International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 7, Issue 1, 2015, pp.-588-597.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

CHARACTERIZATION OF THE BACTERIAL MICROFLORA ON THE SKIN OF BOREAL TOADS, *Anaxyrus (Bufo) boreas boreas*, AND COLUMBIA SPOTTED FROGS, *Rana luteiventris*, IN GRAND TETON NATIONAL PARK, WYOMING USA

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Received: December 23, 2014; Revised: March 05, 2015; Accepted: March 09, 2015

Abstract- Background: Cutaneous bacteria inhabiting the skin of boreal toads *(Anaxyrus* (*Bufo*) *boreas boreas*) and columbia spotted frogs (*Rana luteiventris*) from Grand Teton National Park were isolated and identified using their 16S SSU rRNA gene sequence. We also used a culture-independent method, Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis of the 16S SSU rRNA gene sequence, to characterize and compare the bacterial microbiota of these two amphibian species within and between different collection sites in Grand Teton National Park, Wyoming, USA.

Results: Bacterial isolates belonged to 5 major phylogenetic lineages: the *Actinobacteria*, the *Bacteroidetes*/*Chlorobi* Group, and the *Alpha-*, *Beta-*, and *Gamma-Proteobacterial* lineages. TRFLP analyses showed a high species richness between sites and between amphibian species, as well as a significant amount of diversity. All three measures of diversity used (Margalef Species Richness, the Shannon Index, and the Simpson Index) were higher for frog samples than toad samples, but varied between sites. Additive Main effects and Multiplicative Interaction (AMMI) analysis of the TRFLP results showed more variability in the 3' fragments than in the 5' fragments of the 16S SSU rRNA gene sequences amplified from metagenomic DNA extracted from amphibian skin surface samples. Furthermore, within the 3' fragments one site was shown to be significantly different than the other four sites by AMMI analysis.

Conclusions: This study illustrated the extensive phylogenetic diversity of microorganisms present on the skin of frogs and toads present in GTNP. The identification of some of the bacterial isolates present as belonging to lineages known to produce antifungal or antibiotic compounds (thereby enabling microbial antagonism) forms the basis for a plausible hypothesis for the disease resistance of amphibians to *Batrachochytrium dendrobatidis* in GTNP.

Keywords- bacterial microflora, skin of boreal toads, phylogenetic lineages, AMMI analysis, phylogenetic diversity of microorganisms

Citation: Hillis A.M., et al. (2015) Characterization of the Bacterial Microflora on the Skin of Boreal toads, *Anaxyrus (Bufo) boreas boreas*, and Columbia Spotted Frogs, *Rana luteiventris*, in Grand Teton National Park, Wyoming USA. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 7, Issue 1, pp.-588-597.

Introduction

Amphibians live in moist or aquatic environments and are therefore exposed to a plethora of microorganisms via contact with water, soil, plants, invertebrates, and vertebrates. Due to the high levels of diversity in the bacterial communities in these varied environments, it is likely that the skin of amphibians hosts a subset of the microbiota from these habitats [1-5]. Bacteria can compose over 90% of the cells present on the skin and in the gastrointestinal tracts of vertebrate organisms and these diverse bacteria can be essential for survival of the host they inhabit [5]. Because of this symbiotic relationship, some bacteria can be viewed as mutualists and may have a co-evolutionary relationship with their host species.

In the phenomenon of microbial antagonism, multicellular eukaryotic organisms will promote the growth of a normal flora on the surface of their skin. This normal flora presents a physical barrier to prevent infection by pathogens. Additionally, the production of antimicrobial peptides by the normal flora can serve as a chemical shield that inhibits the growth of pathogenic microorganisms on epithelial surfaces [6]. Some species of bacteria that proliferate on the epidermis of amphibians are known to produce extracellular products which are effective against fungi [1,7]. For example, the bacterial species *Janthinobacterium lividum* produces violacein and indole 3-carboxaldehyde, which have antifungal properties against *Batrachochytrium dendrobatidis* [8]. Brucker *et al.* [8] identified the metabolite 2,4-diacetylphloroglucinol from *Lysobacter gummosus* that is also capable of inhibiting the growth of *B. dendrobatidis* on amphibian. This compound also inhibits the growth of many fungi responsible for plant diseases, indicating it may be a broad spectrum antifungal [9,10].

Batrachochytrium dendrobatidis is the causative agent of the infec-

tious disease chytridiomycosis, which is causing amphibian population declines and extinctions worldwide [11-13,17]. This is the first chytrid fungus known to be parasitic to a vertebrate host. The zoospores of *B. dendrobatidis* are the infectious stage of the organism, infecting keratinocytes in the skin of metamorphosed amphibians and the mouthparts of tadpoles [11,14-16]. *B. dendrobatidis* is infectious to at least 200 amphibian species with aquatic species showing the greatest number of susceptible species, likely because of the ability of zoospores to disperse in water [17,18]. Amphibian species vary in their susceptibility to *B. dendrobatidis*, with susceptibility ranging from subclinical infections to death [4,9,17]. *B. dendrobatidis* has been found in Wyoming, USA, specifically in Grand Teton National Park (GTNP) on the skin of the boreal toad, *Anaxyrus* (*Bufo*) *boreas boreas* [19]. Although these toads are infected, no recent mass mortalities have been attributed to this pathogen, unlike in other amphibian communities in the Pacific Northwest, such as in Colorado.

In 2006, Harris and co-workers [20] conducted a *B. dendrobatidis* challenge assay with three bacterial isolates from the skins of the salamander *Plethodon cinereus* and seven bacterial isolates from the salamander *Hemidactylium scutatum*. These bacteria were found to be capable of inhibiting the growth of *B. dendrobatidis in vitro*. It is worth noting that neither of these two salamander species were exhibiting population declines despite being susceptible to *B. dendrobatidis*. In a more recent study [5], cultivable antifungal bacteria were isolated from the skin of *H. scutatum* [5]. These bacteria represented four phyla, comprising fourteen bacterial families, sixteen genera and forty-eight species, suggesting that antifungal properties can be found in a broad range of bacteria [5].

Only a small fraction of the bacterial diversity of the microbial skin flora of amphibians is known because most research on the microflora of amphibians has focused on cultivable bacterial species, which can be assessed for their antifungal properties. The microbial flora includes both cultivable and non-cultivable organisms. The use of relatively rapid culture-independent techniques such as Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis can provide a "fingerprint" of the diversity present. TRFLP utilizes fluorescent dyes attached to PCR primers and the resolution of sequencing technology to create a quantitative rapid method to analyze a complex microbial community [21].

