

THE IMPORTANCE OF DEEP GENOTYPING IN CROP BREEDING



LA IMPORTANCIA DE LA GENOTIPIFICACIÓN PROFUNDA EN EL MEJORAMIENTO GENÉTICO

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ABSTRACT

One of the greatest challenges facing humanity is the development of sustainable strategies to ensure food availability in response to population growth and climate change. One approach that can contribute to increase food security is to close yield gaps and enhancing genetic gain; to such end, what is known as “molecular breeding” plays a fundamental role. Since a crop breeding program is mainly based on the quality of the germplasm, its detailed genetic characterization is mandatory to ensure the efficient use of genetic resources and accelerating development of superior varieties. Deep genotyping is an essential tool for a comprehensive characterization of the germplasm of interest and, fortunately, the technology is now accessible at a reasonable cost. What must be ensured is the correct interpretation of the genotypic information and on that basis develop efficient practical molecular crop breeding strategies that respond to the real needs of the breeding program.

Key words: breeding population, genetic resources, marker assisted selection, Single Nucleotide Polymorphism (SNP)

RESUMEN

Uno de los mayores desafíos que enfrenta la humanidad es el desarrollo de estrategias sostenibles para asegurar la disponibilidad de alimentos en respuesta al crecimiento de la población y el cambio climático. Un enfoque que puede contribuir a aumentar la seguridad alimentaria es cerrar las brechas de rendimiento y mejorar la ganancia genética; para tal fin, lo que se conoce como “mejoramiento molecular” juega un papel fundamental. Dado que un programa de mejoramiento de cultivos se basa principalmente en la calidad del germoplasma, su caracterización genética detallada es fundamental para garantizar el uso eficiente de los recursos genéticos y acelerar el desarrollo de variedades superiores. La genotipificación profunda es una herramienta esencial para una caracterización integral del germoplasma de interés y, afortunadamente, en la actualidad se puede acceder a la tecnología a un costo razonable. Lo que debe asegurarse es la interpretación correcta de la información genotípica y sobre esa base desarrollar estrategias eficientes y prácticas de mejoramiento molecular de cultivos que respondan a las necesidades reales del programa de mejoramiento.

Palabras clave: población de mejoramiento, recursos genéticos, selección asistida por marcadores, polimorfismo de nucleótido único (SNP)

INTRODUCTION

Crop breeding is a long-term process that usually takes around ten years to develop and release a new variety. Crop breeding is a large-scale logistical operation involving thousands to hundreds of thousands of plants in the initial line fixation stage, but numbers are greatly reduced to a small number of selected advanced breeding lines by the end of the process: approximately 99% of the original starting material in a breeding program is rejected and discarded (Lenaerts *et al.*, 2019). The Food and Agriculture Organization of the United Nations defined modern plant breeding as “the act of using genetic diversity to improve the agronomic performance of plants conducted as a formal endeavor and according to scientific principles” (FAO, 1997). Cooper *et al.* (2014) defined modern plant breeding as an integration of quantitative genetics, statistics, gene-to-phenotype knowledge, and development models, applied to understand the functional diversity of germplasm (Smith *et al.*, 2015).

Crop improvement in a context of continuous population growth and with climatic changes affecting agronomic production has become a major global concern (Hickey *et al.*, 2019). Faced with these threats, current crop improvement strategies are unlikely to achieve genetic gains that satisfy the demand for food both in terms of quantity and quality. In addition, radical changes derived from climate change are causing heat stress and drought, which leads to significant yield losses, so plant breeding strategies need to be adapted to increase their efficiency.

The application of molecular genetics in crop improvement has spread significantly since the appropriate use of the so-known “molecular breeding” (i.e., genotype-based approaches) has demonstrated to contribute to increase genetic gain with a highly favorable cost-benefit ratio (Ismail and Horie, 2017; Xu *et al.*, 2017; Bailey-Serres *et al.*, 2019). The correct choice of genotyping technology allows a fine genetic characterization of germplasm, assisted selection, as well as the implementation of genomic selection strategies.

