# Opportunities and limitations of reduced representation bisulfite sequencing in plant ecological epigenomics

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**Summary**: Investigating the features and implications of epigenetic mechanisms across the breadth of organisms and ecosystems is important for understanding the ecological relevance of epigenetics. Several cost-effective reduced representation bisulfite sequencing approaches (RRBS) have been recently developed and applied to different organisms that lack a well-annotated reference genome. These new approaches improve the assessment of epigenetic diversity in ecological settings and may provide functional insights. We asses here the opportunities and limitations of RRBS in non-model plant species. Well-thought out experimental designs that include complementary gene expression studies, and the improvement of genomics resources for the target group promise to maximize impact of future RRBS studies.

**Key words**: adaptation, bisulfite sequencing, bsRADseq, DNA methylation, ecological genomics, epiGBS, response to environment, RRBS

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## I. Introduction

Ecological epigenetics aims to understand the unique contributions of epigenetic mechanisms to ecological and evolutionary processes (Bossdorf *et al*., 2008; Richards *et al*., 2017). Several studies have shown that DNA methylation variation correlates with ecological factors, suggesting a potential role for epigenetics in adaptation (Verhoeven *et al*., 2016; Richards *et al*., 2017). However, the low genomic resolution of many studies on non-model organisms precludes pinpointing causality of epigenetic effects, and the peculiarities of the few plant and animal model organisms may impede generalizations (e.g., Lyko, 2001; Alonso *et al.*, 2015). Recently, we discussed how high resolution epigenomics tools must be combined with ecologically-relevant experimental settings to explore the importance of epigenetic mechanisms (Richards *et al*., 2017). We also argued that in order to evaluate the relevance of epigenetic mechanisms to ecology and evolution, these approaches need to be applied across a diversity of organisms and conditions. However, powerful epigenomic methodologies have largely been accessible for only a few model species. The 'gold standard' of bisulfite sequencing applied to whole genomes (WGBS) evaluates methylation status for essentially every cytosine in a genome (Frommer *et al*., 1992; Harris *et al.*, 2010; Li & Tollefsbol, 2011; Olova *et al*., 2018), but this approach is still limited in application to species with a high-quality reference genome (e.g. Niederhuth *et al.*, 2016). Furthermore, this approach is prohibitively expensive for large experimental designs, particularly for average to large plant genomes.

Several recently developed approaches based on reduced-representation bisulfite sequencing methods (RRBS) provide cost-effective alternatives to WGBS, by interrogating only a representative fraction of the genome (Gu *et al*., 2011; Wang *et al*., 2015; Trucchi *et al.*, 2016; van Gurp *et al.*, 2016). RRBS approaches are scalable for ecological experimental designs, can be used on organisms without reference genomes, and allow for greater resolution than previous marker-based approaches. RRBS reads can also be mapped to a reference or draft reference genome when available (Gugger *et al*., 2016; Lea *et al.*, 2016; Trucchi *et al*., 2016; Weyrich *et al.*, 2016), and may provide functional insights. For the species that lack a reference genome, RRBS studies can generate accession-specific references for only the loci that are interrogated, either by including non-bisulfite-converted samples in the sequencing (e.g. for bsRADseq, Trucchi *et al.*, 2016) or by inferring the un-converted reference from the bisulfite treated reads themselves (e.g. epiGBS, van Gurp *et al*., 2016). These attributes combined with cost-efficiency make RRBS methods attractive options for studies that address ecological and evolutionary epigenetics in essentially any

organism. However, it is important to evaluate their strengths along with any limitations to identify which research questions can be fruitfully addressed.

