A Perspective on Micro-Evo-Devo: Progress and Potential

Maria D. S. Nunes,* Saad Arif,* Christian Schlötterer,† and Alistair P. McGregor*,[1]
*Department of Biological and Medical Sciences, Oxford Brookes University, Oxford OX3 0BP, United Kingdom, and †Institut für Populationsgenetik, Vetmeduni Vienna, A-1210 Vienna, Austria

ABSTRACT The term "micro-evo-devo" refers to the combined study of the genetic and developmental bases of natural variation in populations and the evolutionary forces that have shaped this variation. It thus represents a synthesis of the fields of evolutionary developmental biology and population genetics. As has been pointed out by several others, this synthesis can provide insights into the evolution of organismal form and function that have not been possible within these individual disciplines separately. Despite a number of important successes in micro-evo-devo, however, it appears that evo devo and population genetics remain largely separate spheres of research, limiting their ability to address evolutionary questions. This also risks pushing contemporary evo devo to the fringes of evolutionary biology because it does not describe the causative molecular changes underlying evolution or the evolutionary forces involved. Here we reemphasize the theoretical and practical importance of micro-evo-devo as a strategy for understanding phenotypic evolution, review the key recent insights that it has provided, and present a perspective on both the potential and the remaining challenges of this exciting interdisciplinary field.

UNDERSTANDING the evolution of phenotypic diversity is one of the main goals of evolutionary biology. Achieving this requires a deeper knowledge of how information encoded in the genome is processed by development to generate phenotypes and of the evolutionary forces that have shaped these processes. In addition, it is essential to describe the dynamics of how the mutations that cause phenotypic variation arise and spread in populations and ultimately contribute to speciation and macroevolutionary change. Since the emergence of the field of evolution and development (evo devo) in the 1990s, it has been recognized and reiterated that a focus on the microevolution of development, or micro-evo-devo, has great potential to help address these important questions (Wilkins 1998, 2002; Raff 2000; Gilbert 2003; Cresko *et al.* 2007; Johnson 2007; Sommer 2009; Stern 2011). Here we define micro-evo-devo as the study of the genetic and developmental bases of natural phenotypic variation within species. Note that although the scope of micro-evo-devo has been described to include the study of closely related species and even differences up to the level of genera (see Wilkins 2002 for discussion), at the core of this branch of evo devo is the study of intraspecific variation, and so, for clarity, we use the stricter definition. While micro-evo-devo also encompasses the study of phenotypic plasticity and developmental robustness (*e.g.*, Braendle *et al.* 2008; Milloz *et al.* 2008), we will concentrate on how this field addresses nonplastic differences in phenotype between populations (Figure 1).

By focusing on intraspecific variation, micro-evo-devo can take advantage of the extensive natural variation in morphology and physiology and patterns of linkage disequilibrium (LD) (Table 1) among populations. This readily allows the application of approaches from population/quantitative genetic and evolutionary developmental biology, particularly when the focal species are established model organisms with powerful experimental tools and genomic resources such as nematodes, flies, mice, sticklebacks, and *Arabidopsis* (Sommer 2009). Therefore, the strength and potential of micro-evo-devo lies in its synthesis of population/quantitative genetics and evolutionary developmental genetics to fully explore the causes and consequences of natural variation (Figure 1).

In this perspective, we first restate the case for micro-evo-devo by highlighting the potential limitations of evo devo without an intraspecific perspective and the incorporation of population genetics. We then discuss the potential benefits of expanding the genotypic focus of population genetics to

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[1]Corresponding author: Oxford Brookes University, Gipsy Lane, Oxford OX3 0BP, United Kingdom. E-mail: amcgregor@brookes.ac.uk.

