Post-orbital color pattern variation and the evolution of a radiation of turtles (Graptemys)

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Post-orbital color pattern variation and the evolution of a radiation of turtles

(Graptemys)

by

Erin M. Myers

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ABSTRACT

One of the most studied areas in the field of evolutionary biology is the formation and maintenance of new species, as well as variation in the rate and extent to which taxa radiate. A range of evolutionary processes, from ecological adaptation to sexual selection and reinforcement, can lead species formation. However, the generation of new species likely results from several isolating mechanisms acting in concert. The map turtle complex (genus: *Graptemys*) is an excellent model system for exploring the nature of speciation given its exceptional species richness and morphological diversity, particularly in facial coloration patterns. This research utilizes an integrative approach to establish the role of post-orbital color patterns in species diversification and maintenance. This multi-faceted approach will incorporate phylogenetics, population and quantitative genetics, morphometrics, and behavior to assess morphological evolution within species and across the genus. The phylogeny of map turtles was characterized by a hard polytomy indicating rapid speciation. Across the genus, morphological evolution occurred parsimoniously. Within species, both morphology and genetics exhibited a pattern of isolation by distance. Temperature significantly influences coloration patterns and multivariate heritability was generally low. Finally, in behavior trials, neither males nor females spent significantly more time with members of their own species. In all projects, the signatures of sexual selection or reinforcement were absent or equivocal where they would be expected if they were the main forces continuing to shape interactions among map turtle species. The results of this research indicate that role of past and on-going selection on coloration pattern within the map turtle clade has been limited, thus post-orbital coloration was not the driving factor in the radiation of this turtle clade. Alternative explanations for map turtle species richness are explored.
CHAPTER 1. INTRODUCTION

One of the most deeply studied areas in the field of evolutionary biology is the formation and maintenance of new species, even 150 years after Darwin first explored this original puzzle (Darwin 1859). In particular, much research has focused on the considerable variation in the rate and extent to which taxa radiate (e.g. Stanley 1998; Arnqvist et al. 2000; Coyne and Orr 2004; Xiang et al. 2004; Kozak et al. 2005). For instance, what processes allow some taxa to form diverse, species-rich lineages (e.g. *Anolis*; Losos 1994), while other clades have remained relatively species-poor over long periods of time (e.g. *Moloch*; Hugall et al. 2008)? A range of evolutionary processes, from ecological adaptation to sexual selection and reinforcement, can lead to the formation of new species and entire books have been devoted to the subject (e.g. White 1978; Howard and Berlocher 1998; Coyne and Orr 2004). However, while individual processes have been studied extensively, their combinations have been less so. The formation of new species likely results from several isolating mechanisms acting in concert, rather than the evolutionary force of one mechanism acting alone (Streelmann and Danley 2003).

Several mechanisms are considered driving forces in speciation processes including sexual selection, genetic divergence, geographic isolation and, potentially reinforcement. Synergistically, these forces can work to produce a host of new species. Geographic isolation can limit gene flow among populations of a given species by restricting migration opportunities (Futuyma and Mayer 1980; Coyne and Orr 2004). Over time, genetic and/or phenotypic differences may accumulate through drift (e.g. Illera et al. 2007; Irwin et al. 2008) or local adaptation (e.g. Mimura and Aitken 2007; Sherman and Ayre 2008). These
differences can be accelerated through differential reproductive success, which in part may be driven by changes in sexually selected signals (Tregenza 2002). Finally, when these isolated populations are in contact once more, reinforcement can strengthen reproductive isolation in response to selection against the reduced fitness of hybrid offspring leading to the formation of two separate species (Servedio and Noor 2003). Selection that leads to reinforcement acts through pre-zygotic isolation to reduce the likelihood of these disadvantageous matings through modifications in signals related to mate identification, courtship and mating behaviors, as well as zygote inviability (Coyne and Orr 1989). Previous work has shown that modifying courtship signals can dramatically influence mating success (Ryan and Rand 1993; Sætre et al. 1997).

This model of speciation makes a series of testable predictions and experiments that can be designed to tease apart these mechanisms and identify evolutionary processes in a particular clade. Reinforcement results in a consistent pattern of higher mate discrimination in sympatric taxa than between allopatric taxa (Coyne and Orr 1997; Noor 1999). Pre-zygotic isolation can increase more quickly at relatively lower genetic distances than post-zygotic isolation, and evolves much more rapidly in sympatric species pairs than in allopatric species (Coyne and Orr 1997). Reproductive character displacement, where there is greater divergence of a sexually selected trait in sympatry than in allopatry, can also result (Noor 1999). While this concept has been widely discussed, its importance in maintaining species boundaries remains controversial (Servedio and Noor 2003; Coyne and Orr 2004). However, reinforcement and reproductive character displacement are eminently testable in comparisons of geographic, genetic, and phenotypic distances among sympatric and allopatric species pairs.
Species radiations, clades with exceptional species diversity relative to sister taxa, provide a unique opportunity to infer the patterns and processes that contribute to species formation. In particular, morphological diversification has often been linked with species radiation (e.g. *Anolis* lizards, Darwin’s finches, and cichlid fish; Losos 1994; Jackman et al. 1997; Schluter 2000), though its role as a driver of speciation versus a by-product can be difficult to establish. Indeed, morphology may contribute to species formation via two means, ecological specialization and diversification through natural selection or, alternatively, by sexual selection on morphological traits tied to species recognition or mate choice (Streelman and Danley 2003; Coyne and Orr 2004). During ecological speciation, speciation occurs via adaptation to utilize different resources which is often accompanied by morphological differentiation. This process has contributed to the diversification of Darwin’s finches and *Anolis* lizards which differ in their food resources and perch types respectively, leading to divergent morphology (Losos et al. 1997; Grant and Grant 2006). In contrast, morphological and other types of phenotypic traits (e.g. acoustic, chemical) may contribute to species formation and maintenance through sexual selection and the trait’s role in species recognition and mate choice (Ryan and Rand 1993; Sætre et al. 1997; Rudh et al. 2007; Seddon et al. 2008). Such sexual selective driven diversification may be the factor contributing to the explosiveness of the cichlid radiation relative to other species radiations (Streelman and Danley 2003). However, differentiating between these two major selective regimes may be complicated by patterns of morphological convergence and often both natural and sexual selection contribute to diversification at different points in the formation of the radiation (Streelman and Danley 2003).
Phylogenetic perspective provides an important tool to tease apart these morphological hypotheses. Using this approach patterns of morphological and species diversification are examined across a phylogeny, rather than a more limited pair-wise comparisons (Harmon et al. 2003; Sueur et al. 2007; Seddon et al. 2008). In this way it is possible to discriminate between patterns of convergence or parsimonious morphological evolution (Revell et al. 2007). Additionally, by examining the relationship of other ecological variables across the phylogeny one can, in turn, differentiate between ecological speciation and speciation via sexual selection. Nonetheless, assessing patterns of morphological diversification within and between clades in this manner requires a resolved phylogenetic hypothesis regarding the relationships among species (Losos et al. 1998). However, recovering the phylogenetic history of rapidly diversified clades can be challenging as branch lengths are often short or poorly supported which can complicate interpretations of morphological evolution (Jackman et al. 1999; Sueur et al. 2007; Sanders et al. 2008).

In combination with a phylogenetic perspective, a comparative assessment of sympatric species pairs can serve as a model system for the entire clade to infer the patterns and processes of morphological evolution in relation to species recognition and sexual selection. Closely related sympatric species are expected to be under stronger selection to correctly identify an appropriate mate in order to minimize hybridization and the production of maladaptative offspring, maintaining the species barrier (Servedio and Noor 2003; Coyne and Orr 2004). It is in such species pairs, where populations occur in both sympatry and allopatry, where patterns of reinforcement should be most readily observable (Coyne and Orr 2004). In addition, the comparison of morphological characters within species between these population types facilitates the discernment of reproductive or ecological character
displacement (Sætre et al. 1997; Adams and Rohlf 2000; Adams 2004). Such species pairs constitute the cornerstone of analyses seeking to uncover the genetic mechanisms of reinforcement and speciation (e.g. Littlejohn 1965; Coyne and Orr 1997; Sætre et al. 1997; Noor 1999).

Intraspecific examinations of genetic, geographic and morphological relationships can also inform about the evolutionary mechanisms contributing to selection on and maintenance of morphological variation. Geographic distance or biogeographic barriers can limit migration events, which in turn limits gene flow between populations, potentially leading to gradual divergence due to differential selection or stochastic processes (Avise 2000). Species with specialized environmental requirements may be more susceptible to population structuring if suitable habitat is distributed non-randomly across the landscape (Nunney 1991). Geographic isolation and population fragmentation may also influence morphological variation and differentiation through a variety of mechanisms. Divergence in morphology may result from stochastic processes via the accumulation of random mutation (Illera et al. 2007), ecological character displacement resulting from competition for shared resources with other members of the community (e.g. Adams and Rohlf 2000; Losos 2000), or from divergence in mate preference and sexual selection for phenotypic character related to mate choice (e.g. Roberts et al. 2007; Rudh et al. 2007). Population divergence in mate preference at low levels of genetic divergence can mark the initial stages of the speciation process (Coyne and Orr 1989; Ryan and Rand 1993; Coyne and Orr 2004). Thus, exaggerated morphological divergence at small genetic and geographic distance within species can be indicative of reinforcement and strong sexual selection on phenotype (Coyne and Orr 1989, 2004).
While selection may be strong on phenotypic traits involved in species recognition, in order for an evolutionary response to occur there must be heritable genetic variation in the trait at hand (Falconer 1989). Without this additive genetic variation, natural and sexual selection can act on the present phenotypic variation, but there would be no concordant change in subsequent generations. However, phenotypes are often influenced by factors beyond strictly additive genetic variance. Indeed, non-additive genetic factors, such as dominance and epistatic effects, can interact with environmental factors as well as, maternal and paternal effects, to generate a host of offspring phenotypes (Pigliucci 2001; Carlborg and Haley 2004; Kaplan and Phillips 2006; Roff and Emerson 2006). For traits that are under strong selection, such as would be expected for traits relating to fitness or species recognition, the enhanced selection pressure may lower estimates of heritable genetic variation in a population as selection can act to decrease the available variation within the genetic pool (Mousseau and Roff 1987) but see (Geber and Griffen 2003). Similarly, high levels of phenotypic variation generated by environmental or non-additive factors will also result in lower heritability, given the low contribution of additive genetic variance to total variation. Genetics and environment can work in concert to produce a suite of phenotypes through phenotypic plasticity and genetic by environment interactions (Pigliucci 2001). Establishing the relative contributions of both heritable, additive genetic and environmental variations to the total phenotypic variation may provide additional insight on the strength of selection and/or the ability to respond to selection on traits potentially involved in recognition and species maintenance.

Finally, explicit examinations of behavioral responses to phenotypic traits provide a direct measure of these traits’ importance in sexual selection and species recognition.
Morphological cues, such as tail length and plumage color in birds, are classic examples of sexually selected traits (Møller 1988; Safran and McGraw 2004). Countless other morphological characters have also been examined in this light (e.g. Jones et al. 2002; Kemp 2007). However, other phenotypic characters including acoustic or chemosensory traits are also subject to natural and sexual selection on species recognition (Ryan 1980; Palmer et al. 2005). Comparisons of phenotype between sympatric and allopatric populations may show enhanced species recognition and discrimination in sympatric populations and relatively poor discrimination in allopatric populations consistent with reinforcement (Massie and Markow 2005). While reinforcement remains controversial in relative extent and contribution to speciation as a whole, its presence in at least some systems has been well documented (Sætre et al. 1997; Noor 1999; Massie and Markow 2005).

The map turtle complex (genus: *Graptemys*) is an excellent model system for exploring the nature of speciation and the potential role of morphological traits in contributing to the speciation process. The genus is the third most species-rich genus within turtles (Figure 2.1; (EMYSystem 2008) and thus represents an exceptional species radiation of turtles. Map turtles are almost entirely aquatic, rarely leaving the water except for short terrestrial forays by females, and are found through much of the central United States (Ernst et al. 1994). All members of the genus are long-lived with a lifespan of 30+ years. Males typically reach maturity at 4-7 years of age, while females may take 10+ years to reach maturity (Ernst et al. 1994). Several species within the clade are characterized by dietary specialization focused exclusively on a variety of molluscan prey, in contrast to the less specialized omnivore species (Ernst et al. 1994). Of the 12-13 currently recognized species, three are widely distributed, including *G. geographica*, *G. pseudogeographica*, and *G.*
ouachitensis (Ernst et al. 1994). The genus also exhibits high levels of river endemism, with nine species occupying a few or single river drainages (Ernst et al. 1994; Lamb et al. 1994). This localization has led to the listing of several species as threatened or endangered at the state, federal, and international levels (Moll and Moll 2004; US Fish and Wildlife Service 2008; Lechowicz 2008). Other aquatic species (fish and mussels) that are co-distributed have similar patterns of endemism (Wiley and Mayden 1985; O'Brien and Williams 2002). Interestingly however, other turtle species are found in these same drainages but do not exhibit the associated levels of speciation (Moll and Moll 2004), suggesting that something unique to map turtles may be contributing to their diversification.

In addition to species richness, map turtles are characterized by extraordinary morphological diversity. First are the post-orbital color patterns, which include a stripe of color along the nose and an area of color behind the eye that can range from a small dot of color, to a stripe, to a full mask of color across the face (Ernst et al. 1994). These patterns vary in color intraspecifically and range from white to yellow (E. Myers, personal observation). Map turtles are also characterized by extreme, female-biased sexual size dimorphism (Ernst et al. 1994; Lindeman 2008). Females of several species have enlarged heads and broad crushing surfaces along their jaws that correspond to dietary specialization on mollusks (Ernst et al. 1994; Lindeman 2008). However, these morphological and dietary specializations are found only in females, while males maintain a more omnivorous diet and small body size. Finally, both sexes in a subset of map turtles known as the sawbacks are characterized by exaggerated ridges along the carapace of the shell (Ernst et al. 1994). However, the functional role of these ridges has not been established. This suite of morphological characters has led to several map turtles being highly prized on the pet turtle
market which has contributed to populations declines (Moll and Moll 2004). Original species
descriptions of these turtles were based on morphological characters, particularly post-orbital
color pattern variations (see McKown 1972 for a review), which have led to a cluttered
taxonomic history with multiple species revisions and re-classifications.

The courtship in two species, *Graptemys ouachitensis* and *G. pseudogeographica*, has
been examined in detail and is consistent with that noted in other closely related emydid turtles.
This courtship consists of an initial interaction, a series of titillation bouts (males drumming their
foreclaws on the ocular region of the female), and copulation (Vogt 1980). These pre-copulatory
interactions occur face-to-face and, in most cases, will not proceed between heterospecific pairs
(Vogt 1978). However, males have also been observed near the cloacal region of females
allowing for the potential of chemical communication, in addition to visual signals (Vogt 1980).
These observations suggest that traits on the facial region may be important signals in species
identification and mate choice which suggests that post-orbital coloration may be important in
species recognition. Studies in closely related species have examined turtle vision and shown that
turtles can discriminate color (Venture et al. 2001) and thus should be able to discriminate among
different coloration patterns. Considerable variation exists between species, and in some cases
within species (Vogt 1980, 1993), however this variation is often reduced in areas of
sympatry such that each species has an identifiable, non-overlapping color pattern (Vogt
1980, 1993; Janzen et al. 1995). This pattern is suggestive of reproductive character
displacement, suggesting that reinforcement may be acting in this group to maintain species
differences.

In order to examine historical and current importance of post-orbital coloration in
species formation and recognition, solid phylogenetic framework is required to reconstruct
the levels of genetic differentiation between species, their times of divergence, and their relationships to one another (Avise 2000). As indicated earlier, map turtles have had multiple revisions during their taxonomic history. Initial characterizations of species relationships relied on post-orbital coloration and mega-cephaly to define two major clades of map turtles, narrow- and broad-headed (McKown 1972). However, these clades reflect dietary relationships more than evolutionary relationships among species. Subsequent assessments have included genetic traits including DNA sequence information (Lamb et al. 1994), as well as morphological characters (Stephens and Wiens 2003). The latter has established the position of map turtles relative to other emydid turtles with both molecular and morphological data supporting the sister taxa relationship of *Graptemys* with the monotypic genus *Malaclemys* (Stephens and Wiens 2003). In contrast to the well-supported relationship outside the genus, the relationships within the map turtle clade itself are generally less resolved (Lamb et al. 1994; Stephens and Wiens 2003). The phylogenies recovered three major lineages. Molecular and morphological data strongly support *G. geographica* as the sister taxon to the rest of the map turtle lineage, which is subsequently split into two major clades, the *pulchra* and *pseudogeographica* clades. However, the relationships of taxa within these clades remain unclear. Unfortunately, it is difficult to determine if the lack of resolution is a function of a limited molecular dataset, the relatively slow pace of molecular evolution in turtles (Avise et al. 1992), or if it reflects rapid species formation. None of these phylogenetic analyses, however, has considered morphological evolution of the post-orbital color pattern or its evolution within the genus. Thus, color pattern’s contribution to diversification of the genus remains unknown.
For the majority of species, relatively little is known about the extent of morphological and genetic differentiation across the species range. For species with limited ranges, differentiation and fragmentation is expected to be relatively low. In contrast, for the widely distributed species (Graptemys geographica, G. ouachitensis, and G. pseudogeographica) this expectation is likely unrealistic. In addition to natural evolutionary processes that can lead to differentiation among populations of the same species, anthropogenic changes to the landscape have created potential barriers to dispersal and gene flow through the damming of the Mississippi River and its major tributaries. The majority of map turtle research for these three widely distributed species has focused on one sympatric population (Stoddard, Wisconsin; Bull and Vogt 1979; Vogt 1980, 1993; Janzen et al. 1995). This population likely does not provide a complete picture of the patterns and processes, for both genes and morphology, operating in these species across their entire ranges. The limited molecular genetic research to assess population differentiation found incomplete evidence of differentiation. In G. geographica, mitochondrial data restriction fragment data found no differences between four populations across the extent of the range (Avise et al. 1992). In contrast, only one allozyme locus, out of 19 examined loci, was useful in within and between species analysis in G. ouachitensis and G. pseudogeographica, which had slight variation in protein migration rates between populations in Wisconsin and those in Arkansas and Louisiana (Vogt 1993). More recent population genetic approaches to estimating divergence, in turtles and elsewhere, have turned to microsatellite analyses as allozymes are limited by having only moderate resolution and may be tied more directly to fitness, in contrast to microsatellites which can have higher resolution and are considered more neutral (Avise
2004). Thus, higher levels of genetic differentiation may be present in map turtle species, though not previously detected.

Morphological variation within map turtles species is widespread. This variation has led to the classification of several sub-species typified by differentiation in post-orbital coloration and geographic location (Vogt 1993). *Graptemys pseudogeographica* is characterized by two subspecies, *G. p. pseudogeographica* and *G. p. kohnii*, although others have speculated that these may be two separate species (Dundee 1974). These species are differentiated on the basis of the post-orbital stripe which forms a complete crescent in *G. p. kohnii* (Vogt 1993; Ernst et al. 1994). Similar sub-specific classifications are also made in *G. ouachitensis* and *G. nigrinoda* (Vogt 1993; Ernst et al. 1994). Interestingly, intraspecific variation between some species is reduced in sympatric populations relative to their allopatric counterparts, suggestive of character displacement on this trait (Vogt 1978; Janzen et al. 1995). Thus patterns of intraspecific variation in morphology may provide insights into both species formation and maintenance.

Establishing the quantitative genetics of post-orbital coloration is an important step towards understanding the ability of the trait to respond to selection pressures. Selection acts at the level of the phenotype, yet any evolutionary changes occur at the level of the genotype (Falconer 1989). However, the effects of selection on shaping future phenotypic variation can be complicated by environmental conditions that could influence color pattern. In many turtle species, including map turtles, temperature can directly influence metabolic rate in both adults and juveniles, as well as incubation length and, in some species, sex of offspring (Bull and Vogt 1979, 1981; Ewert 1985; Rhen and Lang 2004). Earlier research has shown that aspects of these color patterns are associated with incubation temperature (Ewert 1979; Vogt
1980, 1993). Such variation may result from temperature-specific effects, from the suite of differences initiated by the temperature-dependent sex determination cascade and subsequent sex-effect, or a combination of both (Rhen and Lang 2004). Temperature may also indirectly influence variation in these color patterns through its influence on developmental rate and incubation length (Ewert 1985). In addition to temperature, hydric conditions during incubation can impact embryo physiology and size at hatching, which in turn has the potential to influence morphological variation (Janzen et al. 1995). All of these factors can dilute the impact of sexual or natural selection on post-orbital coloration. However, other studies have suggested that at least some aspects of these post-orbital coloration patterns are heritable (Janzen and Ast, unpublished data; Vogt 1993). The relative contribution of additive genetic variance and environmental factors on these traits is unknown and therefore, the ability of these traits to respond to potential sexual and/or natural selection is also unknown.

This project will utilize an integrative approach to establish the role of post-orbital color patterns in species diversification and maintenance. This multi-faceted approach will incorporate aspects of phylogenetics, population and quantitative genetics, morphometrics, and behavior to assess morphological evolution within species and across the genus. The goals of this research are four-fold. First, I will establish the importance of post-orbital coloration in the species formation of *Graptemys* through a phylogenetic assessment of morphological evolution. This assessment will include the reconstruction of ancestral phenotypes, examining morphological evolution across to the phylogeny to identify patterns of parsimonious or convergent evolution of phenotypes. Second, I will examine patterns of morphological and genetic differentiation across the geographic landscape for the three
broadly sympatric map turtle species to examine the concordance or discordance of morphological evolution with genetic differentiation. Third, I determine the relative contributions of additive genetic variance and environmental factors, such as temperature, in generating phenotypic variation in post-orbital coloration using a multivariate heritability approach. Finally, behavioral experiments will be used to directly assess the role of post-orbital coloration in species recognition and preference. These experiments will enable a determination of whether the main factor contributing to species recognition is based solely on post-orbital coloration or involves alternative cues such as titillation frequency or chemical cues.

