Variability of the mc1r Gene in Melanic and Non-Melanic Podarcis lilfordi and Podarcis pityusensis from the Balearic Archipelago

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Abstract

The association between polymorphism at the mc1r locus and colour variation was studied in two wall lizard species (Podarcis lilfordi and P. pityusensis) from the Balearic archipelago. Podarcis lilfordi comprises several deep mitochondrial lineages, the oldest of which originated in the Pliocene, while much shallower mitochondrial lineages are found in P. pityusensis. Here, we examined whether specific substitutions were associated with the melanic colouration found in island populations of these species. Homologous nuclear sequences covering most of the mc1r gene were obtained from 73 individuals from melanic and non-melanic Podarcis from different populations (the entire gene was also sequenced in six selected individuals). MtDNA gene trees were also constructed and used as a framework to assess mc1r diversity. Mc1r showed greater polymorphism in P. lilfordi than in P. pityusensis. However, we observed no substitutions that were common to all melanic individuals across the two species. Only one significant association was detected in the mc1r partial sequence, but this was a synonymous A/G mutation with A alleles being more abundant in melanic populations. In addition, there were no associations between the main dominant phenotypes (green and brown, blue and yellow spots and ventral colour) and synonymous or non-synonymous substitutions in the mc1r gene. There was no statistical evidence of selection on mc1r. This study suggests no relationship between mc1r polymorphism and colour variation in Balearic Podarcis.

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Introduction

Although environmental stimuli can contribute to colour variation within species, most of this variation appears to be genetically controlled [1]. Molecular analyses are starting to reveal mutations associated with melanism in wild populations. In some birds and mammals, melanism seems to be associated with amino acid substitutions in the melanocortin-1 receptor (mc1r), a gene known to control the synthesis of melanin by melanocytes [2,3]. The agouti-melanocortin 1 receptor pathway is a ligand receptor pair that modulates the amount and type of pigment produced by melanocytes (red/yellow pheomelanin or brown/black eumelanin). Genetic subtypes of mc1r genes (of which mc1r is one) have high structural similarity. The majority of them seem to have originated early in vertebrate evolution before the divergence of ray-finned fishes and tetrapods [4]. The main structural properties of these genes have remained remarkably conserved over a period of at least 400 million years [4]. Gain-of function and/or deletion mutations in the mc1r locus are well recognized causes of melanism [5]. For example, a deletion in the mc1r gene explains melanism in squirrels [6]. In birds, Guo et al. [7] reported abundant polymorphism in the mc1r gene which was associated with black plumage in Hebei chickens. Different mutations in the mc1r gene also explain the brown phenotype in the cavefish, Astyanax mexicanus [8].

Several studies have recently addressed the mc1r gene and colour polymorphism in amphibians and reptiles. Three independent mc1r mutations (His208Tyr, Thr170Ile, and Val168Ile) are responsible for blanched coloration of three lizard species on the gypsum dunes of White Sands, New Mexico, where they are associated with melanin production in the species Holbrookia maculata, Aspidoscelis inscripta and Sceloporus undulatus [9]. Although the same gene contributes to light phenotypes in these White Sands populations, the specific molecular mechanisms leading to reduced melanin production appear to be different. In contrast, sequence variation in mc1r does not explain melanism in the widespread amphibian Rana temporaria [10] nor does it appear to be involved in dorsal colour adaptations in two sympatric species of sand lizard (Lacerta) that inhabit the south eastern coast of South America [11] or colour pattern in Uta lizards [12]. Some authors consider blue colouration to be a form of melanism in reptiles [13]. However the blue abdominal skin seen in several lizards is a sexually dimorphic trait that is more pronounced in males [14] and is attributed to eliciting a behavioural response in the observer [15]. Recently, an association between mc1r variants and brown
scale colour phenotypes has been described in the European ocellated lizard, *Lacerta lepida* (*Timon lepidus*) [16].

