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1 ***Deciphering the genetic diversity of landraces with high-***
2 ***throughput SNP genotyping of DNA bulks: methodology and***
3 ***application to the maize 50k array***

4
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15

16 **ABSTRACT**

17 Genebanks harbor original landraces carrying many original favorable alleles for
18 mitigating biotic and abiotic stresses. Their genetic diversity remains however poorly
19 characterized due to their large within genetic diversity. We developed a high-throughput,
20 cheap and labor saving DNA bulk approach based on SNP Illumina Infinium HD array to
21 genotype landraces. Samples were gathered for each landrace by mixing equal weights from
22 young leaves, from which DNA was extracted. We then estimated allelic frequencies in each
23 DNA bulk based on fluorescent intensity ratio (FIR) between two alleles at each SNP using a
24 two step-approach. We first tested either whether the DNA bulk was monomorphic or
25 polymorphic according to the two FIR distributions of individuals homozygous for allele A or
26 B, respectively. If the DNA bulk was polymorphic, we estimated its allelic frequency by using
27 a predictive equation calibrated on FIR from DNA bulks with known allelic frequencies. Our
28 approach: (i) gives accurate allelic frequency estimations that are highly reproducible across
29 laboratories, (ii) protects against false detection of allele fixation within landraces. We
30 estimated allelic frequencies of 23,412 SNPs in 156 landraces representing American and
31 European maize diversity. Modified Roger's genetic Distance between 156 landraces estimated
32 from 23,412 SNPs and 17 SSRs using the same DNA bulks were highly correlated, suggesting
33 that the ascertainment bias is low. Our approach is affordable, easy to implement and does not
34 require specific bioinformatics support and laboratory equipment, and therefore should be
35 highly relevant for large-scale characterization of genebanks for a wide range of species.

36 Keywords: Landraces, DNA pooling, Genetic diversity, 50K Illumina Infinium HD Zea
37 mays L., Allelotyping , genebanks

38 **INTRODUCTION**

39 Genetic resources maintained *in situ* or *ex situ* in genebanks represent a vast reservoir
40 of traits/alleles for future genetic progress and an insurance against unforeseen threats to
41 agricultural production (Tanksley 1997; Hoisington *et al.* 1999; Kilian and Graner 2012;
42 McCouch *et al.* 2012). Due to their local adaptation to various agro-climatic conditions and
43 human uses, landraces are particularly relevant to address climate change and the requirements
44 of low input agriculture (Fernie *et al.* 2006; McCouch *et al.* 2012; Mascher *et al.* 2019). For
45 instance, maize displays considerable genetic variability, but less than 5 % of this variability
46 has been exploited in elite breeding pools, according to (Hoisington *et al.* 1999). However,
47 landraces are used to a very limited extent, if any, in modern plant breeding programs, because
48 they are poorly characterized, genetically heterogeneous and exhibit poor agronomic
49 performance compared to elite material (Kilian and Graner 2012; Strigens *et al.* 2013; Brauner
50 *et al.* 2019; Mascher *et al.* 2019; Hölker *et al.* 2019). Use of molecular techniques for
51 characterizing genetic diversity of landraces and their relation with the elite germplasm is
52 essential for a better management and preservation of genetic resources and for a more efficient
53 use of these germplasms in breeding programs (Hoisington *et al.* 1999; Mascher *et al.* 2019).

54 The genetic diversity of landraces conserved *ex situ* or *in situ* has been extensively studied
55 using various types of molecular markers such as restriction fragment length polymorphism
56 (RFLP) or simple sequence repeat (SSR) in maize (Dubreuil and Charcosset 1998; Dubreuil *et al.*
57 *et al.* 1999; Rebourg *et al.* 1999, 2001; Gauthier *et al.* 2002; Rebourg *et al.* 2003; Reif *et al.* 2005b;
58 Vigouroux *et al.* 2005; Reif *et al.* 2005a; Camus-Kulandaivelu 2006; Dubreuil *et al.* 2006;
59 Eschholz *et al.* 2010; Mir *et al.* 2013), in Pearl Millet (Bhattacharjee *et al.* 2002), cabbage (Dias
60 *et al.* 1991; Divaret *et al.* 1999), Barley (Parzies *et al.* 2000; Backes *et al.* 2003; Hagenblad *et al.*
61 *et al.* 2012), pea (Hagenblad *et al.* 2012), oat (Hagenblad *et al.* 2012), rice (Ford-Lloyd *et al.*
62 2001; McCouch *et al.* 2012), Alfalfa (Pupilli *et al.* 2000; Segovia-Lerma *et al.* 2003) and fonio
63 millet (Adoukonou-Sagbadja *et al.* 2007). SSRs have proven to be markers of choice for
64 analyzing diversity in different plant species and breeding research, because of their variability,
65 ease of use, accessibility of detection and reproducibility (Liu *et al.* 2003; Reif *et al.* 2006; Yang
66 *et al.* 2011). Nevertheless, the development of SSR markers requires a substantial investment
67 of time and money. Allele coding is also difficult to standardize across genotyping platforms
68 and laboratories, a major drawback in a genetic resources characterization context. SNPs have
69 become the marker of choice for various crop species such as maize (Ganal *et al.* 2011), rice
70 (McCouch *et al.* 2010), barley (Moragues *et al.* 2010) and soybean (Lam *et al.* 2010). They are

71 the most abundant class of sequence variation in the genome, co-dominantly inherited,
72 genetically stable and appropriate to high-throughput automated analysis (Rafalski 2002). For
73 instance, maize arrays with approx. 50,000 and 600,000 SNP markers are available since 2010
74 (Illumina MaizeSNP50 array, Ganal *et al.* 2011) and 2013 (600K Affymetrix Axiom, Unterseer
75 *et al.*, 2013), respectively. SNP arrays may however lead to some ascertainment bias notably
76 when diversity analysis was performed on a plant germplasm distantly related from those that
77 have been used to discover SNP put on the array (Nielsen 2004; Clark *et al.* 2005; Hamblin
78 *et al.* 2007; Inghelandt *et al.* 2011; Frascaroli *et al.* 2013). Properties of SNP array regarding
79 diversity analysis have to be carefully investigated to evaluate ascertainment biases. For maize
80 50K Infinium SNP array, only “PZE” prefixed SNPs (so called later PZE SNPs in this study)
81 give consistent results for diversity analysis as compared with previous studies based on SSR
82 markers and are therefore suitable for assessing genetic variability (Inghelandt *et al.* 2011;
83 Ganal *et al.* 2011; Bouchet *et al.* 2013; Frascaroli *et al.* 2013). 50K Infinium SNP array has
84 been used successfully to decipher genetic diversity of inbred lines (van Heerwaarden *et al.*
85 2011; Bouchet *et al.* 2013; Frascaroli *et al.* 2013; Rincent *et al.* 2014), landraces using either
86 doubled haploids (Strigens *et al.* 2013) or a single individual per accession (van Heerwaarden
87 *et al.* 2011; Arteaga *et al.* 2016), or teosinte with few individuals per accession (Aguirre-
88 Liguori *et al.* 2017).

89 Due to high diversity within accessions, characterization of landraces from allogamous
90 species such as maize or alfalfa should be performed based on representative sets of individuals
91 (Reyes-Valdés *et al.* 2013, Segovia-Lerma *et al.*, 2002, Dubreuil and Charcosset., 1998).
92 Despite the recent technical advances, genotyping large numbers of individuals remains very
93 expensive for many research groups. To bring costs down, estimating allele frequencies from
94 pooled genomic DNA (also called “allelotyping”) has been suggested as a convenient
95 alternative to individual genotyping using high-throughput SNP arrays (Sham *et al.* 2002;
96 Teumer *et al.* 2013) or using Next Generation Sequencing (Schlötterer *et al.* 2014). It was
97 successfully used to decipher global genetic diversity within maize landraces using RFLPs
98 (Dubreuil and Charcosset 1998; Dubreuil *et al.* 1999; Rebourg *et al.* 2001, 2003; Gauthier *et al.*
99 *et al.* 2002) and SSR markers (Reif *et al.* 2005a; Camus-Kulandaivelu 2006; Dubreuil *et al.* 2006;
100 Yao *et al.* 2007; Mir *et al.* 2013). Various methods for estimating gene frequencies in pooled
101 DNA have been developed for RFLP (Dubreuil and Charcosset 1998), SSR (LeDuc *et al.* 1995;
102 Perlin *et al.* 1995; Daniels *et al.* 1998; Lipkin *et al.* 1998; Breen *et al.* 1999) and SNP marker
103 arrays in human and animal species (Hoogendoorn *et al.* 2000; Craig *et al.* 2005; Brohede 2005;

104 Teumer *et al.* 2013; Gautier *et al.* 2013). These methods have been successfully used to detect
105 QTL (Lipkin *et al.* 1998), to decipher genetic diversity (Segovia-Lerma *et al.* 2003; Dubreuil
106 *et al.* 2006; Pervaiz *et al.* 2010; Johnston *et al.* 2013; Ozerov *et al.* 2013), to perform genome
107 wide association studies (Barcellos *et al.* 1997; Sham *et al.* 2002; Baum *et al.* 2007), to identify
108 selective sweep (Elferink *et al.* 2012) or to identify causal mutation in tilling bank (Abe *et al.*
109 2012). Genotyping DNA bulks of individuals from landraces with SNP arrays could therefore
110 be interesting to characterize and manage genetic diversity in plant germplasm. SNP arrays
111 could be notably a valuable tool to identify selective sweep between landraces depending on
112 their origin, to manage plant germplasm collection at worldwide level (e.g. identify duplicate),
113 to identify landraces poorly used so far in breeding programs or to identify genomic regions
114 where diversity has been lost during the transition from landraces to inbred lines (Arca *et al.*, *in*
115 *prep*).

