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THE LOCI OF EVOLUTION: HOW PREDICTABLE IS GENETIC EVOLUTION?

David L. Stern[1,2] and Virginie Orgogozo[3,4]

[1]*Department of Ecology and Evolutionary Biology, Princeton University, Princeton, New Jersey 08544*

[2]*E-mail: dstern@Princeton.edu*

[3]*CNRS, Université Pierre et Marie Curie, Bâtiment A, 5ème étage, case 29, 7 Quai Saint Bernard, 75005 Paris, France*

[4]*E-mail: Virginie.Orgogozo@snv.jussieu.fr*

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Is genetic evolution predictable? Evolutionary developmental biologists have argued that, at least for morphological traits, the answer is a resounding yes. Most mutations causing morphological variation are expected to reside in the *cis*-regulatory, rather than the coding, regions of developmental genes. This "*cis*-regulatory hypothesis" has recently come under attack. In this review, we first describe and critique the arguments that have been proposed in support of the *cis*-regulatory hypothesis. We then test the empirical support for the *cis*-regulatory hypothesis with a comprehensive survey of mutations responsible for phenotypic evolution in multicellular organisms. *Cis*-regulatory mutations currently represent approximately 22% of 331 identified genetic changes although the number of *cis*-regulatory changes published annually is rapidly increasing. Above the species level, *cis*-regulatory mutations altering morphology are more common than coding changes. Also, above the species level *cis*-regulatory mutations predominate for genes not involved in terminal differentiation. These patterns imply that the simple question "Do coding or *cis*-regulatory mutations cause more phenotypic evolution?" hides more interesting phenomena. Evolution in different kinds of populations and over different durations may result in selection of different kinds of mutations. Predicting the genetic basis of evolution requires a comprehensive synthesis of molecular developmental biology and population genetics.

KEY WORDS: ***cis*-regulation, development, genetic variation, genome, phenotypic variation.**

> "Curiously, the improved understanding of the nature of gene and mutation has not added, so far, to the understanding of evolutionary phenomena."
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> E. Mayr Animal Species and Evolution (1963, p. 172)
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> "There are many generalizations in biology, but precious few theories."
>
> F. Jacob *The Logic of Life* (1973, p. 13)

Natural selection causes predictable changes in phenotypic variation. This predictability exists at two levels. First, quantitative genetics provides predictions for the short-term response to selection, given estimates of heritability and the selection differential (Falconer and Mackay 1996). Second, selection theory often provides reasonable predictions of how populations will adapt over the long term following a change in the selective regime. These are probabilistic predictions and, due to historical contingency, populations may not evolve as predicted in every case. Nonetheless, in some cases, precise predictions at the phenotypic level have been fulfilled by observation (Herre 1985, 1987). Natural selection thus provides a compelling explanation for phenotypic evolution of life on the earth.

In contrast, the genetic changes underlying these phenotypic changes have historically not been expected to show predictable patterns. For example, it has long been recognized that different genetic causes can generate similar patterns of phenotypic variation (Robertson 1959; Wilkens 1971). Discoveries in molecular and developmental biology over the past 40 years, however, have led some biologists to suggest that mutations that alter the

regulation of gene expression are more likely to contribute to phenotypic evolution, particularly changes in morphological pattern, than mutations that alter the amino acid sequence of a protein. That is, this hypothesis claims that the genetic causes of evolution are predictable, at least at some scales of genomic organization. We use the term predictability in the sense normally implied by evolutionary genetics as probabilistic predictions.

To convince the reader that genetic evolution is predictable in at least some general sense, we point out that there is already an uncontroversial general theory of genetic evolution. Nonsynonymous mutations are predicted to contribute more to phenotypic evolution than synonymous mutations. There is, of course, a good reason for this prediction. Nonsynonymous mutations alter the amino-acid sequence and are thus likely to affect protein structure, stability, activity, or binding properties. In contrast, synonymous mutations do not alter the amino-acid sequence, although they can modify gene function through other mechanisms, such as changes in translation efficiency or mRNA stability. In addition, there is empirical evidence that nonsynonymous mutations have contributed more to phenotypic evolution than synonymous mutations. Due to the genetic code, 24% of nucleotide substitutions in protein-coding DNA are expected to cause synonymous substitutions if mutations occur randomly (Wilke 2004). To our knowledge, only two evolutionary changes in phenotype have been shown to derive from synonymous mutations (Stam and Laurie 1996; Nackley et al. 2006), whereas hundreds of evolutionary changes in phenotype have been shown to involve nonsynonymous mutations (see below). Therefore, evolutionary biologists are already familiar with the kinds of arguments and evidence that support the contention that some types of mutations contribute more to phenotypic evolution than others.

In this review we focus on whether evolutionarily relevant mutations occur preferentially in *cis*-regulatory regions. Since the 1960s various authors have wielded diverse arguments and data to predict that changes in *cis*-regulatory regions are more likely to underlie phenotypic evolution than other types of genetic changes (see for example Jacob and Monod 1961; Wallace 1963; Zuckerkandl 1963; Britten and Davidson 1969, 1971; King and Wilson 1975; Wilson 1975; Jacob 1977; Raff and Kaufman 1983; Carroll 1995; Gerhart and Kirschner 1997; Akam 1998; Stern 2000; Davidson 2001; Wray et al. 2003; Davidson 2006; Wray 2007). This idea has come under attack recently (Alonso and Wilkins 2005; Hoekstra and Coyne 2007). Hoekstra and Coyne have argued that there is no reason to expect a preponderance of evolutionarily relevant mutations in any particular gene regions (Coyne and Hoekstra 2007; Hoekstra and Coyne 2007).

We must first define how we are using the terms "*cis*-regulatory" and "coding." The *cis*-regulatory region of a gene encompasses all of the DNA elements (enhancer, promoter, 5′UTR, 3′UTR, introns, etc.) that regulate its expression in *cis*, in other words that act directly on the gene-coding region located on the same DNA strand, without encoding intermediary factors (Fig. 1). The coding region is the part of a gene that encodes the final gene product, either a protein or a mature RNA (Fig. 1). One can distinguish three main types of mutations: (1) coding changes, which alter the amino-acid sequence or the mature RNA nucleotide sequence; (2) *cis*-regulatory changes, which alter gene expression; and (3) genetic changes that alter both the coding and the *cis*-regulatory regions of one or several gene(s) (gene loss, gene duplication, gene rearrangement, etc.). Coding mutations always occur in coding regions and most *cis*-regulatory mutations occur in *cis*-regulatory regions. However, in rare cases, *cis*-regulatory mutations may arise in coding regions. For example, a few genes are known to contain transcription factor binding sites in exons (*keratin18* in humans and *nonA* in *Drosophila melanogaster* [Wray et al. 2003]). In this review, when we refer to *cis*-regulatory regions, we explicitly mean nucleotides that may be altered to change gene expression irrespective of their precise physical location in a gene region.

The prediction that *cis*-regulatory mutations have played a predominant role in evolution has been stated in many forms. All forms include components of two separate issues. First, most authors have generated predictions specifically for morphological variation, whereas others have considered all kinds of phenotypic changes (morphology, behavior, physiology, etc.). Second, the predominance of *cis*-regulatory mutations has been invoked either relative to coding mutations, what we call the "narrow *cis*-regulatory hypothesis," or relative to any other type of mutation, the "broad *cis*-regulatory hypothesis." For example, the broad *cis*-regulatory hypothesis for all phenotypes predicts that *cis*-regulatory mutations should be the predominant cause of phenotypic evolution, in contrast to coding changes, changes in alternative splice sites, gene duplication events, whole gene deletions, gene rearrangements, gene fusions, etc.

Discussions of developmental evolution have not always distinguished clearly between the effects of *cis*-regulatory and coding mutations. Indeed, another oft-mentioned hypothesis, named hereafter the "regulatory hypothesis," is that phenotypic or morphological evolution is caused mostly by regulatory changes, that is changes in the regulation of gene expression. Regulatory changes, however, can result from mutations in *cis*-regulatory or coding regions, for example in the coding region of a transcription factor that regulates the target gene. The regulatory hypothesis focuses on mutations that alter gene regulation through any means whereas the narrow and broad *cis*-regulatory hypotheses focus on *cis*-regulatory mutations. The regulatory hypothesis predicts simply that phenotypic evolution will be, in most cases, associated with changes in gene expression. It makes no clear prediction about the molecular nature of the mutations underlying evolution. In this review, we will address whether evolution is predictable

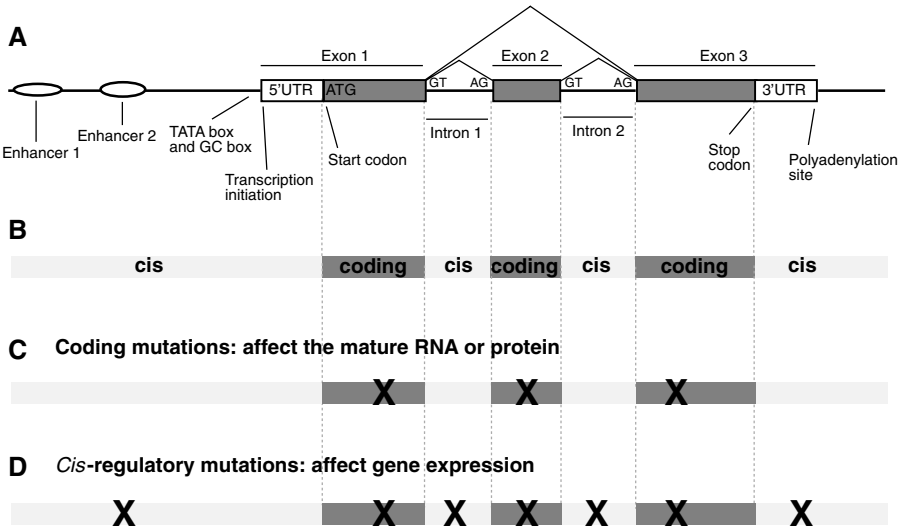

Figure 1. **Gene structure and definitions of *cis*-regulatory and coding regions and *cis*-regulatory and coding mutations. (A) A single gene encodes a complex set of instructions in the DNA sequence. The final gene product can either be a protein, via an mRNA intermediate, or a mature RNA molecule itself (transfer RNA, ribosomal RNA, micro RNA, etc.). Gray boxes indicate DNA regions that encode a protein product. The mRNA molecule is transcribed from the transcription initiation site to the polyadenylation signal and introns are spliced out. Many genes encode alternative mRNA splice variants that can be generated by alternative use of different exons (Graveley 2001; Xing and Lee 2006). This is indicated in the figure by lines above the gene connecting alternative exons. Alternative splice variants are usually expressed in different tissues and at different times in development. The mechanisms regulating splicing are not fully understood, but at least some of the information is encoded in the introns and must be recognized by cell-type-specific splicing factors (Lopez 1998). The mRNA contains 5′ and 3′ untranslated regions (UTRs), which are involved in mRNA stability, mRNA localization, and translation. The basal transcription apparatus binds upstream of the gene-coding region, often at a TA-rich sequence motif called a TATA box. Two enhancer modules are indicated to the left of the exons. Each module can contain binding sites for multiple transcription factors. In some cases, transcription factor binding sites are not clustered into discrete modules. (B) Genes can therefore be divided into coding regions, encompassing all of the exons, and *cis*-regulatory sequences, which include all other DNA that regulates gene expression. *Cis*-regulatory sequences include sequences that regulate transcription, RNA stability and splicing, and translation. (C) We define coding mutations as mutations that alter the amino acid sequence encoded by the mRNA or that alter the nucleotide sequence of a mature RNA molecule. (D) *Cis*-regulatory mutations can occur anywhere in the gene region, including noncoding sequence and coding sequence. In rare cases, synonymous mutations in coding regions alter gene regulation in *cis*, for example through modification of transcription factor binding sites or through modification of RNA stability (see text for further details). In principle, nonsynonymous mutations could alter both the polypeptide sequence and gene regulation, but no such examples have been reported yet. The regulation of gene expression operates at multiple levels: translation, alternative splicing variants, mRNA stability, mRNA cell localization, translation, etc. (Stern 2003; Alonso and Wilkins 2005). All of these levels of gene regulation are, potentially, available for evolutionary modification (Alonso and Wilkins 2005). However, by far the majority of variation in the distribution of gene products during development is controlled at the transcriptional level (Davidson 2006).**

at the genetic level. Therefore, we focus primarily on the two hypotheses that make predictions about the genetic basis of phenotypic evolution, the narrow and broad *cis*-regulatory hypotheses, together referred to as the *cis*-regulatory hypothesis.

