MOLECULAR AND MORPHOLOGICAL EVIDENCE FOR MULTIPLE CRYPTIC CRAYFISH INVASIONS IN THE SOUTHERN APPALACHIAN MOUNTAINS

A Thesis

By

RAYMOND AMANDUS KESSLER IV

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APPROVED BY:

Dr. Michael M. Gangloff, PhD
Committee Chair

Dr. Robert P. Creed, PhD
Committee Member

Dr. Lynn M. Siefferman, PhD
Committee Member

Dr. Susan L. Edwards, PhD
Biology Department Chair

Dr. Edelma D. Huntly, PhD
Dean of the Cratis Williams Graduate School

Abstract

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Raymond A. Kessler IV
B.S., The Citadel
M.S., Appalachian State University

Chairperson: Michael M. Gangloff

The crayfish genus *Orconectes* is widespread in North America and most diverse in the southeastern river drainages. *Orconectes* includes numerous drainage or regional endemics as well as several species considered to be aggressive invaders of freshwater ecosystems. During the last decade, the invasive *O. rusticus* was reported from three western North Carolina and eastern Tennessee streams. I used mtDNA and morphological analyses to examine species boundaries in *Orconectes* populations in the southern Appalachian Mountains and assess the validity of morphological diagnoses of invasive populations. I sequenced and analyzed a portion of the cytochrome *c* oxidase subunit I (COI) gene and compared data with individuals collected from *O. rusticus'* native range in Kentucky as well as from GenBank specimen reference sequences. I evaluated the ability of dichotomous keys and quantitative morphological analyses to correctly classify specimens. Comparisons with reference sequences revealed a high level of cryptic diversity among populations. Furthermore, my data demonstrates the incongruence between using molecular data and morphological identifications for some crayfish taxa.

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Table of Contents

List of Tables

List of Figures

Foreword

Work contained within this thesis will be submitted to the journal Conservation Genetics for publication. My thesis has been formatted according to the style guide for Conservation Genetics.

Introduction

Invasive freshwater species pose a direct threat to ecosystem structure and biodiversity on a global scale (Lodge et al. 1998). Moreover, the potential economic and ecological impacts of the introduction of exotic species can be tremendous (Pimentel 2001). Crayfish (Decapoda: Astacidae, Cambaridae) are important components in many temperate freshwater communities; they play critical roles, in many communities and ecosystems (Creed 1994; Lodge et al. 1994). Because crayfish can dramatically alter the freshwater community in which they reside, exotic crayfish may dramatically affect colonized ecosystems (Momot et al. 1978; Nyström et al. 1996; McCarthy et al. 2006; Lodge et al. 2012). The ability to rapidly and reliably identify exotic crayfish species is important for the containment and management of biological invasions and will improve watershed management.

Although some crayfish species are easily identified, others are relegated to poorly resolved species complexes (Taylor 2000; Mathews et al. 2008; Filipová et al. 2010; Larson et al. 2012). The ability to differentiate morphologically similar members of species complexes is vital for conservation efforts. This is especially true for the '*juvenilis* complex,' which consists of both, know invasive species and a proposed undescribed endemic species. The '*juvenilis* complex' includes six species of the subgenus *Procericambarus*: *Orconectes cristavarius* (Taylor 2000), *O. juvenilis* (Hagen 1870), *O. putnami* (Girard 1852), *O. ronaldi* (Taylor 2000), *O. rusticus* and *O. spinosus* (Bundy 1887). The morphological similarity between members of this complex makes accurate species identification difficult and impedes the implementation of successful management plans.

Orconectes rusticus (Girard 1852), a member of the '*juvenilis* complex,' is an aggressive crustacean native to the upper Ohio River drainage that has become established in freshwater ecosystems in both the United States and Canada (Hobbs 1989; Dresser and Swanson 2012). *Orconectes rusticus*, or the rusty crayfish, is a benthic omnivore that competes strongly for food and shelter resources and may completely replace some native crayfish species (Hill and Lodge 1999; Wilson et al. 2004; Nilsson et al. 2011). Invasive crayfish, such as *O. rusticus*, could possibly affect multiple trophic levels and benthic community structure (Lodge and Lorman 1987; Wilson et al. 2004). Because of the profound effects that *O. rusticus* may have on freshwater community structure, accurate identification of the morphologically similar species within the '*juvenilis* complex' is necessary for the proper management of freshwater ecosystems.

The '*juvenilis* complex' was split apart and regrouped based on morphological variations and uncertainty about species distribution (Ortmann 1931; Hobbs 1972; Bouchard 1976; Taylor 2000). The most recent examination of the '*juvenilis* complex' by Taylor (2000) divided the members of this complex into two major groups based on mandible morphology. *O. juvenilis* and *O. rusticus* have blade-like mandibles whereas *O. cristavarius*, *O. putnami*, *O. ronaldi* and *O. spinosus* have serrated mandibles. Although *O. juvenilis* is morphologically similar to *O. rusticus*, it differs from *O. rusticus* in length of the first gonopod and the ratio of the central projection to gonopod length (Taylor 2000). Due to their morphological resemblance, *O. juvenilis* has been mistaken for *O. rusticus* in both the southeastern United States (personal communications CE Skelton; CE Williams) and Europe (Chucholl and Daudey 2008). *Orconectes rusticus* is a problematic invader but few studies have addressed the effects of *O. juvenilis,* or other members of this complex, on naive ecosystems.

