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Complete List of Authors:	Omony, Jimmy; HelmholtzZentrum München, Plant Genome and Systems Biology Nussbaumer, Thomas; Helmholtz Zentrum Munchen Deutsches Forschungszentrum fur Umwelt und Gesundheit, Institute of Network Biology, Department of Environmental Science; Chair and Institute of Environmental Medicine, UNIKA-T, Technical University of Munich and Helmholtz Center Munich, Research Center for Environmental Health, Augsburg, Germany; CK CARE Christine Kühne Center for Allergy Research and Education, Davos, Switzerland Gutzat, Ruben; Gregor Mendel Institute of Molecular Plant Biology GmbH, Austrian Academy of Sciences, Vienna BioCenter (VBC), 1030 Vienna
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DNA methylation analysis in plants: review of computational tools and future perspectives

Jimmy Omony[1,*$], Thomas Nussbaumer[2,3$] and Ruben Gutzat[4*]

[1]Plant Genome and Systems Biology, Helmholtz Center Munich-German Research Center for Environmental Health, 85764 Neuherberg, Germany.

[2]Institute of Network Biology, Department of Environmental Science, Helmholtz Center Munich, 85764 Neuherberg, Germany.

[3]Chair and Institute of Environmental Medicine, UNIKA-T, Technical University of Munich and Helmholtz Center Munich, Research Center for Environmental Health, Augsburg, Germany; CK CARE Christine Kühne Center for Allergy Research and Education, Davos, Switzerland.

[4]Gregor Mendel Institute of Molecular Plant Biology, Austrian Academy of Sciences, Vienna BioCenter (VBC), 1030 Vienna, Austria.

[$] joint first authors

Correspondence*:

Dr. Jimmy Omony, Helmholtz Center Munich, Germany.
Email: jimmy.omony@helmholtz-muenchen.de

Dr. Ruben Gutzat, Gregor Mendel Institute of Molecular Plant Biology, Austria.
Email: ruben.gutzat@gmi.oeaw.ac.at

Jimmy Omony is a postdoc (Bioinformatician) at the Plant Genome and Systems Biology, Helmholtz Center Munich, Germany. His research interests include plant genomics, epigenetics, machine learning, and biostatistics. He undertook the first postdoc at the University of Groningen (RuG). He holds a PhD in computational systems biology (Wageningen University).

Thomas Nussbaumer is a postdoc (Bioinformatician) at the Institute of Network Biology and also in the Institute of Environmental Medicine. His research interests include epigenomics, plant genomics, protein-protein interaction analysis, and microbiomics. He undertook his first postdoc at the University of Vienna and is currently a Postdoc Fellowship Program holder at the Helmholtz Center Munich.

Ruben Gutzat is a postdoc at the Gregor Mendel Institute of Molecular Plant Biology, Austrian Academy of Sciences, Vienna (Austria). His research interests are in plant epigenetics and developmental biology.

Abstract

Genome-wide DNA methylation studies have quickly expanded due to advances in next-generation sequencing techniques along with a wealth of computational tools to analyze the data. Most of our knowledge about DNA methylation profiles, epigenetic heritability, and the function of DNA methylation in plants derives from the model species *Arabidopsis thaliana.* There are increasingly many studies on DNA methylation in plants – uncovering methylation profiles and explaining variations in different plant tissues. Additionally, DNA methylation comparisons of different plant tissue types and dynamics during development processes are only slowly emerging but are crucial for understanding developmental and regulatory decisions. Translating this knowledge from plant model species to commercial crops could allow the establishment of new varieties with increased stress resilience and improved yield. In this review, we provide an overview of the most commonly applied bioinformatics tools for the analysis of DNA methylation data (particularly bisulfite sequencing data). The performances of a selection of the tools are analyzed for computational time and agreement in predicted methylated sites for *A. thaliana,* which has a smaller genome compared to the hexaploid bread wheat. The performance of the tools was benchmarked on five plant genomes. We give examples of applications of DNA methylation data analysis in crops (with a focus on cereals) and an outlook for future developments for DNA methylation status manipulations and data integration.

Keywords: epigenomics, epigenetics, bisulfite sequencing, DNA methylation, plants, differentially methylated regions.

Introduction

Methylation of cytosine at carbon position 5 (also termed 5-meC) is a hallmark of an epigenetic modification and 5-meC has been described as the 5th base of DNA [1]. Although the extent and context of 5-meC vary considerably between different plant lineages, all plants whose genomes have been sequenced and analyzed so far show substantial DNA methylation [2, 3]. Two major genomic contexts can be distinguished: (i) methylation on gene bodies and (ii) methylation on repeat sequences and transposons. Gene body methylation typically peaks on exons of moderately transcribed genes and, despite a comprehensive body of publications [3-5], its function remains mysterious [6]. Methylation on repeat sequences and transposons is crucial for suppressing transcription and is necessary for establishing heterochromatic domains. Consequently, mutations that abolish most DNA methylation lead to transposon activation and genomic meltdown after several generations in *Arabidopsis thaliana*. However, in early generations, the mutation can be outcrossed and selfed offspring will be isogenic but with different DNA methylation states [7-9]. Experiments along these lines have established that these differences in DNA methylation can be stably inherited over many generations and influence ecologically relevant phenotypic traits [10-15].

In contrast to animals, which only maintain CG methylation, in most plants 5-meC occurs also in several sequence contexts (CG, CHG, and CHH, where H is any of the bases A, T, or C) and is catalyzed by different methyl-transferases acting on different DNA methylation pathways. In *A. thaliana,* CG methylation is maintained by MET1, CHG methylation by CMT3, and CHH by CMT2 and the RNA induced DNA methylation pathway (RdDM). CG methylation occurs in euchromatin and heterochromatin whereas CHG and CHH methylation decorate repeats and transposons [16]. The cross-functioning and redundant DNA methylation pathways form a nuclear/DNA protection system that aids in identifying invading transposons and permanently shutting off their expression (see review by Kim *et al*. [17]).

Lister and Ecker [18] argued that 5-meC should be used as a dynamic fifth letter of the genomic code because of the important implications of methylation. It has become tractable to analyze genome-wide DNA methylation states in populations or across different plant species because of advances in next-generation sequencing (NGS) technologies. Much effort has been undertaken to determine the landscape of DNA methylation changes especially in *A. thaliana* and other land plants such as rice and tomato, which have had reference genomes available for several years [19, 20]. DNA methylation patterns vary widely among animals; *Drosophila* completely lacks CG methylation while the human genome is highly methylated (~75% of the cytosines). In *A. thaliana*, ~24% of the CGs, ~ 6.7% of the CHGs, and ~1.7% of the CHHs are methylated [21, 22].

Plants have varying levels of repeat content, which might be the result of bursts of single-repeat retro-elements, which can amplify rapidly using a reverse transcription step to make multiple copies, or DNA transposons, which use a copy-and-paste strategy [23, 24] and thus can amplify during DNA replication. While the repeat content is only ~20% in *Arabidopsis*, in cereals such as barley and wheat the repeat content can be up to 90%. Together with the presence of three subgenomes in hexaploid wheat, these repeats requires tightly regulated epigenetic mechanisms [25]. Genes have evolved different mechanisms for tolerating transposable elements (TEs) in their vicinity [26, 27]. Hirsch and Springer [28] provide a review of the interactions between TEs and gene expression in plants. They discuss three mechanisms by which transposons influence gene expression, namely: (i) the prevailing evidence that TE insertions within introns or untranslated regions of genes are often tolerated and have minimal impact on gene expression levels or splicing. Conversely, TE insertions within genes lead to aberrant or novel transcripts; (ii) TEs act as novel alternative promoters – with the potential to result in different expression patterns; and (iii) TE insertions near genes can influence gene regulation. In Arabidopsis two genes (IBM1 and IBM2) have been identified that prevent spreading of CHG and CHH methylation from transposons into gene bodies or promotors.