Arguments for the Cis*-Regulatory Hypothesis*

Over the past 50 years, many different arguments have been advanced to support the predominant role of *cis*-regulatory changes in phenotypic or morphological evolution. We believe that these can be parsed into seven discrete arguments. We discuss and critique each one below.

IMPORTANCE OF GENE REGULATION IN LIFE

The origins of the *cis*-regulatory hypothesis can be traced back, ultimately, to classic experiments on gene regulation in the bacterium *Eschericia coli* (Jacob and Monod 1961). These experiments revealed that levels of enzyme activity are determined primarily by transcriptional regulation. Certain gene products, what we now call transcription factors, bind to specific nucleotides adjacent to the target coding sequence and either recruit or block recruitment of the basal transcription apparatus to the promoter. This is, in principle, a generic mechanism to control gene expression in response to external signals.

Over the past 30 years, research in developmental biology has shown that this basic mechanism in bacteria also applies to

development of multicellular organisms (Ptashne and Gann 2002). With a few exceptions, all the cells of a multicellular organism are genetically identical, and the phenotypic differences between cells (heart muscle, neurons, hair follicles, etc.) are determined by gene regulation. Different sets of genes are turned on and off in different parts of the body at different times in development. From these facts, it became reasonable to extrapolate to the hypothesis that much of phenotypic diversity between species is caused by changes in gene expression. This was the essential argument supporting the regulatory hypothesis. However, this argument does not explicitly provide support for the *cis*-regulatory hypothesis, because regulatory changes might arise through either *cis*-regulatory or coding mutations.

CORRELATION BETWEEN PHENOTYPIC CHANGE AND CHANGE IN GENE EXPRESSION

A vast body of comparative data has revealed that changes in expression patterns of developmental patterning genes are often correlated with evolved phenotypes. The singular fact that makes these correlations compelling, beyond their sheer number, is that we understand how transcriptional changes of these genes could, based on their molecular properties, alter the phenotypes being studied. For example, Abzhanov et al. (2004) have discovered that higher levels of *Bone morphogenetic protein 4* (*BMP4*) expression are correlated with deeper beak shapes among Darwin's finches. *BMP4* is a member of the transforming growth factor-ß superfamily of proteins, which are ligands involved in many cell-signaling processes. *BMP4* was previously known to promote bone development in vertebrates and Abzhanov et al. (2004) showed that overexpression of *BMP4* in a chick embryo altered beak development in the predicted direction. Therefore, differential expression of *BMP4* provides a reasonable explanation for changes in finch beak shape. This is but one of many examples in which a correlation between expression of a developmental patterning gene and phenotypic variation makes sense in light of the known developmental function of the gene. However, most of these studies have not discriminated between *cis*-regulatory evolution and coding evolution as the cause of observed changes in gene expression patterns. The mutation(s) underlying the beak size difference have not been identified yet. It is possible, for example, that they affect the coding region of a transcription factor regulating the *BMP4* gene. For example, the increase in *scute* expression associated with the production of extra bristles in a Moroccan population of *D. melanogaster* has been shown to result from a coding change in a transcription factor gene regulating *scute* expression (Gibert et al. 2005). We thus consider the prevalence of evolutionary changes in gene expression patterns as good evidence for the regulatory hypothesis, but as weak evidence for the *cis*-regulatory hypothesis.

CONSERVATION OF CODING SEQUENCES ACROSS TAXA

Comparative DNA sequence data have also been used to support the *cis*-regulatory hypothesis. For example, King and Wilson (1975) argued that the 1% protein sequence divergence observed between humans and chimpanzees could not account for the many phenotypic differences between these species. Instead, they suggested, changes in the regulation of gene expression may have played a large role in phenotypic evolution and they explicitly favored *cis*-regulatory mutations as the cause of regulatory evolution.

> Nucleotide substitutions in a promoter or operator gene would affect the production, but not the amino acid sequence, of proteins in that operon. Nucleotide substitutions in a structural gene coding for a regulatory protein such as a repressor, hormone or receptor protein, could bring about amino acid substitutions, altering the regulatory properties of the protein. However, we suspect that only a minor fraction of the substitutions which accumulate in regulatory proteins would be likely to alter their regulatory properties. (King and Wilson 1975, p. 114)

Many of the genes encoding transcription factors and signaling molecules display sequence conservation across vast phylogenetic distances. This has lent further support to the idea that *cis*-regulatory changes might be more common than coding changes.

Although gene-coding regions are usually remarkably conserved across taxa as divergent as humans and worms, the total number of coding changes between closely related species is not negligible. There are, for example, about 60,000 nonsynonymous differences in gene-coding regions between humans and chimpanzees (Eyre-Walker 2006). Although it is difficult to estimate the number of phenotypic differences between species, in strict numerical terms these numerous coding changes may be sufficient to explain most phenotypic variation. Thus, by itself, this argument does not offer compelling support for the *cis*-regulatory hypothesis.

DIFFERENT CONSTRAINTS ON *CIS*-REGULATORY AND CODING REGIONS

Most genes are expressed in multiple tissues at multiple times in development (Tomancak et al. 2002). These complex expression patterns are generated primarily by binding of the transcription factors expressed and active in each cell at a particular time—the regulatory state—to specific sites in the *cis*-regulatory regions of many genes (Wray et al. 2003; Davidson 2006). Often, independent transcription factor binding sites are clustered in regions of several hundreds of base pairs and together they encode a particular transcriptional output.

Because information is encoded differently in *cis*-regulatory and coding regions, these regions may evolve at different rates. First, the redundancy of the genetic code causes about 24% of

Table 1. Haploid genome sizes and the proportion of coding and noncoding regions for various eukaryotes (modified from tables 3.2 and 3.3 of Lynch 2007).

	Approximate haploid genome size (in Mb)	Proportion coding	Proportion noncoding	Estimated proportion of noncoding DNA that is regulatory[1]
Saccharomyces cerevisiae	12	74.2	25.8	22
Aspergillus nidulans	30	45.9	54.1	2
Plasmodium falciparum	23	52.8	47.2	2
Caenorhabditis elegans	100	26.4	73.6	12
Drosophila melanogaster	137	19.4	80.6	20
Mus musculus	2500	1.4	98.6	2
Homo sapiens	2900	1.4	98.6	2

[1]The amount of regulatory DNA was estimated from islands of conserved DNA sequence between closely related species. See Lynch (2007) for details.

mutations in coding regions to be synonymous changes (Wilke 2004). Similarly, a single transcription factor can bind to multiple similar DNA sequences, rather than to only a single specific sequence. For example, the transcription factor SRY, which is involved in sex determination, binds to the DNA sequence WWCAAW, where W can be either T or A (Harley et al. 1994). It is not yet known if this "transcriptional code" is more or less redundant than the genetic code.

Second, most insertions or deletions in protein-coding regions (those that are not multiples of 3 bp) will disrupt the reading frame and cause a premature stop codon or alteration of amino acids in the protein. These changes are likely to have deleterious consequences. In contrast, insertions or deletions in *cis*-regulatory regions are less likely to alter *cis*-regulatory function because clusters of transcription factor binding sites can be separated by nonfunctional DNA of heterogenous length.

Third, new transcription factor binding sites may evolve more easily than new coding regions. The rate of point mutations is sufficiently high to rapidly generate new transcription factor binding sites (Stone and Wray 2001). For instance, the human genome contains abundant polymorphism in transcription factor binding sites that lead to both loss and gain of expression (Rockman and Wray 2002).

Furthermore, in most cases, the precise arrangement of transcription factor binding sites relative to each other is not critical to function. For example, the *cis*-regulatory region that drives *even-skipped* expression in stripe 2 of the *Drosophila* embryo has gained and lost transcription factor binding sites and retained the same function (Ludwig et al. 1998, 2000). In addition, among different fly species, the *cis*-regulatory region of *hunchback* contains a similar number of binding sites for the transcription factor Bicoid, but the precise number, orientation, and location of the binding sites differs among species (McGregor et al. 2001).

These features cause *cis*-regulatory DNA sequences to evolve faster than coding DNA sequences. However, it is not clear that these rapid changes in *cis*-regulatory regions have produced more phenotypic evolution than coding mutations. It is possible that a large number of the mutations occurring in *cis*-regulatory regions do not alter the phenotype and fitness. There is currently insufficient data to determine whether the higher flexibility in *cis*-regulatory sequence evolution biases the number of mutations causing phenotypic evolution toward *cis*-regulatory regions.

MUTATIONAL TARGET SIZE

The probability of fixation of a new mutation depends on the fitness effect associated with the mutation and the effective and demographic population sizes (Kimura 1962; Bürger and Ewens 1995). In addition, the probability that a mutation with a particular phenotypic effect arises in the first place is a function of the site-specific mutation rate and the mutational target size. If *cis*-regulatory regions encompass a larger mutational target size than coding regions, then we might expect more evolutionarily relevant mutations to accumulate in *cis*-regulatory regions than in coding regions. It is currently difficult to know whether *cis*-regulatory regions in fact have a larger mutational target size than coding regions.

Eukaryotic genomes are divided unevenly between noncoding and coding DNA (Table 1). In some eukaryotes, such as the yeast *Saccharomyces cerevisiae* and the malaria parasite *Plasmodium falciparum*, genomes contain more coding than noncoding DNA. In most eukaryotes, however, noncoding DNA is present in large excess relative to coding DNA. In *D. melanogaster*, about 80% of the genome is noncoding and in humans and mice more than 98% of the genome is noncoding. However, in humans more than 90% of this noncoding DNA is occasionally transcribed into RNA although the functional roles, if any, of most of this RNA are currently poorly understood (Mattick 2003; Birney et al. 2007).

