Journal of Experimental Botany, Vol. 70, No. 15 pp. 3693–3698, 2019
doi:10.1093/jxb/erz191 Advance Access Publication 25 April, 2019
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eXtra Botany

Viewpoint

To clean or not to clean phenotypic datasets for outlier plants in genetic analyses?

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Editor: Christine Raines, University of Essex, UK

Based on case studies, we discuss the extent to which genome-wide association studies (GWAS) are affected by outlier plants, i.e. those deviating from the expected distribution on a multi-criteria basis. Using a raw dataset consisting of daily measurements of leaf area, biomass, and plant height for thousands of plants, we tested three different cleaning methods for their effects on genetic analyses. No-cleaning resulted in the highest number of dubious quantitative trait loci, especially at loci with highly unbalanced allelic frequencies. A trade-off was identified between the risk of false-positives (with no-cleaning and/or a low threshold for minor allele frequency) and the risk of missing interesting rare alleles. Cleaning can lower the risk of the latter by making it possible to choose a higher threshold in GWAS.

An outlier is usually defined as an observation considered to be inconsistent with the distribution of values in the dataset being analysed (Barnett and Lewis, 1994). Observations may be time-points (Grubbs, 1950) or whole time-courses of one or more variables (Hubert *et al.*, 2015). The concept can be extended to ‘outlier plants’, defined here as biological replicates that deviate from the overall distribution of plants on a multi-criteria basis, regardless of the quality of measurements. For example, outlier plants can originate from poor seed quality, from wrong genotype identification, or from fertilization of ovaries by undesired pollen, for example generating a hybrid instead of an inbred line, which can have a large effect if the hybrid is derived from lines with high consanguinity. In field experiments, outlier plants have a low impact on genotypic means because the experimental units (the smallest entity to which a treatment can be applied; http://www.miappe.org) are microplots containing tens of plants (Tollenaar *et al.*, 1984). In phenotyping platforms with hundreds of genotypes, and also in many other experiments in controlled conditions, the experimental unit is frequently an individual plant with 3 to 10 replicates per genotype, so the presence of one or more outlier plants may have a high impact on genotypic means (Estaghvirou *et al.*, 2014).

Whilst numerous methods have been developed for detecting outlier points, involving either individual (Grubbs, 1950; Utz, 2003; Rousseeuw and Hubert, 2011) or multiple traits (Reimann *et al.*, 2008; Rousseeuw and Hubert, 2011; Hubert *et al.*, 2015), the detection of outlier plants is, to our knowledge, still in its infancy. This is probably because the concept of an outlier is less straightforward in plants because it involves a reasoning based on a multiplicity of criteria. Statistical methods based on individual traits are reproducible for a given experiment, but they may exclude different plants depending on the considered trait, resulting in different final trait-specific datasets for each variable. Multi-trait methods based on multivariate procedures can avoid this problem but they are complex to handle and, in our experience, result in non-satisfactory results if traits are not weighted based on expert rules. Visually removing outlier plants based on expert intuition is the most used method, and can result in similar accuracy compared with statistical methods (Bernal-Vasquez *et al.*, 2016). However, criteria for visual elimination can appreciably differ between experimenters. Moreover, whereas visual cleaning can be performed in small datasets, it becomes nearly impossible when thousands of time-courses need to be analysed.

In addition, issues other than the significance of statistical tests on the variable of interest need to be considered for outlier plants. Indeed, the benchmark in this case is rather the degree to which each method affects the results of genetic

