobacter* sp. (D, V)	White	*Bacilli*	-	LF	A, A +,+	-,+	+	+		+	+. -
	Salmonella sp.	White	*Bacilli*	-	NLF	K, A	-,	+	+		-	-

	(D)					+, -	+					
	Pseudomonas sp. (V)	colorless	*Bacilli Mucoid*	-	NLF	K, K -,-	-,+	+	+		-	-
	Enterobacter sp. (V)	colorless	*Bacilli*	-	LF	A, A +,-	-, +	+	+		-	-
	Micrococcus sp. (D)	White	*Cocci*	+						-		
	Citrobacter sp. (D, V)	Pink	*Bacilli Mucoid*	-	LF, LF	A, A +, +	-, +	+	+		+	+
M. stejnegeri	*Proteus* sp. (D,V)	Colorless	*Mucoid*	-	NLF	K,A K,K -, +	+, -	+	+		+	+
	Staphylococcus sp. (D)	White	*Cocci in cluster*	+								
	Enterobacter sp. (D)	Pink	*Bacilli Mucoid*	-	LF	K, A +, -	-, +	+	+		-	+
	Staphylococcus aureus (D)	White	*Cocci in cluster*	+								
M. stejnegeri	*Pseudomonas* sp. (D)	Colorless	*Bacilli Mucoid*	-	NLF	K, K +, -	-, +	+	+		-	+
	Diplococcus sp. (D)	White	*Cocci in pairs*	+						-		

Side obtained: D- dorsal side, V- ventral side; GSR- Gram Stain Reaction; LF-Lactose Fermentation: LF-lactose fermenter, NLF- Non-lactose fermente.

Morpho-Biochemical results of the bacterial isolates

Morphological assesment and biochemical test results revealed nine bacterial isolates from the three frog species sampled (Table 1). *Citrobacter* sp. was the common isolate found in all frog species swabbed which was obtained in both dorsal and ventral sides. Aside from an occasional pathogen, this bacterium can also be isolated from soil (Tortora, Funke, and Case, 2013). Finding this bacterial taxon common in all frog samples is not surprising. The low number of isolates that we obtained despite working with three varied frog species may suggest that frog species harbor host-specific bacterial species (McKenzie *et al*, 2012). Moreover, since the study focused only on bacterial isolates that have grown on the media used, other bacterial isolates thriving on the frogs' skin may have not been accounted.

Two of the bacterial isolates (*Enterobacter* sp., and *Pseudomonas* sp.) reported in this study were previously accounted to be associated with the skin of some Philippine frogs in Luzon (Eda, Diesmos, Vredenburg, and Chan, 2015). These isolates were also found to be present in non-Philippine frog species. *Pseudomonas* sp. was documented in *Atelopus* spp. from Colombia (McKenzie *et al*, 2012; Harris *et al*, 2006). *Citrobacter*, *Micrococcus*, *Enterobacter* were also earlier reported as inhabitants of the skin of amphibians (Carr *et al*, 1976; Hird *et al*, 1983).

The presence of bacterial isolates on frog's skin is considered a mutualistic relationship. Bacterial species inhabiting the skin of various frog species provide positive inducement for release of antimicrobial peptides while others block establishment of certain pathogens (Mangoni *et al*, 2001; Becker *et al*, 2014). However it is not yet fully established if all bacterial taxa residing in the skin of frogs accomplish both these functions. The bacteria seem to benefit from this relationship by the nourishment provided by the mucus secretions of the frog's skin (Lauer *et al*, 2007).

Conclusion

Nine bacterial isolates from the skin of three endemic frog species: *Kalophyrnus sinensis* (Peters, 1867), *Limnonectes magnus* (Stejneger, 1910), and *Megophrys stejnegeri* (Taylor, 1920) were obtained. Isolates which were characterized thorugh morphological and results of some biochemical tests incluuded *Citrobacter* sp., *Salmonella* sp., *Pseudomonas* sp., *Enterobacter* sp., *Micrococcus* sp., *Proteus* sp., *Staphylococcus* sp., *Staphylococcus aureus*, and *Diplococcus* sp. Eight of these isolates were found in *Megophrys stejnegeri* (Taylor, 1920). *Citrobacter* sp. was the common bacterial isolate found in all the frog species on both dorsal and ventral sides. The presence of bacterial isolates on these frogs maybe suggestive of a mutualistic relationship, though this requires further studies.

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References

Ates F, Delima EM. 2008. Assemblage and microhabitats of anurans from Mt. Sinaka, Arakan, Cotabato and Mt. Hamiguitan, Davao Oriental, Mindanao Island, Philippines. Journal of Nature Studies **7(1),** 101-107.

Becker M, Zawacki C, Gratwicke B, and Bleden L. 2014. The effect of captivity on the cutaneous bacterial community of the critically endgangered Panamian golden frog (*Atelopus zeteki*). Biological Conservation **176,** 199-206.

Brown R, De Layola L, Lorenzo A, Diesmos M. 2015. A new species of limestone karst inhabiting forest frog, genus *Platymantis* (Amphibia: Anura: Ceratobatrachidae. Zootaxa **4048(2),** 191-210.