Lack of in-depth analysis when implementing a molecular breeding strategy can lead to failure, generating many undesirable results and discouraging breeders from using the technology. Consequently, before implementing a molecular breeding strategy, a serious analysis of its advantages and disadvantages is strongly recommended, taking into consideration the DNA technology of choice, the genetic diversity of the germplasm, the architecture of the traits of agronomic interest to be improved, and the resources demanded (Zambelli, 2019; Bohar *et al.*, 2020).

GENETIC CHARACTERIZATION OF GERmplasm

The configuration of an efficient molecular breeding strategy must begin with a comprehensive genetic characterization of the germplasm of interest through deep genotyping. Once characterization is complete, the next challenge is to identify useful applications of genotype-based technology to increase genetic gain.

Genetic characterization is relevant for germplasm management and agronomic use of both agricultural crops and their respective wild relative species. The use of genomic tools is today technical and practically more accessible than before, mainly due to the development of next-generation sequencing (NGS) technologies and the reduction of their application costs (Wu *et al.*, 2014; Dempewolf *et al.*, 2017; Milner *et al.*, 2019; Sansaloni *et al.*, 2020; Fu *et al.*, 2021).

The topics to be listed when addressing the genetic characterization of germplasm include SNP deep genotyping, genetic diversity, genetic relationships, linkage disequilibrium, association mapping, and population structure.

SNP deep genotyping

Different types of molecular marker systems have been used for genotyping applied in plant breeding: restriction fragment length polymorphisms (RFLPs), random-amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), Diversity Arrays Technology (DArT) and simple sequence repeats (SSRs). However, currently the most advanced and commonly used marker systems are single nucleotide polymorphisms (SNPs). Their abundance in genomes and the achievability to adapt them to automated platforms have expanded access to deep genotyping at reasonable costs, making SNPs the most widely adopted marker system for different genomic applications (Mondini *et al.*, 2009). With the increasing throughput of NGS technologies, *de novo* and reference-based SNP discovery are today feasible for most crop species.

When applying NGS two variables need to be attended: coverage and sequencing depth. Coverage indicates the average number of reads that cover a specific target genomic region, describing a relationship between the number of reads and a reference region, and can be expressed in terms of average coverage (for example, 10X means that on average the target regions are covered by 10 reads). Instead, sequencing depth describes the absolute number of total usable reads produced by sequencing, usually expressed in number of reads (in millions). Depending on the experimental objective of interest, coverage can vary from the entire genome, one

locus, or random nucleotide positions.

There are several genotyping methods available which are generally offered by commercial parties for which only tissue samples need to be sent for DNA extraction. Widely adopted genotyping options fall into three categories: whole genome resequencing (WGR), reduced representation sequencing (RRS), and SNP arrays (Scheben *et al.*, 2017). WGR and RRS methods are based on NGS technologies and bioinformatics pipelines that align reads to a reference genome and call both SNPs and genotypes (Scheben *et al.*, 2017; Pavan *et al.*, 2020). WGR differs from RRS in the absence of a stage of reduction of genome complexity. RRS usually employs restriction enzymes (RE) to digest genomic DNA prior to sequencing (method identified as RE-RRS) giving rise to genotyping-by-sequencing technology or GBS (Elshire *et al.*, 2011).

SNP arrays rely on allele-specific oligonucleotide (ASO) probes (including target SNP loci plus their flanking regions) fixed on a solid support, which are used to interrogate complementary fragments from DNA samples and infer genotypes based on the interpretation of the hybridization signal. The two leader manufacturers (Affymetrix™ and Illumina™) had developed 46 SNP arrays for 25 crop species with several markers ranging from 3K to 820K, although for their routine application in the molecular breeding of the most prominent field crops, arrays of 25–50K are usually chosen (Rasheed *et al.*, 2017).

The use of WGR, at least for the moment, is not considered financially feasible in large genome crops such as corn (2.5 Gbp), barley (5 Gbp), and wheat (17 Gbp). However, the final decision on the convenience of making the investment will depend on the commercial importance of the crop, the added value of the trait of interest and the expected net return. For most of molecular breeding applications, deep genotyping by using GBS or SNP arrays is recommended as they allow a satisfactory balance among the number of SNP loci genotyped, quality data, and costs.

