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Morphological and Molecular Analyses of the Blacknose Dace Species Complex (Genus Rhinichthys) in a Large Zone of Contact in West Virginia

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Morphological and molecular analyses of the blacknose dace species complex (Genus *Rhinichthys*) in a large zone of contact in West Virginia

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Abstract

Morphological and molecular analyses of the blacknose dace species complex (Genus *Rhinichthys*) in a large zone of contact in West Virginia

Geoffrey D. Smith

The blacknose dace species complex (*Rhinichthys atratulus, Rhinichthys obtusus obtusus*, and *Rhinichthys obtusus meleagris*) are among the most common freshwater fishes in eastern North American. Despite this fact, the taxonomy of this group is still in question. This study focuses on the relationship of the members of this species complex along an unusually large zone of contact in the high Appalachian Mountains of West Virginia. Morphological, distributional, and molecular analysis of the relationships of this complex were conducted in this area to try to shed light on the systematics of this group. Morphological analysis of coloration patterns of nuptual males displayed a strong division between the different forms and suggested that width of coloration of the side of the body, coloration of lateral line stripe, coloration below Lateral Line Stripe, and pectoral fin nuptual pad coloration were the strongest characters in determining the assignment of the individuals to the respective subspecies. Analysis of the distribution of the subspecies revealed a large number were found in drainages outside of their predicted range and in three instances, more than one form existed in a single waterbody. When the pre-Pleistocene distribution was applied to the present drainage, nearly all instances of anomalous distribution or the presence of multiple forms were explained. Molecular analysis of mitochondrial cytochrome *b* gene revealed a strong division between the eastern and western forms and showed evidence of an intermediate form from streams that were subject to stream capture events. Use of coloration patterns proved effective for differentiating between the subspecies and agreed with mitochondrial DNA based clusters, in West Virginia populations but caution must be used in areas where stream capture or interbasin transfer may have occurred.

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Chapter 1: Introduction

The taxonomy of the blacknose dace species complex (family Cyprinidae, genus *Rhinichthys*) has been an area of disagreement for more than 25 years. Several studies have analyzed the morphology, morphometrics, meristics, and breeding behaviors of the members of this species complex in various populations in eastern North America without resolution. Despite the lack of agreement on solid, discriminatory characteristics, reclassification of the members of this species complex has taken place. This study will focus on accepted morphological identifiers for separating the members of the species complex, literature suggested distribution patterns, and then compare them with novel molecular data to try resolve the relationships between the members of this interesting species complex.

In 1982, Matthews *et al*. published a study from Meadow Creek, James River drainage near the Virginia/West Virginia border where the eastern blacknose dace (*Rhinichthys atratulus atratulus*) and southern blacknose dace (*Rhinichthys atratulus obtusus*) were sympatric with little overlap. The presence of both subspecies in a single water body while still displaying little overlap in range raised questions as to the relationship of the two members of the species complex. Analysis of the allopatric populations showed several characters to be different between the subspecies but no single morphometric or meristic character reported that complete separation. Differences were observed in scale count across the back, lateral line scale count, and caudal peduncle scale count but none were statistically significant. When sympatric populations were analyzed, most fish had characters intermediate between the subspecies found in the allopatric populations, suggesting that hybridization or introgression was occurring. Based on their findings, it appeared that some of the fish present were individuals that were not first generation offspring (F1) of the two subspecies, suggesting that the two populations (i.e. subspecies) were not reproductively isolated. Analysis of these characters was also not bimodal in distribution. Instead they found them to rather evenly distributed across the range of characters, suggesting that this population had undergone several successful breeding events as well as repeated introduction of new genetic material from both outside populations. Based on their findings, Matthews *et al*. (1982)

suggested that the three morphological types remain as subspecies . Although evidence suggested that the differences were distinct enough to allow species designation, there was also much evidence to refute this elevation, suggesting that a more robust study of the species complex across its entire range was needed before this question could be answered.

Smith (1985) examined the eastern blacknose dace and western blacknose dace (*Rhinichthys atratulus meleagris*) in an area where they were syntopic in New York, and reported little evidence of sympatry and intergradation. Smith stated that the ranges of the two subspecies overlap little, if any, and that the males (presumed nuptial males) of the two forms were distinct. Smith suggested that in order to best understand the relationship of the two forms, it is crucial to keep information separate and that giving separate species status to these two subspecies would be the best way to go about this until they can be more thoroughly studied.

In order to elevate the western blacknose dace to separate species status as suggested by Smith (1985), the consubspecific form, *R. atratulus obtusus,* designated by Matthews *et al.*(1982) would also have to be elevated. Based on previous revisorship by Jordan and Gilbert (Jenkins and Burkhead, 1994), the nominal species would become *Rhinichthys obtusus*. With this elevation, the type subspecies and *Rhinichthys obtusus meleagris* would fall under this new species designation.

In a review of the <u>Inland Fishes of New York</u> (Smith, 1985), Jenkins (1988) commented on the depth and breadth of the material used for the suggested reclassification. Jenkins suggests that not enough material was presented to warrant the type of action that was called for by Smith.

In <u>Freshwater Fishes of Virginia</u> (Jenkins and Burkhead, 1994), the authors restate that, based on information in Matthews *et al.*(1982), these forms of the species complex all retain subspecies status. Although the suggestions of Smith (1985) are addressed, there is no comment on the validity of this elevation, only the complexities that would arise with the reclassifications based upon previous revisorship stated earlier. Throughout the species description, Jenkins and Burkhead continue to refer to all forms as subspecies under *Rhinichthys atratulus* and comment on studies (Matthews *et al.*, 1982; Adkins *et al.*, 1985) of possible zones of contact that might “markedly improve” the understanding of the relationship of this species group.

In the Common and Scientific Names of Freshwater Fishes of the United States, Canada, and Mexico (2004), the status of the subspecies is changed to reflect the recommendations of Smith (1985). This publication accepted the elevation and created the eastern blacknose dace (*Rhinichthys atratulus* Herman, 1804) and western blacknose dace (*Rhinichthys obtusus* Agassiz, 1854). The cited references for the change in classification are Smith, 1985; Jenkins and Burkhead, 1994; and Matthews *et al.*, 1982. No description of the decision making criteria were presented in this text and no further definitive studies were presented other than those previously mentioned.

Following the reclassification, a study of Canadian populations of eastern and western blacknose dace (Fraser *et al.*, 2005) showed that there were no significant differential characters found to separate the two species. The study focused on both allopatric and sympatric populations using characters described in Matthews *et al.*(1982) and Smith (1985) and found no morphological feature consistently distinguishing between the forms. Based on these findings they refute the elevation of the species elevation and suggest the need for a further ranging study in terms of distribution and the addition of genetic analysis as the given traits are insufficient or are not feasible other than during limited time frames throughout the year (i.e. coloration patterns in breeding males).

Between ca 1988 and 2004, little, if any, published literature pertains to the systematics of the blacknose dace species complex yet the most significant decisions regarding their classification occurred during this time. All of the studies previously conducted identify the need for further study of this species complex but list various foci as important in resolving the issue. Several of the publications identify the need of molecular analysis of the species complex (Matthews *et al.*, 1982; Adkins *et al.*, 1985; Jenkins and Burkhead, 1994; Fraser *et al.*, 2005) yet it appears that no such analysis has been published to date. The history of contradictory conclusions from these studies suggests that further review of physical characteristics be conducted to identify appropriate taxonomical tools for separation of the members of this species complex (Matthews *et al.*, 2005; Jenkins and Burkhead, 1994; Fraser *et al.*, 2005). The inclusion of a comparison of genetic data with more standard morphological practices for aid in determining appropriate characters would help resolve their taxonomy (Fraser *et al.*, 2005). A few studies suggest that zones of contact are important areas to study the relationships (Matthew *et al*., 1982; Adkins *et al*., 1985; Jenkins and Burkhead, 1994) while others call for more widespread studies of the entire distribution of the species complex (Jenkins, 1988; Fraser *et al*., 2005). Regardless of means and direction, all studies identify the need for further research before any resolution can be brought to the issue of classification of the blacknose dace species complex.

For this study, I chose to look at the morphological, distributional, and molecular relationships of the members of this species complex as they exist in a zone of contact in Appalachian Mountains of West Virginia. All three forms are known to exist in a rather large zone of contact in this area (M. Little, Personal Communication) and based on suggestions of previous studies regarding the importance of these contact areas (Matthews *et al*., 1982; Adkins *et al*., 1985; Jenkins and Burkhead; 1994) this is an appropriate area to study these relationships. By coupling molecular and morphological analyses along with analysis of distribution patterns in this zone, I hope to shed some light on the relationships of the members of this species complex and lay the groundwork to developing the tools necessary to start resolving the morphological taxonomy questions at hand.

Chapter 2: Background

An extensive review of the ecology, life history, and systematics of the blacknose dace species complex was completed by Matthews *et al*. (1982) and will be used as the basis for the information in this chapter. All information not explicity cited can be credited to that work. For the purpose of this chapter, I am going to focus on the areas of this review that are directly pertinent to systematics or are conducive to reproductive isolation and subsequently, speciation.

Distribution

The distribution of the three forms of the blacknose dace species complex; *Rhinichthys atratulus*, *Rhinichthys obtusus obtusus*, and *Rhinichthys obtusus meleagris* are largely allopatric with few instances of sympatry. The eastern form, *Rhinichthys atratulus*, is largely confined to the Atlantic drainage and Lake Ontario of the Laurentian drainage (Jenkins and Burkhead, 1994). The southern form, *Rhinichthys obtusus obtusus*, is distributed across the southern portion of the Ohio River Basin and some areas in the southeast on the Atlantic portion of the continental divide (Jenkins and Burkhead, 1994). The western or central form, *Rhinichthys obtusus meleagris*, is distributed over the upper Mississippi and Great Lakes drainages (Jenkins and Burkhead, 1994). Collectively, the latter two forms are referred to as the western blacknose dace, *Rhinichthys obtusus* Aggasiz (Nelson *et al*., 2004) and are loosely described as inhabiting the Upper Mississippi, Ohio, and Great Lakes drainages. Although not explicitly stated, distribution of these forms follows the Mississipian and Atlantic refugium models (Chapleau and Pageau, 1985; Mandrak and Crossman, 1992) and Pliocene flow patterns (Fraser *et al*., 2005)

Breeding Behavior

One of the primary ways in which the members of the blacknose dace species complex were believed to differ was in the breeding behaviors displayed among the subspecies. Comparison of documented breeding behaviors in the eastern blacknose dace in New York, Virginia, and Maine however showed reproductive behaviors to be highly variable within the subspecies. Many of the behaviors such as site selection, male grouping, territoriality, aggressiveness, and courtship appear to be more indicative of populations and show high levels of local variability therefore indication as strict, selective breeding behaviors is questionable, especially concerning potential source of reproductive isolation (Matthews *et al.*, 1982).

Comparison of the behaviors of the western blacknose dace by Raney (1940) were found to greatly differ from the eastern blacknose dace documented by Traver (1929) in the New York populations. Raney reported that repeated spawning events created nest-like depressions in the substrate but actual construction of the depressions was never seen. Based on Raney's observations, Schwartz (1958) stated that the western form, after witnessing the reproductive behaviors of the southern form, was the only form that actually conducted nest building when in fact his interpretation of Raney's comments may have been in error. Bartnick (1970) witnessed a breeding behavior conducted by *R. o. meleagris* in which females pushed snouts into substrate and rooted, possibly, signaling readiness to breed. This may have caused the disturbances documented by Raney and consequently misinterpreted by Schwartz (Matthews *et al*., 1982).

The reproductive behaviors of southern blacknose dace were also found to be different between populations. Schwartz (1958) documented behaviors of *R. obtusus obtusus* in West Virginia populations and found them to be highly different than both the other forms. Males of these populations would lead females to their respective territories to breed. This breeding would take place high in the water column, much different than the other two forms which bred in contact with the substrate (Schwartz, 1958). Jenkins found that small depressions 1-3 cm in depth and 5-10 cm in width that were probably the result of repeated breeding events by *R. obtusus obtusus* in Virginia populations. Jenkins also found that males had established territories and did actively defend those territories. Virginia populations did not lead females to a mating area, instead females chose areas and then were bred by the male who held that territory. The Virginia populations of the southern form shared many of the traits of the other two forms and were quite consistent with the habits displayed by the western form, R. *o. meleagris* (Matthews *et al*., 1982). Because of these findings, it was recommended that the breeding habits of all three forms were more population dependent than subspecies specific and were not adequate for separating the forms. The similarities of the southern and western forms in terms of breeding behavior were strong and “did not differ trenchantly” (Matthews *et al*., 1982).

Collective review by Matthews *et al*. (1982) and later by Jenkins and Burkhead (1994) suggest that breeding behavior among populations was highly variable. No single suitable characteristic of the breeding behavior was exclusive to any one of the forms of this species complex.

Nuptual Coloration Patterns

Male nuptual coloration is by consensus the most distinguishing feature separating the three separate forms of the blacknose dace species complex. Despite being the most predictive means of identifying the different forms, there still are discrepancies in the literature regarding the specific traits.

Pectoral fin and nuptual pad coloration are described by Matthews *et al*. (1982) and Jenkins and Burkhead (1994) as the most accurate characters for distinguishing between *Rhinichthys atratulus* and the two forms of *Rhinichthys obtusus*. Nuptual males of *R. atratulus* possess either bright orange to red fins with bright orange to red nuptual pads or yellow to clear fins with bright orange to red nuptual pads. Regardless of fin coloration, the nuptual pad is a brilliant orange to red on virtually all individuals sampled. The other forms of the species complex are said to possess olive-yellow to yellow, little or no color, or at least less orange than the eastern form. More variability has been documented in these forms of this species complex. Several studies cite the presence of yellow, orange, or red pectoral fin colorations on the western and southern forms of the blacknose species complex as well (Bartnik, 1970; Tarter, 1969; Clay, 1975; Forbes and Richardson, 1920; Trautman, 1957). Matthews *et al*. (1982) state that reported variation may, in fact, be due to the physiology of the breeding act or possibly even to subjectivity in descriptions of colors. They did however state that, although important in their study area (Virginia), this may not be the case everywhere as referenced by the number of independent appearances in the literature.

Coloration patterns are reported to be indicative of the forms of the blacknose dace species complex; at least for separating *Rhinichthys atratulus* from the two forms of *Rhinichthys obtusus*. The dark lateral line stripe in the eastern form is said to be suffused with or masked by cinnamon brown, rust, orange, brick, or red. This coloration pattern is also said to be mainly restricted to the area immediately around the stripe. The coloration of the areas ventral to the lateral line stripe are either silver- or creamy-white to pale gold-yellow occasionally with a pale green tint, including the operculum and cheek. Matthews *et al*. (1982) state that the yellow displayed by the eastern form does not appear to be an 'incipient stage' of the orange coloration seen in the other forms and therefore is an indicative coloration trait for distinguishing the eastern form from the western and southern form. The coloration patterns listed from the other two forms seem to be more variable and more interchangeable than is either with the eastern form.

Rhinichthys obtusus obtusus, as described by Matthew *et al*.(1982) and Jenkins and Burkhead (1994) has a brighter lateral line stripe than *Rhinichthys atratulus*, being brighter or more pure orange to red-orange but varied with size of individual and nearness to spawning. In the areas ventral to the lateral line stripe in *R. o. obtusus*, there is a second center of development which they identify as midlateral (the more common lateral line stripe) and a submidlateral center of coloration, which may or may not be separated by an area of white, which they feel is dependent on the fish's stage of breeding. The orange coloration is also found on the operculum and chin of these fish. Some populations may lack the lower center of color (Matthews *et al.*, 1982; M. Little, personal communication). Jenkins and Burkhead (1994) do not weight this character as heavily and state that there is a faint to translucent orange coloration on the sides of the body. Shontz (1962) reported that nuptual male *R. o. meleagris* differed from *R. o. obtusus* in that the coloration was confined to the immeadiate area of the dark midlateral stripe and does not extend to the origin of the pectoral fins. Several other studies refute this claim and state that nuptual male *R. o. meleagris* possess coloration over large portion of the side of the body (Bartnick, 1970; Forbes and Richardson, 1920; M. Little, personal communication). Based on the variability the coloration patterns of nuptual males, Matthews *et al*. (1982) states that the coloration patterns of the areas ventral to the lateral line stripe are good characters for separating the eastern from the western and southern forms but are not conducive to accurately distinguishing between the western and southern forms.

Morphology and Meristics

Traditional morphological and meristic analyses of this species complex have yielded little in the way of a definitive answer in systematically differentiating between the different forms. Analysis of Meadow Creek (Virginia) populations of *R. atratulus* and *R. o. obtusus* in a zone of syntopy by Matthews *et al*. (1982) found the only significant difference to be that scale count indices (lateral line scales + scales across back) which were higher in *R. o. obtusus* than in *R. atratulus*. However they caution that the reference condition for the *R. o. obtusus* was found at a much higher elevation than the *R. atratulus* reference, a common factor that has been found to influence scale size and number (Matthews *et al.*, 1982). The values generated from this index were not completely separated between these two forms although significantly different. Individuals from the overlap zone displayed characters intermediate of the two reference forms. A robust study of both morphological and merisitic characters of allopatric and sympatric populations of *R. atratulus* and *R. o. meleagris* conducted in Canada found that populations of these two forms were indistinguishable using these characters (Fraser *et al*., 2005). Characters of the allopatric populations of the two forms were found not to differ significantly nor did the variability of these characters differ between allopatric and sympatric populations.

Careful analysis of these factors shows a great deal of variability both among and within populations of each of these forms. There is little or no agreement between the interested parties as to the systematic status of this species complex. Not least of which was the relatively unsubstantiated elevation of two forms of this species complex to a new species, the western blacknose dace *Rhinichthys obtusus* Aggasiz (this includes the type subspecies and *R. o. meleagris*). Lacking from the published studies was a study of the genetic relationships of these forms. Preliminary molecular analysis of West Virginia populations began to show some interesting relationships between the forms but was never published (M. Little, personal communication). Matthews *et al*.(1982) and Jenkins and Burkhead(1994) state that more careful analysis along zones of contact, presumably using molecular and more common morphological methods, will give the greatest insight into the relationships of this group.

