Evolution of Coloration Patterns

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Abstract
There is an amazing amount of diversity in coloration patterns in nature. The ease of observing this diversity and the recent application of genetic and molecular techniques to model and nonmodel animals are allowing us to investigate the genetic basis and evolution of coloration in an ever-increasing variety of animals. It is now possible to ask questions about how many genes are responsible for any given pattern, what types of genetic changes have occurred to generate the diversity, and if the same underlying genetic changes occur repeatedly when coloration phenotypes arise through convergent evolution or parallel evolution.
BACKGROUND

The remarkable diversity of coloration patterns seen in animals is one of the most striking features in the natural world. Examples include *Melanitis* butterflies that resemble dried vegetation, flounder and flatfish that are pigmented on only one-half of their body and alter their appearance to match their surroundings, the bold black and white stripes of zebras, brilliantly colored tropical fish and birds, and cavefish that lack pigment altogether (Figure 1). Countless examples exist of unique colors and patterns for all types of animals. This variation, and the ease of observing it, has made the study of coloration patterns a popular and tractable subject for scientific inquiry. In the past few decades, genetic, molecular, biochemical, and cellular approaches in both model and nonmodel species have allowed us to understand many details of the basis for color patterns during development. In addition, it is possible to surmise, and sometimes experimentally validate, the selective pressures behind animal coloration, allowing one to approach the question of how these patterns evolved.

FUNCTIONS OF COLORATION

When one is studying coloration from an evolutionary perspective, the first question that comes to mind is, What is the function of a particular pattern? In cases such as the dead-leaf butterfly, functional significance is fairly obvious: Appearing leaf-like may cause predators to misidentify potential prey (Figure 2). The functional and evolutionary significance of other examples, such as a zebra’s stripes, is not as obvious. Many functions of coloration have been proposed: concealment, thermoregulation, warning of toxicity, mimicry, sexual selection, and linkage to beneficial characteristics such as immunity and salinity tolerance (reviewed in Roulin 2004). Finally, it is always possible that a certain coloration pattern evolved by chance through processes such as genetic drift.

Concealment is a very common function of coloration. Many animals, for example, sea dragons, when viewed in their natural habitat, blend in almost perfectly with their surroundings (Figure 3a). This is all the more striking when multiple populations of a single species living in different habitats have distinct coloration forms that match each environment (Figure 4). This phenomenon has been observed in many species; rock pocket mice, beach mice, deer mice, and fence lizards are just some examples within the vertebrates (Hoekstra 2006, Hoekstra et al. 2006, Mundy 2005). Rock pocket mice of the southwestern United States and Mexico provide a particularly clear example. Most live predominantly on
Figure 1
Some samples of the diversity of animal coloration patterns. (a) Melanitis butterfly, whose underside resembles the dried vegetation on which it sits. (b) Flatfish that can change coloration on the upper side of its body to blend into the background. (c) A group of zebras with their striking black and white stripes. (d) Blueface angelfish. (e) Rainbow lorikeet. (f) The unpigmented blind cave loach Nemacheilus troglocataractus, from Thailand (image courtesy of R. Borowsky).

lightly colored rocks, and these mice have light-colored fur. However, there are also some populations of rock pocket mice that colonized dark lava flows (some of which are just a few thousand years old), and these individuals have dark fur (Hoekstra & Nachman 2003). It is thought that the melanic form provides better camouflage in the lava flow environment, lessening the chance of predation.

Coloration is often a constant feature of an animal throughout its life. However, there are cases when the animal’s coloration changes at different times and in different environments. This ability to change coloration is often advantageous for animals that move around on different-colored substrates. One classic example is that of the cuttlefish (Figure 3b). Cuttlefish skin changes in both color and texture as the animal moves. It is thought that this change of color and pattern is advantageous for camouflage as well as for signaling to other individuals (Barbato et al. 2007).

Another mechanism by which coloration patterns disguise an animal is by disruptive coloration, a phenomenon in which a color pattern breaks up the animal’s form so that it is difficult to identify the real outline of the animal. A common way of disguising the boundaries of an animal is to hide the eyes by an eye mask pattern and thus distort one of the most identifiable features of a prey item. Also potentially disruptive are black and white lines intersecting the outline of the animal, e.g., tapirs and pandas (Caro 2005).

Whereas many forms of coloration camouflage an animal, there are many examples of conspicuous coloration used as a warning to potential predators. Black, red, orange, and yellow often indicate that the species is distasteful. The yellow and black ant, Crematogaster inflata, produces chemicals, including 5-n-alkyl resorcinols, that make them unpalatable to some predators, and the predators learn to avoid these ants (Ito et al. 2004). Often, multiple
The within-population morphological diversity of dead-leaf butterflies of the species *Kallima inachus*. The butterflies were all collected within a small geographic region. Although all the butterflies resemble dead leaves on their undersides, the variation on the basic leaf pattern is quite remarkable. This variation may help ensure that predators cannot cue on a specific pattern element to distinguish the butterflies from the general leaf litter.

**Müllerian mimicry:** phenomenon in which an unpalatable organism mimics the appearance of another unpalatable one

**Batesian mimicry:** phenomenon in which a palatable organism mimics the appearance of an unpalatable one

Unpalatable species not only will use the same bright colors but will closely mimic other species in pattern as well. This phenomenon, in which multiple species share the same warning coloration pattern, is called Müllerian mimicry and is particularly well studied in butterflies and moths (*Figures 5 and 6*) (reviewed in Parchem et al. 2007). Batesian mimicry, in contrast, refers to instances in which a palatable species mimics the coloration pattern of an unpalatable one and thereby evades predation (*Figure 5*). Ants of the genus *Camponotus* may utilize Batesian mimicry; *Camponotus* individuals overlap in territory with the previously mentioned unpalatable *C. inflata* and have a very similar coloration. Chick-feeding experiments determined that *Camponotus* individuals were palatable whereas *C. inflata* individuals were not. Chicks that had previously eaten *C. inflata*, however, rarely attempted to eat *Camponotus* (Ito et al. 2004).