An analysis of the normal microbial flora of amphibians in GTNP has not been done in depth. Given the findings of other studies that suggest the microflora of amphibians may help their resistance to pathogenic fungal infections, identifying the microflora on these animals may help our understanding of the amphibian populations and their relationship with this pathogen [2,3,5,8,20,24,32]. The objectives of this study were to (i) characterize and compare the phylogenetic diversity of the cultivable cutaneous bacteria on *Anaxyrus boreas* [22,23] and *Rana luteiventris* based on 16S rRNA fragments, and (ii) use a culture-independent method (TRFLP) to characterize and compare the cutaneous bacterial microbiota of *A. boreas* and the frog species *R. luteiventris.*

Materials And Methods

Sample Collection and Site Description

At least 20 individuals of each species were swabbed according to previously described protocols [48] during daylight hours at 5 sites in the Grand Teton area in late spring (Nowlin Pond (A), Schwabacher's Landing (B), Willow Flats (C), Snake River Quarry (D), and Black Rock (G)); [Fig-1]. Polyester swabs were stored in R2 media in the field for 2-3 days at 19°C until processing could be completed in the lab. Samples were then transferred to a microcentrifuge tube containing a 50% glycerol solution and stored at -20ºC until the analysis was conducted. A subset of 5 samples from each amphibian species at each of the 5 locations was randomly sampled from the larger collection, with the exception of Willow Flats or Snake River Quarry, as no frogs were seen at the time of collection.

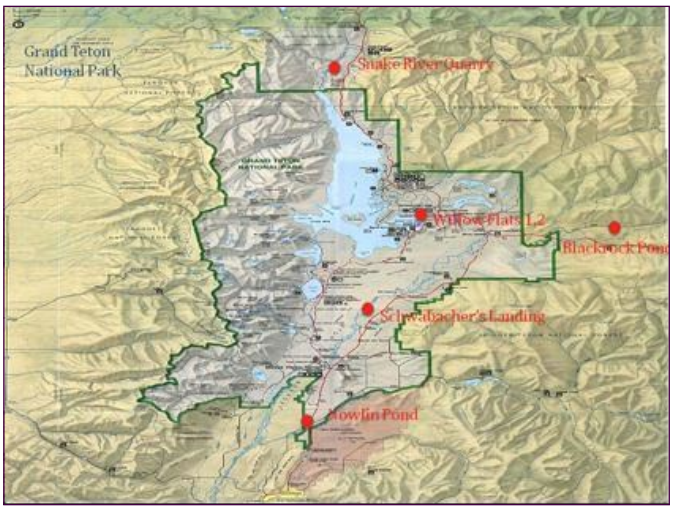

Fig. 1- Locations of 5 amphibian breeding sites in Grand Teton National Park in 2006. Site A: Nowlin Pond, B: Schwabacher's Landing, C: Willow Flats, D: Snake River Quarry, and G: Black Rock Pond.

Bacterial Isolation

Microcentrifuge tubes containing 1 mL of R2 glycerol sample solution were vortexed and 0.1 mL of solution was micro-pipetted onto R2 agar media plates. The plates were incubated at 20ºC for 6-7 days. Bacterial colonies were struck for isolation and upon isolation were transferred to R2 broth media. They were incubated at 20ºC until growth was observed. This process was repeated until cultures were determined to be axenic. Bacterial morphotypes were classified based upon pigmentation and colony morphology, using form, margin, and elevation. At least 10% of the isolates from each group were randomly chosen as representatives within that morphotype.

DNA Extractions

Genomic DNA from each isolate was extracted as follows: 0.5 mL of turbid broth culture was combined with 0.75 mL of lysis buffer solution (10mM Tris, 50 mM NaCL, 100 mM EDTA, 0.5% SDS) and 0.25 milligrams of a 1:1 mixture of 0.1 mm and 0.5 mm diameter glass beads. The mixture was vortexed at maximum speed for 5 minutes and incubated at 80ºC for 30 minutes. The solution was cooled to room temperature and centrifuged for 5 minutes at 3000 rpm. Then 0.75 mL of the supernatant was transferred to a microcentrifuge tube containing 0.45 mL of Isopropanol and incubated at -20ºC overnight. The solution was centrifuged at 13,000 rpm for 10 minutes and the DNA pellets were washed with 0.2 mL 70% Ethanol. The pellets were dried at 37ºC for 30 minutes and resuspended in 0.1 mL of 10 mM Tris buffer (pH 8.0).

Metagenomic DNA was extracted by taking 0.5 mL of the original R2 enrichments and combining them with 0.75 mL of lysis buffer solution and 0.25 mg of a 1:1 mixture of glass beads. The mixture

was mixed for 10 minutes at maximum speed in a Biospec Products Mini-Beadbeater-8 and then the rest of the procedure was done following the same steps used for genomic DNA isolation.

PCR Amplifications

PCR amplifications were done in a final volume of 50 µL. The PCR reaction mixture contained: 0.5 µL of Vent polymerase (New England Biolabs, Ipswich, MA); 5 µL of 10X ThermoPol Reaction Buffer (New England Biolabs, Ipswich, MA); 8 µL (200 µM/ea) dNTPs (New England Biolabs, Ipswich, MA); 2.5 µL (20 µM/L) of primer bacterial 8F (5'-AGAGTTTGATCCTGGCTCAG-3') (IDT DNA Technologies, Inc, San Diego, CA); 2.5 µL (20 µM/L) of primer bacterial 1492R (5'-TTACCTTGTTACGACTT-3') (IDT DNA Technologies, Inc, San Diego, CA); 1 µL of DNA; and 30.5 µL Nuclease Free Water (IDT DNA Technologies, Inc, San Diego, CA). The PCR amplification conditions were as follows: initial denaturation at 95ºC for 10 minutes, followed by 35 cycles of (95ºC for 1 minutes, 58ºC for 1 minutes, 72ºC for 4 minutes), a final extension at 72ºC for 10 minutes, followed by a hold at 4ºC. Amplification products of the appropriate size were purified directly from agarose gels using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). A further clean-up step was done via precipitation with 100 µL of 2.5 M NaCl/20% PEG solution, washing with 70% Ethanol, drying at 37ºC for 30 min and resuspension in 100 µL of 10 mM Tris, pH 8.0 buffer.

