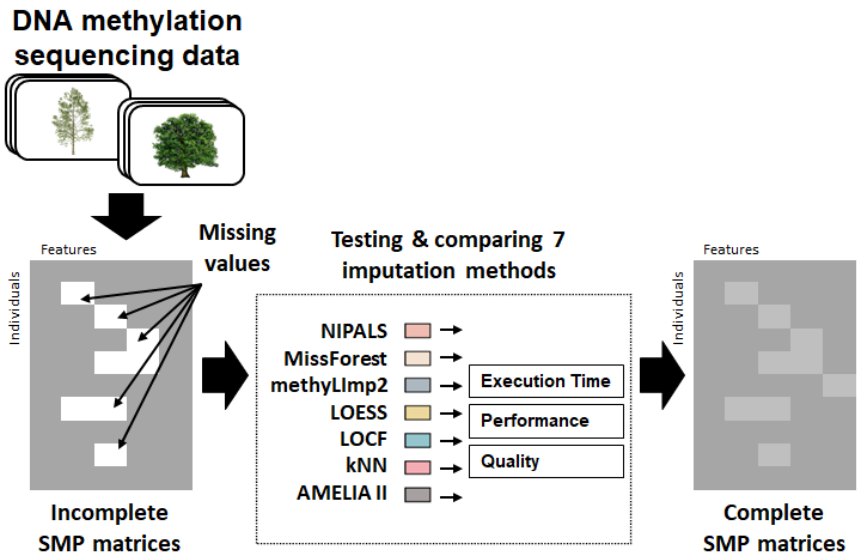
# Imputation strategy for population DNA methylation sequencing data

Alexandre Duplan [1,2], Shannon Brandt [3], Abel Garnier [4], Jorg Tost [4], Leopoldo Sanchez [2], Ludovic Duvaux [3], Stéphane Maury [1], Harold Duruflé [2]*

[1] INRAE, ONF, BioForA, UMR 0588, 45075 Orléans, France
[2] Université d'Orléans, INRAE, P2e, EA 1207 USC 1328, 45067 Orléans, France.
[3] INRAE, Université de Bordeaux, BIOGECO, F-33610 Cestas, France
[4] Centre National de Recherche en Génomique Humaine, CEA-Institut de Biologie, François Jacob, Université Paris-Saclay, F-91000 Evry, France

*Corresponding authors (harold.durufle@inrae.fr)

## Graphical abstract



# Abstract

**Motivation:**

DNA methylation plays a crucial role in gene regulation and epigenetic inheritance, making it essential information for studies of genotype-environment interactions. As whole genome sequencing for DNA methylation analysis is still expensive at the population level, studies focus on regions of interest. However, data matrices may contain missing data for some individuals, which can hamper analyses. Here, several methods for imputing missing data were evaluated for targeted DNA methylation population sequencing data in two species.

**Results:**

We compare seven different imputation methods on targeted methylation sequencing data obtained from 200 and 189 individuals from natural poplar and oak populations, respectively. We evaluate their accuracy and performance to determine the most suitable approach for this type of data. Our results provide a reference for the selection of imputation strategies in targeted sequencing studies, improving the reliability of methylation analyses and broadening the applicability of this type of data in epigenomic research.

**Availability and Implementation:** The data and R Markdown are freely available online at: https://doi.org/10.57745/SUOZVE



**Contact:** harold.durufle@inrae.fr



**Supplementary information:** Supplementary tables and figures are available at Bioinformatics online



**Keywords:** SeqCapBis, Omics, Population, epigenetics, Missing data

# Introduction

Collected datasets often contain some degree of missing or incomplete data, which can arise for various reasons. Managing missing data remains a significant challenge, and researchers and statisticians have long emphasized the importance of avoiding missing values whenever possible (Lin and Tsai, 2020). It is generally accepted that when a dataset has a low percentage of randomly distributed missing data, typically less than 15%, the missing values can be removed without significantly impacting the final analysis results (Strike *et al.*, 2001; Raymond and Roberts, 1987). However, when the missing rate exceeds this threshold, the treatment of these missing data must be done carefully (Acuña and Rodriguez, 2004). Furthermore, in many cases, even a small amount of missing data, present only in a subset of individuals, may hold valuable information that should not be overlooked. Moreover, these missing data may be distributed in a scattered manner, making the elimination decision difficult. It is acknowledged that there is no ideal method for handling missing data (Swalin, 2018) and that understanding the reasons behind missing data is crucial before applying any imputation technique.

Large omics datasets can now be obtained with relative ease. However, their management and pre-processing are not straightforward and often requires careful consideration. Among the various omics techniques available to study biological systems, epigenomics is a more recent and stands out as particularly valuable one (Li, 2021; Kakoulidou *et al.*, 2021). Epigenomics focuses on stable yet reversible chemical modifications to nucleotide bases or histones, which influence gene expression without altering the underlying DNA sequence and that can be inherited. This pseudo-stability makes epigenetic modifications a key factor in understanding causal biological mechanisms under environmental conditions (Aristizabal *et al.*, 2020). Epigenetics encompasses three main types of modifications: DNA methylation, histone modifications and variants and non-coding RNAs. DNA methylation, specifically, involves the reversible addition of a methyl group to the 5' position of cytosines, a process realized by DNA methyltransferase and demethylase enzymes. In plants, DNA methylation takes place in three different contexts (Kakoulidou *et al.*, 2021): CpG, CHG, and CHH (with H representing the bases A, T, or C).