Some animals also employ coloration to startle predators. For example, some moths, when resting, rely primarily on cryptic coloration but, when startled, raise their forewings to expose a brightly colored area on their hindwings (*Figure 3c,d*). A variation of the startle response is to have a striking coloration pattern (false heads, eye spots, or vivid coloration of tails) in a noncritical part of the animal to draw the predators’ attention away from the most vulnerable area of the body.

Coloration also seems to play an important role in sexual selection (*Figure 3e*). Studies in birds and other animals with color polymorphisms show that pigmentation can influence mate choice. In feral pigeons, irrespective of the female phenotype, the most desired male phenotype is that of blue checker males (reviewed in...
Figure 3
Examples of the many functions of coloration. (a) Both the coloration and shape of the leafy sea dragon help it to blend in perfectly with its surroundings. (b) A cuttlefish can quickly change its colors either to blend in or to communicate. (c) While at rest, the moth *Automeris io* is well camouflaged. (d) Shown is the same moth as in panel c, but after being disturbed. By raising its forewings, it displays the brightly colored eyespots of the hindwings, which are thought to startle a would-be predator. (e) A male peacock, whose brilliant coloration is thought to be due to sexual selection. (f) The cavefish *Astyanax mexicanus*, which lacks the pigmentation found on its surface relatives (image courtesy of R. Borowsky).

Roulin 2004). Additionally, in guppies, females prefer males with the most orange color, and this preference is strongest in populations that contain males with a large amount of orange color (Houde & Endler 1990). There is a balance, however, between the advantage of being conspicuous to females and the disadvantage of being conspicuous to predators.

Figure 4
Figure 5
Examples of Batesian and Mullerian mimicry. In the left-most column are heliconid and ithomid butterflies that are thought to be unpalatable. In the middle column are pericopid moths that are also thought to be unpalatable. In the right-most column are pierid butterflies of the genus *Dismorphia* that are thought to be palatable. Thus, within each row, the left and middle Lepidoptera are examples of Mullerian mimics, whereas the right-most specimen is a Batesian mimic.

Figure 6
Heliconius mimicry. Each quadrant shows two *Heliconius* butterflies that look strikingly similar, but within each quadrant, the left specimen is *Heliconius erato*, whereas the right specimen is *Heliconius melpomene*. Thus, the two species show incredible variation, but where their geographic ranges overlap, the two species have evolved to be comimics (Mullerian mimics). For example, the *erato/melpomene* specimens shown in the upper left quadrant are found in the Chanchamayo region of Peru, whereas the ones in the lower left quadrant are found in Ecuador.
A particular pattern may not have an adaptive function. One potential example is the loss of pigmentation in cave animals (Figure 3f). Because of the dark habitat of these animals, pigmentation cannot provide typical functions, e.g., protection from ultraviolet radiation, concealment from predators and prey, and mate choice. Indeed, one of the theories for pigmentation loss in cavefish is that the cave environment predicts no selective advantage for this trait and therefore the trait is lost (Culver 1982).

There are many other examples for which there appears to be no obvious adaptive function of a particular coloration pattern, but it is difficult to know what additional characteristics are affected by, or linked to, coloration.

All these possible functions give us some idea of the evolutionary forces that underlie the remarkable diversity of coloration patterns in animals. In recent years, it has been possible to move beyond observation and begin exploring the evolution of this amazing diversity at the genetic and molecular levels. By the use of both model and nonmodel species, it is now possible to begin addressing the following kinds of questions: What is the molecular basis of coloration patterns? How many genes are involved in orchestrating differences in coloration between species or between individuals within a population? What types of mutations cause phenotypic changes? If the same coloration pattern occurs repeatedly across taxa, will the same genes be responsible?

**EARLY GENETIC STUDIES OF COLORATION AND STUDIES OF COLORATION IN MODEL SYSTEMS**

Genetic methods have been used for many years to study coloration differences. More than a thousand years ago in China, selective breeding was used to create various colors of goldfish. Mice breeding for coat color variation started in China and Japan as early as the eighteenth century. Later, in the nineteenth century, Mendel’s laws of inheritance were formulated by the use of traits in peas that included flower, seed, and pod color. Mendel’s rules were then applied in the early 1900s to examine the genetic basis of coat coloration in rodents (reviewed in Russell 1985).

Since these early days of genetic studies of coloration, extensive work has been performed to study coloration in various model species, including the fly Drosophila melanogaster, the mouse Mus musculus, and the zebrafish Danio rerio. Traditionally, large-scale mutagenesis screens for color mutants were carried out, the mutations were mapped, and the causative (i.e., mutated) genes were identified and cloned. We briefly discuss some of the major players in the pigmentation pathways of these three model systems (focusing on those that have been further studied in nonmodel systems) and then discuss nonmodel systems.

In D. melanogaster, epidermal cells produce and secrete cuticular pigments that give the body its characteristic brownish color with black bristles and dark abdominal stripes. Several structural genes that function in pigment synthesis include yellow, pale, Ddc (dopa decarboxylase), ebony, black, tan, and aaNAT (N-acetyl transferase), whereas regulatory genes, such as optomotor-blind and bric-a-brac, function in establishing the pigment pattern in D. melanogaster (reviewed in Wittkopp et al. 2003a).

