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Genetic and Morphological Diversity Along Altitudinal Gradients in the Genus *Rhoadsia* (Teleostei: Characidae: Rhoadsiinae)

A Thesis Presented in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

By

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November 2015

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Acknowledgements

The completion of this thesis would not have been possible without the support and hard. work of many of my peers, collaborators, and friends. First and foremost I would like to thank my graduate advisor Dr. Windsor Aguirre who supported me through all the ups and downs of both field and lab work on this project. His patience and passion for research is both inspiring to me personally and indispensable to the success of this project. I would also like to thank him for the opportunity to join his field crew in the collection of samples in Ecuador, he was endlessly patient, even after three flat tires. The experience of gaining first-hand knowledge of the system and collecting field data provided me with a learning experience that could not be replaced by any book, it is an education that will influence and provide perspective for the rest of my career in Biology.

I would like to extend my deepest gratitude to all those who helped me in the field, including Virginia Shervette, Ronald Navarrete Amaya, Fredy Nugra Salazar, Jonathan Valdivezo Rivera, and again Windsor Aguirre. Without their tireless assistance despite long hours, challenging collections, sunburns, and language barriers my project could not have been a success. I would also like to thank the Aguirre family for their generous hospitality hosting our field crew and gear during our time in Guayaquil as well as the generous loan of their vehicle for field work.

I would also like to thank my faculty committee members, Dr. Jason Bystriansky and Dr. William Gilliland for their advice and influence throughout my research project. They provided kind support and encouragement and both added unique expertise which contributed considerably to my ability to complete this project. I would also like to thank our Graduate Student Director, Dr. Margaret Silliker for her support and guidance.

I would also like to extend thanks to the Field Museum of Chicago, particularly the Pritzker Genetics Lab and Kevin Feldheim. While much of the work done with them did not end up in my thesis they generously extended the use of their equipment, materials, and expertise, and their generosity was greatly appreciated.

I would like to thank the DePaul Biology Graduate Students Class of 2015 who encouraged me and through editing and many helpful suggestions regarding my project proposal and onwards. Many of them, especially my lab-mate Daniel Reyes, provided help to me throughout my time here in a variety of ways which I am grateful for. I would also like to thank the Department of Biological Sciences for their support and the opportunity to study here as well as their funding through two Graduate Research Opportunity Grants.

I would also like to thank Sigma-Xi for their funding through the Grants in Aid of Research program.

I would also like to thank my wonderful parents Jamie and Stephen, and my brother Sam for their unquestioning support. I could not have finished my project without their constant encouragement and perspective.

Abstract

The Neotropics have the highest fish species diversity of any area in the world, with some experts estimating that as many as 4000 species of fishes are present in freshwater ecosystems. Elevational gradients are partially responsible for this diversity due to the rapidly changing ecological conditions associated with changes in altitude in rivers. One area where elevational gradients are particularly important is Western Ecuador; this region forms part of a biodiversity hotspot extending south from Panama along the western side of the Andes. Fish species diversity is relatively low because of the small size of most of the river drainages and drier conditions but rates of endemism are high. The land area west of the Andes in Ecuador is relatively small, therefore the rivers run short distances between the Andes Mountains and the Pacific Ocean, resulting in steep elevational gradients and allowing for increased interactions between fish species adapted to different elevations. Ecuador is also under increasing threat from anthropogenic factors. Despite this, relatively little is known about the ecology, evolution or status of most of the fishes there, even some of the most abundant fishes such as the members of the genus *Rhoadsia*. There are two species presently recognized as valid in the genus *Rhoadsia*, *R. altipinna* and *R. minor.* They are both relatively small fish with rhomboidal bodies. They are also highly sexually dimorphic, with males being much larger than females and displaying bright breeding colors and long fin rays. *Rhoadsia minor* and *R. altipinna* are differentiated by their size and body depth, the latter typically being expressed as a fineness ratio (FR). *Rhoadsia altipinna* is common in low altitude waters of southwestern Ecuador and has FR values between 2.25-2.5. *Rhoadsia minor* was described from high altitude waters (>1200m) in the Esmeraldas drainage in northwestern Ecuador and has a more elongate body with FR values between 2.8-3.0.

However, previous research has questioned the validity of *R. minor* as a separate species and demonstrated that over a low elevational gradient, FR increases with increasing elevation.

In this study, I examine genetic and morphological divergence of *Rhoadsia* sp. along elevational gradients in two rivers of western Ecuador to determine whether a broader geographic sampling and combined analysis of morphological and molecular data support the recognition of two species in the genus. The two rivers sampled were the Esmeraldas River drainage in northwestern Ecuador and the Jubones River in southwestern Ecuador. A total of 116 specimens were sequenced for the mitochondrial Cytochrome Oxidase I (COI) gene (the DNA barcoding gene) and 278 individuals were used in a geometric morphometric analysis of body shape variation. Based on evaluation of the patterns of genetic variation, pairwise F_{ST} values, AMOVA and clustering analysis, the genetic data provide little support for the recognition of two distinct species. Genetic diversity was relatively low across all samples, haplotypes were generally shared between rivers, and there was no monophyletic sample or group of samples corresponding to the species described as *R. minor*. Haplotype frequencies did differ significantly among river drainages, with similar amounts of genetic variation segregating among individuals within a site and among sites in different rivers. However, these differences were more consistent with population genetic structuring within a single species then with the occurrence of two species. Morphological data show a strong relationship between elevation and morphology, with fish becoming more elongate with increase in elevation across both drainages, although the patterns differ between rivers and body elongation with elevation is much stronger in the Esmeraldas River. Whether this change in body shape has a genetic basis or is due to phenotypic plasticity is not known and an important direction for future research. The description

of *R. minor* was likely a mistake attributable to the limited number of samples available at the time that the species were described.

Background

Molecular Markers and the Inference of Evolutionary History

Molecular Markers are a relatively new tool in the historical context of Biology, but they come from a long line of research looking for tools to address questions beyond the scope of traditional methods of observation. Since the discovery of the structure of DNA (Watson and Crick 1953), DNA has been thought of as an objective tool for answering fundamental questions in biology. Few areas of biology have advanced more rapidly in the last few decades than molecular biology. A better understanding of the genome (Lander 2011) and the development of new molecular tools (e.g., Saiki et al. 1988, Zhang et al. 2011) have contributed to an explosion in the use of DNA data for studying the evolutionary history of populations (Allendorf et al. 2001, Helyar et al. 2011, Ellegren, 2014). DNA can often contribute to answering some questions better than through the use of more traditional methods alone, such as addressing taxonomic concerns, studies of evolution, estimation of demographic parameters, and population dynamics (Allendorf et al. 2010). DNA can also answer some questions we could never have asked before, such as directly measuring a population's inbreeding risk (Haber et al. 1980, Ralls et al. 1988), identifying cryptic species (e.g., Quattro et al. 2006, Hyde et al. 2008), and determining the genetic viability of populations (e.g., Rieman and Allendorf 2001, Allendorf and Ryman 2002). Each of these questions requires different data that can be collected using different types of molecular markers. Molecular markers vary in location in the genome, variability, and cost, which makes different types of markers useful for different types of studies (Parker et al. 1998, Schlötterer 2004).

The popularization of molecular markers began with the use of allozymes (variants of proteins) as an indirect representation of genetic variation (Lewontin and Hubby 1966). They

were highly variable and uncovered both the high level of diversity which exists within most natural populations, as well as the fact that most mutations are likely neutral (Parker et al. 1998). With the invention of PCR (Saiki et al. 1988), DNA became much easier to work with and other molecular markers gained popularity. There have been a variety of molecular markers that have been developed since the fading of allozymes, and each one is used in a different context (Parker et al. 1998). The most popular PCR based DNA marker for addressing questions in population genetics has been the microsatellite (Schlötterer 2004). Microsatellites are short tandemly repeating DNA segments with a high mutation rate in non-coding regions of the genome. These markers transformed population biology by allowing the efficient identification of population structure and rates of gene flow (Schlötterer 2004). More recently, single nucleotide polymorphisms (SNPs) and other genomic approaches have been increasing in popularity as they become easier and cheaper to employ and because they allow a more comprehensive sampling of the genome (Allendorf 2010). This new approach has allowed for researchers to not only examine population structure and gene flow, but also identify candidate loci under natural selection. These loci can then be sequenced and further studied to understand the evolution of specific genes (Allendorf et al. 2010). However, neutral nuclear molecular markers have similar drawbacks; they are generally too variable to identify taxonomic resolution above the population level. The high level of diversity can separate populations well but the markers can either become obfuscated by reverse mutations (the process by which a nucleotide mutates twice, the second time restoring the original nucleotide), or simply no longer be in the same place in the genome as in the case of microsatellites. Conversely protein coding nuclear markers can be too conserved (Allendorf 2010). Therefore, the most popular molecular markers for taxonomic

resolution have historically been those in the mitochondria, which accumulate mutations at a much slower rate than microsatellites but faster than nuclear genes (Hebert et al. 2003).

Several different mitochondrial genes have been used, and any could be used for any given taxonomically study. However the most frequently used are Cytochrome b and Cytochrome Oxidase I (Hebert et al. 2001). Cytochrome Oxidase I (COI), also known as the 'barcoding gene', has been proposed as a standard in taxonomic studies (Hebert et al. 2001, Ward 2003). In the mitochondria, COI codes for a protein which is responsible for hydrolyzing water in the respiratory chain. Therefore, it is highly conserved because it performs an essential function and there is strong selection against negative mutations. As a consequence, there tends to be little variation in this gene within species (Ballard and Whitlock 2004). However, because it is a mitochondrial gene, mutations accumulate more quickly than for nuclear genes, such that different species in the same genus will typically differ for this gene (Hebert et al. 2001). Additionally, it is present in all eukaryotic organisms. COI is highly useful due to the large data set which already exists for comparison as well as accessible primer sets. The existing data sets allow new species to immediately be put in context of an enormous wealth of data and the primers make the acquisition of new data efficient (Hebert et al. 2001). COI has been used with increasing frequency and as it has continued to be demonstrated to identify species reliably (e.g, Jung et al. 2011, Knebelsberger et al. 2014, Pentinsaari et al. 2014). Although a majority of researchers agree on which gene to use to compare species, few agree on the specific rules that should be employed for differentiating two species from one another. This disagreement is known as the species problem (Mayden 1997).

There are currently 24 recognized species concepts, each one representing a different way of defining species (Hey 2006). While they all have a good deal in common, they vary

considerably in their approach to identifying species. One of the underlying issues is that the actual process of speciation is complicated and exists on a continuum of stages, rather than a discontinuous shift between forms. Recent studies indicate that speciation can take place in a series of steps: first some reproductive separation, followed by shifts to create some genetic differentiation, then an increase in differentiation which leads to difficult but not impossible gene flow between the two groups, and lastly complete and irreversible genetic differentiation (Hendry et al. 2009). If we were to take a snapshot in time at any one of these four steps, they would meet and fail various species concepts. This would in part be due to the possibility that any one of these steps may or may not be accompanied by phenotypic divergence, but they certainly would be accompanied by some genetic divergence. How much varies wildly and is greatly influenced by the details of the specific species, especially the time elapsed and the selective pressures involved.

A measure of sequence divergence is traditionally used to determine whether two species are in fact two distinct species, however exactly how much divergence is required is another contested point. The interpretation of this is still a matter of debate as some scientists argue that the biologic, geographic, or morphological differentiation is just as, if not more, important than any DNA variability (Mayden 1997).

Geometric Morphometrics and the Inference of Divergence in Form

Geometric morphometric methods have been used since the 1990s as a way to quantify the difference in body shape with measures that go beyond simple caliper measurements (Rohlf and Marcus 1993, Adams et al. 2003). Traditional morphometrics collected data by a series of linear measures, e.g., length of the fins, body depth and length, etc., and used these as ostensibly

independent measures which combine to make a complete data set (Reyment et al. 1984). However some of the biggest drawbacks were that many of the usual lengths were correlated to one another and that the results were not easily related to specific shape change, so differences between individuals were detectable but these could not be translated back to relevant shape changes or quantified as accurately (Rohlf and Marcus 1993). Geometric morphometrics improves upon this because with the use of two or three-dimensional landmarks, shape can be considered as a whole with the geometric configuration of the landmarks conserved and each feature of the organism is mathematically independent of others. While PCA and CVAs have been used with traditional morphometrics, the results were simply interpreted as shape has or has not changed. With geometric morphometrics, coordinates can be related to relevant shape results and these can be precisely quantified and also visualized (Adams et al. 2003). Geometric morphometrics also allows for a more straightforward treatment of size because it allows for the calculation of a multidimensional measure of size, centroid size. Centroid size, calculated as the square root of the summed squared distances from each landmark to the centroid point (Monteiro 1999), is considered to be the only measure of size that is mathematically independent of shape, and therefore a more complete and reliable measure of size (Adams 1999, Zelditch et al. 2004). Additionally, a number of powerful and easy to use programs have been developed to make geometric morphometrics more accessible (Adam et al. 2003).

Introduction

Elevation gradients are often important components of discussions surrounding the evolution of biological. This is at least partially attributable to the diverse set of factors that covary with elevation (Austin and Gaywood 1994). Some studies attribute elevational gradients with playing a major role in evolutionary diversification in areas with particularly high levels of biodiversity, like those seen in the of the Amazon basin (Anderson and Maldonado-Ocampo 2008). The Neotropics have the highest fish species diversity of any area in the world with some experts estimating that as many as 4000 species of fish occur there (Winemiller et al. 2008). Neotropical fish species exhibit a wider range of ecological diversity, with many species exhibiting behavioral, morphological and physiological specializations not seen anywhere else in the world (Winemiller et al. 2008).

The distribution of species is not homogeneous across Neotropical landscapes. For example, streams in larger drainages have higher diversity. Species diversity also varies with elevation. Traditionally it was though that increases in elevation are associated with decreases in species diversity (Winemiller et al. 2008), however, a recent review found that hump-shaped patterns of species diversity are more common, with diversity peaking at mid-elevations (Sanders and Rahbek 2012). The decrease in diversity at high elevations relative to mid-elevations is thought to be attributable to the need for specialized adaptations to survive at high elevations given the more extreme physiological demands found there (Winemiller et al. 2008).

Elevation itself is not a factor which directly acts upon organisms. However, it is a useful metric for representing shifts in other factors associated with elevation like productivity (Terborgh 1977), temperature (Machac et al. 2011), predation, habitat, food availability, etc. (Machac et al. 2011). This ordered change of factors creates a generally predictable continuum of

abiotic and biotic conditions along rivers (Vannote et al. 1980), which in turn impact biological communities, leading to somewhat predictable patterns of change in biological communities with elevation. Vannote et al. (1980) summarized these notions in the River Continuum Concept (RCC). According to the RCC, rivers are coldest and fastest with the most oxygen at higher elevations, and warmest and slowest with less oxygen at lower elevations. Biological communities vary considerably with elevation because at higher elevations stream production relies primarily on allochthonous input from nearby terrestrial vegetation, which in turn leads to a high proportion of shredding invertebrates (aquatic invertebrates which shred leaves to eat colonizing fungus as their main source of food), little primary productivity, and smaller fish that are rarely piscivorous. Overall, there is more coarse particulate matter (CPOM). Lower reaches have more primary production, less shredders and more filter feeding invertebrates, and larger, more predatory fishes.

