GenoTriplo: A SNP genotype calling method for triploids

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Abstract

Triploidy is very useful in both aquaculture and some cultivated plants as the induced sterility helps to enhance growth and product quality, as well as acting as a barrier against the contamination of wild populations by escapees. To use genetic information from triploids for academic or breeding purposes, an efficient and robust method to genotype triploids is needed. We developed such a method for genotype calling from SNP arrays, and we implemented it in the R package named GenoTriplo. Our method requires no prior information on cluster positions and remains unaffected by shifted luminescence signals. The method relies on starting the clustering algorithm with an initial higher number of groups than expected from the ploidy level of the samples, followed by merging groups that are too close to each other to be considered as distinct genotypes. Accurate classification of SNPs is achieved through multiple thresholds of quality controls. We compared the performance of GenoTriplo with that of fitPoly, the only published method for triploid SNP genotyping with a free software access. This was assessed by comparing the genotypes generated by both methods for a dataset of 1232 triploid rainbow trout genotyped for 38,033 SNPs. The two methods were consistent

for 89% of the genotypes, but for 26% of the SNPs, they exhibited a discrepancy in the number of different genotypes identified. For these SNPs, GenoTriplo had >95% concordance with fitPoly when fitPoly genotyped better. On the contrary, when GenoTriplo genotyped better, fitPoly had less than 50% concordance with GenoTriplo. GenoTriplo was more robust with less genotyping errors. It is also efficient at identifying low-frequency genotypes in the sample set. Finally, we assessed parentage assignment based on GenoTriplo genotyping and observed significant differences in mismatch rates between the best and second-best couples, indicating high confidence in the results. GenoTriplo could also be used to genotype diploids as well as individuals with higher ploidy level by adjusting a few input parameters.

Author Summary

To cultivate plants, fish and shellfish more profitable for both farmers and consumers, one can utilize individuals one can utilize individuals with three chromosome sets instead of the two found in fertile populations that are diploids. These individuals, called triploids, are generally sterile and then often exhibit higher growth and quality of products, such as seedless fruits or better flesh quality for fish and shellfish. To be able to improve performances of the sterile triploids by selective breeding, it is important to know the versions of the genes present in the three chromosome sets of triploids. Until now, few methods existed to identify these three versions, and none have been demonstrated as sufficiently effective. It is the reason why we developed the GenoTriplo software. We demonstrate in this paper the possibility to accurately genotype triploids, as well as how it can be used to reconstruct pedigree information of triploid progeny. Ultimately, we expect that it can help select for reproduction the parents that have the best triploid progeny for the traits of interest such as growth, vigour or product quality.

Introduction

Polyploidy, characterized by the presence of three or more sets of chromosomes in the nucleus, is a phenomenon that occurs spontaneously across various taxa in the tree of life, spanning from plants [1–3] to vertebrates [4]. Certain forms of polyploidy, such as triploidy, exhibit noteworthy attributes relevant to agricultural practices. Triploid individuals, possessing three sets of chromosomes, are generally sterile, impeding the production of sexual tissues and yielding favourable outcomes for farmers. In horticulture, the cultivation of seedless fruits is facilitated by the sterility of triploids, a characteristic appreciated by consumers [5]. Triploidy has also been reported to enhance growth rate and vigour in plants [6]. In aquaculture, triploid fish demonstrate an accelerated growth rate due to the energy savings stemming from the lack of sexual maturation [7]. Additionally, the enhanced flesh quality of triploid fish and shellfish is attributed to the prevention of gonadal maturation [8,9]. From an environmental perspective, the sterility of triploids serves as a barrier against the contamination of wild genotypes by selectively bred genotypes in instances of contact between these populations [10]. Triploidy also can act as a safeguard against theft of genetic progress among competing producers.

The induction of triploidy has been achieved in various plant species [11], like citrus [5] and mulberry [12], as well as in shellfish such as oysters [13] and in finfish, in particular rainbow trout [14,15].

While triploids present advantages over diploids, their widespread production in aquaculture necessitates that selective breeding programs consider their specific performance. Breeding programs obviously require fertile broodstock, and are thus performed with diploid selection candidates. In order to maximize genetic gains on desired traits for triploid production however, it would be necessary to incorporate the performance of triploids sibs in the evaluation of breeding values. Indeed, evaluating only diploid performance may be suboptimal as the genetic correlation for the same trait between diploids and triploids may differ from unity [16–18]. In mixed-family aquaculture breeding programs, families are mixed at hatching and their pedigree is recovered *a*

posteriori using genomic markers [19]. In such designs, selecting for triploid performance implies to be able to genotype triploids and recover their pedigree, in order to be able to rank diploid selection candidates using breeding values from their triploid sibs.

Technically, two platforms, Illumina and Affymetrix, have been used for genotyping SNP arrays in both diploid [20] and polyploid species [21]. As reported by [21], genotype calling is complicated for polyploids because these species have more possible genotypes at a SNP locus than diploid species do (homozygote with reference allele, heterozygote, and homozygote with alternative allele). Theoretically, the number of genotypes can be up to p+1 in a species with a ploidy level of p (i.e. 4 in triploids, 5 in tetraploids, …). So far, genotype calling software accompanying genotyping platforms cannot identify more than 5 clusters for Illumina and 3 clusters for Affymetrix. More specifically, the GenomeStudio software from Illumina is able to provide 5 clusters, but it requires manual adjustment of the cluster boundaries for each marker, which is impractical to use for SNP arrays with several tens of thousands SNP. The Axiom Analysis Suite (AXAS) software, widely used in both plant and fish species, is only designed for genotype calling on diploid luminescence output files from the Thermo Fisher Affymetrix platform, and does not currently support triploids. Up to 2020, there were only two publicly available software, fitTetra and ClusterCall, initially written for tetraploids [22], which could call up over three genotypes using output files with allelic signals from SNP array genotyping platforms. Another software, SuperMASSA, was written for genotype calling from Genotype-By-Sequencing data for all ploidies [22]. Many methods struggle with low-frequency genotypes [23] or lack permissiveness when faced with allelic signal shifts in polyploids [24,25]. For autopolyploids, such as induced triploids in aquaculture, the major complication is distinguishing between different allele dosages (AAA, AAB, ABB, BBB), as in this case only two alleles per locus are normally present in their diploid parents.

Therefore, limited options for genotype calling in triploids exist [26] and open source tools are even more rare. As far as we know, only the R package fitTetra, initially developed for tetraploid

98 individuals [27,28], has been implemented in a more advanced version of the package called fitPoly
99 to consider any other level of auto-polyploidy. However, our first trial yielded some inconsistent
100 results using fitPoly to genotype triploids in rainbow trout. Therefore, the first objective of this study
101 was to devise a clustering method for a better genotype calling of triploid individuals and to compare
102 our results to those of fitPoly genotype calling on our rainbow trout study case. The second objective
103 was to implement and disseminate this new method through an R package deposited on the CRAN to
104 ensure its free accessibility.

105

106 Materials & Methods

107 Available dataset

108 To develop this novel genotype calling method for triploids, we used the allelic signals produced by
109 Thermo Fisher Affymetrix platform for a French research project on genomic selection in rainbow
110 trout [29]. The experimental stock was established from 190 dams and 98 sires of a commercial
111 selected all-female line of Aquaculteurs Bretons breeding company (Plouigneau, France) and 1232
112 triploid offspring and the 190 dams and 98 sires were genotyped for 57,501 SNPs using the medium-
113 density Rainbow Trout Axiom® 57K SNP array from Thermo Fisher [30]. We retained the allelic signals
114 for 38,033 high quality markers present in both SNP array [31,32]. Luminescence values of probsets A
115 and B (S_A and S_B) for each marker and individual were obtained through the AXAS software.

