Combining morphological, acoustic, and genetic techniques to better understand hybridization of the most abundant toad in Alabama: *Anaxyrus fowleri*

by

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Abstract

The *Anaxyrus americanus* species complex has provided an interesting group of species to study hybridization. Two species in particular, *Anaxyrus americanus* and *Anaxyrus fowleri,* are known to hybridize in northern portions of their geographic range. In this study, I examined morphology, male anuran advertisement calls, and genetics for these species to determine the frequency of hybridization between these two species in Alabama and the reliability of morphology to detect genetic hybridization. I examined morphology for three hybridizing species of toads within the *A. americanus* species complex: *A. americanus, A. fowleri* and *A. terrestris. Anaxyrus terrestris* was included in morphological analysis due to its abundance in Alabama. I measured five key morphological characteristics and snout-vent length for all Auburn Natural History Museum specimens that were collected in Alabama: height of the junction of the interorbital and postorbital crests, width of largest tibial wart, size of largest dorsal wart relative to size of dorsal dark spot, and length and width of contact of the postorbital crest with the parotoid gland. Using discriminant function analysis (DFA), morphology revealed approximately 16% hybridization occurring among these three species. I then examined male advertisement calls of *A. americanus* and *A. fowleri* to determine if character displacement specifically occurred for *A. fowleri* in sympatry with *A. americanus* in current Alabama populations. New specimens were collected from extant populations in northern Alabama in order to examine advertisement calls as well as morphology and to perform genetic analysis on current populations of these species. Recordings from males calling in the field were measured using six

call parameters: call duration, pulse rate, length of call, dominant frequency, length of pulse, and time between calls. No evidence of character displacement was seen between *A. fowleri* in allopatry and *A. fowleri* in sympatry with *A. americanus.* Nuclear DNA was extracted from tissue samples of the new specimens, sequenced using the next-generation sequencing technique, genotype-by-sequencing, and analyzed using fastSTRUCTURE. Only 5.4% of individuals genetically examined showed evidence of hybridization. High gene flow was seen between all *A. fowleri* populations regardless of location in Alabama. High genetic and acoustic variation was seen in *A. americanus*. The DFA of call parameter data showed individuals gave species distinct calls regardless of genetic influence. Through comparison of the morphological DFA and genetic analysis of the recently collected specimens, known genetic hybrids weakly correlated with morphological characteristics.

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DFA	Discriminant function analysis
GBS	Genotype-by-sequencing
MDS	Multi-dimensional scaling
mtDNA	Mitochondrial deoxyribonucleic acid
nDNA	Nuclear deoxyribonucleic acid

CHAPTER 1

INTRODUCTION

For decades, philosophers and biologists alike have debated what constitutes a species. As a result, there are many different concepts and ideas that have been used to help explain what we should consider a species. One accepted concept is the Cohesion Species Concept, which categorizes species as "population, or series of populations, that has genetic or demographic cohesion" (Templeton 1989). This concept can be seen in the literature on anuran sexual selection because female anurans are hard wired to detect advertisement calls from males of their species, maintaining species cohesion (Gerhardt and Huber 2002). This process encourages conspecific mating and discourages heterospecific mating; therefore in many cases, anuran mating patterns are consistent with predictions of the Cohesion Species Concept.

Despite the prezygotic barrier of advertisement calls, anuran species are known to hybridize, a feature of reproduction that is broadly distributed among anurans, including *Pseudacris*, *Hyla*, and *Anaxyrus* among local genera. In anurans, hybridization breaks down the cohesion barrier and can make it difficult to classify hybrid individuals to one specific species (Blair 1941; Blair 1963a; Cicort-Lucaciu and Covaciu-Marcov 2011; Templeton 1989). Hybridization can carry many consequences including failure to pass on genetic information or reduced offspring fitness (Fitzpatrick 2004; Lemmon and Lemmon 2010; McDade 1990). If advertisement calls of hybrid males are not as distinct to females as those of conspecific males, hybrids can be less reproductively successful than pure males with species distinct advertisement

calls (McDade 1990). Although hybridization exists, strong selection against hybrids can explain the existence of character displacement in calls of sympatric anurans (Blair 1974). Evidence of such reinforcement, can be seen in studies of *Pseudacris feriarum* and *P. nigrita* (Lemmon 2009; Lemmon and Lemmon 2010) and *Hyla gratiosa* and *H. cinerea* (Höbel and Gerhardt 2003). Specifically, lab-created hybrids of *P. feriarum* and *P. nigrita* had 44% reduced fitness levels due to female selection against male hybrids and reduced fertility of hybrid males (Lemmon and Lemmon 2010). Similarly, in areas where these two species co-occur, call characters of the less abundant species exhibit significant character displacement relative to the more abundant species (Lemmon 2009). Female *P. feriarum* prefer conspecific males in sympatry, reducing hybrid success by 60% compared to the fitness of a conspecific male (Lemmon 2009). In *H. gratiosa*, female preferences for conspecific calls over calls of other male species are greater in areas of sympatry than in areas of allopatry (Höbel and Gerhardt 2003).

Although one may expect character displacement to reduce the chance of hybridization becoming a significant reproductive event, it is not always observed. One example in anurans is the hybridization of *Anaxyrus fowleri* (Fowler's toad) and *Anaxyrus americanus* (American toad), a closely related species also occurring in the southeastern United States. Males of *A. fowleri* produce calls with a whining quality, made up of low pulse rates, whereas males of *A. americanus* produce whistling calls comprised of high pulse rates (Conant and Collins 1998). Despite the fact that these species' possess significantly different advertisement calls, hybrids occur frequently, suggesting that the calls may not be very effective pre-zygotic barriers (Blair; Green 1984; Zweifel 1968). Hybrids involving *A. fowleri* are thought to produce fertile offspring, making this species a good candidate for studying hybridization (Blair 1941; Blair 1963a).

In order to classify anurans as hybrids or pure stock of a particular species, some taxonomists rely on morphology (Conant and Collins 1998; Mount 1975). *Anaxyrus fowleri* is unique among eastern toads in having postorbital crests that directly contact the parotoid glands, typically have two or more warts per dorsal dark spot, and do not exhibit enlarged tibial warts as seen in *A. americanus*. In comparison, *Anaxyrus americanus* possesses a posteriorly directed spur coming from the postorbital crest that contacts the parotoid gland, enlarged tibial warts, and a single wart per dorsal dark spot or no dorsal dark spots. Toads with intermediate or unusual combinations of *A. fowleri* and *A. americanus* morphological characters have been found throughout Alabama, which suggests that these species may hybridize in this area (Blair 1963a; Weatherby 1982).

In addition to morphological analysis, studies of natural hybridization have benefitted from molecular tools, which allow measures of hybridization that can be more precise and indicate a complex picture of hybridizing species. Such methods have improved our knowledge of toad species and hybridization in Alabama (Fontenot et al. 2011; Masta et al. 2002). These observations question the utility of distinguishing species or hybrids based on morphology alone. These problems are particularly acute in morphologically pure *A. fowleri*, which may not constitute a monophyletic taxon (Masta et al. 2002).

In light of these recent observations I propose to ask the following questions: 1) Are putative morphological hybrids of *A. fowleri* and *A. americanus* putative genetic hybrids? 2) Is there evidence of character displacement in the advertisement calls between *A. fowleri* in allopatry and *A. fowleri* in sympatry with *A. americanus*? 3) Do male putative genetic hybrids of *A. fowleri* and *A. americanus* produce advertisement calls with intermediate qualities? And 4) How extensive is gene flow between sympatric populations of *A. fowleri* and *A. americanus*? In

order to address these questions, I will utilize morphology, advertisement calls, and genetics of *A. fowleri* and *A. americanus* individuals from sympatric populations and from allopatric populations of *A. fowleri*.

In Chapter 2, I examined morphology of *A. americanus*, *A. fowleri* and *A. terrestris* museum specimens from the Auburn University Natural History Museum to estimate putative hybridization based on key morphological characteristics. Of all museum specimens collected from Alabama, I found roughly 16% hybridization between the three species based on morphological evidence. In Chapter 3, I examined advertisement calls of *A. americanus* and *A. fowleri* from a sympatric population and *A. fowleri* from an allopatric population and found no evidence of character displacement in *A. fowleri*. Finally, in Chapter 4, I used measurements of key morphological characteristics, measurements of male advertisement calls, and sequences of nuclear DNA of *A. americanus* and *A. fowleri* to examine gene flow and the hybridizing relationship between these two species in Alabama. I found approximately 5.4% genetic hybridization between Alabama populations of *A. americanus* and *A. fowleri* with significantly more genetic hybrids found within the *A. americanus* population than the *A. fowleri* population. Known genetic hybrids were weakly correlated with morphological hybrids, and male genetic hybrids did not give intermediate advertisement calls.

CHAPTER 2

HYBRID MORPHOLOGY OF *ANAXYRUS AMERICANUS, ANAXYRUS FOWLERI* AND *ANAXYRUS TERRESTRIS* IN ALABAMA

INTRODUCTION

Morphology is the primary tool used to identify species in field and museum settings. Taxonomists also rely on morphology to document hybridization between pairs of species or intergradation between pairs of subspecies. For example, Dessauer et al. (2000) demonstrated that individuals within the zone of contact between *Cnemidophorus tigris punctilinealis* and *C. t. marmoratus* had intermediate morphological features, and were most prevalent at the center of the zone where both species were in the highest contact. Similarly, Brede et al. (2000) used morphology to document that an introduced population of *Triturus carnifex* hybridized with a native species (*Triturus cristatus*) at the introduction site, but that the influence of the invasive species has not spread to other populations.

Anaxyrus americanus, *Anaxyrus fowleri*, and *Anaxyrus terrestris* are three species of toads in eastern North America that are difficult to identify because they hybridize (Blair 1941; Blair 1972; Green 1984; Volpe 1952). Four dichotomous morphological features are used to diagnose these species: 1) large dark dorsal spots with more than one wart per spot (*A. fowleri* and *A. terrestris*), or small spots with a single wart or lacking spots entirely (*A. americanus*); 2) postorbital crest touching parotoid gland via a spur—a thin raised region located on the anterior-dorsal region of the head—(*A. americanus* and *A. terrestris*) or touching parotoid gland directly

(*A. fowleri*); 3) junction of interorbital and postorbital crests with enlarged, knob-like projection (*A. terrestris*) or lacking a knob (*A. americanus* and *A. fowleri*); and 4) tibial warts large (larger than femoral or tarsal warts; *A. americanus*) or small (same size as femoral or tarsal warts; *A. fowleri* and *A. terrestris*). Additionally, the three species differ in call pulse rate (high pulse rate whistle in *A. americanus* and *A. terrestris*; low pulse rate whine in *A. fowleri*) and duration (short in *A. fowleri* and *A. terrestris*; prolonged in *A. americanus*) (Conant and Collins 1998; Cook 1983; Green and Parent 2003; Green and Pustowka 1997; Mount 1975). Hybrid individuals with intermediate calls are observed in nature and are known to possess intermediate external morphology as well as suspected to possess intermediate morphology in the larynx which causes them to create intermediate calls (Blair 1941; Blair 1972; Martin 1971; Volpe 1952; Weatherby 1982; Zweifel 1968).

Anaxyrus americanus and *A. terrestris* are separated geographically, with *A. terrestris* restricted to the Coastal Plain of the southeastern United States and *A. americanus* occurring above the Fall Line (Blair 1963a; Blair 1974; Leary 1988). In Alabama, the two species have a narrow zone of overlap along the Fall Line, a boundary between the eastern Gulf Coastal Plains and the Appalachian Highland regions, where populations producing calls intermediate between the two species are present and individuals with intermediate morphology are common (Mount 1975; Weatherby 1982). *Anaxyrus fowleri* is found throughout the state of Alabama without geographic limitations (Mount 1975).

The three species have overlapping breeding seasons, with *A. americanus* beginning at the end of January, peaking in March, and ending in May; *A. terrestris* beginning in the middle of March and ending in July; and *A. fowleri* starting in March and ending in August (Mount 1975). Overlap in both geography and breeding seasons suggest ample opportunities for

hybridization as seen by Blair (1963a) and Mount (1975). Although it is suggested that hybridization exists among all three species, Mount (1975) states there is morphological evidence of gene flow between *A. americanus* and *A. terrestris* along the western portion of *A. americanus* distribution and morphological evidence of *A. fowleri* and *A. terrestris* hybrids throughout the state, but no evidence of *A. fowleri* and *A. americanus* hybridization. The extent of genetic introgression remains unknown among these species given the variation in phenotypes of putative hybrids (Mount 1975).

Masta et al. (2002) recovered a paraphyletic assemblage of *A. fowleri* specimens based on aspects of the mitochondrial genome, with a southern lineage of *A. fowleri* having mitochondrial sequences most closely related to sequences of morphologically-pure *A. terrestris,* and also because of the presence of individuals possessing hybrid morphology and mitochondrial introgression between *A. americanus* and *A. fowleri*. Fontenot et al. (2011) compared nuclear DNA (nDNA) with advertisement calls of the same three species. These authors found one individual that identified morphologically as *A. fowleri,* but possessed portions of the nuclear genome that fall within the same clade as *A. terrestris* individuals. One individual was morphologically *A. americanus* with a whistling advertisement call, but portions of a nuclear genome that fall within the same clade as *A. fowleri* individuals. Four individuals were identified morphologically as *A. terrestris* with a whistling advertisement call, but portions of a nuclear genome that fall within the same clade as *A. fowleri* individuals. These studies create ambiguity in accurate identification of these species. In order to reduce uncertainty, further assessments of morphological methods used to assign individuals to species are needed.

Because most previous studies of toad morphology were based on subjective interpretation of dichotomous features and not measurement of continuous variables, and

because recent genetic information has questioned the monophyly of the morphological cluster assigned to *A. fowleri*, we chose to reexamine the morphological features used to diagnose *A. americanus*, *A. fowleri*, and *A. terrestris*. We restricted our examination to specimens from the state of Alabama, where hybridization between species is acute (Mount 1975) and where part of a mitochondrial clade of *A. fowleri* that shares genetic sequences with *A. terrestris* is found (Masta et al. 2002). We asked three questions of these data: 1) which morphological features best diagnose the three species; 2) what pattern of hybridization is evident among species based on morphology; and 3) how similar are distribution patterns of the three species when based on traditional treatment of morphology compared to assignments based on multivariate analysis of measured characters. Our study represents the first step in an eventual examination of the strength of correlations between measures of gene flow among species and evidence of hybridization measured through morphological characteristics and advertisement calls.

