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PERSPECTIVE

Opportunities and challenges of next-generation sequencing applications in ecological epigenetics

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Evolutionary theory posits that adaptation can result when populations harbour heritable phenotypic variation for traits that increase tolerance to local conditions. However, the actual mechanisms that underlie heritable phenotypic variation are not completely understood (Keller 2014). Recently, the potential role of epigenetic mechanisms in the process of adaptive evolution has been the subject of much debate (Pigliucci & Finkelman 2014). Studies of variation in DNA methylation in particular have shown that natural populations harbour high amounts of epigenetic variation, which can be inherited across generations and can cause heritable trait variation independently of genetic variation (Kilvitis *et al.* 2014). While we have made some progress addressing the importance of epigenetics in ecology and evolution using methylation-sensitive AFLP (MS-AFLP), this approach provides relatively few anonymous and dominant markers per individual. MS-AFLP are difficult to link to functional genomic elements or phenotype and are difficult to compare directly to genetic variation, which has limited the insights drawn from studies of epigenetic variation in natural nonmodel populations (Schrey *et al.* 2013). In this issue, Platt *et al.* provide an example of a promising approach to address this problem by applying a reduced representation bisulphite sequencing (RRBS) approach based on next-generation sequencing methods in an ecological context.

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Platt *et al.* sampled leaf or flower tissue from 11 *Quercus lobata* (Fig. 1) individuals in three populations in southern California that differ in elevation and climatic factors. They used a reduced representation bisulphite sequencing (RRBS) technique to generate 100-bp fragments resulting in base pair resolution of 7991 C/T polymorphisms (labelled as single methylation polymorphisms or SMPs) and 10 388 other single nucleotide polymorphisms (SNPs; A/C, A/G, A/T, C/G or G/T polymorphisms). Using these data, they were able to show greater population differentiation at SMPs than SNPs suggesting a stronger correlation of DNA methylation than other sequence polymorphisms with local site conditions. In addition, they provided evidence of greater differentiation at genetic loci linked to SMPs, providing support for the idea that some of the epigenetic variation is heritable. For ecological epigenetics studies on nonmodel plants, this approach provides an exciting improvement over the current standard of methylation-sensitive AFLP (MS-AFLP) for several reasons. Reduced representation sequencing approaches provide many more fragments (typically thousands to tens of thousands at 10× resolution; Narum *et al.* 2013) than MS-AFLP (typically on the order of hundreds). RRBS also provides base pair resolution of DNA methylation within each of the isolated fragments, while MS-AFLP only provide information about the methylation status of the cytosines in the recognition sequence for the methylation-sensitive enzymes (CCGG for the oft-used *Msp*I and *Hpa*II). SMPs may ultimately be linked to functional sequences (Becker *et al.* 2011; Schmitz *et al.* 2011) and sequence information provides for the direct comparison of genetic and epigenetic information in the same set of fragments, allowing questions to be asked about the relative contribution of SNPs and SMPs to population divergence, which cannot be performed using AFLP and MS-AFLP. Because the mechanisms by which DNA methylation can influence gene expression and phenotype are still not entirely understood, the movement from anonymous loci to functional markers will be important to definitively link DNA methylation variation to phenotypic

Fig. 1 Valley oak, *Quercus lobata*. Photograph courtesy of Dr. Paul Gugger.

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variation, especially in the context of population divergence and local adaptation.

With the application of bisulphite sequencing to ecological epigenetics, it is important to consider both old and new challenges in data analysis and interpretation. We have argued previously that confirming local adaptation can be particularly difficult in studies of ecological epigenetics (Richards *et al.* 2010). Truly demonstrating adaptation requires specific patterns of organismal response in reciprocal transplant studies in the field or studies in a controlled environment. Outlier locus analysis or identifying loci with high F_{ST} (e.g. Platt *et al.* 2015) can suggest selection on ecologically important genomic regions, but demonstrating that the observed variation is adaptive still requires association with actual fitness or performance of whole organisms when evaluated in relevant environments. In addition, epigenetic variation, unlike genetic variation, can be environmentally labile. Therefore, any measures of epigenetic variation in field-collected samples probably contain some component of environmentally induced epigenetic variation. In one of the earliest studies of epigenetic response to different environmental conditions, Verhoeven *et al.* (2010) showed that up to 26% of the methylation changes induced by treatments reverted to previous methylation patterns when offspring were grown in control conditions. Platt *et al.* (2015) suggest that their patterns of differentiation at SMPs are not due to environmentally induced methylation because they found differentiated SNPs in the same fragments. However, their design cannot discriminate between persistent, independently causal methylation polymorphisms from those that are merely downstream read-outs of differentiated SNPs (i.e. either obligatory or facilitated *sensu* Richards 2006). Their analysis does suggest that if epialleles are largely downstream effects of causative SNPs, those causative SNPs are tightly linked to their SMPs. As a follow-up, common garden experiments with offspring from the oaks in this study are necessary to confirm population divergence due to inherited epigenetic variation, rather than epigenetic variation that is induced in local sites but not inherited.