One of the challenges that remains is that these relationships are not as well researched in tropical streams as they are in temperate streams (Dudgeon 2005). Although there are some abiotic shifts that are likely the same everywhere, e.g., temperature generally decreases and water flows faster with an increase in elevation, the specific conditions and pattern of changes can vary from river to river, and even more so between tropical and temperate ecosystems. However, a recent examination of invertebrate communities demonstrated that the distribution of the relative proportions of invertebrate functional feeding groups, one of the foundational components in the RCC, were similar in temperate and tropical rivers, suggesting that the RCC may be applicable to tropical streams as well (Tomanova et al. 2007). One implication of the RCC is that there can be very different selective pressures acting at different elevations in streams, which in turn can lead to a variety of novel ecological opportunities at different

elevations. As a consequence, one would expect species pairs to segregate by elevation as they adapt to local selective pressures along the gradient.

Questions about the effects of elevation are inextricably linked with concerns regarding biodiversity. Since biodiversity decreases at high elevations in the Andes and diversity is important for overall ecosystem functioning and persistence, understanding the relationship between elevation and the distribution of species is of increasing importance (Anderson and Maldonado-Ocampo 2008, Worm et al. 2004). The altered flow regimes with increased water velocity, decreased temperature, and changing food availability associated with high elevations act as selective pressures favoring traits that allow organisms to thrive under specific conditions. In fishes, this leads to fish that can withstand the increased water velocity while developing, foraging for food, and reproducing. This can manifest itself in a wide range of specific traits, but often selects for fishes that are dorsoventrally compressed and have large pelvic and pectoral fins (Winemiller et al. 2008). Overall body shape is under selection because the ability to swim quickly is essential for escaping from predators and hunting prey. Body shape is particularly important for fishes because the high viscosity of water can lead to substantial energetic demands for sustained swimming. Body shape variation is thus often correlated with the ecological performance of fishes and studying body shape variation can provide insight into patterns of ecological divergence among fishes (Langerhans et al. 2010).

In addition to the disproportionately high levels of biodiversity, tropical areas are under particularly severe environmental threat. Western Ecuador constitutes a region of particular concern because of the high rates of endemism in the region and the extent to which ecosystems are being degraded by human activity. The unique fauna in this region is in part due to its relative isolation (Fowler 1911); Western Ecuador and Northern Peru have been isolated for

millions of years from the major South American river systems since the rise of the Andes Mountains (Hoorn et al. 2010). The Andean uprising is thought to be the source of much of the local species diversity because of the magnitude of environmental change that it caused and the amount of time that has elapsed. This pattern of Andean uprising leading to increases in biological diversity is supported by the association between the timing of biotic diversification as seen in mammals, plants, invertebrates, and fishes, and our present understanding of the timing of the Andean rising (Hoorn et al. 2010). Western Ecuador forms part of one of the most important biodiversity hotspots on Earth (Myers et al. 2000) and harbors a unique fish fauna with high rates of endemism (Eigenmann 1918; Albert et al. 2011; Barriga 2012). Unfortunately, ecosystems in the region are under severe threat from anthropogenic factors including habitat loss and transformation (especially for agriculture), pollution from fertilizers, pesticides, mining and human waste, the construction of large dams, the presence of invasive species, heavy exploitation of fishes for food, and the alteration of river banks (Riccardi 1996, Aguirre et al. In Press). Despite the threats, relatively little is known about the ecology, evolution or status of most of the fishes there, even some of the most abundant fishes such as the members of the genus *Rhoadsia*.

Rhoadsia is a genus of fish in the family Characidae, one of the most diverse families of fishes in the world (Böhlke 1958). They are in the subfamily *Rhoadsiinae,* which is a particularly small, geographically restricted subfamily that includes only six species in three genera ranging from Costa Rica, through western Colombia, south into western Ecuador and the northwestern most part of Peru. The three genera are *Parastremma* (with three species: *P. sadina, P. alba, P. pulcha), Rhoadsia* (with two species: *R. altipinna* and *R. minor*)*, and Carlana* (with a single species: *C. eigenmanni*). The subfamily *Roadsiinae* is characterized by having a single

premaxilla tooth series when immature, developing a second tooth series after reaching maturity (Cardoso 2003). Because of its low species diversity and the lack of research on the fishes in the region in which it occurs, not much is known about it. In addition to exhibiting low species richness, there are also considerable taxonomic issues to be resolved. For example, *C. eigenmanni* has been suggested to consist of two separate species (Géry 1977), and *R. minor* has also been suggested to potentially be a high altitude dwarf form of *R. altipinna* (Eignemann and Henn 1914; Géry 1977).

Rhoadsia altipinna and *R. minor* are the only two species found in this genus and both occur in western Ecuador. *Rhoadsia minor* is particularly interesting because it is the only one of the six species in the subfamily to live at high altitudes; all other species are endemic to low elevation waters (Géry 1977). The two species of *Rhoadsia* are quite similar. Both species exhibit extreme sexual dimorphism with males being larger, exhibiting bright mating colors, and developing long, highly ornate fins. *Rhoadsi*a *minor* and *R. altipinna* are differentiated by overall size, the maximum reported size of *R. altipinna* is 17 cm versus 10.4 cm for *R. minor*, and body depth, with *R. minor* being more elongate with a fineness ratio (FR – standard length divided by maximum body depth) between 2.8-3, whereas *R. altipinna* has a fineness ratio between 2.25-2.5. *Rhodsia altipinna* has also typically been reported from lower elevations in southwestern Ecuador, typically 0 to ~600 meters, whereas *R. minor* has been reported from higher elevations in northwestern Ecuador, above 1200 meters (Fowler 1911, Eigenmann and Henn 1914). However, the exact geographic and elevational distributions of the two described species are not clear because of a lack of directed studies. For example, the identity of specimens of *Rhoadsia* collected at low elevations in northwestern Ecuador is not clear; they have typically been identified as *R. minor* simply because the type locality for this species is in northwestern

Ecuador. While these species were described as distinct species, based primarily on differences in size and body shape, more recent studies suggest that they may be ecotypes and not two distinct species. This has been suggested by Eigenmann and Henn (1914) and Géry (1977), both of whom indicated that *Rhoadsia minor* may be simply a "dwarf mountain form" of *R. altipinna* rather than a distinct species. If they were ecotypes, that would suggest that the large difference in body shape described may be due to local adaptation of geographically structured populations to divergent environmental conditions associated with differences in the elevations at which they occur or phenotypic plasticity. In order to examine the possibility that *R. altipinna* and *R. minor* are not two distinct species, a careful study of the genus across replicated elevational gradients is needed, and would ideally include data on both genetic and morphological variation.

This thesis aims to address the question of the relationship between the two species in the genus *Rhoadsia.* For this purpose, sampling was conducted in two river drainages of western Ecuador in the Summer of 2014; the Esmeraldas River drainage in northwestern Ecuador and the Jubones River in southwestern Ecuador (Figure 1). For subsets of the specimens collected, the mitochondrial COI gene, also known as the DNA barcoding gene (Hebert et al. 2003), was sequenced to examine how genetic variation is distributed across the landscape and whether the magnitude of genetic divergence between populations in northwestern and southwestern Ecuador, or at low and high elevations in the rivers, is consistent with the existence of two distinct species. Because the primary morphological difference described between *R. altipinna* and *R. minor* is body depth, geometric morphometric methods were also employed to examine how body shape differs among rivers and with elevation across the landscape.

This thesis will address three basic questions: (1) Are *R. altipinna* and *R. minor* two valid species? (2) How is genetic diversity distributed across the landscape? (3) What is the relationship between elevation and morphological variation across the landscape?

Methods

Sampling Area

The Esmeraldas drainage is located in northwestern Ecuador between the Santiago and Guayas drainages and drains an area from the western slope of the Andes to the Pacific Ocean in a southeast to northwest direction. Just south of it is the Guayas Drainage, which is the largest drainage in Western Ecuador and runs in a north to south direction funneled between the coastal mountain range (Cordillera Chongon-Colonche) and the Andes Mountains into the Gulf of Guayaquil (Gomez 1989). The Jubones River is further south than the Guayas and is separated from it by many other small rivers running east to west between the Andes and the Pacific Ocean. The Jubones River also runs east to west along a relatively steep gradient into the Pacific. Immediately south of it is the Santa Rosa River drainage, which also runs along a relatively steep gradient from east to west between the Andes Mountains and the Pacific Ocean (Figure 1). All of these drainages are isolated, impeding the migration of fishes between rivers, with the exception of flooding during severe rainy seasons that may allow for low rates of migration among the rivers in southwestern Ecuador and historical connections that may have existed before the rise of the Andes Mountains.

The Esmeraldas and the Jubones rivers are quite distinct. Beyond the differences in size (the Esmeraldas River drainage is much larger) and rainfall (the Esmeraldas drainage receives much more rain), plotting elevational gain by distance traveled shows that the Jubones River is much steeper than the Esmeraldas River (Figure 1d). Within the Esmeraldas the southern branch is steeper than the Northern branch, but less steep than the Jubones.

Samples were collected from eight locations across an altitudinal gradient in a Y formation in the Esmeraldas River drainage. These sites were selected to include the broadest altitudinal range of sites that were accessible and for which specimens of *Rhoadsia* were collected. There were four low elevation sites along the main branch of the river sampled at 50m, 94m, 174m, and 282m above sea level respectively (Table 1). Samples were collected from two high altitude sites on each of two branches of the river above where it branched into the Rio Toachi (southern branch) and Rio Blanco (northern branch). The upstream sites were located at 668m and 1095m on the southern branch and 810m and 1260m on the northern branch. The site at 1260m on the northern branch is Mindo, which is the type locality for *R. minor* (Eigenmann and Henn 1914). In the Jubones drainage, five sites were sampled along an altitudinal gradient at 69m, 136m, 251m, 909m, and 1095m (Table 1, Figure 1). Although several attempts were made to collect between the 251m and 909m sites, much of the river in this area was not accessible or did not harbor *Rhoadsia*. The same was true for sites above 1095m. The sites were plotted on a map to help illustrate their locations relative to one another (Figure 1). The incline of the river gradients was plotted to display differences in gradient between rivers by plotting the total distance traveled between sampling sites (as estimated by straight line distances across the map) by elevational gain.

Field Methods

Samples were collected using a Smith-Root Model LR24 electrofishing backpack (in combination with seines and dip nets) and settings were adjusted to maximize catch efficiency based on variation in local environmental conditions. Generally, electrofishing outputs ranged between 200-950v with 25-30hz, with a standard pulse type and approximately 12-30% duty

cycle. At each sampling location, electrofishing continued until 50 specimens were collected or 100m of stream were sampled, whichever came first. The locations varied greatly in the abundance of *Rhoadsia* and 50 specimens could not be collected at all sites. Specimens were euthanized using buffered tricaine methanesulfonate, MS-222, pH 7.5 in water until 10 minutes after opercular movement stopped. The specimens were then individually tagged and the right pectoral fin (and end of the caudal fin for small specimens) were collected and preserved in 1.5ml microcentrifuge tubes of 95% ETOH and stored at -80ºC for long term storage. The specimens were fixed in 10% formaldehyde for at least 24 hours, at which point samples were rinsed thoroughly in water and moved to 70% ETOH for long term storage. Preserved specimens and tissue samples were then transported back to DePaul University for morphometric and genetic analysis. Samples were collected under research permit 013-14 IC-FAU-DNB/MA from the Ministry of the Environment of Ecuador and DePaul IACUC protocol 14-002. Samples were exported under Export Permit MECN-2014-001 from the Museo Ecuatoriano de Ciencias Naturales.

COI Sequencing

A total of 116 specimens were sequenced from 13 sites: six sites in the Esmeraldas River and four in the Jubones River. Additional specimens from the Santa Rosa and Guayas Rivers were added from a prior study of *R. altipinna* in the region (Aguirre et al. in press) to provide more comprehensive geographic representation. Samples were collected in July 2008 using very similar field methods to those employed here. The Santa Rosa River is located just south of the Jubones river and the samples used in this study were collected at 3°30'06.0"S, 29°57'25"W, which is at approximately 86m in altitude, making it the southernmost sample in this study, with

its closest neighbor being the samples from the Jubones River. The Guayas River is the largest river drainage in western Ecuador and is located between the Esmeraldas and Jubones Rivers. Samples were collected at two sites in the drainage, next to the town of Jauneche (01˚14'17.6"S, 79˚40'21.3"W, approximately 75 m in elevation) and at the Río Palenque scientific station (00˚34'26.3"S, 79˚21'43.5"W, approximately 150 m in elevation) (Aguirre et al. in press).

DNA was extracted using a modified phenol-chloroform method with the addition of an RNAse step to eliminate RNA from the DNA extractions, and a fragment of the COI gene was PCR amplified using primers from Hebert et al. (2003). Tissue was digested in 600 µl of Digestion Buffer (10 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.5% SDS) with 10 µl of proteinase K (20mg/ml), vortexed and incubated overnight at 55ºC. After digestion, 10 µl of RNAse A (20mg/ml) was added to each sample, the samples were vortexed, and allowed to digest at 37 ºC for one hour to remove all RNA. After digestion was complete, 600 µl of 25:24:1 phenol:chloroform:isoamyl alcohol solution was added, samples were vortexed, and centrifuged at room temperature at 12,100 rpm for 10 minutes. The supernatant was then removed and transferred into a new 1.5 ml microcentrifuge tube containing 1 ml of 100% ETOH and centrifuged at room temperature for 5 minutes at 12,100 rpm. The supernatant was removed and replaced with 500 µl of 70% ETOH and then centrifuged at room temperature for 5 minutes at 12,100 rpm. The supernatant was again removed and the samples were spun briefly to remove any residual supernatant. Pellets were then dried at room temperature until most of the ethanol evaporated and resuspended in 100 µl of TE (10 mM Tris pH 8, 1 mM EDTA pH8). DNA concentration was measured using a Nanodrop spectrophotometer, and the average concentration was 126 ng/ul (range: 3 ng/ul - 1300 ng/ul).

