Evolution of a snake mimicry complex

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Abstract

George Harper: Evolution of a snake mimicry complex
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Batesian mimicry, the adaptive resemblance of harmless organisms (mimics) to harmful organisms (models) that causes predators to avoid both models and mimics, occurs in diverse taxa. Despite the fascination that mimicry complexes generate, many questions remain unanswered concerning the role of mimicry in evolution.

My Ph.D. research has examined the evolution of a snake mimicry complex in the southeastern United States in which selection on the mimetic phenotype varies spatially in magnitude and direction. The mimic, harmless scarlet kingsnakes (*Lampropeltis triangulum elapsoides*), and the model, venomous eastern coral snakes (*Micrurus fulvius*), vary in absolute and relative abundance such that the model is more common deep within its range and the mimic is more abundant at the edge of the model's range. Also, despite selection against the mimetic phenotype outside the range of the model, the range of the mimic exceeds that of the model (an area termed allopatry). Therefore, I sought to determine: 1) what evolutionary mechanisms maintain the mimic in allopatry, 2) whether there has been an evolutionary response to selection against the mimetic phenotype in allopatry, and 3) whether spatial variation in the relative abundance of models and mimics leads to spatial variation in the degree to which mimics resemble the local model.

A potential confounding factor in the evolution of the mimetic phenotype in *L. t. elapsoides* is interbreeding with non-mimetic conspecifics. Therefore, I looked for gene flow from non-mimetic conspecifics and examined the relationship between *L. t. elapsoides* and the rest of *Lampropeltis*.

My results indicate that the best mimics occur at the edge of the model's range and that gene flow from there into allopatry maintains the mimetic phenotype in allopatry. Despite gene flow, selection against the mimetic phenotype is decreasing the resemblance between allopatric *L. t. elapsoides* and *M. fulvius*. Additionally, gene flow from non-mimetic *L. triangulum* is not altering the phenotype of *L. t. elapsoides*, and, in fact, the scarlet kingsnake diverged from other *Lampropeltis* millions of years ago. Thus, I recommend re-elevating the scarlet kingsnake to full species status and renew the use of *L. elapsoides*.

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Table of Contents

List of Tables

List of Figures

Figure

Chapter 1

General Introduction

When Henry Walter Bates (Bates 1862) published his ideas on how one species could benefit from looking like another, he provided the foundation on which mimicry theory is built. Charles Darwin recognized the significance of Bates' paper and wrote Bates a letter noting, "You have most clearly stated and solved a most wonderful problem."

The form of mimicry that has come to bear Bates' name (Batesian mimicry) involves a harmless, palatable species (mimic) that comes to resemble a dangerous, non-palatable species (model). The resemblance benefits the mimic because predators avoid the models, and the resemblance between the models and mimics dupes predators who then avoid the harmless mimic. Thus, Batesian mimicry is a form of defensive mimicry (Pasteur 1982; Vane-Wright 1976; Wickler 1968) and is found in many diverse taxa (Ruxton et al. 2004; Wickler 1968).

Resemblances between snake species were noted prior to the publication of Bates paper (Cope 1860) and were classified as mimicry shortly after Bates published (Wallace 1867). In particular, an estimated 20% of snake species in the new world resemble coral snakes (Greene 1997) and many have been labeled coral snake mimics. Misunderstandings over how coral snake mimicry, and Batesian

mimicry in general, works led to an ongoing debate over whether coral snake mimicry occurs (Brattstrom 1955; Greene and McDiarmid 1981; Hecht and Marien 1956; Mertens 1956; Wickler 1968), and, if it does occur, how it operates in nature. For example, several researchers (Brattstrom 1955; Gadow 1911; Pough 1976) have suggested that the brightly colored ringed patterns of coral snakes and their putative mimics are actually cryptic rather than aposematic. Crypsis in this case relies upon the snakes appearing to be one solid color when in motion and disappearing when they stop because the transverse rings break up the outline of their bodies (Pough 1976).

Despite the long history of Batesian mimicry in general and of coral snake mimicry in particular, and despite the large number of papers published on both topics, many questions remain unanswered. Pfennig (Pfennig et al. 2001) recently addressed a prediction of Batesian mimicry theory using a coral snake mimicry system. Namely, does protection provided by the model break down in a frequency-dependent manner such that there is no protection where the model is absent? They concluded that the protection does decrease in a frequency-dependent manner and that mimics should receive no protection beyond the geographical range of the model. In another recent study, Pfennig et al. (in press) demonstrated that selection does, in fact, act against the mimetic pattern where the mimics occur outside the range of the model.

The results of these two studies (Pfennig et al. 2001; Pfennig et al. In Press) led me to address three questions concerning the evolution of Batesian mimicry:

1. If selection is against the mimetic pattern outside the range of the model, what maintains mimics in allopatry with their model?

2. If selection acts against mimetic phenotypes in allopatry with their model, has there been a response to selection, so that mimicry begins to break down in such regions?

3. If protection from predation is frequency-dependent, what are the implications for where the best mimics are found (i.e., the mimics that most closely resemble their model)? Are the best mimics actually found where models are present but actually relatively uncommon, because, in such areas, predators are relatively unlikely to make recognition errors? Conversely, can poor mimics persist in regions where models are relatively abundant?

In chapter 2, I address the first two questions. One of the main evolutionary forces that can counteract selection is gene flow. Therefore, I sought to determine whether gene flow from sympatry (where the mimetic pattern is favored) to allopatry (where the mimetic pattern is disfavored) is maintaining the mimetic pattern in allopatry. Also, if gene flow is maintaining the mimetic phenotype in allopatry is it sufficient to overcome the selection against the mimetic pattern?

In chapter 3, I turn my attention to what is happening to the mimetic pattern in sympatry. Sympatry is not a homogenous environment and variation in the absolute and relative abundances of models and mimics is to be expected. Yet, the ratio of models to mimics is a key component in determining the amount of protection provided by the models. One of the other key components in determining the amount of protection that the mimics receive is the resemblance between the

models and the mimics. Therefore, does the geographic variation in the absolute and relative abundances of models and mimics lead to geographic variation in the resemblance between the models and the mimics?

I performed both of the above studies using a coral snake mimicry system in the southeastern United States that is composed of *Micrurus fulvius* (eastern coral snake, model) and *Lampropeltis triangulum elapsoides* (scarlet kingsnake, mimic). A potential confounding factor in my studies of the mimetic pattern has been the possibility that *L. t. elapsoides* interbreeds with three subspecies of *L. triangulum* (*L. t. amaura, L. t. syspila and L. t. triangulum*) where their ranges overlap. Of those three, only *L. t. amaura* is a coral snake mimic. Thus, any gene flow from *L. t. syspila* or *L. t. triangulum* into *L. t. elapsoides* could degrade the mimetic pattern. Therefore, I began to look for gene flow between these four *L. triangulum* subspecies. Chapter 4 is the result of that investigation and goes beyond looking for evidence of gene flow to ask what the relationship is between *L. t. elapsoides* and the rest of *Lampropeltis*.

Finally, chapter 5 pulls together the preceding chapters to look at the overall evolution of the mimetic pattern in *L. t. elapsoides* and discuss the role of mimicry in shaping this organism in the past, present and future. I conclude with suggestions for future studies within this snake mimicry system and within Batesian mimicry in general.

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Chapter 2

Selection overrides gene flow to break down maladaptive phenotypes in a snake mimicry complex

Abstract

Batesian mimics – edible species that evolve to resemble dangerous species that predators avoid – should only occur in sympatry with their model, because predators would not be under selection to avoid the model or any harmless look-alikes in areas where the model is absent. Yet, contrary to this expectation, mimics often occur in allopatry with their model. Here, I focus on one such example – a coral snake mimicry complex – to evaluate whether gene flow carries alleles for mimetic phenotypes from sympatry (where mimics are favored) to allopatry (where mimics are disfavored). Using indirect DNA based methods and paleoecological data, I show that there has been recent (<10,000 years bp) gene flow by mimics into regions where their model is absent. I also show that such gene flow has been much stronger in nuclear genes than in maternally inherited mitochondrial genes, suggesting that dispersal by males may explain the continued presence of maladaptive mimic phenotypes in allopatry. Yet, despite gene flow, selection has begun to erode the maladaptive mimetic phenotypes in allopatry. Thus, although gene flow may carry mimetic phenotypes into areas where their model is absent,

natural selection can break down mimetic phenotypes in such regions rapidly and thereby promote evolutionary divergence between allopatric and sympatric populations.

Introduction

Predators typically avoid dangerous species, and edible species can acquire protection from predation by evolving resemblance to dangerous species through a process known as Batesian mimicry (Ruxton et al. 2004). This protection should break down, however, where the dangerous model is absent, because predators in such areas would not be under selection to avoid the model or any of its mimics (Pfennig et al. 2001). Yet, the geographical distributions of many mimics extend far beyond that of their models (Brower and Brower 1962; Clarke and Sheppard 1975; Greene and McDiarmid 1981; Pfennig et al. In Press). Here, I examined whether mimics often occur in allopatry with their model because of gene flow: the movement of alleles encoding the mimetic phenotype from regions where the model is present (and where mimics are selectively favored) to regions where the model is absent. I also asked if gene flow overwhelms selection against mimetic phenotypes in allopatry or if such selection ultimately breaks down mimetic phenotypes in these areas.

To address these two issues, I focused on a well-known snake mimicry complex. In the eastern U.S., nonvenomous scarlet kingsnakes, *Lampropeltis triangulum elapsoides*, closely resemble highly venomous eastern coral snakes, *Micrurus fulvius* (Greene and McDiarmid 1981). Both species are brightly colored, with rings of red, yellow (or white), and black encircling the body (Williams 1978). Predators avoid such tricolor ringed patterns (Brodie 1993; Pfennig et al. 2001), often without prior experience (Smith 1975), but only in areas where *M. fulvius* actually occur (Pfennig et al. 2001). Yet, despite evidence that mimetic phenotypes

incur a cost in terms of increased predation in allopatry (Pfennig et al. In Press), *L. t. elapsoides* occurs hundreds of kilometers outside the range of *M. fulvius* (Fig. 2-1).

Methods

Gene flow estimation

I estimated gene flow using indirect DNA based methods. I extracted DNA from 108 sympatric and 38 allopatric *L. t. elapsoides* (Fig. 2-1 and Appendix 1). I amplified three mitochondrial genes (*ND4*, *CytB*, and *16s*) using PCR and direct sequenced them on an ABI3730 (for primers, see Table 2-1). Sequences were assembled using Sequencher 4.2 and aligned using ClustalX 1.81 (Thompson et al. 1997). Alignments were checked by eye. I also amplified five microsatellite (nuclear) loci using PCR and fluorescently labeled primers (Table 2-1). Microsatellite samples were genotyped using an ABI3730. Microsatellite genotype data was analyzed using GeneMarker 1.5.

I analyzed the geographic and genetic structure of *L. t. elapsoides* using minimum evolution (ME), maximum parsimony (MP), and maximum likelihood (ML) analyses with PAUP* 4.0 (Swofford 2002). An analysis of molecular variance run on Arlequin 2.0 (Schneider et al. 2000) detected significant genetic structure (F_{ST} = 0.095, P = 0.009). Moreover, the ME, MP and ML analyses of mtDNA haplotypes revealed that all individuals outside of Florida belong to one clade (the "northern clade"). Therefore, estimates of gene flow between sympatric and allopatric populations were restricted to this clade only.

To estimate gene flow, I calculated the number of migrants between populations per generation (N_m) from F_{ST} (for the mtDNA loci) or R_{ST} (for the nuclear microsatellite loci, (Slatkin 1995) values obtained from Arlequin 2.0 using the equation $N_m = 1/4(1/F_{ST} - 1)$ (Wright 1951). Because mtDNA loci are linked to each other, and because some of the microsatellite loci may also be linked, the Arlequin analysis was run by combining the three mtDNA loci together and by combining the five microsatellite loci together. I also used MIGRATE 2.1.3 (Beerli and Felsenstein 1999; Beerli and Felsenstein 2001) to estimate N_m for the mtDNA loci using ML and coalescence.

Range expansion and divergence estimation

To detect the signature of a recent range expansion, I used three methods. First, I calculated Tajima's D using Arlequin 2.0. A significantly negative Tajima's D is a signature of possible range expansion (Tajima 1989a; Tajima 1989b). Second, I calculated mismatch distributions of sequence data from the mtDNA *ND4* locus. A recently expanded population would not be in equilibrium and would therefore be expected to produce a unimodal distribution of mismatches. I calculated the actual mismatch distribution and a model of the expected mismatch distribution under population expansion using Arlequin 2.0. Failure to reject the model points to recent population expansion (Rogers and Harpending 1992). Third, I used Nested Clade Analysis (NCA) (Templeton 1998). NCA tests for nonrandom associations of alleles or haplotypes with geographical location and then interprets the factors (e.g., range expansion) that best explain those nonrandom associations. To perform NCA, I first

produced statistical parsimony haplotype trees of the mtDNA sequences using TCS 1.21 (Clement et al. 2000). I produced a nested clade diagram (Templeton and Sing 1993) from the statistical parsimony tree and tested for nonrandom associations between haplotypes and geographical location using Geodis 2.5 and 10,000 permutations of my data (Posada et al. 2000). I then inferred the role of range expansion in shaping the current genetic population structure of *L. t. elapsoides* using the inference key provided with the Geodis software.

To estimate the timing of range expansion, I calculated the mean Kimura two parameter sequence divergence between sympatric and allopatric individuals in the northern clade and divided that mean by the divergence rate per million years. For this estimate, I used published divergence rates that were calculated for *ND4* and *cytB* in snakes (Pook et al. 2000; Wuster et al. 2002; Zamudio and Greene 1997).

Color pattern analysis

I took digital images of preserved specimens of *L. t. elapsoides* and *M. fulvius* using a digital camera (Appendix 4). All snakes were photographed on the same background material with a ruler included in each photo to establish scale.

I then projected an enlarged photo of each snake onto a 1 m x 1 m whiteboard and measured both color and size characteristics (Table 2-2) using digital calipers. All measurements were converted to actual lengths using the ruler in each photo. Characteristics of the colors themselves were not measured because the specimens were preserved in formalin and/or alcohol, and colors fade in these preservatives.

Pattern characters were analyzed in several ways. First, I selected three pattern characteristics *a priori* as critical measures – the proportion of the middorsum red; the proportion of the middorsum black; the proportion of rings complete – based on a preliminary morphometric analysis, which showed that these characteristics distinguished "good" mimics (see chapter 3). I limited my analysis to red and black, because these two colors are the predominant colors on both models and mimics, and including all three colors would remove the independence of the characters. I compared these characteristics among *M. fulvius* (n = 41), sympatric *L. t. elapsoides* (n = 113), and allopatric *L. t. elapsoides* (n = 57).

Results and Discussion

Five lines of evidence suggest that *L. t. elapsoides* underwent a range expansion into allopatry (i.e., historical gene flow) no earlier than within the last 20,000 years and probably within the past 10,000 years (i.e., within 5,000 generations of *L. t. elapsoides*). First, I used the mtDNA *ND4* locus to calculate Tajima's D, a measure that can differentiate between population expansion (negative values) and population subdivision (positive values; see Methods). Tajima's D for *L. t. elapsoides* in allopatry and adjacent sympatry (i.e., for the "northern clade"; see Methods) was -2.163, which is significantly less than zero ($P < 0.001$). Thus, northern populations of *L. t. elapsoides* appear to have undergone range expansion. Second, a distribution of pairwise sequence mismatches within the northern clade did not differ from the projected distribution of mismatches calculated under a model of population expansion (sum of squared deviation = 4.8×10^{-5}, $P = 0.99$), again,

pointing to a range expansion. Third, a nested clade analysis (NCA) indicated that there had been a contiguous range expansion for the entire northern clade of *L. t. elapsoides*.

Molecular clock estimates and paleoecological data provide two additional lines of evidence that *L. t. elapsoides* expanded its range into allopatry no earlier than 20,000 years ago and probably within the past 10,000 years. Estimates of sequence divergence between populations of *L. t. elapsoides* in allopatry and northern sympatry reveal that the mean genetic distance between these two populations (based on the mtDNA *ND4* locus) is 1.28088 x 10^{-3}. Using two molecular clocks computed for the *ND4* locus of snakes (Pook et al. 2000; Wuster et al. 2002; Zamudio and Greene 1997), I estimate that divergence between allopatry and northern sympatry occurred between 19,407 to 8,895 years bp. Typically, estimates of divergence over such short time periods are overestimates (Ho et al. 2005), leading to inflated divergence time estimates. Therefore, *L. t. elapsoides* likely expanded into allopatry within the past 10,000 years.

Moreover, paleoecological data confirm that range expansion likely occurred within the past 10,000 years.   Until about 10,000 years ago, the climate in the southeastern U.S. was colder and drier than at present. Consequently, forests in present allopatric regions were dominated by northern pines and spruce (Whitehead 1981).   Based on their modern distribution (Conant and Collins 1998; Williams 1978), it seems unlikely that *L. t. elapsoides* would have been present in modern allopatry until longleaf pine and hardwood forests replaced these boreal forests about 10,000 years ago (Whitehead 1981).   Thus, multiple, independent lines of

evidence suggest that *L. t. elapsoides* began to expand into allopatry relatively recently.

I next sought to quantify the strength of gene flow by estimating the number of migrants per generation (N_m) into allopatry. I also asked if any such gene flow was caused by both sexes or primarily by one sex. I began by estimating gene flow using three mtDNA loci (*ND4*, *cytB*, *16S*). Because mitochondria are haploid and passed only from mother to offspring, such markers measure dispersal caused by females only. Based on these markers, estimates of N_m between sympatric and allopatric populations were extremely low, regardless of the populations being compared (Table 2-3). In fact, I detected virtually no such gene flow east of the Appalachian Mountains, and very limited gene flow west of the Appalachians. In addition, the NCA for the northern clade revealed restricted gene flow within this clade in general and within subclades that include allopatric haplotypes in particular.

Because males of many species are often more likely to disperse than females, I also estimated gene flow based on five nuclear (microsatellite) loci, which measure gene flow caused by both sexes. These estimates of N_m between sympatric and allopatric populations were much higher than those based on mtDNA (Table 2-3). Thus, recent gene flow from sympatry to allopatry was most likely driven by the dispersal of males. In addition, gene flow into allopatry is much higher west of the Appalachians than east of the Appalachians (Table 2-3).

Given evidence of recent gene flow, I next explored if such gene flow is sufficient to overcome selection against the mimetic phenotype in allopatry (Pfennig et al. In Press). Whereas selection should favor the maintenance (or enhancement)

of the mimetic phenotype in sympatry, selection should favor its elimination in allopatry (Pfennig et al. In Press). Thus, unless ongoing gene flow is strong enough to overcome such selection against the mimetic phenotype in allopatry, *L. t. elapsoides* should be less similar phenotypically to the model, *M. fulvius*, in allopatry than in sympatry.

An analysis of geographic color pattern variation among mimics in sympatry and allopatry with their model reveals that selection has indeed begun to erode mimetic phenotypes in allopatry. Allopatric *L. t. elapsoides* east of the Appalachians are less similar phenotypically to *M. fulvius* than are sympatric *L. t. elapsoides* (Fig. 2-2). Indeed, a discriminant analysis that compared allopatric and sympatric *L. t. elapsoides* to *M. fulvius* mistakenly classified sympatric *L. t. elapsoides* as *M. fulvius* significantly more often than allopatric *L. t. elapsoides* (36 of 78 sympatric *L. t. elapsoides* vs. 24 of 85 allopatric *L. t. elapsoides* were mistakenly classified as *M. fulvius*; two-tailed Fisher's exact test, P = 0.0228). In addition, *L. t. elapsoides* are increasingly dissimilar to *M. fulvius* as the distance from the sympatry / allopatry boundary increases. This gradual decrease in morphological similarity to the model is significantly correlated with distance from the sympatry / allopatry boundary per se and not with latitude (partial correlation for distance from sympatry / allopatry boundary and proportion of dorsum red controlling for latitude = 0.29, d.f. = 86, $P <$ 0.01; partial correlation for distance from sympatry / allopatry boundary and proportion of dorsum black controlling for latitude = -0.27, d.f. = 86, $P <$ 0.01). Moreover, predators appear to perceive allopatric "mimics" as being less similar to the model than sympatric mimics: when free-ranging predators are given a choice of

attacking plasticine replicas (Brodie 1993; Pfennig et al. 2001; Pfennig et al. In Press) modeled after either allopatric or sympatric *L. t. elapsoides*, they preferentially attack the former (see chapter 3).

