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Short Communication

Sequence variation in the *Mc1r* gene for a group of polymorphic snakes

Christian L. Cox [a,b,*], Alison R. Davis Rabosky [c,d], Paul T. Chippindale [a]

[a] Department of Biology, The University of Texas, Arlington, TX 76010, USA
[b] Department of Biology, The University of Virginia, Charlottesville, VA 22903, USA
[c] Department of Integrative Biology and the Museum of Vertebrate Zoology, University of California, Berkeley, CA 94720, USA
[d] Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109, USA

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ABSTRACT

Studying the genetic factors underlying phenotypic traits can provide insight into dynamics of selection and molecular basis of adaptation, but this goal can be difficult for non-model organisms without extensive genomic resources. However, sequencing candidate genes for the trait of interest can facilitate the study of evolutionary genetics in natural populations. We sequenced the melanocortin-1 receptor (*Mc1r*) to study the genetic basis of color polymorphism in a group of snake species with variable black banding, the genera *Sonora*, *Chilomeniscus*, and *Chionactis*. *Mc1r* is an important gene in the melanin synthesis pathway and is associated with ecologically important variation in color pattern in birds, mammals, and other squamate reptiles. We found that *Mc1r* nucleotide sequence was variable and that within our focal *Sonora* species, there are both fixed and heterozygous nucleotide substitutions that result in an amino acid change and selection analyses indicated that *Mc1r* sequence was likely under purifying selection. However, we did not detect any statistical association with the presence or absence of black bands. Our results agree with other studies that have found no role for sequence variation in *Mc1r* and highlight the importance of comparative data for studying the phenotypic associations of candidate genes.

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1. Introduction

Studying the genetic factors underlying a trait is fundamental in evolutionary biology and can provide insight into dynamics of selection and molecular basis of adaptation (Hoekstra, 2006). However, for many non-model organisms, there are no genomic resources available for such studies. The "candidate gene" approach is a promising method that uses genes with known association to the trait of interest in other species, which then can be easily screened in and applied to non-model systems (Hoekstra, 2006; Rosenblum et al., 2004). This type of study has been especially fruitful in the study of color pattern polymorphism in natural populations (Hoekstra and Nachman, 2003; Nachman et al., 2003; Uy et al., 2009). Color polymorphism is extremely widespread across animals, and it has been implicated in the dynamics of sexual selection (Gray and McKinnon, 2006; Sinervo and Lively, 1996), speciation (Corl et al., 2010; Gray and McKinnon, 2006), mimicry complexes (Campbell and Lamar, 2004; Mallet and Joron, 1999), and adaptive background matching (Hoekstra et al., 2006; Rosenblum et al., 2010).

The snake genera *Chilomeniscus, Chionactis* and *Sonora* exhibit great variation in color pattern, including variation in the presence and absence of black bands. In particular, the North American ground snake, *Sonora semiannulata*, has marked color pattern polymorphism, including polymorphism for the presence of black bands (Fig. 1). Although no breeding studies have addressed the transmission genetics of color pattern in ground snakes, banding and striping are generally under simple genetic control in snakes (Bechtel, 1995; Bechtel and Whitecar, 1983; Zweifel, 1981). We characterized sequence variation in *Mc1r*, a gene that controls melanistic color in squamates and other vertebrates (Hubbard et al., 2010; Rosenblum et al., 2010) in *Sonora, Chionactis* and *Chilomeniscus* and determined the role of *Mc1r* in banding polymorphism for *S. semiannulata*.

Although many genes have been implicated in color pattern development in vertebrates, *Mc1r* has emerged as a particularly important single gene underlying the genetic architecture of color pattern (Hoekstra, 2006; Hubbard et al., 2010; Johnson, 2012; Mundy, 2005; Rosenblum et al., 2004). *Mc1r* is a single-copy gene that encodes for melanocortin-1 receptor, a G-protein coupled receptor with extensive transmembrane domains (Hoekstra, 2006). In mammalian systems, *Mc1r* acts as a molecular switch between eumelanin (black or brown pigment) and pheomelanin (red pigment) production based on binding with either alpha-MSH or agouti ligands, respectively (Hoekstra, 2006). The melanin pathway is not well characterized in reptiles, with only one type of melanin (eumelanin) identified (Rosenblum et al., 2004). Rather than acting as a switch between eumelanin and pheomelanin, it is thought that *Mc1r* controls the amount of melanin deposited in reptiles (Hubbard et al., 2010; Rosenblum et al., 2004). The *Mc1r* gene has been

Abbreviations: *Mc1r*, melanocortin-1 receptor.

