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THE CULTURABLE SKIN MICROBIOME OF THE OCOEE SALAMANDER, *DESMOGNATHUS OCOEE*

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Abstract.—The Ocoee Salamander (*Desmognathus ocoee*) is a streamside salamander endemic to the southeastern USA. Like other salamander species, *D. ocoee* hosts a diverse assemblage of microorganisms on its skin. In this study, we characterized the culturable cutaneous microbiome of six *D. ocoee* individuals using standard microbiological techniques and Sanger DNA sequencing. We isolated 41 bacterial colonies that occurred primarily in the bacterial phyla Proteobacteria (n = 27) and Bacteroidetes (n = 9). A SIMPER analysis indicated that skin bacterial communities on individual salamanders were 42.5% similar to one another. A Kruskal-Wallis test indicated that the individual salamanders did not differ from one another at the bacterial rank of family. This work documents foundational knowledge on the microbiome hosted by the skin of *D. ocoee.*

Key words. — Amphibian, Bacteroidetes, direct colony PCR, probiotics, Proteobacteria, streamside salamander

Global biodiversity is currently experiencing its sixth major extinction event (Gibbons et al. 2000; Wake and Vredenburg 2008), and this represents a major challenge in the field of conservation biology (Thomsen and Willerslev 2015). Currently, there is scientific agreement on the importance of this issue, yet the Earth's biodiversity remains under described, making conserving biodiversity on Earth especially challenging (Hawksworth 1991; Stork 1993; Pons et al. 2006; Schmit and Mueller 2007).

The biodiversity of salamanders of the southeastern United States ranks among the highest in the world (Wake 1991) and is considered a hotspot of salamander biodiversity. Populations of amphibians are suffering from anthropogenic effects like habitat loss, pollution, and emerging fungal pathogens (Daszak et al. 2003). Relative to other pathogens, the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has been responsible for the greatest number of amphibian-population declines worldwide during the last decade (Belden and Harris 2007). The discovery in 2013 of an especially virulent chytrid species in Asia and Europe, *B. salamandrivorans* (*Bsal*), has led to increasing concern for the welfare of salamander populations (Martel et al. 2013, 2014). If *Bsal* should arrive in the southern Appalachian Mountains, it could extirpate vast numbers of salamander species in a global hotspot for biodiversity (Martel et al. 2013; Stokstad et al. 2014).

The skin of amphibians is an interesting interface between the organism and its environment; for most species, it must be kept moist and therefore is excellent habitat for microorganisms, including fungal pathogens (Walke et al. 2014). Amphibians have been documented to have a core skin microbiome made of resident bacterial communities that are in part influenced by environmental microbes

(Fitzpatrick and Allison 2014; Kueneman et al. 2014; Loudon et al. 2014; Walke et al. 2014). Several bacterial members in the core microbial community have been documented to provide resistance against the chytrid fungus *Bd* (Lauer et al. 2007; Harris et al. 2009; Becker and Harris 2010; Bletz et al. 2013; Antwis et al. 2014).

The Ocoee Salamander (*Desmognathus ocoee*) is endemic to the southeastern United States where it is relatively abundant (Petranka 1998; Fig. 1). While often found in seepages and streambeds of larger streams, *D. ocoee* will move away from the streambeds and become part of the terrestrial-salamander community under moist conditions (Hairston 1987; Petranka 1998). The purpose of our study was to produce foundational work on the host-microbe associations of *D. ocoee* by characterizing the culturable skin microbiome. We compared individual salamander microbial assemblages to one another and determined bacterial taxa contributing to community similarity.

METHODS AND MATERIALS

Isolation of skin microorganisms—. Six *D. ocoee* salamanders were collected by C. Camp in 2015 in Sosebee Cove (Blairsville, GA). Individuals were briefly washed with sterile water to remove transient microbes and swabbed as in Walker et al. (2015). Freshly collected swabs were streaked out in triplicate onto quadrant one of a Luria agar Petri plate (90 mm) using a standard microbiological streak-plating technique. Sterile inoculating loops were then used to complete the streak-plate technique in quadrants 2–4. The primary plates were incubated at 30 °C and all morphologically distinct colonies isolated into pure culture for six days.

Microbial genotyping—. Independent fresh bacterial colonies (≈ 24 hours) were aseptically transferred with a sterile needle into 0.2 mL PCR tubes and diluted with 100 µl sterile water. An approximately 1,300 bp fragment of the bacterial 16S rRNA gene was amplified using the following reaction components (25 µl total volume): 12.5 µl BioMix Red (Bioline), 2.5µl (10 mM) of both forward and reverse primers (8F: AGAGTTTGATCCTGGCTCAG and 1492R: GGTTACCTTGTTACGACTT; Lane 1991), 5 µl molecular grade water, and 2.5 µl bacterial cell suspension. PCR amplification conditions were as follows: an initial incubation step at 94 °C for 5 minutes followed by 35 cycles of 94 °C for 30 seconds, 50 °C for 45 seconds, 72 °C for 90 seconds, and a final extension of 10 minutes at 72 °C. PCR amplicons were visualized on a 2% gel for correct amplicon size and purified by combining 3 µl of ExoSapIT (ThermoFisher Scientific) with 13 µl of PCR amplicon and incubating at 37 °C for 15 minutes followed by 80 °C for 15 minutes. Purified PCR product was sent to the University of Chicago DNA Sequencing Facility for Sanger sequencing.