Three crayfish populations that were tentatively identified as *O. rusticus* have been documented in western North Carolina over the past decade. Fullerton and Watson (2001)

reported the presence of *O. rusticus* populations in both the Little Tennessee (Swain County) and Broad Rivers (Rutherford County). These populations were believed to have originated from angler-introductions into the Fontana (Little Tennessee River) and Lure (Broad River) Reservoirs (Cooper and Armstrong 2007). However, in 2005 subsequent North Carolina Wildlife Resource Commission (NCWRC) surveys of the Little Tennessee and Broad River drainages did not detect any *O. rusticus* specimens (Simmons and Fraley 2010). In 2007, NCWRC surveys detected *O. rusticus* in the North Fork of the Catawba and Catawba Rivers in McDowell County, North Carolina. This is believed to be a sizable population as NCWRC personnel collected 41 individuals from four sites in 2011. This population has significant potential for expansion.

In Tennessee, four introduced populations of *O. juvenilis* are present in the Clinch, Cumberland, Holston and Nolichucky Rivers. According to the Tennessee Wildlife Resources Agency (TWRA), these populations appear to be spreading. Introduction of *O. juvenilis* into these ecosystems is most likely an artifact of bait bucket introductions; this hypothesis is supported by the distribution of *O. juvenilis* within the past 15 years as bait in a bait shop near the Holston River in Morristown, Tennessee (CE Williams personal communication).

The Cheoah Crayfish, *Orconectes (Procericambarus)* sp. cf. *spinous,* is considered to be an undescribed, endemic species that is only known to exist within five western North Carolina streams (Cooper and Russ 2012). Because other closely related *Procericambarus* crayfish have been introduced into these western North Carolina and eastern Tennessee areas, I chose to include the Cheoah Crayfish in my study.

The objectives of this study were to use molecular and morphological techniques to examine phylogeographic variability in putative *Procericambarus* taxa in the southern Appalachian Mountains. Specifically, I 1) examined whether the traditional morphological characteristics used to identify crayfish were able to accurately identify members of the

juvenilis complex, 2) examined whether the species in the *juvenilis* complex form monophyletic clades and 3) assessed whether the Cheoah Crayfish is an un-described endemic species or a population of an introduced species.

Materials and Methods

Specimen collection

Crayfish were collected between May 2010 and November 2013 using baited minnow traps, dip nets, and seines (Table 1). I conducted re-surveys of the sites where putative *O. rusticus* were detected in the Little Tennessee, Broad and Catawba Rivers. I collected 32 *Orconectes* specimens in Sawmill Creek (~1 km upstream of the confluence of the Little Tennessee River and Fontana Reservoir) near its confluence with the Little Tennessee River and in the Little Tennessee River proper, but did not encounter this taxon in any adjoining streams or further upstream in the Little Tennessee River. Surveys in the Broad River downstream of Lake Lure failed to detect additional specimens. In addition, I collected 20 *O. rusticus* from the Catawba River near US 221 in McDowell County, North Carolina.

Between August 2011 and February 2012, I collected 11 specimens of the Cheoah Crayfish from Panther Creek at the confluence of the Fontana Reservoir. I also collected 46 specimens from four tributary creeks to the Santeetlah Reservoir in western North Carolina (Table 1). No further specimens were encountered in any nearby tributaries. The vast majority of *Orconectes* spp. were collected within the first 50 m upstream of the stream-reservoir confluence. Few individuals were encountered upstream of this confluence.

For comparison, putative *O. juvenilis* specimens were obtained from four drainages in Tennessee by TWRA staff (Table 1). *Orconectes cristavarius* were collected from five locations in its native range, including 10 specimens from the New River in North Carolina and 12 specimens from four tributaries to the Kentucky River in Kentucky. Twenty-five *O. juvenilis* were also obtained from two tributaries to the Kentucky River in Kentucky.

Morphological analyses

Field identifications of the Little Tennessee and Catawba River *O. rusticus* specimens indicated that each population represented a morphologically distinct species. The Little Tennessee River population lacked the characteristic "rusty spot" that is typically observed on the carapace of *O. rusticus*, although it was present on Catawba River specimens. Furthermore, gonopod length of form I males differed between the two populations. To verify the identity of the collected specimens, I conducted qualitative morphological and genetic analyses on all collected populations and quantitative morphological analyses on the Little Tennessee, Broad and Catawba River populations. I was unable to detect *O. rusticus* in the Broad River; therefore, specimens from this locality are not included in genetic analysis. Museum specimens from North Carolina State Museum of Natural Sciences (NCSM) were included in morphological analyses.