Interestingly, DNA methylation levels can also affect how plants respond to stress. *Arabidopsis* mutants with reduced global DNA methylation show increased expression of defense related genes and enhanced resistance to pathogens [29]. Polymorphisms of CMT2 correlate with DNA methylation variation along a longitudinal temperature gradient in natural populations [30] and *cmt2* plants are more heat tolerant [31]. Isogenic lines with different DNA methylation states show differences in their ability to compete in synthetic plant communities [32]. Similar influences on stress tolerance have also been observed in monocots, and wheat with experimentally reduced DNA methylation show resilience to salt and oxidative stress. The dynamics of the methylation state of genomic elements are tissue-specific (for instance, in *A. thaliana* seedlings [33-35]) and differ between juvenile and mature plants (e.g. in a study of *Acacia mangium* [36]). Reduced DNA methylation also results in abnormal plant development in *A. thaliana* [37]; hence, an optimally regulated level of methylation is vital for normal plant growth and development.

Plant-pathogen invasion can also influence methylation levels in different ways. For instance, genome-wide hypomethylation and hypermethylation influence resistance-related genes [38] and alter gene expression profiles, resulting in plant adaptation to stress. Wang *et al*. [39] showed that drought-induced alterations to DNA methylation in rice influence an epigenetic mechanism that regulates gene expression. As a major modification of the eukaryotic genome, DNA methylation significantly influences gene expression. Methylation of genomic features can lead to different gene regulatory effects. For instance, alteration of a gene’s expression potential is a result of DNA methylation affecting the interaction between transcription factors and DNA with chromatin proteins [40]. Additionally, methylation of the promoter region results in repression of gene expression and gene body methylation leads to the opposite effect [41, 42]. Studies have shown that gene body methylated genes are constitutively expressed in a wide range of conditions and tissues [6].

Chemistry of bisulfite conversion and sequencing

Bisulfite sequencing is generally done in three main steps, namely: (i) denaturing, (ii) bisulfite treatment, and (iii) polymerase chain reaction (PCR) amplification. In bisulfite conversion, DNA is denatured in a process that separates the forward and reverse strands. This is followed by treatment with sodium bisulfite, which converts unmethylated cytosine into uracil – which is then converted to thymine during PCR [43]. Quantification of the abundance of each cytosine can be achieved via Sanger sequencing [44] or NGS technologies [45]. The DNA strands cease to be complementary after bisulfite conversion. Treatment of genomic DNA with sodium bisulfite [46] enables us to distinguish between highly similar (and yet different) methylated cytosine, which has the same base-pairing features as unmethylated cytosine. Mapping read sequences to a reference genome enables the determination of positions with matching and mismatching bases. This process enables identification of methylated and unmethylated bases.

Bisulfite sequencing can be accomplished with different sequencing kits depending on whether whole-genome bisulfite sequencing (WGBS) or reduced representation bisulfite sequencing (RRBS) (WGBS: Lister and Ecker [18], RRBS: Jeddeloh *et al*. [47], Schmidt *et al*. [48]) is performed. Currently, WGBS remains the most informative method for generating DNA methylation data. It provides a huge wealth of data and requires no prior targeting. Unlike WGBS, which is expensive, RRBS can be performed more economically because it is restricted to CpG-enriched regions that make up a smaller portion of the genome. The restriction enzyme *Msp1* cleaves at 5’-C*CGG-3’ targets (base preceding * is methylated), thereby, mainly CpG-rich regions are targeted – which is advantageous for large genomes.

Typical workflow for processing bisulfite sequencing data

Before reads are mapped to a reference genome, the sequencing quality of reads can be checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) or NGS QC Toolkit [49] followed by removing low-quality bases and adapters with, among others, Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), cutadapt [50], or Trimmomatic [51]. However, some WGBS data processing tools integrate various analytic steps - enabling data preprocessing, read alignment, a more robust statistical analysis which output statistics such as read coverage, the percentage of uniquely aligned reads, and statistics on the three methylation contexts (CpG/CHG/CHH). One such tool is gemBS [52], which is a recently published pipeline for processing and analysis of WGBS data. The pipeline integrates data pre-processing and analysis steps from adaptor trimming through downstream statistical analysis of mapping results. gemBS uses the high-performance read aligner GEM3 [53] as a dependency and BScall (embedded in samtools, bcftools; http://samtools.sourceforge.net/) which is a variant caller for bisulfite sequencing data. Both GEM3 and BScall support single and paired-end reads. Further reading on the generic workflow of analyzing WGBS is found in the work of Liang *et al*. [54] and Wrecyzcka *et al*. [55].

Non-bisulfite based methods and related bioinformatics tools

While bisulfite sequencing methods represent the most popular approaches for analyzing epigenomic data, there are other approaches within the field of DNA modification based methods. These approaches include MeDIP-seq and MethylCap-seq, in methylated DNA immunoprecipitation (MeDIP) analyses [56] where the genomic DNA is randomly sheared, sonicated, and immunoprecipitated with an antibody recognizing 5-methylcytidine. Precipitated DNA can either be sequenced or hybridized to microarrays. MethylCap-seq uses the methyl-CpG Binding Domain (MBD) of MeCP2 [57] while oxBS [58] is used to specifically detect 5-methylcysteine (5mC) and 5-hydroxymethylcytosine (5hmC) which can be also done with *Tet*-assisted bisulfite sequencing (Tet) [59]. CAB and fCAB for the recognition of 5caC [60]. Notably, the presence/absence of 5hmC in plants remains contentious. Some scholars claim that 5hmC is present in plants [61, 62] while others claim its absent [63]. A comprehensive overview of the various tools is given at https://omictools.com/medip-seq-category.

Tools for analyzing epigenomics datasets

Bismark [64] and BSMap [65], as one of the first published tools for quantifying epigenomic datasets had to address the challenge of attaining high read mapping efficiency to enable a sensitive sequence search. Bowtie [66], Merman [67], SNAP (http://snap.cs.berkeley.edu/), and Bowtie2 [68] have been used as dependencies in epigenomics tools, for instance, BS-Seeker [69], BS-Seeker2 [70], BS-Seeker3 [71], BRAT-nova [72], WALT [73] and Bismark, which are currently among the most commonly applied tools for mapping bisulfite methylation data. We outlined the most common tools for mapping bisulfite sequencing data along with tools that allow for the detection and analysis of differentially methylated regions (DMRs). The program parameters as well as input and output data formats are specified in Table S1. This table provides an overview of the main tools for mapping and analysis of epigenomic data – particularly for bisulfite sequencing data. Additionally, we also categorized the tools into three major classes, namely: (a) mapping, (b) statistical analysis, and (c) complete pipelines (Table S1). The defining features for each tool, such as their ability to handle single or double-stranded sequence data as well as their ability to process data and perform down-stream statistical analysis, are also provided. Reviews by Adusulalli *et al*. [74], Shafi *et al*. [75] and Wrecyzcka *et al*. [55] complement our overview Table S1. The most frequently applied computational epigenetics methods were applied and tested using DNA methylation data, particularly with data acquired from bisulfite sequencing experiments. Therefore, there are many statistical procedures available for analyzing methylome data – categorized into the parametric and non-parametric approach. Both approaches are widely used in the literature [76]. For instance, MethylMix [77] is an excellent example of a parametric approach which uses Bayesian mixture models to identify