116 Clustering algorithm

117 The clustering process aimed at grouping individuals that share the same genotype. To enhance the
118 efficiency of the clustering method, variable(s) given to the algorithm must be chosen carefully so the
119 different genotypes are well separated along the axe(s) [25]. In our approach, we decided to use 2
120 variables (and so 2 axes): the contrast (Eq.1) and the signal strength (Eq. 2), commonly used by AXAS
121 for diploids.

$$x = Contrast = \log_2\left(\frac{S_A}{S_B}\right) \quad (Eq.\,1)$$

$$y = Signal\ Strength = \frac{\log_2(S_A) + \log_2(S_B)}{2} \quad (Eq.\,2)$$

Thus, each individual was represented by a pair of coordinates (x, y) for each marker (Fig 1, Stage 1). For each SNP, the Rmixmod clustering package (version 2.1.8) [33] was then used on R software (version 4.3.1) [34] to find clusters among individuals for a given marker, with no prior information. The clustering function of Rmixmod initiates the process by randomly picking individuals as starting point and uses an expectation-maximization algorithm (EM) to probabilistically update parameters of the clusters (mean, variance, weight). N_{init} initializations were performed and the one that maximized likelihood passed to the next steps.

During the initialization phase, the clustering function of Rmixmod was asked to find N_{clus} clusters among individuals with N_{clus} greater or equal to the number of possible genotypes for a given SNP (4 in our case) (Fig 1, Stage 2). N_{clus} values of 4, 8 or 12 were tested to find an optimal value.

When the algorithm failed to find N_{clus} clusters among individuals (failure of the EM algorithm to converge with N_{clus} clusters), it was restarted with N_{clus}=N_{clus}-1 clusters and so on, until the algorithm converged and a non-error solution was obtained. For these retries, N_{init} was automatically reduced by 2 (with a minimum value of 1) to limit computing time. Indeed, when the algorithm failed to find the initial number of N_{clus} clusters, it was likely that the marker did not display all possible genotypes. Thus, a high N_{init} was not necessary to find a suitable solution.

As the final N_{clus} might be higher than the maximum number of genotypes, a single genotype could be divided into different clusters. If more than 4 clusters remained (the maximum number of genotypes in triploids), or if two clusters were too close to be considered as distinct genotypes, the two clusters with the weakest distance in Contrast value were merged into a single one (Fig 1, Stage 3 to Stage 4). Two clusters were declared as too close if:

$$D_{Clus1,Clus2} < 0.28 * (1 + abs(\frac{Contrast_{clus1} + Contrast_{clus2}}{2})) \quad (Eq. 3)$$

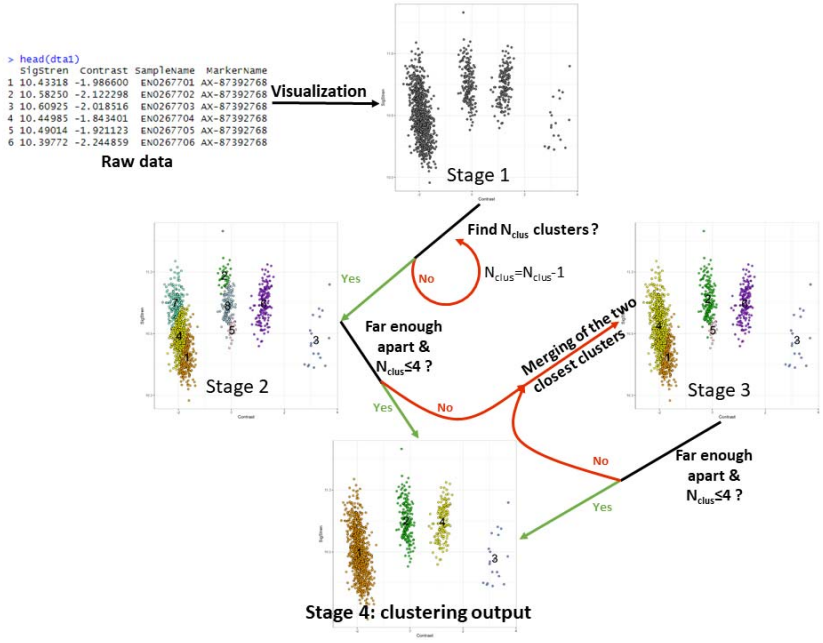
$$Where, \qquad Contrast_{clusi} = Mean(x_{indiv_{clusi}}) \quad (Eq. 4)$$

$$And, \qquad D_{clus1,clus2} = abs(Contrast_{clus1} - Contrast_{clus2}) \quad (Eq. 5)$$

Where $D_{Clus1, Clus2}$ represented the distance between the center of cluster 1 and the center of cluster 2 in Contrast value (abscissa), and $Contrast_{Clusi}$ represented the mean Contrast value of cluster i. As the standard deviation along the Contrast axis of a genotype increased when $Contrast_{Clusi}$ moved away from 0 (to positive or negative value), the distance criteria to merge clusters had to increase the more $Contrast_{Clus1}$ and $Contrast_{Clus2}$ differed from 0. The factor of 0.28 was empirically determined using a trial and error assay.

To assess the impact of the number of initializations i.e. random starting points on the final clustering, the algorithm was tested with three modalities for N_{init}: 1, 5 and 10 different initializations.

The algorithm was also tested for three other modalities to assess the impact of N_{clus} on the outcome: 4, 8 and 12, i.e. a number greater or equal to the number of possible genotypes for a given SNP (4 in our case). Other existing methods for genotyping usually look for a maximum number of clusters which exactly corresponds to the number of possible genotypes. However, by increasing the initial number of clusters (8 and 12), we aimed to enable the algorithm to identify clusters gathering only a few individuals, which can happen frequently in case of a low frequency genotype.



158

159 Figure 1: Algorithm stages for the clustering phase

160 **Genotype calling**

161 Two situations must be accounted for to assign genotypes to clusters depending on the origin of the
162 samples: i) either all samples originated from a same population or ii) they come from various
163 populations that can be genetically distant. The right situation must be specified to our algorithm as
164 they involve different hypotheses. In our case, the samples originated from a single population, and
165 we only used the corresponding method for genotype calling.

166 In the situation of a unique population, genotypes were attributed by considering the mean Contrast
167 of each cluster and its position relative to other clusters. The most extreme cluster, identified by the
168 absolute value of its contrast mean (x), was designated as a homozygous genotype (AAA if mean(x)>0
169 and BBB if mean(x)<0) (Fig 2). Other clusters were ordered by their mean contrast values, and
170 genotypes were subsequently assigned based on the first cluster that had been assigned (Fig 2). For
171 example, if the mean contrast was positive for the most extreme cluster (i.e. assigned as AAA),
172 genotypes were then assigned depending on their mean contrast values in the order AAB, ABB and
173 BBB, from the closest to the furthest cluster from the AAA homozygous genotype. On the contrary, if

the mean contrast was negative for the most extreme cluster (i.e. assigned as BBB), genotypes were then assigned depending on their mean contrast values in the order BBA, BAA and AAA, from the closest to the furthest cluster from the BBB homozygous genotype (Fig 2).

We assumed that when the outcome of clustering was a single cluster for a given SNP, it could only correspond to a homozygous genotype; 2 or 3 clusters indicated a homozygous genotype and the closest heterozygous or the two heterozygous genotypes; and 4 clusters represented all 4 possible genotypes for triploids. Note that our algorithm can also be used for genotype calling in diploids as the same reasoning could be applied with a maximum of 3 possible genotypes for diploids as long as it is specified in the input parameters to the algorithm.

