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SYMPOSIUM

Ecological Epigenetics: Beyond MS-AFLP

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Synopsis Ecological Epigenetics studies the relationship between epigenetic variation and ecologically relevant phenotypic variation. As molecular epigenetic mechanisms often control gene expression, even across generations, they may impact many evolutionary processes. Multiple molecular epigenetic mechanisms exist, but methylation of DNA so far has dominated the Ecological Epigenetic literature. There are several molecular techniques used to screen methylation of DNA; here, we focus on the most common technique, methylation-sensitive-AFLP (MS-AFLP), which is used to identify genome-wide methylation patterns. We review studies that used MS-AFLP to address ecological questions, that describe which taxa have been investigated, and that identify general trends in the field. We then discuss, noting the general themes, four studies across taxa that demonstrate characteristics that increase the inferences that can be made from MS-AFLP data; we suggest that future MS-AFLP studies should incorporate these methods and techniques. We then review the short-comings of MS-AFLP and suggest alternative techniques that might address some of these limitations. Finally, we make specific suggestions for future research on MS-AFLP and identify questions that are most compelling and tractable in the short term.

Introduction

Better knowledge of how the genome responds to natural environments would transform our understanding of the translation from genotype to phenotype. Scientists have abundant information on sequences for a variety of organisms, but have made little progress in understanding how the genome actually functions in creating complex traits that are adaptive in complex environments (Richards et al. 2009, 2012b; Pigliucci 2010; Martin et al. 2011). One area that may contribute to this understanding is Ecological Epigenetics, which studies the relationship between epigenetic variation and ecologically relevant phenotypic variation. Epigenetic mechanisms can alter gene expression and organismal function without altering DNA sequence (Richards 2006) and can cause variation in morphological characters (Cubas et al. 1999; Morgan et al. 1999; Rakyan et al. 2003; Manning et al. 2006; Kucharski et al. 2008). Some epigenetic marks may be stably transmitted across generations (Jablonka and Raz 2009; Johannes et al. 2009; Verhoeven et al. 2010). Epigenetic marks can vary among individuals and populations (Herrera and Bazaga 2010, 2011; Massicotte et al. 2011; Herrera et al. 2012; Liu et al. 2012; Massicotte and Angers 2012; Richards et al. 2012a; Schrey et al. 2012). Thus, epigenetic mechanisms are a potentially important component of an individual's response to its environment (Angers et al. 2010; Verhoeven et al. 2010; Richards et al. 2010); understanding the consequences of epigenetic variation should provide insights at both ecological and evolutionary time scales (Bossdorf et al. 2008; Richards et al. 2008, 2010; Angers et al. 2010).

There are several molecular epigenetic mechanisms that impact gene expression (e.g., remodeling of chromatin, deacetylation of histones, position effects, and interference by small RNAs), but methylation of DNA so far has dominated the Ecological Epigenetics

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literature. Methylation of DNA usually involves a methyl group being attached to a cytosine, where cytosine is immediately followed by a guanine in the DNA sequence (Bossdorf et al. 2008). Methylation of DNA can have variable effects on gene expression, but it often decreases gene activity (Jablonka and Lamb 2006; Bossdorf et al. 2008). There are multiple techniques that can screen for variation in DNA methylation; however, to date, the most commonly employed technique in the Ecological Epigenetics literature is genome-wide surveys for methylation of DNA using methylation-sensitive-AFLP (MS-AFLP; Reyna-Lopez et al. 1997).

MS-AFLP can efficiently and economically screen variation in methylation of DNA at several restriction sites (loci). MS-AFLP identifies a multi-locus epigenotype for each individual by substituting methylation-sensitive isoschizomeric enzymes *Msp*I and *Hpa*II for *Mse*I in a standard AFLP protocol (Vos et al. 1995). The enzymes *Msp*I and *Hpa*II have different sensitivities to cytosine methylation of the CCGG recognition sequence (Reyna-Lopez et al. 1997; Salmon et al. 2008). *Msp*I does not cut when the inner cytosine is methylated; *Hpa*II does not cut when either or both cytosines are fully methylated or hemi-methylated (McClelland et al. 1994; Roberts et al. 2007). Performing the protocol independently for each enzyme for each individual allows detection of the methylation state of a particular locus from the resulting banding pattern (Salmon et al. 2008).