Samples were PCR amplified using the forward primer COI-FishF1 (5'-TCAACYAATCAYAAAGATATYGGCAC-3') and the reverse primer COI-FishR1 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3'), which target a stretch of about 670 bp (Hebert et al. 2003). PCR was performed in 30 µl reactions that included 6 µl of Bovine Serum Albumin (BSA), 3.0 µl of 1x PCR buffer, 1.2 µl of 2 mM $MgCl_2$, 0.75 µl of 0.25 mM dNTPs, 1.05 µl of 10 uM COI-FishF1 primer, 1.05 µl of 10 uM COI-FishR1, .15 µl DNA Taq Polymerase (5 U/ µl) (Invitrogen) and PCR grade water. Samples were then amplified on a BIO-RAD C1000 thermal cycler under the following conditions: 1 min 45 s at 95 ˚C, 45 s at 50 ˚C, and 45 s at 72 ˚C, 4 cycles of 45 s at 94 ˚C, 45 s at 50 ˚C , 45 s at 72 ˚C, 30 cycles of 30 s at 92 ˚C, 45 s at 50 ˚C , and 45 s at 72 ˚C; with a final extension of 7 minutes at 72 ˚C, after which samples were held at 4˚C indefinitely. Amplified samples were then run on a 1.5% agarose gel to confirm successful amplification. Both the forward and reverse strands were sequenced on an Applied Biosystems 3730 DNA Analyzer at the University of Arizona Genetics Core.

Sequencing chromatograms were manually checked using Chromas Lite (Technelysium, Pty Ltd., South Brisbane, Queensland, Australia; http://www.technelysium.com.au/chromas_lite.html) and sequence editing and alignment was done in BioEdit 7.2.3 (Hall 1999). Any samples for which both chromatograms were unreadable or not identifiable, were eliminated. After editing and creating contigs for the forward and reverse sequences of each specimen, the sequence fragment length for all samples was standardized to 606 bp. All sequences were then aligned using the multiple ClustalW alignment tool in Bioedit (Hall 1999). Mutations that resulted in a novel haplotype were verified by reviewing the original sequencing chromatograms again, to ensure that the mutation was not the result of an error in the sequence call.

Basic genetic diversity measures were recorded for each sample, including the haplotype frequencies per sampling site, haplotype richness (reported as the number of haplotypes present per site), the haplotype diversity calculated as $N(1-\Sigma p_i^2)*(N-1)^{-1}$ (Aguirre et al. in press), the number of private haplotypes per site (haplotypes that occurred exclusively at one site), and the percentage of private haplotypes per site (Table 2).

Sequences were imported into MEGA version 6 (Koichiro et al. 2013) for the construction of a haplotype tree and the identification of pairwise distances and diversity indices. Haplotype trees were constructed using the neighbor-joining method with bootstrapping for 100 replicates. The Kimura two parameter (k2p) distances were calculated and used to create the pairwise distance matrices between sampling sites and between haplotypes. The K2p distance was selected as the divergence metric because it has been found to be the best measure of genetic distance when distances are low (Nei and Kumar 2003, Hebert et al. 2003).

The aligned sequences were also imported into the program NETWORK (Fluxus Technology Ltd., Suffolk, England; http://www.fluxus-engineering.com/sharenet.htm) for the construction of haplotype networks. Haplotypes were analyzed using the Draw Network function. The program then created a network map with nodes representing the different haplotypes or sequences, and the lines connecting them representing the number of nucleotide differences between haplotypes. The nodes were made proportional in size to the frequency of the haplotypes and sampling sites were color coded to indicate the number of specimens of each sample corresponding to a particular haplotype.

Morphometrics methods

Geometric morphometric analysis was performed on 278 specimens from the Jubones and the Esmeraldas Rivers to examine body shape variation along altitudinal gradients and between River drainages. A set of 16 anatomical landmarks previously used to examine body shape variation in this species (Figure 4) was used in this study (Aguirre et al. in press). Specimens were straightened with insect pins, if needed, to minimize shape variation related to bending of the body. Landmarks which were difficult to see in lateral pictures were marked with size 000 insect pins to facilitate landmark placement. The left side of each specimen was then photographed using a 10.3 megapixel Nikon Coolpix P100 digital camera attached to a copy stand (Appendix 2). A scale bar was included in each picture for recording of size and measurements from the photos. Landmarks were digitized on the images using TPSdig version 2.17 (Rohlf 2013). Samples were then aligned through Procrustes superimposition in TPSrelwarp version 1.61.

Additionally, the measure function in TPSdig was used to measure standard length (SL), length from tip of the snout to end of the vertebral column, and body depth (BD), the depth of the body at its deepest point measured perpendicularly to standard length. The Fineness ratio (FR), a measure of elongation commonly used with fishes (e.g., Ohlberger et al. 2006), was then calculated as the ratio of standard length divided by body depth (SL/BD). To examine variation in elongation among the samples, FR was plotted for each site and arranged by elevation, allowing examination of how body elongation differs by river drainage and elevation. This also permits evaluation of which samples are morphologically consistent with the traditional descriptions of each species, since the major morphological difference between species is in their relative body depth.

There is a significant relationship between size and body shape in *Rhoadsia,* seen in the initial regression analysis, therefore a size correction method was employed to examine shape variation independent of size differences among samples. To perform size correction, tps files were opened in MorphoJ and size correction was performed according to the MorphoJ manual (Klingenberg 2011). Samples were realigned using the Procrustus fit function along the principal axis. The alignment was then used to perform a regression where the dependent variables were the Procrustus coordinates and the independent variable was the centroid size. Permutations were performed with the default of 10,000 replicates, which represents the number of pairwise comparisons returning reliable data within minimal computing time (Klingenberg 2011). The regression represents the relationship between the shape and centroid size, so the residuals represent the size-corrected data because they correspond to the variation in shape that is not explained by size. The residuals were saved and used in subsequent analyses.

A MANCOVA was also performed in TPSRegr version 1.40 using the body shape data as the dependent variables and centroid size, elevation, and stream as the explanatory variables (Rohlf 2011) to determine if variation in body shape covaries with any of the environmental variables. Significance of the variables was assessed by running the analysis with and without each variable, and examining whether the difference in the variance explained was statistically significant.

A principal component analysis (PCA) was performed in MorphoJ to explore the major patterns of shape variation in the size-corrected data. The PCA treats each residual from the landmark as XY coordinates and plots each of the points as an additional dimension, creating a multi-dimensional cloud representing the variation among individuals across all 16 landmarks in space. PC1 represents the axis through the cloud accounting for the most possible variation along

a straight line, while PC2 is the longest line at a 90˚ angle to PC1, PC3 is the next longest line at a 90˚ angle to both PC1 and PC2, and so on. Using geometric morphometric methods, specific patterns of body shape change associated with variation along the PC axes can be estimated, so deformation grids representing the major patterns of body shape variation along PCs 1 and 2 were created and included in the figures.

Additionally three Canonical variate analyses (CVAs) were performed using MorphoJ on the size corrected data. A CVA follows the same initial steps as the PCA, however, instead of identifying the axis of maximum variation, it identifies the axis of variation which best separates a priori designated groups (Adams 1999). The first analysis was set to identify the different between the two river drainages; the second two were conducted separately on samples from each river with the sites as the groups to examine the major patterns of body shape divergence among sites within rivers. Color coded plots were then created with wireframe grids depicting the shape change associated with the first two axes of variation. Additionally, for all three plots, an additional plot was constructed using the means for each group to facilitate visualization of the distribution of groups in multivariate space and their relation to one another.

*Comparison of genetic and morphological differences among sample*s

In order to more directly compare the patterns of genetic and morphometric variation, cluster analysis was conducted on both data sets using a distance-based neighbor-joining approach. Two pairwise distance matrices between site means were computed. First, the K2P distance was calculated in MEGA and used as the measure of genetic distance between site means. To compute morphological distances, PC scores were calculated from the size corrected residuals and averaged for each group. The Euclidean distance between each group mean was

then found using the program PAST version 3.09 (Hammer et al. 2001). Each matrix was then converted into a neighbor-joining tree using Phylip's Neighbor.exe (Felsenstein 1989). Trees were then compared to one another to examine the extent of correspondence between morphological and genetic variation.

Results

Genetic Differentiation

There were 11 identified haplotypes across the four drainages, resulting from twelve variable nucleotides (1.98% of nucleotides sequenced). Of the 606 base pairs sequenced, the maximum number of pairwise differences between haplotypes was six, which represents 0.99% of the sequenced segment. All 12 mutations are located at third codon positions and none resulted in a change of the protein in the translated sequence. Haplotype sequence names follow Aguirre et al. (in press) with four new haplotypes identified in this study (Figure 2).

Of the thirteen sites sampled the number of haplotypes per site ranges from only one haplotype present in four of the Esmeraldas sites to four haplotypes present in three of the Jubones sites (Table 2). Only four sites have private haplotypes, E06, the highest elevation site in the Esmeraldas river (and the type locality of *R. minor*), J05 and J01, the highest and the lowest elevation sites in the Jubones, and G02 in the Guayas. At each of these locations the private haplotypes represent between 25% and 33% of the total haplotypes per site, although these percentages may decline with greater sampling. While haplotype diversity was highly variable in the Esmeraldas and Jubones Rivers, there is much less diversity present in the Esmeraldas. Four of the six sites there have a haplotype diversity of 0.00. The only two sites with any diversity are the two highest elevation sites, E06 and E08, although they are still lower in diversity than the least diverse site in the Jubones River (Table 2).

The network map revealed information about the distribution of haplotypes among sites and drainages (Figure 3). Most samples from the Esmeraldas river belong to Haplotype 3, which is the most abundant haplotype overall. This haplotype is also shared with G02, but predominantly is made up of individuals from the Esmeraldas. There are two haplotypes that are

one mutation from H3, these are H8 and H4. The Guayas has samples that belong to the H4 haplotype as well. From this position the network branches in two directions: one includes haplotypes from the Guayas and samples from E08 (H1), the other direction is dominated by the Jubones samples (H11, H7, H10 and H6). The Jubones samples encompass seven haplotypes, none of which is particularly dominant in terms of frequency. The Santa Rosa and Jubones samples are similar in both the haplotypes present and their frequencies, such that all haplotypes present in the Santa Rosa are also present in the Jubones. These rivers are also geographically close to one another. The Guayas is intermediate to both the Esmeraldas and the Jubones, sharing several haplotypes with them (H1, H3, and H4). It is also geographically intermediate between the two rivers. This genetic similarity is thus consistent with the geographic proximity of the rivers. There is also one haplotype which differs from all the rest by one base pair and is unique to the Jubones, where it occurs at a frequency of 7% and is present at three sites in the drainage. This is much higher than the frequency of the haplotype which is unique to the Esmeraldas at E06 (0.8%) (Table 2). The network map also revealed a lack of monophyly by river; there is substantial overlap and sharing of haplotypes between all of the drainages. The only haplotype to be unique to a drainage is H10, in the Jubones. This unique haplotype is found throughout the river. If there were monophyly, there would be lineage separation by drainage or by elevation and minimal sharing of haplotypes between distinct populations (DeQueiroz and Donoghue 1985).

F_{ST} values and AMOVA revealed significant population genetic structuring and allowed identification of the major patterns of genetic divergence among samples. Overall, most pairwise comparisons of genetic differentiation among sites (59 of 78) were significant, with pairwise F_{ST} values ranging from 0 to 0.85 (Table 3). The largest F_{ST} values tended to occur between pairs of

sites from different drainages, while lack of significant genetic differentiation tended to occur among geographically neighboring sites within the same river or geographically neighboring sites in different drainages. Almost none of the sites in the Esmeraldas River were differentiated from one another with the exception of E08, which was different from all sites except E07, the other site on the southern branch and its closest neighbor. The sites within the Jubones are variable in the magnitude of their divergence, with two of six pair-wise distance values being significant (J01 vs. J03 and J01 vs. J05). There is no significant difference between G02 and E08, or G01 and either E08 or E07. Additionally, G01 and G02 are not significantly different from each other. The Santa Rosa site is different from all other sites, although it tends to be most similar genetically to sites in the neighboring Jubones River (Table 3). The AMOVA was consistent with the patterns of genetic divergence suggested by comparison of pairwise F_{ST} values, revealing that most of the genetic variation was attributable to differences between Rivers (43.22% of the genetic variation) and variation among individuals within sites (47.72%). Only 9.05% of the genetic variation is attributable to divergence among sites within Rivers.

Body Shape Differentiation

The regression analysis indicated significant ($p<0.00001$) effects of centroid size, elevation, and drainage on body shape variation (Figure 6). Overall, the three variables explained 29.1% of the variation in body shape seen in the study. Centroid size explained approximately 21% of the variation in shape. The largest change in shape associated with size is a drastic shift in landmark 16, which is more anteriorly located reflecting a relatively smaller mouth in small fish, shifting to a more posterior location reflecting relatively larger mouths in larger fish. Additionally, smaller individuals have proportionally smaller eyes. Elevation explained 5% of

the variation in body shape. The landmarks that seemed to be most strongly associated with elevation are landmarks 3, 4, 10, and 11. Individuals at low elevations are deeper bodied with landmarks 3 & 4 and 10 & 11 moving away from one another, while the opposite is seen in specimens collected at high elevations. These two landmark pairs move closer to one another and the fish are more elongate. River explained only 2.1% of the variation in body shape and there was little divergence in body shape that could be directly associated with river drainage.

Plots of FR versus site location indicate a complex relationship between FR and elevation (Figure 5). The lowest site in the Esmeraldas drainage exhibits much greater variation than the rest of the sites in this drainage with a range in FR from about 2.4-2.8 FR tends to be relatively stable through the fourth site (ordered by elevation) at approximately 668m (E07). These sites have average FR close to 2.5. FR then increases relatively linearly from the 810m site (E05) to the 1260m site (E06). All sample sites, with the exception of E06, fall partially within the range indicated for *R. altipinna* of 2.25-2.5 Fowler 1911), and only E06 (Mindo, the type locality of *R. minor*) falls primarily within the range of values set for *R. minor,* 2.8-3 (Eigenmann and Henn 1914). Additionally, all samples have specimens with FR values of 2.5-2.8, which according to the species descriptions (Fowler 1911, Eigenmann and Henn 1914) should be between the two species, and therefore empty. In the Jubones River, the relationship between FR and elevation is quite distinct. While FR increases with elevation along the three low altitude sites, it then appears to plateau quickly, such that the two highest elevation sites (909 and 1095 m) have values that are similar to the third site (251m). As a consequence, fish in the Jubones River never become as elongate as those seen at the highest elevations in the Esmeraldas. Thus, although the pattern of change in the FR with elevation in the Jubones River differs substantially from that in

the Esmeraldas, the general pattern of increasing FR with increasing elevation does generally hold (Figure 5).

The PCA of size corrected body shape data indicates some divergence in body shape by site with substantial variation among specimens within sites (Figure 7). The first six PC axes account for 75% of the variation in body shape, with PC1 representing 24.5% and PC2 21.2%. Shape changes associated with the first two PCs were represented as wireframe graphs. The graphs for PC1 indicate that the major shape changes are in the positions of the eye and the mouth, and in body depth (elongation). From positive to negative, variation along the PC axes is associated with a downward shift of the eye and a decrease in body depth generally associated with landmarks located in the ventral region of the body. Although there was some separation by site, there was also considerable overlap among specimens from different sites, with no complete segregation of any site from the others. This indicates that there is substantial heterogeneity in body shape within samples. Additionally, as seen in the plot of the mean PC scores, there is an overall pattern of association between body shape and elevation. Mean PC scores generally move positive to negative values along PC1 and then negative to positive values across PC 2 with an increase in elevation. These changes in the positions of sample means are associated with body shape changes primarily related to increasing elongation with increasing elevation (Figure 7).

