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Laryngeal apparatus and call structure in North American hylids

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LARYNGEAL APPARATUS AND CALL STRUCTURE IN NORTH AMERICAN HYLIDS

by

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ABSTRACT

Although the ecological implications and structure of frog calls have been the subject of much study, little is known about the association between the laryngeal apparatus morphology and call structure in North American hylids. In this study linear measurements of the laryngeal apparatus were captured and compared to the call structures of thirteen species of North American hylids. Species examined included: *Pseudacris crucifer, P. triseriata, P. ocularis, Acris crepitans blanchardi, Hyla avivoca, H. cinerea, H. gratiosa, H. chrysoscelis, H. versicolor, H. squirella, H. femoralis, H. arenicolor,* and *H. eximia.* Six homologous landmark points were identified, and the lengths between them were measured on the ventral side of five specimens representing each of the 13 species. Angles between the points were also measured. Additionally, measurements were taken of the lateral side of five specimens of each species. Snout-vent length was considered as a covariate, indicative of overall body length. The 18 morphological measurements were compared to 13 call characteristics, which included acoustic (spectral and temporal) units interpreted mechanistically. Morphological results are consistent with the divergence of the three genera following the phylogeny of Faivovich et al. Four call characteristics that correlate with the morphological characteristics are discussed in the context of the phylogeny of the group.

ACKNOWLEDGMENTS

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1. INTRODUCTION

1.1. BACKGROUND

Frogs are an ideal model to examine the role of sexual selection in speciation. In frogs, sexual selection is a strong force of evolution and takes the form of acoustic communication. Frog calls are vital to the continuance of frogs---if frogs are to reproduce from one generation to the next generation, the males must communicate with females through calls (Gerhardt and Huber 2002). Frogs communicate through vocalizations produced by the morphological structures of the laryngeal apparatus and the vocal sacs (Duellman and Trueb 1994). Males call to attract females. In a chorus of frogs a male's call must be within the female's preference range. Thus, calls vary among individuals, and changes in females' preference may lead to reproductive isolation, and in some cases, speciation (Boul et al. 2007). The goal of this thesis is to understand the relationship between laryngeal morphology, call structure and its role in the evolution of frogs.

Frogs in the family Hylidae, also known as tree frogs, are diverse in morphology and call structure (Duellman and Trueb 1994), and thus, are ideal for studying the evolutionary relationship between acoustic communication and morphology. This study concentrates on the North American hylids, which include the genera *Acris, Pseudacris* and *Hyla*. The many studies on acoustic communication in frogs, their morphological structures, and evolution of their calls have either focused on a specific species [e.g., Wilczynski et al. 2001] or on how evolution formed call recognition mechanisms [Ryan et al. 2001]. At the present time, the state of scientific knowledge is incomplete regarding the relationship between the

laryngeal apparatus and the call structure of the frogs, and the impact of this relationship on the biodiversity of frogs. Thus, this study concentrates on the association between the laryngeal apparatus morphology and the call structure of several species of North American hylid frogs.

The main question in this study is what are the underlying laryngeal structures that produce variations in call structures. There are three main questions that are addressed by this research: 1) Does the morphology of the laryngeal apparatus differ among the species; 2) Are variations in morphology associated with variations in the calls; and 3) Is there a phylogenetic pattern to morphology and call variation. By studying calls and the structures associated with those calls, it is possible to learn about the morphological innovations underlying divergence in communication systems and potential speciation.

Although there are differences among the species in their calls, there are some general characteristics that apply to all frog calls. The morphological structures used to generate a call involve the laryngeal apparatus. Cartilaginous structures support the larynx, which include the arytenoid cartilage and the cricoid cartilage. The cricoid cartilage is the structure connected with the vocal chords. The cricoid ring acts as a base for the arytenoid cartilages to pivot around. (Duellman and Trueb 1994). To find an evolutionary change in the laryngeal apparatus that underlies an evolutionary change in the calls leading to the many species of North American hylids requires studying both morphology and call structure in light of a robust phylogenic tree.

1.2. GOALS AND OBJECTIVES

1.2.1. Main Goal. The primary goal of this study is to determine if evolutionary changes in the morphology of the laryngeal apparatus led to evolutionary changes in calls, which associated with speciation within a large clade of frogs, the North American hylids.

1.2.2. Key Objectives. Several objectives exist for this research.

1.2.2.1. First objective. My first objective was to quantify the various morphological shapes and structures comprised in the laryngeal apparatus of select species of North American hylids.

1.2.2.2. Second objective. My second objective was to analyze calls in several representative species in the group.

1.2.2.3. Third objective. My third objective was to compare the call structure with the morphological structures in light of a phylogeny to determine if changes in the morphological structures correlate with changes in calls.

1.2.3. Hypotheses. The hypotheses tested herein are: 1) Evolutionary changes in the laryngeal apparatus led to evolutionary changes in calls of the North American hylids, and 2) The evolutionary changes in the morphology and call structure associated with speciation within a large clade of frogs, the North American hylids

2. REVIEW OF LITERATURE

2.1. SEXUAL SELECTION'S RELATION TO SPECIATION

A major force behind speciation is sexual selection. In frogs this takes the form of acoustic communication. Generally, only males produce advertisement calls. This sexual dimorphism in call production seems to be a consistent pattern among thousands of species of frogs (Emerson 1999). Most frogs use sound to attract females (Gridi-Papp 2003; Duellman and Trueb 1994; Gerhardt and Huber 2002). The female has to choose among the many frogs calling within a chorus. This means not only does the male call have to be unique to attract the female, but of equal or greater importance, it must be within the range of the female preference (Gerhardt 2005). *Hyla chrysoscelis* and *H. versicolor* cannot be distinguished by their external morphology; however, Gerhardt (2005) found that mate choice in these two species was based on the variances in their acoustic signals to the exclusion of any other factor. This divergence in female preferences and the unique male signals can lead to pre-zygotic reproductive isolation. This has been shown to occur among several frog populations, and has been shown to lead to speciation in more than one species (Boul et al. 2007). Two tree frogs, *H. gratiosa* and *H. cinerea*, overlap extensively in their range distribution and have similar call types (Ryan et al. 2001). Because hybridization is costly, females of both species are under strong selection to recognize and have preference for the male calls of their own species.

2.2. MORPHOLOGICAL STRUCTURE

Since sexual selection for calls can lead to speciation, other studies have looked at the underlying morphological structures that selection ultimately acts upon. The laryngeal apparatus constitutes a sound producing structure (Maglia et al. 2007). Located between the lungs and buccal cavity it is made up of a pair of arytenoid cartilages supported by the cricoid ring (Duellman and Trueb 1994). A study conducted by Martin (1972) described the morphology of the laryngeal apparatus. In the hylids the posterior edges of the vocal cords attach to the arytenoid and cricoid cartilage. Pulmonary air pressure forces the arytenoids apart and then they are returned to a closed position by elasticity during each vibratory cycle (Martin 1972). The laryngeal apparatus morphology displays sexual dimorphism (Emerson 1999). Vocalizations in anurans are a result of relationships between the morphology of the larynx and vocal tract and movements of the arytenoid cartilages. The morphology of the larynx can influence the frequencies and other spectral characteristics of the vocalizations in anurans. Some temporal characteristics of the call come from movements of the larynx (McClelland et al. 1996). McClelland's study of *Acris crepitans* (cricket frogs), demonstrated that dominant frequency, call duration and number of pulses were positively correlated with the size of the laryngeal apparatus and body size. Since the components that comprise the laryngeal apparatus are influenced by body size, McClelland et al. (1996) showed that the temporal and spectral properties of calls are interrelated. Changes in the larynx can be an influential factor on the divergence of call characteristics among populations, and in turn, this can have a potential effect

on the course of speciation. However, this study included only populations of one species, *A. crepitans* (McClelland et al. 1996). Since the results had implications for populations of only one species, limitations are placed on how broadly these conclusions can apply to other species.

One study indicated that an ideal group for a comparative analysis of communication would be a group with a robust phylogeny that had well-documented tape recordings that are in museums. One of the groups that fit this ideal is the Hylidae (Cocroft and Ryan 1995).

2.3. CALL CHARACTERISTICS

The advertisement call is the most important and conspicuous of anuran vocalizations (Ryan 1988). The advertisement calls are vocalizations that males use to attract the females in order to mate and to advertise their presence to other males (Ryan 1988). In some instances, frogs will alternate their calls or overlap their calls in time (Ryan 1988). Call characteristics consist of acoustic units (spectral and temporal) that are interpreted mechanistically. Advertisement calls with unique spectral and temporal structures are produced by males, which enable the females of a species to identify and select the high-quality male with which to mate (Welch et al. 1998).

2.3.1. Acoustic Characters. Fundamental frequency, dominant frequency and bandwidth compose spectral characters. The spectral call characteristics, especially dominant frequency, are of special importance to frog evolution (Ryan 1986). Selection does occur in the form of female choice on call frequency. Females are attracted by calls with the proper temporal structure (Feng and Ratnam

2000). However, this by itself is demonstrative of selection, and does not imply evolution. High frequencies are favored when communicating over long distances, however, this acts in opposition to the environment which favors low frequencies (Ryan 1988). In certain species selection on low frequency calls by the female has had some influence on the evolution of the call (Ryan 1986). McClelland et al. (1996) and Ryan (1988) showed that body size (measured using snout-vent length) and the dominant frequency of the mating call usually show a correlation.

Call duration and number of pulses comprise some of the temporal call characteristics. Call duration can be a reliable indicator of heritable genetic quality (Welch et al. 1998). In one study, female gray tree frogs (*Hyla versicolor*) showed preference for long duration calls (Welch et al. 1998). Females generally prefer long calls repeated at slower rates than short calls repeated at a high rate (Gerhardt and Brooks 2009). Fine temporal characteristics can be important such as pulse rise time (Gerhardt and Huber 2002)

Other selective factors can cause call divergence. Temperature affects many call characteristics in a predictable and quantitative way (Cocroft and Ryan 1995). It can affect both the spectral and temporal properties of the calls. Temperature has been shown to affect the dominant frequency (Ryan 1988) and to have a significant correlation with pulse rate (Keller and Gerhardt 2001). A study by Gerhardt (1999) showed that environmental factors can partially account for the evolutionary change in communication systems.

Another study addressed the degradation of signal efficacy over distance (Kime et al. 1999). Degradation is affected by the height at which the call occurs and also where it is received, as well as the environment through which it travels. However, factors other than external ones affect signal degradation. In the 22 species of Central American frogs in this study the amount of degradation differed among habitats and between transmission heights. Additionally, degradation also differed among the species and among the calls that differed in spectral characteristics, specifically dominant frequency. The notable result in this study was that all of the differences in degradation did not relate specifically to the local calling habitat of the species.

2.3.2. Mechanistic Coding. Examining acoustic characters solely without considering the mechanism of sound production can result in misleading phylogenetic information and misleading results in understanding the evolution of vocalizations (Robillard et al. 2006). Acoustic signals are the end-product of calling behavior. To have a clear and accurate picture of homologies in frog vocalization that can be used effectively in phylogenetic analyses, a comparison of calls among species has to involve interpretation of acoustic units mechanistically. The acoustic (pulse, call) units may differ from the mechanistic (note) unit among frogs in closely related species. The note is the amount of sound produced during a single expiration (Robillard et al. 2006). A call can equal one note, or one pulse can equal one note. Robillard's (2006) study showed that three *Hyla* species (*H. squirella, H. cinerea,* and *H. gratiosa*) all have a single-note call. This correlation exists even though one species (*H. squirella*) is pulsed but the other two species are

unpulsed. By looking solely at the acoustic character (pulsed versus unpulsed) a mismatch would occur that can lead to misleading inferences about the evolution of the calls (Robillard et al. 2006). Thus, a comparative study must interpret acoustic units in regard to mechanism of sound production in order to have consistent phylogenetic information.

