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Molecular Analysis of Hybridization in Two North Florida Cricket Frogs, Acris creptians and Acris gryllus

Saisrinivas Ennam

THE FLORIDA STATE UNIVERSITY

COLLEGE OF ARTS & SCIENCES

MOLECULAR ANALYSIS OF HYBRIDIZATION IN TWO NORTH FLORIDA CRICKET FROGS, *ACRIS CREPTIANS* AND *ACRIS GRYLLUS*

By

SAISRINIVAS ENNAM

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Dr. Emily Lemmon

Thesis Director

Dr. Peter Beerli

Outside Committee Member

Dr. Darin Rokyta

Committee Member

Abstract:

Hybridization is the process in which evolutionarily distinct individuals come into contact with each other and interbreed. Hybridization can be evaluated using phenotypic traits from each species. Levels of gene transfer can also be utilized. Hybridization is a universal process and most commonly occurs between closely related species. Cricket frogs (genus: *Acris*) are an anuran species distributed throughout the southeastern U.S. This genus is split into three individual species, *Acris crepitans*, *Acris gryllus,* and *Acris blanchardi*. We aimed to test the levels of hybridization between *A. gryllus* and *A. crepitans* within the Big Bend region of northern Florida where the two species interact in sympatry. We sampled frogs from 10 different sites with 67 samples total. To assess hybridization, we extracted and sequenced DNA for thousands of loci and conducted several population generic and hybridization analyses. We hypothesized that there can be one of three possible outcomes: 1) there are strong species boundaries between *Acris crepitans* and *Acris gryllus*, 2) that there is unidirectional gene flow between *Acris crepitans* to *Acris gryllus* or vice versa, and that the other species has little to no gene flow, or 3) there is equal gene flow between the two species. Overall, we found little support for hybridization where the two species cooccur, suggesting strong reproductive boundaries between *A. crepitans* and *A. gryllus.*

Introduction:

Speciation is a phenomenon that occurs when new distinct species arise along the evolutionary timeline. Although there are many ways in which new species are formed, two of the most well-known examples of speciation are sympatric and allopatric speciation, which are

defined by the presence or absence of interactions between the two new species as they are formed, respectively (Mallet et al. 2009). Generally, allopatric speciation is the most common of the two, however, the geographic separation of the two species is often not permanently maintained, resulting in their secondary contact.

Hybridization commonly occurs when two species come into secondary contact. This phenomenon may result in the collapse of the two species, the formation of new hybrid species (eg. anoles and cichlids, Köhler et al. 2010; Meier et al. 2017), or the reinforcement of species boundaries to prevent hybridization and complete the speciation process (Lemmon 2009; Coyne & Orr 2004). If the two species have diverged ecologically, often their hybrid zones occur in intermediate areas of their preferred habitats, and in some cases, these intermediary hybrid zones may be maintained over long periods (Barton & Hewitt 1985; Engebretsen et al. 2016).

Hybridization can have negative effects on the hybrid offspring's fitness. Prezygotic isolation is one means of preventing hybridization of two species prior to fertilization and may be driven by several differences such as behaviors including songs in birds and frogs or by physical barriers that prevent fertilization by some phenotypic mechanism (Coyne & Orr 2004). Post-zygotic isolation is another way in which hybridization is prevented. In postzygotic isolation, fertilization occurs, but the offspring produced via this hybridization have reduced fitness due to hybrid inviability or sterility that prevents reproduction of hybrids and the continuance of hybrid lineages.

Cricket frogs (*Acris)* consist of three currently recognized species that are widespread across the central and eastern United States. *Acris crepitans* is known to inhabit a wide range of places across the eastern United States but is most associated with closed canopy wetlands (Gamble et al. 2008). Conversely, *A. gryllus* is most associated with open canopy wetlands in

coastal plain ecosystems from the Mississippi River to the Atlantic coast (Gamble et al. 2008). Although these two species have diverged somewhat ecologically, there are several areas where their ranges overlap. In these areas, phenotypic characters that differentiate the two species may become unreliable (Jensen 2008), suggesting the hybridization of two species. However, no study to date has investigated this phenomenon and its genetic signatures.