116 In this study, we developed a new DNA bulk method to estimate allelic frequencies at SNPs
117 based on Fluorescent Intensity data produced by the maize 50K Illumina SNP array (Ganal *et*
118 *al.* 2011). Contrary to previous methods that have been mostly developed for QTL detection
119 purposes, our approach is dedicated to genome-wide diversity analysis in plant germplasm since
120 it protects against false detection of alleles. Additionally, calibration of equations for predicting
121 allelic frequencies of DNA bulks for each SNP is based on controlled pools with variable allelic
122 frequencies rather than only heterozygous genotypes as in previous methods (Hoogendoorn *et*
123 *al.* 2000; Brohede 2005; Peiris *et al.* 2011; Teumer *et al.* 2013). As a proof of concept, we
124 applied our new approach to maize by estimating allelic frequencies of 23,412 SNPs in 156
125 maize landraces representative of European and American diversity present in genebanks
126 (Arca *et al.*, *in prep*). To our knowledge, it is the first time that a DNA bulk approach was used
127 on 50K maize high-throughput SNP array to study genetic diversity within maize landraces
128 germplasm.

129 **RESULTS**

130 We developed a new method to estimate allelic frequencies of SNPs within pools of
131 individuals using the fluorescent intensity ratio (FIR) between A and B alleles from Illumina
132 MaizeSNP50 array. Briefly, allelic frequencies at SNPs belonging to MaizeSNP50 array were
133 estimated within 156 maize landraces by pooling randomly 15 individuals per population and
134 by calibrating a predictive two-step model (Figure 1). We considered only the subsample of
135 32,788 prefixed PZE markers (so called PZE SNPs) that have proven suitable for diversity

136 analyses (Ganal *et al.* 2011). Among these SNPs, we selected 23,412 SNPs that passed weighted
137 deviation (wd) quality criteria ($wd > 50$). This removed SNPs for which estimated allelic
138 frequency deviated strongly from expected allelic frequency (Figure. S1 A, B, C, D, E, F and
139 G for the threshold choice and validation).

140 ***Accuracy of allelic frequency prediction and detection of allele fixation***

141 In order to prevent erroneous detection of alleles within landraces, we first tested for each
142 landrace whether allele A or allele B was fixed at a given SNP locus (Figure 1). We tested for
143 each SNP whether the FIR of the landrace was included within one the two Gaussian
144 distributions drawn from mean and variance of FIR of genotypes AA and BB within the inbred
145 line panel (Figure 1). For landraces that were considered polymorphic after this first step (allele
146 fixation rejected for both alleles), we estimated allelic frequency based on FIR by using a unique
147 logistic regression model for the 23,412 SNPs, calibrated with a sample of 1,000 SNPs (Figure
148 1). Parameters of the logistic model were adjusted on these 1,000 SNPs using FIR of two series
149 of controlled pools whose allelic frequencies were known (Figure S2). We obtained these pools
150 by mixing various proportion of two series of three inbred lines with known genotypes (Table
151 1). The 1,000 SNPs were selected to maximize the allelic frequency range within controlled
152 pools (Table 1). The logistic regression was calibrated on 1,000 SNPs taken together rather than
153 for each SNP individually to avoid the ascertainment bias that would be generated by selecting
154 only SNPs polymorphic in the controlled pools (Figure S3) and to reduce loss of accuracy in
155 prediction for SNPs displaying limited allelic frequency range in two controlled pools (Figure
156 S4). To investigate the loss of accuracy of the prediction curve due to a reduction in allelic
157 frequency ranges in controlled pool, we progressively removed at random from one to 15
158 samples from the calibration set of the 1000 above described SNPs. The mean absolute error
159 (MAE) between 1000 replications increased significantly from 4.14 % to 8.54 % when
160 removing more samples (Table 2). For comparison, MAE was 7.19 % using a cross-validation
161 approach in which the predictive equation was calibrated with a random subsample of 800 out
162 of 1000 SNPs, and then applied to estimate allelic frequencies for the remaining 200 SNPs
163 (Table S1). Calibrating the logistic regression between FIR and allelic frequency in controlled
164 pool based on 1000 SNPs therefore appears well adapted to prevent ascertainment bias while
165 increasing globally prediction accuracy (Figure S4). Finally, we observed that MAE was higher
166 for balanced allelic frequencies than for almost fixed allelic frequencies (Figure 2 and Tables
167 S2). Accordingly, the dispersion of predicted frequencies were larger for expected allelic
168 frequencies near 0.5 than for fixed alleles (Table S2).

169 ***Reproducibility of frequency across laboratories and samples***

170 We evaluated the reproducibility of the method across laboratories by comparing FIR of
171 one series of controlled pools from two different laboratories using all PZE SNPs or 23,412
172 SNPs selected using *wd* criterion (Figure 3). The correlation between the two different
173 laboratories for controlled pools was very high ($r^2 > 0.98$) whether we selected the SNPs based
174 on *wd* criterion or not. Beyond reproducibility across laboratories, the precision of frequency
175 estimation depends on the sampling of individuals within landraces (Table 3). The precision of
176 frequency estimation was addressed both by numerical calculation and by the independent
177 sampling of 15 different individuals (30 different gametes) within 10 landraces (biological
178 replicate). For both numerical calculations and biological replicates, the sampling error was
179 higher for loci with balanced allelic frequencies than for loci that are close to fixation (Table 3,
180 Figure 4). Sampling error also decreased as the number of sampled individuals increased (Table
181 3). Considering a true frequency of 50% within landraces, we expect that 95% of frequency
182 estimates lie between 31.30% and 68.70% when sampling 15 individuals per landrace and 42.9
183 to 57.13% when sampling 100 individuals per landrace (Table 3). Considering biological
184 replicates, allelic frequencies of the two biological replicates over 23,412 SNPs were highly
185 correlated except for population Pol3 (Table S3). When excluding Pol3, 94.5% of points were
186 located within the 95% confidence limits accounting for the effect of sampling alone,
187 suggesting that the error inherent to the frequency estimation for DNA pools was very low
188 compared to the sampling error (Figure 4). Over nine populations with replicates (excluding
189 Pol3), we observed only four situations among 23,412 loci where two different alleles were
190 fixed in the two replicates (Figure 4). Loci for which an allele was fixed in one replicate was
191 either fixed or displayed a high frequency (above 88%) for the same allele in the other replicate
192 in 98% of cases. Moreover, we estimated pairwise roger's genetic distance (MRD) based on
193 23,412 SNPs between the two independent pools from the same landraces. Excluding
194 Population Pol3 (MRD = 0.208), this distance ranged from 0.087 to 0,120 (Table S3). These
195 values provide a reference to decide whether two populations can be considered different or
196 not.

197 ***Effect of SNP number and wd on the relationship of genetic distance*** 198 ***estimated with SNP and SSR***

199 Finally, we evaluated the possible ascertainment bias due to SNP selection with our filtering
200 based on *wd* criterion. MRD obtained with 17 SSR markers (MRD_{SSR}) and MRD based on

201 different set of SNP markers (MRD_{SNP}) were highly correlated (Figure 5), indicating a low
202 ascertainment bias. The selection of SNPs based on *wd* quality criterion strongly increased the
203 coefficient of determination (r^2) between MRD_{SNP} and MRD_{SSR} , from 0.587 to 0.639 (Figure
204 S6). We attempted to define the minimal SNP number required to correctly describe the
205 relationship between maize landraces. While increasing the number of SNPs from 500 to 2500
206 slightly increased r^2 between MRD_{SNP} and MRD_{SSR} from 0.606 to 0.638 (Figure S6 D,E,F), we
207 observed no further increase beyond 2500 SNPs (Figure S6 A, B, C) suggesting that 2,500
208 SNPs are enough to obtain a correct picture of landrace relationships.

