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Discovery of novel beneficial alleles in maize landraces for the improvement of quantitative traits

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There are no miracles in agricultural production.

(Norman Borlaug)

Content

Summary III
Zusammenfassung V
List of figures VII
List of tables VIII
List of abbreviations IX
Publications included in this thesis XI
1 Introduction 1
1.1 Background 1
1.2 Outline 4
2 Material and methods 10
2.1 Genotypic and phenotypic data 10
2.1.1 Plant material 10
2.1.2 Genotypic data 12
2.1.3 Field experiments 14
2.2 Analyses of molecular data 16
2.2.1 Site frequency spectrum 16
2.2.2 Diversity parameters 18
2.2.3 Population structure 19
2.2.4 Linkage disequilibrium 22
2.3 Haplotype identification 23
2.3.1 Haplotype construction 23
2.3.2 Association scans 24
2.3.3 Effect direction and stability of trait-associated haplotypes 26
2.3.4 Haplotypes with effects on multiple traits 27
2.3.5 Comparisons between landraces and breeding lines 27
3 Discussion 29
3.1 Choice of source material 29
3.1.1 Variation within and across landraces 29
3.1.2 Haplotype inventories in landraces and breeding lines 32
3.1.3 Adaptation and genomic background of landraces relative to elite germplasm 34
3.2 Representation of allelic diversity by DH libraries 36
3.3 Identification of novel beneficial variation 39
3.3.1 GWAS based on SNPs and haplotypes 39
3.3.2 Beneficial haplotypes for germplasm improvement 43
3.3.3 Haplotype variation in trait-associated genomic regions 45
3.4 Prospects of germplasm improvement through the use of landraces 47
4 References 51
5 Publications 67
6 Acknowledgements 104

Summary

Genetic variation is a fundamental prerequisite for selection and crop improvement. For most crops, today's breeding material captures only a fraction of the genetic diversity available within the species. Therefore, broadening the genetic base of elite germplasm is considered essential for meeting the increasing demands on agricultural production under changing environmental conditions and resource limitations. Landraces are a rich source of novel diversity, but especially for quantitative traits and cross-pollinating species efficient strategies for their targeted utilization are lacking. Main challenges hampering the utilization of landraces are the lack of genetic and phenotypic information about most accessions, the heterogeneous and heterozygous nature of landraces and their performance gap to modern cultivars. In this thesis, a genome-based strategy was developed for identifying novel beneficial variation in landraces to improve elite germplasm for quantitative traits. The proposed strategy was validated experimentally in the context of European maize.

The choice of source material is of major importance when working with genetic resources. In this thesis, different sampling strategies were evaluated with respect to their influence on genomic parameters affecting the accuracy and efficacy of genome-enabled mapping and prediction. Using dense genotypic data from 35 European maize landraces with more than 20 individuals per landrace, different sampling schemes were assessed. Most of the molecular variation was found within landraces while differences among landraces accounted only for a small proportion of the variation. On average, five landraces captured about 95% of the molecular diversity of the entire dataset. Within individual landraces, absence of pronounced population structure, moderate to low linkage disequilibrium (LD) and consistency of linkage phases were found. When combining landraces, LD decay distances decreased to a range harboring on average less than five genes. In summary, the results suggest that in studies aiming at gene discovery or genomic selection in landraces, the comprehensive sampling of diversity from few pre-selected landraces has many advantages. Pre-selected landraces can be chosen to show high levels of diversity for target traits, avoid confounding effects of strong adaptive alleles, allow optimal control for population structure, provide high resolution in association mapping and facilitate the introduction of relevant landrace alleles into the target breeding pool.

Taking this strategy into practice, more than 1,000 doubled haploid (DH) lines were derived from three populations selected from the 35 European landraces analyzed in this thesis.

DH lines were genotyped at high density and phenotyped for more than 25 traits in up to eleven environments. A statistical framework for the identification of novel beneficial variation in landrace-derived material was developed, involving the construction of haplotypes, the test for haplotype-trait association, the assessment of effect stability and the comparison with elite germplasm. Haplotype-based genome-wide association scans identified many significant associations for the target trait early plant development at high resolution. In contrast, only few associations for flowering were detected, indicating that confounding effects of strong adaptive alleles were avoided. Part of the identified haplotypes with favorable effects on early plant growth were absent in a broad panel of 65 European flint breeding lines, pointing to beneficial novel variation. DH lines carrying these novel haplotypes outperformed breeding lines with alternative haplotypes. Most haplotypes associated with target traits showed stable effects across populations and environments and only limited correlated effects with undesired traits making them ideal for introgression into elite germplasm. The proposed strategy to sample comprehensively individuals from a limited set of pre-selected landraces was successful in linking molecular variation to meaningful phenotypes and identifying novel variation for the genetic improvement of elite germplasm. The strategy and methods developed in this thesis can be extended to other maize germplasm groups and even to other allogamous crop species.

Zusammenfassung

Genetische Variation ist die Grundvoraussetzung für Selektion und die genetische Verbesserung von Kulturpflanzen. Allerdings deckt das heutige Zuchtmaterial in den meisten Kulturpflanzen nur einen kleinen Teil der verfügbaren genetischen Diversität der jeweiligen Spezies ab. Es wird daher als essentiell angesehen, die genetische Basis des Elitematerials zu erweitern, um die pflanzliche Produktion unter sich verändernden Umweltbedingungen und Ressourcenknappheit zu sichern. Landrassen sind reichhaltige Quellen neuer Diversität, aber für quantitative Merkmale fehlen effiziente Strategien für deren gezielte Nutzung. Die größten Hindernisse für die Nutzung von Landrassen sind unzureichende genetische und phänotypische Information über die meisten Akzessionen, ihre Heterogenität und Heterozygotie und der Leistungsrückstand im Vergleich zu modernen Sorten. In dieser Arbeit wurde eine genombasierte Strategie für die Identifizierung neuer, vorteilhafter Variation in Landrassen entwickelt. Die vorgeschlagene Strategie wurde an europäischem Flint Mais experimentell überprüft.

Bei der Nutzung genetischer Ressourcen ist die Auswahl des Ausgangsmaterials von größter Bedeutung. Daher wurden zunächst Schätzwerte genomischer Parameter, die die Genauigkeit und Effektivität genombasierter Methoden beeinflussen, zwischen verschiedenen Stichproben verglichen. Mittels hochauflösender genotypischer Daten von 35 europäischen Maislandrassen wurde die molekulare Diversität von mehr als 20 Individuen pro Landrasse untersucht. Der Großteil der molekularen Diversität war innerhalb von Landrassen zu finden, während Unterschiede zwischen Landrassen nur einen relativ kleinen Teil der Gesamtvariation des Datensatzes erklärten. Im Durchschnitt wurden ca. 95% der Gesamtvariation durch fünf zufällig gezogene Landrassen repräsentiert. Es wurde gezeigt, dass innerhalb der Landrassen keine ausgeprägter Populationsstruktur vorherrscht, das Kopplungsphasenungleichgewicht („linkage disequilibrium“, LD) mäßig bis gering ist und die Kopplungsphasen konsistent sind. Durch die Kombination weniger Landrassen kann eine genetische Auflösung von weniger als fünf Genen erzielt werden. Insgesamt deuten die Ergebnisse darauf hin, dass für die Genidentifizierung oder die genomische Selektion die umfassende Beprobung von Diversität innerhalb weniger vorselektierter Landrassen viele Vorteile hat. Vorselektierte Landrassen können auf genetische Variation für Zielmerkmale selektiert werden, Störeinflüsse durch starke adaptive Allele werden vermieden, die optimale Kontrolle von Populationsstruktur ist möglich, eine hohe Auflösung in der Assoziationskartierung wird erreicht und die

Integration relevanter Landrassenallele in das entsprechende Elitezuchtmaterial wird begünstigt.

Mit dieser neu konzipierten Strategie konnten in dieser Arbeit neue, vorteilhafte Allele aus Landrassen identifiziert werden. Dazu wurden zunächst drei der 35 europäischen Landrassen selektiert um insgesamt mehr als 1.000 doppelhaploide (DH) Linien zu erstellen. Die DH-Linien wurden hochauflösend genotypisiert und für mehr als 25 Merkmale in bis zu elf Umwelten phänotypisiert. Für die Untersuchung der Landrassen auf neue, vorteilhafte Variation, wurden aufeinander aufbauende statistische Modelle entwickelt, welche die Konstruktion von Haplotypen, deren Test auf Merkmalsassoziation, die Untersuchung der Effektstabilität und den Vergleich mit Elitezuchtmaterial erlauben. Mit der haplotypbasierten genomweiten Assoziationsanalyse konnten somit signifikante Assoziationen für das Zielmerkmal frühe Jugendentwicklung in hoher Auflösung auf dem Maisgenom kartiert werden. Für die Blüte wurden nur wenige Assoziationen entdeckt, was dafürspricht, dass Hintergrundeffekte starker adaptiver Allele vermieden werden konnten. Ein Teil der identifizierten Haplotypen mit vorteilhaften Effekten für das frühe Pflanzenwachstum trat in einer Auswahl von 65 europäischen Flint Züchtungslinien nicht auf. Sie repräsentieren somit neue vorteilhafte Variation, was dadurch validiert wurde, dass DH-Linien, die diese Haplotypen trugen, Züchtungslinien mit alternativen Haplotypen phänotypisch überlegen waren. Die meisten Haplotypen, die mit Zielmerkmalen assoziiert waren, zeigten stabile Effekte über Populationen und Umwelten. Korrelierte Effekte mit unerwünschte Merkmalen traten kaum auf, was die Einkreuzung in Elitematerial erleichtert. Die hier vorgeschlagene Strategie, große Stichproben aus einem begrenzten Set an vorselektierten Landrassen zu untersuchen, war erfolgreich. Es gelang molekulare Variation mit aussagekräftigen Phänotypen zu verknüpfen und neue Variation für die Verbesserung quantitativer Merkmale in Elitematerial zu identifizieren. Sowohl die Strategie als auch die hier entwickelten Methoden können auf andere Populationen in Mais sowie auf andere allogame Spezies erweitert werden.

List of figures

Figure 1: Expected changes of genomic parameters depending on the strategy for sampling genetic resources. 5

Figure 2: Map of biogeographical regions of Europe and the origin of 35 maize landraces analyzed in this thesis. 10

Figure 3: Number of haplotypes in each of the 35 landraces and in the panel of 65 flint breeding lines (Flint-BL). 33

Figure 4: Ancestry proportions of 65 flint and 70 dent breeding lines. 36

Figure 5: Comparison of diversity measures and number of haplotypes between DH libraries and original landraces. 38

Figure 6: Genome-wide association scans for PH_V4 based on two different approaches. 43

Figure 7: Effects of alternative haplotypes relative to a favorable focus haplotype for PH_V6. 46

List of tables

Table 1: Landraces under study. 11

Table 2: Overview of genotypic datasets. 12

Table 3: Hierarchical structure in an analysis of molecular variance (AMOVA) 21

Table 4: Parameters of diversity and recombination for varying numbers of SNPs per window 41

List of abbreviations

AMOVA	analysis of molecular variance
BLUE	best linear unbiased estimate
CI	confidence interval
cM	centimorgan
DH	doubled haploid
FDR	false discovery rate
FF	female flowering
EV	early vigor
GBS	genotyping by sequencing
GWAS	genome-wide association scan
H	expected heterozygosity
IBD	identical by descent
IBS	identical by state
indel	insertion/deletion
kb	kilobase pairs
LD	linkage disequilibrium
LO	lodging
LP	line *per se*
Mb	megabase pairs
MF	male flowering
MRD	modified Rogers' distance
nR	minimum number of historical recombination events
PCoA	principal coordinate analysis
PCo	principal coordinate
PH	plant height
PIC	polymorphism information content
PP	proportion of polymorphic markers
π	nucleotide diversity
QTL	quantitative trait locus/loci
RFLP	restriction fragment length polymorphism
S	number of segregating sites
SeeD	Seeds of Discovery
SFS	site frequency spectrum

SNP	single nucleotide polymorphism
SSR	simple sequence repeat
TC	testcross
TILL	tillering
WGS	whole genome sequence

Publications included in this thesis

Mayer et al. (2017)

Mayer M, Unterseer S, Bauer E, de Leon N, Ordás B, Schön CC (2017) Is there an optimum level of diversity in utilization of genetic resources? Theor Appl Genet 130:2283-2295, doi: 10.1007/s00122-017-2959-4.

Abstract

Genome-enabled strategies for harnessing untapped allelic variation of landraces are currently evolving. The success of such approaches depends on the choice of source material. Thus, the analysis of different strategies for sampling allelic variation from landraces and their impact on population diversity and linkage disequilibrium (LD) is required to ensure the efficient utilization of diversity. We investigated the impact of different sampling strategies on diversity parameters and LD based on high-density genotypic data of 35 European maize landraces each represented by more than 20 individuals. On average, five landraces already captured ~95% of the molecular diversity of the entire dataset. Within landraces, absence of pronounced population structure, consistency of linkage phases and moderate to low LD levels were found. When combining data of up to 10 landraces, LD decay distances decreased to a few kilobases. Genotyping 24 individuals per landrace with 5k SNPs was sufficient for obtaining representative estimates of diversity and LD levels to allow an informed pre-selection of landraces. Integrating results from European with Central and South American landraces revealed that European landraces represent a unique and diverse spectrum of allelic variation. Sampling strategies for harnessing allelic variation from landraces depend on the study objectives. If the focus lies on the improvement of elite germplasm for quantitative traits, we recommend sampling from pre-selected landraces, as it yields a wide range of diversity, allows optimal marker imputation, control for population structure and avoids the confounding effects of strong adaptive alleles.

Candidate's contribution

The candidate made major contributions to designing the study, analyzed the data, created figures and tables, wrote the first draft of the manuscript, and made all revisions.

Hölker et al. (2019)

Hölker AC, Mayer M, Presterl T, Bolduan T, Bauer E, Ordás B, Brauner PC, Ouzunova M, Melchinger AE, Schön CC (2019) European maize landraces made accessible for plant breeding and genome-based studies. Theor Appl Genet 132:3333-3345, doi: 10.1007/s00122-019-03428-8.

Abstract

Maize landraces comprise large allelic diversity. We created doubled-haploid (DH) libraries from three European flint maize landraces and characterized them with respect to their molecular diversity, population structure, trait means, variances, and trait correlations. In total, 899 DH lines were evaluated using high-quality genotypic and multi-environment phenotypic data from up to 11 environments. The DH lines covered 95% of the molecular variation present in 35 landraces of an earlier study and represent the original three landrace populations in an unbiased manner. A comprehensive analysis of the target trait plant development at early growth stages as well as other important agronomic traits revealed large genetic variation for line *per se* and testcross performance. The majority of the 378 DH lines evaluated as testcrosses outperformed the commercial hybrids for early development. For total biomass yield, we observed a yield gap of 15% between mean testcross yield of the commercial hybrids and mean testcross yield of the DH lines. The DH lines also exhibited genetic variation for undesirable traits like root lodging and tillering, but correlations with target traits early development and yield were low or nonsignificant. The presented diversity atlas is a valuable, publicly available resource for genome-based studies to identify novel trait variation and evaluate the prospects of genomic prediction in landrace-derived material.

Candidate's contribution

The candidate made major contributions to the generation of genotypic and phenotypic data as well as to the analysis of genotypic data, created one figure, and contributed to the discussion of results and revision of the manuscript.

Mayer et al. (2020)

Mayer M, Hölker AC, González-Segovia E, Bauer E, Presterl T, Ouzunova M, Melchinger AE, Schön C-C (2020) Discovery of beneficial haplotypes for complex traits in maize landraces. Nat Commun 11:4954, doi: 10.1038/s41467-020-18683-3.

Abstract

Genetic variation is of crucial importance for crop improvement. Landraces are valuable sources of diversity, but for quantitative traits efficient strategies for their targeted utilization are lacking. Here, we map haplotype-trait associations at high resolution in ~1,000 doubled-haploid lines derived from three maize landraces to make their native diversity for early development traits accessible for elite germplasm improvement. A comparative genomic analysis of the discovered haplotypes in the landrace-derived lines and a panel of 65 breeding lines, both genotyped with 600k SNPs, points to untapped beneficial variation for target traits in the landraces. The superior phenotypic performance of lines carrying favorable landrace haplotypes as compared to breeding lines with alternative haplotypes confirms these findings. Stability of haplotype effects across populations and environments as well as their limited effects on undesired traits indicate that our strategy has high potential for harnessing beneficial haplotype variation for quantitative traits from genetic resources.

Candidate's contribution

The candidate made major contributions to conceiving the study as well as the generation of genotypic and phenotypic data, developed the statistical framework, analyzed the data, created figures and tables, wrote the first draft of the manuscript, and made all revisions.

1 Introduction

1.1 Background

The global population is predicted to surpass nine billion people within the next 20 years and is expected to plateau at more than ten billion by the end of this century (United Nations 2019). At the same time, growing wealth leads to dietary changes towards higher meat consumption (Tilman and Clark 2014). Together with increasing bioenergy use (Popp et al. 2014) these factors generate an urgent need for a substantial increase in agricultural production. To keep up with the rising demand for food, feed, fiber and fuel, crop production needs to approximately double by 2050 (Tilman et al. 2011). A comparable increase in crop production has been achieved before, during the so-called green revolution, leading to a production gain of 125% from 1960 to 2000 (Khush 2001). However, today the effects of climate change, soil degradation, water and land shortages are threatening current cropping systems. As a consequence, agricultural production must be increased in a sustainable way and adapted to new environmental conditions (Godfray et al. 2010; Lobell and Tebaldi 2014). The main driver of the green revolution was the genetic improvement of crops with genes found in traditional varieties (Khush 2001). Genetic variation is the basis of selection and germplasm improvement. However, for most crops, today's breeding material captures only a fraction of the genetic diversity available within the species (McCouch et al. 2013). Broadening the genetic diversity of elite germplasm through utilization of genetic resources is therefore considered essential for sustainably intensifying agricultural production (Hoisington et al. 1999; Cooper et al. 2001; Ortiz et al. 2010; McCouch et al. 2013; Sood et al. 2014).

Maize (*Zea mays* L. ssp. *mays*) is one of the most important staple crops worldwide (www.fao.org/faostat) and an important model plant for basic and applied research (Strable and Scanlon 2009). On a global scale, maize shows tremendous genetic diversity relative to other crop species (Buckler et al. 2006). In contrast, high levels of relatedness are observed in today's maize elite breeding pools (Romay et al. 2013; Gouesnard et al. 2017). Reduced genetic variation in elite germplasm compared to the species-wide diversity is the result of a history of strong demographic shifts accompanied by adaptation to novel environments and intensive selection for agronomic performance. Maize originated from a wild relative, teosinte (*Zea mays* L. ssp. *parviglumis*), approximately 9,000 years ago in today's Mexico (Matsuoka et al. 2002; van Heerwaarden et al. 2011). Stringent selection for maize-like phenotypes, like increased ear size and reduced lateral branches (Piperno

et al. 2015), led to a reduction of effective population size accompanied with loss of allelic diversity (Wright et al. 2005; Hufford et al. 2012). The post-domestication spread of maize throughout the Americas was characterized by serial founder effects leading to decreasing diversity with increasing distance from the center of origin (Vigouroux et al. 2008; van Heerwaarden et al. 2011; Wang et al. 2017b). After the discovery of the New World by Columbus approximately 500 years ago, maize was introduced in Europe (Tenaillon and Charcosset 2011) and rapidly spread around the world (Mir et al. 2013). Local adaptation and selection by farmers led to the creation of thousands of landraces, also called farmers' varieties. Due to the dynamic genome of maize, outcrossing mating and continued germplasm exchange (Anderson and Brown 1952; Tenaillon and Charcosset 2011), landraces retained a high level of phenotypic and genetic variation (Hufford et al. 2012; Sood et al. 2014).

The transition from open-pollinated to hybrid varieties at the beginning of the last century (Troyer 2004; Barriere et al. 2006) represents a milestone in maize breeding and led to an enormous increase in yield (Duvick 2005). Today's heterotic breeding pools, however, were established by the choice of only few founder lines derived through selfing from important landraces (Messmer et al. 1992; Mikel and Dudley 2006; White et al. 2020). New inbred lines were mainly developed by intercrossing within the respective breeding pools with an excessive use of few very successful elite lines, like e.g. B73, Mo17, PH207 for the US dent breeding pools and DK105, EP1, F2, F7 for the European flint breeding pool. Decades of advanced cycle breeding with high selection intensities shaped the genetic base of today's elite germplasm (Duvick et al. 2004; Reif et al. 2005a). Comparing maize breeding populations from different breeding cycles, Yu and Bernardo (2004) showed that, except for grain yield, losses in genetic variances were greater than expected based on quantitative genetic theory. It can be assumed that for traits that were not primary targets of selection, favorable alleles were lost and unfavorable alleles fixed during the selection process, due to drift and hitchhiking effects (Flint-Garcia et al. 2003; Hartfield and Otto 2011; Voss-Fels et al. 2017). Although annual gains in yield have not yet reached a plateau (Schauberger et al. 2018), limited genetic diversity for other traits, like abiotic stress tolerance and resource-use efficiency, hampers genetic improvement under changing environmental conditions and breeding goals (Lobell and Tebaldi 2014).

Due to their high genetic diversity and local adaptation, landraces can serve as source of novel beneficial alleles for traits with limited variation in elite germplasm (Dwivedi et al. 2016). Despite their assumed high genetic potential for germplasm improvement, the

thousands of landrace accessions stored in gene banks worldwide have remained widely unused so far (Ortiz et al. 2010; Wang et al. 2017a). Besides legal obligations making plant breeders reluctant to the use of genetic resources (CBD 2011), there are practical and genetic obstacles for introducing landrace material into breeding programs. First, limited information on seed bank accessions hampers an informed choice of suitable source material (Sood et al. 2014). While advances in sequencing and genotyping technologies enable a cost-efficient generation of comprehensive molecular inventories of plant genetic resources (Wang et al. 2018; Milner et al. 2019; http://seedsofdiscovery.org), meaningful phenotypic information is lacking (McCouch et al. 2012; Wang et al. 2017a). Second, for allogamous crops like maize, landraces represent collections of heterogeneous and heterozygous individuals, making their maintenance as well as their phenotypic evaluation difficult. Evaluating the breeding potential of individual genotypes or estimating the within-landrace genetic variation requires the generation of reproducible genetic units, like inbred lines. The third obstacle for the utilization of genetic resources for breeding is the performance gap between landraces and modern cultivars (Wilde et al. 2010; Brauner et al. 2019). Landrace-derived lines cannot be directly integrated into modern breeding populations, as the supposed increase in genetic variation would come at a too high cost of reduced performance. Besides the assumed novel favorable alleles, landraces can be expected to harbor many deleterious and unfavorable alleles, which would compromise the elite breeding pools. Therefore, efficient strategies for a targeted utilization of beneficial landrace alleles for the improvement of elite germplasm are needed.

A major factor influencing the prospects of success in the utilization of genetic resources is the genetic architecture of the trait of interest. For mono- or oligogenic traits, phenotypic screening of vast amounts of accessions is feasible (McCouch et al. 2012) and mapping of favorable alleles and their introgression into breeding germplasm should be possible with standard forward genetics and backcrossing approaches (Visscher et al. 1996; Ødegård et al. 2009). In fact, impressive examples exist, where the introgression of single alleles with very large effects led to a substantial improvement of disease resistance (Khush 2001), nutrient uptake (Wissuwa et al. 2002) or submergence tolerance (Bailey-Serres et al. 2010) of elite germplasm and enabled changes in management practices. In contrast, successful examples for the use of genetic resources for the improvement of quantitative traits are scarce (Sood et al. 2014). Many traits of agronomic importance are quantitative in nature, i.e. large-effect quantitative trait loci (QTL) are already fixed while the remaining genetic variation is determined by many genes with comparably small effects. Furthermore, many quantitative traits are strongly influenced by the environment and often

show pronounced genotype by environment (G×E; van Eeuwijk et al. 2016) as well as epistatic interaction (Chandler et al. 2013). Allele mining in genetic resources for the improvement of quantitative traits still is a major challenge up to date, requiring the development of specialized experimental designs and efficient genome-enabled approaches.

1.2 Outline

The first and presumably most important decision to make, when working with genetic resources, is the choice of source material. Given limited capacities for material development, genotyping and phenotyping, different strategies for sampling the native diversity of landraces can be followed. When devising the optimum strategy for particular study objectives, two important aspects need to be considered: The composition of the sampled material regarding the number of landraces and the number of individuals per landrace (Figure 1a) as well as the environmental adaptation of chosen accessions (Figure 1b).

For the investigation of landraces, one can broadly sample material across landraces of various origin, with only one or few individuals per landrace, or one can focus on the diversity within particular pre-selected landraces with large sample sizes per landrace (Figure 1a). Up to date, the genomic characterization of landraces was predominantly based on sampling individuals across a wide range of landraces, aiming at maximizing the level of diversity in the sampled genetic material (Tenaillon et al. 2001; Hufford et al. 2012; Sood et al. 2014). This can be advantageous for studying for example crop evolution (Matsuoka et al. 2002; van Heerwaarden et al. 2011), genomic signals of adaptation to different environments (Takuno et al. 2015; Romero Navarro et al. 2017) and the effects of rare alleles (Krakowsky et al. 2008; Kremling et al. 2018). Using this sampling approach, more than 1,000 candidate genes associated with flowering time and environmental adaptation to altitude and latitude were identified in Central and South American landraces (Romero Navarro et al. 2017). However, such broad samples are characterized by large variation in local adaptation and strong population structure, confounding genetic analyses aiming at linking molecular variation to meaningful phenotypes. In association mapping, these confounding factors limit the power of detecting true associations with non-adaptive traits of agronomic importance, especially if the associated alleles are restricted to particular subpopulations (Zhao et al. 2011; Brachi et al. 2011). As landraces represent self-contained, locally adapted populations (Dwivedi et al. 2016), sampling diversity within rather than across landraces should eliminate the confounding effects of strong adaptive

alleles in genome-wide association scans (GWAS; Figure 1a). Further, due to the random-mating of landraces, samples of individuals from the same landrace can be expected to show absence of pronounced population structure and low levels of linkage disequilibrium (LD), leading to high mapping resolution in GWAS. Although sampling across many landraces can lead to even lower LD levels (Figure 1a), as indicated by Tenaillon et al. (2001), the admixture of many different source populations with varying linkage phases might decrease the accuracy and efficacy of GWAS.

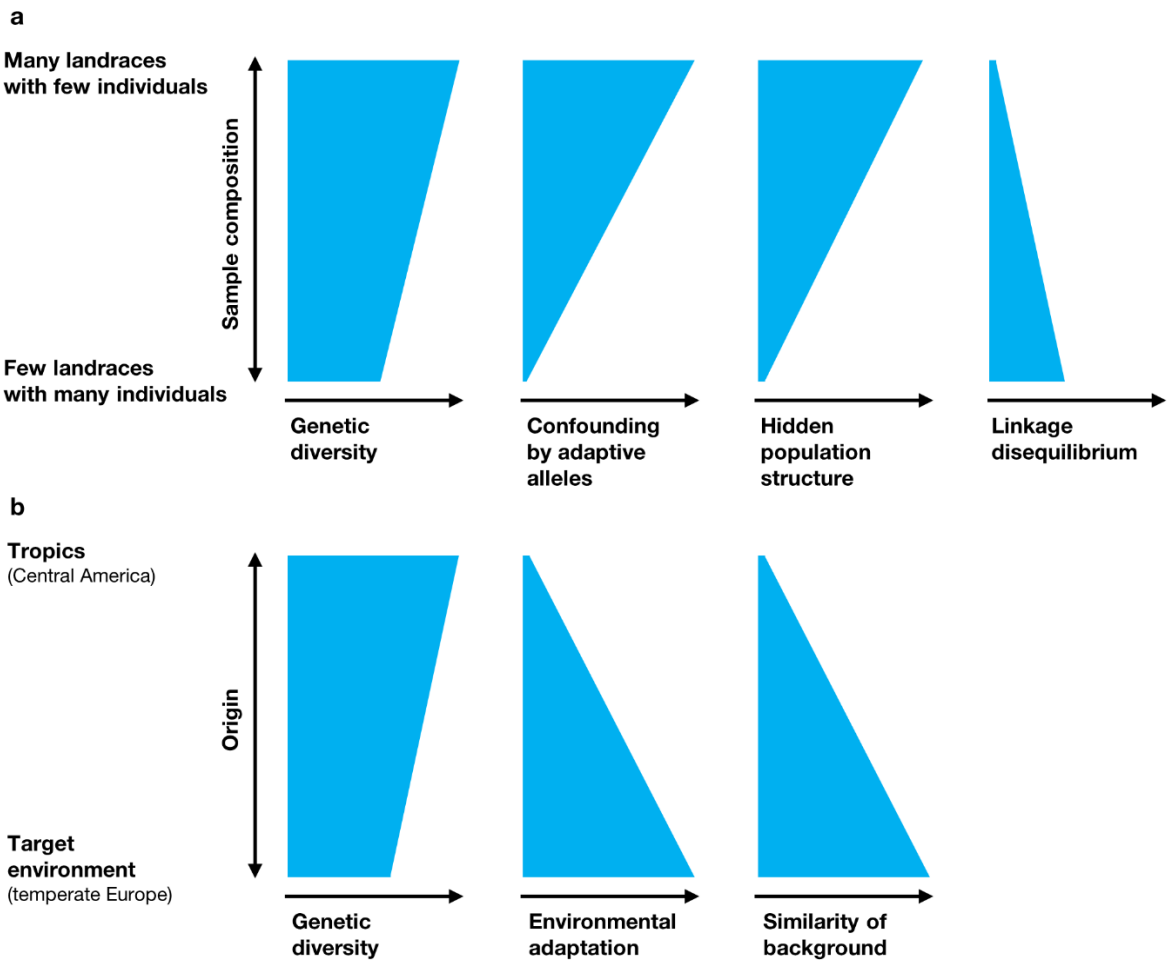

Figure 1: Expected changes of genomic parameters depending on the strategy for sampling genetic resources. a) Assumed trend for the level of genetic diversity, the extent of confounding effects in genetic analyses caused by adaptive alleles and hidden population structure, and the level of linkage disequilibrium, when comparing sampling schemes varying in their number of landraces and individuals per landrace, given a particular total number of individuals. **b)** Assumed trend for the level of genetic diversity, adaptation to the target environment and similarity to the genomic background of the target elite breeding pool, when comparing (sub)tropical landrace material from Central America (close to the center of maize domestication) with temperate European landraces. Here, the target environment as well as the target elite breeding pool are assumed to be in Europe.