The CVA reveals moderate separation between groups in all cases (Figures 8-10). The first CVA separates the two drainages relatively well, with some overlap. CV1 is primarily associated with differences in body elongation and CV2 is associated with minor changes near the head and adipose fin, all of which are uncoordinated and smaller than CV1. The two rivers primarily separate over CV2 however there is variation over CV1 in both systems. The site averages indicate that in both drainages the sites generally separate by elevation across CV1,

with the low sites being deeper bodied and high being more elongate. The second CVA, which only includes sites form the Esmeraldas drainage, also shows moderate separation of specimens by site with some overlap, but with some segregation along CV1. This CV is generally associated with increasing body elongation (Figure 9). The CVA for the Jubones is similar with the sites segregating along CV1 from positive to negative values with elevation, which is also associated with an increase in body elongation (Figure 10).

Comparison of Morphological and Genetic Divergence

Both morphological and genetic data were examined by comparing patterns of site clustering through Neighbor-Joining analysis (Figure 11). Although geographically proximal sites tend to cluster based on both the genetic and morphological data, there are different broader patterns of association that emerge. Genetically, samples clustered by geographic location (Figure 11). The lowest altitude site in the Esmeraldas drainage was distinct from the rest, with the remaining sites in the Esmeraldas clustering together with the exception of E08. E08 is the southernmost high elevation site in the Esmeraldas River and is very close geographically to the headwaters of the Guayas River. E08 grouped with the G01 and G02 sites from the Guayas River drainage. The remaining group encompassed the Jubones and Santa Rosa Rivers with the bottom two sites being more similar to one another than the rest. This is consistent with the close geographic proximity of the Jubones and Santa Rosa rivers. The morphological tree tells a different story with sites predominantly clustering by elevation (Figure 11). E01 is once again distinct and then the high elevation sites (E06 and E08) fall out clustering together. The intermediate to low sites in the Esmeraldas (E03 and E04) then form a cluster with the four lowest elevation sites in the Jubones River, with J01, J02, and E03 forming a cluster and J03 and

J04 clustering together. E04 is at the base of this cluster. The next group is the mid to high elevation sites in the Esmeraldas (E05 and E07) forming a cluster with the highest elevation site in the Jubones. J05 is (1095m) in elevation, higher than the Esmeraldas sites that it clusters with (668 and 810m). However, the relationship among these sites is consistent with the more moderate increase in elongation with elevation seen in the Jubones River samples and suggests that the highest elevation site in the Jubones River generally resembles intermediate elevation sites in the Esmeraldas in terms of body shape.

Discussion

Overall, the data suggest that there is not enough genetic or morphometric evidence to support the distinction of two separate species in the genus *Rhoadsia* in western Ecuador. None of the criteria for distinguishing species based on mitochondrial genes were met for the populations of *Rhoadsia* examined, and body shape tended to vary continuously with elevation, consistent with previous research (Aguirre et al. in press). Although there was significant divergence in haplotype frequencies by river drainage and some populations differed significantly in morphology, the patterns of variation observed are more consistent with geographically divergent populations within a single species that are adapting to heterogeneous environmental conditions across the range of the species.

Evidence for the Lack of Species Differentiation

One tool for differentiating species is the percent divergence between groups at a particular gene. Over time, genes accumulate mutations at a predictable rate and if two groups are different species, these mutations will cause them to become more different over time and therefore percent divergence can be used as a tool to diagnose species differences (e.g., Hubert et al. 2008). For example, Hebert et al. (2003) found that most of the 200 lepidopteran sister species surveyed were separated by at least 3% sequence divergence and therefore 3% divergence represents a reasonable cutoff point to distinguish one species from two species. However, Avise and Walker (1998) found that for 90% of the 252 vertebrates that they surveyed sister species had greater than 2% sequence divergence. They did not recommend that 2% sequence divergence be thought of as diagnostic for species level differences but simply a benchmark to be interpreted in broader context. In *Rhoadsia,* there are six nucleotide differences between the most

distant individuals, which is only about 0.99% divergence. This is lower than would be expected between even two sister species by either Hebert et al. (2003) or Avise and Walker (1998). Additionally, in previous studies the percent divergence is used to distinguish two groups, not individuals within one group. Therefore, the 0.99% is misleading as it is not the difference between two groups, but between the most divergent individuals in a single group.

More recently, due to the strong criticism of the percent divergence benchmark approach, several more holistic approaches have been developed. One approach is to compare within species variation to between species variation, which takes into account the specifics of the system. Specifically, when examining a diverse group of cypraeid marine gastropods, Meyer et Al. (2005) demonstrated that species are delimited when there is ten times more divergence between groups than within them. Another recent review using crustaceans as the model system focused on the number of substitutions between monophyletic groups, indicating that on average 0.16 substitutions per site between groups can be used as a diagnostic tool (Lefébure et al. 2006). These approaches addressed two additional issues, the relationship between magnitudes of intraspecific and interspecific divergence and monophyly. If these two additional approaches are applied to *Rhoadsia,* we continue to see a failure to meet the distinction of two species. In fact, these two thresholds cannot even be applied due to the overall lack of monophyly of the traditionally described species. In a monophyletic group, there would be two distinct groups of individuals forming phylogenetic clusters that are separated by some measurable distance, such as a few fixed nucleotide differences between sequence groups. To qualify as distinct species, they would also differ by some additional criteria like those proposed by Meyer et al. (2005) or Lefébure et al. (2006). In *Rhoadsia* there is no monophyletic grouping of specimens from the type locality of *R. minor* (Site E06), high elevation specimens from the Esmeraldas drainage that

are traditionally considered to be *R. minor*, or even of the specimens from the Esmeraldas drainage (Figures 2, 3). One haplotype, H04, is in fact shared by all drainages, and others are shared by two or three drainages.

On all genetic measures, *R. altipinna* and *R. minor* fail to pass as two distinct species. However, there is evidence for a more nuanced interpretation of the distribution of genetic variation across the landscape. The AMOVA indicates that there is a fairly homogeneous distribution of genetic variation within rivers, with less than 10% of the genetic variation segregating among sites within the same river, but that there is a substantial proportion of the genetic variation (43.22%) segregating among rivers (Table 4). This pattern of genetic divergence is consistent with geographic structuring of genetic variation among geographically separated rivers, a pattern that is relatively common in Neotropical fishes (e.g., Lovejoy and Araujo 2000, Moysés et al. 2002, Cardoso and Montoa-Burgos 2009). The greater genetic similarity between geographically proximal rivers like the Jubones and Santa Rosa Rivers, or the sharing of haplotypes between the Esmeraldas and Guayas Rivers and the Jubones/Santa Rosa and Guayas Rivers are also consistent with the association of genetic variation and geographic distance.

There were some surprises in the analysis of genetic distances, for example, the lack a significant pairwise F_{ST} between E08 and J03. All other pairwise comparisons between the Jubones and the Esmeraldas sites are significantly different with this single exception. There is no obvious reason why these two sites would be more closely related genetically than the rest and it is possible that their similarity simply reflects a historical accident. Another interesting pattern of genetic relationship is that the Guayas sites are different from all sites in the Jubones, but not different from the sites in the southern fork of the Esmeraldas River. This is consistent

with the geographical proximity between site E08 and the head waters of the Guayas River drainage and suggests the possibility of more recent gene flow between the populations in the upper reaches of the Guayas and Esmeraldas rivers.

Despite the ease of the COI barcoding system and the interesting patterns seen in the results in *Rhoadsia* some more general issues need to be considered. One problem with this methodology is the use of a single locus to represent the entire evolutionary history of a species, particularly a locus that is only inherited from the maternal line and may not reflect the evolution of other parts of the genome. This difference can be amplified with the large variation which exists in species life history traits or in their evolutionary history, such as differences in effective population sizes, mutation rates, or selection (Ballard and Whitlock 2004). It can also become confused in systems which have recently speciated or where hybridization has occurred (e.g., Crochet et al. 2003). Lack of research on the basic biology and evolutionary history of *Rhoadsia* makes it difficult to assess the potential impacts of some of these factors. However, the convenience and the wide success of employing COI to help delimit species boundaries has resulted in its continued use on this approach. The second concern is that the imposition of a strict rule for differentiating species, which leaves out the nuances of speciation (especially in systems which have been subject to hybridization or introgression), may prevent some good species from being identified (Hebert et al. 2003; Ballard and Whitlock 2004). The use of strict rules is particularly concerning when considering how speciation occurs in nature, along a continuum (Hendry et al. 2009).

The species continuum reflects the biological process of speciation where geographic separation of populations is followed by differentiation and the evolution of barriers to reproduction. Over time, a population can become more distinct and eventually, by one of many

mechanisms, can cease interbreeding with other populations. At many points along the species continuum, the two groups could be considered two good species. However, they may be difficult to distinguish as such when at earlier stages of the continuum. Additionally they may be at a stage when the potential for interbreeding has not completely been eliminated. This would occur when one population is in the process of becoming two but has not completed the last steps along the process. When two populations have spent little time geographically separated, there will not be enough time for enough mutations to accumulate for the populations to pass the 3% threshold, but they may do so in the near future. If the two populations are not interbreeding, they could be two separate species and be moving toward this level of divergence, but fail all the tests of speciation (Lefébure et al. 2006). In *Rhoadsia,* this is of concern because so little is known about their evolutionary history that their place along the continuum cannot be estimated. This could be in part addressed by examining their relationship to the other genera in the subfamily Rhoadsiinae, but none of the other genera have been examined genetically. This means that *Rhoadsia* may well be in the early stages of speciation, in which case the diagnostic criteria would not detect the occurrence of two distinct species.

Rhoadisa may not consist of two species, but the genetic data suggest the occurrence of genetically divergent populations that are geographically separated by river drainage. Population level differences can be just as important as species level differences from an evolutionary perspective, and therefore necessary to consider from conservation and pure science perspectives (Fraser and Bernatchez 2001). The notion of considering evolutionary differences between populations led to the popularization of the concept of Evolutionarily Significant Units (ESU) by Ryder (1986). An ESU is presently recognized as “A lineage demonstrating highly restricted gene flow from other such lineages within the higher organizational level (lineage) of the

species" (Fraser and Bernatchez 2001). The validity of recognizing this concept from a conservation perspective has been questioned since its inception (e.g., Crandall et al. 2000) and the definition has also changed over time (Fraser and Bernatchez 2001). However, the ESU is now a taxonomic level recognized for conservation purposes in the U.S. endangered species act. These designations can come from geographical or genetic differentiation, but often some level of genetic differentiation is required (Moritz 1994). F_{ST} is one measure of genetic differentiation and represents the variance in gene frequencies segregating among populations (Wright 1943). It provides a quantitative measure of differentiation between populations, and can be tested statistically to examine whether two populations differ significantly in their genetic properties (Brown 1970, Nei 1977). The segregation of a large percentage of the genetic variation among rivers and the patterns of morphological divergence associated with elevation suggest that there are several ESUs that could be recognized for *Rhoadsia* in western Ecuador.

Genetic diversity across the landscape does not appear to be homogeneous in *Rhoadsia*. The Esmeraldas River has much lower diversity than the other rivers surveyed, with most sites only having one haplotype. This could indicate that that the population experienced a strong genetic bottleneck or strong selection at some point in its history. Rapid reductions in population size due to disease, natural disasters or other factors, and strong selective sweeps can all result in declines of genetic variation (Gozlan et al. 2005). This pattern could also indicate that the Esmeraldas River was colonized more recently than the other rivers analyzed and perhaps from a relatively small set of founders, causing a founder effect (Hansen and Mensberg 1996). If the latter is the case, the genetic similarity to populations surveyed from the Guayas River would suggest that the populations in the Esmeraldas River may have been established from those in the Guayas River. The Esmeraldas and Guayas rivers share many freshwater fish species (Barriga

2012, Jiménez et al. 2015), which is consistent with historical migration between these drainages. Ancestral populations also typically tend to be more genetically diverse than descendant populations. For example, human populations in Africa, where humans originated, are more genetically diverse than human populations in other parts of the world, which migrated out of Africa (Merriwether et al. 1991). Greater geographic structuring in other rivers (the Guayas, Jubones, and Santa Rosa) could also be leading to the observed differences in genetic diversity since structuring of subpopulations promotes the accumulation of genetic variation within species (Knight et al. 2009). The analysis of additional genetic markers, especially genomic markers, could help distinguish among these hypotheses (Allendorf and Ryman 2002).

Evidence for Elongation with Elevation

While the genetic data display a pattern of similarity based on geography, the morphometric data do not. First, the regression clearly shows the importance of allometry, change in body shape with size, in this species. Allometry was not only four times more significant in explaining body shape variation than elevation; it also explained 20% of the overall body shape variation in the study. This is not a terribly unusual result; allometry is often an important source of body shape variation in fishes (e.g., Oliveira and Almada 1995, Sidlauskas et al. 2006, Martínez-Montaño 2014). The deformation grids indicate that the largest changes associated with an increase in centroid size are in the posterior shift of the mouth and the decrease in the size of the eye. This is in line with field observations of the organisms, young individuals tend to be small, elongate and have large eyes for their size. As they grow and become adults, the eye becomes proportionately smaller. Changes in the relative size of the eye associated with growth are common across fishes (Klingenberg et al. 1991) so this aspect of

allometry in *Rhoadsia* is following a general change in body form with increasing size seen across fishes. Additionally, as males become mature they become much larger than the females and the mouth becomes substantially larger, occasionally even passing the posterior edge of the eye. This unique trait is noted in the species description as well as more recent research into the species as it is particularly obvious (Glodek 1978, Barriga 1988). The changes in the mouth are likely mostly due to sexual dimorphism, however, since non-sexually mature individuals are indistinguishable, and males are also substantially larger linking size and adult male traits, these changes are treated as allometry. The change in body elongation with size is not unique to *Rhoadsia*, it is a pattern seen often in fishes (e.g., Claverie and Wainwright 2014) and has been described previously for *Rhoadsia* (Fowler 1911, Aguirre et al. In press).