2.4. PHYLOGENETIC ANALYSIS

The North American hylids are an ideal group for comparative studies due to their diversity (Gridi-Papp 2003; Cocroft and Ryan 1995). However, a recent analysis showed an understanding of the relationships among North American hylid frogs was ambiguous despite morphological, molecular and behavioral data (Moriarty and Cannatella 2004). This study did show there were four strongly supported clades within *Pseudacris*, one of which is the *crucifer* clade consisting of *P. crucifer* and *P. ocularis*. Another of these clades is the *nigrita* clade consisting of *P. triseriata*. The hylids underwent two radiations in North America with one including the genus *Hyla* and the other includes the genera *Acris* and *Pseudacris* (Lemmon et al. 2007 from Smith 2005). It was the study done by Faviovich et al. (2005) that helped tremendously to clarify the phylogenetic relationships among the North American hylids. As put forth by Faviovich et al. (2005), *Hyla* consists of four groups, three of which are addressed in this research - *H. cinerea* group, *H. versicolor* group and *H. eximia* group.

2.5. SUMMARY OF LITERATURE REVIEW

A major force behind speciation is sexual selection, which in frogs takes the form of male-male competition and female mate choice of call characteristics.

Studies have examined the underlying morphological structures that selection ultimately acts upon. Additional studies have looked at the acoustic call structures interpreting them mechanistically (Robillard et al. 2006). The North American hylids have been studied a great deal since they are a diverse group with more than 800 different species. The phylogeny of this group has been studied often, and a number of revisions have taken place with additional understanding of the relationships. Most call analyses have concentrated on either the morphological structures in a specific species or how evolution formed call recognition.

The current study continues where some of the other studies have stopped by concentrating on the association between the laryngeal apparatus morphology and the call structure of North American hylids to determine if there is a phylogenetic pattern to morphology and call variation. By including a phylogeny, this comparative study can identify patterns of divergence and the direction of character change (Cocroft and Ryan 1995).

3. MATERIALS AND METHODS I: MORPHOLOGY

3.1. SAMPLING METHODS

3.1.1. Specimens and Preparation. Whole specimens preserved in 70% ethyl alcohol (ETOH) were obtained from the Herpetology Collection at the University of Kansas Natural History Museum and Biodiversity Research Center. The skins of the specimens were removed and each specimen was eviscerated. The specimens then were processed through the clearing and doubled-staining procedure following methods adapted from Taylor and van Dyke (1985). The clearing and double-staining procedure provides a way to view the skeletal system of the frog. Clearing uses digestive enzymes such as potassium hydroxide, ethyl alcohol and the enzyme trypsin to digest the muscle tissues. Chemicals such as alizarin red and alcian blue are added to the specimen. Alizarin makes the bones appear as red, while alcian blue adheres to the chondrin of the specimen causing the cartilage to be stained blue. The specimens are preserved in glycerine with one or two crystals of thymol to prevent fungal growth. A detailed description of the clearing and double-staining protocol used in this study can be found in Appendix A. Figure 3.1 demonstrates a cleared and double-stained specimen of the laryngeal apparatus of one of the North American hylid specimens.

A total of thirteen (13) species were cleared and doubled-stained to visualize cartilage in the vocal apparatus. Each of the species is represented by five (5) adult male specimens for a total of 65 specimens. Appendix B lists the specimens examined. The laryngeal apparatus of each specimen was dissected out and observed using an Olympus SZX12 stereoscope. The laryngeal apparatus is

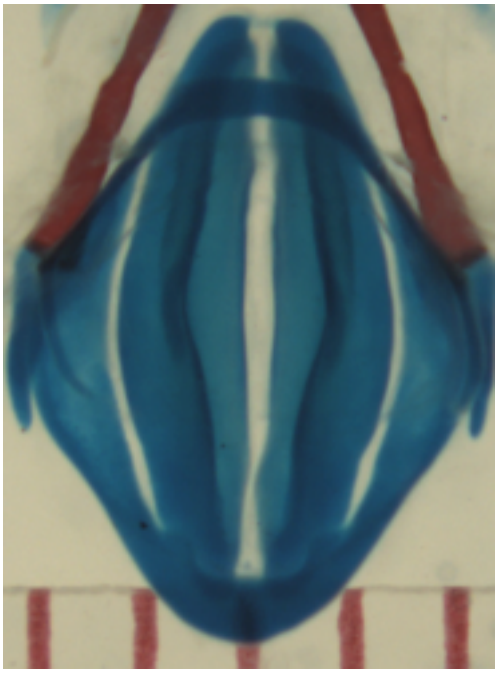

Figure 3.1. Ventral view of laryngeal apparatus of *Hyla squirella* (KU145631) Measurements are in mm.

composed mainly of the cricoid and arytenoid cartilages. The cricoid is composed of a ring that surrounds the larynx. The arytenoid cartilages are composed of a pair of valves that rest on the cricoid cartilages. The cricoid has several processes which include the cardiac, bronchial, lateral and esophageal (Figure 3.2).

3.1.2. Descriptions and Illustrations. The laryngeal apparatus of each specimen was photographed in ventral view (with a 1mm scale bar) using an Olympus 5.0 RTB QImaging camera mounted to an Olympus SZX12 stereoscope with QCapture Pro^{c} software (version 5.0.1.26). Laryngeal apparatus terminology follows that of Trewavas (1933), Martin (1972) and Duellman and Trueb (1994). Taxonomy follows that of Faivovich et al. (2005).

3.1.3. Landmark Points. Six landmark points were chosen on the laryngeal apparatus of all specimens. These points were chosen based on criteria that made the points easy to identify in all the specimens (Larson 2002). Additionally, the landmark points described regions of the laryngeal apparatus whose functions were easy to identify and homologous among the species. The landmark points were digitized on the ventral side of the laryngeal apparatus in the 65 specimens using tpsDIG version 2.12 (Rohlf, 1998, Department of Ecology and Evolution, SUNY, Stony Brook). The landmark points were: 1: pulvinar vocale; 2: cardiac process of the cricoid; 3: bronchial process of the cricoid; 4: lateral process of the cricoid; 5: posterior margin of the arytenoid; and 6: esophageal process of cricoid ring as seen on Figure 3.2.

3.1.4. Linear Morphometrics. Eleven (11) linear measurements were taken using the landmark points digitized on the ventral side of the laryngeal apparatus as seen on Figure 3.3. These measurements include: 1) the distance between the pulvinar vocale to the posterior margin of the arytenoid; 2) the distance between the cardiac process of the cricoid to the bronchial process of the cricoid; 3) the distance between the bronchial process of the cricoid to the lateral process of the cricoid; 4) the distance between the lateral process of the cricoid to the esophageal process of the cricoid; 5) the distance between the cardiac process of the cricoid to the esophageal process of the cricoid; 6) the distance between the pulvinar vocale to the medial margin of the arytenoid; 7) the distance from the right ventral lateral process of the cricoid to the left ventral lateral process of the cricoid

(width of the cricoid ring at the medial margins); 8) the distance between the anterior corners of the bronchial processes; 9) the distance between the medial margin of the arytenoid to the posterior margin of the arytenoid;) 10) the distance between the pulvinar vocales; 11) the distance between the highest anterior curvatures of the cardiac process of the cricoid (Location of landmark point 2 is the highest anterior curvature).

These measurements represent the general shape of the laryngeal apparatus (the length and width of the arytenoid cartilage and the cricord ring). The measurements also represent the shape of structures that are functionally important. Another measurement taken for each specimen included the snout-vent length (SVL), which measures overall body size of the frog specimen. The snout-vent length was measured directly from specimens using vernier calipers. The measurements were taken using the tpsDig program which initially measures in pixels. A one millimeter scale bar was measured in pixels. Then each measurement in pixels was converted into millimeters by dividing the number of pixels of the particular measurement by the number of pixels in one millimeter.

3.1.5. Lateral Measurements. Two lateral measurements were taken of each of the 65 specimens. The two lateral measurements included the lateral height of the laryngeal apparatus and the lateral height of the arytenoid cartilage as seen on Figure 3.4. The measurements were taken using the tpsDig program which initially measures in pixels. A one millimeter scale bar was measured in pixels.

Then each measurement in pixels was converted into millimeters by dividing the number of pixels of the particular measurement by the number of pixels in one millimeter.

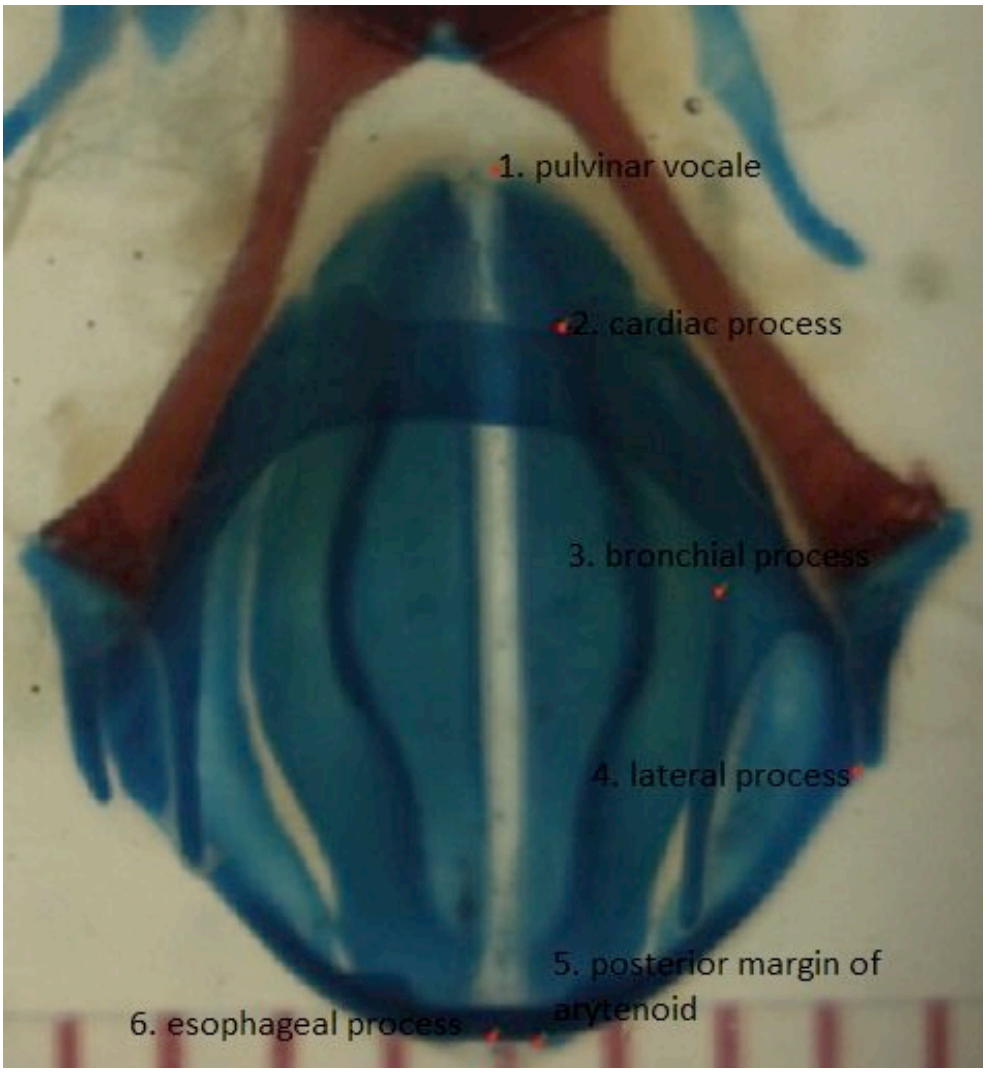

Figure 3.2. Six Landmark points *Hyla arenicolor*: 1. pulvinar vocale 2. cardiac process 3. bronchial process 4. lateral process 5. posterior margin of arytenoid 6. esophageal process.