Based on these recommendations, this study aimed to focus on the relationships of these members of the three forms of the blacknose dace in a zone of contact in the Appalachian Mountains of West Virginia. Important in this study were the areas of known stream capture events, anomalous distributions, and areas along the present day Eastern Continental Divide that separates the Atlantic from the Ohio River drainages, the major factor in the separation of the newly defined species status. By focusing on this region and using both described character traits (nuptual male coloration patterns) and previously unpublished molecular relationships, I hope to shed some light on the relationships of this species complex along this uniquely large zone of contact.

Chapter 3: Methods

Specimen Collection

All specimens were collected by use of backpack electrofishing using a Coffelt Manufacturing Mark 10 Backpack Electrofisher. Candidate stream reaches were electrofished based on likelihood of containing specimens determined by appearance. Only male *Rhinichthys atratulus* and *Rhinichthys obtusus* specimens in full nuptual coloration were collected. Due to the close association of the adult males in nuptual coloration with cover objects, electrofishing practices had to be modified to include the flipping or disturbing of cover objects to allow efficient capture of adequate quality and number of specimens. Following capture, specimens were labeled with an arbitrary numeric code (MU_BND_XXX). All individuals were digitally imaged in a photographic aquarium on site to ensure maximum coloration expression in specimens. Following imaging and data collection, samples were placed in mylar foil pouches and preserved in dry ice to maintain integrity of DNA. Mylar pouches we used as opposed to plastic as the then to hold up better than plastics at -80^{o}C.

Morphological Data Collection

All specimens were identified in the field to subspecies level using characteristics identified as the most predictive characters for taxonomy of the members of this species complex (Matthews *et al.*, 1982; Jenkins and Burkhead, 1994; M. Little, personal communication). Table 1 lists all characters used for morphological portions of this project. Characters were taken with live specimens in the field as well as from digital images. Preference was given to field records as photographs tended to not fully represent some of the lighter colors (e.g. “wash-out” and glare). As there has been little agreement on the relatedness and importance of each of these characters to the taxonomy of each of these subspecies, a substantial portion of this project was to blindly group the individuals based on character combinations and then weight which of these characters best predicted the identity of that cluster. Previous studies have analyzed these relationships using morphometric and meristic characters using multivariate statistic methods (Matthews et al., 1982, Fraser et al., 12005) but none have analyzed the nuptual male coloration patterns as described as the consensus most suitable traits for discrimination, probably because of the subjectivity and categorical nature of these data. Heirarchal and Two-step cluster analyses were performed on this data using SPSS for Windows (SPSS Inc., Chicago, IL, 2002). These analyses were used to arrange clusters based on the categorical nuptual male coloration pattern data and then rate these variables in terms of the importance on the association with the derived clusters, respectively. By rating the importance of the variables, it will be possible to start suggesting best characters and conditions of those characters for field identification of subspecies based on nuptual male coloration patterns.

Table 1: Characters and conditions used to analyze morphological associations of the blacknose dace species complex

Character	Option 1	Option 2	Option 3	Option 4
Lateral Line Stripe Color	Cinnamon	Rust	Orange	Scarlet
Lateral Line Stripe Width	Confined	Wide	Double	
Color below Lateral Line Stripe	Yellow	Orange	White	
Pectoral Fin Color	Orange	Yellow	Clear	
Nuptual Pad Color	Orange	Opaque	Yellow	
Melanophore Presence	Present	Absent		
Melanophore Density	Few	Many	Very Many	
Chin Color	Yellow	Orange	White	
Opercle/ Cheek Color	Yellow	Orange	White	
*Obliterated Lateral Line Stripe	Not obliterated	Obliterated		
*Amount of Obliteration	No obliteration	Complete	Partial	

**The term "obliteration" was used to refer to condition in which dark lateral line stripe was replaced by nuptual color*

Molecular Analysis

A subset of specimens were selected for molecular analysis based on the representativeness of described subspecies coloration patterns and area of capture. In order to get the best analysis of the depth and breadth of genetic variation encountered in the study area, individuals that displayed the best traits of each of the subspecies, individuals that appeared to be either introgressed or hybridized from two or more subspecies, and at least one individual from each of the watersheds sampled as part of the morphological analysis. A total of 16 individuals were included in the molecular analysis. Table 2 lists the fish included in the molecular analysis, their location of their capture, and the subspecies-level taxonomic identification given to them from field and photograph interpretation.

Table 2: Fish included in molecular analysis of the blacknose dace species complex.

Fish ID	Subspecies	Waterbody	Drainage	Major Drainage
MU_BND_001	*R. obtusus meleagris*	Johnnys Run	Greenbrier River	Ohio (Lower)
MU_BND_005	*R. obtusus meleagris*	W. Fk. Greenbrier River	Greenbrier River	Ohio Lower
MU_BND_006	*R. obtusus obtusus*	Files Creek	Monongahela River	Ohio (Upper)
MU_BND_016	*R. obtusus meleagris*	UNT W. Fk. Greenbrier River	Greenbrier River	Ohio (Lower)
MU_BND_017	*R. atratulus atratulus*	UNT Youghiogheny River	Monongahela River	Ohio (Upper)
MU_BND_035	*R. obtusus meleagris*	W. Fk. Greenbrier River	Greenbrier River	Ohio (Lower)
MU_BND_036	*R. atratulus atratulus*	UNT Youghiogheny River	Monongahela River	Ohio (Upper)
MU_BND_043	*R. obtusus meleagris*	W. Fk. Greenbrier River	Greenbrier River	Ohio (Lower)
MU_BND_045	*R. obtusus meleagris*	UNT W. Fk. Greenbrier River	Greenbrier River	Ohio (Lower)
MU_BND_047	*R. atratulus atratulus*	Abernathy Run	S. Br. Potomac River	Atlantic
MU_BND_054	*R. atratulus atratulus*	Abernathy Run	S. Br. Potomac River	Atlantic
MU_BND_056	*R. obtusus meleagris*	W. Fk. Greenbrier River	Greenbrier River	Ohio (Lower)
MU_BND_077	*R. obtusus meleagris*	W. Fk. Greenbrier River	Greenbrier River	Ohio (Lower)
MU_BND_086	*R. obtusus meleagris*	W. Fk. Greenbrier River	Greenbrier River	Ohio (Lower)
MU_BND_088	*R. obtusus meleagris*	W. Fk. Greenbrier River	Greenbrier River	Ohio (Lower)
MU_BND_132	*R. obtusus meleagris*	Laurel Fork Cheat River	Monongahela River	Ohio (Upper)

Based on recommendations made in Pfrender *et al*. (2004), a 677 base pair (bp) segment of the mitochondrial cytochrome *b* gene was analyzed for sequence variation among populations of blacknose dace species complex. Pfrender et al.(2004) studied within and among population genetic variability of a congener of the blacknose dace species complex, the speckled dace (*Rhinichthys osculus* Girard), in the Oregon and found these methods to be adequate for analyzing these relationships. Similarly, other studies have focused on this gene when analyzing relationships among cyprinid fishes (Raley and Wood, 2001; Mesquita *et al.*, 2001; Dowling *et al.*, 2002; Kotlik and Berrebi, 2002; Cunha *et al.*, 2004; Perdices *et al.*, 2005; Girard and Angers, 2006). Whole fish sections weighing between 100 and 300 mg were manually cut and chopped to begin to break tissue down for DNA extraction. Once well masticated, material was placed in a 2 mL screw top microfuge tube with 700 μL of sterile salt homogenizing buffer and approximately 500 μL of 0.1 mm Zicronium beads and beat at 3300 rpm for 1 minute to further break down tissue. Samples were iced for 2 minutes and beat again for 1 minute at 3300 rpms. Following the second beating session, liquid was allowed to settle and 500 μL of tissue and liquid mixture was taken and transferred to a new tube containing 20% SDS and proteinase-K for digestion overnight. Following digestion, protein was precipitated using 100 μL 6M NaCl and centrifugation at 10,000 x *g* for 30 minutes at 4°C. Following centrifugation, 750 μL of supernatant was removed and transferred to sterile tube at which time an equal volume of 100% isopronol was added, contents mixed and chilled for 60 minutes at -20°C. Following chilling, the sample was centrifuged at 14,000 x *g* for 20 minutes at 4°C to precipitate DNA. Supernatant was removed and the precipitated DNA pellet was resuspended in 100 μL sterile deionized water. Mitochondrial DNA (mtDNA) was extracted using phenol/ chloroform and centrifugation and separation by specific gravity using wax emulsion in microfuge tubes. Remaining mtDNA was ethanol precipitated using 100% ethanol and centrifuged at 14,000 x *g* for 30 minutes at 4°C. Ethanol was pipetted off using transfer pipettes and a second 500 μL volume of 70% ethanol was added, to remove left over salt, and centrifuged a second time for 14,000g for 20 minutes. Ethanol solution was then pipetted off and pellet was allowed to completely dry. Purified mtDNA was then resuspended in 100 μL 4% IDTE. The presence of DNA was verified by gel electrofloresis using 1% agarose gels. Purified DNA was stored at -20°C.

DNA was amplified using polymerase chain reaction as described by Pfrender *et al*. (2004). Purified DNA was amplified in 50 μL reactions containing 5 μL of 50pM DNA and 45 μL of master mix containing nucleotides, 25 mM MgCl, 10 x Buffer, 50 pM Primer L15162 (5'-TTCTTCCATGAGGACAAATAT-3'), 50 pM Primer H15915A (5'- CCTCCGTCTTCCGGATTACAAGAC-3'), rTaq polymerase (Takara Bio, Inc., Shiga, Japan), and sterile deionized water. Although all reactions were conducted with primer concentrations of 50 pM, less concentrated primer solutions may be beneficial in future studies. Reactions worked best with rTaq polymerase when compared to all other Taq polymerases tried. Table 3 lists detailed amounts and concentration of reagents for all master mixes for polymerase chain reactions.

PCR products were then cleaned for sequencing using Pall Nanosep 30K microfuge spin columns (Pall Corporation, East Hills, NY) to limit interference of primer in sequencing process. Samples were consolidated and centrifuged at 5,000 rpm for 4 minutes as per manufacturer recommendations. Cleaned PCR products were then placed in labeled, bar-coded 2-D tubes for sequencing. Samples were sequenced in both directions using QuickLane® DNA sequencing process (Agencourt Bioscience

Corporation, Beverly, MA). Results were received via secure FTP site in .ab1 file format. Fragments were assembled using VectorNTI Configexpress (Invitrogen Corporation, Carlsbad, CA) and unknown regions verified and low quality sequence regions trimmed to reduce noise caused by questionable data from areas outside of particular region of interest. Sequences were then transferred to FASTA format and aligned using Clustal W. Aligned sequences were then analyzed for sequence variation analysis using PAUP v4.0b10.

Table 3: Reagents and their respective concentrations and volumes for PCR master mixes

		Total No. of PCRs	1	= ***X***
Reagent	**Concentration of Stock Solution**	**Volume in μl 1 sample**	**Volume *X* samples**	**Final**
Buffer, no Mg*	10x	5	5	
Nucleotides	10 μM	1	1	
$MgCl_2$*	25mM	3	3	
Primer L15162	50 pM*	0.2	0.2	
Primer H15915A	50 pM*	0.2	0.2	
Taq	5 μg/μl	0.5	0.5	
H_2O		35.1	35.1	
		45	45	

Chapter 4: Results

A total of 84 nuptual male blacknose dace of 3 subspecies were captured from 14 different waterbodies in 11 drainages. Table 4 is a list of the all individuals captured as part of this project, subspecies identity and stream where captured.

Distributional Analysis

A total of 54 of the 84 nuptual male blacknose dace captured were located in drainages other than those suggested for that particular subspecies in Matthews *et al*. (1982). Three of the waterbodies sampled contained more than one form of blacknose dace based upon nuptual coloration patterns. Files Creek contained both *Rhinichthys obtusus obtusus* and *Rhinichthys obtusus meleagris,* Laurel Fork contained both *Rhinichthys atratulus* and *Rhinichthys obtusus meleagris,* and Pheasant Run contained all three forms (see Table 4)

Morphological Analysis

The hierarchal cluster analysis yielded a total of three clusters based on the coloration characters of nuptual male blacknose dace. Clustering of individuals strongly resembled the subspecies level identities. Figure 1 is a dendrogram generated using hierarchal cluster analysis based on nuptual coloration patterns in male blacknose dace. The two-step cluster analysis also yielded three distinct clusters based on the nuptual coloration characters analyzed for each of the individuals. A total of 8 of the 12 characters had chi square values for at least two the clusters above the set Bonferroni-adjusted critical value (CL=95%). Figures 2-13 are histograms depicting frequency of each condition as it applied to each cluster for the two-stage cluster analysis. Figures 14-25 are chi-square test results as they relate to each cluster for each of the 12 characters analyzed for the two-stage cluster analysis.

Molecular Analysis

Multiple sequence alignment indicated non-identical sequences among the fish analyzed. Although differences were present, there were a large number of individuals that were remarkably similar (< 0.001 % difference). Table 5 is a multiple sequence alignment generated in Clustal W (v1.83) for all individuals included in this analysis. Table 6 is a Tamura-Nei matrix displaying the comparative differences by multiple sequence alignment between the individuals as generated by PAUP v4.0b10. In this analysis, a 3:1 weight was applied to transversions versus transitions. All analyses were rooted using the outgroup specimen of the longnose dace (*Rhinichthys cataractae*) from NCBI Genbank. Sequence was trimmed from submitted complete cytochrome b sequence of specimen DQ990251 (Dowling et al., 2006). A total of 1, 000 bootstrap replicates were conducted for each analyslis. Figure 26 is a neighbor-joining phylogram displaying differences as branch length. Figure 27 is a neighbor-joining rectangular cladogram displaying clustering of individuals. Figure 28 is a Tamura-Nei phylogram displaying differences as branch length. Figure 29 is a strict consensus rectangular cladogram displaying consensus clustering of individuals.

Chapter 5: Discussion

Taxonomy and Physical Characters of Nuptual Males

As described by previous researchers, I found the taxonomy of the members of the blacknose dace species complex to be highly variable and convoluted. A synthesis of the existing data pertaining to the status of each of the subspecies in regards to coloration patterns in nuptual males led to much confusion. Spatial comparisons of accounts of the same subspecies would yield highly variable results. Also comparisons of different subspecies from different areas would be highly similar. These accounts are directly attributable to the ongoing confusion around proper taxonomy and systematics of this ubiquitous species complex. Analysis of West Virginia populations found them to be no different in regards to the complexity concerning taxonomy.

In this study, a set of key characters were selected for primary identification of the subspecies consistent with literature; primarily what is described in Matthews *et al.* (1982). The eastern form, *Rhinichthys atratulus atratulus,* was the easiest of the 3 members of this species complex to distinguish from the others. This form has a dark cinnamon lateral line stripe that is mostly confined to the immediate area of the normally dark lateral line stripe. It appears to have an orange tint to it compared to its usual chestnut brown to black coloration. When in peak breeding coloration, the lateral line stripe appears to have a slight "halo" of lighter orange around the lateral line stripe. This coloration only barely extends outside the confines of the normally present lateral line stripe. The other primary characters used to distinguish this subspecies from the others was the presence of deep orange to red nuptual pads on the pectoral fins and a yellow, in some cases, almost chartreuse tint to the area below the lateral line stripe. The southern (*Rhinichthys obtusus obtusus*) and western (*Rhinichthys obtusus meleagris*) were slightly harder to distinguish from one another based on the literature. Our diagnostic characters for the southern form varied slightly from those described in Matthews *et al.* (1982). These authors describe bright orange on the side of the body with two centers of coloration on the sides of the body, a midlateral point of origin and a submidlateral origin. Jenkins and Burkhead (1994) however comment on this subspecies as having a bright translucent orange to red lateral line stripe and, if present, a lighter translucent orange below the lateral line stripe. Both authors comment that this lower area of coloration may or may not be present. The description of these characters as observed in West Virginia populations given by M. Little (personal communication) are consistent with those described in Jenkins and Burkhead (1994) and was the primary reason for selection as suitable characters for this analysis. A similar condition exists with description of these same characters in the western form. Matthews *et al.* (1982) states that the colorations on the sides of the body in *R. o. meleagris* was more confined to the lateral line stripe region and the lower body and venter usually lacked color. It is stated that other populations, however, have been known to have nearly the entire side of the body exhibiting bright colorations. Studies conducted by D. Tarter and M. Little (M. Little, personal communication) found West Virginia populations to be more consistent with the latter description and therefore were used in this study. These characters were used for preliminary field identification of the subspecies, however, these identifications were kept independent of the character matrix for cluster analysis of physical characters. Table 7 is a list of all fish included in this analysis and their respective conditions for each of the characters.

Heirarchal Cluster Analysis of the 11 coloration variables resulted in strong separation along the subspecies lines. Figure 1 is the hierarchal cluster analysis dendogram for all fish analyzed. In initiating the hierarchal cluster analysis, the threshold for clustering was set to allow the maximum number of data derived clusters (n=15) as to not bias or limit results. Based on the data at hand, a total of three distinct clusters were formed. When subspecies identities were placed on all individuals included in the analysis, this model clustered the fish strongly along lines as described in the previous methods. A few individuals did separate quickly to group together under another cluster. These individuals did appear to have traits that were intermediate between subspecies and were found in waterbodies that contained more than one form of this species complex. These cases will be covered more in depth in the following section. The strength of the results of this analysis suggests that a combination of the characters of nuptual males is useful in differentiating between the three subspecies in at least West Virginia populations.