We reviewed the use of MS-AFLP in Ecological Epigenetics and highlight the benefits of this technique. We identified the general trends in taxa that have been investigated with MS-AFLP and selected four MS-AFLP-based studies with particular characteristics that allow for the greatest inference to be drawn. Next, we reviewed the short-comings of MS-AFLP and suggest other techniques that might compensate for some of these short-comings. Finally, we make specific suggestions for future MS-AFLP research and identify two compelling questions that could be addressed in the short term using available techniques.

Benefits of MS-AFLP

There are multiple benefits to MS-AFLP (Table 1). First, it allows for research on non-model systems including those that lack sequenced genomes. Second, MS-AFLP is technically similar to AFLP: it requires the same equipment (DNA extraction platform, thermal cycler, and fragment analysis platform), similar protocols, and expertise. Third, the technique is cost-effective, there is minimal start-up, it can be scaled-up easily, and the same reagents can be used on multiple taxa. Fourth, a large number of individuals can be screened at multiple loci concurrently, which potentially generates powerful data for detecting differentiation among populations or treatments. Fifth, MS-AFLP has facilitated the detection of environmentally induced variation in DNA methylation. Thus, in spite of its inferential limitations, it has proven valuable already in a nascent discipline.

Table 1 A review of the beneficial characteristics and short-comings of methylation-sensitive-AFLP (MS-AFLP)

MS-AFLP
Beneficial characteristics
Allows for research on non-model systems
Technically similar to AFLP
Cost-effective with minimal start-up
Screens multiple loci concurrently
Has been used to detect ecologically relevant epigenetic variation
Short-comings
Conceptual
Screens anonymous loci
No model of expected change to explain variation
Complex relationship between DNA methylation and phenotype
Technical
Genetic variation or hyper-methylation could cause one of the MS-AFLP outcomes
Potential for variable PCR to masquerade as epigenetic variation

MS-AFLP in Ecological Epigenetics

We identified 39 peer-reviewed manuscripts using the MS-AFLP technique and that were published from 1999 to 2012 and that addressed a research question in Ecological Epigenetics (Table 2). We expect that the low number of manuscripts reflects the recent birth of this discipline. As indicated in Fig. 1, studies of plants dominate these papers, accounting for 82% (32 of 39) of all studies, but there has been an increase in studies of non-plant taxa; since 2010, 11 studies were plant-based and 6 considered other taxa. The year with the most non-plant studies was 2012 ($n=4$). There has also been a steady increase in Ecological Epigenetics papers since 1999 (Fig. 1), with 17 of the 39 studies published since 2010.

Examples of MS-AFLP-based studies in Ecological Epigenetics

We selected four studies (Herrera and Bazaga 2011; Massicotte et al. 2011; Herrera et al. 2012; Richards et al. 2012a) that highlight particular characteristics

Table 2 Papers published between 1999 and 2012 on Ecological Epigenetics of various species, and that used methylation-sensitive-AFLP (MS-AFLP)