These various methods will be used to address the main hypothesis that post-orbital coloration patterns are the major component of species recognition and species maintenance in map turtles. In this way, post-orbital coloration has been a contributing factor in the diversification of the map turtle clade through their role in species recognition, sexual selection, and reinforcement. From this hypothesis, several predictions are generated. First, these coloration patterns should have a key role during behavioral experiments of species recognition to limit hybrid pairings. Second, across the distribution of species, population variation in phenotype may be related to patterns of reinforcement among populations due to drift in morphological patterns preferred by sexual selection on color pattern. Third, there should be high heritability tied to high additive genetic variation in these traits allowing for a rapid response to selection for different morphologies. In addition, environmental influence on trait morphology should generally be limited as this influence would make these species-specific signals less reliable and dilute the ability of these traits to respond to selection. Finally, if selection on post-orbital coloration contributed to the diversification of the species,
the morphology should be distributed non-parsimoniously across the phylogeny such that

closely related species have enhanced morphological differentiation.

This project integrates several fields of biology, including molecular phylogenetics,
geometric morphometrics, behavioral ecology, and population and quantitative genetics to
address questions about the essential mechanisms of speciation, reinforcement, and
morphological evolution. As a result of my project, I will determine the relationship between
post-orbital color pattern variation and species formation and maintenance. This research can
serve as a framework for future ecological and evolutionary studies within *Graptemys* and in
speciation, sexual selection, and reinforcement research.

**Dissertation Organization**

This dissertation consists of four independent projects addressing morphological
evolution in map turtles from different conceptual vantage points as outlined above. Each
chapter has been organized and written for submission to scientific journals. The first data
chapter details the study of morphological evolution across the entire genus utilizing a
phylogenetic perspective. The second data chapter focuses on the morphological and genetic
differentiation of three sympatric species across their range. The third chapter addresses the
quantitative genetics of post-orbital coloration in three species. Finally, the fourth data
chapter uses a series of behavioral experiments to test the importance of coloration in species
recognition and preference. The dissertation is concluded by a general discussion of the
results from each study and their overall interpretation.
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CHAPTER 2. A PHYLOGENETIC ASSESSMENT OF MORPHOLOGICAL EVOLUTION WITHIN A RADIATION OF TURTLES

Modified from a paper submitted to *Evolution*

Erin M. Myers

Abstract

Morphological and species diversification are often linked in species radiations. However, it is not often clear whether morphological diversification was a driving factor in speciation, or whether it occurred as a by-product of other speciation processes. I addressed this question using a speciose and morphologically rich turtle radiation, the map turtles (*Graptemys*), characterized by unique facial colorations. To assess the role that color pattern variation has played in the diversification of this genus, this project sought to determine the evolutionary relationships among the species within the map turtles and to subsequently assess patterns of morphological evolution across the phylogeny. Map turtles formed a monophyletic group with two major clades. However, species-level resolution was limited, consistent with rapid speciation within the genus. I found parsimonious evolution of four major morphological patterns across the genus and less parsimonious evolution within morphological groups. This pattern suggests possible early sexual selection on these facial coloration traits during the formation of the major species clades and then subsequent stochastic morphological evolution.
Introduction

Morphological diversification has often been linked with species radiations (e.g. *Anolis* lizards, Darwin’s finches, and cichlid fish; Losos 1994; Jackman et al. 1997; Schluter 2000). However, it is not always clear whether phenotypic diversification occurs as a by-product of species diversification or, the alternative, that morphological innovation drives the speciation process. Knowledge of this relationship is at the core of understanding the origins of biological and morphological diversity and is a fundamental component of evolutionary biology. Morphological and other phenotypic traits (e.g. acoustic traits) may contribute to species formation and maintenance through a role in species recognition and mate choice (Ryan and Rand 1993; Sætre et al. 1997; Rudh et al. 2007; Seddon et al. 2008). However, phenotypic differentiation may also result from adaptation to new environments or stochastic processes following species formation via other means (Losos et al. 1997; Sueur et al. 2007). Previous attempts to address this question have examined pairs of sister taxa where one taxon is species-rich and the other is relatively species-poor and compared the origin of morphological traits across these taxa (e.g. Barraclough et al. 1995; Mitra et al. 1996). More recent approaches have examined patterns of morphological and species diversification across a phylogeny, rather than the more limited pair-wise comparisons (Schluter 2000; Harmon et al. 2003; Lovette 2004; Sueur et al. 2007; Seddon et al. 2008). However, assessing patterns of morphological diversification within and between clades in this manner requires a resolved phylogenetic hypothesis regarding the relationships among species (Losos et al. 1998). In rapidly formed species radiations, this process can be complicated by a series of relatively weakly supported short branches (Sanders et al. 2008) or by polytomies (Jackman et al. 1999; Sueur et al. 2007).
One species group that exhibits high species richness and morphological diversity is the map turtle complex (genus: *Graptemys*). Map turtles are relatively unique for their high species richness (12-13 species), making them the third most speciose genus of turtle (Figure 2.1; EMYSSystem 2008). Several species are widely distributed throughout the central United States, though many species also exhibit river endemism (Figure 1 from Lamb et al. 1994; Ernst et al. 1994). Interestingly, while *Graptemys* exhibits high species richness, its sister taxon, *Malaclemys*, is monotypic and broadly distributed (Hauswaldt and Glenn 2005).

While the phylogenetic placement of *Graptemys* within the turtle lineage is generally well established (Stephens and Wiens 2003), the phylogenetic relationships within the genus are less resolved. Previous research has employed both molecular and morphological techniques (McKown 1972; Lamb et al. 1994; Stephens and Wiens 2003) with mixed results. Earlier molecular studies were hampered by limited DNA sequence capability and relied on both sequence and restriction site data. With additional DNA regions and the increased ease of DNA sequencing, larger nucleotide sequence data sets may resolve the relationships within this group. Such a phylogeny is important for addressing the patterns of evolution of phenotypic traits.

While other turtle species are found in the same river drainages as map turtles, they do not exhibit a similar level of speciation (Moll and Moll 2004), suggesting something unique to map turtles is contributing to their diversification. In addition to species richness, map turtles are characterized on the basis of dramatic morphological diversity in both shell characters and in facial color patterns (Ernst et al. 1994). These color patterns range from a small, post-orbital dot of color to a full mask of color across the face. The extent of phenotypic diversification is similar to that seen in other better known and more species rich
radiations (Ernst et al. 1994; Nicholson et al 2007; Grant and Grant 2008). This morphological diversity within the genus has often played a role in species descriptions (McKown 1972; Vogt 1978; Lovich and McCoy 1992; Vogt 1993) and previous research has suggested that at least some aspects of color pattern shape have a heritable genetic basis (Janzen and Ast, unpublished data). It has also been hypothesized that these facial color patterns may play an important role in species identification and mate choice in at least some species, as courtship occurs face to face (Vogt 1978). Thus, morphological differentiation could drive species differentiation in this system through selection on facial coloration. On the other hand, morphology may have diverged stochastically as species formed and became isolated in different river drainages. These alternative hypotheses generate different predictions for how morphology is distributed across the phylogeny. If speciation occurred as a result of allopatric speciation and isolation, one might predict stochastic morphological evolution evidenced by a gradual, parsimonious evolution of morphological types along the phylogeny or no pattern at all. However, if speciation were driven in part by sexual selection or reinforcement on the facial color patterns prior to developing endemic ranges, one might then expect to see more extreme differences in morphology between sister taxa than between more distantly related taxa, similar to patterns observed for other pre-zygotic species recognition traits (e.g. Coyne and Orr 1997). Additionally, if sexual preference were constrained to favor certain morphologies, one might also expect to see repeated evolution of morphological types.

This project seeks to understand the role that facial color pattern morphology has played in the diversification of this speciose and morphologically-rich turtle genus. To do so, the goals of the project were two-fold: 1) to develop a resolved, molecular phylogeny of the
species relationships for this genus, and 2) to assess patterns of morphological evolution across the phylogeny. This approach allows for the potential reconstruction of putative ancestral morphologies in addition to the visualization of existing variation.

Methods

_Taxon Sampling, DNA Preparation, and Amplification_

At least two individuals per species (13) and subspecies (2) of all map turtle species were collected for DNA extraction and sequencing. These samples were supplemented with tissue samples from three outgroup taxa: *Chrysemys picta* (1 sample), *Malaclemys terrapin terrapin* (2 samples), and *Malaclemys terrapin littoralis* (1 sample). Tissues were collected from field caught and/or captive specimens (Appendix 1) and consisted of blood in buffer, blood on filter paper, shell scrapings, tail clips, and frozen or ethanol-preserved liver or muscle. DNA was extracted from the preserved tissue samples using DNeasy tissue extraction kits (Qiagen Inc., Valencia, CA) following standard protocols. An additional *Chrysemys picta* individual was included in all subsequent analyses using the published mitochondrial genome (Mindell et al. 1999) available from GenBank (AF069423).

Three gene regions were selected for amplification and DNA sequencing, two from the mitochondrion (the region spanning NADH-2 to cytochrome oxidase I; ND2-COI, and the control region; CR) and one nuclear gene (recombination activating gene 1; RAG-1). These genes were selected because of their relatively fast rates of molecular evolution and ability to resolve species level relationships (Wolstenholme 1992; Zardoya and Meyer 1996; Krenz et al. 2005; McGaugh et al. 2008). A subset of the control region was previously sequenced in *Graptemys* and showed moderate sequence variability (Lamb et al. 1994).
External and internal primers were selected for polymerase chain reaction on the basis of cross-species amplification in related taxa (Engstrom et al. 2007). In some cases, the primer sequence was modified to match the sequence of the published *Chrysemys picta* mitochondrial genome (Mindell et al. 1999) as the *C. picta* sequence is likely to be more similar to *Graptemys* sequences than more distantly related turtles. Sequences for primers used in this study are given in Table 2.1. Primers DES-1 and DES-2 were used to amplify a 700bp fragment of the mitochondrial control region. Approximately 2100bp were amplified using LGL-562 and LGL-452 encompassing the ND2 (1040bp) gene of the mitochondrion as well as part of the surrounding genes for tRNA-met, -trp, -ala, -asn, -cys, -tyr, and COI (684bp). Finally, an 850bp fragment from the middle third of the RAG-1 gene was selected for amplification with RAGF2 and RAGR2 as this was the most variable portion of the RAG-1 gene (Krenz et al. 2005). PCR conditions were standardized across reactions. Thermal cycling conditions were optimized for each primer set. Following initial amplification, PCR products were visualized using agarose gel electrophoresis. Successful reactions were purified using ExoSap-IT (USB Corp.) following standard protocols. Purified products were then used as template in DNA sequencing reactions. Initial PCR primers were used as sequencing primers for RAG-1 and CR. Additional internal primers were utilized for ND2 (Table 2.1). Sequencing reactions were conducted using Big Dye v3 (Applied Biosystems, Inc.). Sequencing reactions were purified using sephadex G50 columns and then run on ABI 3730xl DNA Analyzer (Applied Biosystems, Inc.) at the Iowa State University DNA Facility. DNA sequences were visualized in BioEdit v.7.0.5.3 (Hall 1998). Sequences were edited by hand and forward and reverse reactions combined. Multiple sequence
alignment was conducted using ClustalW (Thompson et al. 1994) as implemented in BioEdit. All sequences were deposited in GenBank (Appendix 3).

The above sequences were supplemented with 400bp from cytochrome B (CYTB) available from GenBank (U81345, L28772-8, L28780-2, L28784, L28789, L28798) generated by Lamb et al. (1994). The Chrysemys sample was that from the full mitrochondrial genome sequence (Mindell et al. 1999). Sequence data were available for all species of map turtles and Malaclemys terrapin terrapin. Unfortunately, sequence was not available for Malaclemys t. littoralis. Attempts to sequence this gene region following Lamb et al. (1994) for this sub-species and others indicated the possible presence of a nuclear pseudogene. For M. t. littoralis, this gene region was incorporated into the phylogeny two ways. First, this region was coded using N for the entire sequence. Secondly, as Lamb et al. (1994) found no sequence differences between similar sister sub-species pairs for G. ouachitensis ouachitensis – G. o. sabinensis or G. nigrinoda nigrinoda – G. n. delticola, the M. t. terrapin individual’s sequence was used for both Malaclemys sub-species. Both methods recovered the same topology (data not shown).

Phylogenetic Analysis

The combined genetic dataset was analyzed using three criteria: maximum parsimony (MP), maximum likelihood (ML) implemented in PAUP* b10 (Swofford 2002) and a Bayesian analysis implemented in MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001). Heterozygous positions in the RAG-1 gene were relatively few (8 positions: 5 found in one species only, all represented likely cases of incomplete lineage sorting) and did not occur at phylogenetically informative positions. These positions were treated as multi-state traits with
both bases given in the analysis. Under parsimony, the combined dataset was run using the multi-state ‘polymorph’ option, which assumes that variable characters are a heterogeneous terminal group rather than selecting the character that minimizes branch length. Additional analyses were run with these eight positions excluded and the recovered trees had few topological differences (data not shown). A heuristic search was employed to find the most parsimonious tree and 2000 bootstrap replicates were performed using the ‘FastStep’ option to assess confidence in the topology. Prior to conducting the ML analysis, I used Modeltest v. 3.7 (Posada and Crandall 1998) to find the best fitting model of sequence evolution using the Akaike Information Criterion (AIC; Akaike 1973; Posada and Buckley 2004). PAUP does not support multiple partitions in ML analysis. Therefore for the combined dataset (CR, ND2-COI, RAG-1, CYTB), the K81uf + I + Γ model was selected as the best fitting model for the concatenated dataset. Similar to MP, the ML analysis was implemented with an initial heuristic search for the tree with the maximum likelihood followed by 2000 bootstrap replicates using the ‘FastStep’ method. Consensus trees with bootstrap support for each node were generated for both ML and MP analyses.

For the Bayesian analysis, I performed a partitioned analysis implementing different nucleotide substitution models for each gene (McGuire et al. 2007; Sanders et al. 2008). I utilized 6 data partitions: 1 each for the genes CR, RAG-1, CYTB, while ND2-COI was partitioned into 3 subdivisions, ND2, COI, and t-RNAs. To select the model of substitution for each data partition, I employed MrModeltest v2.2 (Nylander 2004) and selected the best-fitting model using AIC. The models selected were as follows: HKY+I+Γ for CR, HKY+I for COI and CYTB, HKY+Γ for ND2, HKY for t-RNA and RAG-1. The Bayesian analysis used 2 million generations, sampling from the chain every 1,000 generations. I checked
stabilization of the chain by comparing log-likelihood scores against generation number. Chain convergence occurred within 15,000 generations, and I conservatively discarded the first 200,000 generations as “burn-in.” I generated a consensus phylogram with mean posterior probability values at each node and estimates of branch lengths.

As polytomies were present in the consensus trees generated by the above analyses, I also conducted an analysis to assess the type of polytomy (‘soft’ vs. ‘hard’). A soft polytomy would likely be resolved into a sequential bifurcation with the accumulation of additional sequence data, while a hard polytomy represents a rapid, nearly simultaneous diversification event that is unlikely to be resolved with additional data (Maddison 1989; Walsh et al. 1999). I conducted a power analysis using the difference of proportion method to estimate the number of base pairs of DNA sequence required to recover a bifurcating phylogeny within the polytomous ‘pseudogeographica’ clade (Walsh et al. 1999). The null hypothesis was that the species of map turtle diversified nearly simultaneously with a single event estimated at 2.6 million years ago during the late Pliocene. This date is in accordance with the estimated divergence time of the pulchra and pseudogeographica clades (Lamb et al. 1994). In contrast, the alternative hypothesis was a sequential bifurcating process with an internode time interval of 100,000 years, consistent with the shortest major glacial or interglacial period during the late Pliocene-early Pleistocene (Miller and Withler 1996; Walsh et al. 1999). The effect size index (h) is a factor of the proportion of bases expected to have undergone substitution and is determined using empirically derived rates of substitution (Walsh et al. 1999). This value is then used to determine the amount of DNA sequence that would be needed to differentiate between the two hypotheses. All available sequence data (CR, ND2-COI, RAG-1 and CYTB) were used to estimate the average DNA substitution
rate. The sensitivity of the result to the initial parameters was also assessed for a range of
divergence dates (3.5 mya to 2.0 mya) and internode intervals (100,000 to 60,000 years).

*Morphological Data Analysis*

Digital images of the dorsal view of the head were obtained for all species of map
turtles using either a Nikon CoolPix 5700 or a Nikon DXM-1200 high resolution digital
camera. The majority of specimens photographed were from museum collections (National
Museum of Natural History, Carnegie Museum of Natural History, Texas Natural History
Collections, University of Kansas Natural History Museum; Appendix 2). These collections
were supplemented with field collected individuals that were part of other on-going research
projects with *Graptemys* (Myers 2008a; 2008c). Photographs from all specimens are
available from the author upon request. The total number of specimens and numbers for each
species are presented in Table 2.2. Sub-species were not used as part of the morphological
analysis because of relatively limited identified samples in the museum collections. Several
specimens classified as *Graptemys pulchra* and collected prior to 1980 were re-identified as
either *Graptemys gibbonsi* or *Graptemys ernsti* on the basis of site of collection and
morphological features (Lovich and McCoy 1992). Finally, outgroup taxa were not included
in the morphological assessment as the color patterns present in map turtles are unique to the
genus and no homologous morphologies are present in either *Chrysemys picta* or *Malaclemys
terrapin*.

Facial color pattern morphology was quantified for each species of map turtle using
landmark-based geometric morphometrics (Rohlf and Marcus 1993). From each image, the $x,$
y coordinates of 19 landmarks were recorded using TpsDig (Rohlf 2004). These landmarks
were selected to capture the range of shape variation present in these 13 species, as well as match those of a previous study (Myers 2008a; 2008b). Landmarks included two fixed points at the base and apex of the central nose stripe, two sliding semi-landmarks on either side of the nose stripe, and 13 sliding semi-landmarks positioned around the eye-bar (Figure 2.2A).

Only the left post-orbital eye-bar was used to avoid any potential singularity in the data resulting from near or perfect symmetry between the sides (Bookstein 1996; Klingenberg et al. 2002) and thus, patterns of asymmetry were not examined. The $x$, $y$ coordinates were used in a Generalized Procrustes Analysis to superimpose the specimens to a common coordinate system and eliminate the effects of non-shape variation from digitizing position, rotation, and scale (Rohlf and Slice 1990). Semi-landmarks were allowed to slide in their positions in order to minimize the bending energy using three iterations (Bookstein 1997). Following alignment of all specimens, the average specimen was calculated (Figure 2.2B). From the $x$, $y$ coordinates, I subsequently generated 34 shape variables as the partial warp scores from the thin-plate spline (Bookstein 1991) and uniform components (Rohlf and Bookstein 2003). These shape variables can then be used to test hypotheses of shape variation using standard multivariate statistics. Both superimposition and shape variable computation were conducted in TPSRelWarp (Rohlf 2007a). Additionally, population mean shape was calculated and differences between means were calculated as the sum of the Euclidean distances between each population at each shape variable.

**Statistical Analysis of Morphology**

Initially, I examined whether morphology was consistent between adults and juveniles within a species using a two-factor multivariate analysis of variance (MANOVA)
with species and age class as the main factors and a species by age class interaction in R v2.6.2 (R Development Core Team 2006). Significance was assessed using residual randomization. To examine patterns of morphological evolution for each species, I performed a MANOVA with species as a fixed effect in JMP v6.0 (SAS Institute). To determine which pair-wise relationships were significant, a randomization procedure was implemented in R to randomly shuffle individuals between species and calculated Euclidean distance among species means (Adams and Collyer 2007; Collyer and Adams 2007). To account for multiple comparisons, 100,000 randomizations were used to assess significance after sequential Bonferroni correction to maintain an overall $\alpha = 0.05$. The average specimen shapes for each species and for the total sample were generated. Morphological patterns were visualized through a principal components analysis (PCA) of shape. To compare amounts of variation across species, I measured morphological disparity (Hollander et al. 2006) in Excel (v 2002, Microsoft). This method calculates the amount of shape difference as a weighted average distance between individuals and their group means (Hollander et al. 2006).

To examine patterns of morphological evolution within the genus, I plotted morphology along the phylogeny using TPSTree v.1.21 (Rohlf 2007b). The Bayesian tree was pruned to include only those species for which I had morphological data. Branch lengths for monophyletic species (A and B sample as a monophyletic pair) were averaged and a single individual retained. The average specimen for each species was used as input for the phylogenetic mapping. Ancestral morphologies were calculated in TPSTree using a squared-change parsimony approach (Maddison 1991; McArdle and Rodrigo 1994). Thin-plate spline deformation grids were generated to graphically describe patterns of shape change between species and for hypothetical ancestors. To determine whether morphological evolution
occurred parsimoniously, I performed a PCA on the average shapes for each species and all hypothetical ancestors generated from the TPSTree analysis and subsequently plotted these shapes using the first two principal components with the phylogeny over-layed (Rohlf 2002). A pattern of none, or few, crosses of the linking phylogeny suggests parsimonious evolution, while numerous criss-crossing indicates a complicated, un-parsimonious evolution of morphology (Rohlf 2002).

Results

Phylogenetic Analyses

I performed phylogenetic analyses on a combined dataset including mitochondrial and nuclear genes comprising 4071 aligned nucleotide positions divided into four genes (695 bp for control region, 1040 bp for ND2, 684 bp for COI, 422 for tRNAs, 825 bp for RAG-1, and 401 bp for CYTB). Of these, 3610 positions were invariant, 85 were parsimony uninformative, and 376 were parsimony informative (approx. 25% of informative characters were from CR, 50% from ND2-COI, and 25% from RAG-1 and CYTB combined). All sequences will be deposited in GenBank.

None of the three phylogenetic analyses fully resolved the topology of the map turtle tree with strong support. For maximum parsimony, the analysis recovered greater than 1000 equally parsimonious trees. Much of the difference between the trees was confined to the most recent nodes and tips with differing membership in clades or polytomies. The bootstrap consensus tree is shown (Figure 2.3A) with clades collapsed to polytomies if bootstrap support was less than 50%. The tree maximizing the likelihood score was similar to parsimony consensus tree, although the relationships among G. caglei, G. versa, G.
*nigrinoda nigrinoda*, and *G. n. delticola* were more resolved (data not shown). However, these were not supported strongly in the bootstrap analysis and were collapsed into the polytomy (Figure 2.3B). Bayesian analysis had slightly more resolution than either of MP or ML topologies (Figure 2.3C & D). This Bayesian tree included the joining of *G. caglei* and *G. versa* as sister taxa. This topology was similar to that recovered from the ML analysis though it was not supported in the final ML bootstrap consensus tree. All three phylogenetic analyses identified *Malaclemys* as the sister taxon to *Graptemys*. Within *Graptemys*, all three phylogenies strongly supported *G. geographica* as the sister clade to all other map turtles. Additionally, there was strong support across all methods for a split between two major clades (the *pulchra* and *pseudogeographica* clades) within the map turtles.

