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Review

Convergence in pigmentation at multiple levels: mutations, genes and function

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Convergence—the independent evolution of the same trait by two or more taxa—has long been of interest to evolutionary biologists, but only recently has the molecular basis of phenotypic convergence been identified. Here, we highlight studies of rapid evolution of cryptic coloration in vertebrates to demonstrate that phenotypic convergence can occur at multiple levels: mutations, genes and gene function. We first show that different genes can be responsible for convergent phenotypes even among closely related populations, for example, in the pale beach mice inhabiting Florida's Gulf and Atlantic coasts. By contrast, the exact same mutation can create similar phenotypes in distantly related species such as mice and mammoths. Next, we show that different mutations in the same gene need not be functionally equivalent to produce similar phenotypes. For example, separate mutations produce divergent protein function but convergent pale coloration in two lizard species. Similarly, mutations that alter the expression of a gene in different ways can, nevertheless, result in similar phenotypes, as demonstrated by sister species of deer mice. Together these studies underscore the importance of identifying not only the genes, but also the precise mutations and their effects on protein function, that contribute to adaptation and highlight how convergence can occur at different genetic levels.

Keywords: adaptation; *Agouti*; colour; *melanocortin-1 receptor*; parallel evolution; *Peromyscus*

1. INTRODUCTION

Convergence—the repeated evolution of similar phenotypes serving the same ecological function in two or more taxa—has long been of interest to evolutionary biologists. On the one hand, phenotypic convergence among populations or species experiencing similar environmental pressures strongly suggests that these traits have evolved by natural selection (Harvey & Pagel 1991). On the other hand, convergence at the genetic level (i.e. the same genes and/or mutations are responsible for similar phenotypes) suggests that genetic constraints limit the available variation upon which natural selection can act, thereby influencing the course of evolutionary change (Wake 1991; Gould 2002). Convergent evolution, thus, informs us about the ultimate and proximate mechanisms generating diversity and can reveal the extent to which the evolutionary process is both repeatable and predictable (Gould 2002; Conway Morris 2003).

Over the last two decades, molecular phylogenetic analyses in a wide range of taxa have shown that natural selection frequently results in the evolution of convergent phenotypes both within (e.g. Brower 1994; Wang & Shaffer 2008) and between species (e.g. Losos *et al.* 1998; Blackledge & Gillespie 2004). More recently, however, increased power to identify genes responsible for adaptive traits in natural populations (reviewed in Ellegren & Sheldon 2008; Stinchcombe & Hoekstra 2008; Mackay *et al.* 2009; Slate *et al.* 2009) has permitted examination of the proximate mechanisms of convergent evolution as well (reviewed in Hoekstra & Coyne 2007; Stern & Orgogozo 2008, 2009; Gompel & Prud'homme 2009). There are multiple cases in which the same genes are responsible for adaptation in distantly related taxa and, conversely, multiple cases in which different genes produce similar phenotypes in closely related taxa (Arendt & Reznick 2008). Thus, evolutionary distance does not necessarily predict genetic mechanisms underlying convergent adaptations (for this reason, we do not distinguish between 'convergent' and 'parallel' evolution; Arendt & Reznick 2008). Because there are relatively few studies that have precisely characterized the molecular changes responsible for adaptive phenotypic change, it is unclear how often 'genetic convergence' (i.e. same gene) is mirrored at the mutational and functional levels.

In terms of taxonomic breadth, genetic and developmental mechanism and ecological function,

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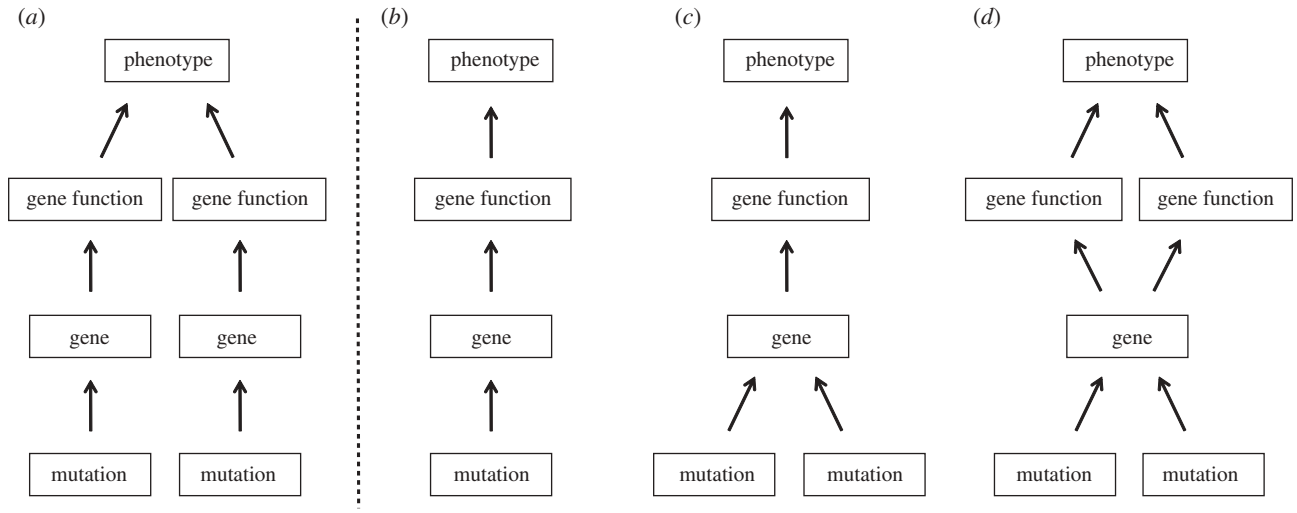

Figure 1. Genetic convergence at multiple levels. Similar phenotypes, serving the same ecological function, can evolve by: (*a*) changes in different genes; (*b*) the same mutation in the same gene; (*c*) different mutations in the same gene that have similar functional consequences and (*d*) different mutations in the same gene that affect gene function or expression in different ways.

pigmentation is one of the best-studied morphological features in natural populations of animals (reviewed in Hoekstra 2006; Protas & Patel 2008; Hubbard *et al.* 2010). This wealth of knowledge about pigmentation allows us to study convergence in colour at multiple levels of biological organization—from phenotype to mutational mechanism. In this review, we describe recent pigmentation studies performed in natural populations that reveal how convergent phenotypes in different taxa can occur by the evolution of (i) different genes; (ii) the same gene and the same mutation; (iii) the same gene but different functionally *distinct* mutations; or (iv) the same gene but different functionally *equivalent* mutations (figure 1). In the discussion, we consider how identifying the precise mutations, genes and functional mechanisms responsible for convergent phenotypes will allow us to address fundamental questions about the genetic basis of adaptation and, more generally, the evolutionary process.

