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Uncovering morphological variation in light of genetic data within the Ozark minnow, Notropis nubilus

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Uncovering Morphological Variation In Light Of Genetic Data Within The Ozark Minnow, *Notropis nubilus*

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Introduction

Notropis nubilus, the Ozark minnow, is a small stream fish distributed in two disjunct regions of the United States—a northern region in southeast Minnesota, northeast Iowa, southeast Wisconsin, and northwest Illinois, and a southern region in the Ozark Plateau of eastern Kansas, northeastern Oklahoma, southern Missouri, and northern Arkansas. A recent study of the genetic diversity of the Ozark minnow revealed significant genetic divergence associated with geographic areas, seen in Figure 1 (Berendzen *et al*., 2010). Within these areas are genetic groups consistent with geographic distribution that make up three clades, or regions: upper Mississippi and northern Ozarks, western Ozarks, and southern Ozarks (Fig. 2). Results of the study suggested that populations were fragmented by repeated glacial advances during the late Pliocene and into the early Pleistocene eras resulting in the modern distribution of genetic diversity (Berendzen *et al*., 2010).

Although there is significant genetic diversity within the species, it is unclear if these distinct groups have morphological differences. Oftentimes, genetic data reveal hidden diversity present within a species, which is labeled as cryptic (Berendzen *et al*., 2009). In other words, cryptic means that there are differences between groups at the genetic level but not at the morphological level. The purpose of this research is to determine if there is morphological variation within *Notropis nubilus* that is consistent with the genetic hypothesis. This study used two methods to measure morphology: meristic and morphometric analyses. From these studies, a conclusion will be drawn as to whether or not significant morphological differences can be found that are consistent with the three genetic clades, upper Mississippi and northern Ozarks, western Ozarks and

southern Ozarks, that were revealed by Berendzen *et al.* (2010).

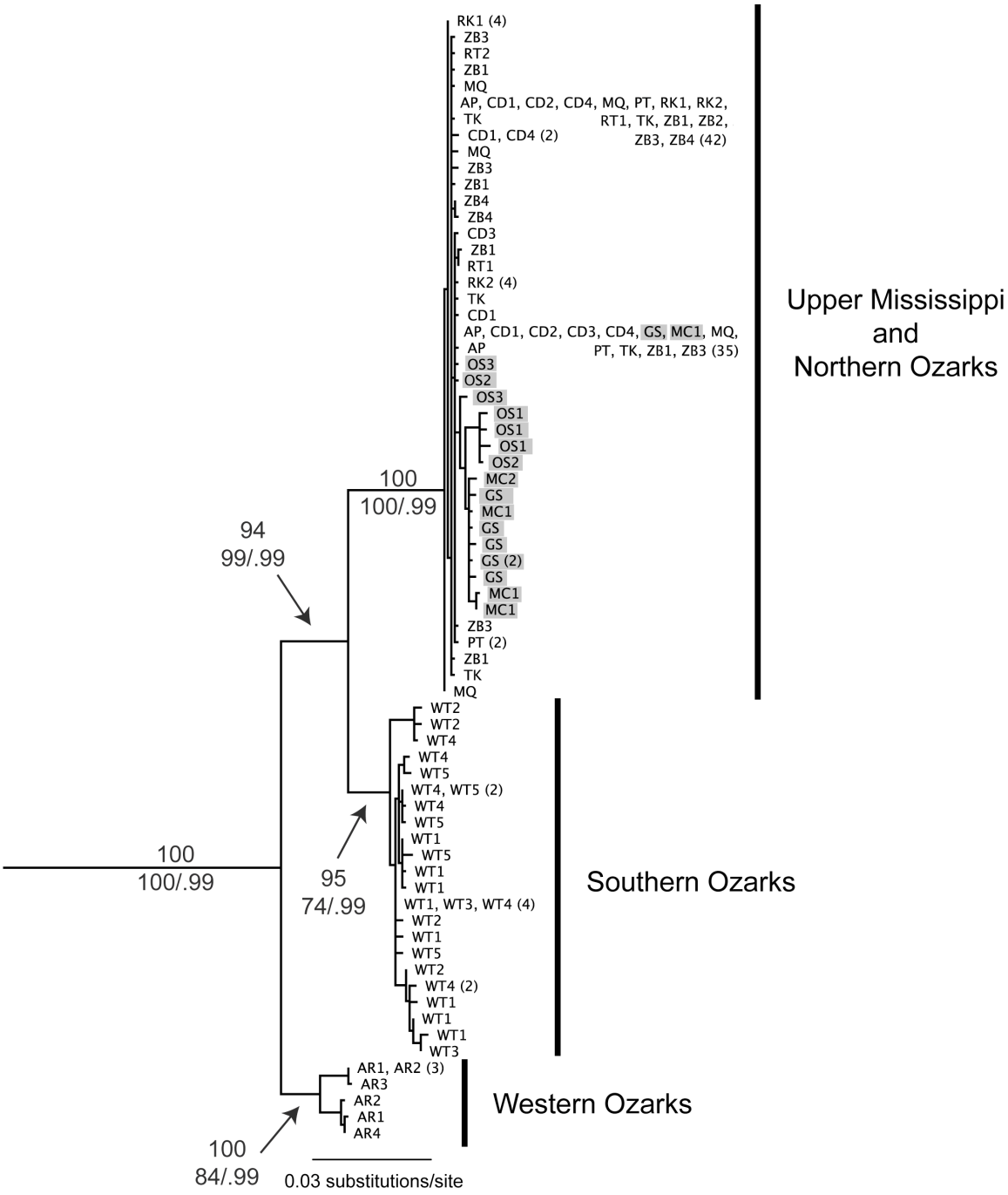

Figure 1. This genetic tree was revealed in the Berendzen *et al.* (2010) study on the genetic variation within the *Notropis nubilus* species. Three distinct groups are present, with western Ozarks being the most genetically dissimilar compared to the two sister groups, upper Mississippi and northern Ozarks, and southern Ozarks.