MATERIALS AND METHODS

Sampling methods

We measured five variables from specimens of *A. americanus*, *A. fowleri*, and *A. terrestris* from the state of Alabama that were housed in the Auburn University Museum of Natural History. We excluded juveniles by including only specimens greater than 40 mm in snout-vent length in order to only account for morphology of sexually mature individuals. For each specimen we recorded sex and snout-vent length (SVL). Additionally, we recorded: 1) wart-to-spot ratio, 2) diameter of largest tibial wart (nearest 0.001 mm), 3) length and 4) width of connection between postorbital crest and parotoid gland, and 5) height (nearest 0.001 mm) of the junction of the interorbital and postorbital crest (Figure 1). Wart-to-spot ratio was estimated by

calculating the diameter of the largest dorsal wart (measured to nearest 0.01 mm) and dividing this by the area of the dark dorsal spot in which it resided (ellipse based on spot length and width). The minimum distance from the anterior edge of the parotoid gland to the lateral edge (including the width) of the postorbital crest (nearest 0.001 mm) was used to characterize the length of the connection between these two structures (i.e. presence or absence of a spur). The width of this connection was the distance between the lateral and medial points connecting the postorbital crest and parotoid gland (nearest 0.001 mm). We found no significant linear relationship between SVL and any measured morphological trait. Therefore, no adjustments of our measurements were required to account for differences in body size.

Preliminary Analysis

Within each species, we selected twenty individuals determined by eye to possess all the distinct morphological characteristics for that species (Conant and Collins 1998; Green and Pauley 1987; Mount 1975). Once these 60 individuals were chosen, their five key morphological characteristics were measured and analyzed in the multivariate statistical program, Primer v6 (Clarke 1993). Distribution graphs, created using R v3.1.0, of each variable separated specimens into two or three groups (Appendix 1). Cluster analysis, based on the Bray-Curtis measurement of similarity, was employed to group individuals. A SIMPROF test detected significant structure for each species and within-group similarities ranged from 72-75% (Appendix 2). Based on these findings, we generated 70% prediction intervals (Whitmore 1986) for each character from the original twenty individuals sampled for each species (Table 1). These intervals allowed us to create a statistical model that would allow us to characterize all 1,041 specimens within the Auburn University collections based on statistically defensible measurements rather than by

evaluation of the dichotomous key characters typically used by field guides (e.g. Conant and Collins 1998).

Model building

For each species, we selected 20 additional individuals known to fall within the 70% prediction intervals of all five morphological characters for that species. These 60 specimens were chosen to represent pure stock of each species. Additionally, we selected 20 individuals to represent putative hybrids. This group contained 15 individuals for which 70% prediction intervals conflicted for three or more characters, and a random sample of 5 individuals for which prediction intervals conflicted for two characters. This approach was taken because morphological hybrids are expected to show conflict across many characters (Tovar-Sánchez and Oyama 2004).

To evaluate the three species as distinct morphological entities, we used cluster analysis with the Bray-Curtis measurement of similarity in order to group individuals and a SIMPROF test to detect significant structure among clusters. A BEST analysis was used to determine which morphological variables most frequently sorted individuals into significant clusters (Clarke and Gorely 2001). A multidimensional scaling (MDS) plot was used to visualize separation among pure stock of the three species.

We then performed a discriminant function analysis (DFA) using the *lda*() function in the package MASS of the statistical program R v3.1.0. This analysis built a model in which the identity of the four groups (pure stock of three species plus hybrids) was based on measurements of the key morphological characteristics from the 80 individuals chosen. The coefficients of linear discriminates were used to assign all 1,041 individuals to one of four possible categories (one of the three species or hybrid). We used MDS to visualize the groupings created by the

DFA model (Clarke and Gorely 2001). To further investigate species influence in putative hybrids, all classified hybrid individuals were run through a DFA created using only 60 specimens to represent each species category. This allowed us to examine percentage of different species' influencing hybrid morphology. Finally, we evaluated the utility of our statistical identity of toads by generating a geographic distribution using R v. 3.1.0 for each pure stock and comparing it to published field guide distributions. We also created a map for putative hybrids to examine their distribution across Alabama.

RESULTS

When based on our sample of morphologically identifiable specimens, cluster analysis revealed three significant groups, each representing a different species (Figure 2). Results of a SIMPROF test revealed these species to represent significant clustering at 78-83% similarity, values that we used to justify use of 77% confidence ellipses below. When compared to the preliminary analysis, these similarity values represent a 5-10% increase in similarity of clusters when identity is based on prediction intervals of measured variables rather than by interpretation of characters by eye (Appendix 2). When examined in MDS space, our sample of pure stock specimens showed complete separation of *A. fowleri* specimens. Additionally, overlap of 77% confidence ellipses for *A. terrestris* and *A. americanus* (Figure 3) was reduced when compared to preliminary identifications made by eye (Appendix 2). The BEST analysis showed tibial wart diameter, followed by the length of connection between postorbital crest and parotoid gland and width of connection between the postorbital crest and the parotoid gland, to be the strongest morphological characteristic sorting individuals into species (Clarke and Gorely 2001). Distributions were as expected for four of the five characteristics. The exception was tibial wart

diameter, which was trimodal: average tibial wart width for *A. terrestris* was 1.737 mm, for *A. americanus* was 3.03 mm, and for *A. fowleri* was 0.98 mm (Appendix 1).

The first coefficient of linear discriminates created by the DFA model separated *A. fowleri* specimens from all others, the second linear discriminate separated *A. americanus* specimens from all remaining specimens, and the third linear discriminate separated *A. terrestris* from hybrids (Table 2). The DFA correctly identified all *A. americanus, A. fowleri*, and *A. terrestris* used to create the model and correctly classified 90% (18/20) of the hybrids used in model construction. Of the misclassified hybrids, one was estimated to be *A. fowleri* and the other to be *A. terrestris*.

When all individuals were classified into one of the four groups created by our DFA model, their distribution in MDS space recovered separation of three species, with hybrids occupying intermediate positions (Figure 4). A heavier concentration of hybrids was seen between the *A. terrestris* and *A. fowleri* clusters than between the *A. americanus* and *A. fowleri* clusters, with the highest concentration occurring between *A. americanus* and *A. terrestris* (Figure 4). After running the DFA classified hybrids back through the DFA model without a hybrid category, approximately 82% of individuals were morphologically identified has having 90% or higher posterior probability of being classified to one specific species (Figure 5).

Specimens classified by DFA to be *A. americanus* were mostly restricted within the geographic range in Alabama traditionally thought to be occupied by that species (Figure 6A). Specimens classified by DFA to be *A. fowleri* were distributed throughout Alabama, but were reduced in abundance in the southwestern portion of the state (Figure 6B). The distribution of *A. terrestris* specimens identified by DFA extended beyond the geographic range traditionally thought to be occupied by this species, including northeastern portions of Alabama (Figure 6C).

Overall, 16.2% (n=169/1041) of specimens were classified by DFA to be hybrids and these individuals were found throughout the state (Figure 6D).

DISCUSSION

Based on morphological measurements of key characteristics *A. fowleri* is consistently a morphologically distinct group; however, there is some overlap between *A. americanus* and *A. terrestris*. This suggests the presence of morphological similarities between *A. americanus* and *A. terrestris* and that morphology can be used to identify *A. fowleri.* Based on the BEST analysis and the results from the tibial wart distribution plot, the tibial wart diameter characteristic is the most prominent morphological character in helping to classify between all three species.

As predicted, the MDS plot including all individuals showed hybrids falling between all three species. However, the MDS plot with all individuals has broader groupings than the MDS plot created from the morphologically pure individuals, which excludes hybrids, implying hybridization influences morphological clarity. In our MDS plot, hybrids are seen bordering every pure species cluster, blurring the lines between morphologically distinguished species and hybrids.

Morphological hybrids had a 16.2% frequency of identification among the museum specimens with the hybrids seen most frequently between *A. americanus* and *A. terrestris* and hybrids seen more frequently between *A. fowleri* and *A. terrestris* than *A. fowleri* and *A. americanus*. Based on the results from the hybrid DFA, many of the classified hybrids had less than 10% evidence of morphology influenced by multiple species. However, this DFA also showed individuals with low percent of major species (<40%) suggesting potential evidence of morphology from all three species. The MDS plot including all individuals gives a visual portrayal of the dominant presence of morphological hybridization between these three species.

With broader overlapping geographic ranges, *A. fowleri* and *A. terrestris* have a higher potential to interbreed than does *A. fowleri* and *A. americanus*. However, the map of Alabama indicating putative hybrid locality shows morphological evidence of potential hybrids among most of the counties in Alabama. Morphologically pure *A. americanus* individuals were found only in the northeast portion of the state as originally described by Mount (1975). Morphologically pure *A. fowleri* individuals were not readily seen in the southwest portion of the state suggesting morphological *A. fowleri* hybrids mainly occupy this area. Presence of *A. woodhousi*, another species of toad known to hybridize with *A. fowleri*, in the west may have influence on the presence of morphological *A. fowleri* hybrids in the southwestern portion of Alabama as well as *A. terrestris* (Conant and Collins 1998). The *A. terrestris* map indicates there is morphological evidence of *A. terrestris* in their expected range and in areas outside of their known geographic range. This suggests *A. fowleri* may be a conduit which serves to transmit gene flow into areas extending beyond *A. terrestris* range or there may be influences of *A. terrestris* from bordering states. Supporting this statement, putative hybrid individuals had locality data throughout the state without any limitation. This suggests hybridization is not limited to the Fall Line of Alabama where *A. americanus* and *A. terrestris* ranges meet.

This study provides quantitative evidence of morphological hybridization among these species. Furthermore, it suggests that morphological hybridization is more frequent than previously expected, and has expanded throughout the state (Mount 1975). To truly determine how reliable morphology is when identifying individuals, examining genetics in tandem with morphology would be a wise approach as seen in previous studies (Brede et al. 2008; Cedeño-Vázquez et al. 2008; Dessauer and Cole 2003; Feder 1979; Green 1984; Green and Pustowka 1997). However in some cases, individuals classified as pure stock based on morphology may

contain genetic evidence of hybridization (Blair 1941; Feder 1979). Using morphological characteristics to identify individuals is often based on a subjective present/absent method. This method alone can cause ambiguity and reduce identification accuracy. Nevertheless, genetic analysis alone can lead to inconsistent conclusions, as seen with a paraphyletic *A. fowleri* group based on mitochondrial DNA, and results from a nuclear phylogenetic tree of this species complex conflicting with results from a mitochondrial phylogenetic tree (Fontenot et al. 2011; Masta et al. 2002). Even though, with increased diversity of molecular tools, the value of morphology in identifying hybridization has come under closer scrutiny, some cases have used genetic information to confirm hybridization identified first by the presence of morphological intermediates (Cedeño-Vázquez et al. 2008; Dessauer et al. 2000; Feder 1979). Therefore, this study improves the method of identification for this species complex by examining morphology on an objective quantitative basis, allowing for a more precise assignment to species. This study has demonstrated the usefulness and limitations of morphology within this group of toads. In future studies, combining objective morphological identification methods with genetic analysis, a better understanding of interbreeding species can be reached.

Table 1-1. Prediction intervals for each morphological characteristic. These intervals are based on 70% provided by the cluster analysis from the 60 preliminary morphologically pure individuals. Intervals are created for three species for each of five morphological characters.

Species	**Wart to spot ratio**	**Tibial wart diameter**	**Length of Connection**	**Width of connection**	**Height of intraorbital crest**
A. terrestris	0.0522-0.1733	1.48-2.18	2.282-3.604	0.9194-1.478	1.459-2.378
A. americanus	0.1777-0.3601	2.41-3.641	2.25-3.434	0.951-1.506	1.041-1.761
A. fowleri	0.04343-0.2003	0.6791-1.359	0.86399-1.54	1.78-2.607	0.7701-1.283

*

Table 1-2. Coefficients of linear discriminants for each key characteristic based on the 80 individuals chosen to represent each category of species present in the model: *A. americanus, A. fowleri, A. terrestris*, hybrid.

Morphological Characteristic	**LD1**	**LD2**	**LD3**
Wart to spot ratio	2.6137083	-11.669812	3.6018676
Tibial wart diameter	1.7275749	-0.8026616	0.9068685
Length of connection	1.6398126	-0.4502951	-2.211692
Width of connection	-1.506219	-1.7855126	-1.003704
Height of intraorbital crest	0.9975305	2.509867	2.1236678

Table 1-3. Specimens (AUM number) used in the cluster analysis of 60 new specimens chosen based on 70% prediction intervals of morphological characteristics.

Label	Specimen number
F1	9607
F2	4185
F3	23729
F4	969
F5	3749
F6	5517
F7	5446
F8	23472
F9	4699
F10	5395
F11	9625
F12	5550
F13	14354
F14	4527
F15	11314
F16	8703
F17	36398
F18	3762
F19	10503
F20	23731
T1	3951
T2	3990
T3	14365
T4	3942
T5	3992
T6	11522
T7	3524
T8	28938
T9	28944
T10	14367
T11	24098
T12	14350
T13	3616
T14	3617
T15	3988
T16	3753
T17	3989
T18	14337
T19	23353
T20	3991
A1	28891
A2	39810
A3	28983

A4	28900
A5	21292
A6	28899
A7	33550
A8	23974
A9	28997
A10	28880
A11	28881
A12	1724
A13	21289
A14	11543
A15	11542
A16	21287
A17	28958
A18	33552
A19	21291
A20	13542

Table 1-4. Specimens (AUM number) used in the cluster analysis of 60 preliminary specimens chosen based on five key morphological characteristics.

Figure Label	Museum number
F1	5447
F2	28951
F3	28953
F4	4700
F5	28946
F6	28948
F7	5358
F8	28952
F9	28950
F10	9605
F11	1285
F12	18447
F13	9606
F14	10217
F15	4317
F16	10215
F17	961
F18	9611
F19	9083
F20	1204
T1	28932
T2	29047
T3	28910
T4	29098
T5	29048
T6	28923
T7	3939
T8	22136
T9	28939
T10	28981
T11	28937
T12	3792
T13	29072
T14	28979
T15	3940
T16	3919
T17	29051
T18	3941
T19	3994
T20	23784
A1	11594
A2	11590
A3	29036

A4	11585
A5	11593
A6	22080
A7	1716
A8	29026
A9	11574
A10	29022
A11	13601
A12	11583
A13	18448
A14	109
A15	18450
A16	29031
A17	11588
A18	29790
A19	29789
A20	29024

Figure 1-1. Morphological measurements taken for every specimen; *A. terrestris* (left); *A. americanus* (upper right); *A. fowleri* (lower right). Red lines represent lengths measured. The red dot represents the diameter of the largest tibial wart measured. Yellow lines represent diameter of largest dorsal wart measure inside spot measured.