An unknown fraction of the noncoding DNA encodes a *cis*-regulatory function. Estimates from first principles imply that in an organism like *D. melanogaster* only about 3% of the

genome encodes *cis*-regulatory function (Alonso and Wilkins 2005), whereas 19% is coding (Lynch 2007). Estimates of *cis*-regulatory DNA based on islands of sequence conservation among closely related species span from 1% to 22% of the noncoding DNA, depending on species (Table 1). Estimates based on evolutionary comparisons of rates of sequence evolution between closely related *Drosophila* species reveal that 40–70% of nucleotides in noncoding regions are more conserved than synonymous sites, suggesting that they are under functional constraint (Andolfatto 2005). However, regions can be conserved for reasons other than *cis*-regulatory function. They could participate in other functions such as DNA structure, chromosome replication, or they might also encode functional RNA transcripts. Given the wide range of current estimates and the many sources of variation, it is premature to generate confident estimates of the fraction of eukaryotic genomes that contains *cis*-regulatory information.

Not all mutations, whether in coding or *cis*-regulatory regions, will generate a functional change at the molecular level (a change in protein or RNA or a change in gene expression, respectively). For a random coding DNA sequence, the percentage of nonsynonymous substitutions equals 76% if all substitutions are equally likely (Wilke 2004). Functional changes in *cis*-regulatory regions can result from complete loss or gain of binding sites or from quantitative changes in transcription factor binding activity. Our current limited understanding of *cis*-regulatory regions prevents an accurate estimate of the percentage of random substitutions that are likely to alter gene expression.

Finally, only a fraction of these functional changes will cause a change in morphology, metabolism, physiology, or behavior (Kacser and Burns 1981). It is not yet clear whether the larger noncoding DNA regions of many eukaryotic genomes has led to a larger role of *cis*-regulatory mutations in phenotypic variation. Most noncoding DNA may not have a *cis*-regulatory function, and most *cis*-regulatory mutations may not have any phenotypic effects.

A POPULATION GENETICS ARGUMENT

Another argument supporting the *cis*-regulatory hypothesis derives from a detailed understanding of molecular and developmental mechanisms and rests, ultimately, on population genetics reasoning. This argument is that natural selection favors *cis*-regulatory mutations because they may have fewer pleiotropic effects than coding mutations. This argument has two core assumptions. The first assumption is that natural selection should favor mutations with fewer pleiotropic effects over those with more pleiotropic effects. The second assumption is that mutations in *cis*-regulatory regions should have relatively specific, less pleiotropic, effects than mutations in coding regions. We first explore both assumptions, which leads to support for this line of reasoning. Then, we critique this argument.

The theoretical consequences of pleiotropy

Does pleiotropy impact evolution? The consequences of pleiotropy for evolution have been explored within several theoretical frameworks: Fisher's geometric model of adaptation (Orr 1998, 2000; Welch and Waxman 2003); a framework in which the total selection acting on an allele is the sum of the positive direct effects and positive or negative pleiotropic effects (Hill and Keightley 1988; Barton 1990; Otto 2004); and a framework in which a mutation can influence one trait under directional selection and a second trait under stabilizing selection, so called "hidden pleiotropy" (Baatz and Wagner 1997). Despite this variety of approaches, all models agree on several results. First, as the degree of pleiotropy increases, the rate of adaptive evolution decreases dramatically. Orr (2000) estimates that this reduction in the rate of adaptation scales as n^{-1}, where n equals the number of phenotypic dimensions along which mutations can move the phenotype. Orr (2000) calls this the "cost of complexity," because universal pleiotropy in complex organisms should greatly reduce the rate of adaptation. Welch and Waxman (2003) show that this result is robust to a wide range of parameter values and to alterations to the model. They show that the reduction in the rate of adaptation is probably even faster than n^{-1}. In other words, mutations that move the phenotype along only one dimension should contribute to adaptation much more often than mutations that move the phenotype along two or more dimensions simultaneously, that is mutations that cause pleiotropic effects. The other modeling approaches reinforce this conclusion. Otto (2004) finds, for example, that under weak selection, pleiotropy reduces the total selection coefficient on an adaptive allele by half on average. She also finds that the probability of fixation for a mutation that improves a trait, but causes pleiotropic effects, is proportional to the square of the selection coefficient, s^2, where $0 < s < 1$. In contrast, the probability of fixation for an advantageous mutation without pleiotropic effects is approximately $2s$ (Haldane 1927; Fisher 1930; Wright 1931; Kimura 1962). Thus, strong pleiotropy will prevent fixation of most potentially adaptive mutations. Similarly, "hidden pleiotropy" dramatically reduces the rate of adaptation (Baatz and Wagner 1997). Thus, pleiotropy does not stop adaptation in its tracks, it just slows it, to a surprisingly large extent.

In real species—where one can get the distinct impression that natural selection has molded dozens, hundreds or thousands of discrete subtle adaptations for each species—the cost of complexity would appear to present a serious hurdle to adaptation. The models seem to imply that complex organisms with universal pleiotropy will have enormous difficulty adapting to novel environments. But complex organisms can evolve quickly both in the laboratory and in the field (Endler 1986). Either the models are wrong or the assumptions are wrong or both (Welch and Waxman 2003).

Given the diverse modeling approaches that have been taken, it seems unlikely that the general results are flawed. It seems more likely that the assumptions are flawed. Because organisms are, rather obviously, complex, it is unlikely that there is a problem with the assumption that organisms must adapt along multiple phenotypic axes simultaneously.

Welch and Waxman (2003) explored one possible way out of this paradox. They recognized the prevalence of developmental modularity in multicellular organisms (Raff and Kaufman 1983; Gerhart and Kirschner 1997) and asked whether modularity might eliminate the cost of complexity imposed by pleiotropy. They divided development into the activity of relatively independent modules. For example, legs develop largely independently from arms and heads. However, modularity at the level of organ development does not eliminate the cost of complexity (Welch and Waxman 2003). This is partly because the effect of pleiotropy on the rate of adaptation is so strong that it kicks in with even modest levels of pleiotropy.

Another possible escape from the cost of complexity is offered by results from several of these models. Strong selection on the main target of selection partially ameliorates the effect of deleterious pleiotropic effects on the rate of adaptation (Baatz and Wagner 1997; Otto 2004). Under strong selection, the response to selection more nearly reflects the sum of the direct and pleiotropic fitness effects. This makes intuitive sense. If a mutation arises in a cow that causes a phenotypic effect cherished by a farmer, this trait can be selected even in the face of severe pleiotropic fitness effects. However, strong selection appears to be rare in natural populations (Hoekstra et al. 2001; Kingsolver et al. 2001; Andolfatto 2007), suggesting that the assumption of relatively weak selection is probably realistic.

It seems most likely that there is a problem with the assumption of universal pleiotropy. How common is pleiotropy? Is it universal?

Gene pleiotropy

Gene pleiotropy, the effect of an allelic variant on multiple different phenotypes rather than just one, has historically been considered universal or at least very common. J. B. S. Haldane (1932, p. 62–63) wrote "Since the gene exists in every cell of the body, it may be expected to affect the organism as a whole, even if its most striking effect is on some particular organ or function." Sewall Wright (1968, p. 61) wrote "The available evidence indicates that pleiotropy is virtually universal." This idea has been repeated throughout the evolutionary literature and underlies all models of the effects of pleiotropy on evolution.

Although these sentiments were based largely on observations of the phenotypic effects of individual mutations in domesticated races and in laboratory strains, recent studies have confirmed the existence of widespread pleiotropy throughout the genome. For example, examination of 501 morphological phenotypes in each of 4710 deletion mutants of nonessential genes in yeast found that about 35% of deletion mutants affected at least two morphological characters (Ohya et al. 2005). A second study assayed relative fitness of these deletion mutants in 21 adverse growth conditions versus a control medium (Dudley et al. 2005). About 58% of genes displayed growth reduction in two or more conditions, which the authors considered to reflect pleiotropic effects in different growth conditions. These two studies have explored a limited number of growth conditions and aspects of morphology. In addition, yeast possess far fewer phenotypic features than multicellular eukaryotes. Therefore, deletion of genes in more complex eukaryotes is likely to cause at least as much pleiotropy as observed in yeast. Although gene pleiotropy may be relatively high in yeasts because they have relatively few genes, it seems likely that the classical view of widespread gene pleiotropy is accurate.

Further analysis of these datasets shows that gene pleiotropy is correlated with the number of biological processes and the number of protein–protein interactions that gene products participate in (He and Zhang 2006). Pleiotropy is not correlated with the number of molecular functions or the number of protein domains per gene. That is, pleiotropy results from the participation of a gene product in multiple cellular processes in which it performs the same molecular function. In multicellular organisms, therefore, considerable pleiotropy is expected to result from expression of genes in multiple tissues during development.

In the growth assays, elimination of genes displaying more pleiotropy reduced fitness to a greater degree than elimination of genes displaying less pleiotropy (Cooper et al. 2007). These results fit comfortably within the intuition embodied in Fisher's geometric model of adaptation (Fisher 1930). In this model, universal gene pleiotropy causes most mutations to move a population away from the fitness optimum.

Finally, the extent of gene pleiotropy discovered in the growth assays is correlated with the level of protein sequence conservation (He and Zhang 2006). This agrees with theoretical expectations that pleiotropy should constrain protein sequences (Waxman and Peck 1998). All together, these observations support the hypothesis that gene pleiotropy constrains protein evolution because gene products participate in multiple cellular functions.

Gene pleiotropy seems, therefore, to be extremely common. How, then, do complex organisms evolve specific adaptations apparently so readily?

Pleiotropic genes versus pleiotropic mutations

It is a striking fact that none of the population genetic models exploring the effects of pleiotropy have incorporated realistic models of gene structure and function. All have assumed that gene pleiotropy is universal and, more importantly, that universal

gene pleiotropy equals universal pleiotropy of mutational effects. However, there is no equivalence between the pleiotropic roles of genes and the effects of individual mutations (Stern 2000). Some of the mutations occurring in coding or in *cis*-regulatory regions of genes are likely to have specific effects, potentially without any pleiotropic effects. That is, the conception of pleiotropy that has historically gripped evolutionary biologists and served as the central assumption for recent theoretical treatments is effectively based on the effects of null mutations (Stern 2000). This conception is incomplete and future evolutionary models should be based on more realistic estimates of the developmental effects of individual mutations. In particular, models should incorporate the potentially differential pleiotropic effects of mutations in *cis*-regulatory and coding regions, as discussed below.

Modularity of cis*-regulatory regions*

In most cases studied in sufficient detail, *cis*-regulatory regions can be divided into small DNA regions, on the order of hundreds of base pairs, each of which encodes a small part of the entire pattern (Davidson 2006). In most cases, each of these *cis*-regulatory modules acts independently of the others. That is, these modules can be experimentally dissected from their native genomic region, artificially coupled to a heterologous promoter and reporter gene, and shown to drive reporter gene expression in a small part of the complete pattern. Each *cis*-regulatory module is a collection of transcription factor binding sites that, together, encode a transcriptional output. Usually, each module contains multiple binding sites for each of several transcription factors.

Cis-regulatory modules appear to evolve as structural features of the genome that are independent of one another (Wray et al. 2003). The simplest observation supporting this contention is that *cis*-regulatory modules are often separated from each other by large DNA regions, thousands to tens of thousands of base pairs long. In addition, although the modules can retain conserved function, the DNA regions separating them often evolve in size. This suggests that the precise distance between these regions is not required for function. This is consistent with the current mechanistic view that the intervening DNA is looped out, allowing the transcription factors bound to the DNA to make contact with the basal transcription apparatus. This looping apparently enables *cis*-regulatory modules to reside very far, sometimes over 100 kbp, from the basal promoter. The modules can reside 5′ or 3′ of the coding region, in introns, or, in rare instances, in exons (Wray et al. 2003).