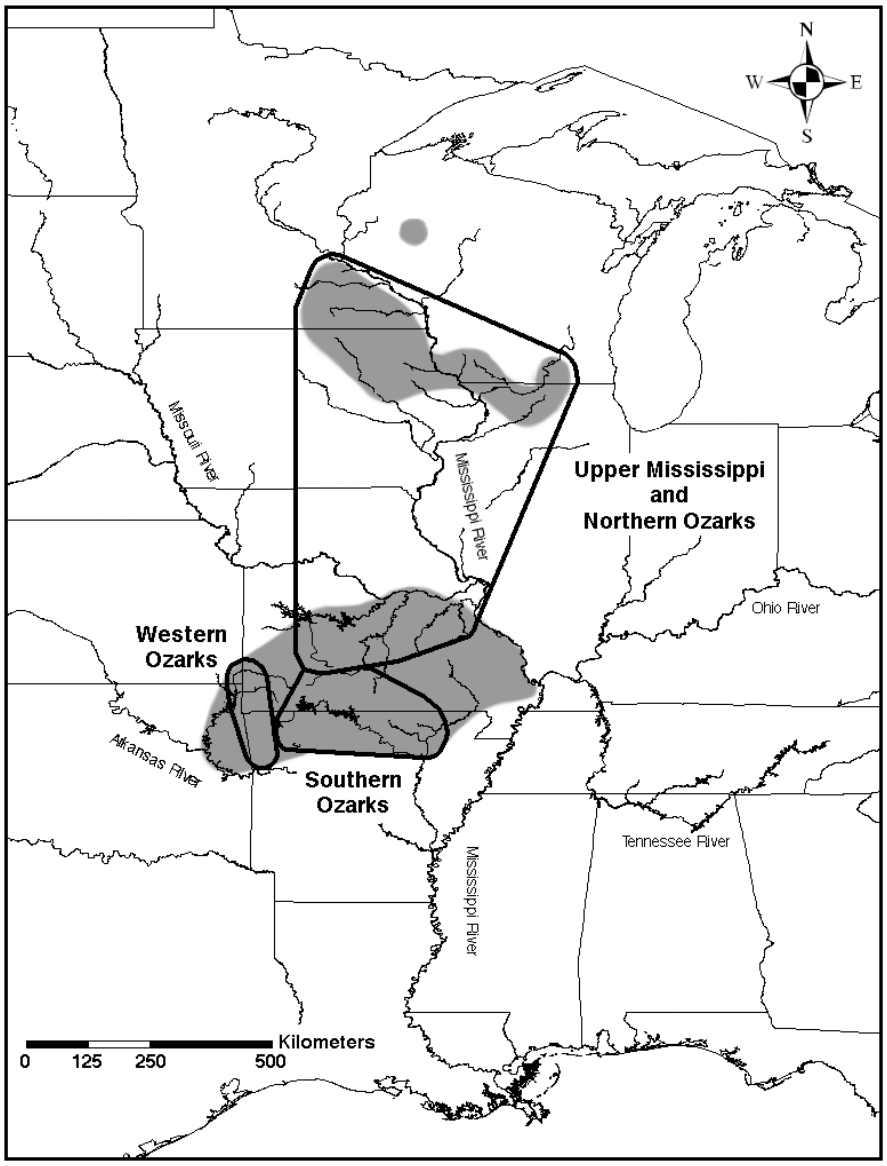

Figure 2. Distribution map of the major localities of each of the three clades defined in Berendzen *et al*. (2010) The shaded regions represent areas where the *Notropis nubilus* minnow is found. The three regions are defined as western Ozarks, southern Ozarks, and upper Mississippi and northern Ozarks.

Literature Review

The Ozark minnow, *Notropis nubilus,* was described by Forbes (1878). The fish lives in clear cool streams and is a slim, terete minnow with a moderately large eye, small head, and slightly oblique mouth (Fig. 3). The color of the body is dusky with silvery

sides and a white belly. A dark lateral band runs from the tip of the snout to the end of the caudal peduncle (Harlan *et al.,* 1987). The species has a blunt snout, eight anal fin rays, eight dorsal rays, a long, coiled intestine and a black peritoneum, the membrane lining the abdominal cavity. The lateral line typically has 33-37 scales and the front of the dorsal fin base is approximately equidistant between the snout tip and caudal base (Pflieger, 1975).

Figure 3. Photograph of a *Notropis nubilus* specimen taken by P. Berendzen.

It has been hypothesized that the modern distribution of the Ozark minnow was shaped by the fragmentation of a widespread ancestral population by periodic glaciations during the late Pliocene and early Pleistocene eras (Berendzen *et al*., 2010). Today, the species comprises three distinct genetic clades that are: upper Mississippi and northern Ozarks, western Ozarks, and southern Ozarks. In light of this hypothesis, the objective of this study is to determine if the morphology of the fish displays differences that parallel the genetic variation. One way to determine these morphological differences within a

population is to look at the geometric morphometrics of the species.

Geometric morphometrics is essentially another way of looking at the shape of the specimen that allows visualization of differences among complex shapes. It is an older field of classifying organisms based on quantitative measurements from certain homologous points, or landmarks, on the fish. Traditionally, fish are identified by meristic analysis, which involves counting scales, however geometric morphometrics has become a more accurate indicator of morphological variation (Zelditch *et al,* 2004). Geometric morphometrics involves a process of taking photographed and landmarked specimens and using computer programs such as IMP to overlay specimens, find the means within data points and compare those means to see if morphological variation exists between both non-predefined and predefined groups of specimens.

Berendzen *et al*. (2009) did a similar study on the *Notropis rubellus* species complex, rosyface shiners. Findings indicated that there was significant natural variation in the fin-ray and scale counts, which resulted in a considerable overlap between the clades. Thus, the meristic analysis did not give any distinct indication of morphological differences between the genetic clades. However, the morphometric analyses yielded a variety of results. The morphometric analysis was useful in that it was able to find subtle morphological differences. The morphometric analyses looked for differences between the mean shapes between groups. Both principal component analyses (PCA) and canonical variates analyses (CVA) were performed. The PCA is a method that looks for differences in shape between all individuals without predefining the groups. PCA reduces the dimensionality of multivariate data by taking out the eigenvectors, or principal components, of the variance-covariance matrix. The principal components are explained

by each axis (Zelditch *et al.,* 2004). The Berendzen *et al.* (2009) study was unable to discern any clustering of groups. The PCA of the geometric morphometric data of the rosyface shiners revealed no clear separation between the seven hypothesized clades of Berendzen *et al*. (2008), which is why the CVA was used.