2. PIGMENTATION AS A MODEL SYSTEM

Colour phenotype in most vertebrates is largely determined by two factors: the spatial distribution of pigments across the body and the type of pigments deposited along individual hairs, feathers or scales. Although the cellular and molecular events governing pigmentation patterning are poorly understood, the genetic and physiological bases of pigment synthesis and deposition have been widely studied in mammals (Jackson 1994; Jackson *et al.* 1994). The type of pigment produced is governed largely by the interaction of two genes: the *melanocortin-1 receptor* (*Mc1r*) and its antagonist, *Agouti* (Barsh 1996). The signalling activity of the transmembrane Mc1-receptor at the surface of pigment-producing cells (i.e. melanocytes) leads to the production of the dark (brown-black) pigment, eumelanin. The binding of its ligand, the secreted molecule Agouti, causes a switch to light (yellow-red) phaeomelanin production. Studies in *Mus* have shown that *Agouti* is likely to be involved in both pigment pattern and pigment-type switching by producing two transcriptional isoforms: the first is expressed in the ventral skin and is associated with dorsal–ventral differences in pigmentation (Bultman *et al.* 1994; Vrieling *et al.* 1994) and the second is expressed in a timed pulse during hair growth and is responsible for the banded hair pattern typical of most rodent hairs (Vrieling *et al.* 1994).

In addition to *Mc1r* and *Agouti*, a growing list of pigmentation genes identified in laboratory models provides a wealth of candidate genes to understand the mechanisms underlying the convergence of colour phenotypes at different levels of biological organization (Hoekstra 2006; Hofreiter & Schöneberg 2010; Hubbard *et al.* 2010). At the genetic level, mutations in genes involved in the same functional pathway may be more likely to produce comparable colour phenotypes (e.g. changes in the regulation of melanocyte migration generally cause differences in the distribution of pigments across the body, whereas changes in the enzymatic reactions governing melanin synthesis generally cause differences in the type and distribution of pigment along individual hairs). At the phenotypic level, different colour variants can serve the same ecological function: for example, both a reduction in the pigmented body regions and a switch to the lighter pigment type in hairs can provide camouflage in light-coloured habitats.

Here, we focus on the convergent evolution of colour phenotypes that confer the same ecological function: crypsis. Cryptic coloration minimizes detection by visually hunting predators. Arguably, the simplest form of crypsis is the correlation between dorsal pigmentation and substrate colour in terrestrial vertebrates (e.g. Dice & Blossom 1937). Because of the wide range of substrate colours—ranging from black lava flows to white sand dunes—that have formed independently in geographically dispersed regions, crypsis provides an ideal opportunity to examine phenotypic convergence.