The genus *Podarcis* is one of the most diverse and abundant reptile groups in southern Europe, with more than 20 currently recognized species [17,18], and since the early works of Einer [19] has been known to contain several species that contain melanic populations. Melanic lizards and darker individuals in general, were originally thought to be associated with older island populations [19,20,21].

Two endemic species of *Podarcis* inhabit the Balearic Archipelago: *Podarcis lilfordi* in the Eastern Gymnesic Islands group (Mallorca, Menorca, Cabrera and their coastal islets) and *Podarcis pityusensis* in the Western Pityusic group (Ibiza, Formentera and coastal islets). Phylogenetic analyses have showed geographical structuring of mtDNA among insular populations of *P. lilfordi* with four main intraspecific lineages, the first of which diverged some 2.6 Ma [22,23]. Divergence within *P. pityusensis* is more recent with the main Ibiza and Formentera clades sharing a common ancestor around 1 Ma ago [22,23].

Eisentraut [21] described melanic populations of *P. lilfordi* from the islands of Aire (Menorca), and Foradada (Cabrera archipelago), as well as *P. pityusensis* from Bleda Plana [19].

### Table 1. Colour patterns of the different lizard populations.

| LOCALIZATION | POPULATIONS | DORSAL COLOUR | VENTRAL COLOUR |
|--------------|-------------|---------------|---------------|
|              |             | Brown | Green | Black | Dominant Spots | Blue | Yellow | Light | Dark | Blue | Orange | Blue Oce. |
| **Podarcis lilfordi** | CABRERA ARCHIPELAGO | Cabrera | x | x | Brown | x | x | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Foradada | x | Black | x | x | x |
| **MALLORCA** | | Dragonera | x | x | Brown | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;El Toro | x | Brown | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Colomer | x | Black | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Guardia | x | Black | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Moltona | x | Black | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Malgrats | x | x | Black | x | x | x |
| **MENORCA** | | Addaia | x | x | Green | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Binicodrell | x | Brown | x | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Rei | x | Brown | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Sanitja | x | Brown | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Aire | x | Black | x | x | x | x |
| **Podarcis pityusensis** | IBIZA | Alga | x | x | Brown | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Bosc | x | x | x | Green | x | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Conillera | x | x | Green | x | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Dau Gran | x | x | Green | x | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Eivissa | x | x | Green | x | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Espanbar | x | Green | x | x | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;S. Josep | x | x | Green | x | x | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Bleda Plana | x | Black | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Escull Vermell | x | Black | x | x | x |
| **FORMENTERA** | | Cap Barbaria | x | x | x | Green | x | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;P.Trocadors | x | x | Brown | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Sa Pujada | x | x | Green | x | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;St F. Xavier | x | x | Green | x | x | x | x | x |
| **Podarcis tiliguerta** | | Foradada | x | x | Brown | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Padodell | x | x | Brown | x | x | x |
| &nbsp;&nbsp;&nbsp;&nbsp;Stramari | x | x | Brown | x | x | x |
| **Podarcis filfolensis** | | Comino | x | x | Brown | x | x | x | x |

Melanic populations are indicated by grey shading.

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hypothesized that dark phenotypes were induced by a higher consumption of plant material. In contrast, Kramer [20] suggested an evolutionary explanation: dark colouration/melanism conveyed adaptive advantages through protection from harmful ultraviolet radiation, while enhancing heat absorption during cooler weather. Later, Hartmann [24] proposed that mutations that caused melanism originated before the coastal islets were separated from the main islands. The high degree of phenotypic variation (body size and colouration) among coastal islets is now well-established [25]. Several coastal islets host melanic populations of \textit{P. lilfordi}, while a smaller number of islets also host melanic or very dark populations of \textit{P. pityusensis}.

The purpose of the present paper was to investigate the putative association between polymorphism at the \textit{mc1r} locus and colour phenotype in \textit{Podarcis} from the Balearic Islands and therefore establish whether specific substitutions were associated with the melanic colouration of these populations.