Sequence processing and taxonomic assignment—. Geneious v. 7.1 was used to construct contigs from the forward and reverse reads. Taxonomy was assigned using UCLUST (Edgar 2010) in QIIME (Caporaso et al. 2010) by comparison to the Greengenes v 13.8 database at 97% sequence similarity. A SIMPER analysis in the software Primer7 was used to determine overall community similarly among salamanders at the taxonomic rank of bacterial family. Statistical differences between skin communities at the rank of bacterial family were determined using a Kruskal-Wallis test preformed in GraphPad Prism (Prism 6, Graphpad). Since 12 of 41 isolates were unidentified at the genus rank, we chose to analyze communities at the bacterial family level to allow for comparison between all isolated taxa.

RESULTS

A total of 41 bacterial isolates were obtained from six *D. ocoee* salamander individuals (Table 1). The minimum number of isolates from a

single salamander was one, and the maximum was 10. The most abundant bacterial phyla were the Proteobacteria (n = 27) and Bacteroidetes (n = 9). The most commonly isolated classes were the Gammaproteobacteria (n = 22) and the Flavobacteria (n = 9). The SIMPER analysis indicated that skin bacterial communities from individual salamanders were 42.5% similar to one another at the rank of bacterial family. The families Enterobacteriaceae, Weeksellaceae, and Pseudomonadaceae contributed 36.8%, 22.3%, and 20.1% to the shared similarity among salamanders, respectively (79.3% total community similarity). A Kruskal-Wallis test indicated that the individual salamanders did not differ from one another at the bacterial rank of family (H (5) = 6.835, P = 0.2332).

FIG. 1. Our study organism, the Ocoee Salamander (*Desmognathus ocoee*).

DISCUSSION

The main objective of this research was to characterize the culturable microbes that associate with the skin of *D. ocoee.* We found that there were no significant differences in culturable microbes between individuals at the rank of bacterial family, and that three bacterial families, Enterobacteriaceae, Weeksellaceae, and Pseudomonadaceae, contributed to overall community similarity. Current research suggests that the skin microbial community of amphibians is most influenced by host-species identity, with site explaining additional variation in composition (McKenzie et al. 2012; Kueneman et al. 2014). The skin microbiome of salamanders removed from their natural habitat and housed in sterile conditions results in community shifts, suggesting that environmental microbes influence the microbiome (Loudon et al. 2014).

The culturable cutaneous microbiome of *D. ocoee* is similar to previously studied salamander species. The most commonly cultured bacterial species found in this study were within the phyla Proteobacteria (n = 27) and Bacteroidetes (n = 9), both of which have been described as core or dominant phyla in other aquatic and terrestrial amphibian species (McKenzie et al. 2012; Kueneman et al. 2014; Becker et al. 2015). The classes most commonly cultured in this study were Gammaproteobacteria (n = 22) and Flavobacteria (n = 9). Species within the class Gammaproteobacteria have been commonly found in the cutaneous microbiome of amphibians, with the genus *Pseudomonas* often found on amphibian skin and in the environment (Kueneman et al. 2014; Loudon et al. 2014). Bacteria from the class Gammaproteobacteria are often cited as having antifungal activity against *Batrachochytrium dendrobatidis* (*Bd*), the causative agent of chytridiomycosis (Lauer et al. 2007). *Chryseobacterium* in the Bacteroidetes, a common isolate in our study, has also been cited for having antifungal activity (Lauer et al. 2007).

The bacterial species *Janthinobacterium lividum* has been bioaugmented in several studies and used as a probiotic to treat chytridiomycosis (Brucker et al. 2008; Becker et al. 2009; Harris et al. 2009). This species is commonly found in the salamander skin microbiome (Lauer et al. 2007); however, we did not isolate this species during our study. The use of culture-dependent methods often fails to

TABLE 1. Bacterial isolates from the skin of Ocoee salamanders based on taxonomic assignment using UCLUST in QIIME by referencing the GreenGenes v13.8 database.

Salamander	Phylum	Class	Order	Family	Genus
1	Firmicutes	Bacilli	Bacillales	Bacillaceae	unknown
1	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	*Pseudochrobactrum*
2	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	*Leucobacter*
2	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Paenibacillus*
2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown
2	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
2	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter*
2	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	*Pseudochrobactrum*
2	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	unknown
2	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown
2	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
3	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	*Chryseobacterium*
3	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	*Chryseobacterium*
3	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	*Chryseobacterium*
3	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	*Chryseobacterium*
3	Firmicutes	Bacilli	Bacillales	Bacillaceae	unknown
3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Enterobacter*
3	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
3	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae	*Pseudochrobactrum*
3	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown
4	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	*Chryseobacterium*
4	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
4	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown
4	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown
4	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
4	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
4	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
4	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown
4	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown
4	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown
5	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	*Chryseobacterium*
5	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	*Chryseobacterium*
5	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	*Chryseobacterium*
5	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*
5	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unknown
6	Bacteroidetes	Flavobacteria	Flavobacteriales	Weeksellaceae	*Chryseobacterium*
6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
6	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Serratia*
6	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Citrobacter*

the majority of bacterial species within the microbiome, and therefore may explain the inability to identify *J. lividum* on the skin of *D. ocoee* salamanders. It has been estimated that fewer than 0.1% of bacterial species can be cultured in laboratory conditions, severely limiting the effectiveness of culture-dependent methods (Torsvik and Ovreas 2002). Next-generation sequencing technologies have increased the resolution at which we can observe complex microbial communities (Claesson et al. 2011). This study has provided foundational work to identify the culturable microbiome of the stream side *D. ocoee* salamanders, which can now be compared to next-generation sequencing data.

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