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analyses. In genome-wide association studies (GWAS), a causal polymorphism (quantitative trait locus, QTL) is considered significant if the values of the studied variable (e.g. leaf area) differ significantly between genotypes carrying alleles A or B at the considered marker on the genome. Because the detection of outliers may interact with allelic frequencies at this QTL, we aimed to identify in which cases the detection of outlier plants may or may not affect the results of genetic analyses. To this end, as a case study, we compared the outputs of a genetic analysis of a raw dataset consisting of daily measurements of leaf area, biomass, and plant height for thousands of plants with those of datasets based on the same experiments but resulting from three different cleaning methods, namely visual elimination based on the experimenter's expertise, a statistical semi-automated method based on single traits, and a statistical composite multi-trait method combined with expert rules (see Supplementary Protocol S1 at *JXB* online). The dataset (Alvarez Prado *et al.*, 2018) consisted of a diversity panel of 254 maize hybrids growing in three experiments (Spr12, Spring 2012; Win13, Winter 2013; and Spr13, Spring 2013) with two irrigation treatments (WW, well-watered; and WD, water deficit treatments) (Supplementary Table S1). Each experiment involved 1680 plants in an image-based phenotyping platform located in a greenhouse (see Supplementary Protocol S1; https://www6.montpellier.inra.fr/lepse/M3P).

Different cleaning methods provide markedly different lists of plant outliers

In the visual method, outlier plants were identified and tagged by the experimenter based on expert criteria. The statistical single-trait method was performed for each trait individually, meaning that distinct outlier datasets associated with each considered trait were obtained. The composite statistical method was based on a multi-trait approach with expert rules that considered two categories of potentially outlier plants, namely plants that were apparently too small or too large. For the detection of unexpectedly small plants that probably have physiological disorders, the progression of leaf stages was considered in addition to the time-course of shoot biomass. Indeed, leaf appearance rate carried non-redundant information compared with biomass (r=0.49, 0.26, and 0.30 in Exp. Win13, Spr13, and Spr12, respectively). It usually presents low plant-to-plant variability except in case of severe disorders, and is relatively insensitive to environmental cues other than temperature, which was already taken into account via the use of thermal time (Parent *et al.*, 2010, 2019). A small plant, which would be difficult to classify as an outlier based on biomass alone because of the continuous distribution of values, was identified by combining the biomass information with that of progression of leaf stages for which one plant unambiguously differed from the others (Box 1A). For the detection of unexpectedly large plants, potentially associated with wrong genotype identification, combining plant height and biomass resulted in an efficient identification, as illustrated in Box 1B and revealed by the statistical approach (see Supplementary Protocol S1 for details).

When comparing the three methods, a trade-off appeared between the resulting heritability and the rate of outlier exclusion. The statistical composite method increased heritability by only 1% for biomass, leaf area, and plant height compared with the raw dataset (Supplementary Tables S2, S3), but it was the most parsimonious with only 0–2.2% of plants identified as outliers depending on the experiment. The statistical single-trait method increased heritability by 8.0, 9.5, and 4.3% for biomass, leaf area, and plant height, respectively, but with higher rates of exclusion (0–4.3%). The visual method resulted in the highest heritability, which was increased by 10.0, 11.0, and 6.2% for the same three traits compared with the raw dataset, at the cost of a high rate of exclusion (3–12.2%). The plants that were identified differed among the methods (Supplementary Table S4), so appreciably different datasets were generated by each one. Hence, the increase in heritability cannot be considered as the unique benchmark for ranking the effectiveness of the cleaning methods.