**Materials and Methods**

**Samples**

A sample of 72 individuals from the genus \textit{Podarcis} was analyzed (Table 1). These were: 1) 46 \textit{Podarcis lilfordi} from 13 islands and islets corresponding to 7 light insular forms and 6 dark/melanic insular forms (from Mallorca, Menorca, and Cabrera), 2) 22 \textit{P. pityusensis} from 14 populations (from Ibiza and Formentera) with only two dark/melanic insular populations of \textit{P. lilfordi}, while a smaller number of islets also host melanic or very dark populations of \textit{P. pityusensis}.

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**Figure 1.** MC1R amino acid substitutions detected in \textit{P. lilfordi} and \textit{P. pityusensis}, and their locations relative to the cell membrane (modified from Garcia-Borron \textit{et al.} [43]). Melanic populations are indicated in bold. doi:10.1371/journal.pone.0053088.g001

DNA Extraction, Amplification and Sequencing

DNA was extracted during previous conservation genetics projects that described the mtDNA diversity within these lizards in order to underpin conservation strategies by the Balearic Islands Autonomous Government [25,26]. A 720 bp fragment of \textit{mc1r} gene was amplified with the forward primer MC1R-PF 5’-GGCNGGATYGTCAANAACCGGAACC-3’ and the reverse primer MC1R-PR 5’-CTCCGRAAGGCRTAAAT-NATGGGGTCCAC-3’ (modified from Pinho et al., 2010 [27]). A second pair of primers was designed from the \textit{Podarcis sicula} sequence to obtain the complete \textit{mc1r} sequence (944 bp). The forward primer was 5’-ATGTCATGTGGCCATACCCCT-3’ and the reverse primer was 5’-GTTCGGGTGCTTCGATTAATGAC-3’. The same PCR conditions were used for both sets of primers.

PCR reactions were performed in 25 µl volumes with 80 ng DNA, 1×PCR Buffer, 0.4 mM dNTPs, 0.3 µM of each primer (Genbank: GU225767). Insular lizard populations were selected so that the most extreme phenotypes were included. All specimens were captured with official permits from national and regional organisms and the lizards released at the point of capture.

**Pigmentation Variation**

Variation in pigmentation of the Balearic populations was classified from previous descriptions [25,26]. Several colour characteristics were noted (within-islet variation was negligible for these characteristics). They included the presence of brown, black, green on the dorsum, dark/light ventral colouration and the presence of blue, yellow and orange spots (Table 1).

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### Table 2. Synonymous and non synonymous (in bold) changes.

| Change positions (according to *P. sicula*) | 180 C | 210 G | 258 G | 261 C | 262 G | 274 G | 275 T | 298 C | 318 T | 329 T | 333 C | 346 G | 351 C | 375 C | 390 A | 402 T | 405 T | 415 A | 438 C | 441 C | 445 T | 480 C | 483 C | 492 C | 540 C | 568 C | 594 C | 604 A | 629 G | 645 C | 681 G | 684 G | 696 T | 726 C | 735 C | 750 C | 764 T | 787 A | 795 G | 804 C |
|---------------------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Populations                                | Cabrera | Dragonera | El Toro | Addala | Binicodrell | Reil | Santja | Bosch | Conillera | Dau Gran | Elvirasa | Espartar | Espalmador | S. Josep | P. Trocader | Sa Pujada | S. F. Xavier | Foradada | Colomer | Guardia | Moltona | Malgrats | Aire | Bleda Plana | E. Vernall |
| Change positions (according to *P. sicula*) | 180 C | 210 G | 258 G | 261 C | 262 G | 274 G | 275 T | 298 C | 318 T | 329 T | 333 C | 346 G | 351 C | 375 C | 390 A | 402 T | 405 T | 415 A | 438 C | 441 C | 445 T | 480 C | 483 C | 492 C | 540 C | 568 C | 594 C | 604 A | 629 G | 645 C | 681 G | 684 G | 696 T | 726 C | 735 C | 750 C | 764 T | 787 A | 795 G | 804 C |
| G                                           | G      | G      | G      | G      | G      | G      | T      | C/T    | C/T    | C/T    | C/T    | C/T    | T      | C/T    | T      | C/T    | C/T    | C/T    | C/T    | T      | C/T    | C/T    | C/T    | C/T    | C/T    | C/T    | T      | C/T    | T      | C/T    | G      | C/T    | T      | C/T    | C/T    | T      | C/T    | C/T    | T      | C/T    |
and 0.5 units of DNA polymerase. PCR conditions were: 5 min at 92°C, 35 cycles of 30 s at 92°C, 30 s at 56°C, 90 s at 72°C; 5 min at 72°C. PCR products were purified using the Invitek MSB® Spin PCRapace (Invitek GMBH, Berlin, Germany). Both heavy and light strands were sequenced on an automated ABI 3130 sequencer using a Big Dye® v3.1 Cycle sequencing kit (Applied Biosystems, Foster City CA, USA). Sequence data have been deposited at the GenBank data library under accession numbers JX126622-JX126693.