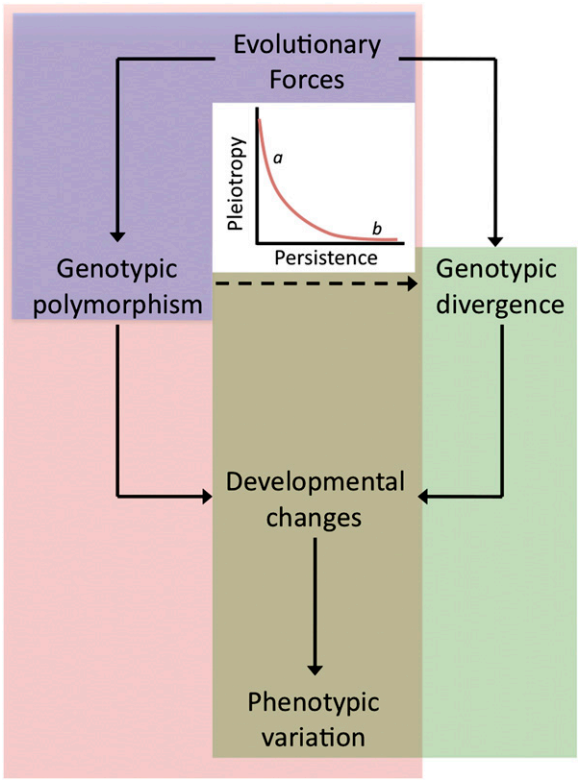

Figure 1 Foci of population genetics, evo-devo, and micro-evo-devo. The main foci of research in population genetics (blue-shaded area), evo devo (green-shaded area), and micro-evo-devo (pink-shaded area). While population genetics investigates mainly the evolutionary forces responsible for patterns of genotypic variation, it generally does not explain the causative genetic polymorphisms underlying phenotypic differences primarily due to a lack of functional and developmental characterization of candidate loci. Most evo devo research focuses on large-scale evolutionary changes, and hence it relies on contrasting the expression and function of broadly conserved genes and/or macroevolutionary changes. Micro-evo-devo allows the identification of the genetic changes underlying phenotypic variation among populations by combining genetic variation information with functional and developmental analysis of identified loci, providing an understanding of the evolutionary processes responsible for the maintenance of that phenotypic variation. In addition, micro-evo-devo can provide insights into macroevolutionary events, if this type of data are available across different species segregating for similar phenotypes. The dashed arrow denotes the conceptual relationship between population-level polymorphisms and species divergence. Given that a higher degree of pleiotropy is one of the factors that can result in increased fitness costs (Fisher 1930; Orr 2000; Cooper *et al.* 2007), a negative relationship is generally expected between the degree of pleiotropy of a mutation and its persistence in a population or species through time (True 2003; Stern 2011). Therefore, all else being equal, highly pleiotropic mutations at position *a*, including protein-coding changes of transcription factors (*e.g.*, possibly *poils au dos* and *FRI*), may be less likely to persist over time than changes at the other end of the spectra at position *b*, representing mutations in modular *cis*-regulatory elements altering expression patterns of specific genes (*e.g.*, possibly *Pitx1* and *scute*).

fully explain phenotypic diversity through the understanding of developmental biology and the functional consequences of genetic and developmental variation brought by evo devo.

To support our view, we then review key studies that fall within our definition of micro-evo-devo that have provided new insights into aspects of the genetics and development of phenotypic evolution and in some cases explore the evolutionary forces involved. We also evaluate how insights from micro-evo-devo can inform evolutionary research at scales above the species level. Finally, we discuss how micro-evo-devo can take advantage of the latest tools and approaches for the mapping and functional analysis of genetic variants and the use of high-throughput phenotyping.

Restating the Case for Micro-Evo-Devo

The emergence of evo-devo gave development an evolutionary context and evolution a developmental genetic perspective (Wilkins 2002; Carroll *et al.* 2005; Arthur 2011; Stern 2011). Evo-devo has subsequently largely studied patterns of conservation and change in gene expression and function over large evolutionary timescales and large taxonomic distances such as those addressed by the fields of paleontology and systematics (Figure 1). From this ground-breaking work, we have learned that the developmental "toolkit" of genes is mostly conserved across distantly related taxa and that phenotypic change across such broad scales is often accompanied by spatial or temporal changes in the expression of these conserved genes (Davidson 2001; Wilkins 2002; Carroll *et al.* 2005). As such, these studies have given and continue to provide valuable insights into both the ancestral features of the genetic toolkit and developmental regulation and how these factors and processes have subsequently evolved in extant lineages.