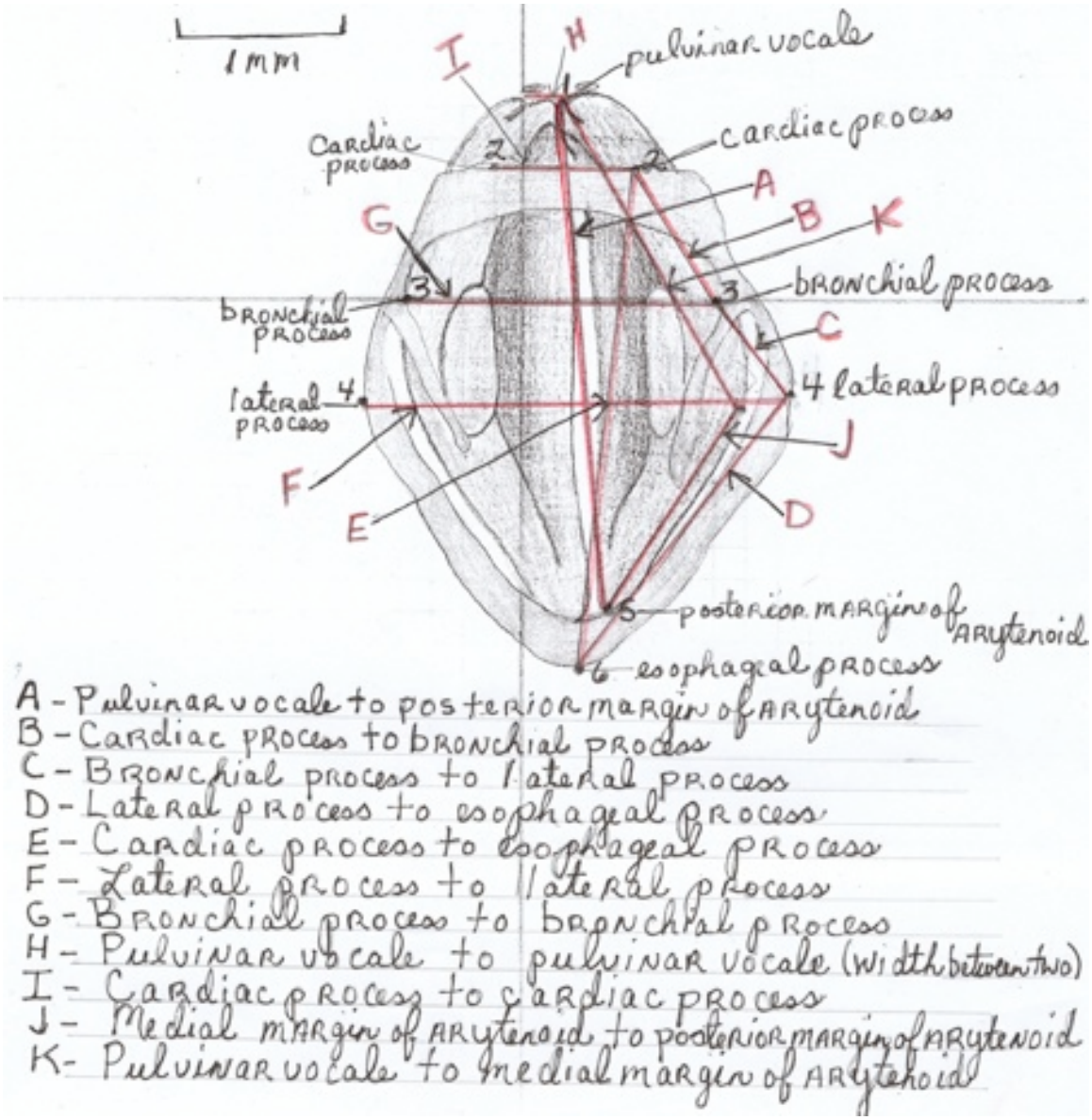

Figure 3.3. Linear Measurements of Laryngeal Apparatus.

3.1.6. Angle Measurements. Four angle measurements were taken of the laryngeal apparatus of each of the 65 specimens. The measurements were taken

using the tpsDig program and the angles represent degrees. The four measured angles included: 1) the pulvinar vocale measured from the posterior margin of the arytenoid to the medial margin of the arytenoid; 2) the medial margin of the arytenoid measured from the pulvinar vocale to the posterior margin of the arytenoid; 3)the bronchial process of the cricoid measured from the cardiac process of the cricoid to the esophageal process of the cricoid; and 4) the lateral process of

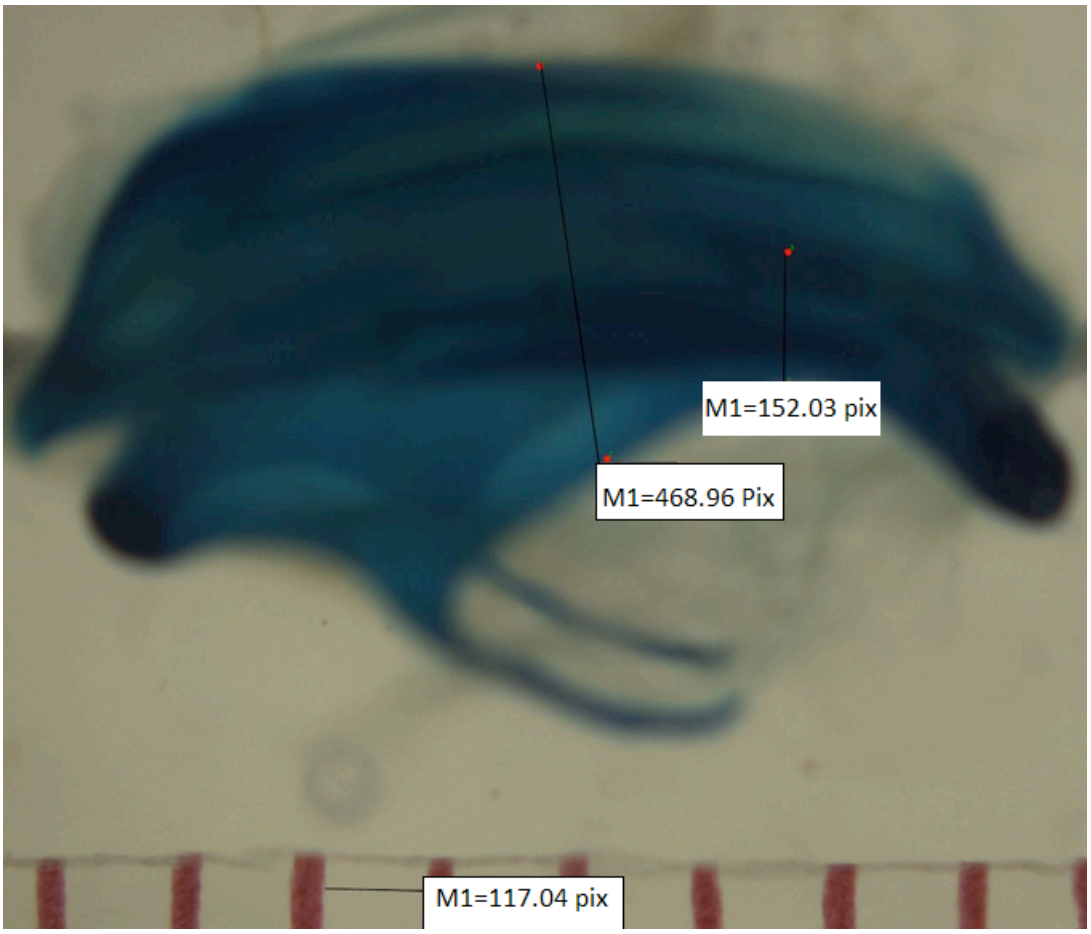

Figure 3.4. Lateral Measurements of Laryngeal Apparatus of *Hyla cinerea.* Lateral height of laryngeal apparatus 468.96 pix (4mm) Lateral height of arytenoid 152.03 pix (1.3mm).

the cricoid measured from the cardiac process of the cricoid to the esophageal process of the cricoid. The measurements are shown on Figure 3.5.

3.2. STATISTICAL ANALYSIS

3.2.1. Multivariate Analysis of Variance (MANOVA). SAS version 9.2 (Statistical Analysis System 2008) was used to determine the significant differences among the species using multivariate analysis of variance (MANOVA). The main focus was to determine which of the 18 morphological characters showed significant differences among the species. This was an attempt to determine whether certain linear, angle and/or lateral measurements were unique to each of the three genera.

3.2.2. Principal Components Analysis (PCA). The same SAS program was used to run a principal components analysis (PCA) on the data set. PCA extracts from a set of variables a reduced set of components that account for most of the variance in the set of variables. The principal components reduce the number of correlated dependent variables and explain the percent of variance among all the specimens. Scatterplots of the factor scores were examined to determine whether the species clustered into distinct genera when considering the morphological characters. The main focus was to determine if the clusters associated with clades that correlated with the phylogeny.