Erosion of the mimetic phenotype in allopatry is not caused by hybridization between mimetic *L. t. elapsoides* and other, less mimetic *L. triangulum* subspecies that occur in allopatry. Studies east (chapter 4) and west of the Appalachians (Armstrong et al. 2001; Collins and Hirschfield 1964; Mount 1975) reveal no evidence of recent hybridization between *L. t. elapsoides* and other *L. triangulum*. Instead, the observed gradual break down of the mimetic phenotype with increasing distance from the sympatry / allopatry boundary is consistent with the results of a previous field experiment (Pfennig et al. 2001), which revealed that protection from predation declines gradually as the model becomes increasingly rare.

Thus, despite gene flow promoting the spread of the mimetic phenotype into allopatry (Table 2-3), selection is apparently breaking down the maladaptive phenotype in such areas, leading to the rapid evolution (i.e., in < 10,000 years) of a less mimetic phenotype in allopatry (Fig. 2-2). My results therefore indicate that, as predicted by Batesian mimicry theory (Pfennig et al. 2001; Ruxton et al. 2004), selection maintains mimetic phenotypes in areas where the dangerous model is present but not where the model is absent.

Finally, the finding that *L. t. elapsoides* are evolving away from the mimetic phenotype in the allopatric population east of the Appalachians contrasts markedly with the pattern in sympatric populations, where selection appears to be maintaining a close match to the model (Fig. 2-2). This divergent pattern of natural selection

between allopatry and sympatry could contribute to divergence between sympatric and allopatric populations. If, as phenotypes become less mimetic in allopatry, immigrants from sympatry are more often preyed upon than individuals from allopatry, then dispersal from sympatry into allopatry would be selectively disfavored and reproductive isolation between such populations might thereby result (Nosil et al. 2005). Indeed, consistent with this hypothesis, I found substantially higher levels of ongoing gene flow among sympatric populations than between sympatric and allopatric populations (Table 2-3). Thus, by selectively favoring reduced gene flow between sympatric and allopatric populations, Batesian mimicry may promote the evolution of reproductive isolation and, possibly, the origin of new species.

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Table 2-1. Primers for PCR, Sequencing and Genotyping.

Locus	Primer	Sequence	Reference
ND4	ND4	TGACTACCAAAAGCTCATGTAGAAGC	(Forstner et al.
	Leu	TACTTTTACTTGGATTTGCACCA	1995)
CytB	L14910	GACCTGTGATMTGAAAAACCAYCGTTGT	(Burbrink et al.
	L14919	AACCACCCGTTGTTATTCAACT	2000)
	L15584	TCCCATTYCACCCATACCA	
	H16064	CTTTGGTTTACAAGAACAATGCTTTA	
	H15149	CCCTCAGAATGATATTTGTCCTCA	
	H15716	TCTGGTTTAATGTGTTG	
16s	16sf	GGCCTAAAAGCAGCCACCTA	
	16se	GGTCTGAACTCAGATCACGTAGGACT	
Le1	Eobu1F	ATCAGTAGGAGTGAGAGCAACT	(Blouin-Demers and
	Eobu1R	CTGCATACTCTTCCAGAACC	Gibbs 2003)
Le2	Eobu2F	CTTGGGGAGAAAGTGTCAT	(Blouin-Demers and
	Eobu2R	TGGCTGGATTCTTACAAGT	Gibbs 2003)
Le3	Eobu3F	ATTTGGTAGCCATCACATC	(Blouin-Demers and
	Eobu3R	CAGTCCTAAATGTTCTGTTGA	Gibbs 2003)
Le4	Eobu10F	ATTGACTTCATAGCACAATGTCA	(Blouin-Demers and
	Eobu10R	CAGAGTCTCCTTGGTGAGAAG	Gibbs 2003)
Le5	Eobu373F	GAGACCATATGCACCAAGAC	(Blouin-Demers and
	Eobu373R	GGCTGAAGTTTACTGGTCTG	Gibbs 2003)

Table 2-2. Body and Pattern Characteristics

Body Characteristics	Pattern Characteristics
Total Length	Number of black, red and yellow rings
Snout-Vent Length	Width of each ring at middle of the dorsum
Head Length	Width of each ring at side
Head Width at widest point	Length and Width of black on head
	Number of black and red rings that
	completely cross ventral surface

Table 2-3. Estimates of gene flow (number of migrants per generation, *Nm*).

Recipient population	Mitochondrial DNA		Nuclear DNA	
	F_{ST}	*Nm*	R_{ST}	*Nm*
Allopatry				
Eastern allopatry (NC, VA)	0.22	0.89 (0)	0.03	7.70
Western allopatry (KY, TN)	0.12	1.79 (0.11)	0.01	19.30
Mean ± s.e.m. =		**1.34 ± 0.87***		**13.50 ± 8.37**
Sympatry				
Northeastern sympatry (NC)	0.02	11.53 (3.78)	0.02	13.93
Eastern sympatry (GA, SC)	0.02	13.65 (0.35)	0.01	38.53
Southern sympatry	0.02	13.15 (9.34)	0.02	12.24
(LA, MS, AL, FL panhandle)				
Southern sympatry (N FL)	0.02	14.86 (30.43)	0.01	30.53
Mean ± s.e.m. =		**13.30 ± 0.62***		**23.81 ± 5.92**

F_{ST}, values are the means of three mtDNA loci; R_{ST}, values are the means of five microsatellite loci. F_{ST}, R_{ST}, and *Nm* for the two allopatric populations estimates gene flow from all sympatric populations and for the four sympatric populations from all other sympatric populations. Numbers in parentheses: *Nm* estimates from MIGRATE. Asterisk: *Nm* values significantly different from each other ($t_4 = 11.17$, $P = 0.004$). State abbreviations: AL – Alabama; FL – Florida; GA – Georgia; KY – Kentucky; LA – Louisiana; MS – Mississippi; NC – North Carolina; SC – South Carolina; TN – Tennessee; VA – Virginia

Figure legends

Figure 2-1. Nonoverlapping distributions of model and mimic in a coral snake mimicry complex. The nonvenomous scarlet kingsnake, *Lampropeltis triangulum elapsoides*, mimics the warning coloration of the venomous eastern coral snake, *Micrurus fulvius*. Although the two species co-occur in the southeastern U.S., the geographical range of the mimic extends far north of the range of its model. Dots: collection sites of samples used for genetic analyses.

Figure 2-2. Color pattern variation among mimics in sympatry and allopatry with their model. Comparison of model, sympatric mimic, and allopatric mimic in **a** – mean ± s.e.m. proportion of dorsum red; **b** – mean ± s.e.m. proportion of dorsum black; **c** – mean ± s.e.m. proportion of rings complete. Different letters indicate means that are significantly different. **d –** photos of model, sympatric mimic, and allopatric mimic; state where snake was collected in parentheses.

Figure 2-1

Allopatry (only mimic present)

Sympatry (model and mimic present)

○ Collection site

Figure 2-2

M. fulvius (NC)   *L. t. elapsoides* (NC)   *L. t. elapsoides* (VA)
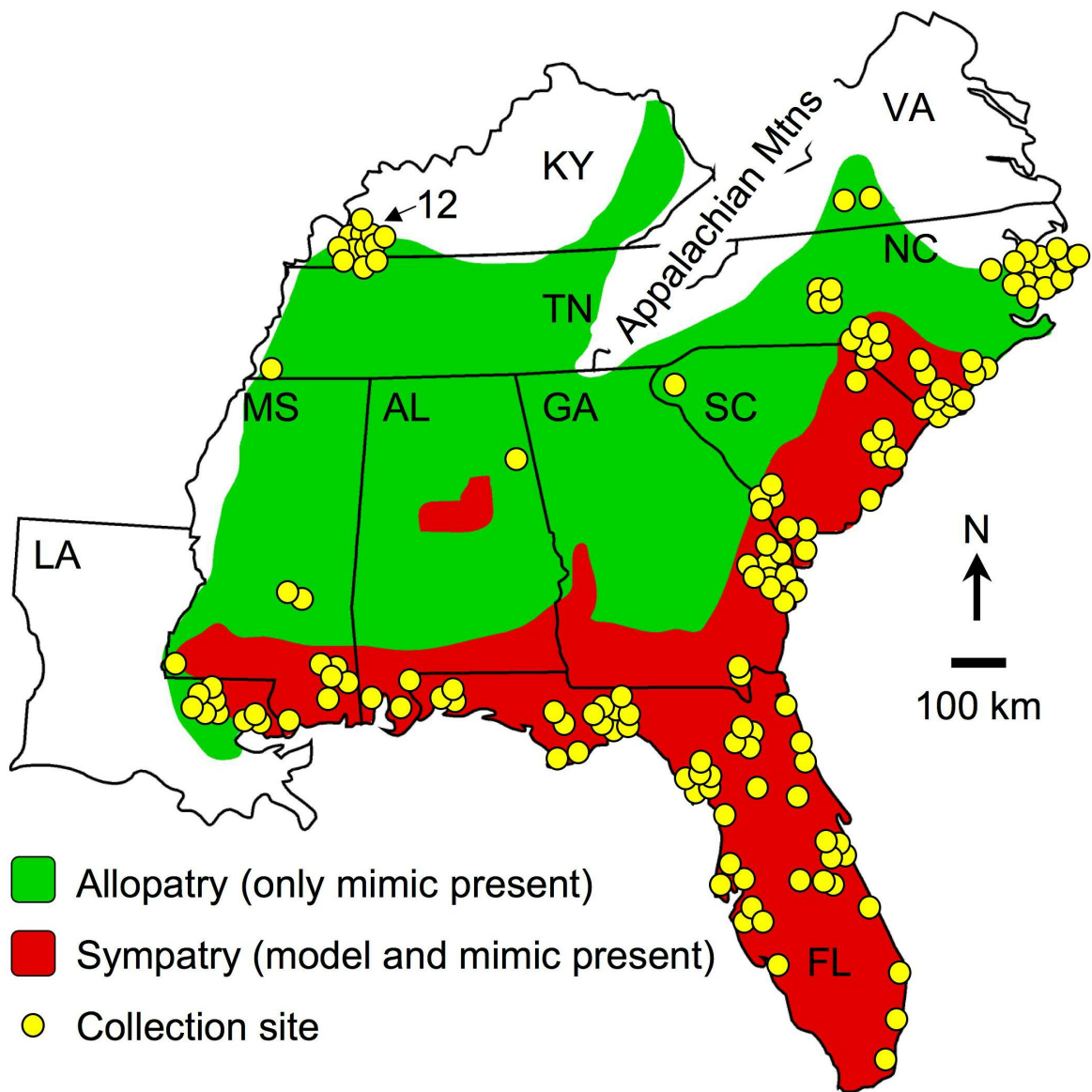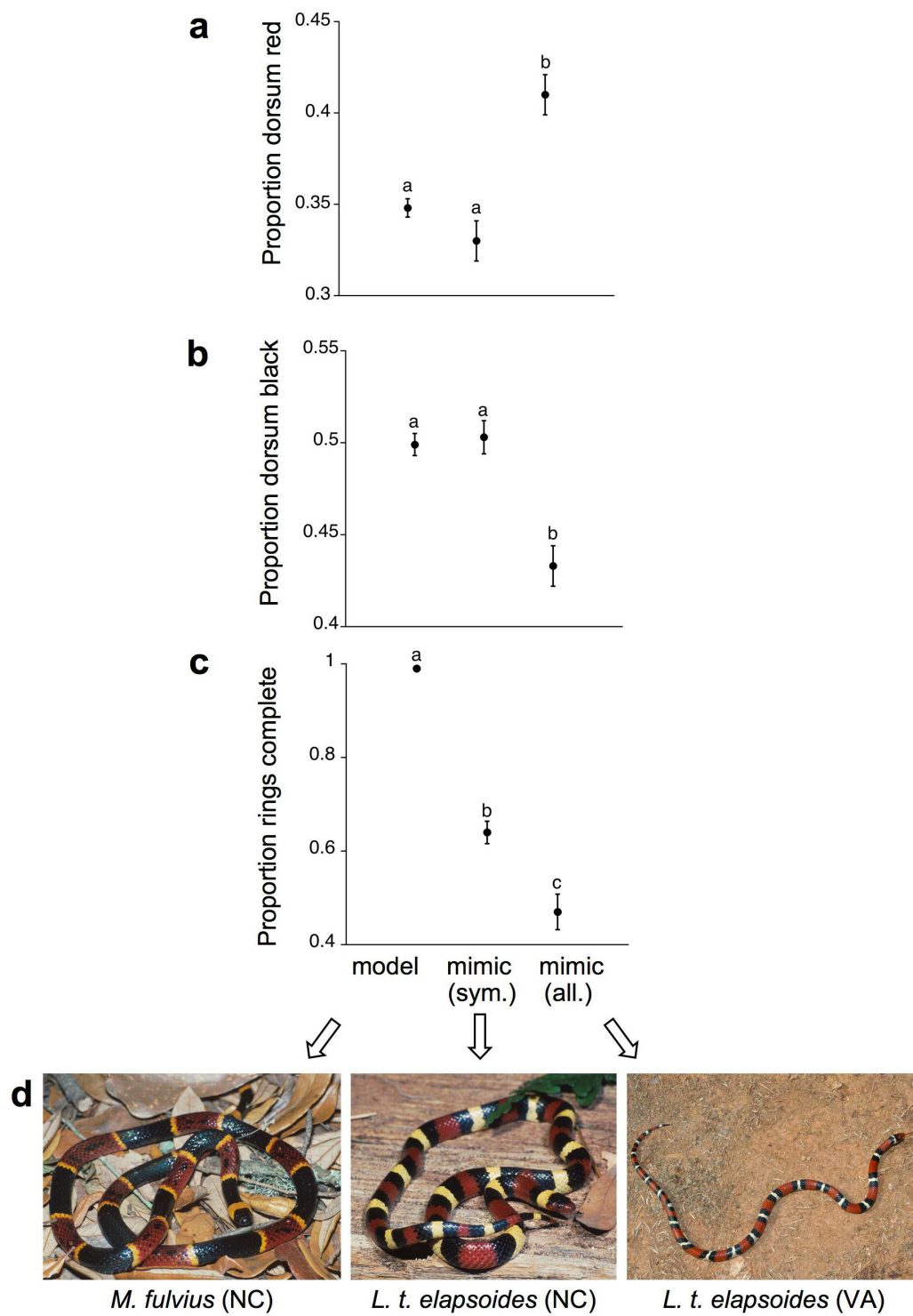
Chapter 3

Mimicry on the edge: Why do mimics vary in resemblance to their model in different parts of their geographical range?

Abstract

Batesian mimics – benign species that predators avoid because of their resemblance to a dangerous species (the "model") – are often imperfect replicas of their model. Theory predicts that mimics do not have to be perfect replicas if the model is relatively common. Here, I tested this prediction in a coral snake mimicry complex where the geographical range of the mimic extends beyond that of its model. I specifically asked whether the best mimics exist on the edge of their model's range. I found that the ratio of models to mimics was greatest in the center of the model's range (where models were more abundant than mimics) and lowest on the edge (where models were less abundant than mimics). Moreover, mimics on the edge more closely resembled the model than did those in deep sympatry. Finally, when given a choice of attacking either good or poor mimics, free-ranging natural predators on the edge of the model's range avoided only good mimics. Thus, the best mimic may generally exist on the edge of their model's range where models are rare and, hence, where all but the best mimics would be selected against. By

contrast, poor mimics may persist in deep sympatry, where predators are more likely to encounter the dangerous model. More generally, these results explain geographical variation in mimic-model resemblance and provide support for a central prediction of Batesian mimicry theory.

Introduction

Batesian mimicry evolves when a palatable species (the "mimic") co-opts a warning signal from a dangerous species (the "model") in order to deceive potential predators (Bates 1862). Such resemblances can be favored by natural selection if predators avoid the model and any look-alikes (reviewed in Ruxton et al. 2004).

Although it is often assumed that selection will tend to favor those mimics that most closely resemble their model, Batesian mimics are often imperfect replicas of their model (Edmunds 2000). Indeed, five hypotheses have been advanced to explain the widespread occurrence of apparently imperfect mimics (Edmunds 2000; Ruxton et al. 2004). First, mimics that appear to be poor from a human's perspective may in fact appear as good mimics to predators (Dittrich et al. 1993). Second, poor mimics may reflect a "breakdown" of mimicry, such as what might occur in areas where mimics become more abundant than models (Brower 1960; see also Chapter 2). Third, even poor mimics can gain protection from predators if the model is relatively common or highly noxious (Ruxton et al. 2004). Fourth, imperfect mimicry may evolve as a consequence of selection to resemble simultaneously more than one model living in separate areas (Edmund 2000; Darst and Cummings 2006). Finally, seemingly poor mimics may evolve through antagonistic coevolution between mimic and model. Because models may suffer increased predation as mimics become more numerous (Fisher 1930; Oaten et al. 1975), selection should favor models that evolve away from their mimics. Such "chase-away evolution" (Gavrilets and Hastings 1998; Holmgren and Enquist 1999) may convert good mimics into poor mimics.

Some of the best mimicry systems for exploring these ideas are those in which a single species of mimics vary in phenotypic resemblance to their model(s) in different parts of their geographical range (e.g., see Greene and McDiarmid 1981; see also Chapter 2). In such systems, there may be a geographical mosaic (sensu Thompson 2005) in mimic-model resemblance, so that good mimics occur in some areas and poor mimics in others (e.g., see Pfennig et al. 2006).

I sought to evaluate the above five hypotheses by testing critical predictions of each (Table 3-1), but I concentrate on testing the third hypothesis above. I focused on a coral snake mimicry system (described below) where the geographical range of the mimic extends beyond that of the model. This pattern is not unusual – in many mimicry complexes, mimics occur where their model is absent (Clarke and Sheppard 1975; Gordon and Smith 1998; Koch et al. 2000; Pfennig et al. 2001; Prudic et al. 2002). In situations where the model's range is nested within that of the mimic, the model to mimic ratio is likely to vary between the edge and center of the model's range, and, as a consequence, the optimal degree of resemblance between mimic and model should also vary geographically. In particular, in the center of the model's range, where the model is likely to be relatively common, even poor mimics are likely to gain protection. In contrast, on the edge of the model's range, where the model is likely to be relatively rare, only good mimics are likely to be protected. Thus, if this hypothesis is correct, then I would expect the best mimics to be present on the edge of their model's range. I tested these ideas by combining population censuses of model and mimic abundances, morphometric analyses of model to mimic phenotypic

similarity, and field experiments to estimate selection on different mimetic phenotypes.

Materials and Methods

Study system

In the southeastern U.S., eastern coral snakes (*Micrurus fulvius*) occur from the Atlantic coast to the Mississippi river with the northern edge of their range in North Carolina, and generally are found in coastal plain and sandhill habitats within 200 km of the Atlantic Ocean and the Gulf of Mexico (Conant and Collins 1998). These highly venomous, aposematically colored elapids serve as models for the scarlet kingsnake (*Lampropeltis triangulum elapsoides*; Pfennig et al. 2001), a harmless colubrid whose range encompasses and exceeds that of the coral snake (Fig. 3-1).

Within the *M. fulvius* / *L. t. elapsoides* mimicry system are areas with many coral snakes (e.g. Florida, hereafter called deep sympatry), few coral snakes (e.g. portions of North Carolina, hereafter called edge sympatry) and no coral snakes (e.g. areas where *L. t. elapsoides* exceeds the range of *M. fulvius*, hereafter called allopatry). Such variation in the ratio of model to mimic is important for testing questions concerning the resemblance between models and mimics.

Previous studies have established that, as predicted by Batesian mimicry theory, attacks on replicas of *L. t. elapsoides* increase as the number of *M. fulvius* decline (Pfennig et al. 2001), and that selection acts against the mimetic pattern in areas where the mimic occurs but the model does not occur (Pfennig et al. *In press*).

In addition, *L. t. elapsoides* in allopatry do not resemble the typical *M. fulvius* as closely as do sympatric *L. t. elapsoides* (Chapter 2).

Focusing on this system, I conducted three separate studies. First, to determine whether the ratio of models to mimics differs between the edge sympatry region and the deep sympatry region, I tallied museum collections of both models and mimics (a proxy for population estimates). Second, I performed a comparative analysis of the color patterns of models and mimics in the edge sympatry region and the deep sympatry region to determine whether the mimics differ in their resemblance to coral snakes from their respective regions. Finally, I conducted a field experiment of predation on 'good' and 'poor' mimic replicas in the edge sympatry region and the deep sympatry region to determine whether predation pressure is different for good and poor mimics in the two regions.