Here, we will investigate the hybridization of *A. crepitans* and *A. gryllus* in the Big Bend region of Florida using next-generation sequencing and modern population genetic analyses. We predicted that the data would reveal one of three possible outcomes may occur. First, that strong isolating barriers between exist the two species of frogs preventing significant gene flow between the two species. Second, that hybridization occurs between the species and that gene flow between the two species is bidirectional and relatively uniform. Third, that there is unidirectional gene flow solely from one species and little to no gene flow from the other species. The results of this study will help us better understand the evolutionary history of *Acris* and will be informative for understanding hybridization in anurans more broadly.

Methods:

Sample Collection: Individuals were collected from four different sites along the Ochlocknee River where potential hybrid zones were present (Rock Bluff in Leon County, Cemetery Pond in Leon County, Near Rock Bluff in Leon County and the Ochlocknee river in Lee County). 42 samples were collected along the Ochlocknee river. To have true allopatric samples from each species we received seven samples of *Acris crepitans* from the North Carolina Museum of Natural Sciences from two locations (Rockingham County and Alamance County, North Carolina) and 16 samples of *Acris gryllus* from four locations (Marion County,

Alachua County, Taylor County Florida and Warren County, Kentucky) from the Florida Museum of Natural History which were collected from two locations. There were a total of 10 (mean= 6.5 individuals per location, range= 3-12 individuals per location) locations where 65 frogs were sampled (Table 1 in Supplementary Tables). Frogs were then anesthetized with benzocaine gel, photographed, and measured for phenotypic characteristics, and dissected for tissue used in DNA extraction. Liver and leg tissue were collected from each sample and placed into respective storage tubes containing tissue buffer at room temperature to preserve tissue. Samples were stored at -10°C before being transferred to -40°C prior to extraction.

DNA extraction and sequencing: DNA was extracted using E.Z.N.A Tissue DNA Kit and was sonicated soon after extraction, sonicated DNA will be 300-800 bp. This was done using Covaris E220 Focused Ultrasonicator with respective Covaris microTUBES. Following extractions samples were then run through gel electrophoresis in 2% agarose gel at 110 V for one hour to establish that our extractions yielded quality DNA. To determine DNA concentration per sample a Qubit fluorometer quantitation will be run and concentrations will be given in ng/ ml. In order to analyze levels of hybridization among *A. crepitans* and *A. gryllus,* we prepared Illumina libraries by 1) fragmenting DNA enzymatically (i.e. ftp; Ignatov et al. 2019), ligating a common adapter (i.e. the i7 adapter of Meyer and Kircher 2010), then amplifying regions flanking the repeat seququence GGCCCCGGCCC. Amplification is performed using a tailed primer, in which the primer consists the repeat sequence and the tail consists of the i5 indexing primer of Meyer Kircher (2010). The repeat sequence was estimated in preliminary analyses to occur 2054 times in 4 gigabases of *Pseudacris feriarum* illumina reads (obtained from Banker et al. 2020). The resulting libraries were pooled and sequenced on an Illumina NovaSeq sequencer

using a PE150 protocol on a SP flow cell. An average of 3.4 million read pairs (1Gb) were collected for each sample.

Population structures, distribution, and genetic variation: After merging overlapping reads via the method of Rokyta et al. (2012), we converted reads from a standard fastq format to a ddRAD-style fastq format by replacing the G2C4G2C3 priming sequencing at the beginning of the read with the sample-specific barcodes followed by the restriction tag TGCAG. The reads were then processed using STACKS (v2.55; Catchen et al. 2011) to produce SNPs. We used these SNPs to estimate the population genetic structure of *Acris* to characterize the distribution of genetic variation across the sampled area. Prior to any analyses the data was filtered to remove SNPs with more than 20% of data that was missing. We then utilized the program STRUCTURE Version 2.3.4 with 176 loci using one SNP per locus for 57 individuals. The structure analysis was run We were able to assign the best fit for variation patterns we found in the samples we collected. (Lawson et al. 2018) We ran the structure analysis at different K levels; which are genetically distinct clusters with ten replicates per K value. (K=1, K=2, K=3) STRUCTURE was run using 200,000 MCMC steps with 20,000 steps of burnin. Depicted in Figure 2. Once genetic clusters have been identified Heirfstat V 0.5-1.1 was used to run a genetic PCA using 2047 SNPs from 57 sampled individuals to better understand the population structures of *A. crepitans* and *A. gryllus* as well as to control the stratification amongst populations, reducing dimensionality between them (Agrawal et al. 2020; Figure 3)

Hybrid indexing: Following the PCA analysis hybrid indexes were utilized to test our hypotheses. Introgress V1.2.3 was used to run the hybrid indexes. Hybrid indexes were quantified for 37 individuals sampled, 88 fixed differences were observed between the samples of *A. crepitans* and *A. gryllus* collected. Finally, phenotypic characters will be analyzed to

identify characters that differentiate the two species and their hybrids. The genotypes for each individual were recorded. (Number of distinct loci with homozygous *crepitans* and homozygous *gryllus,* the number of distinct heterozygous individuals as well as if there was any missing data). For more information refer to Table 3 in the supplemental tables.