The following partial mitochondrial genes were also amplified using PCR and sequenced: 12S rRNA, cytochrome b (two regions obtained separately), control region and an 800 bp (ND) fragment that included part of the ND1 gene, three tRNA genes, tRNAIle, tRNAGln, and tRNAMet and part of the ND2 gene. The total length of mitochondrial sequence analyzed for each animal was 2370 bp. We sequenced individuals from P. pityusensis (GenBank

| Table 2. Cont. |
|----------------|
| **Populations** |
| Cabrera | Dragonera | El Toro | Addaia | Binicodrell | Rei | Santija | Alge | Bosc | Conilera | Da Gru | Eivissa | Espartar | Espladolar | San Josef | P. Trogader | Sa Pujada | St. F. Xavier | P. Molentas | Foradada | Colomer | Guardia | Moltona | Malgrats | Aire | Bleda Planas | E. Vermell |
| 814 T | A/T |
| 879 A | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C |
| 885 C | C/T | C/T | C/T | C/T | C/T | C/T | C/T | C/T |
| 886 G | C |

Melanic populations are indicated by grey shading. doi:10.1371/journal.pone.0053088.t002

Figure 2. Network showing relationships among mc1r haplotypes observed in Balearic populations. Alleles from melanic populations are represented as black circles, and non-melanic populations as white circles. Alleles that were found in both melanic and non-melanic populations are represented as grey circles. doi:10.1371/journal.pone.0053088.g002
Haplotype phases for \(mc1r\) were resolved for heterozygotic individuals using DnaSP software v5.10 [28] which implements an algorithm from the program PHASE [29,30,31]. The same software was used to obtain estimates of sequence diversity and compute the nucleotide diversity at synonymous, non-synonymous, and silent sites, following Nei and Gojobori [32]. Neutrality was tested with Tajima’s D test [33] and Fu’s F test [34] using DnaSP [28].

A \(mc1r\) haplotype network was constructed using the program TCS v.1.21 [35] to examine whether or not melanism was associated with the overall \(mc1r\) genealogy. TCS creates a network using statistical parsimony [36,37]. The probability of parsimony for linking haplotypes was set at the 95% level.

Phylogenetic trees were obtained using Bayesian inference on the haplotypes (MrBayes v.3.1.2 [38]). Two MCMC samplers were run in parallel (4 chains each, temperature (no lo llama el “heating parameter”) parameter set at 0.2) starting from a random tree for \(1.3 \times 10^6\) generations (samples recorded every 100 generations). In both sampling runs, stationarity of the Markov Chain was determined by stable split-standard deviations and stable sampled log likelihood values. The posterior sample of trees that followed burn-in were combined into a majority-rule consensus tree and used to estimate posterior node probabilities.

Results

Assignment of the 27 Balearic populations and other \(Podarcis\) species to melanics and non-melanic sets is shown in Table 1. Individuals from Foradada, Guardia, Moltona, Aire and Escull Vermell islands within the melanics group of 8 populations also show blue spots. Melanic individuals from Aire and Malgrats populations also show brown dorsal spots and blue ventral ocelli. The non-melanics group comprises 19 populations. In this group, the most dominant dorsal colour is green (10 populations), followed by brown (8 populations). The presence of black, blue and/or yellow spots is less common. In general, non-melanics populations have light ventral colour.