In GBS technology, the allele-calling does not require a reference genome, offering an unbiased method to

assess genetic diversity in a large collection of accessions, especially in orphan crops because SNP discovery and genotyping can be done simultaneously with less bias toward genetic backgrounds (Rasheed *et al.*, 2017; Darrier *et al.*, 2019). When the germplasm includes commercial materials combined with exotic materials, GBS would be the most appropriate genotyping. The disadvantage of using SNP arrays is the risk that some SNPs may not be informative for all individuals. Since the ASO probes immobilized in the array are fixed and predefined (identified from a restricted set of genotypes, mostly public) the proportion of useful SNP for capturing the genetic diversity of the germplasm of interest cannot be predicted. Therefore, one of the limitations to work with SNP arrays is the ascertainment bias since they cannot identify marker-trait associations for SNPs that were not present in the population used for array development (Frascaroli *et al.*, 2013; Lachance and Tishkoff, 2013; Rasheed *et al.*, 2017; Negro *et al.*, 2019). Contrarily, GBS ensure that all SNPs discovered will be informative for all the sequenced genotypes of interest, producing high-quality polymorphism data. Although actual relative costs vary with the number of samples and the SNP density required, is widely considered that pricing of genotyping by GBS is lower than SNP arrays (Li *et al.*, 2015; Pavan *et al.*, 2020). An extra complexity of GBS respect to arrays is the necessity of library preparation and bioinformatics analysis (Elshire *et al.*, 2011; Li *et al.*, 2015; Sansaloni *et al.*, 2020; Fu *et al.*, 2021). The good news is that there are many companies that provide the service at reasonable prices. In Table 1, the main features of both genotyping technologies are summarized.

Once genotype information is collected (independently of the technology used), an adequate filtering criterion considering different indicators should be followed to define the high-quality genotyping dataset to avoid inaccuracies and bias in downstream analyses. The presence of SNP loci with a high rate of missing data is considered a feature of inaccurate genotype calls, so those SNPs should be excluded from the analysis. SNP loci characterized by excessive heterozygosity should

Table 1. Comparison of the main features of genotyping by SNP* array and GBS** technologies

Genotyping technology	Cost per sample	Cost per data point	Proportion of informative SNPs	Informative for exotic materials	Ascertainment bias
SNP array	Moderate - High	Low - Moderate	Moderate - High	Variable	Yes
GBS	Moderate	Low	High	Yes	No

*SNP= single nucleotide polymorphic; **GBS=(completar)

also be excluded, as they are indicative of technical artifacts or paralogous/repetitive regions that could not be distinguished through the genotyping procedure. SNP loci displaying very low frequency alleles may derive into genotyping errors and provide poor statistical power to reveal association with phenotypic traits or establishing relative kinship. Thus, the recommended conditions that SNPs should meet are: (i) up to 10% of missing genotype calls; (ii) up to 10% of heterozygous calls (assuming inbred lines are being genotyped); (iii) the number of heterozygous calls does not exceed the number homozygous minor allele counts; and (iv) minor allele frequency (MAF) > 0.05 (Wu *et al.*, 2014; Darrier *et al.*, 2019; Milner *et al.*, 2019; Pavan *et al.*, 2020).

Genetic diversity and genetic relationships

Productivity of most of field crops remains far below the potential due to several factors such as access to high quality seeds, irrigation, and fertilizers, abiotic stresses, high incidence of pests and diseases, and weeds. However, genetic improvement provides an approach to address some of these constraints, but largely depends on the availability of genetic diversity, systematic classification, and efficient use of the available germplasm.

A high-impact activity that contributes to improving germplasm management and utilization is the analysis of patterns of genetic diversity and population structure, which is important for broadening the genetic basis and therefore, to establish successful commercial breeding. Breeders demand a detailed genetics information of germplasm in order to (i) define core subsets of germplasm for specific traits, (ii) select parental combinations for developing progenies with maximum genetic variability for further selection, (iii) identify genetic duplicates for better germplasm management, (iv) enhance the search for unique germplasm with traits of breeding targets for better varietal development, (v) describe heterotic groups (Mohammadi and Presanna 2003; Reif *et al.*, 2003; Flint-Garcia *et al.*, 2009; Ertiro *et al.*, 2017; Ellis *et al.*, 2018; Jeong *et al.*, 2019; Singh *et al.*, 2019; Sansaloni *et al.*, 2020).