Assuming that the penalty for making a mistake was equal between regions, I made predictions concerning the field experiment and the color pattern analysis based on whether a region had a low model to mimic ratio (<1) or a high model to mimic ratio (>1). My overall prediction is that a low model to mimic ratio should indicate a lower probability of a predator making a costly mistake, and therefore mimics may need to resemble their models more closely to receive protection. Specifically, I predicted that predators from areas with low model to mimic ratios should only avoid good mimics, whereas, predators from areas with high model to mimic ratios should avoid both good and relatively poor mimics. In addition, I predicted that mimics from areas with low model to mimic ratios should more closely

resemble their models, but that mimics from areas with high model to mimic ratios would resemble their model less.

Model to mimic ratio

I first asked if the likelihood of making a mistake varies geographically from deep sympatry to edge sympatry. In order to do so, I asked whether the ratio of models to mimics differs between edge sympatry and deep sympatry. I assembled data on numbers of *L. t. elapsoides* and *M. fulvius* collected in Florida from numerous museums. I used the data previously published by Palmer and Braswell (1996) for both species in North Carolina.

I tallied the number of individuals of each species by county and only used counties that had at least one mimic sample. I calculated the ratio of models to mimics for each county and used those ratios to calculate the mean model to mimic ratio for the two regions. Because the data did not meet assumptions for parametric data analysis, the means of the ratio of models to mimics for each region were compared using a Kruskal-Wallace rank sums test.

Color pattern analysis

Next, I evaluated whether mimics vary phenotypically in different geographical areas, and, if so, if they vary in how closely they resemble the local model, such that the phenotypic match between mimic and model is closer in some geographical regions than in other regions. To address this issue, I conducted morphometric analyses of both mimics and models.

Digital photos of 87 *L. t. elapsoides* and 47 *M. fulvius* were taken using a Canon EOS Rebel digital camera and transferred to an Apple G4 Powerbook computer with iPhoto software. All snakes photographed were preserved specimens from museum collections (Appendix 4). All were photographed on the same background material with a ruler included in each photo to establish scale.

I characterized the pattern of each snake by projecting an enlarged photo of each snake onto a 1 m x 1 m whiteboard and measuring numerous pattern and size characteristics (Table 3-2) using digital callipers. All measurements were converted to actual lengths using the ruler in each photo. Characteristics that could be affected by the size of the snake, such as the average width of rings, were standardized for differences among snakes in total length (TL) by using the residuals of the character regressed on TL. Characteristics of the colors themselves (e.g. hue, saturation, etc.) were not measured because the specimens were all preserved in formalin and/or alcohol, and colors fade in these preservatives.

All statistical analyses were performed using JMP 5.1.2 (SAS Institute Inc., Cary, NC). Pattern characters were analyzed in several ways. First, I selected three pattern characteristics *a priori* as critical measures – the proportion of the middorsum red; the proportion of the middorsum black; the proportion of rings complete. I limited my analysis to proportion of the dorsum that is red (hereafter referred to as Pred) and proportion of the dorsum that is black (hereafter referred to as Pblack), because these two colors are the predominant colors on both models and mimics, and including all three colors (red, black and yellow) would remove the independence of the characters. I compared these characteristics between the four

categories of snakes, edge sympatry models (*M. fulvius*, n = 22), deep sympatry models (*M. fulvius*, n = 25), edge sympatry mimics (*L. t. elapsoides*, n = 41) and deep sympatry mimics (*L. t. elapsoides*, n = 46). In addition, to determine how often mimics are mistaken for models and vice versa I performed discriminant analyses of models and mimics in each region based on Pred and Pblack.

Predation experiment

Finally, I sought to determine if selection on good and poor mimics varied in different geographical regions, depending on the ratio of model to mimic. Specifically, I asked whether poor mimics (i.e., mimics that were less phenotypically similar to the model) were more likely to be attacked in regions where models were relatively rare (i.e., on the edge of sympatry) than in regions where models were relatively abundant (i.e., in deep sympatry). In order to address this issue, I used plasticine replicas of snakes to measure selection on different color patterns by exposing these replicas to free ranging predators.

I constructed artificial models of snakes (replicas) similar to those used in two recent studies within this snake mimicry system (Pfennig et al. 2001; Pfennig et al. In Press). The replicas were made of cylinders of precolored, non-toxic plasticine threaded onto S shaped wires. Plasticine remains soft, thus allowing me to record predation attempts by observing beak and teeth imprints left in the plasticine by natural predators (Brodie 1993; Brodie and Janzen 1995; Hinman et al. 1997; Pfennig et al. 2001).

I constructed snake replicas (1.5 x 18 cm) with a tricolor ringed pattern with

proportions of red, black and yellow similar to those of *M. fulvius* (good mimic), a tricolor ringed pattern with more red and yellow and less black than the average *M. fulvius* (poor mimic), and a plain brown pattern. Both the good and poor mimic replicas were modeled after scarlet kingsnakes, and resembled them in size, color hue, color order, and ring width. The two mimetic replicas differed in the proportions of the three colors, with the poor mimic containing 8% more red, 4% more yellow and 12% less black than the good mimic (see Chapter 2). Both mimetic replica patterns were within the range of variation for scarlet kingsnakes. Brown replicas served as controls and resembled several abundant, nonvenomous snakes found in the areas I used for my experiment, including eastern earth snakes (*Virginia valeriae*), northern redbelly snakes (*Storeria occipitomaculata*), brown snakes (*Storeria decayi*), queen snakes (*Regina septemvittata*), and eastern worm snakes (*Carphophis amoenus*).

I conducted experiments during April and May of 2006 at 10 sites in North Carolina and 10 sites in Florida (Fig. 3-1). Each site contained one 750 m transect that was laid following the procedures in Pfennig et al. (2001). Each replica was used only once. I collected replicas four weeks after their placement. Following collection, a person without knowledge of the replica's location scored attacks by noting any impressions corresponding to a predator. I considered a replica to have been 'attacked' only if it contained teeth marks of a carnivore (e.g., black bear, bobcat, coyote, fox, raccoon). There were no bird attacks. Impressions made by rodents or insects were excluded from the analysis, because these animals would not have represented a threat to a live snake.

For the analyses, my response measure was the proportion of good mimic or poor mimic replicas attacked along each transect (= number of good/poor mimic replicas attacked divided by the total number of mimetic and brown replicas attacked). For the statistical analyses, I compared the proportion of good/poor mimic replicas attacked along each transect with the proportion expected if attacks were random with respect to pattern (0.33). Proportion data were arcsine square root transformed prior to analysis to meet parametric assumptions.

Results

Model to mimic ratio

Forty-eight counties in Florida (72% of counties in the state) and 25 counties in North Carolina (25%) had at least one record for *L. t. elapsoides* and were included in my analyses. The ratios of models to mimics for these two regions are significantly different (Kruskal-Wallace rank sums test, $\chi^2 = 20.18$, df = 1, P < 0.0001). The model outnumbers the mimic in Florida (deep sympatry, mean ± s.e.m. = 2.61 ± 0.68). By contrast, the mean model to mimic ratio for North Carolina, and thus for the edge sympatry region, was 0.43 ± 0.28 indicating that mimics were more abundant than models in this region.

Color pattern analysis

Coral snakes in edge sympatry closely resembled those in deep sympatry (Fig. 3 and Table 3). This close resemblance exists for both Pred (2-tailed t Test, |t| = 0.5904, df = 45, P = 0.558) and Pblack (2-tailed t Test, |t| = 0.10601, df = 45, P =

0.916). In addition, coral snakes in both regions showed little variation for either color characteristic (Table 3-3). There was a difference in the variance within the two regions for Pred (Levene Test, $F = 4.601$, $P = 0.037$) with higher variance in the deep sympatry population. There was, however, no difference in variance within the two regions for Pblack (Levene test, $F = 1.358$, $P = 0.250$).

Conversely, *L. t. elapsoides* from the edge sympatry region and the deep sympatry region do not resemble one another as closely (Fig. 3-3 and Table 3-3) and are significantly different for Pred (2-tailed t Test, $|t| = 5.978$, $df = 85$, $P < 0.0001$) and Pblack (2-tailed t Test, $|t| = 3.879$, $df = 85$, $P = 0.0002$). In addition, *L. t. elapsoides* from the deep sympatry region were more variable in these color characteristics than were *L. t. elapsoides* from the edge sympatry region (Pred – Levene Test, $F = 16.554$, $P = 0.0001$; Pblack – Levene test, $F = 16.129$, $P = 0.0001$).

I also compared the color patterns of mimics to models. I predicted that mimics in areas with low model to mimic ratios should closely resemble their models, but that mimics in areas with high model to mimic ratios could bear less resemblance to their models and in addition could safely harbor more variation for the pattern characteristics. As noted above, the edge sympatry area is a low model to mimic ratio area while the deep sympatry area is a high model to mimic ratio area. Thus, I predicted that mimics in edge sympatry (North Carolina) would resemble the local coral snakes more closely than would mimics in deep sympatry (Florida).

Mimics in the edge sympatry region do closely resemble the models in their region (Fig. 3-3 and Table 3-3) for both Pred (2-tailed t Test, $|t| = 0.564$, $df = 61$, $P = $

0.575) and Pblack (2-tailed t Test, |t| = 1.005, df = 61, P = 0.319). The mimics in the edge sympatry region are, however, significantly more variable than the models in the edge sympatry region for both Pred (Levene test, F = 14.168, P = 0.0004) and Pblack (Levene Test, F = 8.882, df = 40, P = 0.0041).

Mimics in the deep sympatry region do not closely resemble the models in that region (Fig. 3-3 and Table 3-3) for either Pred (2-tailed t Test, |t| = 5.475, df = 69, P < 0.0001) or Pblack (2-tailed t Test, |t| = 3.431, df = 69, P = 0.0011). As in the edge sympatry region, when compared to models in their region, mimics in the deep sympatry region are more variable for both Pred (Levene test, F = 18.016, P < 0.0001) and Pblack (Levene test, F = 19.780, P < 0.0001).

Finding no difference between the means for both Pred and Pblack for models and mimics in the edge sympatry region is one way to look at their similarity. Another way is to estimate how often models are mistaken for mimics and how often mimics are mistaken for models. Therefore, I performed discriminant analyses based on Pred and Pblack. My analyses misclassified significantly more models and mimics in the edge sympatry region than in the deep sympatry region (34.93% vs. 18.31%; 2-tailed Fisher's exact test, P = 0.0322).

Predation pressure on good and poor mimics

Of the 300 replicas placed in the field in North Carolina, 21 were attacked (7.0%). Similarly, 20 of 300 replicas placed in the field in Florida were attacked (6.67%). More importantly, eight of the 10 North Carolina transects had attacks, but only five of the 10 Florida transects had attacks. In addition, one transect in Florida

accounted for half of the total attacks in the deep sympatry region. The combination of low attack rate and heterogeneity of attacks in Florida eliminated the power I needed for statistical analysis. Therefore, statistics are only presented for the North Carolina (edge sympatry) field experiment.

Predators in the edge sympatry region attacked significantly fewer good mimics than expected had they shown no color pattern preference (Fig. 3-2, mean proportion attacked = 0.125, N = 8, 2-tailed Wilcoxon signed rank test, P = 0.039). In addition, as I predicted based specifically on the low model to mimic ratio, predators did not show an avoidance of the poor mimic (Fig. 3-2, mean proportion attacked = 0.406, N = 8, 2-tailed Wilcoxon signed rank test, P = 0.633). Rather, predators attacked both the poor mimic and the control replica (Fig. 3-2, mean proportion attacked = 0.469, N = 8, 2-tailed Wilcoxon signed rank test, P = 0.438) at a rate that was not significantly different from random expectation.

Discussion

Researchers have long known that the degree of resemblance between mimic and model is important in determining the effectiveness of Batesian mimicry (Dittrich et al. 1993; Hetz and Slobodchikoff 1988; Holloway et al. 2002; Howse and Allen 1994; Lindstrom et al. 2004; Lindstrom et al. 1997; Sherratt 2002). Predation pressure on mimics should be greater in areas where the ratio of model to mimic is relatively small (Estabrook and Jespersen 1974; Holling 1965; Huheey 1964). The greater predation pressure is due to a decrease in the likelihood of a predator making a recognition "error" (e.g., mistakenly identifying a model as a mimic) when

selecting prey. Conversely, predation pressure on the mimics should be lower in areas where the model is abundant or where the ratio of model to mimic is large. Thus, if the ratio of model to mimic varies geographically, mimics should most closely resemble their models in areas where models are relatively rare.

In the paper, I investigated geographical variation in mimic-model resemblance. I specifically sought to evaluate five hypotheses to explain the evolution of imperfect mimics (Table 3-1). I did so by focusing on a coral snake mimicry system where the geographical range of the mimic extends beyond that of the model.

I found that coral snake mimics, *L. t. elapsoides*, vary phenotypically in different geographical areas, such that mimic-model resemblance is highest on the edge of the model's range ("edge sympatry"), where models are relatively rare, and lowest in the center of the model's range ("deep sympatry"), where models are relatively common. Because predators are less likely to encounter the dangerous model on the edge of the model's range, they are less likely to mistakenly identify a model as a mimic. This low likelihood of making a recognition error should select for a close match between mimic and model. Indeed, I found that predators at the edge of the model's range discriminated between good mimics and poor mimics. Thus, mimics on the edge of their model's range are under selection to closely resemble the models.

Variation within the color pattern characters also supports the hypothesis that poor mimics can be favored as long as their model is relatively common. A corollary prediction of this hypothesis is that selection on the pattern of the mimics will be relaxed in areas with high model to mimic ratios. Both edge sympatry mimics and

deep sympatry mimics were variable for both the proportion of the dorsum that was red and the proportion of the dorsum that was black. However, mimics from deep sympatry were much more variable than were mimics from edge sympatry. In fact, the range of values for deep sympatry for both Pred and Pblack encompassed and exceeded the values for edge sympatry. This finding is consistent with the hypothesis that there has been a relaxation of selection for mimicry in deep sympatry. By contrast, this finding is not consistent with the hypothesis that selection is driving the pattern in a particular direction as would be the case if the mimics were tracking changes in the model's phenotype. The *L. t. elapsoides* in deep sympatry were still mimetic and composed of the same components (rings of black, red and yellow) as those from edge sympatry. Also, the discriminant analysis supports the conclusion that *L. t. elapsoides* in deep sympatry were still mimetic, because 18% of the samples from that region were misclassified, which suggests that predators would still be at risk for making a recognition error.

In contrast, the results do not support chase away evolution. The pattern analyses indicate that mimics in one region (edge sympatry) have the same mean proportion of red and black as do models from both regions, whereas mimics in the other region (deep sympatry) are significantly different from all of the models for mean proportion of red and black. In addition, models in the two regions were not significantly different from each other for either Pred or Pblack, indicating that it is unlikely that chase-away evolution has occurred separately in the two regions for these characters (e.g., see Table 3-1). Models in the two regions did differ from one another in the number of rings on their bodies (not including their tails), and the

number of rings increases from the south to the north for both the models and the mimics. However, the models and mimics in each region have significantly different numbers of rings on their bodies. Moreover, it seems less likely that predators would mistake models and mimics based on the number of rings that they possess rather than on the proportions of the colors.

My analysis assumes that the cost to predators of mistakenly attacking a model was the same in the two regions. This need not be the case. In fact, spatial and temporal variation in venom is common in both vipers and elapids (Alapegiron et al. 1994; Chippaux et al. 1991). However, it could be that the cost to predators for making a mistake is so high in this system that geographic variation in the venom of *M. fulvius* makes no difference to predator behavior.

Finally, what is the significance of geographic variation in the resemblance between mimics and models? First, my results suggest that selection on models, mimics and predators may vary geographically to produce a geographical mosaic (sensu Thompson 2005) of mimic-model resemblance. Second, differing strengths of selection acting on the mimetic pattern in sympatry may select for a reduction in gene flow among mimics between deep sympatry and edge sympatry (see Chapter 2). In particular, if, as phenotypes become less mimetic in deep sympatry, migrants from deep sympatry to edge sympatry are more often preyed upon than individuals from edge sympatry, then dispersal from deep sympatry into edge sympatry would be selectively disfavored and reproductive isolation between such populations might thereby result (Nosil et al. 2005). Future studies should seek to test this idea.

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Table 3-1. Hypotheses and associated critical predictions to explain the occurrence of imperfect Batesian mimics.

_____

Hypothesis	Critical prediction
(1) Mimics that appear to be poor replicas to humans may appear as good mimics to predators.	Both good and poor mimics should receive protection in all geographical areas where they each occur.
(2) The presence of poor mimics may reflect a "breakdown" of mimicry.	Poor mimics should be selected against in allopatry with their model, but not in sympatry.
(3) Even poor mimics can gain protection if the model is relatively common or highly noxious.	Both the degree of protection and dissimilarity to the model should be inversely correlated with the model's relative abundance.
(4) Imperfect mimicry may evolve as a consequence of selection to resemble simultaneously more than one model living in separate areas.	Multiple species should serve as models to the same species of mimics in different geographical areas.
(5) Poor mimics may evolve through antagonistic coevolution between mimic and model.	Assuming that the system is in equilibrium, mimics should match the local model, but not models in other geographical areas.

Table 3-2. Body and pattern characteristics measured on mimics.

Body Characteristics	Pattern characteristics
Total length	Number of black, red and yellow rings
Snout-vent length	Width of each ring at middle of the dorsum
Head length	Width of each ring at side
Head width at widest point	Length and width of black on head
	Number of black and red rings that completel cross ventral surface

Table 3-3. Color pattern proportions.

Population	Mean Proportion of Dorsum Black	s.d.	Mean Proportion of Dorsum Red	s.d.
FL *M. fulvius*	0.474[b]	0.037	0.378[d]	0.041
NC *M. fulvius*	0.476[b]	0.031	0.384[d]	0.027
FL *L. t. elapsoides*	0.535[a]	0.108	0.269[c]	0.123
NC *L. t. elapsoides*	0.465[b]	0.054	0.390[d]	0.057

Different letters indicate means that are significantly different. P < 0.0001.

Table 3-4. Summary of results for different analyses for edge sympatry and deep sympatry.

Test	Edge Sympatry	Deep Sympatry
Model to mimic ratio	Low	High
Predation experiment	Only good mimics avoided	? (unknown because of few attacks)
Discriminant analysis	Many mistakes	Fewer mistakes
Resemblance of mimic to model	High	Low

Figure Legends

Figure 3-1. Coral snake mimicry system in the southeast US. Scarlet kingsnakes (*Lampropeltis triangulum elapsoides*) mimic the highly venomous eastern coral snake (*Micrurus fulvius*). The mimic's range greatly exceeds that of the model. I have marked the sites of my field experiment. Ranges of models and mimics based on Conant and Collins (year).

Figure 3-2. Attacks on replicas. Mean proportion of replicas attacked of each type $\pm$ s.e.m. Dashed line represents the proportion expected (0.33) if attacks were random with respect to color pattern. Asterisk indicates significant difference from random expectation. GM = Good mimic. PM = Poor mimic.

Figure 3-3. Comparison of black and red for models, mimics, and good and bad mimic replicas. Mean proportion of dorsum black (A) or red (B) $\pm$ s.e.m. Data points not connected by the same letter are not significantly different. NC = North Carolina. FL = Florida. Lte = mimic. Mf = model. GM and PM as in figure 2.