## II. RRBS loci as genome-wide epigenetic markers

RRBS methods are more powerful than previous methods (such as methylation-sensitive amplified fragment length polymorphism or MS-AFLP/MSAP; see e.g., Schrey *et al*., 2013) for exploring patterns of DNA methylation variation at a genome-wide scale. The increase in power is due to the fact that RRBS typically reviews multiple cytosine positions within each of tens of thousands of fragments. Unlike earlier methods, RRBS data is quantitative at nucleotide resolution, which reflects the biology of DNA methylation across cells in a tissue sample. RRBS methods provide methylation information at different sequence contexts (CG, CHG, CHH). Methylation in these different DNA contexts is correlated to distinct functions, regulated by different molecular pathways, and known to have different levels of heritability and environmental-sensitivity, thus providing further tools for dissecting natural patterns of DNA methylation (Dubin *et al.*, 2015; Niederhuth *et al*., 2016; Trucchi *et al*., 2016; Niederhuth & Schmitz, 2017). RRBS data can be integrated to quantify overall similarity among individuals in DNA methylation, which supplies a basis for describing patterns of epigenetic variation within and among natural populations.

Such descriptive population epigenetic measures provide useful insights about the distribution of epigenetic variation. For instance, to what extent is natural epigenetic population structure correlated to underlying genetic population structure, and to what extent is it correlated with environmental variation? High correlation between epigenetic and genetic structure has been observed in several studies of natural populations of *Arabidopsis thaliana* (Dubin *et al*., 2015; Kawakatsu *et al*., 2016), experimental populations of maize (Eichten *et al*., 2013), and recombinant inbred lines (RILs) of soybean (Schmitz *et al*., 2013), suggesting that a significant portion of epigenetic variation is under genetic control. These studies rely on detecting associations between differentially methylated regions (DMR) and neighboring genetic polymorphisms to indicate genetic control in *cis* over DNA methylation. Associations in *trans* have also been detected, for example between CHH methylation and genetic variation in the chromomethyltransferase CMT2 (Dubin *et al*., 2015). However, *cis* and *trans* correlations can also arise in the absence of genetic control, when a spontaneous epimutation becomes stably inherited (Taudt *et al*., 2016). In addition, some studies have reported that a component of epigenetic population structure was not explained by genetic structure, and was instead associated with ecological conditions (e.g. Richards *et al.*, 2012;

Dubin *et al*., 2015; Foust *et al*., 2016; Kawakatsu *et al*., 2016). This suggests that epigenetics can contribute directly or indirectly to adaptation: either *i*) because epigenetic changes are induced by environmental conditions, and provide a capacity for phenotypic plasticity or longer-term heritable responses, and/or *ii*) because the epigenetic variation that does not correlate to overall (neutral) genetic population structure can be under control by individual genetic loci.

In addition to characterizing natural DNA methylation variation, the high heritability of cytosine methylation in plants suggests that RRBS-loci could be used as markers for linkage mapping purposes in crosses where genetic variation is limited or absent (Johannes *et al*., 2009; Cortijo *et al*., 2014; Dapp *et al*., 2015; Hofmeister *et al*., 2017). Also, general characteristics of DNA methylation dynamics, such as transgenerational stability, epimutation and reversal rates, and response to experimental or field conditions of interest, can be investigated by looking at many, but not necessarily all, loci in the genome, and may thus be addressed with RRBS approaches.

**III. Exploiting functional annotation of RRBS loci**

The DNA sequence contained in RRBS fragments holds promise for providing information about the function of the genomic regions that show DNA methylation differences (Fig. 1). In particular, epigenetic changes at enhancer elements can drive gene expression alterations and have functional consequences (Taudt *et al*., 2016). RRBS methods were initially developed for studies in mammals where a large fraction of gene promoters are associated with GC-rich areas (CpG islands) and methylation status of these areas has functional consequences for gene regulation (Meissner *et al*., 2005). Using restriction enzymes that bias toward GC-rich sites (such as MspI), RRBS in mammals evaluates methylation in 80-90% of CpG islands by targeting only a small portion of the total genome (Smith *et al*., 2009). Typical plant genomes lack CpG islands and MspI-based reduced representation of genomes does not result in a bias towards gene promoters (Hsu *et al*., 2017). However, bias to either gene bodies or gene promoters can be achieved in plants by selecting different restriction enzymes, and increasing overall genome representation. For instance, RRBS in maize using the frequent cutter MseI provides a representation of >80% of the annotated gene promoters when coverage is increased to ~19% of the total genome. With a different enzyme (CviQI), >80% of gene bodies were included by sequencing ~15% of the genome (Hsu *et al*., 2017). The enrichment for functional components of the genome may not be as extreme as in mammals, but it does allow for functional insights. For instance, Hsu *et*

*al*. (2017) identified genes with tissue-specific expression associated with specific promoter DNA methylation profiles. In addition, complementary expression analyses with RNAseq or qPCR will complement RRBS datasets, potentially uncovering gene expression alterations associated with DNA methylation variation.