Analysis of variation in FR ratios indicate that individuals become more elongate with elevation. There is quite a bit of variation within sites and overlap among sites, but the overall trend of increasing elongation with elevation is clear. E06 is the site with the most elongate specimens and the only one which fits into the parameters set forth by the species description of *R. minor;* a FR between 2.8 and 3 (Eigenmann and Henn 1914). However, while E06, the type locality for *R. minor* (Eigenmann and Henn 1914), is the most elongate overall, it is in line with the pattern of increasing elongation with increasing elevation and overlaps substantially with the next closest site in terms of elevation (E08). Some specimens from E06 span into the ratio space for *R. altipinna* (between 2.4-2.5 in the FR) and almost all other sample locations overlap values of 2.5-2.8 for the FR, which is described as between the two species. If the differentiation of populations followed the species descriptions, this range of FR values should be devoid of specimens. With increased geographic sampling, FR values are not consistent with two well

defined species falling into distinct groups based on a gap in relative body depth (Fowler 1911, Eigenmann and Henn 1914).

FR is a simplified measure of shape that is of functional significance because of its association with swimming performance and survival (Scarnecchia 1988). Body elongation is a functional characteristic because a deeper bodied fish experiences more drag, which in a viscous aquatic environment can be highly energetically costly (Langerhans and Reznick 2010). This can in turn prevent the fish from having the resources to adequately perform other life functions, such as breeding or foraging, or they may not have adequate energetic resources to maintain their position in rapidly flowing water (Langerhans and Reznick 2010). FR has been shown to vary with environmental conditions in fishes previously (Thomas and Wainwright 2014), with high elevation individuals having a larger ratio, making them more streamlined (Poff and Allan 1995, Sidlauskas et al. 2006). Presumably, this would be adaptive since water at high elevations often flows faster than at lower elevations due to the steeper river gradients at high elevations. Studies done in habitats that are similar to those of *Rhoadisia,* highlight changes in morphology that are very similar to those seen in this study. Specifically, higher velocity has been found to be associated with a decrease in body depth in salmonid fishes (Pakkasmaa and Piironen 2000), and velocity is one of the major factors that changes with elevation (Vannote et al. 1980). *Rhoadsia* may be particularly susceptible to selection on body shape in faster flowing waters because they are deep-bodied relative to ecologically similar species. Other characids with more typical, streamlined shapes may be better suited for greater water velocities at higher elevations, making the impacts of adapting to faster flowing waters at higher elevations less obvious than for the deep-bodied *Rhoadsia*. Review papers on the subject also found that FR in fish increases with variability in flow, which itself generally increases with elevation (Schaefer et al. 2011). These

studies focused on the differences between species (fishes of Wisconsin in Poff and Allan 1995, Topminnows in Schaefer et al. 2011), but the same concepts should apply for differences within a polymorphic species. This pattern could explain some of the variation seen in *Rhoadsia*, particularly the differences in elongation with elevation observed between river systems. The Esmeraldas River is a more tropical river, on average it receives 31,217 Hm^3/year of rainfall compared with the Jubones which receives 1,827 Hm^3/year. Additionally, the Esmeraldas has a ratio of volume to area of 1.46 while the Jubones has a ratio of only 0.42 (Gomez 1989, 2001). This means that the Esmeraldas not only receives more rain fall but the ratio of water volume to drainage area is much higher, suggesting that the variability of the water flow may be much higher on average. Western Ecuador as a whole experiences rainy and dry seasons, which means that while they will both have high variability in flow, the Esmeraldas will likely experience an exaggerated difference between seasons. This could lead to the difference in patterns between the two streams, because while they both display an increase in elongation with elevational gain, the increase is much greater in the Esmeraldas.

Analysis of FR across a broader geographic region and with increased sampling along elevational gradients revealed that the primary metric on which the distinction between species was described does not hold up. However, there are more complex patterns associated with elevation that emerge with a more complete examination of overall body shape using geometric morphometrics. After size correction, the CVA reveals similar patterns to those seen in the PCA. The groups separate somewhat by drainage with substantial overlap. They also tend to be separate by elevation, but with a higher level of noise in the main graph (Figures 8-10). The axis of separation is associated predominantly with elongation in all three cases. However, the deformation grids indicate that body shape changes in several ways, with elongation simply

being the most conspicuous change (Figures 8-10). The differences with elevation are maintained in both streams when looking at all sites together. When the differences between streams are compared to elevational shifts, the former appear fairly minimal (Figure 8). This pattern is different than what was seen in the AMOVA results for the distribution of genetic variation, which indicate that there is more differentiation among sites in different rivers than there is among sites within rivers (Table 4). These patterns indicate that there is an important relationship between body shape and elevation, perhaps even stronger than that between genetic distances and morphological differentiation. However, whether the association between body shape and elevation is due to genetic divergence or phenotypic plasticity is not known.

While the mitochondrial DNA data indicate that the high elevation sites are not significantly different from low elevation sites in the same river and therefore support the idea of plasticity, there could be nuclear genes under divergent natural selection at different elevations that are leading to local genetic adaptation and are unlinked to the mitochondrial marker used. A very similar pattern is seen in Lake Whitefish (*Coregonus clupeaformis*) that are known for developing a dwarf shallow water form and a normal deep water form in parallel in several lakes (Pigeon et al. 1997). The ecotypes interbreed and live in close contact with each other, however, mtDNA data show that the largest genetic differences are between lakes, not ecotypes within the same lake (Pigeon et al. 1997). However, nuclear markers indicate a significant relationship between genetic differentiation and levels of morphological differentiation (Lu and Bernatchez 1999). Pumpkinseed sunfish (*Lepomis gibbosus*) show a contradictory pattern for several of their distinguishing ecotype characteristics. Pumpkinseed sunfish have two ecotypes, an open water form which has a thinner body and a shallow water form which is deeper bodied. Robinson et al. (2000) found that this differentiation was much more attributable to environmental (53%) than to

genetic (14%) factors, indicating that phenotypic divergence in this case was primarily attributable to phenotypic plasticity.

This variation of elongation with elevation could be simply attributed to the same factors discussed previously for FR, velocity and flow variability increasing at high elevation and leading to more elongate fish as described by Schaefer et al. (2011). However, the specific relationship between elevation and flow can be complicated by the heterogeneity of stream topology. Schaefer et al. (2011) also found two species of fish that was deeper bodied at high elevation (*Fundulus notatus* and *Fundulus olivaceus*). In that case, the high elevation fish used backwaters to escape from the high flow and the associated energy demands, but backwaters were not as common in low elevation areas, making the high elevation site the slower and less variable environment. *Rhoadsia* were reported as more often being found in pools and backwaters in this and previous collections (Aguirre et al. In press), which raises concerns for flow and variability as overly simplified explanations for the patterns of elongation with elevation observed. An alternative explanation of the pattern of elongation with elevational gain is variability in biological factors, specifically predation. Research on elongation with predation (e.g., Hambright 1991) shows that in areas with high levels of piscivorous predation, prey species develop deep bodies because these provide protection against being swallowed by fish predators. Vannote et al. (1980) indicate that piscivorous fish are much more prevalent at low elevations therefore it is possible that there is a range in selective pressures associated with predation that could lead to the pattern seen in *Rhoadsia* of more elongate individuals at higher elevation. Future work on the availability and use of riffle vs. pool habitats and the type and severity of predation experienced at different elevations and in different rivers may help clarify the patterns of change in body shape seen in *Rhoadsia*.

Comparison of patterns of genetic and body shape variation

If the genetic and morphological data are contrasted, a more complete picture of the major patterns of distribution of genetic and morphological variation in *Rhoadsia* across the landscape emerges. In the cluster analysis of the genetic data, samples cluster primarily based on geography. The geographically proximal sites cluster with one another, including the highest elevation site in the southern branch of the Esmeraldas (E08) with the sites in the Guayas (G01 and G02), and the bottom of the Jubones (J01) with the bottom of the Santa Rosa (SR). If there is a maintained or historical connection between the sites, it is likely at these junctures as they are closest and most vulnerable to watershed capture events in which rivers from distinct drainage systems can connect due to geological changes in the landscape (Young et al. 2011). In contrast, in the cluster analysis of the morphological data, individuals cluster primarily by elevation, not geography. The sites from the two rivers that group together are the mid elevation sites from the Esmeraldas (E05 and E07) with the highest sites in the Jubones (J05), the two high sites in the Esmeraldas alone (E06 and E08), and the lower sites all together (E03, E04, J01, J02, J03, and J04). This shows that individuals are genetically differentiated primarily based on the geographic distances by site, while samples are morphologically differentiated primarily based on the elevation of the sites at which they occur, more than with the geographic or genetic distances among them. This pattern of contrasting genetic and morphological signals is consistent with *Rhoadsia* in western Ecuador consisting of one widely distributed, geographically structured, and morphologically variable species, with the morphological variation being primarily associated with local conditions. As described previously, contrasting patterns of morphological and genetic

variation among populations within species are relatively common (Young et al. 2011), and often reflect the contrasting effects of gene flow and local adaptation in complex landscapes.

Conclusions

The genetic and morphological data analyzed in this study strongly suggest that *Rhoadsia* in western Ecuador consists of a single, widely distributed, geographically structured, and morphologically variable species that should likely be recognized under the name *Rhoadsia altipinna* (Fowler 1911), given the seniority of this species name. The structuring of genetic variation among river drainages and the morphological divergence among samples indicates that this is not a single panmictic population, however, there are possibly several distinct ESUs. The broad ecological and geographic distribution *Rhoadsia*, as well as its morphological variability, may partially be due to ecological opportunity associated with the relatively low diversity of freshwater fish species in western Ecuador (Jiménez et al. 2015). If our findings are confirmed by future studies, than *Rhoadsia* in western Ecuador may serve as an important model for studying local adaptation along elevational gradients in Neotropical streams.

The description of two distinct species based primarily on differences in body shape may have been partially due to the limited availability of samples that formed the basis for the species descriptions. *Rhoadsia altipinn*a was described based on only samples from one location near Bucay, Ecuador which is at an elevation of about 300m in the Guayas River (Fowler 1911). *Rhoadsia mino*r was described later based on 92 individuals from Mindo, site E06 in this study, from about 1260m in elevation in the Esmeraldas River (Eigenmann and Henn 1914). Given this limited set of geographically isolated samples, one can see why it would have been tempting to describe samples from these two populations as two distinct species. They indeed differ

substantially in morphology and present no overlapping values for FR. Unfortunately, these two samples seem to represent the extremes of a continuous distribution of morphological variation, thus leading to erred conclusions when evaluated in isolation from the broader context of morphological and geographic variability across river drainages. Additionally, the relatively low number of specimens analyzed made accounting for allometry and sexual dimorphism difficult in the comparisons of the two species. In the present study, the genetic data, which were not available when the species were originally described, also strongly suggest that there is only a single species of *Rhoadsia* in western Ecuador.

In conclusion, all three questions set forth in the objectives of this study were addressed. (1) Overall, there is sufficient data, both genetic and morphological, to indicate that the genus *Rhoadsia* contains a single species, *Rhoadsia altipinna,* and that the description of *Rhoadsia minor* was likely a mistake attributable to a limited sample set before sufficient geographic sampling was conducted or genetic tools were available. (2) Across the landscape, there are approximately similar amounts of genetic variation segregating among individuals at any given site as there is among rivers, with very little difference between sites within a river. There is some sharing of haplotypes between rivers, which demonstrates that while there is genetic structuring associated with geographic distances among sites, there is considerable shared genetic variation among sites. It is possible that the distribution of genetic diversity indicates a strong bottleneck in the history of *Rhoadsia* in the Esmeraldas River. (3) There is a strong association between elevation and morphology; fish become more elongate with increasing elevation consistently across both of the rivers surveyed although the specific pattern of increasing elongation differs substantially between rivers. The increase in elongation with

elevation could be attributed to the energetic cost of being deeper bodied in high elevation areas with faster flowing water or higher predation at low elevations selecting for deep bodies.

Many questions remain. Without nuclear genetic markers morphological change cannot be directly attributed directly to phenotypic plasticity or genetic differences. Also due to a relatively limited morphological and environmental survey and lack of controlled environmental conditions, specific attributions to elevation, e.g., dose response curves, could not be established. Elevation is used as a proxy for correlated environmental variables, but the patterns of correlation may vary substantially among rivers such that a specific elevation, e.g., 1200m, will not likely represent the same ecological conditions for fishes inhabiting two distinct river systems like the Esmeraldas and Jubones.

This research leads to several potential directions for future research, both genetic and morphological. Genetically, nuclear markers, particularly in coding regions, could be used to test for local adaptation and point at a potential genetic explanation for the variation in morphology seen. This would best be accomplished with a genome level sequencing approach like ddRADSeq (Peterson et al. 2012), because it would allow broad sampling of the genome without the need for a reference genome. This analysis would also return information on gene flow across the landscape, population structure, and potentially allow for the identification of candidate loci which may be associated with the morphological change documented. To address the question of phenotypic plasticity, a common garden or reciprocal transplant experiment could be employed (Kawecki and Ebert 2004). If individuals maintained their morphological differentiation in a controlled environment through the F2 generation (to eliminate maternal effects), this would indicate that the differences are genetic (Einum and Fleming 1999). Similarly, if individuals at low elevations are transplanted to high elevations and become

elongate within a generation, and vice versa, that would indicate that morphological differences are plastic in nature and primarily due to the environment in which individuals develop. A more functional approach could also provide insight. Examining individuals with different body shapes in a controlled swim tunnel and calculating the energetic costs of the various body shapes would allow estimation of the fitness advantage of a more elongate body in high velocity water.

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Tables and Figures

Table 1. Sites for this study. Site Name is the code used for sites in the text, tables and figures, Site ID is the field number that sites were assigned during collections, the location is the nearest identifiable named geographic landmark (i.e., river or town), the altitude is the height above sea level at the sampling site, N is the total number of *Rhoadsia* caught per site, COI N is the number of individuals from that location included in the genetic analysis, Morphometrics N is the number of individuals from that location included in the morphometric analysis.

Site Name	Site Name	Altitude (m)	Latitude	Longitude	N	COI N	Morph N
E01	Afuera	50	00°35'02.8680" N	079°36'38.8800" W	6	6	6
E02	Esmeraldas	94	00°22'25.4280" N	079°29'36.0600" W	1	0	1
E03	Rio Bravo	174	00°02'19.5360" S	079°20'38.7240" W	10	0	10
E04	Valle Hermoso	282	00°04'44.1480" S	079°17'05.8560" W	55	8	30
E05	Rio Blanco	810	00°00'22.9800" S	078°54'10.5000" W	8	4	8
E06	Mindo 1	1260	00°03'28.9200" S	078°46'26.3400" W	84	9	31
E07	Meme Chico	668	00°13'26.1120" S	079°04'19.8120" W	30	2	30
E08	Rio Transito	1093	00°18'40.9680" S	078°54'16.0560" W	22	7	22
J01	Huizho	69	03°19'40.0080" S	079°44'30.2640" W	50	9	30
J02	Casacay	136	03°19'48.2160" S	079°42'40.8960" W	50	0	30
J03	Rio Mollopongo	251	03°18'55.4040" S	079°39'29.7360" W	43	6	30
J04	Rio Minas	909	03°20'47.5440" S	079°22'53.5440" W	20	8	20
J05	Rio Mondur	1095	03°18'50.7240" S	079°16'48.3240" W	33	10	30
G01	Jauneche	x	01°14'17.6"S	79°40'21.3" W	15	15	x
G02	Palenque	x	00°34'26.3"S	79°21'43.5" W	16	16	x
SR	Santa Rosa	86	3°33'31.4"S	79°56'45.5" W	16	16	x
Total						116	278

Table 2. Sample sizes and measures of genetic diversity for the populations sampled in the study. N is the number of specimens per site, H is the number of different haplotypes, Hd is the haplotype diversity index, Hp is the number of private haplotypes at each location, and Hp% is the percentage of private haplotypes.