The assessment of genetic diversity within and between plant populations can be performed by using morphological features, biochemical characterization of allozymes, and DNA markers. DNA markers offer several advantages over phenotype-based alternatives as they are stable and detectable in all tissues regardless of growth, differentiation, or development stage and additionally, are not confounded by environmental, pleiotropic, and epistatic effects. The availability of low cost and high throughput SNP platforms facilitate genetic characterization of germplasm contributing to study the amount and distribution of genetic variation they contain, arising as a potent tool both for hybrid

breeding and inbred breeding. Use of genotype data to study genetic diversity can be mainly performed by calculation of population genetics parameters and analysis of genetic relationships among samples (Govindaraj *et al.*, 2015).

The measuring of genetic diversity is based on comparisons of individual genotypes within and between populations. The analysis starts with the construction of a genotype matrix, sample × sample pairwise and the calculation of the genetic distance (or similarities) that can be done by different statistical methods, such as: (i) Nei and Li's coefficient, (ii) Jaccard's coefficient, (iii) simple matching coefficient, and (iv) modified Rogers' distance (Mohammadi and Presanna, 2003).

The two main ways of analyzing the resulting matrix are principal coordinate analysis (PCoA) and dendrogram (or clustering tree diagram). PCoA is used to produce a 2- or 3-dimensional scatter plot of the samples such that the distances among the samples reflect the genetic distances among them with a minimum of distortion. The second approach is to produce a dendrogram where samples are grouped in clusters according to their genetic similarity. Different algorithms were used for clustering, including Unweighted Pair Group Method with Arithmetic Averages (UPGMA), neighbor-joining, and Ward's method (Govindaraj *et al.*, 2015).

Cluster analysis is of great help for breeders in defining which genotypes should be crossed to develop breeding populations that increase the chances of obtaining novel allelic combinations and to reverse or mitigate the genetic erosion. Besides, the analysis of genetic relationship is particularly useful when identifying the best materials to quickly integrate them into an eroded germplasm pool through exchange, purchase, or in-licensing germplasm (Beckett *et al.*, 2017; Leitão *et al.*, 2017; Vendelbo *et al.*, 2020). Different genetic materials, such as elite lines, ecotypes, landraces, subspecies, or wild relatives, are potential useful sources of genetic variation. Lack of genetic variation for traits of interest within the domesticated genetic pool, imposed a greater exploration of crop wild relatives (CWR). Thus, breeders in barley, maize, wheat, rice, sorghum, and soybean (among other species) reported a lack of variation for traits of interest within the domesticated germplasm, being exploration of CWR a feasible approach to mitigate the genetic erosion (Pourkheirandish *et al.*, 2020). Dempewolf *et al.* (2017) reviewed how CWR contributed to the development of improved crop varieties by crossing them with wild species carrying beneficial allelic variation for traits. Private industry has valued the diversity of CWRs and landraces, which sometimes is preferred as an alternative to the use of transgenic technology associated with high regulatory costs and often resisted by consumers (Dempewolf *et al.*, 2017). The proper use of GBS constitutes a powerful tool to reveal and measure the genetic variation contributed

by wild species, a previous step required for its potential use in crop improvement (Xu *et al.*, 2017).

Existence of heterotic parental gene pools constitutes the cornerstone in hybrid breeding programs as the prerequisite for achieving a high heterosis effect in hybrid crosses. Hybrid crop breeders evaluate the germplasm to assign inbred lines into distinct heterotic groups by studying combining ability, mainly based on grain yield. However, the use of molecular markers for genetic characterization of inbred lines can complement and fine-tune the combining ability data. Genetic distance estimates contribute to the assigning of genotypes to heterotic groups and the exploitation of complementary lines which maximize the outcome of hybrid breeding programs (Wu *et al.*, 2014; Xu *et al.*, 2014; Zhao *et al.*, 2015; Beckett *et al.*, 2017; Labroo *et al.*, 2021; Silva *et al.*, 2021).

Thus, plant breeding community has recognized that exploitation of genetic variability by conventional plant breeding in combination with genomics approaches have contributed to developing high yielding varieties or hybrids reducing the breeding cycle (Varshney *et al.*, 2005, 2021).