It was shown that in allogamous crops like maize, large proportions of the molecular (Sood et al. 2014) and genetic (Böhm et al. 2017; Brauner et al. 2018) variance lie within and not across landraces. Therefore, individual landraces should provide large variation for

mapping quantitative traits. If the landraces are pre-selected for variation in particular target traits, the chances of detecting useful novel alleles can even be increased. Despite these considerations, a comprehensive analysis of the influence of different sampling strategies on important genomic parameters, affecting the accuracy and efficacy of genome-enabled approaches in landraces, has been lacking so far. As part of this thesis, molecular diversity, population structure, LD and persistence of linkage phase were analyzed within and across 35 European maize landraces, with more than 20 individuals per landrace genotyped at high density (Mayer et al. 2017). Based on the change of parameters when varying the number of sampled landraces and individuals per landrace, the suitability of different sampling strategies for genome-enabled approaches, such as GWAS and genomic selection, were evaluated.

In addition to molecular and phenotypic diversity, environmental adaptation is an important factor affecting the suitability of landraces as source of novel variation for elite germplasm improvement. The highest level of diversity can be expected for accessions from Central America close to the center of maize domestication (Vigouroux et al. 2008; Mir et al. 2013). However, for the improvement of temperate elite germplasm, accounting for 60% of today's global maize production (Li et al. 2017), the suitability of (sub)tropical landrace material for mining novel alleles is limited by the lack of adaptation to temperate environmental conditions (Figure 1b). Incorporation of (sub)tropical germplasm into temperate breeding pools can cause maladaptive symptoms like low germination rates, poor seedling vigor, late flowering, high moisture at harvest, and high ear and plant height, which are partly caused by photoperiod sensitivity (Goodman 1999; Tarter and Holland 2006; Sood et al. 2014). Such pronounced effects of adaptive traits hamper the phenotypic evaluation of unadapted genetic resources for traits of agronomic importance in the target environments (Castillo-Gonzalez and Goodman 1989). Examples exist which have overcome these restrictions by slowly adapting tropical material to temperate conditions using mild mass selection (Lewis and Goodman 2003; Teixeira et al. 2015), but these approaches are very time consuming and bear the risk of losing favorable alleles linked to unfavorable alleles at loci under selection during adaptation. As a further obstacle, the efficacy of incorporating favorable alleles of tropical origin into temperate elite germplasm might be decreased due to suppressed recombination between genetically distinct chromosomes (Lonnquist 1974; Rieseberg et al. 1999; Rodgers-Melnick et al. 2015). Even in case of their successful incorporation, favorable alleles of tropical origin might cause unexpected phenotypic effects in temperate germplasm due to their dependency on the genomic background. In view of these challenges, sampling genetic material from

landraces already adapted to the target environments and with shared historical ancestry with the target elite breeding pool can be a preferable strategy (Figure 1b). Among temperate genetic resources, more than 4,500 landraces are conserved in European gene banks (Böhm et al. 2014), which might be of special interest for promoting early plant development and cold tolerance (Peter et al. 2009; Rodríguez et al. 2010). For assessing the diversity of European maize landraces in a broader context, molecular diversity of 35 temperate European landraces was analyzed in Mayer et al. (2017) together with a set of ~4,000 (sub)tropical landrace accessions from Central and South America, which were part of the Seeds of Discovery (SeeD) project (http://seedsofdiscovery.org).

After assembling the source material, the second step is the generation of adequate reproducible experimental units for conducting genome-enabled studies. The highly heterozygous individuals of the original landraces represent unique genotypes, which cannot be reproduced in subsequent generations due to recombination. The problem could be overcome by the development of inbred lines, but inbreeding by recurrent selfing is time consuming and might be hampered by high genetic load (Hallauer et al. 2010). Very large starting populations would be required, as many lines might be lost in advanced selfing generations due to fixation of detrimental or (sub)lethal alleles. Selective forces against deleterious alleles during selfing might further lead to the loss of favorable alleles due to hitchhiking (Hartfield and Otto 2011). Alternative approaches to access the native diversity of landraces include crosses of random samples of landrace individuals with elite genetic material (Stadler 1944; Sood et al. 2014; Gorjanc et al. 2016; Romero Navarro et al. 2017). Depending on the performance gap between the landrace and parental germplasm, such approaches bear the risk of selection in favor of alleles contributed by the elite parent in subsequent steps of line development (Sood et al. 2014; Gorjanc et al. 2016). Further, if phenotypic evaluation is conducted based on heterozygous (Romero Navarro et al. 2017) or partly heterozygous (Stadler 1944) genotypes, the additive genetic variance is decreased compared to fully homozygous lines (Bernardo 2002) and the number of replicates, i.e. the precision of phenotyping, is limited by a finite number of seeds per genotype. Further, when selecting (partly) heterozygous genotypes for pre-breeding, selected genotypes might have to be excluded in subsequent generations due to deleterious recessive alleles masked in the heterozygous state.

A method for obtaining fully homozygous lines but circumventing the process of recurrent selfing is the production of doubled-haploid (DH) lines through *in vitro* or *in vivo* recovery of haploid gametes (Dwivedi et al. 2015). The DH technology is routinely applied in many

crop species, including maize (Chaikam et al. 2019). Success rates of DH production are much lower in landraces compared to elite material (Melchinger et al. 2017), requiring large starting populations similar to the recurrent selfing approach. However, deleterious and (sub)lethal alleles are readily expressed and potentially purged in the haploid stage and thus the time consuming and cumbersome development of large sets of segregating plants/families with various degrees of homozygosity over multiple generations are avoided. A comparative analysis of the allelic composition between original landraces and DH libraries derived from them, indicated that the DH lines captured the molecular diversity of the original landraces well and that purging from genetic load did not act on specific genomic regions (Melchinger et al. 2017). Somewhat contradicting results were obtained by Zeitler et al. (2020) based on the same genetic material but a different analysis framework. Zeitler et al. (2020) concluded that DH lines may be a valuable tool for making landrace variation amenable for elite germplasm improvement, but that the genetic diversity of the original landraces is not fully captured by DH libraries. These and other studies (Wilde et al. 2010; Strigens et al. 2013; Böhm et al. 2017; Brauner et al. 2018) gave important insights for the application of the DH technology to landrace material, but they were based on relatively small sample sizes with limited statistical power for genome-enabled studies. During the course of this thesis, three European maize landraces were selected based on the results of Mayer et al. (2017) and in total ~1,000 DH lines were generated, representing the largest collection of DH lines derived from maize landraces so far. With these DH libraries, the strategy of focusing on a limited set of pre-selected landraces for identifying novel beneficial alleles, as proposed by Mayer et al. (2017), was put into practice and results are described in Hölker et al. (2019) and Mayer et al. (2020). DH lines were genotyped with 600k markers and phenotyped in up to eleven environments, with the main focus lying on traits related to early plant development and cold tolerance. In Hölker et al. (2019), a comprehensive analysis of the phenotypic data was conducted, characterizing the DH libraries in terms of line *per se* (LP) and testcross (TC) performance, trait correlations, and genetic variances. Further, population structure of DH lines and sampled individuals of the respective original landraces was analyzed (Hölker et al. 2019). Using GWAS, Mayer et al. (2020) mapped haplotype-trait associations at high resolution in the DH libraries. The comparison of molecular haplotype inventories between the DH lines and a broad panel of 65 European breeding lines, genotyped with the same 600k array, revealed that the landraces carry novel beneficial haplotype variation for traits related to early plant development. DH lines carrying these novel haplotypes outperformed breeding lines not carrying the respective haplotypes. Most haplotypes associated with

target traits showed stable effects across populations and environments and only limited correlated effects with undesired traits making them ideal for introgression into elite germplasm. The strategy proposed in Mayer et al. (2017) was successful in linking molecular variation to meaningful phenotypes and identifying novel variation for quantitative traits in plant genetic resources, as shown in Mayer et al. (2020).

Here, results presented in Mayer et al. (2017), Hölker et al. (2019) and Mayer et al. (2020) are complemented by additional findings relevant for the development and evaluation of the proposed strategy for accessing the native diversity of landraces for elite germplasm improvement.

2 Material and methods

2.1 Genotypic and phenotypic data

2.1.1 Plant material

The 35 European maize landraces investigated in Mayer et al. (2017) were chosen to represent the diversity of maize cultivated in Central and Western Europe before the hybrid breeding era, covering different biogeographical regions (Figure 2). Part of the landraces represented historically important varieties in terms of acreage (Oettler et al. 1976) and/or served as source of important inbred lines for establishing the European flint elite breeding pool (Messmer et al. 1992). From each landrace, 22 to 48 plants, hereafter referred to S_0-plants, were randomly drawn, resulting in a total of 952 individuals for genotyping (Table 1). The panel of European landraces was extended by data from 4,710 individuals from 4,020 Central and South American maize landrace accessions of the International Maize and Wheat Improvement Center (CIMMYT; Hearne et al. 2012), which were part of the SeeD project (http://seedsofdiscovery.org). After filtering for accessions with known geographical origin, 3,560 individuals from 2,898 accessions remained.

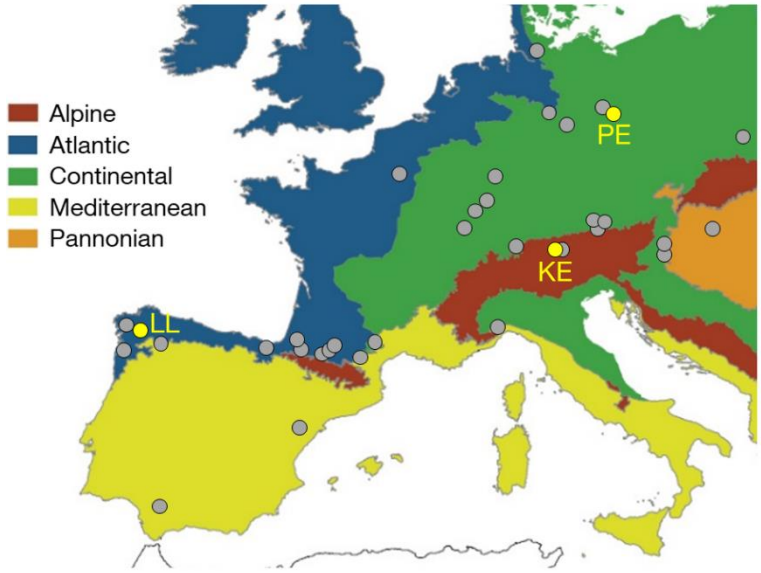

Figure 2: Map of biogeographical regions of Europe and the origin of 35 maize landraces analyzed in this thesis. The map of biogeographical regions was modified after Schmeller et al. (2012). Dots indicate the approximate origin of the landraces based on the coordinates provided in Mayer et al. (2017). The three landraces selected for generation of the DH libraries analyzed in Hölker et al. (2019) and Mayer et al. (2020) are colored in yellow.

Three European maize landraces, Kemater Landmais Gelb (KE), Lalin (LL) and Petkuser Ferdinand Rot (PE), were selected for DH production, based on the population genetic analyses described in Mayer et al. (2017) as well as based on data from preliminary field

trials evaluating the genetic variation for traits related to early plant development. After a seed multiplication step, a total of 1,015 DH lines (516 KE, 67 LL and 432 PE) were generated and multiplied using the *in vivo* haploid induction method (Röber et al. 2005). For comparison of molecular diversity between S_0-plants and DH lines, additional 48 S_0-plants from each of the three landraces were drawn randomly from the same seed batch that served as source for DH production. For a subset of 378 DH lines (190 KE and 188 PE), TCs were produced using the dent line F353 (Institut national de la recherche agronomique, INRA) as female parent.

Table 1: Landraces under study. Name, abbreviation, geographical origin and number of genotyped S_0-plants and DH lines of each landrace after quality filtering. Landraces from which DH lines were derived are highlighted in gray (Table modified after Mayer et al. 2017).

Landrace name	Abb.	Geographical origin	N genotyped	
			S_0-plants	DH lines
Andoain	AN	Northern Spain	24	-
Barisis	BA	Northern France	24	-
Bugard	BU	Southern France (Pyrenees)	22	-
Castellote	CA	Eastern Spain	24	-
Colmar	CO	Eastern France (Alsace)	24	-
Gazost	GA	Southern France (Pyrenees)	24	-
Gelber Badischer Landmais	GB	South Western Germany	46	-
Gleisdorfer	GL	South Eastern Austria	24	-
Kemater Landmais Gelb	KE	Austria (Alpine valley)	(*48) / 48	501
Knillis	KN	South Eastern Austria	24	-
Krajova c29	KR	Slovakia	24	-
Lacaune	LC	Southern France	24	-
Lalin	LL	North Eastern Spain (Galicia)	(*48) / 48	31
Lucq de Bearn	LB	Southern France (Pyrenees)	24	-
Mahndorfer	MD	Northern Germany	24	-
Maleksberger	MB	Central/Northern Germany	24	-
Millette du Lauragais 2	ML	Southern France (Pyrenees)	24	-
Moncassin	MO	Southern France (Pyrenees)	24	-
Nostrano dell Isola	ND	Northern Italy	24	-
Oberhuber Martha	OM	Austria (Alpine valley)	24	-
Oesterreichische Landsorte	OE	Austria	24	-
Petkuser Ferdinand Rot	PE	North Eastern Germany	(*47) / 48	409
Pfarrkirchner	PF	South Eastern Germany	24	-
Polnischer Landmais	PL	Poland	24	-
Rheintaler Monsheim	RM	Western Germany (Rhine valley)	24	-
Rheintaler (St. Gallen)	RT	Switzerland (Lake Constance)	23	-
Rottaler	RO	South Eastern Germany	24	-
Roux de Chalosse	RD	Southern France (Pyrenees)	24	-
Santiago	SA	North Eastern Spain (Galicia)	23	-
Schindelmeiser	SC	North Eastern Germany	23	-
Strenzfelder	SF	Central/Northern Germany	23	-
Tremesino	TR	Southern Spain	24	-
Tuy	TU	North Eastern Spain (Galicia)	24	-
Viana	VI	North Eastern Spain (Galicia)	48	-
Wantzenau	WA	Eastern France (Alsace)	24	-

*In addition to the 48 S_0-plants analyzed in Mayer et al. (2017), another set of S_0-plants (LR3-S_0; Table 2) were genotyped, after seed multiplication, from the same seed batch that served as source for DH production.

The 66 breeding lines described by Unterseer et al. (2014, 2016) served as representatives of the European flint elite breeding germplasm in comparisons with landrace-derived DH lines. In addition to the flint lines used in Mayer et al. (2020), genotypic data of 70 dent lines (16 European and 54 US American lines) were taken from Unterseer et al. (2016) and used in this thesis for extending the analysis of the genomic background of elite germplasm to the dent breeding pool.

2.1.2 Genotypic data

Table 2 gives an overview of the different genotypic datasets investigated in this thesis. For comparative analyses, datasets were combined using the respective overlapping markers.

Table 2: Overview of genotypic datasets. Name, included material, genotyping platform, number of genotypes and markers, and publications in which the datasets were used.

Name	Material	Genotyping platform	N genotypes	N markers	Publications
LR35-S_0	S_0-plants of 35 European landraces	600k array	952	516,797	Mayer et al. 2017; Hölker et al. 2019
LR3-S_0	S_0-plants of 3 landraces selected from LR35-S_0; same seed batch as LR3-DH	600k array	143	499,574	Hölker et al. 2019
LR3-DH	DH-lines derived from three European landraces	600k array	941	501,124	Hölker et al. 2019; Mayer et al. 2020
SeeD-S_0	S_0-plants of 2,601 Central and South American landrace accessions	GBS	3,101	104,223	Mayer et al. 2017
Flint-BL	Breeding lines of European flint pool	600k array, except for two lines with WGS	65	501,124	Mayer et al. 2020
Dent-BL	Breeding lines of Dent pool (mainly USA)	600k array	70	501,124	-

Individuals of the European landraces (Table 1) as well as 64 European flint and 70 dent breeding lines were genotyped with 616,201 markers, using the 600k Affymetrix® Axiom® Maize Array (Unterseer et al. 2014). For all 600k array datasets, markers were filtered for assignment to the best quality class (Poly High Resolution; Unterseer et al. 2014), a call rate ≥ 0.9 and a mapped position on the B73 reference sequence, AGPv2 (Chia et al. 2012) for Mayer et al. (2017) and AGPv4 (Jiao et al. 2017) for Hölker et al. (2019) and Mayer et al. (2020).

In Mayer et al. (2017), markers which were specifically developed to maximize differences among US Stiff-stalk and non-Stiff-stalk material (Ganal et al. 2011; Frascaroli et al. 2013) as well as insertion/deletion (indel) markers were excluded to avoid bias in population genetic analyses. The resulting dataset (LR35-S_0; Table 2) comprised 952 S_0-plants from 35 landraces (Table 1) and 516,797 single nucleotide polymorphism (SNP) markers. The unimputed dataset of the SeeD panel (Hearne et al. 2012) used in Mayer et al. (2017) comprised 955,120 markers generated by genotyping by sequencing (GBS; Elshire et al. 2011). Filtering for bi-allelic SNPs and for a call rate ≥ 0.8 resulted in a set of 3,101 individuals from 2,601 accessions and 104,223 markers (SeeD-S_0; Table 2). When analyses required data with known haplotype phases and without missing genotype calls, imputation and phasing was conducted for each landrace of LR35-S_0 separately and for the entire set of SeeD-S_0 using Beagle version 4.0 (Browning and Browning 2007) with default settings, except for parameter 'nsamples', which was set to 50. Comparative analyses between the European and American landraces were based on 5,045 overlapping markers (Mayer et al. 2017).

For the landrace-derived DH lines used in Hölker et al. (2019) and Mayer et al. (2020), preliminary ancestry analyses were conducted with the software ADMIXTURE (Alexander et al. 2009), using the S_0-plants of the three landraces for defining the ancestry groups. Lines with less than 75% concordance with the presumed landrace of origin were excluded from further analyses. A filter for < 5% heterozygous calls was applied, to exclude markers which potentially mapped to multiple positions in the genome as well as lines derived from diploid embryos. Remaining heterozygous calls (0.19%) likely resulted from technical errors and were set to missing. All missing genotype calls were imputed using Beagle version 5.0 (Browning et al. 2018) with default settings. The resulting dataset (LR3-DH; Table 2) comprised 941 DH lines of three landraces (501 KE, 31 LL and 409 PE; Table 1) and 501,124 markers. From the 144 S_0-plants from the seed batch used for DH production, one line was excluded due to > 10% missing values. Missing genotype calls in these S_0-plants were imputed and gametic phase estimated separately for each landrace, using Beagle version 5.0 (Browning et al. 2018) with parameter settings 'iterations' = 50, 'phase-segment' = 10, 'phase-states' = 500 and with the DH lines of the respective landrace used as reference panel. For comparative molecular analyses between the DH lines and the original landraces, 499,574 markers overlapping between LR3-DH and the set of 143 S_0-plants (LR3-S_0; Table 1 and Table 2) were used in Hölker et al. (2019).

The 600k genotypic data of the breeding lines where filtered for the 501,124 high quality markers of LR3-DH. One flint breeding line (FV66) was excluded due to > 5% of calls indicating heterozygosity. For two flint breeding lines (EZ5 and F64), genotypic data of 424,400 sites overlapping with the 501,124 markers (85%) were extracted from whole genome sequence (WGS) data of the HapMap panel (version 1.2.3; Bukowski et al. 2018). For combining the WGS and 600k array data, all alleles were coded according to the B73 AGPv4 (Jiao et al. 2017) forward strand. After setting remaining heterozygous calls to missing, missing values for the set of 65 flint breeding lines (Flint-BL; Table 2), used in Mayer et al. (2020), as well as the set of 70 dent breeding lines (Dent-BL; Table 2) were imputed separately using Beagle version 5.0 (Browning et al. 2018) with default settings.

2.1.3 Field experiments

LP performance was evaluated for 958 DH lines as well as 14 flint and one dent breeding lines used as checks in up to eleven environments (location × year combinations). Genotypes were randomized using multiple 10 × 10 lattice designs with two replicates (single row plots with 3 m in length), with 1000 entries (958 DH lines plus checks) at four locations in 2017 and 800 entries (756 DH lines plus checks) at three locations in 2018 in Germany. A random subset of lines (500 entries, 458 and 468 DH lines plus checks in 2017 and 2018, respectively) was phenotyped at two locations in Spain in both years. In addition to the line checks, evaluated as duplicate entries, the original landrace populations were included as quadruplicate entries. In total, 899 DH lines phenotyped in the field passed quality filtering of the corresponding genotypic data. Phenotypic data of the remaining lines were excluded from further analyses. Mean temperature per environment for the period of 15th of April to 30th of September ranged between 14.0°C (Oberere Lindenhof; OLI, 2017) and 19.0°C (Tomeza; TOM, 2018), while minimum and maximum temperatures were -6.0°C (OLI, 2017) and 39.5°C (Bernburg; BBG, 2018). TC performance was evaluated at four locations in Germany in 2018 using 10 × 10 lattice designs with two replicates per genotype (400 entries, 378 DH lines × F353 plus checks). Three flint breeding lines were crossed to the same tester and used in addition to two commercial hybrids as checks in the TC trials. Checks were included in each of the four lattices (i.e. quadruplicate entries). For both, LP and TC trials, sowing density, fertilization and plant protection followed standard agricultural practices at the respective trial locations.

More than 25 traits were measured in the LP trials. The focus lied on early-development-related traits like early vigor (EV; at growth stages V3, V4, and V6, score 1-9, 1 = very poor vigor, 9 = very vigorous), early plant height (PH; at V3, V4 and V6, average over three

measured plants per plot, cm) and cold tolerance (1–9 score, 1 = low, 9 = high cold tolerance; symptoms were chlorosis and necrosis on the leaves). Further, agronomic traits like final plant height (PH_final; cm), female (FF; days after sowing until 50% of plants show silks) and male (MF; days after sowing until 50% of plants shed pollen) flowering as well as undesirable traits like tillering (TILL; score 1-9, 1 = no tillers, 9 = many and long tillers) and lodging (LO; 1 = no lodging, 9 = all plants showing severe lodging) were evaluated. Additionally, morphological traits like tassel architecture (tassel length, spike length, number of tassel branches, and tassel angle) as well as physiological traits like maximum efficiency of photosystem II (at V4 and V6, measured with fluorometer OS-30p, Opti-Sciences Inc., USA) and leaf greenness (at V3, V4, and V6, measured with chlorophyll content meter CCM-200, Opti-Sciences Inc., USA) were measured. For TC trials, additionally forage total dry matter yield (dt/ha) and dry matter content (near infrared spectroscopy or drying, in %) were evaluated.

The analysis of phenotypic data was based on the following model (Hölker et al. 2019):

$$y_{ijkopst} = \mu + \gamma_i + d_{ij}\delta_j + u_{k(ij)} + w_o + d_{ij}\delta w_{jo} + uw_{ko(ij)} + k_{p(o)} + r_{s(op)} + b_{t(ops)} + e_{ijkopst} \quad (1)$$

$y_{ijkopst}$	trait observation
μ	overall mean
γ_i	effect of group *i*, with *i* = 1, 2, 3 (DH lines, checks, and S_0-plants) for LP and *i* = 1, 2 (DH lines, checks) for TC
δ_j	effect of landrace *j* in group *i* = 1, with *j* = 1, 2, 3 (KE, LL, PE) for LP and *j* = 1, 2, (KE, PE) for TC
d_{ij}	dummy variable, with $d_{ij} = 1$ for *i* = 1 and $d_{ij} = 0$ otherwise
$u_{k(ij)}$	effect of genotype *k* nested in group *i* and landrace *j*
w_o	effect of environment *o*
δw_{jo}	interaction effect for landrace *j* and environment *o*
$uw_{ko(ij)}$	interaction effect for genotype *k* and environment *o*
$k_{p(o)}$	effect of the lattice *p* nested in environment *o*
$r_{s(op)}$	effect of replicate *s* nested in lattice *p* and environment *o*
$b_{t(ops)}$	effect of block *t* nested in replicate *s*, lattice *p* and environment *o*
$e_{ijkopst}$	residual error

For estimating genotype and genotype × environment interaction variance components, all effects except γ_i and δ_j were treated as random. Variance components for $u_{k(ij)}$ and

$uw_{ko(ij)}$ were modeled individually for the three landraces, assuming that DH lines were unrelated. Residuals were assumed to be normally distributed with mean zero and different variances for DH lines ($d_{ij} = 1$) and checks/S_0-plants ($d_{ij} = 0$), but equal residual variance for all three landraces in all environments. Raw data was manually filtered for outliers based on residual plots. Variance components and their standard errors were estimated using ASReml-R package version 3.0 (Butler et al. 2009). Heritabilities were calculated separately for each landrace on an entry-mean basis according to Hallauer et al. (2010):

$$h^2 = \frac{\sigma_u^2}{\sigma_u^2 + \frac{\sigma_{uw}^2}{n_w} + \frac{\sigma_e^2}{n_w n_r}} \quad (2)$$

h^2	entry-mean heritability
σ_u^2	genotype variance
σ_{uw}^2	genotype × environment variance
σ_e^2	residual variance
n_w	number of environments
n_r	number of replicates

Adjusted genotype means (BLUEs; Best Linear Unbiased Estimates) across environments were calculated using the model in equation 1 without the terms γ_i, $d_{ij}\delta_j$ and $d_{ij}\delta w_{jo}$ and treating u_k as fixed effect. BLUEs within environments were calculated using the same model without environment-related model terms. Phenotypic correlations among traits were calculated based on Pearson correlation coefficients from BLUEs within DH libraries. Genetic correlations were estimated by expanding the model defined in equation 1 to a bivariate model for pairs of traits.

2.2 Analyses of molecular data

2.2.1 Site frequency spectrum

In population genetic theory, the term site frequency spectrum (SFS) refers to the distribution of allele frequencies of all mutations in a population. In accordance with the infinite sites mutation model (Kimura 1969), segregating sites are assumed to be bi-allelic. The SFS is estimated by tabulating the sample allele frequencies of derived alleles at all segregating sites and is given by the vector $f = (f_1, f_2, f_3, \dots, f_{g-1})$, where f_i is the proportion of sites with a derived allele frequency of i/g and g is the number of gametes

within the sample. The expected SFS under the standard coalescence model with infinite sites mutations is given by Nielsen and Slatkin (2013):

$$E[f_i] = \frac{1}{i}\sum_{j=1}^{g-1}\frac{1}{j} \quad , i = 1,2, \dots, g-1 \tag{3}$$

The determination of which allele at a given locus is derived and which is ancestral is usually based on examining outgroups, i.e. closely related species. If outgroup information is not available, like in Mayer et al. (2017), an alternative approach is to fold the SFS by summing up respective frequency classes of reference alleles, which are arbitrarily chosen for each bi-allelic locus. The folded SFS f^* is obtained by $f^* = f_i + f_{g-i}$ for $i < g/2$ and $f^* = f_i$ for $i = g/2$ (Nielsen and Slatkin 2013). Using unimputed datasets for calculating SFS in Mayer et al. (2017), g gametes with non-missing genotype calls were randomly sampled per marker, where $g = 2nc$ with n corresponding to the number of individuals and c representing the minimum call rate in the respective dataset. The folded SFS was calculated by averaging allele frequencies per SNP over 1,000 random samples.

Deviations of an empirical SFS from the expected SFS as defined in equation 3 can be caused by demographic as well as selective processes. While population growth and positive or negative selection shift the SFS towards extreme allele frequencies, population decline, population substructure and balancing selection can lead to an excess of intermediate allele frequencies (Hartl and Clark 1997). Distortions in the SFS can also be caused by nonrandom sampling of the genomic loci under consideration. This so-called ascertainment bias is common for genotyping arrays, where SNPs were previously discovered based on sequencing a relatively small panel of individuals and the resulting genotyping arrays are then applied to a much larger set of individuals. As the probability of discovering rare alleles depends on the sample size, i.e. in this case the size of the discovery panel, SNP array data tend to be enriched for intermediate allele frequencies (Nielsen et al. 2004). Further, ascertainment bias can arise, if the allelic composition of the discovery panel is not representative for the sample under consideration (Frascaroli et al. 2013; Lachance and Tishkoff 2013). Population genetic analyses, like estimation of molecular diversity, LD and population differentiation, depend on allele frequencies and therefore are affected by ascertainment bias (Nielsen and Signorovitch 2003; Albrechtsen et al. 2010). To assess the extent and influence of ascertainment bias in the analyzed data (Mayer et al. 2017), SFS were calculated for different datasets and compared with the expected SFS (equation 3).

2.2.2 Diversity parameters

Various measures can be used for describing molecular diversity. A simple measure of DNA polymorphisms is the number of segregating sites, *S*, in a population (Hamilton 2009). *S* is usually calculated for samples of DNA sequences and can be expressed as the proportion of segregating sites relative to the total number of nucleotide sites. Analogously for the 600k array data, the proportion of polymorphic markers (PP; Nei 1987) was calculated for different datasets in Mayer et al. (2017). While PP is not affected by sample allele frequencies, other measures of diversity are sensitive to allele frequency changes and thus capture different information. Nucleotide diversity (π) is a commonly used measure and defined as the average number of pairwise nucleotide differences between a set of n DNA sequences (Nei and Li 1979; Tajima 1989):

$$\hat{\pi} = \frac{1}{\binom{n}{2}} \sum_{i}^{n-1} \sum_{j>i}^{n} \pi_{ij} \tag{4}$$

π_{ij} number of nucleotide differences between the i-th and j-th DNA sequence

When calculated per site, or as in Mayer et al. (2017) per marker, π is identical to the unbiased estimate of gene diversity, also known as expected heterozygosity (*H*), which is defined as (Nei and Roychoudhury 1974; Tajima 1989):

$$\widehat{H} = \frac{n}{n-1}\left(1 - \sum_{i=1}^{I} \hat{p}_i^2\right) \tag{5}$$

I number of distinct alleles at the locus (i.e. $I = 2$ for bi-allelic SNPs or $I =$ number of distinct haplotypes in a given genomic window)

$\hat{p}_i$ frequency of allele i in the sample

A similar measure of diversity is polymorphism information content (PIC), which is used to assess a marker's usefulness for linkage analysis (Botstein et al. 1980). PIC is the probability that a given marker genotype of an offspring of a heterozygous parent, affected with a dominant disease, will allow to deduce which marker allele the offspring inherited from this parent (Botstein et al. 1980):

$$\widehat{PIC} = 1 - \sum_{i=1}^{I} \hat{p}_i^2 - \sum_{i=1}^{I-1} \sum_{j=i+1}^{I} 2\hat{p}_i^2 \hat{p}_j^2 \tag{6}$$

Both, *H* and PIC, can be calculated based on SNPs (H_{SNP} and $\mathrm{PIC}_{\mathrm{SNP}}$) or haplotypes ($H_{\mathrm{hap}}$ and $\mathrm{PIC}_{\mathrm{hap}}$), where haplotypes refer to particular combinations of jointly inherited nucleotides at neighboring markers in a defined genomic window. In Mayer et al. (2020), H_{SNP} and $\mathrm{PIC}_{\mathrm{SNP}}$ as well as H_{hap} and $\mathrm{PIC}_{\mathrm{hap}}$ were used for assessing the molecular diversity of landrace-derived DH-libraries (LR3-DH) and breeding lines (Flint-BL). In Mayer et al. (2017), H_{hap} was used in addition to PP and π as an haplotype based diversity measure for LR35-S_0 and SeeD-S_0. Haplotype based parameters can be assumed to be less affected by ascertainment bias compared to SNPs (Conrad et al. 2006). In addition to the diversity of individual nucleotide sites comprised in the window under consideration, haplotype diversity depends on the number of recombinations that have occurred in the respective genomic region. Therefore, the minimum number of historical recombination events (nR) was calculated in Mayer et al. (2020) for LR3-DH and Flint-BL, based on the four-gamete test (Hudson and Kaplan 1985).