Pigmentation in mice is also a vast field of research. There are more than 127 loci in mice affecting coloration (Bennett & Lamoreux 2003). Mammalian melanocytes, or pigment cells, are derived from the neural crest and migrate to various areas of the body (Bennett & Lamoreux 2003). Just as in D. melanogaster, there are several classes of genes that affect pigmentation in mice. For example, there are the spotting loci that affect melanocyte development. Examples of these genes are Kit and Mitf. Other genes such as Tyrp1 (Tyrosinase-related protein 1), Tyrosinase, Oca2 (Ocular and cutaneous albinism 2), and Matp (Membrane-associated transporter protein) affect the synthesis of melanin. Yet another group of genes affecting pigment includes those that control the switch between eumelanin and phaeomelanin production.
In some ways, studying coloration patterns in domestic animals provides an intermediate between genetic model systems and natural populations (Andersson & Georges 2004, Schmutz & Berrier 2007). The insights from these studies are highly informative for questions of the evolution of coloration and coloration pattern, but owing to space constraints, this review discusses only examples of naturally occurring populations.

**NATURAL COLOR VARIATION IN NONMODEL SPECIES**

Certain species are better suited than others for investigating questions of natural variation. For example, there is an enormous difference in the color and pattern of a giraffe versus those of a zebra. However, it is difficult to compare these two animals because they differ in so many other ways. But it is useful, at least initially, to examine coloration on a microevolutionary scale—within single species that are polymorphic in coloration or between related species that have diverged in coloration but are still close enough to interbreed. This kind of intraspecific variation in coloration is fairly common; for example, such variation exists in 3.5% of all bird species (Roulin 2004). Data from such microevolutionary studies can then be applied to understand coloration changes on a macroevolutionary scale.

The types of methods used to study coloration on a microevolutionary scale include genetic crosses, gene expression analyses (through in situ hybridization and microarray analysis), complementation studies, and linkage mapping. First, we describe each of these methods along with examples in which color patterns have been investigated. Then, we return to the question of how pigment patterns evolve, and we discuss the insights that recent studies have provided.

Among the first methods used to study coloration in nonmodel species were genetic crosses between individuals from populations with distinct coloration differences. One classic example comes from the snail *Cepea nemoralis*. The lip of the shell can be black, dark brown, pink, or white, whereas the rest of the shell is light tan, pink, orange, or red with one to five
dark bands (Cain & Sheppard 1954). Crosses between individuals with different coloration patterns indicated that a single locus is responsible for shell and lip color, the presence or absence of bands, and band pigmentation (reviewed in Jones et al. 1977). The number of bands, however, appears to be unlinked to this gene.

Crossing individuals with different phenotypes allows investigators to ask how many genes are responsible for a certain phenotype, which alleles are recessive and which are dominant, and what phenotypes are linked to each other. The next step is to delve into the pathways, and ultimately the mutations, that are modified to create variation in coloration. To accomplish this, one can use information known about pigmentation and coloration in model species and apply that information to nonmodel species.

CANDIDATE GENE APPROACH: GENETIC ASSOCIATIONS IN WILD POPULATIONS

The most common method employed in recent years to study the genetic basis of evolutionary variation in color is a candidate gene approach. As discussed above, pigmentation pathways in model organisms are generally well studied, and some of the major genes are conserved. These genes then become candidates for study in nonmodel systems. The disadvantages of using the candidate gene approach include selection bias and potentially long (and, ultimately, unsuccessful) lists of numerous candidate genes. A candidate gene approach, however, is sometimes the only option if one is working with a nonmodel species with few genetic resources.

The most studied coloration gene in nonmodel vertebrates is Mc1r, a transmembrane receptor expressed in melanocytes that, when modified, generally produces changes in coloration of the entire body. Mc1r is thought to be responsible for intraspecies coloration differences in at least four different bird species (Doucet et al. 2004, Mundy et al. 2004, Theron et al. 2001). For example, Caribbean bananaquits have melanic morphs, which generally live in forests, and paler morphs, which usually live in drier lowland areas. Mc1r was sequenced from pale and melanic individuals, and the melanic form of the bird has an amino acid substitution also found in melanic forms of chicken and mice (Theron et al. 2001). In Arctic skuas, another polymorphism in Mc1r correlates with the coloration phenotype (Mundy et al. 2004). The lesser snow goose has six different classes of pigmentation, and the degree of melanism is associated with a different amino acid substitution in Mc1r (Mundy et al. 2004). One final example, in birds, is that of the fairy wren. Male mainland fairy wrens have bright blue nuptial coloring, whereas island populations have black nuptial coloring, a phenotypic difference also associated with polymorphisms in Mc1r (Doucet et al. 2004).

Pocket mice are another example in which the candidate gene approach was used to test if there was an association between coloration and Mc1r. As mentioned above, the sandy-colored mice live in an area that has pale rocks, whereas the melanic form lives on dark lava flows. An association was found between four linked amino acid polymorphisms in Mc1r and coloration in one lava-dwelling population of melanic rock pocket mice (Hoekstra & Nachman 2003). Furthermore, Mc1r is associated with variation in coloration in jaguars, jaguarundis, and reptiles (Eizirik et al. 2003, Rosenblum et al. 2004).

Most of these studies used the approach of sequencing the coding region of Mc1r from wild individuals and comparing genotypes with coloration. This allows for the discovery of associations while avoiding laborious and time-consuming interspecific and intraspecific breeding programs. The primary disadvantage of this approach is that it is often difficult to determine experimentally that the changes in the Mc1r sequence actually cause the coloration differences. Even when the results from this approach are combined with genetic mapping data (see below), the observed association between an Mc1r genotype in the coding sequence and a melanic phenotype may instead be due to
a regulatory mutation in \textit{McIr} or a mutation in a different gene very closely linked to \textit{McIr}.