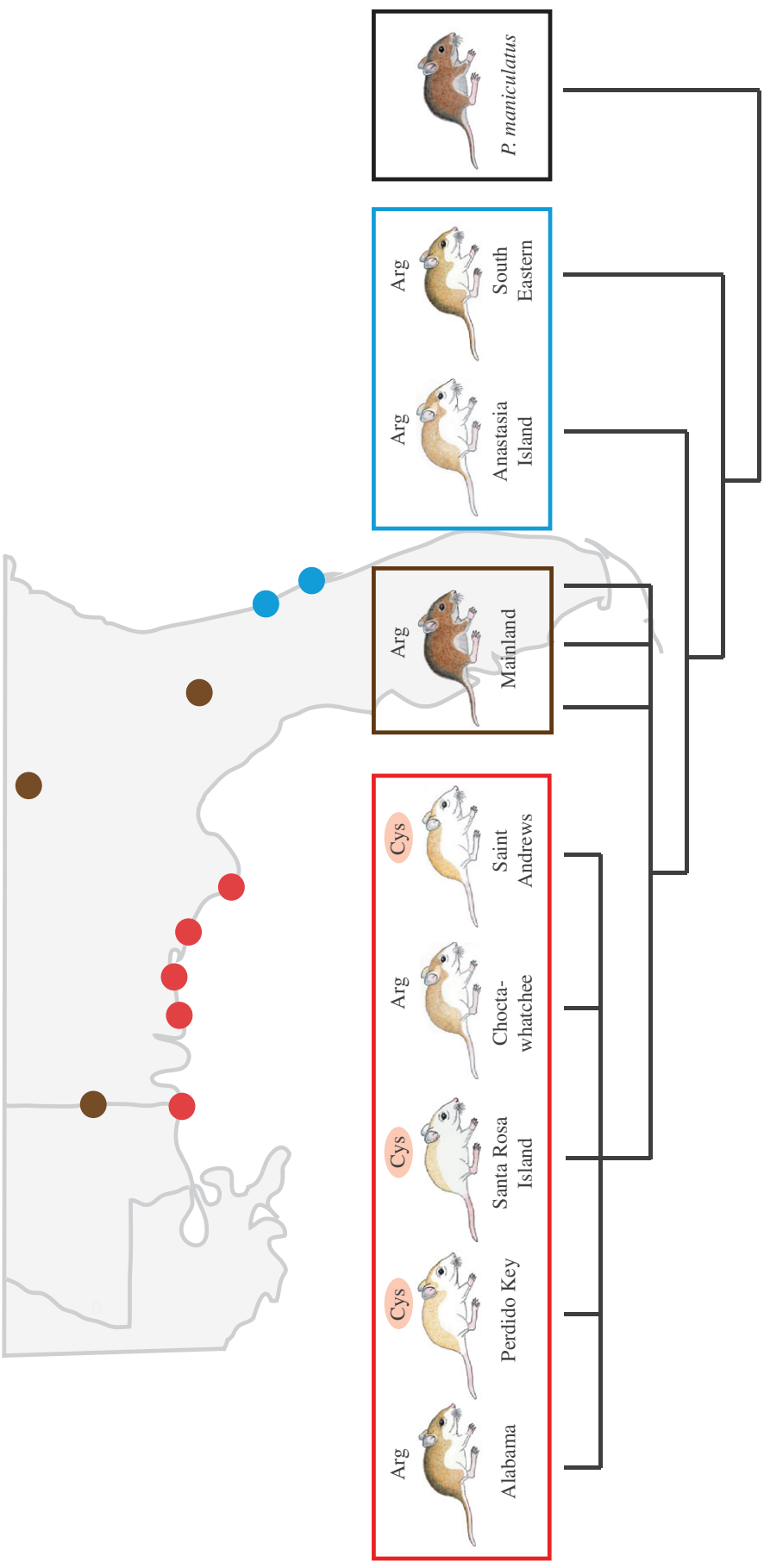

Figure 2. Light coloration in beach mice has evolved independently through changes in different genes. Map of the southeastern USA, in which circles represent collecting locales for *P. polionotus*: three mainland subspecies (brown), five Gulf Coast (red) and the two Atlantic Coast (blue) beach mouse subspecies. Atlantic and Gulf Coast beach mice have independently evolved lighter coloration, compared with their dark ancestor (cartoons). A mutation ($Arg^{65}Cys$) in *Mc1r* contributes to light coat colour in Gulf Coast subspecies, but not in Atlantic Coast subspecies. The most common amino acid at site 65 in each subspecies is shown: ancestral (Arg) and derived (Cys, highlighted in light red). The schematized tree represents the phylogenetic relationships of *P. polionotus* subspecies (*P. maniculatus* is shown as an outgroup; adapted from Steiner *et al.* 2009).

3. DIFFERENT GENES PRODUCE SIMILAR PHENOTYPES

There are several known cases in which mutations in different genes can result in similar phenotypic effects. One striking example involves the dorsal coat colour of oldfield mice (*Peromyscus polionotus*), which closely match their local substrate and thereby reduce the attack rate of predators (Vignieri *et al.* 2010). These mice typically inhabit densely vegetated oldfields with dark loamy soils in the southeastern USA, where they have a dark brown dorsal coat and a grey ventrum. However, mice that have colonized the sandy coastal dunes of the Gulf and Atlantic coasts of Florida have a significantly lighter colour (figure 2; Howell 1920; Sumner 1929; Mullen & Hoekstra 2008). In general, these 'beach mice' (i) differ in the distribution of pigmentation, largely associated with an upward shift in their dorsal–ventral boundary, and (ii) in areas that are pigmented, have both a lower density of pigment and more light phaeomelanin than mainland mice (Steiner *et al.* 2007). Although all beach mice are lighter than mainland mice, different subspecies vary significantly in several pigmentation traits, and remarkably, Atlantic Coast subspecies are more similar in colour pattern to Gulf Coast subspecies than their neighbouring subspecies (Steiner *et al.* 2009). An intraspecific phylogeny of Gulf and Atlantic Coast subspecies, however, shows that beach mice are not monophyletic (figure 2), and the hypothesis that there was a single origin of the light 'beach mouse' phenotype can be rejected statistically (Steiner *et al.* 2009). Thus, the lighter overall colour of Gulf and Atlantic beach mice probably represents the independent evolution of camouflage. In this system, then, one can ask whether the same genes were targets of adaptive change multiple times or whether different genes were modified to produce similar phenotypic results.

As a first step in determining the genetic basis of colour variation in these mice, Hoekstra and co-workers generated a genetic intercross between a mainland subspecies (*P. polionotus subgriseus*) and the Santa Rosa Island beach mouse subspecies (*P. p. leucocephalus*) from Florida's Gulf Coast. Using a quantitative trait loci (QTL) approach, three genomic regions, which together explain most of the colour variation between the two subspecies, were identified (Steiner *et al.* 2007). These regions each contain a candidate pigment gene, *Mc1r* (Hoekstra *et al.* 2006), *Agouti* (Steiner *et al.* 2007) and *Corin* (Jacobs-Palmer *et al.* submitted), which encodes a serine protease acting upstream of *Agouti* signalling (Enshell-Seijffers *et al.* 2008). Changes in both *Agouti* and *Corin* mRNA expression level are statistically associated with colour differences, but the causal mutations in these loci have yet to be identified. Although there are no measurable differences in *Mc1r* expression levels between beach and mainland mice (Steiner *et al.* 2007), a single amino acid substitution in the first intracellular region of the Mc1r protein ($Arg^{65}Cys$) is strongly associated with colour differences. Cell-based functional assays confirm that the Mc1r $Arg^{65}Cys$ mutation reduces ligand binding, causing a decrease in receptor signalling, and consequently a reduction in the production of dark eumelanin (figure 3*b*; Hoekstra *et al.* 2006). This single mutation explains approximately 30 per cent (depending on the specific trait) of the colour difference between mainland and beach mice in this cross. In nature, ^{65}Cys is fixed and derived in Santa Rosa Island beach mice and absent from mainland populations (Hoekstra *et al.* 2006; Mullen *et al.* 2009).

By contrast, despite their similarities in colour pattern, the Atlantic Coast beach mice do not harbour the ^{65}Cys mutation (Hoekstra *et al.* 2006), suggesting that the light pigment phenotype has a different mutational basis. *Mc1r* sequence comparison between the mainland and the Atlantic Coast beach mice revealed four new amino acid mutations. However, none of these mutations overlaps with previously described mutations known to affect coloration, none is fixed in an Atlantic Coast subspecies or strongly correlated with light coloration and none alone affected receptor function measured using *in vitro* assays (figure 3*b*; Steiner *et al.* 2009). Therefore, although it remains formally possible that *Mc1r* is responsible for light coats in Atlantic Coast mice (e.g. via a change in the regulatory region), results to date strongly suggest that different genes are responsible for the convergent phenotypes of the Atlantic and Gulf coasts beach mouse populations.

4. SAME GENE AND MUTATION IN DIFFERENT SPECIES PRODUCE SIMILAR PHENOTYPES

Although different genetic paths can be taken within a species, the same mutation can sometimes contribute to phenotypic convergence between wildly different species (figure 1*b*). Several examples involve *Mc1r* (figure 3). In a recent ancient DNA study, the complete coding sequence of the *Mc1r* gene was sequenced from an approximately 43 000-year-old mammoth (*Mammuthus primigenius*) bone excavated in Siberia (Römpler *et al.* 2006). The sequenced individual was polymorphic at three amino acid sites, including the exact same mutation (C to T nucleotide and Arg to Cys amino acid) at the homologous position identified in the Gulf Coast beach mice ($Arg^{65}Cys$). To functionally verify the effect of this mutation, Römpler *et al.* constructed expression vectors containing the two different *Mc1r* alleles and showed a difference in receptor signalling, similar in magnitude to that observed in beach mice (figure 3*b*). These results raise the possibility that Pleistocene mammoths were polymorphic for hair colour; in fact, both dark and light coloured mammoth hair has been recovered from permafrost mummies. However, the ecological relevance of mammoth colour variation remains unclear.