Exclusion of outlier plants strongly affects the results of genetic analyses

Genome-wide association studies were performed on individual traits for each combination of experiment × water treatment (Supplementary Protocol S1, Supplementary Table S5). The cleaning method had a large effect on the distribution of allelic effects at QTLs identified in all the tested datasets. This is exemplified in Box 2 for one QTL of leaf area on chromosome 10 that was identified in Exp. Win13_WD. Appreciably unbalanced allelic frequencies were observed at this marker (236 versus 18 genotypes for alleles A and B, respectively), which were nevertheless acceptable in GWAS analyses (7%, for a commonly accepted threshold of 5%). The range of phenotypic values was large for both allelic groups, from 0.1–0.45 m^2 for allele A and from 0.05–0.4 m^2 for allele B (Box 2A). In the absence of cleaning, six hybrids carrying allele B had low genotypic means because of plants with low leaf area (Box 2B), so the QTL was found to be significant ($\log_{10}P$-value=5.7). The statistical single-trait method resulted in the same output ($\log_{10}P$-value=5.3). In contrast, visual cleaning resulted in a non-significant QTL ($\log_{10}P$-value=0.139) after elimination of 45 plants with low leaf area, in particular plants of genotypes carrying allele B. The composite statistical method eliminated only 15 plants, but also resulted a in a non-significant QTL ($\log_{10}P$-value=3.44). Interestingly, this specific QTL was found to be non-significant in Exp. Win13_WW, Spr12_WW and WD, and Spr13_WW and WD regardless of the method used (Supplementary Table S5). Hence, the QTL identified in Exp. Win13_WD with either no cleaning or with the statistical single-trait method is likely to be an artefact. Notably, the differences in significance between methods were not linked to the size of samples involved in the GWAS, because the cleaning methods affected the values but not the number of genotypic means (254 in all cases).

At the whole-genome level, a considerably higher number of QTLs was observed in the raw dataset compared with those that were cleaned, in which many QTLs disappeared at genomic positions with highly unbalanced allelic frequencies (Box 3B, Supplementary Table S5). Indeed, 55 QTLs of leaf area were detected without cleaning, 47 with the statistical

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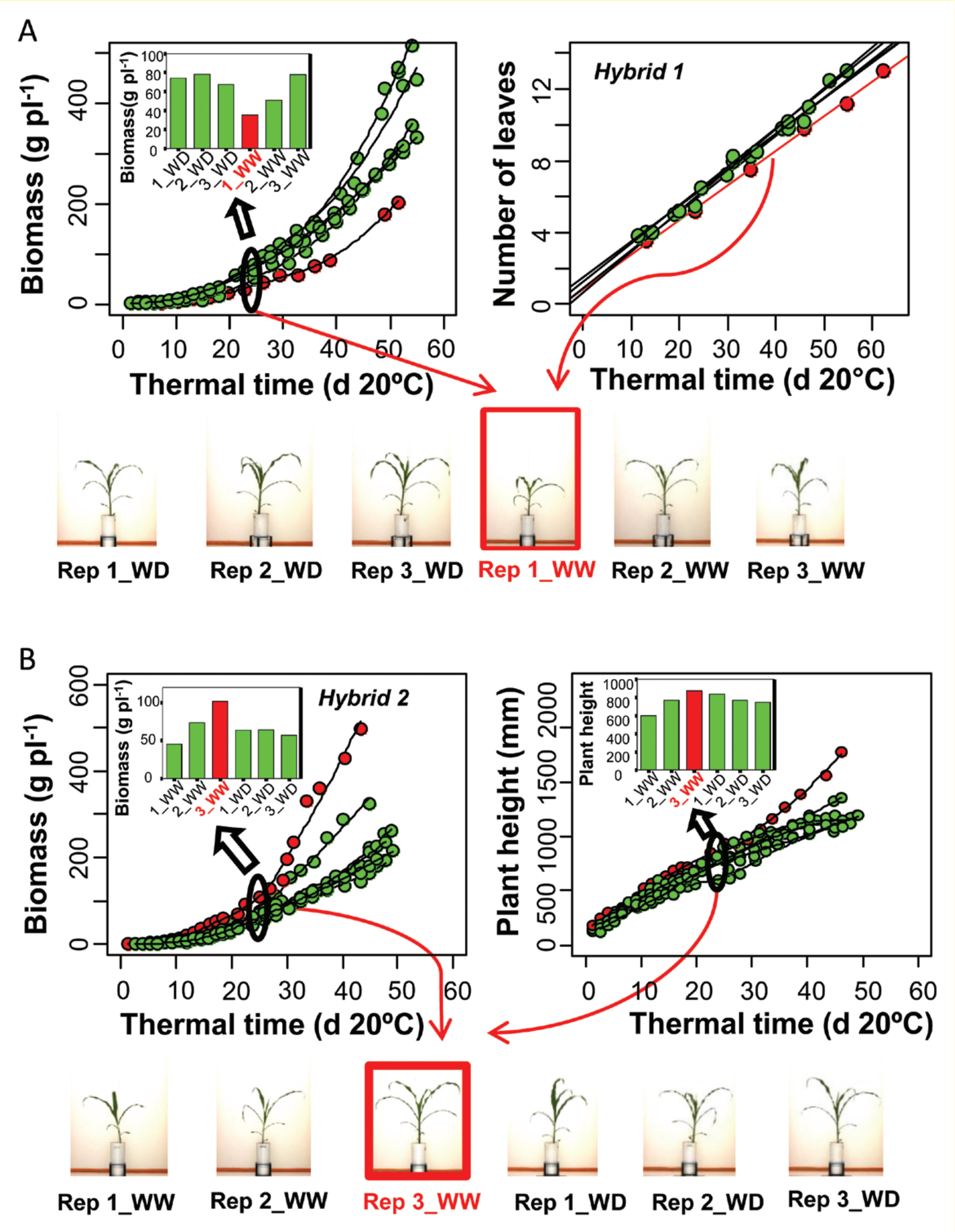
Box 1. An example of multi-trait detection with expert rules