The *cis*-regulatory regions of genes expressed in various tissues often contain multiple modules that act independently to drive part of the total expression pattern. The modules apparently have only weak constraints on position relative to each other and on position relative to the basal promoter. These features imply that modification or loss of a single module will normally alter only a small part of the total transcriptional pattern of the gene.

Coding mutations may either eliminate gene function or alter the gene product. Modifying a single domain of a protein or an RNA molecule is likely to affect the molecular properties of this molecule in many of the cellular contexts where it is present. If the gene encodes a transcription factor, then this change can alter the transcription of many other genes in every cell where the transcription factor is expressed. In contrast, mutations in *cis*-regulatory modules will normally alter gene expression in only a small part of the complete expression pattern.

Because *cis*-regulatory regions are usually more modular than coding regions, and because *cis*-regulatory modules are more independent of each other than the domains composing a protein or RNA molecules, mutations in *cis*-regulatory modules are likely to have fewer pleiotropic effects than mutations in coding regions. Together with the population genetics argument, this implies that *cis*-regulatory mutations are more likely to cause phenotypic evolution than coding changes. This argument thus supports the narrow *cis*-regulatory hypothesis, that phenotypic evolution should result more from *cis*-regulatory mutations than coding changes. It does not, however, provide strong support for the broad *cis*-regulatory hypothesis because the relative importance of mutations that are neither *cis*-regulatory nor coding changes is not explicitly addressed by this argument.

Difficulties with the population genetics argument

The population genetics argument was originally formulated for genes with pleiotropic roles in development and in possession of modular *cis*-regulatory regions. This is certainly the simplest way to conceptualize the problem and is consistent with the observation that many genes contain modular *cis*-regulatory regions. However, many genes may not have *cis*-regulatory regions organized into independent modules and it is not yet clear whether mutations in nonmodular *cis*-regulatory regions have nonpleiotropic effects. Although it is true that the majority of genes that have been examined in detail appear to possess at least some modularity in their *cis*-regulatory regions, it is also true that this modularity does not appear to be always complete. For example, experimental dissection of the *cis*-regulatory region of the *runt* gene in *D. melanogaster* has identified a >10 kb region required for embryonic expression in seven stripes. It was not possible, however, to dissect this region into smaller, independently acting modules (Klingler et al. 1996). The transcription factor binding sites driving expression in seven stripes may be dispersed throughout this region, rather than being compacted into individual modules.

In addition, the "file drawer" effect (Scargle 2000) may have caused an excess of modular *cis*-regulatory regions to be reported in the literature. Several of our colleagues have informed us of their unpublished attempts to dissect *cis*-regulatory regions that

Table 2. The distribution of evolutionarily relevant mutations in plants and animals.

	Plants	Animals
Coding[1]	71	163
Cis-regulatory	26	48
Other[2]	16	7
Total	113	218
Null[3]	67	32

[1]Includes mutations altering mRNA splicing.

[2]Includes gene amplification, gene loss, stable DNA methylation, as well as four cases in plants where the mutations were mapped to a gene but not localized to a coding versus *cis*-regulatory change.

[3]Number of total alleles that are presumed null based on existence of premature stop codons, altered splice sites that disrupt the protein, and deletions of part or all of the protein-coding sequence.

have not uncovered simple, modular *cis*-regulatory regions. These studies normally remain in the "file drawer" (for an exception, see Davis et al. 2007). Because most such studies have not been published, it is currently impossible to accurately estimate the proportion of genes containing independent *cis*-regulatory modules (Scargle 2000).

The lack of *cis*-regulatory modularity in some genes may not really be a vulnerability of the population genetics argument. These genes might still contain *cis*-regulatory regions in which at least some mutations can have specific, nonpleiotropic effects. That is, the *cis*-regulatory regions may be functionally modular without displaying obvious clusters of transcription factor binding sites. For example, although the *runt cis*-regulatory region cannot be dissected into independent modules, it is possible that some individual mutations would influence only one of the seven stripes driven by this enhancer region.

Although the *cis*-regulatory hypothesis was originally formulated as requiring only that mutations in *cis*-regulatory modules have fewer pleiotropic effects than mutations in coding regions, the current cost of complexity theory suggests that the absolute number of pleiotropic effects is the more important parameter. It is possible that individual mutations in *cis*-regulatory modules still have pleiotropic effects. We are aware of no empirical studies of the potential pleiotropic effects of *cis*-regulatory mutations.

Finally, the population genetics argument might not hold when other factors, such as genetic drift in populations of small effective size, strong selection, or differences in mutational target sizes bypass or overcome the cost of complexity.

In conclusion, we cannot find any serious difficulties with the proposal that genes with pleiotropic functions will preferentially accumulate evolutionary relevant mutations in the *cis*-regulatory region of the gene, especially when selection is weak and other parameter values are equal. However, in some circumstances—such as for genes without pleiotropic roles, for evolution by strong selection or in small populations, or for genes with a higher mutational target size for coding mutations than for *cis*-regulatory mutations—this prediction might not hold.

EXPERIMENTAL EVIDENCE

The last argument that has been advanced to support the *cis*-regulatory hypothesis is that many *cis*-regulatory changes have been identified as responsible for evolutionary changes in phenotype, or more specifically in morphology. As Hoekstra and Coyne (2007) justly noted, a comprehensive survey of the experimental evidence is required to test this claim. We have compiled a database of published studies that provide compelling evidence for the individual genetic mutations causing evolved phenotypic variation within and between species of multicellular organisms (Appendix 1). We found a total of 234 mutations in coding regions and 74 mutations in *cis*-regulatory regions (Table 2), including 62 coding changes and 43 *cis*-regulatory changes causing morphological evolution (Table 3). The absolute numbers of reported mutations in coding and *cis*-regulatory regions on their own clearly do not provide support for the *cis*-regulatory hypothesis in its simplest formulation. However, as discussed below (in section "The Data: Evidence for a Predictive Theory of Genetic Evolution"), many sources of ascertainment bias strongly inflate the reported contribution of coding changes to evolution. A more detailed analysis of the data is given in the same section.

WHITHER THE *CIS*-REGULATORY HYPOTHESIS?

The arguments reviewed above do not, on their own or combined together, provide definitive support for the *cis*-regulatory hypothesis. The strongest case against the *cis*-regulatory hypothesis is, currently, that more protein-coding changes are known to cause phenotypic evolution than *cis*-regulatory changes. However, the apparent abundance of protein-coding changes has resulted from several layers of ascertainment bias, which we discuss in more detail in the section "The Data: Evidence for a Predictive Theory of Genetic Evolution." In summary, theoretical arguments provide reasons why phenotypic evolution is likely to be dominated by *cis*-regulatory evolution, but other evolutionary forces such as mutational target size or population demographic history might obscure the expected trend. In any case, the arguments presented above do not prove that phenotypic evolution is dominated by *cis*-regulatory evolution. The question of the importance of *cis*-regulatory evolution will ultimately be settled with empirical data.

The Data: Evidence for a Predictive Theory of Genetic Evolution

We now address what we believe to be the fundamental disagreement between proponents of the *cis*-regulatory hypothesis and

Table 3. Distribution of evolutionary relevant mutations among phenotypic classes and among regulatory network levels.

	Morphology	Physiology	Behavior	DGB member[1]	Non-DGB member[2]
Coding[3]	62	170	2	132	102
Cis-regulatory	43	29	2	34	37
Other[4]	3	20	0	9	14
Total	108	219	4	175	153
Null[5]	41	58	0	22	77

[1]Gene is a known or presumptive member of a differentiation gene battery (DGB).

[2]Gene known or presumed to reside upstream of a DGB. Three genes could not be assigned to the DGB or non-DGB category because their function is unknown.

[3]Includes mutations altering mRNA splicing.

[4]Includes gene duplications, gene losses, stable DNA methylation, and four cases in which the mutations were mapped to a gene but not localized to a coding versus *cis*-regulatory change.

[5]Alleles presumed null based on existence of premature stop codons, altered splice sites, and deletions of part or all of the protein-coding sequence.

Hoekstra and Coyne (Coyne and Hoekstra 2007; Hoekstra and Coyne 2007). Evolutionary developmental biologists have examined the structure of developmental regulatory networks and the structure–function relationship of individual genes and predicted that *cis*-regulatory mutations should play a dominant role in morphological evolution. Hoekstra and Coyne (2007) have argued that these molecular mechanisms might not bias the distribution of evolutionarily relevant mutations and, in any case, that historical contingency might dominate patterns of genetic evolution (see also Appendix 2). We state the problem in stark terms to clarify what is at stake: a theory of genetic evolution. Does the architecture of gene regulatory networks and the structure of genes influence which mutations are favored during evolution? Or, does the historical contingency of the mutational process dominate and cause fundamentally unpredictable patterns of genetic evolution?

So far, debate has focused on the proportion of *cis*-regulatory versus coding mutations causing phenotypic evolution. Empirical data clearly demonstrate that considerable numbers of both types of mutations contribute to phenotypic evolution. We believe that little progress will be made by structuring the debate as an enquiry simply into the proportion of *cis*-regulatory versus coding changes. This superficially attractive dichotomy hides considerable complexity resulting from precisely how development generates the phenotype and in how mutations traverse populations to cause phenotypic variation and population differentiation. It may be more profitable to turn the problem around and ask more specific questions. For example, how do we expect particular kinds of mutations to generate particular kinds of phenotypic variation? How do we expect population genetic parameters to influence the spread and fixation of different kinds of mutations?

In this spirit, we discuss three predictions that derive from the *cis*-regulatory hypothesis and from our current understanding of the molecular basis for development and test them with available data. We chose to focus on multicellular plants and animals. Many studies indicate that unicellular organisms show predictable patterns of genetic evolution (see for example Boucher et al. 1992; Wichman et al. 1999; Riehle et al. 2001; Dunham et al. 2002; Hittinger et al. 2004; Segre et al. 2006; Woods et al. 2006).

THE DATA

Currently, considerable effort is devoted to identifying the genes and mutations underlying phenotypic evolution, particularly in domesticated races and in natural populations of single species. We have compiled a database of published studies that provide compelling evidence for the individual genetic mutations causing evolved phenotypic variation (Appendix 1). We included variation in domesticated species (99 cases), intraspecific variation in wild species (157 cases), and interspecific differences (75 cases). We included domesticated species because, ever since Darwin, they have been considered as potential models for how evolution might occur in the wild (Price 2002; Andersson and Georges 2004). We did not include variation selected in laboratory experiments. The dataset includes many studies from both plants and animals (Table 2). Although we have almost certainly inadvertently overlooked some relevant studies, we have attempted to be comprehensive.