Figure 3-1

sympatry (model and mimic present)

allopatry (only mimic present)

field experiment site
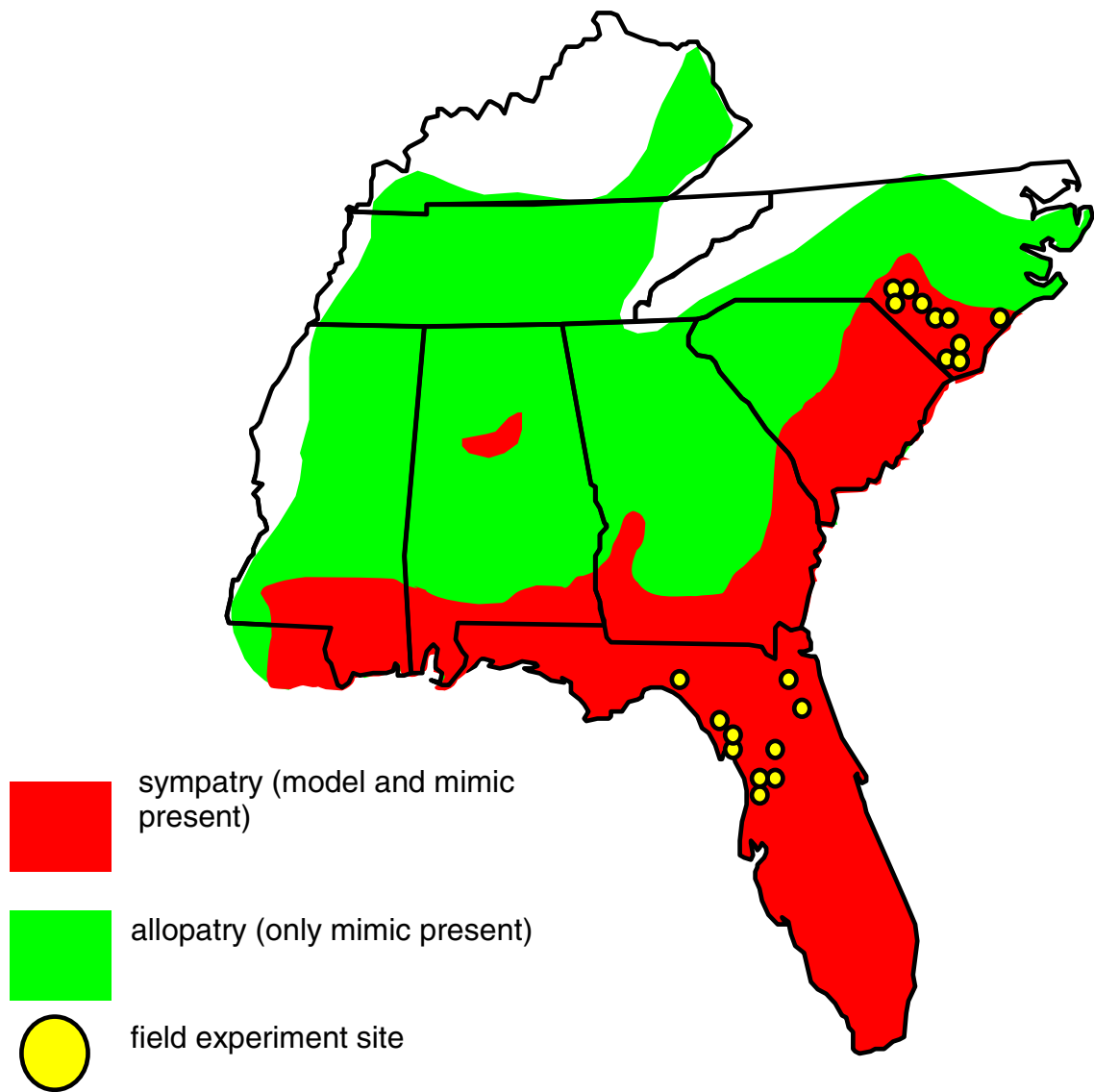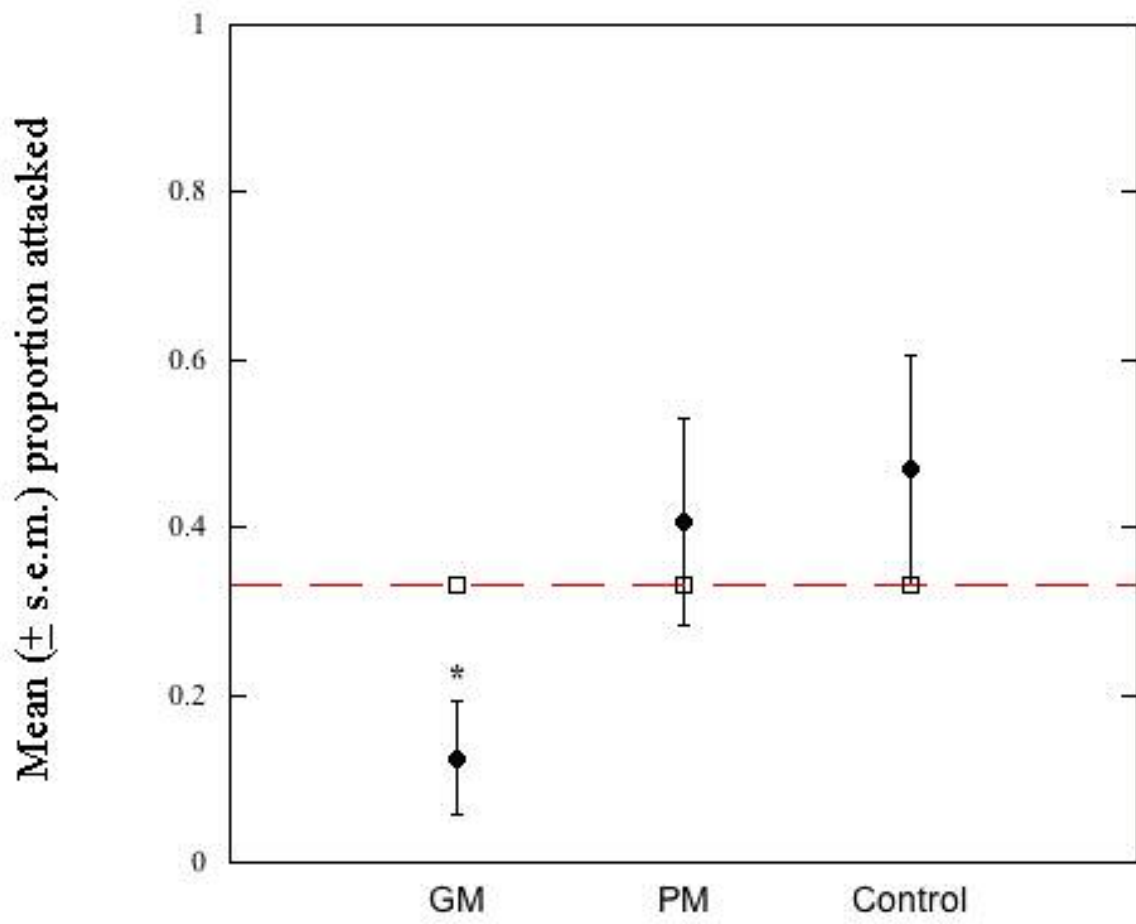
Figure 3-2

Figure 3-3
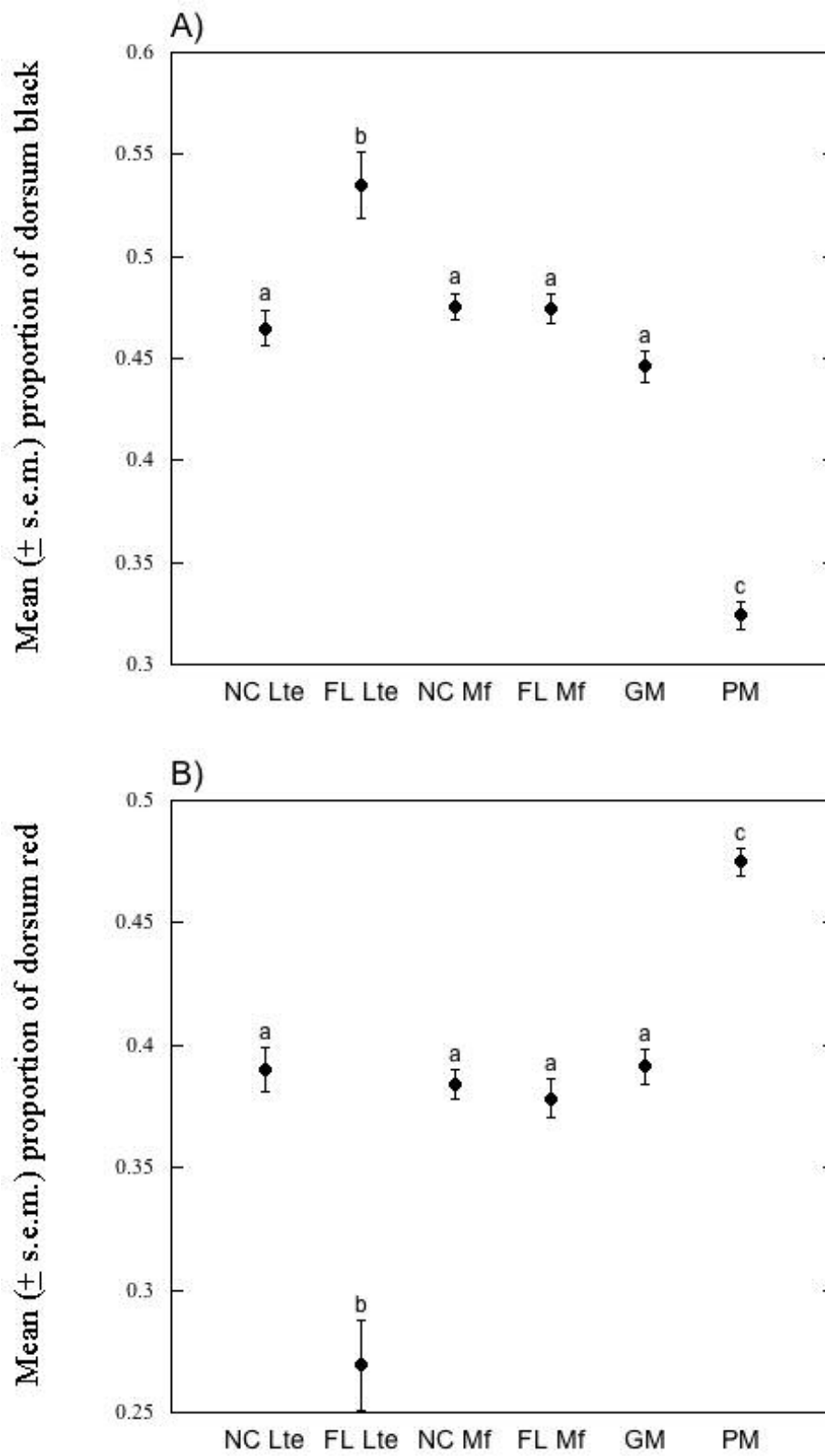
Chapter 4
Mitochondrial phylogenetics of a coral snake mimic, *Lampropeltis triangulum elapsoides*

Abstract

The milk snake, *Lampropeltis triangulum*, has 25 subspecies and ranges from Canada to Ecuador. The validity of some of the 25 subspecies is questionable as is the contention that *L. triangulum* forms a single species. In particular, the scarlet kingsnake, *L. t. elapsoides*, has vacillated between species and subspecies status since it was first described by Holbrook in 1838. The results of previous researchers have been split concerning whether *L. t. elapsoides* interbreeds with other *L. triangulum* where they co-occur. No previous study has used molecular phylogenetics to examine the status of the scarlet kingsnake. Therefore, I obtained 2,700 base pairs from three mitochondrial loci (16s, ND4, cytB) and analyzed them using maximum parsimony, maximum likelihood and Bayesian inference. In addition, I estimated the sequence divergence between *L. t. elapsoides* and other *Lampropeltis* species and used those values to estimate when *L. t. elapsoides* split from other groups. In all of the phylogenetic analyses, *L. t. elapsoides* forms a strongly supported monophyletic clade that is most closely related to *L. mexicana* and *L. pyromelana* and split from those groups approximately six million years ago.

None of the three *L. triangulum* subspecies that co-occur with *L. t. elapsoides* are closely related to it, and there is no evidence of interbreeding between *L. t. elapsoides* and the three *L. triangulum* subspecies over most of the areas in which they co-occur. There is evidence for hybridization in northeastern North Carolina, however, most hybrids there appear to be the result of a single hybridization event that may have occurred thousands of years ago. All of my data suggest that scarlet kingsnakes are an independently evolving lineage worthy of species recognition. Therefore, I recommend re-elevating them to species status and restoring the name *Lampropeltis elapsoides*.

Introduction

The milk snake (*Lampropeltis triangulum*; Lacepede 1788) is one of the most widespread terrestrial vertebrate species with a range extending from Canada to Ecuador (Conant and Collins 1998). Within that geographic range are 25 recognized subspecies of *L. triangulum* (Williams 1978; Williams 1994). The subspecific designations are based mainly on color pattern differences, particularly patterns on the head (Williams 1978). Figure 4-1 illustrates the ten subspecies found in the United States.

The usefulness and validity of subspecies designations has been hotly debated (Cracraft 1983; Frost and Kluge 1994; Frost et al. 1992; Mayr 1942; Mayr 1982; McKitrick and Zink 1988; Wilson and Brown Jr 1953). Some criticize subspecies designations because they are sometimes applied to groups that are not truly distinct (Zink 2004). Others criticize subspecies designations because such designations can mask an organism's evolutionary history (Burbrink et al. 2000). Thus, some subspecies designations erect separations when such separations do not exist, whereas others hide true species.

Doubts concerning the validity of the various subspecies of *L. triangulum* are not new (Conant and Collins 1998; Stebbins 2003). One area of concern is where to draw the lines between subspecies (Stebbins 2003). The major concern, however, is whether *L. triangulum* is in fact one species or actually two or more species (Conant and Collins 1998). The scarlet kingsnake (*Lampropeltis triangulum elapsoides*; Holbrook 1838), in particular, has vacillated between full species and subspecies within the past 120 years (Blanchard 1920; Blanchard 1921; Brimley 1905; Brimley

1920; Conant 1943; Cope 1893; Klauber 1948; Stejneger and Barbour 1917; Williams 1978; Williams 1994; Wright and Bishop 1916). Within that time, the organisms that we now call *L. t. elapsoides* have been classified as a separate species with no subspecies (Stejneger and Barbour 1917), a separate species with two subspecies (Blanchard 1920), a single subspecies of North American milk snake (Williams 1978), and three subspecies of North American milk snake (Cope 1893).

The most recent reviews of *L. triangulum* were conducted by Williams (1978, 1994). He included *L. t. elapsoides* as a subspecies of the milk snake based on alleged interbreeding with the other subspecies of *L. triangulum* in areas where they co-occur (Williams 1978). Conant (1943) suggested that scarlet kingsnakes interbreed with coastal plains milk snakes (formerly *L. triangulum temporalis*) on the coastal plain of northeastern North Carolina and adjacent Virginia. In addition, he concluded that *L. t. triangulum* interbreed with coastal plains milk snakes from southern New Jersey to the Delmarva peninsula. Conant based his conclusion on the fact that color patterns and scale counts of *L. triangulum* in appear to be intermediate between *L. t. elapsoides* and *L. t. temporalis* in the south and *L. t. triangulum* and *L. t. temporalis* in the north.

Williams (1978) went beyond Conant's findings and concluded that the coastal plains milk snake itself resulted from hybridization between *L. t. triangulum* and *L. t. elapsoides* and stripped *L. t. temporalis* of its subspecific status. In addition, Williams (1978) suggested that *L. t. elapsoides* interbreeds with other *L. triangulum* in Louisiana and Kentucky. However, no genetic evidence has been put forth to

support interbreeding between *L. t. elapsoides* and any other subspecies of *L. triangulum*.

Contrary to William's conclusion, Blanchard (1921) specifically stated that *L. t. elapsoides* did not appear to intergrade with *L. t. amaura* in Louisiana or with any other member of the *L. triangulum* group except for the coastal plains milk snake. In addition, Mount (1975) stated that he saw no evidence of interbreeding between *L. t. elapsoides* and any *L. triangulum* in Alabama where their ranges overlap. Most recently, Armstrong et al. (2001) specifically looked for evidence of interbreeding between *L. t. elapsoides* and *L. t. syspila* in Kentucky based on color pattern and scale counts. They concluded that there is likely no interbreeding between these subspecies in Kentucky. Their conclusion comports with the findings of Collins and Hirschfield (1964) who concluded that Kentucky *L. t. elapsoides* show no signs of interbreeding with other *L. triangulum*. As above, there are no published genetic data that would support a lack of interbreeding between *L. t. elapsoides* and other *L. triangulum*.

Are scarlet kingsnakes a subspecies of the milkshake, *L. triangulum*, or are they a separate species? Previous attempts to answer this question have been inconclusive. On the one hand, as noted above, morphological evidence based on individuals from populations east of the Appalachian Mountains appears to support the view that *L. t. elapsoides* interbreeds with other *L. triangulum*. Based on these data, scarlet kingsnakes would be considered a subspecies of *L. triangulum*. On the other hand, as also noted above, genetic evidence based on individuals from populations from west of the Appalachians appears to support reproductive isolation

64

between *L. t. elapsoides* and other *L. triangulum.* Based on these data, scarlet kingsnakes would be considered a separate species. Until now, a comprehensive molecular phylogenetic approach has not been used to determine the species status of *L. t. elapsoides*.

Here, I address three questions regarding *L. t. elapsoides* using mitochondrial DNA sequences:

1. Is *L. t. elapsoides* a subspecies of *L. triangulum* or an independent evolutionary lineage that should be considered a distinct species?

2. Which extant subspecies or species are most closely related to *L. t. elapsoides*?

3. Is there any genetic evidence that *L. t. elapsoides* interbreed with the three *L. triangulum* subspecies with which they co-occur?

Methods

Sample selection

Three subspecies of *L. triangulum* (*L. t. triangulum*, *L. t. syspila* and *L. t. amaura*) have ranges that border or overlap that of *L. t. elapsoides* (Fig. 4-1). Williams (1978) asserted that *L. t. elapsoides* intergrades with each of these subspecies where they co-occur. In addition, debate continues concerning the origin of *L. t. temporalis* (Grogan and Forester 1998). Therefore, I obtained multiple samples of all of these taxa (128 *L. t. elapsoides*, 53 *L. t. triangulum*, 12 *L. t. syspila*, 5 *L. t. amaura*, and 47 putative intergrades between *L. t. triangulum* and *L. t. elapsoides*; Appendix 1).

The *L. t. elapsoides* samples come from throughout the range of this group with special emphasis placed upon obtaining samples from the three putative intergrade zones (24 from NC & VA not including putative intergrades, 12 from KY, 9 from LA). Similarly, the *L. t. triangulum* samples come from most areas of that taxon's current distribution. However, 36 samples come from areas within or near the putative intergrade zones. Nine of the 12 *L. t. syspila* come from the putative Kentucky intergrade zone, and all of the *L. t. amaura* are from the putative Louisiana intergrade zone.

Because doubt exists concerning the status and closest extant relative of *L. t. elapsoides*, I obtained samples of all of *Lampropeltis* species that occur north of Mexico (*L. calligaster*, *L. getula*, *L. pyromelana* and *L. zonata*), as well as samples from one species found only in Mexico (*L. mexicana*; see Appendix 2). Moreover, I obtained mitochondrial DNA sequences from *Lampropeltis* species (to compare to the sequences that I generated; see below) and sequences from closely related genera (for use as outgroups) from Genbank (Appendix 3).

To avoid confusion, I refer to *L. t. triangulum*, *L. t. syspila* and putative *L. t. triangulum* x *L. t. elapsoides* hybrids from Maryland to New Jersey as eastern *L. triangulum*. In addition, I refer to any of the *L. triangulum* subspecies found within the U.S. west of the Mississippi River as western *L. triangulum*, and any *L. triangulum* subspecies from Mexico to South America as southern *L. triangulum*. *L. t. elapsoides* will always be kept separate from these groups and will be referred to by name.

66

Sequencing

Samples consisted of shed skin and blood from live snakes, and muscle and organs from dead snakes. Total genomic DNA was extracted from all samples by first incubating the tissue overnight in a solution of 2X CTAB, proteinase-K and beta-mercaptoethanol. The DNA was then isolated with chloroform and isoamyl alcohol (24:1), precipitated with ethanol, washed, dried, and re-suspended in water.

I amplified 2,700 base pairs of mitochondrial DNA from three genes (16s, ND4 and cytB) using the polymerase chain reaction. The primers used for amplification were all obtained from the literature (Table 4-1). All of the mitochondrial loci were amplified in 50 μl reactions. Each locus varied slightly in the PCR profile used, however, the basic PCR profile involved heating to 94ºC for five to seven minutes to separate the DNA followed by 45 – 50 cycles of heating to 94ºC for 30 – 40 seconds, cooling to the primer annealing temperature for 30 seconds, heating to 72ºC for 30 – 40 seconds to allow the DNA polymerase to replicate the DNA, and finally seven minutes at 72ºC to allow the DNA polymerase to complete replication. The 16s and ND4 PCR products were purified using ExoSAP-IT (USB Corp., Cleveland, OH). The cytB PCR products were purified using the QIAquick gel extraction kit (Qiagen Inc., Valencia, CA). After purification, all three loci were direct sequenced on an ABI3730 (Applied Biosystems Inc., Norwalk, CT), and I obtained 802 base pairs (bp) of *ND4* and flanking tRNAs, 1,100 bp of *cytB* and 798 bp of *16s*. Primers used for PCR and sequencing are listed in Table 4-1.   All sequences were assembled using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI) and aligned using ClustalX 1.81. Alignments were checked by eye.