Although functional validation of *Mc1r* mutations is rare, other studies have shown that mutations at homologous sites of *Mc1r* are statistically associated with colour differences in divergent taxa. For example, bananaquits (Mundy *et al.* 2004), Japanese quail (Nadeau *et al.* 2006) and chickens (Ling *et al.* 2003) all share the same non-synonymous substitution ($Glu^{92}Lys$), probably leading to Mc1r's constitutive activation and ultimately a melanic phenotype. Similarly,

the Asp^{119}Asn mutation is associated with melanism in Monarch flycatchers from the Solomon Island (Uy *et al.* 2009), domestic pigs (Kijas *et al.* 1998) and several strains of sheep (Vage *et al.* 1999). Finally, melanic arctic skuas (Mundy 2005) and melanic pocket mice (Nachman *et al.* 2003) both have an Arg^{233}His mutation. In all of these cases, because the species sharing a common mutation are so divergent, mutational convergence probably represents the independent origin and subsequent selection of the same mutation.

5. SAME GENE BUT DIFFERENT MUTATIONS PRODUCE SIMILAR PHENOTYPES

Similar phenotypes can also be produced by different mutations in the same gene. This can be accomplished in two distinct ways—the mutations either have the same effect on gene function (figure 1*c*) or have distinct effects on gene function but still produce ecologically equivalent phenotypes (figure 1*d*). Here we highlight two recent studies performed in lizards and mice—involving either coding-region mutations that directly affect protein function or regulatory mutations that modify gene expression—that illustrate how different mutations can produce ecologically equivalent phenotypes.

(a) *Mutations in the coding region*

The White Sands of New Mexico represent a geologically young habitat (approx. 6000 years old) comprised of stark white gypsum sands. Three species of lizards inhabiting these sand dunes have evolved blanched dorsal coloration compared with their conspecifics in the surrounding Chihuahuan Desert, most likely as an adaptation for crypsis (Rosenblum 2006). Comparison of *Mc1r* sequences between light and dark forms of each species revealed a derived amino acid change in the blanched morph of all three species (Rosenblum *et al.* 2004). Cell-based functional assays have shown that there is no measurable effect of the derived *Mc1r* mutation in one species, the common lesser earless lizard (*Holbrookia maculata*), despite a strong statistical association between *Mc1r* mutation and colour (figure 3*c*; Rosenblum *et al.* 2010). This result demonstrates two points. First, functional verification of statistical associations is necessary before genes can be implicated in phenotypic change. Second, the blanched coloration in earless lizards is most likely caused by changes in a different gene(s), although, as is the case of the Atlantic Coast beach mice, further investigation is needed to rule out changes in *Mc1r* expression.

However, in the other two White Sands lizard species, the eastern fence lizard (*Sceloporus undulatus*) and the little striped whiptail (*Aspidoscelis inornata*), the derived amino acid replacement causes a change in Mc1-receptor activity (figure 4; Rosenblum *et al.* 2010). Moreover, the amino acid replacements in each species generate blanched phenotypes via entirely different functional mechanisms. In one species, the functional effect of the *Mc1r* mutation is similar to that observed in beach mice. Specifically, in whiptail lizards, the Thr^{170}Ile mutation causes a reduction in Mc1r signalling similar in magnitude to the effects of the Arg^{65}Cys mutation observed in Gulf Coast beach mice and mammoths (figure 3*c*). Thus in the comparison between whiptail lizards and beach mice, different mutations in the same gene produce similar blanched phenotypes via similar effects on gene function, in this case diminished receptor signalling (figure 1*c*). In fence lizards, the *Mc1r* mutation also results in a partial loss of function, affecting the transmembrane domain of the receptor. However, this mutation (His^{208}Tyr) compromises the receptor's function via a different mechanism: the mutation in blanched fence lizards prevents Mc1r from efficiently integrating into the melanocyte membrane (as opposed to integrating efficiently but then impeding proper signal transduction; Rosenblum *et al.* 2010). Thus, in the comparison between fence and whiptail lizards, different mutations in the same gene produce similar blanched phenotypes, but through different functional mechanisms (figure 1*d*).

(b) *Mutations in the regulatory region*

Far from the white sandy Florida beaches where its sister species, *P. polionotus*, evolved a light coat colour, deer mice (*P. maniculatus*) living on the pale dunes of the Nebraska Sand Hills also have dorsal coats that match their local habitat. Like beach mice, this adaptive pigmentation change has evolved recently (less than 8000 years ago), and visual predators are likely the agent of selection favouring a cryptic, light coat colour (figure 5; Dice 1947). The golden colour of Sand Hills mice is caused primarily by a change in the type and distribution of pigments on individual hairs (figure 5*c*). Like the hairs of most rodents, *Peromyscus* dorsal hairs contain a short, subterminal band of light phaeomelanic pigment on an otherwise dark eumelanic background. In Sand Hills mice, this light band is markedly wider than in their darker conspecifics.

Laboratory crosses show that this 'wideband' phenotype (McIntosh 1956) is inherited as a single dominant allele (Dodson 1982) and that there is a perfect association between this phenotype and nucleotide variation in the *Agouti* gene, but not at other loci (Linnen *et al.* 2009). Moreover, *Agouti* mRNA levels are higher in *P. maniculatus* carrying the wideband allele than in their wild-type counterparts. This expression difference is maintained in wideband/wild-type heterozygotes, demonstrating that a *cis*-acting mutation(s) in, or linked to, the *Agouti* gene is causal (Linnen *et al.* 2009). These findings are consistent with *Agouti*'s role in producing banded hairs. Specifically, when *Agouti* is expressed, melanocytes at the base of hair follicles switch from eumelanin to phaeomelanin production. As hairs grow, a pulse of *Agouti* expression results in the formation of phaeomelanin bands (Vrieling *et al.* 1994). In Sand Hills mice, phaeomelanin bands are widened because this pulse of *Agouti* expression during hair growth is both longer and higher than in wild-type mice (Linnen *et al.* 2009).