2.2.3 Population structure

The term population structure describes the grouping of a population into smaller subpopulations in which mating usually takes place (Hartl and Clark 1997). Characteristically, individuals are genetically more related to each other within than across subpopulations. Analyzing population structure and the phylogenetic relationships between sampled individuals can help in understanding evolutionary processes and inferring patterns of historical ancestry (Matsuoka et al. 2002; van Heerwaarden et al. 2011; Mir et al. 2013). Knowledge about population structure in the material under study is important, as population structure can be a confounding factor in genetic analyses like GWAS and genomic prediction (Zhao et al. 2011; Barton et al. 2019).

Genetic dissimilarity between individuals can be estimated based on genetic distance measures calculated from molecular markers (Reif et al. 2005c). Modified Rogers' distance (MRD; Wright 1978; Goodman and Stuber 1983) is a scaled Euclidean distance measure, ranging between 0 and 1:

$$MRD = \frac{1}{\sqrt{2m}} \sqrt{\sum_{i=1}^{m} \sum_{j=1}^{I_i} \left(p_{ij} - q_{ij}\right)^2} \qquad (7)$$

m number of markers

I_i number of alleles at marker *i* (for bi-allelic SNPs, $I_i = 2$)

p_{ij}, q_{ij} allele frequencies of the *j*-th allele at the *i*-th locus in the two populations or individuals under consideration ($p_{ij}, q_{ij} \in \{0, 0.5, 1\}$ for individuals)

Fulfilling the Euclidean distance property, MRD is well suited to elucidate population structure with multivariate analysis methods, such as hierarchical cluster and principal coordinate analysis (PCoA). PCoA is a statistical method for representing a dissimilarity matrix (e.g. MRD) in a low-dimensional, Euclidean space (Gower 1966). PCoA decomposes the dissimilarity matrix into its latent roots and vectors, also called Eigenvalues and Eigenvectors. The Eigenvectors, standardized by the square root of the corresponding Eigenvalue and ordered according to their Eigenvalues, represent the principal coordinates (PCo). In contrast to the columns of the dissimilarity matrix, the PCos are uncorrelated (orthogonal) and the proportion of variance explained per PCo is maximized. PCoA based on pairwise MRD between individuals was calculated in Mayer et al. (2017), Hölker et al. (2019) and Mayer et al. (2020) for analyzing population structure in the respective dataset. In addition, an unrooted neighbor joining tree was constructed for dataset LR35-S_0 (Mayer et al. 2017). The neighbor joining method (Saitou and Nei 1987) is a clustering algorithm applied to genetic (dis)similarity measures (e.g. MRD), resulting in an unrooted phylogenetic tree in which distances, represented by the length of the branches, are additive. The algorithm starts with a star-like tree, in which each sample represents a separate cluster, and iteratively joins two samples to a newly created node. The pair of samples to be joined in each step are chosen based on the principle of minimum evolution (Saitou and Nei 1987), minimizing the total branch length of the resulting tree. Besides such algorithmic approaches, ancestry coefficients can be estimated as parameters of statistical models, as implemented in programs like *structure* (Pritchard et al. 2000) and ADMIXTURE (Alexander et al. 2009). For a pre-defined number of groups, *K*, both programs simultaneously estimate group allele frequencies along with ancestry proportions of each individual, based on the probability of the observed genotypes. ADMIXTURE relies on maximum likelihood estimation, leading to a considerable reduction in computation time compared to the Bayesian approach of *structure*. If no *a priori* information on (sub)populations is available, *K* can be estimated using a cross-validation approach implemented in ADMIXTURE, predicting masked genotype scores and comparing them to observed values. ADMIXTURE can also be used in a supervised mode, where genetic groups are pre-defined by reference individuals. The algorithm was used for analyzing population structure in LR35-S_0 and SeeD-S_0 (Mayer et al. 2017) as well as estimating ancestry proportions for Flint-BL and Dent-BL. As ADMIXTURE assumes linkage

equilibrium between markers, the respective marker sets were pruned based on LD using PLINK (Purcell et al. 2007).

Genetic differentiation among identified or *a priori* known subpopulations can be assessed by partitioning the total molecular variance into within and across subpopulation (co)variance, using an analysis of variance framework (Weir and Cockerham 1984; Excoffier et al. 1992). Analysis of molecular variance (AMOVA) is based on Euclidian distances among gametes and estimates (co)variance components and F-statistic analogs, reflecting the correlation of molecular diversity at different levels of hierarchical subdivision (Excoffier et al. 1992). A typical hierarchical structure is given in Table 3 (Excoffier and Lischer 2010):

Table 3: Hierarchical structure in an analysis of molecular variance (AMOVA)

Source of variation	Degrees of freedom	Expected mean squares
Among subpopulations	$P-1$	$\frac{1}{P-1}\left(2N-\sum_{p\in P}\frac{2N_P^2}{N}\right)\sigma_a^2+2\sigma_b^2+\sigma_c^2$
Among individuals within subpopulations	$N-P$	$2\sigma_b^2+\sigma_c^2$
Within individuals	N	σ_c^2
Total	$2N-1$	σ_T^2

P	number of subpopulations
N	number of diploid individuals
σ_a^2	covariance component due to differences among subpopulations
σ_b^2	covariance component due to differences among individuals within subpopulations
σ_c^2	covariance component due to differences among gametes within individuals
σ_T^2	total molecular variance

The following statistics can be derived from the (co)variance component estimates:

$$F_{ST}=\frac{\sigma_a^2}{\sigma_T^2} \quad (8) \quad , \quad F_{IT}=\frac{\sigma_a^2+\sigma_b^2}{\sigma_T^2} \quad (9) \quad , \quad F_{IS}=\frac{\sigma_b^2}{\sigma_b^2+\sigma_c^2} \quad (10)$$

F_{ST} "fixation index"; proportion of molecular diversity due to allele frequency differences among subpopulations

F_{IT} departure of genotype frequencies from Hardy–Weinberg expectations relative to the entire population

F_{IS} "inbreeding coefficient"; average departure of genotype frequencies from Hardy–Weinberg expectations within subpopulations

Significance of covariance components and F-statistic analogs can be tested using permutations (Excoffier et al. 1992). AMOVA was used in Mayer et al. (2017) for decomposing the total molecular variance of dataset LR35-S_0 into within- and across-landrace components, as well as for estimating the proportion of variance captured by groups of landraces. Further, F_{IS} was estimated separately for each landrace of LR35-S_0. In Hölker et al. (2019), AMOVA was used for estimating proportions of molecular variance within and between the sets of DH lines (LR3-DH) and S_0-plants (LR3-S_0) of each landrace.

2.2.4 Linkage disequilibrium

LD refers to the nonrandom association of alleles at different loci in gametes of a population. Many genetic analyses rely on the concept of LD, such as marker-assisted selection, GWAS and genomic prediction. The extent of LD determines the resolution that can be obtained in GWAS, with lower LD levels leading to higher resolution, assuming marker density is high enough. For two bi-allelic loci with alleles A/a and B/b, respectively, LD can be measured as D, the difference between the observed and expected frequency of haplotype AB (Lewontin and Kojima 1960):

$$D = p_{AB} - p_A p_B \tag{11}$$

p_{AB} frequency of haplotype consisting of the pair of alleles A and B at the first and second locus, respectively

p_A , p_B frequencies of alleles A and B at the first and second locus, respectively

The maximum range of D spans from -0.25 to 0.25. The realized range depends on marker allele frequencies, with the largest positive value being $p_A p_b$ or $p_a p_B$, whichever is smaller, and the largest negative value being -$p_A p_B$ or -$p_a p_b$, whichever has the smaller absolute value. Lewontin (1964) introduced another measure of LD, D', which is scaled by the maximum value of D, given the allele frequencies in the studied material, and thus ranges between 0 and 1. In contrast to D, the standardized measure D' allows comparison of relative values across genomic regions or even across studies. Other standardized

measures of LD are r and r^2 (Hill and Robertson 1968), where r is defined as $D/\sqrt{(p_A p_a p_B p_b)}$ and represents Pearson's correlation coefficient in allelic state between alleles in the same gamete. Whenever it is important to capture the information of gametic phase, as for example in the context of genomic prediction across populations, r, ranging from -1 to 1, is an appropriate measure of LD. In Mayer et al. (2017), r was used for assessing the persistence of linkage phase between landraces of dataset LR35-S_0, calculating the correlation of r values of the respective landraces as well as the proportion of r values with equal sign. In many cases, only the magnitude of LD and not the direction is relevant, like for example for detecting marker-trait associations in GWAS. In such cases, r^2, ranging from 0 to 1, is a commonly used parameter, as it is an intuitive measure of how well the allele at one locus (e.g. the QTL) can be predicted by the allele at another locus (e.g. the marker). Due to recombination, LD decays with physical distance. Therefore, the level of LD within a population under study is often described as an average decay distance. Hill and Weir (1988) derived an equation describing r^2 as a function of physical distance between loci. Using this equation, LD decay distance was estimated for the datasets analyzed in this thesis (Table 2) by fitting a nonlinear regression curve and determining the crossing point with r^2 = 0.2.

2.3 Haplotype identification

2.3.1 Haplotype construction

Haplotypes derived from experimental data represent phased nucleotide sequences in a defined genomic segment comprising multiple SNPs. Therefore, haplotypes are generally more discriminative between individuals than single SNPs and better suited for tracking the inheritance of potentially trait-associated alleles (Stephens et al. 2001; Hayes 2013). Knowing the gametic phase is a prerequisite for haplotype construction. Phases are known when working with fully homozygous lines, as in Mayer et al. (2020). When working with heterozygous material, they can be inferred statistically (Browning and Browning 2011; Pook et al. 2020), as in Mayer et al. (2017). Various methods for haplotype construction exist. Haplotypes can be constructed for sliding windows with a fixed physical length (Hess et al. 2017) or based on a constant number of markers (Calus et al. 2009; Ferdosi et al. 2016). If marker density is high, windows can be chosen according to the boundaries of annotated genes (Yano et al. 2016; Bustos-Korts et al. 2019). Pre-defining genomic windows for haplotype construction facilitates haplotype comparisons across populations, varying in their evolutionary history and extent of LD. Window sizes, however, should not

be chosen too large, for reducing the risk of haplotypes being broken up by recombination. Alternatively, many methods exist for generating haplotype blocks that are specific for the respective population under study. Most of those methods are based on identifying local patterns of increased LD or reduced recombination in the genome (Nothnagel et al. 2002; Gabriel et al. 2002; Barrett et al. 2005; Pattaro et al. 2008; Kim et al. 2017). Other methods infer haplotype blocks based on genomic segments with reduced diversity (Daly et al. 2001; Patil et al. 2001) or based on pairwise (Browning and Browning 2013) or groupwise (Pook et al. 2019) segments identical by descent (IBD). For the analyses conducted in Mayer et al. (2020), comparability across datasets, in this case between landraces and breeding germplasm, was an important factor. Therefore, haplotypes were constructed for sliding windows with a constant number of ten SNPs. Parameters nR and H_{hap} as well as the average physical and genetic window size were considered for evaluating the extent of recombination that might have occurred in the constructed haplotypes. As the marker density of the 600k array roughly follows the average recombination rate (Unterseer et al. 2014), using a fixed number of markers should result in similar probabilities of recombination per window.

2.3.2 Association scans

Mixed linear models are routinely used in GWAS, testing the association of a SNP (or haplotype) with a trait of interest, while simultaneously controlling confounding factors such as population structure and cryptic relatedness. In the standard model, the SNP (or haplotype) tested is fitted as a fixed effect, while additional fixed effects, e.g. principal components of the genotype data, account for population structure. In addition, a random polygenic effect, with a genomic relationship matrix defining the covariance structure, accounts for cryptic relatedness. Many variations of this model, initially proposed by Yu et al. (2006), were developed, for improving computational efficiency and/or GWAS power (Xiao et al. 2017). In Mayer et al. (2020), association analyses were conducted in two steps, following the approach of Millet et al. (2016). First, the genome-wide efficient mixed-model association (GEMMA; version 0.98.1) algorithm of Zhou and Stephens (2012) was used for conducting univariate GWAS within single environments as well as across environments, using the respective environment-specific and across-environment BLUEs as response variable in the model, respectively:

$$\mathbf{y} = \mathbf{W}\boldsymbol{\alpha} + \mathbf{x}\beta + \mathbf{Z}\mathbf{u} + \mathbf{e} \tag{12}$$

$\mathbf{y}$ vector ($n \times 1$) of phenotypic values (BLUEs), with n = number of lines

$\mathbf{W}$ design matrix ($n \times 3$) for fixed effects

$\boldsymbol{\alpha}$ vector (3 × 1) of fixed effects (intercept and landrace effects of KE and LL)

$\mathbf{x}$ vector (n × 1) of genotype scores (0 or 2) of the haplotype tested

β fixed effect of the marker tested

$\mathbf{Z}$ design matrix (n × n) for random effects

$\mathbf{u}$ vector (n × 1) of random genotypic effects, with $\mathbf{u} \sim N(0, \mathbf{K}\sigma_g^2)$; where $\mathbf{K}$ denotes the (n × n) genomic relationship matrix according to Astle and Balding (2009) and σ_g^2 the genetic variance pertaining to the model

$\mathbf{e}$ vector (n × 1) of random genotypic effects, with $\mathbf{e} \sim N(0, \mathbf{I_n}\sigma_e^2)$; where $\mathbf{I_n}$ denotes the (n × n) identity matrix and $\sigma_\mathbf{e}^2$ the residual variance pertaining to the model

Haplotypes were coded as binary presence/absence markers, leading to scores 0 and 2 for fully homozygous lines. Haplotypes present less than three times in the dataset were excluded from GWAS. For each single environment as well as for the across-environment GWAS, significance of haplotype effects was determined using a likelihood-ratio test and a false discovery rate (FDR; Benjamini and Hochberg 1995) of 15%. Significant haplotypes from the same genomic region (within 1 Mb distance) and in high LD ($r^2 \geq 0.8$) were considered as marking the same QTL. The respective trait-associated genomic region was defined as the interval between the start and end position of the first and last haplotype belonging to the QTL, respectively. For subsequent analyses, each QTL was represented by the haplotype with the lowest *P*-value in the respective region, referred to as the focus haplotype.

In the second step, the candidate haplotypes obtained from the first step were tested in a multi-locus, multi-environment model as suggested by Millet et al. (2016):

$$y_{ijk} = \mu + \omega_i + \delta_j + \sum_{q \in \mathbf{Q}} x_{kq}\beta_q^i + u_k + e_{ijk} \quad (13)$$

y_{ijk} phenotypic value (BLUE) of line k belonging to landrace j tested in environment i

μ common intercept

ω_i fixed effect of environment i

δ_j fixed effect of landrace j

x_{kq} genotype score (0 or 2) of line k for haplotype q

β_q^i fixed effect of haplotype *q* in environment *i* comprising the haplotype main and haplotype by environment interaction effect, i.e. $\beta_q^i = \beta_q + (\beta \times \omega_i)_q$

u_k random genotypic effect of line *k*, with $\mathbf{u} \sim N(0, \mathbf{I}\sigma_g^2)$; where $\mathbf{I}$ denotes the identity matrix and σ_g^2, the genetic variance pertaining to the model

e_{ijk} random residual error with environment-specific residual error variance

Step-wise backward elimination of haplotypes based on the Wald test (Kenward and Roger 1997) was performed, leading to a final set of haplotypes, **Q**. At every step, the significance of β_q^i was assessed for each haplotype when it was the last one entering the model and the haplotype with the highest *P*-value was removed, as long as $P \geq 0.01$. The proportion of genetic variance explained by the final set of haplotypes, **Q**, was determined by the reduction in σ_g^2, when comparing the null model (excluding $\sum_{q \in \mathbf{Q}} x_{kq}\beta_q^i$) with the full model. For comparison, GWAS was conducted analogously based on SNPs instead of haplotypes, with a minor allele count of ≥ 3.

Instead of testing each haplotype coded in a binary way separately, as described above, GWAS can also be performed by taking the haplotype window as fixed factor, treating the haplotypes within the respective window as levels of a categorical variable (Bustos-Korts et al. 2019). This approach was used in Mayer et al. (2020) for evaluating the effect of the chosen focus haplotype (element of **Q**) relative to the alternative haplotypes in a given genomic window, extending equation 13 to:

$$y_{ijk} = \mu + \omega_i + \delta_j + \sum_{q \in \mathbf{Q}'} x_{kq}\beta_q^i + x_{kh}\beta_h^i + u_k + e_{ijk} \qquad (14)$$

$\mathbf{Q}'$ set of haplotypes **Q** without the focus haplotype of the window tested

x_{kh} haplotype *h* in the window tested, carried by line *k*

β_h^i effect of haplotype *h* relative to the focus haplotype in environment *i*

The proportion of genetic variance explained by the haplotype window was estimated by comparing the genetic variance component pertaining to the null model (excluding $\sum_{q \in \mathbf{Q}'} x_{kq}\beta_q^i + x_{kh}\beta_h^i$) and the model with the $x_{kh}\beta_h^i$ term, respectively.

2.3.3 Effect direction and stability of trait-associated haplotypes

Significance of environment-specific haplotype effects, fitted by the models defined in equation 13 and 14, respectively, was assessed by constructing 95% confidence intervals (CI = effect estimate ± 1.96 × standard error), following Millet et al. (2016). A CI excluding

0 indicated significance in a given environment. Haplotypes with constant effect direction for all environments where the effect was significant were categorized as 'favorable' or 'unfavorable'. For early plant development related traits (EV_V4, EV_V6, PH_V4, PH_V6) positive (negative) effects were defined as favorable (unfavorable). For the undesirable traits (LO, TILL), positive (negative) effects were defined as unfavorable (favorable). For traits like FF, MF and PH_final, for which breeding goals are varying, no categorization was made. Haplotypes with changing directions of significant effects, depending on the environment, were categorized as 'interacting'.

2.3.4 Haplotypes with effects on multiple traits

Identified haplotypes with significant effects on one trait, according to equation 13, were tested for effects on other traits using a bivariate model, similar to Stich et al. (2008):

$$y_{tijk} = \mu_t + \omega_{ti} + \delta_{tj} + x_k\beta_t + u_{tk} + e_{tijk} \quad (15)$$

y_{tijk} phenotypic value (BLUE) for trait *t* of line *k* belonging to landrace *j* tested in environment *i*

μ_t intercept for trait *t*

ω_{ti} fixed effect of environment *i* for trait *t*

δ_{tj} fixed effect of landrace *j* for trait *t*

x_k genotype score (0 or 2) of line *k* for the tested haplotype

β_t fixed effect of the tested haplotype for trait *t*

u_{tk} random genotypic effect of line *k* for trait *t*, with $\mathbf{u} \sim N(0,\ \mathbf{G} \otimes \mathbf{K})$

e_{tijk} residual with $\mathbf{e} \sim N(0,\ \mathbf{E} \otimes \mathbf{I}_n)$, where **G** and **E** correspond to the genetic and error variance-covariance matrices among traits pertaining to the model, respectively

Significance of haplotypes in each environment was again assessed, using 95% CIs for β_t.

2.3.5 Comparisons between landraces and breeding lines

The frequency of trait-associated haplotypes identified in LR3-DH (equations 13 and 14) was assessed in Flint-BL for evaluating which favorable haplotypes were absent and which unfavorable haplotypes were common in breeding lines. The former represent potentially novel beneficial variation, while the latter represent potential targets for purging in breeding germplasm. Frequency distributions of favorable and unfavorable haplotypes were compared with a random set of 500 haplotypes present at least three times in LR3-DH.

Significance of differences in mean frequencies between groups were assessed using the Mann-Whitney test (two-sided). In addition to the molecular comparisons, the potential effect of landrace haplotypes in elite material was assessed phenotypically based on the 14 flint checks included in the field trials in 2017. Significance for differences in means between germplasm groups as well as between lines carrying or not carrying a particular haplotype was tested based on 10,000 permutations (two-sided test).

3 Discussion

Meeting rising demands on crop production under changing environmental conditions and resource shortages requires genetic improvement of crops for stress resilience and resource use efficiency. For most crops, today's elite breeding germplasm shows only limited genetic variation for such traits. In contrast, genetic resources, such as landraces, are assumed to harbor novel beneficial variation. However, for quantitative traits, efficient strategies for harnessing the native diversity of landraces are lacking. The objective of this thesis was to develop a genome-based strategy for the targeted utilization of genetic resources for elite germplasm improvement and to demonstrate its effectiveness experimentally in the context of temperate European maize. In this chapter, the combined results from the three publications underlying this thesis (Mayer et al. 2017; Hölker et al. 2019 and Mayer et al. 2020) are discussed and complemented by additional findings.

3.1 Choice of source material

3.1.1 Variation within and across landraces

For each major crop, such as maize, wheat and rice, > 50,000 accessions of genetic resources are stored in gene banks worldwide (Hoisington et al. 1999). For allogamous crops like maize, an accession mostly represents a diverse collection of seeds from an individual landrace (Sood et al. 2014). Understanding how the vast native diversity of genetic resources is distributed within and among landraces is essential for finding optimum strategies for sampling landrace material for genome-enabled studies. The molecular diversity of maize landraces of various origin has been studied based on isozyme (Doebley et al. 1988; Sanchez et al. 2000; Sood et al. 2014), restriction fragment length polymorphism (RFLP; Dubreuil and Charcosset 1998; Rebourg et al. 2001, 2003), and simple sequence repeat (SSR; Reif et al. 2005b; Vigouroux et al. 2008; Eschholz et al. 2010; Mir et al. 2013) markers. Part of these studies used multiple individuals per accession (up to twelve, except for Reif et al. 2005b with 30 individuals from five landraces) or DNA bulks of 15 plants for genotyping, enabling the comparison of variation within and among landraces. A common result of those studies was, that most of the molecular variation lies within rather than among landraces (Sood et al. 2014) or analogously within rather than among geographically defined groups of landraces (Vigouroux et al. 2008). This is a general characteristic for allogamous species. Similar observations were made in crops like rye (Persson and von Bothmer 2002), sunflower (Park and Burke 2020) and cassava (Tovar et al. 2016). Recent studies in maize used high density molecular data, based on GBS or

genotyping arrays, but either only one or few individuals per accession (Takuno et al. 2015; Arteaga et al. 2016; Romero Navarro et al. 2017) or a limited number of landraces (Melchinger et al. 2017) were genotyped. In Mayer et al. (2017), a panel of 35 European maize landraces, with 22 to 48 individuals per landrace, was genotyped with 600k SNPs (LR35-S_0; Table 2), allowing a comprehensive analysis of molecular diversity, population structure and LD within and across landraces. In accordance with previous studies based on low density marker data (Sood et al. 2014), AMOVA revealed that 73.1% of the total molecular variance lied within landraces, whereas 26.9% were due to variation across landraces. On average, five landraces captured 95% of the total molecular variance in the panel of 35 landraces. Diversity parameters, such as PP, π and H_{hap}, calculated within landraces were on average lower than in random samples of an equal number of individuals across landraces. However, except for few landraces with substantially reduced diversity (minimum within: PP = 0.410, π = 0.142 and H_{hap} = 0.474), parameter estimates indicated high diversity within landraces, with the most diverse landraces (maximum within: PP = 0.913, π = 0.306 and H_{hap} = 0.787) almost reaching the across landrace level (average across: PP = 0.965, π = 0.323 and H_{hap} = 0.863). Estimates of molecular diversity based on SNP array data might be overestimated due to an overrepresentation of sites with intermediate allele frequencies (Nielsen et al. 2004). Considering that the genetic variance of a quantitative trait is a function of effect sizes and allele frequencies at associated QTL (Falconer and Mackay 2009), the obtained estimates should still reflect the magnitude of the genetic variance. If molecular variation is predictive of genetic variation, the results obtained in this thesis indicate that single landraces can already provide high variation for quantitative traits of agronomic importance and thus could be utilized in mapping studies and pre-breeding approaches. This hypothesis was validated in Hölker et al. (2019), where three landrace-derived DH libraries evaluated for LP and TC performance for more than 25 traits, revealed large genetic variances within landraces and only small differences among landraces.

Population structure analyses in Mayer et al. (2017) indicated that most of the 35 landraces represent largely unstructured populations, as expected under approximate random mating within landraces. Individuals consistently grouped according to their landrace-membership in NJT and PCoA and had high ancestry proportions attributable to their respective pedigree, as estimated using ADMIXTURE. No pronounced sub-grouping of individuals within landraces was observed. Only five landraces showed significant, slightly positive F_{IS} values, indicating deviations from random mating in the last seed multiplication step. Especially for allogamous crops, seed multiplication and regeneration of accessions

in gene banks require special effort with a large number of controlled crosses, to avoid inbreeding or assortative mating and consequently loss of alleles (FAO 2016). The results of Mayer et al. (2017) suggest, that landraces were generally maintained properly, although it cannot be ruled out that the limited molecular diversity of few landraces might have been caused by bottlenecks during seed multiplication. The absence of pronounced population structure within landraces increases the power of association mapping and reduces the risk of spurious associations (Zhao et al. 2011). Further, absence of population structure increases the efficacy of genomic prediction in pre-breeding, because population structure can lead to biased estimates of genomic breeding values, genomic heritability, and prediction accuracy (Windhausen et al. 2012; Guo et al. 2014; Albrecht et al. 2014). Thus, it could be shown here that for samples within landraces, the prediction of breeding values of individual lines will not be masked by their membership to specific sub-populations, in contrast to broad species-wide samples (Yu et al. 2016).

LD levels within the 35 landraces analyzed in Mayer et al. (2017) were low, as indicated by a median r^2-decay distance of 251 kb, a range harboring on average less than six annotated genes according to current versions of flint reference genomes (Haberer et al. 2020). These results show that samples within landraces provide sufficient mapping resolution in GWAS. Only two landraces exhibited r^2-decay distances of > 1 Mb, at the same time also showing the lowest levels of molecular diversity. Such landraces must be excluded in a pre-screening step when choosing landrace material for mapping studies. In Mayer et al. (2017), LD levels were analyzed for sampling schemes varying in the number of landraces and number of gametes per landrace. In general, LD levels decreased with increasing numbers of landraces, with an r^2-decay distance of 50 kb when calculated across the whole set of 35 landraces. Already by combining few landraces a substantial decrease in LD could be observed compared to the within landrace LD levels, with, for example, an average r^2-decay distance of 79 kb when sampling from five landraces. The main reason for the drop in LD with increasing number of landraces might be differences in linkage phases between landraces. While for short physical distances (< 10 kb), high congruency of linkage phases was observed, the similarity in linkage phases decreased rapidly with physical distance, with for example < 65% of marker pairs within 190 – 200 kb showing equal phase between two randomly drawn landraces. The proportion of interchromosomal marker pairs in LD was generally low, but significantly increased for samples across landraces, indicating admixture induced LD. When sampling material from a limited number of landraces, sub-populations can be defined *a priori* in statistical analyses to avoid confounding effects of admixture induced LD. In contrast, for broad

samples across landraces, as for example the SeeD dataset (Romero Navarro et al. 2017), accounting for population structure is challenging, as many individuals show strong population admixture and thus cannot be clearly assigned to particular sub-populations (Mayer et al. 2017).

Overall, the comprehensive analysis of 35 European maize landraces in Mayer et al. (2017) indicated that sampling from pre-selected landraces, with large sample sizes per landrace, is advantageous for genome-enabled studies aiming at gene discovery or genomic selection. Diversity levels are only slightly reduced compared to samples across many landraces. Due to a clear genetic differentiation between landraces and absence of pronounced population structure within landraces, confounding effects of hidden population structure in genetic analyses can be avoided. Low levels of LD within landraces or combinations of few landraces enable high mapping resolution in GWAS. Accordingly, high mapping resolution was obtained for the three landraces analyzed in Mayer et al. (2020), with a median of three annotated genes per trait-associated genomic region. While differences in linkage phases compromise accuracies of genomic prediction between landraces, the prospects of genomic selection within landraces are promising. Consistent linkage phases within landraces further facilitate genotype imputation and haplotype construction, which are important factors for the precise localization of causal genes in the genome.

3.1.2 Haplotype inventories in landraces and breeding lines

Haplotype inventories were analyzed in landrace and breeding germplasm, using 486,887 SNPs overlapping across LR35-S_0, LR3-DH, Flint-BL and Dent-BL. In total, LR35-S_0 comprised 9,391k haplotypes, constructed for non-overlapping windows of ten SNPs. Most of these haplotypes had low frequencies, which is expected under the standard neutral model (Kimura 1968) and in accordance with results based on isozyme markers (Sanchez et al. 2000). In fact, 94% of haplotypes showed frequencies < 0.01 in LR35-S_0. For comparison, 353k and 328k haplotypes were found in Flint-BL and Dent-BL, respectively. Thereof, 340k (96.3%) and 310k (94.4%) of haplotypes were captured by the landrace panel. The probability of detecting a particular haplotype in a sample of gametes is a function of its frequency in the respective population and the sample size. Within each of the 35 landraces, sample sizes of 22 to 48 individuals (44 to 96 gametes; Table 1) were comparable to sample sizes of the breeding line panels. On average, 785k haplotypes were found within individual landraces, varying between 228k and 1,533k (Figure 3). Individual

landraces captured on average 36.5% of the haplotypes of the 65 flint breeding lines, varying between 20.0% and 64.1%.