In order to perform morphological analyses of form I males, I also obtained preserved specimens of each member of the *O. juvenilis* complex (except *O. ronaldi*, which I was unable to obtain) from the Auburn University Museum and the NCSM. I measured carapace, gonopod, and central projection lengths with digital calipers of all form I males to the nearest 0.1 mm, following Hobbs (1981). Morphological characteristics and ratios of measurements were used to assess species identification (qualitatively) based on a key provided by Taylor (2000). For the three North Carolina populations attributed to *O. rusticus,* I used principal components analysis to reduce morphological measures and ratios to orthogonal variables using a variation matrix. Principal Component 1 (PC_1) was plotted against PC_2 to examine discreet (quantitatively) population differences. Quantitative analyses on other populations were omitted as differences in samples sizes between populations and carapace lengths of form I males confounded statistical analyses.

Genetic analyses

I extracted total genomic DNA from gill tissue using a Qiagen dneasy kit (Valencia, CA). A 658 bp portion of the COI gene was amplified using primers LCO-1490 and HCO-2198 (Folmer et al. 1994). PCR products were then sequenced by RetroGen, Inc. (San Diego, CA) with an ABI 3730 DNA Analyzer (Applied Biosystems). Sequences were examined for the presence of pseudogenes, following recommendations made by Song et al. (2008) and Buhay (2009). I compiled and edited forward and reverse sequences in Sequencher 5.0 (Gene Codes Corp.). Next, I compared these sequences to GenBank data for each of the members of the *O. juvenilis* complex as well as other North American crayfish (Table 2).

I collapsed haplotypes in the final data set and aligned by muscle using MEGA5 (Tamura et al. 2011) and determined substitution models using jmodeltest2 (Darriba et al. 2012). I calculated pairwise differences between populations and constructed a maximum likelihood tree with 1000 bootstrap replicates using MEGA5. I then conducted a Bayesian Inference with MRBAYES v3.1.2 (Huelsenbeck and Ronquist 2001) by Metropolis-coupled Markov Chain Monte Carlo (MCMC). Estimates of *a posteriori* probabilities of different models were made using reversible-jump MCMC using both gamma and invariable site parameters which allows Markov chains to search the entire time reversible substitution model instead of relying on *a priori* substitution models (Miller and Bergsten 2012). Recent work suggests that relaxed molecular clock models may produce more accurate estimates of phylogeny than non-clock models for some data sets (Drummond et al. 2006; Miller and Bergsten 2012). To determine whether a relaxed molecular clock model could generate more accurate estimates of phylogenies than a non-clock model, I conducted stepping stone analysis to estimate marginal likelihoods for each model. For each model general parameters were kept the same and two independent searches started with random trees were run using three heated chains (temp=0.1) and one cold chain.

The relaxed molecular clock model, TK02 (Thorne and Kishino 2002) an auto-correlated continuous model, which uses rates of ancestral nodes as the mean of a lognormal distribution from which rates of descendent nodes are pulled. Stepping stone analysis was run utilizing 50 steps where each ran for 1,000,000 generations and sampled every 1000th generation with a burin in of -1. An alpha of 0.4 was used based on recommendation made by Xie et al. (2010). I generated a final tree using the best model from the stepping stone analysis with the same parameters and allowed it to run until split frequencies reached <0.01.

Results

Morphological analyses

Little Tennessee River specimens were morphometrically similar to *O. juvenilis.* Catawba and Broad River specimens were morphometrically similar to *O. rusticus* (sensu Taylor 2000). Principle component analysis run on the morphological data from the Broad, Catawba, and Little Tennessee River populations revealed two principal components that explained ~97% of the total variance in morphology (Table 3). Principal component 1 explained 64.4% of the total variance in morphology and size-related measurements loaded heavily on PC 1 (e.g., carapace length, gonopod and central projection length). Principal component 2 explained 32.5% of the total variance and ratios between morphological measurements loaded heavily on PC2. A plot of PC2 vs. PC1 (Fig. 1) shows distinct separation between *O. juvenilis* and *O. rusticus*. One group is comprised of Little Tennessee River *O. juvenilis* specimens and the other of Broad and Catawba River *O. rusticus* specimens.

In North Carolina, qualitative identification of the Little Tennessee River population demonstrated that this population was morphometrically similar to *O. juvenilis* and that the Catawba and Broad River populations were morphometrically similar to *O. rusticus* (see Taylor 2000). Further, the Catawba River population was the only sampled population with rusty spots on the side of the carapace associated with *O. rusticus*. Due to bleaching from ethanol preservation, no pigmentation was obvious on the Broad River populations.

Neither the Santeetlah Reservoir nor Panther Creek Cheoah Crayfish populations morphologically resembled *O. juvenilis*. I observed clear differences in mandible morphology. *O. juvenilis* has blade-like mandibles and the Cheoah Crayfish has serrated mandibles. The

Cheoah Crayfish more closely resembles *O. cristavarius* in morphology, coloration and the ratio central projection length to total gonopod length (mean=46.8, *N*=20, SD=2.0). All Tennessee populations resembled *O. juvenilis* morphologically. Only subtle variations in pigmentation and chela morphology were found between the Cumberland River population and populations from the Clinch, Holston, and Nolichucky Rivers.