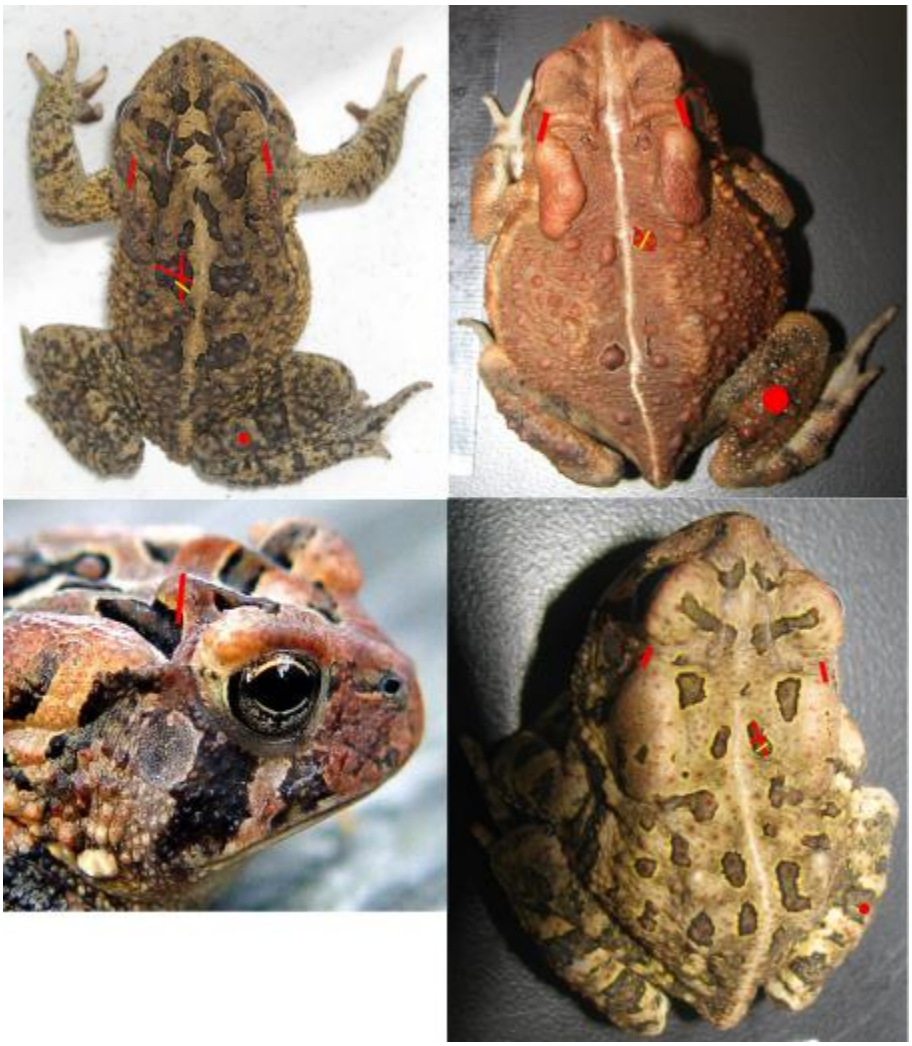


Figure 1-2. Cluster analysis of measurements from pure individuals (N=60) chosen based on 70% prediction intervals (Table 1). Sample labels indicate species (A = *A. americanus*; F = *A. fowleri*; T = *A. terrestris*) and replicate (number; see Table 3 for list of specimens). Black lines represent statistically significant clusters based on a Simprof test (Clarke and Gorley 2001).

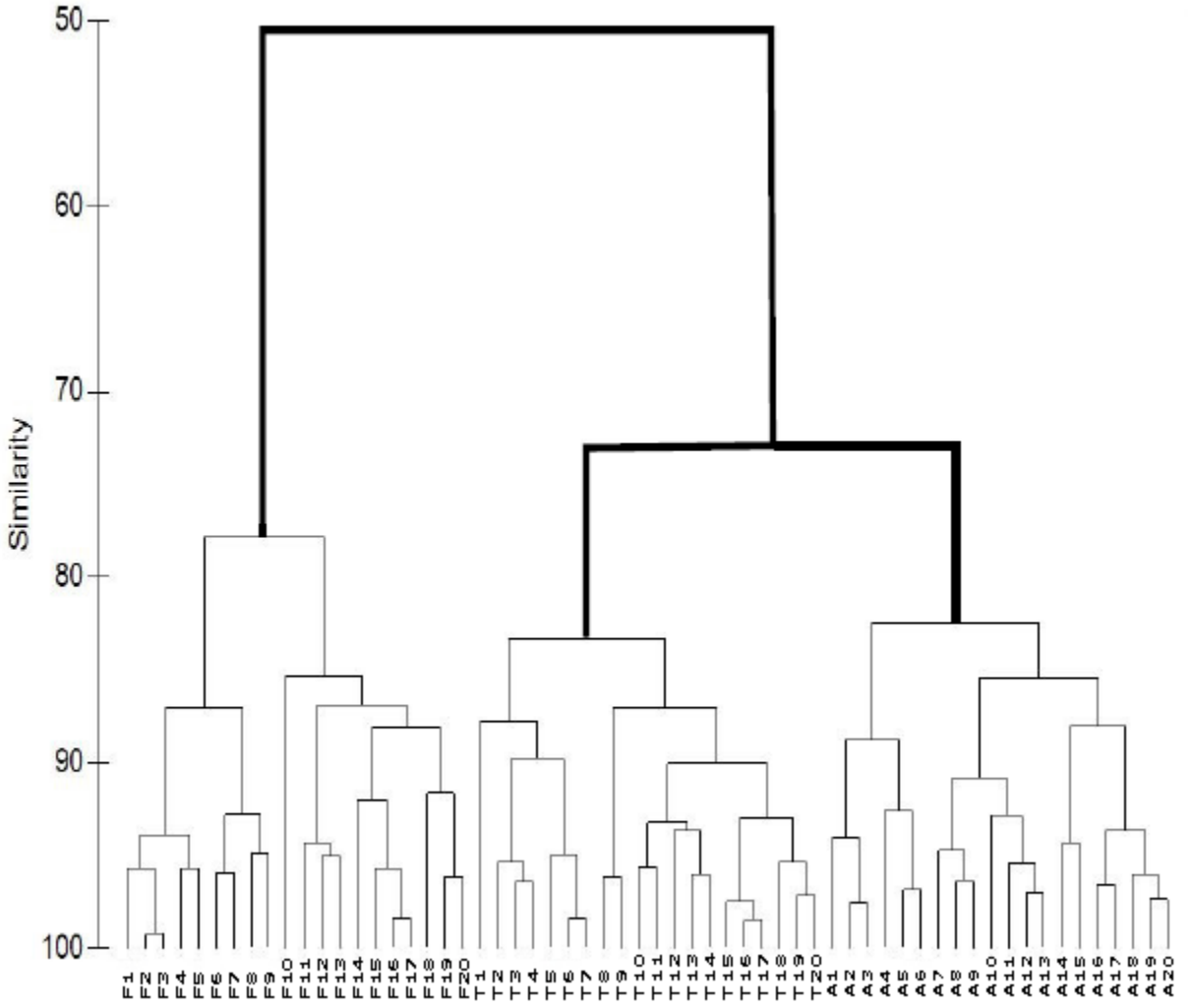


Figure 1-3. Multi-dimensional scaling plot of 60 specimens chosen based on 70% prediction intervals (Table 1) to create DFA model. *A. americanus* = (light blue) squares; *A. fowleri* = (dark blue) downward triangles; *A. terrestris* = (green) upward triangles).

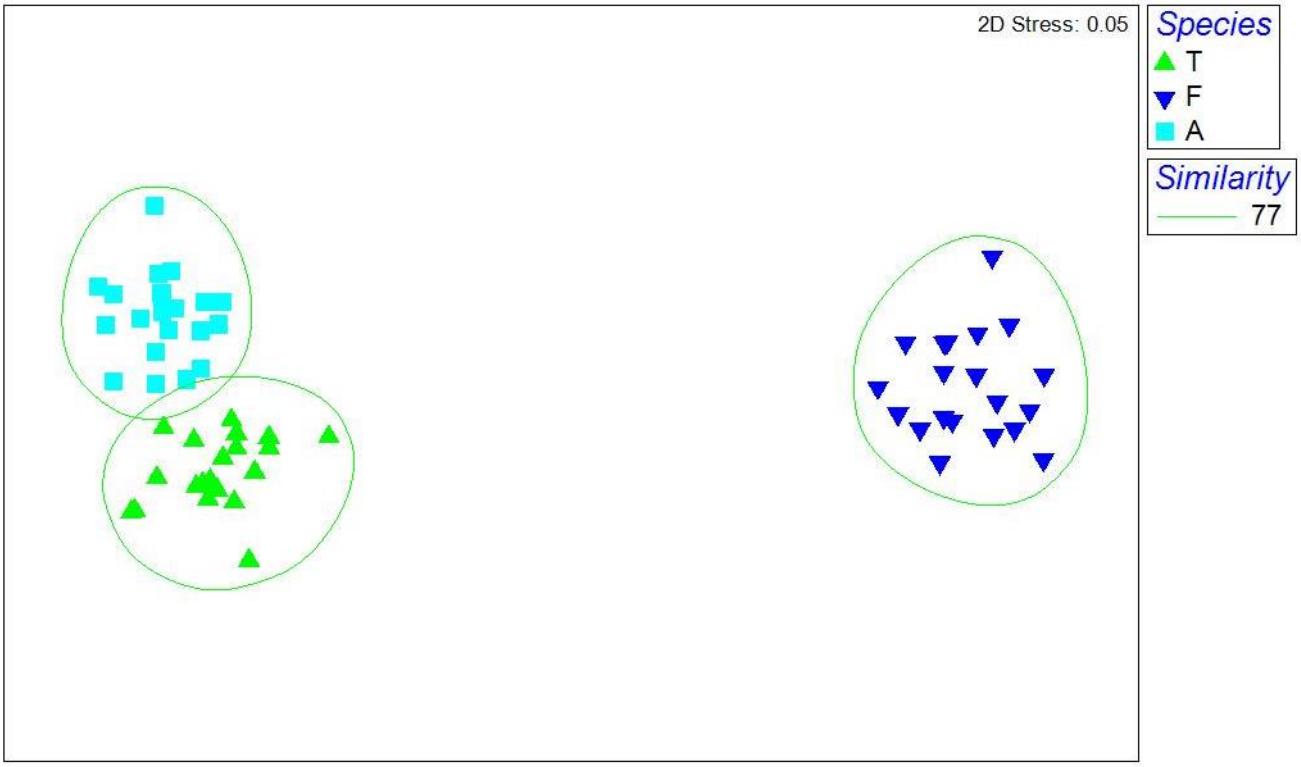


Figure 1-4. Multi-dimensional scaling plot based on assignments given by DFA model of 1041 specimens. *A. americanus* = (light blue) squares; *A. fowleri* = (dark blue) downward triangles; *A. terrestris* = (green) upward triangles); Hybrid = (red) diamonds.

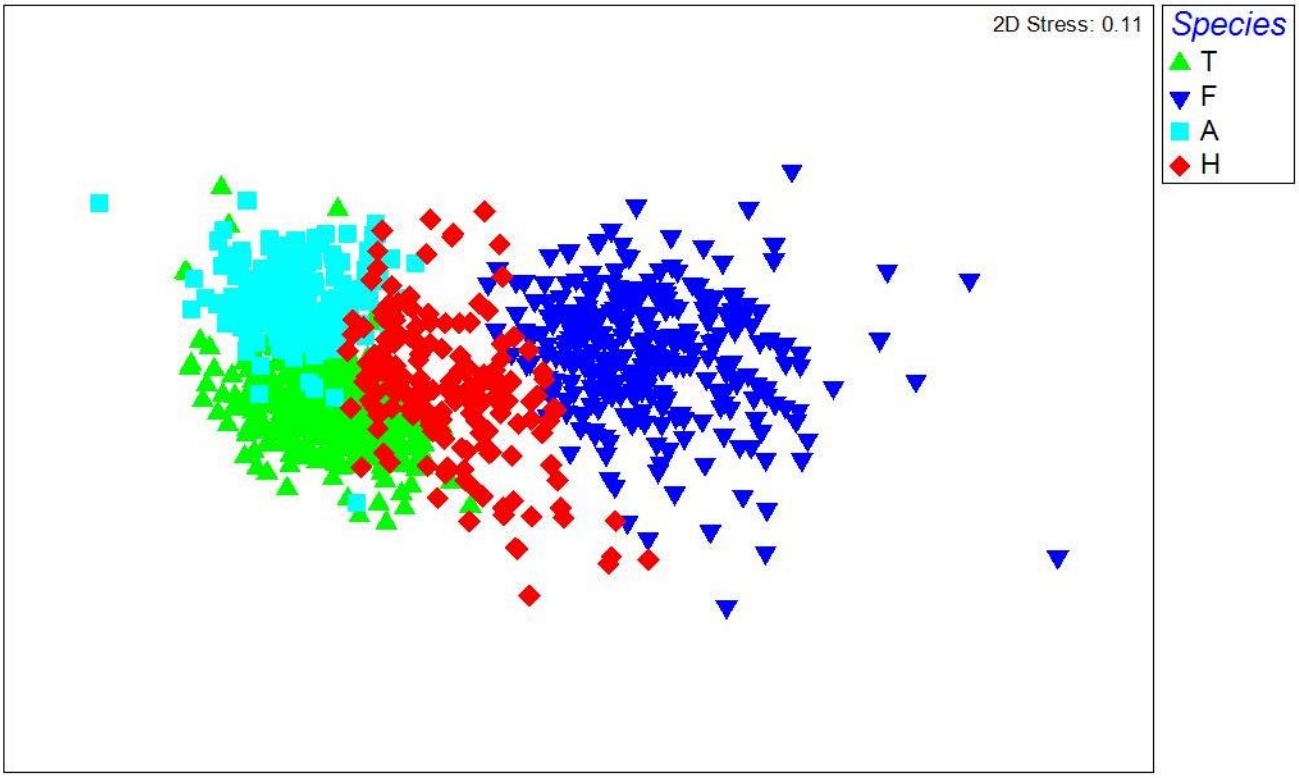


Figure 1-5. Percentage of major species influencing hybrid morphology for every classified hybrid (n=169). Individuals with 90% or less classified to a major species are suspected to have multiple species influencing the morphology.