Polytomy analysis found that for 2.6mya divergence and 100,000 internode interval, the combined dataset (CR, ND2-COI, RAG1, and CYTB) should be sufficient to resolve a bifurcating tree (Table 2.3). This result suggests that diversification of the *pseudogeographica* clade is a hard polytomy. This result is somewhat dependent on the estimates of divergence date and internode interval length, however. If the divergence is more recent, the data should be able to resolve bifurcations with even shorter internode intervals, but the reverse is true for older divergence estimates. Therefore, if speciation occurred near the most recent estimate for the split of the *pseudogeographica* clade (2.0 mya) then the available sequence data should resolve a bifurcating phylogeny with an internode interval as low as 70,000 years (Table 2.3). On the contrary, if the time of speciation was closer to the oldest estimates (3.5 mya) then the available data would not be sufficient to resolve bifurcations with an internode interval of 100,000 years (Table 2.3).
Morphological Analyses

In the combined analysis with both adult and juvenile morphology, species and age classes (Adult/Juvenile) were significantly different (F = 21.698, p<0.0001 and F = 11.627, p<0.0001, respectively). Additionally, the species x age interaction term was also significant (F = 2.159, p<0.0001). Further assessment revealed that juveniles had a significantly different morphology from adults within the same species for some but not all species (significant after Bonferroni correction for *G. flavimaculata, G. gibbonsi, G. pseudogeographica,* and *G. pulchra*). Consequently, remaining analyses used only the adult dataset.

Using the adult dataset only, the MANOVA still found species to be significantly different (F = 17.017, p<<0.0001). In general, the species fell out into four morphological groups (Figure 2.4): a group characterized by a smaller, dot-like post-orbital spot (*G. geographica*), a group characterized by an intermediate sized post-orbital blotch (*G. oculifera* and *G. flavimaculata*), a group with a full mask of coloration (*G. barbouri, G. ernsti, G. pulchra* and *G. gibbonsi*), and finally a group with a post-orbital stripe (*G. caglei, G. kohnii, G. nigrinoda, G. ouachitensis, G. pseudogeographica*, and *G. versa*). The results from the individual pair-wise comparisons are given in Table 2.4 along with the morphological distance between species. *G. geographica* was significantly different from all other species. Most other species were significantly different from one another. However, when species were not different, this was typically for species within the same morphological group. All species had similar levels of variation in morphology, with values for disparity ranging from 0.0174 to 0.0364, and the range of values was independent of sample size.
Average morphology was generated for each species and deformation grids were used to visualize shape differences from the overall average specimen (Figure 2.5). When morphology was plotted onto the phylogeny, several patterns emerged. The “dot” morphology from *G. geographica* was the most basal morphology for the genus, as indicated by the morphology at the ancestral node for all *Graptemys* (Figure 2.5). Examining the color patterns along the internal nodes, post-orbital color pattern mapped parsimoniously onto the phylogeny. The species with a masked morphology clustered together in one clade (consistent with the genetic *pulchra* clade) and the stripe morphology clustered in the other clade (*pseudogeographica* clade; Figures 2.5 & 2.6). The blotch morphology could be considered newly derived within the striped clade or possibly an incomplete reversion to the dot morphology occurring within the *pseudogeographica* clade. Relative to the other morphologies, the dot morphology is quite distinct and relatively distant in shape space which is consistent with *G. geographica* as the sister taxon to all other map turtles (Figure 2.6). Within the two larger clades, morphological evolution proceeded differently once the clade was formed, a parsimonious evolution of morphology variation within type for the masked clade and un-parsimonious evolution with multiple criss-crossing branches of the phylogeny in shape space within the stripe clade (Figure 2.6).

**Discussion**

Morphology and species diversity are often tied together in species radiations. However, it is not often clear how exactly morphology has contributed to this diversification process. To determine the role that color pattern variation has played in the diversification of the map turtles, a speciose and morphologically rich turtle radiation, this project sought to
determine the evolutionary relationships among the species within the map turtles and to subsequently assess patterns of morphological evolution across the phylogeny. Map turtles formed a monophyletic group with two major clades. However, species-level resolution was limited consistent with a hard polytomy and rapid speciation within the genus. I found parsimonious evolution of morphology for four major morphological patterns across the genus. Further interpretation of these combined results is discussed below.

**Phylogenetic Interpretation**

Several features of the map turtle phylogeny were strongly supported across phylogenetic reconstruction methods. In particular, there was strong support for *Malaclemys* as the sister taxon to *Graptemys* and the status of *Graptemys geographica* as the basal taxon to the rest of the lineage. In addition, all phylogenetic methods recovered a deep divergence within the genus, splitting the map turtles into two major clades traditionally referred to as the *pulchra* and *pseudogeographica* clades. These results are consistent with earlier studies (Lamb et al. 1994; Stephens and Wiens 2003). There was some support (Figure 2.3B) for the sister taxa relationship of *G. caglei* and *G. versa* which is concordant with their nearby, Texas endemic distributions (Ernst et al. 1994) and was not detected in earlier reconstructions (Lamb et al. 1994; Stephens and Wiens 2003). Interestingly, individuals from *G. ouachitensis sabinensis* formed a well supported, monophyletic clade that was sister to the clade containing *G. flavimaculata* and *G. oculifera*, as well as the clade of *G. ouachitensis ouachitensis, G. pseudogeographica*, and *G. kohnii*. While the resolution within the *pseudogeographica* clade was limited, the molecular data however, did provide robust
support for the species classifications of *G. pulchra*, *G. ernsti*, and *G. gibbonsi* originally described on the basis of divergent morphological characters (Lovich and McCoy 1992).

The inability to fully resolve the map turtle phylogeny is not completely surprising. Within the time frame of late Pliocene and early Pleistocene glaciations (2.5-3.0 mya), the available data should have been sufficient to resolve a bifurcating topology based on the power analysis of the polytomy if one existed even with relatively short internode intervals of 100,000 years or less. However, if the age of divergence is 3.5 mya or older, additional sequence data from other rapidly evolving genes or new techniques such as single nucleotide polymorphisms (SNPs) may potentially be able to more fully resolve the topology. Previous authors have speculated that such Pliocene and Pleistocene glaciation events were responsible for speciation within the genus (Lamb et al. 1994); however, fossil evidence within the major clades is generally lacking (Ernst et al. 1994). As *Graptemys* are highly aquatic turtles that rarely leave the water except for female terrestrial nesting forays (Ernst et al. 1994), they may be especially prone to isolation and speciation, relative to other turtle species, as a consequence of climate and water level changes induced by glaciations, similar to other types freshwater species (McKown 1972; Avise 2000; Berendzen et al. 2008). Consistent patterns of species endemism have been observed in co-distributed species of fish and mussels (Wiley and Mayden 1985; O'Brien and Williams 2002). Glaciations and changes in sea level could isolate several populations at once and initiate multiple, simultaneous divergence events (Hoelzer and Melnick 1994). Such simultaneous isolation has been implicated in other species during this time frame (Hoelzer and Melnick 1994; Kidd and Friesen 1998; Walsh et al. 1999; Lovette 2004) and thus seems plausible within this genus.
Morphological Interpretation

Whereas the topology of species relationships was not fully resolved, patterns of morphological diversification across the genus were more easily established and not fully dependent on within-clade resolution. It is likely that the ancestral map turtle had a small post-orbital color pattern similar to that of the basal map turtle, *G. geographica*. Unfortunately, it is not possible to reconstruct the ancestral pattern beyond this basal lineage because this morphological character is only present within map turtles (i.e. no homologous trait is observed in outgroup taxa). Therefore, the evolutionary origins of this trait remain obscure. Within the map turtles, it appears that morphology has diversified roughly parsimoniously with respect to major morphological types (e.g. dot, blotch, mask, and stripe), with subsequent diversification in patterns within each of these clades. That is, each morphological group evolved once and that within type, changes in morphology across shape space did not necessarily correspond to phylogeny. This result then sheds light on the potential role of morphology in the diversification of this genus. Based on the initial predictions, the data support a process of allopatric speciation and subsequent stochastic evolution in facial morphology for *Graptemys*. However, it remains possible that sexual selection was an important factor in the early diversification of the genus into the two major clades and morphological groups, with subsequent morphological diversification within groups occurring via stochastic processes. Similarly mixed patterns of phenotypic evolution and species diversification have been seen for acoustic traits (Sueur et al. 2007). Additionally, map turtle species are characterized, to varying degrees, by other unique morphological features such as mega-cephaly in females, extreme female-biased sexual size dimorphism, and an exaggerated ‘sawback’ carapacial ridge (Ernst et al. 1994). Examination
of these additional traits was beyond the scope of the present study, but future work should assess their potential contribution to sexual selective processes.

While the morphological data are informative about patterns of morphological evolution across the genus, they are unlikely to resolve the morphological and taxonomic debates that have followed some species, including *G. kohnii*, *G. ouachitensis*, and *G. pseudogeographica* (Dundee 1974; Vogt 1978, 1993; Ernst et al. 1994). This and other studies have found species-specific differences in morphology between *Graptemys ouachitensis* and *Graptemys pseudogeographica* including differences in post-orbital stripe width and differences in nose-strip characteristics of length and width, beyond the shell and cheek spot characters often used to differentiate these species (Myers 2008a; Vogt 1993; Janzen et al. 1995). In contrast, significant differences were not detected between *G. kohnii* and *G. pseudogeographica*, though there was a difference with *G. ouachitensis*. This result lends some support to the suggestion that *G. pseudogeographica kohnii* should only attain sub-specific status. However, not all species-informative characters can be captured in a single analysis and thus field classifications should continue to rely on the full suite of available characters. In addition, the molecular analysis was unable to resolve the relationships between these three taxa, suggesting that they are either of recent origin or that there is on-going gene flow amongst them, limiting the ability to resolve their relationships.

In summary, this study found rapid speciation and morphological diversification within the map turtle clade. This morphological evolution was generally parsimonious. While sexual selection and reinforcement processes may have contributed to the diversification of the major morphological groups, much of the speciation in the genus was likely allopatric contributing to stochastic morphological evolution within these groups. To more fully
explore the role of facial color pattern in species recognition and its potential for use in mate choice, future research should include behavioral tests to establish the extent that animals use this trait in mating decisions.

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### Tables

Table 2.1. List of primers names, sequence, and references for primers utilized in DNA amplification and sequencing of three gene regions. (int) indicates internal sequencing primer and * indicates primer sequence was modified from the original for this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’-3’</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control Region</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DES-1</td>
<td>GCATTCATCTATTTTCCGTAGCA</td>
<td>(Starkey et al. 2003)</td>
</tr>
<tr>
<td>DES-2</td>
<td>GGATTTAGGGGTGGACGAGAAT</td>
<td>(Starkey et al. 2003)</td>
</tr>
<tr>
<td><strong>ND2 to COI</strong></td>
<td></td>
<td></td>
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<tr>
<td>LGL-562*</td>
<td>TAAGCTATTGGGCCCATTACC</td>
<td>(Osentoski and Lamb 1995)</td>
</tr>
<tr>
<td>LGL-452*</td>
<td>ACTTCGGGGTGACCAAAGAATCA</td>
<td>(Osentoski and Lamb 1995)</td>
</tr>
<tr>
<td>912-COI* (int)</td>
<td>GTGGTTGTAGAGAAAAATCA</td>
<td>(Seutin et al. 1994)</td>
</tr>
<tr>
<td>1613-ND2* (int)</td>
<td>CTTAGCCTATTCTTCTA</td>
<td>(Seutin et al. 1994)</td>
</tr>
<tr>
<td>ND2-IntF (int)</td>
<td>TCATCATAACCTCAACCATCCTCC</td>
<td>this study</td>
</tr>
<tr>
<td>ND2-IntR (int)</td>
<td>GGGTTGTGTAAATTGTGATGGA</td>
<td>this study</td>
</tr>
<tr>
<td><strong>RAG-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAGF2</td>
<td>GAGATCATTYGAAAGGCACC</td>
<td>(Krenz et al. 2005)</td>
</tr>
<tr>
<td>RAGR2</td>
<td>GATGTTCAAGAAGGATTCCT</td>
<td>(Krenz et al. 2005)</td>
</tr>
</tbody>
</table>
Table 2.2: Sample size of specimens used in morphological analyses of map turtles.

<table>
<thead>
<tr>
<th>Species</th>
<th>$N_{\text{Adult}}$</th>
<th>$N_{\text{Juvenile}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Graptemys barbouri</em></td>
<td>21</td>
<td>26</td>
</tr>
<tr>
<td><em>Graptemys caglei</em></td>
<td>26</td>
<td>16</td>
</tr>
<tr>
<td><em>Graptemys ernsti</em></td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td><em>Graptemys flavimaculata</em></td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td><em>Graptemys geographica</em></td>
<td>139</td>
<td>35</td>
</tr>
<tr>
<td><em>Graptemys gibbonsi</em></td>
<td>28</td>
<td>15</td>
</tr>
<tr>
<td><em>Graptemys kohni</em></td>
<td>49</td>
<td>18</td>
</tr>
<tr>
<td><em>Graptemys nigrinoda</em></td>
<td>21</td>
<td>7</td>
</tr>
<tr>
<td><em>Graptemys oculifera</em></td>
<td>22</td>
<td>6</td>
</tr>
<tr>
<td><em>Graptemys ouachitensis</em></td>
<td>66</td>
<td>18</td>
</tr>
<tr>
<td><em>Graptemys pseudogeographica</em></td>
<td>110</td>
<td>30</td>
</tr>
<tr>
<td><em>Graptemys pulchra</em></td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td><em>Graptemys versa</em></td>
<td>17</td>
<td>13</td>
</tr>
</tbody>
</table>

**Total**: 538 $\text{Adult}$, 179 $\text{Juvenile}$

Table 2.3. Results from polytomy power analysis for multiple divergence time and internode interval estimates. The analysis determines the number of base pairs required to resolve a bifurcating tree and then asks whether the data at hand (4071 bp) is greater than this value and thus sufficient to see resolution. If the empirical data are less than the number of bp needed, then more sequence data may resolve the polytomy, however, if the data are greater than the number required then it suggests the polytomy is hard and unlikely to be resolved with additional data.

<table>
<thead>
<tr>
<th>Divergence</th>
<th>internode</th>
<th># bp needed</th>
<th>Data sufficient?</th>
</tr>
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<tbody>
<tr>
<td>2 mya</td>
<td>100,000</td>
<td>2669</td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td>90,000</td>
<td>2965</td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td>80,000</td>
<td>3336</td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td>70,000</td>
<td>3813</td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td>60,000</td>
<td>4448</td>
<td>NO</td>
</tr>
<tr>
<td>2.6 mya</td>
<td>100,000</td>
<td>3469</td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td>90,000</td>
<td>3855</td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td>80,000</td>
<td>4337</td>
<td>NO</td>
</tr>
<tr>
<td>3 mya</td>
<td>100,000</td>
<td>4003</td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td>90,000</td>
<td>4448</td>
<td>NO</td>
</tr>
<tr>
<td>3.5 mya</td>
<td>100,000</td>
<td>4671</td>
<td>NO</td>
</tr>
</tbody>
</table>
Table 2.4. Morphological distance between all species pairs below the diagonal. Significance of species differences assessed by 100,000 randomizations is above the diagonal. Bolded values are significant after sequential Bonferroni correction. Species are abbreviated to first three letters of the species name.

<table>
<thead>
<tr>
<th></th>
<th>bar</th>
<th>cag</th>
<th>ern</th>
<th>fla</th>
<th>geo</th>
<th>gib</th>
<th>koh</th>
<th>nig</th>
<th>ocu</th>
<th>oua</th>
<th>pse</th>
<th>pul</th>
<th>ver</th>
</tr>
</thead>
<tbody>
<tr>
<td>bar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>ern</td>
<td>7.432</td>
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<td></td>
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<tr>
<td>fla</td>
<td>7.594</td>
<td>5.114</td>
<td>10.433</td>
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<tr>
<td>geo</td>
<td>10.540</td>
<td>7.377</td>
<td>13.592</td>
<td>6.762</td>
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<td></td>
<td></td>
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<tr>
<td>koh</td>
<td>8.909</td>
<td>2.378</td>
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<td>4.801</td>
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<tr>
<td>nig</td>
<td>8.564</td>
<td>5.633</td>
<td>12.589</td>
<td>5.179</td>
<td>7.977</td>
<td>15.433</td>
<td>5.259</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ocu</td>
<td>8.714</td>
<td>5.239</td>
<td>11.644</td>
<td>3.800</td>
<td>4.967</td>
<td>15.349</td>
<td>4.983</td>
<td>5.722</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Figures

Figure 2.1. Frequency distribution of species number per genus for all genera of turtles. The position of the map turtles (*Graptemys*) is indicated.
Figure 2.2A. Three representative species indicating the general position of landmarks over the suite of morphological variation in the genus *Graptemys* (from left to right: *G. geographica*, *G. pseudogeographica*, and *G. ernsti*). B. Average configuration of landmarks for all adult map turtles.
Figure 2.3A. Bootstrap consensus topology recovered in maximum parsimony analysis for CR, ND2-COI, RAG-1, and CYTB. Species names are indicated at right. Outgroup taxa are abbreviate with first letter of genus and first 3 letters of species name. Ingroup taxa names are abbreviated to first three letters of species names. Sub-species have first letter of species name and 3 letters of subspecies name. Two individuals were used per species, A and B represent sample number. Values to left of nodes indicate bootstrap support from 2000 bootstrap replicates. Nodes with less than 50% support were collapsed to polytomies.
2.3B. Maximum likelihood bootstrap consensus tree. Species name abbreviations follow those above. Values to left of nodes indicate bootstrap support from 2000 bootstrap replicates. Nodes with less than 50% support were collapsed to a polytomy.
2.3C & D. Topologies recovered from the Bayesian analysis. Species abbreviations follow those above. C shows the tree with branch length scaled per molecular evolution. As branch lengths are short, D depicts the support from 2 million generations.

C.

D.
Figure 2.4. Principal components plot of all individuals. Circles indicate the clusters for each of the general morphological groups: solid circle around light gray triangles outlines the dot morphology of *G. geographica*, dotted circle around stars outlines the blotch morphology of *G. flavimaculata* and *G. oculifera*, dashed circle surrounding black points highlights the masked group of *G. barbouri*, *G. ernsti*, *G. gibbonsi*, and *G. pulchra*, the dashed-dot circle encompasses the gray squares of the striped group which has *G. caglei*, *G. kohnii*, *G. nigrinoda*, *G. ouachitensis*, *G. pseudogographica*, and *G. versa*.

Figure 2.5. Morphological patterns for each of the map turtle species represented by photograph of post-orbital color pattern and deformation grids representing shape change from the average specimen to each species. A and B labels correspond to the sample for species whose two samples did not cluster together. Reconstructed ancestral phenotypes are indicated at nodes along the collapsed Bayesian phylogeny. Morphological groups and clade membership are also indicated.
Figure 5
Figure 2.6. PCA plot of average species morphology and all hypothetical ancestor topologies. Morphologies are connected using the pruned morphology phylogeny. Terminal taxa are indicated with a black circle and hypothetical ancestral morphologies are indicated by white boxes. Ancestor number corresponds to the nodes on the phylogeny with 1 being the most basal. Parsimonious morphological evolution occurred between the 4 major morphological types (dot, blotch, mask, and stripe). However, evolution within the stripe group was non-parsimonious.
## Appendices

### Appendix 2.1. Locality information for specimens

<table>
<thead>
<tr>
<th>Species</th>
<th>Locality</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chrysemys picta</em></td>
<td>Mississippi River, Carroll Co., IL</td>
</tr>
<tr>
<td><em>Malaclemys terrapin terrapin</em></td>
<td>Patuxent River, St. Mary’s Co., MD</td>
</tr>
<tr>
<td><em>Malaclemys terrapin littoralis</em></td>
<td>Nueces Bay, Nueces Co., TX</td>
</tr>
<tr>
<td><em>Graptemys barbouri</em></td>
<td>Captive (2)</td>
</tr>
<tr>
<td><em>G. caglei</em></td>
<td>Captive (2)</td>
</tr>
<tr>
<td><em>G. ernsti</em></td>
<td>Escambia River, Santa Rosa Co., FL</td>
</tr>
<tr>
<td><em>G. Conecuh River, Covington Co., AL</em></td>
<td></td>
</tr>
<tr>
<td><em>G. flavimaculata</em></td>
<td>Ward Bayou/Parish Lake, Jackson Co., MS</td>
</tr>
<tr>
<td><em>G. geographica</em></td>
<td>Captive (1)</td>
</tr>
<tr>
<td><em>G. gibbonsi</em></td>
<td>Mississippi River, Guttenberg, IA</td>
</tr>
<tr>
<td><em>G. kohnii</em></td>
<td>Illinois River, Tazewell Co., IL</td>
</tr>
<tr>
<td><em>G. Yazoo River, Humphreys Co., MS</em></td>
<td></td>
</tr>
<tr>
<td><em>nigrinoda nigrinoda</em></td>
<td>Captive (2)</td>
</tr>
<tr>
<td><em>nigrinoda delticola</em></td>
<td>Captive (2)</td>
</tr>
<tr>
<td><em>G. oculifera</em></td>
<td>Pearl River, Madison Co., MS (2)</td>
</tr>
<tr>
<td><em>ouachitensis ouachitensis</em></td>
<td>Mississippi River, Jersey Co., IL</td>
</tr>
<tr>
<td><em>G. ouachitensis sabinensis</em></td>
<td>Cedar River, Muscatine Co., IA</td>
</tr>
<tr>
<td><em>pseudogeographica</em></td>
<td>Missouri River, Knox Co., NE</td>
</tr>
<tr>
<td><em>G. Cedar River, Muscatine Co., IA</em></td>
<td></td>
</tr>
<tr>
<td><em>pulchra</em></td>
<td>Cahaba River, Bibb Co., AL</td>
</tr>
<tr>
<td><em>G. Tombigbee River, Monroe Co., MS</em></td>
<td></td>
</tr>
<tr>
<td><em>versa</em></td>
<td>Captive (2)</td>
</tr>
</tbody>
</table>
Appendix 2.2- List of specimens used in morphological analyses. Museums abbreviated as follows: Carnegie Museum (CM), United States National Museum (USNM), University of Kansas (KU), University of Texas (UT). Photographs from all specimens indicated, as well as those from field collected turtles, are available from the author upon request.