A Two-Step Cluster Analysis was conducted with the same data to determine which of the variables included played the strongest role in determining the creation of the clusters and subsequently could be used as the most reliable characters for taxonomic identification of the members of this species complex. Similar to what was expected, width of coloration of the side of the body (LLBandWidth), coloration of lateral line stripe (LLColor), coloration below Lateral Line Stripe (ColorBelowLateral), and pectoral fin nuptual pad coloration (NuptualPadColor) were the strongest characters in determining the assignment of the individuals to the respective clusters. Coloration of the operculum/suboperculum (CheekColor), coloration of the chin area (ChinColor), disappearance of the dark lateral line stripe and replacement with nuptual coloration (ObliterationLatLineStripe), and the degree to which the lateral line stripe was replaced by nuptual coloration (AmtObliteration) had less substantial roles in determining associations but these conditions were useful for distinguishing between two separate clusters and subsequently, between species or between subspecies.

Interestingly, three of the more cited characters used in analyses of this species complex; fin color, dark melanophore presence, and dark melanophore density, were not strong identifiers of subspecific identity in West Virginia populations. Fin color, although cited frequently as yellow, orange, or red, did not appear to differ much from normal coloration in West Virginia populations. It could be quite possible that nuptual pad coloration may have been documented as fin coloration in most studies but upon close examination of the pectoral fins, there were areas where membrane was not covered by nuptual tubercles (i.e. nuptual pad) and lack of coloration was evident and led to my distinguishing of these two characters at the recommendation of Matthews *et al.* (1982) and Jenkins and Burkhead (1994). Melanophore presence and density was highly variable both within and among populations and did not appear to follow any patterns and was found to be not discriminating between subspecies of West Virginia populations.

Distribution of the forms within the large zone of contact

Collection of the fish immediately yielded some interesting answers regarding distribution of each of the forms when compared to literature suggested distributions. A total of 54 of the 84 adult male blacknose dace collected were found in waterbodies outside of their literature suggested ranges. Three of the waterbodies sampled were found to contain more than one form of blacknose dace. Table 4 lists all fish collected and their putative subspecies and stream of capture. Figure 30 is a map of collection sites and the subspecies composition of that site. Figure 31 is a map of locations were correct and erroneous distribution of the subspecies occurred

Review of the available literature also suggests that this is the case in other waterbodies in the same area. Schwartz (1958) published the breeding behavior of *R. obtusus obtusus* from tributaries of the Cheat River in the same relative area as was focused upon in this study. Based on the suggestions in other papers, this waterbody, as a tributary to the Monongahela River should contain the western form, *R. o. meleagris*. Similar to Schwartz's (1958) findings, *R. o. obtusus* was found in Pheasant Run, a tributary to Shavers Fork, just downstream of Rattlesnake Run which was included in the study conducted by Schwartz. The collections at Pheasant Run yielded all three forms. Consequently, the form which was native to that stream could not be determined. Collections in Files Creek in the Tygart Valley River drainage, which abuts Shavers Fork, also largely contained *R. o. obtusus*. The Tygart Valley River is also a tributary to the Monongahela River and should contain *R. o. meleagris* based upon literature recommendations. One individual was however recorded as *R. o. meleagris* from Files Creek but the taxonomy of that individual is questionable based upon review of the image of that individual.

The anomalies in distribution were not only limited to those waterbodies and subspecies. Horseshoe Run, a tributary in the lower reaches of the Cheat, Dry Fork Cheat River, and Laurel Fork Cheat River all contained populations the eastern form, *R. atratulus atratulus*. These streams are spatially removed from the Atlantic drainage and the presence of this form in these waters is perplexing. The Laurel Fork Cheat River also contained populations of *R. o. meleagris* and several individuals captured appeared to be intermediate in identity between these two forms. Hendrik et al. (1979) documented the presence of *R. a. atratulus* in the Youghiogheny River system, a tributary to the Monogahela River. Collections conducted as part of this study also verified the presence of this form in the upper reaches of the Youghiogheny River. Collections made in the East and West Forks of the Greenbrier River yielded exclusively *R. o. meleagris*. Based on its status as a tributary of the New River, it would be expected that this stream would contain *R. o. obtusus*, but I did not find that to be the case in the areas sampled as part of this study.

One possible explanation for the anomalies in distribution is stream capture events. Hendrik *et al.*'s (1979) documentation of *R. a. atratulus* in the Youghiogheny system was directly attributed to a stream capture event. Jenkins and Burkhead (1994) identify another stream capture in the upper Youghiogheny near our collection site (see Figure 30). These records give a possible explanation of the mechanism that resulted in the transfer of this subspecies between drainages. Similarly, Hocutt et al. (1978) document that the upper Greenbrier River captured the East and West Fork Greenbrier Rivers from the Cheat River system, providing a possible explanation for the presence of the western form in this area. Jenkins and Burkhead (1994) also document a capture event that has the upper Cheat River (vicinity of Dry Fork) capturing a portion of the South Branch Potomac River drainage, a possible explanation of the presence of *R. a. atratulus* in the upper reaches of the Cheat. When the distribution of the subspecies is compared with the known stream capture records, a large proportion of all erroneous records are explained, including possibly the presence of both the eastern and western forms in the Laurel Fork Cheat River.

The distribution of the subspecies in these areas appears to be more consistent with pre-Pleistocene drainage patterns than present day drainage patterns (Figure 32). Even though this seems like a convenient and logical answer to the problems regarding distribution, more simple explanations such as bait bucket introduction could be the culprit. These fish are popular baitfish and the area of focus for this study is an area with high frequency of recreational fishing. The true source of the dispersal of these subspecies to areas outside of their known distribution will likely go unknown but there is strong evidence to suggest that stream capture events were potential major mechanisms for the dispersal of the forms to areas outside of their suggested distributions.

Molecular Analysis

Molecular analysis of the blacknose dace species complex in this zone of contact demonstrated interesting relationships among the members and the waterbodies. When these populations were compared based on cytochrome *b* sequence analysis, the associations did not always match the phenotypic identify of the fish. I believe that this is due, at least in part, to the incidence of stream capture mentioned previously and the syntopy that it is observed in a number of the study sites. Figures 26 – 29 are the various phylograms and cladograms created by PAUP (v4.0b10) based on the mitochondrial associations by multiple sequence alignment.

The strongest associations generated were in the group of fish from waterbodies that were subject to stream capture events or contained syntopic forms of the blacknose dace species complex. The distance length neighbor-joining phylogram (Fig. 26) and the Tamura-Nei phlyogram (Fig. 28) both had these relationships with an indiscernible genetic distance between them (< 0.001 %). Interestingly, this cluster was not built out of a single subspecies. This cluster contained both *R. a. atratulus* from and UNT Youghiogheny River (1), *R. o. meleagris* from the upper Greenbrier River (11), and *R. o. meleagris* from the Laurel Fork Cheat River (1). Only narrowly separated from this cluster is a single *R. a. atratulus* from UNT Youghiogheny River. The cladograms also indicate similar results (Fig. 27 and Fig. 29). The non-distance based methods also displayed grouping of individuals within specific drainages and strengthened my confidence in the analysis. Based on the waterbodies that this association included, it appears that this group is indicative of an intermediate form and is potentially characteristic of this phenomena and not necessarily characteristic of the *R. o. meleagris* that it is largely comprised of. This group would however all contain some level of genetic material that is representative of *R. o. meleagris* because of either their present or past association with the distribution of that form.

All phylograms and cladograms (Fig. 26-29) created displayed a substantial difference between *R. o. obtusus* (MU_BND_006) and the previously described cluster as was expected (diff.= 0.04). Although this individual shared the same major drainage (Monongahela River) as many of this other fish analyzed, this individual separated from the others indicating that this form is mitochondrially unique. Although no strong assumptions can be made based on the condition of all the *R. o. meleagris* analyzed, it does appear that this individual was not far removed from that group (fig. 26 and fig. 28). Matthews *et al*. (1982) suggested that these two forms were consubspecific and based on its association with the intermediate forms in this study it appears that this may be true.

Similar to the treatment of *R. o. obtusus,* all of the trees removed the two *R. a. atratulus* analyzed and displayed a substantial difference between these and the other forms. These individuals were prime specimens both in appearance and based on their location (Abernathy Run) as not ever being subject to a drainage pattern other than the Potomac River. These individuals exhibited substantial distance separation in both distance length phylograms (fig. 26 and 28). This separation was farther from *R. o. obtusus* than was *R. o. obtusus* was from the intermediate form (diff.= 0.07 – 0.09, compared to 0.04). This is not that surprising when both past and present drainage patterns are considered. This form, at no time in its history, has ever been in a drainage pattern other than the Atlantic while the other two forms have the present Ohio River drainage pattern in common. It is somewhat troubling however that these two individuals (MU_BND_054 and MU_BND_047) expressed such difference between (0.04) them when far less (<0.001) was shown between either the subspecies (MU_BND_036 and MU_BND_035) or drainages (MU_BND_001 and MU_BND_016).

Chapter 6: Conclusion

Despite being a common fish in freshwaters, the members of the blacknose dace species complex have a long history of unclear taxonomy. The relationship among the different forms and their distribution is an intriguing issue. The various studies that have been conducted arrive at many different results and the authors have drastically different stances on the placement of the members of this complex systematically. Once this study began I began to see the reason for the large scale disagreement and the confusion that follows this group.

The most suitable characters for distinguishing between the members of this species complex, nuputal coloration in males, still yielded high levels of variability and uncertainty when arriving at a taxonomic identification. Upon careful review of all available literature, some the traits I chose, as documented earlier, proved to be sufficient for distinguishing between West Virginia populations. By using coloration patterns such as width of coloration of the side of the body, coloration of lateral line stripe, coloration below lateral line stripe, and pectoral fin nuptual pad coloration, it should be possible to identify the members of this complex in West Virginia populations to subspecies level. Other characters such as coloration of the operculum/suboperculum, coloration of the chin area, disappearance of the dark lateral line stripe and replacement with nuptual coloration, and the degree to which the lateral line stripe was replaced by nuptual coloration also are suitable traits for distinguishing between the subspecies. It is uncertain whether these traits will work outside of the immediate area, as shown by the variability of the traits as documented in the literature. These traits are limited in their usage as the breeding period for this species complex covers only roughly one month so other suitable characters will have to be determined, possibly using populations identified in this study.

There is a caveat, however, in distinguishing between the members of this complex; drainage pattern. Stream capture and drainage played a large role in the condition of these coloration patterns and the presence of the forms in the various waterbodies. Most of the individuals captured were found outside of their predicted range including the presence of more than one form in a number of different waterbodies.

This influence was at times evident in the creation of an intermediate color form but most commonly these individuals displayed the coloration pattern consistent with their pre-Pleistocene drainage pattern. This factor could play an even larger influence outside of the breeding season were species or subspecies can be determined solely by drainage. As was the case in the areas where stream capture occurred between Atlantic and Ohio Drainage streams, the primary means for separating *Rhinichthys atratulus* from *Rhinichthys obtusus*. In these areas, care must be use in conducting the taxonomy of these species. Whether working with West Virginia populations or in areas near the continental divide where stream capture may have occurred, strict understanding of the geologic history in regards in drainage patterns would help greatly in identifying these fishes.

Molecular analysis of the West Virginia populations of this species complex did, however, shed some light on the relationships among its members. As described by Matthews *et al*. (1982), Jenkins and Burkhead (1994), and ultimately stated with a reclassification in Nelson *et al*. (2004), there does appear to be a larger difference mitochondrially between *R. atratulus* and *R. obtusus* than there does between *R. obtusus obtusus* and *R. obtusus meleagris*. Unfortunately the *R. meleagris* populations sampled as part of this study appeared to be of an intermediate form so this can not be determined definitively. A more robust sample of all fishes from areas well within their predicted ranges would be much better served to draw conclusions regarding molecular relationships than this study with such a narrow focus and variable geologic history.

This common freshwater fish and the relationships among the members of the complex are quite unique. The disagreement between the breeding behaviors, coloration patterns, morphometry, meristics, and distribution of this species complex has made it fascinating to study and it is a great model for research in speciation and problems in systematics. More in-depth and robust studies of many of the variables listed above may lead to a better understanding of the relationships among this group and the mechanisms for its distribution. Hopefully someday the systematics of this interesting group of fishes will be clarified.

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Tables and Figures

Table 4: All blacknose dace captured, their subspecies identification, and location of capture

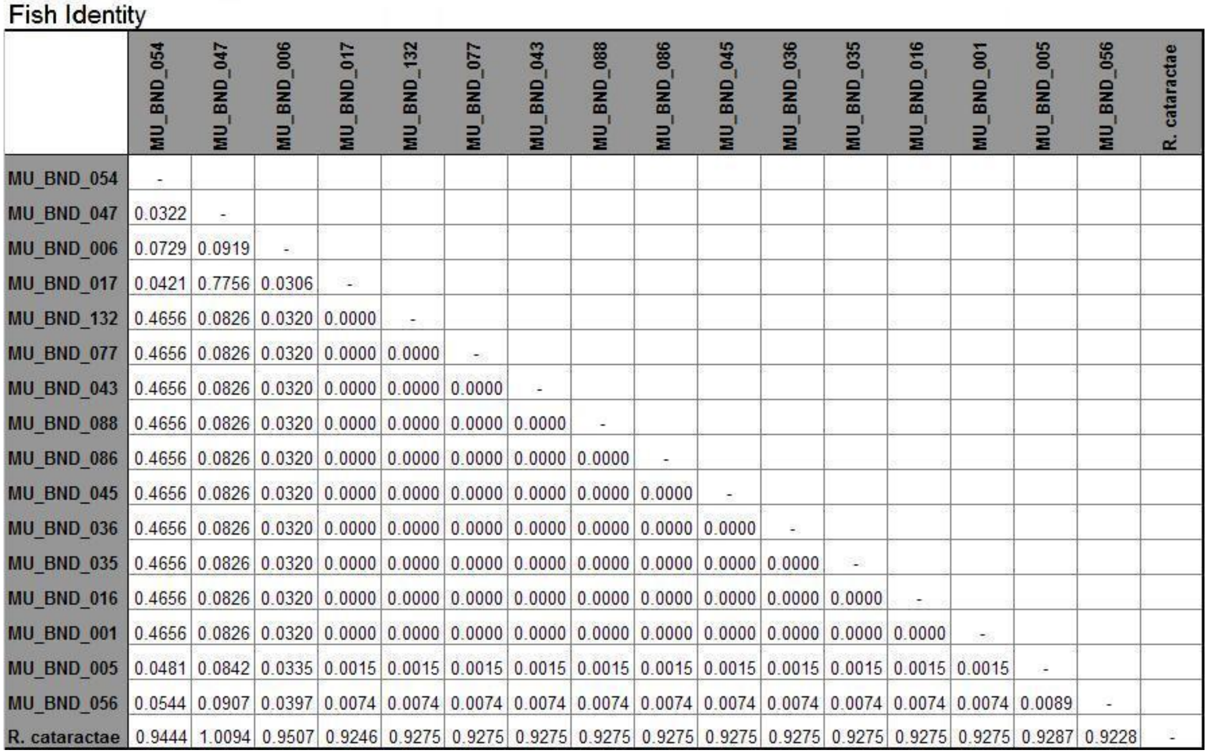
Fish Code	Subspecies	Stream Name	Drainage	Major Drainage
MU_BND_001	R. obtusus meleagris	Johnnys Run/ Johns Run	E. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_002	R. obtusus meleagris	Johnnys Run/ Johns Run	E. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_003	R. obtusus meleagris	Cove Run	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_005	R. obtusus meleagris	West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_006	R. obtusus obtusus	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_008	R. obtusus obtusus	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_009	R. atratulus atratulus	Horseshoe Run	Cheat River	Ohio River (Upper)
MU_BND_010	R. obtusus meleagris	Pheasant Run/ Pleasant Run	Shavers Fork	Ohio River (Upper)
MU_BND_011	R. obtusus obtusus	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_016	R. obtusus meleagris	UNT to West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_017	R. atratulus atratulus	UNT to Youghiogheny River	Youghiogheny River	Ohio River (Upper)
MU_BND_019	R. atratulus atratulus	Pheasant Run/ Pleasant Run	Shavers Fork	Ohio River (Upper)
MU_BND_021	R. obtusus obtusus	Otter Lick Run	Elk River	Ohio River (Lower)
MU_BND_023	R. obtusus meleagris	West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_027	R. atratulus atratulus	Horseshoe Run	Cheat River	Ohio River (Upper)
MU_BND_028	R. obtusus meleagris	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_029	R. obtusus obtusus	Otter Lick Run	Elk River	Ohio River (Lower)
MU_BND_030	R. atratulus atratulus	UNT to Youghiogheny River	Youghiogheny River	Ohio River (Upper)
MU_BND_031	R. atratulus atratulus	Pheasant Run/ Pleasant Run	Shavers Fork	Ohio River (Upper)
MU_BND_032	R. obtusus obtusus	Otter Lick Run	Elk River	Ohio River (Lower)
MU_BND_033	R. obtusus meleagris	UNT to West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_035	R. obtusus meleagris	West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_036	R. atratulus atratulus	UNT to Youghiogheny River	Youghiogheny River	Ohio River (Upper)
MU_BND_037	R. atratulus atratulus	Abernathy Run	S. Br. Potomac River	Atlantic Ocean
MU_BND_038	R. atratulus atratulus	UNT to Youghiogheny River	Youghiogheny River	Ohio River (Upper)
MU_BND_039	R. atratulus atratulus	UNT to Youghiogheny River	Youghiogheny River	Ohio River (Upper)