Site	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	N	H	Hd	Ap	Ap%
E01			6									6	1	0.000	0	0
E04			8									8	1	0.000	0	0
E05			4									4	1	0.000	0	0
E06			7	1				1				9	3	0.417	1	33.3
E07			2									2	1	0.000	0	0
E08	4		2			1						7	3	0.667	0	0
J01				1	2	4			2			9	4	0.778	1	25
J03				2	1		2			1		6	4	0.867	0	0
J04						3	1			3	1	8	4	0.786	1	25
J05							5			5		10	2	0.644	0	0
G01	13						2					15	2	0.248	0	0
G02	11	1	1	3								16	4	0.517	1	25
SR				4	11	1						16	3	0.492	0	0
N=	28	1	30	11	14	9	10	1	2	9	1	116				
Frequency=	0.24	0.01	0.26	0.09	0.12	0.08	0.09	0.01	0.02	0.08	0.01					

Table 3. Pairwise F_{ST} distances among sites (lower left portion of the matrix), and their significance (upper right portion of the matrix) depicted as + for statistically significant F_{ST} values and – for non-significant F_{ST} values. Lines in the matrix separate samples from different river drainages to facilitate comparison.

	E01	E04	E05	E06	E07	E08	J01	J03	J04	J05	G01	G02	SR
E01	0.000	-	-	-	-	+	+	+	+	+	+	+	+
E04	0.000	0.000	-	-	-	+	+	+	+	+	+	+	+
E05	0.000	0.000	0.000	-	-	+	+	+	+	+	+	+	+
E06	0.006	0.046	-0.062	0.000	-	+	+	+	+	+	+	+	+
E07	0.000	0.000	0.000	-0.259	0.000	-	+	+	+	+	+	-	+
E08	0.506	0.557	0.434	0.315	0.300	0.000	+	-	+	+	-	-	+
J01	0.551	0.593	0.494	0.395	0.391	0.225	0.000	+	-	+	+	+	+
J03	0.567	0.619	0.493	0.387	0.355	0.199	0.038	0.000	-	-	+	+	+
J04	0.563	0.607	0.501	0.405	0.391	0.231	0.062	-0.165	0.000	-	+	+	+
J05	0.663	0.696	0.619	0.511	0.545	0.395	0.336	0.070	0.112	0.000	+	+	+
G01	0.826	0.840	0.807	0.685	0.780	0.147	0.532	0.525	0.531	0.593	0.000	-	+
G02	0.627	0.653	0.593	0.489	0.536	0.007	0.358	0.352	0.373	0.467	0.053	0.000	+
SR	0.664	0.688	0.633	0.526	0.581	0.436	0.217	0.354	0.373	0.481	0.628	0.471	0.000

Table 4. AMOVA results for the COI data.

	Degrees of Freedom	Sum of Squares	Variance Components	Percent of Variation Explained
Between Rivers	3	19.79	.20655 Va	43.22%
Among sites in Rivers	9	5.039	.04326 Vb	9.05%
Within sites	103	23.49	.22806 Vc	47.72%

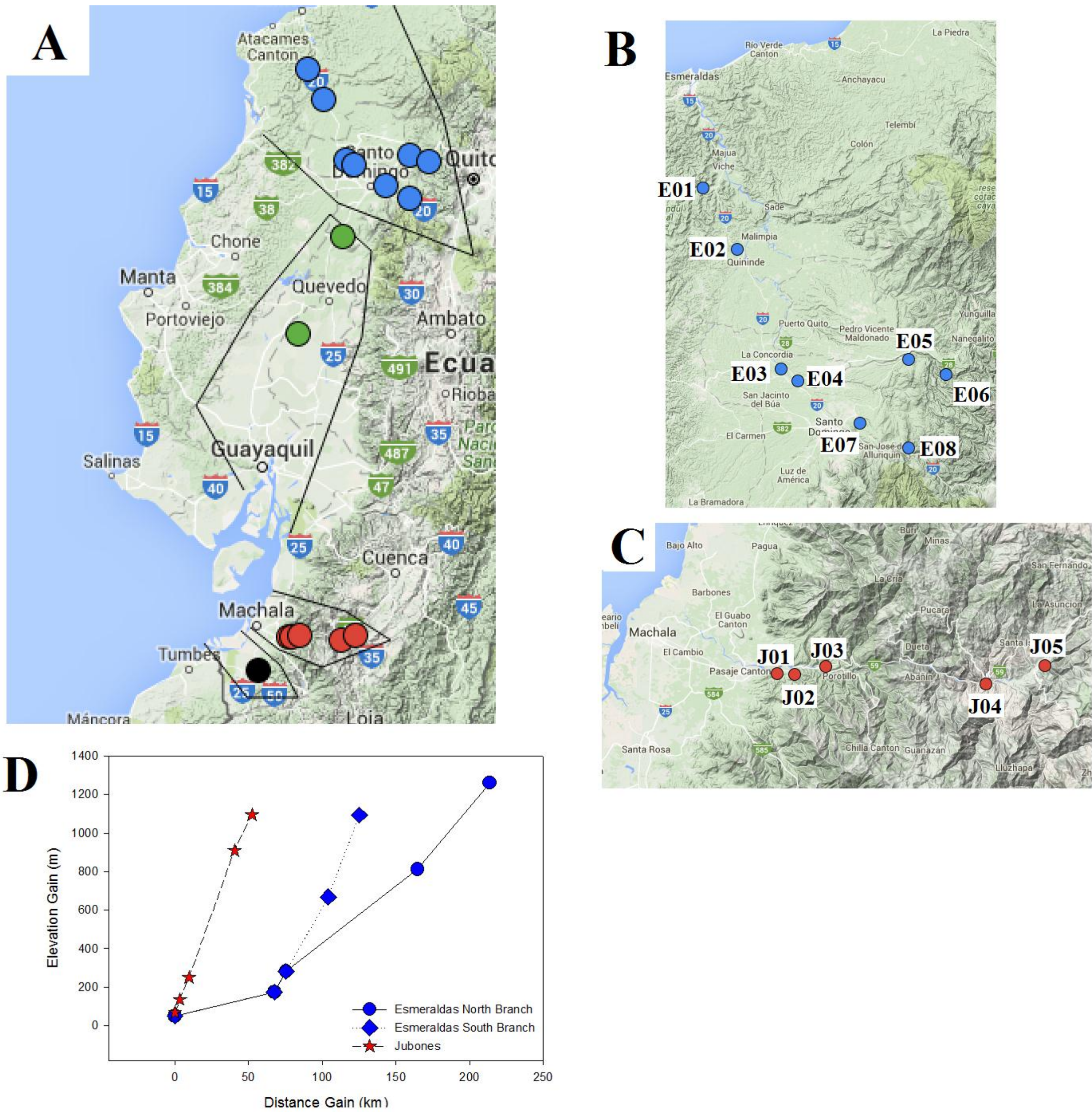

Figure 1. Maps of the sampling locations. A) A general map of where the rivers are located in Ecuador, The Esmeraldas, Guayas, Jubones, and Santa Rosa Rivers form North to South. Drainages are approximated for reference. B) Sites sampled in the Esmeraldas River, labeled. C) Sites sampled in the Jubones River, labeled. D) Plot of elevational gain over distance to visualize gradient.

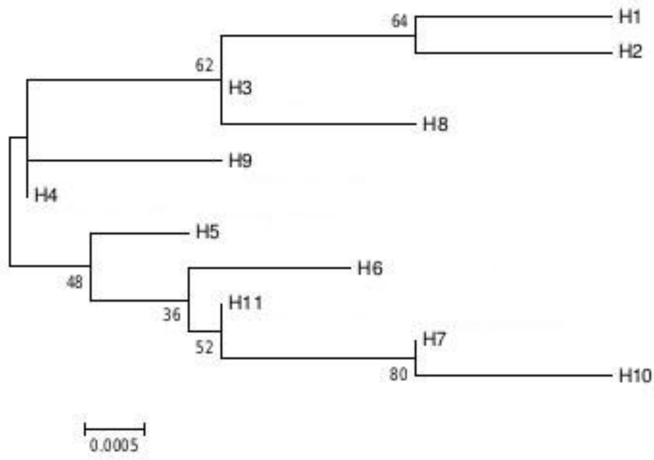

Figure 2. A neighbor-joining tree summarizing the relationship between the 11 haplotypes of *Rhoadsia sp*. Node numbers indicate the bootstrap support for each node.

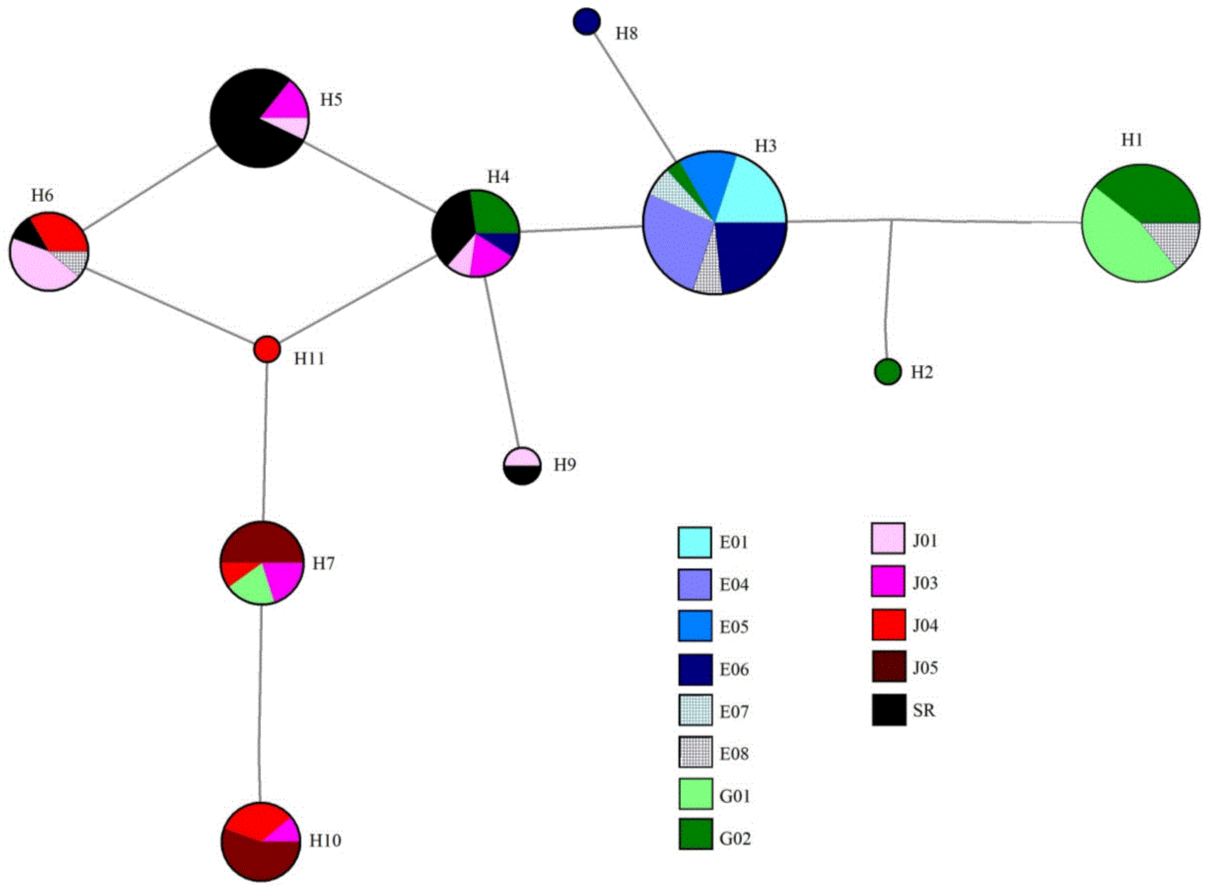

Figure 3. A network map of COI haplotypes of *Rhoadsia sp.* from the Esmeraldas, Guayas, Jubones, and Santa Rosa drainages. Nodes are proportional to the number of individuals sampled at each location and colored to represent the proportion from the given location.

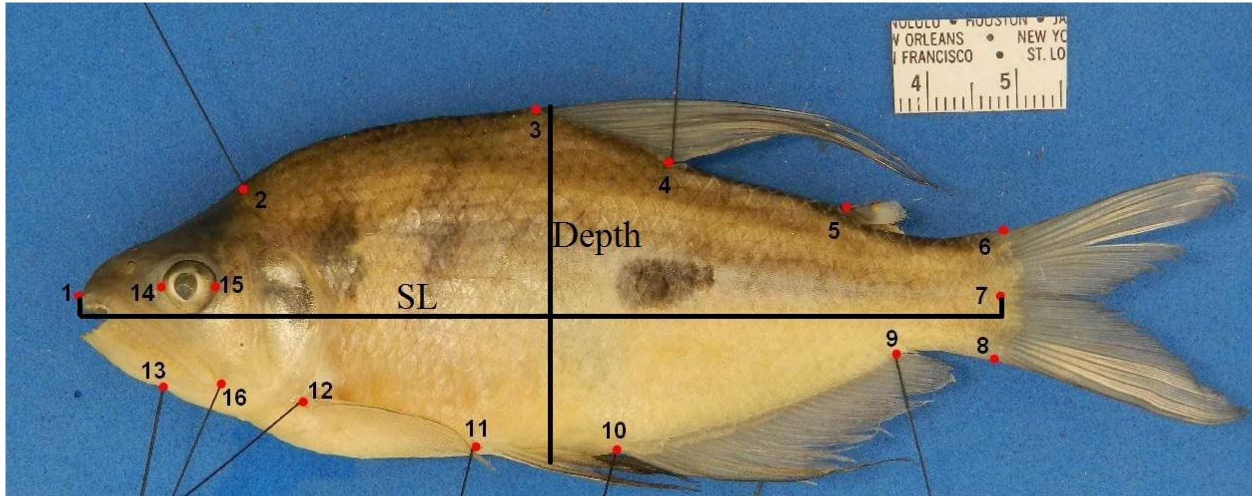

Figure 4. Landmarks used for morphological analysis. Standard length and body depth measures shown. Ruler included for scale.