Primarily examining the expression and function of largely conserved genes across broad phylogenetic scales, however, can be a correlational enterprise that is intrinsically somewhat limited in its capacity to yield new insights into the causes of developmental evolution. This is because comparing distantly related taxa means that it is generally not possible to characterize the precise molecular basis and nature of causative changes (*e.g.*, *cis vs. trans*, additive *vs.* epistatic; see Table 1), given the sheer amount of change that has occurred and the existence of developmental systems drift (Table 1) (True and Haag 2001; Wang and Sommer 2011). This makes the study of how variation can arise and spread (*e.g.*, *de novo* mutations *vs.* standing genetic variation) and the underlying evolutionary forces (*e.g.*, drift *vs.* selection) very difficult to pursue above the level of species. Therefore, as cautioned during the emergence of evo devo (Wilkins 1998), this field needs to better incorporate a focus on intraspecific variation and population genetics. Without that dimension, evo devo risks being pushed to the periphery of evolutionary biology. The following illustrates the scope of the problem: at the most recent meeting of the European Society for Evolutionary Developmental Biology, only ~5% of abstracts could be considered to fall within the paradigm of micro-evo-devo. Since one of the major goals of evo devo is to provide a mechanistic understanding of phenotypic change, and the population level is an obvious scale at which to explore such questions, this is rather surprising.

Table 1 Glossary of terms

Term	Definition
Microevolution	Conventionally defined as allele-frequency changes within a population or several populations connected by gene flow. Microevolutionary processes include mutation, migration, selection, and genetic drift, and microevolutionary studies traditionally fall in the realm of population genetics.
Macroevolution	Processes and patterns generated by fixed genetic differences at or above the species level. The modern synthesis often depicts macroevolution as the result of several rounds of microevolution; however, this is not always the case (Erwin 2000). Macroevolution includes a myriad of patterns and processes investigated by paleontologists, evolutionary ecologists, phylogeneticists, and comparative developmental biologists.
Developmental systems drift	This evo devo theory proposes that so long as adult phenotypic features remain static the molecular components of the underlying GRN are free to evolve.
QTL	QTL are genomic regions associated with variation in particular quantitative phenotypes. Methods to determine QTL are traditionally based on linkage mapping between inbred lines or in families with known pedigrees.
GWAS	GWAS use genome-wide scans for loci associated with particular phenotypes based on linkage disequilibrium between genetic markers and causal genetic variants.
Epistasis	In quantitative genetics, this refers to the statistical phenomenon whereby two or more alleles at different loci exert a non-additive effect on a given phenotype. Developmental biologists use a more mechanistic definition of epistasis based in terms of how components of a GRN interact with one another.
Pleiotropy	The ability of a given genetic locus to influence many different phenotypes.
LD	The nonrandom association of alleles at two different loci. Although LD between two physically linked loci will decay as a function of the recombination rate, LD can persist in populations due to several factors including selection, mutation, drift, and gene flow.
Selection coefficient	The relative fitness of a given phenotype or genotype measured on a scale between 0 and 1, with 0 implying selective neutrality and 1 being complete lethality of the phenotype/genotype.

The reasons for this neglect are not entirely clear and likely multifarious. They could be rooted, however, in both the macroevolutionary traditions of comparative morphology and the omission of embryology from the classic evolutionary synthesis and the emphasis of contemporary evo devo on understanding the development and evolution of body plans and evolutionary novelties.

This is not to say that evo devo has completely ignored population thinking and issues of genetic variation: on the contrary, studies of closely related species have provided important insights into morphological evolution (*e.g.*, Stern 1998; Sucena and Stern 2000; Gompel *et al.* 2005; Jeong *et al.* 2006, 2008; Rebeiz *et al.* 2009b; Frankel *et al.* 2011; Loehlin and Werren 2012). However, an expanded micro-evo-devo effort can extend this work and provide further examples to test existing hypotheses about phenotypic evolution across a wide range of organisms and traits, especially where suitable closely related species are not available for genetic mapping or reciprocal functional analysis.

The field of population genetics is devoted to the analysis of changes in the frequency of genetic variation in populations and traces the genomic signatures of the evolutionary forces involved (Figure 1). Combined with quantitative genetics, population genetics has in many cases described the association between genotypic and phenotypic variation (*e.g.*, Stam and Laurie 1996; Lai *et al.* 2007; Harbison *et al.* 2009; Li *et al.* 2010; Casto and Feldman 2011). At this point it may be argued that a combination of population and quantitative genetics may be all that is required for a comprehensive understanding of phenotypic evolution. However, there remain some inherent limitations in these approaches.