3.3. ELEVATION OF *ACRIS BLANCHARDI*

While this project was ongoing the designation of *Acris crepitans blanchardi* as a subspecies of *Acris crepitans* changed. The morphometrics used to define *A.c. blanchardi* was found to be of doubtful utility (McCallum et al. 2006), and the morphological data that had been used to differentiate *Acris crepitans crepitans* from *Acris crepitans blanchardi* did not adequately discriminate these two (Gamble et al. 2008). DNA analysis showed that the two species, originally designated as subspecies, are two different species. In light of the results Gamble et al. (2008) elevated the populations of *A.c. blanchardi* bordered by the Mississippi and Ohio rivers to *Acris blanchardi.* The specimens analyzed in the present study are from locations within the boundaries designated by Gamble et al. (2008), and thus, by their results, should be considered *A. blanchardi*. However, the call recordings are from *A. crepitans.* To be consistent with the listings given the specimens, reference is made only to *A. crepitans.*

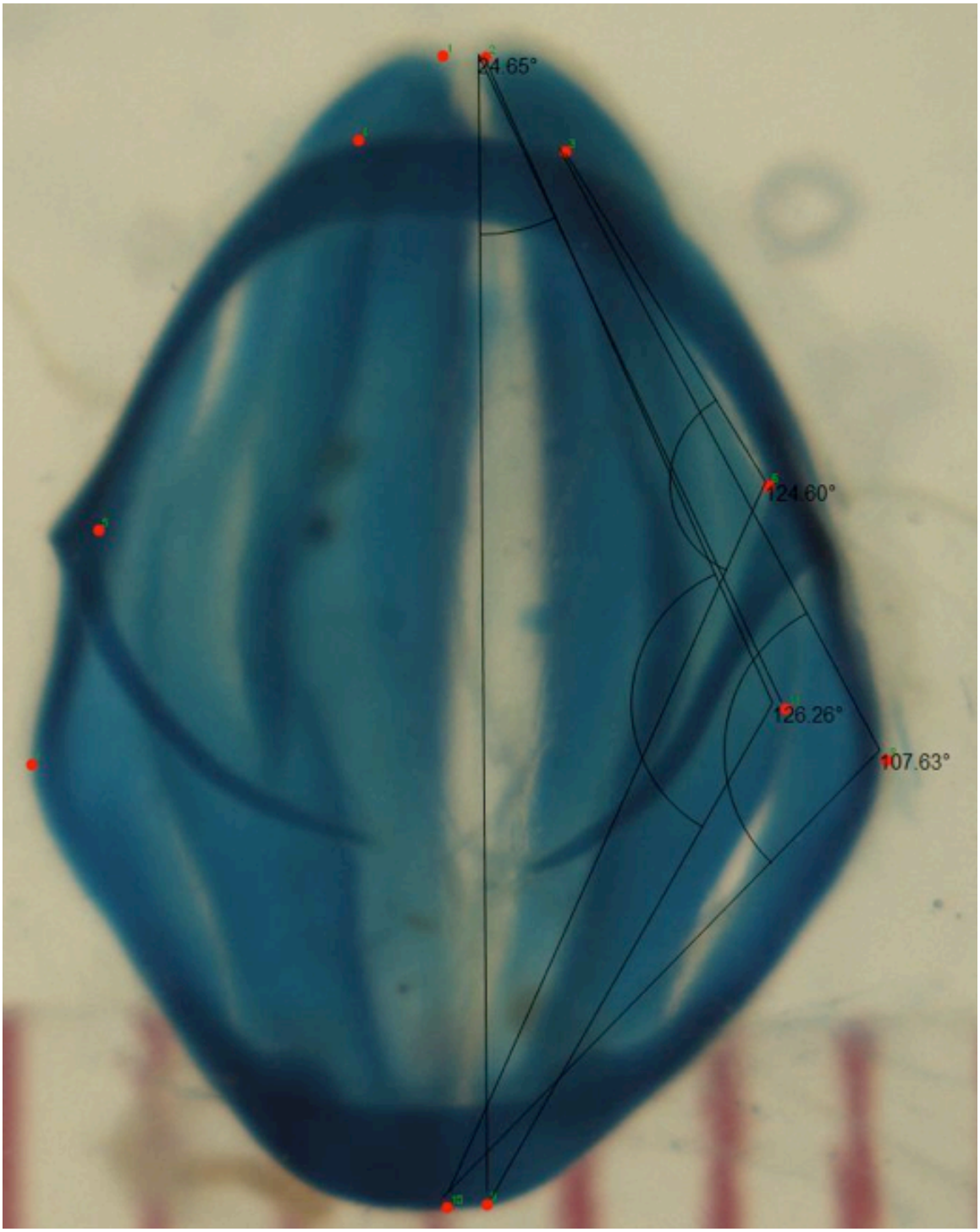

Figure 3.5. Angle Measurements Ventral View *Hyla.cinerea* 1. Pulvinar vocale 24.65^0 2. Medial margin of arytenoid 126.26^0 3. Bronchial process of cricoid 124.60^0 4.Lateral process of cricoid 107.63^0 (Measurements in degrees).

4. RESULTS I: MORPHOLOGY

4.1. MANOVA ANALYSIS

A multivariate analysis of covariance, using the 18 morphological characteristics as the dependent variables and SVL as the covariate, showed statistically significant differences between the three genera (Rao's $R_{36,88} = 7.14$; $P < 0.0001$). The three genera are highlighted on the phylogenetic tree taken from Faivovich et al. (2005) in Figure 4.1. A subsequent MANCOVA of the three hylid groupings also showed statistically significant morphological differences (Rao's $R_{36,48} = 4.29$; $P < 0.0001$). Univariate comparisons showed significant differences among the species groups or species depending on the variable examined. The majority of morphological differences split *Acris* and *Pseudacris*, either together or individually from *Hyla*. Four characteristics evolved in the common ancestor of *Acris* and *Pseudacris*, when they diverged from *Hyla*. Three call characteristics are unique to *Acris* and one or seven (depending on whether you group *A. crepitans* with *P. ocularis*) are unique to *Pseudacris*. Another two are the same in the relatively distant *Hyla* (*H. gratiosa* and *H. arenicolor*) as presented in Table 4.1.

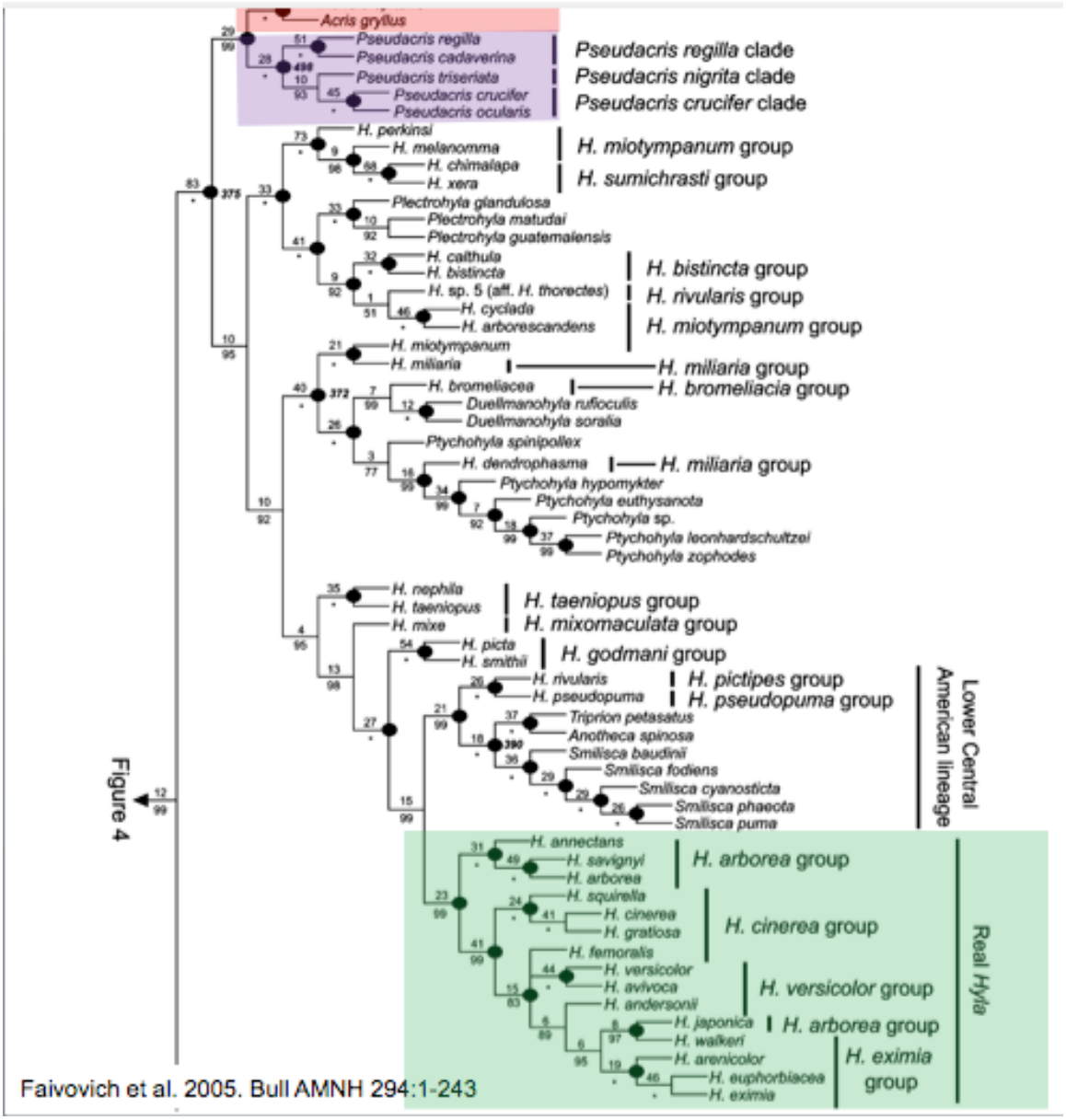

Figure 4.1. Phylogenetic tree taken from Faivovich et al. 2005 showing *Acris* in red, *Pseudacris* in purple and *Hyla* in green.

Table 4.1. Morphology MANOVA Summary.

Acris vs. all others	**Angle 1** - Pulvinar vocale from posterior margin of arytenoid to medial margin of arytenoid **Angle 2** - Medial margin of arytenoid from pulvinar vocale to posterior margin of arytenoid **Angle 4** - Lateral process of cricoid from cardiac process to esophageal process
Pseudacris vs. all others	**Linear D** - Lateral process to esophageal process
Pseudacris vs. all others (*A. crepitans* similar to *P. ocularis*)	**Linear B** - Cardiac process to bronchial process **Linear C** - Bronchial process to lateral process **Linear G** - Bronchial process to bronchial process **Linear H** - Pulvinar vocale to pulvinar vocale **Linear I** - Cardiac process to cardiac process **Lateral 2** - Height of cricoid ring
(*Acris* + *Pseudacris*) vs. *Hyla*	**Linear C** - Bronchial process to lateral process **Linear G** - Bronchial process to bronchial process **Linear H** - Pulvinar vocale to pulvinar vocale **Linear I** - Cardiac process to cardiac process
H. gratiosa + *H. arenicolor* vs. all others	**Linear A** - Pulvinar vocale to posterior margin of arytenoid **Linear F** - Lateral process to lateral process

4.2. PRINCIPAL COMPONENTS ANALYSIS (PCA)

Many of the morphological characteristics are intercorrelated, so the 18 morphological variables were subjected to principal components analysis. The first

two components displayed eigenvalues greater than one, together accounting for 90% of the variance. Corresponding factor loadings are presented in Table 4.2. A variable was considered to load on a given component if the factor loading was 0.7 or greater. Linear measurements were found to load heavily on PC1 while PC2 is comprised of angular measurements. The PCA analysis involved using scatterplots taking all the morphological measurements into consideration to determine the largest variation among the 13 species. The first two principal components (PC1 and PC2) explained 90% of the variation among the species as seen on Figure 4.2. Generally, species within each genus cluster together. Several of the variables discussed in Section 4.1 account for this clustering. One consistent exception is *A. crepitans* that is similar to *P. ocularis* but differs from the other two *Pseudacris* (*P. crucifer* and *P. triseriata*). However, *P. ocularis* is similar to *P. crucifer* and *P. triseriata*. The similarity between *A. crepitans* and *P. ocularis* may be explained by the fact both are miniature species and convergent patterns of morphology are commonly seen in miniatures. The scatterplot showing species within each genus clustering together supports the hypothesis that the morphology of the laryngeal apparatus does differ among the clades in this project.