DNA methylation states of genes as either hypo- or hypermethylated. The method entails fitting a distribution function onto the frequencies of DNA methylation counts. The advantage of using non-parametric models is that no prior knowledge of the data distribution is required. However, when such knowledge is available, then parametric models are the preferred choice for modelling such data. MethylMix quantifies the effect of DNA methylation on genes, which is interesting for integrative studies that aim at establishing the association between the methylation states of the individual genes and their expression profiles. Investigating such associations unravels any hidden variations within and between samples (or tissues) as illustrated in [78-80]. Lea *et al*. [81] discussed the applications of mixed models on DNA methylation in plant epigenetics. They specifically focused on the binomial mixed model with the sampling-based algorithm (MACAU: Mixed model association for count data via data augmentation) for the approximation of parameters and computation of *p*-values. Other modelling frameworks are based on algorithms that integrate various analytical steps resulting in the detection of DMRs across the entire genome, for instance: (i) the weighted optimization algorithm proposed in [82] (which is an extension of MethylKit [83]), and (ii) ChAMP.DMR [84] which applies the Bumphunter [85] or ProbeLasso Algorithm [86]. An example of a non-parametric model is the Bayesian approach based on the Dirichlet-process beta-mixture model – which is used for clustering methylation profiles [76]. The model considers the DNA methylation expressions consisting of an infinite number of beta mixture distributions [87, 88].

DNA methylation: plant physiology and pathophysiology

Investigating the dynamics of DNA methylation in plant growth and development requires the analysis of samples from different plant tissues (e.g. Bartels *et al*. [34]). To our knowledge, no existing software has been developed specifically for the analysis of plant physiology and pathophysiology. However, there are many studies analyzing bisulfite data using samples from different plant developmental stages (from seedlings to mature plants). For instance, Bismark –

in leaf tissues from bread wheat seedlings [89], BSMap – for various datasets from different tissues in *A. thaliana* [90], and BS-Seeker2 – for young *Zea mays* leaves [91]. With rapid advancements in the development of software/tools for analysis of epigenomes, we are optimistic such tools will soon be available to the public.

Differentially methylated regions and their significance

Genomic regions (or bases) with different methylation profiles between samples are known as differentially methylated regions (DMRs). This is also referred to as differentially methylated CpG sites since the CpG-methylated sites occur in much larger numbers compared to the non-CpG contexts (CHG and CHH) [92, 93]. Peak detection enables the identification of CpG islands – which are essential for differentiating methylation profiles between samples (typically between controls and test samples). CpG islands are not randomly distributed in the genome but are instead grouped close together [94]. Long stretches of non-dense CpG sites, known as CpG shores can also be detected. Combining the methylation profiles of both CpG-islands and CpG-shores enables more efficient comparative analysis of DNA methylation profiles between samples.

Various statistical algorithms have been proposed for identifying DMRs – the most popular ones being: methylKit [83], metilene [95], DMRcaller [96], and Bumphunter [85]. For elaborate discussions on the DMR detection methods and a discussion on choosing the right method for DMR detection see Hebestreit *et al*. [97], and Kurdyukov and Bullock [98]. The tools are written and compiled in different programming languages (e.g. R, Python, Perl, Java, C, and C++; Table S1). Essentially, such tools are used to identify DMRs from either targeted regions of the genome or from the whole genome. Critical considerations have to be made, e.g. the choice of experimental designs for experiments and statistical methods for data analysis [99]. DMRs are intricately linked to transcription and the abundance of CpG sites (CpG islands). A high concentration of CpG sites are often found within the promoter regions of genes – so it is essential to accurately identify such sites. Methylation of promoter regions influences the level of transcription – heavy methylation disrupts transcription and de-methylation leads to transcription reactivation [100-102].

Peak identification and normalization are crucial initial steps in analyzing DNA methylation data and visualization and can be useful for comparing datasets and judging the performance and agreement between tools. Post-processing and visualization of (differentially) methylated sites enable high-resolution exploration and comparison of regions in the genome for variations in methylation profiles. Therefore, tools like BiQ [103] and BSeQC [104] have aided quality control and visualization of methylation data, thereby enabling researchers to explore data attributes and perform data quality control before analysis. There are many methods for clustering methylation marks such as the dynamic genome warping [105] approach which uses hierarchical clustering and the combination of different epigenomics analytic platforms and data integrative modules. Dynamic genome warping has been demonstrated to be a reliable way to get more meaningful results from datasets (for instance, Chari *et al*. [106]). To utilize this method, Liang *et al*. [54] developed a web-server to analyze whole-genome bisulfite sequencing data and their platform includes major steps for detection and mapping of the conversion rate, detection of DMRs, and their association to gene expression. Wreczycka *et al*. [55] discussed data requirements and computational attributes for specific software and assess bisulfite sequencing data analysis methods, alignment and data processing, detection of differential methylation, and assess strategies for handling large epigenetic datasets. In contrast, our work highlights existing asymmetries between mapping tools and contrasts their computational capabilities.

Another important aspect in plant epigenetics is how hypomethylation and hypermethylation affects gene expression. The concept of hypomethylation and hypermethylation is not limited to plants as they have also been extensively studied in cancer progression in humans [107], coronary heart disease [108] and eukaryotes in general [109]. The division of DMRs into hypo- and hypermethylated enables investigations into the influence of both types of methylation on gene expression. Many computational tools have integrated modules that enable the extraction and quantification of the extent of hypo- and hypermethylation in genes. One such tool is MethylMix, which requires that changes in a gene's methylation state must also agree with its expression profile. Additionally, it requires a treatment and control sample (for agricultural studies) or healthy and disease conditions (for clinical studies).

Downstream analyses of bisulfite methylome data

After data processing and calling of methylation sites, downstream analysis can be performed – including the functional annotation of differentially methylated regions and analysis of the associated pathways influenced by the targeted genes. Such analysis enables the assignment of functions and gene annotation as seen in the overviews of Bioinformatics omicX tools (https://omictools.com/epigenomics-category). Examples of tools for performing downstream analysis are given in Table 1.

Technical challenges: conversion rate, repetitive regions and differentially methylated regions (DMRs)

The main challenges in the analysis of DNA methylation data include incomplete methylation patterns and overdispersion of read-mappings [110-112]. Here, overdispersion means the presence of variability in the reads compared to the expected read distributions based on a given model structure. When epigenomics marks coincide with repetitive regions in the genome, mapping tools need to keep reads that map to multiple genomic locations – making these tools slower and computationally memory-intensive. This problem can be partly circumvented through parallel computing using multiple threads, especially for larger repetitive plant genomes.

Conversion rates

As a method for studying DNA methylation, bisulfite conversion involves the conversion of cytosine to uracil (while 5-methylcytosine, 5-mC remains unchanged). Bisulfite sequence conversion rates vary for different datasets. It is essential for conversion rates to be determined accurately to ensure the reliability of down-stream data analysis. Reliable results can be obtained from datasets with bisulfite-conversion rates higher than ~0.999 (see e.g. Sun *et al*. [113] – demonstrated using their tool MethQA). However, they urge caution for datasets with lower conversion rates. Modern commercially available bisulfite sequence conversion kits generally indicate conversion efficiencies of 90–100% [114]. An elaborate discussion on methods for estimating conversion rate from bisulfite DNA methylation data is provided in [115, 116].