MU_BND_042	R. obtusus obtusus	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_043	R. obtusus meleagris	West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_044	R. obtusus obtusus	Birch River	Elk River	Ohio River (Lower)
MU_BND_045	R. obtusus meleagris	UNT to West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_046	R. atratulus atratulus	Abernathy Run	S. Br. Potomac River	Atlantic Ocean
MU_BND_047	R. atratulus atratulus	Abernathy Run	S. Br. Potomac River	Atlantic Ocean
MU_BND_048	R. atratulus atratulus	Abernathy Run	S. Br. Potomac River	Atlantic Ocean
MU_BND_049	R. obtusus meleagris	West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_053	R. obtusus meleagris	UNT to West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_054	R. atratulus atratulus	Abernathy Run	S. Br. Potomac River	Atlantic Ocean
MU_BND_055	R. obtusus obtusus	Birch River	Elk River	Ohio River (Lower)
MU_BND_056	R. obtusus meleagris	West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_057	R. atratulus atratulus	Abernathy Run	S. Br. Potomac River	Atlantic Ocean
MU_BND_058	R. obtusus obtusus	Pheasant Run/ Pleasant Run	Shavers Fork	Ohio River (Upper)
MU_BND_062	R. atratulus atratulus	Abernathy Run	S. Br. Potomac River	Atlantic Ocean
MU_BND_065	R. obtusus obtusus	Birch River	Elk River	Ohio River (Lower)
MU_BND_066	R. atratulus atratulus	UNT to Youghiogheny River	Youghiogheny River	Ohio River (Upper)
MU_BND_067	R. atratulus atratulus	Abernathy Run	S. Br. Potomac River	Atlantic Ocean
MU_BND_068	R. obtusus obtusus	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_069	R. obtusus obtusus	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_070	R. obtusus obtusus	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_071	R. obtusus obtusus	Back Fork Birch River	Elk River	Ohio River (Lower)
MU_BND_073	R. obtusus obtusus	Otter Lick Run	Elk River	Ohio River (Lower)
MU_BND_074	R. obtusus meleagris	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_075	R. atratulus atratulus	Pheasant Run/ Pleasant Run	Shavers Fork	Ohio River (Upper)
MU_BND_076	R. obtusus meleagris	West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_077	R. obtusus meleagris	West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_078	R. obtusus obtusus	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_079	R. obtusus meleagris	Pheasant Run/ Pleasant Run	Shavers Fork	Ohio River (Upper)
MU_BND_080	R. obtusus meleagris	West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)

MU_BND_081	R. obtusus meleagris	West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_082	R. obtusus obtusus	Back Fork Birch River	Elk River	Ohio River (Lower)
MU_BND_083	R. atratulus atratulus	UNT to Youghiogheny River	Youghiogheny River	Ohio River (Upper)
MU_BND_084	R. obtusus meleagris	West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_085	R. obtusus obtusus	Back Fork Birch River	Elk River	Ohio River (Lower)
MU_BND_086	R. obtusus meleagris	West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_087	R. obtusus obtusus	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_088	R. obtusus meleagris	West Fork Greenbrier River	W. Fk. Greenbrier River	Ohio River (Lower)
MU_BND_089	R. obtusus obtusus	Pheasant Run/ Pleasant Run	Shavers Fork	Ohio River (Upper)
MU_BND_090	R. obtusus obtusus	Birch River	Elk River	Ohio River (Lower)
MU_BND_091	R. atratulus atratulus	Dry Fork	Black Fork River	Ohio River (Upper)
MU_BND_128	R. obtusus meleagris	Laurel Fork Cheat River	Laurel Fork Cheat River	Ohio River (Upper)
MU_BND_129	R. obtusus meleagris	Laurel Fork Cheat River	Laurel Fork Cheat River	Ohio River (Upper)
MU_BND_130	R. obtusus meleagris	Laurel Fork Cheat River	Laurel Fork Cheat River	Ohio River (Upper)
MU_BND_131	R. atratulus atratulus	Laurel Fork Cheat River	Laurel Fork Cheat River	Ohio River (Upper)
MU_BND_132	R. obtusus meleagris	Laurel Fork Cheat River	Laurel Fork Cheat River	Ohio River (Upper)
MU_BND_133	R. obtusus meleagris	Laurel Fork Cheat River	Laurel Fork Cheat River	Ohio River (Upper)
MU_BND_140	R. obtusus obtusus	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_142	R. obtusus obtusus	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_143	R. obtusus obtusus	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_144	R. obtusus obtusus	Files Creek	Tygart Valley River	Ohio River (Upper)
MU_BND_166	R. atratulus atratulus	Dry Fork	Black Fork River	Ohio River (Upper)
MU_BND_174	R. atratulus atratulus	Dry Fork	Black Fork River	Ohio River (Upper)
MU_BND_175	R. atratulus atratulus	Dry Fork	Black Fork River	Ohio River (Upper)
MU_BND_179	R. obtusus meleagris	Laurel Fork Cheat River	Laurel Fork Cheat River	Ohio River (Upper)
MU_BND_183	R. obtusus meleagris	Laurel Fork Cheat River	Laurel Fork Cheat River	Ohio River (Upper)
MU_BND_184	R. atratulus atratulus	Dry Fork	Black Fork River	Ohio River (Upper)

MU_BND_185	R. atratulus atratulus	Dry Fork	Black Fork River	Ohio River (Upper)

Table 5: Tamura-Nei corrected distance matrix based upon multiple sequence alignment



Fish Identity

	MU_BND_054	MU_BND_047	MU_BND_006	MU_BND_017	MU_BND_132	MU_BND_077	MU_BND_043	MU_BND_088	MU_BND_086	MU_BND_045	MU_BND_036	MU_BND_035	MU_BND_016	MU_BND_001	MU_BND_005	MU_BND_056	R. cataractae
MU_BND_054	-																
MU_BND_047	0.0322	-															
MU_BND_006	0.0729	0.0919	-														
MU_BND_017	0.0421	0.7756	0.0306	-													
MU_BND_132	0.4656	0.0826	0.0320	0.0000	-												
MU_BND_077	0.4656	0.0826	0.0320	0.0000	0.0000	-											
MU_BND_043	0.4656	0.0826	0.0320	0.0000	0.0000	0.0000	-										
MU_BND_088	0.4656	0.0826	0.0320	0.0000	0.0000	0.0000	0.0000	-									
MU_BND_086	0.4656	0.0826	0.0320	0.0000	0.0000	0.0000	0.0000	0.0000	-								
MU_BND_045	0.4656	0.0826	0.0320	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	-							
MU_BND_036	0.4656	0.0826	0.0320	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	-						
MU_BND_035	0.4656	0.0826	0.0320	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	-					
MU_BND_016	0.4656	0.0826	0.0320	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	-				
MU_BND_001	0.4656	0.0826	0.0320	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	-			
MU_BND_005	0.0481	0.0842	0.0335	0.0015	0.0015	0.0015	0.0015	0.0015	0.0015	0.0015	0.0015	0.0015	0.0015	0.0015	-		
MU_BND_056	0.0544	0.0907	0.0397	0.0074	0.0074	0.0074	0.0074	0.0074	0.0074	0.0074	0.0074	0.0074	0.0074	0.0074	0.0089	-	
R. cataractae	0.9444	1.0094	0.9507	0.9246	0.9275	0.9275	0.9275	0.9275	0.9275	0.9275	0.9275	0.9275	0.9275	0.9275	0.9287	0.9228	-

Figure 1: Dendogram based upon Heirarchal Cluster Analysis of morphological traits for 84 nuptual male blacknose dace

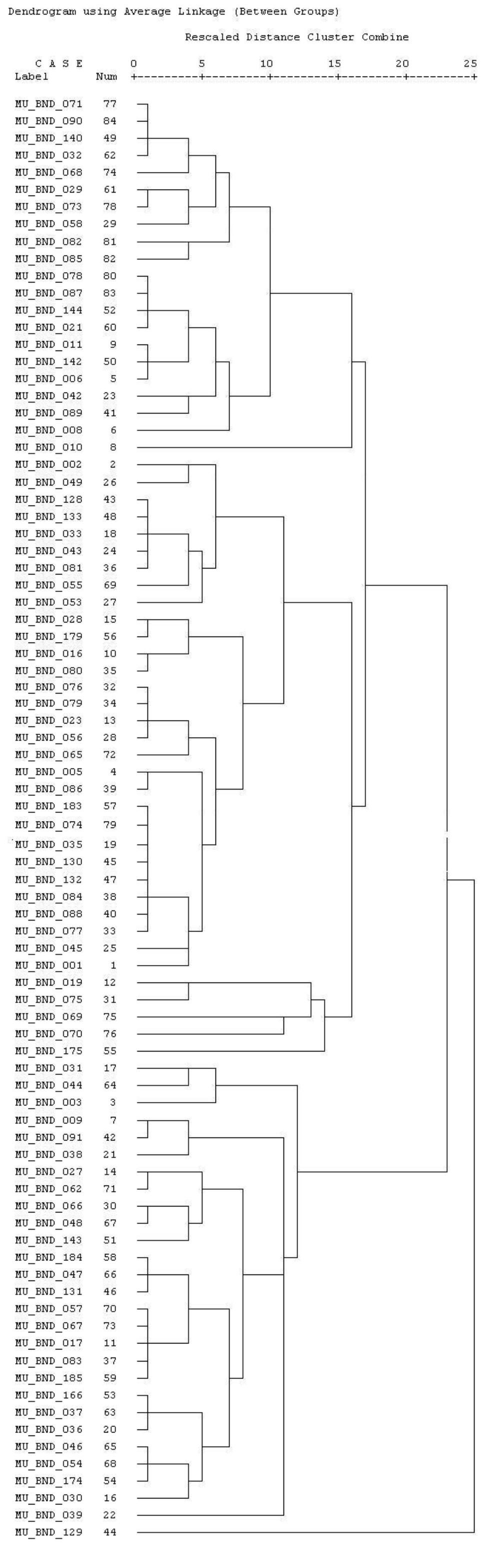


Figure 2: Frequency histogram of each condition of Lateral Line Stripe width to each cluster in the two-step cluster analysis

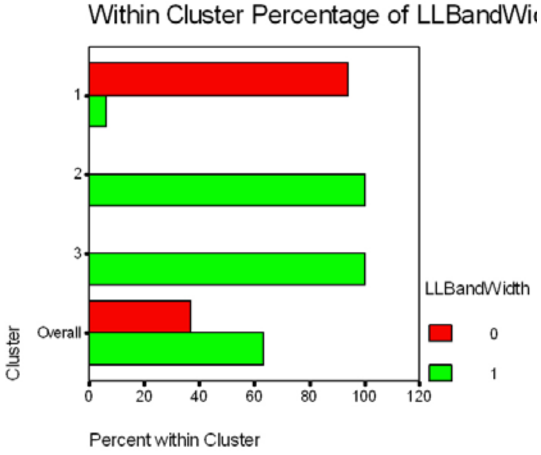


Figure 3: Frequency histogram of each condition of Lateral Line Stripe Color to each cluster in the two-step cluster analysis

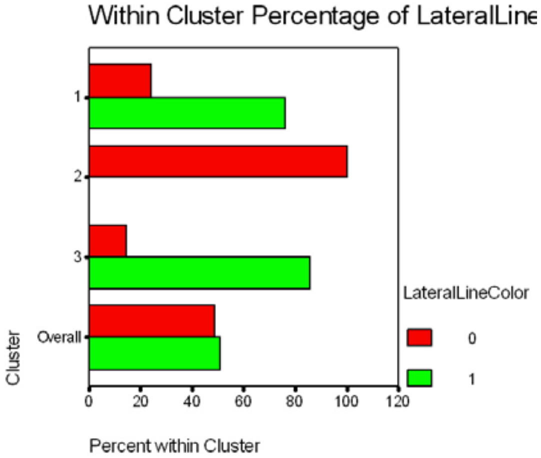


Figure 4: Frequency histogram of each condition of Color Below Lateral Line Stripe to each cluster in the two-step cluster analysis

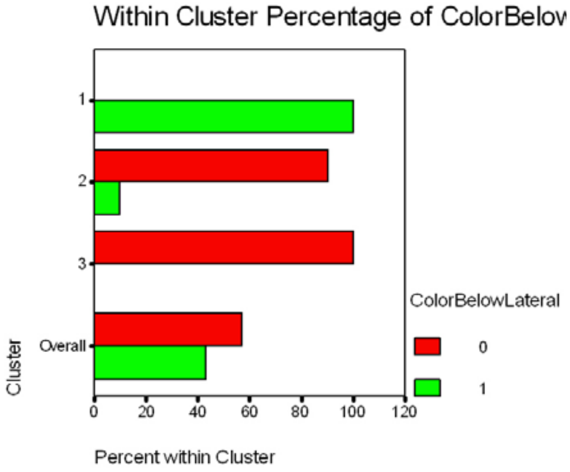


Figure 5: Frequency histogram of each condition of Fin Color to each cluster in the two-step cluster analysis

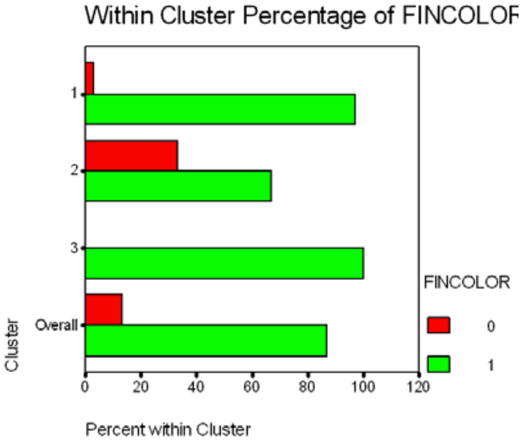


Figure 6: Frequency histogram of each condition of Nuptual Pad Color to each cluster in the two-step cluster analysis

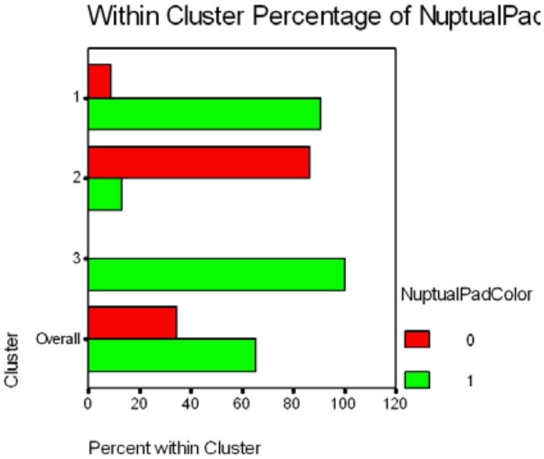


Figure 7: Frequency histogram of each condition of Melanophore presence to each cluster in the two-step cluster analysis

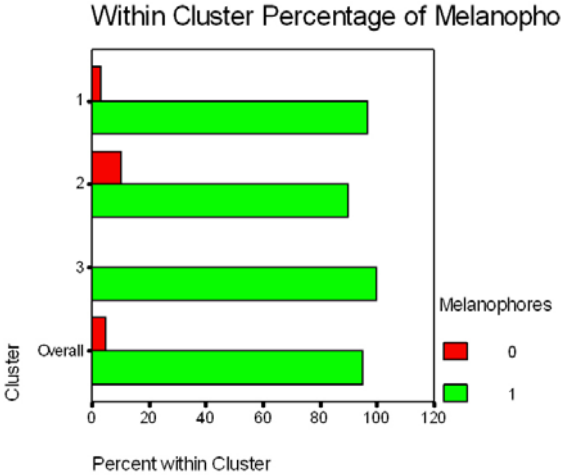


Figure 8: Frequency histogram of each condition of Melanophore Density of less dense patterns to each cluster in the two-step cluster analysis

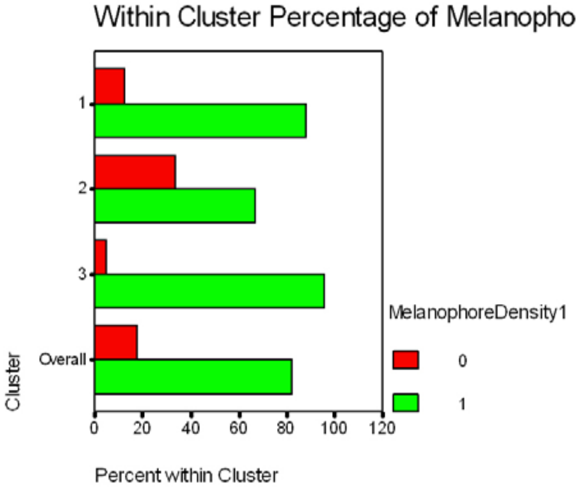


Figure 9: Frequency histogram of each condition of Melanophore Density more dense patterns to each cluster in the two-step cluster analysis

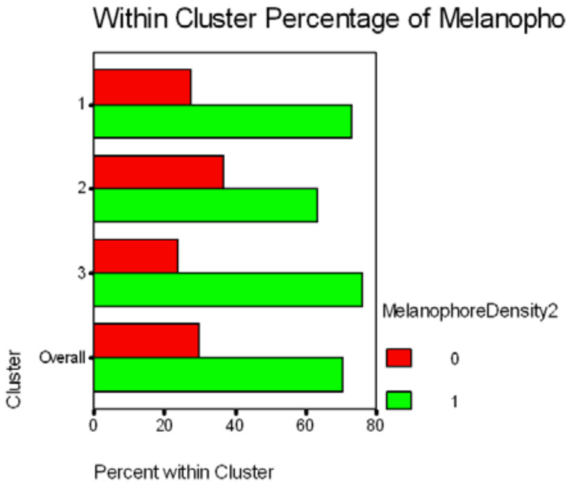


Figure 10: Frequency histogram of each condition of Chin Coloration to each cluster in the two-step cluster analysis

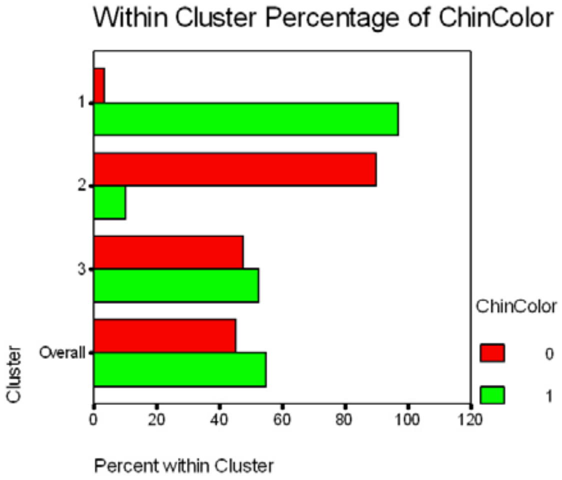


Figure 11: Frequency histogram of each condition of Cheek Coloration to each cluster in the two-step cluster analysis