Morphologic data: Individuals collected were measured using ImageJ. Lengths from the snout to vent, femur, tibia, and head width were measured. A total of 42 individuals were measured (Table 2 in Supplemental Tables). To identify if an individual was either *A. crepitans* or *A. gryllus* diagnostic tools were used, namely the presence of a ventral stripe. (Micancin & Mette 2009) If an individual had a continuous ventral stripe present it is classified as *A. gryllus* and if the stripe was not continuous then the individual was classified as *A. crepitans*.

Results:

DNA extraction and sequencing:

Once DNA was sequenced, we yielded approximately 3.4 million reads per sample. To produce SNPs, we merged reads together utilizing STACKS. We were able to capture 146,423 SNPs which were then filtered to yield 2047 SNPs across 176 loci per individual.

Population structures, distribution and genetic variation:

Because phenotypic diagnostic characteristics are sometimes unreliable where the two species overlap, we expected to observe significant amounts of gene flow where these two species are sympatric. The STRUCTURE analysis revealed that the sampled Acris fall into two distinct clusters corresponding to the two species investigated. There was no significant additional support for clusters outside two corresponding the two species investigated (Figure 2).

Moreover, no individuals or populations had significant cluster proportions outside of that for the identified species or population ecotype (i e open or closed canopy)

The genetic PCA analysis easily distinguishes the two species based on the first principal component (Figure 2). As shown PC1 explains 54.33% of the variance between the samples collected. PC2 was able to explain only 3.9% of the variance. We observed only one sympatric sample that did not tightly cluster with its species. But when investigated further this individual was determined to have missing approximately 15% missing data and this pattern could have affected the clustering of this individual.

Hybrid indexing:

Our hybrid indexing revealed very little if any hybrid genetic content in any of the sympatric samples (Figure 2). These results suggest there is very little if any gene flow occurring between *A. crepitans* and *A.gryllus* where sympatric. However, we also observed that there was a small proportion of SNPs that were fixed in the allopatric populations but were present in the other species in the sympatric populations (Table 1). While this could be evidence of some gene flow, it is possible that the large geographic distance between allopatric and sympatric *A. crepitans* resulted in using fixed differences for our hybrid index that are not in fact fixed throughout all *A. crepitans.*

Morphologic data:

Morphologic and genetic data showed that the diagnostic character and completeness of the thigh stripe was an accurate diagnostic for species and genetic makeup. We found no individual that wasn't easily defined to species by this character, this character corresponded to both the genetic makeup and ecosystem samples.

Discussion:

Our findings suggest the presence of strong species boundaries in the localities in which *Acris crepitans* and *Acris gryllus* habitats converge. We found little evidence of gene flow between the two species in sympatry but based on the presence of few heterozygote SNPs we were able to conclude that the two species could have interbred and converged due to previous migratory events. With this result it is likely that there are strong reproductive isolation barriers preventing hybridization. Increasing the sample size and range of *A. crepitans* being analyzed can allow us to draw better conclusions on the presence of hybridization events. Following the PCA analysis we observed that an individual we collected (Sample ID: 134931) was missing 56 out of the 88 fixed differences between *A. crepitans* and *A. gryllus* making it difficult to draw conclusions about its ancestry. When determining the presence of hybrid zones, we only took into consideration the presence of coinciding habitats between the two species. We did not take into consideration tension zones which are areas within the hybrid zones that have negative selection against individuals with hybrid ancestry. The position of respective tension zones are influenced by density of interacting species (Taylor et al. 2015). Morphological data though deemed inconsistent with hybrid zone, showed to be consistent with the diagnostic leg stripe for populations sampled.