Description of experiment: benchmarking selected tools

We aimed to determine how the well-established computational epigenomics methods perform on a small genome such as *A. thaliana* with ~130 Mbp (TAIR10) compared to a genome with a high repeat content and much larger genome size such as bread wheat – taking chromosome 1A (Chr1A) for demonstration purpose, IWGSC.v1 *et al*. [117]. We used bisulfite sequencing data from two studies (with accession numbers SRR429549 [118, 119] for *A. thaliana* and ERR1141918 [89] for *T. aestivum*, data from NCBI) and applied four methods: BSMap [65], Bismark [64], BS-Seeker3, and segemehl [120]. Our analysis focused on the speed and agreement of common methylated sites between the tools. BS-Seeker3 was the fastest, followed by BSMap, while Bismark and segemehl were the slowest irrespective of genome size – especially for multiple threads (Figure 1: A and B). When using a single thread, segemehl (keeping reads that mapped a maximum of 3 times) performed slowest compared with the other methods. Overall, the computation time required for the *T. aestivum* (Chr1A) dataset is significantly longer than those from *A. thaliana* (Figure 1: A and B). When comparing the reported sites, we found that, for *A. thaliana*, 562,051 sites are shared amongst all four tools. While most sites were overlapping between BSMap, BS-Seeker3 and Bismark, likely because they use the same mapping software, segemehl reported only ~10% of these sites. However, for *T. aestivum*, ~101,944 sites were reported with most of them being reported in segemehl (Figure 1: C and D). The existence of such asymmetries requires more attention and is certainly worth taking into consideration when using the different computational tools. Other studies on comparisons of the performance of epigenetics analysis tools, specifically focusing on mapping short reads for bisulfite sequencing data, can be found in the work of Tran *et al*. [121]. Several studies have also compared run-time and memory consumption of different epigenomics tools, such as Tran *et al.* [121] who compared the five bisulfite short read mapping tools BSMap, Bismark, BS-Seeker, BiSS and BRAT-BW and Bismark performed best on real data, followed by BiSS, BSMap and BRAT-BW and BS-Seeker. Recently, Huang *et al.* [71] proposed BS-Seeker3 – a fast mapping tool for bisulfite data, and compared it performance for run-time and sensitivity to sister tools like Bismark, BRAT-nova, and BSMap. Additional to being accurate and versatile, Huang *et al.* concluded that BS-Seeker3 is an ultra-fast pipeline to process bisulfite-converted reads. The tool also aids visualization of methylation data; hence, justifying its comparability to the other three tools (Bismark, BRAT-nova and BSMap).

We simulated reads from *A. thaliana* and bread wheat using the tool by Sherman (https://www.bioinformatics.babraham.ac.uk/projects/sherman/) to test the performances of the four tools by comparing the precision and sensitivity along all chromosomes (Figure 2). The sensitivity, also sometimes referred to as recall, is defined as TP/(TP+FN). The precision is defined as TP/(TP+FP), where TP – true positive, FN – false negative and FP – false positive. We observed best performances for the Bismark, followed by BSMap and segemehl, while BS-Seeker3 seemed to have a lower sensitivity in *A. thaliana* compared to the other tools. For bread wheat a similar order to performances of tools was observed when reads where simulated for each subgenomes of chromosome 1 with the three genome copies. All scripts were provided in GitHub (https://github.com/jomony/EPItools/blob/master/README.md).

Feature comparison between the tools and related literature benchmarking

To further benchmark the performance of the tools, we used bisulfite sequencing data from five plant genomes. These genomes consist of the dicots: *Arabidopsis thaliana* (genome size ~0.13Gb*,* SRR4295494*)*, *Arabidopsis lyrata* (~0.21Gb, SRR3880297) and *Glycine max (*~1.2Gb, SRR5079790*)*, and also the monocots: *Triticum aestivum* (chromosome 1A, size ~0.67Gb, ERR1141918) and *Oryza sativa (*~0.43Gb, SRR7265433). Figure 3(A) shows the results of a comparative analysis of the memory footprint analysis of the performance of the four tools benchmarked using data from five genomes. These results come from mapping the bisulfite reads data to their respective reference genomes. Association analysis was performed for each of the four tools as seen in the linear regression model fits (Figure 3: B to E). The results show that the genome sizes for each of the five genomes are significantly correlated to the memory footprint analysis (*p*-values < 0.05).

The key attributes and parameters for the four tools are summarized in Table S2. This table presents a summary of the supported features in the four tools (BSMap, BS-Seeker3, Bismark, and segemehl). Such features are essential for deciding on which tool to use for mapping reads and data analysis. Examples of such features can also be found in the work of Guo *et al*. [70] and Tran *et al*. [121]. Lee *et al.* [122] evaluated the mapping accuracy and mapping rates for Bismark, BSMap, and BS-Seeker2 as a function of the error rates. Using whole genome bisulfite sequencing data, they assessed the influence of the error rates on the mapping rates and mapping accuracy and observed that at low error rates (<4%), BSMap had a higher mapping rate than Bismark and BS-Seeker2. On the contrary, BSMap had a lower mapping accuracy than

Bismark and BS-Seeker2. They also showed that mapping accuracy is independent of the methylation level.

A discussion on benchmarking approaches with a focus on short sequence mapping tools is found in the work of Hatem *et al*. [123]. They assess the performance of various aligners for the read mapping tools and benchmark them using criteria such as mapping percentage, running time and memory footprint. Variations in parameters such as seed length, base quality, single- or paired-end reads on the mapping quality are also evaluated. Benchmarking of tools by comparing the performance of each tool based on multiple attributes can be achieved in various ways, for instance, by assessing the: (i) effect of the read length and sequencing error, (ii) effect of data processing, and (iii) effect of varying parameters in the tools. These are some of the approaches discussed by Tran *et al*. [121]. They compared the performance of epigenomic mapping tools such as BSMap, Bismark, BS-Seeker, BRAT-BW [124] and the Bisulfite Sequencing Scorer (BiSS) [125]. Tran *et al.* primarily benchmarked the performance of the tools basing on mapping efficiency (as the percentage of reads that map uniquely to the genome) and the CPU time.

Outlook

In the near future, there is a need for more comparative analyses to explore the epigenomes of diverse plants in different development stages together with various stress factors. This would enable the discovery of exclusive and common epigenetic regulatory mechanisms. Uncovering and exploiting such mechanisms could potentially promote adaptation to changing environmental conditions. Moreover, a large number of methylomes are required to study the effect of the environment and stress conditions on the epigenomic state of a single plant [126, 127]. Resources like the 1001 epigenomes project (https://1001genomes.org/) in *A. thaliana* are exciting datasets to aid in our understanding of the role of the epigenome. However, it remains unclear whether the observations in these studies are directly applicable to crops.

Computational tools are instrumental for bridging the gap between mapping of sequenced reads, the accurate prediction of methylated sites, and their statistical analysis However, this effort is hampered by variations in the size of epigenomic marks and the complexity associated with normalizing peaks. The need to increase crop yield on the same amount, and in some cases dwindling, of arable land is another important aspect that requires advancements in epigenomics studies. Several studies have shown that during seed and grain development, the plant epigenome changes and leads to gene silencing. Therefore, a change in the epigenetic state of a plant would result in an increase in its likelihood of adapting from one geographical location to another or to different environmental conditions.