\textbf{CANDIDATE GENE APPROACH: COMPARATIVE EXPRESSION ANALYSIS}

Above, we discuss the candidate gene approach in the context of testing whether genetic polymorphisms are associated with a certain phenotype. Another method used to investigate a particular candidate gene is to see if the gene's expression pattern is correlated with a coloration phenotype. Such an association does not necessarily mean that a particular gene is the locus modified to produce the genetic change; e.g., changes in a gene's expression pattern may also be the result of regulatory changes in upstream regulators of that gene. An example of the utility of the expression analysis method is a wing spot study done in \textit{Drosophila biarmipes} (Gompel et al. 2005). \textit{D. biarmipes} has a dark pigmented spot on its wing that is absent from the closely related \textit{D. melanogaster}. The gene, \textit{yellow}, is expressed at very low levels throughout the wing of \textit{D. melanogaster} but highly expressed in the area that forms the pigmented spot in \textit{D. biarmipes} (Gompel et al. 2005). The advantage to working with species closely related to a model system (such as \textit{D. melanogaster}) is the myriad of ways to functionally test hypotheses about coloration patterns. Indeed, Gompel et al. (2005) were able to provide evidence that changes in the \textit{yellow} gene itself, as opposed to changes in a different gene that acts as a regulator of \textit{yellow} expression, are responsible for the expression differences. These authors made transgenic \textit{D. melanogaster} that contained the upstream regulatory region of \textit{yellow} from \textit{D. biarmipes} driving expression of the fluorescent protein GFP. These investigators found that this reporter construct was expressed in \textit{D. melanogaster} in a similar manner to that seen for \textit{yellow} in \textit{D. biarmipes}. This provides good evidence that the expression pattern differences seen for \textit{yellow} between the two species are due to evolutionary changes in the regulatory regions of the \textit{yellow} gene. However, when Gompel et al. (2005) rescued \textit{D. melanogaster} \textit{yellow} mutants with a construct of the \textit{D. biarmipes} \textit{yellow} gene that presumably drives expression in the wing, no pigmented wing spot was observed. Because of this, the authors argue that other genetic changes, in addition to a regulatory change in \textit{yellow}, are responsible for the observed coloration difference.

Another example of the candidate gene expression analysis approach was taken with the gene \textit{bric-a-brac} in \textit{Drosophila willistoni} and \textit{D. melanogaster}. \textit{D. melanogaster} has sexually dimorphic pigmentation patterns, whereas \textit{D. willistoni} does not; expression of \textit{bric-a-brac} is correlated with these coloration differences, and the gene maps to a locus accounting for the coloration differences (Kopp et al. 2000). Finally, within cichlid fish, a correlation was seen between the expression of \textit{fms}, a type III receptor tyrosine kinase thought to be a duplicated form of \textit{kit}, and the presence of egg dummies (pigmentation spots that look like eggs) on the anal fins of male cichlids (Mellgren & Johnson 2002, Salzburger et al. 2007).

This sort of expression analysis is a powerful tool, but it is sometimes difficult to perform on nonmodel organisms. Also, coloration occurs relatively late during embryonic development, and it is not always possible to conduct these analyses on late-stage embryos or juvenile animals for technical reasons. As an alternative, other methods, including microarray analysis and quantitative polymerase chain reaction (PCR), can also be used to look at expression levels in different parts of the animal during development (Reed et al. 2008).

\textbf{COMPLEMENTATION CROSSES WITH MUTANTS IN A MODEL SPECIES}

Another clever way to figure out the genetic basis of a coloration pattern is by a complementation test, a method that can be used only to detect recessive genetic determinants. Complementation tests allow one to ask if the same gene is affected in two species (that have similar
phenotypes) by crossing them. If the variation is in the same gene, the offspring will have the same pattern as both parents. If, however, the affected genes are not the same, complementation will occur, and the offspring will look different from either parent.

A modified version of this method has been used to investigate the genetic makeup of coloration in species of the Danio genus. By crossing different Danio species to D. rerio mutants, investigators could assay whether certain genes were responsible for the difference in coloration between different Danio species. D. rerio has four to five horizontal stripes on its body. The closely related Danio albolineatus has no stripes and a more dispersed pattern of pigment cells (Parichy & Johnson 2001). Numerous screens have been performed in D. rerio to isolate coloration mutants. Seventeen D. rerio mutants in genes of the coloration pathway or neural crest development were crossed to D. albolineatus (Quigley et al. 2005). All the mutants complemented except for fms mutants (zebrafish fms and kit are thought to have resulted from an ancient duplication of kit). This indicates that the fms gene or fms pathway underlies the difference in appearance in the two species.

Further experiments addressed whether characteristics of pigment cell development in the D. rerio fms mutant were similar to those of D. albolineatus. D. albolineatus had fewer melanophores, increased melanophore death, and aberrant melanophore migration, all similar characteristics of the D. rerio fms mutant (Quigley et al. 2005). However, D. rerio fms mutants also have fewer xanthophores than does D. albolineatus. Because of this discrepancy, Quigley et al. (2005) suggest that genetic changes in the fms gene are not responsible for the difference in coloration between D. rerio and D. albolineatus. Instead, changes in the fms pathway are responsible for the observed phenotypic differences. This would imply that the reduced functions of two different genes in the fms pathway combined cause the cross between D. albolineatus and the D. rerio fms mutant not to complement.