Linkage disequilibrium

Selection during crop breeding has caused a dramatic loss of genetic diversity in many genome regions of modern varieties. For instance, in major cereals and sunflower, reductions in diversity of 30–40% and 40–50%, respectively were estimated (Buckler *et al.*, 2001; Whitt *et al.*, 2002; Liu and Burke, 2006). Thus, it can be assumed that CWR for most crop species may have retained genetic information before domestication and artificial selection. Linkage disequilibrium (LD) refers to the non-random association of alleles at different loci (SNPs). LD is a common variable in population genetics and evolutionary biology, used among others, to map quantitative trait loci, estimate effective population size and past founder events, or to detect genomic regions under selection (Lucek and Willi, 2021).

Both D' and r^2 statistics have been widely used to quantify LD, differing in how they are affected by marginal allele frequencies and small sample sizes. To identify SNPs significantly associated with phenotypic trait variation, r^2 is the most relevant LD measurement. In small populations, the effects of genetic drift result in the consistent loss of rare allelic combinations, which increase LD levels. When genetic drift and recombination are at equilibrium, $r^2 = 1/(1 + 4N_e c)$, where N_e is the effective population size and c is the recombination fraction between sites (Flint-Garcia *et al.*, 2003). N_e is one of the most important indicators in population genetics for describing the magnitude of genetic drift, inbreeding, and assessing genetic diversity. The smaller the effective population size, the faster the population will become

inbred and thus no longer respond to selection (Cobb *et al.*, 2019). N_e is an important parameter that helps to quantify the magnitude of genetic drift and inbreeding. Thus, it is highly recommended that breeders actively calculate and monitor N_e through successive breeding cycles to ensure the long-term viability of their breeding programs. Knowledge of N_e helps both, to design efficient selection and, if necessary, to modify parental combinations that maintain or increase genetic variation to ensure the identification of future superior candidates. In larger populations more recombination events occur for which it is expected to have lower levels of LD. N_e can be estimated by using both pedigree and marker data, however the latter is presently preferred (Wang, 2016). In practice, N_e is directly related to the effective number of loci (M_e), which can be defined as the number of independent loci that gives the same variance of realized relationship as obtained in the more realistic situation calculated by $M_e = (2N_e L) / \log(4N_e L)$, where L is the genome size in Morgan. A larger M_e (due to a larger N_e , L , or both) will require a proportionally larger number of markers to capture the relatedness structure of the population (Goddard, 2009; Wang, 2016).

If a true functional polymorphism contributes a fraction of the total trait variation, h^2_q , and has a LD value of r^2 with another SNP, then the trait variation that can be explained by this SNP will be $r^2 \times h^2_q$. A similar inference cannot be made using D' (Zhu *et al.*, 2008). Typically, r^2 values of 0.1 or 0.2 are used to describe the LD decay. For instance, in soybean, a mild decline in LD over distances as great as 50 kbp was described (Zhu *et al.*, 2003), whereas in rice it was found that LD approaches $r^2 = 0.10$ for distances from around 100 kbp (Garris *et al.*, 2003). In contrast, in maize and cultivated sunflower r^2 declines to <0.10 within around 1 kb (Remington *et al.*, 2001; Liu and Burke, 2006).

Measuring of the pattern and extent of LD are influenced by different factors such as mating type, genetic drift, gene flow, selection, mutation, population substructure and relatedness, and ascertainment bias (Flint-Garcia *et al.*, 2003). For instance, domestication can induce population bottlenecks producing higher levels of LD (slow decay). Similarly, the increase in homozygosity associated with self-fertilization reduces the effective recombination rate, resulting in elevated LD (rapid decay) across the genome (Nordborg, 2000) or localized around the targeted loci (Clark *et al.*, 2004). Conversely, gene flow and recombination are predicted to reduce LD (Slatkin *et al.*, 2008).