Genetic analyses

Stepping stone analysis determined that the TK02 relaxed molecular clock model performed 118.3 2xLgBF better than the non-clock model and provided strong evidence that the relaxed molecular clock produced better estimates of phylogeny. Analysis of the COI gene resulted in similar topologies for both maximum likelihood and Bayesian analyses (Fig. 2). Three clades were discovered across all sampled populations (Fig. 2). Of the four morphologically distinct taxa sampled, only *O. cristavarius* and *O. rusticus* were found to be monophyletic (Fig. 2). The *Orconectes cristavarius* clade was well supported and coincided with qualitative morphological identification. Three of four Kentucky *O. cristavrius* populations shared haplotypes and all were closely related to New River (NC) populations (Table 4). The *O. rusticus* clade was comprised of only the Catawba River (NC) population and one *O. rusticus* Genbank sequence. Both the Bayesian inference and maximum likelihood analysis (Fig. 2) revealed a distinct divergence between the Catawba and Little Tennessee River populations.

Both the Cheoah Crayfish and *O. juvenilis* had incongruences between morphology and topology. The two Cheoah Crayfish populations in Panther Creek and Santeetlah Reservoir were paraphyletic and did not support individual clades (Fig. 2). The Panther Creek population had fixed haplotypes, while the Santeetlah Reservoir population included three haplotypes, two singleton haplotypes and one haplotype shared by 35 individuals across four localities. Additionally, the Panther Creek and Santeetlah Reservoir haplotypes were not shared.

However, both the Panther Creek and Santeetlah Reservoir populations grouped within the *O. juvenilis* clade and which also included both topotypic (Kentucky River Drainage) *O. juvenilis* populations, the Little Tennessee River (NC) population, the Cumberland River (TN) population and two Genbank sequences attributed to *O. juvenilis* (Fig. 2). The last clade consisted of the Clinch, Holston and Nolichucky river *Orconectes* populations, *O. cristavarius* and two *O. ronaldi* sequences accessioned to GenBank. The Clinch, Holston and Nolichucky River populations were represented by two haplotypes, one of which was shared across all populations (Table 4).

My calculations of pairwise differences resulted in similar conclusions as exhibited by both Bayesian and maximum likelihood analysis (Table 5). Of the material examined, excluding *O. spinosus* which had a high level of divergence from other specimens, the *juvenilis* complex had ~4.5% sequence divergence (95% Confidence Interval 4.08-4.80%). These estimates of pairwise difference coincided with clades discovered by Bayesian and maximum likelihood analysis.

Discussion

Distribution of Invasive Species

The introduction of invasive crayfish into southern Appalachian freshwaters is an under-reported but growing concern for the management of local biodiversity. For the increased efficiency of watershed management, identification of introduced species is necessary. Despite this necessity, morphological similarities, poorly defined species boundaries and possible morphological plasticity complicate species identification and, in turn, have led to the construction of poorly understood species complexes. Additionally, the morphological similarities between crayfish, such as those that exist between *O. rusticus* and *O. juvenilis*, illustrate the difficulty of relying on morphology alone for species identification and elucidating species boundaries.

Phylogenetic resolution of species complexes is further muddied by the information concerning historic introductions and whether purported endemic species may actually be a historically introduced species. This complication is exhibited by the presence of the Cheoah Crayfish, which was first discovered in 1980 (Cooper and Russ 2013). It is possible that this animal was introduced to areas that were under sampled and overlooked, and upon its discovery, led biologists to believe that this was an un-described, endemic species.

Additionally, unique environmental pressures may have induced morphological plasticity in some crayfish species, which may contribute to species misidentification. Misidentifications may prove to be costly, in both time and resources, as exhibited by the misidentification of introduced *O. juvenilis* as *O. rusticus* in Europe. Upon further investigation, this initial identification was corrected after more careful morphological and genetic analyses (Chucholl

and Daudey 2008; Filipová et al. 2011). For biologists to understand implications of species introductions, thorough investigations of crayfish species boundaries are imperative. My genetic and morphological data indicate that putative *O. rusticus* in the Little Tennessee River was actually an *O. juvenilis* population and is part of a still unresolved *juvenilis* species complex.

Both morphological and genetic data suggest that the Broad and Catawba River populations are *O. rusticus*. The Catawba River population is broadly distributed within the upper Catawba River upstream from James Reservoir (Pandolfi et al. unpublished data). The proximity of this population to a popular angling location suggests introduction via bait bucket. The Broad and Catawba River populations appear to be the only confirmed *O. rusticus* occurrences in the southeastern United States and, although other *Procericambarus* populations in the region were originally identified as *O. rusticus*, closer examination of these specimens have indicated that they are not true *O. rusticus* populations (CE Williams personal communication). *Orconectes rusticus'* range in the southeastern United States is presently limited to the Catawba River, yet the potential threat of invasive *O. rusticus* to stream community structure and function warrant continued monitoring of this population.