**MAPPING APPROACH**

The candidate gene approach is very powerful for systems in which many of the genes involved are known and well conserved in nonmodel organisms. Although the candidate gene approach often works well, novel gene(s) may be responsible for the observed coloration differences. For example, candidate genes for coloration differences may include members of the biosynthetic pathway of a pigment. However, previously uncharacterized genes that are upstream of this pathway or that regulate the pathway may also affect the coloration of an animal. A mapping approach is a less biased method that can also uncover novel genes. Such an approach involves examining a cross of individuals with a number of genetic markers, phenotyping the members of the cross, and from this determining regions of the genome where loci responsible for these phenotypes occur. The initial results of such an approach generally narrow down the search significantly, but hundreds of genes can be within the mapped region. Often a candidate gene approach may then further narrow the analysis to a small handful of genes. It is starting to become possible, however, to use a completely unbiased approach (positional cloning) on nonmodel organisms in which sequence information from BAC or whole-genome sequencing is available (e.g., Colosimo et al. 2005, Miller et al. 2007).

Mapping approaches have been used to examine morphological changes in the extremely diverse group of East African cichlid fishes (Figure 7) (Albertson et al. 2003). One coloration phenotype examined is the orange blotch phenotype (Figure 7a), which is found mainly in females and is of unknown function (Streelman et al. 2003). Another coloration phenotype found in the same species is blue with black bars (Figure 7b). A blue-with-black-bar male was crossed to an orange blotch female to generate F1 hybrids that were then crossed to generate F2s (Streelman et al. 2003).

Multiple genetic markers were used to genotype the F2s, and then these genetic data were compared with phenotypes to define a region containing a gene or genes responsible for
Monogenic: a trait that is encoded by one gene

Polygenic: a trait that is encoded by more than one gene

Quantitative trait locus (QTL): an area on a genetic linkage map that is responsible for a certain amount of variance in a measured trait

the orange blotch phenotype. Then, the researchers genotyped approximately 65 wild-caught individuals for the markers flanking this region to see whether this region was associated with the coloration phenotype in the wild. The same genetic marker that showed the highest association to coloration phenotype in the lab-raised cross also showed the highest association to coloration in the wild-caught individuals. Because there was little genomic information available for this species, the markers flanking the orange blotch locus were compared with sequence information from the pufferfish Takifugu rubripes. From this, the researchers identified a syntenic region that contained several genes, some of which are promising candidate genes. Ideally, one would now attempt to test these candidate genes within the cichlids by transgenesis, knockdown, or overexpression. Indeed, in other nonmodel fish species, some of these techniques have already been described (Colosimo et al. 2005, Hosemann et al. 2004, Yamamoto et al. 2004).

Figuring out the genetic basis of phenotypic variation is easiest for monogenic traits. However, some coloration variation appears to be polygenic. One recent example is the old-field mouse, Peromyscus polionotus, which has darkly colored populations and lightly colored populations (Figure 4) (Hoekstra et al. 2006, Steiner et al. 2007). The lightly colored beach mouse lives in dunes and islands, and it is thought that the light color protects the animals from predators by providing camouflage. A large F2 cross between the beach and mainland populations was used to generate a linkage map, which identified three quantitative trait loci (QTLs) (regions where genes responsible for a certain trait reside). The coloration pathway of both mice and humans has been well studied. Ten coloration candidate genes were placed on the map, and of these, Agouti, Mc1r, and Kit mapped to the three QTLs. Mapping to a QTL does not prove that the gene is responsible for a particular QTL because there are many other unidentified genes in that region. However, a
In the previous paper examined the sequence of Mc1r in beach and mainland mice and found that there was an amino acid change in a critical part of the protein (Hoekstra et al. 2006). Comparing the beach and mainland MC1R in an in vitro assay to test MC1R function showed that the beach MC1R had a significantly lower activity level than did the mainland MC1R. The coding sequence of Agouti in the beach and mainland mice was also compared, but no differences were observed (Steiner et al. 2007). The expression level of Agouti was examined, comparing beach and mainland mice by RT-PCR, and it was found that the level of Agouti was higher in the beach versus the mainland mice—consistent with the observation in laboratory mouse strains that higher levels of Agouti lead to light pigmentation (Steiner et al. 2007). The authors hypothesize that a regulatory mutation in Agouti, rather than a coding mutation, is responsible for the phenotypic difference between the two populations.

As outlined above, mapping combined with the candidate gene approach has potential for successfully investigating the genetic basis of coloration pattern. However, it is always possible that no matter how many candidate genes are added to the map, few, if any, will ever coincide with the loci responsible for coloration differences. For example, extensive genetic crosses and mapping have been done between different species and populations of Heliconius to map loci responsible for the position, size, and shape of patterns on the wings (Figure 6) (Jiggins et al. 2005, Kapan et al. 2006, Tobler et al. 2005). Many different genes known to be involved in Drosophila wing patterning or pigment biosynthesis, such as apterous, distal-less, hedgehog, patched, cubitus interruptus, vermilion, and cinnabar, have been placed on the map, and none of them map to the pattern loci being studied (Jiggins et al. 2005, Kapan et al. 2006, Tobler et al. 2005). Therefore, it remains to be determined whether novel wing-patterning genes or candidate genes other than those studied are responsible for the varied coloration phenotypes in Heliconius butterflies.

Similar to the difficulties experienced in predicting the genes responsible for coloration differences in Heliconius butterflies, the majority of studies in drosophilid species have not observed associations between candidate genes and coloration phenotypes (Wittkopp et al. 2003a). However, an association was seen between variation in coloration and the gene ebony in the offspring of crosses between Drosophila americana and Drosophila novamexicana (Wittkopp et al. 2003b), and as mentioned above, sexually dimorphic pigmentation in D. melanogaster is associated with the gene bric-a-brac (Kopp et al. 2003). In cases in which the candidate gene method is not able to predict the genes responsible for a coloration trait, the ideal method to use is a positional cloning approach. The difficulty is that to use this approach one must have either the genome sequence of the species of interest or a large amount of sequence information from BAC sequencing. Within this sequence, it is possible to identify a large number of polymorphic sites that can then be used for high-resolution mapping.