When running the STRUCTURE analysis we yielded two major clusters, but upon further analysis of the hybrid indices we observed that *A. crepitans* displayed greater hybrid ancestry in comparison to *A. gryllus*. This could suggest a potential hybridization event that took place in the past (Fitzpatrick, B.M. 2012). Further investigation of the SNPs yielded through sequencing *Acris crepitans* contained a large proportion of the missing data. The large amounts of missing data made it difficult to assess hybridization between the two species as there was a

fewer number of fixed differences observed between *A. crepitans* and *A. gryllus*. When collecting allopatric samples for each species the distance between the samples was ignored which could have potentially biased our results.

Further work could use Combined Fitness Measure (CFM) for pure allopatric parental populations to compare to the offspring in sympatric F_1 populations where the habitats of the two species can potentially coincide to determine hybrid fitness in sympatry (as in Behrmann-Godel J & Gerlach G. 2008). Additionally, testing models of hybridization using software such as Migrate (Beerli, P. & Palczewski, M. 2010) or Approximate Bayesian Computation (ABC) (Aeschbachler, S. et al 2013) would allow for more direct testing of hypotheses.

Migration frequencies can be used to quantify the number of species and individual's incoming into a certain environmental niches which would then allow us to calculate how many individuals of either species is coming into contact with the other. Increased interspecies interactions can potentially lead to an increased interbreeding between them. Migratory frequency hypotheses can be quantified by the usage of approximate Bayesian computation (ABC) in which nuisance parameters are eliminated. In ABC we could use the spatially subdivided populations of *Acris* in the localities we sampled along with sequencing data we can determine the migration between low and zero migration. (Aeschbachler et al. 2013).

We can observe not only hybrid indexes to quantify levels of hybridization, but we can use methods similar to Good et al. (2015) to determine the levels of nuclear introgression if at certain localities there is hybridization occurring between A. gryllus and A. crepitans. This will also aid in calculating levels of gene transfer between the species and it can help determine if the two species are interbreeding and creating an entirely new species in areas where the habitats can coincide. Another factor we did not take into consideration was the presence of tension zones.

With tension zones we can determine better what locations would be best to sample for hybridization events that occur amongst the two species, the greater the density of the interactions between the two species the greater the potential for hybridization.

Conclusion:

With the results from our study, we are able to better understand the species boundaries of *Acris crepitans* and *Acris gryllus*. This is useful in understanding the reproductive isolation between the two species. We can also understand the evolutionary history of species within the genus *Acris*. Potential future perspectives for our research are that we can use the results to study the process of reinforcement, which is the phenomenon in which reproductive isolation is exacerbated through natural selection.

Acknowledgments:

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Tables:

Table 1: Hybrid index data, 37 individuals were assessed using 88 distinct differences amongst the two species investigated. Homozygous *crepitans* and Homozygous *gryllus* shows the distinct differences attributed to *A. crepitans* and *A. gryllus.* Heterozygous shows the distinct differences present in both species.

Individual ID	Homozygous *crepitans*	Homozygous *gryllus*	Heterozygous	Missing
I34367	82	3	0	4
I34368	84	2	1	2
I34369	83	3	0	3
I34370	82	0	2	5
I34371	70	3	0	16
I34372	84	0	0	5
I34373	81	0	0	8
I34374	80	3	0	6
I34375	78	3	0	8
I34376	79	2	0	8
I34377	82	3	0	4
I34378	0	83	5	1

I34379	0	74	1	14
I34380	0	85	0	4
I34381	0	83	2	4
I34382	0	85	0	4
I34383	1	85	1	2
I34384	1	76	1	11
I34385	1	83	2	3
I34386	0	84	1	4
I34387	0	83	2	4
I34388	0	68	2	19
I34389	0	82	1	6
I34390	0	79	1	9
I34391	0	31	2	56
I34393	0	84	0	5
I34394	0	85	1	3
I34395	0	87	0	2
I34396	0	82	1	6
I34397	74	1	7	7
I34398	84	3	2	0
I34399	79	1	3	6
I34400	81	0	3	5
I34401	71	3	2	13
I34402	81	2	0	6
I34403	82	1	2	4
I34404	79	1	1	8

Table 2: Genetic PCA values, corresponding to individual and population said individual was from. PC1 and PC2 values displayed.