* Corresponding author at: Department of Biology, The University of Virginia, Charlottesville, VA 22903. Tel./fax: +1 434 243 3399.
E-mail address: clcox@virginia.edu (C.L. Cox).

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Fig. 1. Ground snakes (*Sonora semiannulata*) from a single population in western Texas. Note the polymorphism for melanistic banding (banded individual on the far right). Photo by Alison R. Davis Rabosky.

implicated in color pattern polymorphism in mammals (Hoekstra et al., 2006; Nachman et al., 2003), birds (Johnson, 2012; Mundy, 2005; Uy et al., 2009), and reptiles (Rosenblum et al., 2004, 2010). This gene is also known to be relatively free of pleoiotropic effects, and the variation in *Mc1r* among diverse taxa may indicate that selection is free to act on this locus (Hoekstra et al., 2006). Because *Mc1r* seems to act as a binary switch regulating the amount of melanin produced in squamate reptiles, it is a reasonable choice to test for control of a presence or absence of melanistic trait such as black bands in snakes.

Because of minimal pleiotropy, simple structure (a single 1 kbp exon), and excellent characterization in other vertebrates, *Mc1r* is an attractive candidate locus for controlling banding in *Sonora* and it close relatives. Additionally, *Mc1r* has only been well characterized in a few focal squamate reptile species. We sequenced a large dataset of *Mc1r* in *Sonora* and several closely related snake genera to: 1) characterize the molecular evolution of the *Mc1r* gene in *Sonora, Chilomeniscus* and *Chionactis*, 2) determine whether sequence variation in this gene is statistically associated with loss of melanistic banding and test for the molecular signature of selection on *Mc1r* in *S. semiannulata*.

2. Materials and methods

2.1. Taxonomic sampling

We obtained tissues for 6 species of *Sonora*, *Chionactis*, and *Chilomeniscus*, with particular focus on the common and widespread *S. semiannulata* (Table 1). The other species of *Sonora* as well as *Chionactis* and *Chilomeniscus* are included for comparative purposes and phylogenetic context. For *S. semiannulata*, we included individuals from across their geographic range and from multiple individuals from several populations that were polymorphic for banding (Table 1). Additionally, we included individuals with aberrant banding (e.g. faint or incomplete banding). We also included two other snake species (*Thamnophis sirtalis* and *Crotalus tigris*) and three lizard species that have experienced adaptive evolution (Rosenblum et al., 2004, 2010) of *Mc1r* (*Aspidoscelis inornata*, *Sceloporus undulatus*, and *Holbrookia maculata*).

2.2. Molecular methods

Muscle, liver and skin tissue was taken from freshly killed specimens and stored in 95% ethanol or tissue lysis buffer at −80 °C. Genomic DNA was extracted from tissues using the DNAeasy Blood and Tissue Kit (Qiagen) using standard protocol.