Recently, a coloration difference in sticklebacks was investigated by high-resolution mapping and a candidate gene approach (Miller et al. 2007). Some freshwater populations have lightly melanized gills and ventral surfaces, whereas marine populations have heavily melanized gills and ventral surfaces. A major QTL was found for this phenotype and narrowed down by genotyping a total of 1182 F2 fish. The recombination data from these individuals and a draft of the stickleback genome allowed the area to be narrowed down to a 4.5-mb region. Additional genetic markers were designed within this region. By typing the recombinants with the new markers, Miller et al. (2007) narrowed the gene responsible for this QTL to an interval of 315 kb. Fifteen genes resided within this region, and only one had been previously implied to function in pigmentation, Kit ligand. If there had been no promising candidate genes within the 15 genes, the authors would have had to take a functional approach to test each gene and see if each gene affected gill pigmentation. As this example shows,
it is possible to use high-resolution mapping to
narrow down a mapped region to a small num-
ber of genes that contain the loci responsible
for genetic variation in a naturally occurring
vertebrate population.

THE GENETIC BASIS OF
ENVIRONMENTAL EFFECTS
ON COLORATION

The environment can play a large part in deter-
mining an individual’s coloration. Arctic foxes
have a brownish coat during the summer and a
white coat during the winter (Vage et al. 2005).
The different seasonal forms of the butterfly
Precis octavia are so different from each other
that one might easily assume that they were
actually different species (Figure 8). Another
example is Manduca quinquemaculata, a species
closely related to Manduca sexta, the tobacco
hornworm moth (Suzuki & Nijhout 2006).
M. quinquemaculata larvae are black when raised
at 20°C and green when raised at 28°C. In
M. sexta, a species that has only green lar-
vae, a mutation causing a reduction of juve-
nile hormone secretion results in only black
larva. Heat shocking these black larvae often
resulted in a color change to green. The in-
dividuals that showed the most marked color
change upon heat shock were selected and
bred to one another. After 13 generations of
such selection, the larvae mimicked M. quin-
quemaculata in that they were green when raised
at 28°C without heat shock. The researchers
were able to generate environmentally plastic
coloration through reduction of juvenile hor-
mone production and subsequent selection of
the uncovered phenotypic variation (Suzuki &
Nijhout 2006). Further studies determined that
one major gene and several modifier genes
were responsible for the artificially selected
phenotype (Suzuki & Nijhout 2008). It is un-
clear how the coloration system evolved in
M. quinquemaculata, but this system may have
taken a path similar to the artificial selec-
tion in M. sexta. This example demonstrates
a more complicated example of coloration dif-
ferences and shows how the field is adapting
methods to address even these more complex
phenomena.

Having examined the many different ap-
proaches for studying the genetic basis of pig-
mentation patterns, and having sampled some
of the results, we can now return to the evo-
lutionary questions we hope to address by
studying coloration systems.

MANY GENES OR FEW GENES?

How many genes are responsible for the evo-
lution of new forms of coloration? One factor
to consider is that there is a bias in examining

![Figure 8](image_url)

Example of environmentally influenced coloration. These two butterflies are both males of the species *Precis octavia* collected in South Africa. The only difference is that they were collected at different times of the year and represent the two very different seasonal forms of this species. It appears that both temperature and day/night length during the larval period determine the coloration pattern of the adult.
species with a simple genetic basis of coloration because it is easier to study such systems. With that in mind, there are many examples of single genes responsible for coloration differences (Brown et al. 2001, Curtis 2002, Nachman et al. 2003, Trapezov 1997, Wittkopp et al. 2003a). It is easy to see how an overall change in body pigmentation could be affected by modification of one gene in a pigment synthesis pathway. However, differences in the coloration pattern may be easier to explain via a polygenic mode of inheritance. Polygenic modes of inheritance have been identified for wing spots, abdomen, and overall pigmentation in various Drosophila species as well as pigmentation in the oldfield mouse (Carbone et al. 2005, Steiner et al. 2007, Wittkopp et al. 2003a, Yeh et al. 2006). In some cases, observed so far mainly in Lepidoptera, it is unclear whether a mode of inheritance is mono- genic or polygenic with a tightly linked cluster of genes (Clarke & Sheppard 1959, 1960a,b; Joron et al. 2006b). Many recent studies looking at noncoloration variability have also documented that a few genes of large effect account for a large amount of phenotypic difference (Albertson et al. 2003; Colosimo et al. 2004, 2005; Peichel et al. 2001; Shapiro et al. 2004).

REGULATORY VERSUS CODING CHANGES?

What kinds of mutations are responsible for phenotypic changes—regulatory or coding changes? Again, there is bias in the studies that address this question because it is very difficult to find regulatory changes in nonmodel species when there is a lack of knowledge of structure of the gene regulatory elements or when detailed gene expression studies are not possible. Most often, regulatory changes are not identified but are hypothesized when no coding changes are found. An example of a gene that appears prone to coding changes in pigment evolution is Mc1r. Potential coding changes have been found in the rock pocket mouse, bananquit, lesser snow goose, arctic skua, beach mouse, jaguars, jaguarundis, and Neanderthals (Baiao et al. 2007, Eizirik et al. 2003, Hoekstra et al. 2006, Lahueza-Fox et al. 2007, Mundy et al. 2004, Nachman et al. 2003, Rosenblum et al. 2004, Theron et al. 2001). Most of these studies show an association between Mc1r changes and coloration phenotype. Often the changes are in conserved amino acids, implying that the function of the receptor is affected, and recently an in vitro test was devised to test the function of variants of beach mouse Mc1r (Hoekstra et al. 2006). Examples of predicted coding changes in other genes causing pigmentation differences include Agouti in domestic cats, Tyrp1 in Soay sheep, and Oca2 in the Mexican cave tetra (Eizirik et al. 2003, Gratten et al. 2007, Protas et al. 2006).