Authors	Taxa	Authors	Taxa
Xiong et al. (1999)	*Oryza sativa* ssp. *indica*, plant	Zhong et al. (2009)	*Triticum aestivum*, plant
Ashikawa (2001)	*Oryza* cultivars, plant	Gao et al. (2010)	*Alternanthera philoxeroides*, plant
Shaked et al. (2001)	*Aegilops* and *Triticum* wheat, plant	Herrera and Bazaga (2010)	*Viola cazorlensis*, plant
Cervera et al. (2002)	*Arabidopsis thaliana*, plant	Lira-Medeiros et al. (2010)	*Laguncularia racemosa*, plant
Li et al. (2002)	*Malus* × *domestica* cv. *Gala*, plant	Paun et al. (2010)	Dactlorhiza: Orchidaceae, plant
Baurens et al. (2003)	*Musa*, plant	Verhoeven et al. (2010)	*Taraxacum officinale*, plant
Noyer et al. (2005)	Plantain landraces, plant	Chwedorzewska and Bednarek (2011)	*Deschampsia antarctica*, plant
Salmon et al. (2005)	*Spartina* spp., plant	Herrera and Bazaga (2011)	*Viola cazorlensis*, plant
Sha et al. (2005)	*Oryza*, plant	Kou et al. (2011)	*Oryza sativa*, plant
Takata et al. (2005)	*Oryza sativa* and *rufipogon*, plant	Massicotte et al. (2011)	*Chrosomus eos-neogaeus*, fish
Gimenez et al. (2006)	*Musa*, plant	O'Shea et al. (2011)	*Mycobacterium avium*, bacteria
Long et al. (2006)	*Oryza japonica* ssp., plant	Wang et al. (2011)	*Oryza sativa* ssp. *indica*, plant
Fang and Chao (2007)	*Phoenix dactylifera*, plant	Abratowska et al. (2012)	*Armeria maritima*, plant
Lu et al. (2007)	*Brassica napus*, plant	Chwedorzewska and Bednarek (2012)	*Poa annua*, plant
Zhao et al. (2007a)	*Zea mays* hybrids, plant	Herrera et al. (2012)	*Metschnikowia reukaufii*, yeast
Kronforst et al. (2008)	*Hymenoptera* spp., insect	Lui et al. (2012)	*Hipposideros armiger*, mammal
Li et al. (2008)	*Hordeum brevisubulatum*, plant	Massicotte and Angers (2012)	*Chrosomus eos-neogaeus*, fish
Mason et al. (2008)	*Solanum lycopersicum*, plant	Richards et al. (2012a)	*Fallopia* spp., plant
Salmon et al. (2008)	*Brassica oleracea*, plant	Schrey et al. (2012)	*Passer domesticus*, bird
Marfil et al. (2009)	*Solanum ruiz-lealii*, plant		

allowing for the most inferences to be made from MS-AFLP data. These investigations (1) compared contemporaneous genetic and epigenetic data, (2) identified the target of methylation, (3) experimentally demonstrated the consequences of methylation, and (4) extended the topic beyond a mere survey of variation. We do not intend to imply that these are the only good manuscripts; rather, they highlight specific aspects that have great impact on the field and identify common themes.

Herrera and Bazaga (2011)

A wild population of violets, *Viola cazorlensis*, which had been monitored for herbivory for 20 years, was investigated to determine the ecological influence of natural herbivory on genetic and epigenetic characteristics (Herrera and Bazaga 2011). Genetic variation was screened with AFLP and the variation in methylation of DNA was screened with MS-AFLP. There was considerable variation among individuals in their methylation of DNA and this variation was related to damage from browsing. Also, variation at some methylated loci was related to particular AFLP markers that were correlated to herbivory. This study is groundbreaking because both genetic and epigenetic variation was compared contemporaneously in response to different natural environmental conditions. The study identified a potentially complicated relationship among genetic variation, epigenetic variation, and herbivory through structural equation modeling. Also, the high amount of among-individual variation detected in DNA methylation suggests that it may be necessary to scan several MS-AFLP loci to allocate a response to a particular stimulus.

Massicotte et al. (2011)

A study of an all-female clonal fish, *Chrosomus eos-neogaeus*, demonstrated the importance of epigenetic variation for natural populations of a wild vertebrate. Individuals were collected from multiple locations and the variation in DNA methylation was characterized with MS-AFLP. Differences in methylation of DNA were detected among individuals and among tissues within individuals (Massicotte et al. 2011). We highlight this study because 15 MS-AFLP candidate loci were isolated and sequenced, and one of these targets was bisulfite sequenced in order to identify the exact position of the variation in DNA methylation. Four candidate loci could not be identified, whereas 11 had at least some putative similarity in the zebrafish genome. Interestingly, for one candidate locus, bisulfite sequencing indicated that