For DNA methylation analysis (see review of Agius *et al.*, 2023), whole genome bisulfite sequencing (WGBS) is a powerful method to interrogate methylomes at single-base resolution (Lister *et al.*, 2009; Dugé de Bernonville *et al.*, 2022). However, such comprehensive measurements are expensive for medium and large genomes, making it challenging for population-scale studies (Lesur *et al.*, 2024). To overcome this, most published methylomes are sequenced below the saturation threshold, leading to a large proportion of cytosines with missing data or insufficient coverage. Specific methods have been developed to address this type of data. For example, METHimpute (Taudt *et al.*, 2018) is a hidden Markov model-based algorithm that enables the construction of complete methylomes by estimating the methylation state and level of all cytosines across the genome, regardless of coverage depth. BSImp (Chang *et al.*, 2022) is a probabilistic-based algorithm that utilizes information from neighbouring sites to quickly reconstruct partially observed methylation patterns. BoostMe (Zou *et al.*, 2018) is also an imputation method that uses a gradient boosting algorithm named XGBoost, and exploits information from multiple samples of WGBS data for prediction. All these approaches aim to accurately infer methylomes from low-coverage data (*e.g.,* 5X), to achieve a level of accuracy comparable

to high-coverage data (*e.g.,* 50X). DNA methylation BeadChip data is another widely used type of epigenetic data, especially popular for profiling large-scale and population-based studies. For example, the Infinium HumanMethylationEPICv2 was developed for the purpose of studying the human DNA methylome (Noguera-Castells *et al.*, 2023). Methods for imputing missing values from methylation matrices were thus developed based on linear regression, such as methyLImp (Di Lena *et al.*, 2019) and methyLImp2 (Plaksienko *et al.*, 2024). However, other data acquisition methods exist for DNA methylation. Targeted capture bisulfite oligonucleotide sequencing (SeqCapBis) context uses hybridization probes to capture and enrich relevant regions of interest (Masser *et al.*, 2016). This approach has been proposed to study the variation of DNA methylation in natural tree populations (Lesur *et al.*, 2024). First, highly variable DNA methylation regions were identified using WGBS on a subset of genotypes that represent the genetic diversity of the population. Then, variations in DNA methylation within these targeted regions were captured and sequenced at the population level.

Currently, advanced imputation approaches can handle heterogeneous variables, but this often requires substantial computational resources. Additionally, not all imputation methods are appropriate for all data types, especially when dealing with DNA methylation data. In this study, we evaluate several imputation methods on targeted DNA methylation sequencing data for two species, considering the trade-off between accuracy and computational efficiency. The comparative analysis is applied to hundreds of genotypes, from natural populations, of two different tree species, poplar and oak, using the three methylation contexts (CpG, CHG and CHH).

# System and Methods

### *Plant material*

A set of 200 *Populus nigra* genotypes from various natural populations, representative of the natural west European range of this species, were used. The trees were cultivated for four years in a common garden in Orléans (France, 47°50'06.0"N 1°54'39.6"E), as described in previous studies (Guet *et al.*, 2015; Chateigner *et al.*, 2020; Rogier *et al.*, 2023). Young differentiating xylem and cambium tissues were harvested from approximately 80 cm-long stem sections, taken 20 cm above ground level and extending up to 1 m. Samples were collected from two replicates of each genotype in two blocks of the common garden design (described in Chateigner *et al.*, 2020). To ensure sufficient material quantity, the DNA from both replicates was pooled before being sequenced.

For oak, the dataset consisted of 189 *Quercus petraea* genotypes from 22 populations, spanning a South-west to North-east gradient across France and Germany. These trees were cultivated in a common garden experiment located in Sillégny (France, 48°59'24.0"N 6°07'55.2"E) as described in Ducousso *et al.*, 2022. Bud samples from each tree were harvested at the end of ecodormancy (Lesur *et al.*, 2024).

### *Genomic DNA extraction and targeted methylated sequence capture*

The methods for genomic DNA extraction have been described in the previous studies (Sow *et al.*, 2023) and the methods for sequencing analyses have been previously described in

detail (Lesur *et al.*, 2024) with a step-by-step bioinformatics manual available in the public repository protocols.io at dx.doi.org/10.17504/protocols.io.8epv5xw4ng1b/v1.
Briefly, a subset of individuals (20 for poplar and 10 for oak), considered representative of the entire population, was selected and subjected to whole-genome sequencing (WGS), enabling the detection of SNPs. Subsequently, WGBS was performed to identify regions with high methylation variation across populations. Finally, all individuals in the population were sequenced using targeted DNA methylation sequencing (SeqCapBis) approaches to focus on methylation-variant regions that were not caused by SNPs. Each methylation context was considered separately, and methylation was quantified in 1 kb sliding windows across the entire reference genome. Each window and its associated methylation level were considered as potential candidate regions for targeting, with only regions showing high levels of differential methylation (DMR) between populations being used for further analysis, as previously described in detail (Lesur *et al.*, 2024). A set of 120 bp probes was selected to capture 18 Mb of the genome. All the capture experiments were performed at the PGTB and sequencing was performed at the GeT-PlaGe facility on an Illumina NovaSeq 6000. Mapping was performed with BSMAPz 1.1.3 (Li, 2013) against the *Populus trichocarpa* V4.1 reference genome for poplar (Tuskan *et al.*, 2006) and the *Q. robur* reference genome (Haplome V2.3) for oak (Plomion *et al.*, 2018). DNA methylation analysis was performed using the methylKit package 1.18.0, and the data were filtered to retain only sites with at least 5X coverage (Ziller *et al.*, 2014).