Association mapping and population structure

Investigating the magnitude of LD decay determines the resolution of association mapping (AM) and marker-assisted breeding for which studying the LD

pattern contribute to estimate the required numbers of SNPs. AM (also known as LD mapping) is a method of mapping quantitative trait loci (QTLs) using historical meiotic recombination events performed over several generations to associate phenotypes with genotypes in large germplasm populations. AM provides relevant information into the genetic basis of complex traits and is a valued approach to identify the genes underlying agronomically important traits. AM is based on the LD between molecular markers (SNPs) and functional loci, requiring detailed understanding of the pattern of LD. AM of a trait-associated allele is based on the slow decay of LD with closely linked markers (Slatkin *et al.*, 2008; Zhu *et al.*, 2008). For instance, resequencing of cultivated and wild soybeans showed that LD decayed relatively slowly; given the high LD, only a small subset of SNPs would be required for marker-assisted breeding. However, the high LD introduces limitations for association studies using genetic populations (Lam *et al.*, 2010).

Germplasm with a recombination history producing a limited gene flow can result in a structured breeding population with an uneven distribution of alleles across subgroups. Therefore, the use of AM in such stratified populations may lead to non-functional and spurious associations. However, statistical analysis that estimate the effects of population structure-induced linkage disequilibria allowed to expand the proper use of AM (Pritchard *et al.*, 2000).

The domestication of crops has generated new population structures, some of which were geographic. Crops moved from their center of origin to a wide range of environments, where natural selection drove genetic adaptation to the new ones. Equally important are the genetic structures associated with end-use or cultural preferences that lead to the increase of the frequency of favorable alleles. Although they might become fixed within populations, would still be polymorphic in worldwide collections of cultivars or landraces and should be characterized as QTL in mapping studies of diverse material (Hamblin *et al.*, 2011).

PRACTICAL APPLICATIONS OF DEEP GENOTYPING

As discussed, the application of deep genotyping data in the genetic characterization of a germplasm base is important to assess genetic diversity, genetic relatedness, and population structure, contributing to a better understanding of the materials included in a breeding program. One molecular breeding application requiring high-density markers is genomic selection (GS). Although it is not currently used routinely, its importance and consideration are clearly growing. The great advantage of GS use is the ability to accurately

select individuals of higher breeding value without the requirement of collecting phenotypes pertaining to these individuals. This can facilitate a shortening of the breeding cycle and enable rapid selection and intercrossing of early-generation breeding material.

GS consists of the prediction of the genomic estimated breeding value (GEBV) of individuals based on genomic data (Meuwissen *et al.*, 2001). Typically, is performed among the progeny of a biparental cross between two elite inbreds (breeding population) where phenotypes and genome-wide genotypes are investigated in the training population (a subset of the breeding population) to predict significant relationships between phenotypes and genotypes using statistical approaches. Marker effects estimated on the training population will be used to predict the performance of the best candidates in the rest of the breeding population solely based on GEBV (Daetwyler *et al.*, 2013; Heslot *et al.*, 2015). One question that arises is: how many SNP loci should be genotyped to achieve a reasonable prediction accuracy (e.g., 0.6 correlation between true breeding value and GEBV)? There is no single answer, however there are some aspects to consider that can bring us closer to it. Simulation studies showed that the relationships between the individuals in the training population and the individuals in the prediction population had a major impact on the accuracy of the GEBV. Accurate predictions could be obtained with a small number of markers (e.g., 300–500) and a small number of phenotypes (e.g., 200–1000) when the phenotypes were collected from closely related biparental populations. To generate accurate predictions from nominally unrelated individuals many more phenotypes (e.g., 20,000) and many more markers (e.g., 10,000) were required (Hickey *et al.*, 2014). GS provides tremendous opportunities to increase genetic gain in plant breeding. Early empirical and simulation results are promising, but for GS to work, consideration of the cost-benefit balance is needed.

Although deep genotyping allows for the identification of thousands of informative SNPs, most routine molecular breeding applications do not require such a large number of markers. Therefore, the selection of a subset of SNP markers suitable for the chosen breeding strategy and their conversion to a more cost-effective genotyping technology is recommended. Kompetitive Allele Specific PCR (KASP) is a user-friendly SNP platform that is cost efficient for smaller numbers of markers (<200) which is what is needed for marker-assisted recurrent selection, marker-assisted backcrossing, and quality control analysis. KASP is one of the uniplex SNP genotyping platforms that has evolved to be a global benchmark technology for conversion of selected SNP (Semagn *et al.*, 2014).