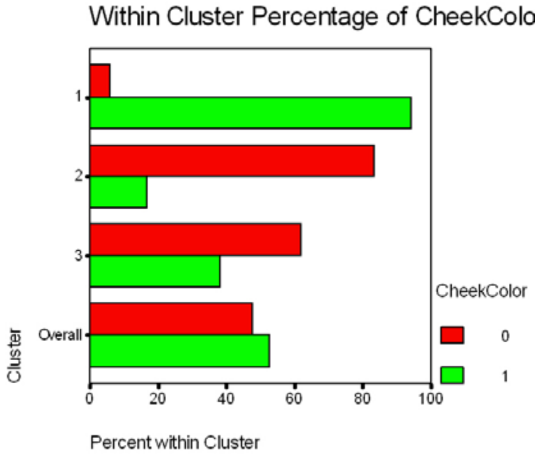


Figure 12: Frequency histogram of each condition of Lateral Line Stripe Obliteration to each cluster in the two-step cluster analysis

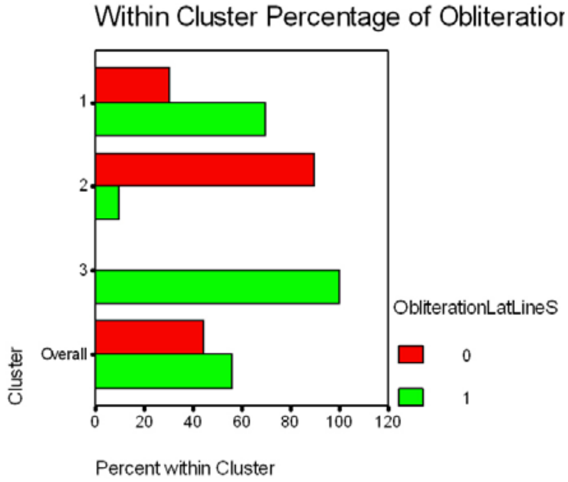


Figure 13: Frequency histogram of each condition of the Amount of Lateral Line Stripe Obliteration to each cluster in the two-step cluster analysis

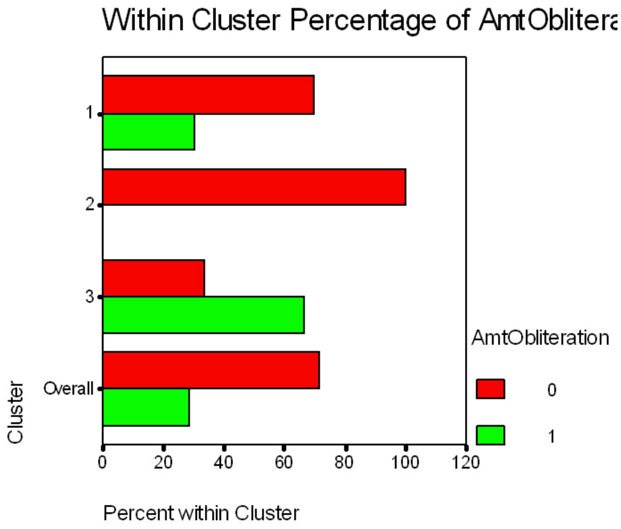


Figure 14: Chi-square analysis of Lateral Line Stripe Width as it relates to each cluster

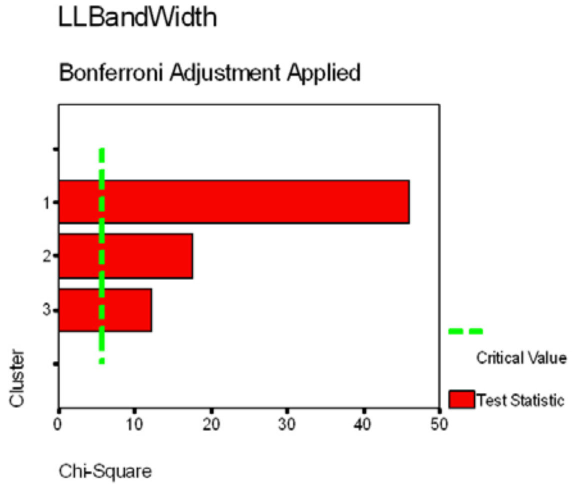


Figure 15: Chi-square analysis of Lateral Line Stripe Color as it relates to each cluster

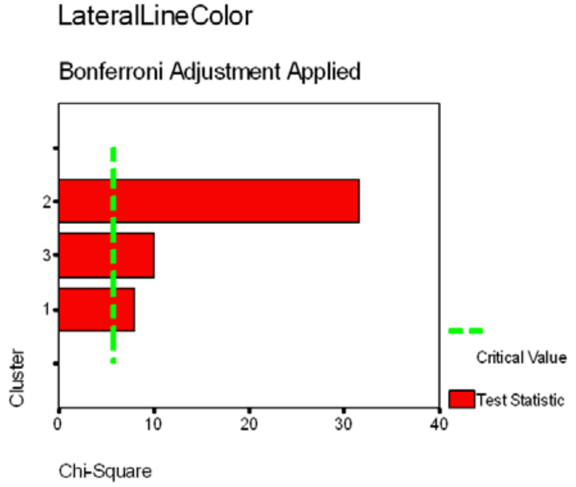


Figure 16: Chi-square analysis of Color Below Lateral Line Stripe Color as it relates to each cluster

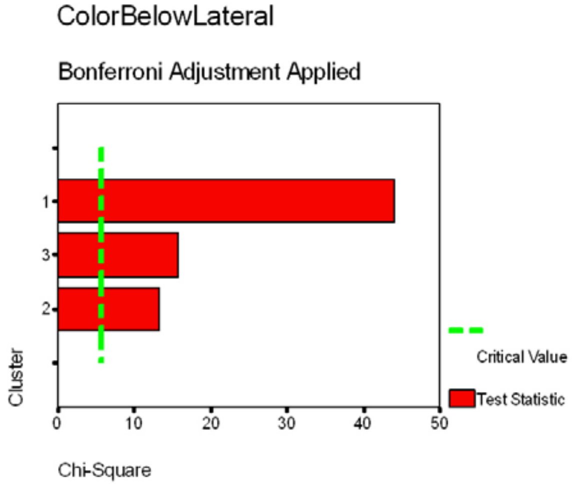


Figure 17: Chi-square analysis of Fin Color as it relates to each cluster

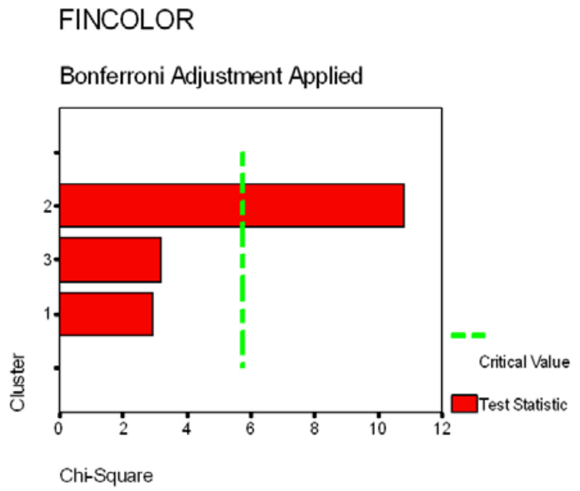


Figure 18: Chi-square analysis of Nuptual Pad Color as it relates to each cluster

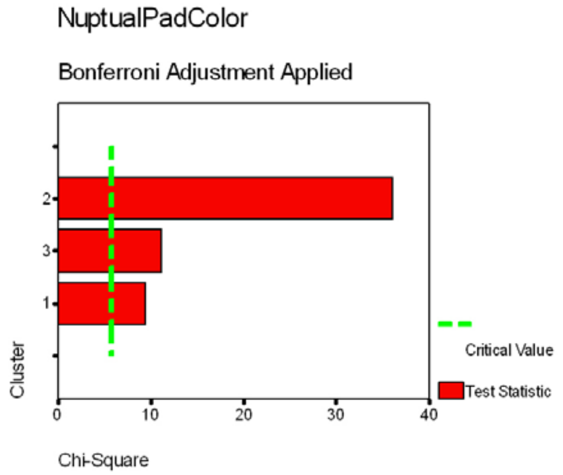


Figure 19: Chi-square analysis of Melanophore presence as it relates to each cluster

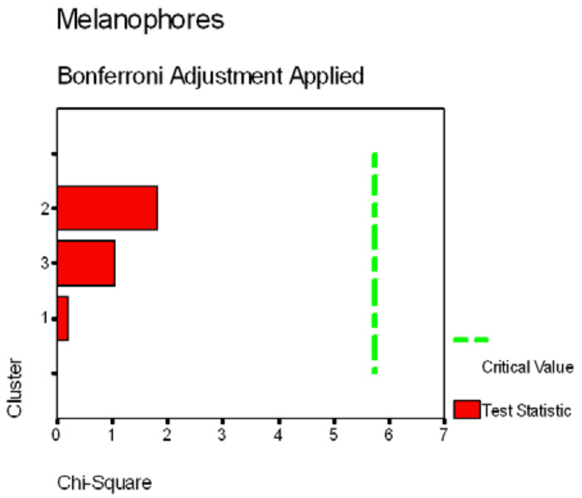


Figure 20: Chi-square analysis of Melanophore Density of Less Dense Patterns as it relates to each cluster

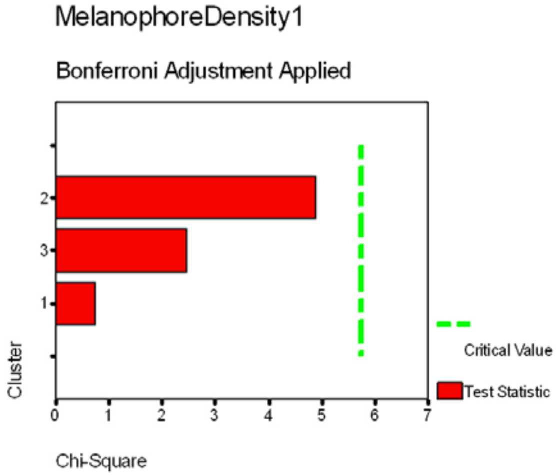


Figure 21: Chi-square analysis of Melanophore Density of More Dense Patterns as it relates to each cluster

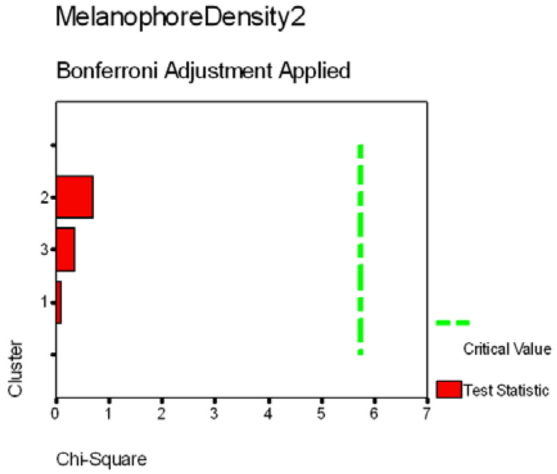


Figure 22: Chi-square analysis of Chin Coloration as it relates to each cluster

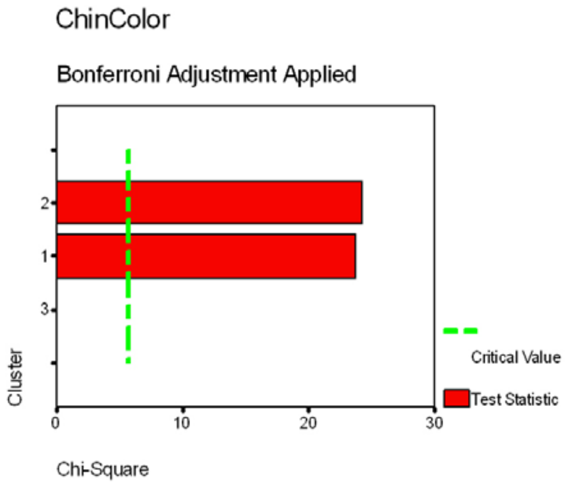


Figure 23: Chi-square analysis of Cheek Coloration as it relates to each cluster

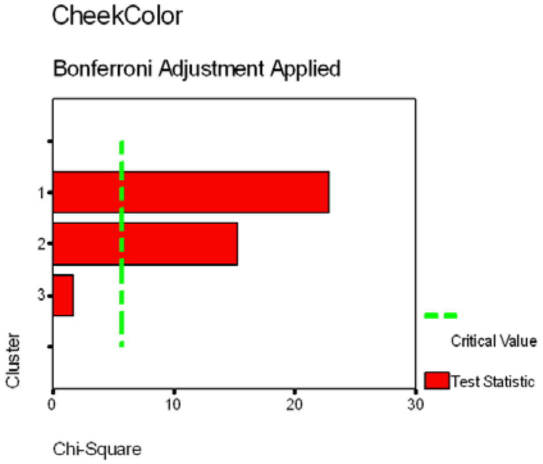


Figure 24: Chi-square analysis of Obliteration of Lateral Line Stripe as it relates to each cluster

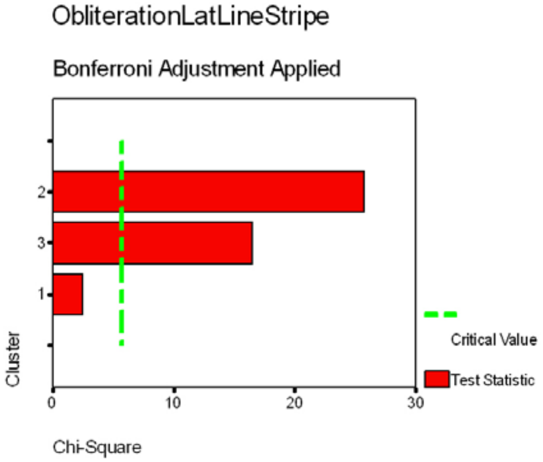


Figure 25: Chi-square analysis of Amount of Obliteration of Lateral Line Stripe as it relates to each cluster

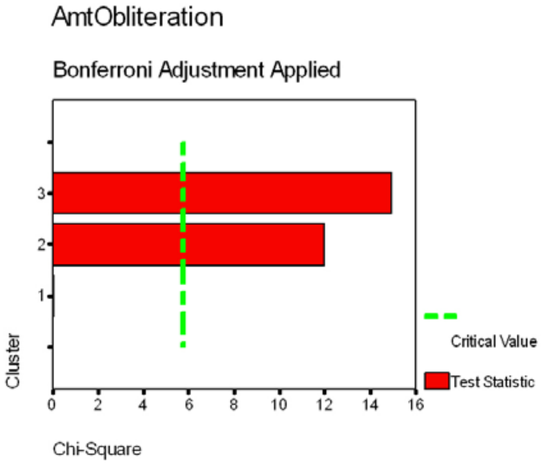


Figure 26: Neighbor-joining phylogram displaying genetic distance in branch length

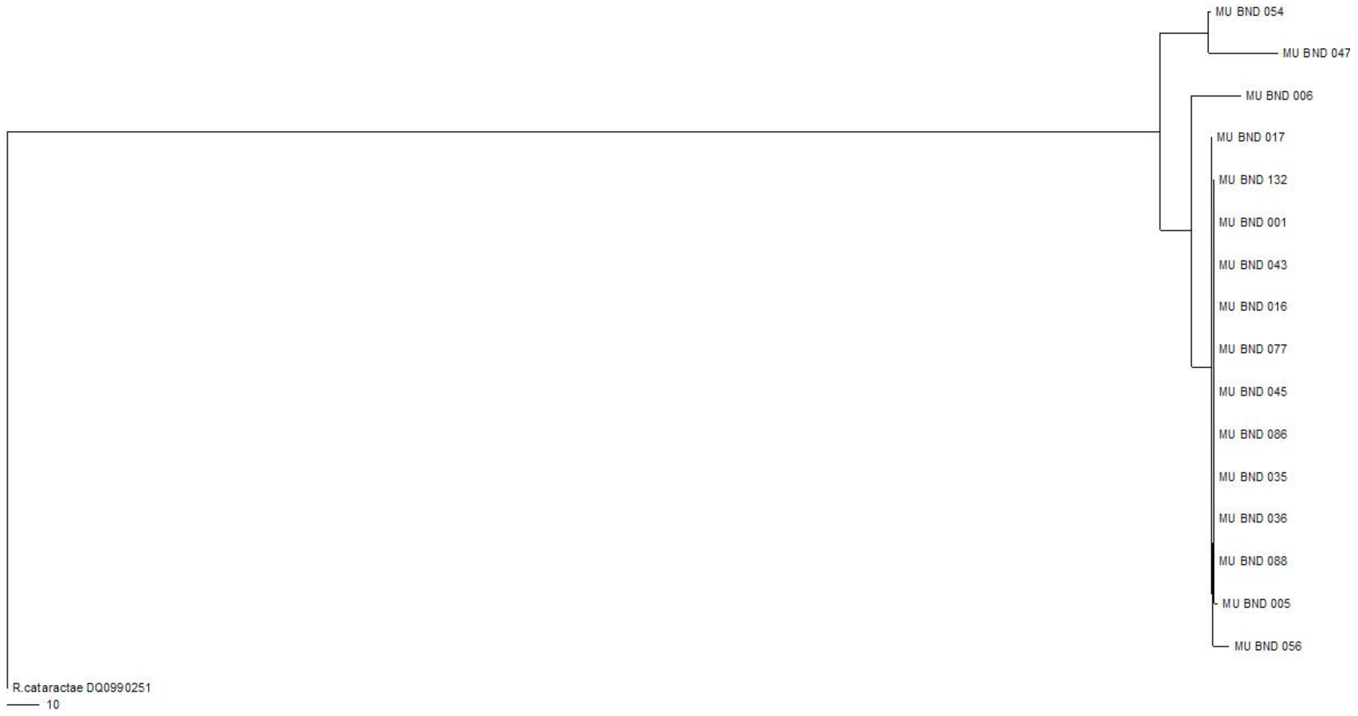


Figure 27: Neighbor-joining rectangular cladogram displaying most likely clustering based on sequence alignment