Lämke and Bäur [128] argued that such modifications have the potential to provide a mechanistic basis for stress memory in plants. This enables plants to respond more efficiently to recurring stress from the environment, for instance drought and salinity stress [129], a topic that was reviewed by Golldack *et al*. [130] (and more recently by Yang and Guo [131] and Abhinandan *et al*. [132]). This might enable plants to prepare their offspring for future attacks from stressors and to improve their adaptation to specific stress factors [130]. Plant adaptation to stress might enable us to explore new ways to improve yield, for instance by shortening or prolonging the time for grain development, by finding ways to regulate the expression of the three homeologs in wheat, or by interfering with fruit ripening (as seen in tomatoes [133-135] and other fruits like peach, apples, and strawberries [136]). A more intriguing discussion on the epigenetic mechanisms of plant stress response and adaptation to different environmental conditions was reviewed in [137-139].

In this review, we have discussed the use of bioinformatics tools to study DNA methylation data in plants. Notably, several studies in humans and mouse were successfully performed using popular tools like BSMap, BS-Seeker/BS-Seeker2/BS-Seeker3, Bismark, in mouse and segemehl in human cancer cell lines. For the analysis of bisulfite sequence data, most of the fundamentals of the chemical background and methylation principles are the same; however, the major difference between the use of such tools in plants, and animals (specifically, in humans and mouse) is the genome structure organization and the presence of predominantly more CHG/CHH methylation contexts in plants. The most predominant context of DNA methylation in mammals is the symmetric CG – estimated to be at ~70-80% of CG dinucleotides genome-wide [140]. The mechanisms of regulation and function of DNA methylation vary in animals and plants [141, 142]. These variations in regulation and function mechanism, coupled with genome structure differences and complexity levels is a motivating factor for integrating small subtle differences in mapping and analysis tools for epigenome data. Another important difference of plants and animals is how they are able to demethylate their genome. So far, enzymes removing directly the methyl group from cytosines have not been identified in plants, but they are important components of mammalian DNA methylation homeostasis. Plants use either passive mechanisms (not maintaining methylation during DNA replication) or base-excision and subsequent repair for direct removal of methylated cytosines. Unlike with the human genome, the CHG/CHH contexts which are more abundant in plants [143] need to be integrated into the mapping and analysis of methylome data. Many plants have large and repetitive genomes compared to that of humans. Such large genomes are a limiting factor in the analysis since they require a lot of computational resources. The sequence mapping to references and statistical computational time for large genomes such that of bread wheat (~17Gb) and barley (~5.3Gb) is likely to scale linearly.

Concluding remarks

In the last decade, there has been tremendous progress in the development of tools for analyzing epigenomic data; however, numerous challenges remain. For instance, the visualization capacity of many tools remains either inadequate or lacks essential modules for handling and displaying statistical outcomes from the resulting analysis. Additionally, the of these tools to scale to handle large genomes remains an issue for further exploration. Technically, most computational tools for analyzing epigenomic data perform well for datasets from organisms with a genome size that is smaller than the human genome (~3Gb). For much larger and complex genomes, more computational resources are required and the genome structure (whether diploid, hexaploidy, or tetraploid) and repetitive nature of the genome has to be taken into consideration during mapping to a reference genome. This is demonstrated in our example where we compared the mapping efficiency for *Arabidopsis* and a wheat chromosome; however, the complexity in genome structure, the presence of transposable elements, and the lack of consistent gene annotations for some plants remain a major obstacle to advancing epigenetic research.

In the next decade, there is likely to be a steady improvement in sequencing methods and performance of already existing computational algorithms. Recently, it was shown that even well-established sequencing methods might be prone to errors, leading to misleading results, e.g. DNA immunoprecipitation sequencing (DIP-seq) [144]. Discovering and amending such errors can lead to new findings from the previous studies and limit these errors' damage to future studies. This will aid further epigenetic research not only in plants but also in life sciences in general. Additionally, a few tools have the capability to effectively get more information out of low-coverage data. Developing new tools or improving on existing ones to attain optimal results using low coverage data and fewer replicates would save experiment and sequencing costs. A high sequence coverage allows for good data quality and enables robust statistical analysis [145]. Achieving high sequence coverage can be quite expensive and the minimum desired coverage can depend on the research objectives at hand. Typically, a coverage value of 5-10X is sufficient for many comparative studies and for achieving reliable methylation calls [145]. However, studies have demonstrated that coverage values as low as 2X is sufficient [146]. Accurate identification of DMRs in large samples, especially between multiple conditions, remains a challenge – despite tremendous progress already made in this area.

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J.O and T.N initiated the study, initiated the analysis framework, performed the data analysis, simulations and drafted the manuscript. R.G stream-lined the manuscript content and write-up.

Key points

- We introduce the concepts of epigenetics in plants and discuss commonly used tools – with a focus on their capabilities.
- Integration of bioinformatics tools needed to understand epigenomics datasets in crops.
- The presence of repetitive elements in the genome influences the prediction of methylated sites.

- We list the runtime and computational requirement for a small and large complex genome and demonstrate their overlaps in four most applied tools.
- Different tools have different levels of asymmetry with regards to their mapping and methylation call statistics.

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Figures

Figure 1. Selection of epigenomics tools.

Figure panels A and B: Results of the calculation user times for four common tools, Bismark, BSMap, BS-Seeker3, and segemehl. We used data for *Arabidopsis thaliana* and chromosome 1A in bread wheat (*Triticum aestivum*). *n.a*: values not available. Figure panels C and D: Overlap of detected sites in the two reference genomes for the four mapping tools.

Figure 2. Precision and sensitivity analysis.

Precision and sensitivity analysis for the *A. thaliana* data based on read mapping of simulated reads using the tool by Sherman (https://www.bioinformatics.babraham.ac.uk/projects/sherman/) – with the parameters (CG=24, CH=8, e=0.5). (A) There is a large difference in the sensitivity of the four tools. BS-Seeker3 was the least sensitive (sensitivity averaging ~ 48%) – Bismark was the most sensitive (sensitivity ~99.9%). The sensitivity values for BSMap and segemehl averaged ~97% and 90%, respectively. (B) For bread wheat (*T. aestivum*), BSMap appears to be marginally less precise and less sensitive than segemehl. There is consistency in the precision and sensitivity values for the subgenomes A, B and D in chromosome 1 of *T. aestivum*. Overall, the results from both (A) and (B) are in agreement. Notably, BS-Seeker3 has a wide range of precision compared to the other three tools. Each data point represents the precision-sensitivity value based on a simulation run for an individual tool. The precision and sensitivity values for Bismark, BSMap, BS-Seeker3 and segemehl averaged approximately (99%, 99%), (94%, 82%), (86%, 38%) and (97%, 87%), respectively. Five (5) simulation runs were performed for each tool – one for each of the *A. thaliana* chromosomes. The elliptical rings around each set of (same colored) data points represent the confidence bounds.