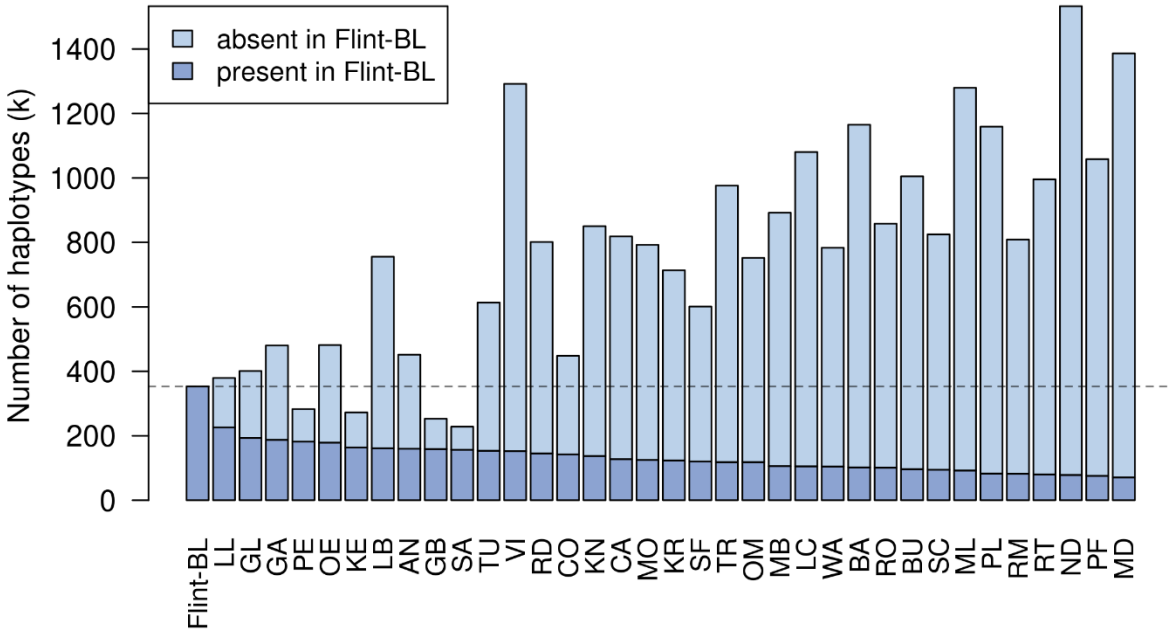

Figure 3: Number of haplotypes in each of the 35 landraces and in the panel of 65 flint breeding lines (Flint-BL). Haplotypes were constructed for non-overlapping windows of ten SNPs. The y-axis shows the number of haplotypes in thousands (k). Colors indicate the proportions of haplotypes present and absent in the Flint-BL panel, respectively. The dashed horizontal line marks the number of haplotypes (353k) detected in the Flint-BL panel.

Almost all landraces showed a comparable or higher number of haplotypes compared to the breeding line panels. Further, all landraces harbored a large amount of haplotypes not found in the breeding lines (Figure 3; minimum 71k for SA). Most of these haplotypes can be expected to have neutral effects (Kimura 1968), part of them might be disadvantageous, but some of them might represent useful novel variation. If landraces are pre-selected for variation in target traits, for example in preliminary field trials, the proportion of potentially novel favorable haplotypes associated with the respective traits should be increased.

As reported in Mayer et al. (2020), haplotype frequencies were correlated between landrace-derived material and breeding lines. Haplotypes absent from breeding germplasm tend to have low frequencies in landraces. Accordingly, also for LR35-S_0, large differences in allele frequencies between haplotypes present and absent in the flint breeding lines were observed, showing mean frequencies of 0.085 (median = 0.041) and 0.002 (median = 0.001), respectively. Only 270k of the novel haplotypes (i.e. absent in breeding lines; Figure 3) showed frequencies > 0.01 in the panel of 952 S_0-plants. When looking at mean haplotype frequencies within individual landraces, values increased to 0.155 (median = 0.127) and 0.028 (median = 0.021), respectively. On average, an individual landrace showed 225k novel haplotypes appearing at least twice in the landrace, i.e.

having a frequency of > 0.021 $\left(\frac{2}{96}\right)$ or up to > 0.045 $\left(\frac{2}{44}\right)$ within the respective landrace, depending on the sample size. As the power for detecting trait-associations in GWAS depends on the allelic effect size and allele frequency, these results indicate that novel favorable alleles might show high enough frequencies for detection within a pre-selected set of landraces, whereas they might remain undetected in broad species-wide samples (Brachi et al. 2011).

When constructing haplotypes based on heterozygous material, the influence of phasing has to be taken into account. Phasing errors could generate false haplotypes leading to an overestimation of the number of haplotypes in the dataset. On the contrary, phasing algorithms favor parsimonious solutions (Browning and Browning 2011) and thus rare haplotypes might remain undetected. High phasing accuracies were reported when analyzing pseudo S_0-plants generated from the DH libraries of LR3-DH (Pook et al. 2020), suggesting that the overall influence of phasing on haplotype construction for the S_0-plants might be minor. When applying a conservative filter with haplotypes being present at least three times in LR35-S_0, the number of haplotypes in the landrace panel decreased from 9,391k to 3,756k. Still, 29 out of the 35 landraces comprised more haplotypes than the breeding line panels, and two landraces showed only < 4k less haplotypes than the Flint-BL dataset. Another factor that could influence comparisons of molecular diversity between the landraces and breeding line panels is ascertainment bias. Although haplotype-based analyses should be less affected by ascertainment bias than SNPs (Conrad et al. 2006), it has to be considered that the 65 flint and 70 dent breeding lines were part of the discovery and/or validation panels when generating the genotyping array (Unterseer et al. 2014), whereas landrace material was not included. This could lead to an overestimation of diversity of the breeding lines compared to the landraces (Lachance and Tishkoff 2013). Thus, the landrace panel might carry additional novel haplotypes, which remained undetected in the analyses.

3.1.3 Adaptation and genomic background of landraces relative to elite germplasm

For avoiding unexpected allelic effects when incorporating novel alleles from landraces into elite germplasm and for increasing the incorporation efficacy, it is advantageous if the source material is adapted to the target environments and shows a similar genomic background compared to the elite breeding pool to be improved. For assessing the molecular diversity of the 35 European landraces (LR35-S_0) in a broader context, molecular analyses were extended to the Central and South American landraces of the SeeD project (SeeD-S_0; Mayer et al. 2017). The SeeD-S_0 dataset showed higher haplotype diversity, as

measured by H_{hap}, compared to LR35-S_0 (Mayer et al. 2017). This fits with theoretical expectations, considering the proximity of the American landraces to the center of maize domestication (Matsuoka et al. 2002; van Heerwaarden et al. 2011) and is in accordance to previous results based on RFLP (Rebourg et al. 2003) and SSR (Mir et al. 2013) markers. Despite their presumably higher genetic variation, the sub(tropical) Central and South American landraces might be less suitable for improving temperate dent and flint elite breeding pools, because they are not adapted to temperate environmental conditions (Sood et al. 2014). Flowering time played a key role in the local adaptation of maize (Bouchet et al. 2013; Romero Navarro et al. 2017; Huang et al. 2018). Adaptation to higher latitudes and temperate environments led to earlier maturity, which is a major characteristic of the European flint breeding germplasm. It was previously shown that genes presumably involved in promoting early flowering in European flint breeding lines had been under selection in European flint landraces (Unterseer et al. 2016). This indicates similarity in the allelic composition between European flint breeding and landrace material, for genes with major contribution to local adaptation.

In addition to similarities in adaptation, it can be assumed that temperate European maize landraces show a more similar genomic background to temperate elite germplasm than sub(tropical) Central and South American landraces. In previous analyses with the 65 flint and 70 dent breeding lines, six major genetic groups were defined (Unterseer et al. 2014, 2016): 'Northern Flint', 'Non-Northern Flint', 'Iodent', 'Lancaster', 'Non-Stiffstalk' and 'Stiffstalk'. Here, ancestry proportions for the breeding lines were estimated using the 35 European landraces of LR35-S_0 as well as the 16 genetic groups of the SeeD-S_0 dataset as reference groups, as defined in Mayer et al. (2017). Calculations were conducted using the software ADMIXTURE in supervised mode based on 4,754 SNPs overlapping among datasets. As expected, the European flint breeding lines were almost exclusively assigned to genetic groups defined by European flint landraces (Figure 4). The landrace Gelber Badischer Landmais showed the most pronounced contribution to 'Northern Flint' breeding lines, which is in accordance to historical records (Oettler et al. 1976; Messmer et al. 1992). Landraces from the Pyrenean region and Galicia showed most pronounced contributions to 'Non-Northern Flint' breeding lines. To a minor extent, the 'Non-Northern Flint' breeding lines had ancestry proportions attributable to Central and South American landraces, mainly from Argentina and the Caribbean. This is in accordance with previous reports, suggesting that southern European maize partly originated from Caribbean and South American material, whereas northern European maize originated most likely from North American Northern Flint maize (Tenaillon and Charcosset 2011). Interestingly,

ancestry proportions of the 70 dent breeding lines were mainly attributable to European maize landraces with dent-like kernel morphology (Unterseer et al. 2016), whereas Central and South American landraces contributed to a lesser extent and mainly only to 'Lancaster' and 'Non-Stiffstalk' lines (Figure 4). The here obtained ancestry proportions cannot be interpreted as ancestry by pedigree, as most of the analyzed landraces did not serve as source for today's heterotic breeding pools. However, the results indicate that the genomic background of temperate breeding lines is more similar to temperate European landraces than to (sub)tropical landraces.

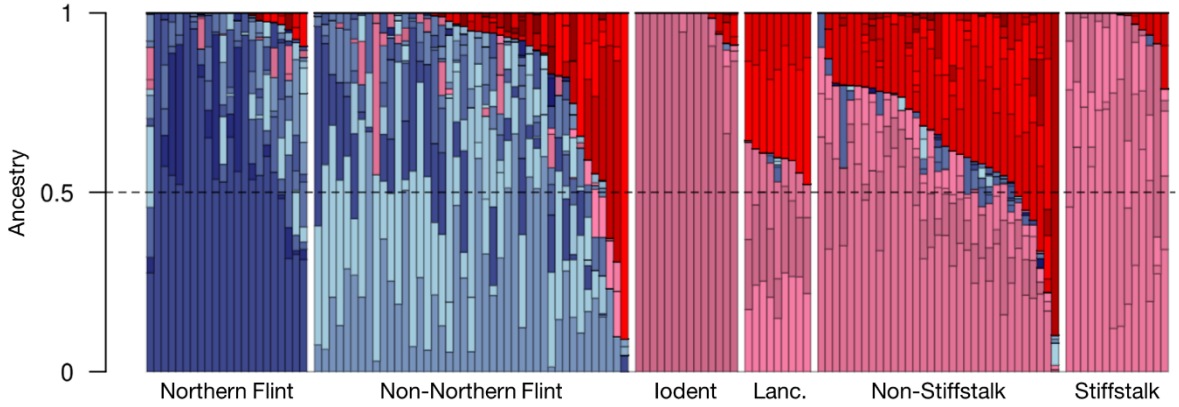

Figure 4: Ancestry proportions of 65 flint and 70 dent breeding lines. The 35 European landraces and 16 genetic groups of the Central and South American landraces were used as templates in calculations with ADMIXTURE in supervised mode. Only landrace individuals with clear group assignment (> 50% ancestry; Mayer et al. 2017) were used as reference. Each bar represents one breeding line consisting of up to 51 colors according to their ancestry proportions attributable to each of the 51 groups. The 29 and six European landraces with flint-like and dent-like kernel morphology (Unterseer et al. 2016) are represented by shades of blue and pink, respectively. The 16 groups of the SeeD-S_0 dataset are colored in shades of red. The x-axis shows the group membership of the breeding lines, as defined by Unterseer et al. (2014, 2016).

3.2 Representation of allelic diversity by DH libraries

Performing genome-enabled studies with landrace material requires the generation of reproducible genetic units. For this thesis, DH lines were derived from three landraces selected based on the results of Mayer et al. (2017). DH lines represent "immortalized" genetic units, allowing for *ad libitum* seed multiplication and thus evaluation in repeated experiments with any degree of precision desired (Hölker et al. 2019). It can be expected that the instantaneous inbreeding associated with DH production leads to a purge of recessive deleterious alleles (Charlesworth and Willis 2009). As an allogamous species, maize harbors many deleterious, partly recessive alleles, mostly at low frequency (Mezmouk and Ross-Ibarra 2014; Yang et al. 2017). Modern inbred lines have been shown to have an overall lower proportion of deleterious alleles compared to landraces (Yang et

al. 2017). This is in accordance with considerably higher success rates of DH production in elite material compared to landraces (Melchinger et al. 2017; Romero Navarro et al. 2017; Hölker et al. 2019). Substantial differences in DH production success rates were observed for the three landraces analyzed in the course of this thesis, with one landrace (LL) yielding only 31 DH lines, compared to several hundred for the other two landraces (KE and PE), despite approximately equal efforts per landrace (Hölker et al. 2019). The different success rates might point to differences in population history, as some landraces might have undergone mild inbreeding and/or anthropogenic selection in the past, decreasing the number of deleterious, (sub)lethal alleles in the population. In accordance with the low DH production efficiency, LL showed higher inbreeding depression in LP evaluations compared to KE and PE (Hölker et al. 2019).

Concern was arising that the selection against deleterious alleles during DH production might lead to loss of diversity in DH libraries compared to the original landraces (Zeitler et al. 2020). While the purging of deleterious alleles itself is desired, it could also lead to the loss of potentially useful alleles linked to deleterious sites. Comparing genome-wide diversity statistics, such as π and H_{HAP}, Zeitler et al. (2020) found consistently lower diversity levels in DH libraries compared to five European landraces from which they were derived. The findings contradict results of Melchinger et al. (2017), who found comparable values of H_{SNP} between DH libraries and original landraces for the same material. The different results can be explained by the use of different marker sets (38k vs. 28k), due to a minor allele frequency filter applied by Melchinger et al. (2017). Here, genome-wide estimates of PP, π, H_{HAP} and LD were compared between S_0-plants of two different seed batches (LR35-S_0, LR3-S_0) and DH lines (LR3-DH) for the three landraces under study, using no minor allele frequency filter. As in Zeitler et al. (2020), H_{HAP} was calculated for non-overlapping windows of 50 kb harboring at least five SNPs. Overall, diversity measures were comparable in LR3-DH and the batch of S_0-plants from which they were derived (LR3-S_0; Figure 5), with only PE showing slightly decreased values of π and H_{HAP} in the DH lines. LD levels were highest in the DH libraries, for all three landraces, but for KE and LL the difference to LR3-S_0 was minor. Interestingly, LR3-S_0 showed slightly lower values of PP, π and H_{HAP} and higher values of LD compared to the preceding generation, LR35-S_0, consistently for all three landraces. At the level of haplotypes, the samples of LR35-S_0 were most diverse compared to LR3-S_0 and the DH library for all three landraces (Figure 5). While for KE and LL, S_0-plants of LR3-S_0 and the DH library showed comparable numbers of haplotypes, the number was slightly reduced in the DH library of PE. Haplotypes not

captured by the DH libraries mostly had very low frequencies in LR3-S_0, with 50%, 25% and 70% of haplotypes occurring only once for KE, LL and PE, respectively.

KE	LR35-S0	LR3-S0	LR3-DH
n	96	96	501
PP	0.818	0.718	0.773
π	0.254	0.227	0.248
H_{Hap}	0.589	0.509	0.542
LD	420	932	984

LL	LR35-S0	LR3-S0	LR3-DH
n	96	96	31
PP	0.917	0.818	0.847
π	0.295	0.271	0.288
H_{Hap}	0.699	0.638	0.659
LD	90	182	207

PE	LR35-S0	LR3-S0	LR3-DH
n	96	94	409
PP	0.837	0.813	0.807
π	0.245	0.244	0.232
H_{Hap}	0.583	0.573	0.529
LD	287	329	490

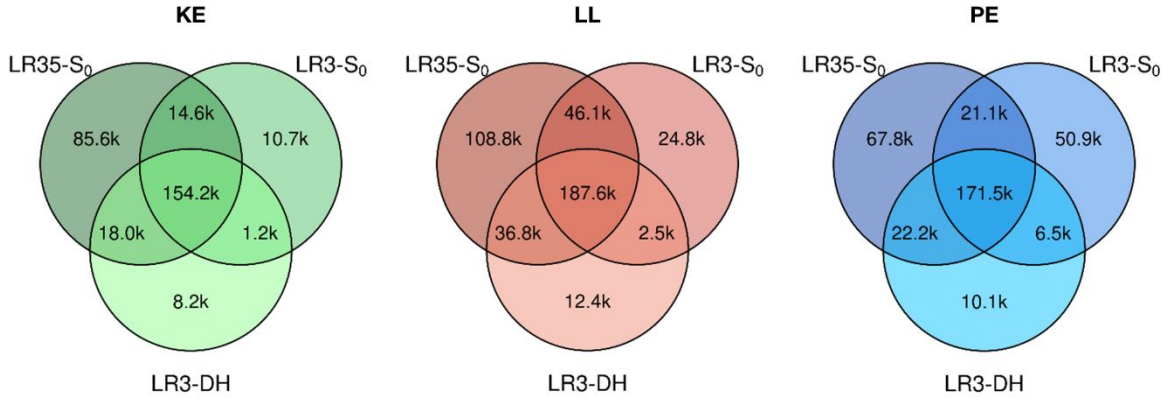

Figure 5: Comparison of diversity measures and number of haplotypes between DH libraries and original landraces. *Top*: Number of gametes (*n*), proportion of polymorphic markers (PP), nucleotide diversity (π), haplotype heterozygosity (H_{HAP}) and r^2-decay distance ($r^2 < 0.2$; in kb; LD) for the DH library (LR3-DH), the batch of S_0-plants from which they were derived (LR3-S_0) and a preceding batch of S_0-plants (LR35-S_0), for landraces KE (*left*), LL (*middle*) and PE (*right*). *Bottom*: Venn diagrams show the number of haplotypes, constructed in windows of ten SNPs, occurring in LR3-DH, LR3-S_0 and LR35-S_0, for landraces KE (*left*), LL (*middle*) and PE (*right*). Analyses were based on 486,887 SNPs.

Changes in allele frequencies and loss of alleles during the course of seed multiplication in gene banks have been reported previously for allogamous crops (Holden et al. 1984; Chebotar et al. 2003). Genetic changes can be caused by unintended selection based on the environmental conditions during seed multiplication, especially upon occurrence of abiotic and biotic stresses (Chebotar et al. 2003). Also *in situ* conservation of crop genetic resources involves dynamic changes in a population's allelic composition, due to natural or farmer-induced selection (Bellon et al. 2017). Selective forces undoubtedly also act during the generation of DH lines (Charlesworth and Willis 2009; Melchinger et al. 2017; Hölker et al. 2019; Zeitler et al. 2020). However, as shown in this thesis, DH libraries capture large proportions of the molecular diversity of the initial landraces, as indicated by AMOVA (Hölker et al. 2019). Genetically, DH libraries can clearly be assigned to their respective landrace, as indicated by PCoA (Hölker et al. 2019). Previous reports suggest, that selection against deleterious alleles acts across the whole genome, although it might be

slightly increased in regions of low recombination (Melchinger et al. 2017; Zeitler et al. 2020). Most signals of selection were landrace-specific (Melchinger et al. 2017; Zeitler et al. 2020), which was also observed for the three landraces under study, supporting the assumption of no systematic directional selection. Phenotypically, large genetic variances in landrace-derived DH libraries were observed (Hölker et al. 2019), in accordance with results of Strigens et al. (2013) and Brauner et al. (2019). When comparing original landraces with synthetic populations resulting from randomly intermating thereof derived DH lines, differences in agronomic trait performance were mostly small or not significant (Strigens et al. 2013).

In conclusion, the results presented here and from previous studies suggest that the DH technology is a valuable tool for making the native diversity of landraces amenable for crop improvement, provided that landrace-specific success rates of DH production allow the generation of sufficiently high numbers of DH lines. Even though the loss of potentially useful alleles, linked to negatively selected deleterious alleles, cannot be excluded, landrace-derived DH libraries harbor high genetic variation for mapping quantitative traits. In fact, large sample sizes of the DH libraries under study enabled the identification of trait associations at high resolution and for QTL with low or moderate allele frequencies (Mayer et al. 2020). Some of the identified trait associated haplotypes were absent or at low frequency in the panel of breeding lines and are assumed to represent novel beneficial variation (Mayer et al. 2020).

3.3 Identification of novel beneficial variation

3.3.1 GWAS based on SNPs and haplotypes

Exploiting molecular diversity and historical recombination events captured in a population, GWAS assesses the degree of association between genetic variants and a trait of interest. GWAS is based upon the concept of LD (Zondervan and Cardon 2004). LD is generated by evolutionary forces such as mutation, migration, drift and selection and is broken down by recombination (Hartl and Clark 1997). The probability of recombination decreases with decreasing genetic distance between two loci and thus, in general, tightly linked loci exhibit stronger LD than loci far apart from each other. Assuming absence of population structure, genetic markers associated with a trait of interest should point to (unobserved) QTL in their proximity. Commonly used GWAS models use single biallelic SNP markers for detecting trait associations. Alternatively, haplotypes can be constructed, representing combinations of jointly inherited alleles at neighboring markers. In theory,

haplotypes increase the power of GWAS if they show higher LD with QTL alleles than single SNP markers (Hamblin and Jannink 2011). Haplotypes capture information about genomic segments IBD between individuals better than SNPs (Meuwissen and Goddard 2000; Hayes 2013). In other words, haplotypes represent segments of the genome tracing back to a common ancestor without being separated by recombination. If haplotypes are IBD, they will carry the same alleles at a potential QTL within that segment. With increasing probability of two identical haplotypes being IBD, the proportion of QTL variance explained by the haplotypes increases, particularly for QTL with low minor allele frequencies (Hayes 2013). An additional advantage of the use of haplotypes over SNPs might be the consideration of short-range epistasis in the analysis (Schaid 2004). Examples exist, where different haplotype variants in the same genomic segment were shown to be associated with specific phenotypes (Yang et al. 2013; Jiang et al. 2015; Si et al. 2016; Yano et al. 2016). Previous studies comparing the power of SNP and haplotype based GWAS reported contrasting results and suggest that it has to be assessed on a case-by-case basis weather the use of haplotypes is beneficial (Long and Langley 1999; de Bakker et al. 2005; Lorenz et al. 2010). Empirical studies reported cases where haplotype based GWAS detected trait associations which remained undetected in SNP based GWAS (Trégouët et al. 2009; Pryce et al. 2010). Other studies reported large overlaps in identified QTL between the two approaches (Liu et al. 2019) or trait associations, which were only detected with SNPs but not with haplotypes (Sato et al. 2016; Li et al. 2019). In this study, overall similar patterns of trait associations along the genome were observed for GWAS with SNPs and haplotypes of ten SNPs (Mayer et al. 2020). For the univariate GWAS (equation 12) using the adjusted genotype means across environments as response variable, on average ~80% of the trait associations detected with SNPs were located in regions also detected with haplotypes, ranging from ~68% for MF to ~86% for PH_V4. Vice versa, on average ~65% of trait associations detected with haplotypes were also detected with SNPs, ranging from ~47% for TILL to ~80% for LO. This indicates a slightly increased number of identified trait associations by the use of haplotypes compared to SNPs. However, the main reason for the focus on haplotypes for the analyses in Mayer et al. (2020) was that haplotypes are more suitable for tracking potentially shared ancestral alleles between landraces and breeding lines.

Various methods for haplotype construction exist. Methods constructing haplotype blocks based on patterns of LD (Nothnagel et al. 2002; Gabriel et al. 2002; Barrett et al. 2005; Pattaro et al. 2008; Kim et al. 2017), linkage (Pook et al. 2019) or diversity (Daly et al. 2001; Patil et al. 2001) might capture the haplotype structure specific for the population under

consideration better than window based approaches. In contrast, fixed window based approaches might be better suited for conducting comparisons among populations varying in their evolutionary history and extent of LD, as for example landraces and breeding lines. Therefore, in Mayer et al. (2020) haplotypes were constructed for non-overlapping windows with a fixed number of markers. The choice of window size influences the number of haplotypes per window as well as the risk of haplotypes being broken up by recombination. Further, the chosen window size has to be adjusted according to the available marker density. For evaluating which window size might provide the best balance between a high probability of haplotypes indicating IBD (in contrast to identity by state; IBS) and at the same time low probability of recombination, the average physical and genetic length of haplotypes as well as different diversity parameters were calculated for varying window sizes (Table 4). The average number of distinct haplotypes per window, H_{HAP}, and nR were assessed for dataset Flint-BL.

Table 4: Parameters of diversity and recombination for varying numbers of SNPs per window. Average physical (kb) and genetic (cM) length of haplotypes constructed for non-overlapping genomic windows with varying numbers of SNPs as well as the average number of distinct haplotypes, haplotype heterozygosity (H_{HAP}) and the number of historical recombination events (nR) per window, calculated for the panel of 65 flint breeding lines (Flint-BL). Measures of genetic length are according to a genetic map generated from a F_2 mapping population of a cross of EP1 × PH207 (Haberer et al. 2020).

N SNPs	Physical size (kb)	Genetic size (cM)	N haplotypes	H_{HAP}	nR
2	4.2	0.003	2.75	0.44	0.07
5	16.8	0.012	4.69	0.59	0.44
10	37.8	0.026	7.25	0.67	1.20
20	79.7	0.055	11.06	0.74	2.79
40	163.7	0.113	16.46	0.81	6.00
80	331.6	0.229	23.38	0.87	12.43
160	667.7	0.460	30.92	0.91	25.29
320	1341.3	0.907	37.67	0.94	50.99
640	2693.9	1.761	43.06	0.95	102.37

Consider two extreme cases of window sizes of two and 640 SNPs (Table 4). In the case of two SNPs, haplotypes have a low probability of recombination, but a high probability to occur just by chance in any population (IBS instead of IBD) and thus they are not predictive for a potential underlying QTL allele of interest. In the case of 640 SNPs, haplotypes are extremely specific and predictive for a potential QTL allele, but the probability that they have been broken up by recombination is very high and thus an absence of the respective haplotype of interest does not imply an absence of the underlying QTL allele. In this study, a window size of ten SNPs provided high enough haplotype diversity and specificity, as

indicated by an average number of 7.25 haplotypes per window, while having a low probability of recombination, as indicated by low physical and genetic window sizes as well as moderate H_{HAP} and low nR values. Therefore, a window size of ten SNPs was used in the analyses in Mayer et al. (2020).

After the construction of haplotypes, various approaches can be used for conducting haplotype-based GWAS. Two main types of tests can be distinguished: i) testing each haplotype within a specified genomic window separately against all remaining haplotypes, or ii) jointly testing all haplotypes within the respective window in a global test. In the former case, each haplotype is treated as a presence/absence variable, which can be coded as 0/1/2 for diploid individuals, and thus it is similar to SNP based tests (Abdel-Shafy et al. 2014; Howard et al. 2017; Li et al. 2019; Srivastava et al. 2020; Mayer et al. 2020). In the latter case, each haplotype represents one level of a categorical variable and the effect of each haplotype is estimated relative to an arbitrary reference haplotype (Yano et al. 2016; Chen et al. 2016; Bustos-Korts et al. 2019; Maldonado et al. 2019; Abed and Belzile 2019). Alternatively, haplotype effects within a window can be treated as a random variable (Druet and Georges 2010; Zhang et al. 2012). The prospects of each method for QTL detection depends on the distribution of haplotype effects within the respective window. If there are groups of multiple haplotypes having opposing effects on a trait, a global test might have greater power for detection compared to the single haplotype test. In contrast, if there is only one haplotype (potentially at low frequency) strongly differing from the remaining haplotypes in its corresponding phenotype, the single haplotype test might be advantageous. In many cases, both approaches might detect the underlying QTL. Here, both approaches were tested for conducting haplotype-based GWAS in the landrace data (Figure 6). Overall, similar patterns of trait associations along the genome were observed for both approaches, as exemplarily shown in Figure 6 for PH_V4, using the adjusted genotype means across environments. In total, 487 and 455 genomic windows showed significant associations for the single and joint haplotype approach, respectively. Thereof, 422 were overlapping. For the 65 windows significant in the single haplotype approach but not in the joint approach, 58 (89%) had *P*-values smaller than the 1% quantile of non-significant windows in the joint approach, indicating similar tendencies. Nevertheless, some genomic regions showed significant trait associations (FDR < 15%) only in one of the two approaches, as for example two regions on chromosomes 6 and 7 detected only in the joint haplotype approach and two regions on chromosomes 1 and 3 only detected in the single haplotype approach (Figure 6). The main goal of the GWAS in Mayer et al. (2020) was the detection of particular haplotypes with strong effects on the traits of

interest, representing potentially useful novel alleles for elite germplasm improvement. Therefore, the single haplotype based approach was used for screening the landrace-derived material for novel variation in Mayer et al. (2020). The single haplotype based approach further facilitated the performed backward elimination in the multi-locus model described in equation 13. In a later step, for obtaining a deeper understanding of the haplotype variation at trait-associated loci, the effects of all haplotypes within the respective genomic window relative to the identified favorable or unfavorable focus haplotype were estimated in a joint haplotype approach (equation 14).