Nebraska *P. maniculatus* and Florida *P. polionotus* (beach mice) thus represent an example of phenotypic convergence, in which overall light colour is an adaptation for crypsis in light-coloured environments.

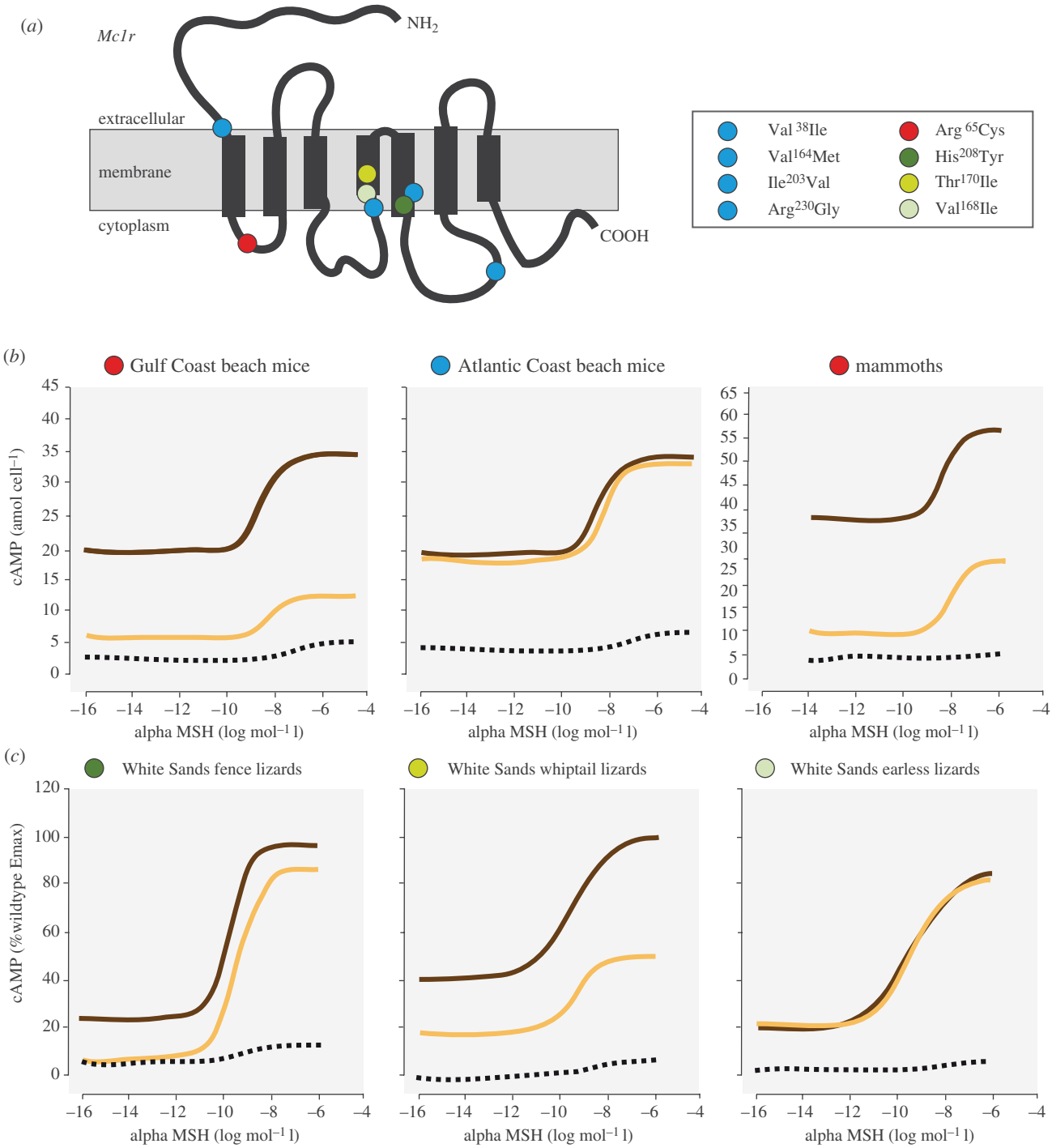

Figure 3. Mutations in *Mc1r* change receptor signalling in some, but not all, species with light coloration. (*a*) Schematic of the Mc1r protein showing the position of amino acid variants in beach mice from Florida's Gulf (red) and Atlantic (blue) coasts, mammoths (red) and blanched lizards from White Sands, New Mexico (shades of green). Functional analysis of *Mc1r* alleles in (*b*) beach mice and mammoths and (*c*) lizards. Intracellular cyclic adenosine monophosphate (cAMP) accumulation was measured in response to increasing concentrations of the agonist alpha melanocyte-stimulating hormone (alpha MSH). For each taxon, the response curves for the dark *Mc1r* allele (brown), light allele (yellow) and control (black) are shown. Some mutations, but not all, cause a decrease in receptor signalling associated with lighter pigmentation (data taken from Hoekstra *et al.* 2006; Römpler *et al.* 2006; Steiner *et al.* 2009; Rosenblum *et al.* 2010).

However, in this case, the same gene, *Agouti*, causes light coloration through distinct mutations leading to distinct functional changes (recall that a change in *Agouti* expression also contributes to light coloration in beach mice). Moreover, contrary to the case of the White Sands lizards, the functional change in both mice species appears to alter patterns of gene expression, not protein function. These changes in *Agouti* expression produce ecologically equivalent, but distinct, phenotypes: although the light beach mouse pelage is due in large part to a pronounced change in pigment patterning (i.e. a change in dorsal–ventral coloration), the light Sand Hills coat is produced by an altered distribution of pigments on