While population genetic tools allow the identification of genomic regions that have been selected within a population, on their own, they do not explicitly reveal how genetic variation maps to phenotypic variation because the developmental genetic function of the identified variants may be unknown and/or the phenotype affected may not be obvious or predictable (Figure 1). Conversely, in quantitative genetics, a major hindrance has been the failure of quantitative trait loci (QTL) (Table 1) mapping approaches to identify genes or nucleotide variants that are of functional importance (Rockman 2012). This is in part because QTL and genome-wide association studies (GWAS) (Table 1) often identify a large number of putative causative loci and verifying their effects and contribution is a daunting task. Recent advances in genotyping have improved the resolution that can be achieved with GWAS and QTL approaches, but even so, functional validation of candidate loci is still rare in such studies, with the caveats that the identification of true causal variants can still be hindered by levels of LD and sample size (but see Tishkoff *et al.* 2007; Schmidt *et al.* 2008; Faraji *et al.* 2012). Hence, population genetics, with its focus on broad patterns of the dynamics of gene frequencies, often neglects the functional role of specific variants and ignores development, even when their study is

experimentally feasible, thereby foregoing insights that can be provided by the combination of developmental studies and the mapping of genetic changes to phenotypic variation. Where causal variants have been identified following mapping studies, this has often relied on using knowledge of developmental genetic functions or direct functional analysis of the role of candidate variants during development, which is essentially the micro-evo-devo strategy (Figure 1). Therefore, we argue that the application of experimental tools commonly used in evo devo to test natural genetic variation identified in population genetic studies can at least address some of these difficult challenges and provide a better understanding of how genetic variation leads to phenotypic variation.

Insights from Micro-Evo-Devo

Here we will highlight studies that demonstrate the great potential of micro-evo-devo not only to reveal the genetic basis of phenotypic change at the population level but also to provide novel insights into developmental regulation. Furthermore, we show how studies of intraspecific variation that incorporate an understanding of the developmental functions of the underlying genes allow us to gain better insights into the evolutionary forces involved.

The genetic basis of phenotypic change

The gene *Pituitary homeobox transcription factor 1* (*Pitx1*) is required for the development of hindlimbs in vertebrates among other important developmental functions (Lanctot *et al.* 1999; Logan and Tabin 1999; Szeto *et al.* 1999). Consistent with this role, it was shown that recurrent deletions of a specific *Pitx1* enhancer lead to the loss of *Pitx1* expression specifically in the developing pelvic structures of freshwater populations of sticklebacks and consequently to a reduction in adult pelvic spines in these fish without affecting the other functions of *Pitx1* (Chan *et al.* 2010). It is thought that these changes are involved in the adaptation of these fish from a marine environment to freshwater (Shapiro *et al.* 2004).

The role of regulatory changes at the *Pitx1* locus was first determined using a combination of genome wide linkage mapping and analysis of *Pitx1* expression patterns during development (Cresko *et al.* 2004; Shapiro *et al.* 2004; Coyle *et al.* 2007). Allele-specific expression analysis in hybrids was then used to verify the contribution of *cis*-regulatory mutations at the *Pitx1* locus (Chan *et al.* 2010). Ultimately, however, the identification of the causal changes was possible only through a combination of high-resolution mapping, taking advantage of recombination in natural populations and positional cloning and functional analysis of different fragments from the identified candidate region (Chan *et al.* 2010).