The CVA is a discriminate function analyses. It is a method for finding axes along which groups are best discriminated. These axes, or canonical variates, are used to maximize the between-group variance relative to the within-group variance (Zelditch *et al.,* 2004). In these analyses the groups were defined, in this case by genetic clade. Of the hypothesized six possible canonical variates, the CVA revealed six distinct and statistically significant canonical variates for all individuals. The conclusion was there were statistically significant differences in shape, but that the differences were very subtle. As in the Berendzen *et al*. (2009) study, this study will first perform a PCA and then a CVA defining groups based on a previously published molecular hypothesis found in Berendzen *et al.* (2010).

Should this study on the Ozark minnow find that there are no apparent morphometric differences between clades or that the differences are inconsistent with already established genetic differences, then this will point to something different, known as cryptic diversity. Species are considered cryptic when they are morphologically indistinguishable yet represent independent evolutionary lineages, based on either molecular or phylogeographic evidence (Bickford *et al.,* 2006). However, many are falsely led, either by not using advanced morphological analyses or solely relying or meristics, to the conclusion that if a species appears to look the same morphologically, then the genetic differences point toward cryptic diversity.

Several studies on various other organisms have followed similar methods as the methods utilized in the Berendzen *et. al,* (2009) study. In a study looking at the multigenic and morphometric differentiation of ground squirrels in Turkey, Gündüz *et al*. (2007) found that the morphometric differentiation in skull and mandible shape among the three species studied was incomplete but statistically significant. This study was used to uncover new Anatolian species and to reconstruct their phylogeographic history. The results indicated that the discrimination between *Spermophilus taurensis, Spermophilus xanthroprymnus* and *Spermophilus citelus* using geometric morphometric methods, especially with skulls, contrasts with a previous inability to separate *S. citellus* and *S. xanthoprymnus.* The previous methods used phenetic distances based on 10 skull measurements (Krystufek and Vohralik, 2005). The study also found shape differences in the skull to be very small, suggesting that size could have easily confounded the differences with the previous analysis (Gündüz *et al.*, 2007). This study on the Ozark minnow will also use geometric morphometrics to determine if there are shape differences between groups, even if the differences are very small.

There are many additional examples of comparisons of genetic variation to morphology. A study performed by Phillimore *et al.* (2008) looked at the patterns of genetic and phenotypic divergence in an island bird, *Zosterops flavifrons.* Results indicated that there were multiple monophyletic island groups within the species, indicating long periods of isolation from one another and little gene flow. The major axis of morphological divergence was body size among the specimens, and phenotypic divergence between populations generally exceeded neutral genetic divergence. Similarly, the Ozark minnow species has undergone geographic isolation, which may

have contributed to potential differences in morphology.

A study done by Douglas *et al*. (2001) looked at the use of geometric morphometrics to differentiate *Gila* within the Upper Colorado River Basin. The study found significant differences in shape among populations as well as three statistically significant variates of the five total being assessed, which accounted for 95.9% of the among-population variation.

In this study on the Ozark minnow, is important to perform meristic and morphometric analyses because there could be morphological differences present that may be hidden to the naked eye. Since significant genetic diversity consistent with geographical distribution has been revealed in the *Notropis nubilus* species, if the morphometric and meristic studies do not reveal any morphological differences between genetic clades, then the findings would be indicative of cryptic diversity. However, if, like in the aforementioned *Anatolian* ground squirrel study and in the Berendzen *et al.* (2009) study on the *Notropis rubellus* species complex, slight morphological changes are found to be consistent with the already discovered genetic differences, then these findings would not be indicative of cryptic diversity, but rather the possibility of a different species.

The main focus of this research is to answer the question of whether or not isolation of the three major clades of the species, *Notropis nubilus,* has led to morphological differences in individuals from the three different regions. This was tested using meristics and geometric morphometrics. Conclusions were made depending on whether or not shape differences exist among specimen from the upper Mississippi and northern Ozarks, western Ozarks and southern Ozarks regions. If they do exist, such data

will support the genetic differences already found by Berendzen *et al*. (2010). However, the idea of cryptic diversity will not be supported. Additionally, morphological differences may be found in the meristic scale counts. If these scale counts are consistent with genetic findings, this would also not support the hypothesis of cryptic diversity.

Materials and Methods

Specimens examined—A total of 286 specimens were borrowed from several natural history museums across the United States (Table 1). Specimens were selected from 40 localities throughout the Upper Mississippi and Ozark regions, with each locality typically represented by 5-20 specimens. These localities span across the three clades so that a relatively equal sampling was taken from each clade. The specimens chosen come from localities consistent with the predefined geographical regions identified in Berendzen *et al.* (2010). Specimen distribution is as follows: Upper Mississippi, n=75; Western Ozarks, n=100; Southern Ozarks, n=111. No specimens were ordered from the northern Ozarks due to difficulty in finding an institution with specimens from that region. All specimens were stored in jars containing 70% ethanol, along with their catalog numbers and general locality information.

Table 1. A compiled list of specimens used, including region, institution that lent specimens, catalog number, number of specimens used (count), and river drainage. Institutions are abbreviated as UAIC=University of Alabama Ichthyological Collection, JFBM=Bell Museum of the University of Minnesota, and U of W=University of Wisconsin.

Region	Inst.	Catalog #	Count	Drainage
Southern Ozarks	UAIC	10073.05	4	Buffalo River
	UAIC	10464.07	10	Current River
	UAIC	13695.04	10	Current River
	UAIC	10344.05	6	St. Francis River
	UAIC	10709.03	10	North Fork White River
	UIAC	10270.08	10	Strawberry River
	UAIC	10096.07	10	Gasconade River
	UAIC	10064.07	9	Black River
	UAIC	10294.11	5	Black River
	UAIC	10341.07	5	St. Francis River
	UAIC	10360.06	9	Current River
	UAIC	10333.02	5	White River
	UAIC	10317.04	10	James River
	JFBM	42330	5	White River
	JFBM	44697	5	Black River
Western Ozarks	UAIC	7939.03	4	Neosho River
	UAIC	12549.04	5	Neosho River
	UAIC	10072.06	20	Neosho River
	JFBM	42949	20	Spring River
	JFBM	42430	10	Arkansas River
	JFBM	37899	5	Elk River
	JFBM	41791	10	Elk River
	JFBM	41527	9	Richland Creek
	JFBM	42939	18	Spring River
Upper Mississippi	JFBM	45627	5	Cedar River
	JFBM	31477	4	Cedar River
	JFBM	43639	14	Pecatonica River
	JFBM	24031	4	South Fork Zumbro River
	JFBM	24917	6	Cedar River
	JFBM	23952	8	Cedar River
	JFBM	24042	8	Zumbro River
	UofW	10517	5	Apple River
	UofW	11550	1	Darien Creek
	UofW	11732	2	Brill River
	UofW	11942	3	Brill River
	UofW	10758	3	Legett Creek
	UofW	8953	5	Pat's Creek
	UofW	11927	4	Brill River
	UofW	10514	5	Platte River
	UofW	11537	5	Little Turtle Creek