The 720 bp \(mc1r\) sequence provided 146 haplotypes, corresponding to 45 segregating sites, across \(P.\) \(lilfordi\), \(P.\) \(pityusensis\), \(P.\) \(fifolensis\) and \(P.\) \(tiliguerta\) species. The observed changes are displayed against the \(P.\) \(sicula\) reference sequence (Table 2). There were 32 synonymous and 13 non-synonymous substitutions. We show the locations of non-synonymous substitutions on the \(MC1R\) genealogy. The TCS creates a network using statistical parsimony [36,37]. The probability of parsimony for linking haplotypes was set at the 95% level.

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Table 3. Genetic diversity parameters based on mc1r gene sequences (720 bp) and mtDNA (2370 bp).

| Gene       | N  | S   | h   | Hd  | K  | Pi  | D   | F  |
|------------|----|-----|-----|-----|----|-----|-----|----|
| P. lilfordi| 92 | 30  | 35  | 0.955 (0.009) | 3.634 | 0.005 (0.001) | -1.242** | -1.237** |
| P. pityusensis | 44 | 14  | 23  | 0.958 (0.014) | 3.050 | 0.004 (0.001) | -0.163** | -0.324** |
| Other species | 8  | 12  | 6   | 0.929 (0.084) | 4.464 | 0.006 (0.001) | -0.178** | 0.025** |
| Melanic     | 44 | 22  | 18  | 0.900 (0.031) | 3.476 | 0.005 (0.001) | -1.029 | -1.143** |
| Non melanic | 92 | 34  | 43  | 0.968 (0.007) | 3.582 | 0.005 (0.001) | -1.493** | -1.972** |

N = number of sequences; S = number of segregating sites; h = number of haplotypes; Hd = haplotype diversity; K = number of pairwise differences; Pi = nucleotide diversity; D = Tajima’s D (1989); F = Fu’s F and Li’s F (1993). SE is indicated in parentheses.

**not significant, *P < 0.05.

The melanic individuals belong to P. lilfordi and P. pityusensis insular populations.

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Discussion

Substitutions in the mc1r gene of endangered Balearic Island Podarcis lizards do not appear to be related to either melanism or other components of the considerable colour pattern variation among islands.

Recent studies on pigmentation genes and their functions have provided evidence that pigment gene function is largely conserved across vertebrate taxa and can influence adaptive coloration, often in predictable ways [1]. The mc1r gene is highly conserved among vertebrates and has a relatively simple genetic structure. This has facilitated its identification in a diversity of taxa, including lizards. The majority of these studies try to associate a punctual non-synonymous sequence change with a discrete colour polymorphism. In some cases, identical mutations at homologous positions in diverse taxa have been found to lead to the same or similar phenotypes [1].

The mc1r gene is polymorphic in the studied populations. We found 45 variable positions with respect to the published mc1r gene sequence of P. sicula and three of these positions could be considered as hot spots due to their high mutation frequency. As expected under neutral evolution, synonymous changes are most numerous but thirteen substitutions encode for different amino acids, and most of them correspond to the transmembrane domain of the protein. Much of the genetic diversity in mc1r appears to reflect the patterns observed in the mtDNA, which have been
interpreted in terms of the historical biogeography of these species [22,23]. For example, *P. lilfordi* shows much greater genetic diversity (in both loci) than *P. pityusensis*. Previous mtDNA analyses showed that it was likely to have originated from ancient isolation on the major islands of Mallorca, Menorca and Cabrera during the Pliocene [22,23].

Nunes et al. [16] detected two associations between *mc1r* variants and ecologically relevant phenotypes in the European oscillated lizard *Lacerta lepida*, a genus related to *Podarcis*. The first is a non-conserved and derived substitution (T162E) associated with the presence of brown scales ("nevadensis" phenotype), while the second substitution (S172C) was associated with the presence of black scales in both *L. l. lepida* and *L. l. iberica*. However, they did not detect mutations associated with the higher proportion of black scales in *L. l. iberica*. Here, the nucleotide positions 162 and 172 were not variable among the very polymorphic populations of *Podarcis*.