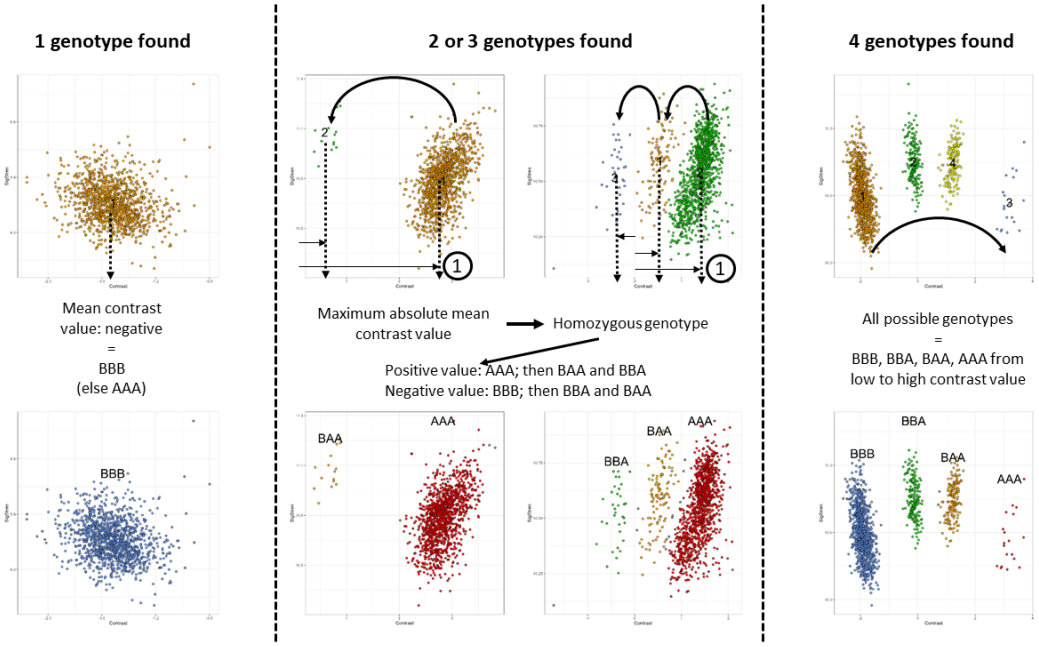


Figure 2: Illustration of genotype determination for 1; 2 or 3; and 4 clusters identified for a given SNP

In case of 3 clusters encountered for a given SNP in triploids, an additional step was added to address the case of a highly shifted signal. This implies markers where genotypes are all shifted toward either positive or negative contrast value making, leading to having a cluster corresponding to a heterozygous genotype in the most extreme position, and thus being wrongly identified as a cluster corresponding to a homozygous genotype. To minimize the error due to that rare behaviour, if the

most extreme cluster had less than half the number of individuals as the opposing cluster, it was assigned as a heterozygous genotype, and the opposite cluster was designated as the homozygous genotype (Fig 3, Before to After). In this case however, the next step of the algorithm concerning SNP quality control and decision criterion to retain or remove a SNP would frequently reject the marker. However, we had to first decide the most likely genotypes in this case. In a population in which the number of apparent AAA is less than half the number of apparent ABB (equivalent to freqA < 0.55) (ex. Fig 3), the probability to have no BBB in the population (freqB ≥ 0.46) is extremely low as the expected frequency of BBB is ≥ 0.1, i.e. it is more probable that apparent AAA might be an AAB shifted genotype and apparent ABB might be a BBB shifted genotype. In this corrected situation, the frequency of A was less than 0.2 making the AAA genotype extremely rare (with an expected frequency < 0.01 and even not present here) and B higher than 0.8 (explaining the high number of BBB) (Fig 3).

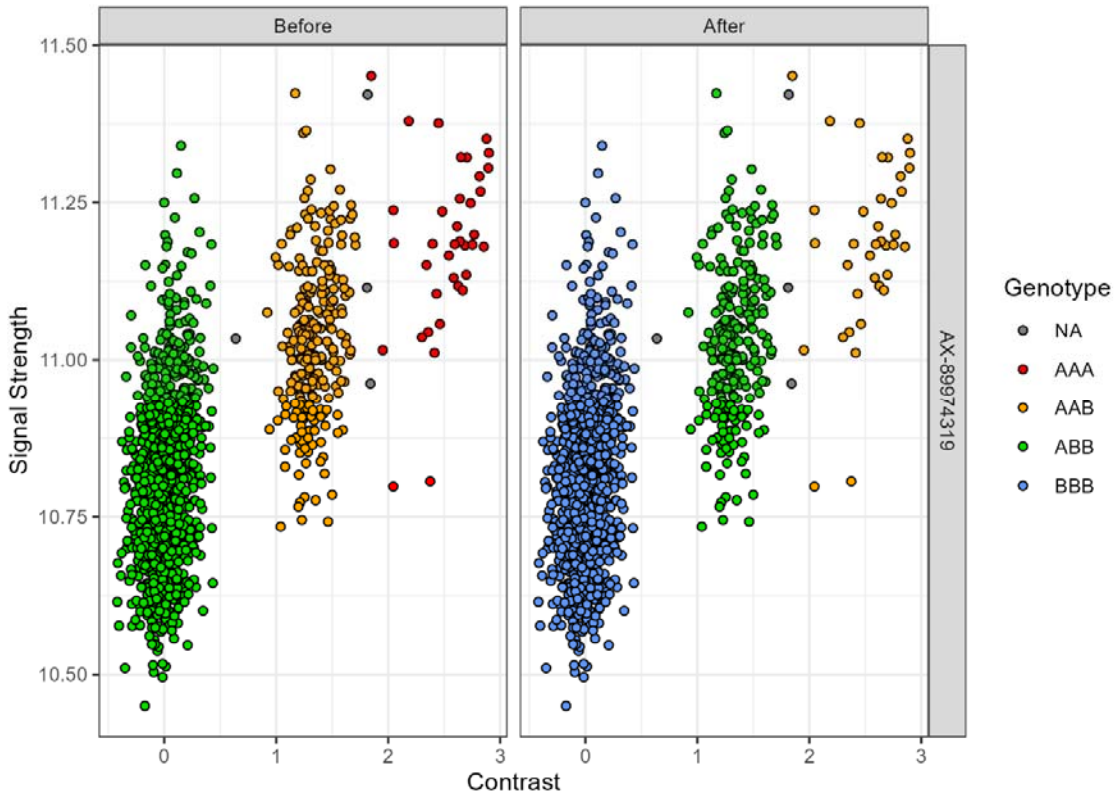


Figure 3: Example of implementation of the additional step to account for highly shifted contrast signal

In the situation where samples originate from distinct populations, there is an additional issue to solve for genotype calling when only two clusters are identified for a SNP. In that case, it is likely that the two clusters correspond to the two homozygous genotypes and not to a SNP to be put in the rare category of "No Minor homozygote". Indeed, the SNP is likely to be monomorphic within a given population, but different populations may have fixed alternative alleles.

To solve this case, we used the approach proposed by [26). We derived reference values for the mean contrasts of all possible genotypes by averaging them across markers with the maximum number of clusters identified (i.e. 4 for triploids). These reference values were used to attribute genotypes for the remaining markers (with a number of clusters below the maximum). For these latter markers, the mean contrast of each cluster was compared to the reference set of values, and the genotype was assigned based on the closest reference value. If two clusters pointed to the same reference value, the genotypes were assigned based on their relative positions. For example, if two clusters pointed toward the negative reference value corresponding to BBB homozygote, the one with the most negative contrast was assigned to the BBB homozygote while the other was assigned to the nearest possible heterozygous genotype BBA.

All the steps of our algorithm (from clustering to genotype calling) can also be used for diploids as the approach can be applied with a maximum of 3 possible genotypes for diploids by indicating the ploidy level of the population under study.

Quality control for genotypes and SNP categorization

Following the approach proposed by AXAS, seven criteria were employed to enhance cluster precision and identify low-quality markers in the genotype calling phase. Three criteria were used to decide whether or not individuals or clusters were assigned to a given genotype or not assigned (NA):

1) No call for individuals. During clustering, individuals were assigned to a cluster number with a certain probability. Individuals with a probability of belonging to their cluster below 0.85 for a given marker were marked as NA to limit incorrect genotyping.

2) Distance between individual and its cluster center. This criterion aimed to avoid wrong genotyping by identifying individuals far from all clusters while still assigned to a cluster. The distance between an individual and the center of its cluster was monitored to not exceed 2.8 times the standard deviation of the cluster along the Contrast axis ($SD_{cluster}$). An individual genotype was set to NA above this threshold. The choice of a 2.8 factor was based on the property that under the assumption of a normal distribution of individuals within a cluster, 99.5% of the observed values should fall within ±2.8 times the standard deviation. This factor can be modified in the R package to allow for more flexibility.