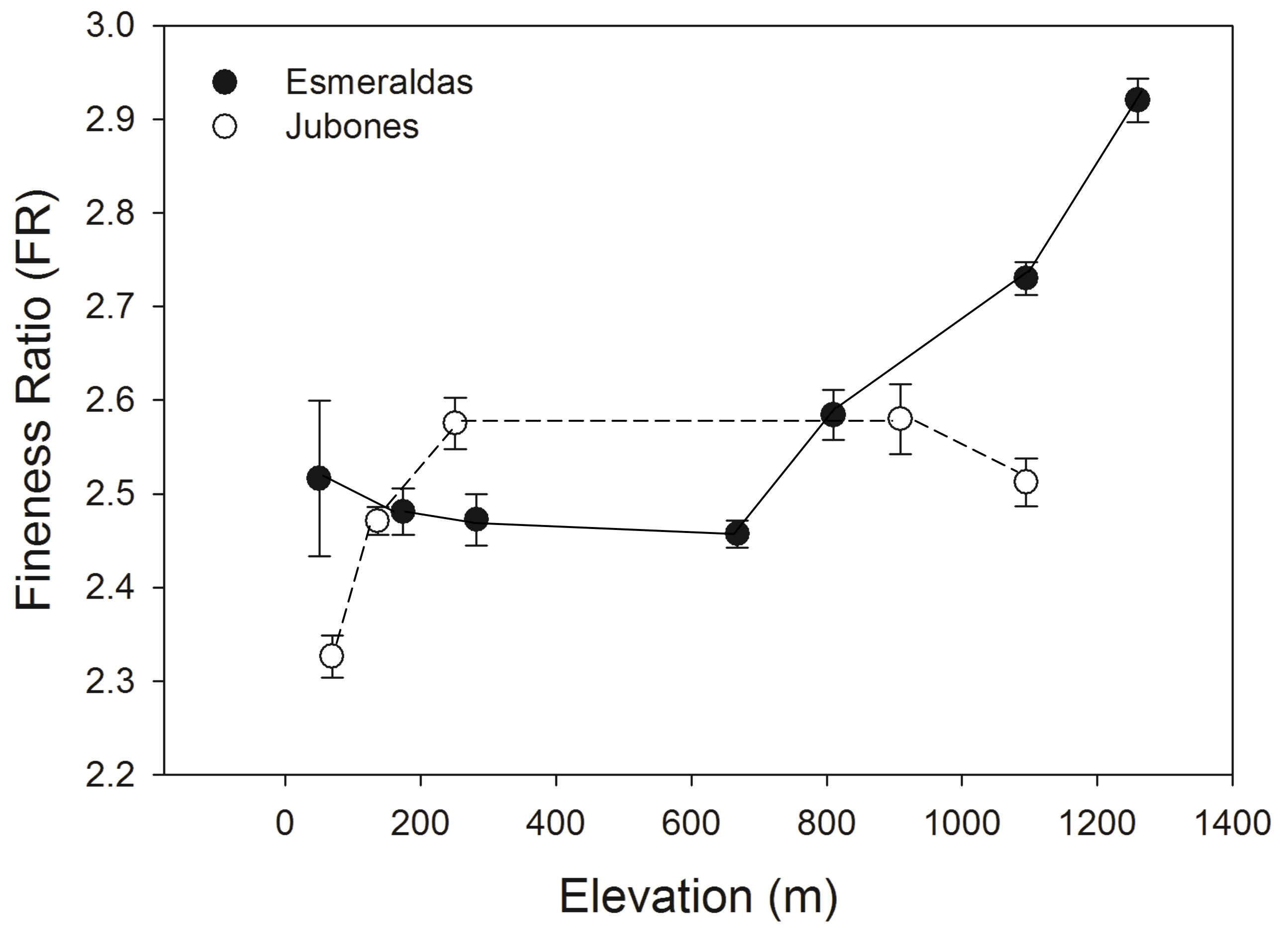

Figure 5. Plots of the mean Fineness Ratio (FR) for each sample location ordered by elevation with standard error bars.

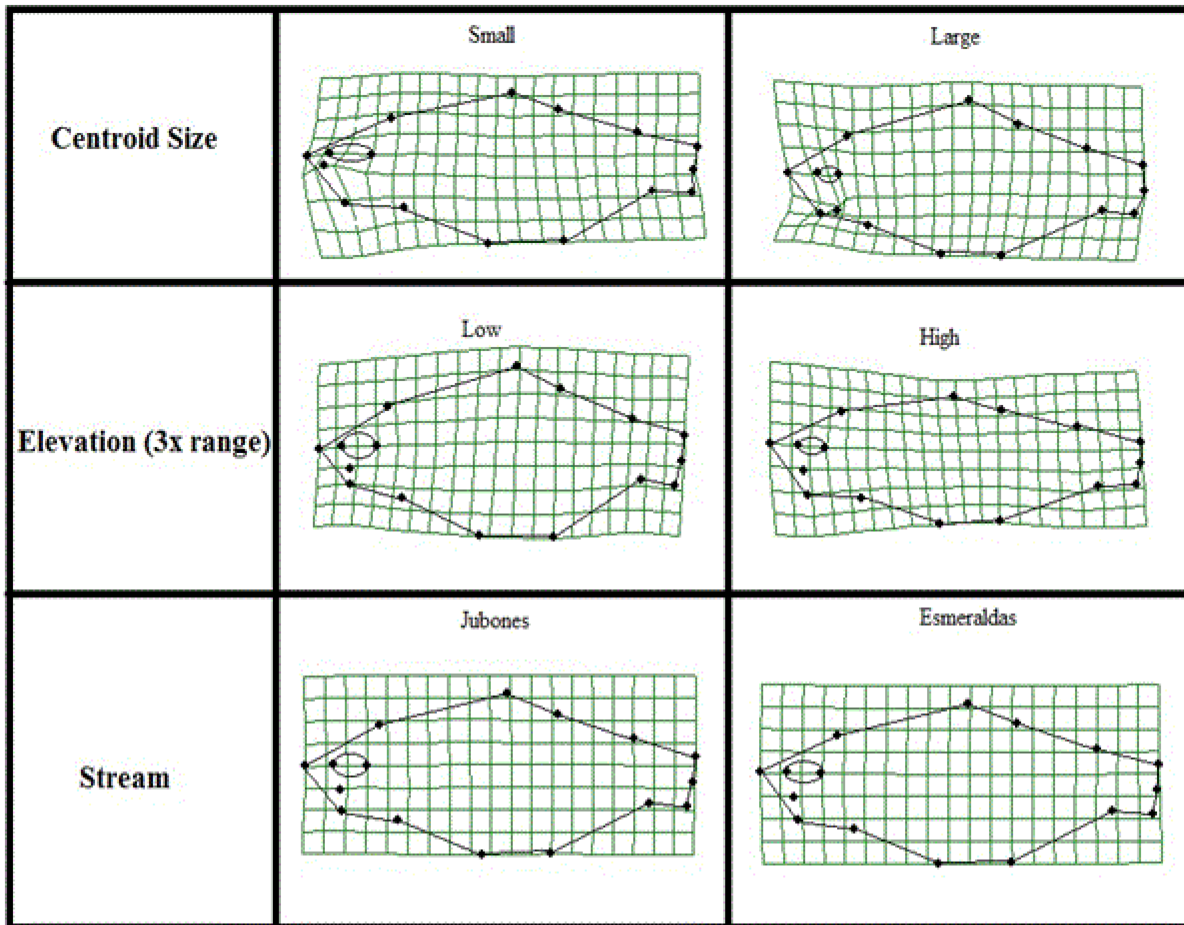

Figure 6. The deformation grids associated with a regression analysis of the relationship between body shape, elevation, centroid size, and river. Deformations are representative of the changes seen over the observed range for the centroid size and stream differences. The body shape deformation associated with elevation is presented with a 3 X exaggeration over the observed range to facilitate visualization.

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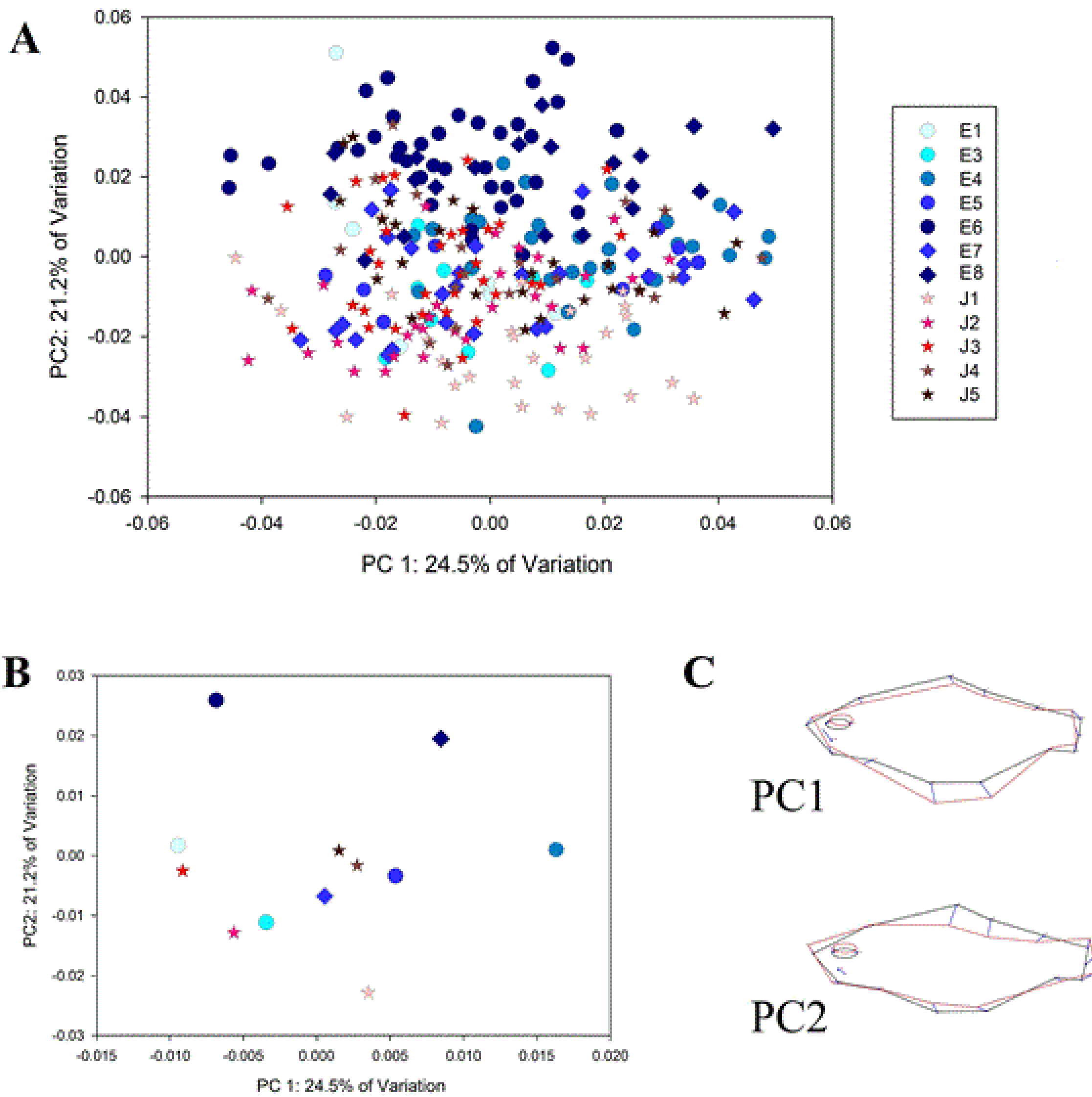


Figure 7. A PCA of all samples after size correction. A) The PCA with all data color coded by site with colors becoming darker with elevation. B) A graph displaying the site averages using the same color scheme. C) Wireframe graphs depicting the changes in body shape associated with PC1 (on top) and PC2 (on the bottom), going from negative scores depicted by the black figure to positive red scores depicted by the red figure.

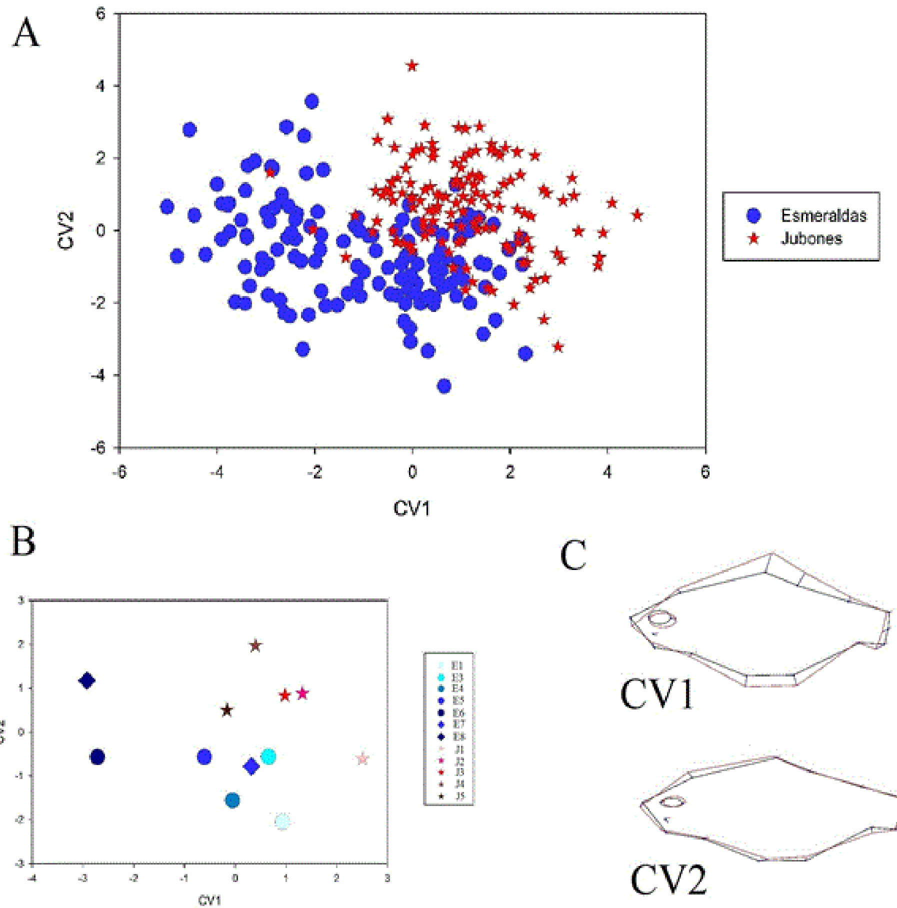


Figure 8. A) A CVA depicting the differences in body shape between the Esmeraldas and Jubones rivers. B) Average CV scores for each site. C) Wireframe graphs depicting the change in body shape associated with each CV axes from negative (black) to positive (red).