Examples of regulatory changes causing pigmentation differences are much fewer in number. However, likely examples are yellow and the wing pigmentation spot and male-specific pigmentation in drosophilids (Gompel et al. 2005, Jeong et al. 2006, Prud’homme et al. 2006). Another predicted regulatory change is in Agouti in beach mice (Steiner et al. 2007), and a regulatory mutation in Kit ligand is probably responsible for the gill pigmentation differences between marine and freshwater sticklebacks (Miller et al. 2007). Coding changes involved in the evolution of coloration may be prevalent because genes involved in pigment biosynthesis are relatively free to alter biochemical function without causing lethality. Genes involved in other processes during development often have pleiotropic effects, and coding changes would likely result in lethality. Whether coding or regulatory sequences are more common agents of evolutionary change is still hotly debated (Hoekstra & Coyne 2007).

SAME GENES OR DIFFERENT GENES?

The third question that can be addressed by genetic studies of coloration is, If the same phenotype comes up multiple times, is it the same or different genes that are responsible? Above we see that Mc1r appears to be involved in pigmentation differences in multiple cases. However, we must keep in mind that Mc1r is
Parallel evolution: the independent evolution of similar characteristics coming from a similar starting point

A highly conserved gene with only one exon and is therefore relatively easy to amplify from a nonmodel organism. Regardless of the bias, Mc1r seems to be involved in pigmentation differences between closely related species or populations in many different types of vertebrates. However, Mc1r polymorphisms are not always associated with pigmentation differences. For example, pocket gophers with coat colors corresponding to the color of the substrate that they live on do not show an association of coloration phenotype with Mc1r genotype (Wlasiuk & Nachman 2007). Also, leaf warblers with small, unmelanized patches and multiple nonhuman primates do not show an association of coloration with Mc1r (MacDougall-Shackleton et al. 2003, Mundy & Kelly 2003). Other examples can be found in populations of rock pocket mice, as well as in beach mice, in which one population does show an association between Mc1r variants and the melanic phenotype, whereas another population does not (Hoekstra & Nachman 2003, Hoekstra et al. 2006). This implies that the melanic phenotype evolved by a different mechanism in the separate populations. So, if there is a bias for mutation in Mc1r, it is not a complete bias.

In the Mexican cave tetra (Figure 3f), the genetic basis of albinism was studied in three different cave populations: Molino, Japonés, and Pachón (Protas et al. 2006). The gene Oca2 was linked to the phenotype of albinism in crosses from the Molino and Pachón populations. These two cave populations both had deletions, but in different parts of the Oca2 coding region. To test whether the cave forms of Oca2 were functional, constructs of the surface Oca2 and Oca2 with the two deletions were transfected into a mouse melanocyte cell line deficient in Oca2. The surface form was able to rescue pigmentation in the cell line, but the two cave forms were unable to rescue pigmentation. Therefore, it appears that these two deletions cause the protein to be nonfunctional. As for the Japonés population, complementation tests were performed with the Molino and Pachón cave populations, and the offspring did not complement, indicating that the Japonés population was likely also deficient in Oca2. A construct containing the coding sequence of Oca2 in the Japonés population was able to rescue pigmentation in the Oca2-deficient melanocyte cell line, suggesting a possible regulatory change. Therefore, it is likely that albinism arose three times separately, in an example of parallel evolution, and that, in each case, the same gene was targeted. This is different from the previously discussed role of Mc1r in the evolution of pigmentation phenotypes in beach mice and rock pocket mice. In this latter case, some mouse populations utilized Mc1r, whereas others used something else. It is unclear why this bias is seen with Oca2, perhaps because the gene is very large and therefore more vulnerable to mutation. It is also important to keep in mind that the different types of coloration changes, such as loss of coloration, gain of coloration, and change in coloration pattern, are quite different from one another and may come about by the alteration of very different genetic programs.

Another example of parallel evolution is coloration patterns from Heliconius butterflies (Figure 6). As discussed above, Heliconius butterflies are a classic example of Müllerian mimicry. Each species has many different geographical races with varied pigmentation patterns, but where races of different species coexist they have matching pigmentation patterns. This provides a unique scenario in which several species have evolved virtually the same pigmentation pattern, allowing investigators to ask whether the same genetic path or different genetic paths were taken to achieve this result. This scenario is different from that of Mc1r in rock pocket mice and Oca2 in cavefish because here we have multiple species—rather than multiple populations of species—evolving the same phenotypes.

Heliconius erato and Heliconius melpomene are two of the better-studied Heliconius species—they are distantly related but have very similar coloration patterns for 23 geographical races (Figure 6) (Joron et al. 2006a). Initial genetic experiments suggest that approximately a dozen loci are responsible for the variation seen within
each species. Genetic linkage maps have been generated, and the positions of a number of coloration loci, including four to five alleles of major effect (Joron et al. 2006a), have now been mapped for both *H. erato* and *H. melpomene* (Jiggins et al. 2005, Kapan et al. 2006). In another species, *Heliconius numata*, most of the variation in wing patterning appears to be controlled by a single genomic locus (possibly one or more tightly linked genes). By investigating the placement of flanking genetic markers relative to the position of each of the color pattern loci in *H. erato*, *H. melpomene*, and *H. numata*, Joron et al. (2006b) determined that the loci responsible for specific color patterns were located in homologous chromosomal regions. Similarly, three color patterning loci were compared between the Heliconius bimera/*H. erato* group and the Heliconius cydno/Heliconius pachinus group and were also found to be located in homologous chromosomal regions (Kronforst et al. 2006). Given the current resolution of the mapping data, these results do not yet prove that the same genes are affected but do support the idea of parallel evolution.