3) Cluster Standard Deviation ($SD_{cluster}$). A cluster was set to NA if its $SD_{cluster}$ exceed $0.28*(1+0.5*abs(Mean_{cluster}))$. This criterion imposed a maximal standard deviation to a cluster to limit the risk of genotype calling for a cluster gathering multiple genotypes (in case the algorithm failed to do the correct clustering). The factor of 0.28 was empirically determined through a trial and error assay. The objective was to establish a minimal $SD_{cluster}$ of 0.28 and to progressively increase this minimum as the cluster moved farther away from 0.

The remaining four criteria acted as filters to assess the SNP quality, similar to criteria implemented in the AXAS software, before categorization of the markers:

4) Marker Call Rate (CR). The minimum CR was fixed to 0.97.

5) Marker Fisher's Linear Discriminant (FLD). The FLD is a measure of the distance between the two nearest genotypes along the x axis (Contrast) and the quality of the clusters. It is defined as:

$$FLD = \frac{abs(Contrast_{Geno1} - Contrast_{Geno2})}{SD_{Geno1,Geno2}} \quad (Eq.\ 6)$$

Where $Contrast_{Genoi}$ represented the mean Contrast of genotype i and $SD_{Geno1,Geno2}$ represented the pooled standard deviation of genotype 1 and 2. If the FLD was 3.4 or lower, two genotypes were considered too close to be reliable.

6) Marker Heterozygous Strength Offset (HetSO). The HetSO measures the offset between homozygous and heterozygous genotypes along the y axis (Signal Strength). Heterozygous clusters are expected to be positioned higher on the y axis than homozygous clusters (i.e. HetSO value > -0.3).

7) Marker Homozygous Ratio Offset (HomRO). The HomRO represented the position of the homozygous cluster along the x axis (Contrast). The threshold value depended on the number of clusters like so: 0.6, 0.3, 0.3, -0.9 for 1, 2, 3 and 4 clusters, respectively (adapted from [35]).

Markers failing to pass one of these criteria were labelled according to the filter they failed: “Call rate below threshold” for call rate threshold, “Off target variant” for HetSO threshold, and “Others” otherwise. Those are rejected markers, meaning markers with low genotyping confidence that should not be used for further analyses.

Markers passing all four filters were categorized based on their number of genotypes: “Mono high resolution”, “No minor homozygote” and “Poly high resolution” for respectively, 1 genotype, 2 or 3 genotypes, and 4 genotypes. Those are accepted markers, meaning markers with high genotyping confidence that could be used for further analyses.

Comparison strategy between GenoTriplo and fitPoly

To evaluate the efficiency of our method in contrast to an existing alternative, we conducted a comparative analysis between GenoTriplo and fitPoly, the sole package available on the CRAN that handles triploid genotyping.

First, we assessed the overall concordance between GenoTriplo and fitPoly by comparing the genotypes assigned by both methods per individual and marker. Then, we examined the number of genotypes identified by each method for all markers and categorized markers by a pair of integers

representing the respective number of genotypes identified by GenoTriplo and fitPoly (for instance category (2;3) corresponded to 2 genotypes found by GenoTriplo and 3 by fitPoly) separating markers in 16 categories.

Categories of equal integer pair (both methods found the same number of genotype) were visually and numerically compared based on the overall genotype concordance rate and the mean contrast value of each genotype for the 4 corresponding categories from (1;1) to (4;4). For the visual comparison, mean cluster position of each genotype for each marker was displayed on a graph to compare genotype global position for each 4 categories.

The genotypes given by GenoTriplo and fitPoly were compared marker-by-marker and the best one was noted based on human visual observation. This was done for all markers in categories gathering 200 or more markers except when both methods found the same number of genotypes. Among the 12 remaining categories, 8 were analysed.

For categories exceeding 1,000 markers, a subset of 1,000 random markers was retained for visual inspection.

For these 8 tested categories, we compared markers acceptance (when a marker passed all quality threshold) and rejection (when a marker did not reach all quality threshold) by the methods to identify any differences. For each category, markers were split into two groups according to the best method to genotype them (GenoTriplo or fitPoly) and an overall genotype concordance rate between the two methods for all the 16 categories was computed.

Both methods had high marker call rate on average (0.98 (± 0.044) for GenoTriplo and 0.97 (± 0.122) for fitPoly). To ensure fair comparison, all NA were removed and not considered as different between methods, recognizing that some NA may be attributed for quality purpose when samples did not clearly belong to a genotype while others may result from misidentification of clusters by one or the other method. This approach aimed to provide a robust comparison while considering the nuances of missing data especially for those methods that provided few NA.

Parentage assignment assessment

To validate the utility of GenoTriplo, we conducted a parentage assignment of the triploid individuals using the R package APIS with the newly available function that enables parentage assignment on triploids (https://cran.r-project.org/web/packages/APIS/index.html). The assignment was done using the 1,000 best markers selected based on their Minor Allele Frequency (MAF) and CR. These markers were chosen from the 32,325 markers that successfully passed through all applied filters, including "Poly high resolution", "Mono high resolution" and "No minor homozygote".

While the true parents of the offspring were not available to fully validate the parentage assignment, we had access to the mating plan, which is composed of 10 independent factorial matings, each being composed of 8 to 10 sires crossed one-by-one with 17 to 24 dams, producing a theoretical number of 1862 full-sib families (or 1862 valid parent pairs). However, parental assignment by exclusion considers all possible parental pairs from the 98 sires and 190 dams [36], and thus a theoretical number of 98*190=18620 possible parent pairs, which is 10 times more than the valid ones. In case of inaccurate assignments, we would thus expect that approximately 9 out of 10 would fall out of the declared mating plan.

R package and shiny application

For enhanced accessibility, we developed a R package called 'GenoTriplo' available on CRAN. The package incorporates functions for executing both the clustering phase ('Run_Clustering') and the genotype calling phase ('Run_Genotyping'). Additionally, to make the usage easier for beginners and experts, a shiny interface was implemented ('launch_GenoShiny'), organized into four steps.

First, the raw dataset from AXAS requires formatting before progressing through the clustering phase. A list of markers or/and a list of individuals can be provided to select specific markers or/and individuals.

The clustering phase starts with the refined dataset obtained at the previous step. Users are prompted to input the ploidy level (default set to 3) of the population and the number of cores for parallelization (default set to $N_{computer\_cores}$-2). An option to fine-tune parameters is available through the 'Add more control' button, allowing adjustments of the number of initializations for the Rmixmod clustering function (default set to 5) and the minimal contrast distance between two clusters (default set to 0.28).

The genotype calling process is applied to the output of the clustering phase. Users have the option to provide a CSV file containing the correspondence between A/B signals of AXAS and ATCG bases. Inputs such as the ploidy of individuals (default set to 3), the number of cores for parallelization (default set to $N_{computer\_cores}$-2), and whether or not individuals originate from the same population are requested (default set to same population). The latter is introduced for simplification, assuming that individuals from the same population cannot exhibit both homozygous genotypes without a heterozygote (as described in **Genotype Calling** section). This step provides flexibility with various adjustable parameters, including no-call threshold for individuals, distance between cluster centres, cluster standard deviation threshold, FLD threshold, HetSO threshold, and CR threshold for markers.

The final step is optional and enables users to visualize the genotyping results through graphs and statistics.

All graphics were made using ggplot2 [37] via R code [34].