Meristic analyses—Meristic data was collected using methods from Hubbs *et al.* (1929). Five scale counts were taken under a microscope from the left side of each specimen. Scale counts taken include: above the lateral line, below the lateral line, predorsal scales, caudal peduncle scales and lateral line scales. Anal and dorsal fin-ray counts were also obtained. The scale counts of specimens from the different localities were recorded and analyzed. Although a natural overlap in scale counts exists, the means of each scale count for each region were calculated to use as comparison between each of the predefined clades.

Morphometric analyses—Photos of each specimen were taken using a Canon SLR camera with a macro lens. Using a photo stand to keep angles consistent, pictures of each specimen were taken and transferred to TpsDig (Rohlf, 2006), a computer program used to plot landmarks on the specimens. The morphometric landmarks were as follows: 1=anterior part of head where premaxillary bones extend the furthest; 2=anterior border of epiphyseal plate at midline dorsal neurocranium; 3=anterior dorsal fin insertion into body; 4=posterior insertion of dorsal fin into body; 5= base of caudal fin on dorsal midline; 6=posterior termination of hypural bones of caudal fin where the muscle tissue extends to the ventral fin ray closest to the lateral line; 7= base of caudal fin on ventral midline; 8=posterior insertion of anal fin into body; 9=anterior insertion of anal fin into body; 10=insertion of pelvic fin into body; 11=insertion of pectoral fin into underlying skeletal girdle dorsolateral to ventral midline; 12=ventral side of articulation between quadrate and mandible, vertical to ventral midline; 13=intersection of maxillary bone and infraorbital bone, the point lies well lateral to midline; 14=most anterior portion of eye at

midline; 15=most posterior portion of eye at midline; 16=most posterior point of bony opercle, usually coexisting with lateral line (Berendzen *et al.,* 2009).

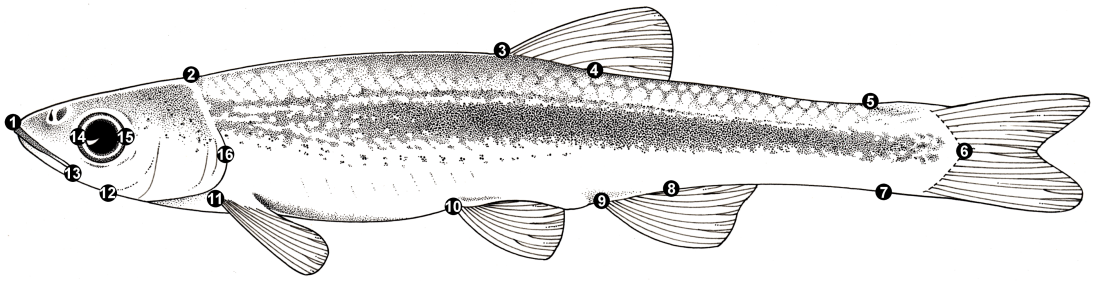

Figure 4. Each number on the fish shown above represents a homologous point, or landmark, on the fish.

After hand-placing landmarks on the specimens, appended files were created for all individuals, all males only, Upper Mississippi and Southern Ozarks specimens, and Upper Mississippi and Southern Ozarks specimens--males only. Males were separated from females and juveniles by looking to see if they had tubercles on their snout. This was to see if any potential sexual dimorphism in the fish would display shape differences more clearly than in an analysis containing all individuals. The coordinates of the landmarks of each appended file were superimposed using generalized Procrustes superimposition using the program CoordGen7.14, part of the Integrated Morphometrics Programs (IMP; Sheets, 2004).

Multivariate analyses were performed, using the morphometric results from the Procrustes. First, a principle component analysis (PCA) was performed using PCAGen to see if evident shape differences between groups existed in the specimens' morphology. Then a discriminate function analysis was performed to decipher the shape differences between groups using CVAGen. This canonical variates analysis (CVA) used canonical

variates (CV) to record and measure shape variation. Plots of the scores of each analysis were generated to see if there was clear separation between groups.

Results

Meristic Analyses—The mean, range, and standard deviation for all five scale counts and both fin-ray counts are presented in Table 2. Results showed considerable overlap in scale counts among clades for each count. Figure 5 presents graphs of each individual scale count. The scale count with the most variation was the predorsal scale count. Figure 6 presents graphs of each individual fin ray count.

Table 2. Scale counts for the three hypothesized clades of Berendzen *et al.,* (2010).

Scale count	Upper Mississippi	Western Ozarks	Southern Ozarks
Predorsal			
Range	12-17	12-16	12-16
Average	13.69	14.10	14.18
SD	1.17	0.96	0.98
Lateral line			
Range	33-38	33-38	30-38
Average	35.98	36.19	36.13
SD	0.93	0.86	1.20
Caudal Peduncle			
Range	12	12-13	11-12
Average	12.00	12.03	11.99
SD	0	0.224	0.1
Above lat. line			
Range	4-5	5-6	4-7
Average	4.98	5.24	5.25
SD	0.13	0.43	0.5
Below lat. line			
Range	3-4	3-5	3-5
Average	3.99	4.04	3.85
SD	0.09	0.295	0.411

Table 3. Fin-ray counts for the three hypothesized clades of Berendzen *et al.,* (2010).