While sequence data promises to be more powerful due to sheer numbers of fragments and ultimately lend insight into function, technology does not alleviate the complexity of disentangling genetic from epigenetic contributions to population differentiation. Platt *et al.* (2015) suggest four possibilities: (i) populations might show epigenetic divergence if epiallelic variation, independent of genetic variation, is the cause of locally adaptive phenotypes, (ii) DNA polymorphisms co-occur with methylation variation to cause locally adaptive phenotypes, (iii) methylation variation within gene bodies and promoters causes locally adaptive phenotypes, or (iv) methylation polymorphisms are linked to adaptive variation but not causative of phenotype. While conceptual categories like these may contribute to the biological interpretation of SMP and SNP data, these categories do not emphasize the complexities of the interaction between genetic and epigenetic variation. Richards (2006) identified obligatory, facilitated and pure epigenetic variation, which is controlled in a deterministic, probabilistic and stochastic manner by genetic variation, respectively. To apply these categories in the context of adaptation, what matters is how these different types of variation contribute to phenotype. Like genetic variation, epigenetic variation can have no effect on phenotype or fitness or may be the product of evolutionary drift or linkage disequilibrium. Additionally, because epigenetic variation may be caused by genetic variation, it may not be the ultimate cause of phenotypic change. Richards' (2006) framework helps to clarify when epigenetic variation might contribute a previously unappreciated source of heritable phenotypic variation: either facilitated by or independent of genetic variation. Subsequently, any hypotheses about selection on phenotypes caused by epigenetic variation must be confirmed in much the same way they are confirmed in genetic experiments, with measures of phenotype and fitness (Richards *et al.* 2010).

Finally, some technical limitations are specific to the RRBS approach in Platt *et al.* (2015). Although Platt *et al.* (2015) make good use of a limited sample size, they do not use control (non-bisulphite-treated) samples for each individual. This experimental design limits their ability to discriminate methylation polymorphisms from C/T SNPs —they suggest only 58% of these polymorphisms are actually likely to be methylation polymorphisms. However, this estimate is based on a single individual. Measures of epigenetic variation are often high both within and among populations, and numbers of SMPs may be drastically different from individual to individual. More importantly, the conflation of SMPs and C/T SNPs potentially clouds measures of epigenetic differentiation and interferes with our ability to understand differences between sequence contexts; any conclusions about population divergence due to epigenetic differentiation will be less accurate when 42% of the variation assumed to be epigenetic is actually sequence based. A solution to this issue is use of paired non-bisulphite-treated controls to better disentangle methylation polymorphisms from C/T SNPs.

The study by Platt *et al.* (2015) represents an exciting first attempt at the application of next-generation sequencing in ecological epigenetics, an avenue which has been attractive but unlikely due to the cost of sequencing at the sample sizes required to draw robust conclusions about natural populations. RRBS makes sequencing feasible for nonmodel systems with large genomes, which will provide much needed data to advance the field. To apply this exciting new methodology effectively in natural systems, we suggest the following approaches to experimental design: (i) use non-bisulphite-treated controls to distinguish between C/T SNPs and SMPs, (ii) use common garden design to distinguish between induced and inherited epigenetic variation and (iii) use reciprocal transplants and measures of phenotype and fitness to confirm local adaptation. The ecological and evolutionary importance of DNA methylation can be explored in greater detail than

ever before with this powerful new technology, but can only be interpreted with an understanding of its limitations.

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