A similar combination of approaches was also successfully applied to investigate the genetic basis of phenotypic variation in *Arabidopsis* (Hilscher *et al.* 2009). Natural populations of *Arabidopsis* harbor a large amount of variation in trichome number that is thought to be maintained by selection for protection against insect herbivores and resistance to environmental stress factors (Handley *et al.* 2005). Genetic dissection of this trait, through QTL mapping and characterization of introgression lines carrying the alternative low- and high-density alleles in a mutant background, identified three candidate genes with demonstrated or predicted roles in trichome development: *ENHANCER OF TRY AND CPC2* (*ETC2*), *TRICHOMELESS1* (*TCL1*), and *TCL2* (Hischer *et al.* 2009). Overexpression and sequence analysis of these genes, however, ruled out the involvement of *TCL2* and *TCL1*, leaving *ETC2*. Subsequently, population-level sequence data for *ETC2* further narrowed the evolved region to two candidate nucleotides: one single-nucleotide polymorphism (SNP) in the 5′ UTR and one nonsynonymous replacement in the coding region. Site-directed mutagenesis showed that only the coding change had an effect on trichome number (Hischer *et al.* 2009). Importantly, *ETC2* had not been identified in previous studies of variation in trichome number using knockouts because these mutants had been generated in a genetic background with the weak *ETC2* allele; thus the effect could not be detected. Hence, the findings described above were made possible only by taking advantage of natural variation.

As evidenced by these examples, the dissection of the genetic basis of phenotypic variation very often involves crossing strains with extreme phenotypic values to generate genetic-linkage maps of the trait of interest. An alternative approach is to look for genomic signatures of selection in candidate genes with known roles in the development of the trait of interest. Taking such an approach, Pool and Aquadro (2007) obtained sequence polymorphism data at the *ebony* locus from populations sampled along an altitudinal cline for abdominal pigmentation and found evidence for a partial selective sweep spanning the upstream noncoding region of *ebony*. Using transgenic complementation tests, Rebeiz *et al.* (2009a) confirmed the contribution of variation at *ebony* to body melanization. A combination of functional tests of sequence variants and population sequence analysis was then carried out. The results revealed that darker pigmentation was caused by new mutations in a previously assembled haplotype containing standing variation that also affected pigmentation, and it was this combination of existing and *de novo* mutations that gave rise to a large-effect allele that was then swept to high frequency (Rebeiz *et al.* 2009a).

The study of hair color in vertebrates provides another example of the success of micro-evo-devo. The genetic architecture of body-hair-color differences between two subspecies of oldfield mice (*Peromyscus polionotus*), *P. p. subgriseus* (found in coastal areas with light soil and has light body color), and *P. p. leucocephalus* (distributed over the mainland and has a darker body color) was determined using genome-wide linkage mapping followed by a candidate gene approach that took advantage of the well-characterized developmental pathway for pigmentation (Steiner *et al.* 2007). Three QTL regions were found to affect several pigmentation traits, each of them containing only one gene with a known role in pigmentation. Analysis of the

spatial expression of two of these genes, *Mc1r* and *Agouti*, indicated that coding changes in *Mc1r* and regulatory changes closely linked to *Agouti* contribute to the pigmentation differences between these mice (Hoekstra *et al.* 2006; Steiner *et al.* 2007).

Further analysis of the *cis*-regulatory changes affecting *Agouti* expression also provided new insights into the development of these pigmentation patterns (Manceau *et al.* 2011). The apparently simple difference between these mice is caused by a change in the distribution pattern of four different hair types across the dorsal–ventral adult body axis. Manceau *et al.* (2011) showed that *cis*-regulatory changes in the *Agouti* light allele result in a new expression domain that moves the dorsal–ventral pigmentation boundary upward and in higher ventral expression that prevents melanocyte maturation and therefore the development of nonpigmented ventral hairs.

Insights into developmental regulation and evolution

Developmental biology has greatly enriched our understanding of the genetic programs that build plants and animals. Classically, evo devo has used this knowledge as the basis for candidate gene approaches to reveal the extensive conservation of the genetic regulation of development among organisms. Furthermore, this has also allowed associations to be made between changes in these programs and animal body plans across large phylogenetic distances (Carroll *et al.* 2005).

The studies of *Pitx1* and *Agouti* in pelvic reduction and pigmentation, respectively, discussed above, show that micro-evo-devo can provide a deeper understanding of the role and regulation of genes previously known to be involved in particular developmental processes. However, micro-evo-devo has also given novel insights into the regulation of development that would probably not have been achievable with a candidate gene approach and even in the absence of candidate genes.