**IV. Limitations of RRBS methods for non-model species**

Although RRBS can target functional regions of a genome, identification of the genomic context of RRBS loci in non-model species is typically restricted to fragments that have homology to annotated species (Fig. 1). This approach has known limitations since genes and their function evolve over time, which may prevent proper interpretation of the information obtained in non-model species (Pavey *et al*., 2012; Alvarez *et al*., 2015). In the absence of a reference genome, a reference transcriptome could help improve functional predictions. In practice however, even if a large portion of the RRBS fragments overlap with a well annotated transcriptome (e.g., ca 10,000 of 36,000 RRBS fragments in *Spartina*, Alvarez *et al*., in review; >40% of bsRADseq in *Heliosperma*; Trucchi *et al*., 2016) or can be annotated with known plant genes (10-15% of epiGBS loci in six grassland species, Van Moorsel *et al*., 2018), only some of the fragments actually overlap the 5' end of genes (Alvarez *et al*., in review) where increased DNA methylation has been correlated with gene silencing (Niederhuth *et al*., 2016; Niederhuth & Schmitz, 2017). The functional relevance of gene body methylation outside of the 5' end in plants appears to vary by context and across taxa (Niederhuth & Schmitz, 2017), and often gene body methylation is not correlated or only weakly correlated to gene expression (Bewick & Schmitz, 2017). While methylation of the promoter region is highly correlated with silencing, without a well-annotated reference genome of the target species or a close relative, it is difficult or even impossible to identify those RRBS fragments that overlap promoter regions. Further, obtaining sufficient coverage of the genome may be prohibitive for plants with average to large genomes.

RRBS approaches allow for more power than earlier methods to detect outlier loci or identify loci with high $F_{ST}$ (e.g., Platt *et al*., 2015), which suggests selection on ecologically important genomic regions (see also McKinney *et al*., 2017 for a discussion of such an approach). Unlike DNA sequence information that is generally organised in large (e.g. several megabases) linkage blocks shaped by recombination, co-inheritance of epigenetic signals may be less stable and only locally restricted to one or a few neighboring genomic elements that may be missed in reduced representation methods. Similarly, for traits that are

controlled by relatively few functional loci, these may simply be missed in RRBS. This problem is amplified in species with large and complex genomes.

A further difficulty for all RRBS approaches is the identification of differentially methylated regions (DMRs). Studies in a variety of species have found that methylation changes across large chromosomal stretches (DMRs) are more likely to influence transcriptional activity at nearby loci, and contribute to phenotypic change than at single cytosines (DMPs) (reviewed in Richards *et al.*, 2017). However, DMRs are still difficult to define even in whole genome studies. The short fragments interrogated with RRBS methods (typically less than 500 bp) will contain only a few cytosine positions, and calling DMRs with statistical confidence with such data will be difficult in most cases (Fig. 1). Hence, most RRBS studies will be limited to calling DMPs, with their inherent stochasticity.

The evolutionary history of all plant genomes has been shaped by polyploidization and hybridization, which have played a major role in plant diversification and adaptation (e.g., Van de Peer *et al*., 2017). Sequencing approaches generally will require more sequencing depth to identify all copies of duplicated loci, particularly for those with high levels of heterozygosity, and this problem may be exacerbated with short fragments that have less discriminatory power. This means that the number of polymorphisms that define different copies at a given locus will grade into the number of polymorphisms that define different locations within the genome. This level of information about the genomic landscape is limited to very few non-model species, but could have profound impacts on understanding the importance of both sequence level and methylation variation for ecology and evolution.