Although the establishment of exotic species into local freshwater ecosystems is a concern for biologists and managers alike, periodic sampling conducted by the NCWRC indicate that *Procericambarus* introductions are frequently (and fortunately) unsuccessful. For example, despite frequent follow-up sampling in the Broad River, no *O. rusticus* specimens were detected subsequent to 2002, suggesting that this population most likely failed to become established. It is possible that interspecific competition between *O. rusticus* and the region's species-rich native crayfish assemblage or differences in environmental factors between southern Appalachia and Ohio Valley streams where *O. rusticus* evolved may have led to the failure of this *O. rusticus* population.

Similar to southeastern *O. rusticus* populations, the Little Tennessee River *O. juvenilis* population appears to be highly localized. Despite my extensive surveys and additional sampling by the NCWRC in the upper Little Tennessee watershed, there is no evidence that *O. juvenilis* occurs beyond a short reach of the Little Tennessee River (~1 km) and a nearby tributary, Sawmill Creek. Populations are unknown from Fontana Reservoir but this deep, oligotrophic impoundment's crayfish fauna has not been extensively sampled.

Tennessee *O. juvenilis* populations (*n*=4) span two different clades. The Cumberland River population was qualitatively identified as *O. juvenilis* and genetically grouped with the *O. juvenilis* clade. The second clade from the Clinch, Holston and Nolichucky Rivers was morphologically identified as *O. juvenilis*; however, this clade was genetically most closely related to GenBank *O. ronaldi*. Clinch, Holston and Nolichucky River populations also had limited genetic diversity and included two haplotypes, one of which was shared by all populations within these three rivers.

Orconectes ronaldi has a narrow distribution range throughout western Kentucky and is morphologically distinct (based on form I male gonopods and mandible morphology) from the Clinch, Holston and Nolichucky River populations (Taylor 2000). Despite differences in mandible morphology and currently described range, my analyses suggest that the Clinch, Holston and Nolichucky populations are *O. ronaldi*, which in turn leads to the speculation that the *O. juvenilis* sold as bait near the Holston River may have actually been O. *ronaldi*. Although the species of the crayfish sold for bait is unknown, the marketing of crayfish for bait supports the hypothesis that many of these populations were introduced via bait buckets.

The Cheoah Crayfish

Taylor (2000) did not examine material from the Cheoah Crayfish, *Orconectes (Procericambarus)* sp. cf. *spinous*, but speculation amongst biologists is that this taxon is an un-

described species (Cooper and Russ 2012). This North Carolina endemic species is only known to exist within five western North Carolina creeks (Cooper and Russ 2012) and is morphologically similar to Taylor's (2000) description of *O. cristavarius* in mandible and gonopod morphology. Form I Cheoah crayfish males, like *O. cristavarius*, are observed to have a central projection that constitutes on average 46.5% (*N*=18, SD=1.9) of total gonopod length, which is similar to the 45-50% of total length metric described by Taylor (2000). The pigmentation of the Cheoah Crayfish also resembles Kentucky and North Carolina *O. cristavarius* populations. However, the Cheoah Crayfish appears distinct from *O. cristavarius* in my phylogenetic analysis (Fig. 2).

Despite pronounced differences in mandible morphology, the Cheoah Crayfish groups within the *O. juvenilis* clade. Both the Panther Creek and Santeetlah Reservoir populations were not distinct from *O. juvenilis* and likely do not represent a unique species but rather a population of an introduced but perhaps cryptic species. Both the Santeetlah Reservoir and Panther Creek populations appear to be more closely related to *O. juvenilis* in pairwise distances than to each other (Table 4). The Panther Creek population contained a single COI haplotype and the Santeetlah Reservoir population had three, one of which was shared by all four collection locations (Table 5). Finally, the Cheoah Crayfish has an odd geographic distribution. Specimens have been located in only five tributaries near their confluence with reservoirs. No Cheoah Crayfish populations have been encountered more than ~1 km upstream from reservoirs, suggesting that the Cheoah Crayfish was possibly introduced via bait buckets to these systems.

Juvenilis complex species boundaries

The conflicting genetic and morphological evidence encountered during this study of the *juvenilis* complex illustrates the challenges inherent in identifying species boundaries within

closely related species complexes. Although the presence of species complexes are widely recognized among North American crayfish taxonomists (Mathews et al. 2008; Larsen et al. 2012), their taxonomic and geographic boundaries are poorly understood. Sinclair et al. (2004), examined freshwater crayfish from across the globe and found that the COI gene is ~6% divergent among crayfish species. Although my estimates of COI sequence divergence among species are lower than that of Sinclair et al. (2004), this is the result of comparison of closely related groups of crayfish where as Sinclair et al. (2004) is based on crayfish taxa from across the globe. Using this >4.0% for defining species boundaries for the *juvenilis* complex further demonstrates that the *juvenilis* complex has incongruent morphological and genetic species boundaries (Table 5). The magnitude of historical and contemporary introductions further complicates analyses of genetic patterns within the *juvenilis* complex. Additionally, convergent morphologies resulting from environmental heterogeneity appear to be a problematic trend across many invertebrate taxa (Moore and Willmer 1997). Recent work on North American crayfish suggests that morphological characteristics commonly used to identify crayfish (e.g., rostrum length, gonopod morphology) may be affected by convergent evolution and phenotypic plasticity (Crandall and Fitzpatrick 1996; Breinholt et al. 2012). As a result, mandible morphology, the trait most frequently used to separate *O. juvenilis* and *O. rusticus* from other members of the complex may be the result of convergent, rather than divergent, evolution (Fig. 2).