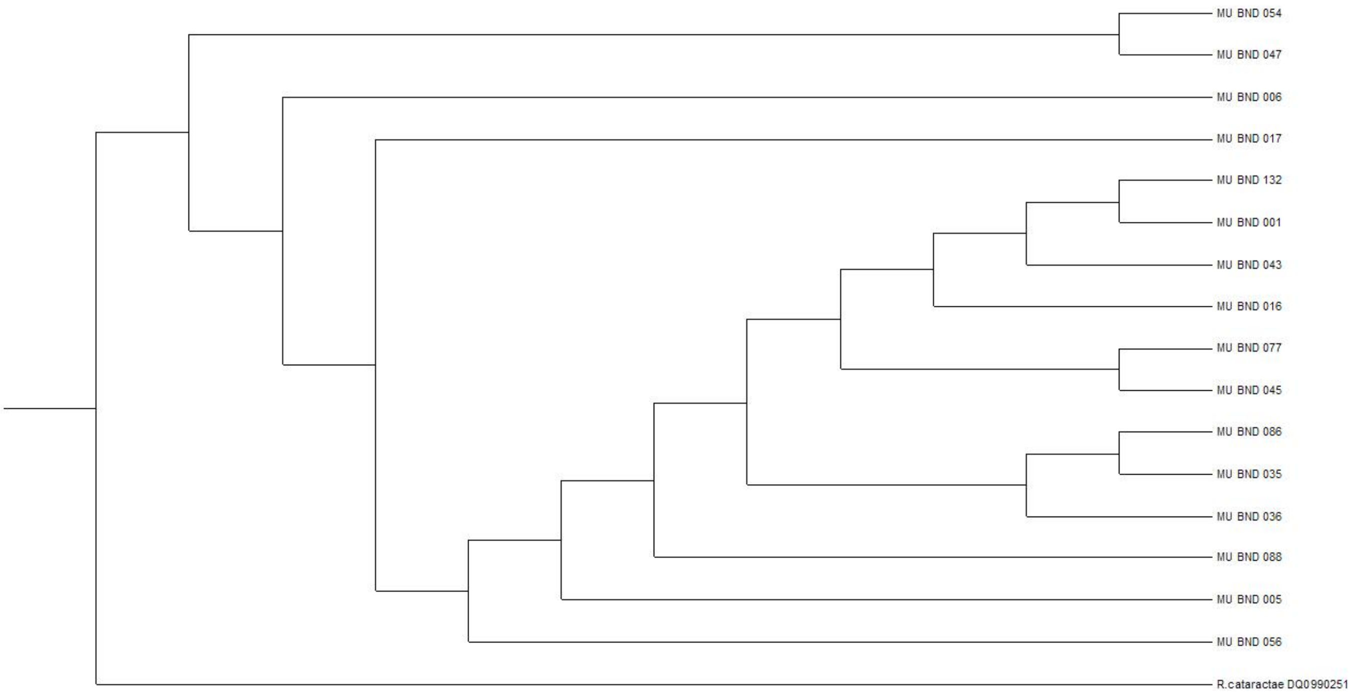


Figure 28: Tamura-Nei phylogram displaying genetic distance in branch length

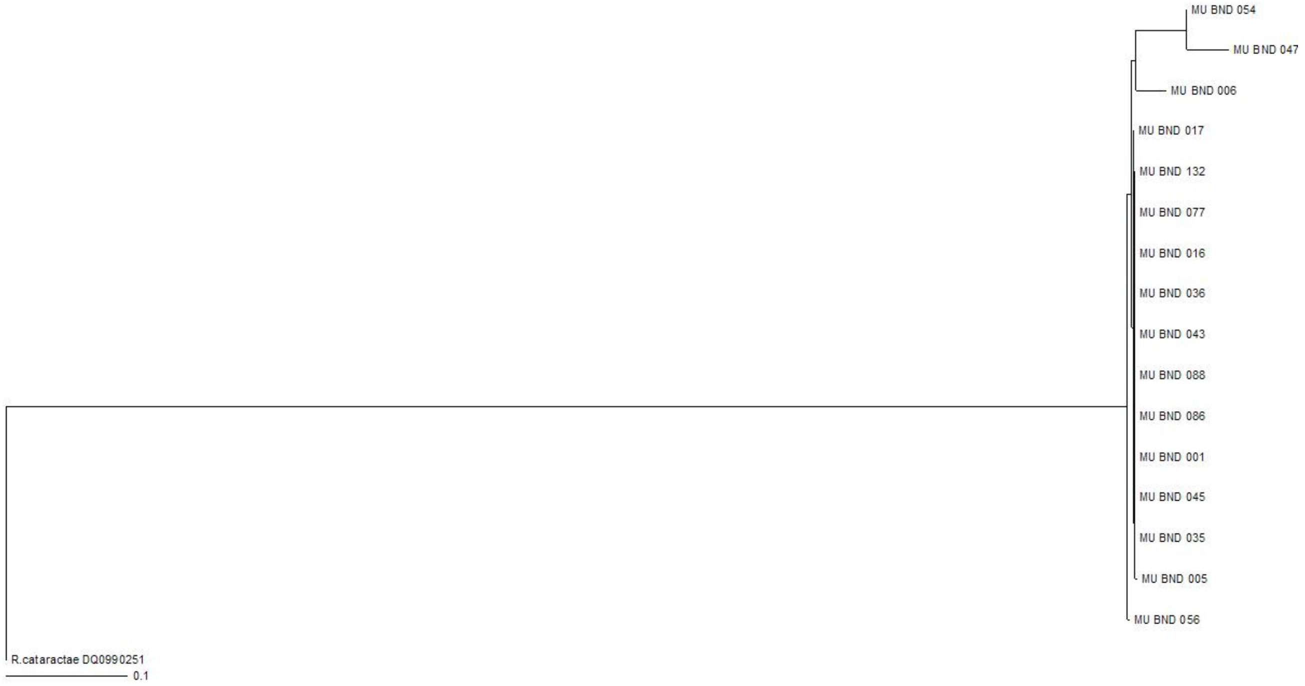


Figure 29: Strict consensus cladogram displaying most likely clustering based on sequence alignment

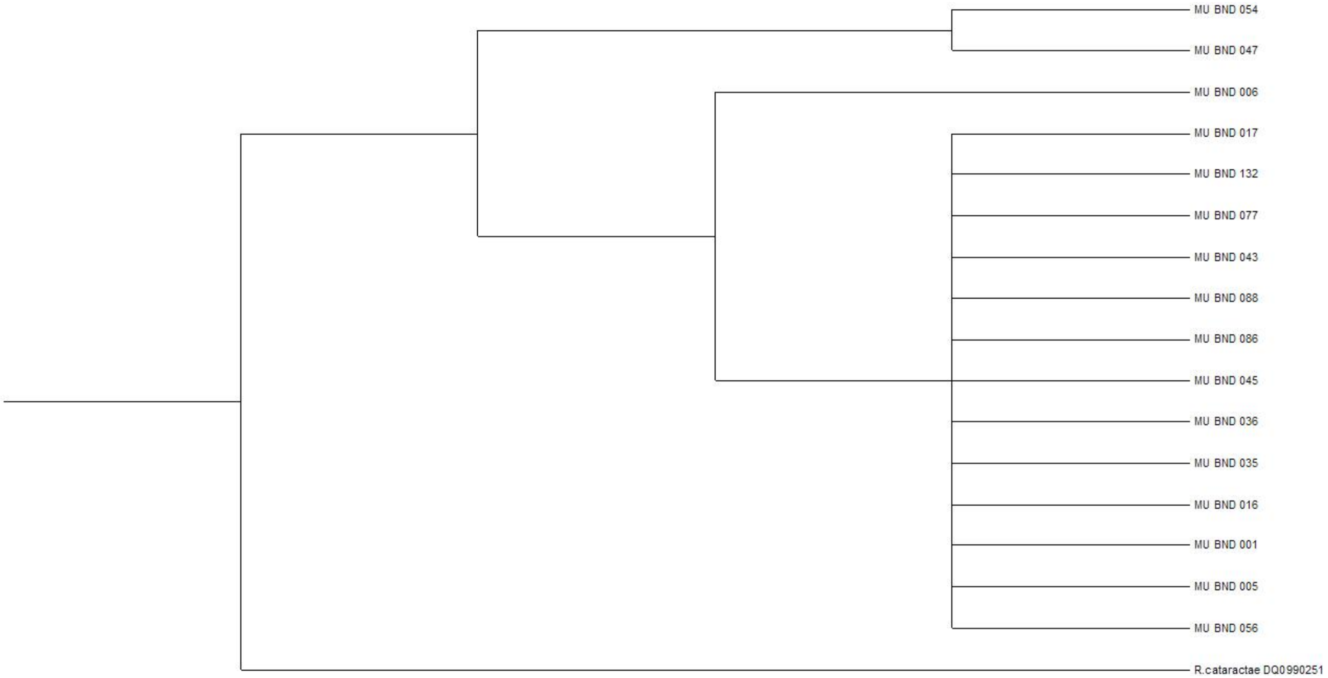
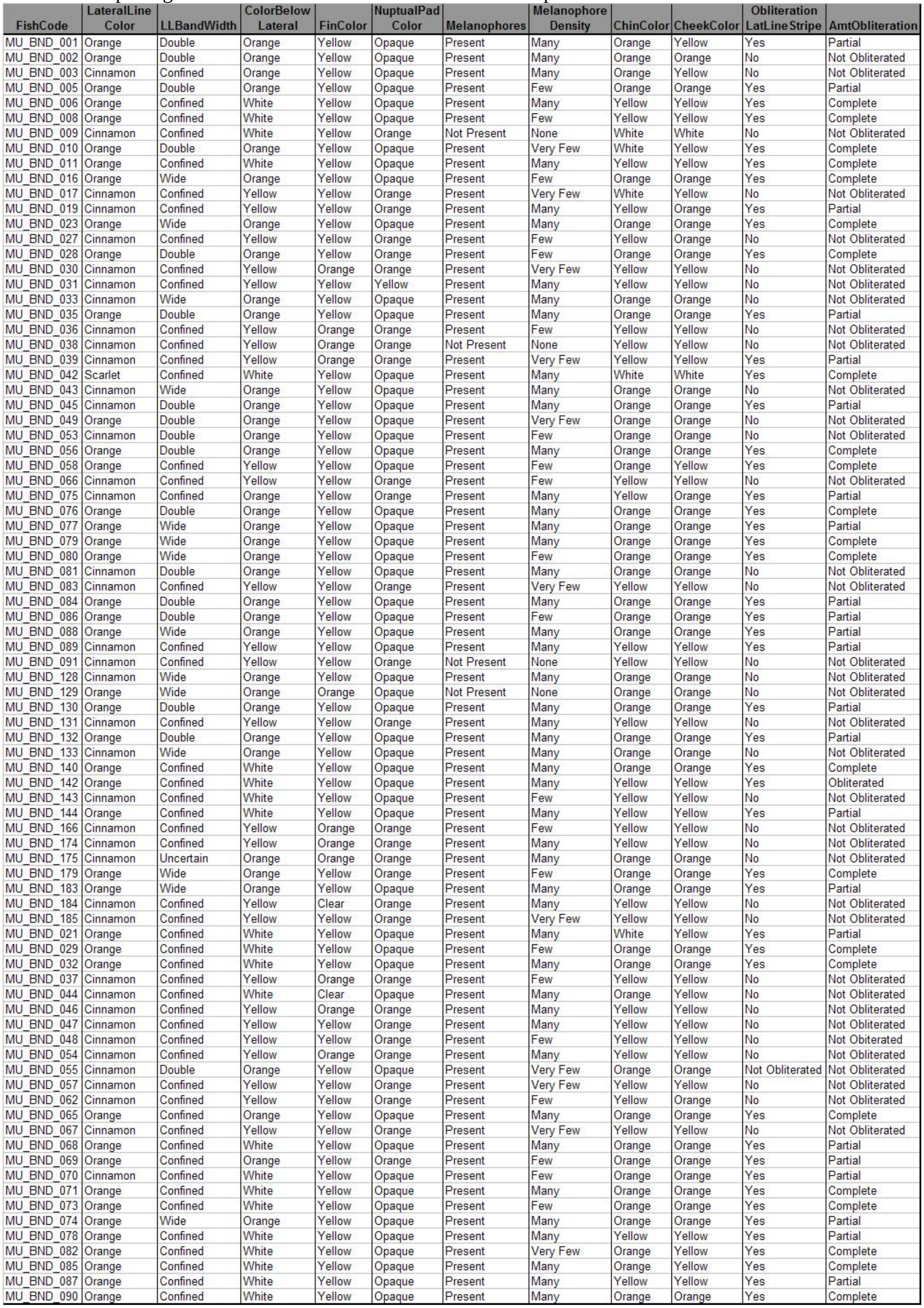
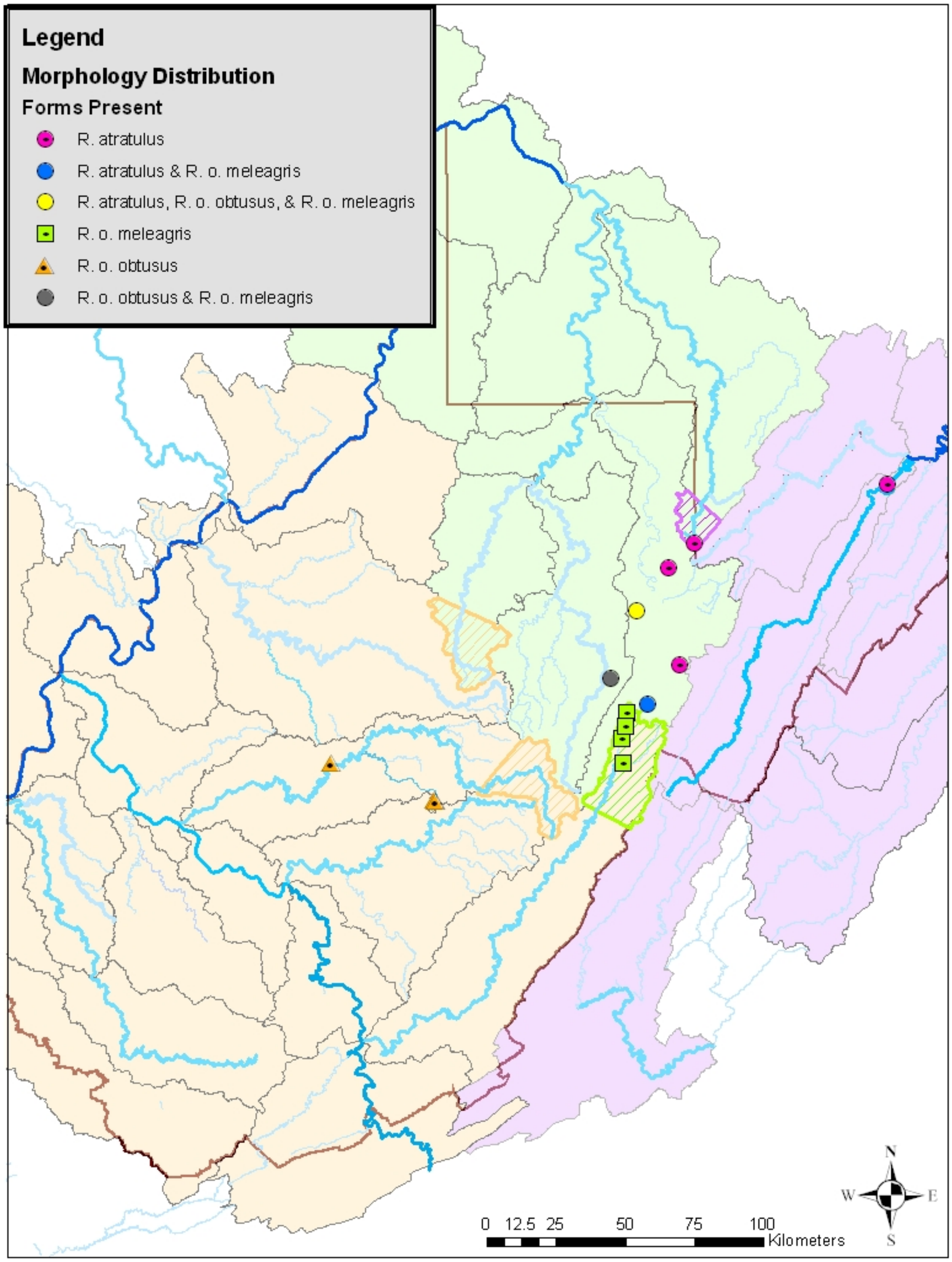