While the internal segment of *Mc1r* is relatively conserved, the external sequence of the genes can be quite variable. Therefore, universal primers are not available and acquiring novel sequence data for *Mc1r* requires genomewalking. This is a standardized, ligation based approach for acquiring additional sequence from a known sequence. We adopted this approach using the Clontech Genomewalking Kit. First, we sequenced a ~450 bp internal fragment of *Mc1r* for a subset of *Sonora* tissues (Table 2). We used this known sequence to design group-specific internal primers for *Sonora* and close relatives (Table 2). We then used the Clontech kit to genomewalk the sequences upstream and downstream of the internal *Mc1r* fragment (see the Clontech Genomewalker Universal Kit User manual for more details). Briefly, we digested Qiagen kit extracted tissue samples using one of four different restriction enzymes (Dra WE, EcoR V, Pvu II and Stu I) to create four different digestion libraries. Digested libraries were purified and extracted using a phenol chloroform extraction and were rehydrated in 1 X TE buffer. Universal adaptors (see Clontech Genomewalker Universal Kit User manual for sequence) were ligated to the digested product using T4 DNA ligase (3 units) at 16 °C for 12 hrs. We then amplified from the purified and digested product using the internal *Mc1r* primers and universal adaptor specific primers (see Clontech Genomewalker Universal Kit User manual for sequence) using a simplified two step touchdown thermal cycle (94 °C for 25 sec followed by 3 min at 72 °C for 7 cycles and 94 °C for 25 sec followed by 3 min at 67 °C for 32 cycles with final 7 min 67 °C extension). We then performed a nested PCR using an aliquot of the primary PCR with similar thermal cycling parameters (94 °C for 25 sec followed by 3 min at 72 °C for 5 cycles and 94 °C for 25 sec followed by 3 min at 67 °C for 20 cycles with final 7 min 67 °C extension). Results for each step were visualized on a 1–1.5% ethidium bromide stained agarose gel, and all experiments included a positive (human genomic DNA) and negative (deionized water) control. This process yielded fragments ranging from 200 to 1500 bp upstream and downstream of the internal *Mc1r* fragment for a total of 1700 bp spanning the approximately 1 kb coding sequence. PCR products were prepared for sequencing by the ExoSAP-IT kit (United States Biochemical) and we used the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems Inc.) to sequence the PCR products following the manufacturer's protocol. The sequenced products were precipitated using an ethanol/sodium acetate method and rehydrated in HPLC purified formamide (Hi-Di). The sample was then analyzed either on an ABI PRISM 3100xl Genetic Analyzer in the Genomics Core Facility at the University of Texas-Arlington. Sequences were edited and assembled using Sequencher (Genes Code Corps., Inc.). We then designed 4 different primer pairs that captured the entire coding region and found one primer pair that was reliable and yielded 1150 bp including the entire coding sequence (Table 2). These primers were used to amplify the entire *Mc1r* coding sequence for all tissues. Individual sequences were exported to MEGA (Tamura et al., 2011) and aligned in MEGA using the CLUSTAL algorithm (Larkin et al., 2007) with default parameters and manually adjusted if necessary.

2.3. Analytical methods

We assessed molecular evolution of the *Mc1r* gene within *Chilomeniscus*, *Chionactis* and *Sonora* by determining the distribution of indels, synonymous, and nonsynonymous mutations of the coding sequence and the amino acid sequence. Because our greatest sampling was for the genus *Sonora* and *S. semiannulata*, we also determined average percent divergence for these groups. We then focused the remainder of our analyses on our dataset of 51 *S. semiannulata*. We tested nonsysnonymous mutations for association with banding in *S. semiannulata* using Fisher's Exact Test. Because heterozygosities were relatively rare, we completely phased all sequences with heterozygosities and included each phase of the heterozygous sequence in our analysis. Additionally, we reanalyzed by treating heterozygous sites as a separate character state (H). In all cases, the results of these different strategies were statistically identical. The few specimens with pattern abberations such as faint or broken bands were coded as both banded and unbanded, with statistically indistinguishable results. We specifically scrutinized the amino acid residues that are associated

Table 1
Specimen information and Genbank accession number for all samples in this study. In the Bands column, the presence of bands is indicated with Y, and absence with N. The two specimens with aberrant or faint bands are noted in this column as FB.