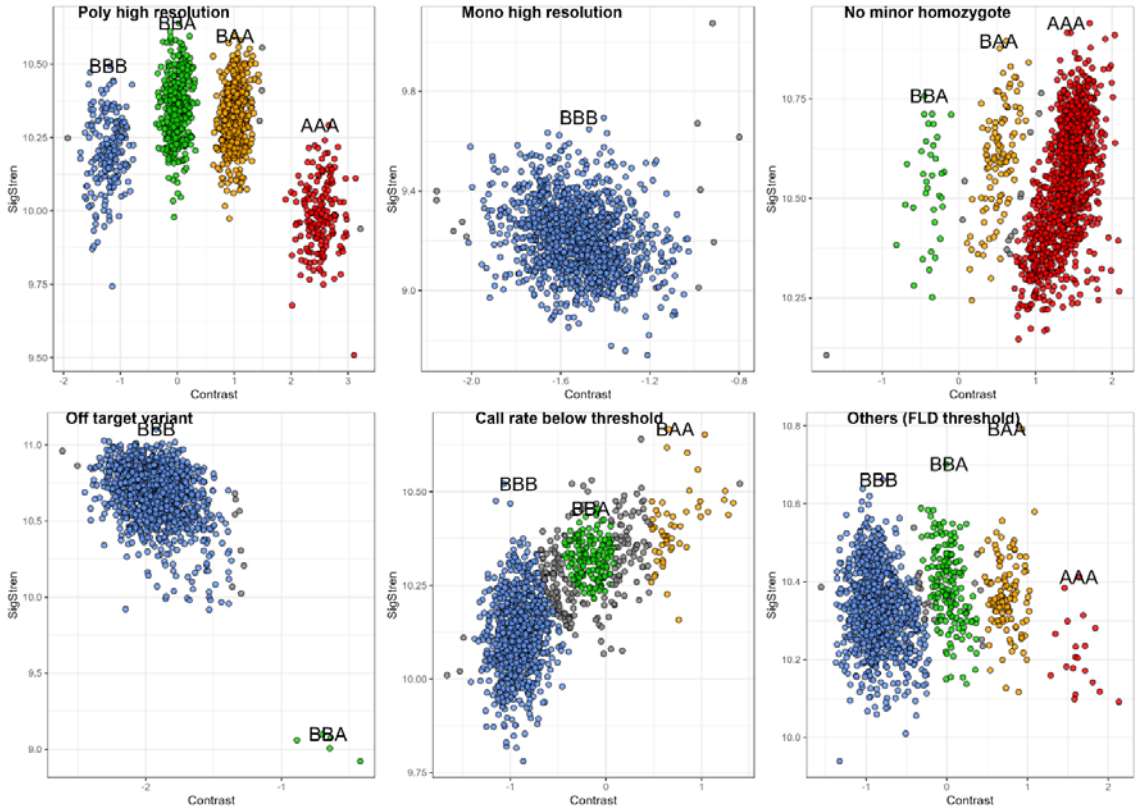
Results

Clustering and genotype calling phases

A “Poly high resolution” marker was characterized by the maximum number of genotype and well-separated clusters (Fig 4, “Poly high resolution”) whereas a “No minor homozygote” marker shared these characteristics but lacked one of the homozygous genotypes (Fig 4, “No minor homozygote”).

345 Occasionally, despite apparent separation of clusters, they failed to meet all established thresholds
346 and did not pass filters; for example, in Fig 4, "Others (FLD threshold)" exhibited a FLD of 3.36,
347 slightly below the set threshold of 3.4.

348 The methodology demonstrated robustness in identifying issues related to the position of the
349 heterozygote cluster (Fig 4, "Off target variant"), where the BBA genotype exhibited lower signal
350 strength than the BBB genotype, and in detecting mixed or uncertain clusters by augmenting the
351 number of NA among individuals between clusters (Fig 4, "Call rate below threshold").



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353 Figure 4: Examples of distribution on the axes of contrast and signal strength of genotypes identified
354 by GenoTriplo for each category of markers

355

356 **Number of initializations and maximal number of clusters**

To assess the impact of the numbers of initializations and of maximum clusters in GenoTriplo, we conducted a quantitative comparison of the marker distribution across various categories following the completion of the clustering and genotyping phases.

The number of initializations positively impacted the performance of the algorithm. The number of markers in “Poly high resolution” category increased steadily from 1 to 10 initializations (+18% from 1 to 5 and +5% from 5 to 10), while numbers in “No minor homozygote” and “Call rate below threshold” categories decreased. The supplementary “Poly high resolution” markers identified with 10 initializations, compared to 5, originated partly from the “Call rate below threshold” category. This subset of markers may have encountered call rate issues due to cluster standard deviation thresholds. If the low-frequency genotype was not found, it might have been erroneously grouped with another genotype, significantly increasing the standard deviation of the cluster and resulting in NA assignments for all individuals in that cluster. Another subset originated from the “No minor homozygote” category, where individuals belonging to a smaller, low-frequency genotype might have been inaccurately grouped with a higher frequency genotype. This led to a lesser increase in standard deviation or NA assignments due to the distance-to-centre threshold. “Others” category showed less sensitivity to changes in the number of initializations (Table 1).

Increasing the initial number of clusters defined for Rmixmod clustering function also helped to get more markers included in the “Poly high resolution” category, especially when increasing from 4 to 8 clusters and, to a lesser extent, from 8 to 12 clusters (Table 1). Conversely, the number of SNPs in the “No minor homozygote” category decreased, respectively from 8,480 to 4,452 markers with 4 and 12 initial clusters, respectively. Notably, the number of markers in the “Mono high resolution” category decreased substantially for 12 clusters (3,132), while it remained stable around 4,300 for 4 and 8 initial clusters. The number of markers in the “Call rate below threshold” category strongly decreased from 4 to 8 initial clusters (12,513 to 4,734), but increased from 8 to 12 initial clusters (4,734 to 6,516), indicating an optimal number of initial clusters of 8 as compared to 4 and 12 clusters.

Although the number of SNPs put in "Others" category increased with the number of clusters, it did not counterbalance the decrease of SNPs in "Call rate below threshold" category, indicating that some markers were pulled out of the low-quality categories towards the high-quality categories (Table 1).

In summary, utilizing 5 initializations, 8 clusters, and default parameters and thresholds for quality control of the genotyping resulted in 85% of markers falling into high quality marker categories i.e. "Mono high resolution", "No minor homozygote" and "Poly high resolution".

Table1: Number of markers by categories for the different parameters used in clustering phase

Runs		**Categories**					
N_{init}	N_{clus}	Poly high resolution	No minor homozygote	Mono high resolution	Call rate below threshold	Off target variant	Others
1	8	18307	7126	4315	7451	411	423
5	8	21715	6233	4377	4734	421	553
10	8	22501	5838	4299	4344	438	613
5	4	11867	8480	4612	12513	400	161
5	12	22875	4452	3132	6516	403	655