Individual	Population	PC1	PC2
I34367	1	-10.296022	-2.1747663
I34368	1	-10.725603	-2.8612336
I34369	1	-10.521271	-2.7166244
I34370	1	-10.192738	-1.76529
I34371	1	-9.4593879	-2.5897315
I34372	1	-10.347751	-1.2958298
I34373	1	-10.151174	-2.0516175
I34374	1	-10.355228	-2.4774651
I34375	1	-10.240375	-3.0277068
I34376	1	-10.267639	-2.4704619

I34377	1	-10.315315	-3.1893663
I34378	2	9.20224817	-0.0508959
I34379	2	8.89835131	-0.1908604
I34380	2	9.80569946	0.30305444
I34381	2	9.46861415	-0.4914876
I34382	2	9.66064168	-0.2973622
I34383	2	9.7544309	0.04534999
I34384	2	8.52998341	-0.0031523
I34385	2	9.56463295	0.00710037
I34386	2	10.180324	-0.2929688
I34387	2	9.56088681	-0.3263576
I34388	3	7.67046951	-0.0023515
I34389	3	10.0617295	0.19604586
I34390	3	9.08567129	-0.0242578
I34391	3	3.99792165	-0.1050966
I34393	3	9.2085201	-0.2550688
I34394	3	9.87157044	0.34258696
I34395	3	9.30055096	-0.5018043
I34396	3	9.45237796	0.0756558
I34397	4	-9.5806441	-2.2938184
I34398	4	-10.448109	-1.8776682
I34399	4	-9.9958793	-1.2466233
I34400	4	-10.15512	-1.1351379
I34401	4	-9.5703282	-1.8139549
I34402	4	-10.408236	-2.050494
I34403	1	-10.038527	-2.3475336
I34404	1	-10.008382	-1.5206916
I34406	6	9.44184255	0.01922701
I34407	7	-5.9915814	1.23534899
I34408	5	10.113317	-0.1906902
I34409	8	9.53867424	-0.2700599
I34410	5	9.17253275	-0.0615424
I34412	7	-3.3031771	1.01258493
I34413	5	9.79149825	-0.1349909
I34414	6	8.88108428	-0.2419272
I34415	6	6.52472107	-0.716285
I34416	8	9.41396376	-0.1293965
I34417	5	9.92670984	-0.1651944
I34418	6	7.95598887	-0.0989236
I34419	8	9.31812879	-0.1385817
I34421	9	-7.5983942	5.27212881

I34422	0	-9.8128664	7.03050952
I34423	9	-9.4012371	6.27423629
I34424	0	-6.9026293	5.35883216
I34425	0	-9.4081784	6.53092731
I34426	0	-9.9562567	6.47019428
I34427	0	-7.9010356	5.4214882

Figures:

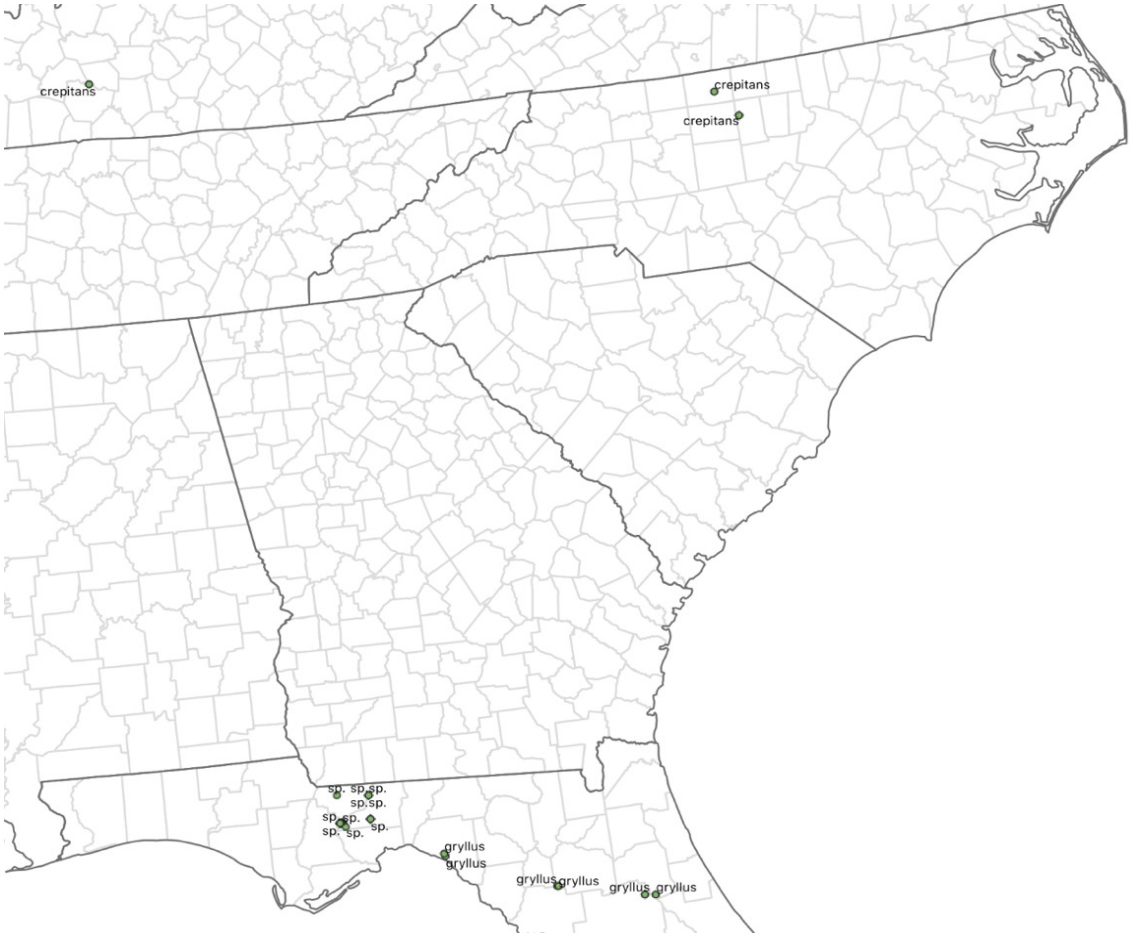

Figure 1: 66 individuals were collected at locations marked upon the map. Species names are given only to those that are true allopatric species *(A. gryllus* or *A. crepitans*). Sympatric samples are denoted as Sp.