(A) Detection of plants that probably have physiological disorders. Detection based on the time-course of biomass only is difficult because of the continuous distribution of values, whereas considering it together with leaf appearance rate allows unambiguous identification (red points). Here, the plant architecture itself would not provide any extra information (images). (B) Detection of plants that probably have different genotypes. Detection based on biomass only is ambiguous, whereas combining it with plant architecture (height) identifies the plant represented by the red dots. The data are from six replicates in one experiment for one genotype.



single-traits method, 13 with the composite method, and 12 with the visual method (Box 3A; Supplementary Table S5). The difference between methods depended on allelic frequencies, but was still appreciable for some QTLs displaying more than 15% of minor allele frequency (Box 3B).

When and where do cleaning methods affect the outputs of GWAS analyses?

The above results suggest that the method for managing outliers can cause a 'QTL×Method interaction' in the same way as

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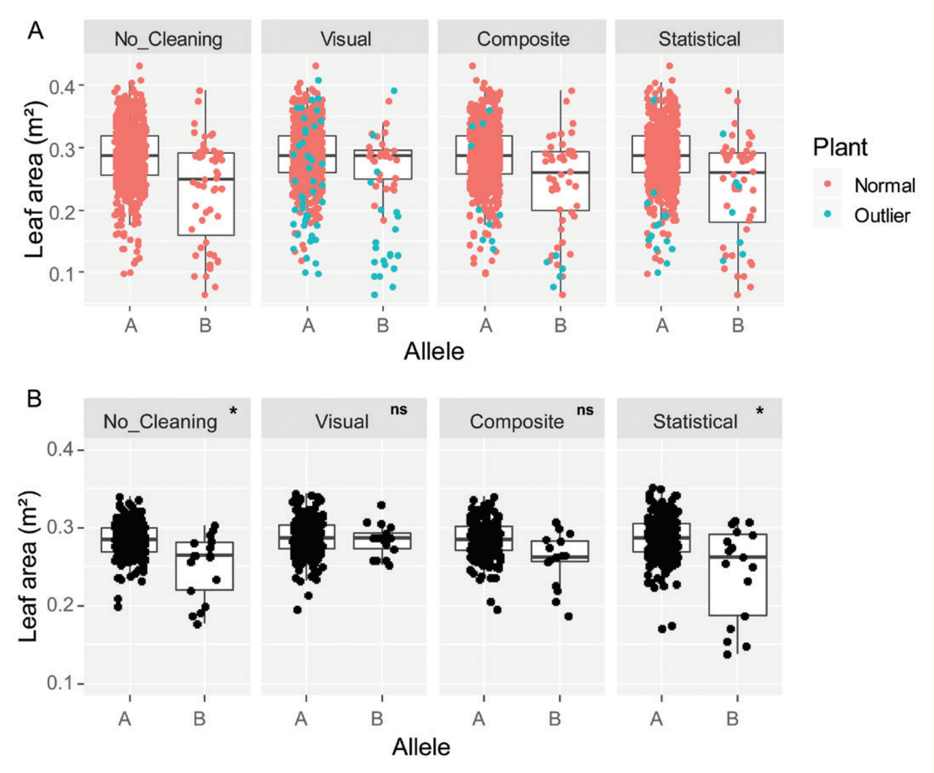
Box 2. The method used for data cleaning affects the apparent allelic effects on leaf area at a given genomic position