With some exceptions [5] melanism is also associated with substitutions at the *mc1r* locus in a variety of mammals and birds, including domestic [39,40] and wild species [41]. In this case structural mutations (deletions) are thought to be responsible for the melanic phenotype. There were no deletions in the *mc1r* gene sequence in melanic populations of *Podarcis lilfordi* and *Podarcis pityusensis*, suggesting that this is not the case here. The unique substitutions that we have observed in the melanic populations: Foradada, Colomer, Guardia and Moltona (*P. lilfordi*), are synonymous changes at Thr117 and Ser227. However, the *P. lilfordi* Aire and Malgrats island populations, and the *P. pityusensis* Escull Vermell and Bleda Plana island populations, are melanic, but do not share these substitutions. It is therefore very difficult to believe they play a role in melanin in any of these species. Similar findings have recently been reported for the side-blotched lizard, *Uta stansburiana* [12].

The presence of a dark phenotype is thought to be a relic character in corydile lizards [42]. However, it has been hypothesized that this character is under quite strong selection in *Podarcis* due to its impact on thermoregulation [20]. Given the low prevalence of melanism in other *Podarcis*, it seems unlikely that melanism is the ancestral condition for these species. If we assume that the mtDNA branching pattern reflects the true species/population history then the most parsimonious explanation is that the ancestral condition is the non-melanic colour seen in most other *Podarcis*. If this is the case then melanism has clearly evolved several times within Balearic *Podarcis*. However, our statistical tests on *mc1r* provided no support for the hypothesis that that this can be attributed to different selection regimes on different melanic populations.

*Podarcis* coloration therefore seems to be attributable to other loci. For example, agouti signaling protein (*asip*) is important in melanin synthesis and multiple mutations in this gene are associated with colour variation. However, compared with *mc1r*, far fewer studies have been carried out on wild populations and also the molecular changes associated with colour variation are different with both coding and regulatory regions being implicated. To date, agouti-like sequences have not been reported in reptiles. In addition to melanin pigments, animal coloration can involve carotenoid pigments and pterins, but the genetic mechanisms involved in these pathways are poorly understood [1].

Balearic Island *Podarcis* populations exhibit a wide variety of colour variants, with morphs ranging from completely melanic to quite light-coloured individuals, although green-brown pigmentation is the most frequent morph. Sequencing of the *mc1r* gene in individuals with different morphological phenotypes has not revealed a clear correlation between mutations and/or deletions and these different colour morphs.

### Author Contributions

Conceived and designed the experiments: MMR JAC RPB. Performed the experiments: JMB VR VPM AP. Analyzed the data: JMB VR MMR. Contributed reagents/materials/analysis tools: VPM BT MMR. Wrote the paper: JMB VR RPB MMR.