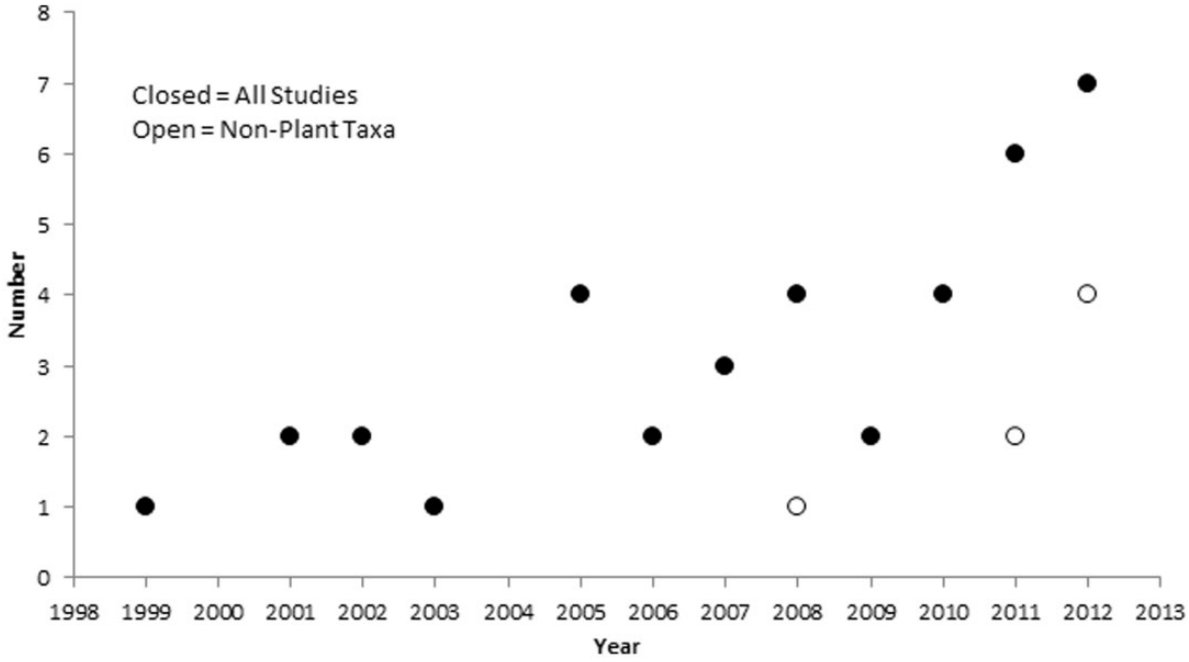

Fig. 1 Number of Ecological Epigenetics studies published by year from 1999 to 2012. The number of non-plant studies is indicated by open circles beginning in 2008.

there was additional variation in DNA methylation at adjacent sites, some sites being variable and others either always methylated, or always non-methylated.

Herrera et al. (2012)

Studies of the nectar-living yeast, *Metschnikowia reukaufii*, showed how methylation of DNA contributes to exploitation of resources from a broad range of environments (Herrera et al. 2012). To demonstrate the importance of environmentally-induced changes in DNA methylation in response to nectar environments with different sugar compositions and concentrations, Herrera et al. (2012) experimented with cultures of five strains of *M. reukaufii*, which were obtained from floral nectar of different host species. Strains were grown on media mimicking the variation in concentration and composition of sugar in natural nectars. Sugar composition, sugar concentration, and their interaction significantly influenced the probability of MS-AFLP loci changing from non-methylated to methylated, yet methylation status did not vary significantly among strains. The study is remarkable because by tracking the growth of the colony after treatment with the methylation inhibitor 5-Aza-2-deoxycytidine (5-AZA), the changes in methylation of DNA were demonstrated to be causally active in the ability of *M. reukaufii* to exploit resources. Herrera et al. (2012) not only demonstrated that plasticity in the use of resources was correlated with changes in methylation but they also found that inhibition of methylation significantly depressed the growth of *M. reukaufii* in certain nectar-sugar environments.

Richards et al. (2012a)

Invasive Japanese knotweeds provided an opportunity for exploring the importance of epigenetic effects in a species' response to novel environments (Richards et al. 2012a). Japanese knotweeds have been introduced into multiple habitats and they maintain significant differences within and among populations for most traits, yet have almost no genetic diversity (Richards et al. 2008). Using AFLP and MS-AFLP markers, Richards et al. (2012a) tested the hypothesis that differentiation in response to new habitats (beach, salt marsh, and roadside) is correlated with epigenetic variation. Only 4 out of 200 AFLP loci were polymorphic and only 8 different genotypes were detected. One of these genotypes corresponded to the single giant clone of *Fallopia japonica* found across Europe and in the United States (Richards et al. 2008, 2012a; Hollingsworth and Bailey 2000). The AFLP markers differentiated the two species, *F. japonica* and *Fallopia* × *bohemica*, but there was no differentiation among habitats. There was greater epigenetic differentiation; 19 polymorphic sites among 180 MS-AFLP sites created 128 epigenotypes, and some epigenetic loci were

differentiated by habitat conditions (Richards et al. 2012a). This study is particularly noteworthy because the plants were collected from multiple habitats and grown under common garden conditions. This allowed the MS-AFLP-based results to support inferences beyond just environmentally induced short-term variation to variation that persists in a common environment.