Phylogenetic analyses

The aligned sequences for each of the three loci were analyzed separately using maximum parsimony (MP) and maximum likelihood (ML) as implemented in PAUP* 4.0 (Swofford 2002). Each locus was also analyzed using Bayesian inference (BI) as implemented in Mr.Bayes 3.0 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). In addition, ND4 and cytB loci were concatenated and analyzed using all three phylogenetic methods.

For the MP analyses, the starting trees were assembled by random stepwise addition and the branches were swapped using the tree-bisection-reconnection (TBR) method. The MP analyses were run with all sites weighted equally, and each MP analysis consisted of 100 heuristic searches. The reliability of the clades on the shortest trees was tested using 1,000 replicates of nonparametric bootstrapping with heuristic searches as above.

Both ML and BI require a pre-selected model of evolution that is appropriate for the sequence data. I used Modeltest 3.7 (Posada and Crandall 1998), which uses both hierarchical likelihood ratio tests and the Akaike Information Criterion (AIC), to determine the appropriate model of evolution for each locus separately and for the concatenated ND4 and cytB sequences. The 16s locus evolves at a slower rate than either of the other loci and had the least parameter-rich model of evolution, HKY85. The appropriate model of evolution for both the ND4 and cytB loci was the general time-reversible (GTR) model of evolution.

The BI analyses consisted of two runs with four chains each of Markov chain

Monte Carlo (MCMC; Hastings 1970; Metropolis et al. 1953) run with uniform prior probabilities and default chain heating values, and the program was allowed to estimate all of the parameters. All BI analyses were run until the average standard deviation of split frequencies was below 0.01 (16s – 0.009323, 750,000 generations; ND4 – 0.009106, 750,000 generations; cytB – 0.009623, 750,000 generations; concatenated ND4/cytB – 0.009417, 400,000 generations) in order to allow the program to converge on the optimal tree and parameter values. The first 25% of generations were discarded as burn-in for the estimation of parameters and for the calculation of clade reliability scores (posterior probabilities).

Pairwise Genetic Distances and Dating Divergence

The phylogenetic analyses produced distinct clades, both within *L. t. elapsoides* and between *L. t. elapsoides* and other groups. I therefore used these clades to calculate pairwise genetic distances. I calculated both intraclade and interclade distances using Kimura's two-parameter (K2P) model of evolution. The mean distance for the ND4/cytB concatenated sequences were used to estimate the divergence of the clades. This was done at the 'species' level by comparing *L. t. elapsoides* to the other *Lampropeltis* clades (*L. pyromelana*, *L. zonata*, eastern U.S. *L. triangulum* and western U.S. *L. triangulum*), and at the 'intraspecific' level by comparing the clades found within *L. t. elapsoides*. Estimates of divergence are based on two molecular clocks calculated for the ND4 and cytB loci in snakes that estimated divergence rates of 0.66% per million years (Pook et al. 2000; Wuster et al. 2002; Zamudio and Greene 1997) and 1.44% per million years (Wuster et al.

2002). The faster of the two rates was calibrated using the closing of the isthmus of Panama (Wuster et al. 2002). These estimates of divergence provide a general estimate of how long ago each of these groups diverged from one another.

Evidence for or against hybridization

To determine if two populations of organisms hybridize with each other, and to determine the direction of hybridization, I would need to use both nuclear and mitochondrial DNA with species-specific alleles. However, because I do not have species-specific nuclear alleles, I used the congruence, or lack thereof, of mtDNA sequences and taxon-specific morphological traits as the criteria for calling an individual (that does not possess an intermediate phenotype) a hybrid or pure species. For example, *L. t. elapsoides* is a small snake with rings of red, black and yellow/white and has a red snout (Fig. 4-1; Williams 1978). No other subspecies of *L. triangulum* possess that combination of traits (Williams 1978, 1994). Thus, any snakes with these characteristics should possess *L. t. elapsoides* specific mitochondrial sequences. Taxon-specific trait combinations for each of the taxa are listed in Table 4-2.

Any individuals that were intermediate for species-specific traits were categorized as hybrids regardless of the mtDNA sequences they possessed. Thus, all *L. t. temporalis* from North Carolina were categorized as hybrids because they are ringed snakes with red snouts that are larger than *L. t. elapsoides*. This method of classifying hybrids should catch the majority of first generation hybrids as well as many backcrosses because several of the size and color traits being examined are

thought to be continuous and should show intermediate phenotypes in most individuals for the first few generations after hybridization.

Results

16s Phylogenetic Analysis

I obtained and aligned 798 bp of 16s sequence for 89 *L. t. elapsoides*, 25 *L. t. triangulum* (including putative hybrids), seven *L. t. syspila*, and four *L. t. amaura*. I added *L. pyromelana* and *L. zonata* sequences generated for a separate project. There were 167 variable sites and 110 parsimony informative sites. The ML and BI trees (Fig. 4-2) had the same topology. However, the MP (Fig. 4-3) tree differed slightly. All three trees agree that *L. t. elapsoides* forms a clade with North Carolina coastal plains milk snakes within a larger clade that includes *L. pyromelana* and *L. zonata*. This clade does not include eastern *L. triangulum* (as defined above) or western *L. triangulum*. The MP analysis placed *L. t. elapsoides* as the sister clade to the clade composed of the monophyletic *L. pyromelana* and the monophyletic *L. zonata*. The ML and BI analyses created a polytomy with one branch leading to the *L. t. elapsoides* clade, a second branch leading to the *L. pyromelana* clade and the third branch leading to the *L. zonata* clade. In all three analyses, eastern *L. triangulum* and western *L. triangulum* are sister clades.

Monophyly of 16s sequences for both *L. pyromelana* and *L. zonata* is supported by strong bootstrap values, as well as high Bayesian clade reliability scores. Conversely, *L. triangulum* does not form a monophyletic group. Rather, it forms three major clades that correspond to *L. t. elapsoides* and the North Carolina *L. t.*

71

temporalis, eastern *L. triangulum* and western *L. triangulum*. Strong bootstrap values and clade reliability scores support each of these groups. Henceforth, when I refer to *L. t. elapsoides* (clades or haplotypes), I am including the North Carolina coastal plains milk snakes (see Discussion section below concerning hybridization and introgression)

Within *L. t. elapsoides*, there were 24 haplotypes among the 89 individuals. The most common haplotype accounted for 54% of individuals and was found in all parts of the geographic range. The five most common haplotypes combined accounted for 78% of individuals, while the other 19 of haplotypes represented only one individual each.

The eastern *L. triangulum* group had 19 haplotypes among 31 individuals, while the western *L. triangulum* group had only four individuals and each had a unique haplotype. Within the eastern *L. triangulum* clade, the most common haplotype was found in 35% of individuals and included *L. t. triangulum*, *L. t. syspila* and putative *L. t. triangulum* x *L. t. elapsoides* hybrids. Sixteen of the 19 haplotypes represented only one individual each.

Thus, the data from the 16s locus indicate that *L. t. elapsoides* forms a distinct clade separate from *L. triangulum*.

ND4 Phylogenetic Analysis

The ND4 sequences are composed of the ND4 locus as well as part of the neighboring tRNA loci. I obtained 802 bp sequences for 89 *L. t. elapsoides*, 45 *L. t. triangulum*, 8 *L. t. syspila*, and 4 *L. t. amaura*. I added *L. pyromelana* and *L. zonata*

sequences that I generated for a separate project. Moreover, I added *L. alterna*, *L. calligaster*, *L. getula*, *L. mexicana*, *L. pyromelana*, *L. ruthveni*, *L. zonata* and southern *L. triangulum* sequences from Genbank. The ND4 sequences had 274 variable sites and 210 parsimony informative sites. The MP analysis produced three most parsimonious trees that differed only in the branches within the *L. t. elapsoides* clade. As with the 16s data above, the ML and BI trees had the same topology (Fig, 4-4). The MP tree, however differed slightly (Fig. 4-5). All three analyses were run twice, once with the full set of haplotypes and a second time with a subset of haplotypes picked from each clade in the full analysis. There were no interclade differences between these two data sets. The smaller set of haplotypes is presented in figures 4-4 and 4-5.

All trees show *L. t. elapsoides* to be a monophyletic group. Unlike the 16s trees, a clade composed of *L. mexicana*, *L. ruthveni* and southern *L. triangulum* (*L. mexicana* clade) forms the sister group to *L. t. elapsoides*. I have no 16s sequences for *L. mexicana*, *L. ruthveni* or southern *L. triangulum* so their position in that analysis is unknown. The *L. t. elapsoides – L. mexicana* clade is sister to the *L. pyromelana – L. zonata* clade in the ML and BI analyses. This differs slightly in the MP analysis in that the *L. t. elapsoides – L. mexicana* clade is sister to the *L. zonata* clade, and the *L. pyromelana* clade is sister to that group (*L. t. elapsoides – L. mexicana* and *L. zonata*). The monophyly of each of these clades is highly supported by both nonparametric bootstrap values and clade reliability scores.

Eastern *L. triangulum* forms a monophyletic group. Western *L. triangulum*, however, does not form a monophyletic group when I add sequences from *L.*

alterna, *L. t. gentilis* and *L. t. celaenops*. Rather, western *L. triangulum* and *L. alterna* are interspersed within a highly supported clade. In addition, eastern *L. triangulum* and the western *L. triangulum* – *L. alterna* group form a clade with another *Lampropeltis* sp. (*Lampropeltis getula*) and an outgroup taxon (*Stilosoma extenuatum*). In fact, *S. extenuatum* is the sister taxon to eastern *L. triangulum* in both trees.

There are 33 ND4 haplotypes among the 89 *L. t. elapsoides* samples. Seven of those haplotypes represent more than one individual. The most common haplotype is found in 43% of the samples, and is found in each state from which I have samples.

Within the 53 eastern *L. triangulum* samples there are 19 haplotypes, while there are three haplotypes among the four western *L. triangulum* samples. As with the 16s locus, individuals from *L. t. triangulum*, *L. t. syspila* and the putative *L. t. triangulum* x *L. t. elapsoides* hybrids share some ND4 haplotypes. Within eastern *L. triangulum*, one haplotype is found in 45% on the samples. Samples with the most common haplotype come from CT, MA, MD, NJ, NY, PA and VA.

Similar to the 16s locus, the data from the ND4 locus indicate that *L. t. elapsoides* forms a distinct clade that is separate from L. triangulum.

CytB Phylogenetic Analysis

I obtained and aligned 1,100 bp of cytB sequence from 50 *L. t. elapsoides*, 13 *L. t. triangulum*, two *L. t. syspila* and two *L. t. amaura*. I added *L. alterna*, *L. calligaster*, *L. getula*, *L. mexicana*, *L. pyromelana*, *L. ruthveni*, *L. zonata*, and both western and

southern *L. triangulum* sequences from Genbank. The cytB sequences had 365 variable sites and 287 parsimony informative sites. The MP analysis produced 97 equally parsimonious trees that differed only in intraclade placement of sequences. As with the 16s and ND4 loci, the ML and BI trees (Fig. 4-6) had the same topology, while the 50% majority consensus MP tree (Fig. 4-7) was slightly different.

All three analyses placed *L. t. elapsoides* into a highly supported monophyletic group. Likewise, *L. pyromelana* and *L. zonata* each formed highly supported monophyletic groups. Unlike the ND4 analysis, *L. mexicana*, *L. ruthveni* and southern *L. triangulum* did not form a monophyletic group in the MP analysis. Rather, *L. mexicana* and one member of the southern *L. triangulum* group formed a clade and *L. ruthveni* and the other two members of the southern *L. triangulum* group formed a second clade. The MP analysis placed the *L. pyromelana* clade, *L. zonata* clade, *L. mexicana* clade and *L. ruthveni* clade together in the sister clade to *L. t. elapsoides*.

As with the other two loci, eastern *L. triangulum* forms a highly supported monophyletic clade in all of the analyses that is outside the clade that includes *L. t. elapsoides*. Rather, eastern *L. triangulum* forms a clade with western *L. triangulum*, *L. alterna* and *L. getula*. The western *L. triangulum* again forms a clade with *L. alterna*. In the MP analysis, the combined western *L. triangulum* – *L. alterna* clade is the sister to the *L. getula* clade.

The 50 *L. t. elapsoides* samples produced 28 unique haplotypes. Six haplotypes represent two or more individuals and account for 58% of the individuals. The single

most common haplotype represented 24% of the *L. t. elapsoides* samples, and was found in every state for which I have samples.

The 16 eastern *L. triangulum* individuals produced eight unique haplotypes, with the single most common haplotype representing five individuals from MA, MD, NJ and PA. Each of the two western *L. triangulum* samples had a unique haplotype.

Thus, all three mitochondrial loci agree that *L. t. elapsoides* forms a distinct clade that is separate from *L. triangulum* and all of the other taxa used in this study.

Combined Locus Phylogenetic Analysis

I concatenated the ND4 and cytB loci into 1,902 bp sequences for 49 *L. t. elapsoides*, seven eastern *L. triangulum*, and two western *L. triangulum* and ran all three phylogenetic analyses with the concatenated sequences. I combined the ND4 and cytB loci because Modeltest returned the same results for these two loci. I did not add any *Lampropeltis* species samples to this analysis from Genbank.

Consistent with all of the results above, *L. t. elapsoides* formed a strongly supported monophyletic clade in both the MP (Fig. 4-8) and the ML/BI (Fig. 4-9) analyses. Likewise, *L. pyromelana* and *L. zonata* each formed strongly supported monophyletic clades. In all three analyses (MP, ML and BI), *L. pyromelana* and *L. zonata* form a clade that is sister to the *L. t. elapsoides* clade. Also consistent with all of the above results, eastern *L. triangulum* forms a strongly supported monophyletic group clade that is closely related to the western *L. triangulum* clade.

Throughout all of the phylogenetic analyses, neither eastern *L. triangulum* nor western *L. triangulum* is ever included in the sister clade to *L. t. elapsoides*. Instead,

eastern and western *L. triangulum* forms a separate clade with *L. getula* and *S. extenuatum*.

Genetic distance and divergence dates

As indicated by the phylogenetic analyses above, *L. t. elapsoides* forms a distinct clade that is more closely related to *L. pyromelana*, *L. zonata* and *Lampropeltis* species from Mexico to South America than it is to other *L. triangulum* subspecies north of Mexico. Interestingly, however, *L. t. elapsoides* is not genetically close to any of the taxa that I analyzed (Table 4-3).

For each locus separately and for the combined ND4 and cytB loci, the interclade range of values between *L. t. elapsoides* and each of the other groups far exceeds the range of values within *L. t. elapsoides*. In addition, *L. t. elapsoides* is relatively distant from all of the taxa in the comparison. Indeed, estimates of divergence dates between the various clades (Table 4-4) suggest that *L. t. elapsoides* split off from the other taxa millions of years ago.

Hybridization between L. t. elapsoides and other taxa

Comparisons between phenotype-based subspecies designations and mitochondrial sequences for 30 individuals from Kentucky (12 *L. t. elapsoides*, 9 *L. t. triangulum* and 9 *L. t. syspila*) yielded no potential hybrids between *L. t. elapsoides* and either of the eastern *L. triangulum* with which they co-occur. There were, however, four cases of *L. t. triangulum* sharing a haplotype with *L. t. syspila*.

Similarly, there was no evidence of hybridization between *L. t. elapsoides* (nine samples) and *L. t. amaura* (four samples) from Louisiana.

The putative hybrid zone from North Carolina to New Jersey produced mixed results. All of the samples from Maryland (N = 12) and New Jersey (N = 7) were classified as coastal plains milk snakes (based on their phenotypes) and yielded eastern *L. triangulum* mitochondrial sequences as expected. Similarly, two samples from southern Virginia classified as *L. t. triangulum* yielded eastern *L. triangulum* mitochondrial sequences, and two samples of *L. t. elapsoides* from the same Virginia county (Bedford) yielded *L. t. elapsoides* mitochondrial sequences.

Conversely, 17 samples from northeastern North Carolina were classified as coastal plains milk snakes, yet all of these individuals yielded *L. t. elapsoides* associated sequences. Sixteen of these 17 samples shared a 16s haplotype with most of the *L. t. elapsoides* from North Carolina; the one individual that did not had a very closely related haplotype not found in any *L. t. elapsoides*. Ten of the 17 coastal plains milk snakes shared an ND4 haplotype that was closely related to but not found in any animals classified as *L. t. elapsoides*. In addition, three coastal plains milk snakes had unique ND4 haplotypes that were closely related to the *L. t. elapsoides* ND4 sequences. The other four coastal plains milk snakes shared the most common *L. t. elapsoides* ND4 haplotype. Only three of the individuals were haplotyped at the cytB locus. None of those sequences were found in any *L. t. elapsoides* individuals, but as with the 16s and ND4 loci the unique sequences were closely related to the sequences from *L. t. elapsoides*.

Discussion

Is *L. t. elapsoides* a subspecies of *L. triangulum*, or is it an independent evolutionary lineage deserving of species status? Three independent lines of evidence suggest that *L. t. elapsoides* is an independent evolutionary lineage deserving of species status: (1) phylogenetic analyses of mitochondrial DNA, in which *L. triangulum* were never included in the sister clade to *L. t. elapsoides*; (2) estimates of divergence times, which suggest that *L. t. elapsoides* split off from the other taxa millions of years ago, and (3) the absence of hybrids between *L. t. elapsoides* and other *L. triangulum*.   Below, I discuss each of these lines of evidence in more detail.

The phylogenetic analyses place *L. t. elapsoides* in a clade with two or three other *Lampropeltis* species and never with the eastern and western *L. triangulum* groups. This placement indicates that the *L. t. elapsoides* mtDNA sequences are more closely related to sequences from *L. pyromelana*, *L. zonata* and *L. mexicana*. These results could indicate that *L. t. elapsoides* is more closely related to those species than it is to *L. triangulum* or that there has been either convergent sequence evolution or introgression of mitochondria from one of these other species into *L. t. elapsoides*.

Convergence seems highly unlikely for two reasons. The first is that mitochondria are involved in energy production and metabolism, and the energetic needs of *L. t. elapsoides* are different from those of the other three species. For instance, L. t. elapsoides is not found in an environment that is similar to those

where *L. pyromelana*, *L. zonata* and *L. mexicana* live. Each of these three species lives in arid regions and often in higher altitude locations. Conversely, *L. t. elapsoides* is found at low elevations in the southeastern U.S., an area that is much more mesic. In addition, each of these closely related species is much larger than *L. t. elapsoides*.

The second reason that convergence seems unlikely is that each of three loci produces the same relatedness patterns. Mitochondrial loci are all linked. However, the three loci used evolve at different rates and produce products that do not directly interact with one another. In addition, the diversity of mitochondrial sequences in vertebrates indicates that these loci can have many different sequences and still produce viable products that successfully interact with the other components.

Introgression also seems highly unlikely unless it occurred millions of years ago. Introgression requires interbreeding between the donor and recipient lineages. The ranges of *L. t. elapsoides* and *L. pyromelana*, the geographically closest of the three species that group with *L. t. elapsoides* in all of the phylogenetic analyses, are separated by over one thousand kilometers. The distance between *L. t. elapsoides* and the other two species with which it groups in the phylogenetic analyses is even greater. In addition, the sequences of *L. t. elapsoides* and *L. pyromelana* diverged millions of years ago (Table 4-4). The same is true for the sequences of *L. t. elapsoides* and either *L. mexicana* or *L. zonata*.

The mitochondrial sequences of *L. t. elapsoides* indicate that they have very low intra-clade genetic divergence and relatively high inter-clade genetic divergence. In other words, *L. t. elapsoides* are very closely related to one another and distantly

related to all other *Lampropeltis* species. The intra- and inter-clade divergences of *L. t. elapsoides* are indicative of an evolutionarily independent lineage.

In addition, *L. triangulum* does not constitute a monophyletic group, regardless of the status of *L. t. elapsoides*. Both *L. getula* and *S. extenuatum* group more closely with *L. t. triangulum* than do *L. t. elapsoides* and southern *L. triangulum*, and *S. extenuatum* groups more closely with *L. t. triangulum* than does western *L. triangulum*.

In sum, based on all of the data presented above, I recommend that scarlet kingsnakes be removed from *L. triangulum* and returned to species status under their former name, *Lampropeltis elapsoides* (Holbrook 1838; Stejneger and Barbour 1917).