### *Benchmark datasets*

To limit computational times for imputation analysis, only chromosome 16 of the poplar genome was used because of its average size. This chromosome has a size of 14,802,566 bp (Tuskan *et al.*, 2006) including in this study 665,168 of single methylation polymorphisms (SMP) distributed in 59,070 CpG (including 12,957 without missing data), 97,748 CHG (including 27,023 without missing data), and 508,350 CHH (including 120,025 without missing data) along the chromosome.
For oak, computational time was also limited by using a subset of the oak genome. The CpG SMP dataset was split into 20 similarly sized bins, ensuring that known scaffolds were not split. In this study, Bin 1 is used as it contains the least amount of scaffolds (Sc0000001, Sc0000047, Sc0000011, Sc0000017, Sc0000003, Sc0000027, Sc0000005). This subset contains 443,687 SMP distributed in 42,504 CpG (including 6,509 without missing data), 55,272 CHG (including 7,230 without missing data) and 345,911 CHH (including 33,024 without missing data).
The value of an SMP is defined as the ratio of the number of reads showing a methylated cytosine to the total number of reads at a specific cytosine position in the genome. This value therefore ranges from 0 (unmethylated) to 1 (methylated), reflecting the degree of methylation at that particular position.

### *Computational information*

All statistical and genetic analyses were run using R v4.4.0 (R Core Team, 2021) and analyses were carried out on the Genotoul Bioinformatics platform (http://bioinfo.genotoul.fr).

*Imputation Algorithm*

Amelia II

This approach performs multiple imputations of missing observations simultaneously using an EMB Expectation-Maximization with Bootstrapping algorithm. This algorithm is designed to be faster and more flexible than Markov Chain Monte Carlo based methods (Honaker *et al.*, 2011). Multiple imputations serve to create several independent versions of the dataset, each with different imputed values. This allows for the estimation of the variability and uncertainty introduced by the missing data, providing more robust and reliable results. The algorithm is particularly efficient in handling large and complex datasets with missing values that may not be missing at random (King *et al.*, 2001). For this approach, the R package `Amelia` v1.8.3 was used (Honaker *et al.*, 2011), with the parameter M set to 3. This parameter specifies the number of imputations to generate and sets it to 3 to maintain a realistic computational time.

k-nearest neighbour (kNN)

The kNN method (Batista and Monard, 2002) replaces a given missing value with the mean of the nearest neighbours, based on a similarity measure of the observations of that same variable. Distance functions can thus be used to select neighbours to allow for the inclusion of numerical variables. An aggregation of the k values of the kNN is used as the imputed value. For this study, the parameter for the number of (k) neighbours was fixed at 8. The calculation of the distance to define the nearest neighbours is based on an extension of the Gower distance (Gower, 1971), where the distance between two observations is the weighted average of the contributions of each variable. The aggregation of the k values into an imputed value is the default median for continuous variables. The main advantages of the kNN model are: 1) imputed values are real values and not constructed; 2) it uses auxiliary information provided by the values themselves, which preserves the initial structure of the data; 3) it is non-parametric and therefore does not require the specification of a predictive model (Beretta and Santaniello, 2016). For this approach, the R package `VIM` v6.2.2 was used (Kowarik and Templ, 2016). To reduce computational time while providing a preliminary assessment of the model's performance, the evaluation of the kNN imputation model in the context of CHH methylation was carried out on a subset of the first 15,000 SMPs.

Last Observation Carry Forward (LOCF)

When an observation is missing for a sample, it is replaced by the last available observation for that same sample. The method assumes that the result remains constant at the last observed value after the observation (Das *et al.*, 2019). For this approach, the R package `zoo` v1.8.12 was used (Zeileis and Grothendieck, 2005).

LOcal regrESSion (LOESS)

Local regression imputes missing data using a low-degree polynomial fitted around the missing data by weighted least squares, giving more weight to values close (*i.e.* a neighbourhood in the space of independent variables) to the missing data (Cleveland and Devlin, 1988). For this approach, the R package `locfit` v1.5.9.10 was used (Loader and Liaw, 2024) as described in (Loader, 1999).

methyLImp2

methyLImp is a missing value imputation method developed for DNA methylation BeadChip data (Di Lena *et al.*, 2019). This algorithm is based on solving many multiple linear regressions with the aim of improving the observed inter-sample correlation at cytosine methylation levels. This method allows parallelizing the imputation by chromosomes in order to impute missing values using only probes on the same chromosome for the CpG context. For this approach, the R package `methyLlmp2` v1.0.0 was used (Plaksienko *et al.*, 2024).

MissForest

This approach is a random forest imputation algorithm for missing data. For each variable with missing values, the algorithm fits a random forest on the observed part using other variables as explaining variables before predicting the missing part. This training and prediction process repeats iteratively until a stopping criterion is met or a user-specified maximum number of iterations is reached. The reason for these multiple iterations is that, from iteration 2 onwards, the random forests performing the imputation will be trained on higher quality data. For this approach, the R package `missForest` v1.5 was used (Stekhoven and Bühlmann, 2012), with the parameters maxiter = 10 and ntree = 10, to limit computational time.

Nonlinear Iterative Partial Least Squares (NIPALS)

The NIPALS algorithm (Wold, 1973) is an iterative Jacobi-type method that estimates the elements of a PCA of a finite-dimensional random vector. This can be done even in the presence of missing data, without the need to remove the affected individuals or impute these missing values (Preda *et al.*, 2010), making it a method specifically adapted to this type of data (Tenenhaus, 1998). NIPALS then uses the components estimated by PCA to reconstruct the missing elements in the data. In this study, we set the default parameter (ncomp = 10), which specifies the number of principal components to extract from the data. For this approach, the R package `mixOmics` v6.28.0 was used (Rohart *et al.*, 2017).

# Results

In this study, we compared the performance in terms of accuracy for seven single methylation polymorphism (SMP) imputation methods using targeted DNA methylation sequencing data. The value of an SMP ranges from 0 (unmethylated) to 1 (methylated), reflecting the degree of methylation at that particular position. The benchmark was carried out by using data from the three methylation contexts present in plants (CpG, CHG and CHH) for two sets of natural populations of poplar and oak.