### References

1. Hubbard JK, Uy JAC, Hauber ME, Hoekstra HE, Safran RJ (2010) Vertebrate pigmentation: from underlying genes to adaptive function. Trends Genet 26: 231–239.
2. Robbins LS, Nadeau JH, Johnson KR, Kelly MA, Roselli-Rehfuss L, et al. (2009) Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. Cell 72: 827–834.
3. Takeuchi S, Suzuki S, Hirose S, Yabuuchi M, Sato C, et al. (1996) Molecular cloning and sequence analysis of the chick melanocortin-1-receptor gene. Biochim Biophys Acta 1306: 122–126.
4. Schioth HB, Hattina T, Ling MK, Fredriksen R, et al. (2005) Evolutionary conservation of the structural, pharmacological, and genomic characteristics of the melanocortin receptor subtypes. Peptides 26: 1816–1900.
5. Anderson TM, vonHoldt BM, Candille SJ, Musiani M, Greco C, et al. (2009) Molecular and evolutionary history of melanism in North American gray wolves. Science 323: 1339–1343.
6. McRobie H, Thomas A, Kelly J (2009) The genetic basis of melanism in the gray squirrel (Sciurus carolinensis). J Hered 100: 769–714.
7. Guo XL, Li XL, Li Y, Gu ZL, Zheng CS, et al. (2010) Genetic variation of chicken *MC1R* gene in different plumage colour populations. Br Poult Sci 51: 734–739.
8. Gros JG, Borowsky R, Tabin CJ (2009) A novel role for *McIr* in the parallel evolution of depigmentation in independent populations of the caviafe *Ayous mexicanus*. PLoS Genet 5(7): e1000326.
9. Rosenblum EB, Rompler H, Schoneberg T, Hoekstra HE (2010) Molecular and functional basis of phenotypic convergence in white lizards at White Sands. Proc Natl Acad Sci USA 107: 2113–2117.
10. Herzog G, Matsuha C, Merila J (2010) Sequence variation in the melanocortin-1 receptor gene (*Mc1r*) does not explain variation in the degree of melanin in a widespread amphibian. Ann Zool Fennici 47: 37–45.
11. Corso J, Gonçalves GL, de Freitas TRO (2012) Sequence variation in the melanocortin-1 receptor (*MC1R*) pigment gene and its role in the cryptic coloration of two Southern American sand lizards. Genet Mol Biol 35: 81–87.
12. Mieletti S, Parra E, Rountman EJ (2012) Adaptive Color Polymorphism and Unusually High Local Genetic Diversity in the Side-Blotted Lizard, *Uta stansburiana*. PLoS One 7: e47694.
13. Raya P, Guarino FM, Turano M, Polasek G, Rippa D, et al. (2010) The blue lizard spandrel and the island syndrome. BMC Evol Biol 10: 209.
14. Bagnara JT, Fernandez PJ, Fujii R (2007) On the blue coloration of vertebrates. Pigment Cell Res 20: 14–26.
15. Parker AR (1998) The diversity and implications of animal structural colours. J Exp Biol 201: 2343–2347.
16. Nunes VL, Miraaldo A, Beaumont MA, Butlin RK, Paulo OS (2011) Association of *Mc1r* variants with ecologically relevant phenotypes in the European oscillated lizard, *Lacerta lepida*. J Evol Biol 24: 2259–2266.
17. Harris LJ, Arnold EN (1999) Relationships of Wall Lizards, *Podarcis* (Reptilia: Lacertidae) Based on Mitochondrial DNA Sequences. Copeia 1999: 749–754.
18. Arnold EN, Arrabis O, Carranza S (2007) Systematics if the Palaearctic and Oriental lizard tribe Lacertini (squamata: Lacertidae: Lacertinae), with descriptions of eight new genera. ZooKeys 1430: 1–86.
19. Eizirik E, Morard M (1984) Der inselmelanismus der Eidechsen und seine Entstehung im Streit der Meinungen. Zool Anz 152: 317–321.
20. Kramer G (1949) Uber Inselmelanismus bei Eidechsen. Z Indukt Abstamm Ver 83: 157–164.
21. Eizirik E (1949) Die Eidechsen der spanische Mittelmeeinseln und ihre Rassenauflsaltung im Lichte der Evolution. Mitt Zool Mus Berlin 26: 1–225.
22. Brown RP, Terrasa B, Perez-Mellado V, Castro JA, Hoskisson PA, et al. (2008) Bayesian estimation of post-Messinian divergence times in Balearic Island lizards. Mol Phylogenet Evol 46: 350–359.
23. Terrasa B, Perez-Mellado V, Brown RP, Picornell A, Castro JA, et al. (2009) Foundations for conservation of intraspecific genetic diversity revealed by analysis of phylogeographical structure in the endangered endemic lizard *Podarcis lilfordi*. Diversity Distrib 15: 207–221.
24. Hartmann M (1953) Die Rassenauflsaltung der Blearischeninsel Eidechsen. Zool Jb Phys 64: 86–96.