If *L. elapsoides* constitutes a separate species, which *Lampropeltis* species is its sister? In all of the phylogenetic analyses, *L. pyromelana* and *L. zonata* are included within the same clade as *L. elapsoides*. In addition, *L. mexicana* and southern *L. triangulum* also group closely with *L. elapsoides*. The estimated divergence dates indicate that *L. elapsoides* split from *L. mexicana* and *L. pyromelana* at approximately the same time. Thus, there does not appear to be a clear sister species to *L. elapsoides*. Rather, *L. elapsoides* appears to be most closely related to both *L. pyromelana* and *L. mexicana*, which both are more closely related to other members of *Lampropeltis*.

There may be a definitive sister species to *L. elapsoides* among the 16 southern *L. triangulum* subspecies. While I did not sample most of those groups, molecular phylogenetic analyses that include those groups should clarify the relationship of *L.*

elapsoides to the rest of the genus. However, the independence of *L. elapsoides* as an evolutionary lineage is not dependent upon which group constitutes its sister species, as interbreeding between that group and *L. elapsoides* is not possible due to the thousands of kilometers that separate them.

There was no genetic evidence of hybridization between *L. elapsoides* and *L. triangulum* in either Kentucky or Louisiana, which contradicts with the conclusions of Williams (1978) but is consistent with several other researchers (Armstrong et al. 2001; Blanchard 1921; Collins and Hirschfield 1964). In addition, samples from Virginia and Alabama where both *L. elapsoides* and *L. triangulum* occur show no evidence of hybridization based on mitochondrial sequences. The only area in which I found evidence of possible hybridization between *L. elapsoides* and *L. triangulum* was in northeastern North Carolina.

Evidence of hybridization between *L. elapsoides* and *L. triangulum* in North Carolina could appear problematic for the conclusion that *L. elapsoides* is an independent evolutionary lineage. However, a closer look at the hybridization data indicates that contemporary hybridization is most likely rare. Most of the coastal plains milk snake samples from North Carolina (13 of 17) have haplotypes at the ND4 and cytB loci that are not found in any *L. elapsoides*. In particular, 10 of the 17 North Carolina coastal plains milk snakes share one ND4 haplotype that does not occur in any *L. elapsoides* samples and could be the result of a single hybridization event that occurred over 87,000 years ago. That estimate is based on the same molecular clocks used for the inter-clade genetic divergences above.

Should possible interbreeding between scarlet kingsnakes and coastal plains milk snakes in North Carolina prevent re-elevating scarlet kingsnakes to species status? The answer depends on whether scarlet kingsnakes hybridize or intergrade with other *L. triangulum*. Hybridization is interbreeding between species, and it occurs among many currently recognized species (reviewed in Barton and Hewitt 1985). Interbreeding between subspecies is more properly called intergradation, and typically occurs wherever two subspecies come together. There is no genetic data to indicate that scarlet kingsnakes interbreed with *L. triangulum* in most areas where they co-occur. Rather, they appear to interbreed with a form of *L. triangulum* only in northeastern North Carolina, suggesting that interbreeding between scarlet kingsnakes and *L. triangulum* reflects hybridization and not intergradation. Thus, interbreeding between scarlet kingsnakes and coastal plains milk snakes in North Carolina should not preclude re-elevating scarlet kingsnakes to species status.

An additional line of evidence that indicates that hybridization between *L. elapsoides* and *L. triangulum* in northeastern North Carolina is most likely rare comes from an analysis of the color patterns of *L. elapsoides* from Kentucky, Tennessee, Virginia, and areas of North Carolina outside the range of *M fulvius* (part of the analyses done in chapter 2). That analysis shows no difference in the patterns of *L. elapsoides* from those four areas. That result would be unlikely if *L. elapsoides* were frequently hybridizing with *L. triangulum* east of the Appalachians but not hybridizing with them west of the Appalachians.

As noted in the Introduction, a major criticism of the subspecies concept is that such designations may "hide" populations that are so distinct from one another that they would be considered as evolutionarily independent populations (Burbrink et al. 2000). Such appears to be the case with scarlet kingsnakes, which differ greatly from *L. triangulum* in morphological (Williams 1978) and molecular (this study) characteristics, and, thus, should be considered as a distinct species.

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Table 4-1. Primers for PCR and Sequencing.

Locus	Primer	Sequence	Reference
ND4	ND4	TGACTACCAAAAGCTCATGTAGAAGC	(Forstner et al. 1995)
	Leu	TACTTTTACTTGGATTTGCACCA	
CytB	L14910	GACCTGTGATMTGAAAAACCAYCGTTGT	(Burbrink et al. 2000)
	L14919	AACCACCCGTTGTTATTCAACT	
	L15584	TCCCATTYCACCCATACCA	
	H16064	CTTTGGTTTACAAGAACAATGCTTTA	
	H15149	CCCTCAGAATGATATTTGTCCTCA	
	H15716	TCTGGTTTAATGTGTTG	
16s	16sf	GGCCTAAAAGCAGCCACCTA	
	16se	GGTCTGAACTCAGATCACGTAGGACT	

Table 4-2. Taxon-specific morphological traits.

Character	*L. t. elapsoides*	*L. t. triangulum*	*L. t. syspila*	*L. t. amaura*
Adult size	36 – 69 cm	66 – 132 cm	53 – 107 cm	41 – 79 cm
Mid-body Scale Rows	17 – 19	21 – 23	21	21
Snout color	Red	Gray/Brown	Gray/White/Red	Black
Ringed or Blotched	Ringed	Blotched	Blotched	Ringed

Morphological traits used to determine sub-specific identity of samples. Taken together, each gives a unique combination of traits. *L. t. temporalis* from MD and NJ have characters that match *L. t. syspila*. *L. t. temporalis* from NC do not match any of these taxa for all four characters. Data taken from Williams (1978) and Conant and Collins (1998).

Table 4-3. Minimum and maximum genetic divergences

Clade	L.t.e.	E.L.t.	W.L.t.	Lm	L.p.	L.z.
Lte						
16s	0.00 – 1.01%					
ND4	0.00 – 1.39%					
cytB	0.00 – 2.42%					
ND4/cytB	0.00 – 1.44%					
ELt						
16s	3.62 – 4.96%	0.00 – 0.88%				
ND4	8.31 – 9.49%	0.00 – 1.14%				
cytB	10.05 – 12.12%	0.00 – 2.03%				
ND4/cytB	9.56 – 10.74%	0.00 – 1.11%				
WLt						
16s	3.04 – 3.73%	3.33 – 4.15%	0.00 – 0.39%			
ND4	8.87 – 10.19%	5.21 – 6.60%	0.00 – 2.04%			
cytB	10.47 – 12.87%	6.81 – 8.45%	0.00 – 2.22%			
ND4/cytB	10.28 – 11.35%	6.60 – 7.01%				
Lm						
16s						
ND4	7.31 – 9.67%	9.34 – 10.83%	8.42 – 10.94%	0.00 – 7.74%		
cytB	7.34 – 9.23%	10.17 – 11.60%	10.69 – 11.90%	0.00 – 1.47%		
ND4/cytB						
Lp						
16s	1.86 – 3.35%	3.10 – 4.48%	3.23 – 4.34%		0.00 – 1.87%	
ND4	7.09 – 10.71%	8.85 – 10.70%	8.16 – 10.41%	8.38 – 11.21%	0.00 – 6.00%	
cytB	7.88 – 10.24%	9.20 – 10.91%	9.59 – 12.20%	7.59 – 8.40%	0.00 – 4.65%	
ND4/cytB	8.20 – 10.06%	9.51 – 10.52%	9.83 – 10.80%		0.00 – 5.14%	
Lz						
16s	3.23 – 4.72%	3.64 – 4.88%	4.03 – 4.60%		1.73 – 4.34%	0.00 – 2.14%
ND4	8.47 – 12.22%	8.45 – 10.52%	8.75 – 12.06%	7.62 – 12.39%	6.66 – 10.48%	0.00 – 5.94%
cytB	8.80 – 10.99%	10.58 – 12.00%	10.90 – 12.65%	7.66 – 9.71%	7.46 – 10.24%	0.00 – 4.65%
ND4/cytB	9.02 – 10.56%	10.01 – 11.52%	10.81 – 11.68%		8.49 – 9.58%	0.00 – 6.22%

Table 4-4. Estimated range of divergence dates

Clade	Lte	ELt	WLt	Lm	L.p.	L.z.
Lte	0.6 – 1.3 ma					
ELt	6.9 – 15.1 ma	0.3 – 0.7 ma				
WLt	7.5 – 16.3 ma	4.7 – 10.3 ma	0.8 – 1.7 ma			
Lm	5.9 – 12.8 ma	6.9 – 15.2 ma	6.7 – 14.5 ma	2.3 – 4.9 ma		
Lp	6.2 – 13.6 ma	6.9 – 15.1 ma	7.2 – 15.7 ma	6.1 – 13.3 ma	2.7 – 6.0 ma	
Lz	6.8 – 14.7 ma	7.4 – 16.1 ma	7.8 – 16.9 ma	6.4 – 13.9 ma	6.1 – 13.3 ma	2.7 – 5.8 ma

Estimates of intra- and inter-clade divergence are based upon the mean pairwise K2P distance for the concatenated ND4 and cytB loci except for the intraclade WLt estimate and all estimates involving Lm. Those estimates are based on the mean of the mean pairwise K2P ND4 distance and the mean pairwise K2P cytB distance because there were too few individuals with concatenated sequences. Both methods for computing mean pairwise K2P for the combined sequences give similar results. The upper limit to the divergence date is based upon a divergence rate of 0.66% per million years (Pook year, Zamudio and Greene year), and the lower limit is based upon a divergence rate of 1.44% per million years (Wüster et al. year). Lte = L. t. elapsoides, ELt = eastern L. triangulum, WLt = western L. triangulum, Lm = L. mexicana, Lp = L. pyromelana, and Lz = L. zonata.

Figure Legends

Figure 4-1. Ranges of different color pattern variants (subspecies) of milk snakes, *L. triangulum*, within the United States (a-j) and also of the eastern coral snake, *Micrurus fulvius* (**k**), which serves as a model in a Batesian mimicry complex with some of the *L. triangulum*. Subspecies designations of *L. triangulum* are as follows: **a** - *L. t. taylori*, **b** - *L. t. multistriata*, **c** - *L. t. gentilis*, **d** - *L. t. celaenops*, **e** - *L. t. annulata*, **f** - *L. t. amaura*, **g** - *L. t. syspila*, **h** - *L. t. elapsoides*, **i** – nee *L. t. temporalis*, **j** - *L. t. triangulum*. Range map from Conant and Collins 1998. Photos a, h, k by R. W. Van Devender; b, c, g, i, j by R. D. Bartlett; d by G. and C. Merker; e, f by M. J. Bowerman.

Figure 4-2. Maximum likelihood and Bayesian Inference phylogram of select *Lampropeltis* 16s sequences. Values above the branches are clade reliability scores generated via Bayesian inference. This phylogram and all of the subsequent phylograms and cladograms were first generated using all of the haplotypes within the data set. All of the major clades were distinct. For clarity, the data set was pared down and the analyses were re-run. All of the reduced data set analyses produced the same clades.

Figure 4-3. Maximum Parsimony 50% majority consensus cladogram of select *Lampropeltis* 16s sequences. Values above the branches are nonparametric bootstrap values from 1,000 bootstrap replicates.

Figure 4-4. Maximum likelihood and Bayesian Inference phylogram of select *Lampropeltis* ND4 sequences. Values above the branches are clade reliability scores generated via Bayesian inference.

Figure 4-5. Maximum Parsimony 50% majority concensus cladogram of select *Lampropeltis* ND4 sequences. Values above the branches are nonparametric bootstrap values from 1,000 bootstrap replicates.

Figure 4-6. Maximum likelihood and Bayesian Inference phylogram of *Lampropeltis* cytB sequences. Values above the branches are clade reliability scores generated via Bayesian inference.

Figure 4-7. Maximum Parsimony 50% majority concensus cladogram of select *Lampropeltis* cytB sequences. Values above the branches are nonparametric bootstrap values from 1,000 bootstrap replicates.

Figure 4-8. Maximum likelihood and Bayesian Inference phylogram of concatenated *Lampropeltis* ND4/cytB sequences. Values above the branches are clade reliability scores generated via Bayesian inference.

Figure 4-9. Maximum Parsimony 50% majority concensus cladogram of concatenated *Lampropeltis* ND4/cytB sequences. Values above the branches are nonparametric bootstrap values from 1,000 bootstrap replicates.

Figure 4-1

Figure 4-2

L t elapsoides A
L t elapsoides T
L t elapsoides B
L t elapsoides C
L t elapsoides D
L t elapsoides S
L t elapsoides H
L t elapsoides N
L t elapsoides R
L t elapsoides X
L t elapsoides E
L t elapsoides F
L t elapsoides G
L pyromelana A
L pyromelana N
L pyromelana C
L pyromelana D
L pyromelana U
L zonata A
L zonata D
L zonata C
L zonata B
E L triangulum U
E L triangulum K
E L triangulum H
E L triangulum I
E L triangulum M
E L triangulum N
E L triangulum B
E L triangulum C
W L triangulum A
W L triangulum B
W L triangulum C
W L triangulum D
E obsoleta

— 1 change

96

Figure 4-3

Lampropeltis 16s Maximum parsimony tree
Majority rule
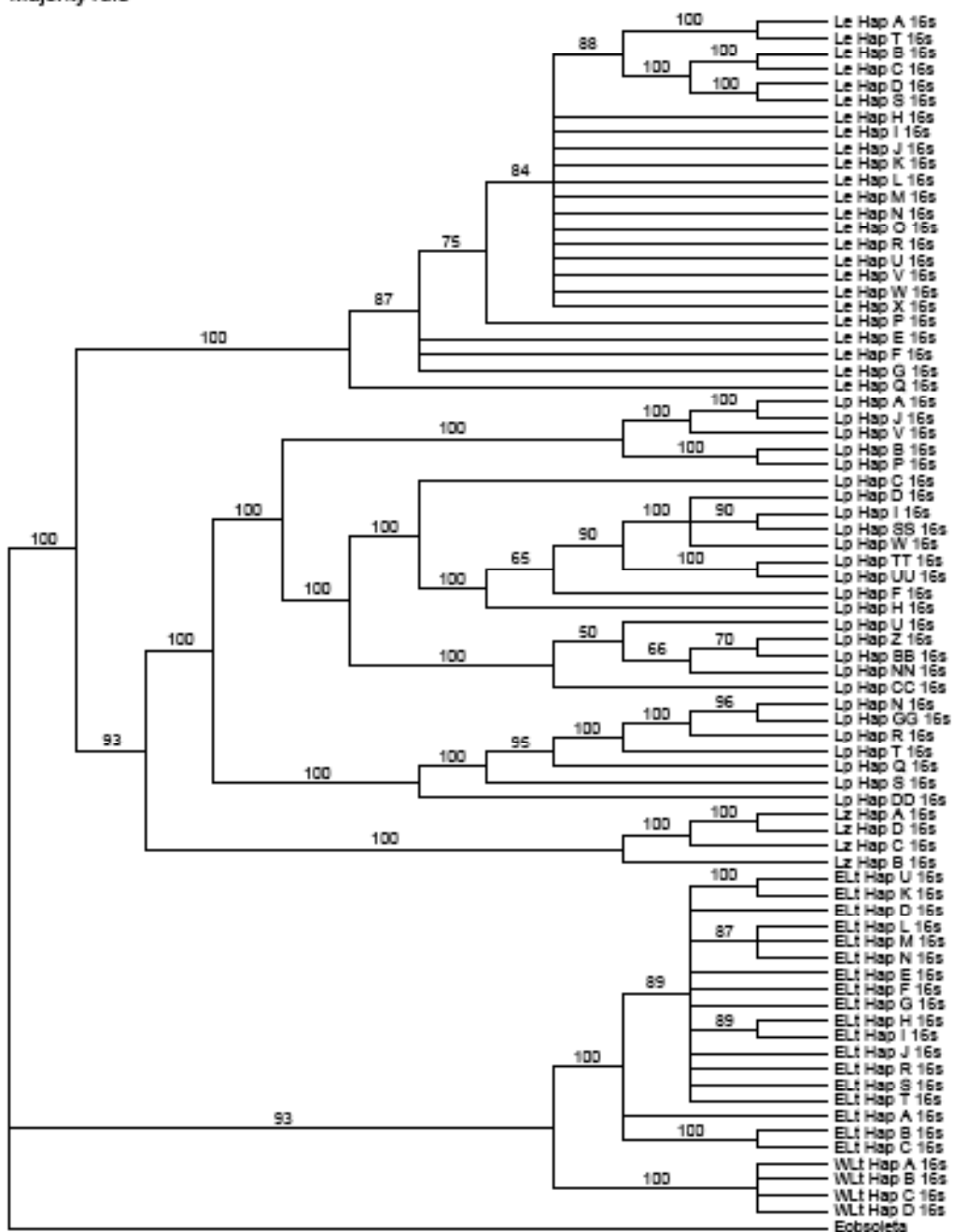
Figure 4-4

Figure 4-5

Figure 4-6

L t elapsoides A
L t elapsoides H
L t elapsoides C
L t elapsoides I
L t elapsoides L
L t elapsoides P
L t elapsoides V
L t elapsoides W
L t temporalis D
L mexicana A
L mexicana C
L mexicana B
L mexicana D
S L triangulum C
L ruthveni
S L triangulum A
S L triangulum B
L pyromelana A
L pyromelana B
L pyromelana D
L pyromelana C
L zonata A
L zonata B
L zonata C
L zonata G
L zonata D
L zonata E
L zonata F
L alterna A
L alterna B
W L triangulum E
W L triangulum D
W L triangulum C
W L triangulum A
W L triangulum B
L getula A
L getula B
L getula C
E L triangulum A
E L triangulum D
E L triangulum F
E L triangulum G
L t temporalis B
E L triangulum K
L t temporalis A
L t temporalis C
E L triangulum I
E L triangulum L
E L triangulum J
L calligaster

—— 10 changes

100

Figure 4-7

L t elapsoides A
L t elapsoides H
L t elapsoides C
L t elapsoides I
L t elapsoides L
L t elapsoides P
L t elapsoides V
L t elapsoides W
L t temporalis D
L mexicana A
L mexicana C
L mexicana B
L mexicana D
S L triangulum C
L pyromelana A
L pyromelana B
L pyromelana D
L pyromelana C
L ruthveni
S L triangulum A
S L triangulum B
L zonata A
L zonata B
L zonata C
L zonata G
L zonata D
L zonata E
L zonata F
L alterna A
L alterna B
W L triangulum E
W L triangulum D
W L triangulum C
W L triangulum A
W L triangulum B
L getula A
L getula B
L getula C
E L triangulum A
E L triangulum D
E L triangulum F
E L triangulum G
L t temporalis B
E L triangulum K
L t temporalis A
L t temporalis C
E L triangulum I
E L triangulum L
E L triangulum J
L calligaster

Figure 4-8

—— 10 changes

Figure 4-9

Chapter 5

Conclusions

In this thesis, I examined the evolution and maintenance of Batesian mimicry in a coral snake mimicry complex. Longstanding theory (reviewed in Ruxton et al. 2004) and recent experiments (Pfennig et al. 2001) both predict that attack rates by predators on Batesian mimics should depend on the relative abundance of mimics and models. Indeed, Bates (1862, p. 514) himself noted, "It may be remarkable that a mimetic species need not always be a rare one, although this is very generally the case." Yet, despite this central prediction of mimicry theory, the ratio of models to mimics may vary greatly geographically, even to the point at which there are mimics but no models (Brower and Brower 1962; Clarke and Sheppard 1975; Greene and McDiarmid 1981; Pfennig et al. In Press).

Here, I focused on a system that showed such variation in the ratio of models to mimics to ask three questions:

1. If selection is against the mimetic pattern outside the range of the model, what maintains mimics in allopatry with their model?

2. If selection acts against mimetic phenotypes in allopatry with their model, has there been a response to selection, so that mimicry begins to break down in such regions?

3. If protection from predation is frequency-dependent, what are the implications for where the best mimics are found (i.e., the mimics that most closely resemble their model)? Are the best mimics actually found where models are present but actually relatively uncommon, because, in such areas, predators are relatively unlikely to make recognition errors? Conversely, can poor mimics persist in regions where models are relatively abundant?

I addressed these questions in a coral snake mimicry system in the southeastern U.S. in which the eastern coral snake, *Micrurus fulvius*, is the model and the scarlet kingsnake, *Lampropeltis triangulum elapsoides*, is the mimic. Moreover, because *L. t. elapsoides* co-occurs in various parts of its range with three other subspecies of *L. triangulum* that vary in their degree of mimicry, I also examined whether there is frequent interbreeding between the mimic and other less mimetic subspecies. This last project led to a broader investigation of the phylogenetic relationships between *L. t. elapsoides* and the rest of the genus *Lampropeltis*.