*Missing data and details of the benchmark datasets*

The poplar dataset has fewer missing data than the oak dataset (Figure 1). In detail, for poplar, 12,957 CpG SMPs (22%), 27,023 CHG SMPs (28%), 120,025 CHH SMPs (24%) had no missing data across all 200 genotypes. We observed 22,304 CpGs (38%), 43,747 CHG (45%), 204,471 CHH (40%) with 10 or less NAs across all 200 genotypes (hereafter referred to as "10NA" positions). For oak, 6,509 CpG SMPs (15%), 7,230 CHG SMPs (13%), 33,024 CHH SMPs (10%) had no missing data across all 189 genotypes. We observed 12,412 CpGs (29 %), 15,197 CHG (27 %), 73,974 CHH (21%) with 10 or less NAs.

Methylation ratios in the CpG context exhibit high variability, with values ranging from 0 to 1 (Figure S1) for both species. In the CHG context, variability is comparable to that of CpG, with a greater number of SMPs in this dataset. The CHH context shows lower variability, with values most often ranging from 0 to 0.2, but this dataset contains much more data, with approximately ten times the number of SMPs compared to the other contexts.
The density distribution of SMPs with respect to the number of missing values reveals a distinct pattern across both species and tree contexts (Figure S2). About a quarter of SMPs exhibit low levels of missing data (*i.e.,* concerning less than 10 individuals). Finally, another peak in the distribution highlights a subset of SMPs with a high number of missing values. For example, in the case of the oak, 5,853 SMPs were found in CG, 7,427 in CHG, and 53,686 in CHH that were detected in fewer than 10 individuals. Similarly, for the poplar, 9,075 SMPs in CG, 13,156 in CHG, and 70,225 in CHH were identified under the same criteria."

*Imputation accuracy assessment*

We evaluated the performance of the seven imputation methods described above using the benchmark datasets in poplar and oak. To do this, we started with the largest subset of data with no missing values in each species, which represented 22% and 15% of the total SMP in poplar and oak, respectively (Figure 1). On this basis, we generated 12 alternative datasets for each species in which we randomly masked the values of 10 individuals for 30% of the SMP. Imputation performances were calculated as the correlation between the imputed data and the actual data in each dataset and for each method (Figure 2).

Overall, the imputation result showed a trend towards higher coefficient of determination ($R^2$) in the CHG methylation context compared to the other contexts, with an average of 0.936 and 0.952 for poplar and oak, respectively. The CpG datasets show lower $R^2$ than CHH for poplar and the opposite for oak, where the $R^2$ for CpG is just as high as CHG. The range of $R^2$ values varied between 0.75 to 0.97 for all methods and data types except for the methyLImp2 method, which showed $R^2$ values of 0.68 for poplar in CpG context. $R^2$ values were all relatively high across the various methods, with little variation between the 12 replicates.
The NIPALS imputation method showed the highest $R^2$ values across all three contexts, for both species datasets, in poplar (CpG: 0.880, CHG: 0.970, CHH: 0.907) and in oak (CpG: 0.970, CHG: 0.973, CHH: 0.885). The methyLImp2 algorithm had the highest MAE and RMSE value and the lowest $R^2$ value in three contexts for both species (Table 1) except for oak in the CHH context, where it ranked second lowest (0.773) with the LOCF method showing the lowest $R^2$ (0.751). For the poplar datasets, NIPALS and MissForest showed the highest $R^2$ of 0.880. Moreover, MissForest shows the lowest RMSE value (0.146) followed by NIPALS (0.147). Concerning the MAEs, the kNN algorithm showed the lowest value (0.081), followed by NIPALS and then MissForest (0.085 and 0.086 respectively). For oak datasets, results were similar. NIPALS and LOESS showed the highest $R^2$ of 0.970, followed by MissForest (0.969). NIPALS had the lowest RMSE (0.076) followed by MissForest (0.078). Concerning the MAEs for oak, the kNN algorithm also shows the lowest value (0.041), followed by NIPALS (0.043) and MissForest and LOESS (0.045 for both). Globally,

the same patterns observed in the CpG context were also observed for the CHG and CHH context in both species (Table S1 and S2).

Concerning calculation time, the imputation models followed the order: LOCF, LOESS, Amelia II, methyLImp2, MissForest, NIPALS, and kNN, for both species, in the three methylation contexts (Table 1). In the CHH context, the times were relatively low for kNN because the dataset was reduced as too resource- and time-consuming (Table S1).

# Discussion

Because population-level DNA methylation sequencing data are specific and require specialized approaches, only a few imputation methods have been specifically adapted to these data. In this study, we evaluate seven different imputation methods to address the challenge of imputing targeted DNA methylation sequencing data for populations, for which no established operational method is currently recommended. These were evaluated and compared using the three distinct contexts of plant methylation. The targeted DNA methylation sequencing data come from two tree species, poplar and oak, and are classified according to the three methylation contexts (CpG, CHG, CHH). This distribution of missing data show that imputing SMP values for positions captured in the large majority of individuals (~90%) can be a relevant approach to maximize the use of available data. Moreover, this suggests that some positions are widely represented in the dataset and indicates that another part appears to be specific to certain individuals or populations. Furthermore, the three methylation contexts show expected variability, with high variability in the CpG, and to a lesser extent in the CHG context, and low variability for the CHH data (Figure S1). The amount of data is also expected, with about ten times more SMPs in CHH than in the other two contexts. To compare imputation methods, we rely on the performance, accuracy, and computation time of seven different algorithms performed on 12 independent tests, imputing 10 missing data in 30% of SMPs.