Table 4.2. Factor Loadings (Morphology).

Morphological Variable	PC1	PC2
A	**0.99**	<0.01
B	**0.88**	-0.06
C	**0.78**	0.22
D	**0.97**	0.01
E	**0.98**	-0.09
F	**0.96**	0.20
G	**0.94**	0.11
H	**0.73**	0.31
I	**0.88**	0.28
J	**0.99**	0.01
K	**0.99**	0.07
L	**0.90**	0.20
Angle 1	-0.35	**0.86**
Angle 2	0.29	**-0.86**
Angle 3	0.66	-0.49
Angle 4	0.33	**-0.85**
Lateral 1	**0.92**	0.08
Lateral 2	**0.74**	0.03

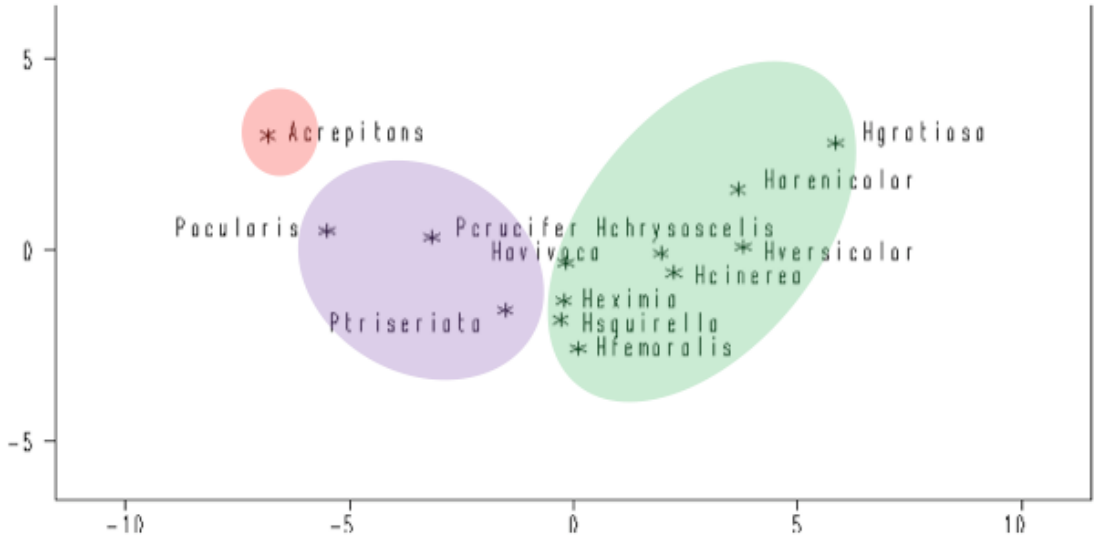

Figure 4.2. PCA of Morphological Measurements Showing Species Within Each Genus Clustering Together.

5. MATERIALS AND METHODS II: CALL STRUCTURE

5.1. ACOUSTIC ANALYSIS

5.1.1. Acoustic Data. For the acoustic analysis, prerecorded advertisement calls were used. These calls were recorded by H.C. Gerhardt and colleagues at the University of Missouri-Columbia, Division of Biological Sciences. The acoustic analysis was performed using Raven Pro version 1.3 for Mac OS X (Cornell Laboratory of Ornithology Bioacoustics Research Program 2008). The acoustic measurements included both spectral and temporal call characteristics. An example of a sonogram, from which temporal call characteristics were measured, is shown in Figure 5.1. An example of a power spectrum that was used to measure the spectral characteristics is shown in Figure 5.2. Analysis was done of five calls (averaged) for each of the five specimens for each of the 13 species. The spectral measurements were fundamental frequency, dominant frequency, and bandwidth at -18dB. The temporal measurements included call duration, call period, call rise time, note duration, note rise time and note period. The acoustic units were interpreted mechanistically. Explanations of these measurements are in Table 5.1.

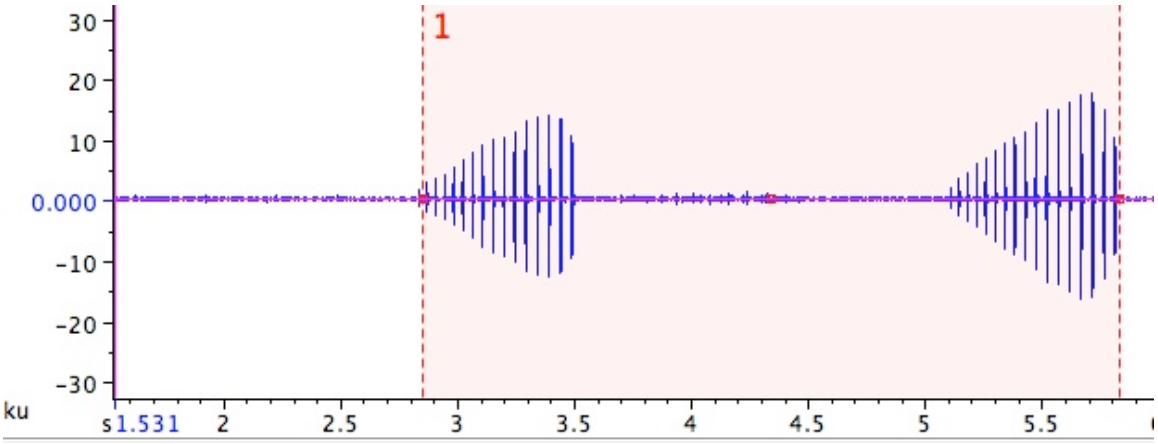

Figure 5.1. Sonogram of *Pseudacris triseriata* (pulsed call).

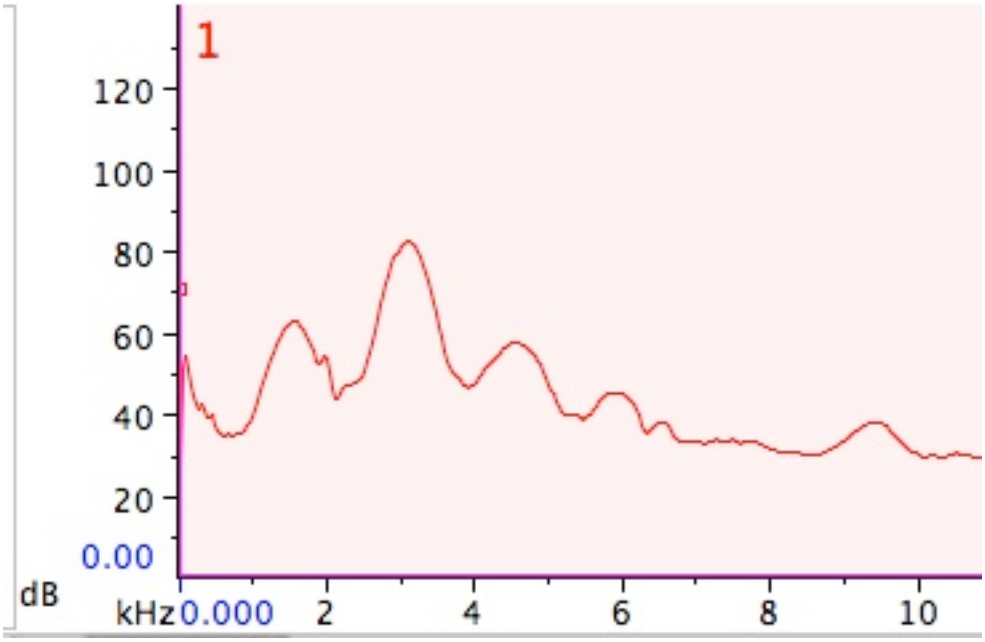

Figure 5.2. Spectogram of *Pseudacris triseriata* calls.

Table 5.1. Acoustic Measurements.

Spectral Measurements (Hz)	
Fundamental Frequency	Lowest frequency in a harmonic series
Dominant Frequency	Frequency peak with most energy
Bandwidth at -18dB	Range of frequencies with amplitudes within -18 dB the amplitude of the dominant frequency
Temporal Measurements (Sec)	
Call Duration	Time from start of call to end of call
Call Rise Time	Amount of time from start of call to maximum amplitude in call
Call Period beginning	Amount of time from beginning of one call to of next call
Note Duration	Time from start of note to end of note
Note Rise-Time	Amount of time from start of note to maximum amplitude in the note
Note Duty Cycle	Percent of time taken by sound

5.1.2. Mechanistic Coding. The units used in the mechanistic analyses are notes. A note is considered the amount of sound produced during a single expiration [Robillard et al. 2006]. The same prerecorded calls from the laboratory of H.C. Gerhardt were used for the mechanistic analyses, which determined whether one call equaled one note or whether one pulse equaled one note. Note duty cycle

and note rise time also were determined from previous measurements. Note duty cycle describes the percent of time taken by the sound. The advertisement calls of several of the species demonstrate single note calls, whereas the advertisement calls of some of the other species examined have multinote calls composed of a number of pulses with each pulse equal to a single note as presented on Table 5.2.

Table 5.2. Mechanistic Unit of Each Species.

Pseudacris crucifer	1 note = 1 call
Pseudacris triseriata	1 note = 1 pulse
Pseudacris ocularis	1 note = 1 call for the pulsed part
	1 note = 1 call for the unpulsed part
Acris crepitans	1 note = 1 call
Hyla eximia	1 note = 1 call
Hyla femoralis	1 note = 1 call
Hyla arenicolor	1 note = 1 pulse
Hyla versicolor	1 note = 1 pulse
Hyla chrysoscelis	1 note = 1 pulse
Hyla squirella	1 note = 1 call
Hyla gratiosa	1 note = 1 call
Hyla cinerea	1 note = 1 call
Hyla avivoca	1 note = 1 pulse

5.1.3. Temperature Data. Temperature was used as a covariate. Temperatures were recorded at the time of the recording of the calls.

5.2. MULTIPLE REGRESSION ANALYSIS

Multiple regression analysis was conducted using SAS. MINITAB Student version 14, 2005 was used to generate graphs. Multiple regression analysis is a method of data analysis used to analyze a dependent variable(s) relationship to an independent variable(s) (predictor variable). To determine which independent (predictor) variables should be included in the multiple regression analysis, Best Subsets Regression analysis was used. The model that maximized R^2 (adjusted) value and minimized the C-p Mallows value was selected for further interpretation. Multiple regression analysis was used to determine whether there was a correlation between call characteristics and morphological characteristics and temperature. The dependent variables (call characteristics) were considered in relationship to the independent variables (morphological characteristics and temperature). The five call characteristics considered were fundamental frequency, dominant frequency, call duration, note duration and note duty cycle. The independent variables were PC1 and PC2 of morphology and temperature. The main focus was to determine if any correlation existed between the calls and the morphology taking into account temperature.

6. RESULTS II: CALL STRUCTURE

6.1. MANOVA RESULTS

A multivariate analysis of covariance using the 9 mechanistic characteristics as dependent variables and temperature as the covariate showed statistically significant differences between the three genera (Rao's $R_{18,118} = 12.21$; $P < 0.0001$). A subsequent MANCOVA of the three hylid groupings also showed statistically significant call differences (Rao's $R_{18, 66} = 9.83$; $P<0.0001$). Significant ($P < 0.05$) univariate comparisons among species showed unclear relationships and are harder to summarize.