Short-comings of MS-AFLP

Conceptual

The biggest short-coming of MS-AFLP is that it screens anonymous loci (Table 1). The AFLP framework was designed to screen a large number of random sites throughout the genome, thereby generating sufficient data to make comparisons among populations or taxa. Thus, MS-AFLP operates under a similar framework and variation at MS-AFLP loci could be statistically linked to traits. The adjacent sequence to each locus is unknown; therefore; MS-AFLP cannot specify the region or gene influenced by methylation; rather, it infers genome-wide epigenetic variation via the production of various fragment lengths. However, it is possible to extract and sequence the fragment (if electrophoresis is done on acrylamide gels) and identify homologous sequences in the database (e.g., Salmon et al. 2005; Massicotte et al. 2011).

The second short-coming is that there are no known expectations for change in DNA methylation among groups, treatments, or even loci, and there is no model available to predict consequences of changes in methylation state at particular loci identified with MS-AFLP to changes in phenotype. Thus, some loci likely have different stabilities; that is, some may be highly labile changing often over time, while others are more stable (e.g., Verhoeven et al. 2010).

The third short-coming is that there is likely a complex relationship between methylation and phenotype. When methylation of DNA directly affects gene expression, the phenotypic consequences may be clearer; however, there is not always an obvious connection between DNA methylation and gene expression. Some of the observed variable sites are likely to be of more value to changes in phenotype than others. It may be possible to begin to address this issue by performing association mapping to link phenotype to epigenetic states at particular loci (Zhao et al. 2007b). Further, investigating the relationship between outlier loci and epigenetic states may help to understand the relationships among genetic variation, epigenetic variation, phenotypic variation, and the environment (Herrera and Bazaga 2011). The second and third short-comings create a situation similar to the different mutation rates and consequences expected in the evolution of gene sequence, but we lack sophisticated models to characterize the behavior of MS-AFLP.

The fourth short-coming to the MS-AFLP technique is that it resolves a dominant banding pattern. A band is either present or absent at each position; thus, it is not possible to distinguish heterozygote epigenotypes. Because some methylation states can be masked by the dominant marker, it is likely that detecting a relationship between methylation state and differential gene expression will be inhibited in certain conditions. This problem can be resolved with the genetically-based AFLP technique by recovering the proportion of expected heterozygote genotypes via Hardy–Weinberg frequencies. However, this is not possible for methylation states because there is no known relationship between homozygote and heterozygote frequencies in methylation states.

Technical

There are two technical short-comings of the technique. The first is that the banding pattern observed when both *Msp*I and *Hpa*II fail to cut (Type-IV; Salmon et al. 2008) can be generated by both genetic (point mutation to the restriction site, or changes to adjacent restriction sites) and epigenetic (hypermethylation, methylation of all cytosines in the restriction site) causes. Thus, some methylated states are missed. The second technical short-coming is that scoring AFLP-type banding patterns can be challenging, in part, because of variation in PCR making it difficult to standardize results among laboratories. Also, two reactions must be compared for every individual and it is possible that in some cases the differences between *Msp*I and *Hpa*II reactions could be generated by inconsistent restriction digests, or variation in PCR, rather than by differential methylation.