Although my study focused on elucidating the identity of introduced populations, it also provides insight into complex genetic structures of species flocks. Moreover, my research illustrates that morphological plasticity may encourage biologists to construct inaccurate species boundaries within species complexes. Future studies addressing species boundaries and relationships between morphology, gene flow, and environmental factors should use geomorphometric landmarks to quantify morphological responses to habitat parameters across

each species range and provide a more comprehensive assessment of inter and intra-specific morphological diversity.

Management Implications

Quantitative morphological analysis of form I males was useful for distinguishing *O. rusticus* in the Catawba and Broad Rivers from *O. juvenilis* in the Little Tennessee River. This finding is expected as *O. rusticus* is distinguished by its much shorter gonopod and central projections compared to other members of this complex. For managers encountering these taxa in the field, an examination of central projections can provide a quick and reliable way to identify *O. rusticus* from other members of the complex.

Genetic and morphological data confirm the presence of introduced *O. rusticus* in the Catawba and Broad Rivers. Although I was unable to resolve the likely origins of these introduced populations, I discovered that members of the *O. juvenilis* complex have likely been introduced to numerous southern Appalachian streams including tributaries of Fontana and Santeelah Reservoirs. All populations examined occur in close proximity to reservoirs and likely represent bait bucket (i.e., angler-mediated) introductions. It is possible that this and other historical introductions may be confounding our understanding of crayfish diversity and distributions and closely related species complexes may be especially problematic. Future surveys for invasive crayfish in the southeastern United States should target reservoirs and transitional habitats between reservoir and stream ecosystems, as these habitats appear to facilitate the establishment of invasive crayfish.

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Collected material	State	County	Watershed	Water body	Lat	Long
Orconectes cristavarius **Serrated mandibles**	KY	Lee	Kentucky R	Blaine Br	37.57276	-83.68169
	KY	Breathitt	Kentucky R	Hurst Fk	37.66894	-83.41686
	KY	Wolfe	Kentucky R	Mandy Holland Fk	37.67855	-83.34654
	KY	Breathitt	Kentucky R	Lower Negro Br	37.63549	-83.38718
	NC	Watauga	New R	South Fk	36.20653	-81.65171
Orconectes juvenilis **Strait mandibles**	TN	Campbell	Cumberland R	Elk Fk	36.53894	-84.17992
	TN	Grainger	Clinch R	Indian Cr	36.39328	-83.40726
	TN	Hawkins	Holston R	Big Cr	36.46413	-82.9368
	TN	Hamblen	Nolichucky R	Bent Cr	36.20848	-83.13418
	NC	Swain	Little Tennessee R	Little Tennessee R	35.35553	-83.50640
	KY	Madison	Kentucky R	Tates Cr	37.7792	-84.386
	KY	Madison	Kentucky R	Silver Cr	37.6918	-84.3611
Orconectes spcf spinosus **"Cheoah Crayfish" Serrated mandibles**	NC	Swain	Little Tennessee R	Panther Cr	35.39473	-83.62305
	NC	Graham	Cheoah R	Snow Bird Cr	35.30965	-83.85502
	NC	Graham	Cheoah R	W Buffalo Cr	35.32798	-83.89428
	NC	Graham	Cheoah R	Santeetlah Cr	35.36835	-83.9102
	NC	Graham	Cheoah R	Sweetwater Cr	35.32702	-83.79713
Orconectes rusticus **Serrated mandibles**	NC	McDowell	Catawba R	Catawba R	35.70718	-82.03465

Table 1. List of collection locations for crayfish specimens examined in this study. Taxa are reported based on their initial morphological diagnoses.

Specimen Identification	Genbank Number
Cambarus bartonii	JX514435
Cambarus striatus	JX514447
Orconectes juvenilis	AY701233.1
Orconectes juvenilis	JF437985.1
Orconectes luteus	JX514454.1
Orconectes negelectus	JX514455.1
Orconectes ronaldi	AY701247.1
Orconectes ronaldi	JX514456.1
Orconectes rusticus	AY701248.1
Orconectes spinosus	AY701251.1
Procambarus clarki	JF737582.1
Procambarus paeninsulanus	JF737557.1
Pacifastacus leniusculus	EU921148

Table 2. List GenBank accession numbers for specimens included in analyses of DNA gene fragments.

Variable	PC 1	PC 2
Carapace Length	0.978	
Right Central Projection Length	0.847	
Left Central Projection Length	0.876	
Right Gonopod Length	0.992	
Left Gonopod Length	0.993	
Right CPL/PL		0.976
Left CPL/PL		0.982
% of total variation	64.4	32.5

Table 3. PCA Loading factors from a principal components analysis.