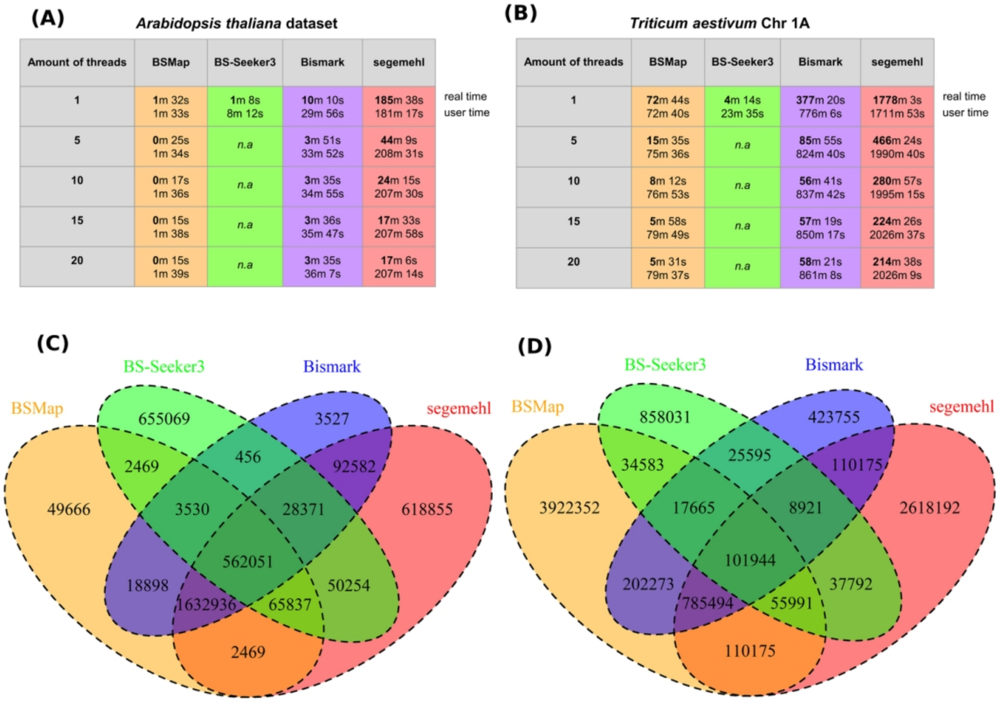
Figure 3. Memory footprint analysis for the four tools – benchmarked on five genomes. (A) Barplots showing variation in attained memory footprint between the tools benchmarked on different genomes. (B to E) Correlation analysis of genome size and memory footprint analysis. A benchmark of the four tools, (B) BSMap, (C) BS-Seeker3, (D) Bismark, and (E) segemehl. The genome sizes are all significantly correlated to the memory footprint analysis (*p*-values < 0.05). Red dotted line: fitted regression line, green-dots: data points.

Table 1. Examples of some down-stream analysis software.

Supporting information

Table S1. A selection of popular packages and tools for epigenome data analysis. Unranked list compiled based on high (≥100) citation and usage (October 2018). Most of these tools are freely available for download (non-commercial) and some are embedded into a web-server.

Table S2. Summary of key attributes and parameters for the four benchmarked tools.



(A) *Arabidopsis thaliana* dataset

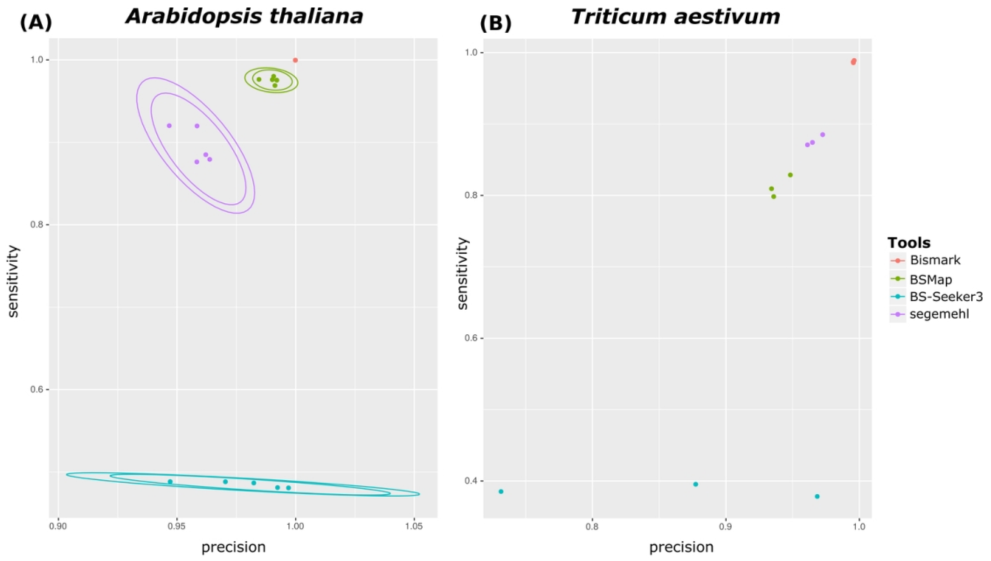
Amount of threads	BSMap	BS-Seeker3	Bismark	segemehl	
1	**1**m 32s 1m 33s	**1**m 8s 8m 12s	**10**m 10s 29m 56s	**185**m 38s 181m 17s	real time user time
5	**0**m 25s 1m 34s	*n.a*	**3**m 51s 33m 52s	**44**m 9s 208m 31s	
10	**0**m 17s 1m 36s	*n.a*	**3**m 35s 34m 55s	**24**m 15s 207m 30s	
15	**0**m 15s 1m 38s	*n.a*	**3**m 36s 35m 47s	**17**m 33s 207m 58s	
20	**0**m 15s 1m 39s	*n.a*	**3**m 35s 36m 7s	**17**m 6s 207m 14s	

(B) *Triticum aestivum* Chr 1A

Amount of threads	BSMap	BS-Seeker3	Bismark	segemehl	
1	**72**m 44s 72m 40s	**4**m 14s 23m 35s	**377**m 20s 776m 6s	**1778**m 3s 1711m 53s	real time user time
5	**15**m 35s 75m 36s	*n.a*	**85**m 55s 824m 40s	**466**m 24s 1990m 40s	
10	**8**m 12s 76m 53s	*n.a*	**56**m 41s 837m 42s	**280**m 57s 1995m 15s	
15	**5**m 58s 79m 49s	*n.a*	**57**m 19s 850m 17s	**224**m 26s 2026m 37s	
20	**5**m 31s 79m 37s	*n.a*	**58**m 21s 861m 8s	**214**m 38s 2026m 9s	