The bacterial SSU rDNA gene sequences from the metagenomic DNA extracted from amphibian skin swabs were amplified using the primer set bacterial 8F HEX (5'-HEX-AGAGTTTGATCCTGGTCAG-3') and bacterial 1492R FAM (5'-FAM-TTACCTTGTTACGACTT-3'). The PCR amplifications were performed in a 50 µL PCR reaction mixture containing 0.5 µL Vent polymerase (exo-) (New England Biolabs, Ipswich, MA); 5 µL of 10X ThermoPol Reaction Buffer (New England Biolabs, Ipswich, MA); 8 µL (200 µM/ea) dNTPs (New England Biolabs, Ipswich, MA); 2.5 µL (20 µM/L) of each primer; 3 µL Template; and 27.5 µL Nuclease Free Water (IDT DNA Technologies, Inc, San Diego, CA). The PCR amplification conditions were as follows: initial denaturation at 95ºC for minutes, followed by 30 cycles of (95ºC for 1 minutes, 53ºC for 1 minutes, 72ºC for 4 minutes), a final elongation step at 72ºC for 10 minutes. Fluorescently-labeled amplicons were purified using Millipore Montage PCR Centrifugal Filter Devices (Billerica, MA) per manufacturer's instructions.

RFLP

Bacterial PCR amplicons (10 µL) were digested with 5U of the restriction endonuclease MspI (C/CGG) (Promega Corporation, Madison, WI) at 37ºC for 2 hours. The restriction fragments were separated, visualized, and analyzed using a 1% agarose gel stained with ethidium bromide. The visualized bands lengths were measured and compared to assess for similarities in patterns.

TRFLP Digestions and Analysis

Fluorescently-labeled amplicons (10 µL) were digested with 5U of the restriction endonuclease *Msp* I (C/CGG) (Promega Corporation, Madison, WI), at 37ºC for 2 hours. The restriction fragments were precipitated with 0.2 volumes of 3 M sodium acetate and 4 volumes of 70% Ethanol. The fragment sizes were determined on an Applied Biosystems 3130 XL Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Idaho State University Molecular Core Facility using 1 µL of the restriction digest.

Table 1- A listing of the bacteria isolated from the backs of frogs and toads in GTNP as well as what site they were collected from and the percent identity the 16S sequences had in comparison to already identified species.

Completed 16S rRNA gene sequences of the bacterial isolates				
Isolate #	Site	16S sequence ID	# of bp	% Identity
1	D	*Stenotrophomonas rhizophila*	1418	99
4	D	*Serratia fonticola*	1419	99
7	G	*Bosea lathyri*	1367	99
8	G	*Sphingobacterium faecium*	1404	99
11	A	*Chryseobacterium antarcticum*	1390	98
16	D	*Stenotrophomonas rhizophilia*	1309	99
18	D	*Pseudomonas proteolytica*	1430	99
19	D	*Stenotrophomonas rhizophilia*	1418	99
20	D	*Delftia tsuruhatensis*	1396	99
23	G	*Enterobacter amnigenus*	1420	99
25	B	*Stenotrophomonas rhizophilia*	1418	99
27	D	*Aeromonas salmonicida*	1415	100
30	G	*Enterobacter amnigenus*	1348	99
34	G	*Arthrobacter oxydans*	1388	99
36	A	*Acinetobacter calcoaceticus*	1234	100
38	G	*Aeromonas salmonicida susp. salmonicida*	1415	99
39	D	*Comamonas testosteroni*	1395	100
41	G	*Acinetobacter calcoaceticus*	1400	99
43	D	*Serratia fonticola*	1406	99
47	D	*Acinetobacter beijerinckii*	1420	99
50	D	*Acinetobacter calcoaceticus*	1400	100
51	D	*Serratia fonticola*	1412	99
55	G	*Serratia fonticola*	1186	99
56	G	*Alcaligenes faecalis*	1427	99
60	A	*Pseudomonas lini*	1388	99
62	G	*Acinetobacter calcoaceticus*	1312	99
70	D	*Aeromonas media*	1416	99
72	D	*Serratia plymuthica*	1200	99
80	D	*Stenotrophomonas maltophilia*	1417	99
81	D	*Stenotrophomonas maltophilia*	1416	99
82	G	*Serratia plymuthica*	1408	99
83	A	*Serratia plymuthica*	1394	99
85	B	*Bosea massiliensis*	1367	99
86	G	*Pedobacter terrae*	1367	99
87	G	*Adhaeribacter aerolatus*	1397	96
90	G	*Pedobacter terrae*	1400	99
92	B	*Rhodococcus corynebacteroides*	1115	99
93	B	*Kocuria rosea*	1403	100
99	A	*Mycobacterium diernhoferi*	1219	98
101	D	*Methylobacterium brachythecii*	1210	99
104	G	*Chryseobacterium jejuense*	1390	99
105	D	*Chryseobacterium jejuense*	1389	99
107	A	*Flavobacterium columnare*	1353	98
108	D	*Stenotrophomonas maltophilia*	1430	99
109	D	*Chryseobacterium indologenes*	1385	98
110	D	*Stenotrophomonas rhizophilia*	1412	99
113	A	*Chryseobacterium soldanellicola*	1339	99
120	G	*Adhaeribacter aerolatus*	1394	96
122	D	*Delftia tsuruhatensis*	1390	99
123	D	*Pseudomonas fluorescens*	1406	99
125	A	*Chryseobacterium antarcticum*	1293	98
127	D	*Pseudomonas putida*	1405	99
142	B	*Microbacterium foliorum*	1402	98
146	A	*Kocuria rosea*	1403	99
152	B	*Bosea massiliensis*	1364	99
153	G	*Xanthomonas translucens*	1411	99
156	D	*Stenotrophomonas maltophilia*	1416	99
157	D	*Pseudomonas putida*	1399	99
158	D	*Bosea massiliensis*	1364	99
161	D	*Pseudomonas koreensis*	1405	99

Terminal restriction fragment sizes and relative peak heights for each sample were determined using PeakScanner Software v.1.0 (Applied Biosystems). Primer 6 (PRIMER-E Ltd, Plymouth, United Kingdom) was utilized to generate Margalef (Species Diversity), Shannon Index, and Simpson Index values. Values for each diversity index were determined separately for the 5' and 3' restriction fragments for each collection site and species of amphibian. Peak values that were present from samples at each site were compiled into tables and these peaks were compared against a dataset of SSU rRNA genes from cultivated bacterial isolates from GTNP which were subjected to an *in silico* "digestion" to generate *Msp* I fragments for each sequence. These *Msp* I "fragment" sequences were used to generate potential identifications of the cultivable organism(s) represented by each peak [Table-1].