Maybe not surprisingly, LOESS algorithms are able to impute CpG DNA methylation data with high $R^2$ in both species. It is well-established that neighbouring CpG sites often exhibit similar methylation levels, a phenomenon referred to as co-methylation (Affinito *et al.*, 2020; Law and Jacobsen, 2010; Zhang *et al.*, 2018). However, the kNN algorithm is by far the slowest method tested here. This may be related to the computational time needed to calculate the nearest neighbour average, making the method inappropriate for large datasets.

On the contrary, using local regressions to impute missing data in methods like LOESS or simply replacing them with the last available observation for the same sample (LOCF) is very fast. However, the low $R^2$ obtained by the LOCF method (second lowest $R^2$) demonstrates that this algorithm is not suitable to impute missing data for targeted DNA methylation sequencing experiments. This limitation can be explained by the fragmented nature of the data due to the capture design. Since the sequencing does not provide continuous coverage, the last observed cytosine is not necessarily adjacent to the missing site on the chromosome. As a result, LOCF assumes spatial continuity where there is none, likely leading to inaccurate imputations and contributing to the low $R^2$ observed. The strategy to replace missing data by the last available observation (LOCF) could be relevant for SMPs, if continuous data is used.

The Amelia II imputation method shows diverse $R^2$ s between methylation context and tree type, but it is generally the fourth and sixth most efficient model between the seven tested here. Thus, the higher RMSE and MAE scores observed at these lower $R^2$, compared to other algorithms, do not encourage the use of this type of imputation method for methylation data. The random forest-based imputation method, MissForest, demonstrates superior performance in imputing SMP data, achieving high $R^2$ and the low RMSE and MAE score for the three methylation contexts and in both species. However, it is the second-slowest method for poplar and requires a substantial amount of computational time.

Surprisingly, the results of methyLImp2 are largely inferior compared to the other approaches. Additionally, this algorithm displays a unique behaviour for poplar data, which contrasts with the patterns observed in the analysis using the other six methods. The methyLImp2 algorithm has been developed for missing value imputation specific for DNA methylation BeadChips for human data. We think that these results are due to the fact that this algorithm needs to be properly calibrated for targeted DNA methylation sequencing data for plants. The NIPALS imputation method showed the highest $R^2$ and the lowest RMSE values in all three contexts, for both species datasets. The performance of this method is similar to kNN, while showing reasonable running times.

# Conclusion

In this study, we tested and compared seven methods for missing data imputation in the specific context of targeted DNA methylation sequencing data in plants. These methods are based on different imputation strategies, including k-nearest neighbours (kNN) averaging, bootstrapping (Amelia II), low-degree polynomial fitting (LOESS), random forest approaches (MissForest), and iterative Jacobi-type algorithms (NIPALS). Additionally, methyLImp2, which uses a linear regression approach to leverage inter-sample correlations inherent to methylation data, demonstrated strong performance on human DNA methylation matrices but was found to be unsuitable for DNA methylation targeted sequencing data in plants. Based on our results, we recommend the NIPALS method as the most effective approach for imputing missing values across the three plant methylation contexts.

Given the critical role of DNA methylation data in functional and predictive genomics, this study provides a framework for optimizing the usability of such datasets, even in the presence of missing values, an issue frequently encountered in targeted sequencing experiments. However, caution is still required when interpreting biological analyses that incorporate imputed methylation values. Despite the robustness of modern imputation techniques, mis-imputed values are inevitable and can introduce bias, potentially affecting downstream analyses and biological conclusions.

# Acknowledgment

Capture experiments were performed at the PGTB facility (https://doi.org/10.15454/1.5572396583599417E12) and sequencing was performed at the GeT-PlaGe facility with a special thanks to Christophe Boury and Erwan Guichoux (GeT, https://doi.org/10.15454/1.5572370921303193E12). We are grateful to the genotoul bioinformatics platform Toulouse Occitanie (Bioinfo Genotoul, https://doi.org/10.15454/1.5572369328961167E12) for providing help and/or computing

and/or storage resources. We also thank GBFOR (INRAE, 2018. Forest Genetics and Biomass Facility, https://doi.org/10.15454/1.5572308287502317E12) for support for the cultivation and quality production of poplar trees. We also thank Odile Rogier and Isabelle Lesur for the development of the bioinformatics pipeline as well as Romane Callarec for the preliminary analyses carried out on the oak data. The authors are also very grateful to Mathieu Emily for his kind proofreading.

## Funding

This work was supported by the ANR (EPITREE ANR-17-CE32-0009-01). AD received PhD grant from the 'Conseil régional Centre Val de Loire'. SB received a postdoctoral fellowship funded by the ECODIV department, INRAE.

## Author Contributions

Conceptualization: AD, HD, SM, LS.
Data acquisition: JT, AG
Data curation: AD, SB.
Formal analysis: AD, SB.
Funding acquisition: SM.
Investigation: AD, HD.
Writing – original draft: HD, AD
Writing – review & editing: All authors.

## References

Acuña,E. and Rodriguez,C. (2004) The Treatment of Missing Values and its Effect on Classifier Accuracy. *Classif. Clust. Data Min. Appl.*, 639–647.

Affinito,O. *et al.* (2020) Nucleotide distance influences co-methylation between nearby CpG sites. *Genomics*, **112**, 144–150.

Agius,D.R. *et al.* (2023) Exploring the crop epigenome: a comparison of DNA methylation profiling techniques. *Front. Plant Sci.*, **14**, 1181039.

Aristizabal,M.J. *et al.* (2020) Biological embedding of experience: A primer on epigenetics. *Proc. Natl. Acad. Sci. U. S. A.*, **117**, 23261–23269.

Batista,G.E. and Monard,M.C. (2002) A study of K-nearest neighbour as an imputation method. *His*, **87**, 251–260.