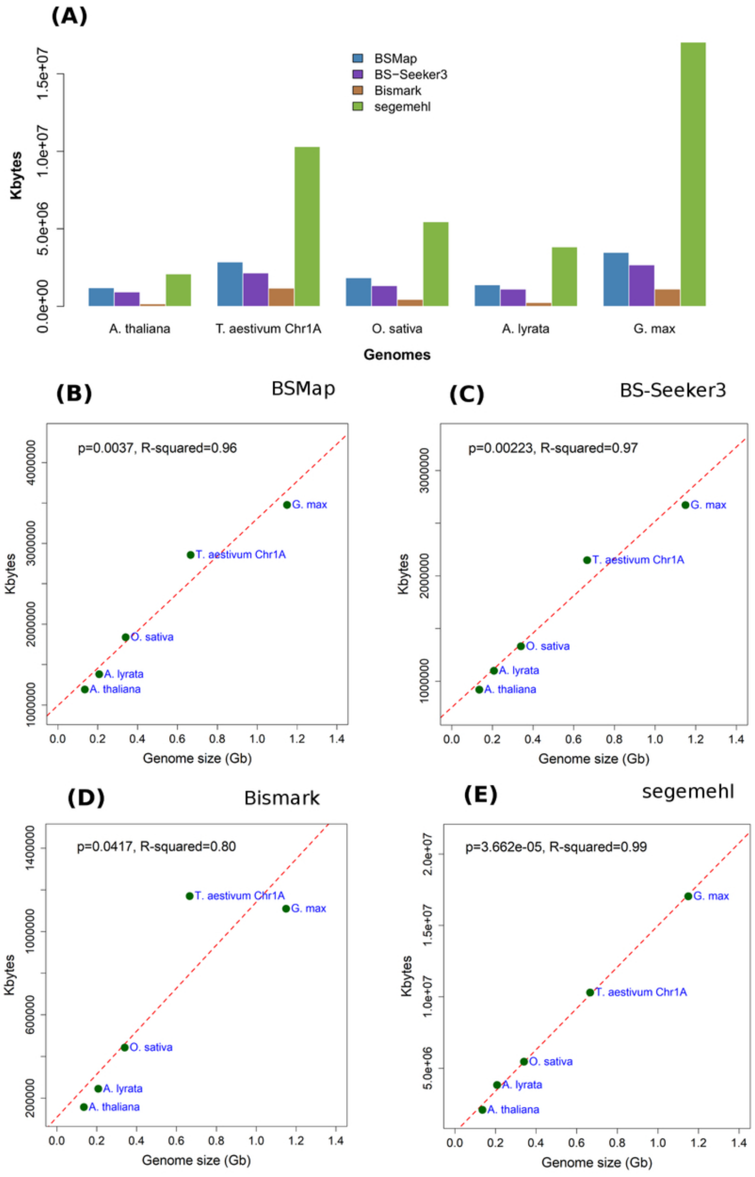
Selection of epigenomics tools. Figure panels A and B: Results of the calculation user times for four common tools, Bismark, BSMap, BS-Seeker3, and segemehl. We used data for Arabidopsis thaliana and chromosome 1A in bread wheat (Triticum aestivum). n.a: values not available. Figure panels C and D: Overlap of detected sites in the two reference genomes for the four mapping tools.

126x95mm (300 x 300 DPI)



Precision and sensitivity analysis. Precision and sensitivity analysis for the A. thaliana data based on read mapping of simulated reads using the tool by Sherman (https://www.bioinformatics.babraham.ac.uk/projects/sherman/) – with the parameters (CG=24, CH=8, e=0.5). (A) There is a large difference in the sensitivity of the four tools. BS-Seeker3 was the least sensitive (sensitivity averaging ~ 48%) – Bismark was the most sensitive (sensitivity ~99.9%). The sensitivity values for BSMap and segemehl averaged ~97% and 90%, respectively. (B) For bread wheat (T. aestivum), BSMap appears to be marginally less precise and less sensitive than segemehl. There is consistency in the precision and sensitivity values for the subgenomes A, B and D in chromosome 1 of T. aestivum. Overall, the results from both (A) and (B) are in agreement. Notably, BS-Seeker3 has a wide range of precision compared to the other three tools. Each data point represents the precision-sensitivity value based on a simulation run for an individual tool. The precision and sensitivity values for Bismark, BSMap, BS-Seeker3 and segemehl averaged approximately (99%, 99%), (94%, 82%), (86%, 38%) and (97%, 87%), respectively. Five (5) simulation runs were performed for each tool – one for each of the A. thaliana chromosomes. The elliptical rings around each set of (same colored) data points represent the confidence bounds.

127x70mm (300 x 300 DPI)



Memory footprint analysis for the four tools – benchmarked on five genomes. (A) Barplots showing variation in attained memory footprint between the tools benchmarked on different genomes. (B to E) Correlation analysis of genome size and memory footprint analysis. A benchmark of the four tools, (B) BSMap, (C) BS-Seeker3, (D) Bismark, and (E) segemehl. The genome sizes are all significantly correlated to the memory footprint analysis (p-values < 0.05). Red dotted line: fitted regression line, green-dots: data points.

71x107mm (300 x 300 DPI)

Tool	Citation and descriptions
ADMIRE: Analysis and visualization of differential methylation in genomic regions using the Infinium HumanMethylation450 Assay	Preussner *et al*. [109]; Online and offline. Adds experimental settings, quality control, automatic filtering, normalization, multiple testing, and differential analyses genome-browser tracks, table outputs, summary files.
BATMAN: Bayesian automated metabolite analyser for NMR spectra	Hao *et al*. [110]; Uses Markov chain Monte Carlo algorithm for sampling. Bayesian based approach.
KEGG: Gene Ontology Pathways	It is a database for mining and analysis of high-level functions. KEGG enables analysis and data mining on different biological scales (e.g. cellular and molecular-level information, whole organism, at ecosystem level, etc – using data from high-throughput experiments; see https://www.genome.jp/kegg/).
IPA: Ingenuity Pathway Analysis	Krämer *et al*. [111]; Platform enables exploration and visualization of complex omics data (e.g. microarrays including miRNA, metabolomics, proteomics, RNA-seq, small RNA-seq and SNP, and small scale experiments). See https://www.qiagenbioinformatics.com/
DAVID: Database for Annotation, Visualization and Integrated Discovery	Huang *et al*. [112]; DAVID enables pathway mining and gene function classification. Input is gene list from high-throughput genomic experiments; https://david.ncifcrf.gov/