AMMI Analysis of T-RFLP data

The Additive Main Effects and Multiplicative Interaction (AMMI) model was used to look at the variation in microbial communities and the effects of the sample location within GTNP on the microbial flora. The peak values from the T-RFLP fragments were analyzed using T-RFLP analysis expedited (T-REX) [25]. The data was then processed by an analysis of variance (ANOVA), which is the additive segment of the AMMI analysis. An interaction principal component analysis (IPCA) was then performed on the resulting ANOVA values in order to reduce the dimensionality of multivariate data. Corresponding IPCA values 1 and 2 were then graphed to yield a representation of peak variability with regards to the 5' and 3' TRFLP fragments.

Sequencing and Phylogenetic Analysis

The purified PCR products were sequenced at the Idaho State University Molecular Research Core Facility on an ABI 3100 automated capillary sequencer (Applied BioSystems, Foster City, CA). The bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3'), 704F (5'-GTAGCCGTGAAATGCGTAGA), 907R (3'-CCGTCAATTCCTTT), and 1492R (5'-TTACCTTGTTACGACTT-3') were used to generate a SSU (16S) rRNA double stranded contig for each bacterial isolate. These double stranded contigs were used in a search of the GENBANK (http://www.ncbi.nlm.nih.gov/) database to identify the closest relative to each isolate in the validated nomenclature from the International Journal of Systemic and Evolutionary Biology (http://www.bacterio.net/), and these sequences were downloaded from GENBANK. The SSU rRNA (16S) genes for each of the bacterial isolates and their most closely related recognized species were aligned using CLUSTALX [26]. Distance, Maximum Likelihood, and Maximum Parsimony analyses were performed on this alignment utilizing the PAUP 4.0 Beta 10 Package [27] after parameters were optimized using ModelTest [28]. Bootstrap analysis (1000 replicates) were performed using the Maximum Likelihood model in PAUP. Phylograms were visualized using TreeView v.1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Nucleotide Sequence Accession Number

All 16S rRNA gene sequences of the bacterial isolates analyzed in this study were deposited in the GenBank database under accession numbers KM114906-KM114965.

Results

Bacterial isolates and RFLP analysis

Over 300 morphologically distinct bacterial colonies were cultured from toad and frog skin samples taken from geographically distinct sites in Grand Teton National Park [Fig-1]. At least 10% of the isolates in each morphotype were randomly chosen for further analysis (165 isolates). Approximately 110 unique band patterns were observed from 165 bacterial isolates subjected to RFLP analysis of their SSU rRNA (16S) gene sequence.

TRFLP Analyses

TRFLP analyses showed a high species richness within sites and within amphibian species. The Margalef Index shows that there are no significant differences between frogs and toads in any of the sites in the 3' fragments [Fig-2](A). Shannon Diversity Index showed that Site G had a greater diversity in the frog samples than in the toad samples [Fig-2](B), but there were no significant differences between frogs and toads at the other sites. It should be noted however that site G only had two frogs sampled while four toads were sampled. Similarly the Simpson Diversity Index indicated a significant difference between frogs and toads at site G, but no significant difference between the amphibians at the other sites [Fig -2](C). For the 5' fragments there were no statistically significant differences in the diversity between frogs and toads for any of the sites in any of the indices [Fig-3].

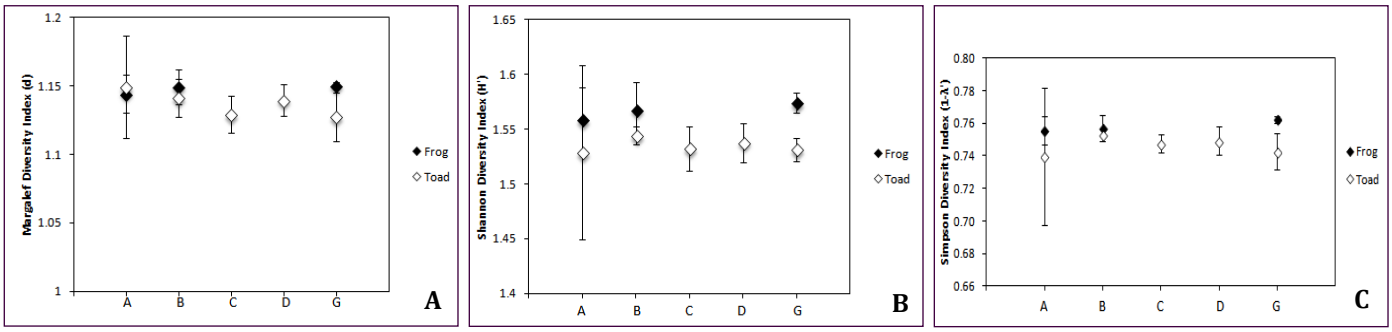

Fig. 2- Comparison plot of the 3' fragments for (A) Margalef Species Diversity, (B) Shannon Diversity Index, and (C) Simpson Index between sites and between amphibian species.

AMMI Analyses

AMMI analysis of the TRFLP 5' fragments indicated no significant difference in the diversity of the bacterial population at any of the sites [Fig-4]. AMMI analysis of the TRFLP 3' fragments showed no significant difference for sites A, B, C and D in the diversity of the bacterial populations between frogs and toads, however there was

a significant difference in the diversity in frogs and toads for Site G. The cumulative IPCA percent variation for all samples was over 75% (88.98%), thus showing an accurate representation of the diversity present [Fig-4].

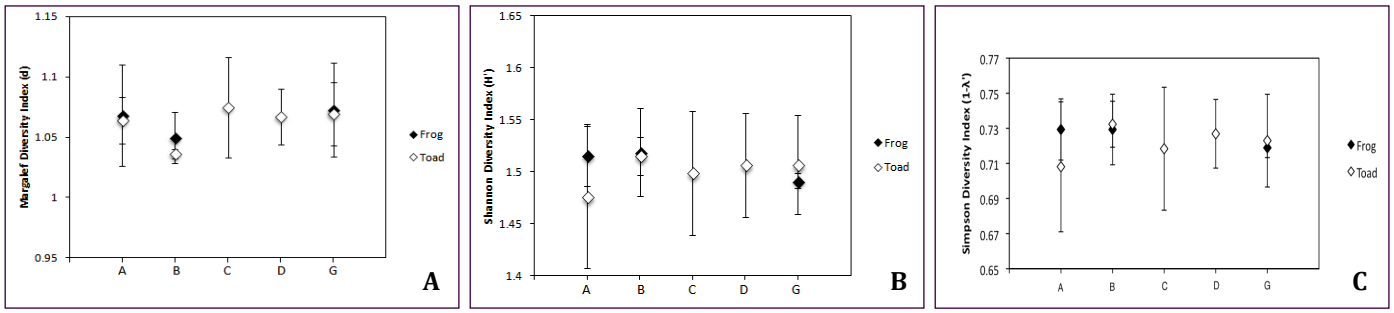

Fig. 3- Comparison plot of the 5' fragments for (A) Margalef Species Diversity, (B) Shannon Diversity Index, and (C) Simpson Index between sites and between amphibian species.