209 **DISCUSSION**

210 A molecular approach for diversity analysis of landraces needs to answer several criteria
211 (i) an accurate estimation of allelic frequency in each population, (ii) a robust and reproducible
212 measurement of allelic frequency across laboratories in order to facilitate comparison of genetic
213 diversity of accessions across genebanks, (iii) a reliable estimate of genetic distance between
214 landraces with no or little ascertainment bias (iv) an affordable, high-throughput and labor
215 efficient method, due to both strong financial and human constraints in plant genebanks. Four
216 main sources of errors affect the accuracy of allelic frequency estimation of a locus in a
217 population using a DNA pooling approach: (i) the sampling of individuals (so called “sampling”
218 errors), (ii) the procedure to mix DNA from individuals (so called “DNA mixing” errors) (iii)
219 the imprecision of quantitative measurement used by the model for the prediction (so called
220 “experimental” errors) and (iv) the predictive equation used to predict allelic frequency in a
221 population (so called “approximation” errors).

222 ***A two-step model to protect against erroneous detection of*** 223 ***polymorphism and predict accurately allelic frequencies in DNA bulk***

224 Approximation errors due to predictive equation depend on (i) the model used to predict
225 allelic frequencies and (ii) the set of individuals and SNPs used to calibrate the predictive
226 equation. In this study, we used a two-step modeling using inbred lines and controlled pools as
227 sets of calibration to test for polymorphism and then predict allelic frequency for polymorphic
228 markers. Detection of allele fixation in a population is an important issue for deciphering
229 and managing genetic diversity within plant and animal germplasm. We used two Student
230 tests based on fluorescent intensity ratio (FIR) distribution of lines homozygous for allele
231 A and B to determine polymorphism of a SNP in a given landrace (Figure 1). In this first
232 step, we preferred a method based on FIR distribution rather than the clustering
233 approach implemented in genome studio because it is possible to control the type I risk
234 of false allele detection (at 5% in our study). Using this two-step approach reduces
235 strongly the erroneous detection of polymorphisms in a population compared to previous
236 methods: MAE for fixed locus <0.1% in our approach (Table S2) vs ~2-3% using PPC
237 method (Brohede 2005) or ~2-8% using different k correction from (Peiris *et al.* 2011).
238 This is not surprising as previous methods focused on an accurate estimation of the
239 difference in allele frequencies between DNA bulks of individuals contrasted for a

240 quantitative trait of interest (Sham *et al.* 2002; Craig *et al.* 2005; Kirov *et al.* 2006; Teumer
241 *et al.* 2013) and did not focus specifically on protecting against false detection of alleles.

242 For loci that were detected as polymorphic, we predicted allelic frequencies from
243 FIR in landrace DNA pools by using a unique logistic regression for 23,412 SNPs passing
244 *wd* quality criterion. The relationship between FIR and allelic frequency was modelled
245 using a quasi-logistic regression for different reasons. First, the logistic function ensures
246 that the predicted frequencies take value in (0,1), a property that is not satisfied by
247 polynomial regression (PPC) or tan transformation (Brohede 2005; Teumer *et al.* 2013).
248 Second, one could observe that the relationship between the fluorescent intensity ratio
249 and allelic frequencies within controlled pools was not linear (Figure S2).

250 This two-step approach led to a low global error rate in allelic frequency prediction
251 (MAE = 3% for polymorphic and monomorphic loci considered jointly; Figure 2, Table S2).
252 It is comparable to the most accurate previous pooling DNA methods for SNP array that
253 used a specific model for each SNP: (i) MAE ranging from 3 to 8 % (Peiris *et al.*, 2011) or
254 5-8% (Brohede *et al.*, 2005) depending of k-correction applied (ii) MAE ~ 3% for PPC
255 correction (Brohede *et al.*, 2005; Teumer *et al.*, 2013) (iii) MAE ~ 1% for tan-correction
256 (Teumer *et al.*, 2013). Several factors can explain this relative good global accuracy of our
257 approach. First, almost half of the loci were fixed on average in each landrace, which
258 contributed positively to global accuracy since our method over-performed previous
259 methods for fixed locus (see above). Second, *wd* quality criterion removed SNPs for which
260 allelic frequencies were poorly predicted using FIR. We observed indeed that increasing
261 the threshold for *wd* quality criterion led to a global increase in accuracy at both steps
262 (Figure S1). While 90% of SNPs have a MAE<2% for *wd* criterion >10, only 50% of SNPs
263 have a MAE<2% for *wd* criterion <10. Taking into account differential hybridization by
264 using a specific logistic regression for each SNP could be a promising way to further
265 improve the accuracy of allelic frequencies prediction, notably for balanced allelic
266 frequencies (Brohede *et al.*, 2005, Peiris *et al.*, 2011, Teumer *et al.*, 2013). To limit possible
267 ascertainment bias and errors in allelic frequency estimation, it requires however to
268 genotype additional series of controlled pools for SNPs for which current controlled pools
269 were monomorphic or have a limited range of allelic frequency (Figure S3 and S4).

270 To estimate the parameters of the logistic regression, we used two series of controlled
271 pools rather than heterozygous individuals for both technical and practical reasons.
272 Controlled pools cover more homogeneously the frequency variation range than
273 heterozygous and homozygous individuals only, which therefore limits the risk of
274 inaccurate estimation of logistic model parameters. Different studies showed that
275 accuracy of allelic frequency estimation strongly depends on accuracy of FIR estimation
276 for heterozygous individual and therefore the number of heterozygous individuals (Le
277 Hellard *et al.* 2002; Simpson 2005; Jawaid and Sham 2009). Between 8 and 16
278 heterozygous individuals are recommended to correctly estimate FIR mean for
279 heterozygous individuals, depending on FIR variance (Le Hellard *et al.* 2002). In maize,
280 we can obtain heterozygote genotypes either by crossing inbred lines to produce F1
281 hybrids, by planting seeds from maize landraces, or by using residual heterozygosity of
282 inbred lines. Using residual heterozygosity to calibrate model is not possible since half
283 SNPs show no heterozygous genotype in the 327 inbred lines of our study. Obtaining at
284 least 16 heterozygous individuals for each SNP therefore requires to genotype a few
285 dozens of F1 hybrids or individuals from landraces considering that expected
286 heterozygosity in a landrace is comprised between 3 and 28% (Arca *et al.*, in prep). This
287 represents additional costs since maize researchers and breeders genotyped
288 preferentially inbred lines to access directly haplotypes without phasing and because
289 genotypes of F1 hybrids can be deduced of that of their parental inbred lines. Beyond
290 allogamous species as maize, genotyping heterozygous individuals could be time
291 demanding and very costly in autogamous cultivated plant species for which genotyped
292 individuals are mostly homozygotes (wheat, tomato, rapeseed). On the contrary, one can
293 easily produce controlled pools whatever the reproductive system, either by mixing DNA
294 or equal mass of plant materials, which allows producing a wide range of allelic
295 frequencies.

296 ***Effect of DNA mixing procedure on accuracy allelic frequency estimation***

297 There are two main errors coming from DNA mixing procedure: (i) the “sampling error”
298 that is directly connected to the number of individuals sampled in each population (Table 3),
299 and (ii) the “bulking error” associated with the laboratory procedure to mix equal DNA amounts
300 of sampled individuals.

301 We evaluated sampling and bulking errors by comparing 10 independent biological
302 replicates from 10 different landraces obtained by independently sampling and mixing
303 equal leaf areas of young leaves of 15 individuals. Allelic frequencies estimated for both
304 biological replicates from a same landrace were highly correlated. Excluding Pol3, 94.5%
305 of difference of allelic frequencies between replicates was of included within 95%
306 confidence limits originated from sampling effect only Figure 4). This suggests that the
307 “bulking error” is low compared to the “sampling error”. Consistently, Dubreuil et al.,
308 (1999) observed a low “bulking error” for RFLP markers using the same DNA pooling
309 method, with a coefficient of determination of 0.99 between allelic frequencies based on
310 individual genotyping of plants and those predicted using DNA bulks. Several studies also
311 showed that the effect of bulking errors on allelic frequencies measured by comparing
312 DNA pool and individual genotyping of plant of this DNA pool is very low compared with
313 other sources of errors (Le Hellard *et al.* 2002; Jawaid and Sham 2009). Additionally, the
314 mixing procedure starting from leaf samples strongly reduced the number of DNA
315 extractions for each DNA bulk as compared to first extracting DNA from each individual,
316 and then mixing by pipetting each DNA samples to obtain an equimolar DNA mix (“post-
317 extraction” approach). Since the cost of DNA extraction becomes non-negligible when the
318 number of individual increases, mixing plant material based on their mass before
319 extraction is highly relevant to save time and money. This can be done without losing
320 accuracy as shown in this study for SNP array and previously for RFLP by Dubreuil et al.,
321 (1999).

322 We highlighted the critical importance of the number of individuals sampled per
323 landrace on allelic frequency estimation (Table 3). By using DNA pooling, accuracy can be
324 gained with very little additional cost by increasing number of sampled individuals.
325 Whereas a high accuracy of allelic frequency estimation within landraces is required to
326 scan genome for selective sweeps, it is less important to estimate global genetic distance,
327 due to the large number of SNPs analyzed. Sampling fifteen plants per population (30
328 gametes) appears convenient to obtain an accurate estimation of frequencies in a
329 population and analyze genetic diversity (Reyes-Valdés *et al.* 2013).