Category	Tool	Year	Software	Mapping tool	Method	Input	Output	Reference/web-page
Mapping	Bismark	2011	Perl	Bowtie	Integrates Bowtie, running four alignment processes simultaneously	FASTA/FASTQ	SAM format, BAM format, one entry (or line) per cytosine	https://github.com/FelixKrueger/Bismark
	BSmooth	2012	R/Perl	Merman, Bowtie, I	Identification of DMRs, Walch t-test, improving previous work based on Fisher's exact test to simulate biological replicates using merman mapper	FASTQ	DMRs	https://github.com/BenLangmead/bsmooth-align
								https://github.com/hansenlab/bsseq
	BSMap	2009	C++	SOAP	Using HASH table seeding + Bitwise masking, bisulfite seq. data mapping program	FASTA/FASTQ/BAM - supports paired end reads	BSP/SAM/BAM	https://code.google.com/archive/p/bsmap/
	RRBSMap	2012	C++	RRBSMAP	RRBS short read alignment tool	BAM files	n.a.	http://rrbsmap.computational-epigenetics.org/
	BS Seeker	2010	Python	Bowtie2	Pipeline for mapping bisulfite sequence data, genome indexing. Accepts both RRE	FASTA/FASTQ, qseq, pure sequence, IGV input	BAM, SAM, BS_seeker and WIG files	https://guoweilong.github.io/BS_Seeker2/index.html
	BS Seeker2	2013	Python	Bowtie2	Pipeline for mapping bisulfite sequence data, genome indexing. Accepts both RRE	FASTA/FASTQ, qseq, pure sequence, IGV input	BAM, SAM, BS_seeker and WIG files	https://guoweilong.github.io/BS_Seeker2/index.html
	BS Seeker3	2018	Python	SNAP-aligner	Pipeline for mapping bisulfite sequence data, genome indexing. Accepts both RRE	FASTA/FASTQ, qseq, pure sequence, IGV input	BAM, SAM, BS_seeker and WIG files	https://github.com/khuang28jhu/bs3
	Metilene	2015	Perl	n.a.	DMR finder (from whole genome and targeted sequencing data)	FASTQ	DMRs	http://genome.cshlp.org/content/26/2/256.short
	segemehl	2012	Perl	segemehl	read mapper. Analysis of: COV, MET, TXN, SNP and CNV.	FASTA/FASTQ	SAM format	http://www.bioinf.uni-leipzig.de/Software/segemehl/
	BRAT-BW	2012	Free and Open Sour	FM-index (Burrow	Mapping of bisulfite reads, supports paired-end libraries, indels, mismatches, …	FASTQ for reads, FASTA for reference sequence	Text files of mapping results	http://compbio.cs.ucr.edu/brat/
	BRAT-nova	2016	Free and Open Sour	FM-index (Burrow	Mapping of bisulfite reads, improved implementation of the mapping tool BRAT-BV	FASTQ for reads, FASTA for reference sequence	Text files of mapping results	http://compbio.cs.ucr.edu/brat/
	WALT	2016	Free and Open Source under GPLv3		Mapping bisulfite sequencing reads	FASTQ for reads, FASTA for reference sequence	SAM or MR files	https://github.com/smithlabcode/walt
Statistical analysis								
	MethGo	2015	Python	n.a	global and gene level scalemethylation pattern around TSS sites. Accepts both RR	FASTA, BAM, GTF and CGmap	SNP, CNV tables, methylation profile summaries/plots/ta	http://paoyangchen-laboratory.github.io/methgo/
	EpiGRAPH	2009	Java	n.a	software for genome and epigenome analysis, uses machine learning algorithms	FASTA/FASTQ, genomic seq data	Enable prediction of genomic regions having similar char	https://epigraph.mpi-inf.mpg.de/WebGRAPH/
	CyMATE	2008	Perl and C	n.a	Unique mapping tool for CpG and non-CpG methylation (web-based tool)	multiple sequence alignment	Text files of per sequence mC, per position mC, global m	http://www.cymate.org/
	MethylMapper	2005	Perl	MethylMapper	High through-put mapping (web-based tool), performs QC analysis	DNA methylation seq. data	File with counts/tallies of methylated sites	https://sourceforge.net/p/methylmapper/wiki/Home/
	RnBeads	2014	R	n.a., using bed file	Supported assays: WGBS, RRBS. Data QC and filtering, DMR finder, Data explor	Illumina microarray platform bisulfite sequencing	bed, bigBed and bigWig file	https://rnbeads.org/
	BISMA	2010	PHP code, Perl and	Uses ClustalW alg	Analysis of bisulfite sequence data. Supports analysis of repetitive sequences (we	ABI, text and single multi-FASTA file formats	Outputs multiple sequence alignment. Web-presented ou	http://services.ibc.uni-stuttgart.de/BDPC/BISMA/
	BSPAT	2015	Java, Tomcat	Bowtie	Online service to analyze methylation patterns in bisulfite sequencing data. Has int	FASTA, FASTQ	SAM	https://github.com/lancelothk/BSPAT
	MethylMix	2015	R	n.a.	Detects hyper- and hypomethylated regions. Uses Bayesian Information Criterion (BIC) to select number of methylation states by itera		Differentially methylated regions (DMRs)	https://www.bioconductor.org/packages/release/bioc/html/MethylMix.html
	bumphunter	2012	R	n.a	Detection of DMRs	FASTA, FASTQ	BED, BigWig files	https://github.com/rafalab/bumphunter
	DMAP	2014	Bismark alignment	n.a	Differentially methylated region detection. Accepts both RRBSand WGBS data	SAM file from aligner tool	DMRs, identifies genes and CpG features; distances to D	http://biochem.otago.ac.nz/research/databases-software/
Complete pipeline								
	SAAP-RRBS	2012	BSMap: modules de	uses hashing/bitw	Includes FASTQC, duplicate read removal, read alignment, and methylation calls	FASTQ	Bed files with CpG sites, annotation files	http://bioinformaticstools.mayo.edu/research/saap-rrbs/
	gemBS	2018	C, Python	n.a	Analysis of whole genome bisulfite sequence data (both WGBS, RRBS).	FASTQ/FASTA, SAM/BAM files	Supports DMRs, Yes (bigWig, bedGraph)	http://statgen.cnag.cat/GEMBS/
	BiQ Analyzer HiMod	2014	web server	n.a	Upgrade of BiQ Analyzer HT	FASTA, FASTQ	BED, bigBed, ... and BigWig files	https://biq-analyzer-himod.bioinf.mpi-inf.mpg.de/
	BiQ Analyzer HT	2011	web server	n.a.	Bisulfite sequence quality assessement tool. Analysis of unique sequences	FASTA or BAM files	Outputs multiple sequence alignment. Web-presented ou	https://biq-analyzer-ht.bioinf.mpi-inf.mpg.de/
	QUMA	2008	n.a.	EMBOSS packag	Interactive web-based tool	FASTQ data, bisulfite sequence	Outputs multiple sequence alignment, statistics summary	http://quma.cdb.riken.jp/

Abbreviations	**Explanation**
RRBS	Reduced representation bisulfite sequencing
WGBS	Whole-genome bisulfite sequence
SAM	Sequence Alignment Map
BAM	Binary Alignment Map
TFBS	Transcription factor binding site
COV	Coverage distribution of methylation sites
MET	Methylation profiling
TXN	Cytosine methylation levels at transcription factor binding sites (TFBSs)
SNP	Single-nucleotide polymorphism
CNV	Copy number variation
GTF	Gene transfer format
HT	High through-put
QC	Quality control
DMRs	Differentially methylated regions
SAAP-RRBS	Streamlined Analysis and Annotation Pipeline for RRBS data
n.a	Not available (no explicitly specified)

Comparison of fearures between the four tools

Feature	BSMap	BS-Seeker3	Bismark	segemehl
Allows for multiple threads	Yes	No	Yes	Yes
Supports single-end(SE)/paired-end(PE) reads	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes
Variable read length (SE/PE)	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes
Allows for mismatches during mapping	Yes	Yes	Yes	Yes
Allows for adaptor trimming	Yes	No	No	No
Supports gapped alignments	Yes	Yes	Yes	Yes
Supports RRBS/WGBS	Yes/Yes	Yes/Yes	Yes/Yes	Yes
Outputs methylation by context (CpG/CHG/CHH)	Yes	Yes	Yes	Yes
Multiple adjustable mapping parameters (e.g. seed size, byte size, ...)	Yes	Yes	Yes	Yes
Strategy used for mapping	wild-card	3-letter	3-letter	3-letter
Supports directional/non-directional libraries	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes
Allows for priliminary quality control analysis	not specified	Yes	Yes	not specified
Provides tabular/visual summary mapping statistics	Yes	Yes	Yes	Yes
Maximum read length allowed	144nt	not specified	variable size	not specified

Abbreviations	Explanation
RRBS	Reduced representation bisulfite sequencing
WGBS	Whole-genome bisulfite sequencing

Tool	Reference/web-page
BSMap	https://code.google.com/archive/p/bsmap/
BS-Seeker3	https://github.com/khuang28jhu/bs3
Bismark	https://github.com/FelixKrueger/Bismark
segemehl	http://www.bioinf.uni-leipzig.de/Software/segemehl/