(A) The leaf area of plants carrying alleles A and B at a QTL detected in experiment Win13_WD, either in the absence of cleaning, or with visual cleaning, single-trait statistical cleaning, or a composite multi-trait method for cleaning. Note that the plants considered as outliers (blue) differ in number between the methods. (B) The mean leaf area of genotypes carrying each allele. The mean values differ between methods for a given hybrid because they were obtained from non-outliers (red points in A). ns, non-significant; and * significant. The $\log_{10}P$-values were 5.7, 0.1, 3.4, and 5.3 for no cleaning, visual, composite, and statistical single-trait methods, respectively. The threshold for considering a QTL as significant was 5. Allelic effects are calculated for SNP952509 on chromosome 10.



multiple environments generate a QTL×Environment interaction (Malosetti et al., 2013). Intuitively, cleaning methods may be seen as aiming to obtain more numerous and more precise QTLs. On the contrary, the results presented here suggest that excluding outlier plants instead serves to avoid the detection of artefact QTLs. For every potential QTL, an artefact is most likely to occur for lowest numbers of genotypes carrying the minor allele, as suggested by Box 2. Because GWAS involve hundreds of thousands of markers over the genome, with varying allelic frequencies between markers, an accumulation of outlier trait values is bound to occur at some markers for the minor allele. This causes an artefact QTL at the corresponding marker position. The number of such problematic markers depends on the total number of genotypes in the panel, but also on the threshold, chosen in all GWAS methods, for the frequency of the minor allele below which a marker is excluded. A high number of non-eliminated outliers has relatively low consequences on the detection of artefact QTLs if the studied population is large (e.g. >400 genotypes) and/or if a high threshold eliminates markers with unbalanced allelic frequencies, as suggested by Box 3B. Elimination of outliers becomes essential if the population is smaller and/or if researchers are interested by rare alleles with unbalanced allelic frequencies (Yang et al., 2010; Ingvarsson and Street, 2011).

Large trades-off associated with each cleaning method therefore appeared between the increase in heritability, the rate of outlier exclusion, and the risks of either identifying artefact QTLs or missing real QTLs. The consequences of no cleaning (which may tend to generate false-positives) may be counteracted by the choice of a higher threshold for minor allele frequency, itself associated with a decreased power of QTL detection. Conversely, a high rate of plant exclusion, observed here using a visual method, is a problematic feature in any analysis but it may allow a lower threshold for minor allele frequency to be chosen. The composite multi-trait method that