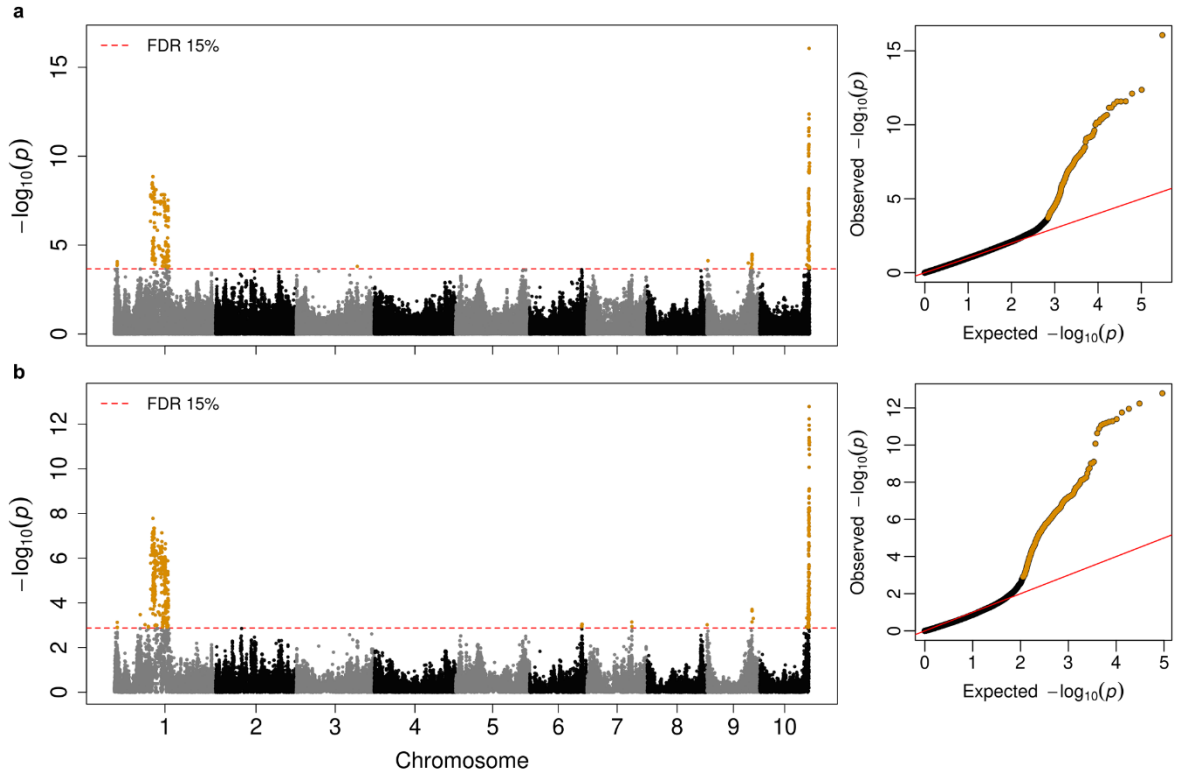

Figure 6: Genome-wide association scans for PH_V4 based on two different approaches. Manhattan plots (left) and corresponding QQ plots (right) when testing haplotypes within non-overlapping genomic windows of ten SNPs **(a)** individually or **(b)** jointly, using adjusted genotype means across eleven environments for 899 landrace-derived DH lines. Significant associations (FDR 15%) are colored in orange. Results are based on **(a)** 154,104 non-collinear single haplotypes and **(b)** 46,049 non-collinear haplotype windows, respectively.

3.3.2 Beneficial haplotypes for germplasm improvement

As commonly assumed (Ortiz et al. 2010; McCouch et al. 2013; Sood et al. 2014; Hellin et al. 2014; Melchinger et al. 2017) but demonstrated only for qualitative traits (Khush 2001; Wissuwa et al. 2002; Bailey-Serres et al. 2010), the results of Mayer et al. (2020) show convincing evidence that landraces harbor novel beneficial variation for agronomically important quantitative traits like early plant development. The complexity of haplotype structures in maize, the masking effects of adaptation and the generally small effects of

novel variants have so far hindered this kind of study. In Mayer et al. (2020), confounding effects of adaptation were mostly eliminated by focusing on a limited set of pre-adapted landraces, and the use of sufficiently large samples of homozygous DH lines allowed reliable estimation of haplotype effects. Most haplotypes were identified for traits related to early plant development, reflecting the success of pre-selecting landraces for variation in target traits. In contrast, only few haplotypes were detected for flowering time, explaining together only 2% (FF) and 33% (MF) of the total genetic variance (Mayer et al. 2020). It can be assumed that alleles with large effects on flowering time were fixed during adaptation of the respective landraces to local environmental conditions. The remaining variation for flowering time results most likely from highly polygenic effects too small to be detected in GWAS with the given sample sizes. The low number of associations for flowering time in Mayer et al. (2020) compared to studies with different sampling strategies (Romay et al. 2013; Romero Navarro et al. 2017; Gouesnard et al. 2017), supports the assumption that the study design successfully eliminated confounding effects of strong adaptive alleles.

The usefulness of trait-associated landrace haplotypes for elite germplasm improvement is determined by the size and direction of their effects as well as their dependency on the environment and genetic background. The haplotypes identified in Mayer et al. (2020) were categorized into ‘favorable’, ‘unfavorable’ and ‘interacting’ according to their effect sign and stability across environments. Most haplotypes showed moderate to high effect stability across a wide range of test environments, including 53 haplotypes with consistently favorable effects on early plant development. The average frequency of these 53 haplotypes in the panel of 65 flint breeding lines was increased compared to randomly drawn haplotypes (Mayer et al. 2020), suggesting that part of these haplotypes might have been positively selected during the last decades of breeding. Part of the haplotypes, however, showed low frequencies in the breeding line panel, with six haplotypes being completely absent, representing potentially novel beneficial variation. Most trait-associated haplotypes explained only a small proportion (< 5%) of the genetic variance of the respective trait in the landrace-derived DH lines, as expected for self-contained populations and absence of pronounced effects of population structure and adaptive alleles. The genetic variance explained is a function of allele frequency and effect size (Falconer and Mackay 2009). Frequencies of haplotypes absent in breeding germplasm tend to be small also in landraces with shared historical ancestry, as shown in section 3.1.2 and Mayer et al. (2020). In contrast, absolute effect sizes of these haplotypes can be substantial, as exemplarily shown for a novel favorable haplotype on chromosome 9 increasing early plant height (Mayer et al. 2020). The relative advantage of individuals

inheriting a favorable allele compared to the population mean is highest if the allele is initially absent in the population (Falconer and Mackay 2009), as would be the case when introducing novel landrace haplotypes into breeding germplasm. When transferring alleles across populations, however, it is well recognized that dependencies on the genetic background can lead to unexpected allelic effects (Chandler et al. 2013; Boyle et al. 2017). Unlike specifically designed mapping populations, like multi- or biparental populations (Lee et al. 2002; McMullen et al. 2009; Dell'Acqua et al. 2015), in which the number of founder lines is limited, landraces represent natural open-pollinated populations. This should result in a less pronounced background dependency of identified trait-associated alleles. The fact that most trait-associated haplotypes had equal effect signs across landraces supports this hypothesis (Mayer et al. 2020). Further, if landraces are chosen for sharing historical ancestry with the elite germplasm to be improved, genetic background effects might be minimized when introducing landrace haplotypes into breeding material. Nevertheless, the final validation of the supposed effects of landrace haplotypes in elite germplasm will require specific crosses between landrace-derived material and lines of the particular target breeding pool to be improved.

3.3.3 Haplotype variation in trait-associated genomic regions

In addition to the effect size and direction as well as the stability over environments and populations, the potential of novel favorable landrace haplotypes for elite germplasm improvement depends on the haplotype inventory of the elite breeding material. If the breeding germplasm to be improved comprises alternative haplotypes at high frequencies, which have comparable or even greater effects on the target trait as the proposed landrace haplotype, an allele substitution might have negligible or even undesired effects. Therefore, within a given trait-associated genomic window, the effect of each landrace haplotype was estimated relative to the selected focus haplotype and their frequencies were assessed in the panel of 65 flint breeding lines (Mayer et al. 2020). Further, the phenotypic performance of landrace-derived DH lines carrying particular haplotypes of interest was compared with a subset of 14 phenotyped breeding lines carrying alternative haplotypes. As expected, the identified trait-associated focus haplotypes exhibited distinct phenotypes compared to the remaining haplotypes, which in most cases showed opposite effect signs. Figure 7 shows exemplarily the haplotypes and their corresponding effects for a genomic window on chromosome 3, carrying a favorable focus haplotype significantly increasing PH_V6 as well as PH_V4 in ten out of eleven environments. The focus haplotype (A) had a frequency of 4.1% in the landraces and was absent in the panel of breeding lines. The genomic window comprised eight alternative haplotypes (B-I), occurring at least three times in the

landrace panel, as well as one additional haplotype (J) solely occurring in the breeding line panel. The six most frequent haplotypes in the landrace panel (B-G) showed significant negative effects on PH_V6 relative to the focus haplotype in almost all environments. Overall, 93.8% of breeding lines carried one of these inferior haplotypes, suggesting that a targeted substitution with the favorable focus haplotype could lead to improvement of the breeding lines. For haplotype J, carried by 6.2% of the breeding lines but none of the DH lines, no effect could be estimated, but it showed a distinct nucleotide sequence compared to the focus haplotype (9/30 nucleotide differences in the surrounding genomic region; Figure 7).

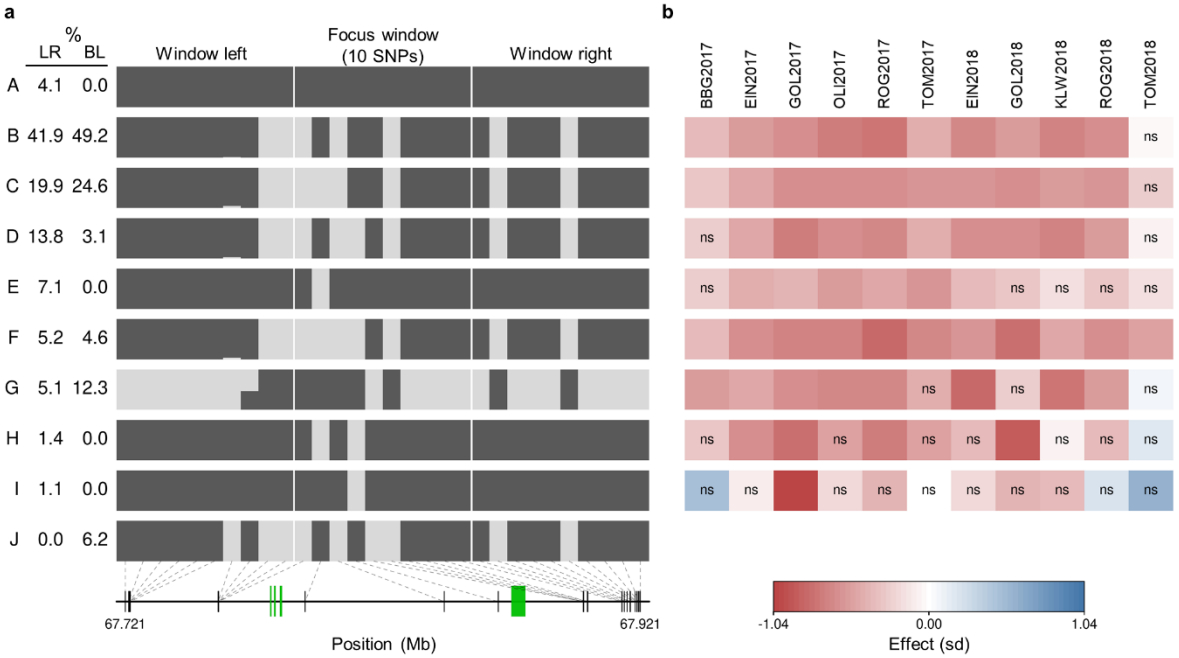

Figure 7: Effects of alternative haplotypes relative to a favorable focus haplotype for PH_V6. **(a)** Haplotype sequences for the focus window as well as the two neighboring windows. Nucleotides equal to the focus haplotype are colored in dark, alternative nucleotides in light gray, respectively. The focus haplotype (A) is at the top, the remaining haplotypes (B-J) are ordered according to their frequency in the panel of 941 DH lines derived from three landraces (LR). Numbers indicate haplotype frequencies (%) for LR and the panel of 65 flint breeding lines (BL). The positions of each SNP are indicated at the bottom. The green rectangles indicate coding regions of annotated genes. **(b)** Effect of each haplotype relative to the focus haplotype for each tested environment. Values are in units of environment-specific phenotypic standard deviations. Non-significant effects are indicated by "ns". For haplotype J, no effects were estimated, as it is only present in BL.

DH lines carrying beneficial landrace haplotypes outperformed significantly the set of 14 phenotyped breeding lines carrying alternative haplotypes, especially in cold environments, as exemplarily shown for two haplotypes on chromosome 3 and 9 associated with early plant growth (Mayer et al. 2020). These results strengthen the conclusion that a targeted introduction of the identified novel beneficial haplotypes into elite breeding germplasm might lead to the improvement of early plant development,

especially under cold conditions. In addition, TCs of the landrace-derived DH lines outperformed commercial hybrids in early plant development (Hölker et al. 2019).

3.4 Prospects of germplasm improvement through the use of landraces

Despite the potential of landraces for broadening the genetic base of elite germplasm (Cooper et al. 2001; McCouch et al. 2013; Sood et al. 2014), the direct integration of landrace-derived lines into elite breeding pools is hampered by the large performance gap to modern cultivars (Wilde et al. 2010; Brauner et al. 2019; Hölker et al. 2019). Instead, more targeted strategies have to be employed for the utilization of landraces for elite germplasm improvement. While molecular inventories of germplasm repositories are growing steadily, the link to meaningful phenotypes is missing (McCouch et al. 2012; Wang et al. 2017a; Mascher et al. 2019). Deciphering the genotype-to-phenotype map (Lewontin 1974) should be the long-term goal for an efficient use of genetic resources (Mascher et al. 2019). In Mayer et al. (2020), a strategy for linking the molecular variation of landraces to meaningful phenotypes and identifying novel favorable haplotypes for quantitative traits was proposed and demonstrated successfully with European flint maize.

The proposed study design (Mayer et al. 2017, 2020) provides an ideal basis for fine mapping and functional characterization of genes controlling agronomically important quantitative traits. By focusing on variation within pre-selected landraces high power and resolution in GWAS can be obtained, as reflected by a large number of identified genomic regions associated with target traits and a limited number of annotated genes per region (Mayer et al. 2020). Trait-associations identified within landraces might remain undetected in elite breeding pools, because relevant loci are likely to show extreme frequencies or even fixation of alleles and increased LD levels decrease mapping resolution (Van Inghelandt et al. 2011; Brauner et al. 2018; Allier et al. 2019; White et al. 2020). In addition to identification and characterization of favorable variants, it is important to generate functional knowledge on alleles with unfavorable effects, making it possible to avoid inadvertent selection for unfavorable traits, like tillering and lodging, thus making germplasm improvement from genetic resources more efficient. Furthermore, elite material could be screened for and if applicable purged from newly identified unfavorable alleles, potentially fixed in the respective breeding pool due to hitchhiking effects (Voss-Fels et al. 2017). Functional validation of relevant haplotypes will allow a more effective mining of germplasm sources for beneficial variants and can facilitate designs of complex crosses

among complementary genotypes. Further, functional knowledge is the prerequisite for genome editing approaches, allowing targeted modifications of single genes or gene combinations (Ran et al. 2013; Scheben et al. 2017). Biotechnological tools like CRISPR/Cas9 allow multiplex editing of several genomic sites at once or deletion/insertion of whole gene clusters (Xing et al. 2014), and thus represent a well-suited approach for gene introgression and improvement of quantitative traits (Jenko et al. 2015; Chen et al. 2019). The approach also allows trait stacking, which means that several beneficial genes from potentially different germplasm sources can be linked and will subsequently be inherited as a single locus. A successful example for the use of genetic engineering on a functionally characterized gene is given by Wu et al. (2019), who altered the expression of the native maize gene, *zmm28*, leading to a consistent increase in grain yield over multiple environments.

Apart from genome editing and transgenic approaches, a limited number of identified beneficial landrace haplotypes, preferably the ones with largest effects, can be readily introgressed into elite breeding pools through established forward breeding procedures, using marker assisted backcrossing (Servin et al. 2004; Ødegård et al. 2009; Han et al. 2017). Alternatively, favorable alleles could be accumulated and unfavorable alleles depleted in landrace material in a genome-wide approach, bridging the performance gap between landraces and elite cultivars through pre-breeding (Simmonds 1993; Sood et al. 2014). Pre-breeding can be described as the recurrent improvement of a donor population harboring novel variation, for obtaining improved genotypes that can be crossed with the elite breeding pool. In case of very large performance gaps between donor and elite germplasm, theoretical (Gorjanc et al. 2016; Allier et al. 2020) and practical (Sood et al. 2014) pre-breeding schemes often involve a bridging population resulting from preliminary crosses with elite breeding lines. This bears the risk, however, of reconstructing the elite parent genome in subsequent selection based on genome-wide predicted breeding values (Sood et al. 2014; Gorjanc et al. 2016). Pre-breeding progress might be accelerated through the use of genomic selection, but except for studies with comparably small sample sizes (Brauner et al. 2018) and simulation studies (Gorjanc et al. 2016; Allier et al. 2020) its implementation in that context is an unexplored area of research. The unique experimental data generated during the course of this thesis (Hölker et al. 2019; Mayer et al. 2020) is ideal for studying and optimizing genome-based pre-breeding schemes. The goal of both, backcrossing and pre-breeding approaches, is to disentangle favorable and unfavorable alleles to avoid linkage drag. The results in Hölker et al. (2019) and Mayer et al. (2020) indicated only limited genomic and locus-induced correlation between desirable and

undesirable traits. Therefore, it can be assumed that unfavorable pleiotropic effects are limited and that for breaking up unfavorable allele combinations in linkage a few cycles of recombination might be sufficient.

3.5 Conclusions

In this thesis, a genome-based strategy was developed for the identification of novel beneficial variation in genetic resources and demonstrated experimentally in the context of European maize. The main conclusions from this work can be summarized as follows:

- The comprehensive analysis of high-density genotypic data of 35 European maize landraces, with large sample sizes per landrace, revealed that most of the molecular variation can be found within landraces while differences among landraces account only for a small proportion of the variation. Absence of pronounced population structure and moderate to low LD levels were found for samples from individual landraces. Together, these findings indicate high power and resolution for GWAS within landraces.

- Consistency of linkage phases within landraces facilitates genotype imputation and construction of haplotypes. In combination with the observed limited population structure and high diversity levels, consistent linkage phases lead to high accuracy and efficacy of genomic prediction within material derived from the same landrace. In contrast, low prediction accuracies can be assumed across landraces, due to varying linkage phases.

- Comparing molecular inventories of European with Central and South American landraces showed that European landraces represent a distinct set of diversity. Integrating results from landraces and diverse panels of temperate elite material revealed that the allelic composition of temperate elite germplasm is more similar to temperate European than to (sub)tropical American landraces.

- The optimal strategy for sampling landrace material depends on the study objective. If the goal is the identification and utilization of novel variation for the improvement of elite germplasm for quantitative traits, sampling the diversity of pre-selected landraces with large sample sizes per landrace is recommended. This sampling strategy may have multiple advantages for genome-enabled studies compared to sampling individuals across a wide range of landraces: i) avoiding confounding effects of strong adaptive alleles, ii) increasing the frequency of alleles associated with the traits of interest in the material under study, iii) absence of

pronounced population structure, iv) low levels of LD, v) consistency of linkage phases, and vi) easier integration of landrace alleles into the target breeding pool.

- The phenotypic evaluation of landrace material requires the generation of reproducible genetic units. It was shown that the generation of DH lines can be a valuable approach for making the native diversity of landraces accessible, provided that landrace-specific success rates of DH production are sufficient. Although the potential loss of alleles during the DH production process cannot be excluded, DH libraries retain high levels of molecular and genetic diversity and represent the original landraces in an unbiased way.

- In total, more than 1,000 DH lines derived from three landraces were generated and evaluated with high-quality genotypic and multi-environment phenotypic data for more than 25 traits. The generated datasets represent an unprecedented resource for genome-based studies in landraces.

- Using haplotype-based GWAS, many associations for target traits but only few associations for flowering were identified. Some of the landrace haplotypes significantly improving early plant growth were absent in a broad panel of 65 breeding lines. Phenotypically, landrace-derived DH lines carrying these haplotypes outperformed breeding lines with alternative haplotypes, demonstrating the potential for germplasm improvement. Haplotype-trait associations were mostly stable across populations and environments and had no or only limited undesired effects on other traits, making them ideal for introgression into breeding germplasm.

- The proposed strategy to sample comprehensively individuals from a limited set of pre-selected landraces was successful in linking molecular variation to meaningful phenotypes, and in identifying novel beneficial alleles for quantitative traits. Demonstrated experimentally in the context of European flint maize, the approach may be extended to other maize germplasm groups and even to other allogamous crop species.

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5 Publications

This section includes reprints of the three publications underlying this thesis. The three publications, including supplementary material, can be accessed via the following links:

Mayer et al. 2017

https://link.springer.com/article/10.1007/s00122-017-2959-4

Hölker et al. 2019

https://link.springer.com/article/10.1007/s00122-019-03428-8

Mayer et al. 2020

https://www.nature.com/articles/s41467-020-18683-3

Theor Appl Genet (2017) 130:2283–2295
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ORIGINAL ARTICLE

Is there an optimum level of diversity in utilization of genetic resources?

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Abstract

Key message **Capitalizing upon the genomic characteristics of long-term random mating populations, sampling from pre-selected landraces is a promising approach for broadening the genetic base of elite germplasm for quantitative traits.**

Abstract Genome-enabled strategies for harnessing untapped allelic variation of landraces are currently evolving. The success of such approaches depends on the choice of source material. Thus, the analysis of different strategies for sampling allelic variation from landraces and their impact on population diversity and linkage disequilibrium (LD) is required to ensure the efficient utilization of diversity. We investigated the impact of different sampling strategies on diversity parameters and LD based on high-density genotypic data of 35 European maize landraces each represented by more than 20 individuals. On average, five landraces already captured ~95% of the molecular diversity of the entire dataset. Within landraces, absence of pronounced population structure, consistency of linkage phases and moderate to low LD levels were found. When combining data of up to 10 landraces, LD decay distances decreased to a few kilobases. Genotyping 24 individuals per landrace with 5k SNPs was sufficient for obtaining representative estimates of diversity and LD levels to allow an informed pre-selection of landraces. Integrating results from European with Central and South American landraces revealed that European landraces represent a unique and diverse spectrum of allelic variation. Sampling strategies for harnessing allelic variation from landraces depend on the study objectives. If the focus lies on the improvement of elite germplasm for quantitative traits, we recommend sampling from pre-selected landraces, as it yields a wide range of diversity, allows optimal marker imputation, control for population structure and avoids the confounding effects of strong adaptive alleles.

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Introduction

Maize (*Zea mays* L. ssp. *mays*) landraces are a rich source of untapped allelic variation, but efficient strategies for exploring their genetic diversity are lacking. The successful use of landraces for improving elite germplasm has been hampered by insufficient genetic and phenotypic information and their heterogeneous and heterozygous nature (Sood et al. 2014). Linking genotypes to meaningful phenotypes by genome-enabled studies will pave the way for accessing the native diversity of landraces in a targeted way (McCouch et al. 2013; Tanksley and McCouch 1997). The success of these studies strongly depends on the choice of genetic material.

Genome-enabled studies with landrace material have successfully investigated crop evolution (Hufford et al. 2012; Matsuoka et al. 2002; van Heerwaarden et al. 2011), genomic signals and marker-trait associations for adaptation

to different environments (Romero Navarro et al. 2017; Takuno et al. 2015) as well as the effects of rare alleles (Krakowsky et al. 2008). As such studies capitalize on maximizing diversity, mostly few individuals are sampled from many landraces covering a wide range of geographic regions. For the improvement of elite germplasm, an alternative approach might be more suitable, namely sampling many individuals from few pre-selected landraces. This sampling strategy comes at the expense of diversity, but might be advantageous for identifying novel alleles adapted to a specific set of environments and to the genetic background of a target elite breeding pool (Goodman 1999; Tarter and Holland 2006). Pre-selecting a representative set of landraces facilitates collection of meaningful phenotypes in the given environments and increases the incorporation efficacy of favorable alleles by reducing the risk of unexpected allelic effects (Lonnquist 1974; Sood et al. 2014). For allogamous crops such as maize, it has been shown that a large proportion of the molecular and phenotypic variation can be found within individual populations, whereas differences between major groups of landraces account only for a small proportion of the total variation (Sood et al. 2014; Vigouroux et al. 2008). In addition, the within-landrace sampling approach capitalizes upon the genomic characteristics of long-term random mating populations such as absence of hidden population structure and consistency of linkage phases. These factors can increase the accuracy and efficacy of genome-enabled approaches, such as genome-wide association studies (GWAS) and genomic selection. Thus, we hypothesize that in studies aiming at gene discovery or genomic selection based on landrace-derived material, an optimum rather than a maximum level of diversity might be beneficial. The comprehensive sampling of diversity within a few pre-selected landraces can be especially promising if the focus lies on the improvement of elite germplasm for quantitative traits. Recently, different strategies have been proposed for accessing the native diversity of landraces (Gorjanc et al. 2016; Melchinger et al. 2017) but a comprehensive comparison of within- and across-landrace estimates of genomic parameters with impact on the power of genome-enabled approaches has been lacking so far.

In this study, we analyzed genetic diversity, population structure, linkage disequilibrium (LD) and persistence of linkage phase within and across 35 European maize landraces with more than 20 individuals per landrace genotyped at high density. We investigated the effect of varying the number of sampled landraces and individuals per landrace on these parameters and give practical recommendations for assembling datasets for genome-enabled studies. We extended our analyses to Central and South American landraces of the Seeds of Discovery (SeeD) project (http://seedsofdiscovery.org) to assess the genetic diversity of European landraces in a broader context.

Materials and methods

Plant material and genetic data

We investigated 35 European maize landraces which were carefully chosen to cover a broad geographical region of Europe comprising different agro-ecological conditions (Fig. 1a). The panel included landraces with major historical relevance in terms of acreage (Oettler et al. 1976) and landraces from which important inbred lines of the European Flint elite breeding pool were derived (Messmer et al. 1992). Each landrace was represented by 22 to 48 plants, resulting in a total of 952 individuals. Name, abbreviation, geographical origin, seed source and the number of genotyped individuals (n_{LR}) for each landrace are listed in Table S1. After DNA extraction following the protocol of Saghai-Maroof et al. (1984), each sample was genotyped with the 600k Affymetrix® Axiom® Maize Array (Unterseer et al. 2014). Markers designed to specifically differentiate between two Dent lines (Ganal et al. 2011) and indels were excluded. Analyses were performed based on markers assigned to the best quality class (Unterseer et al. 2014), with a call rate ≥0.9 and known physical position in the B73 reference sequence (AGP_v2; Chia et al. 2012). All individuals exhibited a call rate ≥0.9, consequently the dataset EU-Array consisted of 952 individuals and 516,797 SNPs.

The publicly available unimputed dataset of the SeeD maize GWAS panel (Hearne et al. 2014) of the International Maize and Wheat Improvement Center (CIMMYT) comprises 4710 individuals from 4020 Central and South American maize landrace accessions (with different CIMMYT germplasm IDs) and 955,120 markers generated by genotyping by sequencing (GBS; Elshire et al. 2011). The dataset was filtered for landraces with known geographical origin, bi-allelic SNPs with a minimum call rate of 0.8 and individuals with a minimum call rate of 0.8. Thus, dataset SeeD-GBS consisted of 3101 individuals from 2601 accessions (Fig. 1b) and 104,223 SNPs. The CIMMYT germplasm IDs and the number of individuals per accession are listed in Table S2. For comparing European and American landraces, the two datasets EU-OL and SeeD-OL were created, each comprising the 5045 SNPs which overlapped between EU-Array and SeeD-GBS. The distribution of SNPs in the different marker sets is shown exemplarily for chromosome 10 in Fig. S1. A summary of the different datasets is given in Table S3.

If not denoted otherwise, analyses within landraces were based on samples of 22 to 24 individuals (24 individuals were randomly sampled for $n_{LR} > 24$; Table S1) and for analyses across landraces individuals were randomly sampled under the side condition that each individual originated from a different landrace. Analyses were done using R version 3.0.1 (R Core Team 2013).

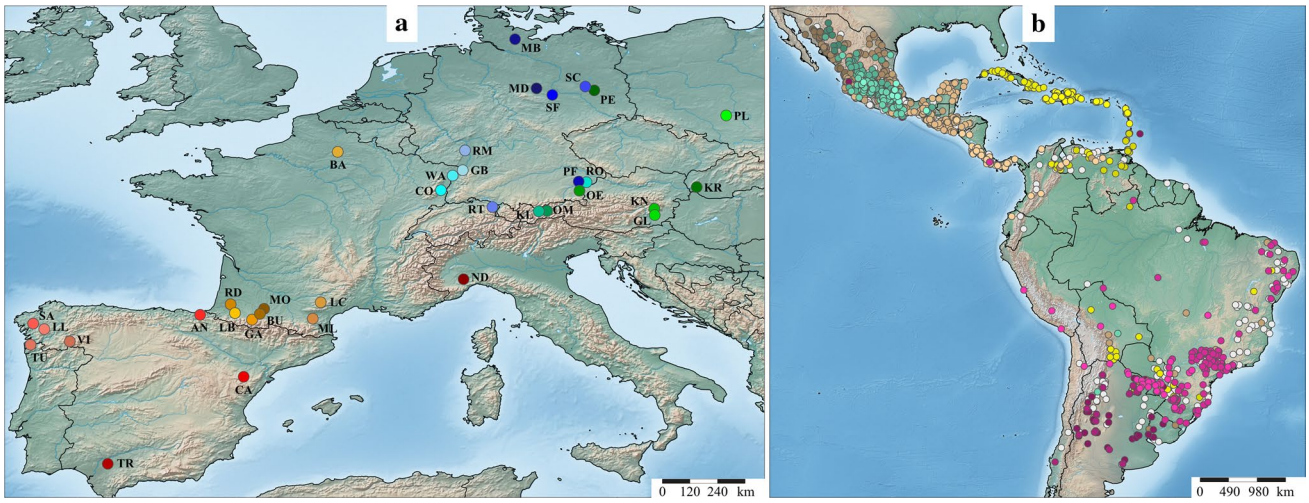

Fig. 1 Geographical origin of European (**a**) and American (**b**) maize landraces investigated in this study. **a** North-eastern and south-western European landraces (Table S1) are colored in *blue*/*green* and *red*/*orange*, respectively. **b** The coloring of the American landraces from the SeeD project (Table S2) refers to different geographical macro regions: Caribbean islands (*yellow*), Central American and Mexican lowlands (*brown*), South America (*violet-red*) and Mexican highlands (*aquamarine*). The grouping of landraces was inferred by the analysis of population structure using ADMIXTURE with 16 genetic groups. Admixed landraces with less than 50% of their ancestry attributable to one of the 16 genetic groups are shown in *light gray*

Site frequency spectrum

The term site frequency spectrum (SFS) refers to the distribution of allele frequencies for a given set of SNPs. Let f_i be the proportion of SNPs with a derived allele frequency of i/g in a sample of g gametes. The (unfolded) SFS is then given by the vector $f = (f_1, f_2, \ldots, f_{g-1})$. Following Nielsen and Slatkin (2013), the expected SFS under the standard neutral coalescence model with infinite sites mutations is given by:

$$E[f_i] = \frac{1}{i \sum_{j=1}^{g-1} \frac{1}{j}} \quad i = 1, 2, \ldots, g-1. \tag{1}$$

Here, we calculated a folded SFS f^* which describes the distribution of minor allele frequencies and is obtained by $f_i^* = f_i + f_{g-i}$ for $i < g/2$ and $f_i^* = f_i$ for $i = g/2$. For a given dataset, g gametes with non-missing genotype calls were randomly sampled per SNP, where g corresponds to $2n \times c$ with n referring to the respective number of individuals and c to the minimum call rate ($c = 0.8$ and $c = 0.9$ for American and EU landrace datasets, respectively). For the estimation of the folded SFS, the number of minor alleles per SNP was averaged over 1000 random samples.