Techniques for addressing short-comings of MS-AFLP

To transition from screening anonymous genome-wide variation in DNA methylation with MS-AFLP, it is necessary to collect data at identified target sequences. Multiple techniques are available that screen variation in DNA methylation at specific targets, with several resulting in single-nucleotide resolution: metAFLP, bisulfite sequencing, *McrBC* digestion and PCR, quantitative analysis of DNA methylation using real-time PCR (qAMP), microarrays, and next-

generation sequencing. Another option is to chemically manipulate the methylation state and assess the effect on phenotype. The major difference between these techniques and MS-AFLP is in the required start-up initial knowledge and additional equipment. Simply put, you either need to already know where to look (sequenced promoters or genes known to be relevant), or you need the platform and capability to look at a wide array of specific targets simultaneously (having a microarray or next-generation sequencing platform and bioinformatics skills). This may require a significant amount of money and time. Below, we summarize the benefits and limitations of each of these gene-specific techniques.

metAFLP

A variation of the standard MS-AFLP protocol, metAFLP uses different isoschizomeric restriction enzyme combinations *Acc*65I and *Kpn*I with *Mse*I. Enzyme activity of *Acc*65I is blocked by *dam* and CpG methylation (Chwedorzewska and Bednarek 2011, 2012). Because *Kpn*I is not sensitive to methylation, this combination of restriction enzymes does not have the same technical short-comings as MS-AFLP. This technique has many of the same positive characteristics as MS-AFLP, and it has been used successfully in epigenetic studies of plants (Chwedorzewska and Bednarek 2011, 2012; Abratowska et al. 2012). There are several restriction enzymes that are sensitive to methylation (López Castel et al. 2011), which suggests that the standard MS-AFLP protocol could be modified by including multiple enzyme combinations in order to increase the power of analysis. However, this technique still screens anonymous loci, which are not easily related to function.

Bisulfite sequencing

Bisulfite sequencing is considered the ''gold-standard'' for analyzing methylation of DNA because it produces results with single-nucleotide resolution (Suzuki and Bird 2008). This technique requires that genomic DNA be treated with sodium bisulfite, which causes deamination of unmethylated cytosines and results in the conversion of these unmethylated cytosines to uracil, leaving methylated cytosines unconverted (Laird 2010). For gene-specific quantification of DNA methylation, PCR amplification of bisulfite-treated DNA replaces uracil with thymine and gene-specific primers amplify fragments that can be analyzed using sequencing platforms to identify methylated cytosines (Zilberman and Henikoff 2007; Suzuki and Bird 2008). Thus, bisulfite sequencing can be applied to nearly any sample and can be utilized for either genome-wide or gene-specific analyses of DNA methylation. Despite the high resolution, there are a few limitations to this technique. Bisulfite sequencing is expensive, time consuming, and labor intensive, especially for those interested in assessing DNA methylation across the entire genome (Suzuki and Bird 2008). Regardless of the scale, there is a risk of incomplete conversion of unmethylated cytosines to thymines (Dahl and Guldberg 2003), as well as of degradation of DNA via depurination (due to high incubation temperatures and high concentrations of bisulfite) (Grunau et al. 2001), both of which potentially could produce misleading results.

McrBC digestion and PCR

Digestion with *McrBC*, an endonuclease that cleaves methylated DNA sequences (Oakes et al. 2006; He et al. 2011), followed by PCR is a more economical and efficient alternative to bisulfite sequencing, which still allows for locus-specific analysis of methylation. DNA is digested with one or more methylation-dependent restriction enzymes (Dahl and Guldberg 2003) followed by PCR amplification of a target. Fragment analysis indicates whether the target has been digested by the methylation-dependent restriction enzymes. This technique was used to detect a high level of epigenetic variation across chromosome 4 in 96 accessions (Vaughn et al. 2007) as well as 22 epi-recombinant inbred lines (Johannes et al. 2009) of *Arabidopsis thaliana*.

Quantitative analysis of DNA methylation using real-time PCR

qAMP is another technique that identifies sequence-specific changes in DNA methylation through digestion with methylation-dependent restriction enzymes and methylation-sensitive restriction enzymes (Oakes et al. 2006). Genomic DNA is distributed into five separate reactions: one containing only DNA (the sham), one digested with a methylation-dependent restriction enzyme (*McrBC*), and the remaining three digested with different methylation-sensitive restriction enzymes (e.g., *Not*I, *Hha*I, and *Hpa*II). Real-time PCR amplifies targets-of-interest using specific primers in each of the five reactions. The resulting cycle threshold values (C_t values) are compared with the sham to determine the percentage of methylation for that target (Oakes et al. 2006). This technique benefits from generating a region-specific assay of methylation and results are highly comparable with those obtained by bisulfite sequencing (Oakes et al. 2006).