However, another way to look at this is by looking at the groups within the different genera. There are three groups within *Hyla*, the *H. cinerea* group, composed of *H. squirella, H. cinerea, H. gratiosa*; the *H. versicolor* group, consists of *H. versicolor, H. chrysoscelis, H. avivoca*; and the *H. eximia* group consisting of *H. arenicolor* and *H. eximia.* According to Favovich et al. (2005) *H. femoralis* is unresolved regarding its relationship with the *H. versicolor* and *H. eximia* groups. Thus, *H. femoralis* is not assigned to a group. By considering each group separately, the results show that the three species in the *H. cinerea* group are not similar regarding four characteristics: call duration, call period, call rise time and temperature. Two of the species, *H. cinerea* and *H. gratiosa,* are similar regarding dominant frequency, bandwidth and note duration. Additionally, *H. squirella* and *H. cinerea* are similar regarding note rise time and note duty cycle, but *H. cinerea* alone shares these two characteristics with *H. gratiosa.* The end result is that in

the *H. cinerea* group four call characteristics are similar when considering this group separately from the other *Hyla* groups.

When considering the *Hyla versicolor* group only dominant frequency and note duty cycle are similar for all three species. The third group, *H. eximia,* has only two characteristics that are similar between the two species in this group.

H. femoralis differs from other *Hyla* regarding fundamental frequency and pulse number.

In summary, by looking at call characteristics among species there is no clear phylogenetic pattern. However, when looking at the three *Hyla* groups, there are similar characteristics among the species in each group. Additionally, *H. femoralis* differs from the other *Hyla* species in two of the call characteristics.

According to Faivovich et al. (2005), *Pseudacris* includes three clades, of which two are included in this research. The two clades are the *P. nigrita* clade, represented here by *P. triseriata,* and the *P. crucifer* clade, represented here by *P. crucifer* and *P. ocularis*. Looking at the two clades together, all three species are similar regarding bandwidth and note duty cycle. Additionally, *P. triseriata* and *P.crucifer* are similar regarding fundamental frequency and dominant frequency. *P.crucifer* and *P. ocularis* are similar regarding call duration, call period, call rise time and note rise time.

Finally, *A. crepitans* differs from *Hyla* and *Pseudacris* regarding dominant frequency.

6.2. PRINCIPAL COMPONENTS ANALYSIS (PCA) RESULTS

Figure 6.1 illustrates the results of the PCA of call characters. PC1 through PC3 together explain 76% of the variation in the dataset.

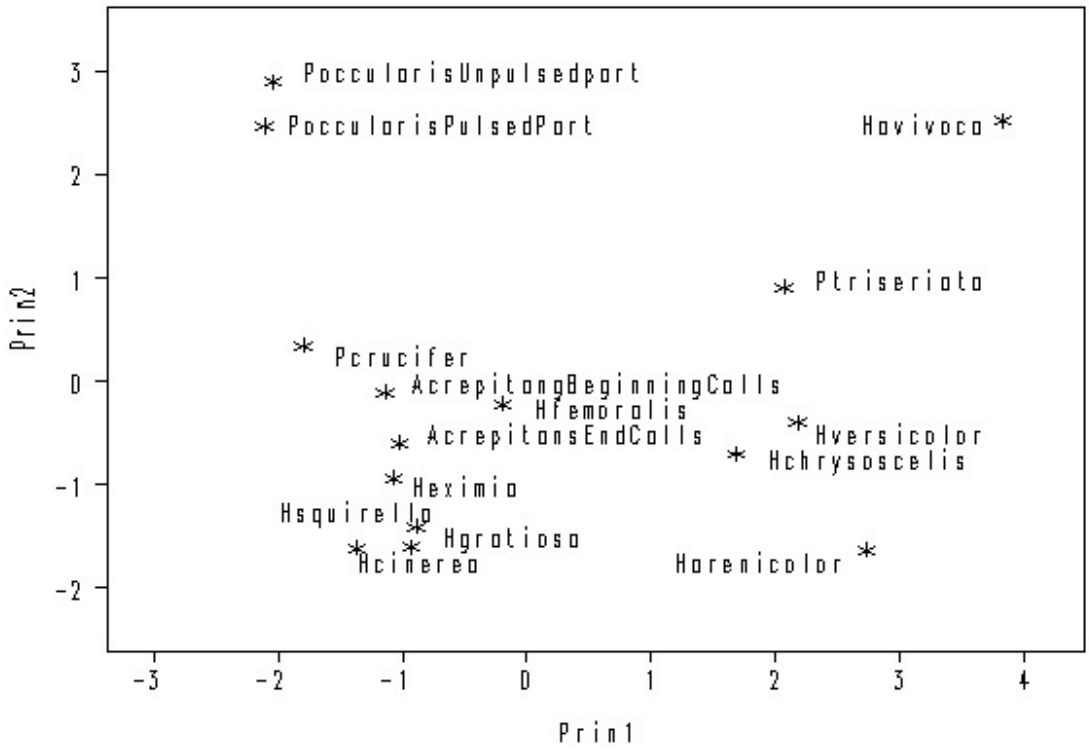

Figure 6.1. PCA of Call Characteristics.

Many of the call characteristics are intercorrelated, so the 10 call variables were subjected to principal components analysis. The first three components displayed eigenvalues greater than one, together accounting for 76% of the variance. Corresponding factor loadings are presented in Table 6.1. A variable was considered to load on a given component if the factor loading was 0.7 or

greater. Call duration, rise time and period were found to load heavily on PC1 while fundamental and dominant frequency loaded heavily onPC2 while PC3 is comprised of temperature.

Table 6.1. Factor Loadings (Calls).

Call Characteristic	PC1	PC2	PC3
Fundamental Frequency	-0.13	**0.86**	0.07
Dominant Frequency	0.30	**0.79**	-0.22
Bandwidth @-18dB	0.02	-0.40	-0.68
Call Duration	**-0.91**	0.11	-0.20
Call Rise Time	**-0.77**	0.38	-0.18
Call Period	**-0.91**	0.17	-0.08
Note Duty Cycle	-0.62	-0.42	-0.22
Note Duration	0.62	-0.27	-0.35
Note Rise Time	0.61	0.53	-0.35
Temperature	-0.01	-0.08	**0.71**

Convergent evolution may be another explanation for the scatterplot results. It is an interesting pattern of evolution in which similar characteristics can develop in organisms even though their ancestors are dissimilar or unrelated

(Enger et al. 2005). Convergent evolution can create much confusion when attempting to find the evolutionary history of species since similarity does not mean relationship between the species (Wallace 1990). In this case the five species on the scatterplot in Figure 6.1 above that share an analogous call characteristic stand apart on the right side. The one call characteristic *H. avivoca, H. arenicolor, H. chrysoscelis, H. versicolor* and *P. triseriata* share is a mechanistic unit of the call where one note = a pulse. The remainder of the species on the left side share the characteristic where one note = one call. It is of utmost importance to interpret the acoustic characteristics in relation to the mechanistic characteristics for consistent phylogenetic results (Robillard et al. 2006). By looking at the call characteristics of note duration and note rise time, *P. triseriata* is similar to *H. chrysoscelis, H. versicolor* and *H. arenicolor*. *H. avivoca* shares the similar mechanistic characteristics of note duration and note rise time with *H. versicolor*. Whereas, *H. avivoca* shares note duty cycle with *P. triseriata, H. chrysoscelis, H. versicolor,* and *H. arenicolor.* Although these five species do share similar characters with the other species, convergent evolution is a possible explanation for the similarities in characteristics among distantly-related species.

6.3. MULTIPLE REGRESSION ANALYSIS RESULTS

Multiple regression analysis was used to determine if there was a correlation between the call characteristics and the morphological characteristics. Five call characteristics (dependent variables) were considered: fundamental frequency, dominant frequency, call duration, note duration, and note duty cycle. These five

call variables were chosen based on the significant differences among the three genera. Best subsets regression was performed to determine the best model to use for multiple regression analysis. After using Best subsets regression to run different models for each of the five dependent variables, the best model for each individual variable was chosen based on the highest R^2 value (adjusted) and low C-p Mallows. It was determined that quadratic models generally provided a better correlation than the linear models. Each of the dependent variables was analyzed to determine if there was a correlation with the independent (predictor) variables, PC1 and PC2 of morphology and temperature. Numbers 1 through 13 in Table 6.2 refer to the numbers as seen on the graphs in Figures 6.2 through 6.5. These numbers represent the species discussed below. The graphs show the residuals, which explain the difference between the actual (true) value of Y (dependent variable) and the value predicted by the regression equation. Each graph shows some of the species with residuals of nearly 0, while a number of other species had positive or negative residuals. The residual plot helps to find extreme outliers or departures from the linearity. When plotting the residuals, multivariate outliers can be found; these indicate that call characters of certain species are not explained well by vocal morphology. However, determining whether an observation is an outlier is ultimately subjective. The value of the response variable is dependent upon the value of the predictor variables. The statistical analysis is used to explain how the response variables are influenced by the predictor values. Adjusted R^2 values can be interpreted as the proportion of Y (dependent character) variance that is

explained by the independent characters (predictors). It measures how well Y (dependent variable) can be predicted from the set of X (independent) variables.

Table 6.2. List of Species For Multiple Regression Analysis.

Number	Species
1	*Pseudacris crucifer*
2	*Pseudacris triseriata*
3	*Hyla avivoca*
4	*Hyla cinerea*
5	*Hyla gratiosa*
6	*Hyla chrysoscelis*
7	*Hyla versicolor*
8	*Hyla squirella*
9	*Hyla femoralis*
10	*Hyla arenicolor*
11	*Hyla eximia*
12	*Acris crepitans*
13	*Pseudacris ocularis*

Fundamental frequency was best explained by a significant regression model incorporating PC1, PC1 squared and temperature squared ($F_{3,9} = 6.70$; $P = 0.011$;

R^2 = 58.8%). Figure 6.2 shows the residuals of the regression of the fundamental frequency component on the independent variables. Morphology explains 58.8% of the variance in the fundamental frequency. As seen on the graph in Figure 6.2, the fundamental frequency of *P. ocularis, H. arenicolor and A. crepitans* is not explained well by vocal morphology, with residuals ranging between 500 and 700. Variation is due to something other than morphology.