ID	Species	Country	State	County/municipality	Bands	Genbank #
BAL1	*Sonora semiannulata*	USA	AZ	Yavapai	Y	JX305468
CER826	*Sonora semiannulata*	USA	TX	Presidio	N	JX305469
CLC011	*Sonora semiannulata*	USA	TX	Shackleford	N	JX305470
CLC012	*Sonora semiannulata*	USA	TX	Shackleford	N	JX305471
CLC013	*Sonora semiannulata*	USA	TX	Shackleford	N	JX305472
CLC031	*Sonora semiannulata*	USA	TX	Shackleford	Y	JX305473
CLC035	*Sonora semiannulata*	USA	TX	Shackleford	Y	JX305474
CLC093	*Sonora semiannulata*	USA	TX	San Saba	Y	JX305475
CLC137	*Sonora semiannulata*	USA	TX	Stephens	Y, FB	JX305476
CLC140	*Sonora semiannulata*	USA	TX	Stephens	N	JX305477
CLC141	*Sonora semiannulata*	USA	TX	Stephens	N	JX305478
CLC142	*Sonora semiannulata*	USA	TX	Stephens	N	JX305479
CLC151	*Sonora semiannulata*	USA	TX	Shackleford	N	JX305480
CLC152	*Sonora semiannulata*	USA	TX	Shackleford	Y	JX305481
CLC204	*Sonora semiannulata*	USA	TX	Crockett	N	JX305482
CLC206	*Sonora semiannulata*	USA	TX	Crockett	N	JX305483
CLC218	*Sonora semiannulata*	USA	TX	Shackleford	Y	JX305484
CLC220	*Sonora semiannulata*	USA	TX	Shackleford	N	JX305485
CLC258	*Sonora semiannulata*	USA	CO	Otero	Y	JX305486
CLC259	*Sonora semiannulata*	USA	CO	Otero	Y	JX305487
CLC261	*Sonora semiannulata*	USA	CO	Otero	Y	JX305488
CLC262	*Sonora semiannulata*	USA	CO	Otero	Y	JX305489
CLC267	*Sonora semiannulata*	USA	CO	Otero	Y	JX305490
CLC328	*Sonora semiannulata*	USA	TX	Stephens	Y, FB	JX305491
CLC369	*Sonora semiannulata*	USA	TX	Jeff Davis	Y	JX305492
CLC371	*Sonora semiannulata*	USA	TX	Hidalgo	N	JX305493
CLC375	*Sonora semiannulata*	USA	TX	Hidalgo	N	JX305494
CLC403	*Sonora semiannulata*	MX	BCS	San Ignacio	N	JX305495
CLC428	*Sonora semiannulata*	USA	AZ	Maricopa	N	JX305496
CLC431	*Sonora semiannulata*	USA	AZ	Santa Cruz	Y	JX305497
CLC432	*Sonora semiannulata*	USA	AZ	Cochise	Y	JX305498
CLC436	*Sonora semiannulata*	USA	TX	Shackleford	N	JX305499
CLC453	*Sonora semiannulata*	USA	OK	Tulsa	Y	JX305500
CLC454	*Sonora semiannulata*	USA	OK	Tulsa	N	JX305501
CLC476	*Sonora semiannulata*	USA	KS	Elk	N	JX305502
CLC478	*Sonora semiannulata*	USA	KS	Elk	N	JX305503
CLC494	*Sonora semiannulata*	USA	OK	Blaine	N	JX305504
CLC495	*Sonora semiannulata*	USA	OK	Blaine	N	JX305505
CLC496	*Sonora semiannulata*	USA	OK	Blaine	Y	JX305506
CLC497	*Sonora semiannulata*	USA	OK	Blaine	Y	JX305507
CLC498	*Sonora semiannulata*	USA	OK	Blaine	N	JX305508
CLC500	*Sonora semiannulata*	USA	OK	Blaine	Y	JX305509
CLC501	*Sonora semiannulata*	USA	OK	Blaine	Y	JX305510
CLC503	*Sonora semiannulata*	USA	OK	Blaine	N	JX305511
CLC505	*Sonora semiannulata*	USA	OK	Blaine	Y	JX305512
CLC506	*Sonora semiannulata*	USA	OK	Blaine	N	JX305513
CLC732	*Sonora semiannulata*	USA	TX	Crockett	Y	JX305514
CLC733	*Sonora semiannulata*	USA	TX	Crockett	N	JX305515
EBU1S	*Sonora semiannulata*	USA	NM	Sierra	N	JX305516
EBU2S	*Sonora semiannulata*	USA	NM	Sierra	N	JX305517
HIL1	*Sonora semiannulata*	USA	AZ	Yavapai	Y	JX305518
JMM660	*Chilomeniscus stramineus*	USA	AZ	NA	Y	JX305519
JMMC10	*Chionactis occipitalis*	MX	SON	Badiraguato	Y	JX305520
JMMC6	*Chionactis occipitalis*	USA	CA	Imperial	Y	JX305521
JRV127	*Sonora mutabilis*	MX	JAL	Huaxtla	Y	JX305522
JRV128	*Sonora mutabilis*	MX	JAL	Huaxtla	Y	JX305523
MJI32	*Sonora semiannulata*	USA	TX	Jeff Davis	N	JX305524
MJI84	*Sonora semiannulata*	USA	TX	Shackleford	Y	JX305525
MZFC 23956	*Sonora michoacanensis*	MX	GRO	Campo Morado	Y	JX305526
ROM RWM875	*Sonora semiannulata*	MX	BCS	San Pedro de la Presa	N	JX305527
UANL 6976	*Sonora aemula*	MX	SON	Navajoa	Y	JX305528
TS16	*Thamnophis sirtalis*	NA	NA	NA	NA	AY586157
NA	*Crotalus tigris*	NA	NA	NA	NA	EU526278
EBR50	*Aspidoscelis inornata*	NA	NA	NA	NA	AY586069
EBR88	*Aspidoscelis inornata*	NA	NA	NA	NA	AY586073
EBR91	*Aspidoscelis inornata*	NA	NA	NA	NA	AY586074
EBR358	*Holbrookia maculata*	NA	NA	NA	NA	AY586104
EBR47	*Holbrookia maculata*	NA	NA	NA	NA	AY586110
EBR53	*Holbrookia maculata*	NA	NA	NA	NA	AY586112
EBR138	*Sceloporus undulatus*	NA	NA	NA	NA	AY586150
EBR174	*Sceloporus undulatus*	NA	NA	NA	NA	AY586153
EBR98	*Sceloporus undulatus*	NA	NA	NA	NA	AY586148