This dataset includes extensive ascertainment bias, both in the choice of genes studied and in the gene regions examined. Most researchers have focused on candidate genes. Even genome-wide mapping studies usually include a search for candidate genes in the mapped region, rather than functional surveys of all genes in the mapped region. But the most important consequence of investigator bias is that the relative number of coding versus *cis*-regulatory mutations causing phenotypic evolution is almost certainly inflated. Indeed, it is easier to identify potentially important coding changes, especially nonsense mutations, than potentially

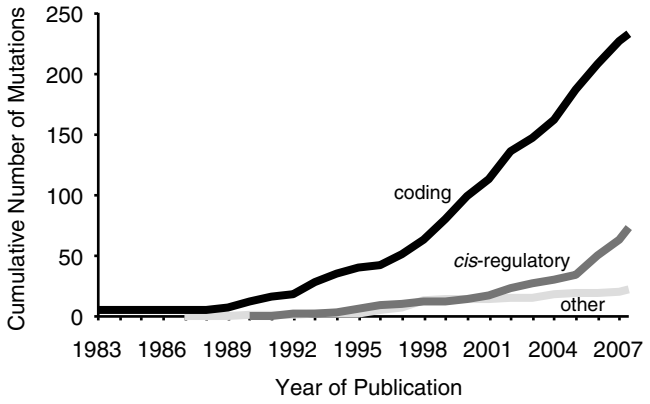

Figure 2. Cumulative number of coding mutations, *cis*-regulatory mutations and other types of mutations (gene amplification, gene loss, etc.) that have been identified over time as responsible for phenotypic evolution. Results are from data in Appendix 1. Note that the slope for *cis*-regulatory mutations has increased in recent years. The current discovery rate of *cis*-regulatory mutations approximately equals the discovery rate of coding mutations. If this reflects the long-term trend, then we expect ultimately to observe approximately equal numbers of *cis*-regulatory and coding mutations.

relevant changes in *cis*-regulatory regions by simple examination of the DNA sequence. The recent surge in examples of *cis*-regulatory evolution (Fig. 2) may be due to the fact that more powerful experimental approaches for identifying *cis*-regulatory mutations have been developed recently (e.g., McGregor et al. 2007).

Therefore, we cannot estimate, based on our dataset, the real overall frequency of *cis*-regulatory versus coding mutations causing phenotypic evolution. Twenty-two percent of mutations in our dataset occurred in *cis*-regulatory regions for all types of phenotypic change (22% with domesticated examples excluded), and 40% for morphological evolution (55% with domesticated examples excluded). These values are almost certainly minimum estimates of the frequency of *cis*-regulatory changes causing phenotypic evolution.

Although we cannot confidently test the *cis*-regulatory hypothesis based on our compilation, it is likely, although not certain, that similar ascertainment bias has been applied to different kinds of traits and to different taxonomic levels. We can therefore use the experimental data to compare the relative importance of *cis*-regulatory evolution between types of traits and between taxonomic levels.

We now examine three predictions derived from the *cis*-regulatory hypothesis and, to the extent possible, test them with available data.

PREDICTION 1: MORPHOLOGICAL EVOLUTION RESULTS FROM MORE *CIS*-REGULATORY CHANGES THAN PHYSIOLOGICAL EVOLUTION

The *cis*-regulatory hypothesis has been applied traditionally to the evolution of morphology. Occasionally, the idea that physiological evolution might involve more coding changes than morphological evolution has been made explicit (Carroll 2005). Hoekstra and Coyne (2007) claim that the *cis*-regulatory hypothesis should apply to all adaptation or not at all. As they argue, and we agree, the division between anatomy and physiology may be a misleading dichotomy. At the very least, there is not a clear boundary between genetic mechanisms generating morphology and physiology. Both result from activity of genes that are embedded within gene regulatory networks. Developmental processes are influenced by physiological processes, and vice versa. For example, development can be regulated by steroid hormones and by sugar and lipid metabolism (Wilkins 2002) whereas endocrine glands are formed through developmental mechanisms.

The distinction between morphological and physiological evolution that has been largely implicit in discussions within the evolutionary developmental biology community is that physiological traits tend to result from genes located at or near the terminal points of regulatory networks, whereas anatomy usually results from the activity of genes embedded deeper in the developmental network. Genes that act at or near the terminal points of regulatory networks, named differentiation gene batteries (Davidson 2006), represent genes expressed in differentiated tissues to fulfill cell-type-specific functions. These gene products build muscle cells, make skeletal biominerals, mediate synaptic transmission, etc. They do not regulate other genes, and they do not control the progressive formation of spatial patterns of gene expression that underlie development. They receive rather than generate developmental instructions. Even though they may be expressed in various tissues, their pleiotropic roles are more limited than gene products that function higher in the regulatory network. For example, a coding mutation in the *D. melanogaster* gene *forked*, a gene involved in terminal differentiation of bristles, can cause every bristle to develop differently, but all in the same way. In contrast, a coding mutation in *wingless*, a signaling molecule involved in diverse regulatory networks in various tissues, will alter the development of segments, legs, wings, genitalia, and eyes in *Drosophila*, all in different ways. Therefore, it should be more difficult to evolve a coding change in *wingless* that somehow provides an advantage than a coding change in *forked*. Because the position of a gene within a gene regulatory network is likely to influence its pleiotropic roles, we might thus predict that genes at terminal positions in regulatory cascades, members of differentiation gene batteries, should result from coding changes more often than genes embedded deeper within regulatory networks.

To test these hypotheses, we have divided the data in several ways. First, we examine the traditional morphology versus physiology hypothesis by classifying mutations, as we felt most biologists would, as contributing to morphology, physiology, or behavior. Just as the "morphology" and "physiology" categories are poorly defined, the "behavior" category also hides considerable diversity in mechanisms. Behavior might evolve because an odorant receptor, a member of a differentiation gene battery (DGB), evolves affinity for a new odorant. Or behavior might evolve because neurons are connected differently as a result of a new pattern of development. To our knowledge, less than 10 alleles causing evolved behavior have been identified so far, so no generalizations about behavioral evolution are possible yet.

Second, we divided the data into genes that are known or are likely to be members of a DGB versus genes that are known or are likely to be embedded more deeply within the developmental regulatory network (non-DGB).

As expected, we found that the proportion of *cis*-regulatory to coding mutations is significantly higher for morphological traits compared to physiological traits (Table 3: Fisher's exact test, two-tailed, $P < 10^{-6}$). This supports the intuition of evolutionary developmental biologists that *cis*-regulatory mutations have more often been reported for morphological variation than for physiological variation.

However, the proportion of *cis*-regulatory to coding mutations for non-DGB genes is not significantly different from the proportion for DGB genes (Table 3: Fisher's exact test, two-tailed, $P < 0.23$). This is despite the fact that morphological evolution for the most part involves mutations in non-DGB genes (80 mutations in non-DGB genes vs. 26 in DGB genes) whereas physiological variation mostly involves changes in DGB genes (148 mutations in DGB genes vs. 70 in non-DGB genes). Although we cannot exclude the possibility that our classification of genes into the DGB and non-DGB categories is erroneous, this suggests that factors others than those discussed here influence the proportion of evolutionary relevant *cis*-regulatory mutations that are identified for different kinds of genes. For example, it is possible that evolutionary developmental biologists studying morphological variation are more likely to study evolution of *cis*-regulatory regions than are physiologists. In any case, this observation fails to support the hypothesis that evolution of genes embedded within regulatory networks are more likely to result from changes in *cis*-regulatory regions than are DGB genes. In both cases, about 20% of reported mutations are *cis*-regulatory.

PREDICTION 2: THE STRENGTH OF SELECTION AND THE EVOLUTIONARY TIME SCALE SHOULD INFLUENCE THE SPECTRUM OF EVOLUTIONARILY RELEVANT MUTATIONS

Over the past 50 years, various authors have suggested that population structure and the strength of selection might influence the kinds of mutations that are selected (Crow 1956; Lande 1983; Liu et al. 1996). Weak selection is expected to bias the spectrum of selected mutations toward those with few or no pleiotropic effects (Otto 2004). In contrast, strong selection, such as that encountered during laboratory selection experiments and perhaps during rapid local adaptation, can overcome pleiotropic deleterious effects of mutations (Baatz and Wagner 1997; Otto 2004). Domestication may also sometimes involve strong selection (Wang et al. 1999). Based on the function of different gene regions, we expect that, on average, *cis*-regulatory mutations will have fewer pleiotropic effects than missense mutations, which in turn should have fewer pleiotropic effects than nonsense mutations or gene deletions.

In addition, short-term evolution may lead to a different spectrum of mutations than long-term evolution. During long-term evolution, mutations may be tested in a variety of environments. Thus, mutations advantageous in one environment, but deleterious in others, may be eliminated over time. In addition, mutations that maintain adaptive plasticity will also be favored over longer time scales when environments vary. Both heterogeneous fitness in multiple environments and loss of plasticity can be considered pleiotropic effects of mutations. *Cis*-regulatory mutations are more likely to limit such pleiotropic effects than coding changes. Thus, *cis*-regulatory mutations may be favored over longer time scales.

We asked whether there is a different distribution of *cis*-regulatory, missense, and nonsense mutations that cause phenotypic differences in domesticated populations, segregating within wild species and between species. These taxonomic categories are expected to vary in the strength and duration of selection and perhaps in other uncontrolled variables. Our compilation reveals that domesticated races and intraspecific variants show striking differences from interspecific comparisons (Table 4).

Thirty-seven percent of the identified mutations underlying intraspecific variation in domesticated species and in a few wild species, especially *Arabidopsis thaliana*, cause the elimination of gene function (Table 4, Appendix 1). The null mutations in domesticated and some wild species often have large phenotypic effects. Many of these traits are closely related to fitness, like flowering time and growth rate, so it is likely that the mutations experienced strong selection. This is a striking result, because most of these genes display strong evolutionary conservation across vast taxonomic distances. Because the elimination of gene function, especially through insertion or deletion events, is largely inconsistent with conservation of these gene sequences through purifying selection, we must conclude that most of these mutations reflect recent selection. This is entirely consistent with the recent origin of domesticated species. It is also consistent with the recent spread of some species, like *A. thaliana*, to novel habitats and the relative rarity of most of these alleles (Le Corre et al. 2002).

Table 4. Distribution of evolutionary relevant mutations among taxonomic levels.

	Domesticated	Intraspecific	Interspecific[1]	Higher taxonomic level[2]
Coding[3]	65	122	28	19
Cis-regulatory	23	24	24	3
Other[4]	11	11	1	0
Total	99	157	53	22
Null[5]	55	39	3	2

[1]Includes recently diverged populations that experience reproductive isolation and are often considered different species, such as divergent stickleback populations.

[2]Comparisons of species that are not sibling species.

[3]Includes mutations altering mRNA splicing.

[4]Includes gene duplications, gene losses, stable DNA methylation, and four cases in which the mutations were mapped to a gene but not localized to a coding versus *cis*-regulatory change.

[5]Includes alleles presumed null based on existence of premature stop codons and deletions of part or all of the protein-coding sequence.

In contrast, it is striking that only about 7% of comparisons above the species level (five cases out of 75) have so far identified null alleles (Table 4). Instead, studies above the species level have found mostly coding mutations that modify but do not eliminate protein function and *cis*-regulatory mutations that alter only part of the gene's function (Table 4).

The similarities in the mutational spectra of domesticated species and *Arabidopsis* populations suggest that population structure and history and the strength and duration of selection can influence which mutations are selected by natural selection. This implies one of two things, or perhaps a mixture of both. The first possibility is that domesticated species and, to a certain extent, *A. thaliana* are poor proxies for genetic evolution happening in most wild populations. Alternatively, many wild populations may experience selective regimes for loss of function alleles similar to those experienced by domesticated populations and *A. thaliana* on the short time scale, but these mutations are not fixed. Instead, other mutations must arise that are fixed to cause differences between species. In most cases, both the domesticated populations and the *A. thaliana* populations are still segregating for the ancestral, conserved alleles of the genes causing phenotypic variation, in addition to the derived loss-of-function alleles. These loss-of-function alleles may not ultimately contribute to species differences if they carry pleiotropic fitness costs (Scarcelli et al. 2007). More specific alleles—at the same or different loci—that impart the advantageous effect without the pleiotropic consequences may replace the original allele and eventually become fixed.