Comparison between GenoTriplo and fitPoly genotyping

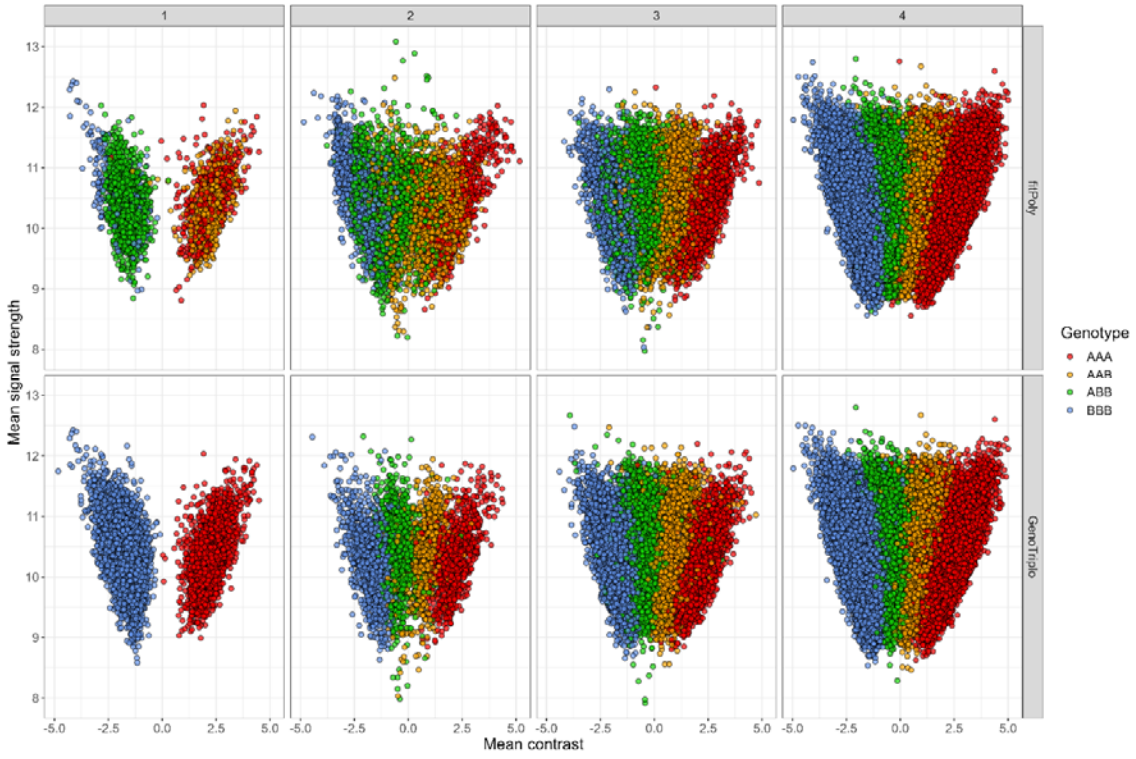
The overall concordance rate between genotypes derived from GenoTriplo and fitPoly was 85%, reaching 89% after exclusion of all NA. Notably, 26% of the SNPs showed differences in the number of genotypes identified by the two methods. GenoTriplo found less SNPs with four genotypes, while fitPoly found less monomorphic SNPs (Table 2).

Table 2: Table with the respective number of SNPs with 1, 2 3 or 4 genotypes identified with GenoTriplo or fitPoly.

GenoTriplo\fitPoly	**1 genotype**	**2 genotypes**	**3 genotypes**	**4 genotypes**
1 genotype	2333	1429	644	86
2 genotypes	28	493	542	783
3 genotypes	28	210	2289	4333
4 genotypes	38	640	966	23001

399 In categories for which both GenoTriplo and fitPoly identified the same number of genotypes, the
400 genotype concordance was not as high as expected. For a single genotype found, the concordance
401 was 25%, increasing to 81% with two genotypes found, 94% with three genotypes found, and
402 exceeding 99% with four genotypes found. The difference in the case of a unique genotype assigned
403 was due to fitPoly frequently assigning a heterozygous genotype rather than a more likely
404 homozygous genotype. Out of 2428 markers with a single genotype assigned by fitPoly, 1752 were
405 identified as heterozygous (Fig 5).

406 A similar pattern emerged, to a lesser extent, when fitPoly identified two genotypes. In contrast,
407 GenoTriplo exhibited the expected behaviour, with each distinct genotype forming distinct clusters,
408 displaying distinct mean contrast values regardless of the number of genotypes identified (Fig 5).



409

410 Figure 5: Mean contrast and signal strength values for genotypes of SNP with 1, 2, 3 and 4 different
411 genotypes (from left to right) for fitPoly (above) and GenoTriplo (under) methods

When the numbers of possible genotypes were different across the two methods, two discernible patterns emerged from the analysis based on visual observation of the clusters, showcasing scenarios where fitPoly outperformed GenoTriplo and *vice versa* (Table 3). FitPoly showed better results in categories where it identified a greater number of genotypes compared to GenoTriplo, specifically in categories (2;3), (2;4), and (3;4). For these 3 categories however, the genotypes provided by GenoTriplo closely matched those from fitPoly when ignoring NA calls, with concordance rates of 99%, 99%, and 97%, respectively. Notably, for 292 markers out of the 1,000 in the (3;4) category, fitPoly identified a lone individual for the minor homozygous genotype, which GenoTriplo categorized as NA.

Conversely, in categories where GenoTriplo exhibited superior performance (categories (1;2), (3;2), (4,2), and (4,3)), fitPoly's genotypes deviated significantly from the expected outcomes, resulting in concordance rates of 49%, 49%, 34%, and 40%, respectively.

In the (1;3) category, a balanced performance between the two methods was observed. When fitPoly outperformed, GenoTriplo's genotypes closely matched fitPoly's (achieving 100% concordance after removing all instances of "NA"). However, when GenoTriplo was better, only 30% of fitPoly's genotypes aligned with the decisions made by GenoTriplo.

Table 3: Number of markers visualized per category, number best genotyped by GenoTriplo, by fitPoly; and corresponding rate of concordant genotypes between methods.

	Number of markers				**Rate of concordant genotypes for markers with**	
Category (GT;FP)	Total visual observation	Best genotyping: GenoTriplo	Best genotyping: fitPoly	No best method or bad marker	Best genotyping: GenoTriplo	Best genotyping: fitPoly
(1;2)	1000	946	6	48	0.49	1
(1;3)	644	330	282	32	0.30	1
(2;3)	542	61	354	127	0.69	0.99
(2;4)	783	37	657	89	0.60	0.99
(3;2)	210	126	5	79	0.49	0.78
(3;4)	1000	105	784	111	0.89	0.97
(4;2)	640	582	0	58	0.34	-
(4;3)	966	841	50	75	0.40	0.72

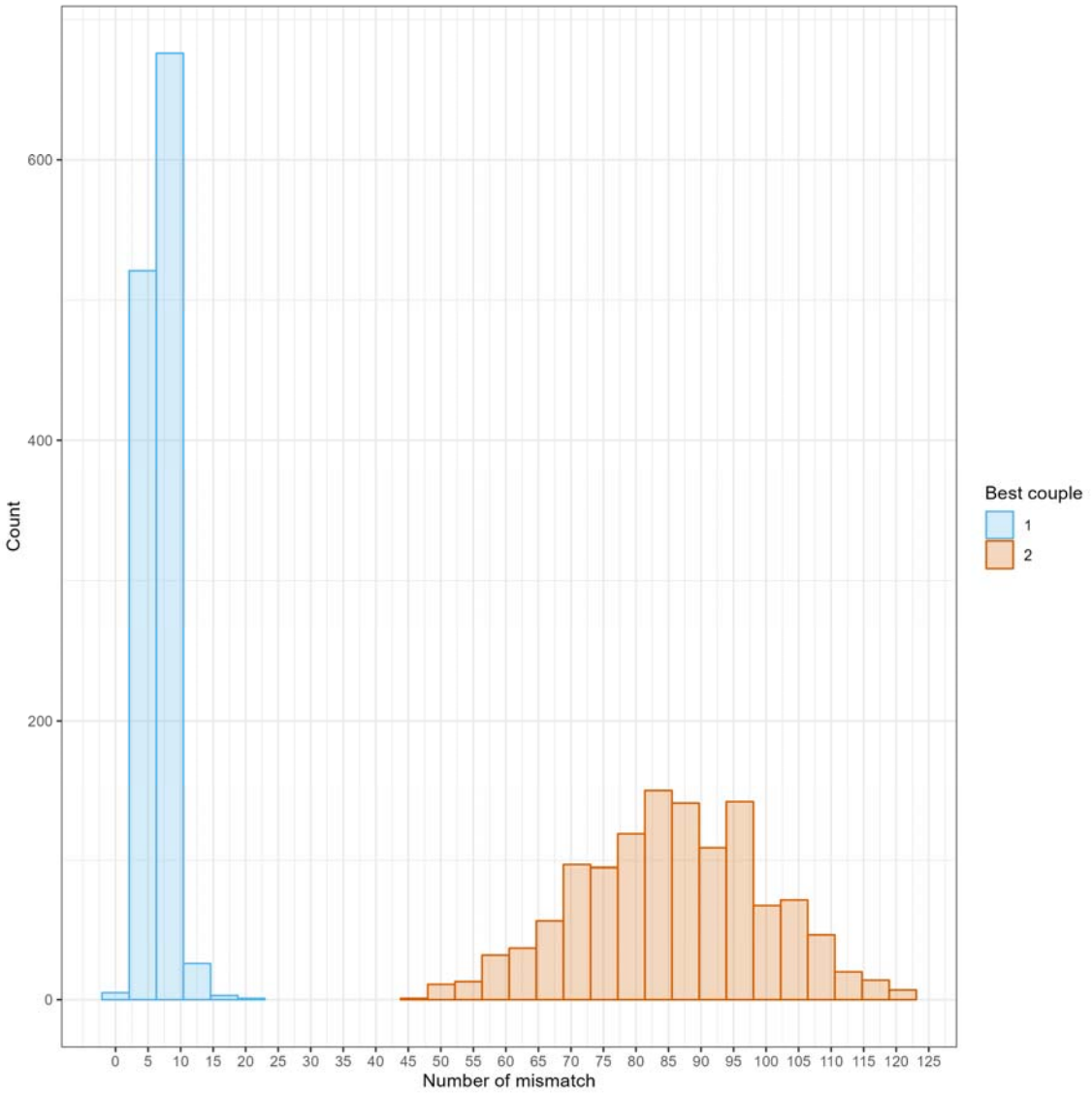
When examining the SNP acceptance/rejection categorization, we found that GenoTriplo retained the majority of SNPs where fitPoly performed better, aligning with expectations due to the close similarity between GenoTriplo and fitPoly. However, most SNPs within the (2;4) category were rejected by GenoTriplo and no by fitPoly, particularly for call rate considerations. Notably, most markers rejected by fitPoly in these categories were also rejected by GenoTriplo.