Microarrays

Microarrays are very efficient at examining a large number of target sequences simultaneously (Gibson 2003). A recent study by Nätt et al. (2012) examined differences in DNA methylation and gene expression between the domesticated chicken and its wild ancestor, the Red Junglefowl (*Gallus gallus*). Nätt et al. (2012) identified promoter sequences upstream of more than 3000 genes with the highest fold change in expression and created a custom tiling array. Then, methylated-DNA immunoprecipitation was used to enrich experimental samples for methylated fragments of DNA and these fragments were competitively hybridized against reference samples to identify variation in DNA methylation among the more than 3000 promoter sequences. Given the number of specific target sequences that can be screened for variation in DNA methylation, microarray-based studies may be well suited to describe broader patterns of expression and methylation between states, species, or environments. However, microarray experiments can be much more expensive than MS-AFLP, and these studies require much more preliminary information in order to develop the array.

Next-generation sequencing

There are several next-generation sequencing platforms that are capable of generating enormous datasets of sequences. These platforms have been used to screen methylation of DNA across the genome (Hawkins et al. 2010). Although it may not be practical to approach large-scale ecological questions at the genomic level, platforms exist that could use next-generation sequencing to collect methylation data among non-model organisms. For example, the use of restriction-site-associated DNA sequencing (RAD-seq) has expanded the possibilities for systems that have no reference genome. RAD-seq is a relatively new protocol that reduces the complexity of the genome sampled by targeting sequences flanking restriction sites for deep sequencing, thereby increasing the power to identify unique sequences (Etter et al. 2011). RAD-seq has not been used to study methylation patterns *per se*, but methylation-sensitive enzymes have been used to target low-copy-number, gene-rich regions, assuming that repetitive DNA regions are uniformly highly methylated (Chutimanitsakun et al. 2011). This indicates that the same methodology could be used in an experimental context to compare replicates of the same genotype exposed to different conditions and thereby allow for a genome-wide probing of changes in methylation. An alternative to using methylation-sensitive enzymes would be to incorporate a sample treated with bisulfite along with control DNA in the RAD-seq protocol with a replicated genotype design, which could also identify variation in methylation patterns across the genome at single base-pair resolution. Yet another possible alternative is to use the single-molecule real-time sequencing protocol, which is able to distinguish DNA bases altered by methylation during a single run (Flusberg et al. 2010).

Pharmacological manipulation of epigenetic state

A different way to study the effect of DNA methylation on an organism's phenotype is to alter patterns of DNA methylation via pharmacological manipulation with zebularine (Zhou et al. 2002), trichostatin A (TSA), or 5-AZA, in order to discern whether methylation (or demethylation) causes observable phenotypic consequences (Bossdorf et al. 2008, 2010). The non-specific use of these agents would likely cause global demethylation, thus making it difficult to ascertain exactly where and how methylation of DNA is affecting phenotype. However, studies have shown that a more directed/specific approach can have profound effects on both gene expression and phenotype. For example, central infusion of TSA within the hippocampus caused DNA demethylation of the glucocorticoid receptor promoter, thus altering the stress reactivity phenotype in rats (Weaver et al. 2004). Furthermore, gene-specific differences in gene expression were found in isolated peripheral blood mononuclear cells from chicken leukocytes after treatment with both TSA and 5-AZA (Gou et al. 2012). Future work utilizing pharmacological manipulation of patterns of DNA methylation may further elucidate the connection between epigenetic variation and phenotypic variation (Bossdorf et al. 2008).