Table 6: Morphological characteristics of all blacknose dace sampled



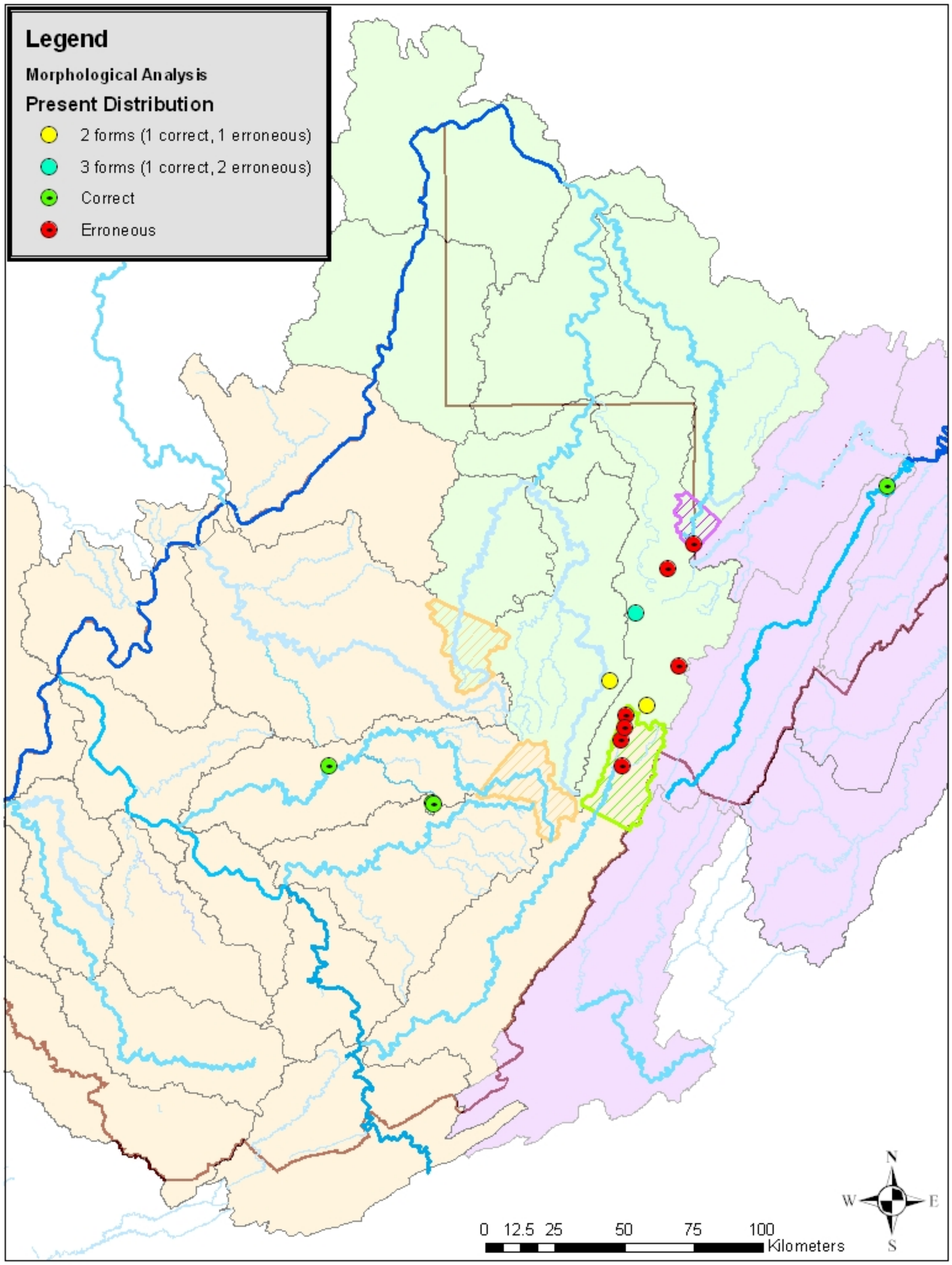
FishCode	LateralLine Color	LLBandWidth	ColorBelow Lateral	FinColor	NuptualPad Color	Melanophores	Melanophore Density	ChinColor	CheekColor	Obliteration LatLine Stripe	AmtObliteration
MU_BND_001	Orange	Double	Orange	Yellow	Opaque	Present	Many	Orange	Yellow	Yes	Partial
MU_BND_002	Orange	Double	Orange	Yellow	Opaque	Present	Many	Orange	Orange	No	Not Obliterated
MU_BND_003	Cinnamon	Confined	Orange	Yellow	Opaque	Present	Many	Orange	Yellow	No	Not Obliterated
MU_BND_005	Orange	Double	Orange	Yellow	Opaque	Present	Few	Orange	Orange	Yes	Partial
MU_BND_006	Orange	Confined	White	Yellow	Opaque	Present	Many	Yellow	Yellow	Yes	Complete
MU_BND_008	Orange	Confined	White	Yellow	Opaque	Present	Few	Yellow	Yellow	Yes	Complete
MU_BND_009	Cinnamon	Confined	White	Yellow	Orange	Not Present	None	White	White	No	Not Obliterated
MU_BND_010	Orange	Double	Orange	Yellow	Opaque	Present	Very Few	White	Yellow	Yes	Complete
MU_BND_011	Orange	Confined	White	Yellow	Opaque	Present	Many	Yellow	Yellow	Yes	Complete
MU_BND_016	Orange	Wide	Orange	Yellow	Opaque	Present	Few	Orange	Orange	Yes	Complete
MU_BND_017	Cinnamon	Confined	Yellow	Yellow	Orange	Present	Very Few	White	Yellow	No	Not Obliterated
MU_BND_019	Cinnamon	Confined	Yellow	Yellow	Orange	Present	Many	Yellow	Orange	Yes	Partial
MU_BND_023	Orange	Wide	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Complete
MU_BND_027	Cinnamon	Confined	Yellow	Yellow	Orange	Present	Few	Yellow	Orange	No	Not Obliterated
MU_BND_028	Orange	Double	Orange	Yellow	Orange	Present	Few	Orange	Orange	Yes	Complete
MU_BND_030	Cinnamon	Confined	Yellow	Orange	Orange	Present	Very Few	Yellow	Yellow	No	Not Obliterated
MU_BND_031	Cinnamon	Confined	Yellow	Yellow	Yellow	Present	Many	Yellow	Yellow	No	Not Obliterated
MU_BND_033	Cinnamon	Wide	Orange	Yellow	Opaque	Present	Many	Orange	Orange	No	Not Obliterated
MU_BND_035	Orange	Double	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Partial
MU_BND_036	Cinnamon	Confined	Yellow	Orange	Orange	Present	Few	Yellow	Yellow	No	Not Obliterated
MU_BND_038	Cinnamon	Confined	Yellow	Orange	Orange	Not Present	None	Yellow	Yellow	No	Not Obliterated
MU_BND_039	Cinnamon	Confined	Yellow	Orange	Orange	Present	Very Few	Yellow	Yellow	Yes	Partial
MU_BND_042	Scarlet	Confined	White	Yellow	Opaque	Present	Many	White	White	Yes	Complete
MU_BND_043	Cinnamon	Wide	Orange	Yellow	Opaque	Present	Many	Orange	Orange	No	Not Obliterated
MU_BND_045	Cinnamon	Double	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Partial
MU_BND_049	Orange	Double	Orange	Yellow	Opaque	Present	Very Few	Orange	Orange	No	Not Obliterated
MU_BND_053	Cinnamon	Double	Orange	Yellow	Opaque	Present	Few	Orange	Orange	No	Not Obliterated
MU_BND_056	Orange	Double	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Complete
MU_BND_058	Orange	Confined	Yellow	Yellow	Opaque	Present	Few	Orange	Yellow	Yes	Complete
MU_BND_066	Cinnamon	Confined	Yellow	Yellow	Orange	Present	Few	Yellow	Yellow	No	Not Obliterated
MU_BND_075	Cinnamon	Confined	Orange	Yellow	Orange	Present	Many	Yellow	Orange	Yes	Partial
MU_BND_076	Orange	Double	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Complete
MU_BND_077	Orange	Wide	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Partial
MU_BND_079	Orange	Wide	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Complete
MU_BND_080	Orange	Wide	Orange	Yellow	Opaque	Present	Few	Orange	Orange	Yes	Complete
MU_BND_081	Cinnamon	Double	Orange	Yellow	Opaque	Present	Many	Orange	Orange	No	Not Obliterated
MU_BND_083	Cinnamon	Confined	Yellow	Yellow	Orange	Present	Very Few	Yellow	Yellow	No	Not Obliterated
MU_BND_084	Orange	Double	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Partial
MU_BND_086	Orange	Double	Orange	Yellow	Opaque	Present	Few	Orange	Orange	Yes	Partial
MU_BND_088	Orange	Wide	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Partial
MU_BND_089	Cinnamon	Confined	Yellow	Yellow	Opaque	Present	Many	Yellow	Yellow	Yes	Partial
MU_BND_091	Cinnamon	Confined	Yellow	Yellow	Orange	Not Present	None	Yellow	Yellow	No	Not Obliterated
MU_BND_128	Cinnamon	Wide	Orange	Yellow	Opaque	Present	Many	Orange	Orange	No	Not Obliterated
MU_BND_129	Orange	Wide	Orange	Orange	Opaque	Not Present	None	Orange	Orange	No	Not Obliterated
MU_BND_130	Orange	Double	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Partial
MU_BND_131	Cinnamon	Confined	Yellow	Yellow	Orange	Present	Many	Yellow	Yellow	No	Not Obliterated
MU_BND_132	Orange	Double	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Partial
MU_BND_133	Cinnamon	Wide	Orange	Yellow	Opaque	Present	Many	Orange	Orange	No	Not Obliterated
MU_BND_140	Orange	Confined	White	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Complete
MU_BND_142	Orange	Confined	White	Yellow	Opaque	Present	Many	Yellow	Yellow	Yes	Obliterated
MU_BND_143	Cinnamon	Confined	White	Yellow	Opaque	Present	Few	Yellow	Yellow	No	Not Obliterated
MU_BND_144	Orange	Confined	White	Yellow	Opaque	Present	Many	Yellow	Yellow	Yes	Partial
MU_BND_166	Cinnamon	Confined	Yellow	Orange	Orange	Present	Few	Yellow	Yellow	No	Not Obliterated
MU_BND_174	Cinnamon	Confined	Yellow	Orange	Orange	Present	Many	Yellow	Yellow	No	Not Obliterated
MU_BND_175	Cinnamon	Uncertain	Orange	Orange	Orange	Present	Many	Orange	Orange	No	Not Obliterated
MU_BND_179	Orange	Wide	Orange	Yellow	Orange	Present	Few	Orange	Orange	Yes	Complete
MU_BND_183	Orange	Wide	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Partial
MU_BND_184	Cinnamon	Confined	Yellow	Clear	Orange	Present	Many	Yellow	Yellow	No	Not Obliterated
MU_BND_185	Cinnamon	Confined	Yellow	Yellow	Orange	Present	Very Few	Yellow	Yellow	No	Not Obliterated
MU_BND_021	Orange	Confined	White	Yellow	Opaque	Present	Many	White	Yellow	Yes	Partial
MU_BND_029	Orange	Confined	White	Yellow	Opaque	Present	Few	Orange	Orange	Yes	Complete
MU_BND_032	Orange	Confined	White	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Complete
MU_BND_037	Cinnamon	Confined	Yellow	Orange	Orange	Present	Few	Yellow	Yellow	No	Not Obliterated
MU_BND_044	Cinnamon	Confined	White	Clear	Opaque	Present	Many	Orange	Yellow	No	Not Obliterated
MU_BND_046	Cinnamon	Confined	Yellow	Orange	Orange	Present	Many	Yellow	Yellow	No	Not Obliterated
MU_BND_047	Cinnamon	Confined	Yellow	Yellow	Orange	Present	Many	Yellow	Yellow	No	Not Obliterated
MU_BND_048	Cinnamon	Confined	Yellow	Yellow	Orange	Present	Few	Yellow	Yellow	No	Not Obiterated
MU_BND_054	Cinnamon	Confined	Yellow	Orange	Orange	Present	Many	Yellow	Yellow	No	Not Obliterated
MU_BND_055	Cinnamon	Double	Orange	Yellow	Opaque	Present	Very Few	Orange	Orange	Not Obliterated	Not Obliterated
MU_BND_057	Cinnamon	Confined	Yellow	Yellow	Orange	Present	Very Few	Yellow	Yellow	No	Not Obliterated
MU_BND_062	Cinnamon	Confined	Yellow	Yellow	Orange	Present	Few	Yellow	Orange	No	Not Obliterated
MU_BND_065	Orange	Confined	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Complete
MU_BND_067	Cinnamon	Confined	Yellow	Yellow	Orange	Present	Very Few	Yellow	Yellow	No	Not Obliterated
MU_BND_068	Orange	Confined	White	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Partial
MU_BND_069	Orange	Confined	Orange	Yellow	Orange	Present	Few	Orange	Orange	Yes	Partial
MU_BND_070	Cinnamon	Confined	White	Yellow	Opaque	Present	Few	Orange	Orange	Yes	Partial
MU_BND_071	Orange	Confined	White	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Complete
MU_BND_073	Orange	Confined	White	Yellow	Opaque	Present	Few	Orange	Orange	Yes	Complete
MU_BND_074	Orange	Wide	Orange	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Partial
MU_BND_078	Orange	Confined	White	Yellow	Opaque	Present	Many	Yellow	Yellow	Yes	Partial
MU_BND_082	Orange	Confined	White	Yellow	Opaque	Present	Very Few	Orange	Yellow	Yes	Complete
MU_BND_085	Orange	Confined	White	Yellow	Opaque	Present	Many	Orange	Yellow	Yes	Complete
MU_BND_087	Orange	Confined	White	Yellow	Opaque	Present	Many	Yellow	Yellow	Yes	Partial
MU_BND_090	Orange	Confined	White	Yellow	Opaque	Present	Many	Orange	Orange	Yes	Complete

Figure 30: Collection sites of nuptual male blacknose dace and subspecies present at each location



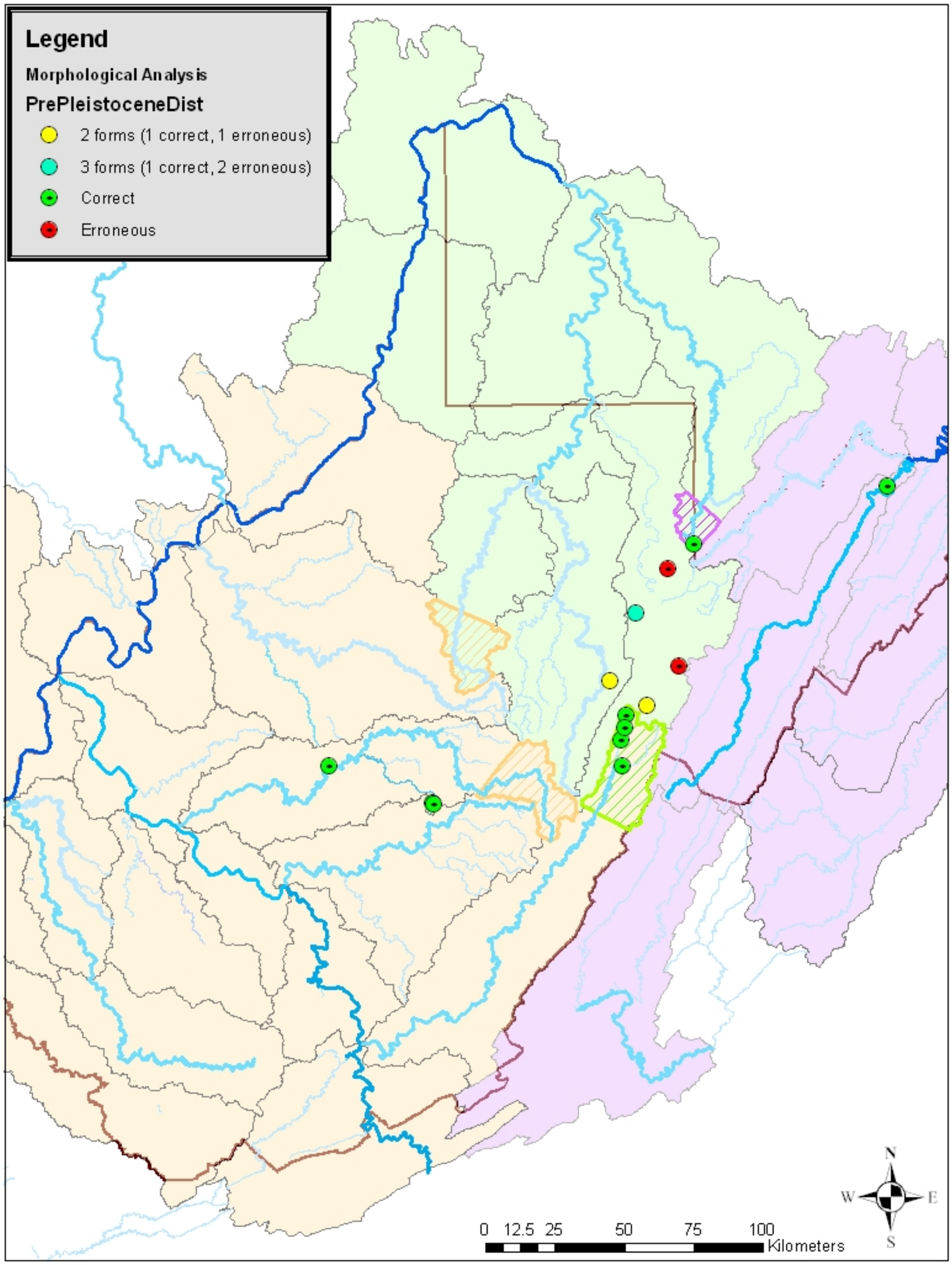
*Shading is representative of literature suggested distribution of each subspecies. Orange depicts range of *R. obtusus obtusus*, Green depicts *R. obtusus meleagris*, and Purple depicts *R. atratulus atratulus* .

Figure 31: Correct and erroneous distribution of subspecies based on individuals collected with present flow regimes.



*Shading is representative of literature suggested distribution of each subspecies. Orange depicts range of *R. obtusus obtusus*, Green depicts *R. obtusus meleagris*, and Purple depicts *R. atratulus atratulus*

Figure 32: Correct and erroneous distribution of subspecies based on individuals collected when stream capture and pre-Pleistocene flow regimes applied



*Shading is representative of literature suggested distribution of each subspecies. Orange depicts range of *R. obtusus obtusus*, Green depicts *R. obtusus meleagris*, and Purple depicts *R. atratulus atratulus*.

Appendix

Appendix A: Multiple sequence alignment of mitochondrial cytochrome *b* sequences of 16 blacknose dace from a large zone of contact in West Virginia (Clustal W v.1.83).