**Graptemys barbouri**
CM: 67334, 95190, 95997-8; USNM: 127235, 252551, 252553, 252563-4, 252566, 322849, 322862, 322864-6, 322980, 326215-6, 328065, 252550, 252556, 252559-60, 252562, 252567, 252569, 260815, 318525, 322850-6, 322858-60, 322863, 322867, 322869; UT: 34041, 48304-5

**Graptemys caglei**

**Graptemys ernsti**
CM: 122403-4, 122407-8, 122411, 122405-6, 122409-10; KU: 69073; USNM: 300604-5, 326217-20, 322998-9

**Graptemys flavimaculata**
CM: 67437, 67446-9, 67451-3, 94958-9, 94969, 95353, 95574-6, 95876, 101574-6, 101578-9; USNM: 221790-1; UT: 48297-8, 48300, 34027-9, 34066-7

**Graptemys geographica**
CM: 112871, 125143-5, 35322, 43862, 43864, 57105-8, 57110, 87498, 87501, 87503-4, 87506, 87510, 87512, 87517, 87519-21, 87526, 95088-9, 95121-3, 95191, 95601, 57761, 57763-4, 96823, 96827, 96842-3, 96835, R3199k, R3040; KU: 15881, 3225, 3265, 3742, 69070-2, 88732-4, 177136-8, 3267, 47474; USNM: 12062, 13589, 15995-8, 16373, 17821, 21123-4, 21624-5, 24697, 55695-7, 55700, 60051, 79451, 118145, 141525-8, 213716-7, 322981, 322996, 326211-2, 334932-8, 519515-7, 519519, 521309; UT: 57258, 34025-6

**Graptemys gibbonsi**
CM: 67440-2, 67460, 94904, 94966-7, 94977-80, 95056, 95563, 95570-2, 95632, 95879, 95361-2, 95559, 95577; USNM: 252584-7, 252589; UT: 34040, 34069-70, 48265-73, 35028

**Graptemys kohnii**
CM: 107612, 60401, 61711, 61714-5, 61717, 61719, 61725, 94822, 94825-6, 94853, 96165, 96168-9, 96172, 96174, 96177, 96180, S4252, S4257-8, S4261, 105293, 105297, 39951g; KU: 3107-8, 3110, 3112, 3233, 3236-40, 3254, 3257, 3287, 3344, 3360, 3375, 3377, 3405, 3795, 3800, 3803, 46746, 88735-7, 88751, 88754-5, 88758, 187862, 188350, 204364, 206479, 217261-3, 288640, 288649; UT: 55113-4
Graptemys nigrinoda
CM: 67423-3, 95000, 95009, 95019, 95769-72, 95838-9, 95843, 95848-9, 95851; USNM: 221789, 292526, 252580-1, 252583; UT: 48301-3, 34033, 34071-3

Graptemys oculifera
CM: 67470, 94882, 94885, 94888, 94915, 94931, 95049, 95557, 95641, 95666, S7518, 101581-2; USNM: 15508-9, 15511, 17820, 292527, 328068-9; UT: 48284-9, 54053-4

Graptemys ouachitensis
CM: 31246, 61733-5, 61744, 61965, 94829-30, 94876, 95113, 95126-7, 95130, 95133-6, 95157, 95212, 95402, 107234-5, 107243-44, 107624, 107662, 107770, 112830, 112884, 112893, 112906, 112925, 112927, 112932, 112956; KU: 224654; USNM: 100213-6, 118143-4, 134312, 139733, 138945, 288152, 81992, 88800

Graptemys pseudogeographica
CM: 87558-9, 87561, 87563, 87566, 94875, 95085-6, 95098-9, 95112, 95114, 95116, 97222, 97254-5, 107600, 107602, 107605, 107637-8, 107649, 107651, 107657-8, 107755, 112870, 112872, 112944; KU: 3162, 3781, 3799, 88738-42, 88744-50, 88752-3, 88757, 88759-63, 156273, 187863, 187866-7, 193299, 199737, 211376, 214302-6, 214308-310, 218669, 218788-9, 220810, 221190-1, 221474-5, 289710; USNM: 7610, 7751, 14669, 16494, 17818-9, 20958, 22718-9, 46000-1, 55259, 55529-34, 59962-3, 99888, 100075-8

Graptemys pulchra
CM: 94095, 94940, 94997-8, 95007, 95010-1, 95616, 95739, 95797, 95856, 101613, 101620, 101626; USNM: 8808, 247946-7

Graptemys versa
CM: 62164, 64080, 150949-50; KU: 88764; USNM: 198054, 323000-1, UT: 28631, 28648, 28654, 28659-60, 28863, 32917, 34249, 44279, 48250-60, 48264, 49815
CHAPTER 3. THE CONCORDANCE OF GENES, GEOGRAPHY, AND MORPHOLOGY IN THREE SPECIES OF AQUATIC TURTLES

Modified from a paper submitted to Molecular Ecology

Erin M. Myers

Abstract

Geography can have a large impact on population structuring of both genetic and morphological diversity. The differential action of selection and stochastic processes, such as drift, may potentially lead to discordant spatial patterns of genetic and morphological differentiation. Alternatively, morphological and genetic divergence may coincide as an early step in the speciation process. To address these possibilities, I examined genetic and morphological structure across the ranges of three aquatic turtle species, *Gratetmys geographica*, *G. pseudogeographica*, and *G. ouachitensis*. I found significant population genetic structuring, and all species exhibited a pattern of genetic isolation by distance, suggesting reduced gene flow over their ranges. Additionally, I found significant morphological differences among species. However, there was little support for a pattern of character displacement. In two species, morphological distance was correlated with genetic and geographic distance in a pattern of isolation by distance. Combined, these results suggest that morphological differentiation among populations within species may have evolved stochastically, rather than through the directed action of sexual or natural selection.
Introduction

Geographic isolation can have a considerable impact on structuring the genetic and morphological landscapes of populations and species. In particular, geographic distance or barriers can limit migration events, which in turn limit the amount of gene flow between populations, potentially leading to gradual divergence due to differential selection or stochastic processes (Avise, 2000). Species with specialized environmental requirements may be more susceptible to population structuring if suitable habitat is distributed non-randomly across the landscape (Nunney, 1991). Additionally, anthropogenic changes to the environment can exacerbate the situation by further restricting gene flow and migration (Riley et al., 2006; Wofford et al., 2005). For example, the addition of dams to the Mississippi River and its major tributaries has fragmented populations of many aquatic species, including fish and turtles, into small, isolated populations by dividing the river into lentic (still water) and lotic (flowing water) habitats. This process has fragmented formerly continuous populations into a series of small, isolated populations, resulting in patterns of reduced allelic variation and increased isolation by distance (Bessert, Orti, in press), as well as increased levels of inbreeding (Lamer et al., 2001).

Geographic isolation and population fragmentation may also influence morphological variation. Morphology can vary considerably across the landscape (Endler, 1977). Divergence in morphology may result from stochastic processes via the accumulation of random mutation (Illera et al., 2007) or through local adaptation (Jarrett 2008). Alternatively, phenotypic traits may vary depending on the other species in the community, manifested as a character displacement from an intermediate morphology in allopatry to a distinct, non-overlapping morphology in sympatry (Adams, Rohlf, 2000; Losos, 2000). Then again,
phenotypic characters related to mate choice may experience selection if mate preference within a population diverges (Roberts et al., 2007; Rudh et al., 2007). Such population divergence in mate preference at low levels of genetic divergence can mark the initial stages of the speciation process (Coyne, Orr, 1989; Coyne, Orr, 2004). Phenotypic differentiation can also be associated with species radiation. Consequently, it is essential to compare the observed differentiation of phenotypic characters, which may be experiencing natural or sexual selection, with those of neutral genetic markers (Rudh et al., 2007). As such, examining the relationships among genetic, phenotypic, and geographic distance can provide insight into the evolutionary patterns and processes operating within and between species, i.e. stochastic divergence or deterministic evolution (Clegg et al., 2002; Irwin et al., 2008).

The map turtle complex (genus: *Graptemys*) is an ideal group in which to examine the interaction of potential genetic and morphological fragmentation and structuring. Map turtles (*Graptemys*) are a clade of highly aquatic turtles distributed throughout the central United States. The genus consists of 12 to 13 species, the majority of which are isolated in small, separated river drainages. Three species however, exhibit broadly distributed ranges and occur in sympathy throughout the majority of their distributions. *Graptemys geographica*, *G. ouachitensis*, and *G. pseudogeographica* are similar in size and general ecological requirements, although *G. geographica* is a mollusk specialist whereas the other two species have more general dietary requirements (Ernst et al., 1994, Myers, personal obs.). Their distribution includes virtually the entire extent of the Mississippi River and many of its major tributaries (Ernst et al., 1994; Iverson, 1992). The lock and dam system placed on many of these rivers has the potential to eliminate migration across dams and fragment populations. Additionally, river traffic has increased channelization and removal of tree snags and sand
bar islands, reducing the amount of optimal nesting and basking habitat available to turtles (Moll, Moll, 2004). Previous attempts to assess the population genetic structure of *Graptemys* have been relatively limited. The use of allozymes to examine population level processes in *G. geographica*, *G. ouachitensis*, and *G. pseudogeographica* was severely limited by monomorphism (Vogt, 1978; Vogt, 1993). Therefore, the effect of habitat fragmentation on the genetic structure of these species is unknown.

In contrast, morphological variation within this species complex is well-documented. The map turtle clade is characterized by the unique color patterns of their facial region; they have a distinct yellow- to white-colored nose stripe along the central line and a region of post-orbital color (eye-bar) which merge to form a mask of color in some species. Considerable variation in both size and shape of eye-bars exists across species. As a species-specific character, this variation is presumed to have a heritable genetic component (Janzen and Ast, unpublished data), although additional research suggests that incubation conditions can exert influence on head patterns (Ewert, 1979; Vogt, 1980; Vogt, 1993). *Graptemys geographica* is characterized by a small, post-orbital dot, whereas *G. ouachitensis* and *G. pseudogeographica* have more rectangular bars behind the eye. These post-orbital color patterns are hypothesized to be involved in species recognition and mate choice (Vogt, 1978). In particular, *G. ouachitensis* and *G. pseudogeographica* exhibit a pattern suggestive of character displacement, with reduced or no overlap in eye-bar size in sympatry (Janzen *et al.*, 1995; Vogt, 1978). Because the geographic ranges are large, and some populations are isolated in individual drainages, additional conditions exist for population-specific morphologies to evolve which may inform about the processes involved in the morphological diversification and species radiation of the genus.
Investigations of genes and morphology may shed light on patterns and processes occurring within and among these three species of turtles. The goals of this study were twofold: 1) to examine the relationships of genetic, morphological and geographic divergence within each species, and 2) to examine the concordance of these patterns across three co-distributed species. Through the use of multiple lines of evidence, I infer the processes structuring morphological and genetic diversity within these species. Additionally, by examining the correlations of genes, geography, and morphology across these three wide-ranging species, I infer the processes that may have contributed to the radiation of this genus.

Methods

Tissue Collection

Three species of map turtles, the common, false, and Ouachita map turtles (Graptemys geographica, G. pseudogeographica, and G. ouachitensis, respectively) were collected during the summer in 2004-2006 at sites in Iowa, Illinois, Missouri, Nebraska, and Alabama (Table 3.1, Figure 3.1) using a combination of hand capture, and box, fyke, and basking traps. Turtles were transported to Iowa State University for a concurrent behavior experiment (Myers 2008c). Each turtle was identified to species by three independent researchers using a variety of morphological characters including shell characteristics, cheek spots, and stripes reaching the orbit (Ernst et al. 1994). A tissue sample was taken from each specimen (blood in buffer, tail clip, or thigh muscle). These samples were supplemented with 42 preserved liver samples collected from hatchlings in 1995 from Wisconsin and Louisiana (Table 3.1, Figure 3.1) as part of a prior experiment (Janzen et al., unpublished data) and five tail clips from Kentucky collected in 2001 (Table 3.1, Figure 3.1). These eight localities
represent a longitudinal sample across the range of each species and encompass several river drainages and multiple locations within some drainages.

**DNA Preparation, Amplification, and Genotyping**

DNA was extracted from tissue and blood samples using DNeasy tissue extraction kits (Qiagen). DNA was subsequently used as template for amplification. Twenty-seven microsatellite loci were previously isolated and primers developed for bog turtles (*Glyptemys muhlenbergi*) and later screened for cross-species amplification in the common map turtle (*Graptemys geographica*). From these loci, fifteen were selected for assessment across each of the three map turtle species used in the study on the basis of amplification in *Graptemys geographica*, size, and putative polymorphism (D28, D114, D90, D70, D95, D21, D55, D87, D88, B12, D16, D51, D79, B08, and D121). One individual was randomly selected from each species to use as a control to screen amplification success and polymorphism across species. D88 did not cross amplify, while D55 and D95 were monomorphic for all three species. D51, was hyper-variable with allele sizes from 230-486 and 46 discrete alleles and was excluded from subsequent analyses. From the remaining loci, five were selected for further use: D121, D114, B08, D90, and D87. These loci were chosen based on allele size classes to allow for multiplex scoring and moderate levels of polymorphism.

Polymerase chain reactions (PCR) were conducted using single locus reactions. Reaction conditions were standard across all reactions as follows: 0.12ul dNTPs (10mM), 1.25ul 10x reaction buffer, 0.5ul MgCl₂ (50mM), 0.2ul each of 50mM forward and reverse primer, 0.08ul Biolase Taq DNA polymerase, 6.65ul ddH₂O, and 3ul DNA for a final reaction volume of 12ul. Forward primers were fluorescently labeled with either 6-FAM or
HEX. Thermal cycling conditions were standard for all reactions as follows: initial
denaturation at 94° for 2 minutes, 3 cycles of 94° for 30 seconds, 58.5° for 30 seconds, 72°
for 1 minute, followed by 30 cycles of 94° for 30 seconds, 57° for 30 seconds, and 72° for 1
minute, and a final extension of 72° for 15 minutes. PCRs were performed on either an
Eppendorf Mastercycler Gradient or Techne TC-412 thermocycler. PCR products were
subsequently diluted and combined into multiplexes of 1-3 loci prior to genotyping.

All samples were genotyped on an ABI 3100 Genetic Analyzer (Applied Biosystems,
Inc.) at the Iowa State University DNA Facility. A negative control, with no template, was
used on each genotyping run and at least one individual was repeated across genotyping runs
to control for slight variation in allele sizing. Alleles were hand scored using Peak Scanner
v.1.0 (Applied Biosystems, Inc.) and assigned to allele bins.

*Genetic Data Analysis*

Multiple collection sites in proximity to one another were pooled to increase the
sample sizes of each population prior to subsequent analyses. Populations were defined by
pooling sites within the same state of collection. Analyses did not detect differentiation
between sites (see below for STRUCTURE methods and results). GenAlEx6 (Peakall,
Smouse, 2006) was used to calculate allele frequencies, observed and expected
heterozygosities. I assessed deviations from Hardy-Weinberg equilibrium and the potential
for null alleles using MicroChecker v. 2.2.3 (van Oosterhout *et al.*, 2004) and calculated null
allele frequencies (Brookfield, 1996).

Genetic distances between populations were determined using both F<sub>ST</sub> (Weir,
Cockerham, 1984) in Arlequin v. 3.11 (Excoffier *et al.*, 2005) and Nei’s genetic distance (D;
Nei, 1972) in GenAlEx6. Significance of pair-wise $F_{ST}$ values was assessed using a null distribution generated from 1,000 permutations in Arlequin. To assess population structure, an analysis of molecular variation (AMOVA; Excoffier et al., 1992) was implemented in Arlequin with 1,000 permutations. Additionally, to examine population differentiation, I utilized the program STRUCTURE v2.2 (Pritchard et al., 2000), which employs Bayesian clustering techniques to determine the number of populations (K) in the dataset. K was calculated for 20 iterations, each with 100,000 runs per iteration after a 100,000 run burn-in. Simulations were run for $K = 1$ through $K = N+2$ (where $N$ is equal to the a priori estimated number of populations based on collection location). I used the population admixture model and allowed allele frequency correlation. In addition to determining the most likely number of clusters per species using LnP(D) (hereafter referred to as L(K)) as suggested by (Pritchard et al., 2000), I also followed the method suggested by (Evanno et al., 2005), which uses an ad hoc statistic $\Delta K$ based on the rate of change in the log of probability between successive K values. The $\Delta K$ statistic, however, cannot identify cases in which K=1 is the most likely number of clusters.

**Morphological Data Analysis**

Post-orbital color pattern morphology was quantified using landmark-based geometric morphometrics (Rohlf, Marcus, 1993). These methods capture shape information after mathematically holding constant non-shape variation such as digitizing position, rotation, and scale (Adams et al., 2004). First, digital images of the dorsal view of the head were obtained using either a Nikon CoolPix 5700 or a Nikon DXM-1200 high-resolution digital camera. Images of collected specimens used in the genetic analysis were
supplemented with images of preserved museum specimens collected as part of an additional study examining map turtle morphology (Myers, in prep.). From each image, the x, y coordinates of 19 landmarks were recorded using TpsDig2 (Rohlf, 2006). Only the left post-orbital eye-bar was used to avoid any potential singularity in the data resulting from near or perfect symmetry between the sides (Bookstein, 1996; Klingenberg et al., 2002). As such, patterns of asymmetry were not examined. Landmarks included two fixed points along at the base and apex of the central nose stripe, two sliding, semi-landmarks on either side of the nose stripe, and 13 semi-landmarks positioned around the eye-bar (Figure 3.2A).

Subsequently, the x, y coordinates were used in a Generalized Procrustes Analysis to superimpose the specimens to a common coordinate system and eliminate the effects of non-shape variation (Rohlf, Slice, 1990). Semi-landmarks were allowed to slide in their positions in order to minimize the bending energy and this process was iterated three times (Bookstein, 1997). Following specimen alignment, the average specimen was determined (Figure 3.2B). Shape variables were generated as partial warp scores from the thin-plate spline (Bookstein, 1991) and the two uniform components (Rohlf, Bookstein, 2003), which can be used to test hypotheses of shape variation using standard multivariate statistics. Superimposition and shape variable computation was conducted in TPSRelWarp (Rohlf, 2007). Population mean shape was also calculated and differences between means were calculated as the sum of the Euclidean distances between each population at each shape variable.

Geographical Data Analysis

To compare the relationship of genetics and morphology with geography, I calculated geographic distance between populations for each species using two methods. The first
method employed was straight-line, Euclidean distance calculated in Google Earth (v. 4.2, Google Inc., Mountain View, CA) using the line function. As map turtles are mostly aquatic and only nesting females display terrestrial forays, migration events along this path may be unlikely. Consequently, geographic distance was also calculated as river distance, which may be more ecologically relevant. A similar approach has been employed elsewhere and found to be important (Roberts et al., 2007). Distance was measured in Google Earth utilizing the path function, which sums the distance along a series of points. In this case, satellite photos were used and the creeks and rivers joining populations were digitized to capture the meandering nature of their passage. Pair-wise distance matrices were then calculated for each species. Because genetic data were pooled by state (see Genetic Data Analysis pg 5), population pair-wise geographic distances were calculated using a weighted average approach. This method was employed so that the centroid of the population reflected the sampling intensity. Distances were measured between collection sites across populations. Population pair-wise geographic distance was subsequently determined by averaging the inter-site distances weighted according to the number of individuals from each site within the population.

Statistical Analyses

To examine patterns of morphological variation for each species, I performed a two-factor multivariate analysis of variance (MANOVA) with species and population as fixed effects along with a species x population interaction term as implemented in JMP v6.0 (SAS Institute) and R v.2.6.2 (R Development Core Team 2006). Populations were considered fixed effects as they were selected to represent a latitudinal gradient along the species distribution. For each species, I conducted an individual MANOVA to examine differences
between populations within a single species and to compare allopatric and sympatric populations in JMP. Additionally, I examined whether there was significant morphological divergence between populations using the phenotypic vector comparisons approach (Adams, Collyer, 2007; Collyer, Adams, 2007) and residual randomization. This procedure was implemented in R (R Development Core Team, 2006). The observed morphological differentiation between populations within species was calculated as Euclidean distance between least squares means from the MANOVA. The MANOVA was then reduced to species as the only factor in order to generate predicted values and residuals. These residuals were then randomly assigned to predicted values to generate “random” phenotypic values and random differentiation between populations. This procedure was repeated 9,999 times, and the fraction of random values greater than the observed was treated as the significance level (see Collyer, Adams, 2007). To compare amounts of variation across species and across populations within species, I measured disparity (Hollander et al., 2006) in Excel (v 2002, Microsoft). Morphological patterns were visualized through a principal components analysis (PCA) of shape. Thin-plate spline deformation grids were generated to graphically describe patterns of shape variation.

Population pair-wise distance matrices were generated for each species as described above to compare patterns of morphological variation with those of genetic and geographical variation. Comparisons of genetic and geographical distance were used to assess patterns of isolation by distance (Wright, 1943) or intraspecific reinforcement versus a null model (Figure 3.5). Under a null model, the prediction would be that no relationship exists between either phenotypic or genetic distance with geography. In contrast, isolation by distance is characterized by a positive linear relationship between geographic distance with either
morphological or genetic distance (Rousset, 1997). Finally, as a first step, reinforcement via mate choice discrimination utilizing eye-bars may be indicated when morphological distance is much greater than genetic distance at relatively shorter geographic distances at the intraspecific level (Coyne, Orr, 1997), although additional comparisons would need to be made with morphological features that are not suspected to be involved in mate choice. A 3-way Mantel test (Smouse et al., 1986) implemented in NTSYS (Rohlf, 2002) with 1,000 permutations was used to assess correlations of genetic and phenotypic distances, accounting for geographic distance. For each species, the analysis was performed four ways to assess the robustness of results to the different measures of genetic and geographic distance. In cases where the 3-way tests were not significant, a standard 2-way Mantel test (Mantel, 1967) was conducted on each possible 2-way pairing of distance matrices. Values of $F_{ST}$ were converted to $F_{ST}/(1 - F_{ST})$, hereafter denoted as $F_{ST}^{*}$ to differentiate from analyses using strict $F_{ST}$, prior to their use in the Mantel tests (Rousset, 1997). Following the Mantel test, a visual examination of the data patterns will be conducted for evidence of non-linearity that may be consistent with reinforcement, and a second analysis conducted incorporating this curvilinearity.