FB = faint bands.

Table 2
Primers for sequencing *Mc1r* in *S. semiannulata*.

Name	Description	Sequence (5′–3′)
Mc1RLprim1	Internal Primer	CTGCTTGCCATACATGGTTGAAATCT
Mc1RRprim1	Internal Primer	CTATCACAGTATCATGACCATACAGC
Mc1RLprim2	Nested Internal Primer	AACATGTGGATGTAGAGCCCTGCAATG
Mc1RRprim2	Nested Internal Primer	ATCCTCTTCATTGTCTACGACAGCACT
Mc1RSoseUpstr2	Primer for entire coding sequence	GAAAGCTGCTGACGGAG
Mc1RDstr3	Primer for entire coding sequence	GTCACCTGCTTGCCCTGAATG

with melanic polymorphism in *Sceloporus*, *Holbrookia* and *Aspidoscelis*. Finally, we tested for the presence and type of selection on *Mc1r* for *S. semiannulata* using three different tests. First, we used Tajima's D, which compares patterns of mutation in putatively neutral loci to the focal loci. Second, we used the codon-based Fisher's Exact test, which compares the proportion of synonymous and nonsynonymous mutations. Finally, we used a maximum-likelihood approach (HyPhy) to test for the presence of selection on specific codons. All selection analyses were performed in MEGA5 (Tamura et al., 2011).

3. Results

3.1. Molecular variation in Mc1r sequence

We found that the coding sequence of *Mc1r* for *Chilomeniscus*, *Chionactis*, and *Sonora* was 948 bp, confirmed by the presence of both start and stop codons. Relative to the outgroups, *Chilomeniscus*, *Chionactis* and *Sonora* possess a 12 bp indel near the beginning of the coding sequence at 78 bp. *Holbrookia maculata* and *S. undulatus* possess a 3 bp indel at 54 bp relative to all other taxa. *Mc1r* was variable, with 4.4% maximum sequence divergence across *Chilomeniscus*, *Chionactis*, and *Sonora*. Maximum sequence divergence within the genus *Sonora* was also 4.4%, and 2.7% within *S. semiannulata*. We found that amino acid variation was minimal. *Sonora semiannulata* possessed three fixed amino acid substitutions and 8 heterozygosities that resulted in amino acid substitutions. All amino acid substitutions were rare, occurring in one or two specimens (a maximum of five individuals possessed one substitution).

3.2. Association of Mc1r variation and banding

We found that amino acid variation was not associated with banding or aberrant banding patterns for *S. semiannulata* (all *P*'s for 8 heterozygous sites and 3 fixed amino acid changes >0.5). Indeed, we found both banded and unbanded individuals for each amino acid change. Additionally, we found no sequence variation in *S. semiannulata* in the amino acid residues responsible for melanin polymorphism in *Holbrookia*, *Sceloporus* and *Aspidoscelis* (i.e., Rosenblum et al., 2004).

3.3. Selection on Mc1r

Tajima's D for *Mc1r* was -2.08, consistent with purifying selection. We found that *Mc1r* sequences did not differ significantly from neutrality according to the codon-based Fisher's exact test. Using HyPhy, around 258 codons (82% of the coding sequence) appeared to be evolving under purifying selection, and seemed to be regionally localized in the middle of the coding region.

4. Discussion

We found that while *Mc1r* coding region was variable among *Chilomeniscus*, *Chionactis*, and *Sonora*, this resulted in relatively few amino acid substitutions. These low frequency amino acid substitutions were not significantly associated with banding, and our analyses suggest that purifying selection is acting on this nuclear gene. Below we discuss the implications and applications of our results.