For example, in the well-characterized gene regulatory network (GRN) for trichome development, *shaven-baby* (*svb*) serves as an input/output device that responds to upstream signals to direct trichome patterning and appearance through a battery of downstream target genes (Stern and Orgogozo 2008). Furthermore, it has been shown that changes in *cis*-regulatory sequences of *svb* and changes at the *Ultrabithorax* locus underlie the evolution of larval and leg trichome patterning, respectively, between species (Stern 1998; McGregor *et al.* 2007). However, a recent study has shown that intraspecific variation in leg trichome patterns in *Drosophila melanogaster* is caused by changes in the expression of a microRNA, *mir-92a* (Arif *et al.* 2013), which represses expression of *shavenoid*, a downstream target of *svb* (Schertel *et al.* 2012; Arif *et al.* 2013). The study by Arif and colleagues, therefore, not only revealed the genetic basis for morphological change, but also provided new insights into a well-characterized developmental GRN.

A combination of mapping and functional analysis of genes during development in natural populations was also successfully applied to understand the developmental basis of adaptive variation in wing pigmentation patterns in butterflies. In this study, Martin and coworkers (2012) found a previously unknown role for *WntA* in wing pigmentation in *Heliconius* butterflies and discovered that *cis*-regulatory variation at this locus was probably responsible for extensive intraspecific and interspecific variation in pigmentation patterns (Martin *et al.* 2012).

Insights into evolutionary forces and effects on fitness

In the previous section, we discussed recent studies that illustrate the potential of micro-evo-devo to dissect the genetic and developmental bases of phenotypic differences. Here we show how information about the specific developmental roles of genes underlying phenotypic differences can also help to illuminate the evolutionary forces and fitness consequences of those differences.

Mapping and subsequent developmental expression and functional analyses have shown that the reduction in lateral plate armor in freshwater sticklebacks compared to marine populations is caused by changes in the expression of the gene *Ectodysplasin* (*Eda*) (Colosimo *et al.* 2005), a gene with no previously known role in the development of this trait. It was thought that the differences in armor plating between freshwater and marine populations may have evolved due to selection against high plate number in the freshwater environment (Cresko *et al.* 2004; Marchinko and Schluter 2007; Barrett *et al.* 2008). However, when the dynamics of changes in frequency of the alternative alleles of *Eda* for low and high plate numbers was followed over a generation after experimental release of marine sticklebacks heterozygous at the *Eda* locus in freshwater ponds (Barrett *et al.* 2008), it was found that the low plate allele was strongly selected against [the selection coefficient (Table 1), *s*, was 0.5] during early stages of development. The low plate allele did increase in frequency in the population but only later in the life cycle of the fish, after formation of the lateral plates. These results showed that changes at or linked to the derived low-plate allele are likely to have pleiotropic effects on other traits relevant to fitness (Barrett and Hoekstra 2011), a result that would not have been anticipated given the repeated and rapid evolution of this trait.

Arabidopsis thaliana provides a further example how micro-evo-devo can link genetics, development, and morphological phenotype to fitness. In this plant, the gene *FRIGIDA* (*FRI*) encodes a scaffold protein required for the assembly of a protein complex that activates the expression of the transcription factor encoded by *FLOWERING LOCUS C* (*FLC*), which in turn represses flowering (Caicedo *et al.* 2004; Shindo *et al.* 2005; Choi *et al.* 2011). The recurrent evolution of loss-of-function *FRI* alleles (FRI^{Δ}) among *A. thaliana* ecotypes was thought to be indicative of adaptation to environments with short or unpredictable seasons due to their effect in reducing flowering time (Scarcelli *et al.* 2007). Controlled field studies showed that selection on the *FRI* locus varies depending on the season of germination and

genetic variation at *FLC* (Korves *et al.* 2007), probably explaining why genetic variation is maintained at that locus. Further work using an outbred population and testing these plants under different treatments simulating seasonal conditions failed to find an association between the *FRI* genotype and fitness (measured as fruit production) (Scarcelli *et al.* 2007). The authors postulated that this lack of association might be due to antagonistic pleiotropic (Table 1) effects of *FRI* on the number of nodes and branches, traits that are strong predictors of fitness in this species. However, Lovell *et al.* (2013) recently carried out further functional analysis of *FRI* nulls and measured the effects on additional aspects of development and physiology. They found that *FRI* nulls had higher stomatal conductance, which results in decreased water use efficiency and increases growth as well as shortening flowering time. This suggests that the evolution of *FRI* nulls may be a drought-escape strategy representing adaptive pleiotropy (Table 1) at this locus (Lovell *et al.* 2013). As these authors point out, more broadscale phenotyping is essential to understand the evolution of particular genetic changes, especially where they are likely to be pleiotropic and the obvious phenotypic change is not necessarily the one being selected.