Practical applications that require around 200 markers include quality control analysis (genetic identity, genetic purity, and parentage verification), linkage mapping of

QTL, marker-assisted recurrent selection, and marker-assisted backcrossing.

Quality control

Control of the genetic purity (in terms of identity of the parental inbred lines and progeny testing of the resulting F_1 hybrids) is an essential quality control (QC) parameter in hybrid breeding, as maintaining high levels of genetic purity is critical to guarantee a robust and stable agronomic performance of the genotype. Genetic purity evaluation is also relevant to meeting the strict intellectual property requirements that govern plant breeding and variety registration in many countries (Chen *et al.*, 2016; Josia *et al.*, 2021). Genetic purity can be proved using different approaches such as grow out test, use of biochemical markers and use of molecular markers. The grow out test is based on the use of a set of morphological descriptors and the biochemical marker approach analyzes electrophoretic protein (isozymes) profiles. Molecular marker approaches detect the variation of genotypes directly at the DNA level and have several advantages including high polymorphism, high-throughput detection methods, and they are unaffected by environmental conditions or the physiological stage of the plant (Chen *et al.*, 2016; Josia *et al.*, 2021). The main purpose of routine QC genotyping is to identify contamination or mislabeling of germplasm during regeneration, seed increase or seed distribution. To achieve a cost-effective QC test, a balance between accuracy of detection and efficiency needs to be maintained, for which optimization of the balance between accuracy and cost is the main concern when choosing a set of markers for QC.

In maize, was proposed the use of two separate sets of markers, each focusing on different types of QC. The first was a broad QC focusing on identity of a sample employing a minimum of 80 KASP markers (which were selected based on MAF, coverage and chromosome distribution) to distinguish each of the entries from one another. It is important to conduct this type of QC before starting new breeding crosses to ensure the identity and purity of the founding parents and to evaluate the levels of residual heterogeneity within them. The second approach was rapid QC for seed production using a smaller sub-set of only ten selected KASP markers (Chen *et al.*, 2016).

QTL mapping and marker-assisted recurrent selection

The nature of a trait may sometimes suggest that much of the quantitative variation is controlled by a few genes with large effects. In this situation, the objective of QTL mapping is finding a few major QTL. The subsequent breeding strategy is to introduce or pyramid these QTL, via standard breeding procedures, into elite germplasm

to develop improved cultivars. Exploiting a few major QTL therefore requires both gene discovery (i.e., QTL mapping) and selection (Bernardo, 2008).

QTL mapping involves identification of a subset of markers that are significantly associated with one or more QTL influencing the expression of the trait of interest. The main steps in linkage-based QTL mapping include (1) selecting and/or developing appropriate bi-parental mapping populations; (2) phenotyping the population for the trait of interest under greenhouse and/or field conditions; (3) choosing the molecular marker system, genotyping the parents of the mapping population and F_1 with larger numbers of markers, and selecting markers exhibiting polymorphism between the parents; (4) choosing a genotyping approach (entire population, selective genotyping, or bulk segregant analysis) and generating molecular data for an adequate number of uniformly-spaced polymorphic markers; and (5) identifying the molecular markers associated with the QTL using statistical programs (Semagn *et al.*, 2010, 2014). There is no clear consensus regarding the number of markers demanded for genotyping bi-parental populations but depending on the species and its genetic map, most researchers use around 200 and 400 markers. Once a significant QTL is identified, a second round of genotyping can be performed by saturating the chromosome region with additional polymorphic SNPs around the QTL of interest (fine mapping). Chromosome position of the QTL will be established relative to closely spaced flanking SNPs, and these markers can potentially be used for marker assisted selection (MAS) of the QTL associated to the trait.

The nature of a trait may sometimes suggest that much of the quantitative variation is controlled by many genes with small effects. Two related approaches have been proposed and used to increase the frequency of favorable QTL alleles at multiple loci: (i) F_2 enrichment followed by inbreeding and (ii) marker-assisted recurrent selection (MARS) (Bernardo, 2008). MARS refers to the improvement of an F_2 population by one generation of phenotypic selection in the target set of environments followed by 2–3 generations of selection based on significant marker genotypes. MARS has been applied for improving a breeding population with respect to QTLs exerting smaller effects on the phenotype (Gokidi *et al.*, 2016).