We did not detect a statistical association of *Mc1r* sequence variation with banding. This finding is supported by the fact that we detected zero nucleotide or amino acid variation in the sites associated with adaptive variation in *Mc1r* in mice and other squamate reptiles. Additionally, the major indels in *Mc1r* were not associated with banding, although one indel was common to all *Mc1r* sequences from *Chilomeniscus*, *Chionactis* and *Sonora* regardless of color pattern. However, we cannot rule out a role for *Mc1r* in banding polymorphism in *Sonora*. It is possible that sequence variation in upstream and downstream regulatory regions and transcriptional regulation are all potential mechanisms controlling color pattern. Additionally, we note that we only tested for the role of sequence variation in *Mc1r* for one type of melanistic polymorphism (the presence or absence of bands). However, *S. semiannulata* displays additional color pattern variation in ground color and degree of melanic maculation, and even unbanded snakes can have a dark or melanistic appearance. Undoubtedly *Mc1r* is important for the production of melanin in snakes (Hubbard et al., 2010; Rosenblum et al., 2010), and may be important for melanistic coloration other than bands in *S. semiannulata*. Beyond *Mc1r*, other genes in the melanin synthesis pathway may be fruitful candidates for controlling melanistic coloration in snakes. Indeed, genes in this pathway such as *agouti* (an *Mc1r* ligand), tyrosinase related protein-1 (*TyrP1*) and dopochrome tautomerase (*Dct*) among many others regulate color pattern in other vertebrates (for details see Hoekstra, 2006). Other studies in squamate reptiles have found mixed results for the role of *Mc1r* in a melanistic phenotype (Rosenblum et al., 2004), which emphasizes the importance of evaluating other genes in the melanin synthesis pathway. Beyond genes in the melanin synthesis pathway, genes that regulate the migration and distribution of pigment cells and precursors may be important in producing a banded phenotype (Bechtel, 1978). We suggest that future studies should examine other types of variation in melanism, sequence variation outside of the coding sequence, transcriptional regulation of *Mc1r*, and other genes in the melanin synthesis pathway to clarify the genetic mechanisms color pattern variation in snakes.

Many nuclear protein-encoding genes bear the statistical signature of purifying selection (Graur and Li, 2000). Given the important role of *Mc1r* in color pattern development in squamates, it is unsurprising that much of the *Mc1r* gene appears to be evolving under purifying selection. Maximum likelihood analysis suggests that the beginning and final codons are not evolving under purifying selection, and this may explain the lack of effective universal primers for this gene. These results are consistent with other studies that suggest that this conserved gene is subject to purifying selection (Harding et al., 2000; Hubbard et al., 2010). The presence of purifying selection on this gene could support a role for *Mc1r* in color pattern variation other than the presence or absence of black bands (e.g. variation in ground color, variation in melanistic maculation outside of bands).

The *Mc1r* gene has proved useful for understanding the molecular control of ecologically and evolutionarily important color pattern variation pattern (Hoekstra, 2006; Hubbard et al., 2010; Mundy, 2005; Rosenblum et al., 2004). Although research within multiple study systems has identified the role of *Mc1r* in color pattern variation associated with background matching, mate choice, and speciation (Hoekstra et al., 2006; Johnson, 2012; Rosenblum et al., 2004; Uy et al., 2009), a number of studies have found that sequence variation in *Mc1r* was not associated with the color pattern feature of interest (e.g., Dorn et al., 2011; Herczeg et al., 2010; Rosenblum et al., 2004). Accordingly, publishing negative results (i.e., instances where sequence variation is not associated with the trait of interest) is important to gain an accurate depiction of the role of *Mc1r* variation for color pattern polymorphism or color pattern variation in natural populations. Our results provide a

valuable contrast to the research that documents a role of *Mc1r* in color pattern variation in squamate reptiles and other vertebrates.

Snakes are often models for natural history studies on the evolution and ecological function of color pattern (Brodie, 1992, 1993; Pfennig et al., 2001; Sanders et al., 2006; Wuster et al., 2004). However, research on the ecological genetics of adaptation in this group is rare (Rosenblum et al., 2004). Although we did not detect a role for *Mc1r* sequence variation in color pattern polymorphism in ground snakes, the large genetic data set contributed in this paper has great utility for primer design and comparative purposes in other functional or phylogenetic studies of this gene in snakes (e.g., Austin et al., 2010).

Disclosure statement

None of the authors have any actual or potential conflicts of interest to disclose.

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