**Results**

**Genetic Data Analysis**

In total, 203 specimens were genotyped (55, 82, and 66 respectively for *G. geographica*, *G. pseudogeographica*, and *G. ouachitensis*) from 8 populations. Most loci had moderate levels of polymorphism (9-24 alleles). Allele frequency information for all three species is summarized in Appendix 1.
Data were analyzed for species to assess patterns of allelic variation within each species. Using MicroChecker, *Graptemys geographica* did not conform to Hardy-Weinberg expectations at three loci (D114, B08, and D87), *G. ouachitensis* at two loci (D121 and D87), and *G. pseudogeographica* at two loci (D90 and D87). Deviations from expectations resulted from a deficiency of heterozygotes and excess in homozygotes (p<0.05 in all cases, after adjusting confidence intervals for Bonferroni correction). Further investigation examined patterns within each population for each species to determine if null alleles or a Wahlund effect due to pooling populations might be contributing to the pattern observed. In *G. geographica*, the excess of homozygosity is driven in part by the Missouri population, where null alleles may be present at loci D114 and D87 at estimated frequencies of 0.17 and 0.10 respectively. Null alleles were not detected at locus B08 such that the heterozygote deficiency at this locus is likely the result of a Wahlund effect. In *G. ouachitensis*, deviations seem to be due to the presence of null alleles operating in multiple populations. In Iowa and Wisconsin, null alleles were present at locus D87 (estimated frequencies of 0.18 and 0.14 respectively) and in Illinois, null alleles were present at locus D121 at estimated frequency 0.14. Finally, in *G. pseudogeographica*, null alleles were only detected at locus D87 in the Nebraska populations (estimated frequency 0.11) and not at locus D90. Consequently, species level deviations at locus D90 are likely the result of a Wahlund effect. Inbreeding is not indicated as deviations did not occur consistently across all loci.

Genetic distances in the form of $F_{ST}$ and Nei’s D were calculated between each population within species (Table 3.2). Considerable variation was present in all species for both genetic measures. In *G. geographica*, the AMOVA found significant variation at all levels of analysis (Table 3.3). In both *G. ouachitensis* and *G. pseudogeographica*, the
variation was significant at the within individual and within population level but not among populations (Table 3.3).

The analysis of genetic population substructure implemented in STRUCTURE showed that for *G. geographica*, the most likely number of clusters was $K=4$ for $L(K)$ and $K=2$ for $\Delta K$. With two clusters, the Iowa population and Missouri populations generally fell out as their own clusters while individuals from Illinois and Alabama were dispersed within each of them (Figure 3.3). Interestingly, two individuals within the Missouri population showed strong support for membership in the Iowa/Illinois cluster. These individuals were collected at the Moreau River, Missouri site which is closer to the Illinois population by river distance, than the other Missouri localities. In *G. ouachitensis*, $L(K)$ identified $K=4$ or $5$ as nearly identical likelihoods, while $\Delta K$ supported $K=4$. However, the clusters did not correspond to geographic population and most individuals were admixed (Figure 3.3). Lastly, in *G. pseudogeographica*, $L(K)$ found one population to be the most likely while $\Delta K$ found two groups to be the most likely, however, the clusters did not correspond to population of origin (Figure 3.3). Within each species, I examined each population for indications of substructure; however, no signal of population substructure was detected. $K=1$ was the most likely number of populations suggesting that pooling of genetic data is not masking any substructure signal.

**Morphological Data Analysis**

Using a MANOVA, I found significant differences in eye-bar morphology between the species ($F = 21.674, p < 2.2 \times 10^{-16}$) and between populations ($F = 1.463, p = 4.929 \times 10^{-05}$). However, the interaction between species and population was non-significant ($F = 1.057,$ 05).
p = 0.329), suggesting no substantive geographical discordance in morphological variation among the species. Relative to the overall average specimen, *G. geographica* was characterized by a rounded post-orbital spot located more distantly from the central nose stripe (Figure 3.4). *G. ouachitensis* and *G. pseudogeographica* were generally similar in having a post-orbital stripe rather than spot. However, there were several morphological differences including a thicker eye-bar located more closely to the nose stripe, and a shorter, wider nose stripe in *G. ouachitensis* (Figure 3.4). In contrast, *G. pseudogeographica* had a thinner eye-bar, and a long, thin nose stripe (Figure 3.4). For *G. ouachitensis* and *G. pseudogeographica*, I found significant differences between populations within each species (F = 2.007, p <0.0001, F = 1.552, p = 0.012, respectively; Figure 3.4). However, for *G. geographica*, populations were not significantly different (F = 1.071, p = 0.375; Figure 3.4). Using MANOVA, there was no significant difference in morphology between sympatric and allopatric populations for *G. geographica* or *G. pseudogeographica* (F = 1.062, p = 0.437, F = 1.150, p = 0.361, respectively). There was no allopatric population for comparison in *G. ouachitensis*. The inclusion or exclusion of museum specimens did not alter the results (data not shown).

Population pair-wise morphological distance was calculated for each species (Table 3.4). Using residual randomization, most pair-wise differences between species-population groups were not significant (Table 3.4). For a subset of cases where each species was present in the same two populations (WI-IA, IA-IL, IL-LA), I also compared the magnitude and direction of phenotypic shape change across species between the populations to test for any similarities in morphological change across the landscape. However, none of these comparisons was statistically significant (p>0.07, most with p>0.25). Lastly, I examined
patterns of morphological variation within the dataset. Morphological variance was not
distributed evenly. Across species, *G. ouachitensis* and *G. pseudogeographica* had nearly
identical values of disparity, while *G. geographica* had much less variance (D= 0.0085 vs.
0.024 and 0.023 for *G. geographica*, *G. ouachitensis* and *G. pseudogeographica* respectively,
Figure 3.4). Variance from the mean, as measured by disparity, was similarly variable across
species-population groups (D ranged from 0.0066 to 0.032). There was a slight trend for
increased disparity with increasing sample size.

*Geographic Distance Analysis*

Geographic pair-wise distance was calculated between each population within each
species (Table 3.5). Distance varied considerably for some populations depending on the
distance metric used (river distance vs. straight-line distance). For each species a 3-way
Mantel test was performed, using each combination of genetic and geographic distance
measure, along with morphology. For *G. ouachitensis*, the Mantel statistic was significant in
all four analyses (F_{ST}/Morph/River: r = 0.674, p = 0.018; F_{ST}/Morph/Straight: r = 0.768, p
= 0.005; Nei/Morph/River: r = 0.660, p = 0.021; Nei/Morph/Straight: r = 0.776, p = 0.031).
For *G. pseudogeographica*, two analyses were significant and two were nearly significant
(F_{ST}/Morph/River: r = 0.589, p = 0.05; F_{ST}/Morph/Straight: r = 0.529, p = 0.052;
Nei/Morph/River: r = 0.729, p = 0.023; Nei/Morph/Straight: r = 0.652, p = 0.009). Both
species exhibited a pattern of isolation by distance for both genes and morphology (Figure
3.5).

However, for *G. geographica*, none of the 3-way test values was significant
(F_{ST}/Morph/River: r = -0.326, p = 0.74; F_{ST}/Morph/Straight: r = -0.878, p = 1.000;
Nei/Morph/River: $r = 0.807$, $p = 0.253$; Nei/Morph/Straight: $r = 0.626$, $p = 0.139$). When deconstructed into pair-wise comparisons, some genetic and geographic distance comparisons were significant, no morphology and geography comparisons were significant (Figure 3.5), and no morphology and genetic distance comparisons were significant ($F_{ST}^*/$River: $r = 0.417$, $p = 0.237$; $F_{ST}^*/$Straight: $r = 0.562$, $p = 0.215$; Nei/Straight: $r = 0.829$, $p = 0.036$; Nei/River: $r = 0.377$, $p = 0.294$; Morph/Straight: $r = 0.552$, $p = 0.218$; Morph/River: $r = 0.00639$, $p = 0.494$; Morph/$F_{ST}^*$: $r = -0.294$, $p = 0.693$; Morph/Nei: $r = 0.750$, $p = 0.127$).

**Discussion**

The goals of this project were to assess the correlation and concordance of genetic, geographic and morphological patterns across three congeneric species of aquatic turtles throughout much of their distribution to determine if and how morphology changes across populations for each species. All three species exhibited a genetic pattern of isolation by distance. Additionally, while species were significantly different morphologically, there were few significant differences among populations within species. Finally, in two species, morphological differences were correlated with both genetic and geographic distances. The interpretation of these combined results is discussed below.

*Genetic Interpretation*

Given the large distribution of these three species, the levels of population differentiation were quite high. $F_{ST}$ values for map turtles were consistent with those of other related emydid turtles exhibiting habitat and genetic fragmentation (up to 0.465 in *Emys*...
blandingii; Mockford et al., 2007). Other river turtles have low $F_{ST}$ values within rivers (0.003-0.04) and higher values between basins (0.04-0.22; Pearse et al., 2006). In contrast, the data here show equally high divergence both within a single river (along the Mississippi River) and across multiple tributaries (Missouri River, Mississippi River, Jack’s Fork River, Current River, and Tennessee Rivers). Additionally, all species exhibited a genetic pattern of isolation by distance (Figure 3.5), though this pattern was more noticeable in *Graptemys pseudogeographica* and *G. ouachitensis*. This pattern suggests that gene flow is restricted to some degree in these turtles across all three species. It seems likely that impoundments can restrict gene flow between populations. Earlier studies revealed that gene flow may occur via aquatic or terrestrial migration in other riverine turtle species (Scribner et al., 1986). However, map turtles rarely leave the water and thus movement around the impoundments is likely to be minimal at best, and potentially confined to flood events. Finally, current levels of genetic divergence are likely to underestimate future fragmentation levels as these turtles are relatively long-lived (30+ yrs; Ernst et al., 1994) while dams have only been in place for approximately 75 years. Thus, current fragmentation is the result of any pre-existing divergence and only a few generations of separation and therefore, these patterns may be exacerbated in future years as the number of generations experiencing fragmentation continues to increase (Kuo, Janzen, 2004).

Interestingly, the amount of genetic population structuring was not consistent across all groups. This counters the initial expectation that the amount of genetic structuring within species should be similar given their overlapping distributions and shared habitats. *Graptemys pseudogeographica* and *G. ouachitensis* exhibited a strong pattern of isolation by distance. This likely contributed to the admixed nature of individuals in the STRUCTURE
analysis, as STRUCTURE is not well suited to analyses for isolation by distance and is more likely to overestimate the number of populations (Pritchard et al. 2008; Schwartz and McKelvey, in press). However, *G. geographica* seemed to be more genetically structured than the other two species. In particular, genetic distances were greater between populations of *G. geographica* when compared to *G. pseudogeographica* or *G. ouachitensis* for the same comparisons (i.e. comparison of IA to IL). Additionally, STRUCTURE found more fully resolved clusters and the AMOVA was significant for variation among populations in *G. geographica* compared to the other species. These results may not be surprising given the more restrictive dietary needs of *G. geographica*. Specialization may result in a patchier distribution and use of the habitat and may be more likely to elicit higher genetic divergence for specialists versus generalists (Nunney, 1991). Therefore, *G. geographica* is likely to be more restricted in its habitat choices and may not utilize as much of the available riverine habitat as its congeners. In addition, because fragmentation of the rivers by impoundments can reduce dissolved oxygen and increase dissolved CO$_2$ and siltation, detrimental to the mollusk prey of *G. geographica*, they are likely to affect *G. geographica* more than its congeners (Moll, Moll, 2004).

*Morphological Interpretation*

Original species descriptions of map turtles were based on morphological characters, particularly post-orbital color pattern variations (see McKowan, 1972 for a review). Consistent with current species descriptions (Ernst *et al.*, 1994), I found significant morphological differences between species. *Graptemys geographica* was quite different from the other two species in having a rounded post-orbital spot. *G. ouachitensis* and *G.
pseudogeographica are quite similar morphologically and have had a cluttered taxonomic history (Vogt, 1978; Vogt, 1993). My results support these earlier studies in highlighting the width of the eye-bar as a potential factor for use in species discrimination for taxonomy. In addition, these results have also shown that the length and width of the central nose stripe may also be useful. However, considerable variation remains within these species for these traits, and as such those seeking field identifications should continue to use the suite of available characters including number of lines entering the orbit and the presence and size of cheek spots above and below the jaw.

In addition to species level morphology, I examined morphological differences within species to determine if phenotype may be involved in population differentiation; however, population-level differences were difficult to detect. While sample size was small for some populations, it is unlikely that low power was a major factor due to considerable within-group variation. Analyses of dispersion indicated that within-population variance increased as sample size increased, suggesting that a great deal of variation in morphology may not be strictly related to geographic location. These results are similar to those for other turtle species that exhibit substantial morphological variation within populations and relatively little divergence between populations throughout the species range (Reynolds, Seidel, 1983). In map turtles, differences in incubation temperature could potentially cause variation in size of blotches on the heads of both G. ouachitensis and G. pseudogeographica (Vogt, 1978; Vogt, 1980). Therefore, much of the observed morphological variation may have an environmental, rather than genetic, basis. Further research should attempt to examine the relative effects of genetic and environmental factors on morphological variation of the post-orbital color patterns within these species.
All three species did not exhibit the same correspondence across genetic, geographic, and morphological distances. Significant correlations among all three measures characterized both *G. pseudogeographica* and *G. ouachitensis* but such patterns were not evident in *G. geographica*. Further assessment revealed that morphology was not correlated with either genetic structure or geography in *G. geographica*. Thus, the morphological signal was considerably stronger in *G. pseudogeographica* and *G. ouachitensis*. This result may be consistent with the hypothesis that eye-bar morphology is a key factor in species recognition and mate choice in these two species. Several lines of evidence suggest instead that a stochastic process such as drift has resulted in the patterns of morphological differentiation observed. First, the data do not support character displacement as a mechanism for the variation in post-orbital color pattern. For those species (*G. geographica* and *G. pseudogeographica*) with both allopatric and sympatric populations, there was no significant difference in morphology between allopatric and sympatric populations to suggest that a character shift occurred. However, it was not possible to examine character shifts in the third species (*G. ouachitensis*), as there are no allopatric populations in this species. Secondly, following Clegg et al. (2002), comparisons of the patterns of morphological differences across species did not show any consistent pattern, lending support to the hypothesis that drift may have been involved. When comparing the matrices of morphological, genetic, and geographic distance, they do not support enhanced morphological differences at shorter geographic distances, which would have been consistent with reinforcement between populations (Figure 3.5). On the contrary, one species did not show any correlation with morphology potentially indicating a neutral trait or stabilizing selection. Two species had patterns of isolation by distance suggesting a more stochastic process for phenotypic
differentiation between populations similar to patterns observed elsewhere (Irwin et al., 2008), including other types of radiations, e.g. island diversification (Illera et al., 2007). It is also possible that the pattern may represent local adaptation; however there was no consistent pattern of morphological differentiation along a latitudinal gradient as might have been expected under this scenario (Figure 3.4)

In summary, this study detected significant patterns of isolation by distance and population genetic structuring across three wide-ranging congeneric species of turtles. However, habitat specialization may have led to increased divergence in the specialist species versus its generalist counterparts. In establishing the potential role of eye-bar variation in diversification of this genus, I found significant differences in trait morphology consistent with post-orbital color pattern as a species-specific trait, but that variation exists within species. The concordant morphological and genetic patterns of isolation by distance suggest that stochastic drift is most likely driving the differentiation of both phenotype and genotype in these species, rather than natural or sexual selection. This result implies that morphology may not have been a driving factor in the speciation and radiation of this species-rich turtle genus, though future research employing behavioral tests should examine if this morphological trait is truly used for species or mate recognition and similarly should examine the relationship of genealogical and morphological patterns across the entire genus.

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**Figure Legends**

Figure 3.1. Map of collection localities for three species of map turtles. The species are distributed throughout the area shown in the figure and are sympatric in the majority of their distribution.

Figure 3.2. (A) Location of landmarks along the nose stripe and post-orbital markings of three species of map turtle (*G. geographica*, *G. ouachitensis*, and *G. pseudogeographica* from left to right). (B) Procrustes Alignment of specimens and (C) final consensus configuration of landmarks averaged across all specimens.

Figure 3.3. Clustering results for each map turtle species from STRUCTURE. A) Results for *Graptemys geographica* for two clusters, B) results for *G. ouachitensis* for four clusters, and C) results for *G. pseudogeographica* for two clusters. Cluster membership is represented along the Y-axis and individuals are organized along the X-axis according to population of origin.

Figure 3.4. Canonical variates plot of mean morphology for each species-population group. Symbol and color signifies species: gray circles represent populations of *Graptemys geographica*, white squares represent *G. ouachitensis*, and black triangles represent *G. pseudogeographica*. Labels indicate populations with abbreviations following Table 3.1. Mean morphology for each species is shown using deformation grids.
Figure 3.5: A) Theoretical outcomes of correlation among phenotypic, genetic, and geographic distances under three potential models: null model- no relationship of genetic of phenotypic distance with geographic distance, isolation by distance (IBD)- positive relationship of genetic and/or phenotypic distance with geographic distance, and reinforcement (Rfmnt)- greater phenotypic distance compared to genetic distance at relatively short geographic distances . B) Correlation among phenotypic and geographic (River) distances in the first row and genetic (Nei’s D) and geographic (River) distances in the second row for three species of turtles. Results were similar for other comparisons (FST and Straight distances).
Table 3.1: Summary of collection localities and specimen numbers for genetic and morphological analyses. Locality indicates pooled population used in analyses, while site represents collection locality. Totals for each locality are presented with abbreviation for each pooled locality. \( n_{\text{gen}} \) is the number of turtles for which tissue samples were taken and used in genetic analysis. \( n_{\text{morph}} \) is the number of turtles for morphological analyses. \( n_{\text{gen}} \) may not equal \( n_{\text{morph}} \) due to the inclusion of museum specimens and not all turtles could be successfully photographed. G indicates *Graptemys geographica*, O is *G. ouachitensis*, and P is *G. pseudogeographica*.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Drainage</th>
<th>Lat (N)</th>
<th>Lon (W)</th>
<th>( n_{\text{gen}} )</th>
<th>( n_{\text{morph}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>G, O, P</td>
<td>G, O, P</td>
</tr>
<tr>
<td>Wisconsin</td>
<td>Mississippi River</td>
<td>43° 39’ 49”</td>
<td>91° 13’24”</td>
<td>0, 27, 10</td>
<td>0, 20, 9</td>
</tr>
<tr>
<td></td>
<td>(WI)</td>
<td></td>
<td></td>
<td><strong>Total: 0, 27, 10</strong></td>
<td>0, 20, 9</td>
</tr>
<tr>
<td>Iowa</td>
<td>Mississippi River</td>
<td>42° 46’ 43”</td>
<td>91° 5’ 28”</td>
<td>21, 7, 0</td>
<td>12, 4, 0</td>
</tr>
<tr>
<td>Iowa</td>
<td>Mississippi River</td>
<td>41° 56’ 24”</td>
<td>90° 7’ 36”</td>
<td>3, 3, 0</td>
<td>0, 1, 0</td>
</tr>
<tr>
<td>Iowa</td>
<td>Cedar River</td>
<td>41° 32’ 12”</td>
<td>91° 8’ 29”</td>
<td>0, 11, 6</td>
<td>0, 4, 1</td>
</tr>
<tr>
<td></td>
<td>(IA)</td>
<td></td>
<td></td>
<td><strong>Total: 24, 21, 6</strong></td>
<td>12, 9, 1</td>
</tr>
<tr>
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<td>Missouri River</td>
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<td>98° 3’ 40”</td>
<td>0, 0, 38</td>
<td>0, 0, 29</td>
</tr>
<tr>
<td></td>
<td>(NE)</td>
<td></td>
<td></td>
<td><strong>Total: 0, 0, 38</strong></td>
<td>0, 0, 29</td>
</tr>
<tr>
<td>Illinois</td>
<td>Mississippi River</td>
<td>38° 57’ 52”</td>
<td>90° 26’ 1’</td>
<td>3, 25, 12</td>
<td>10, 25, 14</td>
</tr>
<tr>
<td></td>
<td>(IL)</td>
<td></td>
<td></td>
<td><strong>Total: 3, 25, 12</strong></td>
<td>10, 25, 14</td>
</tr>
<tr>
<td>Missouri</td>
<td>Moreau River</td>
<td>38° 32’ 29”</td>
<td>92° 6’ 25”</td>
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<td>2, 0, 0</td>
</tr>
<tr>
<td>Missouri</td>
<td>Crane Creek</td>
<td>36° 48’ 0”</td>
<td>93° 32’ 24”</td>
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<td>5, 0, 0</td>
</tr>
<tr>
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<td>93° 14’ 24”</td>
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<td>1, 0, 0</td>
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<tr>
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<td>91° 18’ 0”</td>
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<td>32, 0, 0</td>
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<tr>
<td></td>
<td>(MO)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>(LA)</td>
<td></td>
<td></td>
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<td>0, 4, 7</td>
</tr>
<tr>
<td>Alabama</td>
<td>Tennessee River</td>
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<td>2, 0, 0</td>
</tr>
<tr>
<td></td>
<td>(AL)</td>
<td></td>
<td></td>
<td><strong>Total: 1, 0, 0</strong></td>
<td>2, 0, 0</td>
</tr>
<tr>
<td>Kentucky</td>
<td>Kentucky Lake</td>
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<td>88° 7’ 59”</td>
<td>0, 5, 0</td>
<td>0, 4, 0</td>
</tr>
<tr>
<td></td>
<td>(KY)</td>
<td></td>
<td></td>
<td><strong>Total: 0, 5, 0</strong></td>
<td>0, 4, 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>Species Totals:</strong></td>
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<td>55, 82, 66</td>
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Table 3.2: Genetic distance between populations in three species of map turtle. Pair-wise $F_{ST}$ values below the diagonal and Nei’s D above the diagonal. $F_{ST}$ values in bold, significant at $p = 0.05$. Locality abbreviations follow those presented in Table 1.

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<thead>
<tr>
<th></th>
<th>IA</th>
<th>IL</th>
<th>MO</th>
<th>AL</th>
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</thead>
<tbody>
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<td><strong>Geographica</strong></td>
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<tr>
<td>IA</td>
<td>-</td>
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<tr>
<td>AL</td>
<td><strong>0.242</strong></td>
<td>-0.033</td>
<td>0.033</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>WI</th>
<th>IA</th>
<th>IL</th>
<th>LA</th>
<th>KY</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ouachitensis</strong></td>
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<td>-</td>
<td>0.216</td>
<td>0.161</td>
<td>0.896</td>
<td>0.286</td>
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<tr>
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<td>0.021</td>
<td><strong>0.065</strong></td>
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</tr>
<tr>
<td>KY</td>
<td>0.052</td>
<td>-0.013</td>
<td>-0.031</td>
<td><strong>0.138</strong></td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>WI</th>
<th>IA</th>
<th>IL</th>
<th>LA</th>
<th>NE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pseudogeographica</strong></td>
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<td></td>
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<tr>
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<td>0.402</td>
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<tr>
<td>IL</td>
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<td><strong>0.029</strong></td>
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<tr>
<td>LA</td>
<td>0.098</td>
<td>0.241</td>
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<td>-</td>
<td>1.139</td>
</tr>
<tr>
<td>NE</td>
<td>0.028</td>
<td>0.046</td>
<td>0.005</td>
<td>0.031</td>
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</table>

Table 3.3: AMOVA results for each species of map turtle. Amount of variation explained by each partition is shown and significance is indicated with an asterisk.