330 ***A low ascertainment bias to estimate genetic distance between landraces***

331 There are two possible sources of ascertainment bias using a DNA pooling approach on a
332 SNP array. The first one relates to the design of array because the set of lines to discover SNPs
333 may not well represent genetic diversity and a threshold in allelic frequency was possibly
334 applied to select SNPs. The second one relates to the selection of a subset of SNPs from the
335 array regarding the genetic diversity of samples in calibration set used to predict allelic
336 frequencies.

337 To avoid risk of ascertainment bias due to selection of markers genotyped by the
338 array, the logistic regression model was adjusted on 1,000 SNPs with the largest allelic
339 frequency range rather than for each of the 23,412 PZE SNPs individually. Using a specific
340 model for each SNP would indeed conduct to discard markers monomorphic in controlled
341 pools and therefore select only markers polymorphic between parents of controlled pool.
342 Note that the same issue would be raised by using heterozygous individuals since 8 to 16
343 heterozygotes were recommended to adjust a logistic regression. Using heterozygous
344 individuals and SNP specific equations could lead to systematically counter-select SNPs
345 with low diversity. It could also lead to systematically remove SNPs that are differentially
346 fixed between isolated genetic groups, because no or very few heterozygote individuals
347 are available.

348 We also evaluated ascertainment bias by comparing Modified Roger's Distance (MRD)
349 between the 156 landraces obtained using SNPs (MRD_{SNP}) and SSRs (MRD_{SSR}) (Camus-
350 Kulanidavelu 2006; Mir *et al.* 2013), which display no or limited ascertainment bias.
351 MRD_{SNP} was highly correlated with MRD_{SSR} ($r^2 = 0.64$; Figure 5). This correlation is high
352 considering that SSR and SNP markers evolve very differently (mutation rate higher for
353 SSRs than SNPs, multiallelic vs biallelic), that the number of SSR markers used to estimate
354 genetic distance is low and that errors in allelic frequency prediction occur for both SNPs
355 and SSRs. For comparison, correlation was lower than between Identity By State
356 estimated with 94 SSRs and 30k SNPs in a diversity panel of 337 inbred lines ($r^2 = 0.41$)
357 , although very few genotyping errors are expected in inbred lines (Bouchet *et al.* 2013).
358 Using the *wd* criterion significantly increased the correlation between MRD_{SNP} and MRD_{SSR}
359 markers for 156 landraces (Figure S5). It suggests that the *wd* criterion removes SNP
360 markers that blurred the relationships between landraces. We can therefore define a
361 subset of 23,412 SNPs to analyze global genetic diversity in landraces. This is in

362 agreement with previous studies in inbred lines showing that PZE SNPs are suitable to
363 analyze the genetic diversity in inbred lines (Inghelandt *et al.* 2011; Ganal *et al.* 2011;
364 Bouchet *et al.* 2013; Frascaroli *et al.* 2013). These studies showed that diversity analysis
365 based on PZE SNPs give consistent results with previous studies based on SSR markers
366 (Inghelandt *et al.* 2011; Bouchet *et al.* 2013; Frascaroli *et al.* 2013).

367 The DNA pooled-sampling approach therefore provides a reliable picture of the
368 genetic relatedness among populations that display a large range of genetic divergence
369 and opens a way to explore genome-wide diversity along the genome.

370 ***An affordable, high-throughput, labor-efficient and robust method*** 371 ***compared to SSR / RFLP markers and sequencing approaches***

372 Using SNP arrays instead of SSR/RFLP marker systems or sequencing approaches
373 has several advantages. First, SNP genotyping using arrays is very affordable compared
374 to SSR/RFLP or resequencing approaches because it is highly automatable, high-
375 throughput, labor-efficient and cost effective (currently 30-80€ / individual depending of
376 array). Obtaining accurate estimations of allelic frequencies using a whole genome
377 sequencing (WGS) approach requires high depth and coverage for each individual
378 because of the need of counting reads (Schlötterer *et al.* 2014). To estimate allelic
379 frequency in DNA bulks, WGS remains costly compared to SNP arrays for large and
380 complex genomes of plant species as maize. Different sequencing approaches based either
381 on restriction enzyme or sequence capture make it possible to target some genomic
382 regions and multiplex individuals, reducing the cost of library preparation and
383 sequencing while increasing the depth for the selected regions (Glaubitz *et al.* 2014).
384 However, these sequencing approaches remain more expensive than SNP arrays and
385 require laboratory equipment to prepare DNA libraries and strong bioinformatics skills
386 to analyze sequencing data. These skills are not always available in all genebanks. With
387 the maize 50K array, FIR measurement used to predict allelic frequencies were highly
388 reproducible both across laboratories and batches ($r^2 = 0.987$; Figure 3). We can
389 therefore consistently predict allelic frequencies using 50K array in new DNA pools
390 genotyped in other laboratories, by applying the same parameters of presence /absence
391 test and logistic regression as in this study. This will greatly facilitate the comparison of
392 accessions across collections and laboratories. This is a strong advantage over SSRs for

393 which a strong laboratory effect has been observed for the definition of alleles, leading to
394 difficulties for comparing genetic diversity across seedbanks and laboratories (Mir *et al.*
395 2013). Similarly, one can expect some laboratory effect for sequencing approaches due to
396 preparation of library and bioinformatics analysis. However, there is some disadvantage
397 to use SNP arrays instead of SSR markers or sequencing approach. First, SNP marker are
398 bi-allelic whereas SSRs are multi-allelic. At a constant number of markers, using SNPs
399 rather than SSRs therefore leads to less discriminative power (Laval *et al.* 2002; Hamblin
400 *et al.* 2007). This disadvantage is largely compensated by the higher number of SNPs and
401 the fact that SNPs are more frequent and more regularly spread along the genome than
402 SSR/RFLP, allowing genome wide diversity analyses. Second, contrary to SSR / RFLP
403 markers and sequencing approach, SNP array does not allow one to discover new
404 polymorphisms, which may lead to ascertainment bias for diversity analysis of new
405 genetic groups (Nielsen 2004; Clark *et al.* 2005; Hamblin *et al.* 2007; Inghelandt *et al.*
406 2011; Frascaroli *et al.* 2013). Comparison with SSRs results showed that PZE SNPs
407 provide reliable genetic distances between landraces, suggesting a low ascertainment
408 bias for a global portrayal of genetic diversity (see above). Sequencing techniques may be
409 interesting in a second step to identify, among preselected accessions, those which show
410 an enrichment in new alleles.

411 The number of SNPs affects the estimates of relationship between landraces and
412 population structure (Moragues *et al.* 2010). In our study, the correlation coefficient
413 between MRD_{SNP} and MRD_{SSR} increased with increasing number of SNPs and reached a
414 plateau for 2,500 SNPs (Figure S6). This suggests that increasing the number of SNPs
415 above 2,500 does not provide further improvement in precision to estimate relationships
416 between landraces as compared to 17 SSRs. Our approach could therefore be made further
417 cost efficient by selecting less loci for studying global genetic relationships and genetic
418 diversity. For maize, a customizable 15K Illumina genotyping array has been developed that
419 includes 3,000 PZE SNPs selected for studying essential derivation (Rousselle *et al.* 2015) and
420 12,000 others selected for genetic applications such as genomic selection. Alternatively, the
421 same approach could be applied to other genotyping arrays with higher density as the 600K
422 Affymetrix Axiom Array (Unterseer *et al.*, 2013) to gain precision in detection of selective
423 footprints.

424 **CONCLUSION**

425 The DNA pooling approach we propose overcomes specific issues for genetic diversity
426 analysis and plant germplasm management purposes that were not or partially addressed by
427 previous methods which were mostly focused on QTL analysis and genome wide association
428 studies (Hoogendoorn *et al.* 2000; Craig *et al.* 2005; Brohede 2005; Teumer *et al.* 2013). As
429 proof of concept, we used the DNA pooling approach to estimate allelic frequencies in maize
430 landraces in order to identify original maize landraces in germplasm for pre-breeding
431 purposes and selective footprints between geographic and/or admixture groups of
432 landraces cultivated in contrasted agro-climatic conditions (Arca *et al.*, in prep). Our
433 approach could be very interesting for studying plant germplasm since time, money and
434 molecular skills can be limiting factors to study and compare large collections of landraces
435 maintained in seedbank (Mir *et al.* 2013). Applications could be expanded to QTL
436 identification in pools (Gallais *et al.* 2007), detecting signatures of selection in multi-generation
437 experiments, or detection of illegitimate seed-lots during multiplication in genebanks. The DNA
438 pooling approach could be easily applied to decipher organization of genetic diversity in
439 other plant germplasm since Infinium Illumina HD array have been developed for several
440 cultivated plant species, including soybean, grapevine, potato, sweet cherry, tomato,
441 sunflower, wheat, oat, brassica crops but also animal species.