The occurrence of mimics where there are no models is surprising given that experiments show that mimics receive no protection in such areas (Pfennig et al. 2001) and are, in fact, selected against (Pfennig et al. In Press). Therefore, using indirect DNA methods to estimate migration rates, in chapter 2 I sought to determine whether gene flow from areas where the mimetic pattern is favored (sympatry) to areas where it is disfavored (allopatry) maintains the mimetic pattern in allopatry. I found evidence of limited gene flow from sympatry to allopatry based on mitochondrial DNA (mtDNA), and low to moderate amounts of gene flow from

sympatry to allopatry based on nuclear DNA. Differences between estimates of gene flow based on mtDNA and nuclear DNA results suggest that males are responsible for carrying the mimetic phenotype into allopatry. Despite this likely male-mediated gene flow, the mimetic pattern is breaking down in allopatry and becomes less mimetic the farther one goes from the sympatry/allopatry border. Thus, although gene flow may carry mimetic phenotypes into areas where their model is absent, natural selection can break down mimetic phenotypes in such regions rapidly and thereby possibly promote evolutionary divergence between allopatric and sympatric populations.

Within sympatry, the ratio of models to mimics varies geographically with more mimics than models at the edge of the model's range and far more models than mimics deep within the range of the model. Therefore, in chapter 3, I asked whether geographic variation in the model to mimic ratio – and, thus, in the amount of protection from predation that the mimic receives – favors varying degrees of resemblance between mimic and model. I found that mimics at the edge of the model's range (where models are relatively rare) resemble their model more closely than do mimics from deep within the model's range (where models are relatively common). Furthermore, predators at the edge of the model's range discriminate between good and poor mimics and avoid only the good mimics. Thus, "imperfect" mimics may evolve in areas (e.g., deep sympatry) where the likelihood of encountering a deadly model is high, but not in areas (e.g., edge sympatry) where this likelihood is low. By contrast, natural selection should maintain only "good"

mimics (i.e., mimics that are a close phenotypic match to the local model) in areas, such as edge of the model's range, where models are relatively rare.

Gene flow resulting from hybridization with less mimetic species could also affect the evolution of a mimetic phenotype. In chapter 4, I used genetic markers to determine if there is any evidence of interbreeding between *L. t. elapsoides* and any of the three *L. triangulum* subspecies with which it co-occurs. Surprisingly, analysis of the sequences of three mitochondrial loci (16s, ND4, cytB) revealed that *L. t. elapsoides* diverged from *L triangulum* and all other *Lampropeltis* spp. at least six million years ago. Moreover, I found no evidence of interbreeding between *L. t. elapsoides* and *L. triangulum* over the vast majority of its range. However, there is a small area of northeastern North Carolina in which a single hybridization event may have occurred thousands of years ago. Because there is no evidence for widespread or frequent contemporary hybridization, I therefore recommend that the scarlet kingsnake be re-elevated to species status and resume use of the name *L. elapsoides*.

Taken together, the results of this thesis and of two recent studies (Pfennig et al. 2001; Pfennig et al. In Press) strongly implicate Batesian mimicry in the evolution of the color pattern of the scarlet kingsnake. Specifically, in accord with Batesian mimicry theory, these studies demonstrate that (1) mimetic *L. elapsoides* receive protection from predation in areas where their coral snake models occur (Pfennig et al. 2001); (2) the mimetic pattern is selected against in areas outside the range of the model where mimics are relatively common (Pfennig et al. In Press); (3) gene flow maintains the mimetic pattern in allopatry (chapter 2); (4) but, despite such gene

flow, natural selection rapidly (< 10,000 years) breaks down the mimetic pattern outside the range of the model (chapter 2); (5) resemblance between mimics and models varies depending on the relative abundance of the model (chapter 3); and (6) the breakdown of the pattern outside the range of the model is not due to gene flow from non-mimetic forms of *L. triangulum* (chapter 4).

The occurrence of mimics outside the range of their models has been used to dispute whether this and other systems are in fact examples of mimicry (Brattstrom 1955; Grobman 1978). Now we have a better understanding of why mimics occur in allopatry. Gene flow from sympatry (where the mimetic pattern is favored) into allopatry (where the mimetic pattern is selected against) is carrying alleles for the mimetic pattern into such areas (chapter 2). This process may have begun about 10,000 years ago after the last glacial maximum when both females and males expanded out beyond the range of their coral snake model. More recent gene flow appears to be mainly male-mediated (chapter 2). Moreover, because the best mimics occur at the edge of the model's range (chapter 3), a potentially short migration distance is needed to carry color pattern genes of the best mimics into allopatry.

Why do predators outside the range of the coral snake not extirpate the brightly colored, harmless migrants? Two possible reasons come from the receiver psychology of the predators. First, mimics in allopatry may be subject to apostatic (i.e., frequency dependent) selection. When mimics are rare, predators ignore them and selection against the pattern is relaxed. However, when mimics become common (perhaps because of migration from sympatry) predators may learn or

108

evolve a preference for brightly colored, harmless prey. Thus, selection against the mimetic pattern is elevated until the mimics again become rare. In that way, apostatic selection may promote temporal oscillations in the abundance of mimics in allopatry, where there are periods in which mimics become common in allopatry (due to gene flow and initially weak selection against rare mimetic phenotypes), and then become rare again (as predators learn or evolve a preference for initially more common mimetic phenotypes), followed by becoming rare once again, and so on.

The second possibility for why predators in allopatry do not extirpate the brightly colored, harmless migrants is that predators in allopatric areas may initially exhibit neophobia, such that they avoid attacking novel prey. Similar to apostatic selection, neophobia would allow populations of mimics to grow initially. However, once predators learn or evolve a preference for brightly colored, harmless prey, selection would not be relaxed due to decreases in population size. Therefore, neophobia would be more likely to cause the eventual elimination of individuals with mimetic phenotypes from allopatry and only subsequent migration from sympatry would restore the mimetic pattern to allopatry.

The evolution and maintenance of Batesian mimicry is thus complex. Mimic behavior (sex-biased dispersal), receiver psychology (apostatic predation or neophobia by predators), and model ecology (factors determining the relative abundance of models) all affect the probability of a mimic being attacked, and, thus, the evolution and maintenance of mimicry. The complexity that I detected in the coral snake mimicry system likely extends to other systems as well, particularly those in which models are highly dangerous.

Finally, a number of issues regarding the evolution and maintenance of Batesian mimicry in kingsnakes require further clarification. Five in particular promise to be fruitful areas for future research:

(1) What is the relative importance of mimic behavior and receiver psychology on the maintenance of mimics in allopatry with their model?

Allopatric mimics do not occur in many mimicry systems, and their presence in the *M. fulvius – L. elapsoides* system deserves further exploration. I argued above that both mimic behavior (sex-biased dispersal) and receiver psychology (apostatic predation) promote the maintenance of mimics in allopatry with their model. What is the relative importance of these two factors in maintaining allopatric mimics?

(2) Can Batesian mimicry promote the origin of new species?

The finding that mimics experience higher than random predation in allopatry (Pfennig et al. in press) contrasts markedly with the pattern of predator avoidance of mimics observed in sympatry (Pfennig et al. 2001). Whereas selection should favor the breakdown of this phenotype in allopatry, selection should favor the maintenance (or enhancement) of the mimetic phenotype in sympatry. This divergent pattern of natural selection between allopatry and sympatry could contribute to divergence between sympatric and allopatric populations. If, as phenotypes become less mimetic in allopatry, individuals from one population are more often preyed upon in the alternative population, then gene flow between allopatry and sympatry would be selectively disfavored (chapter 2) and reproductive isolation might thereby result. In

this way, Batesian mimicry may promote the origin of new species (see also Jiggins et al. 2004; Jiggins et al. 2001; Naisbit et al. 2001; Naisbit et al. 2003; Servedio 2004).

(3) What is the significance of geographic variation in mimicry systems?

The finding of geographic variation in the resemblance between models and mimics is not trivial. Rather, it suggests that there may be geographic variation in all of the components that combine to produce the protection that mimics receive and that selection on the models, mimics and predators may dramatically differ within any mimicry system. For example, the low model to mimic ratio at the edge of the coral snake's range appears to have led to the mimic evolving to more closely resemble the model. However, a predator is more likely to encounter a mimic at the edge of sympatry, and thus predators may lose their aversion to the aposematic pattern of the model. Coral snakes could potentially prevent that by increasing the cost a predator pays for mistakenly attacking a model. Thus, coral snakes may differ geographically in venom potency based on geographic variation in other components of the mimicry system.

(4) What is the genetic basis of the mimetic pattern?

Researchers of butterfly mimicry have learned a great deal about the genes that underlie the mimetic pattern (Mallet 1989; Scriber et al. 1996; Sheppard et al. 1985; Tobler et al. 2005). Their research suggests that mimetic phenotypes are regulated by few genetic loci. No parallel studies have been done within snake mimicry

111

systems. Yet, snake breeders are numerous and often mate snakes specifically based on color pattern characters, and thus a great deal of data already exists for examining this topic. In particular, *Lampropeltis* species are favorites among breeders and many different breeders have developed true-breeding lines for different color pattern characters. Such data could be used to determine if mimetic patterns are regulated by just a few or many genetic loci. Moreover, breeding experiments could be used to determine the extent to which the environment modifies the development of the mimetic pattern (i.e., to determine if snake color patterns are canalized or subject to phenotypic plasticity).

(5) What are the phylogenetic relationships within Lampropeltis?

The phylogenetic analyses in chapter 4 clearly show that the relationships between members of the genus *Lampropelis* are far from resolved. Currently there are eight species within the genus (*L. alterna, L. calligaster, L. getula, L. mexicana, L. pyromelana, L. ruthveni, L. triangulum,* and *L. zonata*) and the elevation of *L. elapsoides* to species status would make that nine species. However, the status of the subspecies of *L. triangulum* are questionable. In addition, *L. alterna* mtDNA sequences are indistinguishable from those of *L. triangulum* subspecies found west of the Mississippi River. Thus, additional phylogenetic studies should make use of both mtDNA and nuclear DNA to resolve the relationships within this genus.

Finally, the origin of the coastal plains milk snake (formerly *L. t. temporalis*) needs to be resolved. Williams (1978) claimed that *L. t. temporalis* resulted from hybridization between *L. t. triangulum* and *L. t. elapsoides*. Yet, coastal plains milk

snakes near the center of their distribution look much like *L. t. syspila*, and thus may represent a disjunct portion of that subspecies that subsequently interbred with *L. t. triangulum* in the northern part of their range and *L. elapsoides* in the southern part of their range.

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Appendices

Appendix 1 – Samples for molecular analyses

L. elapsoides and NE North Carolina hybrid samples

Sample number	Collection location	Source	Sample number	Collection location	Source
AL-1	Cleburne Co., AL	J. Apodaca	KY-6	Lyon Co., KY	E. Zimmerer
AL-2	Baldwin Co., AL	CM	KY-7	Trigg Co., KY	E. Zimmerer
AL-3	Mobile Co., AL	CM	KY-8	Trigg Co., KY	E. Zimmerer
FL-1	Franklin Co., FL	J.Collins	KY-10	Lyon Co., KY	E. Zimmerer
FL-2	Pinellas Co., FL	M.Kenderdine	KY-11	Lyon Co., KY	E. Zimmerer
FL-3	Lee Co., FL	M.Kenderdine	KY-12	Lyon Co., KY	P. Peak
FL-4	Brevard Co., FL	B. Grout	LA-1	St. Helena Parrish, LA	CM
FL-5	Pasco Co., FL	M.Kenderdine	LA-2	St. Helena Parrish, LA	CM
FL-6	Seminole Co., FL	M.Kinderdine	LA-3	St. Helena Parrish, LA	CM
FL-7	Brevard Co., FL	M.Kenderdine	LA-4	St. Helena Parrish, LA	CM

FL-8	Hillsborough Co, FL	M.Kenderdine	LA-5	St. Helena Parrish, LA	CM
FL-9	Baker Co., FL	T.Davis	LA-6	St. Helena Parrish, LA	CM
FL-10	Baker Co., FL	T.Davis	LA-7	St. Tammany, LA	CM
FL-11	Brevard Co., FL	B.Grout	LA-8	St. Tammany, LA	LSUMNS
FL-12	Manatee Co., FL	G.Binczik	LA-9	St. Tammany, LA	LSUMNS
FL-13	Levy Co., FL	G.Binczik	MS-1	Perry Co., MS	W. Grogan
FL-14	Leon Co., FL	G.Binczik	MS-2	Perry Co., MS	W. Grogan
FL-15	Manatee Co., FL	G.Binczik	MS-3	Stone Co., MS	LSUMNS
FL-16	Wakulla Co., FL	G.Binczik	MS-4	Wilkinson Co., MS	LSUMNS
FL-17	Leon Co., FL	G.Binczik	MS-5	Perry Co., MS	E. Zimmerer
FL-18	Leon Co., FL	G.Binczik	MS-6	Greene Co., MS	CM
FL-19	Levy Co., FL	G.Binczik	MS-7	Hancock Co., MS	CM
FL-20	Alachua Co., FL	G.Binczik	MS-8	Hinds Co., MS	T. Vandeventer

FL-21	Liberty Co., FL	G.Binczik	MS-9	Hinds Co., MS	T. Vandeventer
FL-22	Levy Co., FL	G.Binczik	NC-1	Scotland Co., NC	D. Pfennig
FL-23	Levy Co., FL	G.Binczik	NC-2	Scotland Co., NC	D. Pfennig
FL-24	Levy Co., FL	G.Binczik	NC-3	Scotland Co., NC	D. Pfennig
FL-25	Manatee Co., FL	G.Binczik	NC-4	Scotland Co., NC	W. Van Devender
FL-26	Leon Co., FL	G.Binczik	NC-5	Scotland Co., NC	T. Thorpe
FL-27	Leon Co., FL	W. Grogan	NC-6	Randolph Co., NC	M. Lewis
FL-28	Brevard Co., FL	M.Lewis	NC-7	Bladen Co., NC	D. Lockwood
FL-29	St. Johns Co., FL	M. Frase	NC-8	Bladen Co., NC	D. Lockwood
FL-30	Duval Co., FL	M. Frase	NC-9	Hyde Co., NC	J. Sliwinski
FL-31	St. Johns Co., FL	M. Frase	NC-10	New Hanover Co., NC	S. Allison
FL-32	Levy Co., FL	FMNH	NC-11	New Hanover Co., NC	S. Allison

FL-33	Liberty Co., FL	FMNH	NC-12	New Hanover Co., NC	S. Allison
FL-34	Alachua Co., FL	FMNH	NC-13	Randolph Co., NC	M. Lewis
FL-35	Alachua Co., FL	FMNH	NC-14	Tyrell Co., NC	B. Johnson
FL-36	Polk Co., FL	FMNH	NC-15	Tyrell Co., NC	B. Johnson
FL-37	Indian River Co., FL	FMNH	NC-16	Pender Co., NC	K. Farmer
FL-38	Columbia Co., FL	E. Zimmerer	NC-17	Pender Co., NC	K. Farmer
FL-39	Columbia Co., FL	E.Zimmerer	NC-18	Pamlico Co., NC	S. M. Quint
FL-40	Citrus Co., FL	CAS	NC-19	Hyde Co., NC	E. Zimmerer
FL-41	Gulf Co., FL	CAS	NC-20	Hyde Co., NC	E. Zimmerer
FL-42	Santa Rosa Co., FL	LSUMNS	NC-21	Currituck Co., NC	E. Zimmerer
FL-43	Santa Rosa Co., FL	LSUMNS	NC-22	Tyrell Co., NC	E. Zimmerer
FL-44	Santa Rosa Co., FL	LSUMNS	NC-23	Pender Co.,	D. Herman
FL-45	Columbia Co., FL	E. Zimmerer	NC-24	Randolph Co., NC	NCSM

Sample number	Collection location	Source	Sample number	Collection location	Source
FL-46	Columbia Co., FL	E. Zimmerer	NC-25	Scotland Co., NC	NCSM
FL-47	Columbia Co., FL	E.Zimmerer	NC-26	Tyrell Co., NC	P. Weaver
FL-48	Not assigned		NC-27	Tyrell Co., NC	P. Weaver
FL-49	Baker Co., FL	E.Zimmerer	NC-28	Tyrell Co., NC	P. Weaver
FL-50	Manatee Co., FL	FMNH	NC-29	Tyrell Co., NC	P. Weaver
FL-51	Manatee Co., FL	FMNH	NC-30	Tyrell Co., NC	P. Weaver
FL-52	Manatee Co., FL	FMNH	NC-31	Tyrell Co., NC	P. Weaver
FL-53	Manatee Co., FL	FMNH	NC-32	Carteret Co., NC	S. M. Quint
FL-54	Manatee Co., FL	FMNH	NC-33	Hyde Co., NC	S. M. Quint
FL-55	Manatee Co., FL	FMNH	NC-34	Montgomery Co., NC	NCSM
FL-56	Manatee Co., FL	FMNH	NC-35	Pamlico Co., NC	NCSM
FL-57	Manatee Co., FL	FMNH	NC-36	Pender Co., NC	NCSM
FL-58	Manatee Co., FL	FMNH	SC-1	Aiken Co., SC	J. Hohman
Sample number	Collection location	Source	Sample number	Collection location	Source
FL-59	Manatee Co., FL	FMNH	SC-2	Berkeley Co., SC	W. Van Devender

GA-1	Liberty Co., GA	D. Stevenson	SC-3	Charleston Co., SC	W. Van Devender
GA-2	Liberty Co., GA	D. Stevenson	SC-4	Aiken Co., SC	M. Green
GA-3	Liberty Co., GA	D. Stevenson	SC-5	Jasper Co., SC	Zoo Atlanta
GA-4	Liberty Co., GA	D. Stevenson	SC-6	Jasper Co., SC	Zoo Atlanta
GA-5	Bryan Co., GA	D. Stevenson	SC-7	Jasper Co., SC	M. Khan
GA-6	Liberty Co., GA	D. Stevenson	SC-8	Berkeley Co., SC	B. Moulis
GA-7	Evans Co., GA	B. Moulis	SC-9	Berkeley Co., SC	B. Moulis
GA-8	Bryan Co., GA	B. Moulis	SC-10	Berkeley Co., SC	B. Moulis
GA-9	Chatham Co., GA	B. Moulis	SC-11	Oconee Co., SC	C. Putnam
GA-10	Bryan Co., GA	B. Moulis	SC-12	Aiken Co., SC	P. Peak
GA-11	Charlton Co., GA	S. M. Quint	SC-13	Aiken Co., SC	P. Peak

GA-12	Charlton Co., GA	CM	SC-14	Berkeley Co., SC	E. Zimmerer
KY-1	Lyon Co., KY	J. Young	SC-15	Chesterfield Co., SC	J. Camper
KY-3	Lyon Co., KY	E. Zimmerer	TN-1	Shelby Co., TN	CM
KY-4	Trigg Co., KY	E. Zimmerer	VA-1	Chesapeake Co., VA	E. Zimmerer
KY-5	Lyon Co., KY	E. Zimmerer	VA-2	Bedford Co., VA	G. Woodie

L. triangulum samples

Sample number	Collection location	Source	Sample number	Collection location	Source
LA-1	Ascension, LA	LSUMNS	LTT41	Fayette Co., KY	P.Peak
LA-2	Calcasieu, LA	LSUMNS	LTT42	Unknown Co., KY	P.Peak
LA-3	Iberville, LA	LSUMNS	LTT43	Unknown Co., KY	P.Peak
LA-4	Iberville, LA	LSUMNS	LTT44	Unknown Co., KY	P.Peak

LA-5	Jefferson, LA	LSUMNS	LTT45	Unknown Co., KY	P.Peak
LA-6	Jefferson, LA	LSUMNS	LTT46	Unknown Co., KY	P.Peak
LA-7	La Salle, LA	LSUMNS	LTT47	Houston Co., MN	E.Zimmerer
LTS1	Trigg Co., KY	E.Zimmerer	LTT48	Houston Co., MN	E.Zimmerer
LTS10	Unknown Co., MO	E.Zimmerer	LTT49	Erie Co., NY	E.Zimmerer
LTS11	Dekalb, Co., AL	E.Zimmerer	LTT5	Hardin Co., KY	R.Todd
LTS12	Trigg Co., KY	E.Zimmerer	LTT50	Erie Co., NY	E.Zimmerer
LTS17	Trigg Co., KY	E.Zimmerer	LTT51	Orange Co., NY	E.Zimmerer
LTS2	Trigg Co., KY	E.Zimmerer	LTT52	Mineral Co., WV	E.Zimmerer
LTS3	Trigg Co., KY	P.Peak	LTT53	Bullitt Co., KY	E.Zimmerer
LTS4	Hickman Co., KY	P.Peak	LTT54	Bullitt Co., KY	E.Zimmerer
LTS5	Hickman Co., KY	P.Peak	LTT55	Bedford Co., VA	G.Woodie