Fin-ray count	Upper Mississippi	Western Ozarks	Southern Ozarks
Dorsal			
Range	8	7-9	7-9
Average	8.00	8.11	8.3
SD	0	0.390	0.560
Anal			
Range	7-8	7-9	7-9
Average	7.99	8.03	8.17
SD	0.095	0.527	0.473

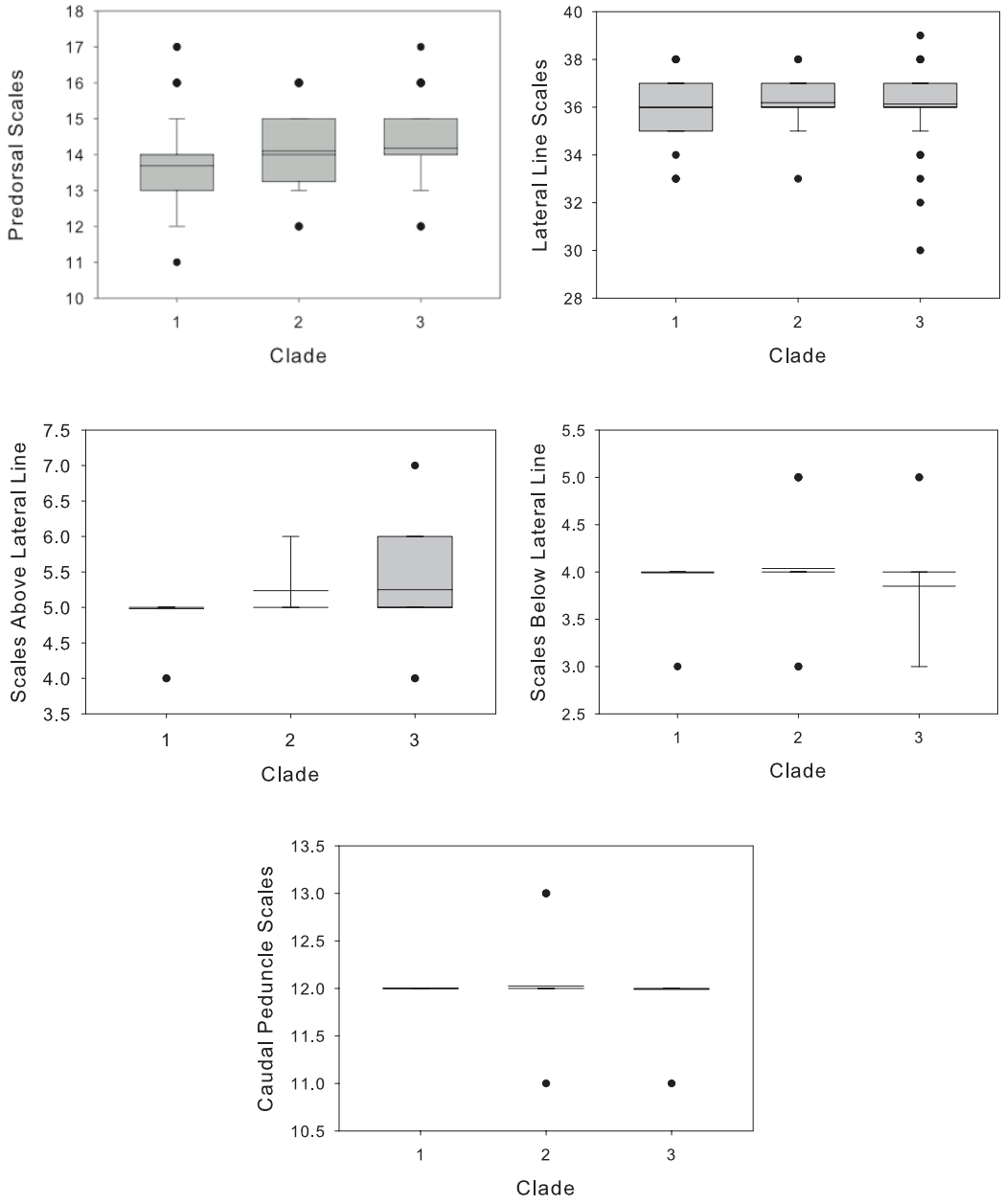

Figure 5. Box plots of each of the five scale counts among the three clades. Clade 1=upper Mississippi. Clade 2=western Ozarks. Clade 3=southern Ozarks. The horizontal line = mean and vertical bar-line = 5th and 95th percentile.