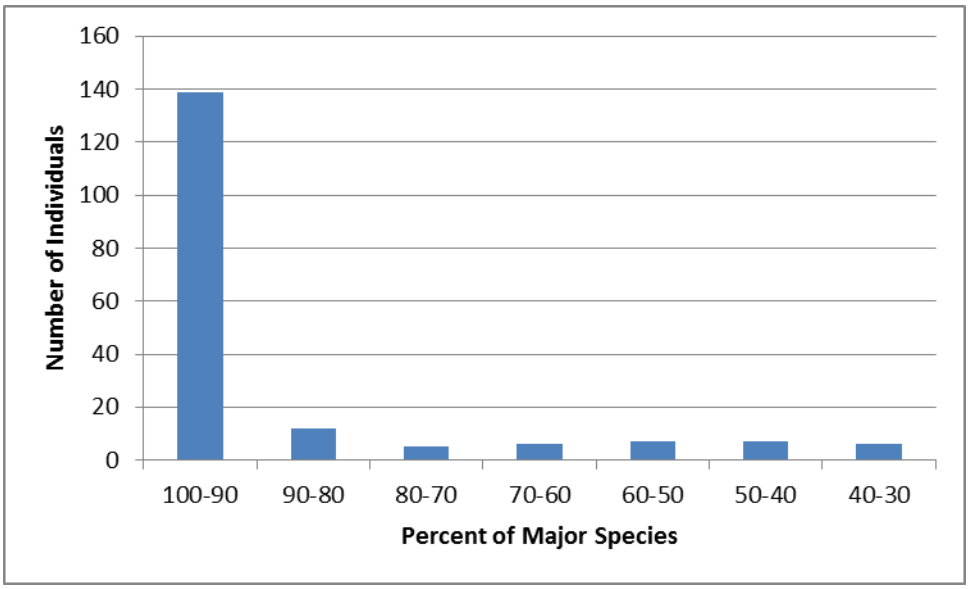
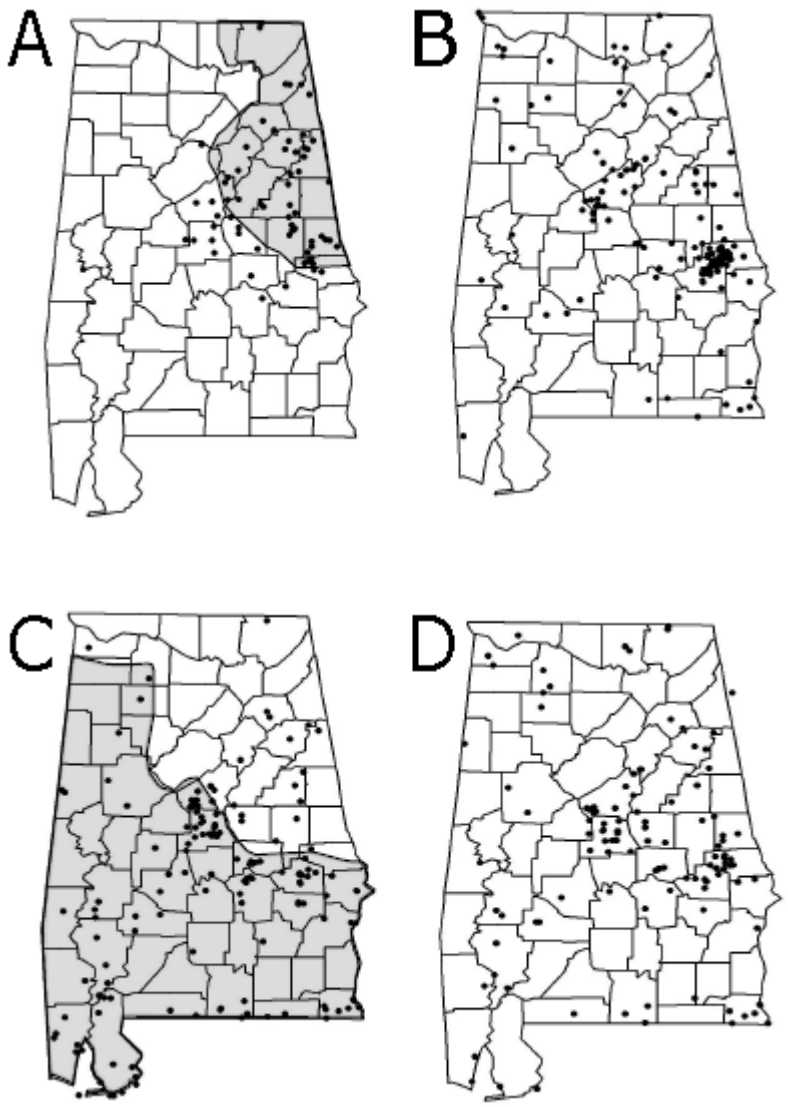


Figure 1-6. Distribution of specimens throughout Alabama identified by DFA to be *A. americanus* (A), *A. fowleri* (B), *A. terrestris* (C), and hybrids (D) in Alabama. Shaded regions in maps A and C indicate approximate distribution of each species as interpreted by Mount (1975).



CHAPTER 3

DO *ANAXYRUS FOWLERI* ALTER MATING CALL CHARACTERISTICS IN SYMPATRY WITH *ANAXYRUS AMERICANUS*?

INTRODUCTION

During the breeding season, male anurans rely on species-specific advertisement calls to attract conspecific females (reviewed in Arak 1983; Blair 1958; Gerhardt and Huber 2002). Every species' call is comprised of a combination of frequencies, durations, and harmonics that are specific to that particular species (Gerhardt 1988; Nevo and Capranica 1986; Ryan and Wilczynski 1991). Within each species, variability can be found among individuals, which allows females to identify males individually as well as by species (Gerhardt 1982; Gerhardt and Huber 2002; Ryan and Wilczynski 1991). The complex structure of a species' call should be a strong prezygotic barrier that isolates each species, but individual variation can blur the line distinguishing species, resulting in hybridization. Hybrid individuals with intermediate calls may not be distinct from purebred individuals with highly variable calls; therefore, hybrids calling in conjunction with purebred individuals may confuse females (Blair 1941; Blair 1972; Gerhardt and Klump 1988a; Green 1984; Martin 1971; Volpe 1952; Weatherby 1982; Zweifel 1968). In this female-choice mating system males typically call in a chorus of the same species and additional species (Wells 1977; Wells and Schwartz 1984). In the case of *Hyla cinerea*, females prefer a mixed chorus over a conspecific chorus (Gerhardt and Klump 1988b). If this preference is a general characteristic for female anurans, then one would infer increased opportunities for

hybridization, especially for congeners (Blair 1941; Blair 1958; Green 1983, 1984; Green and Parent 2003; Green and Pustowka 1997; Martin 1971; Mount 1975; Volpe 1952; Zweifel 1968). Overall, a chorus of calling males makes it difficult for females to detect individuals; therefore, males with similar advertisement call parameters can become less distinct to a female, which increases the potential for hybridization (Gerhardt and Klump 1988a; Wollerman 1999).

In zones of overlap one strategy used to prevent hybridization involves altering advertisement calls in males in ways that increase the ability of females to correctly identify conspecific males, a feature that may be relaxed in allopatry. This process is known as character displacement. In the case of calling males, character displacement seeks to reduce hybridization by selecting against individuals with intermediate calls (Brown and Wilson 1956).

One well-established example of hybridization involves the *Anaxyrus americanus* complex (Blair 1941; Blair 1958; Green 1983, 1984; Green and Parent 2003; Green and Pustowka 1997; Martin 1971; Volpe 1952; Zweifel 1968). This complex currently consists of *A. americanus, A. charlesmithi, A. fowleri, A. hemiophrys, A. houstonensis, A. microscaphus, A. terrestris, A. velatus,* and *A. woodhousii* (Pauley et al. 2004). Based on morphological information for specimens from Alabama, as much as 16% of individuals may be hybrids of various combinations of *A. americanus, A. fowleri,* and *A. terrestris*. Given this high rate of hybridization, we chose to examine call features of *A. americanus* and *A. fowleri*. These two species are found together in the northeastern portion of the state above the Fall Line (Mount 1975). Although there is some separation between their breeding seasons (*A. americanus* from January to May and *A. fowleri* from March to August), differences in advertisement calls are thought to be the main mechanism by which females correctly identify conspecific males, with *A. fowleri* creating a short whining call with low pulse rates and *A. americanus* creating a long

whistling call with high pulse rates (Conant and Collins 1998; Cook 1983; Green and Parent 2003; Green and Pustowka 1997; Mount 1975). Here, we characterize quantitative variation in advertisement calls of *A. fowleri* and *A. americanus* in northeastern Alabama, where the two species are found in sympatry. Additionally, we hypothesis that character displacement changes at least one parameter in male advertisement calls between *A. fowleri* in allopatry and sympatry.

MATERIALS AND METHODS

Sampling methods

We recorded calls of *A. americanus* and *A. fowleri* males in the field using a ZOOM© H2 handy recorder. Recordings of each specimen were taken from approximately 0.5-1.0 meters and at least three consecutive calls were recorded for every individual. Specimens were then captured and identified based upon advertisement call and the presence or absence of morphological features described in field guides (e.g. Mount 1975). *Anaxyrus americanus* (n = 19) and *A. fowleri* (n = 4) individuals were collected at a sympatric site in Jackson County, Alabama between the months of February and June of 2014 and 2015. *Anaxyrus fowleri* (n = 16) individuals were also collected at an allopatric site in Winston County, Alabama between the months of April and June of 2014 and 2015. Every collection took place between the hours of 8:00 and 11:00 pm when temperatures ranged between16.6 and 21.1°C.

Measuring acoustic parameters

We analyzed recorded calls using the sound analysis program Cool Edit Pro v1.2a ©1992. The following six acoustic parameters were measured for each recorded call, as well as ambient air temperature (C°) and snout-vent length (SVL; mm): call duration (pulses/call), pulse

rate (pulses/s), dominant frequency (Hz), length of one pulse (s), length of call (s), and time between calls (s).

Statistical Analysis

Boxplots were created to determine the overall minimum, first quartile, mean, third quartile, and maximum for every parameter measurement for each of three groups: *A. americanus* in sympatry, *A. fowleri* in sympatry, *A. fowleri* in allopatry. These plots allowed us to examine every parameter individually to look for the extent of variance within these groups, areas of overlap, and to visually compare means between all groups. An analysis of covariance (ANCOVA) was first used to test for differences in call parameters among the three groups while accounting for the effects of temperature and SVL. This preliminary analysis revealed that body size affected no call parameter and temperature affected pulse rate. Therefore, we used analysis of variance (ANOVA) to test for differences in call duration, length of one pulse, dominant frequency, length of call, and time between calls among the three groups, followed by a Tukey's HSD to determine whether differences between allopatric and sympatric *A. fowleri* populations were significant. We use ANCOVA to test for differences in pulse rate between sympatric and allopatric *A. fowleri*, while accounting for the effect of temperature on this variable. A non-significant interaction term allowed us to compare elevations of the two regressions. To characterize overall differences in the characteristics of advertisement calls of *A. americanus* and *A. fowleri* in Alabama, we created a multi-dimensional scaling (MDS) plot based on all call parameters considered together.

RESULTS

Call duration, pulse length, call length, dominant frequency, and time between calls differed significantly among populations (call duration $F = 7.3$, $df = 2$, $p < 0.00215$; pulse length $F = 172$, $df = 2$, $p < 0.0001$; call length $F = 66$, $df = 2$, $p < 0.0001$; dominant frequency $F = 21$, $df = 2$, $p < 0.0001$; time between calls $F = 6.9$, $df = 2$, $p < 0.00285$), but did not differ between sympatric and allopatric *A. fowleri* (call duration HSD = -7.9, $p < 0.970$; pulse length HSD = -0.00059, $p < 0.848$; call length HSD = -0.40, $p < 0.864$; dominant frequency HSD = 82, $p < 0.140$; time between calls HSD = -0.88, $p < 0.878$). When controlled for the effect of temperature, pulse rate did not differ between *A. fowleri* in sympatry and *A. fowleri* in allopatry (Figure 2) ($F = 1.25$ $df = 1$, $p < 0.28$).

Overall, advertisement calls of male *A. americanus* were much more variable than those of *A. fowleri* (Figure 3). In the MDS plot, call characteristics differed much more strongly for *A. americanus* than for *A. fowleri*, with calls of *A. americanus* encompassing variation in *A. fowleri*.

DISCUSSION

We did not find evidence of character displacement in any of the call parameters measured. Based on the results from our ANOVA, we are able to reject our hypothesis that character displacement changes at least one parameter in male advertisement calls between *A. fowleri* in allopatry and sympatry. When character displacement occurs in anurans it frequently involves pulse rate (e.g. Gerhardt and Doherty 1988, Lemmon 2009; Smith et al. 2003). Although sympatric male *A. fowleri* did have faster mean pulse rates than allopatric males, this resulted from *A. fowleri* in sympatry being captured at warmer temperatures than those in allopatry. When temperature was accounted for statistically, no evidence of character displacement for pulse rate was evident. Multiple studies have provided evidence that higher

temperatures increase pulse rate (Gayou 1984; Gerhardt 1978; Gerhardt and Huber 2002; Zweifel 1968).

Our data support Leary (2001), who found no evidence of character displacement for advertisement calls in replicate populations of *A. americanus* and *A. fowleri* in Alabama. Zweifel (1968) demonstrated that pulse rate of hybrid males *A. americanus* x *fowleri* are intermediate between pulse rates of the two parent species, creating an opportunity for females to select against hybrids. At our sympatric site, males of both *A. americanus* and *A. fowleri* called side by side at the same pond. The fact that no character displacement in calls occurs at this site suggests that females correctly identify conspecific males via other mechanisms or that their ears are refined enough to distinguish conspecific from heterospecific calls without character displacement. Alternatively, our results may support findings of Green and Parent (2003) who noted that *A. americanus* x *fowleri* hybrid zones represent a mosaic in which hybridization appears and disappears over time and location. Long-term samples at our sympatric site will be necessary to evaluate this alternative.