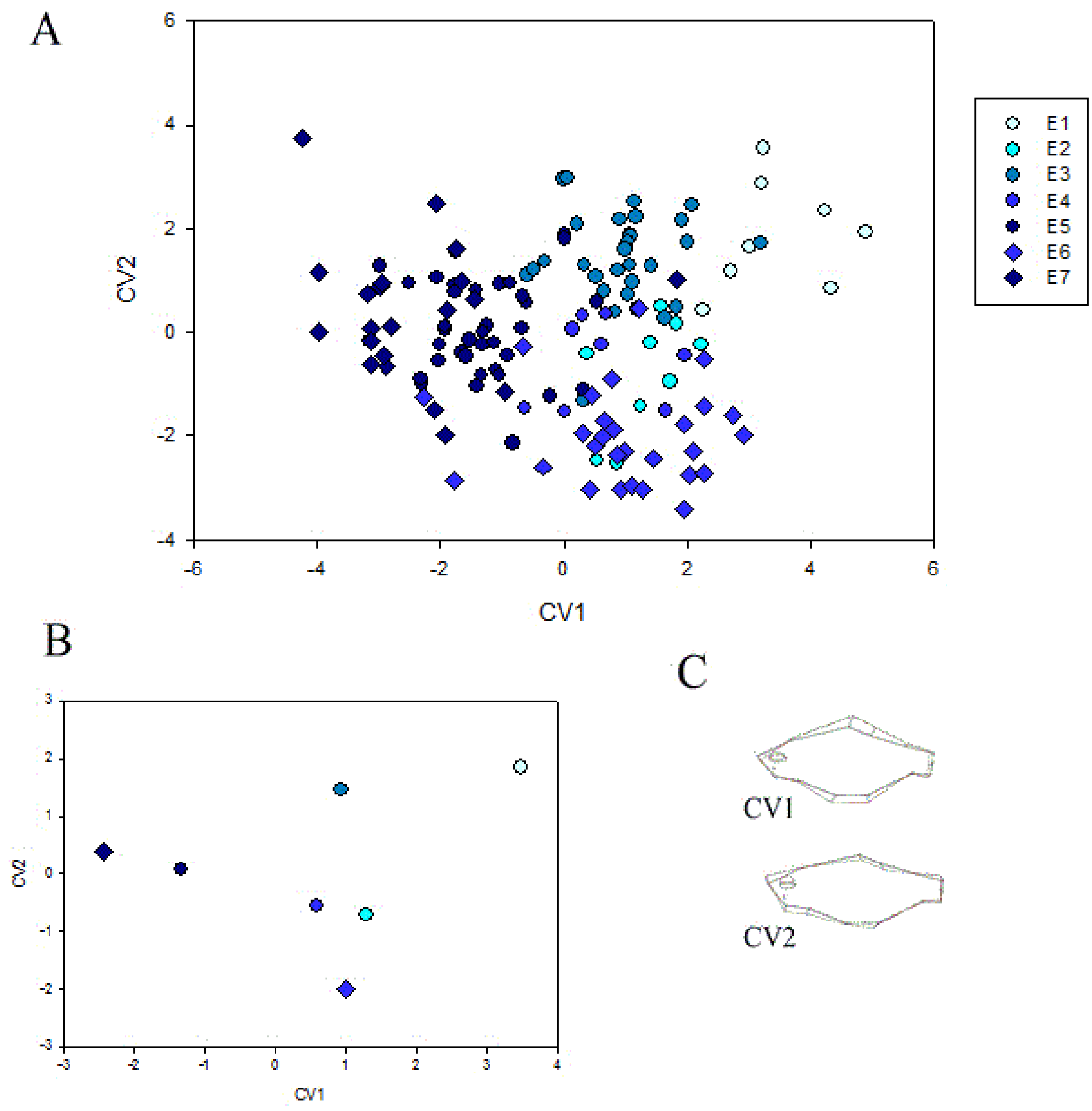


Figure 9. A) A CVA depicting the differences in body shape among sites in the Esmeraldas River. B) Average CV scores for each site. C) Wireframe graphs depicting the change in body shape associated with each CV axes from negative (black) to positive (red).

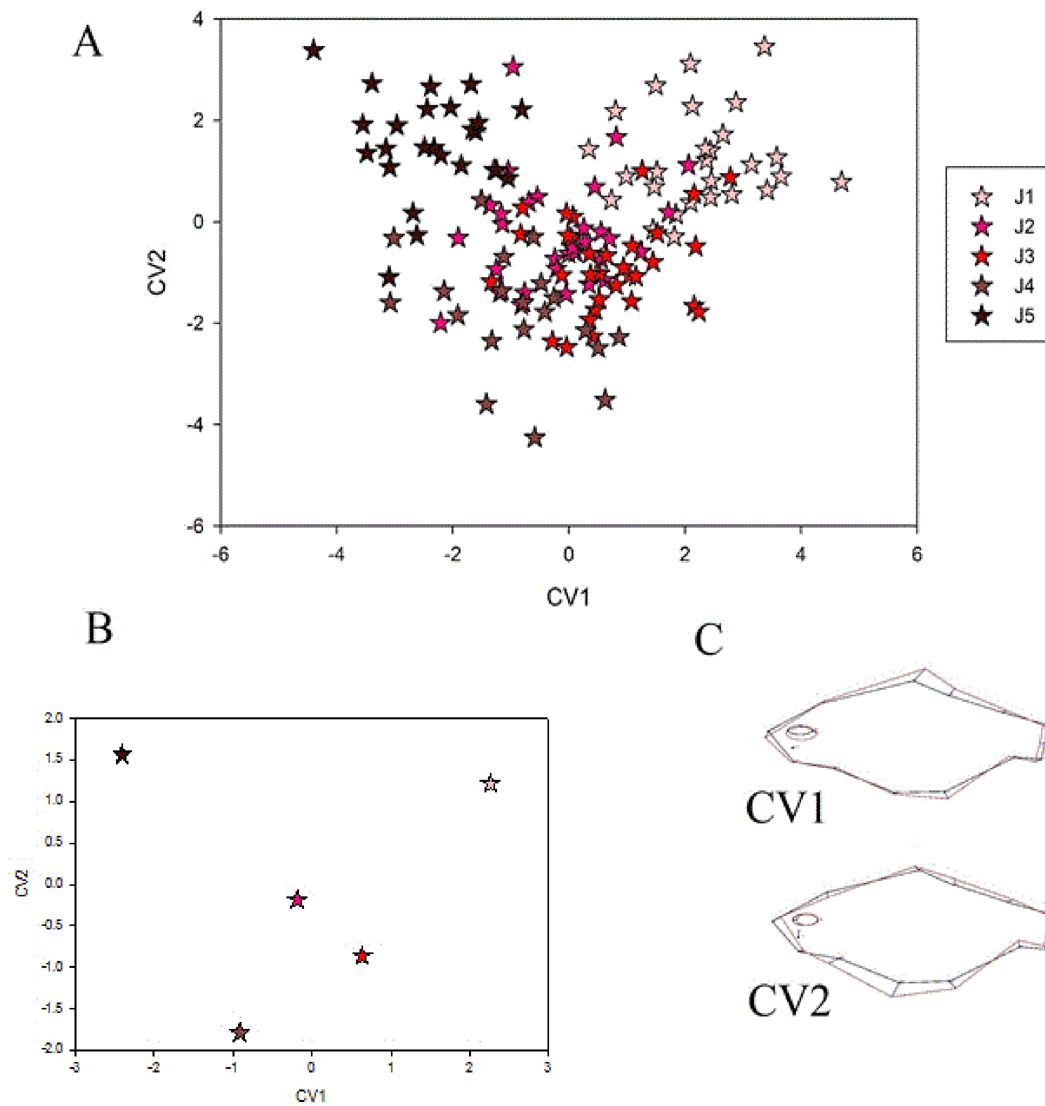


Figure 10. A) A CVA depicting the differences in body shape among sites in the Jubones River. B) Average CV scores for each site. C) Wireframe graphs depicting the change in body shape associated with each CV axes from negative (black) to positive (red).

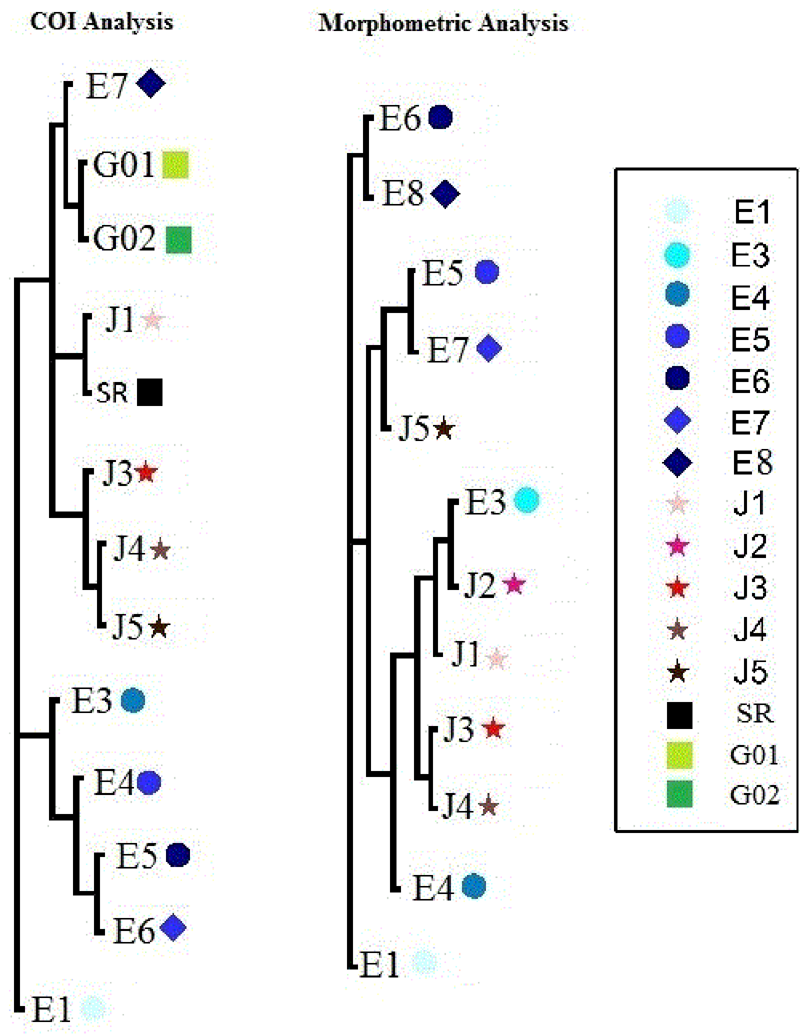


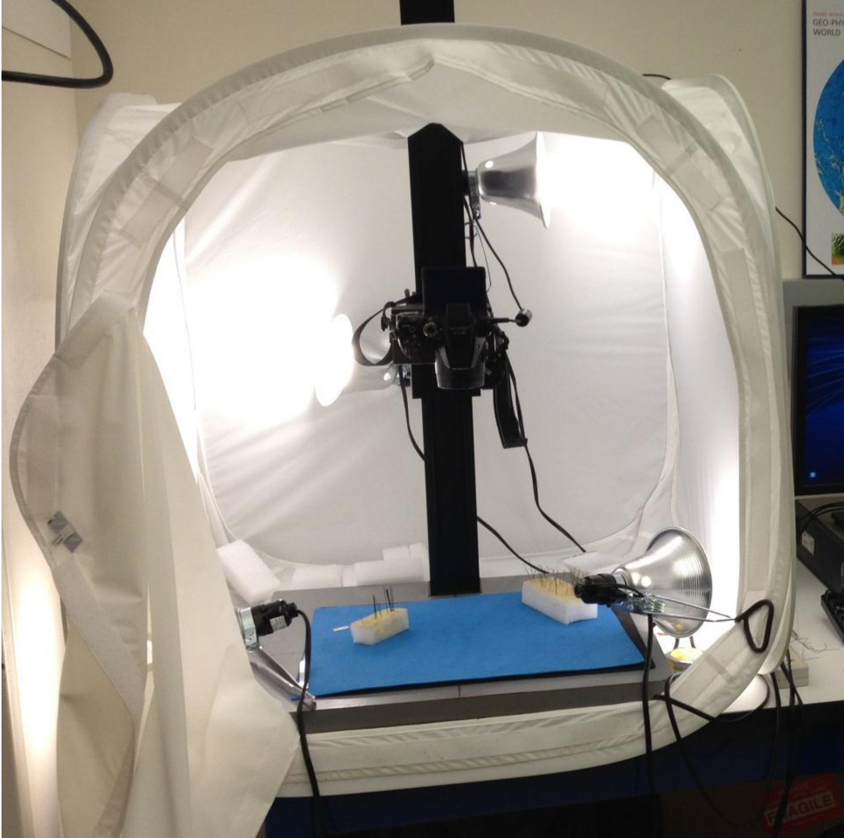
Figure 11. Two neighbor-joining trees comparing the divergence between sites. The tree on the left is calculated from the Euclidean distances of the size corrected sample mean PC scores for the geometric morphometric shape data. The tree on the right is constructed from the K2P distances between sample means calculated from the COI data.

Appendices

Appendix 1. A matrix showing the k2p distances between haplotypes. The distances were calculated in MEGA.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11
H1											
H2	0.0033										
H3	0.0033	0.0033									
H4	0.0050	0.0050	0.0017								
H5	0.0066	0.0066	0.0033	0.0017							
H6	0.0083	0.0083	0.0050	0.0033	0.0017						
H7	0.0083	0.0083	0.0050	0.0033	0.0050	0.0033					
H8	0.0050	0.0050	0.0017	0.0033	0.0050	0.0066	0.0066				
H9	0.0066	0.0066	0.0033	0.0017	0.0033	0.0050	0.0050	0.0050			
H10	0.0100	0.0100	0.0066	0.0050	0.0066	0.0050	0.0017	0.0083	0.0066		
H11	0.0066	0.0066	0.0033	0.0017	0.0033	0.0017	0.0017	0.0050	0.0033	0.0033	

Appendix 2. The copy stand used for sample photography.

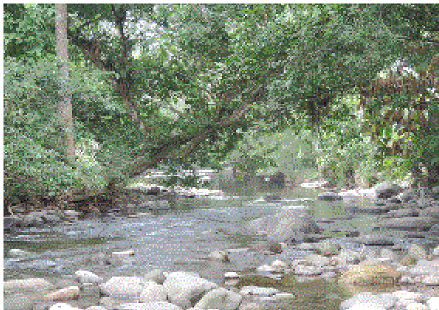


Appendix 3. A representative photograph from each of the Sites in the Esmeraldas.

E01 Afuera

E03 Rio Bravo

E04 Valle Hermoso



E05 Rio Blanco

E06 Mindo

E07 Meme Chico

E08 Transito

Appendix 4. A representative photograph from each of the Sites in the Jubones.

J01 Huizho

J02 Casacay

J03 Mollopongo

J04 Rio Minas

J05 Rio Mondur