Genetic diversity

Genetic diversity was assessed based on proportion of polymorphic markers (PP), nucleotide diversity (π) per marker (Nei and Li 1979) and haplotype heterozygosity (H; Nei and Tajima 1981). H was measured for sliding windows of 100 kb, with steps of 1 SNP and a minimum number of 5 SNPs per window. To obtain genome-wide estimates, mean π over all markers and mean H over all windows were calculated. Average deviation of genotype frequencies from Hardy–Weinberg expectations within populations was calculated using Weir and Cockerham's F_{is} (Weir and Cockerham 1984). For dataset EU-Array, genetic diversity parameters and F_{is} were estimated within each landrace and for 1000 random samples of 24 individuals across landraces. To assess the effects of sample size on diversity estimates, the parameters were calculated for 24 randomly sampled as well as for all genotyped individuals within the five landraces with $n_{LR} > 24$. The results were compared between EU-Array and EU-OL to evaluate the effects of marker number and distribution. For datasets EU-OL, SeeD-OL and SeeD-GBS, diversity parameters and F_{is} were estimated based on 1000 random samples of 35 individuals across landraces. Using the R-package ade4 (Dray and Dufour 2007) version 1.6.2, an analysis of molecular variance (AMOVA; Excoffier et al. 1992) was performed to partition the total molecular variation of dataset EU-Array into within- and between-landrace components. Furthermore, AMOVA was used to estimate the proportion of the total molecular variance captured by groups of l landraces, with $l = 1, 2, 3, 4, 5, 6, 7, 9, 18$. For each l, landraces of dataset EU-Array, with 22 to 24 individuals per landrace (24 individuals were randomly sampled for $n_{LR} > 24$; Table S1), were randomly assigned to groups of l landraces, with the number

of groups being the smallest integer ≥35/*l*. If 35 was not a multiple of *l* (for $l = 2, 3, 4, 6, 9, 18$), one group comprised only $l - 1$ landraces. For each *l*, we conducted 10,000 random repeats. Following Excoffier et al. (1992), significance for AMOVA and F_{is} was evaluated based on 1000 permutations, respectively.

Population structure

To analyze the genetic relationship between individuals, an unrooted neighbor joining tree (NJT; Saitou and Nei 1987) was constructed and principal coordinate analysis (PCoA; Gower 1966) was performed, using the R-package ape (Paradis et al. 2004) version 3.4. NJT and PCoA were based on pairwise modified Rogers' distances (MRD; Wright 1978) between individuals. NJT was constructed for dataset EU-Array. PCoA was calculated for each individual dataset as well as for a combined dataset based on SeeD-OL and one representative of each of the 35 landraces sampled from EU-OL. The correlations between MRD matrices obtained by datasets EU-Array/EU-OL and SeeD-GBS/SeeD-OL were evaluated using a Mantel test (Mantel 1967). PCoA patterns for the first three axes were compared between EU-Array and EU-OL and between SeeD-GBS and SeeD-OL via Procrustes analysis, using R-package ade4 (Dray and Dufour 2007) version 1.6.2. The software ADMIXTURE (Alexander et al. 2009) version 1.23 was used to analyze population structure. The algorithm implemented in ADMIXTURE assumes linkage equilibrium between SNPs, therefore, we pruned SNPs based on pairwise LD using the sliding window approach of PLINK (Purcell et al. 2007) version 1.7 with a window size of 50 SNPs, in steps of 5 SNPs and with an r^2 threshold of 0.8. For the estimation of the most likely number of genetic groups *K* in a given dataset a fivefold cross-validation (CV) approach was applied as implemented in ADMIXTURE. In dataset EU-Array we performed one run for each *K* varying from 1 to 25 and 20 runs with different seed settings for each *K* varying from 26 to 50. Additionally, for $K = 35$, 20 runs were conducted in a supervised mode, in which 35 genetic groups were pre-defined by choosing one individual per landrace as representative of the respective genetic group. In dataset SeeD-GBS, we performed 20 runs with different seed settings for each *K* varying from 1 to 25 and one run for each *K* varying from 26 to 50. For $K = 35$ (EU-Array) and $K = 16$ (SeeD-GBS) population structure according to the model with the lowest CV error of the respective 20 runs was visualized using a customized R-script.

Linkage disequilibrium

Following Hill and Robertson (1968), LD was estimated as r^2. We calculated r^2 for pairs of SNPs with a maximum distance of 1 Mb and investigated the decay of r^2 with physical distance using non-linear regression according to Hill and Weir (1988). An r^2 of 0.2 was used as the threshold to obtain the physical LD decay distance. For EU-Array, mean r^2 and r^2 decay distance were estimated within each landrace and for 1000 random samples of 24 individuals across landraces. For datasets EU-OL, SeeD-OL and SeeD-GBS, mean r^2 and r^2 decay distance were estimated for 1000 random samples of 35 individuals across landraces.

For dataset EU-Array, interchromosomal LD was estimated for 24 individuals sampled from $l = 1, 2, 3, 4, 6, 8, 12, 24$ landraces, with an equal number of individuals per landrace and 10 random repeats per *l*. To obtain comparable results, SNPs were binned according to their minor allele frequency in the respective sample of individuals in steps of 0.05 and for each chromosome 100 SNPs were randomly sampled per bin. The resulting 1000 polymorphic SNPs per chromosome were used for the calculation of interchromosomal LD. The significance of higher fractions of marker pairs with $r^2 > 0.2$ across landraces ($l > 1$) compared to within landraces ($l = 1$) was assessed using the two-sided Wilcoxon rank sum test (Wilcoxon 1945) with Bonferroni correction.

The effect of sample size on LD estimates was evaluated by calculating LD decay distances within the five landraces of dataset EU-Array with $n_{LR} \geq 46$ (Table S1). In addition to calculations including all individuals within the respective landrace, the number of individuals was varied from 5 to 45 in steps of 5. The effect of sample composition on LD estimates was assessed based on dataset EU-OL. LD calculations were performed for sampling schemes varying in the number of landraces *l* and the number of gametes *g* per landrace. In steps of 1, *l* varied from 1 to 35 and *g* from 1 to 44, as 44 was the minimum number of gametes per landrace in EU-OL. For each $g \times l$ combination, LD decay distances were averaged over 10 random samples. Calculations were performed for sampling schemes with $g \times l \geq 12$. To evaluate the effects of marker distribution on LD estimation, LD calculations for varying *g* and *l* were performed analogously for dataset EU-Array, with *g* and *l* varying in steps of 5.

To assess the persistence of linkage phase between landraces of dataset EU-Array, marker pairs were binned according to their physical distance in steps of 10 kb. For each bin and each pair of landraces, we calculated the correlation between the *r* values of the respective landraces and the proportion of marker pairs with equal phase (*PEP*), i.e. with equal sign of *r* (Technow et al. 2012). Both parameters were also estimated for 100 random samples of half of the individuals within each of the five landraces with $n_{LR} \geq 46$ (Table S1) compared to the second half.

Imputation and phasing

For AMOVA, population structure analyses using ADMIXTURE, and the estimation of H, F_{is}, MRD and LD, missing genotype calls were imputed and the haplotype phase inferred using BEAGLE (Browning and Browning 2009) version 4.0 with default settings except for parameter *nsamples*, which was set to 50. Phasing and imputation for dataset EU-Array were done for each landrace separately, while for Seed-GBS they were performed based on the entire dataset. For datasets EU-OL and SeeD-OL haplotype information and imputed genotypes were extracted from EU-Array and SeeD-GBS, respectively.

Availability of data and materials Genotype calls for the European and the SeeD datasets are available at figshare (https://doi.org/10.6084/m9.figshare.4789414.v1) and the CIMMYT Seeds of Discovery dataverse repository (http://hdl.handle.net/11529/10034; Hearne et al. 2014), respectively.

Results

Genetic diversity and population structure within and across European maize landraces

Dataset EU-Array comprised 952 individuals from 35 landraces (Fig. 1a; Table S1) and 516,797 SNPs with an overall call rate of 0.991. As expected for SNP array data, an excess of intermediate allele frequencies compared to the neutral expectation was observed (Fig. S2a), with an average minor allele frequency of 0.239. PP, π and H estimated across all 952 individuals were 0.999, 0.323 and 0.831, respectively. Landraces varied in their level of genetic diversity (Fig. 2; Table S4), with PP, π, and H ranging from 0.410 to 0.913, 0.142 to 0.306 and 0.474 to 0.787, respectively. Within landraces, the average levels of PP, π, and H were 0.735, 0.234 and 0.669, respectively. The average levels of PP, π, and H for 24 individuals randomly sampled across landraces were 0.965, 0.323 and 0.863, respectively. Genetic diversity parameters were on average higher within south-western compared to north-eastern European landraces (Table S5), though the three landraces with the highest PP and π values originated from Austria (GL, KN, OE; Table S4). For the five landraces with $n_{LR} \geq 46$ (Table S1), estimates of diversity parameters for 24 randomly sampled individuals were comparable to levels observed including all individuals within these landraces (Table S6). Values of F_{is} were low and not significant for most landraces, ranging from −0.064 to 0.118 with a mean of 0.006 (Table S4). Five landraces showed a small but significant excess of homozygotes at the 0.05 significance level suggesting deviation from Hardy–Weinberg equilibrium due to inbreeding and/or population structure. AMOVA revealed that 73.1 and 26.9% of the total molecular variance of the 35 landraces originated from within and across landrace variation, respectively. The

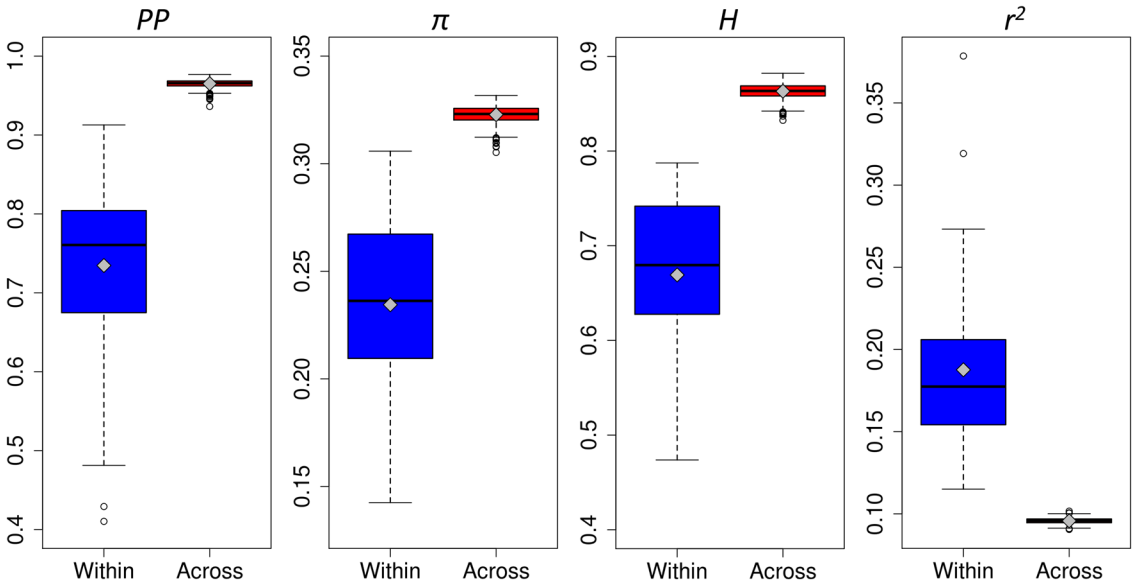

Fig. 2 Genetic diversity and LD within and across European landraces. Proportion of polymorphic markers (PP), mean nucleotide diversity per marker (π), mean haplotype heterozygosity (H) and LD (mean r^2) were calculated based on dataset EU-Array. Boxplots represent values for samples of 22 to 24 individuals within each landrace (*blue*), and for 1000 random samples of 24 individuals across landraces (*red*). Boxplots show the upper and lower quartile, median (*horizontal bar*), mean (*gray diamond*) and whiskers (*vertical bars*) of the respective statistic. Points above and below the whiskers indicate values ±1.5 times the interquartile range

across landrace variance component was significant with $p < 0.001$. On average, ~95% of the molecular variation of the entire dataset (EU-Array) was already captured by groups of five landraces (Fig. 3).

The NJT revealed a clear genetic differentiation between the 35 landraces of dataset EU-Array, with a landrace-specific grouping of individuals (Fig. S3). Different levels of relatedness between landraces were indicated by the formation of geographical clusters, e.g. for landraces from the Alsace region (CO, GB, WA), from Galicia (LL, SA, TU, VI) and from the French Pyrenees (BU, GA, LB, MO, RD). Plotting the first and second principal coordinates (PCo) of the PCoA, a group of north-eastern European landraces was located in the first and fourth quadrant and a group of south-western European landraces in the second quadrant (Fig. S4). The third quadrant contained landraces from both regions. With the exception of ND, these landraces differed from the remaining landraces in their kernel morphology. While most landraces in dataset EU-Array showed typical Flint-like kernels with a thick, hard and vitreous outer layer, these landraces (CA, GL, KN, OE, PL, TR) displayed kernels with a small indentation, characteristic for Dent maize. Analogously, the NJT (Fig. S3) showed groups of Dent-like

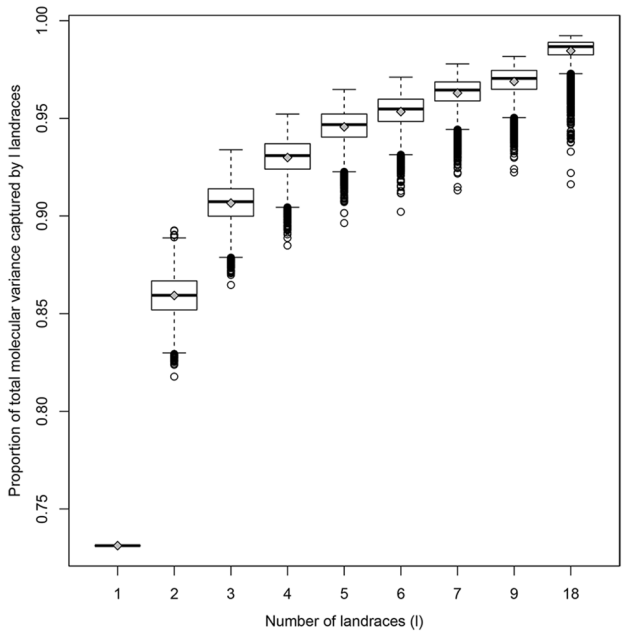

Fig. 3 Proportion of the total molecular variance captured by different numbers of landraces. Landraces of EU-Array, with 22 to 24 individuals per landrace, were randomly assigned to groups comprising *l* landraces, with $l = 1, 2, 3, 4, 5, 6, 7, 9, 18$. The proportion of the total molecular variance of the panel of 35 landraces captured by groups of *l* landraces was estimated using AMOVA. Boxplots show the upper and lower quartile, median (*horizontal bar*), mean (*gray diamond*) and whiskers (*vertical bars*) for 10,000 random repeats per *l*. Points above and below the whiskers indicate values ±1.5 times the interquartile range

north-eastern European (GL, KN, OE, PL) and Dent-like Spanish landraces (CA, TR), respectively.

Population structure of the landraces in the EU-Array panel was analyzed using the software ADMIXTURE. The most likely number of genetic groups *K* in the dataset could not be resolved unambiguously for *K* ranging from 1 to 50. CV errors decreased until $K = 35$, showed only minor differences for $35 \leq K \leq 40$ and reached a plateau for $K > 40$ (Fig. S5). Therefore, given the 35 landraces in the panel, we chose one individual per landrace to represent the genetic composition of the respective landrace. A distinct separation of the 35 landraces was detected, whereas within landraces only limited evidence of population structure was observed (Fig. 4). The five Austrian landraces (GL, KL, KN, OE, OM) as well as AN, GA, LB and PE exhibited higher levels of admixture than the remaining landraces, but for almost all individuals more than 50% of their ancestry was attributed to the respective landrace.

Linkage disequilibrium within and across European maize landraces

Based on 22 to 24 individuals per landrace of dataset EU-Array, mean r^2 of SNP pairs within 1 Mb distance ranged from 0.115 to 0.379 with a mean of 0.188 (Table S4). Mean r^2 estimates calculated for 1000 random samples of 24 individuals across landraces were on average 0.096 and showed substantially less variation compared to within-landrace estimates, ranging from 0.091 to 0.102 (Fig. 2). Within landraces, LD decay distances ranged from 99 to 1809 kb with a mean of 342 kb (Fig. 5a; Table S4). For the majority of landraces, LD decay distance estimates were smaller than 500 kb, with north-eastern European landraces showing on average higher LD levels than south-western European landraces (Table S5). Compared to within-landrace estimates, smaller LD decay distances were obtained for samples across landraces (Fig. 5a; Table S4) ranging from 56 to 73 kb with a mean of 63 kb.

Persistence of linkage phase within and across European maize landraces

The persistence of linkage phase for all pairwise comparisons of the 35 landraces of dataset EU-Array was evaluated based on the correlation of *r* values as well as *PEP*. For marker pairs with distances smaller than 10 kb, both parameters were high with a mean correlation of *r* values of 0.783 (Fig. 5b) and a mean *PEP* of 0.889 (Fig. S6). However, values of both parameters decreased rapidly with increasing physical distance between markers and reached moderate to low levels for marker pairs within distances of 990 to 1000 kb (mean correlation of *r* values = 0.238, mean $PEP = 0.549$). The persistence of linkage phase between

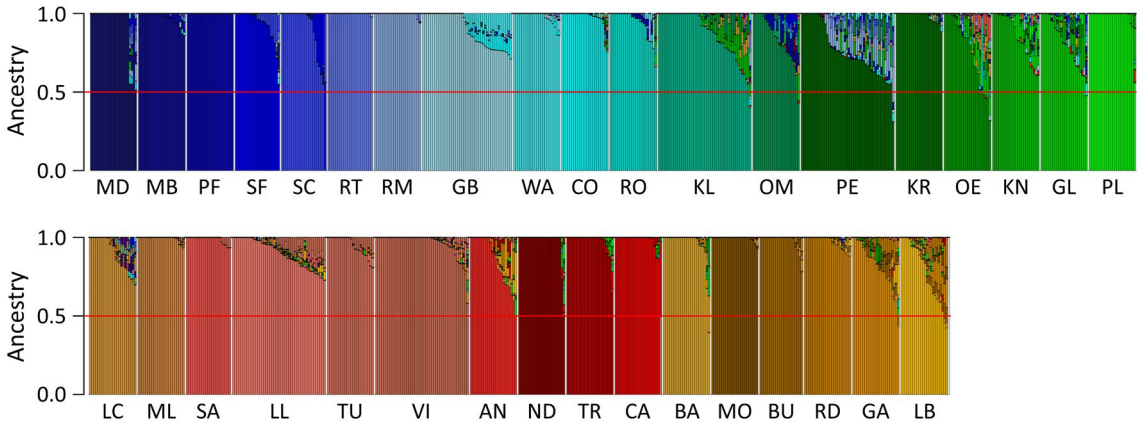

Fig. 4 Population structure in European landraces. Population structure within dataset EU-Array was inferred using ADMIXTURE for 35 pre-defined genetic groups, colored according to Fig. 1a. Each *bar* represents one individual consisting of up to 35 *colors* according to their ancestry proportions attributable to each of the 35 genetic groups. The *red horizontal line* indicates an ancestry proportion of 50%. Landraces are ordered according to their position in the neighbor joining tree (Fig S3), with north-eastern and south-western European landraces at the *top* and *bottom*, respectively

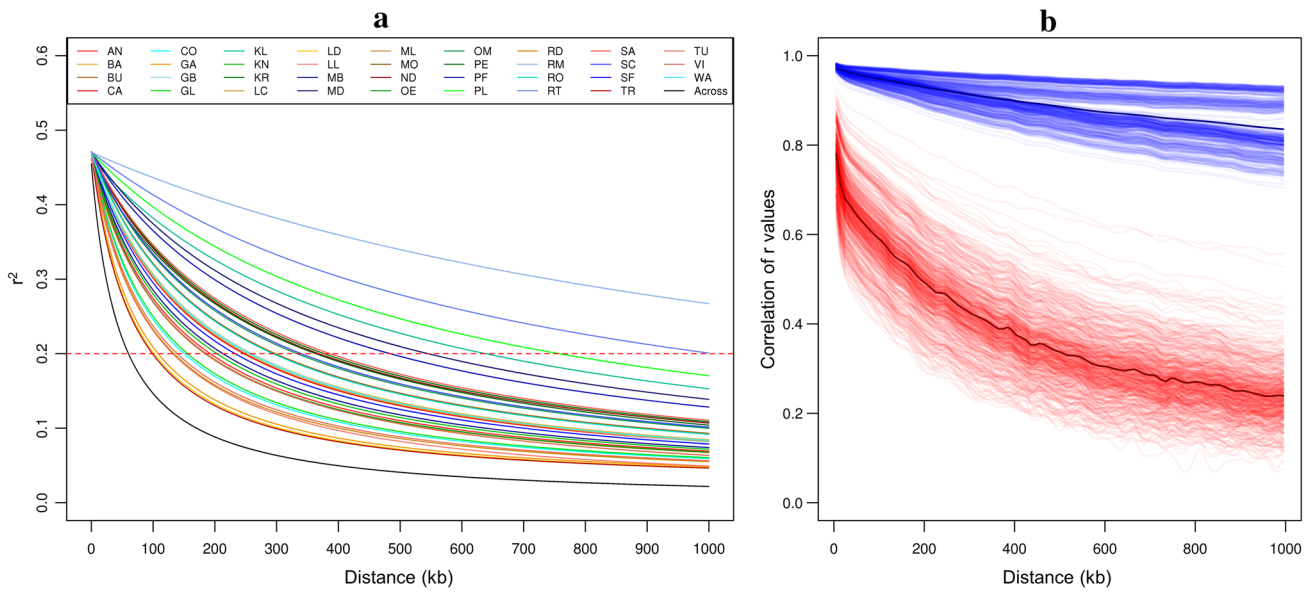

Fig. 5 Decay of LD with physical distance and correlation of *r* within and across European landraces. **a** The decay of LD was estimated via non-linear regression using r^2 values for marker pairs within a maximum distance of 1 Mb. Based on dataset EU-Array, estimates for samples of 22 to 24 individuals within each landrace (*colored* according to Fig. 1a) and the mean over 1000 random samples of 24 individuals across landraces (*black*) are shown. The *red dashed line* indicates the threshold of $r^2 = 0.2$ for calculating the physical LD decay distance. **b** Cubic smoothing spline fits are shown for the correlation of *r* values between samples within (*blue*) and across (*red*) landraces as a function of physical distance, based on dataset EU-Array. For the within-landrace estimates, 100 times half of the individuals within each of the five landraces with $n_{LR} \geq 46$ (Table S1) were randomly sampled and compared with the second half. Across-landrace estimates are based on pairwise comparisons of all 35 landraces. Mean values for within- and across-landrace estimates are shown in *dark blue* and *dark red,* respectively

pairs of landraces was associated with proximity of geographical origin and kernel type. The correlation of *r* values for marker pairs within 1 Mb distance was lowest for the comparison of the northern European Dent-like landrace PL and the southern European Flint-like landrace ND (0.298) and highest for a pair of two Flint-like German landraces (SC, SF; 0.747). *PEP* for marker pairs within 1 Mb distance was lowest for the comparison of the southern European Dent-like landrace TR and the northern European Flint-like landrace CO (0.564) and highest for a pair of two Dent-like

Austrian landraces (GL, KN; 0.749). As expected, when comparing samples within each of the five landraces with $n_{LR} \geq 46$ (Table S1), the two parameters were consistently high, for marker pairs within distances smaller than 10 kb (mean correlation of r values = 0.977, mean $PEP = 0.972$) as well as for marker pairs within distances of 990 to 1000 kb (mean correlation of r values = 0.836, mean $PEP = 0.815$; Fig. 5b; Fig. S6).

Comparison of European and American landraces

To compare the molecular variation of the 35 temperate European landraces in this study with tropical Central and South American landraces and to assess specific properties of these datasets with respect to the use of different genotyping technologies, we extended our analyses to the SeeD maize GWAS panel. Dataset SeeD-GBS comprised 3101 individuals from 2601 accessions (Fig. 1b; Table S2) and 104,223 SNPs with an overall call rate of 0.907. Comparisons between European and American landraces were based on marker subsets of EU-Array and SeeD-GBS, each containing 5045 overlapping SNPs (datasets EU-OL and SeeD-OL). Compared to SeeD-GBS, an overrepresentation of intermediate allele frequencies pertained in these two subsets (Fig. S2b-d).

For each dataset, we estimated PP, π, H, mean r^2 and r^2 decay distance (Table S7) based on 1000 random samples of 35 individuals across landraces. All five parameters differed significantly between datasets ($p < 0.001$), as revealed by two-sided t tests with Bonferroni correction. The levels of PP and π were highest for EU-OL, slightly lower for SeeD-OL and lowest for SeeD-GBS. SeeD-GBS showed the highest level of H and only slightly lower values were observed for SeeD-OL, whereas H was lowest for EU-OL. Mean r^2 for marker pairs within 1 Mb distance and r^2 decay distances were highest for EU-OL, substantially lower for SeeD-OL and lowest for SeeD-GBS.

We used ADMIXTURE to identify major genetic groups within the American landrace panel (SeeD-GBS). CV errors decreased for the number of genetic groups K varying from 1 to 16 and reached a plateau for $K > 16$ (Fig. S7). Thus, we defined 16 genetic groups within SeeD-GBS. The resulting groups reflected the geographical origin of the respective landraces (Fig. S8). Five groups originated from the Mexican and Central American lowlands, four groups comprised landraces from the Mexican highlands, four groups referred to landraces from South America and three groups originated from the Caribbean islands and north-eastern South America. Individuals showed high levels of admixture, especially between geographically adjacent groups.

In the joint PCoA of SeeD-OL and one representative of each of the 35 European landraces sampled from EU-OL (Fig. S9), the first two PCos mainly separated South American from Mexican highland landraces with tropical Caribbean and Central American lowland landraces at the center. A group of north-eastern European Flint landraces was clearly separated from the American landraces. Part of the temperate European landraces, mainly from the south-west, grouped together with part of south-eastern South American landraces, but was clearly separated from the remaining groups. The genetic distance of European landraces to tropical Caribbean and Central American lowland landraces increased with increasing geographical distance to Mediterranean regions and was larger for Flint-like than for Dent-like landraces.

To evaluate the representation of population structure by the reduced marker sets EU-OL and SeeD-OL, we compared MRDs and PCoA between EU-OL and EU-Array and between SeeD-OL and SeeD-GBS, respectively. MRDs between individuals obtained by the respective reduced and full marker sets were highly correlated (correlation of 0.991 and 0.942 for EU and SeeD datasets, respectively; with a significance of $p < 0.001$; Fig. S10). Consistently larger MRDs were observed for SeeD-OL compared to SeeD-GBS. For the first three principle coordinates, the correlation-like statistic of Procrustes analyses was 0.994 for the comparison between EU-OL and EU-Array, and 0.991 between SeeD-OL and SeeD-GBS, respectively ($p < 0.001$).

Influence of sample size, sample composition and marker distribution on LD estimates

Based on dataset EU-Array, we analyzed the effect of sample size on LD estimates by calculating LD decay distances for random samples of individuals within each of the five landraces GB, KL, LL, PE and VI with $n_{LR} \geq 46$. For sample sizes smaller than 20 individuals (40 gametes), a strong increase in mean and variance of LD decay distance estimates was observed with decreasing sample size (Fig. S11). We also calculated LD decay distances for sampling schemes varying in the number of landraces l and the number of gametes g per landrace, based on dataset EU-OL. As expected, estimates of LD decay distance increased with decreasing total number of gametes (Fig. 6). For a given total number of gametes, LD decay distances were larger within landraces ($l = 1$) than across landraces ($l > 1$). For example, an LD decay distance of 174.3 kb was observed for 35 gametes sampled from one landrace in contrast to 8.3 kb when sampling 35 landraces with one gamete each. In general, LD decay distances decreased for increasing l, with the largest decrease observed for l from 1 to 10, and only marginal changes for l larger than 10. Analogously, LD calculations for varying g and l were performed for dataset EU-Array. A decrease in LD estimates with increasing g and l was also observed for EU-Array, but with substantially higher

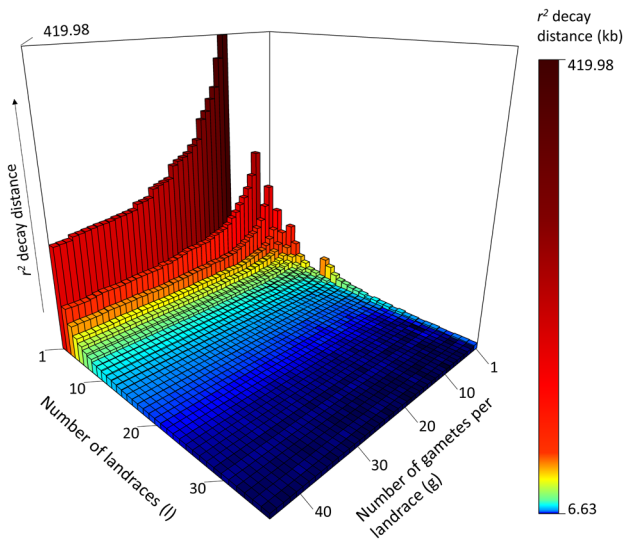

Fig. 6 Effects of sample size and sample composition on the estimation of LD decay distances. Based on dataset EU-OL, LD decay distances were calculated using non-linear regression and an r^2 threshold of 0.2 for sampling schemes varying in the number of landraces l and the number of gametes g per landrace. *Bars* and *colors* represent the average LD decay distance for 10 random samples per $l \times g$ combination

overall levels of LD compared to EU-OL (Fig. S12). The different levels of genome-wide LD estimates between EU-OL and EU-Array can be explained by differences in the distribution of markers, with EU-OL showing a higher marker density in telomeric regions compared to EU-Array (Fig. S1). However, LD estimates of landraces relative to each other were comparable between EU-OL and EU-Array (Table S6).

In admixed populations, LD can appear between unlinked markers due to differences in allele frequencies of subpopulations. To assess the extent of admixture-induced LD, we calculated interchromosomal LD for 24 individuals sampled from $l = 1, 2, 3, 4, 6, 8, 12, 24$ landraces. Overall, the proportion of interchromosomal marker pairs with $r^2 > 0.2$ was low (Table S8), but the Wilcoxon rank sum test revealed significantly higher proportions of marker pairs with $r^2 > 0.2$ across landraces ($l > 1$) than within landraces ($l = 1$).