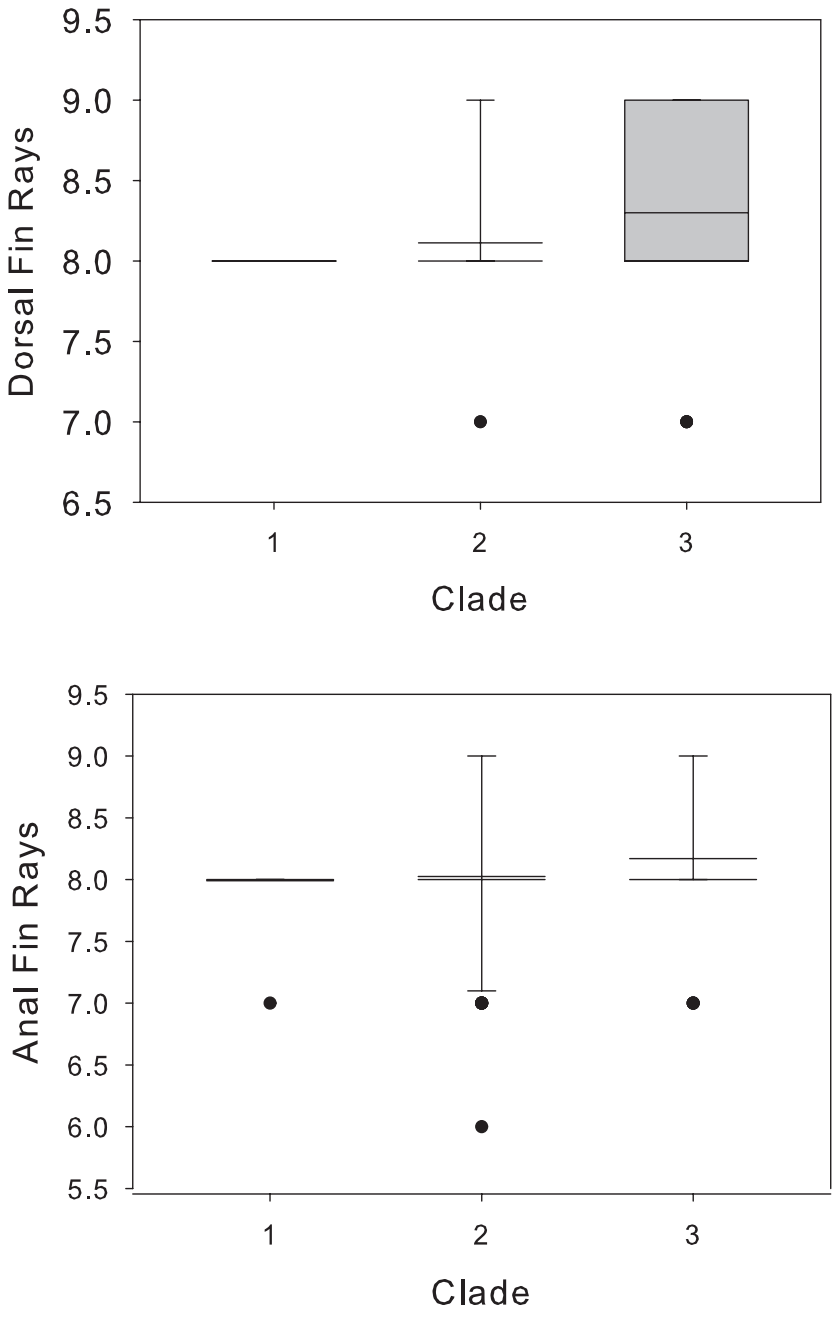

Figure 6. Box plots of the fin-ray counts of each of the three clades. Clade 1=upper Mississippi. Clade 2= western Ozarks. Clade 3=southern Ozarks. The horizontal line = mean and vertical bar-line = 5th and 95th percentile.

Morphometric Analyses— Principal component analyses were performed on all three groups for all individuals and all three groups for males only. Results are presented in Figures 7a and 7b. The results show complete overlap between all groups, which is why the CVA analyses were necessary.

Canonical variates analyses of the grouped data are displayed in Figures 8a-8e. The first canonical variate (CV1) is representative of the greatest morphological variation between the groups, while CV2 is the second greatest variation between groups. The Upper vs. Southern vs. Western CVA analysis found two separate and distinct canonical variates with Eigenvalues of 1.52 and 1.24. Graph A in Figure 8a shows distinction of the groups, with some overlap. The males-only CVA analysis of all three clades found one significant canonical variate with an Eigenvalue of 4.92 and graph B in Figure 8b shows clear separation of the Upper group from the Southern and Western groups. The Upper+South vs. West CVA analysis revealed one canonical variate with an Eigenvalue of 1.29. Graph C in Figure 8c shows distinction with overlap between groups. The males-only CVA analysis of the Upper+South vs. West clades found no significant canonical variates but had an Eigenvalue of 3.61. Graph D in Figure 8d shows distinction between groups. The Upper vs. South CVA analysis found one distinct canonical variate with an Eigenvalue of 2.51. Graph E in Figure 8e shows distinction and a little overlap between groups. The Upper vs. South males-only CVA analysis could not be performed due to a small sample size of males.

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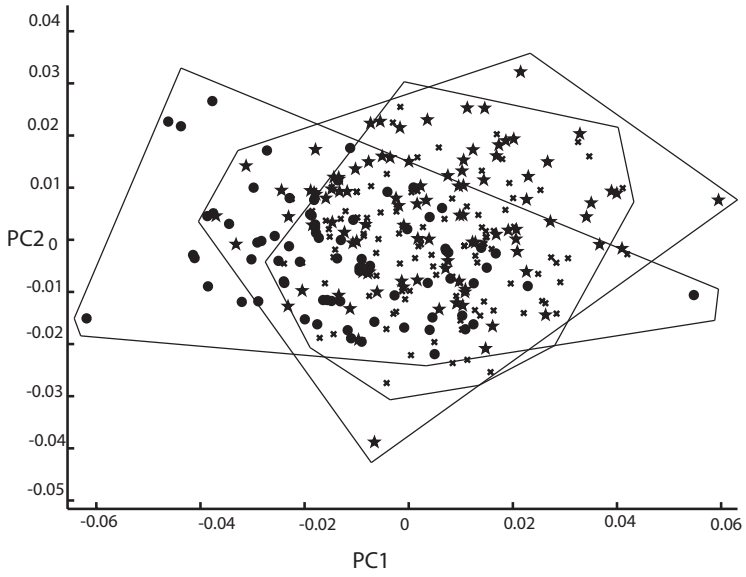