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MU_BND_005      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGACAAGAAGGAAGAGT
MU_BND_056      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
MU_BND_077      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
MU_BND_132      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
MU_BND_043      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
MU_BND_088      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
MU_BND_086      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
MU_BND_045      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
MU_BND_036      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
MU_BND_035      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
MU_BND_016      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
MU_BND_001      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
MU_BND_017      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
MU_BND_006      GCTCATTTCAGTGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGGAGGAAGAGT
MU_BND_054      GCTCATTTCAATGCTTTATTCTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
MU_BND_047      GCTCATTTCAATGCTTTATTTTCTGCCCACCCTGCGAGCGGGGCGAGAAGAAGGAAGAGT
                ********** ********* ************************* *** *********

MU_BND_005      GCGAAGTACAGCACCGACGCGATTTGGCCGATGATGATATATGGGTGTTCTACGGGCATG
MU_BND_056      GCGAAGTACAGCACCGACGCGATTTGCCCGATGATGATATATGGGTCTTCTACGCCCATG
MU_BND_077      GCGAAGTACAGCACCGACGCGATTTGGCCGATGATGATATATGGGTGTTCTACGGGCATG
MU_BND_132      GCGAAGTACAGCACCGACGCGATTTGGCCGATGATGATATATGGGTGTTCTACGGGCATG
MU_BND_043      GCGAAGTACAGCACCGACGCGATTTGGCCGATGATGATATATGGGTGTTCTACGGGCATG
MU_BND_088      GCGAAGTACAGCACCGACGCGATTTGGCCGATGATGATATATGGGTGTTCTACGGGCATG
MU_BND_086      GCGAAGTACAGCACCGACGCGATTTGGCCGATGATGATATATGGGTGTTCTACGGGCATG
MU_BND_045      GCGAAGTACAGCACCGACGCGATTTGGCCGATGATGATATATGGGTGTTCTACGGGCATG
MU_BND_036      GCGAAGTACAGCACCGACGCGATTTGGCCGATGATGATATATGGGTGTTCTACGGGCATG
MU_BND_035      GCGAAGTACAGCACCGACGCGATTTGGCCGATGATGATATATGGGTGTTCTACGGGCATG
MU_BND_016      GCGAAGTACAGCACCGACGCGATTTGGCCGATGATGATATATGGGTGTTCTACGGGCATG
MU_BND_001      GCGAAGTACAGCACCGACGCGATTTGGCCGATGATGATATATGGGTGTTCTACGGGCATG
MU_BND_017      GCGAAGTACAGCACCGACGCGATTTGGCCGATGATGATATATGGGTGTTCTACGGGCATG
MU_BND_006      GCGAAGTATAGCACTGACGCGATTTGGCCGATGATAATATATGGGTGTTCTACGGGCATG
MU_BND_054      GCGAAGTACAGCACCGACGCGATTTGGCCAATGACGATATATGGGTGCTCTACGGGCATG
MU_BND_047      GCGAAGTATAGCACCGACGCGATTTGGCCAATGACGATATATGGGTGCTCTACGGGTATG
                ******** ***** *********** ** ****  **********  ******   ***

MU_BND_005      CCCCCAATTCATGTCAAGATGGCTATATCTGCCACCAGGGTTCAGAACAAGAACTGAGTG
MU_BND_056      CACCCAATTCATGTCAAGATGGCTATATCTGCCACCAGGGTTCAGAACAAGAACTGAGTG
MU_BND_077      CCCCCAATTCATGTCAAGATGGCTATATCTGCCACCAGGGTTCAGAACAAGAACTGAGTG
MU_BND_132      CCCCCAATTCATGTCAAGATGGCTATATCTGCCACCAGGGTTCAGAACAAGAACTGAGTG
MU_BND_043      CCCCCAATTCATGTCAAGATGGCTATATCTGCCACCAGGGTTCAGAACAAGAACTGAGTG
MU_BND_088      CCCCCAATTCATGTCAAGATGGCTATATCTGCCACCAGGGTTCAGAACAAGAACTGAGTG
MU_BND_086      CCCCCAATTCATGTCAAGATGGCTATATCTGCCACCAGGGTTCAGAACAAGAACTGAGTG
MU_BND_045      CCCCCAATTCATGTCAAGATGGCTATATCTGCCACCAGGGTTCAGAACAAGAACTGAGTG
MU_BND_036      CCCCCAATTCATGTCAAGATGGCTATATCTGCCACCAGGGTTCAGAACAAGAACTGAGTG
MU_BND_035      CCCCCAATTCATGTCAAGATGGCTATATCTGCCACCAGGGTTCAGAACAAGAACTGAGTG
MU_BND_016      CCCCCAATTCATGTCAAGATGGCTATATCTGCCACCAGGGTTCAGAACAAGAACTGAGTG
MU_BND_001      CCCCCAATTCATGTCAAGATGGCTATATCTGCCACCAGGGTTCAGAACAAGAACTGAGTG
MU_BND_017      CCCCCAATTCATGTCAAGATGGCTATATCTGCCACCAGGGTTCAGAACAAGAACTGAGTG
MU_BND_006      CCCCCAATTCATGTTAAGATGGCTATATCTGCCACCAGGGTTCAGAATAAGAACTGAGTG
MU_BND_054      CCCCCAATTCACGTCAAGATAGCCATATCTGCCACCAGGGTTCAGAACAAAAACTGAGTG
MU_BND_047      CCTCCGATTCACGTCAAGATAGCCATATCTGCCACGAGGGTTCAGAATAAAAACTGAGTG
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MU_BND_005      ATTGGGCGGAAGGTTAATCCACGCTGTTTTGAGGTGTGTAAGATGGGGACTACCAGTAAC
MU_BND_056      ATTGGGCGGAAGGTTAATCCACGCTGTTTTGAGGTGTGTAAGATGGGGACTACCAGTAAC
MU_BND_077      ATTGGGCGGAAGGTTAATCCACGCTGTTTTGAGGTGTGTAAGATGGGGACTACCAGTAAC
MU_BND_132      ATTGGGCGGAAGGTTAATCCACGCTGTTTTGAGGTGTGTAAGATGGGGACTACCAGTAAC
MU_BND_043      ATTGGGCGGAAGGTTAATCCACGCTGTTTTGAGGTGTGTAAGATGGGGACTACCAGTAAC
MU_BND_088      ATTGGGCGGAAGGTTAATCCACGCTGTTTTGAGGTGTGTAAGATGGGGACTACCAGTAAC
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MU_BND_086      ATTGGGCGGAAGGTTAATCCACGCTGTTTTGAGGTGTGTAAGATGGGGACTACCAGTAAC
MU_BND_045      ATTGGGCGGAAGGTTAATCCACGCTGTTTTGAGGTGTGTAAGATGGGGACTACCAGTAAC
MU_BND_036      ATTGGGCGGAAGGTTAATCCACGCTGTTTTGAGGTGTGTAAGATGGGGACTACCAGTAAC
MU_BND_035      ATTGGGCGGAAGGTTAATCCACGCTGTTTTGAGGTGTGTAAGATGGGGACTACCAGTAAC
MU_BND_016      ATTGGGCGGAAGGTTAATCCACGCTGTTTTGAGGTGTGTAAGATGGGGACTACCAGTAAC
MU_BND_001      ATTGGGCGGAAGGTTAATCCACGCTGTTTTGAGGTGTGTAAGATGGGGACTACCAGTAAC
MU_BND_017      ATTGGGCGGAAGGTTAATCCACGCTGTTTTGAGGTGTGNAAGATGGGGACTACCAGTAAC
MU_BND_006      ATTGGGCGGAAGGTTAATCCGCGTTGTTTTGATGTGTGTAAGATGGGGACTACCAGTAAC
MU_BND_054      ATTGGGCGGAAAGTTAATCCCCGCTGCTTTGAGGTGTGCAAAATGGGGACCACCAATAAC
MU_BND_047      ATTGGGCGGAAAGTTAGTCCCCGTTGCTTTGAGGTGTGCAAAATGGGGACCACTAATAGT
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MU_BND_005      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGATCGTAGA
MU_BND_056      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGATCGTAGA
MU_BND_077      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGATCGTAGA
MU_BND_132      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGATCGTAGA
MU_BND_043      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGATCGTAGA
MU_BND_088      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGATCGTAGA
MU_BND_086      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGATCGTAGA
MU_BND_045      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGATCGTAGA
MU_BND_036      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGATCGTAGA
MU_BND_035      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGATCGTAGA
MU_BND_016      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGATCGTAGA
MU_BND_001      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGATCGTAGA
MU_BND_017      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGATCGTAGA
MU_BND_006      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTTGGAATGGACCGTAGA
MU_BND_054      ACCAGAATGCTAAATAACAATGCCAGGACCCCTCCTAGCTTGTTCGGAATGGATCGTAGA
MU_BND_047      ACTAGGATGCTAAATAATAATGCCAGGACCCCTCCTAGCTTGTTCGGAATGGATCGTAGA
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MU_BND_005      ATGGCGTAGGCAAACAAGAAGTATCACTCTGGTTGGATGTGTGGTGGAGTAACCAGGGGG
MU_BND_056      ATGGCGTAGGCAAACAAGAAGTATCACTCTGGTTGGATGTGTGGTGGAGTAACCAGGGGG
MU_BND_077      ATGGCGTAGGCAAACAAGAAGTATCACTCTGGTTGGATGTGTGGTGGAGTAACCAGGGGG
MU_BND_132      ATGGCGTAGGCAAACAAGAAGTATCACTCTGGTTGGATGTGTGGTGGAGTAACCAGGGGG
MU_BND_043      ATGGCGTAGGCAAACAAGAAGTATCACTCTGGTTGGATGTGTGGTGGAGTAACCAGGGGG
MU_BND_088      ATGGCGTAGGCAAACAAGAAGTATCACTCTGGTTGGATGTGTGGTGGAGTAACCAGGGGG
MU_BND_086      ATGGCGTAGGCAAACAAGAAGTATCACTCTGGTTGGATGTGTGGTGGAGTAACCAGGGGG
MU_BND_045      ATGGCGTAGGCAAACAAGAAGTATCACTCTGGTTGGATGTGTGGTGGAGTAACCAGGGGG
MU_BND_036      ATGGCGTAGGCAAACAAGAAGTATCACTCTGGTTGGATGTGTGGTGGAGTAACCAGGGGG
MU_BND_035      ATGGCGTAGGCAAACAAGAAGTATCACTCTGGTTGGATGTGTGGTGGAGTAACCAGGGGG
MU_BND_016      ATGGCGTAGGCAAACAAGAAGTATCACTCTGGTTGGATGTGTGGTGGAGTAACCAGGGGG
MU_BND_001      ATGGCGTAGGCAAACAAGAAGTATCACTCTGGTTGGATGTGTGGTGGAGTAACCAGGGGG
MU_BND_017      ATGGCGTAGGCAAACAAGAAGTATCACTCTGGNTGGATGTGTGGTGGAGTAACCAGGGGG
MU_BND_006      ATGGCGTAGGCAAACAAGAAGTATCACTCCGGTTGGATGTGTGGTGGAGTAACCAGGGGA
MU_BND_054      ATAGCGTAGGCAAACAAGAAGTATCACTCCGGCTGGATGTGCGGTGGAGTAACCAGGGGG
MU_BND_047      ATAGCGTAGGCAAACAAGAAGTATCACTCCGGCTGGATGTGCGGTGGAGTAACCAGGGGG
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MU_BND_005      TTTGCTGGGGTAAAATTCTCTGGGTCACCTAGAAGGGTGGGGGAGAACAACGTTAGGGAT
MU_BND_056      TTTGCTGGGGTAAAATTCTCTGGGTCACCTAGAAGGGTGGGGGAGAACAACGTTAGGGAT
MU_BND_077      TTTGCTGGGGTAAAATTCTCTGGGTCACCTAGAAGGGTGGGGGAGAACAACGTTAGGGAT
MU_BND_132      TTTGCTGGGGTAAAATTCTCTGGGTCACCTAGAAGGGTGGGGGAGAACAACGTTAGGGAT
MU_BND_043      TTTGCTGGGGTAAAATTCTCTGGGTCACCTAGAAGGGTGGGGGAGAACAACGTTAGGGAT
MU_BND_088      TTTGCTGGGGTAAAATTCTCTGGGTCACCTAGAAGGGTGGGGGAGAACAACGTTAGGGAT
MU_BND_086      TTTGCTGGGGTAAAATTCTCTGGGTCACCTAGAAGGGTGGGGGAGAACAACGTTAGGGAT
MU_BND_045      TTTGCTGGGGTAAAATTCTCTGGGTCACCTAGAAGGGTGGGGGAGAACAACGTTAGGGAT
MU_BND_036      TTTGCTGGGGTAAAATTCTCTGGGTCACCTAGAAGGGTGGGGGAGAACAACGTTAGGGAT
MU_BND_035      TTTGCTGGGGTAAAATTCTCTGGGTCACCTAGAAGGGTGGGGGAGAACAACGTTAGGGAT
MU_BND_016      TTTGCTGGGGTAAAATTCTCTGGGTCACCTAGAAGGGTGGGGGAGAACAACGTTAGGGAT
MU_BND_001      TTTGCTGGGGTAAAATTCTCTGGGTCACCTAGAAGGGTGGGGGAGAACAACGTTAGGGAT
MU_BND_017      TTTGCTGGGGTAAAATTCTCTGGGTCACCNAGAAGGGTGGGGGAGAACAACGTTAGGGAT
MU_BND_006      TTTGCTGGGGTGAAATTCTCTGGGTCGCCCAGAAGGGTGGGGGAGAACAACGTTAGGGCT
MU_BND_054      TTCGCTGGGGTAAAATTCTCTGGGTCACCCAGAAGGGTGGGGGAGAACAACGTTAGGGAT
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MU_BND_047      TTCGCTGGGGTGAAATTCTCTGGGTCACCCAGAAGGGTGGGGGAGAACAATGTTAGGGAT
                ** ******** ************** ** ******************** ******* *

MU_BND_005      GTGAGAGCTAGTAGTATTAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_056      GTGAGAGCTAGTAGTATTAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_077      GTGAGAGCTAGTAGTATTAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_132      GTGAGAGCTAGTAGTATTAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_043      GTGAGAGCTAGTAGTATTAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_088      GTGAGAGCTAGTAGTATTAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_086      GTGAGAGCTAGTAGTATTAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_045      GTGAGAGCTAGTAGTATTAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_036      GTGAGAGCTAGTAGTATTAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_035      GTGAGAGCTAGTAGTATTAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_016      GTGAGAGCTAGTAGTATTAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_001      GTGAGAGCTAGTAGTATTAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_017      GTGAGAGCTAGTAGTATTAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_006      GTGAGAGCTAGTAGTATGAGTACAAAGCCAAGGAGGTCCTTATATGAGAAGTATGGTGG
MU_BND_054      GTGAGAGCCAATAGTATTAGTACAAAGCCAAGGAGGTCTTTATATGAGAAGTATGGTGG
MU_BND_047      GTGAGAGCCAATAGTATTAGTACAAAGCCGAGGAGGTCTTTATATGAGAAGTATGGTGG
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MU_BND_005      AAAGAGATTTTATCCGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_056      AAAGAGATTTTATCCGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_077      AAAGAGATTTTATCCGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_132      AAAGAGATTTTATCCGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_043      AAAGAGATTTTATCCGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_088      AAAGAGATTTTATCCGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_086      AAAGAGATTTTATCCGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_045      AAAGAGATTTTATCCGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_036      AAAGAGATTTTATCCGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_035      AAAGAGATTTTATCCGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_016      AAAGAGATTTTATCCGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_001      AAAGAGATTTTATCCGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_017      AAAGAGATTTTATCCGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_006      AAAGAGATTTTATCTGCGTCGGAGTTTAGCCCGGCAGGGTTGTTTGACCCGGTCTCGTGA
MU_BND_054      AAAGAGATTTTATCCGCGTCGGAGTTTAACCCGGCAGGGTTATTCGACCCGGTCTCGTGA
MU_BND_047      AAAGAGATTTTATCTGCGTCGGAGTTTAACCCGGCAGGGTTATTCGATCCGGTCTCGTGA
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MU_BND_005      AGAAATAGTAAATGCAGGACTGTTGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_056      AGAAATAGTAAATGCAGGACTGTTGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_077      AGAAATAGTAAATGCAGGACTGTTGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_132      AGAAATAGTAAATGCAGGACTGTTGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_043      AGAAATAGTAAATGCAGGACTGTTGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_088      AGAAATAGTAAATGCAGGACTGTTGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_086      AGAAATAGTAAATGCAGGACTGTTGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_045      AGAAATAGTAAATGCAGGACTGTTGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_036      AGAAATAGTAAATGCAGGACTGTTGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_035      AGAAATAGTAAATGCAGGACTGTTGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_016      AGAAATAGTAAATGCAGGACTGTTGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_001      AGAAATAGTAAATGCAGGACTGTTGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_017      AGAAATAGTAAATGCAGGACTGTTGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_006      AGAAATAATAAATGCAGGACTGTTGCACCGGCGATAACGAACGGGAATAAGAAGTGAAAG
MU_BND_054      AGAAATAGTAAATGCAGGACGGTCGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
MU_BND_047      AGAAATAGTAAATGGAGGACGGTCGCACCGGCGATAACGAATGGGAATAAGAAGTGAAAG
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MU_BND_005      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_056      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_077      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_132      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_043      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_088      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
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MU_BND_086      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_045      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_036      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_035      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_016      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_001      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_017      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_006      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_054      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
MU_BND_047      GCGAAGAATCGTGTTAACGTTGCGTTATCTACTGAAAAGCCACCTCAAATCCACTGGACA
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MU_BND_005      AGGGTGTCTCCCATATA
MU_BND_056      AGGGTGTCTCCCATATA
MU_BND_077      AGGGTGTCTCCCATATA
MU_BND_132      AGGGTGTCTCCCATATA
MU_BND_043      AGGGTGTCTCCCATATA
MU_BND_088      AGGGTGTCTCCCATATA
MU_BND_086      AGGGTGTCTCCCATATA
MU_BND_045      AGGGTGTCTCCCATATA
MU_BND_036      AGGGTGTCTCCCATATA
MU_BND_035      AGGGTGTCTCCCATATA
MU_BND_016      AGGGTGTCTCCCATATA
MU_BND_001      AGGGTGTCTCCCATATA
MU_BND_017      AGGGTGTCTCCCATATA
MU_BND_006      AGGGTGTCTCCCATATA
MU_BND_054      AGGGTGTCGCCCATATA
MU_BND_047      AGGGTGTCGCCCATATA
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