Beretta,L. and Santaniello,A. (2016) Nearest neighbor imputation algorithms: A critical evaluation. *BMC Med. Inform. Decis. Mak.*, **16**, 197–208.

Chang,Y.T.S. *et al.* (2022) BSImp: Imputing Partially Observed Methylation Patterns for Evaluating Methylation Heterogeneity. *Front. Bioinforma.*, **2**, 815289.

Chateigner,A. *et al.* (2020) Gene expression predictions and networks in natural populations supports the omnigenic theory. *BMC Genomics*, **21**.

Cleveland,W.S. and Devlin,S.J. (1988) Locally weighted regression: An approach to regression analysis by local fitting. *J. Am. Stat. Assoc.*, **83**, 596–610.

Das,D. *et al.* (2019) Missing Value Imputation-A Review. *Int. J. Comput. Sci. Eng.*, **7**, 548–558.

Ducousso,A. *et al.* (2022) Long-term and large-scale Quercus petraea population survey conducted in provenance tests installed in France. *Ann. For. Sci.*, **79**, 1–10.

Dugé de Bernonville,T. *et al.* (2022) From Methylome to Integrative Analysis of Tissue

Specificity. *Methods Mol. Biol.*, **2505**, 223–240.
Gower,J.C. (1971) A General Coefficient of Similarity and Some of Its Properties. *Biometrics*, **27**, 857.
Guet,J. *et al.* (2015) Genetic variation for leaf morphology, leaf structure and leaf carbon isotope discrimination in European populations of black poplar (Populus nigra L.). *Tree Physiol.*, **35**, 850–863.
Honaker,J. *et al.* (2011) Amelia II: A Program for Missing Data. *J. Stat. Softw.*, **45**, 1–47.
Kakoulidou,I. *et al.* (2021) Epigenetics for Crop Improvement in Times of Global Change. *Biol. 2021, Vol. 10, Page 766*, **10**, 766.
King,G. *et al.* (2001) Analyzing Incomplete Political Science Data: An Alternative Algorithm for Multiple Imputation. *Am. Polit. Sci. Rev.*, **95**, 49–69.
Kowarik,A. and Templ,M. (2016) Imputation with the R Package VIM. *J. Stat. Softw.*, **74**, 1–16.
Law,J.A. and Jacobsen,S.E. (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat. Rev. Genet.*, **11**, 204–220.
Di Lena,P. *et al.* (2019) Missing value estimation methods for DNA methylation data. *Bioinformatics*, **35**, 3786–3793.
Lesur,I. *et al.* (2024) A strategy for studying epigenetic diversity in natural populations: proof of concept in poplar and oak. *J. Exp. Bot.*, **75**, 5568–5584.
Li,H. (2013) Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv*, 1303.3997.
Li,Y. (2021) Modern epigenetics methods in biological research. *Methods*, **187**, 104–113.
Lin,W.C. and Tsai,C.F. (2020) Missing value imputation: a review and analysis of the literature (2006–2017). *Artif. Intell. Rev.*, **53**, 1487–1509.
Lister,R. *et al.* (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*, **462**, 315–322.
Loader,C. (1999) Local Regression and Likelihood Springer. Berlin.
Loader,C. and Liaw,M.A. (2024) Package ‘locfit’.
Masser,D.R. *et al.* (2016) Bisulfite oligonucleotide-capture sequencing for targeted base- and strand-specific absolute 5-methylcytosine quantitation. *Age (Omaha).*, **38**, 1–14.
Noguera-Castells,A. *et al.* (2023) Validation of the new EPIC DNA methylation microarray (900K EPIC v2) for high-throughput profiling of the human DNA methylome. *Epigenetics*, **18**, 2185742.
Plaksienko,A. *et al.* (2024) methyLImp2: faster missing value estimation for DNA methylation data. *Bioinformatics*, **40**.
Plomion,C. *et al.* (2018) Oak genome reveals facets of long lifespan. *Nat. Plants*, **4**, 440–452.
Preda,C. *et al.* (2010) The NIPALS algorithm for missing functional data. *Rev. Roum. Mathématiques Pures Appliquées*, **55**, 315–326.
R Core Team (2021) R: A Language and Environment for Statistical Computing.
Raymond,M.R. and Roberts,D.M. (1987) A Comparison of Methods for Treating Incomplete Data in Selection Research. *Educ. Psychol. Meas.*, **47**, 13–26.
Rogier,O. *et al.* (2023) RNAseq based variant dataset in a black poplar association panel. *BMC Res. Notes*, **16**, 248.
Rohart,F. *et al.* (2017) mixOmics: An R package for ‘omics feature selection and multiple data integration. *PLOS Comput. Biol.*, **13**, e1005752.
Sow,M.D. *et al.* (2023) Epigenetic Variation in Tree Evolution: a case study in black poplar (Populus nigra). *bioRxiv*, 2023.07.16.549253.
Stekhoven,D.J. and Bühlmann,P. (2012) MissForest—non-parametric missing value imputation for mixed-type data. *Bioinformatics*, **28**, 112–118.
Strike,K. *et al.* (2001) Software cost estimation with incomplete data. *IEEE Trans. Softw. Eng.*, **27**, 890–908.