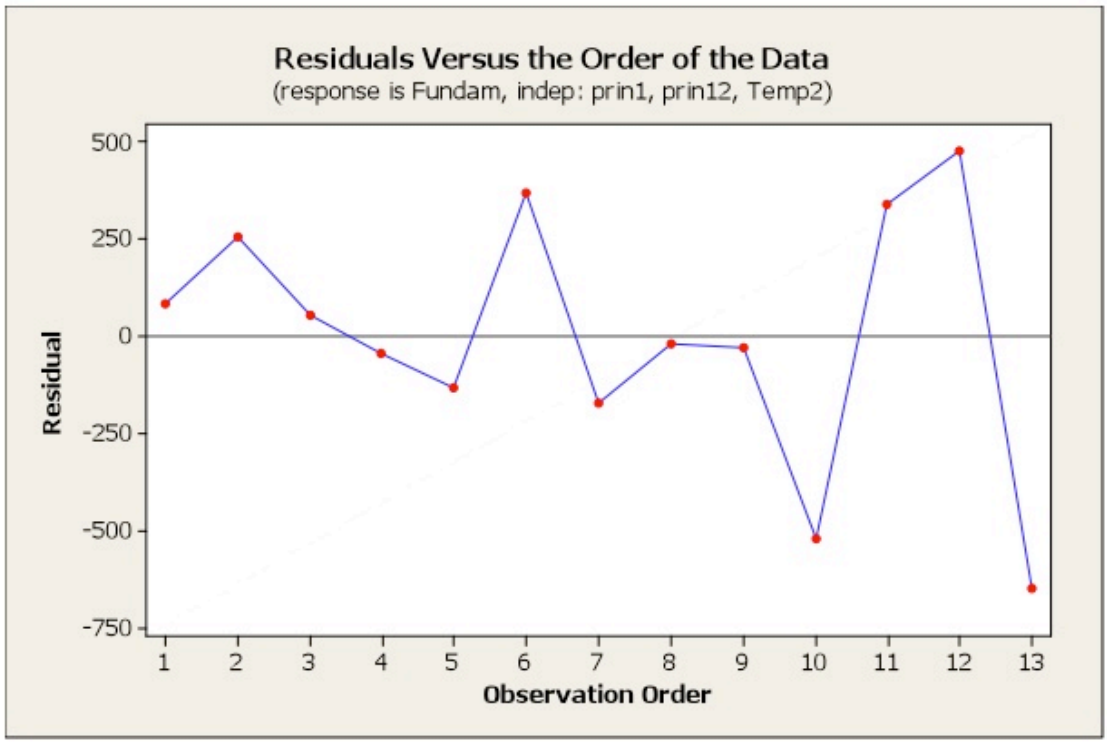

Figure 6.2. Residuals of the Regression of Fundamental Frequency Component on the Independent Variables.

Dominant frequency was best explained by a regression model incorporating all the independent variables except PC1*PC2 ($F_{6,6}$ = 11.70; P = 0.004;

R^2 = 84.3%). Figure 6.3 shows the residuals of the regression of the dominant frequency component on the independent variables. Morphology explains 84.3% of the variance in the dominant frequency. As seen on the graph in Figure 6.3, the dominant frequency of *H. femoralis, H. eximia,* and *P. ocularis* are not explained well by the morphology with residuals ranging between 600 and 750. Variation is due to something besides morphology.

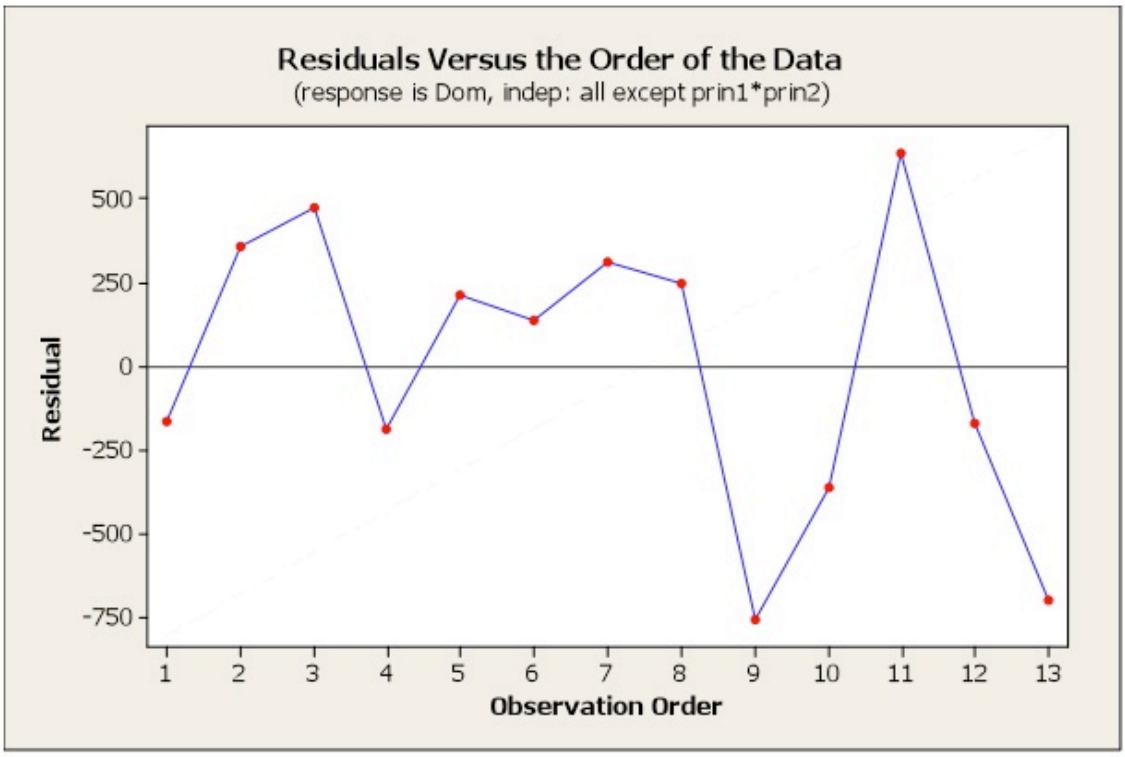

Figure 6.3. Residuals of the Regression of the Dominant Frequency Component on the Independent Variables.

Call duration was best explained by a regression model incorporating PC1, PC2 , PC1 squared ($F_{3,9}$ =1.94; P = 0.193; R^2 = 19.1%). P is greater than 0.05, so there is not a significant relationship between laryngeal morphology and call duration. Figure 6.4 shows the residuals of the regression of the call duration component on the independent variables. Morphology explains 19.1% of the variance in the call duration. As seen on the graph in Figure 6.4, the call duration of *P. crucifer* and *A. crepitans* is not explained well by the morphology with residuals ranging between -0.5 and 1.1. Variation is due to something other than morphology.

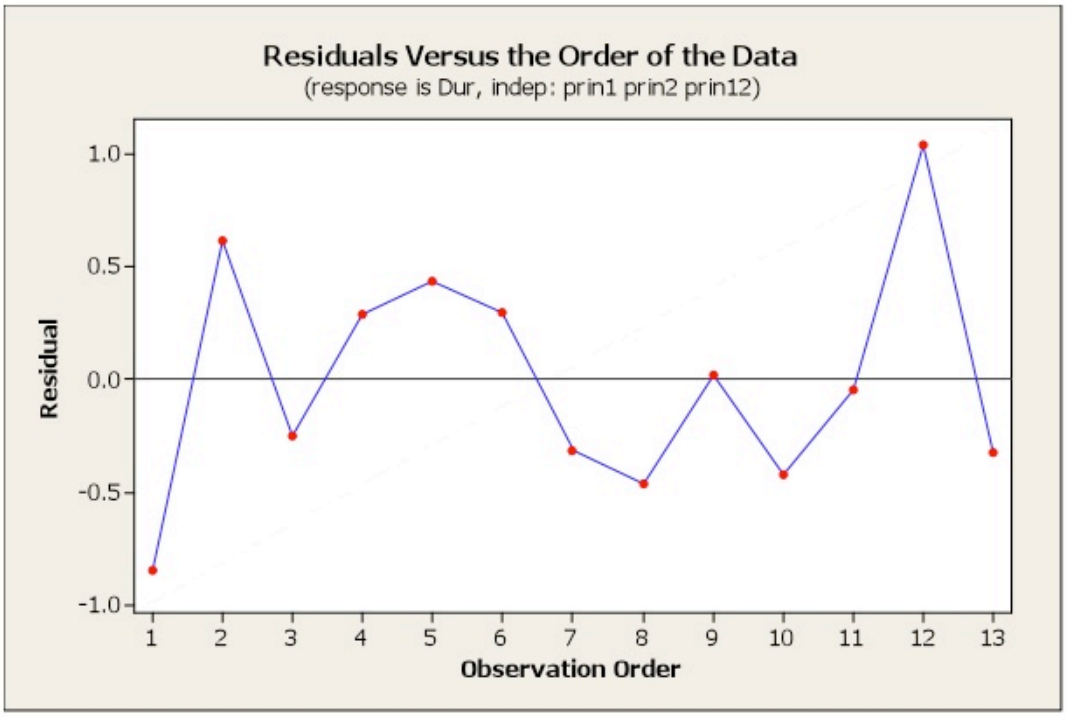

Figure 6.4. Residuals of the Regression of the Call Duration Component on the Independent Variables.

Note duty cycle was best explained by a regression model incorporating all the independent variables except PC2 squared ($F_{6,6} = 5.26$; $P = 0.032$; $R^2 = 68.1\%$). Figure 6.5 shows the residuals of the regression of the note duty cycle component on the independent variables. Morphology explains 68.1% of the variance in the note duty cycle. As seen on the graph in Figure 6.5, the note duty cycle of *H. femoralis, H. arenicolor* and *H. eximia* is not explained well by the morphology with residuals ranging between -10.0 and +10.0. Variation is probably due to something other than morphology.

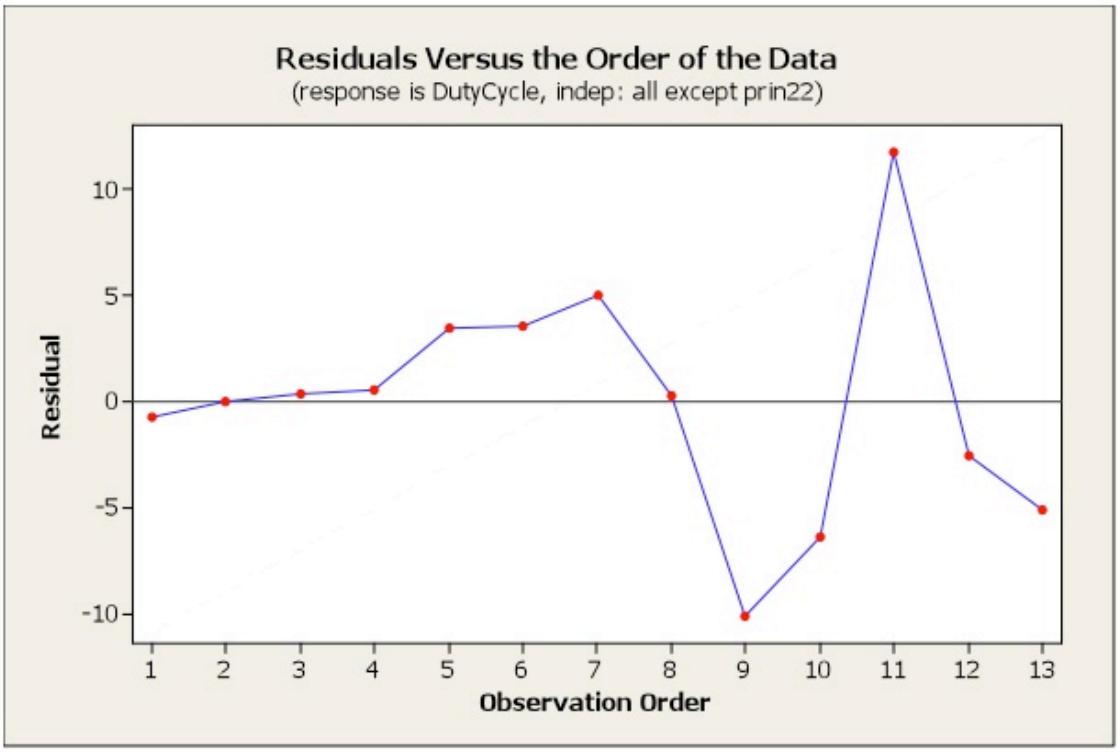

Figure 6.5. Residuals of the Regression of the Note Duty Cycle Component on the Independent Variables.