<table>
<thead>
<tr>
<th>Species</th>
<th>w/in Individual</th>
<th>Individual w/in Population</th>
<th>Among Populations</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. geographica</em></td>
<td>0.726*</td>
<td>0.082*</td>
<td>0.192*</td>
</tr>
<tr>
<td><em>G. ouachitensis</em></td>
<td>0.878*</td>
<td>0.099*</td>
<td>0.023</td>
</tr>
<tr>
<td><em>G. pseudogeographica</em></td>
<td>0.886*</td>
<td>0.082*</td>
<td>0.032</td>
</tr>
</tbody>
</table>
Table 3.4: Pair-wise morphological distances between populations within each species of map turtle are below the diagonal. Significance of pair-wise differences in morphology between species-population groups following residual randomization is indicated above the diagonal. Locality abbreviations follow those presented in Table 1.

<table>
<thead>
<tr>
<th>Geographica</th>
<th>Ouachitensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>WI</td>
</tr>
<tr>
<td></td>
<td>- 0.193 0.221 0.514</td>
</tr>
<tr>
<td>IL 1.785</td>
<td>IA 3.604</td>
</tr>
<tr>
<td></td>
<td>- 0.014 0.195 0.037</td>
</tr>
<tr>
<td>MO 1.870</td>
<td>IL 4.535 2.671</td>
</tr>
<tr>
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<td>- 0.549 0.034</td>
</tr>
<tr>
<td>AL 4.920</td>
<td>LA 6.323 5.365 6.361</td>
</tr>
<tr>
<td></td>
<td>- 0.377</td>
</tr>
<tr>
<td></td>
<td>KY 4.226 3.307 3.515</td>
</tr>
<tr>
<td></td>
<td>5.932 -</td>
</tr>
</tbody>
</table>

Table 3.5: Geographic distance between populations within each species of map turtle. River distance is below the diagonal and straight-line distance is above the diagonal. Locality abbreviations follow those presented in Table 1. Distance is measured in kilometers.

<table>
<thead>
<tr>
<th>Geographica</th>
<th>Ouachitensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA 416 620 961</td>
<td>WI - 186 524 1455 790</td>
</tr>
<tr>
<td>IL 603 - 243 580</td>
<td>IA 317 - 343 1274 614</td>
</tr>
<tr>
<td>MO 2183 1580 - 542</td>
<td>IL 725 497 - 935 299</td>
</tr>
<tr>
<td>AL 1495 892 1849 -</td>
<td>LA 2549 2321 1824 - 759</td>
</tr>
<tr>
<td></td>
<td>KY 1180 952 455 1607 -</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pseudogeographica</th>
</tr>
</thead>
<tbody>
<tr>
<td>WI - 0.224 0.278 0.023</td>
</tr>
<tr>
<td>IA 4.137 - 0.346 0.417</td>
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<tr>
<td>IL 2.934 4.040 - 0.417</td>
</tr>
<tr>
<td>LA 7.105 7.542 6.583 -</td>
</tr>
<tr>
<td>NE 2.353 4.079 1.691 7.100 -</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pseudogeographica</th>
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</thead>
<tbody>
<tr>
<td>WI - 236 524 1455 561</td>
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<tr>
<td>IA 471 - 291 1223 588</td>
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<tr>
<td>IL 725 424 - 935 770</td>
</tr>
<tr>
<td>LA 2549 2248 1824 - 1494</td>
</tr>
<tr>
<td>NE 2047 1746 1322 3076 -</td>
</tr>
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</table>
Figure 3.3
Figure 3.4
Figure 3.5
Appendix

Appendix 3.1: Allele frequencies across populations for all three species of map turtle, *G. geographica* (Geo), *G. ouachitensis* (Ouach), and *G. pseudogeographica* (Pseudo). Locus names are to the left, followed by allele size classes, and frequency within each species.

<table>
<thead>
<tr>
<th></th>
<th>Geo</th>
<th>Ouach</th>
<th>Pseudo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D121</strong></td>
<td>128</td>
<td>0.045</td>
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<td></td>
<td>132</td>
<td>0.336</td>
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<td></td>
<td>136</td>
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<td>0.139</td>
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<tr>
<td></td>
<td>140</td>
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<tr>
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<th>Pseudo</th>
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CHAPTER 4. QUANTITATIVE GENETICS OF SPECIES-SPECIFIC COLOR PATTERNS IN THREE SPECIES OF TURTLES

Modified from a paper to be submitted to the Journal of Evolutionary Biology

Erin M. Myers

Abstract

Morphological traits tied to species recognition are subject to strong natural selection to prevent maladaptive hybrid offspring. Morphological traits typically have high heritabilities indicating rich standing genetic variation and an ability to respond to selection. However, morphological traits may also be influenced by environmental factors such as temperature and moisture. I estimated the multivariate heritability of post-orbital coloration patterns in three sympatric species of turtles and analyzed the influence of temperature and hydric conditions experienced during embryonic development on variation in these traits. Hydric conditions did not significantly influence morphology but temperature did. Heritability was similar in all three species, ≈ 0.3. These results suggest that there is both a genetic and thermal effect on morphology in these species, consistent with previous studies. The relatively low heritability for this morphological character suggests either low additive genetic variance for the trait or low genetic variation as a result of selection. Additional research is needed to differentiate between these hypotheses.
Introduction

Morphology often plays a key role in adaptation within species and in differentiation among species (e.g. Losos 1994; Nagel and Schluter 1998; Coyne and Orr 2004; Grant and Grant 2006; Roberts et al. 2007). In particular, phenotypic traits tied to mate choice and species recognition may have a key role in the speciation process by impacting patterns of reinforcement (Coyne and Orr 2004). Traits that are important for species recognition likely have a substantial genetic basis (e.g. Noor et al. 2001). During the speciation process and subsequent species maintenance, traits involved in species recognition are probably under strong natural selection, or a combination of both natural and sexual selection (Coyne and Orr 2004). This strong selection may erode heritable genetic variation in a population by fixing favorable alleles and eliminating unfavorable ones (Mousseau and Roff 1987). However, environmental factors can also influence morphology, creating a variety of phenotypes upon which selection can act, through phenotypic plasticity and genetic by environment interactions (Pigliucci 2001). Higher levels of environmental influence or relatively low contribution of genetic variance to total phenotypic variance can result in lower heritability. Establishing which processes shape morphological variation requires both an examination of the trait in question, its potential environmental influence and, ideally, other morphological traits for comparison.

Morphological traits typically have high heritability values (e.g. 0.71-0.84 for digitation in Taeniopygia, Forstmeier 2005; 0.4-0.7 for shell shape in Littorina, Conde-Padín et al. 2006; 0.56 for tarsus length in Parus, Hadfield et al. 2007), suggesting high levels of standing genetic variation and consequently, an ability to respond evolutionarily to selection, and a generally weaker direct link to fitness (Mousseau and Roff 1987; Merilä and Sheldon...
However, phenotypic variation within species is widespread and often correlated with environmental factors such temperature and rainfall (Roff and Mousseau 2005). In ectotherms, temperature can directly influence metabolic rate in both adults and juveniles, as well as incubation length and, in some species, sex of offspring (Bull and Vogt 1979; Ewert 1985; Rhen and Lang 2004). A common garden experiment allows one to partition this existing phenotypic variation of a trait to determine the relative contributions of specific, controlled environmental factors, as well as determine the heritable genetic component (Roff et al. 2004). The ultimate goal is to establish the proximate mechanisms shaping the phenotypic variation seen in nature.

Map turtles (genus: *Graptemys*) are characterized by a number of unique morphological features ranging from extreme sexual size dimorphism, exaggerated carapacial ridges, and species-specific coloration patterns on their heads (Ernst et al. 1994; Lindeman 2008; Myers 2008a). Three species, *Graptemys geographica*, *G. ouachitensis* and *G. pseudogeographica*, are widely distributed and sympatric throughout most of their range (Ernst et al. 1994). The post-orbital coloration patterns, in particular, have been of interest for this genus because of their suspected role in species recognition (Vogt 1980; Myers 2008a; 2008b; 2008c). These patterns are characterized by species-specific morphologies, ranging from a small dot to stripes located behind the eye (Figure 4.1A-C), and variable levels of intraspecific variation (Myers 2008a; 2008b). Earlier research has suggested that at least some aspects of these morphologies are heritable (Janzen and Ast, unpublished data; Vogt 1993). In contrast, other research has shown that these traits are subject to environmental variation, in particular associated with incubation temperature (Ewert 1979; Vogt 1980, 1993). Map turtle sex is determined by temperature during embryonic incubation (Bull and
Vogt 1979, 1981). Therefore, such variation may result from temperature-specific effects, from the suite of differences initiated by the temperature-dependent sex determination cascade and subsequent sex-effect, or a combination of both (Rhen and Lang 2004). Temperature has been shown to influence skin coloration patterns in a closely related species of turtle, *Trachemys scripta* (Etchberger et al. 1993). Temperature also may indirectly influence variation in skin coloration by affecting developmental rate and incubation length (Ewert 1985). In addition to temperature, hydric conditions experienced by the embryo during incubation can impact embryo physiology and size at hatching, which in turn has the potential to influence morphological variation (Janzen et al. 1995). Thus, the relative contribution of each of these environmental and genetic effects on the post-orbital coloration is unknown.

The aim of this project was to determine the relative contribution of temperature and hydric environmental factors as well as quantitative genetic variation to variation in post-orbital coloration in map turtles. There were several potential outcomes, which were not necessarily mutually exclusive. Given that these traits are species-specific, I hypothesize that they have a substantial genetic underpinning. If heritability is high, environmental factors play a relatively minor role in shaping phenotypic variation, implying high levels of standing additive genetic variation and low selection. In contrast, low heritability suggests that environmental effects are the relatively major factors producing phenotypic variation. However, low heritability may also indicate that strong selection has reduced additive genetic variation for these traits (Mousseau and Roff 1987). To address these potential outcomes, I used a series of *Graptemys* families reared in a common garden environment that allow for a controlled assessment of phenotypic, genetic, and environmental variance.
Methods

Specimens

Multivariate heritability of post-orbital coloration was examined in three species of map turtle, *Graptemys geographica, G. ouachitensis*, and *G. pseudogeographica*. Average clutch size in the wild ranges from ≈ 10 in *G. geographica* and *G. ouachitensis* to ≈ 14 in *G. pseudogeographica* (Vogt 1980; Ernst et al. 1994). In total, 1512 hatchlings from 191 families were used in the analyses (292 specimens in 37 clutches for *G. geographica*, 649 specimens in 84 clutches for *G. ouachitensis*, and 571 specimens from 70 clutches for *G. pseudogeographica*; Table 4.1). All families were collected from the same population near Stoddard, Wisconsin. Specimens derived from three large data sets and represented hatchlings grouped according to clutch. A series of clutches was obtained from the Carnegie Museum, as deposited by M. A. Ewert (hereafter referred to as Ewert clutches). These clutches spanned multiple years (1973-1985) and each clutch was split and incubated at a variety of temperatures ranging from 22°C to 30°C. The second data set (Vogt clutches), also from Carnegie Museum, included clutches from a single year (1978) used as part of a project on temperature-dependent sex determination (Bull and Vogt 1979). These clutches were incubated at either 25°C or 30.5°C for the whole clutch. Finally, a remaining series of clutches (Janzen clutches) were used from a 1991 experiment on hydric conditions during incubation (Janzen et al. 1995). These clutches were incubated at 29°C, while water potential was varied (-150 kPa or -950 kPa). All 191 clutches were laboratory incubated under a constant thermal profile. This dataset incorporated clutches across multiple years to increase overall sample size. Though not known for certain, given the long-lived nature of these species, relatively long age to maturity (Ernst et al. 1994), and the very large population size
at this site (Vogt 1980, 1993), it was extremely unlikely that a nest from the exact same
female was sampled more than once, yet likely that the same females nested in most of the
years sampled.

Data Collection

Post-orbital color pattern were quantified using landmark-based geometric
morphometrics (Rohlf and Marcus 1993). These methods capture shape information while
mathematically holding constant non-shape variation in the form of digitizing position,
rotation, and scale (Adams 2004). For the Ewert and Vogt clutches, digital images of the
dorsal view of the head were obtained using a Nikon DXM-1200 high-resolution digital
camera. For the Janzen clutches, photographic slides were obtained for each turtle. Each slide
was scanned using a Nikon SuperCool digital slide scanner to generate digital images for
subsequent digitization. Once digital images were generated for all specimens, the x, y
coordinates of 19 landmarks for each specimen were recorded using TpsDig2 (Rohlf 2006).
Only the left post-orbital coloration was used to avoid numerical singularity resulting from
near or perfect symmetry between sides of the head (Bookstein 1996; Klingenberg et al.
2002). Therefore, patterns of asymmetry were not examined in this dataset. The digitized
landmarks included two fixed points along the apex and base of the central nose stripe. These
landmarks were supplemented by 17 sliding, semi-landmarks, two on either side of the nose
stripe and 13 positioned around the post-orbital region (Figure 4.1A-C). The x,y coordinates
were subsequently used in a Generalized Procrustes Analysis to superimpose specimens to a
common coordinate system and eliminate non-shape variation (Rohlf and Slice 1990). Semi-
landmarks were allowed to slide in their position to minimize Procrustes’ distance (Bookstein
1997). Each species was analyzed separately, as the focus of the study was within-species variation, which would likely be overwhelmed by between-species variation during the sliding process. Shape variables (34) were generated as partial warp scores from the two uniform components (Rohlf and Bookstein 2003) and the thin-plate spline (Bookstein 1991). Superimposition and shape variable generation were conducted in TpsRelWarp (Rohlf 2007). Shape variables were then used to test hypotheses of shape variation and heritability using multivariate statistics. Finally, following alignment, the average shape for each species was determined.

_Heritability Analysis_

To determine the degree to which variation in post-orbital coloration had a quantitative genetic basis, I calculated multivariate heritability. Multiple methods for this calculation have been proposed (Klingenberg and Leamy 2001; Monteiro et al. 2002; Klingenberg 2003; Klingenberg and Monteiro 2005). However, recent work has shown that the method based on the multivariate generalization of the breeders’ equation is preferable, as this utilizes the original multivariate data set and, as a result, allows one to examine both the magnitude and direction of heritability (Klingenberg 2003; Klingenberg and Monteiro 2005; Myers et al. 2006). To estimate the genetic and phenotypic variance components for each species, I utilized restricted maximum likelihood (REML; Lynch and Walsh 1998; Klingenberg and Leamy 2001). This approach can accommodate unbalanced designs commonly found in studies incorporating wild populations (Milner et al. 2000). In addition, REML and animal model approaches allow for the inclusion of fixed effects that may be known sources of variation (Wilson 2008). Using the software package WOMBAT (Meyer
2006a, 2006b, 2007), I employed a full-sib animal model with temperature, year, and hydric condition as fixed effects. Given that parental phenotypes were unknown for these clutches, and the potential for multiple paternity within clutch (discussed below), this experimental design only permits the estimation of total genetic variation, rather than strictly additive genetic variation. Because this method is computationally intensive and the shape data are of high dimension relative to the number of families, the shape data were transformed via Principal Components Analysis (PCA) in TpsRelWarp (Rohlf 2007). Only a subset of the shape data principal components (PCs) was used in the REML analysis (7 for *G. geographica*, 7-9 for both *G. ouachitensis* and *G. pseudogeographica*) which captured 85-95% of the shape variation in the data (see Results). The phenotypic (*P*) and genetic (*G*) variance-covariance matrices of shape were determined. From these I calculated $GP^{-1}$ which is essentially equivalent to multivariate heritability based on the multivariate extension of the standard breeder’s equation, $\Delta z = GP^{-1}S$ (Lande 1979; Lande and Arnold 1983). I then calculated a single metric of heritability ($H^2$) as the dominant eigenvalue $\lambda_1$ (Klingenberg 2003) divided by the sum of all eigenvalues (see also Myers et al. 2006). To assess the significance of the heritability estimates, I utilized a re-shuffling procedure to reassign individuals to clutches, estimate the new *G* and *P* matrices, and recalculate $H^2$. Family size and number we held constant, only individual membership was shuffled. 1000 permutations were implemented to generate a random distribution of heritability values where full datasets were used, while 100 permutations were used with subsets of the data due to an increased frequency in non-convergence in these smaller, randomized datasets. P values are given as the number of random values larger than the actual value.
Statistical Analysis

All 34 shape variables were used to assess the relative importance of various fixed effects on shape variation. Analyses were conducted for each species separately. A multivariate analysis of variance (MANOVA) was conducted with temperature and year as fixed effects for all samples within each species. A second MANOVA was performed on the subset of data for which hydric information was available using hydric condition and temperature as fixed effects. The significance of the interaction terms could not be examined given the sampling regime of the clutches (i.e. not all year x temperature x hydric combinations were present in the data). MANOVAs were conducted in JMP v.6.0 (SAS Institute).

Results

I examined the general trends of shape variation in post-orbital coloration within each species. In *G. geographica*, shape differences were generally less pronounced; however, there was a trend between turtles with a circular post-orbital dot located slightly closer to the nose stripe and turtles with a more elongated spot located more toward the side of the head (Figure 4.2A). For *Graptemys ouachitensis*, movement along the first PC axis indicates change in the shape of the post-orbital stripe. In addition, there is a trend for a short, wide nose stripe with the post-orbital stripe placed near the median versus a long, thin nose stripe with a marginally placed post-orbital stripe (Figure 4.2B). Finally in *G. pseudogeographica*, the major shape change occurred in the shape of the post-orbital stripe and in the relative nearness of the post-orbital stripe to the central nose stripe (Figure 4.2C).
For the REML analysis, the model for *Graptemys geographica* achieved convergence of the likelihood scores when the first 7 principal components (PC) were used, representing 91.33% of shape variation. *Graptemys ouachitensis* and *G. pseudogeographica* were both analyzed with the first 7 PCs. These encompassed 85.87% and 91.67% for *G. ouachitensis* and *G. pseudogeographica*, respectively. The higher sample size for these latter two species allowed for analysis with up to 9 PCs, which represented 90.88% of variation for *G. ouachitensis* and 93.89% of variation for *G. pseudogeographica*.

Multivariate heritability ($H^2$) for *Graptemys geographica* was estimated as 0.2538 when year and temperature were incorporated as fixed effects for the first 7 PCs, although this value was not significantly greater than random ($p = 0.988$). In comparison, for the same 7 PCs, over the same years (Ewert and Vogt clutches only), and with the same fixed effects, $H^2$ was 0.3070 ($p=0.881$) and 0.2988 ($p = 0.908$) for *G. ouachitensis* and *G. pseudogeographica*, respectively. When the Janzen clutches were included in the model for *G. ouachitensis* and *G. pseudogeographica*, the heritability values increased to 0.5183 ($p = 0.365$) and 0.3902 ($p = 0.583$), respectively. Hydric conditions during incubation were only known for the Janzen and Vogt clutches. When these clutches were analyzed together in a model that included year, temperature, and hydric conditions as fixed effects the $H^2$ was 0.6423 ($p = 0.030$) and 0.3200 ($p = 0.842$) compared to 0.3574 ($p=0.931$) and 0.3210 ($p=0.366$) when hydric conditions were not included in the model for *G. ouachitensis* and *G. pseudogeographica*, respectively. *Graptemys geographica* was not analyzed for this effect because the Vogt clutches were all treated with the hydric condition and there were no Janzen clutches, which varied hydric conditions, for this species. Finally, I estimated heritability for *G. ouachitensis* and *G. pseudogeographica* for 9 PCs with year and
temperature as fixed effects; $H^2$ was 0.1719 ($p = 0.999$) and 0.3498 ($p = 0.999$) respectively, for all available clutches. In all cases, the null distribution was greater than zero, indicating that heritability values were significantly greater than zero.

For *Graptemys geographica*, the MANOVA found significant effects of temperature and year on color pattern variation in all PCs (Table 4.2). The results were similar for *G. ouachitensis* and *G. pseudogeographica*. Both species also had significant effects for temperature and year (Table 4.2). In contrast, using the subset of data for which hydric conditions during incubation were known, there was no significant influence of hydric condition on morphology of post-orbital coloration in either species (Table 4.2).

**Discussion**

The aim of this project was to estimate the multivariate heritability of post-orbital coloration pattern in three species of turtle. These traits have been implicated in species recognition and thus should have a substantial genetic basis. I found that heritability was similar and moderate in magnitude in all three species, though not greater than expected by random. Additionally, I examined the contribution of various external factors experienced during embryonic development on morphological estimation. Incubation temperature and year significantly influenced morphology, but hydric conditions during incubation did not.

For all species, the heritability values generated using the first 7 principal components were equivalent and near 0.3. This value indicates that there is relatively modest genetic component to post-orbital color pattern shape in these three species. However, heritability values for morphological traits are generally higher than those found here (Mousseau and Roff 1987; Conde-Padin 2006; Visscher et al. 2008). Indeed other morphological traits have
been examined in a closely related turtle species, finding plastron shape heritability in *T. scripta* estimated at ~0.5-0.6 (Myers et al. 2006). In contrast, the lower values of heritability estimated here suggest either a reduced genetic influence on this morphological trait and/or that this trait experiences strong selection, which can limit the available pool of additive genetic variation (Mousseau and Roff 1987). Given the species-specific nature of these traits (Myers 2008a) and their suggested role in species recognition (Vogt 1980), the latter hypothesis may be plausible. However, none of the heritabilities estimated were greater than expected from a random assemblage of turtles suggesting a limited role of additive genetic variation in generating phenotypic diversity. Interestingly, several heritability estimates, including both of the 9 PC estimates, were significantly smaller than expected by chance. This intriguing finding may be indicative of strong selection reducing genetic variation in the face of on-going environmental influences continuing to generate phenotypic variation. In contrast, research utilizing alternative approaches to examine the selective importance of these coloration patterns has found limited evidence to support a strong selective influence (Myers 2008a; 2008b; 2008c). Further research examining other morphological traits for comparison, as well as phenotypic variation at multiple life stages will be needed to fully resolve this puzzle.