442 **MATERIALS AND METHODS**

443 ***Plant material***

444 ***Landraces***

445 A total of 156 landrace populations (Table S4) were sampled among a panel of 413
446 landraces capturing a large proportion of European and American diversity and analyzed in
447 previous studies using RFLP (Dubreuil and Charcosset 1998; Rebourg *et al.* 1999, 2001, 2003;
448 Gauthier *et al.* 2002) and SSR markers (Camus-Kulandaivelu 2006; Dubreuil *et al.* 2006; Mir
449 *et al.* 2013).

450 Each population were represented by a bulk of DNA from 15 individual plants, mixed in
451 equal amounts as described in Reif *et al.* (2005) and Dubreuil *et al.* (2006). In order to analyze

452 the effect of individual sampling on allelic frequency estimation (see below), ten populations
453 were represented by two DNA bulks of 15 plants sampled independently (Table 3).

454 ***Controlled DNA Pools***

455 To calibrate a prediction model for SNP allelic frequencies in populations, we considered
456 two series of nine controlled pools derived from the mixing of two sets of three parental inbred
457 lines: EP1 – F2 – LO3 (European Flint inbred lines) and NYS302– EA1433 – M37W (Tropical
458 inbred lines).

459 For each set of three parental lines, we prepared nine controlled pools by varying the
460 proportion of each line in the mix (Table 1), measured as the number of leaf disks with equal
461 size according to Dubreuil et al., (1999). The proportion of lines 2 and 3 (EA1433 and M37W
462 or F2 and LO3) varies similarly whereas line 1 (EP1 or NYS302) varies inversely. The genotype
463 of the inbred lines and the proportion of each inbred line in each pool give the expected allelic
464 frequencies as shown in Table 1. Combination of genotypes in parental lines can conduct either
465 to monomorphic or polymorphic controlled pools if the genotypes of 3 parental lines are the
466 same or not, respectively. If we exclude monomorphic controlled pools and heterozygote SNPs
467 in parental lines, these different combinations conduct to four different polymorphic
468 configurations in the 9 controlled pools, corresponding to four ranges of allelic frequencies: 1-
469 33% (R1), 33-50% (R2), 51-67% (R3), 67-99% (R4), (Table 1). Combination of R1 and R4
470 configurations in two series of controlled pools displayed the largest allelic frequencies range
471 (1% to 99%) while combination of R2 and R3 displayed a more reduced allelic frequency range
472 (33% to 67%).

473 ***Inbred lines***

474 To test for allele fixation within landraces, we used a panel of 333 inbred maize lines
475 representing the worldwide diversity well characterized in previous studies (Camus-
476 Kulandaivelu 2006; Bouchet *et al.* 2013) (Table S5). This panel includes the six inbred lines
477 used to build two series of controlled pools.

478 ***Genotyping***

479 We used the 50K Illumina Infinium HD array (Ganal *et al.* 2011) to genotype (i) 166 DNA
480 bulks representing 156 landraces (ii) 18 DNA bulks representing 2 series of controlled DNA
481 pools (iii) 333 inbred lines. 50K genotyping was performed according to the manufacturer's
482 instructions using the MaizeSNP50 array (IlluminaInc, San Diego, CA). The genotype results

483 were produced with GenomeStudio Genotyping Module software (v2010.2, IlluminaInc) using
484 the cluster file MaizeSNP50_B.egt available from Illumina. The array contains 49,585 SNPs
485 passing quality criteria defined in (Ganal *et al.* 2011).

486 We also used 17 SSRs genotyping data from 145 and 11 landraces analyzed by Camus *et*
487 *al.* (2006) and Mir *et al.* (2013), respectively.

488 ***Measurement variable: fluorescence intensities ratio***

489 The MaizeSNP50 array has been developed into allele-specific single base extension using
490 two colors labeling with the Cy3 and Cy5 fluorescent dyes. The fluorescent signal on each spot
491 is digitized using GenomeStudio software. Data consist of two normalized intensity values (*x*,
492 *y*) for each SNP, with one intensity for each of the fluorescent dyes associated with the two
493 alleles of the SNP. The alleles measured by the *x* intensity value (Cy5 dye) are arbitrary, with
494 respect to haplotypes, are called the A alleles, whereas the alleles measured by the *y* intensity
495 value (Cy3 dye) are called the B alleles.

496 We assumed that the strength of the fluorescent signal of each spot is representative of the
497 amount of labeled probe associated with that spot. The amount of labeled probes at each spot
498 relies upon the frequency of the corresponding alleles of PCR product immobilized on it. Based
499 on this assumption, the fluorescent intensity ratio (FIR) of each spot ($y/(x+y)$) can be employed
500 to estimate the allele frequency of DNA bulk immobilized on it.

501 To test the reproducibility of the measurement the controlled pool of European lines was
502 genotyped twice in two platforms, at CNG Genotyping National Center, Evry 91, France, and
503 at Trait Genetics.

504 ***SNP filtering and quality control***

505 For the purpose of this study, we used only the subset of 32,788 markers contributed by the
506 Panzea project (<http://www.panzea.org/>), so called PZE SNPs, developed on the basis of US
507 NAM founders (Zhao 2006). These SNPs represent a comprehensive sample of the maize
508 germplasm and are therefore suitable for diversity analysis (Ganal *et al.* 2011).

509 The following equation (1) was then used to create a rank score (weighted deviation, *wd*)
510 for each SNP in order to identify and remove those of poor quality,

$$511 \quad wd = \frac{|\mu_{AA} - \mu_{BB}|}{\sqrt{\frac{N_{AA} \cdot \sigma_{AA}^2 + N_{BB} \cdot \sigma_{BB}^2}{N_{AA} + N_{BB}}}} \quad (1)$$

512 where μ_{AA} and σ_{AA} and μ_{BB} and σ_{BB} are the mean and the standard deviation for the
513 fluorescence intensity ratios of AA and BB genotypes for the 327 inbred lines panel and N_{AA}
514 and N_{BB} is the number of inbred lines with genotype AA or genotype BB respectively. To avoid
515 selection bias, loci which were monomorphic within the reference inbred lines population were
516 selected using the wd equations (1), assuming $\mu_{AA}=0$ and $\sigma_{AA}=0$ for monomorphic BB SNPs
517 or assuming $\mu_{BB}=1$ and $\sigma_{BB}=0$ for monomorphic AA SNPs.

518 This criterion removes from analysis those SNPs for which distributions of fluorescence
519 signal ratios for AA and BB genotypes of 327 inbred lines panel overlap or have large variances.
520 To analyze genetic diversity, we first selected 23,656 with wd above 50 among 32,788 PZE
521 SNPs. This threshold removed SNPs displaying high error rate in allelic frequency prediction
522 (Figure S1). In addition, we removed 244 SNPs that were heterozygous in one of parental lines
523 of controlled pools and that displayed high error rate in allelic frequency prediction (data not
524 shown).

525 ***Alleles detection and allele frequency estimation***

526 Allele frequency estimation within DNA pools was implemented as a two-step process. We
527 first determined the fixation of alleles A and/or B by comparing the fluorescent ratio of DNA
528 pools at a given SNP locus with the distribution of the fluorescent signal of inbred lines (see
529 above) which have AA or BB genotypes at the same locus. We assumed Gaussian distributions
530 for the fluorescent intensities and tested for fixation using a Student's t-tests with a 5% type I
531 nominal level.

532 In second step, for each SNP for which alleles A and B were both declared present, the
533 allelic frequency f_B of allele B was inferred using the following generalized linear model:

$$534 \quad g(f_B) = \alpha + \beta \frac{y}{x+y} \quad (2)$$

535 where x and y are the fluorescent intensities at SNP for alleles A and B respectively, α and
536 β are the parameters of a logistic curve, calibrated on fluorescent ratio data from controlled
537 pools for 1000 SNPs and ε_i is a noise term. As allele B frequency is a binomial variable, GLM
538 was set with a logit link function (R, version 3.0.3).

539 The calibration sample of 1,000 SNPs consists in 250 randomly selected SNPs for each
540 possible configuration (R1, R2, R3, R4 defined in Table 1). It was preferred to a calibration
541 sample of all SNPs or to a specific prediction curve for each SNPs, in order to have a
542 homogeneous distribution of observations into each class of expected frequency. Calibrating

543 the model for each SNP would lead to high error in allelic frequency prediction, notably for
544 monomorphic controlled pools as exemplified by Figure S3 and S4. Calibrating model for all
545 SNPs would give strong weight to fixed allele in calibration due to large number of
546 monomorphic controlled pools that are homozygous either for allele A or B.

547 ***Accuracy of allelic frequency estimation***

548 We assessed the accuracy of allele frequency estimates from pooled DNA samples by
549 calculating the absolute difference between allelic frequencies of the B allele predicted by our
550 two-step model and those expected for controlled pools from the genotype of their six parental
551 lines. We obtained expected allelic frequencies for two series of controlled pools by weighting
552 the allelic frequency of each parental line (0 or 1) by their relative mass in the mix (Table 1).
553 We obtained genotypes of inbred lines from clustering by genome studio. This absolute
554 difference was averaged over SNPs and samples in order to obtain mean absolute error (MAE).