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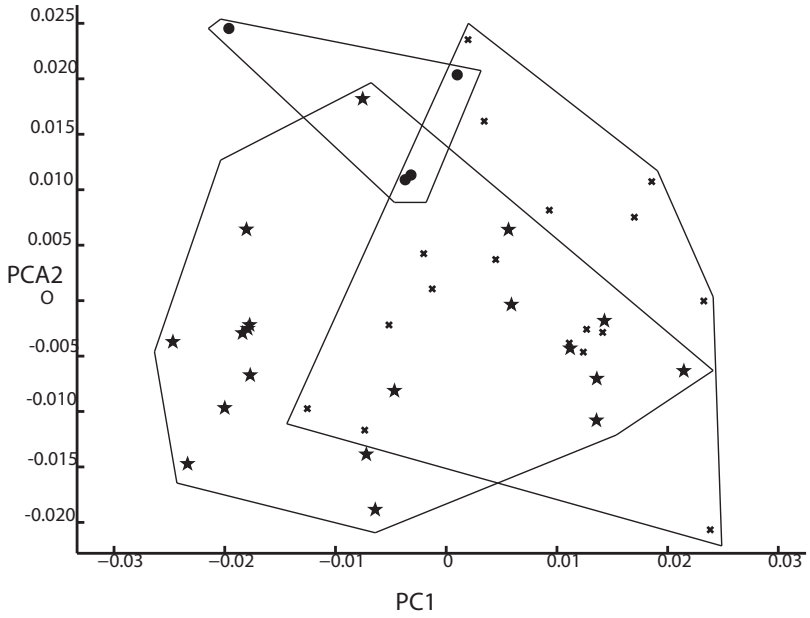
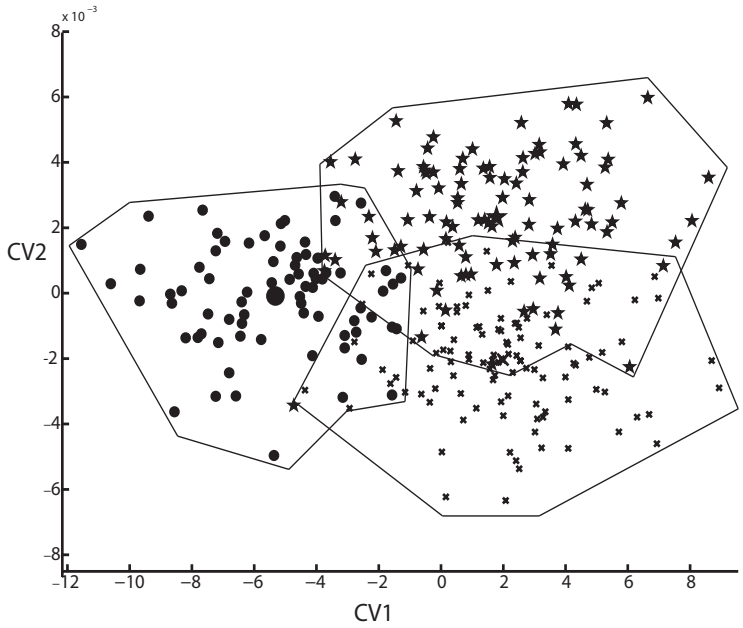
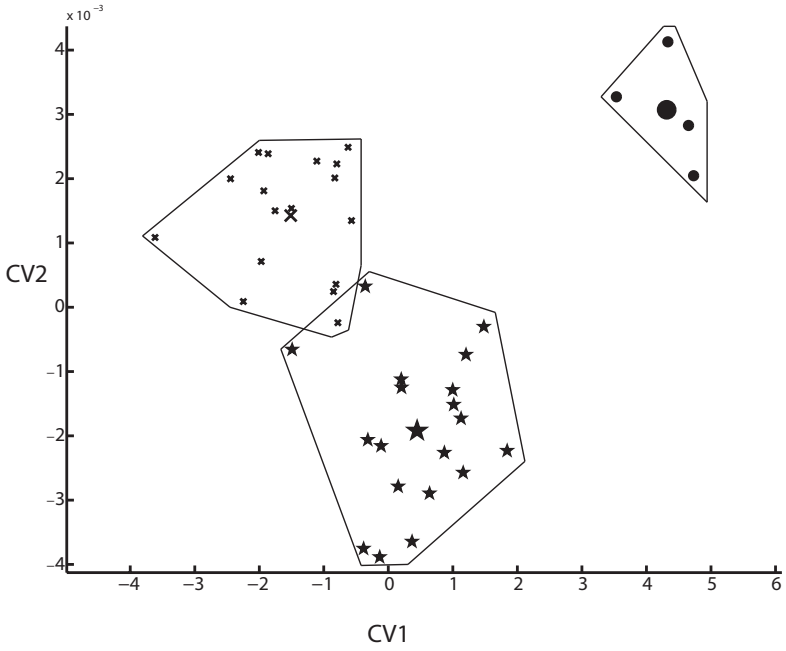


Figures 7a and 7b. Plots of PCA scores. A) all three clades (all individuals) and B) the males of all three clades. Circles represent upper Mississippi, X's represents southern Ozarks, and stars represent western Ozarks.

A)

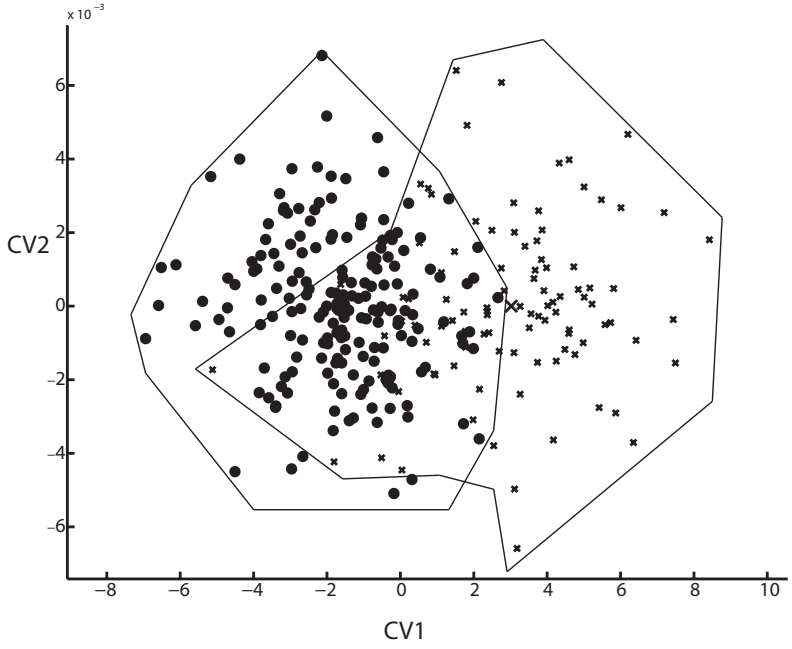


B)

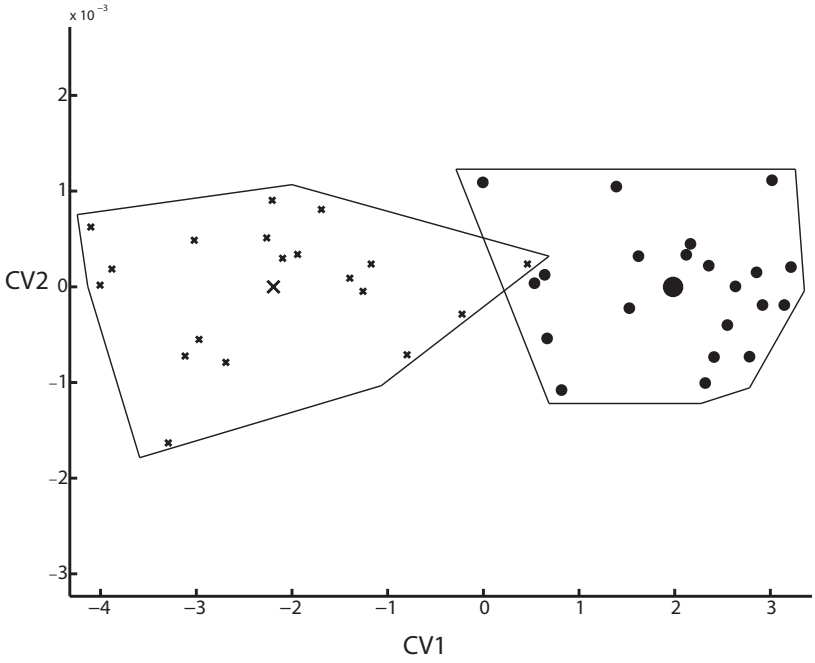


Figures 8a and 8b. Plots of CVA scores. Larger symbols indicate the mean of each group. (A) upper(circles) vs. southern(X's) vs. western clades(stars). (B) upper(circles) vs. southern(X's) vs. western(stars) clades—males only.