The fifth dependent call variable, note duration, had a R^2 value of 0 in all the best subsets, indicating that there was no correlation with the morphological characters. Morphology does not explain the variance in note duration.

In summary, the multiple regression analysis did show a correlation with the morphological characters in 3 of the 5 call variables. Call duration is not a significant variable with $P > 0.05$. Morphology does not explain the variance in note duration.

7. RESULTS III: PHYLOGENETIC INTERPRETATIONS

7.1. CORRELATION OF MORPHOLOGY AND CALLS

When examining the morphological results and the results of the call analysis in light of a phylogeny, a pattern does exist between the morphology and calls of the 13 species studied in this project. There is a clear picture of the unique morphological characteristics and their evolution within the genera, although the patterns for the calls are somewhat less obvious. After detailed examination of the call structures, and appropriate cautions regarding mechanistic sound production, call characters contribute useful phylogenetic information and there is a phylogenetic pattern to the morphology and call variation. This study showed that *Acris, Pseudacris* and *Hyla* when they broke off from the common ancestor had morphological and call characteristics unique to each of the genera. These characteristics were associated with speciation within this large clade of frogs, the North American hylids.

8. CONCLUSIONS

The intention of this study was to determine whether the morphology of the laryngeal apparatus differed among the North American hylid species, and whether variations in the morphology of the North American hylids correlated with the variations in the calls. Then the study looked at whether there was a phylogenetic pattern to the morphology and call variation.

The morphology data clearly showed significant differences among the three genera, *Acris, Pseudacris,* and *Hyla.* The majority of morphological differences split *Acris* and *Pseudacris* from the *Hyla,* together or individually. This aspect of the study was strongly supported by the data.

Analysis of the calls presented a greater challenge. There were pulsed and unpulsed calls and single note and multinote calls. Call data was much less clear and was more difficult to summarize. MANOVA results showed significant differences among the species in the three genera, but the call characteristics that were not significantly different (similar) were often between two species from different genera, or from species within a genus but far apart on the phylogenetic tree. However, when looking at the three groups that formed the *Hyla* genus, a clearer pattern became apparent. Most species in each of the 3 *Hyla* groups shared several similar call characteristics. Additionally, the species within *Pseudacris* share several similar characteristics. *Acris* was significantly different from *Pseudacris* and *Hyla* regarding dominant frequency. The principal components analysis (PCA) did not show the species clustering together in a nice

pattern. However, convergent evolution in five of the species, one from the *Pseudacris* genus and four from the *Hyla* genus, present a possible explanation for the lack of clustering. Multiple regression analysis gave better results when certain call characteristics showed a correlation with the morphological characteristics.

Overall the study did show a phylogenetic pattern between the morphology and the calls of the thirteen species of the North American hylids. This project showed that the behavior of animals is very complicated and requires examination of many different factors. Other features of the environment besides temperature could potentially influence the calling behavior of the frogs and thus their evolution. Additionally, only the laryngeal apparatus was considered in this study. By combining the data from this study with data collected by others who have studied soft tissues, such as the vocal sac, which is also involved in the frog calls, a more complete picture of the morphology may be obtained. To further enhance a study such as was done in this project, a larger number of specimens within each species could give a better understanding of the relationship between the morphology and the calls that led to the large diversity of frogs in the North American hylids within a relatively short period of time. By building on the results in this study, future research could prove useful in presenting a much clearer and more detailed picture of the phylogenetic pattern of the calls. Thus, this study has presented initial interesting results that should prove promising for future projects that study the evolution of this diverse group of frogs.

APPENDIX A

CLEARING AND DOUBLE-STAINING PROTOCOL

Protocol adapted from: Taylor, W.R., and Van Dyke, G.C. 1985. Revised procedures for staining and clearing small fishes and other vertebrates for bone and cartilage study. Cybium 9(2):107-119. This method assumes starting the process with specimens that have already been fixed in formalin and are stored in 70% ethyl alcohol (ETOH). (The specimens used in this study were stored in 70% ETOH.)

Dehydrating the specimens: Place the specimens in several times their volume of 95% ethyl alcohol to remove as much water as possible. Depending on the size of the specimens, this should not take more than 24 hours.

Staining in Alcian blue for cartilage tissue: Place specimens in approximately 10 times their volume of stock Alcian blue staining solution (80 parts 95%ETOH; 20 parts glacial acetic acid; 9-30 mg alcian blue powder per 100 milliliters solution). The time required for staining of cartilage will depend on the size and condition of the specimen. A good rule of thumb is to look for known cartilaginous elements (e.g. articular cartilages in the digits, hyoid elements, and articular cartilages in the vertebrae). Generally this will require several days.

Neutralization: Transfer specimens to 50 times their volume of saturated borate solution (excess sodium borate powder to several liters distilled water; heating allows you to supersaturate the solution) for at least 24 hours but no more than 2 days.

Bleaching: Place neutralized specimens in approximately 30 times their volume of bleaching solution (10-20 parts hydrogen peroxide; 80-90 parts 1% potassium

hydroxide solution). Specimens should be left in until soft tissues are not blue, but without losing blue stain from the cartilaginous structures.

Clearing/Digestion: Transfer specimens to 10-40 times their volume of stock enzyme solution (3 parts saturated borate solution; 7 parts distilled water; 1/4 teaspoon trypsin). Specimens should be left to clear until soft tissues are cleared. If the larger specimens have not cleared sufficiently after 7 to 10 days, then provide a new digestion solution. However, too long in the solution will cause the bony elements to become disjointed.

Staining with alizarin red S: Transfer digested specimens to approximately 20 times their volume of stock solution of alizarin red S (small quantity alizarin red powder in 1% potassium hydroxide solution; solution should be a deep purple) until the bony elements have been stained red.

Rinsing: Transfer specimens to a moderate volume of distilled water for no more than 24 hours.

Final clearing and staining: Soak specimens in stock enzyme solution with trypsin until all (or nearly all) muscle tissue has disappeared. Some clearing will continue once the specimens are transferred to glycerine. In some of the larger, thicker regions it is impossible to digest all of the muscles without destroying the more delicate regions of the specimen such as the laryngeal apparatus.

Glycerine storage: Transfer specimens to 40% glycerine solution for 3-4 days. Then transfer specimens to 70% glycerine solution for 3-4 days. Finally, transfer specimens to 100% glycerine solution (placing 1-2 single crystals of thymol to the glycerine to prevent fungal growth) for permanent storage.

APPENDIX B

LIST OF SPECIMENS

Species	KU Number	Location
Pseudacris crucifer	90865	Taney County Missouri
Pseudacris crucifer	90870	Taney County Missouri
Pseudacris crucifer	90873	Taney County Missouri
Pseudacris crucifer	90874	Taney County Missouri
Pseudacris crucifer	90878	Taney County Missouri
Pseudacris triseriata	296009	Douglas County Kansas
Pseudacris triseriata	296010	Douglas County Kansas
Pseudacris triseriata	216181	Hodgeman County Kansas
Pseudacris triseriata	216182	Pawnee County Kansas
Pseudacris triseriata	224574	Douglas County Kansas
Pseudacris ocularis	295949	Lake County Florida
Pseudacris ocularis	295957	Dade County Florida
Pseudacris ocularis	295962	Dade County Florida
Pseudacris ocularis	295964	Dade County Florida
Pseudacris ocularis	295973	Dade County Florida
Hyla avivoca	22790	East Baton Rouge County La
Hyla avivoca	221049	Ballard County Kentucky
Hyla avivoca	221050	Ballard County Kentucky
Hyla avivoca	221052	Ballard County Kentucky
Hyla avivoca	221057	Ballard County Kentucky
Hyla cinerea	10493	Prairie County Arkansas
Hyla cinerea	10494	Prairie County Arkansas
Hyla cinerea	10506	Prairie County Arkansas
Hyla cinerea	10525	Prairie County Arkansas
Hyla cinerea	10533	Prairie County Arkansas
Hyla gratiosa	55487	Washington County Louisiana
Hyla gratiosa	55488	Washington County Louisiana
Hyla gratiosa	55489	Washington County Louisiana
Hyla gratiosa	60329	Marion County Florida
Hyla gratiosa	109911	East Feliciana County Louisiana
Hyla chrysoscelis	171016	Crawford County Kansas
Hyla chrysoscelis	171019	Crawford County Kansas
Hyla chrysoscelis	174780	Elk County Kansas
Hyla chrysoscelis	312461	Daviess County Missouri
Hyla chrysoscelis	312467	Ray County Missouri
Hyla versicolor	38885	Barry County Missouri
Hyla versicolor	222916	Campbell County Virginia
Hyla versicolor	222918	Campbell County Virginia
Hyla versicolor	222919	Goochland County Virginia
Hyla versicolor	222923	Goochland County Virginia
Hyla femoralis	60285	Marion County Florida
Hyla femoralis	60310	Marion County Florida
Hyla femoralis	60313	Marion County Florida
Hyla femoralis	60320	Marion County Florida
Hyla femoralis	145593	St. Tammany County Louisiana
Hyla squirella	17591	Hancock County Mississippi
Hyla squirella	145629	St. Helena County Louisiana

Hyla squirella	145631	St. Helena County Louisiana
Hyla squirella	145634	St. Helena County Louisiana
Hyla squirella	145635	St. Helena County Louisiana
Hyla arenicolor	10364	Valencia County New Mexico
Hyla arenicolor	10366	Valencia County New Mexico
Hyla arenicolor	10367	Valencia County New Mexico
Hyla arenicolor	10390	Valencia County New Mexico
Hyla arenicolor	97356	Cochise County Arizona
Hyla eximia	38126	Michoacan Mexico
Hyla eximia	38129	Michoacan Mexico
Hyla eximia	38133	Michoacan Mexico
Hyla eximia	43491	Michoacan Mexico
Hyla eximia	43513	Michoacan Mexico
Acris crepitans	197714	Douglas County Kansas
Acris crepitans	197724	Douglas County Kansas
Acris crepitans	197818	Douglas County Kansas
Acris crepitans	197827	Douglas County Kansas
Acris crepitans	307945	Maries County Missouri

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VITA

Barbara Ann C. Fears was born in St. Louis, Missouri. She grew up in St. Louis Missouri while attending Nerinx Hall High School and graduating second in her class. She raised a family of three sons while completing a Bachelor of Science in Business with an emphasis in accounting from the University of Maryland in College Park, Maryland in 1984 with a 3.94 grade point. She was accepted into Saint Louis University Law School in 1988 graduating in 1991 with a Juris Doctorate. She actively practiced law from 1992 to 2004 in Missouri. Some of that time was as an assistant Public Defender in the trial office of the Missouri Public Defender system in Columbia, Missouri. Other years she had her own law practice handling cases involving domestic law as well State and Federal criminal defense cases. She handled many felony trials and argued cases before one of the second highest courts in the United States, the Eighth Circuit Court of Appeals. In 2005 she began an undergraduate program in Geology at the University of Missouri-Rolla [now Missouri University of Science and Technology (Missouri S & T)] and transferred into the undergraduate program in Biology that same year. In 2008 she was accepted into the Master of Science program in Applied and Environmental Biology at Missouri S & T. With completion of her M.S. in 2010 she plans to continue studying the behavior of animals.