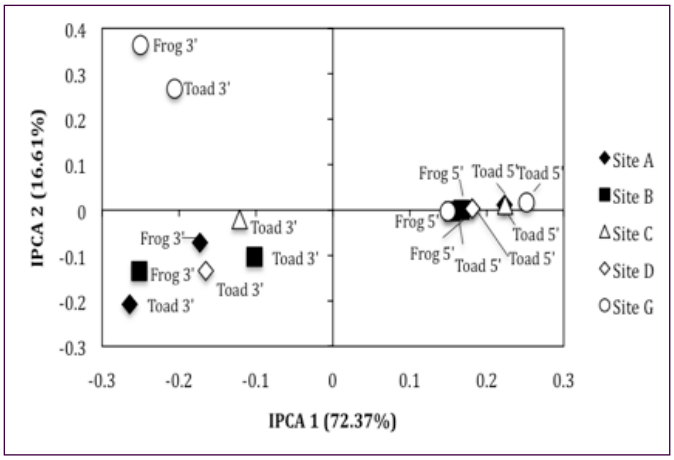

Fig. 4- Comparison plot of the 5' and 3' fragments by an AMMI analysis

Phylogenetic Analysis of Bacterial 16S rRNA Gene Sequences

The 110 bacterial isolates containing distinct banding patterns determined via RFLP analysis were chosen for sequencing. We were unable to produce sequence from all four primers for 50 of the 110 isolates so they were not included in further analysis. Only the 60 sequences in which we were able to generate double stranded contigs and eliminate sequence ambiguities were used in downstream analyses.

The 16S SSU rRNA sequences produced from the 60 bacterial isolates represented a wide diversity of Gram-negative and Gram-positive bacteria from several major phylogenetic groups including the *Actinobacteria*, the *Bacteroidetes*/*Chlorobi*, and the *Alpha Proteobacteria*, *Beta Proteobacteria*, and *Gamma Proteobacteria* [Fig-5]. The phylogenetic identification of each bacterial isolate is given in [Table-1]. The most frequently occurring bacterial species were related to the *Gamma Proteobacterial* group at >50% (32 of 60), followed by the *Bacteroidetes/Chlorobi* Group ~18% (11 of 60), then evenly distributed among the *Actinobacteria*, the *Alpha Proteobacterial* group, and the *Beta Proteobacterial* group. The fewest cultivated isolates sequenced were the Gram-positive (7 of 60), however they represented phylogenetically diverse genera.

***In silico* Comparative Digest**

The calculated fragment lengths generated by the *in silico* digest using the recognition sequence of the restriction enzyme *Msp* I of the 60 bacterial isolates is given in [Table-2]. Isolates from divergent phylogenetic lineages had calculated fragments of identical length for both 5' fragments and 3' fragments, making it difficult to correlate experimentally determined fragments with our bacterial isolates.

Using the TRFLP data for the forward fragments [Table-3], peak lengths of 6-11, 16, 17, 27, 31, 45, and 490 base pairs were found at all sites, peak lengths of 2, 13, 24, 104, 283, 298, 424, and 492 base pairs was found at four of the sites (A,B,C,G), (A,C,D,G), (A,B,C,D), (A,C,D,G), or (B,C,D,G), peak lengths of 23, 36, 162-164, 234, 279, 308, 337, 405-423, 437, 440, 489, 500, 550, and 700 base pairs were found at three sites (A,B,G), (A,B,D), (B,D,G), (A,D,G), (A,B,C), (C,D,G), (A,C,G), or (A,C,D), peak lengths of 34, 83, 100, 126,161, 174-202, 316, 336, 360-400, 450, 491, 494, 514, 538, and 563-650 base pairs were found at two sites (B,G), (A,C), (B,D), (A,D), (A,G), (A,B), or (B,C), and one peak length of 150 was found only at site D. Using the TRFLP data for the reverse fragments [Table-4], peak lengths of 7-10, 25, 27, 41-46, 80, 113, 120-123, and 700 base pairs were found at all sites, peak lengths of 5, 21, 37, 100, 111, 125, 205, and 335-339 base pairs were found at four sites (A,B,D,G), (A,B,C,D), (A,B,C,G), or (B,C,D,G), peak lengths of 16, 101, 116, 490, 750, and 949 base pairs were found at three sites (A,B,G), (A,C,D), (B,C,D), or (A,B,C), peak lengths of 23, 48-70, 98, 243, 322, 493, and 999 base pairs were found at two sites (B,D), (A,C), (B,C), (C,D), or (A,G), and two peak lengths of 90 and 99 were found at one site C and G respectively. Three of the fragments could be tentatively identified using our 5' fragment data from the *in silico* digest and four of the fragments could be tentatively identified using our 3' fragment data.

Discussion

We report here the phylogenetic analysis of bacterial isolates from skin samples taken from boreal toads and columbia spotted frogs at 5 different sites in Grand Teton National Park. Five major taxonomic groups of bacteria were represented: the *Alpha-*, *Beta-*, and *Gamma-Proteobacteria* (68.3%), the *Bacteroidetes*/*Chlorobi* (20%), and the *Actinobacteria* (11.7%) [Fig-5]. The isolates cultured were found on both amphibian species and these major lineages were represented at all sites.

Within the taxonomic group *Proteobacteria*, bacterial isolates were cultured from only the *Alpha-, Beta-,* and *Gamma-* lineages, and the majority of bacterial isolates cultured belonged to the *Gamma Proteobacteria* (53%), with 3 major genera represented. *Stenotrophomonas* (7 of 32) and *Serratia* (7 of 32) were equally prominent fol-

lowed by the genus *Pseudomonas* (6 of 32). Based on the phylogenetic analysis, the *Gamma- Proteobacteria* isolates were not monophyletic, but were split by the *Beta-ProtebBacterial* lineage, although this is likely a tree-building artifact (as shown by the very short, unsupported branch leading to the *Beta/Gamma-Proteobacterial* subset). The genus *Stenotrophomonas* clustered with the genus *Xanthomonas*, which diverged from the rest of the cultured organisms' sequences. The remaining members of the *Gamma Proteobacterial isolates* belong to the genera *Acinetobacter*, *Aeromonas*, *Enterobacter, Serratia*, and *Pseudomonas*.