Swalin,A. (2018) How to Handle Missing Data–Towards Data Science.
Taudt,A. *et al.* (2018) METHimpute: Imputation-guided construction of complete methylomes from WGBS data. *BMC Genomics*, **19**, 1–14.
Tenenhaus,M. (1998) La régression PLS: théorie et pratique TECHNIP. Paris.
Tuskan,G.A. *et al.* (2006) The genome of black cottonwood, Populus trichocarpa (Torr. & Gray). *Science (80-. ).*, **313**, 1596–1604.
Wold,H. (1973) Nonlinear Iterative Partial Least Squares (NIPALS) Modelling: Some Current Developments. *Multivar. Anal.*, 383–407.
Zeileis,A. and Grothendieck,G. (2005) zoo: S3 Infrastructure for Regular and Irregular Time Series. *J. Stat. Softw.*, **14**, 1–27.
Zhang,H. *et al.* (2018) Dynamics and function of DNA methylation in plants. *Nat. Rev. Mol. Cell Biol.*, **19**, 489–506.
Ziller,M.J. *et al.* (2014) Coverage recommendations for methylation analysis by whole-genome bisulfite sequencing. *Nat. Methods*, **12**, 230–232.
Zou,L.S. *et al.* (2018) BoostMe accurately predicts DNA methylation values in whole-genome bisulfite sequencing of multiple human tissues. *BMC Genomics*, **19**, 1–15.

# Figures

**Figure 1:** Number of SMPs per missing values allowed in CpG context of the chromosome 9 of poplar and the Bin 1 of oak.The percentage shown above the curves indicates the proportion of SMPs captured across all data within the CG context.
**Figure 2:** Comparison of the results of seven imputation models using the Pearson correlation coefficient between imputed and real data for a) Chromosome 16 in poplar b) Bin 1 in oak. In both cases, 30% of SMPs have 10 missing data points across 12 independent tests.

# Supplementary Data

**Supplemental Figure S1:** Variability of DNA methylation data on chromosome 16 in poplar and the Bin 1 in oak across the CpG, CHG, and CHH contexts.
**Supplemental Figure S2:** Density distribution of missing values per SMPs site in the CG, CHG, and CHH methylation contexts in Bin 1 for oak and chromosome 16 for poplar. The red dotted line indicates the threshold of 10 missing values per site.
**Supplemental Table S1:** Overall CHG imputation performance was evaluated across 12 tests conducted on 200 genotypes of poplar and oak, with 30% of SMPs containing 10 missing data points.
**Supplemental Table S2:** Overall CHH imputation performance was evaluated across 12 tests conducted on 200 genotypes of poplar and oak, with 30% of SMPs containing 10 missing data points.

# Tables

**Table 1**: Overall CpG imputation performance was evaluated across 12 tests conducted on 200 genotypes of poplar and oak, with 30% of SMPs containing 10 missing data points.

| | Method | Execution Time (h:min:s) | RMSE | MAE | $R^2$ |
|---|---|---|---|---|---|
| **Poplar** | LOCF | **00:00:01** | 0.209 | 0.110 | 0.757 |
| | LOESS | 00:00:11 | 0.157 | 0.091 | 0.863 |
| | Amelia II | 00:02:14 | 0.177 | 0.123 | 0.826 |
| | MissForest | 00:43:43 | 0.147 | 0.086 | **0.880** |
| | methyLImp2 | 02:28:56 | 0.237 | 0.124 | 0.689 |
| | NIPALS | 02:33:31 | **0.146** | 0.085 | **0.880** |
| | kNN | 19:43:41 | 0.160 | **0.081** | 0.858 |
| **Oak** | LOCF | **00:00:01** | 0.113 | 0.057 | 0.938 |
| | LOESS | 00:00:04 | 0.080 | 0.045 | **0.970** |
| | Amelia II | 00:01:04 | 0.093 | 0.063 | 0.957 |
| | MissForest | 00:06:22 | 0.078 | 0.045 | 0.969 |
| | methyLImp2 | 00:10:44 | 0.183 | 0.077 | 0.832 |
| | NIPALS | 00:46:53 | **0.076** | 0.043 | **0.970** |
| | kNN | 02:26:04 | 0.084 | **0.041** | 0.966 |

Note: The results have been averaged over the 12 random replicas per percentage of missing data. Best results per metric are highlighted in bold for each dataset. RMSE: Root Mean Square Error. MAE: Mean Absolute Error. PCC: Pearson Correlation Coefficient.