In the case of SNPs where GenoTriplo exhibited superior performance, fitPoly retained nearly half of them, despite having low concordance with GenoTriplo. For instance, in the (1;2) category, out of the 936 SNPs retained by GenoTriplo, 632 were also retained by fitPoly, even though they were likely incorrect, given the 50% concordance with GenoTriplo. Notably, almost every SNP rejected by GenoTriplo was also rejected by fitPoly.

Parentage assignment assessment by APIS

To evaluate the genotyping performance for pedigree retrieval, we utilized the exclusion method of APIS (https://cran.r-project.org/web/packages/APIS/index.html) for parentage assignment of triploid offspring genotyped with the described method, alongside parents genotyped by AXAS software. All offspring were successfully assigned to a couple of parents belonging to the correct factorial mating plan.

For the best couples assigned, a maximum of 19 mismatches occurred among the 1000 markers, with a mean mismatch of 6.9, representing less than 1% of mismatches between parents and progeny (Fig 6). The second-best couples exhibited a minimum of 47 mismatches, with a mean of 85.6. Therefore, a substantial gap in mismatch numbers existed between the best and second-best couples, with distributions clearly exhibiting no overlap, showing the very high quality of the assignments obtained (Fig 6).



453

454 Figure 6: Number of offspring as a function of the number of mismatches for the best couple (blue)

455 and the second-best couple (red) found by APIS parentage assignment

456

457 # Discussion

458 Our method for genotype calling of triploids from luminescence datasets demonstrated its quality to

459 genotype triploid fish, leading to its integration into the R package GenoTriplo, freely accessible to

460 the scientific community: https://cran.r-project.org/web/packages/GenoTriplo/index.html.

Our approach demonstrated a good accuracy for parentage assignment of triploid offspring with diploid parents. This was validated using the top 1000 markers based on MAF and Call Rate. The method performed well even with fewer markers or randomly selected markers (as few as 200). Although the true pedigree was unknown, the very low numbers of mismatches for the best couple suggested highly accurate assignments.

The method did not depend on prior information on genotype position relative to their own contrast value when identifying genotypes among SNP. This characteristic enhanced efficiency, particularly when contrast values were shifted from the expected values as a same genotype would manifest at different value of contrast dependant on the marker [24,25]. This also allows to genotype new SNPs with no need of human action to set reference genotypes for each SNP, in this way differentiating it from AXAS that relies on reference genotype.

The clustering method underlying the genotyping call was efficient using well-fitted input parameters. Notably, the number of initializations significantly enhanced the clustering algorithm's efficiency by identifying clusters with few individuals, i.e. representing low-frequency genotypes. In our case study, the occurrence of markers with low-frequency genotypes was limited, and most of the different genotypes were thus well-identified with only 5 initialization runs.

Increasing the number of initializations will maximize the probability of identifying clusters corresponding to low-frequency genotypes. However, this increase results in longer computation time, forcing a trade-off between computation time and additional identification of very low-frequency genotype for few SNPs. In our case, using 5 initializations was a good compromise, but this parameter should be optimized for other triploid populations and species.

In addition, the initial number of clusters also significantly influenced the clustering algorithm outcomes. Requesting only 4 clusters for triploids resulted in miss-detection of low-frequency genotypes, leading to a shortage of “Poly high resolution” SNPs and an excess of “No minor homozygote” markers. Conversely, too high a number of clusters led to inappropriate creation of

clusters composed of very few individuals, and resulting in a scarcity of the "Mono high resolution" category. Optimal results were achieved with an intermediate number of clusters, specifically twice the number of possible genotypes (8 for triploids). This configuration allowed for the identification of most of the low-frequency genotypes without generating artefacts. Therefore, our strategy using twice the maximum number of possible genotypes facilitated genotype calling for low-frequency genotypes without the need for of large number of individuals to genotype together as suggested by [23,24].

In the genotyping process, the method employed assumed that individuals originated from the same population. Using Hardy-Weinberg hypothesis, our approach did not accept that both homozygous genotypes coexisted without the two heterozygous genotypes for a given SNP, contributing to the efficiency of our genotype attribution. When informed that the samples can come from various populations, our method involved the comparison of mean contrast values of each current cluster to the values of reference clusters. Those reference values are derived on the same dataset from markers with the maximum number of genotypes. Given the common occurrence of contrast value shifts (when all contrast values of a SNP are all shifted toward positive or negative value), the recommended approach, when possible, is to analyse together pools of individuals originated from the same population.

The overall concordance of genotypes between GenoTriplo and fitPoly was notably high. However, differences emerged when comparing the number of genotypes identified by each method. When both methods identified the same number of genotypes, differences were the result of the fundamentally different approaches to assigning genotypes to clusters of individuals. GenoTriplo relied on stringent assumptions, like assigning a homozygous genotype when only one cluster was identified. In contrast, fitPoly lacked such guidelines, leading to substantial discordance, especially in cases where only one genotype was expected.

GenoTriplo encountered difficulties in identifying all 4 genotypes, often settling for 3 when very few individuals formed the second homozygous genotype. Those few individuals usually were not assigned a genotype, avoiding genotyping errors. Besides, for 292 markers among the 784 markers where fitPoly identified 4 genotypes while GenoTriplo found only 3, a single individual represented the homozygous low frequency genotype in FitPoly. The credibility being low for a single individual to represent a genotype, we consider it preferable to assign the individual to NA, thus avoiding a possible genotyping error.

On the contrary, fitPoly faced difficulties in identifying a limited number of genotypes (below the maximum possible) for a given SNP, particularly when the SNP was monomorphic. This challenge could come from the method per se which prioritizes a high number of genotypes, leading to the creation of unwanted clusters. While some of these SNP were rejected by fitPoly for excess of NA, half were retained even for those with low concordance with GenoTriplo, causing substantial genotyping errors.

While most of the disagreement were minor when fitPoly performed better, GenoTriplo's accuracy outperformed fitPoly's, especially for low number of genotypes and detection of wrong genotypes.

This paper focuses on the genotyping of triploids, but it is essential to note that the method was also successfully tested on diploids, providing similar results to the AXAS software. Furthermore, its application could potentially be extended to higher ploidy levels. The key parameter for the clustering phase would be the minimal distance between two clusters. Notably, the mean contrast value for a homozygous diploid genotype matched that of a triploid homozygous genotype. Consequently, with higher ploidy levels, the insertion of additional heterozygote genotypes is expected between the contrast values of homozygotes, resulting in diminishing distances between clusters as ploidy levels increase, making the discrimination between different allelic dosages more difficult. Currently, the genotyping phase is implemented for diploid and triploid individuals, and further work would be required to extend it to higher ploidy levels.