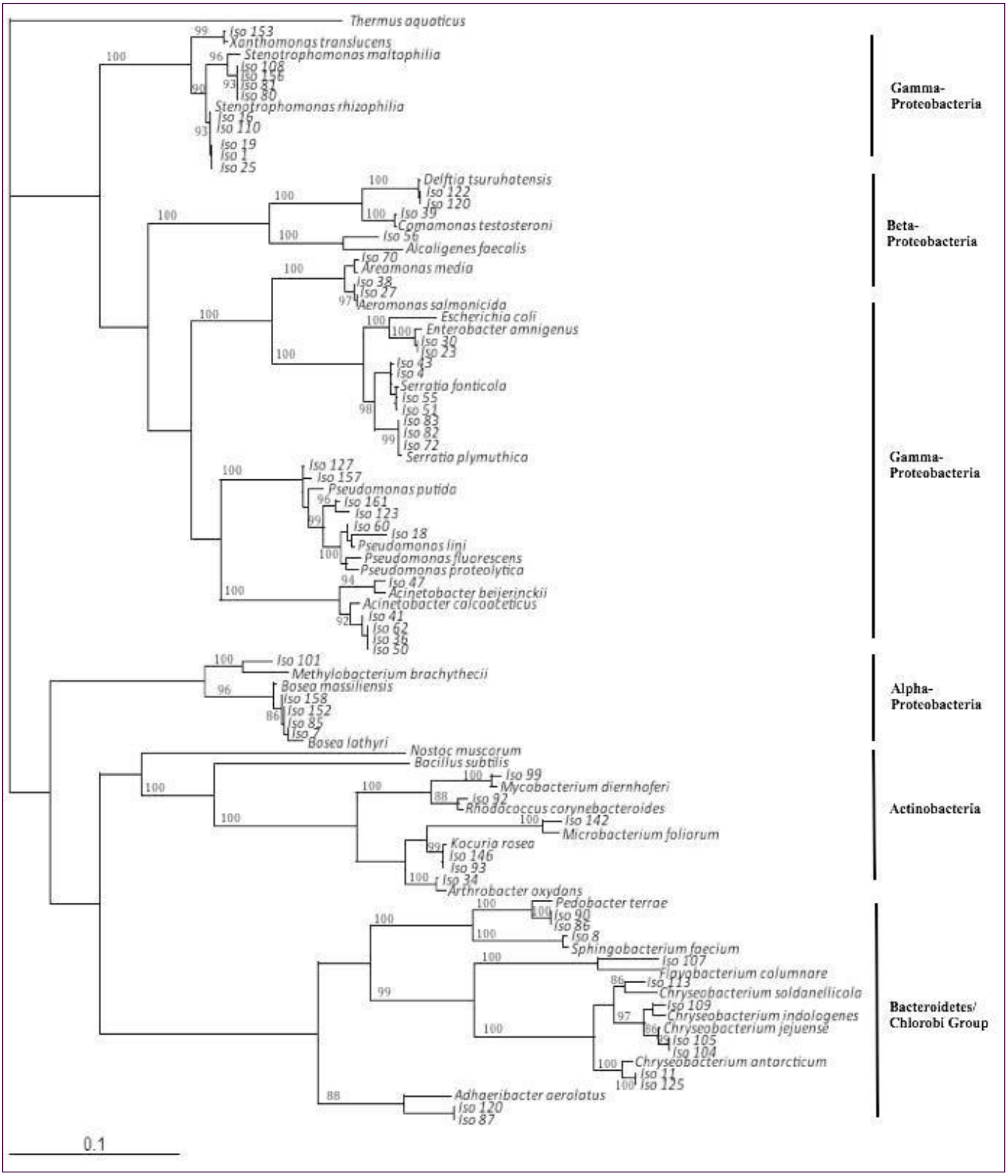

Fig. 5- A Maximum Likelihood Phylogenetic tree (1,000 bootstrap replicates) of the 60 16S SSU rRNA gene sequences from GTNP bacterial isolates and 38 of their closest related species.

Table 2- *in silico* Digests of isolated bacteria from the backs of frogs and toads in GTNP, showing the fragment lengths found with the 5' or 3' primers for comparison to the TRFLP data.

	in silico Digest				
	5' Fragment Length	3' Fragment Length		5' Fragment Length	3' Fragment Length
Isolate 1	455	78	Isolate 82	448	78
Isolate 4	449	87	Isolate 85	109	85
Isolate 7	109	85	Isolate 86	99	41
Isolate 8	145	72	Isolate 87	443	75
Isolate 11	159	73	Isolate 90	76	99
Isolate 16	346	78	Isolate 92	29	96
Isolate 18	93	107	Isolate 93	34	238
Isolate 19	455	78	Isolate 99	50	34
Isolate 20	436	78	Isolate 101	109	154
Isolate 23	451	87	Isolate 104	154	77
Isolate 25	455	78	Isolate 105	159	72
Isolate 27	47	77	Isolate 107	38	41
Isolate 30	387	79	Isolate 108	410	88
Isolate 34	110	28	Isolate 109	159	67
Isolate 36	439	793	Isolate 110	455	71
Isolate 38	47	77	Isolate 113	159	21
Isolate 39	405	78	Isolate 120	443	72
Isolate 41	439	78	Isolate 122	436	73
Isolate 43	451	85	Isolate 123	446	77
Isolate 47	448	90	Isolate 125	150	78
Isolate 50	439	78	Isolate 127	444	78
Isolate 51	451	78	Isolate 142	26	32
Isolate 55	451	62	Isolate 146	34	238
Isolate 56	445	105	Isolate 152	106	85
Isolate 60	427	78	Isolate 153	448	78
Isolate 62	350	79	Isolate 156	409	78
Isolate 70	47	77	Isolate 157	438	78
Isolate 72	437	92	Isolate 158	107	84
Isolate 80	410	78	Isolate 161	78	443
Isolate 81	409	78	Isolate 161	78	443

The genera *Stenotrophomonas* and *Serratia* contain members commonly found in soil and plants [29]. There are several reports indicating the potential of *Stenotrophomonas* species to be biological control agents of oomycete, fungal and bacterial pathogens [30-32]. Antifungal properties have also been reported for *Serratia marcescens* through the production of chitinase [34]. The genus *Pseudomonas* ranges across the *Alpha-*, *Beta-*, *Delta-* and, *Gamma-* lineages and ranges in environmental niches from aquatic to plant associations. These organisms have been used as biological control agents since the 1980's and *P. fluorescens* strains are the best understood [35]. Other *Pseudomonas* species with antifungal activity against fungal plant pathogens are *P. chlororaphis* [36] and *P. aurantiaca*. The *Alpha-* and *Beta- Proteobacterial* lineages had equal representation with five bacterial isolates each. The *Alpha Proteobacteria* had only two genera, with one isolate from the genus *Methylobacterium* while the other four isolates belonged to the genus *Bosea.* The bacterial species representing these two genera have been found in tap water [37]. Bacterial isolates from the genera *Delftia*, *Comamonas*, and *Alcaligenes* represent the *Beta Proteobacteria*. The organisms from these genera commonly occupy water and soil. The genus *Delftia* had two isolates identified as *D. tsuruhatensis*. *Comamonas* had only one species identified and represented from the cultivated skin flora. The genus *Alcaligenes* had only one species identified as *Alcaligenes faecalis*.