Discussion

When building GWAS discovery panels or training sets for genomic prediction from landraces, large data sets of several hundreds or even thousands of individuals are required to obtain sufficient power of QTL detection and high accuracy of prediction. Different sampling strategies can be devised depending on the aim of the study. When aiming at elucidating mechanisms of plant adaptation or discovering novel alleles for disease resistance or quality traits, maximizing the allelic diversity of the discovery panel is crucial. Thus, individuals might be sampled from many landraces covering a wide range of diversity with each landrace being represented by one or few individuals. An alternative strategy is to sample many individuals from each of a few pre-selected landraces, which might be especially promising for broadening the genetic diversity of elite material for quantitative traits.

In this study, we compared estimates of genomic parameters with impact on the power of genome-enabled approaches between different sampling strategies, using dense genotyping data from 35 European maize landraces with more than 20 individuals per landrace. We show for this unique set of landraces covering a wide range of eco-geographic conditions in the temperate maize growing regions of Europe that the majority of the landraces represented unstructured populations as indicated by low F_{is} values, a consistent landrace-specific grouping of individuals in NJT and PCoA, and high ancestry proportions of individuals attributable to their respective landrace (Fig. 4; Fig. S3–S4). With current advances in assembling complex genomes de novo (Unterseer et al. 2017) generating high-quality reference sequences that represent the diversity of a defined set of landraces is within reach. Given that linkage phases were highly consistent within landraces over fairly long genomic distances, imputation of missing genotypes from skim whole-genome sequencing should be possible with high accuracy for a broad range of allele frequencies. This should allow efficient characterization of haplotype variation within and across landraces.

Sampling individuals from a limited number of pre-selected landraces yields only slightly reduced levels of molecular diversity compared to sampling from the entire set of 35 European landraces. On average more than 70% of the total molecular variance present in the 35 landraces was found within landraces and about 95% was captured by samples of five landraces. Based on this high molecular variation, we can assume high genetic variation for quantitative traits of interest within a pre-selected set of landraces, which is in concordance with phenotypic investigations of landrace-derived material (Böhm et al. 2017; Wilde et al. 2010). LD levels within landraces were comparable to or lower than levels reported previously for diverse collections of temperate maize elite lines genotyped with the same array (Unterseer et al. 2014), thus yielding comparable mapping resolution in gene discovery studies. Moreover, mapping resolution for gene discovery can be increased by combining data from several landraces (Fig. 6). When sampling individuals from 10 landraces, LD decay distances of a few

kb were observed, comparable to the level of the entire set of 35 European landraces and sufficiently low for candidate gene identification. Diversity and LD parameters varied between landraces with the majority of landraces retaining high levels of diversity and moderate to low levels of LD during their maintenance by farmers, their recollection and/or their preservation in gene banks. When adding a pre-screening step, the molecular and genetic variance in the data can be increased, as landraces deviating from expectations with respect to diversity, inbreeding or population structure can be excluded. Our results suggest that genotyping 24 individuals per landrace with 5k SNPs was sufficient for obtaining representative estimates of diversity and LD levels for each population (Fig. S11; Table S6). The usefulness of the data set can be further increased by evaluating a broad panel of landraces well adapted to a given target environment in the pre-screening step and by assuring that the selected landraces are segregating for target traits.

We found a gradually decreasing level of relatedness of European to Central and South American landraces with increasing geographical distance to Mediterranean regions (Fig. S9) consistent with previous observations (Dubreuil et al. 2006; Rebourg et al. 2003). This indicates that European landraces represent a broad spectrum of allelic variation, shaped by local adaptation to different agro-ecological zones. Haplotype diversity in the 35 European landraces was lower compared with the SeeD data but still sufficiently high to warrant high genetic variance for quantitative traits of interest. This was also confirmed by a recent study by Böhm et al. (2017) who described high levels of genetic variance for a suite of quantitative traits in doubled-haploid libraries derived from landraces of similar origin as those investigated in this study. While the haplotype based parameter H was presumably less affected by ascertainment bias than single SNP measures (Conrad et al. 2006), an enrichment of intermediate allele frequencies as well as an increase in PP, π and r^2 estimates indicated an overestimation of these parameters in the SeeD dataset when filtering for SNPs overlapping with the 600k array (Fig. S2; Table S7). Array-derived SNPs are restricted to the initial SNP discovery panel and affected by subsequent filtering steps, leading to an enrichment of intermediate allele frequencies compared to GBS-derived SNPs. As the array was optimized for temperate maize, PP, π and r^2 estimates were likely overestimated in European relative to American landraces. In both, the EU-Array and the SeeD-GBS datasets, SNPs were called using the B73 reference sequence and are, therefore, restricted to genomic regions present in B73. GBS-derived data depend on restriction enzyme cutting sites and hence are highly overrepresented in telomeric regions (Romay et al. 2013), as it was also observed in this study when comparing the distribution of SNPs between the Seed-GBS and EU-Array datasets. The differences in marker distributions were likely the main reason for the observed differences in genome-wide LD estimates between EU-Array and EU-OL (Fig. S1, S12) as the two datasets showed similar SFS (Fig. S2). Thus, comparisons of diversity parameters and LD between datasets analyzed with different genotyping technologies need to be interpreted with caution. However, inferences within the respective datasets of European or American landraces should be affected to a minor extent by these limitations and as can be seen from Fig. S9 the results of the PCoA obtained with the SNPs represented in the SeeD-OL dataset were consistent with those presented by Romero Navarro et al. (2017).

Within the European dataset, the grouping of the 35 landraces (Fig. S3–S4) with respect to their geographical origin and kernel type was clearly reflected in the genomic analyses. The level of interchromosomal LD induced by admixture was overall low, but, as expected, varied significantly depending on the sampling strategy (Table S8). However, when constructing data sets by sampling individuals from pre-selected landraces, the clear differentiation between populations allows a priori definition of subpopulations in statistical analyses to avoid false-positive marker-trait associations or inflation of prediction accuracies. In addition, when sampling a sufficiently high number of individuals within landraces, specific marker effects can be estimated using appropriate statistical models as suggested by Lehermeier et al. (2015).

Even though only one or few individuals were sampled from individual landraces in the SeeD-GBS data set, population structure was prevalent with 16 genetic groups mainly representing the geographic origin of the landraces (Fig. S8). With a high proportion of individuals exhibiting strong population admixture, accounting for population structure in the SeeD data set is challenging. Furthermore, the consistency of allelic effect estimates of samples of landraces covering a wide range of geographic regions with respect to a given target elite breeding pool warrants further research. It has been shown that strong correlations of geographic coordinates and specific adaptive traits persist in these data sets (Romero Navarro et al. 2017; Zhao et al. 2007). As these authors pointed out, disentangling associations of target traits from adaptation as well as estimation of genotype × environment interactions is difficult in highly diverse landrace collections. Thus, we conclude, that the incorporation of favorable alleles from landraces into elite germplasm can be expected to be most efficient if landraces are chosen not solely based on maximum allelic diversity but also with respect to a similar environmental adaptation and genomic background as the target elite breeding pool.

Conclusions

We show that sampling a limited number of pre-selected landraces should provide high genetic variance for quantitative traits of interest and high mapping resolution in gene discovery. Absence of pronounced population structure within landraces and clear genetic differentiation between landraces allows a priori definition of subpopulations in statistical analyses and consistency of linkage phases facilitates genotype imputation and haplotype characterization. Thus, for broadening the genetic diversity of elite material for quantitative traits, we recommend capitalizing upon the genomic characteristics of long-term random mating populations and the genetic diversity within a pre-selected set of landraces adapted to a comparable environment as the target elite breeding pool.

Author contribution statement CCS, EB, SU, NdL and MM conceived the study and discussed the results; MM investigated genotypic data and performed analyses; CCS and BO acquired funding; BO contributed part of the Spanish landrace data; MM drafted the manuscript; CCS, EB and SU edited the manuscript; all authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical standards The authors declare that this study complies with the current laws of the countries in which the experiments were performed.

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ORIGINAL ARTICLE

European maize landraces made accessible for plant breeding and genome-based studies

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Key message **Doubled-haploid libraries from landraces capture native genetic diversity for a multitude of quantitative traits and make it accessible for breeding and genome-based studies.**

Abstract Maize landraces comprise large allelic diversity. We created doubled-haploid (DH) libraries from three European flint maize landraces and characterized them with respect to their molecular diversity, population structure, trait means, variances, and trait correlations. In total, 899 DH lines were evaluated using high-quality genotypic and multi-environment phenotypic data from up to 11 environments. The DH lines covered 95% of the molecular variation present in 35 landraces of an earlier study and represent the original three landrace populations in an unbiased manner. A comprehensive analysis of the target trait plant development at early growth stages as well as other important agronomic traits revealed large genetic variation for line per se and testcross performance. The majority of the 378 DH lines evaluated as testcrosses outperformed the commercial hybrids for early development. For total biomass yield, we observed a yield gap of 15% between mean testcross yield of the commercial hybrids and mean testcross yield of the DH lines. The DH lines also exhibited genetic variation for undesirable traits like root lodging and tillering, but correlations with target traits early development and yield were low or nonsignificant. The presented diversity atlas is a valuable, publicly available resource for genome-based studies to identify novel trait variation and evaluate the prospects of genomic prediction in landrace-derived material.

Introduction

Maize (*Zea mays* L. ssp. *mays*) seed banks around the world harbor thousands of landrace accessions, representing a rich resource of currently untapped native diversity that could be harnessed for plant improvement and adaptation to environmental changes (Hoisington et al. 1999; Ortiz et al. 2010; McCouch et al. 2013; Hellin et al. 2014; Wang et al. 2017). European flint maize went through several bottlenecks, the first of which occurred in the Americas (Doebley et al. 1986), followed by the introduction to Europe (Rebourg et al. 2003). In the course of maize breeding, landraces were replaced by hybrids. For the establishment of hybrid breeding, only a limited set of founder landraces was sampled, and the inbred lines produced were subjected to second cycle breeding (Messmer et al. 1992; Barrière et al. 2006). Subsequent selection at high intensities has led to an additional decline in genetic diversity of elite germplasm, especially within the flint heterotic pool important for European maize breeding (Messmer et al. 1992; Reif et al. 2005a, b; Lu et al. 2009). Revisiting the vast diversity of landraces stored in seed banks is considered a promising approach

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for broadening the genetic base of current germplasm pools (Pollak 2003; Salhuana and Pollak 2006; Warburton et al. 2008; Strigens et al. 2013; McCouch et al. 2013; Navarro et al. 2017). However, opening this avenue for quantitative traits entails considerable challenges, and efficient strategies are still lacking.

In a first step, the most promising landraces have to be identified from several thousand stored in seed banks, even if only the flint pool is of interest. Information on stored landraces is limited, and the choice has to be based either on passport data from seed banks, or the per se and/or testcross performance of the landraces has to be evaluated in field trials (Pollak 2003; Salhuana and Pollak 2006; Böhm et al. 2014). In allogamous species like maize, landrace collections represent populations of heterogeneous and heterozygous individuals. Thus, the evaluation of populations either per se or in testcrosses would disregard the large genetic variation found within landraces, and without prior self or cross, it is not possible to evaluate the breeding potential of individual genotypes. In order to harness the genetic diversity within landraces, reproducible genetic units such as libraries of doubled-haploid (DH) lines from landraces have been suggested to overcome some of the aforementioned drawbacks since they are suitable for genotyping and high-precision phenotyping (Wilde et al. 2010; Strigens et al. 2013; Melchinger et al. 2017). Diversity from landraces captured in such DH libraries could help in improving traits such as plant development at early growth stages, for which genetic variation is small in breeding material. However, improving quantitative traits by utilizing lines derived from landraces is complex because the targeted introgression of favorable alleles at major genes is not possible (Bernardo 2002). Any introgression of landrace material therefore carries the risk of an undesired correlated response in traits other than the trait under selection due to the overall poor agronomic performance of the landrace material. To achieve a targeted utilization of natural diversity, an exhaustive characterization of line per se and testcross performance for the trait of interest and as many other agronomic and morphological traits as possible has to be carried out in order to develop a pre-breeding strategy that allows introgression of favorable diversity into elite germplasm without introducing major disadvantages in other traits (Sood et al. 2014).

In the research at hand, we employed large-scale production of DH lines to make native diversity for quantitative traits in maize landraces accessible for the purpose of germplasm improvement and genome-based studies. Our objectives were (i) to create a publicly available diversity atlas of European flint maize by characterizing landrace-derived DH libraries genotypically and phenotypically for line per se and testcross performance, (ii) to provide a comprehensive analysis of the DH libraries in terms of population structure, performance level, trait correlations, and genetic variances for a broad range of traits, and (iii) to gain insights into potential strategies for capturing native diversity for use in germplasm improvement.

Materials and methods

Plant material

The three landraces Kemater Landmais Gelb (KE, Austria), Petkuser Ferdinand Rot (PE, Germany), and Lalin (LL, Spain) were chosen for the production of DH lines because they showed phenotypic variation for early development as well as low levels of linkage disequilibrium (LD) and population structure within populations. They were selected from a set of 35 European maize landraces covering a broad geographical region of Europe that was described in detail by Mayer et al. (2017). Together, they represented 95.0% of the molecular variance of the full set of 35 landraces. From the selected landraces, 1015 DH lines (516 KE, 432 PE, 67 LL) were produced and multiplied using the in vivo haploid induction method (Röber et al. 2005). Phenotyping of lines per se (LP) was conducted in 2017 and 2018. Testcrosses (TC) of a subset of 378 DH lines from landraces KE and PE were evaluated in 2018. To warrant successful TC evaluation, the shortest, earliest, and late maturing lines as well as lines with a high score for lodging were not included in the TC production. The dent line F353 (Institut national de la recherche agronomique, INRA) was used as the female parent in TC production to ensure uniform seed quality across DH lines and because variation in tassel architecture of DH lines hampered detasseling.

Analysis of genotypic data and population structure

The 1015 DH lines and 144 S_0 plants (48 per landrace) from the landraces KE, PE, and LL were genotyped using the 600 k Affymetrix® Axiom® Maize Array (Unterseer et al. 2014). Only markers assigned to the best quality class (Unterseer et al. 2014), with a call rate of ≥ 0.9 and with a known physical position in the B73 reference sequence [AGPv4, (Jiao et al. 2017)], were used for the analyses. One S_0 plant from landrace PE was excluded due to an insufficient call rate (≤ 0.9). Assignment of lines to their respective landrace was performed using the ADMIXTURE software tool (Alexander et al. 2009) in supervised mode with three pre-defined groups (KE, PE, and LL) that were determined from S_0 plants. DH lines with less than 75% concordance with the landrace to which they were assigned by pedigree records were excluded from further analysis. Markers and individuals with > 10% missing values were removed. In DH lines, markers and individuals with > 5% heterozygous genotype calls were discarded, and all remaining heterozygous

calls were set to missing values. Missing values in the DH lines were imputed separately for each landrace using BEAGLE 5.0 (default parameters) (Browning et al. 2018). Missing values in the S_0 plants were imputed, and two gametes were phased from each S_0 plant separately in each landrace using BEAGLE 5.0 (iterations = 50, phase-segment = 10, phase-states = 500) and a reference panel consisting of the corresponding DH lines. Pairwise modified Rogers' distances [MRD; (Wright 1978)] were calculated, and DH lines showing a pairwise MRD of < 0.05 were assumed to be duplicates and excluded from further analyses. Markers were identified which overlapped between DH lines and S_0 gametes. Quality filtering and imputation resulted in 941 DH lines (501 KE, 409 PE, and 31 LL) and 286 S_0 gametes (96 KE, 94 PE, and 96 LL) genotyped with 499,574 common markers.

We performed a principal coordinate analysis [Gower (1966), R-package ape] based on MRD for DH lines and S_0 plants. The MRD matrices of DH lines and S_0 plants were hierarchically clustered using the unweighted pair group method with arithmetic mean (UPGMA) implemented in the hclust function in R and are displayed as 1-MRD. In order to estimate the proportion of molecular variance explained by the three landraces under study, an analysis of molecular variance [AMOVA; Excoffier et al. (1992)] was performed to partition the molecular variation into within- and between-landrace components. This analysis used the panel of 35 European landraces described by Mayer et al. (2017) for comparison. In addition, a second AMOVA decomposing the variance within and between DH lines and S_0 gametes was performed to investigate how much of the molecular variance lies within and between those two groups.

Field experiments and phenotypic analysis

Line per se (LP) performance was evaluated in Germany during 2017 using ten separate 10 × 10 lattice designs in four locations (1000 entries: 958 DH lines plus checks) and during 2018 using eight 10 × 10 lattice designs in three locations (800 entries: 756 DH lines plus checks). A randomly chosen subset (five 10 × 10 lattice designs, 458 and 468 DH lines plus checks in 2017 and 2018, respectively) was evaluated in two locations in Spain in both years. The trial locations were Einbeck (EIN, Germany, 2017 + 2018), Roggenstein (ROG, Germany, 2017 + 2018), Bernburg (BBG, Germany, 2017), Klein Wanzleben (KLW, Germany, 2018), Oberer Lindenhof (OLI, Germany, 2017), Golada (GOL, Spain, 2017 + 2018), and Tomeza (TOM, Spain, 2017 + 2018). See Table S1 for a detailed description of the test locations [geographical coordinates, elevation, precipitation, temperature; the climate data was obtained from the Bavarian State Research Center for Agriculture, Landwirtschaftliches Technologiezentrum Augustenberg, and Menne et al. (2012)]. Each combination of year and location was considered to be one environment in later analyses. The number of lines tested had to be reduced between 2017 and 2018 due to seed shortage and the exclusion of lines that did not pass the quality control described above for the genotypic data analysis. In 2017, 14 flint (CH10 provided by Agroscope Changins-Wädenswil (Switzerland); D152, DK105, UH006, UH007, and UH009 provided by the University of Hohenheim (Germany); EP1 and EP44 provided by Misión Biológica de Galicia, Consejo Superior de Investigaciones Científicas, (CSIC, Spain); F03802, F2, F283, F64, and F7 provided by Institut national de la recherche agronomique (INRA, France); EC49A provided by Centro de Investigaciones Agrarias Mabegondo, Instituto Galego da Calidade Aumentaria (CIAM-INGACAL, Spain) and one dent (F353, INRA, tester in testcross evaluation) inbred line served as checks and were included as duplicate entries. The checks were chosen in order to exhibit variation in plant development at early growth stages and flowering time. In 2018, the number of checks was reduced to four lines (DK105, EP1, F2, and F353) included in each lattice design per location (eight in Germany, five in Spain). In both years, the three landraces were included as quadruplicate entries. Plots were single rows 3 m in length with a distance of 0.75 m between rows and twenty plants per plot, corresponding to a sowing density of about 9 plants m^{-2}.

The testcrosses (TC) were evaluated in four 10 × 10 lattice designs in four locations in Germany in 2018 (EIN, KLW, ROG, OLI). In the TC trials, testcrosses of lines DK105, EP1, and F2 as well as testcrosses of the two landraces KE and PE and two commercial hybrid varieties (CH1 = KWS Stabil, CH2 = KWS Figaro) were planted as checks. The testcrosses of landraces KE and PE were planted in one lattice only, while all other checks were planted in every lattice. In TC, plots were double rows 5 m in length at locations ROG and OLI and 6 m in length at locations KLW and EIN, in both cases with 0.75 m distance between rows. Sowing density followed local practice at the experimental stations and varied between 9 and 11 plants m^{-2}. Fertilization and plant protection were carried out according to standard agricultural practices in both the LP and the TC trials.

In the LP trial, a total of 25 morphological, agronomic, and early-development-related traits were measured (Table S2 provides detailed information on trait × environment combinations). The traits that were scored in ≥ 10 environments included emergence (EME, ratio of emerged plants to sown seeds, %), early vigor (EV, at three different growth stages V3, V4, and V6, 1–9 score, 1 = very poor vigor, 9 = very vigorous), early plant height (PH, at V4 and V6, average over three measured plants per plot, cm), final plant height (PH_final, cm), and female flowering (FF, d). Root lodging at the R6 stage (RL, 1 = no lodging, 9 = all plants showing severe lodging) was scored in six environments;

tillering (TILL, 1 = no tillers, 9 = all plants showing many and long tillers) and male flowering (MF, d) were scored in five environments. The anthesis-silking interval (ASI, d) was calculated for the environments in which both MF and FF were scored. Ear height (EH, cm) was measured in four environments. In the Spanish environments, physiological traits like the maximum efficiency of photosystem II [Fv/Fm, using a fluorometer (OS-30p, Opti-Sciences Inc., USA)] were measured at stages V4 (2017 + 2018) and V6 (only 2017), and leaf greenness (SPAD) was measured by a chlorophyll content meter (CCM-200, Opti-Sciences Inc., USA; V3, V4 in both years, V6 only 2017). Reaction to stress was scored as cold tolerance (CT, 1–9 score, 1 = low cold tolerance, 9 = high cold tolerance; symptoms were chlorosis and necrosis on the leaves) after a very cold night with a slight frost at OLI 2017, drought/heat tolerance (DT, 1–9 score, 1 = low drought/heat tolerance, 9 = high drought/heat tolerance; symptoms were dry leaves and tassels) at EIN 2018, and rust susceptibility (binary) at TOM 2018. Traits related to tassel architecture were measured in ROG 2018. Tassel length was measured from the lowest tassel branch to the tassel tip (TL, cm), spike length was measured as the length of the top spike (SL, cm), the number of branches was counted (NB), and the tassel angle was scored on a 1–9 scale (TA, 1 = completely upright, 9 = branches horizontal). In the TC trial, EME, EV, PH, EH, PH_final, FF, TILL, and RL were scored as was described for LP. In addition, TC plots were harvested with a forage harvester to measure total dry matter yield (TDMY, dt/ha) and dry matter content (DMC, through near infrared spectroscopy or drying, in %).

The statistical model for estimating genotype and genotype × environment interaction variance components for lines derived from the same landrace was

$$y_{ijkopst} = \mu + m_i + \delta_{ij} l_j + g_{k(ij)} + u_o + \delta_{ij} lu_{jo} + gu_{ko(ij)} + k_{p(o)} + r_{s(op)} + b_{t(ops)} + \varepsilon_{ijkopst} \quad (1)$$

where $i = 1, 2, 3$ denotes three groups, i.e., DH lines from landraces (DHL), checks (CH), and landrace populations (LR_S$_0$); $j = 1, 2, 3$ denotes the three landraces KE, PE, and LL; μ is the overall mean; m_i is the effect of group i; l_j is the effect of landrace j in group $i = 1$; δ_{ij} is a dummy variable with $\delta_{ij} = 1$ for $i = 1$ and $j = 1, 2, 3$ and $\delta_{ij} = 0$ otherwise; $g_{k(ij)}$ is the effect of line k nested in group i and landrace j; u_o is the effect of environment o; lu_{jo} is the interaction of landrace j and environment o; $gu_{ko(ij)}$ is the interaction effect for genotype k and environment o. The effects $k_{p(o)}$, $r_{s(op)}$, $b_{t(ops)}$, and $\varepsilon_{ijkopst}$ refer to the effect of the lattice (nested in environments), replicate (nested in lattices in environments), incomplete block (nested in replicates in lattices in environments), and the residual error, respectively. All effects except m_i and l_j were treated as random. Genotype and genotype × environment ($gu_{ko(ij)}$) variance components were modeled individually for the three landraces ($j = 1, 2, 3$), assuming that DH lines across and within landraces were unrelated. Residuals were assumed to be normally distributed with mean zero and two heterogeneous variances, one for $\delta_{ij} = 1$ and one for $\delta_{ij} = 0$ assigning the same residual variance to all three landraces in all environments. Raw data and outliers were manually curated by inspection of residual plots. Since genotyping and the first year of phenotyping were carried out in parallel, some lines were evaluated in the field during 2017 that did not pass quality control in the genotypic data analysis. Measurements for those entries were treated as missing values in the data analysis. The same model was used for the analysis of TC experiments, except that $i = 1, 2$ referred to DHL and CH and $j = 1, 2$ referred to landraces KE and PE. Restricted maximum-likelihood estimation implemented in the ASReml-R package (Butler et al. 2009) was used for estimating variance components and their standard errors. Differences among means l_j were tested with pairwise t-tests using the R-package asremlPlus. Trait heritabilities were calculated on an entry-mean basis within landraces (Hallauer et al. 2010), and standard errors of heritability estimates were derived from standard errors of corresponding variance components using the delta method (Holland et al. 2010). Heritabilities and variance component estimates exceeding twice their standard errors were considered significant. Best linear unbiased estimates (BLUEs) of the genotype mean for each trait and DH line were obtained from a simplified version of the model in Eq. (1), dropping factors m_i, $\delta_{ij} l_j$ and $\delta_{ij} lu_{jo}$ and treating genotype (g_k) as a fixed effect. This model was also used to form linear contrasts used to test for significant differences (t-tests) between original landraces and the mean of the corresponding DH library (LP and TC) and between the mean of the two check hybrids and the mean of the DH library (TC only). We calculated the predicted response from selection within DH libraries (LP and TC) according to Falconer and Mackay (1996) as $\Delta G_{(\alpha)} = i_{(\alpha)} h \sigma_G$, where $i_{(\alpha)}$ = selection intensity for selection with $\alpha = 10\% \left(i_{(10\%)} \approx 1.76\right)$, h = square root of heritability, and σ_G = genetic standard deviation. To account for mean differences and different selection responses, we calculated the usefulness criterion (Schnell 1983) as $U_{(10\%)} = \bar{x} \pm \Delta G_{(10\%)}$ where $\bar{x}$ = mean of the respective DH library. Phenotypic correlations among traits were calculated from BLUEs as Pearson correlation coefficients within libraries in LP and TC, respectively. For evaluating the prospects of selection on LP performance in this material, we calculated Spearman rank correlations for same traits across LP and TC. To adjust for multiple testing, Bonferroni–Holm correction was applied for significance tests of phenotypic correlations in each DH library (Holm 1979). For estimating genetic covariances and genetic correlations between traits, the model in Eq. (1) was expanded to a bivariate model with pairs of traits. Genetic correlations were considered

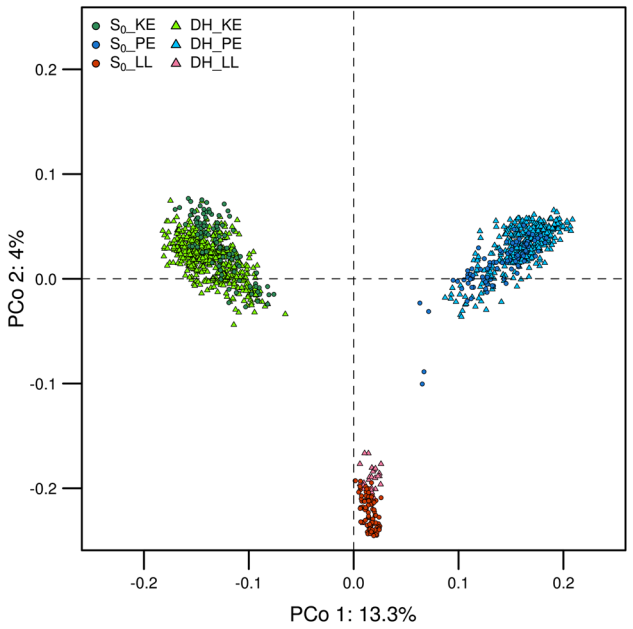

Fig. 1 Principal coordinate analysis (PCoA) of DH libraries and S_0 gametes based on modified Rogers' distances between individuals. Landrace KE is colored in *green*, PE in *blue*, and LL in *red*. Darker colors were used for S_0 gametes and brighter ones for DH. S_0 gametes were plotted as *filled circles* and DH lines as *filled triangles*. Axis labels show the percentage of explained variance per principal coordinate (PCo)

significant if they exceeded twice their standard error. The same method was applied for estimating genetic correlations between LP and TC performance.

In summary, high-quality phenotypic line per se data are available from up to 11 environments for 899 DH lines (471 KE, 402 PE, and 26 LL) and for a subset of 378 lines (190 KE, 188 PE) that were evaluated as testcrosses in four environments. For all lines, data on almost 500,000 SNP markers are available.

Results

Population structure and molecular variation

The principal coordinate analysis clearly separated the three landraces, with the first two coordinates explaining 13.3% and 4% of the total molecular variance, respectively (Fig. 1). DH lines and S_0 gametes derived from the same landrace clustered together except for four gametes from S_0_PE, which fell outside the PE-cluster. Complementing our data with those from Mayer et al. (2017) revealed that S_0 gametes sampled from landraces KE, PE, and LL individually accounted for 77, 75, and 89% of the total molecular variance captured in the collection of 35 European landraces used in their study. The AMOVA on S_0 gametes and DH lines from the same landrace confirmed the results from the PCoA. While 95.3, 96.6, and 96.7% of the molecular variance were found within S_0 and DH of KE, PE, and LL, respectively, less than 5% of the molecular variance was explained by differences between S_0 gametes and DH lines of different landraces. Matrices of 1-MRD

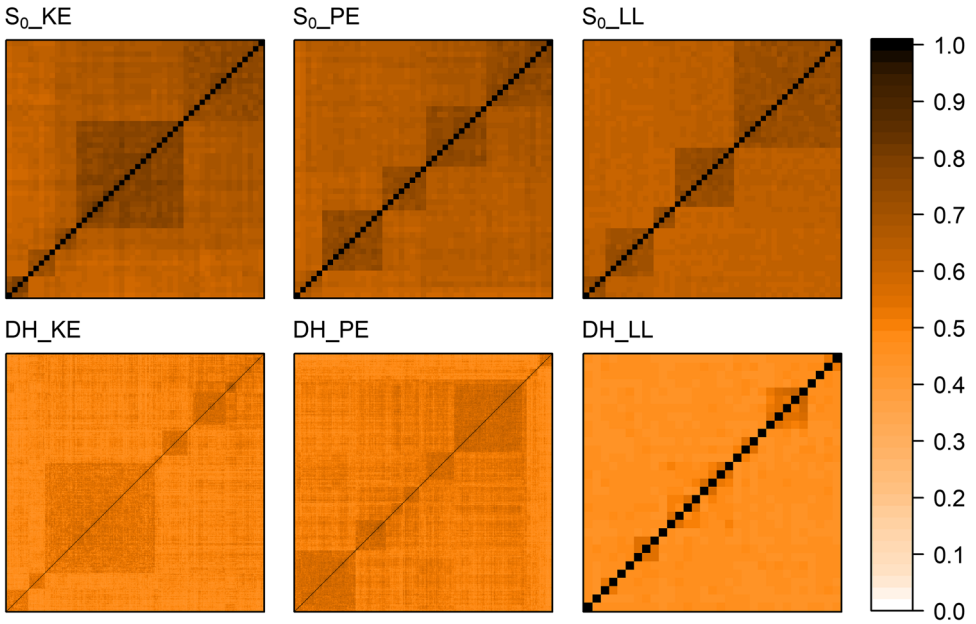

Fig. 2 Heatmaps of 1-MRD matrices S_0_KE ($N=48$ individuals), DH_KE ($N=471$ lines), S_0_PE ($N=47$ individuals), DH_PE ($N=409$ lines), S_0_LL ($N=48$ individuals), and DH_LL ($N=31$ lines). Matrices were ordered according to hierarchical clustering with UPGMA

(Fig. 2) gave no indication of pronounced population structure for either DH or S_0 plants. As expected, the similarity between S_0 plants within landraces was on average higher than in DH lines due to the higher level of heterozygosity in the former.