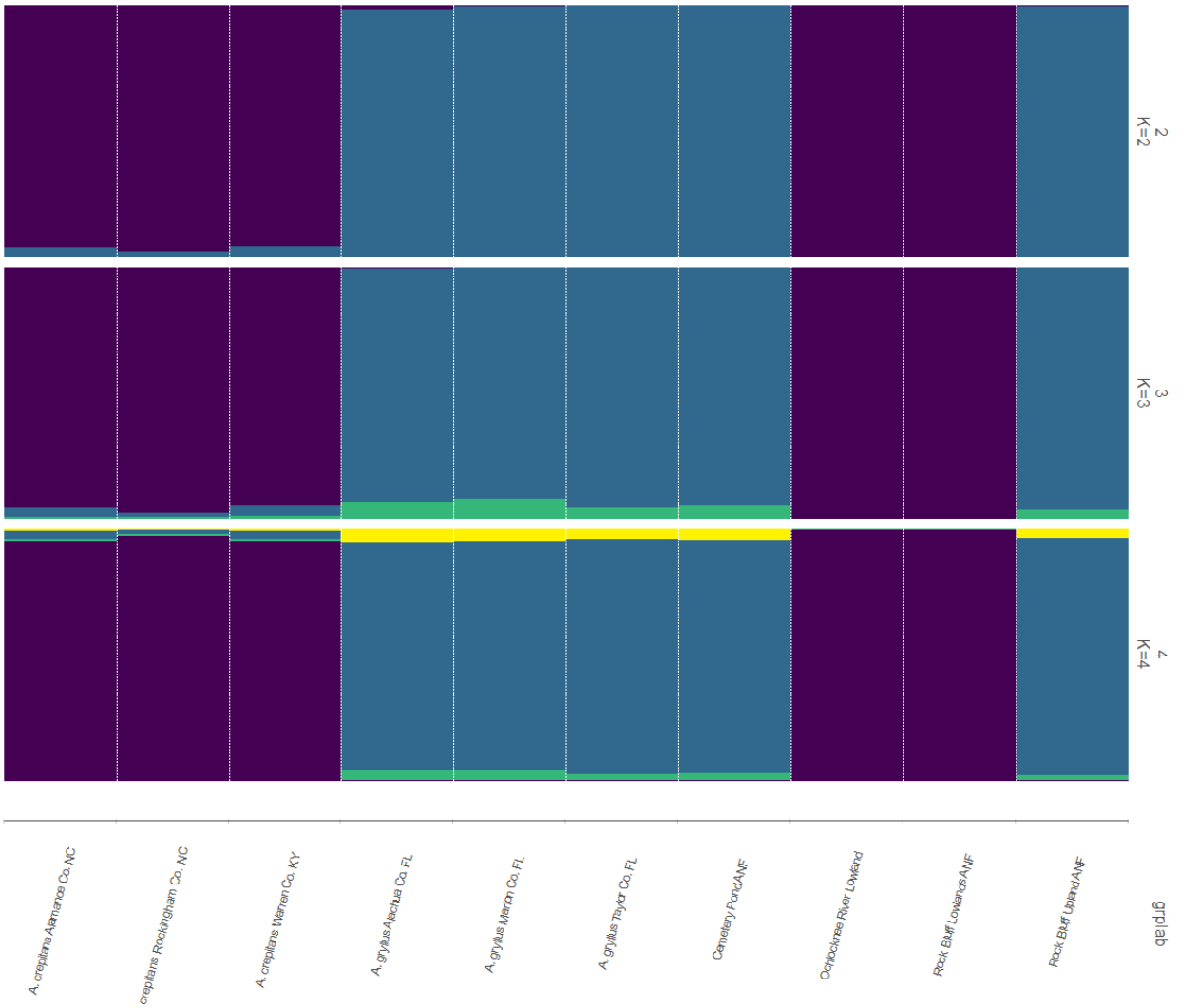

Figure 2: STRUCTURE results at K=1, K=2 and K=3. Populations are depicted on the X-axis. Two colors that make up the bulk of the cluster proportions represent the two species, with blue represents *A. gryllus* and purple representing *A. crepitans*.