Anuran sexual selection is based on a female mate choice system; females are required to recognize conspecific males in order to maximize their fitness (Abt and Reyer 1993; Blair 1974; Gerhardt 1988; Ryan and Rand 1993). In cases of high variation, overlap can exist in certain characteristics of advertisement calls, thus muddling species distinction (Blair 1941; Conant and Collins 1998; Gerhardt and Huber 2002; Haddad et al. 1994; Marquez et al. 1993). When pooled across all of our samples, our measured call parameters document that *A. americanus* occupies a much larger area of call space than does *A. fowleri*. Leary (2001) found a comparable pattern of greater variation in calls of *A. americanus* across the geographic range of the species. High variance seen in the examined parameters for *A. americanus* could ultimately encompass

intermediate calls that overlap with *A. fowleri*. Conversely, *A. fowleri* is shown to have strong similarity in advertisement calls among individuals. The low variance seen suggests precise communication for *A. fowleri* in this region of Alabama. We interpret this to indicate that, if hybridization is occurring at our study site, female *A. americanus* are more likely to hybridize with *A. fowleri* males than *A. fowleri* females are to hybridize with *A. americanus* males. We hypothesize that the great variation in male *A. americanus* advertisement calls, even within a population, makes similar selection against hybridization more difficult for *A. americanus* females.

Figure 2-1. Individual advertisement call parameters of species from different populations. From left to right boxes: *Anaxyrus americanus* in sympatry (box1), *Anaxyrus fowleri* in allopatry (box 2), and *A. fowleri* in sympatry (box 3). These box plots show averages (thick black bars inside boxes) and variance (all between the two small dashed lines) for every call parameter measured: call duration (A), pulse rate (B), length of pulse (C), length of call (D), dominant frequency (E), average time between calls (F). Statistical significance between *A. americanus* and *A. fowleri* is illustrated by a black star. Statistical significance between *A. fowleri* in allopatry and *A. fowleri* in sympatry is indicated by a black diamond.

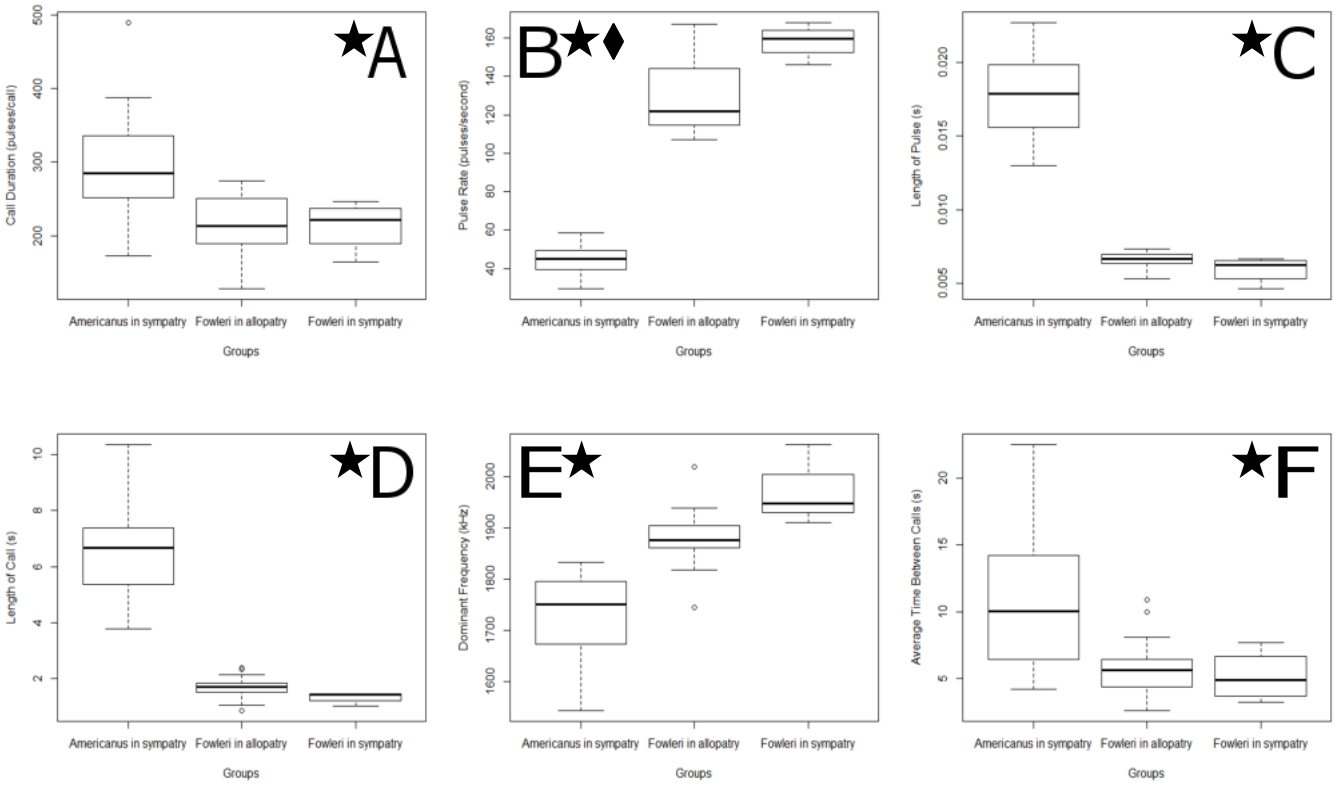


Figure 2-2. Scatterplot of pulse rate (pulses/second) graphed against temperature to show the significant difference seen between the two *A. fowleri* populations were a result of temperature differences. The slopes of both *A. fowleri* populations are insignificantly different from each other based on our ANCOVA test ($F = 1.25$ $df = 1$, $p < 0.28$). Open circles represent *A. fowleri* in allopatry with a tread line ($y=18.743x-224.91$; $R^2=0.7238$). Closed circles represent *A. fowleri* in sympatry with a tread line ($y=9.8667x-45.852$; $R^2=0.8134$). Closed squares represent *A. americanus* in sympatry with a tread line ($y=3.395x-15.208$; $R^2=0.3765$).

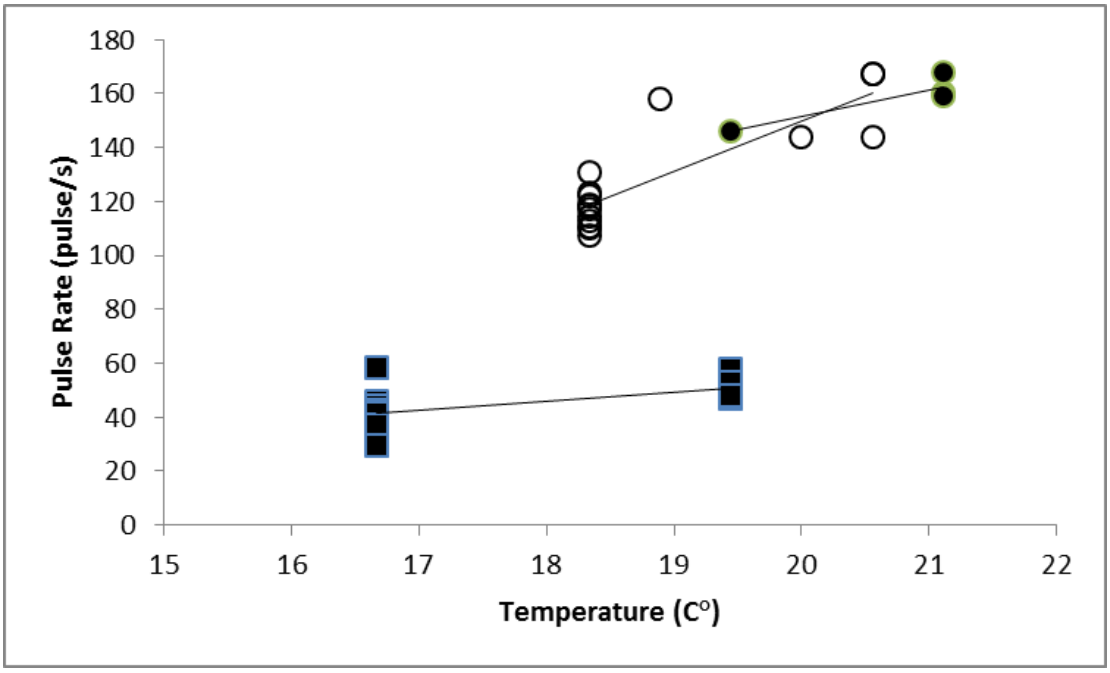
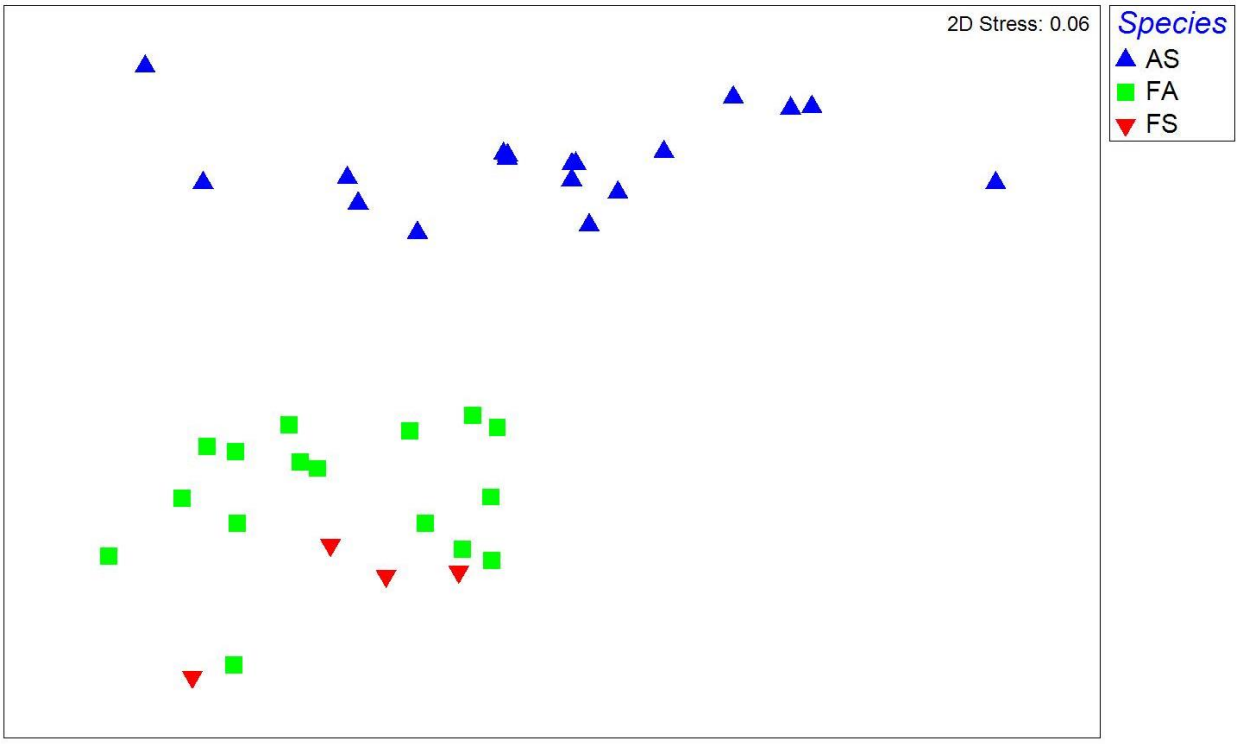


Figure 2-3. Multi-dimensional scaling plot of all male individuals based on acoustic parameter measurements (N=39). Classifications based on field observation. *A. americanus* = (blue) upward triangles; *A. fowleri* in allopatry = (green) squares; *A. fowleri* in sympatry = (red) downward triangles.



CHAPTER 4

ESTIMATES OF HYBRIDIZATION BETWEEN *ANAXYRUS AMERICANUS* AND *ANAXYRUS FOWLERI* IN NORTHERN ALABAMA

INTRODUCTION

The *Anaxyrus americanus* complex (*Anaxyrus americanus, A. charlesmithi, A. fowleri, A. hemiophrys, A. houstonensis, A. microscaphus, A. terrestris, A. velatus,* and *A. woodhousii* (Blair 1972; Conant and Collins 1998; Dixon 2000; Pauley et al. 2004)) has been a major focus of genetic study because many species are known to hybridize with other members of the complex (Blair 1941; Blair 1963a, 1972, 1974; Fontenot et al. 2011; Green 1984, 2003; Masta et al. 2002; Volpe 1952; Zweifel 1968). Many studies have explored the hybridizing relationship of this species complex using allozymes, mitochondrial and nuclear DNA (mtDNA and nDNA), morphology, geographic location, and male advertisement calls (Fontenot 2011; Green and Parent 2003; Masta et al. 2002). These studies suggest the presence of hybridization is evident in morphology, but the extent of genetic introgression remains unknown among these species given the variation in phenotypes of putative hybrids (Blair 1963a; Fontenot et al. 2011; Green 1984; Masta et al. 2002; Mount 1975).

In an earlier study using allozymes, Green (1984) found hybrid individuals with intermediate morphology and genotype, but potentially only first-generation hybrids, with no evidence of extensive introgression between *A. americanus* and *A. fowleri*. However, according to Mallet (2005), use of allozymes to identify hybrids could be problematic due to the fact that

variants of an allele could be derived independently through mutation, causing a change in the protein. Masta et al. (2002) recovered a paraphyletic assemblage of *A. fowleri* specimens with three genetically different clades based on aspects of morphology and mtDNA. This assemblage revealed a southern clade with mitochondrial haplotypes most closely related to sequences of morphologically-pure *A. terrestris,* a central clade with morphological evidence of hybridization with *A. americanus* (suggesting genetic introgression between the species), and a northern clade without any strong ties to another species. Uncovering this paraphyletic relationship of *A. fowleri* muddled the relationships previously thought to exist within this complex. Further work done by Fontenot et al. (2011) used geographic location, male advertisement call, and morphology to classify individuals to one of the following species: *A. americanus, A. charlesmithi, A. cognatus, A. fowleri, A. terrestris, A. velatus,* and *A. woodhousi*. Nuclear DNA analyses of these individuals using the amplified fragment length polymorphisms (AFLP), as well as sequencing of mtDNA genes, revealed the following: a morphologically pure *A. fowleri* that possessed nuclear evidence consistent with an *A. terrestris* clade, a morphologically pure *A. americanus* with a whistling advertisement call and with a nuclear genome consistent with an *A. fowleri* clade, and four morphologically pure *A. terrestris* individuals with a whistling advertisement call, but with nuclear evidence consistent with *A. fowleri*. A mtDNA Bayesian phylogenetic tree estimated by these authors showed paraphyly for *A. americanus* and *A. fowleri*, with some apparent *A. americanus* and *A. terrestris* individuals falling within a clade of *A. fowleri*. Their results suggest mitonuclear discordance and the ineffectiveness of mtDNA in detecting recent events of hybridization. These studies create ambiguity in accurate identification of these species and suggest a weak delineation of species; however, as

technology advances, complex genetic techniques become increasingly able to aid in deciphering complicated questions concerning hybridization.