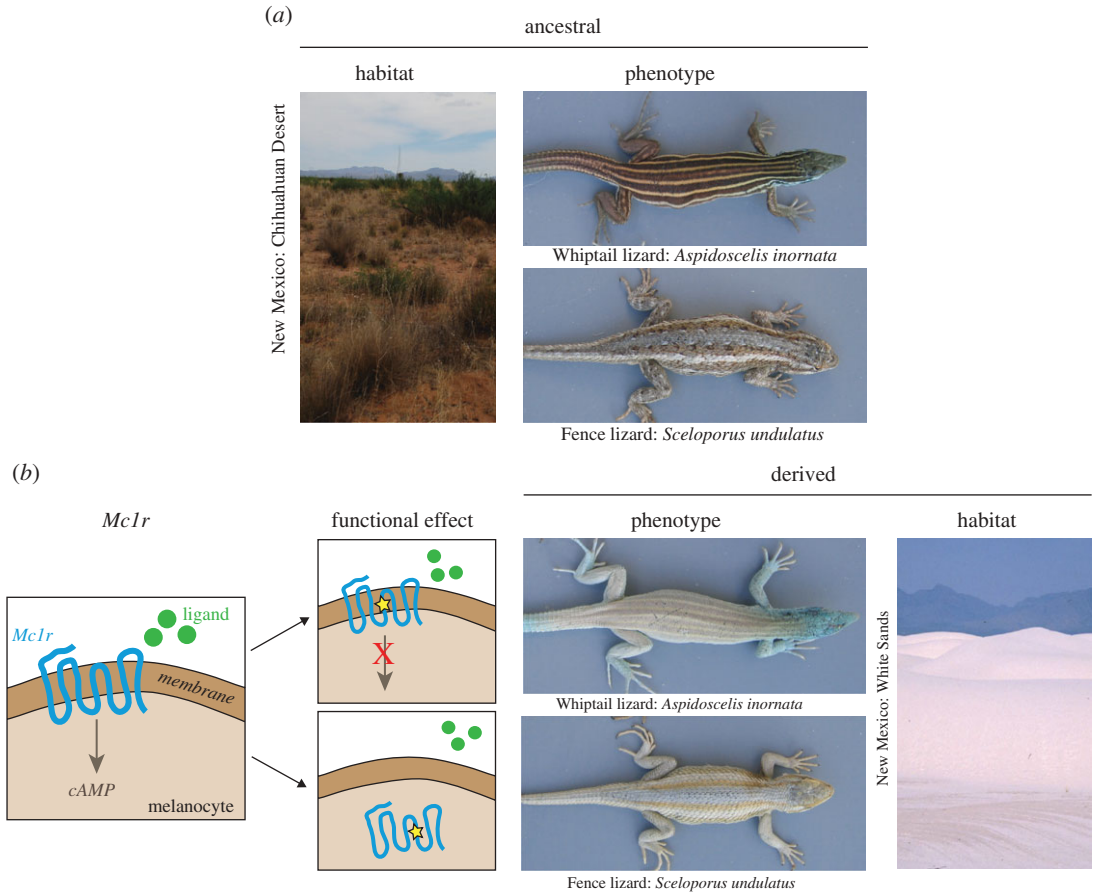

Figure 4. Genetic convergence and functional divergence in lizards. (*a*) In the Chihuahuan Desert of New Mexico, both the little striped whiptail (*A. inornata*) and the eastern fence lizard (*S. undulatus*) have a dark dorsal colour that closely matches the local soil colour. (*b*) Compared with their dark counterparts, a blanched phenotype has independently evolved in whiptail and fence lizards that colonized the dunes of White Sands. In both species, the derived phenotype results from an amino acid mutation in Mc1r (schematized). In the whiptail lizard, the derived mutation impairs Mc1r signalling activity (top right panel). In the fence lizard, a different mutation diminishes the efficiency of Mc1r integration into the melanocyte membrane (bottom right panel). Phenotypic convergence (blanched colour) is reflected at the genetic level (mutations in the same gene, *Mc1r*), but not at the functional level (mutations have different effects on protein function).

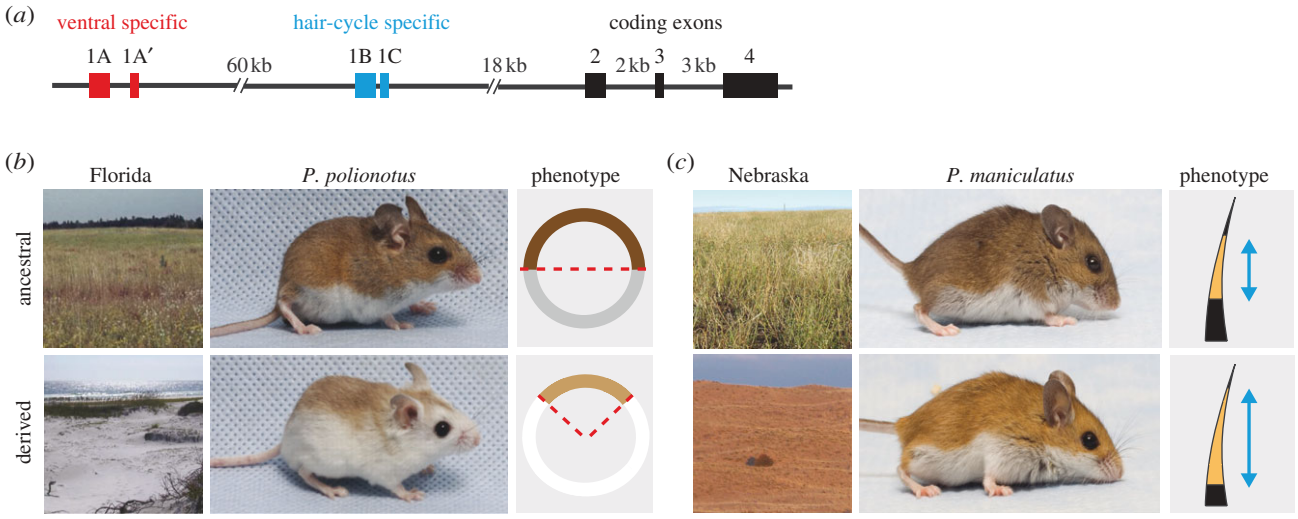

Figure 5. Genetic convergence and functional divergence in deer mice. (*a*) The *Agouti* gene produces two transcriptional isoforms. Both contain exons 2 to 4, but they differ in the first, untranslated exons—the ventral-specific isoform contains exons 1A and 1A′, whereas the hair-cycle-specific isoform contains exons 1B and 1C. In populations of two sister species, (*b*) *P. polionotus* and (*c*) *P. maniculatus*, derived mutations in the *Agouti* gene result in an overall lighter coat colour, which is cryptic in novel habitat (the sand dunes of the Florida Coast or Nebraska's Sand Hills, respectively). However, *Agouti* mutations act through different changes in gene expression: in beach mice, changes in *Agouti* expression may primarily modify colour pattern, whereas in Sand Hills mice, *Agouti* mutations mainly modify pigment type and distribution on individual hairs (i.e. a wider phaeomelanin band). These results suggest that different isoforms might be targeted—the ventral *Agouti* isoform in beach mice and the hair-cycle-specific isoform in Sand Hills mice.

individual hairs (figure 5). One simple hypothesis that explains how expression changes in the same gene produce these different phenotypic outcomes is that the causal mutations affect the two different *Agouti* mRNA isoforms (figure 5*a*). While the causal *Agouti* mutations in both *P. polionotus* and *P. maniculatus* remain to be identified, association studies in both species are underway.