In both approaches the base generation is usually an F_2 population from the cross between two inbreds, although backcrosses, three-way crosses, or double crosses may also be used. The objective is to develop a recombinant inbred with superior *per se* performance for self-pollinated crops or with superior testcross performance for hybrid crops. Whereas F_2 enrichment usually involves only one generation of marker-based selection, MARS involves several cycles of marker-based selection (Bernardo, 2008).

Marker-assisted backcrossing

Marker-assisted backcrossing (MABC) is used for transferring genes which are responsible for favorable agronomic traits from a donor line into the genome of a recipient (recurrent) line. Introgression of a QTL by successive backcrosses is used to improve elite lines (recurrent parent) by introducing alleles from exotic material (donor parent). Besides to maintain the donor allele at the QTL in the progenies, the process pursues two objectives: reduction of the size of the donor genetic background around the target locus, and recovery of the recurrent parent genetic background (Hospital, 2005).

In the absence of selection, the proportion of the donor genome decreases by half at each generation. Thus, it is expected that after five backcross generations (BC_5), 98.4% of recurrent parent background is recovered. However, since selection is for the donor allele at the QTL, elimination of the donor genome around that QTL will be much slower than in the rest of the genome. As a result, the proportion of the donor genome will decrease less for the chromosome carrying the target locus than for the others. This is the so-called linkage drag problem (Naveira and Barbadilla, 1992).

Marker-assisted selection (MAS) in introgression of favorable alleles at QTL usually comprises selection for presence of the donor allele at two markers delimiting the interval in which the putative QTL was detected, and the recurrent parent allele at markers outside the QTL interval (foreground selection). The use of tightly-linked flanking markers for recurrent parental alleles helps to decrease linkage drag more rapidly resulting in short donor chromosome segments attached to the target gene. To optimize the positions of a limited number of markers that flank the target locus was concluded that the larger the population, the closer the markers should be to the target locus (Frisch and Melchinger, 2005).

Marker distance and distribution for genome-wide background selection will impact significantly on the efficiency of MABC method. Contrary to common belief, high marker densities are not required. To efficiently identify the backcross individuals with the smallest percentage of donor genome, a marker distance of 10 cM is sufficient. Decreasing the marker distances below 10 cM had only marginal effect on the recipient genome recovery. One explanation for this result is that, in general, one crossing over by meiosis and chromatid occurs for each chromosome segment 1 M in length. In two- or three-generation backcrossing programs, the number of recombination events resulting in chromosome segments of different parental origin is therefore limited (Herzog and Frisch, 2011, 2013). Computer simulations were conducted to evaluate and optimize the resource requirements of conversion programs of different crop genetic models with chromosome numbers (from $n=7$ to $n=17$) demonstrating how MABC contributes to reduce

the time and costs demanded for gene introgression. The results showed that depending on the genome size of the crop of interest, recovering 10% quantile with 98% of recurrent background can be reached in BC_3 working with population sizes comprised between 10 to 30 individuals per generation and around two to three SNP markers per chromosome equally distributed across each linkage group (Herzog and Frisch, 2013). A further considerable reduction of the costs could be achieved if the population size in the first backcross generation is twice the population size in generations BC_2 and BC_3 of a three-generation backcrossing program (Herzog and Frisch, 2013).

CONCLUSIONS

One of the greatest challenges facing humanity is the development of sustainable strategies to ensure food availability in response to population growth and climate change. Different foresight studies have concurrently argued that current food production practices would not be sufficient and therefore a transformation of the food system is required. One approach that can contribute to increase food security is to close yield gaps and enhancing genetic gain, for which solutions based on multiple disciplines should be found. Among these, clearly the genetic improvement of crops and specifically molecular breeding plays a fundamental role.

There is no doubt that a crop breeding program is fundamentally based on the quality of the germplasm. However, if a detailed genetic characterization is not available, there is a risk of underusing genetic resources or delaying the development of superior varieties. As stated, deep genotyping is an essential tool for a comprehensive characterization of the germplasm of interest and, fortunately, the technology is now accessible at a reasonable cost. What must be ensured is the correct interpretation of the genotypic information and on that basis develop efficient crop breeding strategies that respond to the real needs of the breeding program.

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