Data availability statement

The GenoTriplo package is available on the CRAN at https://cran.r-project.org/web/packages/GenoTriplo/index.html

The data supporting the article are available at: https://doi.org/10.57745/7IMQDS

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Bibliography

1. Adams KL, Wendel JF. Polyploidy and genome evolution in plants. Current Opinion in Plant Biology. 2005 Apr;8(2):135–41.

2. Nishiwaki A, Mizuguti A, Kuwabara S, Toma Y, Ishigaki G, Miyashita T, et al. Discovery of natural Miscanthus (Poaceae) triploid plants in sympatric populations of Miscanthus sacchariflorus and Miscanthus sinensis in southern Japan. American Journal of Botany. 2011 Jan 1;98(1):154–9.

3. Soltis PS, Marchant DB, Van De Peer Y, Soltis DE. Polyploidy and genome evolution in plants. Current Opinion in Genetics & Development. 2015 Dec;35:119–25.

4. Gregory TR, Mable BK. Polyploidy in Animals. In: The Evolution of the Genome [Internet]. Elsevier; 2005 [cited 2023 Sep 12]. p. 427–517. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780123014634500103

5. Gmitter FG, Ling XB, Deng XX. Induction of triploid Citrus plants from endosperm calli in vitro. Theoret Appl Genetics. 1990 Dec;80(6):785–90.

6. Wang X, Cheng ZM, Zhi S, Xu F. Breeding triploid plants: a review. Czech J Genet Plant Breed. 2016 Jun 30;52(2):41–54.

7. Sheehan RJ, Shasteen SP, Suresh AV, Kapuscinski AR, Seeb JE. Better Growth in All-Female Diploid and Triploid Rainbow Trout. Transactions of the American Fisheries Society. 1999 May 1;128(3):491–8.

8. Nell JA. Farming triploid oysters. Aquaculture. 2002 Jul 31;210(1):69–88.

9. Piferrer F, Beaumont A, Falguière JC, Flajšhans M, Haffray P, Colombo L. Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. Aquaculture. 2009 Aug;293(3–4):125–56.

10. Benfey TJ. Effectiveness of triploidy as a management tool for reproductive containment of farmed fish: Atlantic salmon (*Salmo salar*) as a case study. Rev Aquacult. 2016 Sep;8(3):264–82.

11. Thomas TD, Chaturvedi R. Endosperm culture: a novel method for triploid plant production. Plant Cell Tiss Organ Cult. 2008 Apr;93(1):1–14.

12. Thomas TD, Bhatnagar AK, Bhojwani SS. Production of triploid plants of mulberry (Morus alba L) by endosperm culture. Plant Cell Reports. 2000 Mar 13;19(4):395–9.

13. Stanley JG, Allen SK, Hidu H. Polyploidy induced in the American oyster, Crassostrea virginica, with cytochalasin B. Aquaculture. 1981 Apr;23(1–4):1–10.

14. Chourrout D. Thermal induction of diploid gynogenesis and triploidy in the eggs of the rainbow trout (Salmo gairdneri Richardson). Reprod Nutr Dévelop. 1980;20(3A):727–33.

15. Lincoln RF, Scott AP. Production of all-female triploid rainbow trout. Aquaculture. 1983 Jan;30(1–4):375–80.

16. Blanc JM, Poisson H, Vallée F. Covariation between diploid and triploid progenies from common breeders in rainbow trout, *Oncorhynchus mykiss* (Walbaum): Familial covariation between di- and triploid trout. Aquaculture Research. 2001 Jul;32(7):507–16.

17. Friars GW, McMillan I, Quinton VM, O'Flynn FM, McGeachy SA, Benfey TJ. Family differences in relative growth of diploid and triploid Atlantic salmon (Salmo salar L.). Aquaculture. 2001 Jan;192(1):23–9.

18. Leeds TD, Vallejo RL, Weber GM, Gonzalez-Pena D, Silverstein JT. Response to five generations of selection for growth performance traits in rainbow trout (Oncorhynchus mykiss). Aquaculture. 2016 Dec;465:341–51.

19. Vandeputte M, Haffray P. Parentage assignment with genomic markers: a major advance for understanding and exploiting genetic variation of quantitative traits in farmed aquatic animals. Front Genet [Internet]. 2014 Dec 12 [cited 2023 Sep 7];5. Available from: http://journal.frontiersin.org/article/10.3389/fgene.2014.00432/abstract

20. LaFramboise T. Single nucleotide polymorphism arrays: a decade of biological, computational and technological advances. Nucleic Acids Research. 2009 Jul 1;37(13):4181–93.

21. You Q, Yang X, Peng Z, Xu L, Wang J. Development and Applications of a High Throughput Genotyping Tool for Polyploid Crops: Single Nucleotide Polymorphism (SNP) Array. Front Plant Sci. 2018 Feb 6;9:104.

22. Pereira GS, Garcia AAF, Margarido GRA. A fully automated pipeline for quantitative genotype calling from next generation sequencing data in autopolyploids. BMC Bioinformatics. 2018 Dec;19(1):398.

23. Rabbee N, Speed TP. A genotype calling algorithm for affymetrix SNP arrays. Bioinformatics. 2006 Jan 1;22(1):7–12.

24. Shah TS, Liu JZ, Floyd JAB, Morris JA, Wirth N, Barrett JC, et al. optiCall: a robust genotype-calling algorithm for rare, low-frequency and common variants. Bioinformatics. 2012 Jun 15;28(12):1598–603.

25. Teo YY, Inouye M, Small KS, Gwilliam R, Deloukas P, Kwiatkowski DP, et al. A genotype calling algorithm for the Illumina BeadArray platform. Bioinformatics. 2007 Oct 15;23(20):2741–6.

26. Grashei KE, Ødegård J, Meuwissen THE. Genotype calling of triploid offspring from diploid parents. Genet Sel Evol. 2020 Dec;52(1):15.

27. Voorrips RE, Gort G, Vosman B. Genotype calling in tetraploid species from bi-allelic marker data using mixture models. BMC Bioinformatics. 2011 Dec;12(1):172.

28. Zych K, Gort G, Maliepaard CA, Jansen RC, Voorrips RE. FitTetra 2.0 – improved genotype calling for tetraploids with multiple population and parental data support. BMC Bioinformatics. 2019 Dec;20(1):148.

29. Prchal M, D'Ambrosio J, Lagarde H, Lallias D, Patrice P, François Y, et al. Genome-wide association study and genomic prediction of tolerance to acute hypoxia in rainbow trout. Aquaculture. 2023 Feb;565:739068.

30. Palti Y, Gao G, Liu S, Kent MP, Lien S, Miller MR, et al. The development and characterization of a 57K single nucleotide polymorphism array for rainbow trout. Molecular Ecology Resources. 2015;15(3):662–72.

31. D'Ambrosio J, Phocas F, Haffray P, Bestin A, Brard-Fudulea S, Poncet C, et al. Genome-wide estimates of genetic diversity, inbreeding and effective size of experimental and commercial rainbow trout lines undergoing selective breeding. Genetics Selection Evolution. 2019 Jun 6;51(1):26.

32. Bernard M, Dehaullon A, Gao G, Paul K, Lagarde H, Charles M, et al. Development of a High-Density 665 K SNP Array for Rainbow Trout Genome-Wide Genotyping. Front Genet. 2022 Jul 18;13:941340.

33. Lebret R. Rmixmod: A MIXture MODelling R package. In: 1ères Rencontres R [Internet]. Bordeaux, France; 2012 [cited 2023 Sep 12]. Available from: https://hal.science/hal-00717551

34. R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2023. Available from: https://www.R-project.org/

35. Affymetrix. Axiom® Genotyping Solution.

36. Vandeputte M. An accurate formula to calculate exclusion power of marker sets in parentage assignment. Genet Sel Evol. 2012 Dec;44(1):36.

37. Wickham H. ggplot2: ggplot2. WIREs Comp Stat. 2011 Mar;3(2):180–5.