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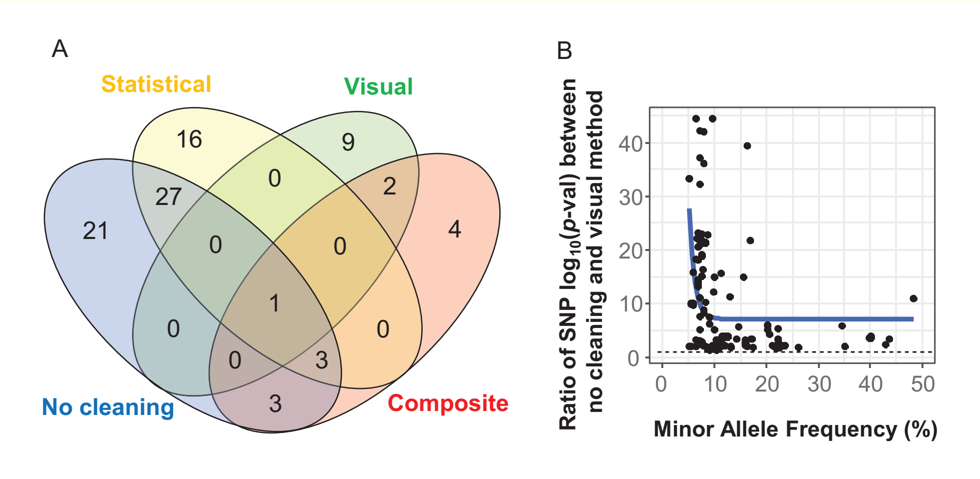
Box 3. QTL detection using datasets originating from different methods as a function of the allelic frequency at the considered genomic position

(A) Venn diagram showing the number of QTLs for leaf area detected without cleaning, with statistical single-trait cleaning, with visual cleaning, or with the composite multi-trait method of cleaning. (B) The relationship between the frequency of minor alleles at the 55 QTLs detected in our case study and the effect of the cleaning method on QTL significance, estimated by the ratio of the $\log_{10}$(P-value) of the considered QTLs detected in the datasets with either no cleaning or with visual cleaning. An exponential decay, $y \sim \exp(-x)$, was fitted to the data in order to represent the trend (blue line).



minimized the rate of outlier exclusion, but also the number of potentially artefact QTLs, appeared to be a promising optimum in the dataset presented here.

The choice of one method or another thus depends on an optimization of criteria and on strategic decisions for genetic analyses. Attempting to standardize this choice, for instance with regards to placing data in repositories (Ćwiek-Kupczyńska *et al.*, 2016), may lead to interminable discussions whose relevance probably depends on specific questions and datasets. In any case, the method of outlier identification, or its absence, is an essential criterion in GWAS analyses. Datasets should be organized and stored in such a way that they can be re-analysed either by the same group some years later in the light of further results, or by different groups (Wilkinson *et al.*, 2016). This requires that detected outliers are identified as such but are not deleted in the information system, and that the rules for outlier detection are kept as meta-data of the GWAS analysis. Recent information systems for phenomic data allow these two conditions to be fulfilled (Neveu *et al.*, 2019).

Keywords: Allele frequency, genetic analysis, outliers, phenomics, quantitative trait loci, statistical analysis.

Supplementary data

Supplementary data are available at *JXB* online.

Protocol S1. Details of the three methods used for outlier detection in our case study.

Table S1. Basic details of the experiments included in the case study.

Table S2. Summary of results for the different cleaning methods when applied to data for leaf area.

Table S3. Summary of results for the different cleaning methods when applied to data for plant height and biomass.

Table S4. Number of common outliers between the three cleaning methods for leaf area, biomass, and plant height.

Table S5. Complete set of QTLs detected for biomass, plant height, and leaf area.

Acknowledgements

This work was supported by the European Union Framework Program 7 ‘Drought-tolerant yielding plants’ (DROPS) project (FP7-KBBE-244374); the ‘Infrastructure Biologie Santé’ Phenome supported by the National Research Agency and the ‘Programme d’Investissements d’Avenir’ (PIA) (ANR-11-INBS-0012), and by the UE H2020 project No 731013 (EPPN^{2020}). SAP was funded by the European Union’s Seventh Framework Program under grant agreement no. FP7-609398, through the award of an AgreenSkills+ fellowship. We are grateful to Stéphane Berthézène, Nicolas Brichet, Thomas Laisné, Vincent Nègre, Carine Palaffre, Pauline Sidawy, and Benoît Suard for their help in conducting the experiments.

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