Although it is striking to see similar phenotypes mapping to similar genomic regions in *H. erato* and *H. melpomene* (something we see in examples above), the mapping of very different phenotypes to the same location (e.g., when comparing *H. erato/H. melpomene* with *H. numata*) is remarkable. Future work will hopefully show how the same region can encode such different phenotypes and uncover the specifics of the genetic architecture controlling these color patterns.

A very detailed example of convergence and parallel evolution is that of wing spot evolution in drosophilids. We discuss above one instance of wing spot difference between *D. melanogaster* and *D. biarmipes* (Gompel et al. 2005). Another example of wing spot gain is *Drosophila tristis* (Prud’homme et al. 2006). A *D. tristis* sequence homologous to that responsible for wing spot gain in *D. biarmipes* did not cause a wing spot pattern when inserted into *D. melanogaster*. Only when a *D. tristis* yellow intronic sequence was included in the reporter construct did the sequence drive wing spot–specific expression. Therefore, the same gene seems to be involved in wing spot gain in two species of *Drosophila*, but different regulatory elements are involved (Prud’homme et al. 2006). Prud’homme et al. (2006) suggest that the evolution of these novel patterns came about by co-option of regulatory elements with preexisting functions.

**STANDING VERSUS NOVEL VARIATION?**

Our last question regarding the evolution of morphological change is whether change comes about by standing variation in the ancestral population or novel variation. Answering this type of question requires a system for which one knows the direction of morphological change. For example, in the Mexican cave tetra, we know that the albino cave form evolved from the pigmented surface form. The example of *Oca2* in cavefish showed that albinism was likely caused by novel variation in the cave populations because, although the same gene was affected, there were at least two different mutations (Protas et al. 2006). Another system in which the question of standing versus novel variation is being examined is with old-field mice. Again, we know the direction of change—the lightly pigmented beach mice are derived from the more highly pigmented mainland mice. As stated above, three QTLs were found for pigmentation in this species, and two of them mapped to the genes *Agouti* and *Mc1r* (Steiner et al. 2007). *Agouti* is epistatic to *Mc1r*. Epistasis may allow the beach *Mc1r* allele to remain hidden in the mainland population as standing variation (Barrett & Schluter 2007). To examine this idea further, *Mc1r* needs to be sequenced from many individuals of the mainland population to see if the beach *Mc1r* alleles are preexisting in that population. Another likely evolution via standing variation is gill pigmentation in sticklebacks (Miller et al. 2007). Three freshwater populations of sticklebacks have *Kit ligand* alleles associated with light pigmentation. Even though the populations are geographically separate, they share the same
alleles at Kit ligand. One possible explanation for this is that standing variation in the ancestral (marine) population was fixed in each of the freshwater populations. To investigate this hypothesis, Kit ligand was sequenced in 107 marine individuals; the light allele was present at a frequency of 12%, supporting the idea that the evolution of lightly colored gills occurred by the repeated fixation of standing variation.

**CONCLUSION**

There has been much progress in understanding the genetic basis of morphological change in the evolution of pigmentation and coloration patterns. The advantages to studying the genetic basis of coloration include the extreme ease of observing this phenotype, the vast knowledge of pigment synthesis pathways in model organisms, and the great diversity of coloration and patterns within and between species. Pigmentation is hence a relatively simple system in which we can explore the possible processes by which phenotypes evolve. Although broad trends in the evolution of pigmentation patterns emerge from the discussion above, there are clearly multiple ways in which evolution of coloration occurs. Also, most of the examples we discuss above are relatively simple pigmentation differences that are more easily examined with existing tools. However, there is a huge diversity of more complicated pigmentation systems being studied that will yield a more complete picture as to how coloration differences evolve.

**SUMMARY POINTS**

1. There are many advantages to studying coloration and coloration patterns in nature, including ease of observation and great morphological diversity within and between species.
2. Molecular and genetic techniques can now be applied to nonmodel organisms to ask questions about evolution.
3. Variation in coloration can evolve by single gene changes or by multiple gene changes.
4. Differences in coloration can evolve by regulatory or coding changes.
5. The evolution of similar phenotypic changes can be accomplished by the same genes or by different genes.
6. Changes in coloration can evolve by selection on standing variation or on novel mutations.

**FUTURE ISSUES**

1. There is a need to identify genes responsible for more quantitative trait loci encoding a small-percent variance of a coloration trait as well as those responsible for a large-percent variance of a trait.
2. Systems that have a more complicated coloration phenotype, for example, those with partially environmentally determined coloration, should be examined.
3. More cases of parallel and convergent evolution of coloration variation require examination.
4. Regulatory changes responsible for coloration variation should be determined.
5. Investigators need to determine other characteristics that evolve in tandem with color variation and how the genetic architecture of these traits interacts with the genetic architecture of coloration traits.

6. The genetics behind examples in which alleles of a single locus can create many different coloration phenotypes merits investigation.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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LITERATURE CITED


The authors performed a quantitative trait and candidate gene analysis of a polygenic pigmentation trait in the oldfield mice.

Investigated a pigmentation trait that changes with the environment by carrying out artificial-selection experiments in a related species.


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