555 We first evaluated the mean absolute error for 23,412 SNPs in the two series of controlled
556 pools (Table S2, Figure 2). In order to estimate the effect of the calibration set of individuals
557 and SNPs on the accuracy of allelic frequency prediction, we applied two cross-validation
558 approaches on the 1000 SNPs and the two series of controlled pools and six parental inbred
559 lines (24 samples) used to calibrate parameters of the common logistic regression. In order to
560 evaluate the effect of SNP calibration set (Table S1), we repeated five time a K-fold approach
561 in which 1000 SNPs were split randomly in a training set of 800 SNPs on which we calibrated
562 our two-step model and a validation set of 200 SNPs on which we predicted allelic frequency
563 using this model in same two series controlled pools and estimated MAE. In order to evaluate
564 the effect calibration samples (Table 2), we repeated 1000 times a K-fold approach on 1000
565 SNPs in which 1, 3, 5, 8, 10, 15 samples among 18 from controlled pools were randomly
566 removed from the calibration set. We used the remaining samples to estimate parameters of the
567 logistic regression, and then predicted allelic frequencies using this predictive equation in these
568 K removed samples (Table 2).

569 To estimate sampling error (Table 3), we estimated the 95% confidence interval of the
570 allelic frequency in the population considering various observed allelic frequency obtained by
571 sampling either 15, 30, 100 or 200 individuals from this population. To obtain the lower and
572 upper bound of the 95% confidence interval for allelic frequency in the population, we
573 considered the binomial probability to obtain various number of allele B in 15, 30, 100, 200
574 individuals (estimated allelic frequencies) from a population (true allelic frequencies) by using

575 binom.confint function implemented in R package “binom”. We used the following parameters:
576 binom.confint(x = number of alleles observed, n = 2*number of individuals, conf.level=95%,
577 methods = exact) with x = number of successes and n = number of trial in the binomial
578 experiment.

579 ***Comparison of genetic distance between SNP and SSR markers***

580 We calculated the modified Roger’s distance (MRD) (Rogers 1972) based on allelic
581 frequency data between landraces using different sets of markers to analyze the effect of the *wd*
582 criterion (Figure S5) and of the number of markers (Figure S6) on the estimation of relatedness.
583 To analyze the effect of *wd* criterion, we selected four random sets of 2,000 SNPs with different
584 *wd* ranges (0-20, 20-40, 40-60, 60-80) among 32,788 PZE SNPs. To analyze the effect of SNP
585 number, we selected six random sets of SNPs with various number of SNPs (15,000, 10,000,
586 5000, 2500, 1000, 500) among 23,412 SNPs with *wd* above 50. In order to test if the genetic
587 distance is robust when changing the type and the number of markers, we compared MRD
588 between landraces estimated with different SNP datasets with that estimated with 17 SSR
589 markers (Figure 5, Figure S5 and Figure S6). Missing allele frequencies within accession were
590 replaced by corresponding average frequencies within the whole set of accessions before
591 running this analysis. Allelic frequencies of two samples for replicated landraces were averaged
592 before estimating MRD distance except for Pol3 for which one of two samples was removed
593 (WG0109808-DNAH04).

594 Coefficient of determination between the distance matrices based on different subsets of
595 SNP (MRD_{SNP}) and 17 SSR markers (MRD_{SSR}) was determined by using linear regression.

596

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612 ***Author's contribution***

613 S.D.N, A.C and B.G designed and supervised the study and selected the plant material

614 M.A, S.D.N, A.C drafted and corrected the manuscript

615 D.M, V.C and A.B extracted DNA and managed genotyping of landraces and inbred lines

616 C.B, B.G and A.C collected, maintained landraces, and inbred lines collection

617 S.D.N, M.A, A.C and T.M-H developed the statistical methods and scripts for predicting
618 allelic frequency from fluorescent data

619 M.A, B.G and S.D.N analyzed genetic diversity of landraces panel.

620 All authors read and approved the manuscript.

621 ***Data availability***

622 R scripts and fluorescent intensity data of 327 inbred lines and two series of controlled pools
623 used for predicting allelic frequency in DNA bulks of maize landraces by our two-step
624 approaches are available at <https://doi.org/10.15454/GANJ7J>. Fluorescent Intensity data and
625 allelic frequencies of 20 samples corresponding to 10 duplicated landraces were also available
626 at <https://doi.org/10.15454/GANJ7J>. Allelic frequencies of new DNA bulks for new maize
627 populations genotyped by maize 50K array could be predicted by using these datasets with R
628 scripts. Note that these datasets and R scripts will become available when the publication would
629 be accepted in a peer review journal.

630 ***Conflicts of interest***

631 No

632

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Table 1: Expected frequencies of allele B for the nine controlled pools obtained by varying the proportions of leaf weights of three inbred lines (L1, L2, L3) according to their genotypes at a bi-allelic SNP coded A/B. Heterozygous genotypes for inbred lines were not considered in this table.

Pools	Proportion of leaf weights			Genotypes of parental lines L1, L2, L3 in controlled pools					
	L1	L2	L3	AA,AA,AA	BB,AA,AA	AA,AA,BB OR	BB,AA,BB or	AA,BB,BB	BB,BB,BB
#1	0.01	0.495	0.495	0%	1%	50%	51%	99%	100%
#2	0.02	0.49	0.49	0%	2%	49%	51%	98%	100%
#3	0.03	0.485	0.485	0%	3%	49%	52%	97%	100%
#4	0.05	0.475	0.475	0%	5%	48%	53%	95%	100%
#5	0.07	0.465	0.465	0%	7%	47%	54%	93%	100%
#6	0.1	0.45	0.45	0%	10%	45%	55%	90%	100%
#7	0.15	0.425	0.425	0%	15%	43%	58%	85%	100%
#8	0.2	0.4	0.4	0%	20%	40%	60%	80%	100%
#9	0.333	0.333	0.333	0%	33%	33%	67%	67%	100%
Configuration of controlled pools				Monomorphic	R1	R2	R3	R4	Monomorphic

Table 2: Mean absolute error (MAE) in frequency estimation for 1,000 SNPs used to calibrate logistic regression equations. MAE is estimated by a cross-validation procedure in which a number of pools comprised between 1 and 15 among 18 is removed at random from the calibration set. This procedure was repeated 1,000 times for each SNP.

# of removed samples	# of repetitions	Mean absolute error (MAE)	
		Mean	SD*
1	1000	0.0414	0.0219
3	1000	0.0428	0.0226
5	1000	0.0447	0.0232
8	1000	0.0484	0.0245
10	1000	0.0522	0.0257
12	1000	0.0582	0.0274
15	1000	0.0854	0.0309

* SD = Standard deviation

Table 3: Sampling error estimated by numerical calculation for one or two biological replicates with independent sampling of 15 or 100 individuals within landraces. Lower and upper bounds indicate the 95% confidence interval for the allelic frequency in the population, based on the binomial probability of the frequency estimated with the corresponding sample size.

Allelic Frequency	15 individuals						100 individuals					
	One biological replicate			Two biological replicates			One biological replicate			Two biological replicates		
	# Alleles	Lower bound	Upper bound	# Alleles	Lower bound	Upper bound	# Alleles	Lower bound	Upper bound	# Alleles	Lower bound	Upper bound
0	0	0	0.116	0	0	0.06	0	0	0.018	0	0	0.009
0.03	1	0.001	0.172	2	0.004	0.115	6	0.011	0.064	13	0.017	0.055
0.1	3	0.021	0.265	6	0.038	0.205	20	0.062	0.15	40	0.072	0.134
0.2	6	0.077	0.386	12	0.108	0.323	40	0.147	0.262	80	0.162	0.243
0.3	9	0.147	0.494	18	0.189	0.432	60	0.237	0.369	120	0.256	0.348
0.4	12	0.227	0.594	24	0.276	0.535	80	0.332	0.472	160	0.352	0.45
0.5	15	0.313	0.687	30	0.368	0.632	100	0.429	0.571	200	0.45	0.55
0.6	18	0.406	0.773	36	0.465	0.724	120	0.529	0.669	240	0.55	0.648
0.7	21	0.506	0.853	42	0.568	0.812	140	0.631	0.763	280	0.653	0.745
0.8	24	0.614	0.923	48	0.677	0.892	160	0.738	0.853	320	0.757	0.838
0.9	27	0.735	0.979	54	0.795	0.962	180	0.85	0.938	360	0.866	0.928
1	30	0.884	1	60	0.94	1	200	0.982	1	400	0.991	1