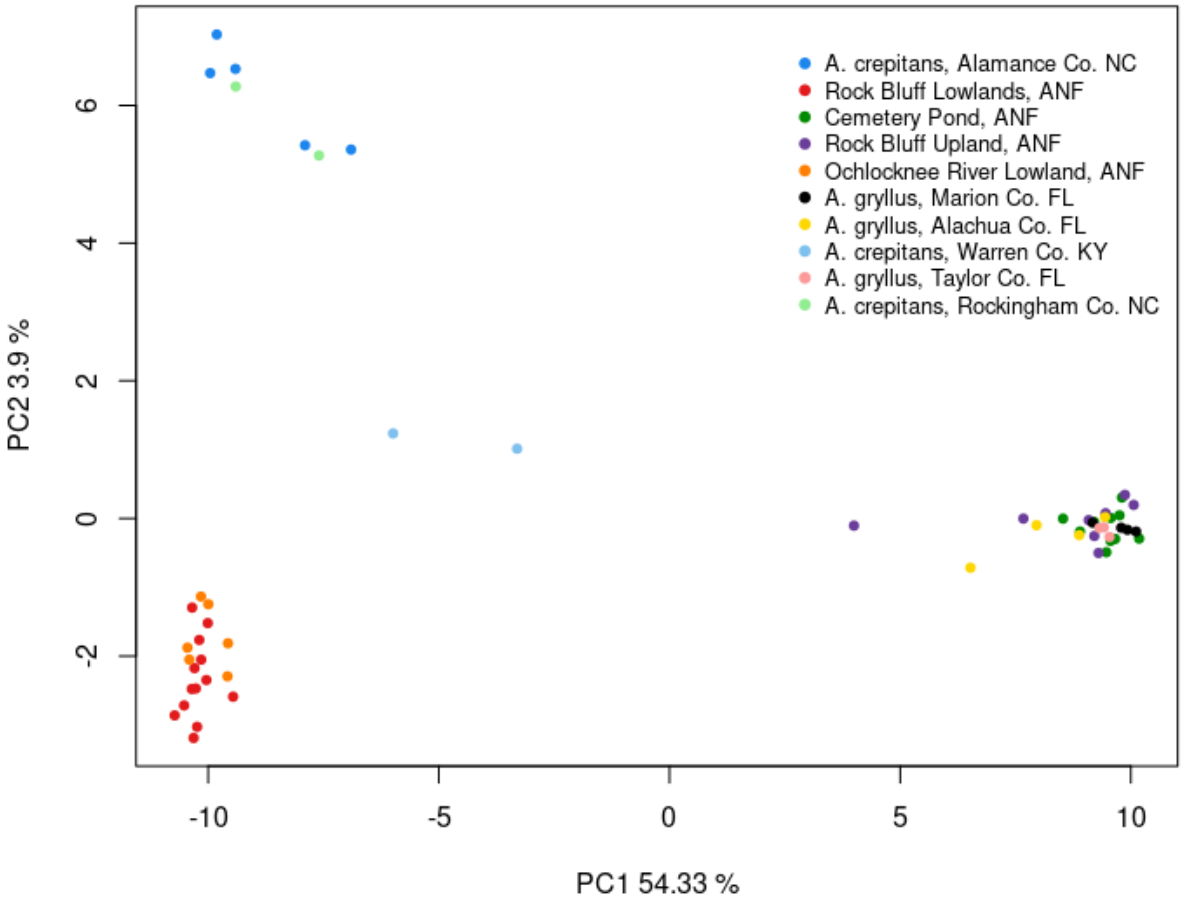

Figure 3: PC1 and PC2 values from the genetic PCA analysis for all allopatric and sympatric samples. Percentages next to PC axis represent the amount of variation explained by the principal component. Each point represents a single individual and points are colored by population.

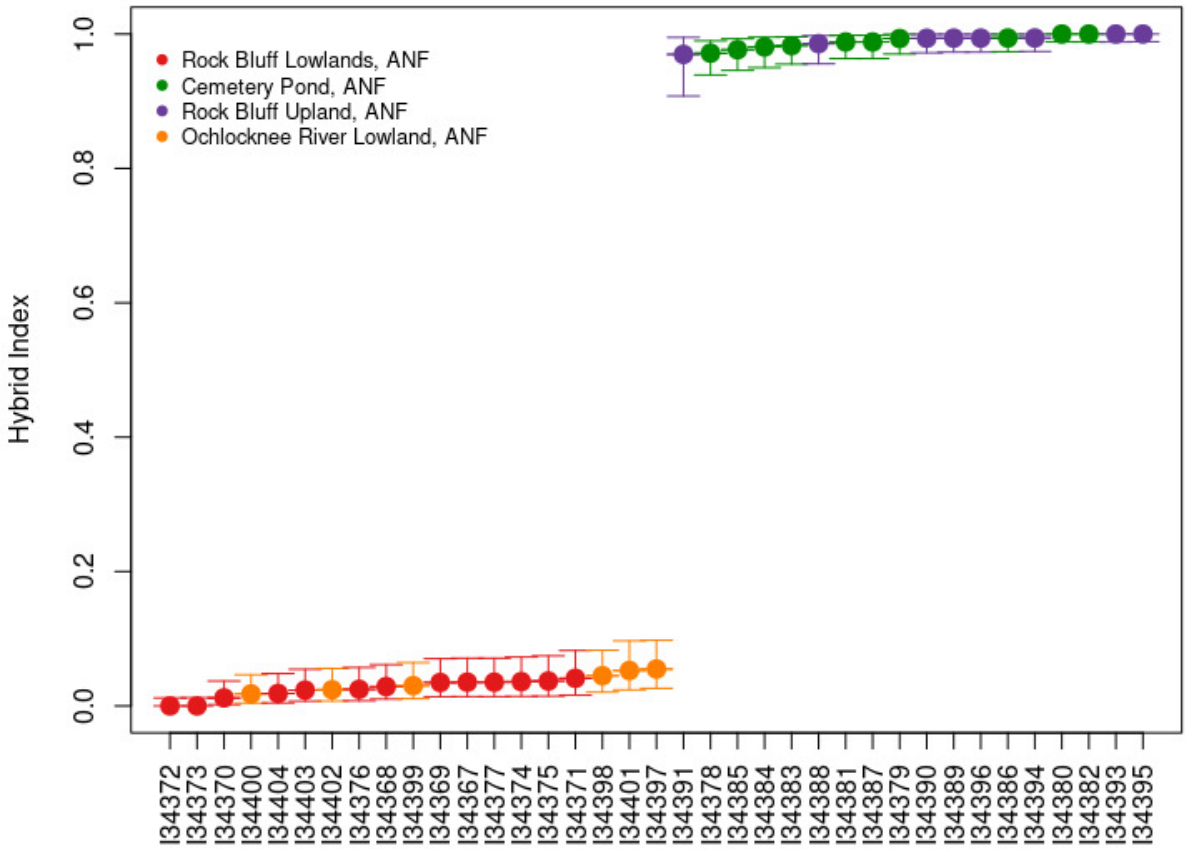

Figure 4: Hybrid Indices for each sympatric individual. Hybrid index values closer to zero represent *A. crepitans* and values closer to one represent *A. gryllus.* Error bars represent the 95% confidence interval and colors represent the populations sampled.