Micro-Evo-Devo and Macroevolution

The above sections demonstrate the potential of micro-evo-devo to expand our knowledge of the genetics and development and evolutionary forces relevant to natural intraspecific variation. However, since the advent of micro-evo-devo, it has often been claimed that this field can also provide crucial insights into macroevolutionary processes (*e.g.*, Wilkins 1998, 2002). By macroevolution, we mean all evolutionary changes above the species level (Table 1). Here we discuss and evaluate the contribution and potential of micro-evo-devo to studies of macroevolution.

Whether macroevolution is a simple extrapolation of microevolution (Table 1) or the transition is actually more complicated has been much debated (Erwin 2000; Leroi 2000; Stern 2011). In the extreme scenario that microevolution rarely contributes to longer-term evolutionary change, then studying micro-evo-devo may have little impact on our understanding of macro-evolutionary processes (Figure 1). Indeed, much of the variation in segregating in natural populations may be due to large-effect alleles, with highly pleiotropic and deleterious effects that are eliminated in the longer term and therefore do not contribute to species differences (Figure 1). The empirical data pertaining to this question has been cataloged previously by Stern and Orgogozo (2008) who listed a large number of loss-of-function alleles underlying phenotypic variation within populations but not between species. This may suggest that, while the study of loss-of-function alleles found in populations (*e.g.*, in the *FRI* gene in *A. thaliana* populations as described above) is informative about short-term adaptation, it may have limited use in understanding longer-term evolution (Stern 2011).

However, while we may have to be cautious in interpreting macroevolution using micro-evo-devo, direct comparisons of QTL identified within and between species for a few traits suggest that intraspecific and interspecific in these traits might be caused by variation in the same genes in about half of the cases studied (Wittkopp *et al.* 2009). The caveats are that interspecific comparisons may inflate the number of QTL for any particular trait because further genetic changes are likely to accumulate after speciation and QTL studies lack the resolution to definitely compare the precise genetic bases.

Studies that provide a higher resolution of the genetic basis of intraspecific and interspecific differences, however, offer a useful insight. The genetic basis of variation in bristle patterns between *D. melanogaster* strains is caused by a presumably deleterious mutation that makes a truncated version of the transcription factor Poils au dos that regulates *scute* (Gibert *et al.* 2005), while variation in the same bristles between species is caused by *cis*-regulatory changes in an enhancer of *scute* (Marcellini and Simpson 2006). However, Rebeiz *et al.* (2009a) showed that putatively adaptive intraspecific variation in pigmentation in *D. melanogaster* is caused in part by several single-nucleotide changes, generally each of small effect in the regulatory region of the gene *ebony*, differences in the regulation of which also contribute to interspecific variation in pigmentation (Wittkopp *et al.* 2003). Similarly, analysis of sequence variation at *tan* showed that the changes fixed in *Drosophila novamexicana* segregate and contribute to pigmentation variation in *Drosophila americana*, indicating that these alleles were probably present in the most recent common ancestor of these two species (the alternative hypothesis of recent introgression being less likely) (Wittkopp *et al.* 2009). Hence, in these species, new mutations seem to have little effect on a phenotypic difference compared to the contribution of standing genetic variation.

Thus, while micro-evo-devo can provide insights into macroevolutionary differences, the contribution of microevolutionary changes to longer-term evolution likely depends on their pleiotropic effects (Figure 1), other factors including epistasis (Table 1), and their overall effects on fitness (Stern 2011). It follows that micro-evo-devo studies are also important to understand convergent evolution and can contribute to the identification of genetic "hotspots" underlying the recurrent evolution of particular phenotypes (Richardson and Brakefield 2003; Gompel and Prud'homme 2009; Kopp 2009; Manceau *et al.* 2010; Kronforst *et al.* 2012; Martin and Orgogozo 2013). This is because micro-evo-devo can be used not only to survey the frequency of changes among widespread populations and to investigate patterns of convergent genetic changes, but also to readily facilitate functional analysis of variants to help elucidate why some changes in developmental regulation have contributed repeatedly to evolution while others do so more rarely. Therefore, micro-evo-devo can make an important contribution to building a predictive framework for understanding the evolution of development generally (Stern 2011).