We can generate a more specific prediction by combining knowledge of gene network positions and the likely history of selection. Weak selection and selection across multiple environments are more likely to have acted upon species differences than on phenotypic variants under domestication and perhaps in some recently evolved populations, like *Arabidopsis*. It is under these conditions that we expect the population genetics argument to become more important. We thus expect to observe more *cis*-regulatory mutations in regulatory genes (non-DGB genes) for interspecies comparisons than for domesticated races or recently evolved populations. This hypothesis receives significant support from published studies.

The proportion of *cis*-regulatory mutations identified at various taxonomic ranks for morphological and physiological traits and for DGB and non-DGB genes is shown in Figure 3. Morphological differences at the interspecies level or higher involve significantly more *cis*-regulatory changes than coding changes (Fig. 3A; Table 5). Similarly, for non-DGB genes, phenotypic differences between species involve significantly more *cis*-regulatory mutations than coding mutations (Fig. 3C; Table 5). For physiological traits and for DGB genes, all mutations responsible for intergeneric differences have been found in coding regions (Fig. 3). To reduce potential bias introduced by the discovery of multiple mutations in the same genes in studies following an initial report, we also analyzed a restricted dataset, where only one or two mutations were included per gene. (Two mutations, one coding and one *cis*-regulatory, were included in the analysis only if both coding and *cis*-regulatory mutations were found for a single gene [Appendix 1].) The trends reported above are also observed for this restricted dataset. (compare Fig. 3A with 3B and 3C with 3D). The restricted dataset contains relatively few mutations causing phenotypic evolution between species, which highlights the need for more data. A recent analysis has shown that evolutionary variation in gene expression levels is more often caused by *cis*-regulatory changes in the target gene between species than within species (Wittkopp et al. 2008). This observation is consistent with our analysis of phenotypic differences within and between species.

These observations provide a possible explanation for the disagreement between most evolutionary developmental biologists and Hoekstra and Coyne (2007). Studies of physiological traits

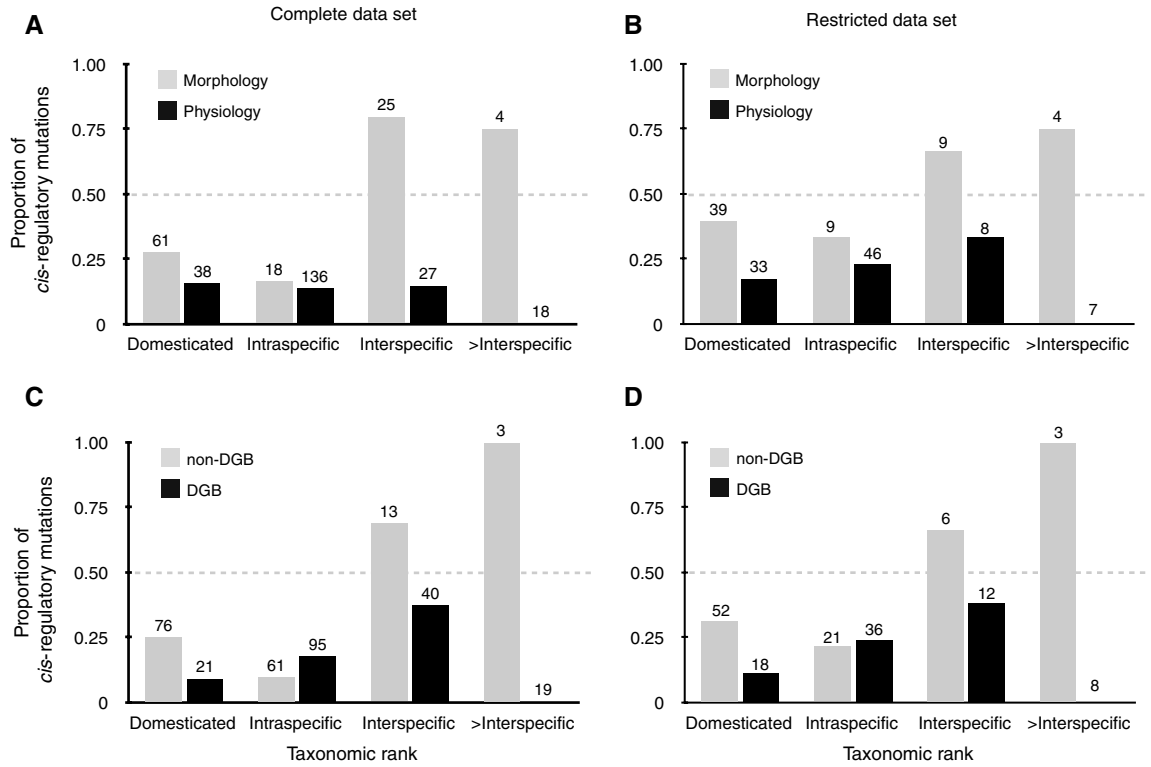

Figure 3. **Evolutionarily relevant *cis*-regulatory mutations are more frequently found in interspecific comparisons than in intraspecific comparisons or among domesticated races. (A) The proportion of all mutations that are *cis*-regulatory mutations for morphological and physiological traits in the complete dataset. (B) Proportion of *cis*-regulatory mutations for morphological and physiological traits in the restricted dataset, where only one or two mutations per gene were included. Two mutations were included only if both coding and *cis*-regulatory mutations were found for a single gene. (C) Proportion of *cis*-regulatory mutations for DGB versus non-DGB genes in the complete dataset. (D) Proportion of *cis*-regulatory mutations for DGB versus non-DGB genes in the restricted dataset. The total number of mutations for each category is shown above the bars.**

and DGB genes between species and of all genes within species have usually provided evidence for a predominance of coding changes. Conversely, comparisons of developmental regulatory genes across species have provided support for the prevalence of *cis*-regulatory changes causing morphological evolution. The data make more sense when both gene function and population genetics are considered simultaneously. The current evidence suggests that strong selection often results in selection of mutations with

Table 5. **Statistical comparisons of the frequency of *cis*-regulatory and coding mutations for different phenotypic classes, different gene-network classes and different taxonomic levels.**

	Complete dataset			Restricted dataset		
	G[1]	*P*[2]	Intraspecific vs. interspecific[3] Fisher's exact[4]	G[1]	*P*[2]	Intraspecific vs. interspecific[3] Fisher's exact[4]
Morphology	25.9	<0.00002	<0.00007	6.2	<0.11	<0.35
Physiology	6.5	<0.09	<0.45	4.0	<0.26	1
DGB	16.8	<0.0008	<0.42	6.2	<0.11	1
Non-DGB	27.0	<0.000006	<0.000002	7.6	<0.06	<0.05

[1]Value of G test of independence for the number of *cis*-regulatory versus coding mutations for domesticated, intraspecific, interspecific, and intergeneric taxonomic levels.

[2]*P* values for all G tests of independence were calculated using three degrees of freedom.

[3]A test of the frequency of *cis*-regulatory versus coding mutations in intraspecific versus interspecific populations. Data from domesticated races were excluded and the interspecific and higher taxonomic level data were pooled.

[4]The *P* value for a Fisher's exact test of independence is reported.

strong and pleiotropic effects, such as those often observed in domesticated populations and in *A. thaliana*. Conversely, evolution over longer time periods apparently leads to fixation of mutations with more subtle and specific effects. Incorporating these observations into a coherent explanation for genetic evolution is an outstanding problem in evolutionary biology.

PREDICTION 3: THE POSITION OF A GENE WITHIN A REGULATORY NETWORK SHOULD IMPACT THE DISTRIBUTION OF EVOLUTIONARILY RELEVANT MUTATIONS AMONG GENES IN THE NETWORK

As discussed above, we believe that the position of a gene in a regulatory network is an important parameter to consider when determining whether *cis*-regulatory or coding mutations are more likely to contribute to phenotypic evolution. In this section we argue that the structure of regulatory networks may also influence which genes in the network are more likely to accumulate evolutionarily relevant mutations. This is a vast topic (Davidson 2006) and we focus here on particular parts of regulatory networks, where a single transcription factor serves as a key regulator of cell differentiation. These cases allow us to see most clearly how developmental regulatory architecture might help us to predict the genetic causes of phenotypic evolution.

This idea is best explained with two examples from *Drosophila*: trichome patterning and bristle patterning. Trichomes are cuticular extensions produced by insect epidermal cells. Larval trichomes may aid movement (Inestrosa et al. 1996). Bristles are pluricellular sensory organs produced through cell division of a single sensory precursor cell selected from a field of epidermal cells (Lai and Orgogozo 2004). Trichomes and bristles are produced during development at specific positions on the fly body (Fig. 4). Summaries of the regulatory networks that generate the final pattern of trichomes and bristles are shown in Figure 5.

For both trichomes and bristles, all of the information from patterning genes is ultimately integrated within each cell by a single gene: *shavenbaby/ovo* (*svb*) for most trichomes and *scute* for most bristles. We can consider *svb* and *scute* as input/output devices (Davidson and Erwin 2006). They integrate an extensive array of inputs, the regulatory state, and they produce an on or off transcriptional output. *Svb* and *scute* are transcription factors that each regulate at least dozens of terminal differentiation genes. Expression of these input/output device genes determines whether a cell differentiates a trichome, a bristle, or smooth cuticle. They therefore occupy bottleneck positions in their respective gene regulatory networks. All patterning information must flow through them and they then regulate multiple downstream genes.

Multiple changes in the pattern of trichomes and bristles have occurred during fly evolution. Based on our current understanding of developmental regulatory networks, we predict that most of these evolutionary changes have probably occurred through *cis*-regulatory mutations at *svb* and *scute*, respectively. The reasoning is as follows. Mutations causing evolutionary changes in trichome or bristle position are likely to result from changes in genes already involved in trichome and bristle development, respectively. Because genes that act downstream of *svb* and *scute* must act in combination with other genes to generate a trichome or bristle (Hartenstein 2004; Chanut-Delalande et al. 2006), mutations in these genes are unlikely to produce a change in trichome or bristle position (mutations in these genes may alter trichome or bristle shape or size). Mutations in patterning genes acting upstream of *svb* or *scute*, whether *cis*-regulatory or coding, are also unlikely to be favored because they will alter development of other structures. For example, the genes regulating *svb* and *scute* expression determine the pattern of multiple epidermal structures and features in addition to trichomes and sensory bristles: muscle attachment cells, oenocytes, epidermal glands, cuticle pigmentation, etc. (Calleja et al. 2002). Therefore, *cis*-regulatory mutations in *svb* and *scute* are likely to have the most specific, least pleiotropic effects of any mutations in any genes that might alter the pattern of trichomes and bristles.