Recently, high-throughput sequencing techniques, described as next-generation sequencing (NGS), allow for a wider examination of the genome at a more rapid rate, all while being cost effective (Liu et al. 2012). Morphological evidence of hybridization has been coupled with investigations of evolutionary genetics, frequently confirming the usefulness of morphology in determining the degree of hybridization (Pallares Amaya 2015; Mallet 2005). Some studies have used next-generation sequencing techniques in tandem with morphology to understand population genetics, hybrid zones, and to test for consistency between the two identification methods (Baldassarre et al. 2014; Mason and Taylor 2009; Moyle 2007; Taylor et al. 2006). In the case of *Quercus crassipes* and *Quercus crassifolia*, two species of oak, molecular markers and morphological characters were highly similar in identifying hybrid individuals (Tovar-Sanchez and Oyama 2004).

The recently-developed technique employed in this study, genotype-by-sequencing (GBS), is an NGS technique that utilizes restriction enzymes to reduce a genome into smaller fragments and uses Illumina sequencing for SNP discovery and genotyping (Elshire et al. 2011). GBS does not require reference genomes to be used for aligning SNPs, and has been successful on a variety of organisms that do not have a genomic reference such as plants, cattle, and foxes (De Donato et al. 2013; Johnson et al. 2015; Lu et al. 2013; Poland and Rife 2012). Morphology and GBS has also been utilized to look at introgression in hybrid zones of the red-backed fairy wren *Malurus melanocephalus* (Baldassarre et al. 2014). Using this tool might allow us to identify hybrids with greater precision than using a single gene or just morphology. It might also

allow us to refine our understanding of how hybridization affects morphological features used to identify species and behavioral features, such as advertisement calls, used as prezygotic barriers.

In this study we focus on hybridization between two species, *A. americanus* and *A. fowleri*, in the northeast portion of the state of Alabama above the Fall Line (Mount 1975). Males of these species produce distinct advertisement calls, with *A. americanus* creating a long whistling call with high pulse rates and *A. fowleri* creating a short whining call with low pulse rates (Conant and Collins 1998; Cook 1983; Green and Parent 2003; Green and Pustowka 1997; Mount 1975). These advertisement calls create a prezygotic barrier between species when they are found in sympatry; however, in areas of sympatry individuals have been observed that produce intermediate calls and possess intermediate external morphology (Blair 1941; Blair 1963a, 1972, 1974; Green 1984; Martin 1971; Mount 1975; Volpe 1952; Weatherby 1982; Zweifel 1968). Based on morphology, we estimated up to 16% of individuals to be hybrids (Chapter 2). Here, we use GBS to determine hybridization and correlate these known hybrids with morphological and call features used previously to assess hybridization. This study aims to test the ability of morphology to predict levels of hybridization and to determine the effect of hybridization on variation in advertisement calls. We expand on previous research to better understand the hybridizing relationship between these two species through quantitative measures of DNA, morphology, and male advertisement calls. We hypothesize that morphological hybrids will indicate genetic hybrids and that calls of hybrids will be intermediate between parameters of purebred parent species.

MATERIALS AND METHODS

Specimen collection

Anaxyrus americanus (N=31) and *A. fowleri* (N=33) individuals were collected at two sympatric sites in Jackson and Chambers Counties, Alabama between the months of February and June of 2014 and 2015. *Anaxyrus fowleri* (N=31) individuals were also collected at an allopatric site in Winston County, Alabama between the months of April and July of 2014 and 2015. Every collection took place between the hours of 8:00 and 11:00 pm when temperatures ranged between 16.6 and 21.1°C. Captured specimens were identified based on advertisement call and the presence or absence of morphological features described in previous field guides (e.g. Mount 1975). Photo vouchers were taken of every specimen with an 8 megapixel Canon PowerShot Pro S5 IS camera. Tissue samples were collected from each specimen (N=95) by severing the medial toe on the left hind leg.

DNA sequencing

Nuclear DNA (nDNA) was extracted from each tissue sample using an Omega BioTek E.Z.N.A Tissue DNA extraction kit (omegabiotek.com, product #D3396-02) following the protocol provided by the manufacturer. Samples were then prepped and approved via the standard protocol for the GBS technique (Elshire et al. 2011) to have sequencing carried out by the Institute of Genomic Diversity at Cornell University. In order to determine which restriction enzyme would best digest and reduce the complexity of the toad genome, 500ng of nDNA from three individuals was combined and digested, separately, with three different restriction enzymes (*Ape*KI, *Eco*T22I, and *Pst*I) according to the Institute of Genomic Diversity (Cornell University) protocol. The restriction enzyme, *Eco*T22I, a six base-pair cutter enzyme, was chosen because it 1) reduced the complexity of the toad genomes; 2) provided deep coverage ability; 3) generated repeated association peaks in DNA profiles, and; 4) has been used to digest other amphibian genomes. Once the nDNA was digested, pooled GBS sequencing libraries were created through

ligation of two sets of adapters, one with specific nucleotide sequences (barcode adapters) followed by a second set comprised of a more generalized sequence to the digested nDNA fragments using standard protocol (Cornell University) (Elshire et al. 2011). Following adapter ligation, polymerase chain reaction (PCR) was utilized to amplify nDNA fragments in preparation for sequencing. Sequencing was accomplished using Illumina HiSeq 2000/2500. The final number of sequencing reads was 331,486,102 with a final number of 11,496,637 tags. Two samples from *A. americanus* (AS17 and AS29) failed to yield adequate sequences and, therefore, were removed from further analysis.

Genetic analysis

From the raw sequence reads, SNPs were called using the GBS non-reference genome Universal Network Enabled Analysis Kit (UNEAK) pipeline from TASSEL v3.0173 at the Institute of Genomic Diversity (Cornell University). The UNEAK pipeline was chosen because these toad species do not have reference genomes (Glaubitz et al. 2014). For implementing this pipeline, the following parameters were set and run on a computer system with 12G of RAM and 8 core processors: FastqToTagCountPlugin –c was 1, MergeMultipleTagCountPlugin –c was 3, UTagCountToTagPairPlugin –e was 0.03, FastqToTBTPlugin –c was 1, tbt2vcfPlugin –mnMAF was 0.01, tbt2vcfPlugin –mnLCov was 0, tbt2vcfPlugin –ak was 3. Tags were merged and reduced to a total of 1,601,783. The sequence provider (Cornell University) also used VCFtools, version v0.1.12a, and PLINK, version v1.07, to generate an MDS plot from the filtered SNP file in variant call format (VCF). Any biallelic SNPs in the file were converted to PLINK format for this analysis. After the SNP data were called, they were filtered for minor allele frequencies >0.01 reducing the raw SNPs count of 45,590 to 36,748. Final SNP data were output as a VCF file.

Once returned from the manufacturer, two sets of SNPs were produced from the VCF file using TASSEL v5.2.17 (Bradbury et al. 2007). One set was filtered for 32% (30/93) of individuals sharing the same SNPs, while the second set was filtered for 10.8% (10/93) of individuals sharing the same SNPs. Both were filtered with a minimum frequency of 0.0 and a maximum frequency of 1.0. These filters were chosen to represent the highest conservation, filtering only species, and the lowest conservation, filtering individuals. After filtering, SNPs were not reduced in the 10.8% filter, but were reduced to 19,120 in the 32% filter.

Following filtering, SNP genotype calling data were exported from TASSEL v5.2.17 as a standard text file and manually converted to fastSTRUCTURE file format using Notepad ++ v6.9 for analysis of population structure for these three populations of toads using the Bayesian inference program fastSTRUCTURE (Raj et al. 2014). fastSTRUCTURE uses an algorithm that clusters individuals based on their SNPs. The clusters created represent populations that are defined based on a set of allele frequencies at each locus (*K*). In order to determine the appropriate *K*, the dataset was originally run in Structure v2.3.4 (Pritchard et al. 2000) with burin-in length of 5,000 and a run length of 50,000 with 5 interactions measuring *K* 1-4 and then run through STRUCTURE HARVESTER (Earl 2012) to determine which *K* best represented the three toad populations. STRUCTURE HARVESTER (Earl 2012) produced *K*=2 for the highest maximum likelihood, with the two populations representing *A. americanus* and *A. fowleri* samples. Therefore, the *K* parameter chosen for fastSTRUCTURE was 2 for both datasets. Default settings were maintained for running fastSTRUCTURE and the following commands were used to run the dataset: */faststructure structure.py -K 2 --input=30faststructure --output=K2_30_output --format=str.*

Structure Plot

Mean Q results were graphed because Q is a measurement of the proportion an individual is assigned to a population (*K*). This allowed us to identify all individuals showing evidence of genetic hybridization. Since each individual was classified to species based on external morphology, this allowed us to count the number of individuals of each morphological species that contained genes of the other species. The mean Q output files for both the 10.8% filter and the 32% filter from the fastSTRUCTURE results were formatted for STRUCTURE PLOT and loaded onto the website (http://btismysore.in/strplot/) to create a structure bar plot with a *K* of 2. A chi-square test was performed to test the hypothesis that *A. americanus* and *A. fowleri* were equally likely to show genetic evidence of hybridization.

Morphological and acoustic measurements

Morphological measurements of the five key characteristics listed in Chapter 2 were taken for every individual (N_{total}=95). We recorded calls of 19 *A. americanus* males and of 32 *A. fowleri* males in the field using a ZOOM© H2 handy recorder. Recordings of each specimen were taken from a distance of approximately 0.5 to 1.0 m and at least three consecutive calls were recorded for every individual. For every call, we measured the six call parameters listed from Chapter 3 using the sound analysis program Cool Edit Pro v1.2a ©1992.

Morphological and acoustic analysis

Once measurements were recorded for morphology and advertisement call, we then performed two discriminant function analyses (DFA) using the *lda*() function in the package MASS of the statistical program R v. 3.1.0, as described in Chapter 2. These two analyses gave posterior probability of assignment to species based on the combined morphological measurements and the combined call parameters for every individual. The results were then graphed separately, for a visual representation of individual assignment. These graphs were

compared to determine the consistency between identifying individuals based on morphology and advertisement call parameters.

In addition to the multi-dimensional scaling (MDS) plot generated from the SNP data, two additional MDS plots were created using Primer v6 in order generate visual representations of the similarity between individuals. The first plot examined similarity between all individuals based on morphological measurements. The second plot specifically examined male individuals based on morphological measurements, allowing us to determine whether any patterns existing specifically within males. Cluster analysis, based on the Bray-Curtis measurement of similarity, was applied to the MDS plots to group individuals within 70% similarity.

RESULTS

Population genetics

Of the 331,486,102 reads generated by GBS sequencing and analyzed via the UNEAK pipeline, only 291,851,738 were classified as good-barcoded reads (ones that do not contain missing data). The total number of tag networks identified was 475,466 with invariants of 459,390. Tag pairs with one or more variants were pruned using the error rate threshold parameter 16,076. The number of reciprocal Tag Pairs identified and used for SNP calling was 1,111,192. From these, a raw count of 45,590 SNPs were recovered. Further filtering of minor allele frequencies resulted in a reduction of SNPs to 36,748. For the filtered VCF file used, the mean individual depth was 2.525 with a standard deviation (sd) of 0.366. The mean of site depth in this file was 2.165 with an sd of 1.593. The individual missingness and site missingness both had a mean of 0.593 (sd= 0.056; sd= 0.251). Average proportion of heterozygosity was moderate for all populations in the study: *A. americanus* population was 0.065; *A. fowleri* in allopatry was

0.055; *A. fowleri* in sympatry was 0.05. An MDS plot created from the filtered SNP data showed genetic dissimilarity between the two species. All but two individuals of both *A. fowleri* populations were clustered tightly (Figure 1). The one individual excluded from this cluster was FS17, which was indicated as a genetic hybrid (Figure 1). *Anaxyrus americanus* specimens were distributed much more broadly than *A. fowleri* (Figure 1). A STRUCTURE PLOT from the mean Q results showed 5.4% (5/93) of individuals had evidence of gene flow between species (Figure 2). The plot also showed consistent gene flow existing between the two *A. fowleri* populations, with no distinction between populations based on the SNPs examined here. This plot only shows the results from K=2 due to the fact that even at K= 3 and 4, population structure did not separate *A. fowleri* in allopatry from *A. fowleri* in sympatry (Figure 2). The contingency table created from the plot contained 63 genetically pure *A. fowleri* individuals, one individual *A. fowleri* sharing alleles with *A. americanus*, 25 genetically pure *A. americanus* individuals, and 4 *A. americanus* individuals sharing alleles with *A. fowleri*. A contingency chi-square test allowed rejection of the null hypothesis of equal proportions of genetic hybrids between the two species (Figure 2; X^2=5.86; p = 0.05).

Morphology

Our DFA model created from morphological measurements revealed 5.4% (5/95) of all individuals collected from current populations possessed intermediate or entirely opposite species morphology based on posterior probability of assignment (Figure 3). Of the individuals classified as hybrid based on morphology (AS17, AS29, AS34, AS35, FA13), and individuals classified as hybrid based on SNP data (FS17, AS6, AS41, AS31, AS34), only one individual matched based on the SNP data (AS34). AS17 and AS29 did not have genetic data available. An MDS plot based on morphology for all individuals showed consistent results with the DFA

model from Chapter 2, recovering two distinct species groups, one representing *A. fowleri* in allopatry and sympatry and the other representing *A. americanus* (Figure 4). The MDS plot for only male individuals showed two distinct species groups with little overlap in cluster grouping (Figure 5).

Advertisement Call

Our DFA model, created based on call parameters, gave posterior probabilities for every individual that produced an advertisement call, and revealed that no specimen gave intermediate calls (Figure 6). When compared to the morphological DFA, 3 of 52 males (AS17, AS34, AS35) had morphological evidence of hybridization, but each of these gave advertisement calls lacking evidence of hybridization. When compared to the genetic DFA, 3 of 52 males (AS6, AS31, AS34) had genetic evidence of hybridization, but each gave advertisement calls lacking evidence of hybridization.