Finally, unravelling the extent to which epigenetic variants are genetically controlled by or at least partly autonomous from genetic variants in different systems and environmental settings remains a key challenge for understanding the ecological and evolutionary role of epigenetic variation (Richards *et al*., 2017). In principle, this issue is better addressed with whole-genome data than with a reduced representation data. Reduced representation data provide adequate information to estimate overall genetic and epigenetic population structure, and thus to evaluate the overall correlation between the two. But in absence of full genome data RRBS is limited in helping pin point the genetic determinants of individual epigenetic variants.

## V. Maximising the impact of RRBS in plants

RRBS approaches allow for great power to detect patterns of variation in the epigenomic landscape, either as heritable signals or as plastic responses to environmental

conditions or experimental treatments. However, confirming local adaptation requires detecting specific patterns of organismal response in reciprocal transplant studies in the field or in experiments in controlled environments, ideally by controlling for any genetic influence (Richards *et al.*, 2010). A careful design of experimental conditions is particularly important for assessing epigenetic variation, which has fractions that are environmentally or developmentally labile (Richards *et al*., 2010, 2017). Measuring epigenetic mechanisms with a proper experimental design can provide information about changes that underlie phenotypic plasticity, and identify changes that are correlated to genetic differences or habitat of origin. Analyzing progeny across at least three generations will also better assess transgenerational heritability of the markers surveyed, but careful consideration of the environment in which to grow these offspring is required.

The flexibility of the RRBS protocols allows for several technical ways to improve impact. For instance, the choice of restriction enzymes determines what portions of the genome are sampled. Methylation-sensitive enzymes will target areas in plant genomes that are biased away from heavily methylated repetitive regions, and thus enrich towards coding regions. But using these enzymes will increase the probability of missing data, particularly within DMRs since individuals that are methylated at the recognition site will not be represented. An enzyme with a GC-rich restriction site will also bias against repetitive regions since several transposable elements (TEs) are associated with regions of the genome that are more AT-rich (Le *et al*., 2000). Furthermore, more frequent cutters (with shorter recognition sequences) will increase representation of the genome regardless of genomic context. Improvements can also come from using platforms that produce longer reads (e.g. MiSeq instead of HiSeq) or by optimizing libraries to get information across larger regions with paired-end sequencing approaches (e.g., up to 800 bp for bsRADseq using Illumina). This increases the likelihood of annotation and calling of DMRs, and potentially identifying promoters.

DNA methylation analyses require some additional considerations since it can be difficult to distinguish among substitutions and epiallele changes in bisulfite sequencing. In particular, mutations of either C or G to an A or T can appear to be an epimutation since bisulfite treatment deaminates un-methylated cytosines to uracil, which then pairs with thymine through PCR amplification. A single reference genome does not provide the information needed to discriminate these possibilities. Because of the cost advantage of RRBS compared to WGBS, unconverted references for each accession can be sequenced

even for large experimental designs for differentiating genetic variation from methylation variation among individuals.

## VI. Conclusions

In the past few years, several RRBS methodologies have emerged, increasing the potential and broadening the scope for epigenetics studies in non-model species. While these methodologies improve resolution for marker-based approaches, they have limitations for functional conclusions in species that lack a good reference genome (Fig. 1). These limitations result from the difficulty of targeting the RRBS fragments towards the most functionally relevant contexts for DNA methylation: the promoter regions and the 5' end of transcribed regions. Although approaches exist to enrich for these specific portions of the genome, generating a draft reference genome will be imperative to locate the promoter regions, and allow for better exploitation of RRBS data. Improving genomics resources in a variety of organisms is an essential next step for understanding the importance of epigenetic mechanisms in ecology and evolution.

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**Figure legend.**

**Figure 1.** Typically, studies in ecological epigenetics will be interested in methylation differences that are associated with two different environmental conditions as shown in this cartoon. RRBS data can provide functional insight in non-model species (e.g. the left most RRBS locus), but detecting the genomic context of RRBS fragments is difficult in the absence of a reference genome. In addition, the number of cytosines contained per RBBS locus may be insufficient to call DMRs, hence RRBS analyses will be limited to calling DMPs.