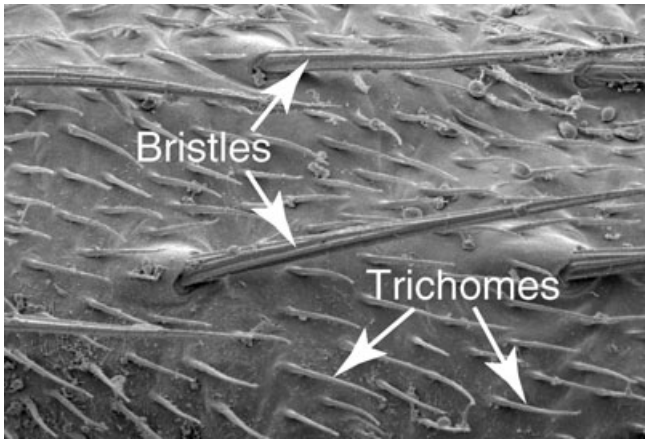

Figure 4. Scanning electron micrograph of trichomes and bristles on a leg of *Drosophila melanogaster*. Trichomes are nonsensory cuticular extensions. Bristles are sensory organs innervated by single neurons.

Cis-regulatory changes have been shown to cause a loss of dorsal larval trichomes in *D. sechellia* for *svb* (Sucena and Stern 2000; McGregor et al. 2007) and a gain of thoracic bristles in *D. quadrilineata* for *scute* (Marcellini and Simpson 2006). Furthermore, other changes in trichome and bristle patterns have been shown to correlate with changes in *svb* expression (Sucena et al. 2003) and *scute* expression (Wülbeck and Simpson 2000; Pistillo et al. 2002; Skaer et al. 2002b), respectively, whereas genes that act upstream of *svb* and *scute* show unchanged patterns of expression (Dickinson et al. 1993; Wülbeck and Simpson 2002; Richardson and Simpson 2006; Simpson et al. 2006).

The positions of *svb* and *scute* in their respective networks are like the positions of light switches in an electrical circuit. There are

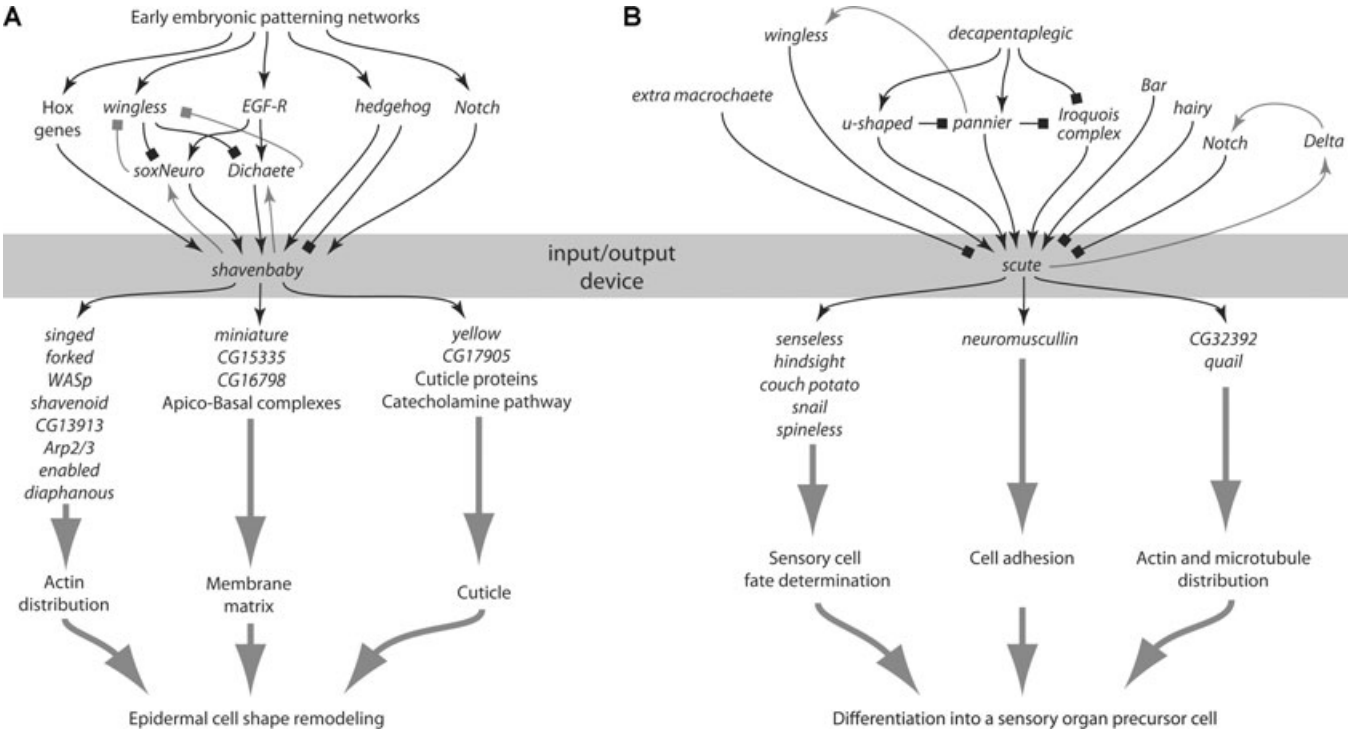

Figure 5. Partial regulatory networks patterning (A) trichomes (modified from results in Chanut-Delalande et al. 2006; Overton et al. 2007) and (B) bristles in *Drosophila melanogaster* (modified from Calleja et al. 2002; Hartenstein 2004).

multiple ways to turn off a light in a room. One could shut down the power generating station, cut the power line to the house, shut off the main breaker in a house, cut wires leading to the switch, flip the light switch, or break the light bulb. Clearly, of all of these options, flipping the light switch is both the most specific and the easiest to reverse. *Svb* is the switch that flips trichomes on or off and *scute* is the switch that flips bristles on or off. The multiple *cis*-regulatory modules of each gene are like the individual light switches in each room of a house. They provide great precision in evolutionary changes with minimal or no pleiotropic effects.

These examples illustrate the explanatory power that arises from a detailed understanding of the molecular mechanisms underlying cell differentiation and development. Of course, our "prediction" based on regulatory networks is really a post hoc explanation developed only after accumulating several pieces of evidence that support the predominance of *cis*-regulatory mutations in *svb* and *scute*. A real test of this hypothesis requires study of additional examples of trichome and bristle pattern evolution. But the true value of a predictive theory of genetic evolution will emerge only when novel predictions are made based on an understanding of other regulatory networks that are then tested with studies of natural variation.

The concept of an input/output gene resembles the concept of "cell-type specific selector gene" (Garcia-Bellido 1975; Mann and Carroll 2002) and is based on a more detailed understanding of development regulatory networks (Davidson 2006). Recognition of such a gene category was fundamental to the prediction discussed here. New compelling predictions for genetic evolution will probably require the development of new concepts and new gene categories based on a more detailed understanding of developmental biology.

Conclusions

In the absence of a population genetics framework, evolutionary developmental biologists have inferred from (1) our current understanding of gene regulatory networks, (2) our understanding of gene structure and function, and (3) the extensive conservation of developmental genes, that mutations in the *cis*-regulatory regions of developmental patterning genes are likely to underlie most of phenotypic evolution. However, no single argument proposed so far provides definitive proof that *cis*-regulatory mutations constitute the predominant cause of phenotypic evolution. By considering development and population genetics simultaneously, a survey of published data suggests that patterns of genetic evolution are not entirely obscured by historical contingency. Population genetics and development must be considered simultaneously to make sense of the data.

It may be unhelpful to pose the coding versus *cis*-regulatory debate as a quantitative question: do coding changes explain more of phenotypic evolution than *cis*-regulatory changes? It may be more productive to turn the question around and ask what kinds

of phenotypic changes are expected from particular coding versus *cis*-regulatory changes in specific genes. As we show, patterns in the currently available data imply that morphological and physiological traits are caused by different frequencies of coding and *cis*-regulatory changes. This is consistent with our molecular understanding of how coding and *cis*-regulatory changes might influence physiology and morphology.

We also found that different spectra of evolutionarily relevant mutations segregate within populations and between species. Interspecific differences in morphology seem to be more often caused by *cis*-regulatory changes than intraspecific variation. This result is not predicted by a traditional neo-Darwinian view of the contribution of intraspecific variation to interspecific differences. Instead, it appears that evolution over longer time scales results in fixation of a specific subset of the genetic variation contributing to intraspecific phenotypic variation.

By fusing developmental and evolutionary genetics, evolutionary biologists may be able to predict, in a probabilistic sense, the mutations underlying phenotypic evolution. Fortunately, scientists are rapidly identifying the genetic causes of phenotypic evolution, providing abundant data for testing new predictions about the genetic basis of evolution.

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Appendix 1

A database of studies providing compelling evidence for genetic changes contributing to evolution in domesticated races, within species and between species is provided as Supplementary Material on the Evolution website. We did not include studies that identified mutations resulting from selection experiments. We included studies that provided compelling genetic and functional evidence for the role of gene regions or individual mutations in generating an evolved phenotypic difference. We excluded most association studies that did not provide further genetic or functional evidence implicating gene regions or individual mutations. We included association studies that identified mutations—such as deletions or nonsense mutations—that are likely to generate complete protein loss of function alleles with obvious phenotypic consequences. The database includes all studies that we examined, together with information about whether they were included or excluded from analysis and why. We would appreciate learning about studies that we inadvertently overlooked.

Appendix 2

The main goal of Hoekstra and Coyne's (2007) commentary was to sow doubt about whether *cis*-regulatory evolution is really the dominant mode of genetic evolution. They attacked the *cis*-regulatory hypothesis from many directions. We address here their most important arguments that were not discussed in the body of our article.

GENE DUPLICATION AND THE *CIS*-REGULATORY HYPOTHESIS

Hoekstra and Coyne (2007) argued that gene duplication has been important for phenotypic evolution and that for two reasons this diminishes the importance of *cis*-regulatory evolution. They argued first that gene duplication seems to be an important source of phenotypic evolution, challenging the broad *cis*-regulatory hypothesis that states that most phenotypic evolution is caused by *cis*-regulatory changes. Second, they argued that gene duplication allows one gene copy to retain an ancestral function and the other to evolve a new function. This may reduce pleiotropic effects associated with coding mutations and thus lessen the importance of the population genetics argument.

How important is gene duplication? Gene duplication and the broad cis*-regulatory hypothesis*

In 1970, Ohno suggested that gene duplication provided important material for the evolution of novel phenotypes (Ohno 1970). Over the entire sweep of evolution, duplication has created many new genes. At particular periods during life history, gene duplication may have played important roles in phenotypic evolution (Lynch 2007). Particular families of genes have sometimes been duplicated as an apparent adaptive response to novel ecological challenges. In mammals, the olfactory receptor family has expanded to include about 1000 genes (Mombaerts 2001; Zhang and Firestein 2002). The nematode, *Caenorhabditis elegans*, contains about 1000 genes that may serve as chemoreceptors (Robertson and Thomas 2006).