In all cases, the heritability estimates in this study are likely to be somewhat conservative given that a full-sib pedigree was assumed. While the incidence of multiple paternity has not been examined in map turtles, similar species have notable levels of multiple paternity, varying from 20-40% in painted turtles (Pearse et al. 2002; McGaugh and Janzen, unpublished data) to 10% in pond turtles (Roques et al. 2006). Thus, at least some proportion of *Graptemys* clutches likely represent half-sibs rather than full-sibs. Therefore,
hatchlings used in this study probably are less genetically related than assumed, meaning that some of the phenotypic variation may have been attributed to the residual effects rather than to genetic variation. Additionally, the experimental design does not permit the partitioning of genetic variation into its constituent components of additive genetic variance, and the dominance and epistatic effects, nor can it identify any potential phenotypic contributions from non-genetic maternal effects. As such, this analysis estimates only the broad-sense heritability. However, additive genetic variance is often close to the total genetic variance (Hill et al. 2008), especially for morphological traits where the effects of dominance and epistasis are reduced (Roff and Emerson 2006). In addition, other research has found limited influence of maternal effects on heritability estimates for morphological traits (Forstmeier 2005). Therefore, the broad-sense heritability estimates are likely similar to the narrow-sense heritability.

Estimates of phenotypic variation and heritability for morphological traits generated in the lab typically are not significantly different from field-based estimates (Weigensberg and Roff 1996; St. Juliana and Janzen 2007). In addition, a laboratory common garden incubation design allows one to selectively influence developmental conditions to test hypotheses regarding their relative importance in generating morphological variation. In this way, I was able to partition phenotypic variance into year, temperature, and hydric condition effects, as well as variation due to genetic differences. Hydric conditions during incubation exerted relatively little influence on morphology for *Graptemys ouachitensis* or *G. pseudogeographica*, the two species for which hydric condition was controlled (Table 4.2). Increases in heritability values associated with the inclusion of hydric condition in the model may reflect an artifact of the animal model parameter estimates rather a biological reality.
Morphologies for each species were significantly different at different temperatures (Table 4.2). This finding is consistent with earlier research suggesting a potential role for temperature in generating phenotypic variation in these traits (Vogt 1978; Ewert 1979) and elsewhere (Murray et al. 1990; Etchberger et al. 1993; Rhen and Lang 2004). However, temperature effects in species with temperature-dependent sex determination may be confounded with sex-specific effects, or an interaction between the two (Rhen and Lang 2004). Additionally, interactions between clutch and temperature effects are common (Bull et al. 1982; Janzen 1992) and result from additive, dominance and epistatic genetic effects, as well as non-genetic maternal effects (Falconer 1989; Rhen and Lang 2004). Given the available data, it is not possible to tease these individual factors apart. If environmental factors generate comparatively more of the variation in phenotype, then it suggests that overall phenotypic variation is generally stochastic and that the role of such traits in sexual selection may be limited.

This analysis represents the best attempt to date to estimate the relative environmental and quantitative genetic effects on post-orbital coloration pattern. A dataset large enough to include all 34 available PCs is unlikely in a natural system. The three datasets incorporated in this analysis (Ewert, Vogt, and Janzen clutches) represent the largest available pools of specimens for hatchlings grouped to nest for these three species. Few additional specimens are available from other populations. Clutches that implement a fluctuating or temperature shift regime are available (Bull and Vogt 1981), but the comparability of these regimes to a constant temperature regime is unclear, as these different thermal regimes can influence phenotypic traits (Ashmore and Janzen 2003; Mullins and Janzen 2006). Finally, some studies have examined just one of the three species, precluding a cross-species comparison of
heritability values (Bull et al. 1982). While all PCs were not utilized in estimating heritability, my results should be robust nonetheless, as the PCs incorporated represented 85-95% of the variation in shape. Additionally, the multivariate geometric morphometrics approach, even with a reduction in PC number, enables one to capture more of the overall shape variation and change than a standard analysis examining length or width of the post-orbital coloration alone. The analysis here showed that, even within the first two PCs, there was variation in size and shape of post-orbital coloration, nose stripe, and relative positions of these features to each other (Figure 4.2A-C). These estimates are likely to more accurately reflect the heritability of the entire coloration pattern than univariate estimates of individuals traits (Vogt 1978, Janzen and Ast, unpublished data).

In summary, I found similarly modest heritable genetic variation for post-orbital coloration in three species of map turtle. These low values relative to other types of morphological traits in related species suggest that environmental effects may be a comparatively significant contributor to variation in color patterns, although a reduction in additive genetic variation due to selection is also possible. Temperature and year significantly influenced morphology, while differences in hydric condition did not. Overall, these results suggest that selection acting on these morphological traits, either through natural or sexual selective processes, could generate a measured evolutionary response.

Acknowledgements

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Literature Cited


### Tables

Table 4.1. Number of specimens used in heritability analysis for each species of map turtle.

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<td>277</td>
<td>37</td>
</tr>
<tr>
<td><strong>Graptemys pseudogeographica</strong></td>
<td>571</td>
<td>70</td>
</tr>
<tr>
<td>Ewert clutches</td>
<td></td>
<td></td>
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<tr>
<td>1973</td>
<td>53</td>
<td>5</td>
</tr>
<tr>
<td>1976</td>
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<td>5</td>
</tr>
<tr>
<td>1984</td>
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<td>4</td>
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<tr>
<td>1985</td>
<td>9</td>
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<tr>
<td>Vogt clutches</td>
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<tr>
<td>1978</td>
<td>293</td>
<td>43</td>
</tr>
<tr>
<td>Janzen clutches</td>
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<tr>
<td>1991</td>
<td>115</td>
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Table 4.2. Results of the GLM analysis examining several fixed effects on post-orbital color pattern for each species.

**Graptemys geographica**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Wilk’s Λ</th>
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<th>df</th>
<th>P</th>
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<td>2.1093</td>
<td>132, 902.02</td>
<td>&lt;0.0001*</td>
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<tr>
<td>Year</td>
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<td>1.7461</td>
<td>99, 677.52</td>
<td>0.0006*</td>
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**Graptemys ouachitensis**

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<tr>
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<tr>
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**Graptemys pseudogeographica**

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Figures

Figure 4.1. Location of landmarks along the nose stripe and post-orbital markings of three species of map turtle A) *G. geographica*, B) *G. ouachitensis*, and C) *G. pseudogeographica*.
Figure 4.2. Plots of the first two principal components for each species of map turtle. The shapes corresponding to morphologies along the axes are represented with deformation grids.

A) *Graptemys geographica*, B) *Graptemys ouachitensis*, C) *Graptemys pseudogeographica*.

A)
Appendix 4.1. List of specimens from the Carnegie Museum used in the heritability analysis.

Ewert clutches

*Graptemys geographica:* 156038-127
*Graptemys ouachitensis:* 156128-281
*Graptemys pseudogeographica:* 155843-156010

Vogt clutches

*Graptemys geographica:* 96552-7, 96559-62, 96570-80, 96585-6, 96621-4, 96644, 96646, 96648-9, 96654-6, 96658-71, 96673-80, 96682, 96693-721, 96724-5, 96727-8, 96732-7, 96741-5, 96760-6, 96768-9, 96771-4, 96776-9, 96781-2, 96784, 96786-8, 96790-6, 96798-800, 96802-15, 96817-34, 96836-42, 96845-7, 96849
*Graptemys ouachitensis:* 97707-12, 97717-20, 97722, 97725, 97757-60, 97762-63, 97777-82, 98323-8, 98330-2, 98334-5, 98344-6, 98348-9, 98352-69, 98371-2, 98430-1, 98434-8, 98450, 98452-4, 98458, 98465-7, 98469-72, 98489-91, 98493-6, 98498, 98500-1, 98504-7, 98509-19, 98530-43, 98547, 98549, 98551-6, 98558-9, 98561-3, 98565, 98567-71, 98574-82, 98584-5, 98587-8, 98590, 98592-9, 98601, 98603-17, 98619-23, 98625, 98627, 98629, 98632-9, 98641-9, 98651, 98672-7, 98685-90, 98692, 98694-701, 98704
CHAPTER 5. THE ROLE OF COLOR PATTERN IN MATE CHOICE OF THREE SPECIES OF MAP TURTLES (*GRAPTEMYS*) AND A DESCRIPTION OF A NOVEL MATING BEHAVIOR

Modified from a paper to be submitted to *Herpetologica*

Erin M. Myers

Abstract

Morphological characters involved in species recognition have the potential to play an important role in species formation and maintenance. Map turtles, in the genus *Graptemys*, are characterized by unique, species-specific coloration patterns on their facial regions. This coloration has been previously implicated in species recognition and mate choice, in addition to possible chemical/pheromone cues. This project attempts to determine the relative importance of color pattern in species recognition using three-way male and female choice experiments involving three sympatric species of turtles. There were no significant differences in time allocation for focal turtles among the three species for any focal species. In addition, males and females did not significantly differ in their species preference and there were no significant differences between allopatric and sympatric populations. These results suggest that morphology may not be the primary mechanism of species recognition in these species. Finally, an unusual courtship behavior was observed in males of *G. geographica* and its implications are discussed.
**Introduction**

Morphology has often been implicated as a factor involved in shaping the formation and diversification of new species (e.g. Losos 1994; Schluter 2000; Coyne and Orr 2004). In particular, traits that are involved in mate choice and species recognition may be involved in helping to form and maintain a reproductive barrier through patterns of increased reproductive isolation via reinforcement (Sætre et al. 1997; Coyne and Orr 2004). Identifying the direct role of a particular trait in species diversification is difficult, as this process can be confounded by other factors such as niche partitioning or divergent selection pressures that may also cause morphological differentiation (Nagel and Schluter 1998; Coyne and Orr 2004). Therefore, behavioral experiments involving direct observation and/or manipulation offer the best opportunity to determine the importance of a trait in mate choice, and as an extension, potential speciation. Additionally, species that occur in both sympatry and allopatry provide an ideal system with which to potentially observe increased species recognition and reinforcement within a species in sympatry compared to its allopatric counterparts.

The genus *Graptemys* (map turtles) contains a unique radiation of turtles, featuring both high species richness and morphological diversity (Myers2008a; Ernst et al. 1994). Most species of map turtle are river endemics found in only one or two river basins (Ernst et al. 1994; Lamb et al. 1994). In contrast, three species, *G. geographica*, *G. ouachitensis*, and *G. pseudogeographica*, are broadly distributed throughout the majority of the Mississippi River drainage system and are sympatric throughout much of their range (Ernst et al. 1994). All map turtles are characterized by a unique, species-specific facial color pattern that ranges from a small post-orbital dot (*G. geographica*) to a stripe located behind the eye (e.g. *G.*
pseudogeographica and G. ouachitensis) to a full mask of color (e.g. G. pulchra; Myers 2008a; Ernst et al. 1994). This trait can be variable within species (Myers 2008b) and in areas of sympatry the degree of morphological overlap between species is reduced (Janzen et al. 1995). This finding suggests that this color pattern may be involved in species recognition, and furthermore, is suggestive of reproductive character displacement (Janzen et al. 1995). Additionally, behavioral research has indicated a potential role for the post-orbital coloration as a species identification cue used in mate choice and recognition in some species (Vogt 1978).

Courtship in map turtles is similar to other emydid turtles in that it occurs face-to-face. This provides an opportunity for males and females to observe the facial coloration patterns of potential partners and use this information in evaluating a potential mate. While vision has not be examined in map turtles explicitly, the closely related slider turtle has been shown to have one of the most complex cone systems in a vertebrate and should be able to visualize color differences and patterns (Loew and Govardovskii 2001). In most cases, these pre-copulatory interactions will not lead to copulation in heterospecific pairings (Vogt 1978).

In G. geographica, previous reports have indicated that males follow this initial observation period with head bobbing and then subsequently mount the female and attempt to mate (Vogt 1980). In contrast, G. ouachitensis and G. pseudogeographica both have elongated foreclaws in reproductively mature males. Males use these to stimulate the female in a “titillation” display similar to that seen in other closely related species (Cagle 1950; Davis and Jackson 1970; Jackson and Davis 1972; Vogt 1980; Ernst et al. 1994). During this display, the male drums the elongate foreclaws on the ocular region of the female and, following a variable number of titillation bouts, the male then seeks to mate with the female (Vogt 1980).
However, at various points during courtship, males often swim near the cloaca of the female, which may expose males to species-specific pheromonal, or other chemical cues (Vogt 1980). While males often initiate courtship, females also play a significant role in mate choice by allowing (or not) a male to mount and copulate. Given the large female-biased size dimorphism, uninterested females can easily out-swim and evade pursuant males or simply not allow insemination by manipulating her tail position (Baker and Gillingham 1983; E. Myers, personal observation).

Males of two species, *G. ouachitensis* and *G. pseudogeographica*, can accurately discriminate between heterospecific and conspecific females (Vogt 1978). However, their interactions with the third sympatric species, *G. geographica*, or *G. geographica*’s own mate discrimination ability have not been examined. *G. pseudogeographica* and *G. ouachitensis* are closely related sister species that are more closely to each other than either is to *G. geographica*, the sister taxon to the rest of the map turtles (Lamb et al. 1994; Stephens and Wiens 2003; Myers 2008a). In addition, hybrids are occasionally formed between these three species, though hybrids are not formed between all species pairs among *G. geographica*, *G. pseudogeographica*, and *G. ouachitensis* group (Vogt 1978; Ernst et al. 1994). The frequency of hybridization is higher between *G. geographica* and the other two species while *G. ouachitensis* and *G. pseudogeographica* rarely, if ever, hybridize (Vogt 1978). This finding is atypical as it is often the case that hybridization occurs more readily between closely related species than between more distant taxa (Coyne and Orr 2004). *Graptemys geographica* is only sympatric with the other two species through the northern two-thirds of their ranges (Ernst et al. 1994). In contrast, *G. ouachitensis* is sympatric throughout its entire range, much
of it with *G. pseudogeographica*, suggesting that selection on reproductive isolation may be acting more strongly in the latter two species (Vogt 1980; Ernst et al. 1994).

The net result of earlier research on mating and courtship in these species implicates a role for facial coloration, pheromones, or both in species recognition (Vogt 1978, 1980). Therefore, the goal of this project was to determine the relative importance of post-orbital coloration in species recognition within this earlier framework. I tested the hypothesis that given a choice, a turtle would spend more time near a conspecific, rather than a heterospecific, member of the opposite sex based on color pattern alone. Additionally, I predicted that species recognition would be higher in *G. ouachitensis* and *G. pseudogeographica*, as their color patterns are more similar, character displacement has been indicated, and reduced hybridization between them has been documented. Finally, I hypothesized that, if coloration is important, then sympatric populations should have higher species recognition and greater preference than allopatric populations, consistent with reinforcement.

**Methods**

**Turtle Collection and Care**

Three species of map turtles, *Graptemys geographica*, *G. pseudogeographica*, and *G. ouachitensis*, were collected in 2005 and 2006 at sites in Iowa, Illinois, Missouri, and Nebraska (Table 5.1). Turtles were trapped using a combination of hand capture, box and basking traps, as well as fyke nets, and were subsequently transported to Iowa State University. Species were differentiated on the basis of several morphological characteristics (e.g. size and shape of the post-orbital color pattern, presence/absence of cheek spots, shell
characters; Figure 5.1.) and each individual was identified to species by at least two experienced researchers. All turtles were individuals numbered with a waterproof, non-toxic sharpie on their carapace.

Turtles were housed in tanks of varying sizes ranging from a 10 gallon (38 L) aquarium with one individual to a 300 gallon (1136 L) cattle tank with 16 individuals. Each tank contained water at a depth to allow swimming, as well as a log or platform for basking. Males and females were kept in separate tanks to prevent courtship and mating prior to the behavioral experiments. Additionally, turtles from different localities were also housed in separate tanks to reduce the potential for germ or parasite transmission. Animals were maintained on a mixed diet consisting of ReptoMin (Tetrafauna) turtle chow supplemented with fresh mussels and snails as available. The day:night cycle was kept consistent with actual day length at Ames, Iowa through the study period and similar to the natural conditions from the populations from which they were collected. I used lighting consisting of both UVA/UVB bulbs (Reptisun 5.0; ZooMed) and standard fluorescent bulbs. To encourage basking, this lighting was supplemented with heat lamps. Ambient temperature was similarly adjusted to follow natural conditions with temperature in early fall near 27° C gradually lowering to 21° C by the completion of experiments. The animals collected in 2005 were over-wintered for two to three months at 5° C, followed by a return to warmer temperatures for the spring and summer of 2006. At the conclusion of the behavior experiments in fall 2006, all turtles were over-wintered and subsequently released at point of capture in spring 2007.

Behavior Experiments
To assess the role of post-orbital color pattern in species recognition and preference, a three-way mate choice chamber was constructed (Figure 5.2A & B). This chamber featured a central, triangular tank with walls (~ 500 cm in length) of clear Plexiglas, hereafter referred to as the “choice” tank. Connected to each wall was a rectangular, side tank with one side open to the Plexiglas and the remaining sides of gray plastic to prevent turtles from being distracted by other turtles or the surrounding environment. This tank was large enough to permit the turtle to move freely. To simulate a natural substrate, each side tank and the choice tank had a layer of small rocks and sand. Tanks were filled ½ to ¾ full of water (~ 20 gallons, 76 L). The tanks were sealed using an epoxy and duct tape to prevent fluid flow between the central chamber and the side chambers. This was to ensure that only visual cues, not potential pheromones, were used during mate choice by the focal animal. The entire chamber was filled and emptied several times prior to the onset of behavioral trials to reduce potential odor from the sealants. Experiments were conducted between 3-21 October 2006. This timeframe is consistent with the timing of courtship and mating in the wild (Vogt 1980).

In the first set of experiments (Female Choice) a single female was placed into the choice tank while one male from each of the three species was placed individually into one of the three side tanks. To prevent position effects, the position of the various species in the side tanks was randomized for each trial. Similarly, the order of which species were used as the focal subject in the choice tank was also randomized. To minimize disruption, an observer was not present during the experiment. Instead, a video recording using a tripod mounted Sony handycam DCR-HC36 was made of the entire mate-choice chamber. Each trial was recorded for thirty minutes and the trial began as soon as the focal turtle was released in the chamber. The procedure for the second set of experiments (Male Choice) was the same as the
first except a male was placed into the choice chamber, and a female of each species was randomly placed in a side tank. In both the female and male choice experiments, the individuals placed into the side tanks were size matched to preclude choice based only on size and the same individuals were used for all trials. A series of controls was also utilized, no-choice and same-species only choice. In the no-choice control (n = 4), a turtle was placed in the choice tank with no turtles placed in the side tanks. In the same-species choice (n = 4), I placed an individual in the choice chamber and three members of the same species, opposite sex in the side chambers. Both controls were designed to examine biases in the way turtles utilized the central chamber. No individuals were used more than once as a focal turtle in the choice experiments.

At the conclusion of choice experiments, a series of post-hoc tests were conducted to gauge reproductive interest, because a lack of interest could potentially explain any negative results. Representative males and females of each species were observed in an open access tank to verify reproductive state. Reproductive interest by males was defined by attempts to chase, titillate or mount a female. Female interest was determined by allowing males to interact with the female rather than swim away from males and remain solitary. These trials were similarly recorded. Qualitative data on behavior (e.g. mounts and copulation attempts) was taken. However, quantitative data, in the form of time spent with any particular female or male, was not taken as this was not the focus of these tests.

Experimental Analysis

Each experiment was analyzed in the following manner. The amount of time spent by the focal individual in any given position of the tank was recorded. The positions were as
follows: Sides (A, B, or C), Corner (1, 2, or 3), or center (Figure 5.2A). The fraction of time that the turtle in the choice tank (focal turtle) spent in proximity or far from the side turtle while on that side was also recorded (Figure 5.2B). Time spent in either corner position was scored as a choice of both adjoining sides. Time spent in the center of the tank was scored as ‘no choice’. Time was recorded in seconds and two metrics were employed; “Near” time consisted only of time spent in close proximity to the turtle in the side tank (Figure 5.2B), while “Total” time was the total time the focal turtle spent at one side of the tank (both in proximity and distant from the side turtle). In both cases, because turtles could choose two turtles simultaneously while in a corner, the amount of time allocated to the three side turtles could sum to more than the total time of the experiment. Additional qualitative notes were taken concerning courtship behaviors (e.g. titillation display, head bobbing series).

For each focal, choice turtle, six values were used in analyses: Total time spent near each species in the side tank (G. geographica, G. ouachitensis, and G. pseudogeographica), and the Near time spent near each species in the side tank. To measure differences in time allocation to different sides of the tank as a proxy for species preference, an analysis of variance (ANOVA) was conducted using both Total time and Near time. To determine if any of the mean time allocations to each species were different from one another, I used a post-hoc Tukey-Kramer HSD test. A separate ANOVA was conducted for males and females as the focal turtle and for each of the three species. A multivariate analysis of variation (MANOVA), which included time allocation of a given focal turtle to all three possible species, was used to determine if the sexes differed in their time allocation. Similarly, a MANOVA was used to test for differences in species preference between sympatric and
allopatric populations. In both cases, the MANOVA was conducted for each species separately. All statistical analyses were conducted in JMP (v. 6, SAS Institute).

**Results**

I observed turtles attempting to swim through the Plexiglas but not through the opaque barriers surrounding the side tanks demonstrating that turtles were able to see through the Plexiglas. Turtles within the choice tank and side tanks also frequently exhibited paired swimming, tracking members of the opposite sex as they moved in their tank. Indeed, in one trial, a male attempted to titillate a female’s head through the glass (Figure 5.4, Video 1), demonstrating that males and females could adequately visualize members of the opposite sex. During the experiments, males were generally more active than females (more time spent in active swimming). Additionally, though not assessed quantitatively, there were species-specific differences in activity; *G. geographica* was the most active for both males and females, *G. ouachitensis* was moderately active, and *G. pseudogeographica* was less active than either of the other two species. However, there were no differences between species or sex in the qualitative metrics of reproductive interest. Only one male was observed in a titillation display and no individuals were observed using head bobbing.