Suggestions for future studies

General suggestions

Reviewing MS-AFLP-based studies in Ecological Epigenetics identifies four general suggestions for increasing the scope of a study. First, genetic and DNA methylation should be collected contemporaneously. These data will be important for understanding and teasing apart the interaction between epigenetic and genetic components of an organism's response to its environment (Herrera and Bazaga 2011; Richards et al. 2012a). Also, each of the four selected studies controlled for genetic variation when investigating the role of DNA methylation. Second, using techniques in addition to MS-AFLP to identify the

targets of methylation can significantly add to the results. Extracting MS-AFLP bands and bisulfite sequencing (Massicotte et al. 2011) may be a good first step in addressing the major short-coming of MS-AFLP. However, at some point, the addition of a large-scale program of bisulfite sequencing to identify the anonymous targets of MS-AFLP would be outweighed by other techniques that identify known targets (i.e., those discussed above). Third, when possible, experimentally inhibiting methylation by pharmacological manipulation can demonstrate the effects of DNA methylation on phenotype (Bossdorf et al. 2010; Herrera et al. 2012). Fourth, studies benefit from incorporating designs beyond surveys of natural variation; experimentally treating specimens (Herrera et al. 2012), using a common garden (Richards et al. 2012a), and investigating invasive species (Richards et al. 2012a), or by identifying clear environmental stimuli (Herrera and Bazaga 2011) and distinct environmental conditions (Herrera and Bazaga 2011; Herrera et al. 2012; Richards et al. 2012a).

Model of variation

One compelling effort that could be undertaken is to investigate a model of MS-AFLP variation. As more researchers rely on MS-AFLP to characterize the role of DNA methylation, it will be critical to understand if some loci change rapidly under local conditions, while others are more stable throughout the lifetime of an individual or between generations (Richards et al. 2010a; Richards 2011). The objective would be to characterize the proportion of loci that vary in response to environmental conditions, that respond to general stressors, that are specific to particular stimuli, and that are stably transmitted to subsequent generations. This would determine how liable epigenotypes are to environmental changes, if the induced changes are consistent, and whether epigenetic variation is partitioned among individuals, populations, or environmental states. This dataset could be used to tease apart the epigenetic and genetic contribution of an organism's response to the environment by testing several models that predict the underlying mechanisms of this variation (*sensu* Herrera and Bazaga 2011).

This effort would connect the MS-AFLP technique with current research that investigates the role of epigenetic effects on evolution. For example, Day and Bonduriansky (2011) proposed a model that allows for the fact that some genetic contexts are more likely to acquire methyl groups than are others. This model could be parameterized with information about the behavior of AFLP and MS-AFLP in natural populations and over generations that would allow for an efficient and economical genome-wide characterization of the stability and behavior of different methylation marks, how they affect phenotype, and how this varies by genotype.

Ecological Epigenetics and changing environments

Multiple studies show how epigenetic mechanisms may support populations that are invading a new area and expanding their range (Chwedorzewska and Bednarek 2012; Richards et al. 2012a; Schrey et al. 2012; Liebl et al. 2013, this issue). These data indicate a need for future work addressing the role of DNA methylation in the ability of certain populations to respond appropriately to new environments, such as those caused by global climatic change. By addressing variation in multiple populations throughout a range of environments, MS-AFLP could identify specific populations (or species) that have the greatest capacity to adjust to changing environments. This may inform biologists and managers which populations are most at risk of declining because of a lack of underlying variation and therefore flexibility.

In a larger context, diagnosing the response of methylation to the environment has implications for an array of biological questions from implications of global change to understanding problems of human health. These questions are ultimately a problem of deciphering the translation from genotype to phenotype. MS-AFLP studies could be used in combination with the target-specific techniques discussed above, and thereby begin to address the role that methylation of DNA plays in an individual's response to the environment.

Conclusion

MS-AFLP has proven to be a useful technique that can efficiently generate data on methylation of DNA in ecological settings among organisms. All the studies reviewed here detected a great deal of variation among individuals, and many detected greater epigenetic than genetic variation (Massicotte et al. 2011; Herrera et al. 2012; Richards et al. 2012a). The benefits of MS-AFLP (Table 1) make it an excellent first step in a new Ecological Epigenetics research program. Data from MS-AFLP can indicate whether there is variation in DNA methylation among the experimental units. Also, MS-AFLP can identify the level at which DNA methylation varies, that is, does the variation in DNA methylation occur among individuals within experimental units, among units, or

in response to a particular stressor? This information can indicate whether it is worth pursuing a target-specific technique that is generally more expensive and effort intensive. However, it is apparent that large-scale next-generation sequencing projects will be required to fully understand the way environmental stimuli affect epigenetic states and the relationship between epigenetic states and phenotypes.

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