Carr AH, Amborski RL, Culley DD, Amborski GF. 1976. Aerobic bacteria in the intestinal tracts of bullfrogs (*Rana catesbeiana*) maintained at low temperatures. Herpetologica **32,** 239–244.

Conlon J. 2011. The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. Cell Tssue Res **343,** 201-212.

Dacalus C, Calunsag A, Hoshino L, Peralta D, Baron E. 2017. Anuran assemblage on forest edges of Datu Salumay, Davao City, Philippines. University of Mindanao International Multidisciplinary Research Journal. https://doaj.org/article/38a3a4293c7247f1a8115b8acd90 2ce2.

Diesmos A, Brown R, Gee G. 2003. International Union for the Conservation of Preliminary Report on the Amphibians and Reptiles of Balbalasang-Balbalan National Park in Luzon Island, Philippines. Slyvatrop The Technical Journal of Philippine Ecosystems and Natural Resources **13,** 63-80.

Eda A, Diesmos A, Vredenburg V, Chan M. 2015. Isolation, Screening, and Identification of Frog Cutaneous Bacteria for Anti-Batrachochytridium dendrobatidis Activity. Interantional Journal of Bioengineering and Life Sciences **9(4)**.

Flechas S, Sarmiento C, Cardenas M, Medina E, Restrepo S, Amezquita A. 2012. Surviving Chytridiomycosis: differential anti-*Batrachochytridum dendrobatidis* activity in bacterial isolates from three lowland species of *Atelopus*. Plos One **7(9),** 1-7.

Harris R, James T, Lauer A, Simon M, Patel A. 2006. Amphibian pathogen *Batrychochytridium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. EcoHealth **3,** 53-56.

Hird DW, Diesch SL, McKinnell RG, Gorham E, Martin FB, Meadows CA, Gasiorowski M. 1983. Enterobacteriaceae and Aeromonas-Hydrophila in Minnesota frogs and tadpoles (*Rana pipiens*). Applied and Environmental Microbiology **46(6)**, 1423-1425.

Inger RF. 1954. Systematics and zoogeography of Philippine amphibia. Fieldiana **33,** 181-531.

International Union for the Conservation of Nature, Conservation International and Nature Reserve. 2004. Global amphibian assessment. 15 April 2018. www.Globalamphibians.org.

Lauer A, Simon MA, Banning JL, André E, Duncan K, Harris R. 2007. Common cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. Copeia **3,** 630–640.

Mangoni M, Miele R, Renda T, Barra D, Simmaco M. 2001. The synthesis of antimicorbial peptides in the skin of *Rana esculenta* is stimulated by microorganisms. FASEB Journal **15(8),** 1431-1432.

McKenzie V, Bowers R, Fiere N, Knight R, Lauber C. 2012. Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. International Society for Microbial Ecology Journal **6,** 588-596.

Mcleod D, Siler C, Diesmos A, Diesmos M, Garcia V, Arkonceo A, Balaquit K, Uy C, Villaseran M, Yarra E, Brown R. 2011. Apmhibians and Reptiles of Luszon Island V: The Herpetofauna of Angat Dam Watershed, Bulacan Province, Luzon Island, Philippines. Asian Herpetological Research **2(4),** 177-198.

Rahman M, Somsiri T, Tajima K, Ezura Y. 2004. Distribution of *Aeromonas* spp. Emphasizing on a newly identified species *Aeromonas* sp. T8 isolated from fish and aquatic animals in Southeast Asia. Pakistan Journal of Biological Sciences **7(2),** 258-268.

Sanguilla M, Cobb K, Siler C, Diesmos A, Alcala A, Brown R. 2016. The amphibians and reptiles of Mindanao Island, southern Philippines, II: the herpetofauna of northeast Mindanao and adjacent islands. ZooKeys **642,** 1-132.

Siler C, McVay J, Diesmos A, Brown R. 2009. A new species of fanged frog, genus *Limnonectes*, (Amphibia: Anura: Dicroglossidae) from Southeast Mindanao Island, Philippines. Herpetologica **65(1),** 105-114.

Siler C, Swab J, Oliveros C, Diesmos A, Averia L, Alcala A, Brown R. 2012. Amphibians and reptiles , Romblon Island Group, central Philippines: comprehensive herpetofaunal inventory. Checklist **8(3),** 443-462.

Supsup C, Guinto F, Redoblado R, Gomez R. 2017. Amphibians and reptiles from the Mt. Hamiguitan Range of eastern Mindanao, Philippines: New distribution records. Checklist **13(3),** 2121.

Tortora G, Funke B, Case L. 2013. Microbiology an introduction 11th edition. Pearson, Boston USA. Pp. 975.

Warguez D, Mondejar E, Demayo C. 2013. Frogs and their microhabitat preferences in the agricultural and secondary forest areas in the vicinity of Mt. Kalatungan Mountain, Bukidnon, Philippines. International Research Journal of Biological Sciences **2(10),** 51-63.

Woodhams D, Vredenburg V, Simon M, Billheimer D, Shakhtour B, Shyr Y, Briggs C, Smith L, Harri R. 2007. Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. Biological Conservation **138**, 390-398.