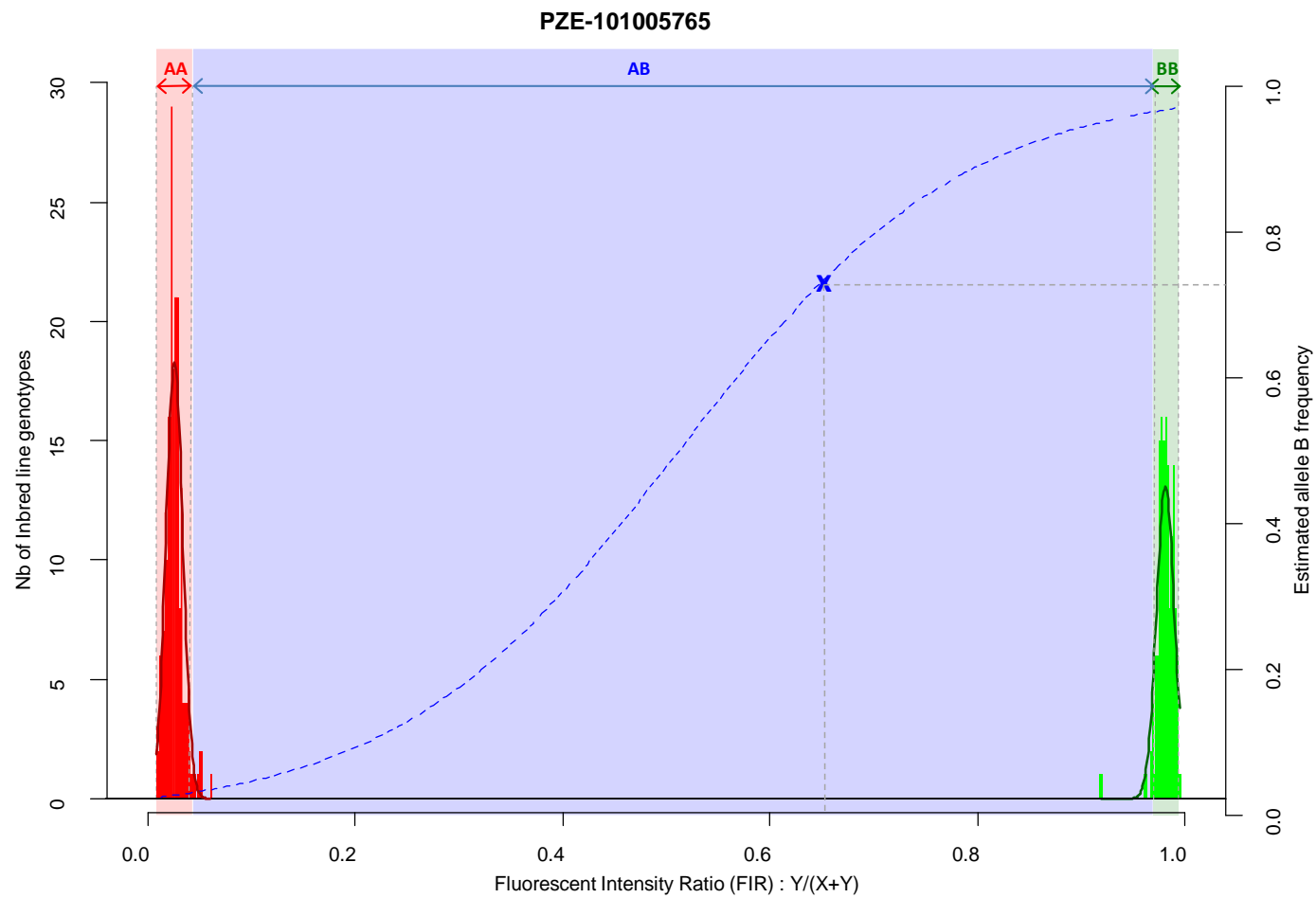


Figure 1: Two-step approach for estimating allelic frequency in DNA pools, exemplified by marker PZE-101005765. Red and green histograms correspond to the fluorescent intensity ratio (FIR) distribution for inbred lines homozygote for allele A (AA) and B (BB), respectively. Red and green curves indicate the corresponding Gaussian distributions. Red, Blue, and Green areas correspond to the FIR for which landraces are declared homozygous for allele A, polymorphic and homozygous for allele B after testing for fixation of alleles A and B. Dotted blue line corresponds to the curve of the logistic regression adjusted on 1,000 SNPs and two series of controlled pools. Blue cross corresponds to a landrace represented by a DNA bulk of 15 individuals, with its observed FIR on X axis and predicted frequency on Y axis.

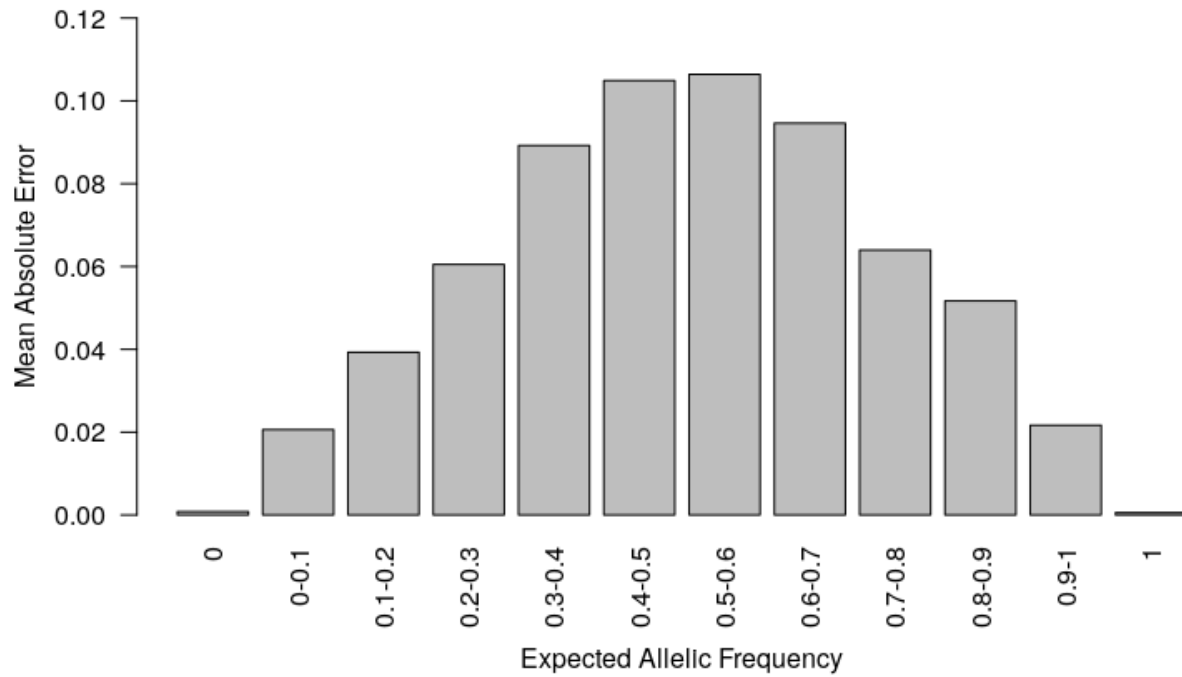


Figure 2: Mean absolute error (MAE) according to the known allelic frequency in two series of controlled pools. MAE measured the absolute difference between allelic frequencies predicted by the two-step approach and those expected from the genotypes of parental lines in two series of controlled pools for 23,412 SNPs. MAE is averaged for each interval of expected allelic frequency across all SNPs.

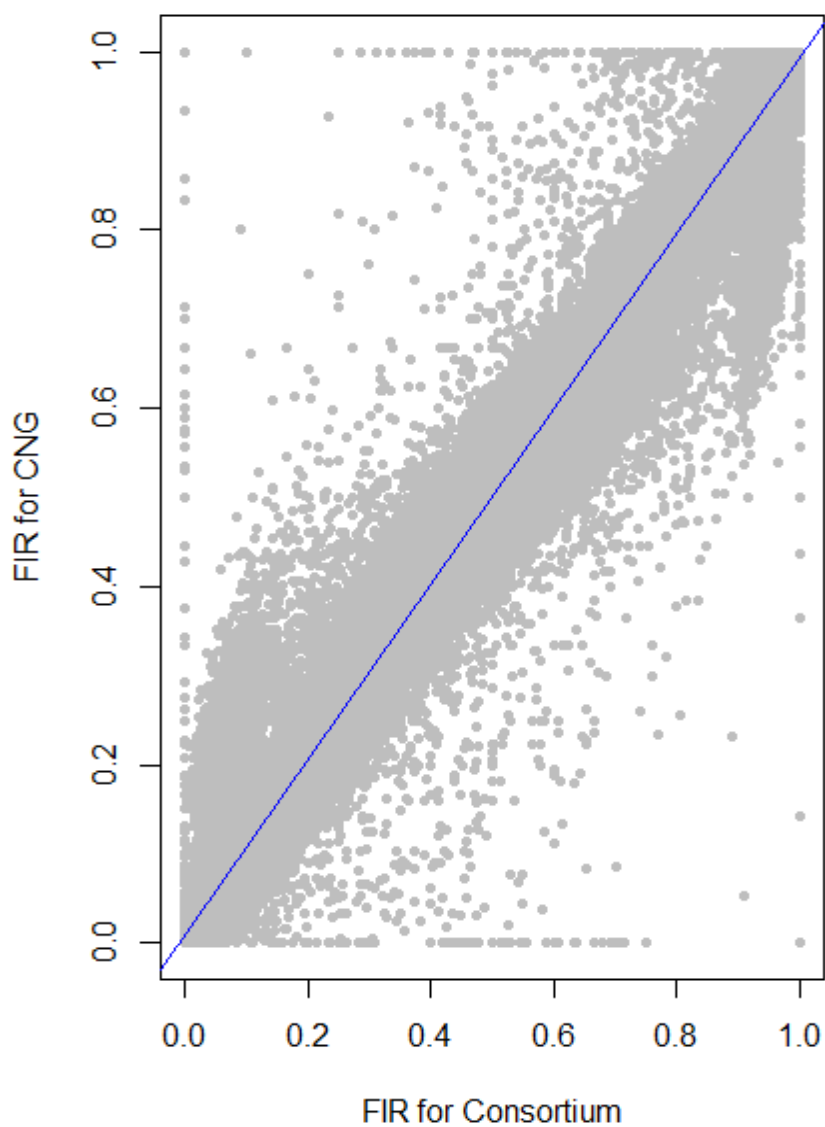


Figure 3: Relationship between fluorescent intensity ratio of European Flint controlled pools genotyped in two different laboratories: CNG and Consortium. Each dot represents the combination of one out 9 controlled pools and one out of 23,412 PZE SNPs. Coefficient of determination (r^2) between FIR of two laboratories is 0.987.