				Hickman	
LTS6	Trigg Co., KY	E.Zimmerer	LTT6	Co., KY	M.Gumbert
LTS7	Lake Co., TN	E.Zimmerer	LTT7	Hickman Co., KY	?
LTS8	Calloway Co., KY	E.Zimmerer	LTT8	Hickman Co., KY	M.Gumbert
LTS9	Trigg Co., KY	E.Zimmerer	LTT9	Hickman Co., KY	J.Collins
LTT1	Hardin Co., KY	R.Todd	MD-1	Unknown Co., MD	J.White
LTT10	Menifee Co., KY	J.Collins	MD-10	Calvert Co., MD	E.Zimmerer
LTT11	Menifee Co., KY	Settles & Gumbert	MD-11	St. Mary's Co., MD	E.Zimmerer
LTT12	Obion Co., TN	USFWS	MD-12	St. Mary's Co., MD	E.Zimmerer
LTT13	Nantucket Co., MA	J.Schofield	MD-13	St. Mary's Co., MD	E.Zimmerer
LTT14	Ile Perrot, Quebec	M.Bouchard	MD-14	Wicomico Co., MD	E.Zimmerer
LTT15	Erie Co., PA	B.Gray	MD-15	Wicomico Co., MD	E.Zimmerer

LTT16	Erie Co., PA	B.Gray	MD-16	Wicomico Co., MD	E.Zimmerer
LTT17	Chester Co., PA	L.Luciano	MD-17	Wicomico Co., MD	W.Grogan
LTT18	Chester Co., PA	L.Luciano	MD-18	Worcester Co., MD	
LTT19	Susquahanna Co., PA	T.Davis	MD-2	Calvert Co., MD	J.Sliwinski
LTT2	Hardin Co., KY	R.Todd	MD-3	Calvert Co., MD	W.Grogan
LTT20	Ocean Co., NJ	T.Davis	MD-4	Calvert Co., MD	B.Johnson
LTT21	Bath Co., KY	T.Davis	MD-5	St. Mary's Co., MD	B.Johnson
LTT22	Anderson Co., KY	T.Davis	MD-6	St. Mary's Co., MD	D.Allen
LTT23	Anderson Co., KY	T.Davis	MD-7	St. Mary's Co., MD	D.Allen
LTT24	Kent Co., MI	J.Sliwinski	MD-8	St. Mary's Co., MD	D.Allen
LTT25	Unknown Co., VA	J.Sliwinski	MD-9	St. Mary's Co., MD	D.Allen

LTT26	Pike Co., PA	J.Sliwinski	NJ	Ocean Co., NJ	S.Brown
LTT27	Pike Co., PA	J.Sliwinski	NJ-1	Ocean Co., NJ	B.Johnson
LTT28	Bucks Co., PA	J.Sliwinski	NJ-10	Ocean Co., NJ	E.Zimmerer
LTT29	Laporte Co., IN	C.Cumings	NJ-2	Ocean Co., NJ	B.Johnson
LTT3	Hardin Co,. KY	R.Todd	NJ-3	Ocean Co., NJ	B.Johnson
LTT30	New Haven Co., CT	C.Annicelli	NJ-4	Ocean Co., NJ	TJ Hilliard
LTT31	Harrison Co., KY	P.Peak	NJ-5	Atlantic Co., NJ	E.Zimmerer
LTT32	Harrison Co., KY	P.Peak	NJ-6	Atlantic Co., NJ	E.Zimmerer
LTT33	Harrison Co., KY	P.Peak	NJ-7	Atlantic Co., NJ	E.Zimmerer
LTT34	Hardin Co., KY	P.Peak	NJ-8	Ocean Co., NJ	E.Zimmerer
LTT35	Hardin Co., KY	P.Peak	NJ-9	Ocean Co., NJ	E.Zimmerer

LTT36	Bullitt Co., KY	P.Peak	SC11	Oconee Co., SC	C.Putnam
LTT37	Bullitt Co., KY	P.Peak	VA-2	Bedford Co., VA	G.Woodie
LTT38	Jefferson Co., KY	P.Peak			
LTT39	Madison Co., KY	P.Peak			
LTT4	Hardin Co., KY	R.Todd			
LTT40	Trimble Co., KY	P.Peak			

CAS = California Academy of Sciences, CM = Carnegie Museum of Natural History, FMNH = Florida Museum of Natural History; LSUMNS = Louisiana State University Museum of Natural Science; NCSM = North Carolina Museum of Natural Sciences.

Appendix 4 – Samples for morphological analyses

L. elapsoides samples

Sample number	Museum	State/County Collected
USNM 23807	Smithsonian National Museum of Natural History	Wake Co., NC
USNM 8957	Smithsonian National Museum of Natural History	Kinston, NC
USNM 192954	Smithsonian National Museum of Natural History	Brunswick Co., NC
USNM 234446	Smithsonian National Museum of Natural History	Hyde Co., NC
USNM 325175	Smithsonian National Museum of Natural History	Carteret Co., NC
USNM 325176	Smithsonian National Museum of Natural History	Carteret Co., NC
USNM 345497	Smithsonian National Museum of Natural History	Hyde Co., NC
USNM 345498	Smithsonian National Museum of Natural History	Brunswick Co., NC
NCSM 30065	North Carolina Natural History Museum	Randolph Co., NC

NCSM 25818	North Carolina Natural History Museum	Montgomery Co., NC
NCSM 26272	North Carolina Natural History Museum	Montgomery Co., NC
NCSM 25717	North Carolina Natural History Museum	Richmond Co., NC
NCSM 25823	North Carolina Natural History Museum	Currituck Co., NC
NCSM 17034	North Carolina Natural History Museum	Scotland Co., NC
NCSM 60054	North Carolina Natural History Museum	Pender Co., NC
NCSM 15005	North Carolina Natural History Museum	Bladen Co., NC
NCSM 21384	North Carolina Natural History Museum	Onslow Co., NC
NCSM 21385	North Carolina Natural History Museum	Onslow Co., NC
NCSM 21386	North Carolina Natural History Museum	Onslow Co., NC
NCSM 20610	North Carolina Natural History Museum	Brunswick Co., NC

NCSM 21098	North Carolina Natural History Museum	Onslow Co., NC
NCSM 20580	North Carolina Natural History Museum	Onslow Co., NC
NCSM 21881	North Carolina Natural History Museum	Brunswick Co., NC
NCSM 18774	North Carolina Natural History Museum	Bladen Co., NC
NCSM 16594	North Carolina Natural History Museum	Pender Co., NC
NCSM 14929	North Carolina Natural History Museum	Columbus Co., NC
NCSM 16697	North Carolina Natural History Museum	Pender Co., NC
NCSM 14930	North Carolina Natural History Museum	Columbus Co., NC
NCSM 14928	North Carolina Natural History Museum	Columbus Co., NC
NCSM 20168	North Carolina Natural History Museum	Brunswick Co., NC
NCSM 17913	North Carolina Natural History Museum	Brunswick Co., NC

NCSM 12536	North Carolina Natural History Museum	Pender Co., NC
NCSM 10066	North Carolina Natural History Museum	Brunswick Co., NC
NCSM 13052	North Carolina Natural History Museum	Bladen Co., NC
NCSM 10228	North Carolina Natural History Museum	Pender Co., NC
NCSM 9896	North Carolina Natural History Museum	New Hanover Co., NC
NCSM 12496	North Carolina Natural History Museum	Brunswick Co., NC
NCSM 23319	North Carolina Natural History Museum	Hoke Co., NC
NCSM 28858	North Carolina Natural History Museum	Harnett Co., NC
NCSM 28546	North Carolina Natural History Museum	Scotland Co., NC
NCSM 30012	North Carolina Natural History Museum	Moore Co., NC
NCSM 25182	North Carolina Natural History Museum	Onslow Co., NC

NCSM 23698	North Carolina Natural History Museum	Bladen Co., NC
NCSM 9513	North Carolina Natural History Museum	Carteret Co., NC
NCSM 9515	North Carolina Natural History Museum	Carteret Co., NC
NCSM 9516	North Carolina Natural History Museum	Carteret Co., NC
NCSM 9512	North Carolina Natural History Museum	Carteret Co., NC
NCSM 32065	North Carolina Natural History Museum	Chowan Co., NC
NCSM 20256	North Carolina Natural History Museum	Carteret Co., NC
NCSM 17031	North Carolina Natural History Museum	Richmond Co., NC
NCSM 21880	North Carolina Natural History Museum	Jones Co., NC
NCSM 16701	North Carolina Natural History Museum	Richmond Co., NC
NCSM 20972	North Carolina Natural History Museum	Randolph Co., NC

NCSM 19781	North Carolina Natural History Museum	Montgomery Co., NC
NCSM 20209	North Carolina Natural History Museum	Montgomery Co., NC
NCSM 14956	North Carolina Natural History Museum	Jones Co., NC
NCSM 15087	North Carolina Natural History Museum	Carteret Co., NC
NCSM 14860	North Carolina Natural History Museum	Carteret Co., NC
NCSM 14955	North Carolina Natural History Museum	Jones Co., NC
NCSM 15088	North Carolina Natural History Museum	Carteret Co., NC
NCSM 15037	North Carolina Natural History Museum	Tyrell Co., NC
NCSM 9252	North Carolina Natural History Museum	Carteret Co., NC
NCSM 12525	North Carolina Natural History Museum	Beaufort Co., NC
NCSM 9253	North Carolina Natural History Museum	Carteret Co., NC

NCSM 12002	North Carolina Natural History Museum	Carteret Co., NC
NCSM 11197	North Carolina Natural History Museum	Tyrell Co., NC
NCSM 9222	North Carolina Natural History Museum	Hyde Co., NC
NCSM 14586	North Carolina Natural History Museum	Hyde Co., NC
NCSM 11946	North Carolina Natural History Museum	Craven Co., NC
NCSM 9273	North Carolina Natural History Museum	Jones Co., NC
NCSM 9259	North Carolina Natural History Museum	Carteret Co., NC
NCSM 10280	North Carolina Natural History Museum	Hyde Co., NC
NCSM 9540	North Carolina Natural History Museum	Stanly Co., NC
NCSM 15050	North Carolina Natural History Museum	Montgomery Co., NC
NCSM 9539	North Carolina Natural History Museum	Stanly Co., NC

NCSM 7997	North Carolina Natural History Museum	Stanly Co., NC
NCSM 16867	North Carolina Natural History Museum	Montgomery Co., NC
NCSM 1898	North Carolina Natural History Museum	Montgomery Co., NC
NCSM 22736	North Carolina Natural History Museum	Montgomery Co., NC
NCSM 25400	North Carolina Natural History Museum	Randolph Co., NC
NCSM 24185	North Carolina Natural History Museum	Richmond Co., NC
NCSM 23699	North Carolina Natural History Museum	Bladen Co., NC
NCSM 23075	North Carolina Natural History Museum	Bladen Co., NC
NCSM 23858	North Carolina Natural History Museum	Brunswick Co., NC
NCSM 41283	North Carolina Natural History Museum	Moore Co., NC
NCSM 62457	North Carolina Natural History Museum	Moore Co., NC

NCSM 44105	North Carolina Natural History Museum	Moore Co., NC
NCSM 33817	North Carolina Natural History Museum	Scotland Co., NC
NCSM 33818	North Carolina Natural History Museum	Brunswick Co., NC
NCSM 33816	North Carolina Natural History Museum	Brunswick Co., NC
MCZ R 127729	Harvard Museum of Comparative Zoology	Franklin Co., TN
MCZ 14007	Harvard Museum of Comparative Zoology	Knox Co., TN
MCZ 60851	Harvard Museum of Comparative Zoology	Franklin Co, TN
NCSM 27402	North Carolina Natural History Museum	Berkeley Co., SC
NCSM 46295	North Carolina Natural History Museum	Sumter Co., SC
NCSM 27045	North Carolina Natural History Museum	Berkeley Co., SC
NCSM 27403	North Carolina Natural History Museum	Berkeley Co., SC

NCSM 33822	North Carolina Natural History Museum	Charleston Co., SC
NCSM 46293	North Carolina Natural History Museum	Berkeley Co., SC
MCZ R 177904	Harvard Museum of Comparative Zoology	Jasper Co., SC
USNM 2384	Smithsonian National Museum of Natural History	Charleston, SC
USNM 218903	Smithsonian National Museum of Natural History	Jasper Co., SC
USNM 218898	Smithsonian National Museum of Natural History	Jasper Co., SC
USNM 218902	Smithsonian National Museum of Natural History	Jasper Co., SC
USNM 218899	Smithsonian National Museum of Natural History	Jasper Co., SC
USNM 218900	Smithsonian National Museum of Natural History	Jasper Co., SC
USNM 218901	Smithsonian National Museum of Natural History	Jasper Co., SC
USNM 218904	Smithsonian National Museum of Natural History	Jasper Co., SC

USNM 267088	Smithsonian National Museum of Natural History	Aiken Co, SC
USNM 307604	Smithsonian National Museum of Natural History	Charleston, SC
USNM 330074	Smithsonian National Museum of Natural History	Jasper, SC
USNM 330075	Smithsonian National Museum of Natural History	Jasper, SC
USNM 5560	Smithsonian National Museum of Natural History	Muscogee Co., GA
NMNH 130146	Smithsonian National Museum of Natural History	Charlton Co., GA
USNM 307594	Smithsonian National Museum of Natural History	Grady, GA
USNM 12927	Smithsonian National Museum of Natural History	New Orleans, LA
USNM 12926	Smithsonian National Museum of Natural History	New Orleans, LA
USNM 12928	Smithsonian National Museum of Natural History	New Orleans, LA

MCZ 16271	Harvard Museum of Comparative Zoology	Indian River Co., FL
USNM 2305	Smithsonian National Museum of Natural History	Volusia Co., FL
USNM 7851	Smithsonian National Museum of Natural History	Apalachicola, FL
USNM 13644	Smithsonian National Museum of Natural History	Brevard Co., FL
USNM 16700	Smithsonian National Museum of Natural History	Fernandina, FL
USNM 23806	Smithsonian National Museum of Natural History	Hillsboro Co., FL
USNM 69665	Smithsonian National Museum of Natural History	Lake Co., FL
USNM 42127	Smithsonian National Museum of Natural History	Brevard Co., FL
USNM 38160	Smithsonian National Museum of Natural History	Dade Co., FL
USNM 22322	Smithsonian National Museum of Natural History	Polk Co., FL
USNM 55903	Smithsonian National Museum of Natural History	Dade Co., FL

USNM 85322	Smithsonian National Museum of Natural History	Monroe Co., FL
USNM 28251	Smithsonian National Museum of Natural History	Dade Co., FL
USNM 26303	Smithsonian National Museum of Natural History	Dade Co., FL
USNM 28910	Smithsonian National Museum of Natural History	Dade Co., FL
USNM 30945	Smithsonian National Museum of Natural History	Dade Co., FL
USNM 10743	Smithsonian National Museum of Natural History	Santa Rosa Co., FL
USNM 9689	Smithsonian National Museum of Natural History	Duval Co., FL
USNM 36566	Smithsonian National Museum of Natural History	Dade Co., FL
NMNH 85323	Smithsonian National Museum of Natural History	Monroe Co., FL
NMNH 85324	Smithsonian National Museum of Natural History	Dade Co., FL
NMNH 129387	Smithsonian National Museum of Natural History	Duval Co., FL

USNM 204238	Smithsonian National Museum of Natural History	Monroe Co., FL
USNM 210070	Smithsonian National Museum of Natural History	Brevard Co., FL
USNM 218773	Smithsonian National Museum of Natural History	Broward Co., FL
USNM 325172	Smithsonian National Museum of Natural History	Wakulla Co., FL
YPR 205	Yale Peabody Museum	Dade Co., FL.
MCZ 150093	Harvard Museum of Comparative Zoology	Pasco Co., FL.
MCZ 170332	Harvard Museum of Comparative Zoology	Seminole Co., FL.
MCZ R 168515	Harvard Museum of Comparative Zoology	Seminole Co., Fl.
MCZ 12770	Harvard Museum of Comparative Zoology	Dade Co., FL
MCZ 56921	Harvard Museum of Comparative Zoology	Hernando Co., FL.
MCZ 12640	Harvard Museum of Comparative Zoology	Dade Co., FL
MCZ 13496	Harvard Museum of Comparative Zoology	Dade Co., FL

MCZ 14457	Harvard Museum of Comparative Zoology	Jupiter, FL.
MCZ 45234	Harvard Museum of Comparative Zoology	Dade Co., FL
MCZ R 166232	Harvard Museum of Comparative Zoology	Osceola Co., FL.
MCZ 45235	Harvard Museum of Comparative Zoology	Dade Co., FL
YPR 206	Yale Peabody Museum	Palm Beach Co., FL.
YPR 2791	Yale Peabody Museum	Marion Co., FL.
MCZ 6799	Harvard Museum of Comparative Zoology	Brevard Co., FL.
MCZ 14008	Harvard Museum of Comparative Zoology	Duval Co., FL
USNM 17924	Smithsonian National Museum of Natural History	FL
USNM 20137	Smithsonian National Museum of Natural History	Alachua Co., FL
USNM 17391	Smithsonian National Museum of Natural History	Putnam Co., FL

NCSM 27404	North Carolina Natural History Museum	Highlands Co., FL

M. f. fulvius specimens

Museum Code	Museum	State/County Collected
NCSM 60231	North Carolina Natural History Museum	New Hanover Co., NC
NCSM 25054	North Carolina Natural History Museum	Bladen Co., NC
NCSM 37878	North Carolina Natural History Museum	Pender Co., NC
NCSM 20272	North Carolina Natural History Museum	New Hanover Co., NC
NCSM 19854	North Carolina Natural History Museum	New Hanover Co., NC
NCSM 15886	North Carolina Natural History Museum	Brunswick Co., NC

NCSM 20614	North Carolina Natural History Museum	New Hanover Co., NC
NCSM 943	North Carolina Natural History Museum	New Hanover Co., NC
NCSM 944	North Carolina Natural History Museum	Moore Co., NC
NCSM 948	North Carolina Natural History Museum	Scotland Co., NC
NCSM 946	North Carolina Natural History Museum	Bladen Co., NC
NCSM 1832	North Carolina Natural History Museum	New Hanover Co., NC
NCSM 942	North Carolina Natural History Museum	Hoke Co., NC
NCSM 5931	North Carolina Natural History Museum	New Hanover Co., NC
NCSM 3978	North Carolina Natural History Museum	Brunswick Co., NC
NCSM 8144	North Carolina Natural History Museum	Aiken Co., SC
NCSM 66320	North Carolina Natural History Museum	Marion Co., FL

NCSM 23323	North Carolina Natural History Museum	Marion Co., FL
NCSM 3361	North Carolina Natural History Museum	Columbia Co., FL
NCSM 63281	North Carolina Natural History Museum	Putnam Co., FL
NCSM 64436	North Carolina Natural History Museum	Marion Co., FL
NCSM 64437	North Carolina Natural History Museum	Marion Co., FL
NCSM 64439	North Carolina Natural History Museum	Hernando Co., FL
NCSM 8160	North Carolina Natural History Museum	Clay Co., FL
NCSM 15321	North Carolina Natural History Museum	Volusia Co., FL
NCSM 9179	North Carolina Natural History Museum	Volusia Co., FL
UF 51197	Florida Museum of Natural History	Hillsborough Co., FL
UF 74446	Florida Museum of Natural History	Hillsborough Co., FL

UF 81634	Florida Museum of Natural History	Hillsborough Co., FL
UF 74445	Florida Museum of Natural History	Hillsborough Co., FL
UF 120531	Florida Museum of Natural History	Monroe Co., FL
UF 84485	Florida Museum of Natural History	Collier Co., FL
UF 118833	Florida Museum of Natural History	Highlands Co., FL
UF 141636	Florida Museum of Natural History	Highlands Co., FL
UF 146575	Florida Museum of Natural History	Dade Co., FL
UF 43546	Florida Museum of Natural History	Seminole Co., FL
UF 84063	Florida Museum of Natural History	Seminole Co., FL
UF 19380	Florida Museum of Natural History	Palm Beach Co., FL
UF 19379	Florida Museum of Natural History	Palm Beach Co., FL
UF 146098	Florida Museum of Natural History	Dade Co., FL
UF 120480	Florida Museum of Natural History	Dade Co., FL