Principal component loading factors and associated eigenvalues for each morphological measurement and ratio (CPL = Central Projection Length, PL = Pleopod Length). Principal component 1 was closely related to size and explained 64.4% of the total variance. Principal component 2 was closely related to the ratios of measurements and explained an additional 32.5% of the total variance.

Taxa Collected material	State	Watershed	Water body	Number of haploypes	Populations with Shared Haplotypes
Orconectes cristavarius **Spiny stream crayfish**	KY	Kentucky R	Blaine Br	2	Hurst, L. Negro
	KY	Kentucky R	Hurst Fk	2	Blaine, L. Negro
	KY	Kentucky R	Mandy Holland Fk	1	X
	KY	Kentucky R	Lower Negro Br	2	Blaine, Hurst
	NC	New R	South Fk	2	X
Orconectes juvenilis **Kentucky River crayfish**	TN	Cumberland R	Elk Fk	3	X
	TN	Clinch R	Indian Cr	2	Nolichucky, Clinch
	TN	Holston R	Big Cr	2	Nolichucky, Holston
	TN	Nolichucky R	Bent Cr	1	Clinch, Holston
	NC	Little Tennessee R	Little Tennessee R	5	X
	KY	Kentucky R	Tates Cr	2	Silver
	KY	Kentucky R	Silver Cr	2	Tates
Orconectes spcf spinosus **Cheoah Crayfish"**	NC	Little Tennessee R	Panther Cr	1	X
	NC	Cheoah R	Snow Bird Cr	1	All Cheoah R populations
	NC	Cheoah R	W Buffalo Cr	2	All Cheoah R populations
	NC	Cheoah R	Santeetlah Cr	1	All Cheoah R populations
	NC	Cheoah R	Sweetwater Cr	2	All Cheoah R populations
Orconectes rusticus **Rusty crayfish**	NC	Catawba R	Catawba R	4	*O. rusticus* AY701251.1

Table 4. List of unique haplotypes encountered in study populations.

Species names are the qualitative morphological identities from each population. The number of haplotypes sampled at each collection location and collection locations where shared haplotypes where found. X in the shared haplotype column indicates that the location did not share haplotypes with any other location.

Taxon	A	B	C	D	E	F	G	H	I	J	K	L	M
A. *O. cristavarius* KY	0.6												
B. *O. cristavarius* NC	0.7	0.2											
C. *O. juvenilis* AY701233.1	4.1	4.4	x										
D. *O. juvenilis* JF437985.1	4.2	4.6	0.5	x									
E. *O . juvenilis* KY	4.9	5.2	2.3	2.6	0.3								
F. *O . juvenilis* Little Tennessee R.	4.4	4.6	0.5	1.0	2.7	0.6							
G. *O . juvenilis* Clinch Holston Nolichucky	4.2	3.7	4.4	4.6	5.5	4.6	0.2						
H. *O . juvenilis* Cumberland R.	5.2	5.4	3.1	3.6	3.4	3.5	5.7	0.3					
I. *O . juvenilis* Panther Cr.	4.8	5.0	2.4	2.6	3.0	2.8	4.8	3.9	x				
J. *O . juvenilis* Santeetlah Res.	4.7	4.7	1.9	1.8	2.5	2.3	4.2	3.0	3.0	0.2			
K. *O. ronaldi* AY701247.1	5.6	5.1	6.3	6.6	6.0	6.5	3.2	6.7	5.6	5.3	x		
L. *O. ronaldi* JX514456.1	5.3	4.8	6.1	6.3	6.7	6.4	3.0	6.9	5.2	5.2	4.9	x	
M. *O. rusticus* Catawba R. + AY701248.1	5.6	5.3	4.8	5.0	5.1	5.1	6.0	6.2	6.0	5.5	7.1	6.3	0.5
N. *O. spinosus* AY701251.1	9.2	8.6	9.1	9.8	9.8	9.5	8.7	10.6	9.1	9.2	9.6	9.5	10.1

Table 5. Estimates of COI sequence divergence between groups.

The number of base differences per site from averaging overall sequence pairs between groups. The analysis involved 44 nucleotide sequences. Positions on the diagonal (shaded) represent pairwise divergence within sampled populations. All positions containing gaps and missing data were eliminated. There were a total of 657 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