6. DISCUSSION

Together, these studies on adaptive colour evolution performed in mice, mammoths and lizards illustrate how convergence at one level (i.e. the gene) does not necessarily imply convergence at other mechanistic levels (e.g. the mutation, gene function or gene expression). Evaluating convergence across levels is thus not merely academic: even when the same gene is involved, differences in the functional effects of different mutations can have important organism-level consequences. As we discuss here, extending the study of convergence to these additional levels has the potential to reveal novel insights about both the proximate and ultimate mechanisms underlying phenotypic convergence.

7. CONVERGENCE AT THE GENETIC LEVEL: LESSONS FROM STUDIES OF *Mc1r*

Just as pigmentation is an excellent model system in which to study genetic convergence, the case studies we present here highlight that *Mc1r*, with its simple genetic structure (i.e. a single coding exon) and well-understood protein function, is emerging as a model gene for understanding convergence at the mechanistic level. To date, *Mc1r* studies in a wide range of taxa have unequivocally demonstrated that the same gene can be responsible for convergent phenotypes. Beyond demonstrating widespread convergence at the gene level, these studies allow for an investigation of how often the same mutation versus a different mutation with an equivalent functional effect gives rise to similar phenotypes.

(a) *Mutational convergence*

For convergence to span all levels of biological organization, the same mutation must occur more than once, have the same functional and phenotypic consequences on different genetic backgrounds and be positively selected in different populations or species experiencing similar ecological pressures (figure 1*b*). Examples of mutational convergence (e.g. mice and mammoths) suggest that evolution is highly repeatable. However, we must be cautious in claiming convergence at the mutational level because observing the same causal mutation in multiple taxa does not necessarily imply that the mutation arose more than once. Two alternatives to mutational convergence include: (i) the causal mutation was present in an ancestral population (e.g. Colossimo *et al.* 2005; Barrett & Schluter 2008) or (ii) the causal mutation arose in one lineage and was transferred to another lineage via hybridization and introgression (e.g. Anderson *et al.* 2009). Although these two alternatives might still represent convergence at the phenotypic level (i.e. there have been independent bouts of selection), they are not caused by independent mutational events.

Fortunately, we can distinguish between ancestral variation, introgression and mutational convergence by examining evolutionary histories for both the causal alleles and the populations/species in which they occur. Specifically, both ancestral variation and introgression predict that haplotypes bearing the causal mutation will form a monophyletic group, whereas mutational convergence predicts that haplotypes will not be monophyletic. For *Mc1r*, several studies identified the exact same mutation in taxa distantly related enough that shared ancestral variation and introgression are highly unlikely, providing examples of 'complete' convergence (figure 1*b*). Mechanisms for the same mutation appearing repeatedly in different taxa could be: mutational hotspots (e.g. Chan *et al.* 2010), few mutational options for achieving particular phenotypes (e.g. Bull *et al.* 1997) and/or a relatively large net selection coefficient for a particular mutation on multiple genetic backgrounds.

(b) *Mutational divergence (but functional convergence)*

Different mutations—either in the same gene or in different genes—can lead to the same functional effect on protein function and/or expression and thus to the same phenotypic change. Mutational divergence but functional convergence is illustrated by the identification, in mammals and lizards, of different *Mc1r* amino acid substitutions that result in the same effect on Mc1r signalling activity (figure 3). Perhaps the simplest, and most common, type of functional convergence at the genetic level is a complete disruption of gene function caused by separate mutations (e.g. a premature stop codon or deletion that produces a non-functional protein product). For example, independent deletions in the *OCA2* gene lead to repeated loss of protein function and thus pigmentation in cavefish (Protas *et al.* 2006), and deletions in *Agouti* repeatedly lead to melanism in deer mice (Kingsley *et al.* 2009; discussed subsequently). Surprisingly, despite the large number of potential knock-out mutations in *Mc1r* (i.e. it is a large mutational target), such mutations have not been reported in natural populations. Three potential explanations include: *Mc1r* null alleles (i) exist in nature but have not yet been described, (ii) are likely to be completely recessive and therefore more likely to be lost to drift, and (iii) have negative pleiotropic effects on fitness (e.g. in addition to its role in pigmentation, *Mc1r* affects pain tolerance; Mogil *et al.* 2005). Distinguishing among these alternatives will require identifying additional *Mc1r* alleles in natural populations and characterizing their phenotypic effects (on colour and other traits) in both heterozygotes and homozygotes. By focusing on the genetic bases of functional convergence (i.e. determining what types of mutations exist in nature and are selectively

favoured and what types of mutations do not appear in the wild due to their patterns of dominance or pleiotropic effects), we can gain insight into constraints acting at the genetic level to shape the evolution of genes and populations.

(c) *Mutational divergence (and functional divergence)*

As the functional dissection of genetic and phenotypic convergence in lizard *Mc1r* and mouse *Agouti* vividly illustrates, the same genes can create ecologically equivalent traits via different functional mechanisms. Moreover, these examples show that separate mutations can have divergent functional effects on gene/protein function (*Mc1r* in lizards) and on gene expression (*Agouti* in mice). Importantly, mutations with different functional effects, even when they occur in the same gene, can lead to differences in trait expression. For example, in the White Sands lizards, *Mc1r* mutations with distinct functional effects—impaired protein signalling or membrane integration efficiency—can lead to blanched coloration. However, the functional consequences of the mutations lead to differences in allelic dominance: light colour is dominant in one species, but largely recessive in the other (Rosenblum *et al.* 2010). These differences in dominance can affect the chance that a new allele is lost due to drift, the rate of allele frequency change driven by selection and the spatial distribution of alleles in nature. In addition to dominance, other important genetic parameters are allele-specific, such as epistasis and pleiotropy, which also contribute to net selection coefficients. Therefore, understanding the functional effects of mutations can inform our understanding of the likelihood a particular allele will be favoured and thus be involved in adaptive phenotypic change.