Challenges

There is great potential for micro-evo-devo to continue to produce a broad and detailed understanding of organismal diversity. However, there are still major challenges in providing the necessary genetic, developmental, and phenotypic resolution, as well as in identifying the evolutionary forces and fitness effects involved.

In particular, identifying all loci contributing to a given phenotype, particularly those of small effect that interact epistatically, remains a serious problem (Rockman 2012). However, many QTL studies show that numerous traits, even the most complex ones, are usually in part explained by at least a few of large-effect loci consistent with models of phenotypic evolution involving adaptive jumps (reviewed by Orr 2005). Moreover, investigating the role of loci of apparently large effect is essential since higher-resolution mapping shows that such loci are usually made up of multiple mutations of small effect that can be functionally validated, and it has been shown in some cases how additive and epistatic interactions among these mutations contribute to phenotypic evolution (McGregor *et al.* 2007; Rebeiz *et al.* 2009a; Frankel *et al.* 2011).

The increasingly cheaper cost of sequencing technologies means that genotyping, with very high resolution, is possible for virtually any species (*e.g.*, Andolfatto *et al.* 2011; Bastide *et al.* 2013). However, several challenges remain in mapping this genotypic change to phenotypic differences and in characterizing the developmental processes involved. The functional characterization of DNA sequence variants depends on the availability of genetic tools and therefore has been mostly limited to model organisms. However, while simply using a null mutant or knocking down the expression of a gene of interest (*e.g.*, using RNA interference) in such a model system may initially help us to understand its larger role in development, such methods do not allow us to assess the functional effect of natural variation in that gene. Fortunately, the advent of recently developed tools for genome editing such as the CRISPR/Cas9 system or transcription activator-like effector nucleases expand the application of reverse genetic approaches for targeted disruption or modification of endogenous loci to a broad range of species (Sanjana *et al.* 2012; Bassett *et al.* 2013; Chang *et al.* 2013; Cong *et al.* 2013; Friedland *et al.* 2013; Gratz *et al.* 2013; Hwang *et al.* 2013; Yu *et al.* 2013). Notably, these genetically engineered animals can be used not only to directly test the functional effect of nucleotide changes during the development of the trait of interest, but also in experiments designed to estimate the effect of these changes on fitness. Moreover, the particular advantage of these approaches is that mutations of interest can be introduced into and then tested in any genetic background.

Probably the biggest limiting step in understanding the genetic and developmental bases of phenotypic differences lies in having enough phenotypic resolution to map precisely the underlying genetic differences that generate those differences and then being able to analyze them functionally. For most morphological, behavioral, and physiological traits, acquiring phenotypic information from a large sample size is extremely laborious and time-consuming. Yet, progress is being made: different research consortia have already gathered phenotypic information for a number of large data sets. These include health and disease in humans and dogs; morphology, physiology, and behavior in *Drosophila*, rat, and mouse; and a variety of quantitative traits in plants (see Houle *et al.* 2010 for a review). However, these data sets are often not ideal for answering many specific questions, and we concur with Houle *et al.* (2010) that joint efforts to develop new technologies and methods for high-throughput data acquisition would, in the longer run, also be useful to the whole research community. One strategy that has proven to be successful in measuring the effect of individual nucleotides underlying variation in some traits and even in exposing cryptic variation is to use sensitive genetic backgrounds, although this application may be limited to particular model organisms (Dworkin and Gibson 2006; Dworkin *et al.* 2009; Gibert *et al.* 2011).

Despite the increasing number of experimental field studies of fitness for focal traits, in many cases these experiments are not possible, for example, when the environmental variables cannot be properly controlled. In these situations, experimental evolution experiments are a good alternative, since the selective agent is known and can be manipulated, adequate controls can be set up, replication is possible, multidimensional phenotype data can be acquired, and allele-frequency changes can be followed, allowing for direct estimates of selection coefficients of any given trait. With the caveat that these experiments have to be conducted in easily cultured, small, and fast-developing organisms, experimental evolution holds great promise for giving a more complete picture of many adaptation processes.

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