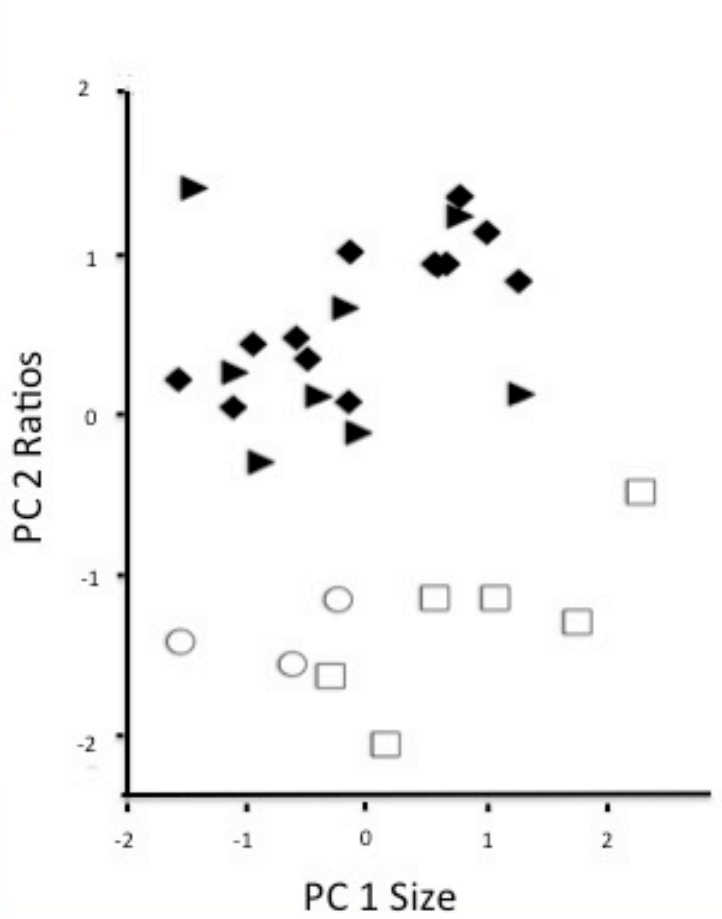

Figure 1. Scatter plot of PC2 (ratios of morphological measurements) vs PC1 (body size) generated using SPSS 17.0. *Orconectes juvenilis* is represented by open symbols: Little Tennessee River (LTR) = open squares and Kentucky (KY) = open circles. *Orconectes rusticus* is represent by solid symbols: Broad River (BR) = solid triangles and Catawba River (CR) = solid diamonds.

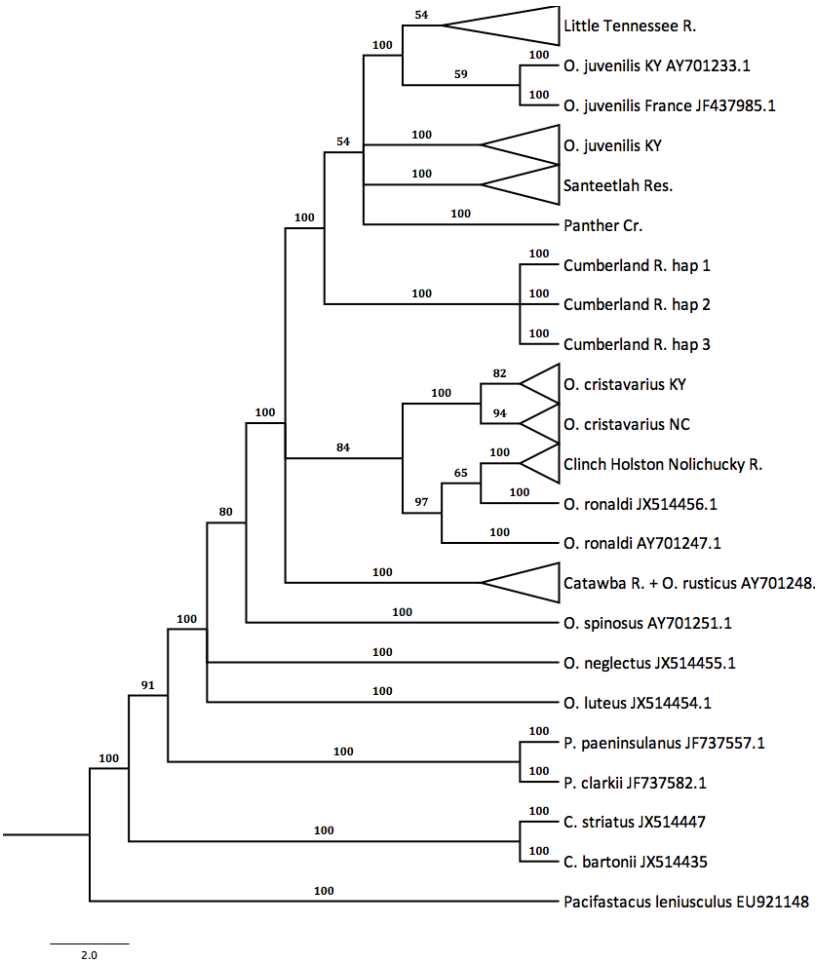

Figure 2. Results of Bayesian inference and maximum likelihood analysis of crayfish CO1 mtDNA. Posterior support is provided as 0.00-1. Nodes with non-significant posterior support or less than 0.50 were collapsed and renamed with the populations they represent. The scale represents the estimated divergence.

VITA

Raymond A Kessler IV was born in Charlotte, North Carolina, to Dr. Raymond and Lois Kessler. He received a Bachelor of Science degree from The Citadel, The Military College of South Carolina in August 2008. After completing his Master's degree in biology, Ray intends to pursue a Doctorate degree in Medicine.