For females of both *G. geographica* and *G. pseudogeographica*, there were no significant differences in time allocation to the three species of male for either Total or Near time (*G. geographica*: $F_{total} = 1.7915$, $p_{total} = 0.1813$, $F_{near} = 2.0805$, $p_{near} = 0.1396$; *G. pseudogeographica*: $F_{total} = 0.0422$, $p_{total} = 0.9587$, $F_{near} = 0.8379$, $p_{near} = 0.4393$). However, for *G. ouachitensis* females there was significant difference between means for Total time but not Near time ($F_{total} = 4.7923$, $p_{total} = 0.0571$; $F_{near} = 2.0853$, $p_{near} = 0.2053$). The Tukey-
Kramer test indicated that time spent with *G. geographica* and *G. pseudogeographica* males were significantly different in Total time but neither was different from *G. ouachitensis*, with more time spent with *G. pseudogeographica* than *G. geographica* (Figure 5.3). The results were similar for males. *G. geographica* and *G. pseudogeographica* had no significant differences in time allocation (*G. geographica*: $F_{\text{total}} = 0.0256$, $p_{\text{total}} = 0.9748$, $F_{\text{near}} = 0.5948$, $p_{\text{near}} = 0.5563$; *G. pseudogeographica*: $F_{\text{total}} = 0.0201$, $p_{\text{total}} = 0.9801$, $F_{\text{near}} = 0.2949$, $p_{\text{near}} = 0.7461$). In contrast, *G. ouachitensis* had significant differences between time spent with females in Near time but not Total time ($F_{\text{total}} = 4.4699$, $p_{\text{total}} = 0.0648$, $F_{\text{near}} = 6.1763$, $p_{\text{near}} = 0.0349$). Similar to the females, the means for time spent with female *G. pseudogeographica* and *G. geographica* were significantly different from each other but not with respect to *G. ouachitensis*, and this pattern was in the same direction with more time spent with *G. pseudogeographica* than *G. geographica* (Figure 5.3). Interestingly, males from both *G. pseudogeographica* and *G. geographica*, as the focal turtle, allocated the most Near time to their conspecific female though this pattern was non-significant (Figure 5.3). Both males and females of *G. ouachitensis* allocated the most Total and Near time to the heterospecific *G. pseudogeographica* rather than conspecifics.

For all species, there was no significant difference between males and females in their time allocation and species preference for either Near or Total time (Near: *G. geographica* $F = 0.7003$, $p = 0.5049$; *G. ouachitensis* $F = 0.2470$, $p = 0.7956$; *G. pseudogeographica* $F = 0.4730$, $p = 0.6286$; Total: *G. geographica* $F = 0.0015$, $p = 0.9985$; *G. ouachitensis* $F = 2.0254$, $p = 0.2775$; *G. pseudogeographica* $F = 0.6174$, $p = 0.5475$). Additionally, there were no significant differences among allopatric and sympatric populations in species preference for either *G. geographica* ($F_{\text{total}} = 0.4233$, $p_{\text{total}} = 0.6590$; $F_{\text{near}} = 1.0234$, $p_{\text{near}} = 0.3724$) or *G.
**Graptemys pseudogeographica** (\( F_{\text{total}} = 0.1578, p_{\text{total}} = 0.8548; F_{\text{near}} = 0.0330, p_{\text{near}} = 0.9676 \)). *G. ouachitensis* was not analyzed as no allopatric populations for this species are present.

In the post-hoc tests to ensure male and female interest, females did not actively attempt to swim away from males. In addition, males from each species showed at least some aspects of courtship or mating behavior indicative of interest in females, though the degree to which males pursued females varied considerably among species. *Graptemys pseudogeographica* showed the least interest, *G. ouachitensis* was occasionally interested, and *G. geographica* actively pursued females and attempted to copulate on multiple occasions.

During the post-hoc tests, and elsewhere (E. Myers, personal observation) males of *G. geographica* utilized an unusual courtship behavior. Males approached female *G. geographica* from a number of positions. Typically, interested males would proceed toward the posterior of the female to investigate her cloacal region or attempt to mount and copulate. On multiple occasions however, males “mounted” at her anterior region (Figure 5.5, Video 2). This behavior was then followed by an attempt to mount at the posterior, an investigation of the cloacal region, or swimming away. Males never mounted other males while in their general care tanks or during post-hoc tests. This behavior was only exhibited by males (\( n = 4 \)) from the Missouri population of *G. geographica* but not by males from the Iowa population and was observed on multiple occasions (exhibited again in spring prior to release, E. Myers, personal observation).
Discussion

This objective of this project was to determine if facial coloration patterns, as opposed to pheromones, are involved in species recognition and preference for three map turtle species. In both male and female choice experiments, turtles did not show a preference for conspecific compared to heterospecific members of the opposite sex regardless of the focal turtle’s species. While it is possible that species recognition could be manifested in other behaviors besides time spent in proximity, this seems unlikely given that only one individual exhibited behavior consistent with courtship in these species. Therefore, it seems likely that morphology is not responsible for species recognition and preference.

Morphology and other phenotypic cues, when tied to species recognition, can be an important factor in species divergence and maintenance (Ryan and Rand 1993; Sætre et al. 1997; Coyne and Orr 2004). Modifications to these species-specific signals could potentially lead to the formation of new species (Coyne and Orr 2004). Traits such as feather color and courtship song have previously demonstrated the importance of phenotype in species recognition (Ryan and Rand 1993; Sætre et al. 1997). Patterns similar to morphological, reproductive character displacement have been documented in map turtles, suggesting that they might use facial color pattern as a species recognition cue (Vogt 1993; Janzen et al. 1995). However, the results of this project indicate that this morphological color pattern alone does not lead to preferential time spent with conspecific potential mates, suggesting that this trait is may not be involved in species recognition prior to mating.

In addition to morphological or acoustic cues, individuals can often differentiate species based on pheromonal or chemical cues (Higbie et al. 2000; Mas and Jallon 2005). Earlier research on map turtles has suggested that species recognition may be based on
morphological cues, pheromonal cues, or a combination of both (Vogt 1980). Map turtles inhabit an aquatic environment that can vary in water quality and turbidity across locations and throughout the year, potentially limiting the effectiveness of visual signals (Myers, personal observation; Ernst et al. 1994). Therefore, pheromones may serve as a species-specific attractant for members of the opposite sex in *Graptemys*, similar to those seen in other vertebrate systems (e.g. *Plethodon*; Palmer et al. 2005). Such signals may potentially be transmitted and received over larger distances than a visual signal in a turbid or murky environment (Twitty 1955; Toyoda et al. 1994; Wabnitz et al. 1999; Munoz 2004; Toyoda et al. 2004), although the evidence for long distance pheromones in reptiles is limited (Vogt 1978; Shine 2005). However, aquatically transmitted chemical cues have been shown to influence behavior in at lease one species of aquatic turtles and is consequently plausible as mechanism in this system (Munoz 2004).

*Graptemys geographica*, unlike *G. ouachitensis* and *G. pseudogeographica*, does not utilize a titillation display as part of courtship (Vogt 1980; Ernst et al. 1994). This titillation display may serve as a third species recognition mechanism, in the latter two species, because males of *G. ouachitensis* and *G. pseudogeographica* drum using a species-specific frequency (Vogt 1978, 1980). Males from the Missouri population of *G. geographica* were observed mounting females from an anterior position on the female, in addition to a more ‘traditional’ posterior position. These males had only been in captivity four months at the time of observation and were some of the largest males in the experiment; therefore, it is unlikely that this behavior, observed more than once, was the result of immaturity or an accident. During the time spent in anterior mounting, the male was not rigid but rather had some pelvic thrusting. This behavior may serve to move water across the females face, similar to the
titillation in other species. Alternatively, this movement may be used as a mechanism to increase pheromone transfer. In particular, this behavior may serve as a mechanism to increase female receptivity as males were observed alternating from a posterior mounted position, to the anterior, and then back to the posterior. In a terrestrial turtle species, the experimental elimination of olfactory function resulted in a 60-70% reduction in mating behavior (Chelazzi and Delfinio 1986). Such pheromone transfer to increase receptivity is seen in salamanders where it is an integral part of the overall courtship experience (Houck and Arnold 2003; Palmer et al. 2005). As yet however, it is unclear whether this behavior is representative of the species as a whole or indicative of a behavioral modification utilized by only one population, as it was not observed in a population from Wisconsin (Vogt 1978; Vogt, personal communication).

In summary, this research demonstrated that color pattern alone does not dictate species recognition and preference prior to choice of mate during the courtship season. However, it is unclear whether this trait is still an important factor in species recognition, in combination with chemical or pheromonal cues. Therefore, future laboratory studies in this species should combine manipulative experiments on the facial color patterns with an experimental design that enables the transmission and/or manipulation of potential pheromone signals to more definitively disentangle the role of visual, morphological signals in species recognition in map turtles.

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Literature Cited


### Tables

Table 5.1: Number of map turtles used in the female and male choice experiments.

<table>
<thead>
<tr>
<th>Species</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Graptemys geographica</em></td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td><em>Graptemys ouachitensis</em></td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><em>Graptemys pseudogeographica</em></td>
<td>15</td>
<td>13</td>
</tr>
</tbody>
</table>

| Total:                   | 33   | 32     |
Figures

Figure 5.1: Facial coloration patterns including the nose stripe and post-orbital markings of three species of map turtle (*G. geographica*, *G. ouachitensis*, and *G. pseudogeographica* from left to right).
Figure 5.2: Schematic of three-way mate choice chamber used in the behavioral trials featuring a central, choice chamber and three side tanks, each holding an individual from one of the three map turtle species. A) figure indicates the criteria used for the classification of the focal turtle’s position within the choice tank. B) indicates criteria for the classification of Near and Total time. Focal turtle at side C would be counted as Near time while focal turtle at side A would be considered far and time spent in this orientation would only count in Total time.

A.

B.
Figure 5.3. Results of female choice (left) and male choice (right) experiments grouped by the focal turtle’s species. Bars represent time spent with each species in the side tank, dark gray stipple for G. geographica, light gray for G. ouachitensis, and white stipple for G. pseudogeographica. Error bars represent +/- 1 standard deviation and arrows indicate conspecific interaction. Top panel uses the Total time metric, while the bottom panel uses Near time.
Figure 5.4: Male *G. pseudogeographica* attempting to titillate a female *G. pseudogeographica* through the Plexiglas barrier (taken as a still screen shot from video recording). Video of entire interaction is available as an electronic supplement (Video 1).

Figure 5.5: Male *G. geographica* engaged in a mounting behavior at the head of a female *G. geographica* (taken as a still screen shot from video recording). Video of entire interaction is available as an electronic supplement (Video 2).
CHAPTER 6. GENERAL CONCLUSIONS

Discussion

The goal of this integrative research was to examine morphological evolution within the species- and morphology-rich, map turtle genus (*Graptemys*). Post-orbital coloration patterns have been implicated by prior researchers in the species recognition and mate choice of this genus. This work has led to the hypothesis that this morphological color pattern has had an important role in the speciation process of this radiation of turtle. To address this hypothesis, I utilized a multi-faceted approach incorporating phylogenetic, population and quantitative genetics, behavior, and morphometrics. I examined patterns of morphological evolution across the entire genus. Focusing on a subset of three broadly sympatric species, I examined patterns of genetic, geographic and morphological differentiation within species, estimated multivariate heritability and the relative contribution of environmental factors in generating variation in post-orbital color patterns, and tested the importance of these morphological traits in species preference trials. The results of each project alone provide insights into aspects of map turtle biology. However, their combined results provide more powerful insights into the evolutionary processes driving morphological evolution in this genus.

In Chapter 2 (Myers 2008a), I estimated the phylogenetic history of map turtles and plotted their post-orbital coloration patterns onto the phylogeny to infer the patterns and processes governing morphological evolution and diversification within the genus. *Graptemys geographica* was strongly supported as the sister taxon to the rest of the map turtle clade. The rest of the clade was divided into two major clades, the *pulchra* clade and
the *pseudogeographica* clade. A supported, resolved topology was recovered for the *pulchra* clade; however, a resolved topology was not recovered for the *pseudogeographica* clade. Further analysis indicated that this lack of structure comprised a hard polytomy based on estimates of the age of the divergence for this clade and for the interval between Pliocene and Pleistocene glaciation events. The phylogeny alone suggests that speciation was relatively rapid, as branch lengths were short or not supported throughout most of the genus.

Across the genus, morphological color patterns on the head were generally species-specific. These patterns were classified into four morphological types: dot (belonging to *G. geographica*), mask (belonging to *G. barbouri, G. ernsti, G. gibbonsi*, and *G. pulchra*), blotch (belonging to *G. flavimaculata* and *G. oculifera*), and stripe (found in *G. caglei, G. kohnii, G. nigrinoda, G. ouachitensis, G. pseudogeographica*, and *G. versa*). Evolution of these morphological types occurred in a parsimonious fashion. However, particularly within the stripe group, subsequent evolution occurred in a non-parsimonious fashion. These results suggest that, whereas the role of sexual selection and/or reinforcement in contributing to the initial formation of the morphological groups is equivocal, such processes did not contribute to subsequent diversification within the groups. It is also possible that the species-specific morphologies developed across the genus in a stochastic manner as a result of allopatric speciation.

Beginning in Chapter 3 (Myers 2008b; 2008c; 2008d), I focused on three members of the *Graptemys* clade to examine genetic and behavioral processes in more detail. These three species, *G. geographica, G. ouachitensis*, and *G. pseudogeographica*, are sympatric throughout the majority of their ranges. Thus, if post-orbital coloration is critical in species
recognition and sexual selection, the effect of selection should be strongest and most
detectable in these three species that regularly encounter congeneric species.

In Chapter 3 (Myers 2008b), I examined the relationship between genes, geography,
and morphology across all three species. Genetically, I found genetic structuring and patterns
of isolation by distance across the range of the species. Population structuring was greatest in
*G. geographica*, consistent with its relative dietary restriction as a mollusk specialist
compared to the essentially omnivore diet of *G. ouachitensis* and *G. pseudogeographica*.
Morphologically, *G. geographica* had less variation in the post-orbital color pattern across
populations than the other two species. There was no relationship between morphological
and geographic distance suggestive of either stabilizing selection on the trait or neutral
variation. Meanwhile, there was a significant relationship between morphology and
geography in *G. ouachitensis* and *G. pseudogeographica*. Rather than exaggerated
morphological distance over short genetic and geographic distances indicative of
reinforcement, these species exhibited a pattern of isolation by distance for genetics and
morphological traits. There was no evidence for character displacement in post-orbital
coloration between allopatric and sympatric populations for *G. geographica* or *G.
pseudogeographica*, which would have been expected if this trait was involved in species
recognition and reinforcement. Unfortunately, no allopatric populations of *G. ouachitensis*
are available for comparison. These results support stochastic processes shaping
morphological variation across the species range rather than sexual or natural selection
processes.

Chapter 4 (Myers 2008c) examined the relative contributions of genetic and
environmental factors in generating the total phenotypic variation in post-orbital coloration in
each of the three sympatric species. Hydric conditions during incubation had a non-significant impact on morphology for both *G. ouachitensis* and *G. pseudogeographica*. The effect of hydric conditions was not examined in *G. geographica* as this variable was not varied in the available clutches. Both temperature and year were found to significantly affect morphology in all species. Heritabilities estimated for all species were approximately equal and near 0.3. None of the estimates were greater than expected by chance, and in some cases, were significantly smaller than expected by chance. This moderate value indicates that, while genetic variance may be present, it is lower than that consistently found in other morphological traits in other taxa. This result implies that additive genetic variation may be reduced due to selection or that environmental and other non-genetic factors provide the majority of the contribution to the standing shape variation in post-orbital color pattern in *G. geographica*, *G. ouachitensis*, and *G. pseudogeographica*.

Finally, in Chapter 5 (Myers 2008d), I explicitly examined the role of the post-orbital coloration in species recognition and preference using behavioral experiments. These experiments were designed to compare male and female species preference for members of the opposite sex during three-way choice. Species and sex-specific differences were observed in activity level; however, there were no differences in qualitative measures of reproductive interest. Males and females, for each species, did not significantly differ in their association patterns. In general, for both *G. pseudogeographica* and *G. geographica* males and females, there were no differences in time allocation to the three species using either the Total or Near time metric. *G. ouachitensis* males and females had different time allocation, using one time metric, significantly differing in their time allocation with the most time spent with *G. pseudogeographica*. There were no significant differences between allopatric and sympatric
populations for either *G. geographica* or *G. pseudogeographica*, in contrast to expectations if reinforcement of speciation using post-orbital coloration was in operation. In addition, a novel mating behavior was observed in males of *G. geographica* from Missouri. Males were observed to mount females both anteriorly and posteriorly. This behavior was interpreted as a potential means to increase pheromone exposure of females as a mechanism to enhance reproductive interest.

The underlying hypothesis of this research program has been that post-orbital coloration is a species recognition character used in mate choice. Thus, this trait could have an important pre-zygotic role in limiting the formation of hybrid offspring. Such offspring may be unfit if their post-orbital coloration does not yield a signal that is recognized by potential mates. Given this, the interpretation can be further extended to suggest that patterns of reinforcement and sexual selection on these traits contributed to the high species-richness and morphological diversity. Accordingly, each project provides valuable insight into different aspects of this potential evolutionary history, however taken together, these results can more thoroughly inform about the patterns and processes governing the map turtles, as a clade, and their morphological evolution.

Unfortunately, the origins of this morphological trait are unknown. All species of *Graptemys* are characterized by the presence of post-orbital coloration of some type. However, such coloration is not present in the sister taxon, *Malaclemys*, and its homology to coloration patterns in closely related emydids (*Chrysemys* and *Trachemys*) is tenuous. Nonetheless, once this post-orbital coloration came to be in the map turtle ancestor, it diverged into quite distinct morphological groups. While sexual selective processes may have contributed to this initial divergence of groups, it may have also occurred stochastically.
during the formation of *G. geographica* and the *pulchra* and *pseudogeographica* clades.

Either way, this hypothesis explains only the formation of the morphological groups, not the complete diversification of the genus. The phylogenetic and population genetic data seem to strongly suggest that morphological evolution within clades and within species occurs in a stochastic manner. Thus, the species-specific nature of these coloration patterns suggests a genetic underpinning for the trait in general, but that variation within the trait is neutral with respect to natural or sexual selection.

Even if post-orbital coloration did not contribute to the species radiation of *Graptemys*, color patterns may still have played a fundamental role in species recognition and maintenance. However, the signatures of sexual selection or reinforcement were absent or equivocal in all cases where they would be expected if they were the main forces continuing to shape interactions among map turtle species. At the within-species scale, morphological differentiation between populations was correlated with genetic differentiation. This result is not consistent with patterns of reinforcement among populations, which may be expected if variation in post-orbital coloration were tied to mate choice and sexual selection. The alternative explanation is one of little phenotypic variation or neutral variation in phenotype as a result of stochastic, drift-based processes creating random variation in traits. In addition to drift-based variation, phenotypic variation in post-orbital coloration is driven by environmental and non-genetic effects. Temperature and year were significant factors in shaping morphological patterns within species. This result, in combination with the relatively low heritability for a morphological trait suggests that non-genetic factors may have a comparatively more important role than additive genetic variation in shaping phenotypic variation within species. Finally, in direct experiments to test the
importance of color pattern in species recognition, a key component to any model involving sexual selection and reinforcement, there was no indication of species recognition and preference based solely on morphological color pattern variation. Without this component, and in combination with the results from the other components, hypotheses based on sexual selection and reinforcement for species formation, reinforcement, and maintenance based solely on this trait are seriously weakened.

Thus, while there is a small heritable genetic component to variation within post-orbital coloration, indicating a potential ability to respond to selection pressures but also a stronger role for environment effects, the results of this research indicate that role of past and on-going selection on coloration pattern within the map turtle clade has been limited. Consequently, it appears that post-orbital coloration was not the driving factor in the radiation of this turtle clade. If this is the case, the question then becomes, what factors did contribute to this unusually species-rich group of turtles? It seems likely that at least one piece of the equation is rapid, concurrent allopatric speciation as a result of water level rise and fall during the late Pliocene and early Pleistocene glaciations. However, other turtle genera are found in the same or similar river drainages as map turtles, and thus would be expected to have experienced the same conditions, yet these clades do not show nearly the same level of species richness or river endemism (Ernst et al. 1994).

Several additional features of map turtle biology may have contributed to their species richness. One mechanism could be habitat specialization. Map turtles generally prefer flowing rivers with a plentiful supply of basking sites and nearby sandbars upon which to nest (Ernst et al. 1994; Moll and Moll 2004). Males of these species never leave the water, while females are only observed to utilize the terrestrial landscape during typically short
nesting forays (Ernst et al. 1994; Moll and Moll 2004). This extremely limited use of the terrestrial landscape may make map turtles more susceptible to river drainage isolation than species whose members are willing to make substantial terrestrial forays (e.g. Blanding’s turtles; Kasuga and Janzen 2008). Many species of map turtle are also dietary specialists with a diet consisting almost entirely of molluscan prey (Ernst et al. 1994). These mollusks can be sensitive to environmental conditions and even within river drainages, they may be patchily distributed (Moll and Moll 2004). Such patchy distribution can therefore influence the distribution of the turtles. Indeed, as noted in chapter 3 (Myers 2008b), the dietary specialist *Graptemys geographica* had greater population fragmentation and structuring than the generalists *G. ouachitensis* and *G. pseudogeographica*. Over a greater evolutionary time scale, this fragmentation process could contribute to speciation, although the former species belongs to a single species subclade of map turtles, in contrast to the two generalists. Finally, pheromones may play an important and underappreciated role in mate choice and species recognition in map turtles. These species-specific chemicals are an important part of species recognition and mate selection in plethodon salamanders (Houck and Arnold 2003; Palmer et al. 2005), and many other species (e.g. Chelazzi and Delfinio 1986; Munoz 2004; Mas and Jallon 2005). Pheromones and other chemical cues have been implicated in courtship and species recognition for a subset of map turtle species (Vogt 1980; Myers 2008d). Sexual selection on these chemical cues, rather than on morphological color patterns, could contribute to diversification. It seems, however, that the most likely explanation of *Graptemys* species richness is that no single, specific factor has contributed to the enhanced speciation rate of this clade. Rather, a combination of factors including biogeographic
processes, ecological properties, and sexual selection on alternative cues, working synergistically, may have created the optimal conditions for species formation.

In conclusion, it unlikely that sexual and natural selection on post-orbital coloration patterns was the driving factor contributing to the radiation of the map turtle clade. This process likely was motivated by biogeographic factors associated with glaciation events; however, additional ecological or sexual selective processes may also have contributed. Future research should examine these additional processes in a phylogenetic framework to assess their potential role. Additional research should also be undertaken to isolate potential map turtle pheromones to more firmly establish their role in courtship and species recognition.

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