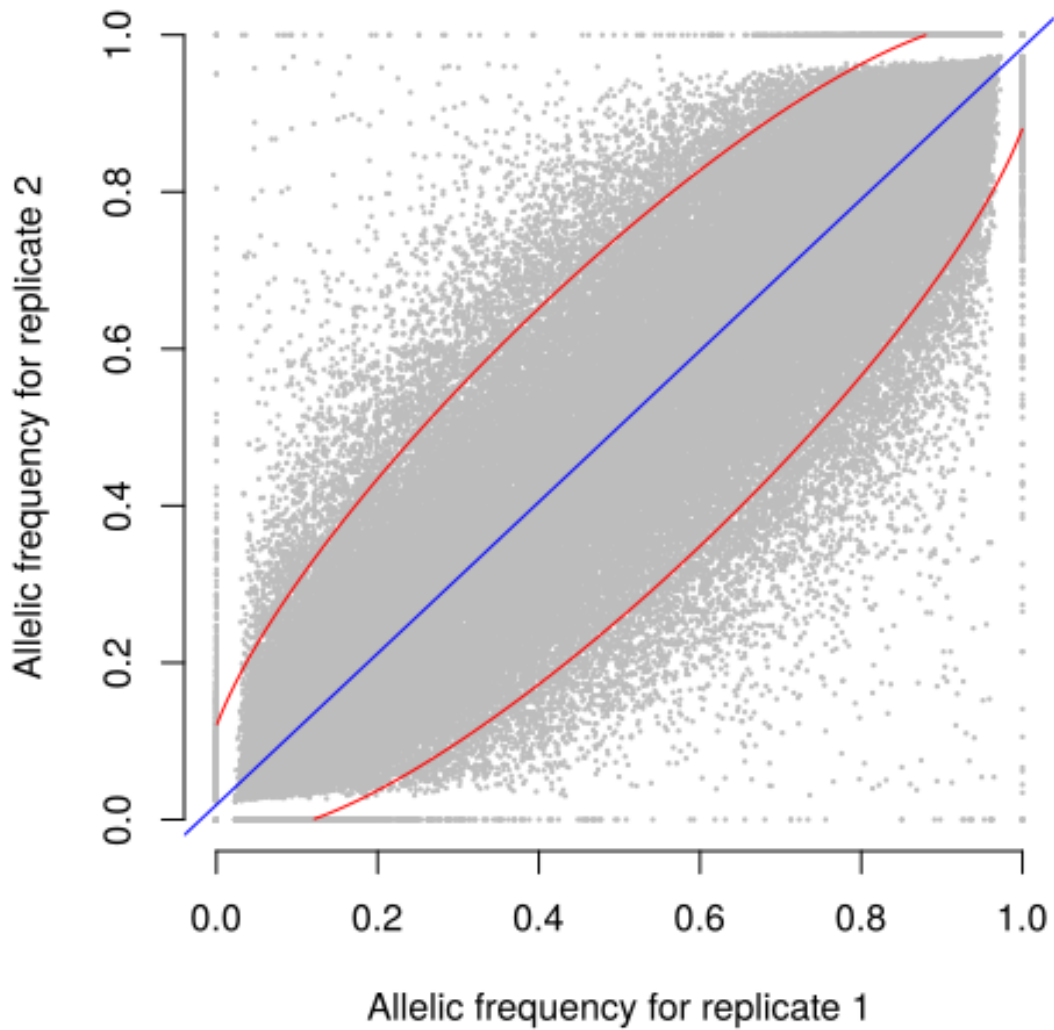


Figure 4: Relationship between allele frequencies predicted for two biological replicates of 9 landraces over 23,412 selected SNPs. Each dot represents one landrace and one SNP, with allele frequency of replicates 1 and 2 on X and Y axes, respectively. Blue line indicates linear regression. 94.5% of points are included in the red ellipse that represents the 95% confidence limit accounting for the effect of sampling alone. r^2 between replicates is 0.93

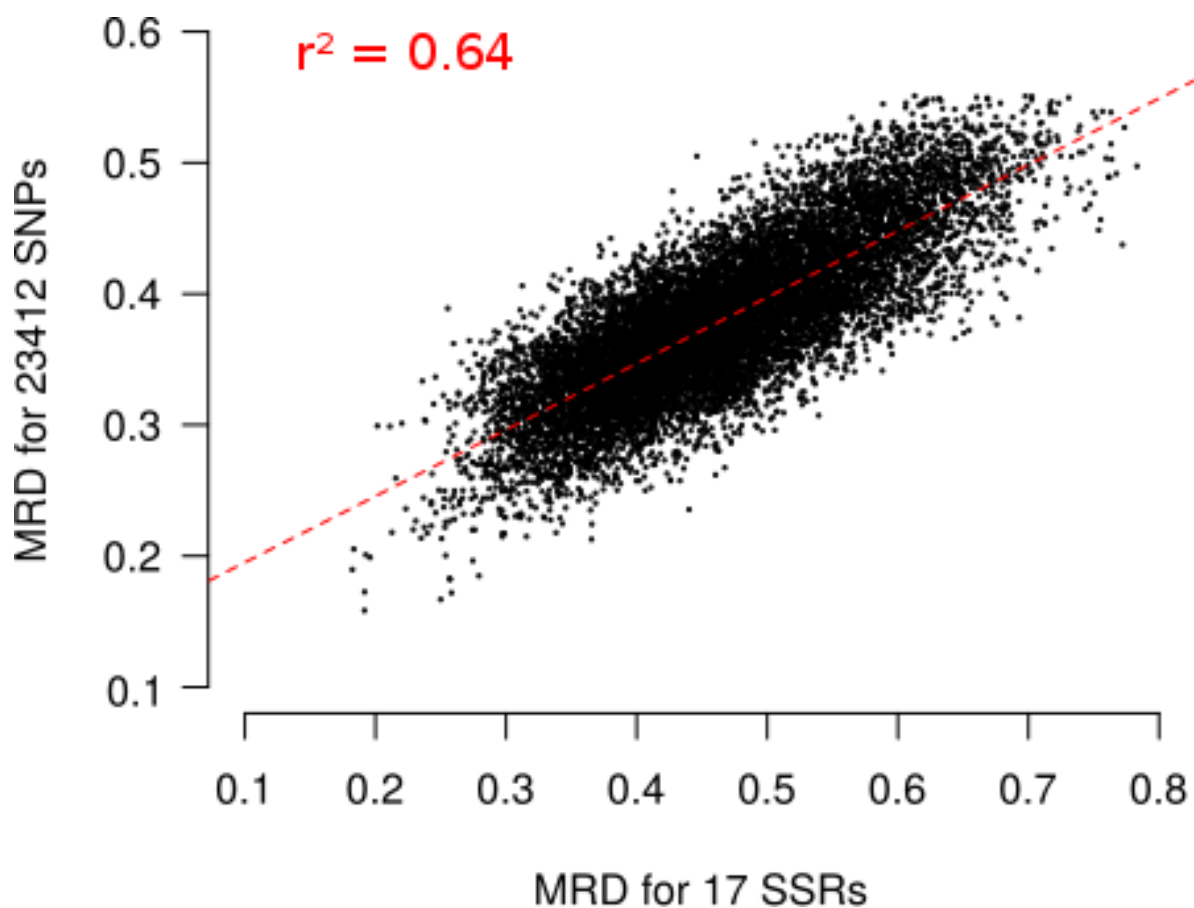


Figure 5: Relationship between Modified Roger's Distances (MRD) obtained with 17 SSRs and 23,412 SNPs for 156 landraces. Each dot represents one pair of landraces. Red dotted lines represents linear regression between MRD_{SSR} and MRD_{SNP} . Coefficient of determination (r^2) is reported on the plot.