DISCUSSION

Genotype-by-sequencing (GBS) revealed 5.4% of individuals to be hybrids, a percentage that is virtually identical to that revealed by morphology (5.3%). Unfortunately, only a single individual was identified both as a genetic hybrid and a morphological hybrid. Two individuals classified as *A. americanus*, but with morphological evidence of hybridization, failed to sequence, limiting our ability to find concordance between the two methods of identifying hybrids. Regardless, this concordance appears to be weak. This is not surprising since morphological hybrids were classified from a limited number of features that can vary based on phenotypic plasticity, developmental anomalies, or random mutations (Mason and Taylor 2015). Additionally, the genes associated with these features may represent such a limited portion of the

genome that evidence of past hybridization would not be evident in them while being found in other genomic regions. Such features should increase the numbers of both false positive and false negative identification of hybrids, when based on morphology. Therefore, we require further investigation to significantly support our hypothesis that morphological hybrids will indicate genetic hybrids. The broad sampling of the genome offered by the GBS method should minimize both methods of false identification of genetic hybrids.

The percentage of hybrids identified by GBS is similar to the 3.4% hybridization found by Fontenot et al. (2011) for *A. americanus* and *A. fowleri* base on nuclear AFLPs. A low percentage of hybridization seems to be the pattern that exists between these two species. Fontenot et al. (2011) used similar sequencing techniques as our study, but they examined random individuals from throughout the geographic ranges of these two species. Our study focused on three main populations within Alabama, allowing us to examine hybridization at a population level. Continuing to examine population gene flow could confirm or uncover a higher percentage of hybridization seen between these two species.

Four of the 29 *A. americanus* individuals showed evidence of gene flow from *A. fowleri*, with only one *A. fowleri* showing evidence of gene flow from *A. americanus*. Our results show evidence of significantly more gene flow in the direction of *A. fowleri* into *A. americanus.* Due to the overall greater variation in male *A. americanus* advertisement calls seen in Chapter 3, we originally hypothesized selection against hybridization would be more difficult for *A. americanus* females and the genetic data from this study supports this suggestion. Although overlap of some call parameters was seen between species, *A. americanus* males were less consistent in their call parameters than *A. fowleri,* thus further supporting potential inaccuracy of female *A. americanus* to differentiate between males. Our genetic evidence further supports this

suggestion based on the direction of gene flow of *A. fowleri* into *A. americanus*, potentially indicating female *A. americanus* confuse male *A. fowleri* with conspecific males. This could be the mode of gene flow existing between these two species, as suggested by results from Chapter 3.

Interestingly, all males identified as genetic hybrids did not give any intermediate advertisement calls; therefore, we reject our hypothesis that calls of hybrids will be intermediate between parameters of purebred parent species. Three of the four hybrid *A. americanus* specimens were males with advertisement calls classified as 100% *A. americanus.* Although our sample size is small, our results could suggest potential gene flow via genetically mixed males giving species specific advertisement calls. In his book, Hauser (1996) states that larynx and muscle structure are the main influences in anuran advertisement calls. Based on this information, if the genetic makeup of this anatomical structure is not directly altered by gene flow, there would not be any intermediate advertisement calls and they would not indicate evidence of gene flow. This means that heterospecific genetic influence may not always alter the portion of the genome that influences advertisement calls. In a study by Zweifel (1968), morphologically hybrid individuals of *A. americanus* and *A. fowleri* produced an intermediate call based on pulse rate. Based on intermediate calls found in Zweifel's (1968) study, the acoustic DFA created in our study should be able to identify any F1 hybrids that possess intermediate calls. Conversely, our study did not find individuals with intermediate calls, and further sampling is required to determine if this indicates potential selection against any intermediate call parameters or that we simply did not sample enough to find a hybrid individual with an intermediate call. The presence of gene flow suggests hybridization at one point in time, but any selection against intermediate calls could be decreasing chances for this event to reoccur.

Our SNP data also document allopatric and sympatric populations of *A. fowleri* to be genetically indistinguishable. Our replicate sympatric sites were separated by approximately 278.4 km, suggesting consistent gene flow among *A. fowleri* populations in distant parts of Alabama. *Anaxyrus fowleri* is one of the most common species of toads found in Alabama, and is known to be found breeding in ponds and other bodies of water scattered throughout the state. Considering this species abundance and efficient ability to reproduce, it was not surprising to find evidence of high gene flow across the state.

Overall, the average proportion of heterozygosity found in all three populations suggests there is moderate genetic diversity in each population, with slightly more diversity seen in *A. americanus* (Hartl et al. 1997). An amphibian individual is heterozygous for roughly an average of 8.0% of all its loci (Hartl et al. 1997). The heterozygosity seen within these three populations suggests adequate genetic diversity exists within each population, but not at a high enough rate to suggest convergence of populations between these two species. The MDS plot created based on SNP results showed genetically tightly clustered *A. fowleri* from both populations while *A. americanus* individuals remained at further distances from each other. This leads us to believe that the two *A. fowleri* populations are more closely related to each other individually and collectively as two populations than is *A. americanus* as one population. This suggests as a species *A. fowleri* is more genetically structured and less genetically diverse, narrowing the path for gene flow from other species into these populations (Kruskal and Wish 1978).

Use of advanced molecular techniques allows for deeper testing for hybridization and gene flow (Mallet 2005). Applying multiple strategies will allow for clearer interpretation of results found from both genetic sequencing and morphology in order to detect if morphological intermediates are true hybrids or if the morphology is simply attributed to phenotypic plasticity

(Mason and Taylor 2015). Detecting specific loci that control or influence phenotypes is difficult, but through genetic analysis, it may be possible to uncover how differences in phenotypes come about within species (Mason and Taylor 2015). Additional investigation into this topic by increasing population studies and expanding genomic coverage will help us to test how morphology and call structure change in species exhibiting low levels of hybridization.

Figure 3-1. Multi-dimensional scaling plot created from the filtered SNP call data of all sequenced individuals (N_{total}=93). This plot was created at Cornell University. This plot clusters genetically similar individuals. High variation can be seen in *A. americanus* specimens while tight clustering is seen in *A. fowleri* specimens.

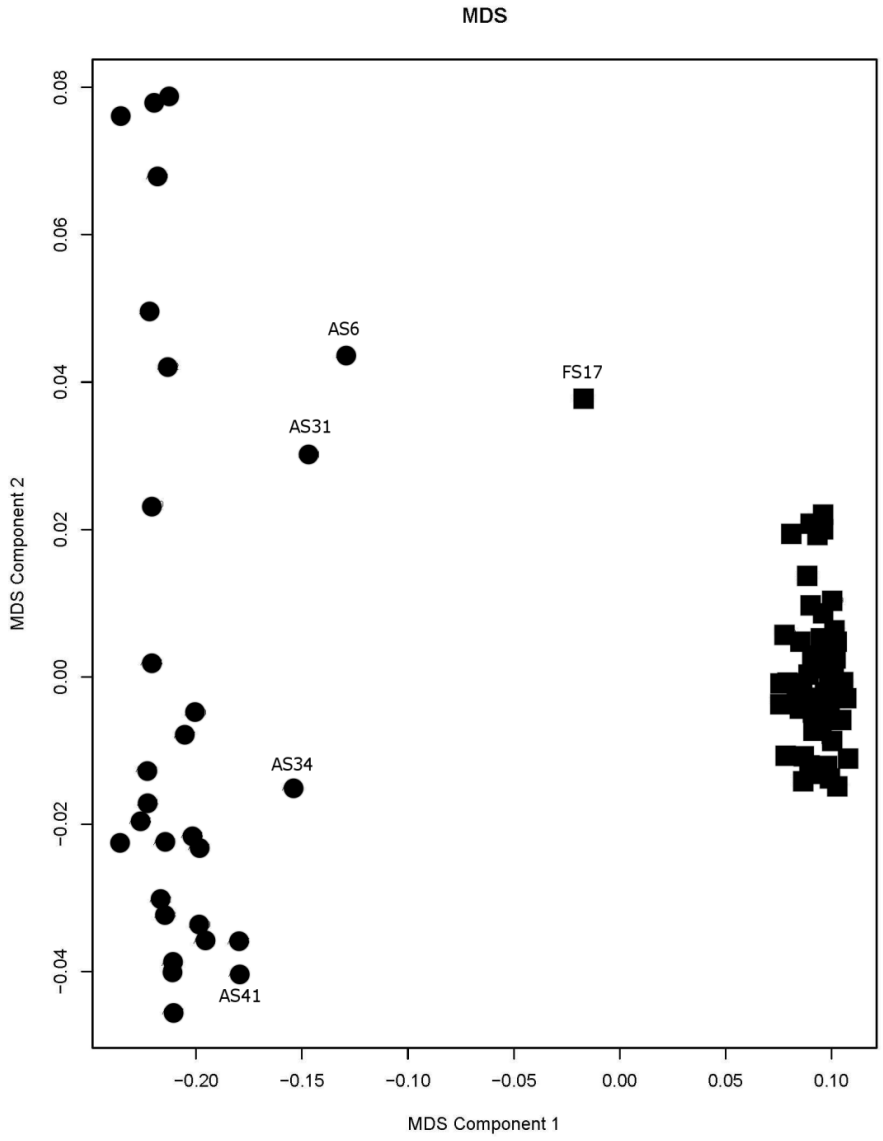


Figure 3-2. STRUCTURE PLOT produced this graph of all individuals genetic sequencing (N_{total}=93) based on filtering of the SNP call data at the 32% filtering (30/93). This filter selected for SNPs common between at least 30 individuals to represent the three populations present. It provides a visual of gene flow between populations. Four *A. americanus* (blue) individuals are seen with some *A. fowleri* genetic influence. Two *A. fowleri* (red) individuals are seen with some *A. americanus* genetic influence.

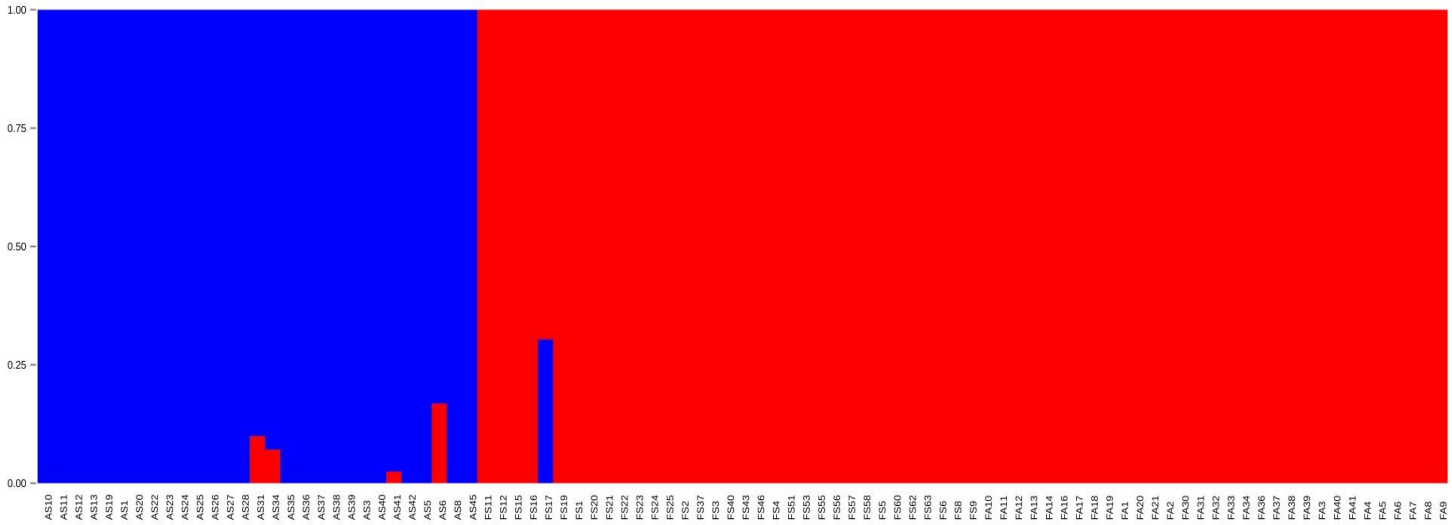


Figure 3-3. Posterior probabilities for all individuals (N_{total}=95) based on the discriminant function model created from combining morphological measurements of species representative specimens from Chapter 2. Percentage of *A. americanus* is represented by blue and *A. fowleri* is represented by red.

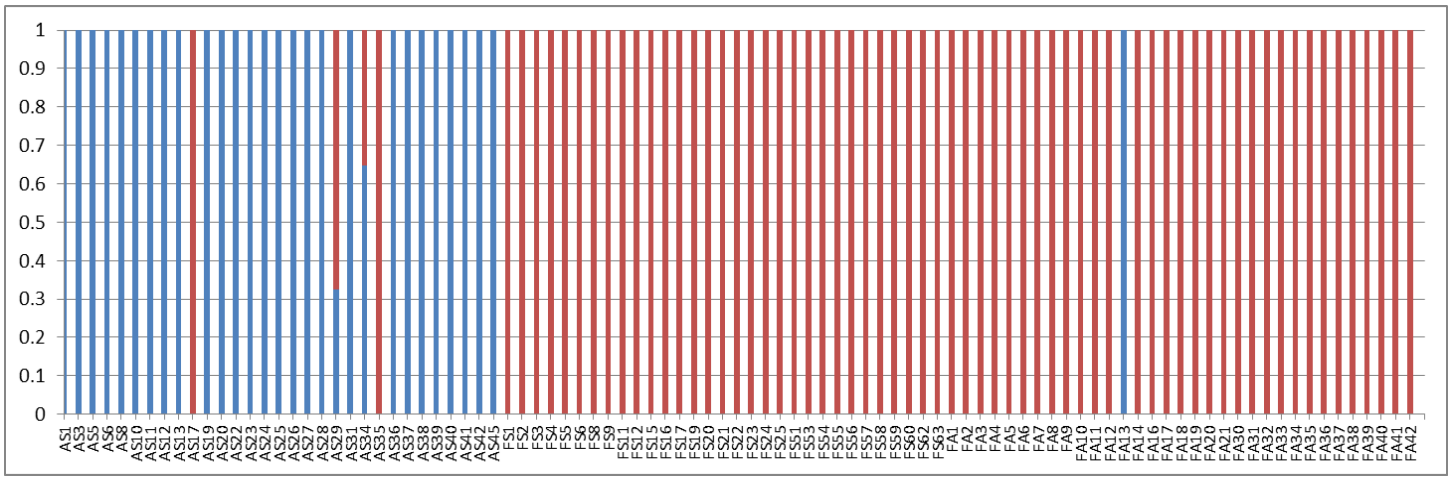


Figure 3-4. Multi-dimensional scaling plot of all individuals based on morphological measurements (N=95). Original classifications based on field observation. *A. americanus* = (blue) upward triangles; *A. fowleri* in allopatry = (green) squares; *A. fowleri* in sympatry = (red) downward triangles.