C)



D)



Figures 8c and 8d. Plots of CVA scores. Larger symbols indicate the mean of each group. (C) upper+southern(circles) vs. west(X's) clades. (D) upper+southern(circles) vs. west(X's) clades males only.

E)

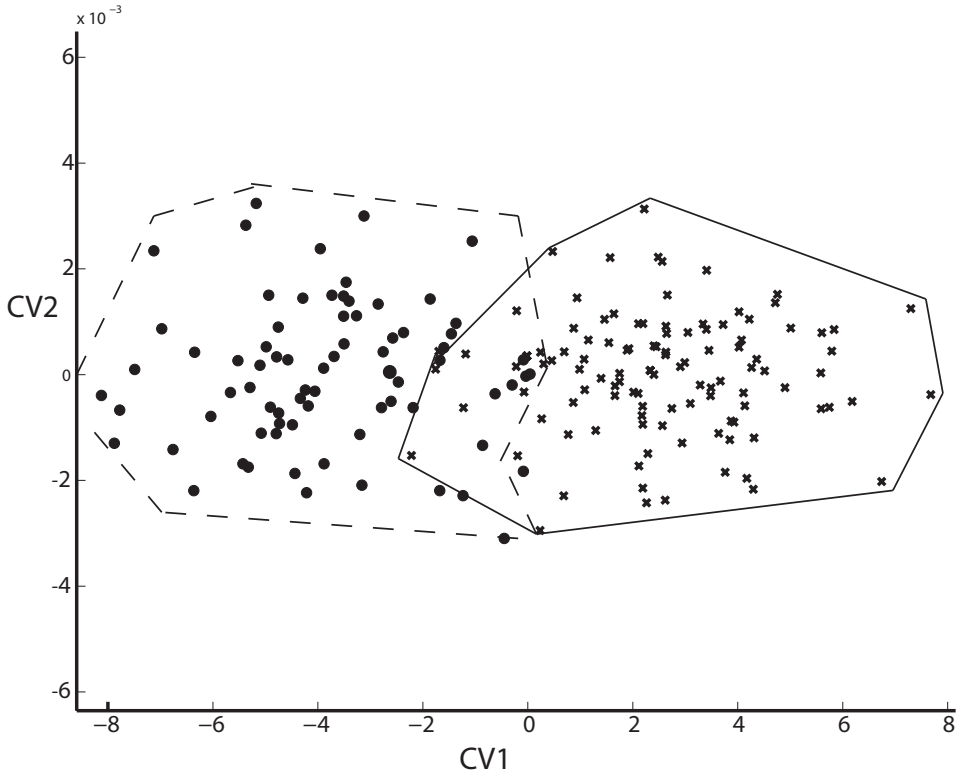


Figure 8e. Plot of CVA scores. upper(circles) vs. southern(X's) clades.

Discussion

The findings of this study do not support the hypothesis of cryptic diversity within the species. Cryptic diversity would have shown little variation between the groups. The meristic analysis showed considerable overlap between groups and was not indicative of separation between groups. The morphometric analyses were then performed, beginning with the PCA. Since the PCA analyses revealed quite a bit of overlap between the groups, a series of canonical variates analyses were performed. CVA of the specimens of *Notropis nubilus* from throughout the distribution of the species revealed geometric morphometric variation. Although there was overlap in the majority of the canonical variates analyses, distinct groups could be distinguished and there was significant variation between clades. Berendzen *et al.* (2010) found genetic differences between the three clades and the findings in morphometric differences in this study are consistent with the genetic differences found previously.

Although overlap occurred heavily in the meristic analyses, that was to be expected and is similar to the findings of the Berendzen *et al.* (2009) study on *Notropis rubellus*. There is significant variation in scale counts even within a clade, so it would be expected that there would be a great deal of overlap between clades. There is not enough distinction between the clades in order to be able to classify a specimen and assign it to a clade solely based on scale counts. The greatest amount of variation was seen in the predorsal scale counts (Fig. 5). There is a little variation seen in the lateral line scale counts, as well as the scales above the lateral line (Fig. 5). This indicates that much of the variation displayed in the CVA analyses is most likely present in the predorsal area of the specimens.

The PCA analyses displayed considerable amount of overlap between groups. Because of this overlap, CVA analyses were performed in order to determine if defining groups would uncover variation between groups. Results of the CVA analyses revealed fairly clear separation between the three groups.

There were several possible sources of error throughout this experiment. The males-only CVA analysis of the Upper vs. Southern clades was unable to be performed due to the small sample size. The CVA analysis only works on a sample size of so many specimens, and since there were only 21 total males among the Upper and Southern regions combined, the number was not sufficient. There are some outliers in the CVA and PCA graphs, and these anomalies could be attributed to malformation due to years in a preservation jar. Although malformed specimens were excluded from the study, it is possible that a malformed specimen did not visually present at such.

Since few males were included in this study, the males-only CVA and PCA analyses may not be indicative of a larger trend in the population. Males were identified based on tubercles on the snout. It is possible that years of preservation made these tubercles less evident and that there were more inconspicuous males than conspicuous. The purpose of looking at males in this study was to determine if there were any more pronounced differences due to sexual dimorphism. The males-only CVA analyses did show more separation in the groups, indicating some form of sexual dimorphism, but this could also be attributed to the small sample size in males.

The determination of whether a species is cryptic or not oftentimes is influenced by the methods used. If this study had only used meristic analyses to determine crypsis, results would have pointed toward a cryptic species. However, the results of the

geometric morphometric analysis showed that the clades do, in fact, have minute morphological differences that were not evident in the meristic analysis and are not visible to the eye.

This study reiterates the importance of doing a geometric morphometric analysis when faced with multiple populations with already known genetic differences. A species with genetic differences between populations and no meristic or morphometric differences to parallel those findings is classified as having cryptic diversity. However, that was not the case in this study. The results of the geometric morphometric analysis in this study parallel the previously found genetic differences found by Berendzen *et al.* (2010). Further studies could be performed to determine whether or not these populations should be classified as separate species. That is ultimately the significance of this study—the broader purpose of knowledge of diversity and classifying organisms by determining separation at the morphological level naked to the eye.

Acknowledgements

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