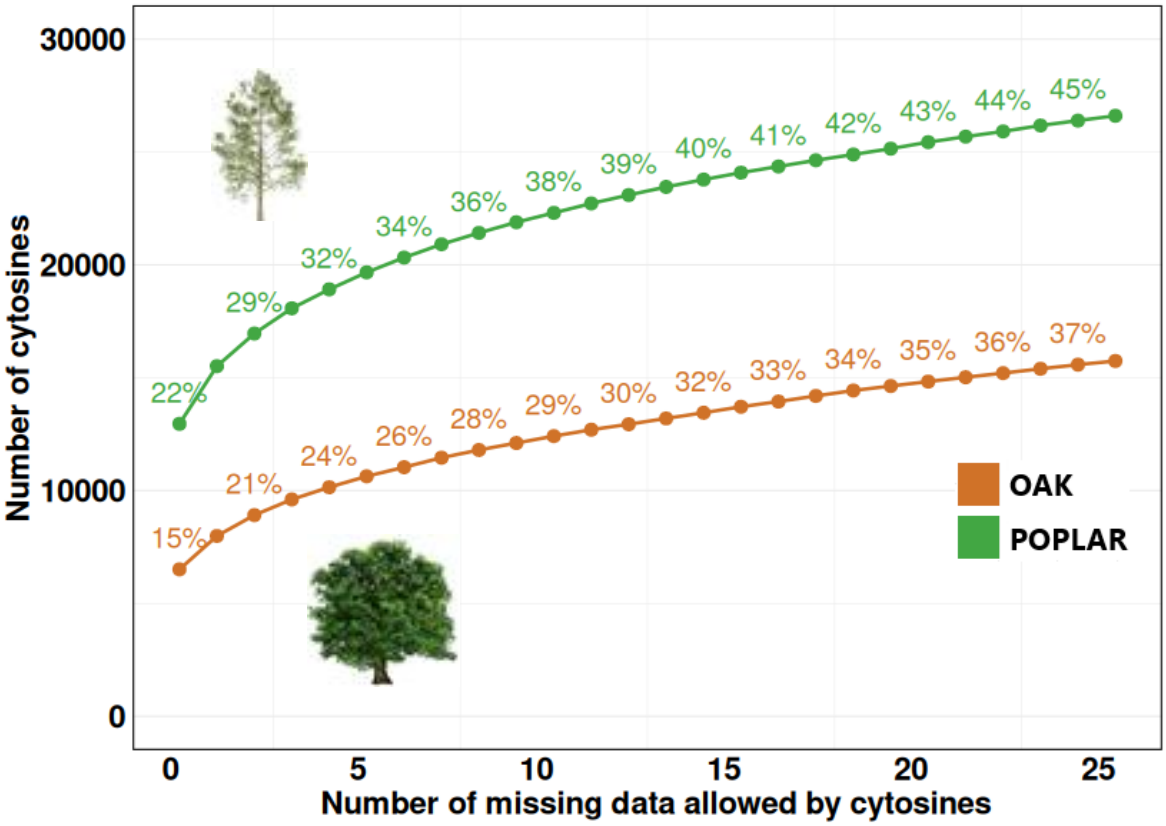



**Figure 1**: Number of SMPs per missing values allowed in CpG context of the chromosome 9 of poplar and the Bin 1 of oak.The percentage shown above the curves indicates the proportion of SMPs captured across all data within the CG context.

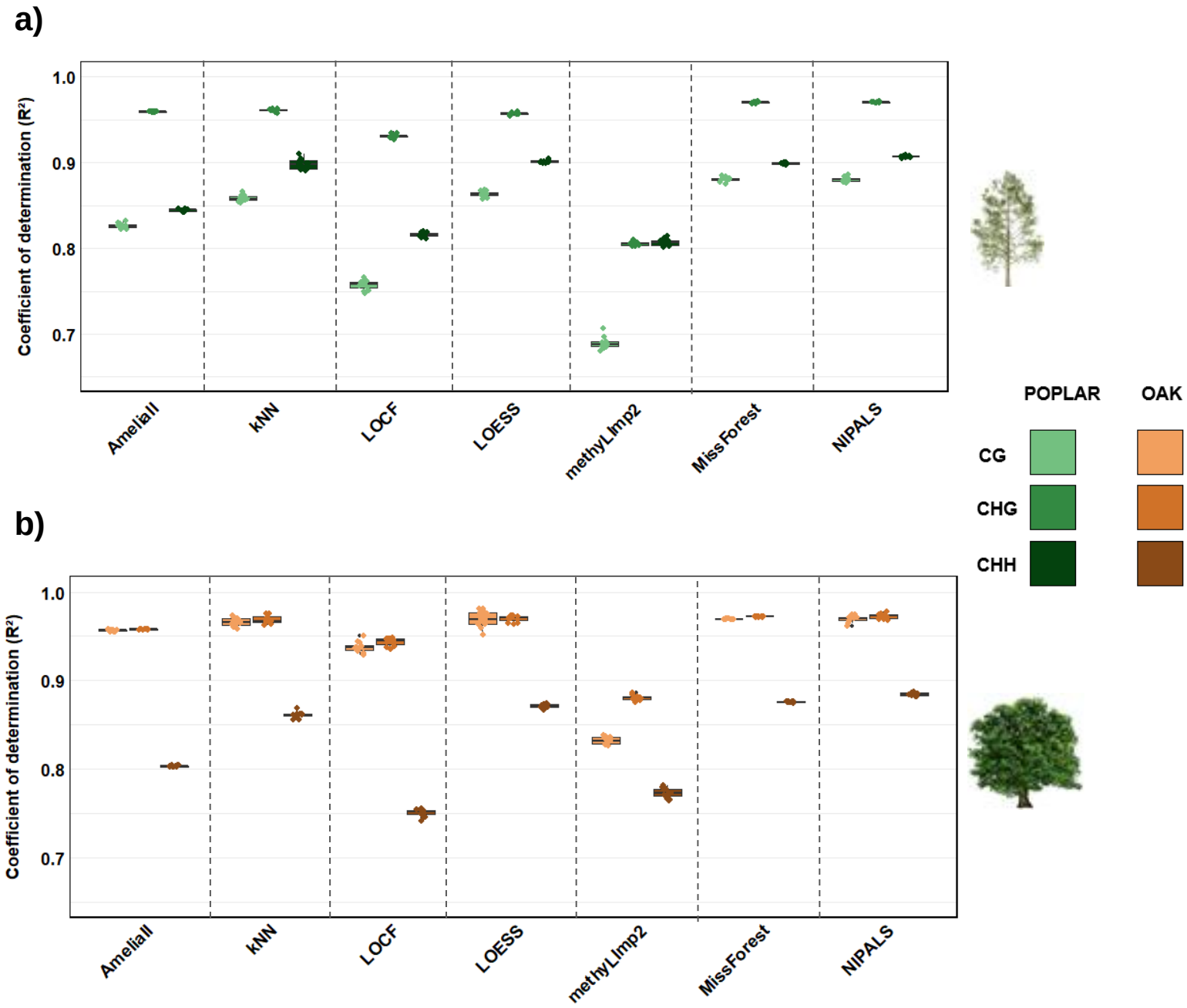



**Figure 2**: Comparison of the results of seven imputation models using the Pearson correlation coefficient between imputed and real data for a) Chromosome 16 in poplar b) Bin 1 in oak. In both cases, 30% of SMPs have 10 missing data points across 12 independent tests.