Over long time scales, there is clear evidence that gene duplication has provided new material for developmental evolution. For example, the different genes of the Hox complex all originated by gene duplication from a single ancestral Hox gene and each of the extant genes has different DNA-binding specificity derived from the different amino acid sequences in the homeodomain (Akam 1995, Akam 1998; Carroll 1995; Averof et al. 1996; Holland and Garcia-Fernandez 1996; Hurley et al. 2005). Presumably such events occur rarely because it is difficult for new genes with pleiotropic roles to establish novel regulatory linkages without altering multiple processes simultaneously. Nonetheless, these events have occurred, albeit rarely. These rare events may have been more important to generating novel patterns of development than their rarity would imply. For example, evolution of the *bicoid* gene through duplication in higher dipterans may have promoted rapid early embryonic patterning (McGregor 2005) and duplication of the *achaete-scute* genes during insect evolution might have allowed the development of stereotyped bristle patterns (Skaer et al. 2002a).

Gene duplicates are fixed in populations at a rate of about one per gene per 100 million years (Lynch and Conery 2000). However, many of these duplicates eventually become pseudogenes and are eliminated from genomes. In general, the rate of gene duplication appears to be lower than the rate of coding or *cis*-regulatory mutations (Carroll 2005). For example, since the divergence of chimpanzees and humans, the human lineage has accumulated about 720 genes by gene duplication (Zhang 2003) whereas approximately 60,000 nonsynonymous differences and even more noncoding differences are found between humans and chimpanzees (Eyre-Walker 2006). Nevertheless, it is difficult to estimate how many of these duplicated genes and coding and *cis*-regulatory changes have contributed to phenotypic evolution.

Gene duplication versus cis*-regulatory and coding changes*

How relevant is gene duplication to the *cis*-regulatory hypothesis? Gene duplication usually causes simply an increase in gene expression. Indeed, few cases of gene duplications have been reported to cause phenotypic evolution. They include gene amplification of esterase genes in mosquitoes and aphids that are resistant to organophosphate and carbamate insecticides (Hemingway et al. 2004) and *amylase* gene amplification in humans associated with increased starch consumption (Perry et al. 2007).

Perhaps more importantly, gene duplication often contributes to phenotypic evolution through the novel *cis*-regulatory and coding mutations that occur subsequently or concomitantly. Here are three examples.

(1) *RNase* —Colobine monkeys eat leaves, rather than fruit and insects, and have evolved a fermenting foregut, similar to ruminants. Symbiotic bacteria in the foregut digest the leaves and the monkeys digest the bacteria. Colobine monkeys have evolved a duplicated RNase1 gene that is expressed specifically in the pancreas and secreted into the small intestine, where it operates in a relatively acidic environment. The duplicated gene has evolved multiple amino-acid substitutions, each of which increases its efficiency in an acidic environment (Zhang et al. 2002). The duplicated gene has also lost the ability to digest double-stranded RNA, which the original gene retains. Thus, this adaptation required gene duplication together with altered expression pattern (expression in the pancreas) and multiple amino-acid substitutions.

(2) *Eye lens crystallins*—Vertebrate eye lens crystallins have evolved repeatedly from enzymes and heat shock proteins (Wistow and Piatigorsky 1988; Piatigorsky and Wistow 1991; Yang and Cvekl 2005). These proteins have lost their enzymatic activity and are expressed specifically in lens cells at very high levels. Although the loss of enzymatic activity is probably relevant to their function in lens, the regulatory change—expression specifically in the lens and at very high levels—was critical. In most cases, it is thought that the regulatory change occurred first, followed by changes in protein structure and gene duplication (Piatigorsky and Wistow 1991).

(3) *Antifreeze proteins*—Fish that live in subzero waters have evolved proteins that capture ice in the bloodstream to prevent ice crystals from puncturing cells. In Antarctic notothenioids, this antifreeze protein evolved from a pancreatic trypsinogen (Chen et al. 1997). The protein has undergone extensive modification resulting from selection for binding ice. At some point, the protein became expressed in liver to allow secretion into the bloodstream. Thus, this evolutionary innovation required changes in both protein structure and expression pattern.

In these three examples, the novel gene functions probably evolved after gene duplication and they required changes in both the protein-coding regions and in the expression patterns. Because both duplicated genes are found in the same *trans*-regulatory environment, their change in expression pattern must result, at least in part, from *cis*-regulatory changes. The alternative—a change only in upstream regulator(s)—would affect the expression pattern of both duplicated genes.

Gene duplication and cis*-regulatory architecture*

Although duplicated genes can evolve novel functions through both protein-coding and *cis*-regulatory changes, it is currently thought that the key feature that allows gene duplicates to persist long enough to evolve novel functions is the fact that genes possess multiple modular *cis*-regulatory elements (Averof et al.

1996; Force et al. 1999; Lynch and Force 2000; Lynch et al. 2001; Force et al. 2005). Some of these modules can be lost either during the duplication process itself (Averof et al. 1996) or subsequently through accumulation of neutral mutations that eliminate complementary *cis*-regulatory enhancers in the original and the duplicated gene (Force et al. 1999; Lynch and Force 2000; Force et al. 2005). Complementary loss of these enhancer modules then generates selection to maintain both duplicates, setting the stage for future functional divergence.

The three examples mentioned above are of genes expressed near or at the end of terminal differentiation. The combination of three facts—the relatively terminal position of these genes in the regulatory hierarchy, the derived genes are duplicates that are not required to maintain the original gene function, and the derived genes have no obvious deleterious effects in the cellular context of the original gene—may provide extensive flexibility in the mutational changes that caused the evolution of novel functions. In contrast, genes that are not at terminal positions within regulatory hierarchies may undergo fewer changes in coding regions following gene duplication. For example, after duplication of the *achaete-scute* genes and of the *paired-gooseberry-gooseberry neuro* genes, the new genes acquired different expression patterns but, in *D. melanogaster*, their gene products can still substitute for one another (Li and Noll 1994; Skaer et al. 2002a). This suggests that in these cases, few or no evolutionarily relevant mutations have occurred in the coding regions of the duplicated genes; they have occurred mostly in their *cis*-regulatory regions.

In conclusion, gene duplication is not really a competitor with the *cis*-regulatory hypothesis because gene duplication simply generates an increase in gene expression. With current data it seems premature to judge whether mutations that follow gene duplication are more likely to occur in coding or in *cis*-regulatory regions. Most examples of gene duplication providing adaptive new functions have involved important changes in both coding and *cis*-regulatory regions.

ADAPTATION AND EVOLUTION

In their critique of the *cis*-regulatory hypothesis, Hoekstra and Coyne (2007) sometimes implicitly equated morphological evolution and adaptation and other times argued that the focus of genetic studies should be on adaptations rather than morphological evolution per se. Thus, their compilation of genes and mutations that contribute to phenotypic evolution comprises almost exclusively traits that are generally recognized to increase fitness or that are maintained by selection.

There are several problems here. First, Hoekstra and Coyne (2007) excluded a few traits from consideration not because they have been shown to be neutral or deleterious, but because they have not been proven to be adaptations. Imagine that we required that only traits with clear adaptive significance were suitable subjects for genetic and developmental analysis. We would greatly reduce the spectrum of admissible traits because the vast majority of tests for natural selection in the wild find very small selection coefficients (Hoekstra et al. 2001; Kingsolver et al. 2001). Unfortunately, most studies are unable to demonstrate that these small selection coefficients are significantly different from zero. This is because either the phenotypic variants are neutral or because sample sizes or selection coefficients are too low to allow experimental detection. The latter model is consistent with recent molecular population genomics analyses (Eyre-Walker 2006; Andolfatto 2007).

The temptation to tell adaptive stories about phenotypic traits has a long and checkered history in evolutionary biology (Gould and Lewontin 1979). In addition, the desire to tell adaptive stories leads to the equally nefarious tendency to dismiss adaptive explanations for traits when our imagination fails us. Darwin warned us of this problem (Darwin 1859). In Chapter 6 of the Origin of Species, in a section entitled "Organs of little apparent Importance, as affected by Natural Selection" he wrote

> In the first place, we are much too ignorant in regard to the whole economy of any one organic being, to say what slight modifications would be of importance or not.

This remains true today. Therefore, traits with no obvious adaptive value should still be considered when investigating the molecular basis of evolutionary changes in phenotype (Nei 2007).

But there is an even deeper problem with studying only clearly adaptive traits. This might bias our understanding of the genetic causes of variation toward traits under strong selection. As we showed in the section "The Data: Evidence for a Predictive Theory of Genetic Evolution," traits under strong selection may be caused by an unusual distribution of mutations. For example, some natural genetic variation underlying apparently adaptive polymorphisms is caused by mutations that eliminate or severely disrupt the gene product. Many of these genes are otherwise strongly conserved in other species, suggesting that these recent adaptive polymorphisms have poor long-term prospects. It is thus not entirely clear that focusing only on adaptive traits, particularly traits under strong selection—which are, of course, the easiest to identify—necessarily identifies alleles that are most relevant to long-term evolution.

There is currently a fundamental disconnect between population genomics approaches to studying adaptation and genetic studies of "obviously" adaptive traits, especially of polymorphisms maintained in populations. Population genomics approaches generate estimates of very small selection coefficients, on the order of 10^{-5} for most adaptive fixations in *Drosophila* (Andolfatto 2007). In contrast, when measured, selection in the wild is often about four orders of magnitude greater (Hoekstra et al. 2001; Kingsolver et al. 2001). Studies of clearly adaptive traits are,

therefore, clearly unrepresentative of the vast majority of substitutions fixed by selection.

Despite our objection to Hoekstra and Coyne's focus on clearly adaptive traits, we do think that there would be enormous value in determining the selection coefficients associated with phenotypic evolution. This would then allow a robust test of whether phenotypic variants with different selection coefficients are caused by different kinds of mutations. However, given the great difficulty of measuring, let alone detecting, weak selection in natural conditions, we are not convinced that in practice it will be possible to gather the required data. Nonetheless, the population genetics argument supporting the *cis*-regulatory hypothesis is, at heart, an argument about the action of natural selection, as shown in the section "Arguments for the *Cis*-regulatory hypothesis."

ARE OBSERVED EXAMPLES OF *CIS*-REGULATORY EVOLUTION BIASED TOWARD TRAIT LOSSES?

Hoekstra and Coyne (2007) parenthetically suggested that the available genetic data might be biased toward trait losses and that trait loss may be easier via *cis*-regulatory changes than through protein-coding changes (p. 1004). They did not provide a molecular explanation for their hypothesis and it is not obvious how *cis*-regulatory evolution would more easily generate phenotypic loss than coding changes. For example, loss of a transcription factor binding site for an activator can lead to loss of expression, but loss of a repressor binding site can cause gain of expression. In addition, a loss of gene expression might be associated with a trait gain and vice versa.

In addition, it is not always clear that a human subjective assignment of trait "gain" or "loss" is appropriate. For example, "loss" of trichomes might just as easily be called "gain" of naked cuticle. Most insect epidermal cells differentiate into one of these two alternative states and both states require the regulation of a large set of genes in a regulatory network. It is not at all clear that one represents a gain or loss relative to the other in any objective sense.

Finally, the abundance of studies of trait loss observed by Hoekstra and Coyne (2007) may reflect the more mundane fact that when comparing closely related species, where analysis is more straightforward, phenotypic loss is more common than gain (Adamowicz and Purvis 2006). That is, the apparent bias noted by Hoekstra and Coyne (2007) may reflect the frequency of trait gain versus loss during evolution, rather than a fundamental bias in the way *cis*-regulatory regions evolve.

Supporting Information

The following supporting information is available for this article:

Database for Appendix 1.
References for Appendix 1 Database.

This material is available as part of the online article from:
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