Table 3- Comparison of the peak lengths with the known fragment lengths of bacteria isolates, using the forward strand.

Peak (bp)	Cultivable Bacterial Tentative ID	Genus	Frog Only	Toad Only	Both Species	Sites
2					X	A,B,D,G
6					X	ALL
7					X	ALL
9					X	ALL
11					X	ALL
13					X	A,B,D,G
16					X	ALL
17					X	ALL
23					X	A,B,G
24					X	A,C,D,G
27					X	ALL
31					X	ALL
34	Isolate 93 & 146	Kocuria	X			B,G
36			X			A,B,G
45					X	ALL
83					X	A,C
100					X	B,D
104					X	A,B,C,D
126					X	B,D
150	Isolate 125	Chryseobacterium		X		D
161					X	A,D
162					X	A,B,D
163					X	A,B,C
164					X	A,B,D
174					X	B,D
176					X	B,D
200				X		A,C
202					X	A,C
234					X	B,D,G
279					X	A,D,G
283					X	A,B,C,G
298					X	A,C,D,G
308					X	A,B,C
316			X			A,G
336					X	A,D
337					X	C,D,G
360					X	B,D
362					X	B,D
378					X	A,D
398					X	A,B
400					X	A,D
405					X	A,B,D
421				X		C,D,G
423					X	A,C,G
424					X	B,C,D,G
437	Isolate 72	Serratia			X	A,B,D
440					X	A,B,C
450				X		A,D
489					X	A,B,C
490					X	ALL
491					X	A,B
492					X	A,B,C,G
494					X	A,B
500					X	A,B,D
514					X	B,C
538					X	B,D
550				X		A,B,C
563					X	A,G
567				X		A,B
603				X		B,C
613					X	B,C
650				X		A,D
700				X		A,C,D

Table 4- Comparison of the peak lengths with the known fragment lengths of bacteria isolates, using the reverse strand.

Peak (bp)	Cultivable Bacterial Tentative ID	Genus	Frog Only	Toad Only	Both Species	Sites
5					X	A,B,D,G
7					X	ALL
9					X	ALL
10					X	ALL
16					X	A,B,G
21	Isolate 113	Chryseobacterium			X	A,B,C,D
23				X		B,D
25					X	ALL
27					X	ALL
37					X	A,B,C,D
41	Isolate 86	Pedobacter			X	ALL
43					X	ALL
46					X	ALL
48					X	A,C
55					X	B,C
70				X		C,D
80					X	ALL
90	Isolate 47	Acinetobacter		X		C
98					X	A,C
99	Isolate 90	Pedobacter			X	G
100					X	A,B,C,D
101					X	A,C,D
111					X	A,B,C,D
113					X	ALL
116					X	B,C,D
120					X	ALL
121					X	ALL
123					X	ALL
125					X	A,B,D,G
205					X	A,B,C,G
243					X	B,C
322			X			A,G
335					X	B,C,D,G
336					X	A,B,C,D
339					X	A,B,C,D
490					X	A,B,C
493					X	A,G
700					X	ALL
750					X	A,C,D
949					X	A,C,D
999					X	A,C

The next major phylogenetic lineage represented was the Bacteroidetes/Chlorobi group (11 of 60 isolates). The predominant representation of bacterial species belonged to the genus *Chryseobacterium* (5 of 11), followed by *Pedobacter* (2 of 11), *Adhaeribacter* (2 of 11), and the genera *Flavobacteria* and *Sphingobacteria* with one isolate each. The species are all organisms occupying soil and aquatic environments. Some species in the genus *Chryseobacterium* produce antimicrobial substances active against pathogenic fungi that infect amphibians, [3] as do species in the genus *Pedobacter* [39]. Of the cultivated organisms identified only one bacterial isolate belongs to the genus *Flavobacterium*.

Lastly the Actinobacteria represent the Gram-positive organisms cultured (11%) with only seven bacterial isolates distributed over five genera (*Rhodococcus*, *Mycobacterium*, *Microbacterium*, *Kocuria*, and *Arthrobacter*). These bacteria are found in aquatic and terrestrial environments. Interestingly, no isolates were identified to represent the Gram-positive lineage Firmicutes. In similar studies, bacteria from this lineage were identified on the skin of Red-backed salamanders [3]. The authors of this salamander study found a diverse group of bacteria with antifungal activity [3]. In a study conducted on the Mountain Yellow-legged Frog, *R. muscosa,* the authors found a large group of antifungal bacteria against *B. dendrobatidis* that also represented genera from the Firmicutes lineage [40].

In addition to being identified via Small Subunit rRNA gene sequencing and phylogenetic analysis, all bacteria identified were used in challenge assays against *B. dendrobatidis in vitro* [38]. Of the 60 tested, 11 isolates were found to be inhibitory to this pathogen and they were species belonging to the genera, *Stenotrophomonas*, *Xanthomonas*, *Pseudomonas*, *Delftia*, and *Chryseobacterium*. All of the organisms found to be inhibitory were Gram-negative and were also from the *Gamma-Proteobacteria* lineage [38].

A high percentage of bacteria isolated in our study have been reported in soil and aquatic environments, therefore they most likely reflect what is found in the habitat of the amphibians sampled. The boreal toad spends the majority of its life in terrestrial environments with the exception of breeding season [41]. The overwhelming representation of Gram-negative bacteria is not surprising considering the numerous bacterial genera found in soil by cultivable methods such as members of *Pseudomonas*, *Flavobacterium*, and the *Chryseobacterium*. Soil is considered to exhibit a stable community structure when there are representation of bacteria from at least nine phyla. Several were represented in this study, including: *Proteobacteria* (*Alpha*-, *Beta*-, and *Gamma*- subdivisions), *Actinobacteria*, and *Bacteroidetes* [42,43]. The stability of community structure in soil is surprising when incorporating several factors such as variation in temperatures, pH, land usage, vegetation, and other community members.