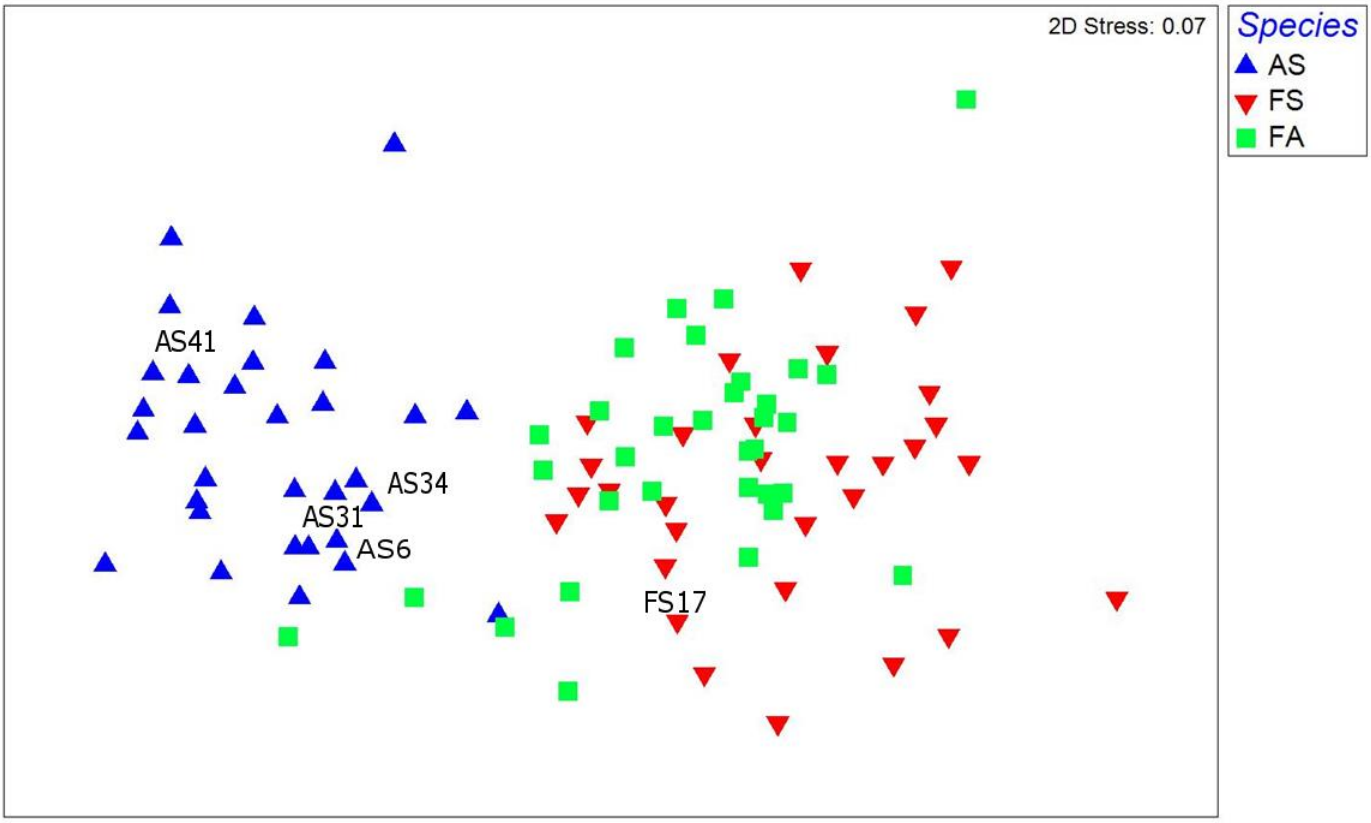


Figure 3-5. Multi-dimensional scaling plot of all male individuals based on morphological measurements (N=52). Classifications based on field observation. *A. americanus* = (blue) upward triangles; *A. fowleri* in allopatry = (green) squares; *A. fowleri* in sympatry = (red) downward triangles.

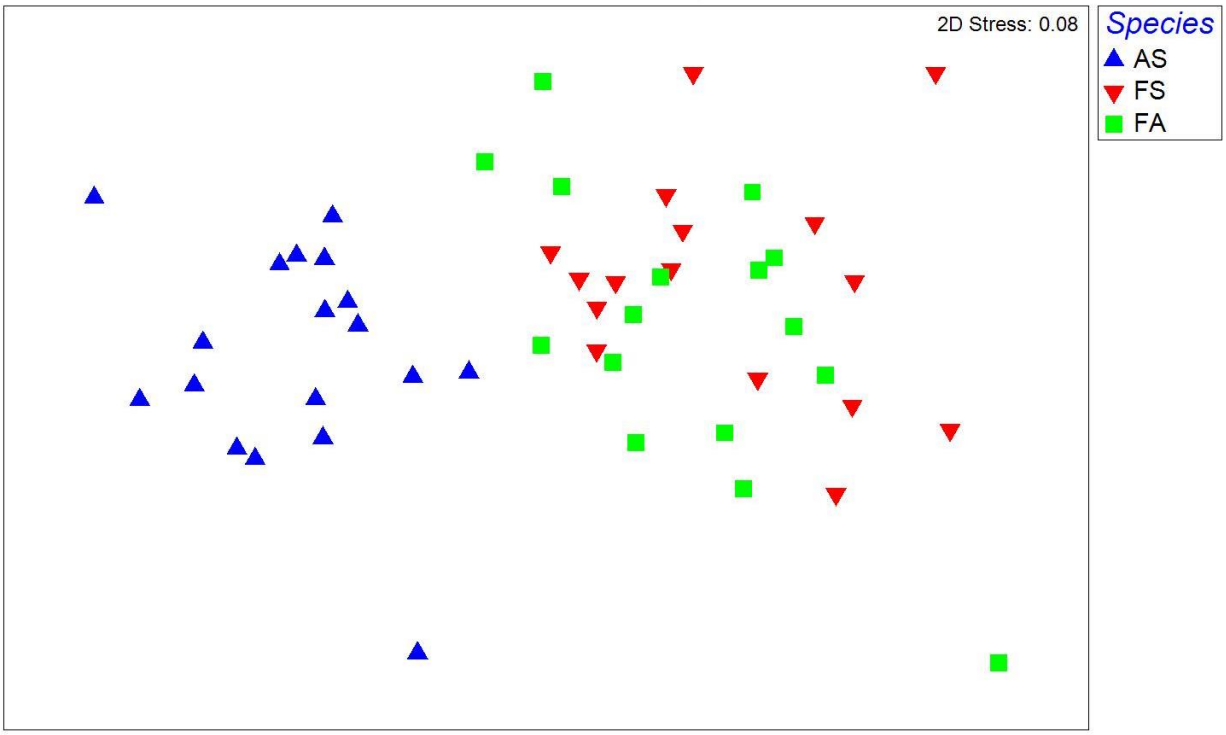
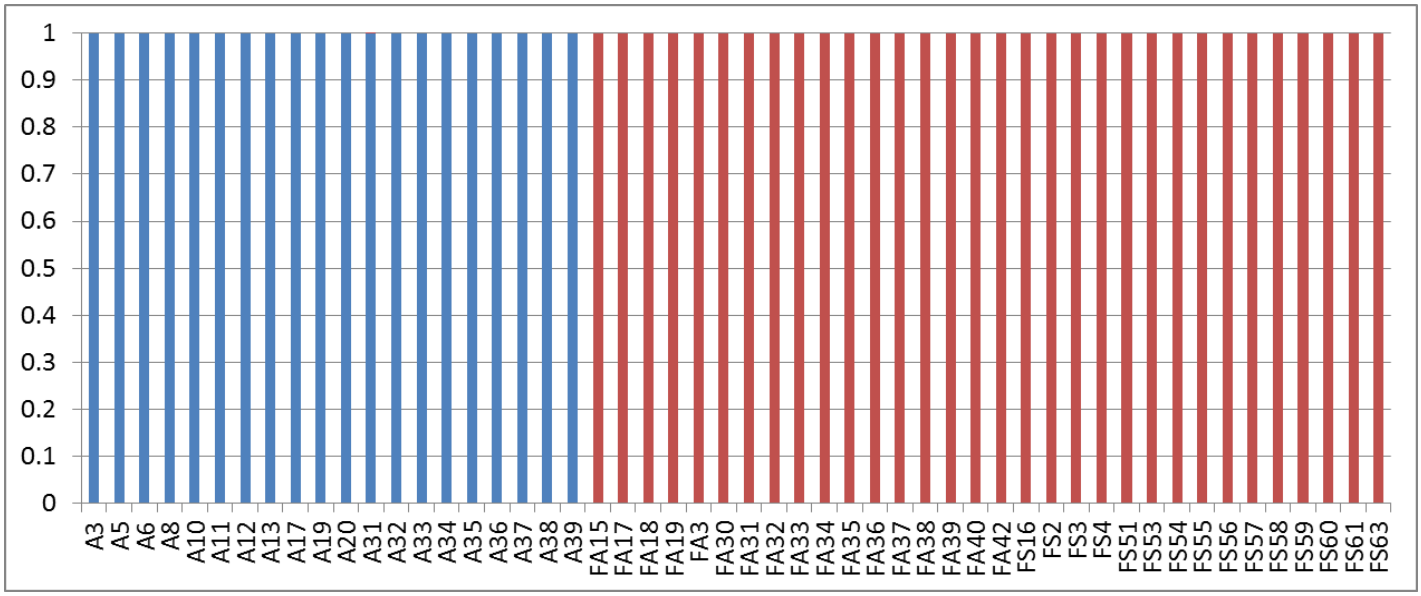


Figure 3-6. Posterior probabilities for every calling male (N_{total}=52) based on the discriminant function model created from combining all call parameter measurements. Percentage of *A. americanus* is represented by blue and *A. fowleri* is represented by red.



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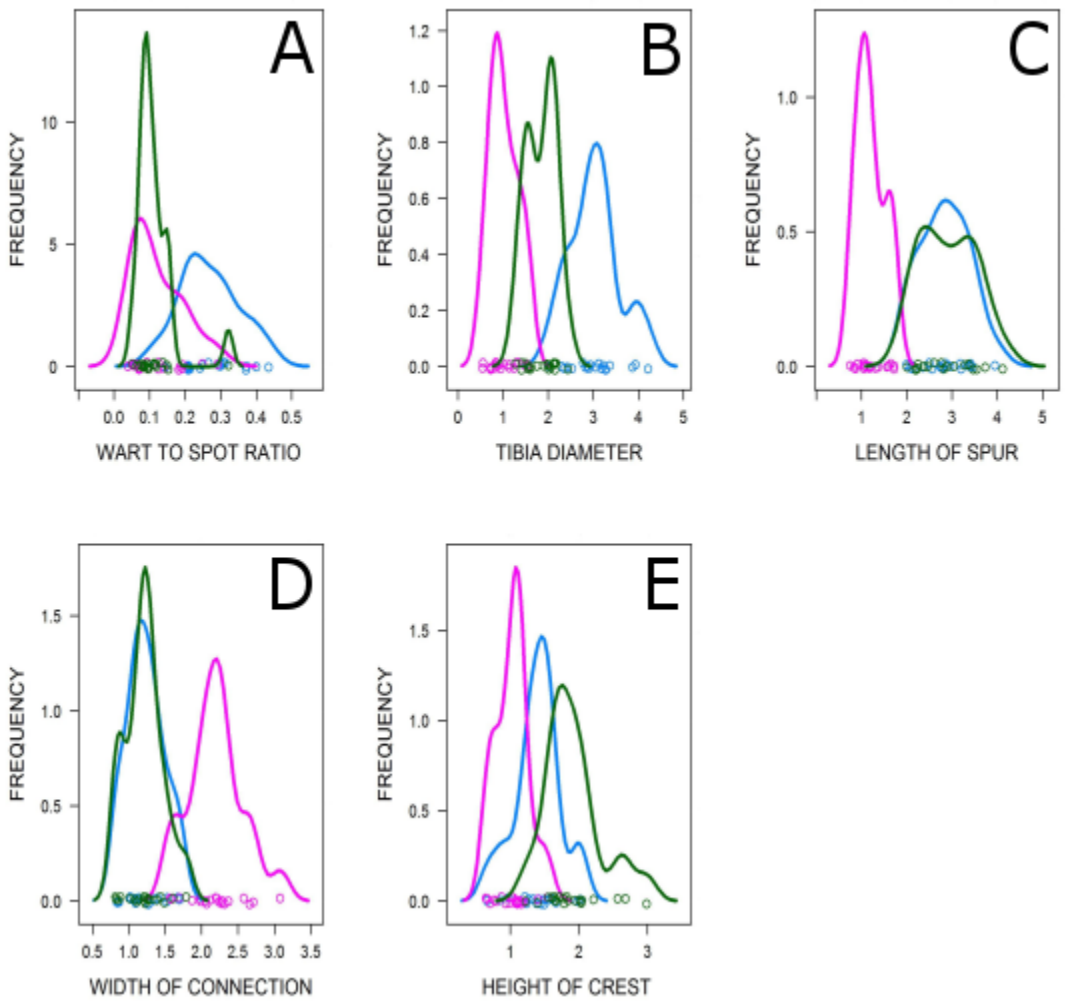
Appendix 1- Frequency distribution of wart-to-spot ratio (A), tibial wart diameter (B), spur length (C), spur width (D), and height of crest junction (E). Red line and hollow circle [Black line and hollow circle] = *A. fowleri*; blue line and hollow circle [short-dash line and hollow triangle] = *A. americanus*; green line and hollow circle [long-dash line and hollow square] = *A. terrestris*.



Appendix 2 Cluster analysis of measurements from pure individuals (N=60) chosen based on five morphological characteristics described in Jones (1973), Mount (1975), and Conant and Collins (1998). Sample labels indicate species (A = *A. americanus*; F = *A. fowleri*; T = *A. terrestris*) and replicate (number; see Table 4 for list of specimens). Black lines represent statistically significant clusters based on a Simprof test (Clarke and Gorley 2001).