8. DIVERGENCE AT THE GENETIC LEVEL: COMPARISONS AMONG PIGMENTATION GENES

(a) *Comparison of* Mc1r *and* Agouti

Like *Mc1r*, *Agouti* has also been implicated in pigmentation differences in several organisms (Kingsley *et al.* 2009 and references therein), and interesting parallels—and differences—emerge by comparing phenotypic convergence caused by these two loci. First, just as we observe functional convergence between two different mutations in *Mc1r* in lizards and mice, independent changes that similarly affect *Agouti* function have been identified in deer mice. For example, convergence at the functional level for *Agouti* was reported in several populations of *P. maniculatus*: melanic phenotypes are repeatedly produced by independent deletions in *Agouti* (Kingsley *et al.* 2009). These deletions eliminate *Agouti* expression and function. As noted earlier, full loss-of-function mutations have not been described in nature for the more intensively studied *Mc1r*. Second, like *Mc1r*, we observed functional divergence between two different mutations in *Agouti* that contribute to adaptive colour variation (in beach and Sand Hills mice). Unlike *Mc1r*, however, these mutations alter gene expression, not protein function, suggesting that adaptive changes in expression may be more common in *Agouti* than in *Mc1r*. Together, these examples illustrate how comparison of convergence and divergence in mutation and function across different genes can shed light on the functional flexibility of the genes of interest, thereby allowing us to hypothesize about the relative role that different genes play in adaptive evolution. In contrast, because selection acts on mutations (i.e. alleles, not genes) that can have different functional consequences, and hence can vary in dominance and pleiotropy, which in turn depend on the genetic background where they occur, evolution may be exceedingly difficult to predict. Testing these alternatives requires that we identify genes (and mutations) that contribute to natural phenotypic variation in an unbiased manner (Kopp 2009).

(b) *Other pigmentation genes*

The examples discussed in this review, along with several other studies (reviewed in Hubbard *et al.* 2010), point to the recurrent role of a few genes (i.e. *Mc1r*, and to a lesser extent, *Agouti*) in producing colour differences among vertebrates. Although the repeated implication of *Mc1r* in colour variation may reflect real biological processes (e.g. constraint, dominance and minimal pleiotropy), it may also stem, at least in part, from ascertainment bias. Thus, in addition to performing detailed functional analyses of candidate pigmentation genes, we also need to perform genome-wide studies in a diverse group of taxa to identify new pigmentation alleles (Kopp 2009). In fact, the two approaches are very complementary: genomic approaches provide a non-biased way to evaluate the relative importance of different pigmentation genes in generating natural colour variation and allow us to test the predictions generated by functional studies of candidate genes. To date, several genes underlying differences in pigmentation have been identified using an unbiased genome-wide approach (table 1). These genomic studies show that a diversity of genes, beyond just *Mc1r* and *Agouti*, contribute to colour variation in natural populations.

9. CONCLUSION

Biologists have long wondered how often the same genes are responsible for the repeated evolution of similar traits. However, the studies we highlight here demonstrate how pinpointing the mutations—not just the genes—underlying convergent phenotypes allows us to test for convergence at levels both below (mutational convergence) and above (functional convergence) the level of the gene. Expanding our view to these additional levels not only deepens our understanding of the proximate mechanisms that generate adaptive phenotypic variation, but also provides novel insights into why (e.g. mutational target size, mutational hotspots, dominance, epistasis and pleiotropy) some evolutionary outcomes are more common than others. Of course, convergence is not limited to the levels of biological organization we discuss here. For example, between the levels of mutation and gene, the same or different nucleotide

Table 1. Genes involved in vertebrate pigment variation identified by an unbiased genomic approach.

species	phenotype	gene	reference
oldfield mouse (*Peromyscus polionotus*)	pigment pattern	*Agouti*	Steiner *et al.* (2007)
		Mc1r	
		Corin	Jacobs-Palmer *et al.* (submitted)
cavefish (*Astyanax* species)	lack of pigment	*Oca2*	Protas *et al.* (2006)
		Mc1r	Gross *et al.* (2009)
threespine stickleback fish (*Gasterosteus aculeatus*)	reduced pigment	*Kitlg*	Miller *et al.* (2007)
Lake Malawi cichlid fish (*Labeotropheus trewavasae*, *Metriaclima zebra*, *M. xantomachus*, *Tropheops* sp.)	increased pigment	*Pax7*	Roberts *et al.* (2009)
North American gray wolf (*Canis lupus*)[a]	increased pigment	*K locus*	Anderson *et al.* (2009)
Soay sheep (*Ovis aries*)	pigment pattern and type	*Tyrp1*	Gratten *et al.* (2006)
		Agouti	Beraldi *et al.* (2006)
human (*Homo sapiens*)	pigment type	*Slc24A5*	Stokowski *et al.* (2007)
		Slc45A2	Sabeti *et al.* (2007)
		Slc24A4	Han *et al.* (2008)
		Tyrp1	Sulem *et al.* (2007, 2008)
		IRF4	
		Mc1r	
		Agouti	
	pigment type	*Kitlg*	Sulem *et al.* (2007, 2008)
		Slc24A4	Han *et al.* (2008)
		Oca2	
		TPCN2	
		IRF4	
		Mc1r	
		Agouti	

[a]The *K locus* was identified by a QTL study as a contributor to pigmentation differences in dogs (Kerns *et al.* 2007). Introgression of this locus in wolf has been shown subsequently.

mutations can produce convergent amino acid substitutions, and above the level of the gene, the same or different genes in the same developmental pathway can have similar impacts on gene interactions and ultimately phenotype. Likewise, phenotypic convergence can be examined at different levels—at a fine grain, we can ask how similar the distribution of pigments are on individual hairs, and at a coarse grain, we can ask how similar the phenotypes are in ecological function. Thus, while evolutionary change is still far from being predictable, the well-studied cases presented here show that evolution can sometimes be repeatable—often the same genes are targeted for adaptive change, but the precise mutations and their effects on protein function can differ.

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