The fact that the spotted frogs and boreal toads shared cultivable bacteria raises the hypothesis that communally shared breeding sites are the source for the resident bacterial members found in this study. These similarities are interesting given that the spotted frog spends the majority of its life in aquatic environments [44] and the boreal toad does not; although the boreal toads were collected close to or in the same body of water as the frogs. It is possible that we would have observed a greater variation between the two amphibian species if they were sampled at other times of the year when they do not share the same habitat (*i.e.* in the summer when toads are found more often on land). Another limitation of our culture study, which may have reduced the variability between sites and species, was that we only sequenced a subset of the isolated bacteria based on morphological characteristics. We may therefore have missed some bacteria. We also only cultured at room temperature and did not employ any other carbon sources than those found in R2 medium.

To address the microflora diversity question in another way we also conducted TRFLP. The AMMI analysis of the TRFLP fragments indicated that there was a definite difference in variation of species in Black Rock Pond (site G) with the 3' fragments for both frogs and toads when compared to other sites. This correlates to the fact that Site G was outside of Grand Teton Valley, instead it is located higher in the mountains and thus may have a different set of natural microbial flora that exists on the skin of frogs and toads [Fig-4]. No significant differences were seen using the 5' fragments for Site G as each data point clustered with the 5' fragments from the other

sites. Comparisons between frogs and toads could not be done for sites C and D because no frogs were sampled from these sites, resulting in fewer samples taken and an underrepresentation could have resulted.

Comparisons of average Margalef (Species Diversity), Shannon Index, and Simpson Index values between sites show that spotted frogs generally have higher values than the boreal toads; however, there was a greater possibility of error in the analysis of the 5' fragments, given the value of standard deviation seen with the 3' samples. This suggests that the 3' fragments offer a more representative look at the different diversity indexes for each of the sites, frog versus toad [Fig-2]. The standard deviation seen in Site A of the 3'fragments indicates that the diversity seen at that site might be less representative than the diversity seen in other sites for the 3' fragments due to its relative size in comparison to the values depicted at the other sites [Fig-2]. The 3' fragments show a significant difference in the diversity between frogs and toads at Site G for both the Shannon and Simpson indices. At Site G it is possible that there is greater diversity in the microbial flora on the backs of columbia spotted frogs than boreal toads, perhaps due to the slight variation of climate preventing the species from being in the same habitat for the same length of time found at the other sites. Both boreal toad and spotted frog values seem to be lower in the 5' strand data than in the 3' strand data. This observation was confirmed in the patterns seen in the *in silico* digest of the cultivated bacterial isolates. For both amphibian species, Schwabacher's Landing (site B) demonstrated higher peak heights than the other sites for Shannon and Simpson indexes [Fig-2], [Fig-3].

The TRFLP data showed some peaks that could be tentatively identified by comparison with the *in silico* digest of our cultivated bacterial isolates using the same restriction enzyme, *Msp* I [Table-3], [Table-4]. Peaks were found on samples from both amphibian species for both the forward and reverse strands, but sites varied in representation. The peaks found from the forward strand of the *in silico* TRFLP digest tentatively identified four bacteria in the cultivation independent analysis of bacterial diversity, at three peak lengths. At peak length 34 two isolates were found, identified as belonging to the genus *Kocuria* and were found from frogs at sites B and G. At peak length 150 one isolate was identified as belonging to the genus *Chryseobacterium* and it was on toads at site D. At peak length 437 one isolate was identified as belonging to the genus *Serratia* and was only found at three of the sites but on both frogs and toads [Table-3]. The peaks found from the reverse strand tentatively identified four bacteria at four peak lengths. At peak length 21 one isolate was identified as belonging to the genus *Chryseobacterium* and was found on frogs and toads from four sites. At peak length 41 one isolate was identified as belonging to the genus *Pedobacter* and was found on both frogs and toads from all sites. At the peak length 90 one isolate was identified as belonging to the genus *Acinetobacter* and was found on toads from site C. At last peak length of 99 one isolate was identified as belonging to the genus *Pedobacter* and was found on frogs and toads from site G [Table-4]. However, these identifications must be considered extremely tentative, as correlating unique TRFLP peaks with individual bacterial genera or species in an environmental sample is highly suspect due to the probability that a single TRFLP peak will contain multiple Operational Taxonomic Units (OTUs) increases as the phylogenetic diversity of a sample increases. The lack of generic-specific and species-specific discriminatory power of the TRFLP technique has been extensively documented [45-47]. The limited number of peak lengths in common from the *in silico* digest and the TRFLP data is likely an underrepresentation of the diversity of the bacteria present on the skin of boreal toads and spotted frogs in part because of our culturing methods as well as the limitation of using one restriction enzyme. Using more than one restriction enzyme might have provided a slightly broader understanding of the non-cultivable microbial community present.

In this project, we confirmed that skin microflora on amphibians in GTNP was diverse. We were able to group 60 bacterial isolates present in the microflora of amphibian skin from GTNP into 5 major taxonomic lineages and 22 genera. Several studies have examined the microflora of other amphibian species using culture techniques but few, until this point, have looked at the bacterial microflora of boreal toads and spotted frogs within the same sites and compared isolates between various sites in the same general geographical region. Studies of the microbial ecosystem involving amphibians are important not only because they provide information about the functional and phylogenetic groups in these environments, but also because they may identify microorganisms that are mutualists with the animals enabling them to co-exist with pathogens such as *B. dendrobatidis*. Further studies, including non-cultivation based experiments with environmental samples, could help answer questions about the antifungal activity of some of these bacteria in Grand Teton National Park, many of which may have the potential to be used as biological control agents.

Author's Contributions: SP carried out cultivation of the bacterial colonies, the molecular genetic studies, the TRFLP study, the sequence alignment, the phylogenetic analysis, and drafted the manuscript. **SP**, **AH**, and **LC** conducted the TRFLP analysis and interpretation. **AH** and **LC** performed an AMMI analysis of the TRFLP data and interpreted it. **MA** participated in the sequence alignment and the TRFLP analysis interpretation. **AH** drafted and edited the manuscript. **SS** participated in the design and coordination of the study, and helped draft the manuscript. **PS** designed and coordinated the study; participated in the sequence alignment, the phylogenetic analysis, and drafting and editing of the manuscript. All the authors read and approved the final manuscript.

Acknowledgements: This research was supported USGS Park-Oriented Biological Support (#77-NRMS) and the Office of Research at Idaho State University. We thank Peter J. Murphy and Sara Bruer for assistance in the collection of skin samples.

Conflict of Interest: None declared

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