Phenotypic variation within and across landraces

In the following, we will refer to a subset of traits as "core traits" since they are considered most important for improvement of early plant development in elite germplasm. These traits were EV_V4 and PH_V4 as representatives for early development, RL and TILL as representatives for traits for which genetic variation is not acceptable in elite germplasm, PH_final and FF as important agronomic traits, and DMC and TDMY for evaluating yield performance. Phenotypic variation for core traits within and across landraces is shown in Fig. 3 (LP) and Fig. 4 (TC) and for all other traits in Fig. S1 (LP) and Fig. S2 (TC). Phenotypic means, variance components, and heritabilities for all traits are provided in Table S3 and Table S4 for LP and TC performance, respectively. The DH libraries exhibited considerable phenotypic variation for all traits. In LP and TC, a similar range of trait values was observed for all DH libraries. Probably due to the small sample size, distribution of phenotypes in LL deviated slightly from the other two landraces, e.g., for traits EV and TILL. Mean performance differed significantly ($P < 0.05$) across landraces for 20 out of 25 traits in LP and for 5 out of 14 traits in TC, which was a result of the high-quality phenotypic data and large sample sizes of KE and PE. As expected, mean LP performance of the DH libraries was significantly ($P < 0.05$) lower than the respective landraces for almost all traits. The reduction was most pronounced for early development traits, final plant height, and photosynthetic efficiency (Fig. 3, Fig. S1). Flowering time of the DH library was delayed by 10 (LL) and 6 (KE, PE) days compared to the non-inbred material. While the LL DH library had consistently lower mean performance in early development traits, ear height, and final plant height compared to KE and PE, this was not true for the original landraces.

When choosing DH lines to be evaluated as TC, we had applied mild selection for flowering time, plant height, and lodging (see "Materials and methods" for details). Mean TC performance of the DH libraries KE and PE did not differ significantly from the TC mean of their respective landrace populations for all traits except for TDMY in PE, indicating that DH lines evaluated as TC represented a random sample

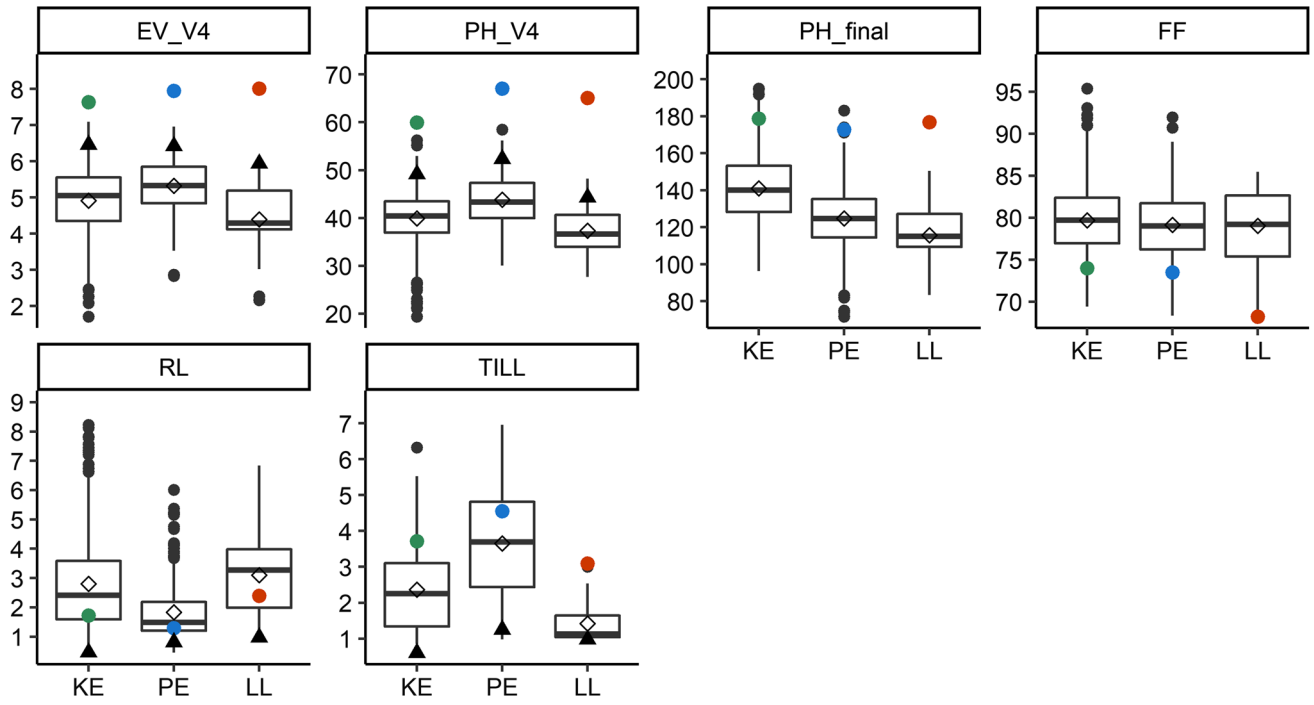

Fig. 3 Boxplots of phenotypic data for line per se (LP) performance for the DH libraries from landraces KE, PE, and LL. Boxplots show the upper and lower quartiles, median (*horizontal bar*), mean (*open diamond*), whiskers (*vertical bars*), and the performance of the respective landrace (*filled circle* in *green*, *blue*, and *red* for KE, PE, and LL, respectively). Points above and below the whiskers indicate values ± 1.5 times the interquartile range. Usefulness for a selection intensity of 10% ($U_{10\,\%}$) is indicated with *black filled triangles*. Traits are early vigor and early plant height at stage V4 (EV_V4, PH_V4), final plant height (PH_final), female flowering (FF), root lodging (RL), and tillering (TILL)

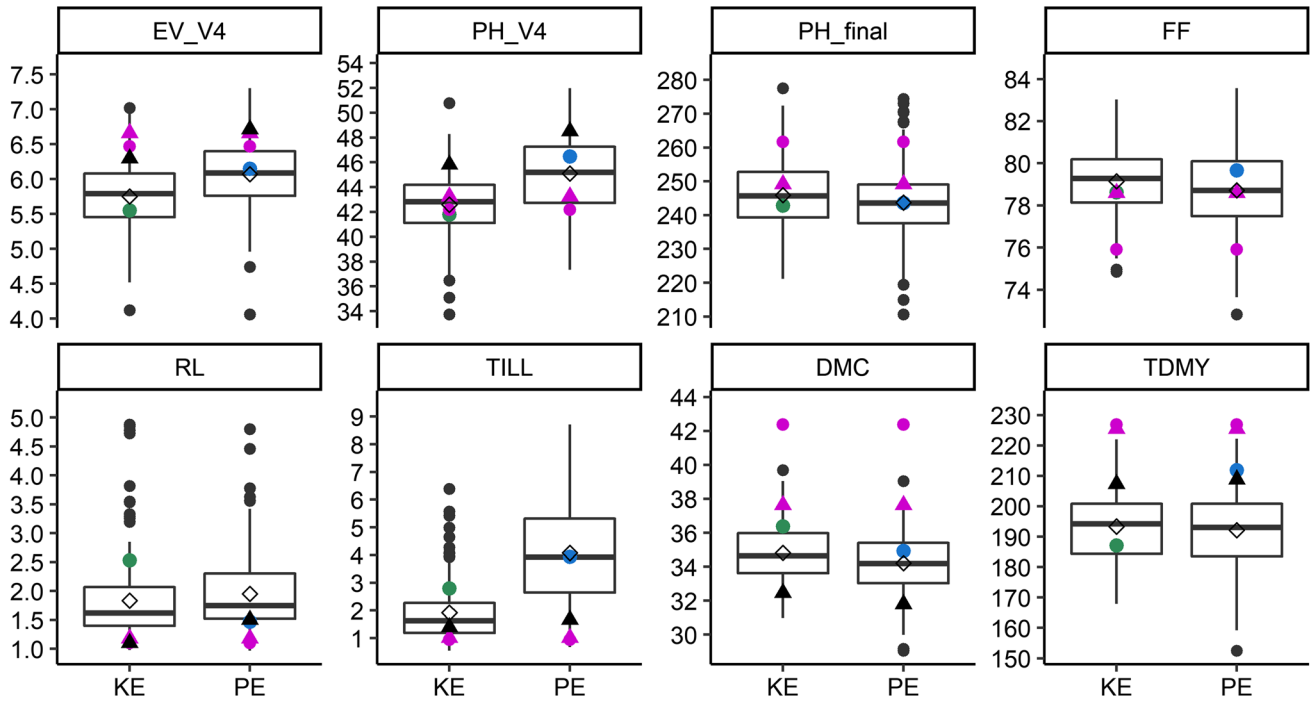

Fig. 4 Boxplots of phenotypic data for testcross (TC) performance for DH libraries from landraces KE and PE. Boxplots show the upper and lower quartiles, median (*horizontal bar*), mean (*open diamond*), whiskers (*vertical bars*) and the performance of the respective landrace (*filled circle* in *green* and *blue* for KE and PE, respectively). Points above and below the whiskers indicate values ± 1.5 times the interquartile range. Performance of the two commercial check hybrids is indicated with a *filled circle* and *filled triangle* in *magenta* for CH1 and CH2, respectively. Usefulness for a selection intensity of 10% ($U_{10\ \%}$) is indicated with *black filled triangles*. Traits are early vigor and early plant height at V4 stage (EV_V4, PH_V4), final plant height (PH_final), female flowering (FF), root lodging (RL), tillering (TILL), dry matter content (DMC), and total dry matter yield (TDMY)

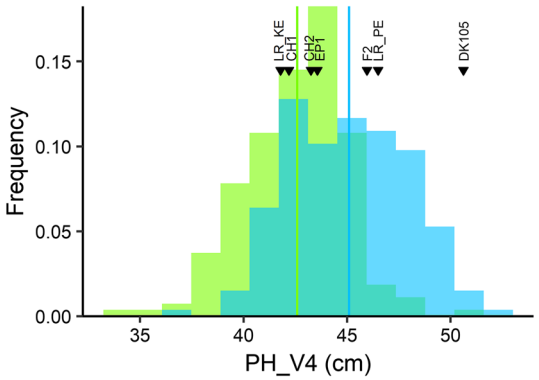

Fig. 5 Histogram of testcross (TC) performance of DH lines from landraces KE ($N = 190$, colored in *green*) and PE ($N = 188$, colored in *blue*) for trait early plant height at V4 stage (PH_V4), including the mean of the DH lines per population (*green* and *blue horizontal bar*) and the performance of testcrosses of lines EP1, F2, DK105, the landrace populations (LR_KE, LR_PE), as well as two commercial check hybrids (CH1, CH2) indicated by labeled *black triangles*

of the entire DH library. The TC of many DH lines outperformed the commercial hybrids as well as the TC of founder lines and landraces for the target trait early development, as is shown for PH_V4 in Fig. 5. Only the testcross of inbred DK105 fell into the upper 10% of the distribution of PH_V4. As expected, the commercial hybrids significantly ($P < 0.05$) outperformed the TC mean of DH lines for TDMY by about 15% and, in contrast to the DH lines, showed no TILL or RL (Fig. 4).

Genetic variances were highly significant in LP and TC for most traits under investigation (Table S3, Table S4). Variance component estimates for LL were similar to the other two libraries, but, due to the small sample size, they were estimated with considerably larger error, resulting in nonsignificant genetic variances for PH_V3, TILL, ASI, photosynthesis-related traits, and SPAD. As expected from quantitative genetic theory, genetic variance component estimates were smaller in TC than in LP. In the statistical model, we allowed for heterogeneity of genetic variances estimated within landraces, but only a few traits (e.g., DT, RL) showed strong differences (> twofold) in genetic variance estimates between KE and PE in LP, which were even alleviated in TC.

In LP, trait heritabilities were generally high and similar across landraces, ranging from 0.35 to 0.96. Except for PH_V3, TILL, ASI, Fv/Fm, and SPAD in LL, the

heritability estimate always exceeded twice its standard error (Table S3). In TC, heritabilities were slightly lower overall than in LP (Table S4), ranging from 0.31 to 0.92, which was expected from the lower number of testing environments and the lower genetic variance compared to LP.

Variation across environments

DH libraries were evaluated in a total of 11 environments comprising seven different locations and two years. Locations covered a geographical region spanning from northern Germany to northwestern Spain at altitudes ranging from 29 to 706 m above sea level (Table S1). Average temperatures differed by 5 °C between the coldest (OLI 2017, 14.0 °C) and the warmest (TOM 2018, 19.0 °C) environments, and precipitation varied from 159 (KLW 2018) to 548 mm (ROG 2018) during the vegetation period. The ratio of genotype by environment and genotype variance components depended on the trait under study. In LP, values ranged from 0.11 (EH in KE) to 1.22 (ASI in PE), but varied between 0.2 and 0.7 for most traits with a mean of 0.51 (Table S3). Similar ratios were observed in TC (Table S4).

Correlations between locations for traits measured in at least five environments ranged from 0.40 to 0.87 in 2017 and from 0.19 to 0.86 in 2018 (Table S6). Correlations between years of a given trait and location ranged from 0.31 to 0.83 (Table S6).

Trait correlations

In LP and TC, phenotypic correlations among early development traits measured at different growth stages were high and stable across DH libraries, ranging from 0.58 to 0.95 (Fig. 6). The corresponding genetic correlations were slightly higher, ranging from 0.65 to 1 (Fig. S3). For LL in LP, only phenotypic correlations among early development traits (ranging from 0.82 to 0.93, data not shown), PH_final and EH (0.75), and FF and MF (0.69) were significant.

In LP, the early development traits showed intermediate to high positive phenotypic and genetic correlations with final plant height (phenotypic 0.4 to 0.6, genetic 0.4 to 0.7). In TC, only the phenotypic correlation between PH_V6 and

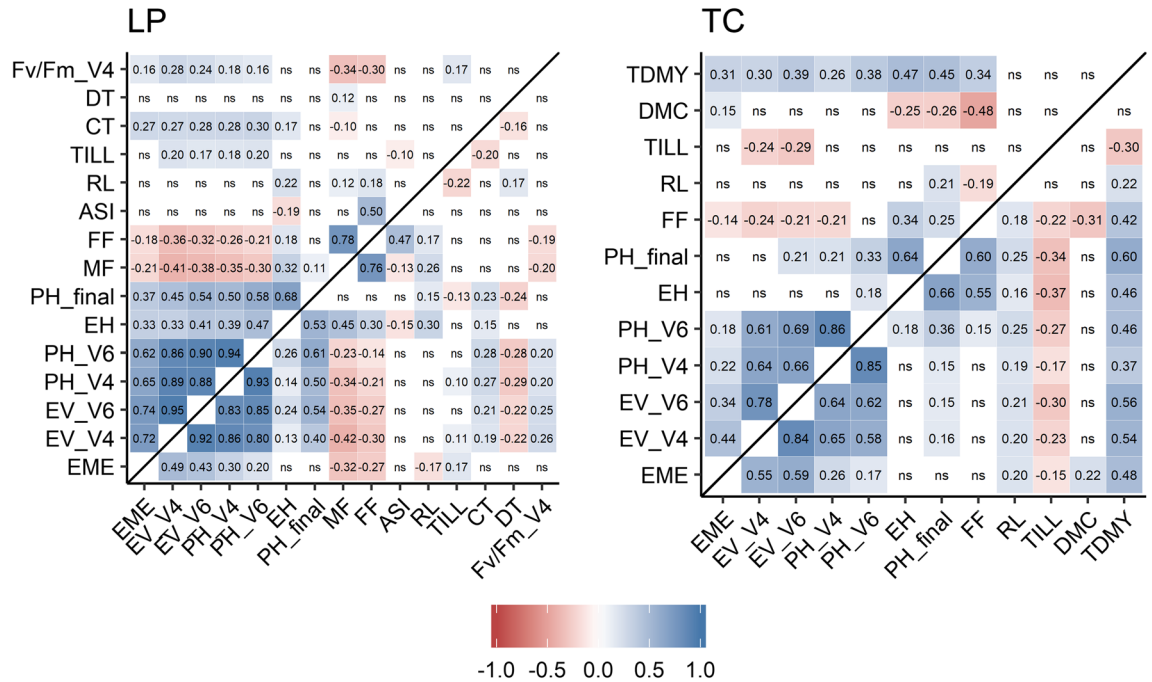

Fig. 6 Phenotypic Pearson correlation coefficients for line per se [LP, *left*, $N = 471$ (KE) and 402 (PE)] and testcross [TC, *right*, $N = 190$ (KE) and 188 (PE)] data within DH libraries KE (above diagonal) and PE (below diagonal) for the traits emergence (EME), early vigor, and early plant height at stages V4 and V6 (EV_V4, EV_V6, PH_V4, PH_V6), ear height (EH), final plant height (PH_final), male flowering and female flowering (MF, FF), anthesis-silking interval (ASI), root lodging (RL), tillering (TILL), cold tolerance (CT), drought/heat tolerance (DT), maximum photosynthetic efficiency at V4 stage (Fv/Fm_V4), dry matter content (DMC), and total dry matter yield (TDMY). *P*-values were adjusted using Bonferroni–Holm correction for multiple testing. Nonsignificant correlations are labeled with ns

final plant height was significant, but it was lower than in LP. Genetic correlations for EV_V4, EV_V6, PH_V4, and PH_V6 with PH_final ranged between 0.2 and 0.5. Intermediate positive correlations were found between early development traits and TDMY in TC (phenotypic 0.3 to 0.6, genetic 0.4 to 0.8) as well as negative correlations of early development with flowering time in LP (FF and MF, phenotypic −0.2 to −0.4, genetic −0.2 to −0.5) in KE and PE libraries. Phenotypic and genetic correlations of RL with all other traits were nonsignificant or small in LP and TC. The same was true for TILL except for TC of PE, where intermediate correlations with early and late plant height, ear height, and TDMY were observed.

Phenotypic correlations between LP and TC performance were significant for all traits except EME. Genetic correlations between LP and TC were intermediate (early development traits, 0.35 to 0.68) to high (PH_final and FF > 0.78) (Table S5).

Discussion

Our study is part of a long-term research project which aims to make maize landrace diversity amenable to plant breeding (www.europeanmaize.net). We produced DH libraries from three landraces for obtaining reproducible genetic units for phenotyping and genotyping and characterized them comprehensively to build a publicly available, immortal genetic resource that is ready to use for pre-breeding and for investigations on functional diversity and the prospects of genomic prediction.

DH libraries capture native diversity for germplasm improvement

The three landraces were chosen to represent the molecular variance of the European landraces characterized by Mayer et al. (2017). Individually, they accounted for more than 75% of the molecular variance in this collection, together for 95%. These findings corroborate results from the literature where it has been shown for several outcrossing species, including maize, that a large proportion of the molecular variation can be found within landraces, while differences between landraces account only for a small proportion (Böhm et al. 2014; Greene et al. 2014; Monteiro et al. 2016). Genotyping with the SNP array technology might have led to an overestimation of the captured molecular variance due to an enrichment of markers with intermediate allele frequencies. For truly quantitative traits, however, the contribution of rare alleles to the additive genetic variance is small and the molecular variance assessed with array data should translate directly into genetic variation observable in phenotypes. With only three (LP) or two (TC) landraces in the statistical model, decomposition of the genetic variance within and across landraces is not meaningful, but from Figs. 3, 4 and Figs. S1, S2 it becomes obvious that differences in trait means across landraces were small compared to the range of values within landraces. Although each landrace accounted for a large proportion of molecular variance individually, we still advise to analyze progenies from several landraces for capturing the genetic variance segregating in a germplasm pool. Molecular variance might be a good indicator for genetic variance averaged across traits, but variation for individual traits must be evaluated for each landrace specifically, as was shown here for TILL, RL, DT, and CT. Different landraces may also differ with respect to their success rates in DH production (Melchinger et al. 2017), pointing to different multiplication histories. While KE and PE may have encountered bottlenecks or inbreeding in the past, LL seems to carry a much higher genetic load that limited the production of fully homozygous DH lines for this landrace. This assumption is also supported by the significantly lower LP mean performance of the LL DH library for early development, ear height, and final plant height compared to KE and PE that was not observed for the original landraces.

The DH libraries generated in this study represented their respective landraces accurately in terms of molecular variance. DH lines and S_0 gametes from the same landrace overlapped nicely in the PCoA (Fig. 1) and the AMOVA showed that almost all molecular variation was found within S_0 gametes and DH lines (> 95%) and not between them. Individuals sampled from a maize landrace are assumed to be unrelated, but pairwise comparisons share different numbers of alleles alike in state, leading to variation in similarity between them. Patterns of variation in similarity were comparable for S_0 plants and DH lines (Fig. 2), corroborating that the two types of progeny represent their original landraces in a similar way. We thus conclude that the three DH libraries derived from KE, PE, and LL represent a valuable resource for genetic improvement of elite flint germplasm, since they cover a large proportion of the genomic and genetic variance of the landrace collection described in Mayer et al. (2017).

Improving early plant development

In many growing regions worldwide, maize encounters low to moderate temperatures during the early vegetative phase. Under these conditions, accelerated early development can increase final biomass yield. Genetic enhancement of early growth can also improve resource efficiency, preserve soil fertility, and reduce the need for herbicide treatment. European flint maize germplasm has been adapted to the temperate climate conditions of Northern and Central Europe through breeding, but genetic variation for early

development under cool temperatures has been depleted simultaneously (Greaves 1996; Rodríguez et al. 2010).

In LP and TC of the DH libraries, the target trait early development assessed through early vigor scores and early plant height measurements showed ample genetic variation (Figs. 3, 4, Figs. S1, S2). In TC, the majority of DH lines outperformed the commercial hybrids for PH_V4, and only one check (F353 × DK105) ranged among the best 10% DH lines, suggesting that the DH libraries can serve as a valuable source of alleles for improving early development traits of the elite European flint pool (Fig. 5).

Identifying maize flint germplasm with superior early growth has been the objective of several studies in both, field and controlled environments (Peter et al. 2009a, b; Rodríguez et al. 2010; Revilla et al. 2016). In most studies, early development was assessed as a visual score, which delivers ordinal endpoints and can be rather subjective. On the other hand, early plant height measurements consume considerable resources. Early vigor scores showed a substantially higher correlation with plant emergence compared to early plant height in this research (Fig. 6, Fig. S3). Even though all TC seed was produced on inbred line F353, the higher phenotypic and genetic correlation of early vigor and EME was maintained. For PH_V4, the commercial hybrids were on average not different from the TC mean of the DH libraries, but they scored better for EV. Thus, the early plant height measurement neglects information that can be accounted for by EV scores, such as differences in leaf coloration or the overall lower EME of the DH library testcrosses. In addition, genetic correlations between TDMY and EV were substantially higher compared to between TDMY and PH_V4 supporting the hypothesis that, although highly correlated, the two types of measurements target different components of early development. For a comprehensive characterization of early growth development, it seems advisable to assess both, EV and early plant height. To allow dissection of early growth development into its genetic components and consequently provide a better understanding of the underlying genetic mechanisms, we propose establishing growth models by monitoring early development at high resolution in time using remote sensing in the field (Huang et al. 2013; Bendig et al. 2015) or in controlled conditions (Gioia et al. 2017). The three DH libraries KE, PE, and LL are most suitable for further investigation on this topic as they exhibit more pronounced genetic variation in early growth traits than can be expected from elite material (Revilla et al. 1999; Peter et al. 2009a).

Comprehensive phenotypic characterization of DH libraries

The prospects for the genetic improvement of elite germplasm for early growth development through the use of landrace-derived material have to be evaluated in a multi-trait context. Comprehensive data on trait correlations are crucial in order to avoid undesired selection response in traits of agronomic importance.

In LP, EV_V4 and PH_V4 showed intermediate negative genetic correlations with flowering time and positive genetic correlations with PH_final, corroborating results of Böhm et al. (2017) on DH lines derived from landraces. Thus, selection for accelerated early development will lead to increased plant height and early flowering which, depending on the target environment, might not be desirable. The DH libraries also showed variation for RL and TILL. Given the low levels of genetic correlations with early development traits and the usefulness of the best 10% of DH lines being close to zero, a simultaneous reduction or removal of lodging and tillering should be possible in a recurrent selection program devoted to the improvement of early development traits. In TC, correlations between early development traits and TDMY were positive. However, the commercial hybrids significantly outperformed the DH lines for TDMY, while testcrosses of founder lines (F2, EP1, DK105) lay well within the range of the DH libraries for both traits (Fig. 4, Fig. S4). The yield gap between the mean testcross yield of the DH lines and the mean testcross yield of two commercial hybrids amounted to about 15% and was comparable to what was reported in the literature for other European landraces (Wilde et al. 2010; Brauner et al. 2019). The usefulness of the best 10% DH lines in KE and PE, respectively, remained 8% below the performance level of the commercial hybrids for TDMY (Fig. 4). Given that the inbred line F353 used as tester for the DH libraries was developed about 20 years ago (year of release 2001, C. Bauland, personal communication) and that the parental components of the commercial check hybrids were highly selected based on their general and specific combining ability, the difference in TDMY between commercial hybrids and the top 10% DH lines seems small and could likely be reduced by the use of modern testers (Hölker et al. 2019). In many material groups, a negative correlation between DMC and TDMY is expected. In our research, phenotypic correlations between TDMY and DMC were nonsignificant when averaged across environments (Fig. 6, Fig. S4) as well as in all four individual environments where TC performance was evaluated (data not shown). This outcome can most likely be attributed to the exceptionally hot and dry conditions during the 2018 growing season (Table S1), the genetic material under study, or an interaction of both. Thus, an additional year of TC evaluation, including more and also later maturing commercial check hybrids, will be conducted for investigating the DMC/TDMY relationship in material derived from genetic resources more closely and for evaluating the overall yield potential of the DH libraries.

Multi-environment testing

One of the aims of this study was to assess trait differentiation in diverse environments and to estimate the magnitude of genotype × environment interactions of landrace-derived material. Thus, the chosen environments covered a broad spectrum of target regions for European flint material (Table S1). Despite locations with very different climatic conditions (e.g., OLI and TOM) and large differences in temperature and precipitation in 2017 and 2018, the ratio of genotype × environment and genetic variance (σ^2_{gu} : σ^2_g, Tables S3, S4) was moderate for most traits. If landraces from which DH libraries are derived are adapted to similar environmental conditions as the target elite breeding germplasm, the confounding effects of adaptive alleles and strong genotype × environment interactions can be avoided and meaningful phenotypes obtained. Thus, our results are encouraging with respect to the prospects of incorporating environmentally stable alleles from pre-selected DH libraries into elite germplasm.

Evaluating landrace-derived material in 11 environments might not be practicable for applied pre-breeding programs. In this study, the large number of test environments was highly useful because we detected the segregation of unfavorable alleles in specific environments such as segregation for rust in TOM (Fig. S5) and drought susceptibility in EIN (Fig. S6), both in 2018. Although infections with rust or severe drought may not occur frequently, it would be devastating if these susceptibilities were transferred inadvertently to elite germplasm through the introgression of landrace-derived material. If evaluating the landrace-derived material in a large number of environments is not possible, prioritized testing in environments known for high disease pressure, abiotic stress, or frequent occurrence of undesirable traits like RL is highly advisable.

DH libraries from landraces make native diversity accessible

The DH libraries presented in this study link the large molecular diversity present in landraces to meaningful phenotypes. DH lines from landraces outperformed flint founder lines and commercial hybrids in early development, and as immortal genetic units they are directly accessible for plant breeding. Improving one or several target traits and simultaneously closing the performance gap between elite and landrace-derived genetic material for multiple traits of agronomic importance requires efficient recurrent population improvement. In this context, knowledge of trait correlations is crucial in order to broaden the narrow genetic base of the elite flint germplasm pool without introducing undesired traits from landraces into elite breeding populations. To obtain maximum selection gain per unit time, theory offers different strategies, such as multi-stage or index selection (Bernardo 2002), which need to be evaluated in the framework of the respective breeding programs. Optimal strategies may vary conditional on species, budget, and short-term or long-term perspectives. Böhm et al. (2017) suggested multi-stage phenotypic selection of landrace-derived DH libraries. In a simulation study, Gorjanc et al. (2016) compared different scenarios for initiating pre-breeding for maize landraces using genomic prediction (GP) and suggested starting directly from landraces (e.g., without crossing to elite lines).

The implementation of GP in pre-breeding of landrace-derived material is still underexploited. The comprehensive phenotypic data and derived quantitative genetic parameters presented for the three DH libraries in this study provide an excellent basis for optimizing genome-based pre-breeding schemes. Multi-environment phenotypic data are available for model training in LP and TC. Sample sizes and marker densities are large, allowing to investigate the effects of population size and required marker densities in populations with relatively low linkage disequilibrium compared to elite germplasm. In addition to investigating the prospects of genome-based prediction, our data provide a comprehensive framework for the discovery of genes controlling favorable and unfavorable traits as well as for the genetic analysis of additional relevant traits such as nutrient efficiency, photosynthesis-related traits, and additional biotic and abiotic stress tolerances.

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Author contribution statement CCS, EB, MO, TP, and AEM conceived the study; CCS, MO, and AEM acquired funding for the study; MO, TP, and TB developed the plant material; MM investigated genotypic data and performed analyses; ACH, MM, EB, TB, TP, BO, and PCB collected the phenotypic data; ACH performed phenotypic data analysis and drafted the manuscript; CCS edited the manuscript; all authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical standards The authors declare that this study complies with the current laws of the countries in which the experiments were performed.

Availability of data and materials All data and material are available through material transfer agreements upon request.

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Discovery of beneficial haplotypes for complex traits in maize landraces

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Genetic variation is of crucial importance for crop improvement. Landraces are valuable sources of diversity, but for quantitative traits efficient strategies for their targeted utilization are lacking. Here, we map haplotype-trait associations at high resolution in ~1000 doubled-haploid lines derived from three maize landraces to make their native diversity for early development traits accessible for elite germplasm improvement. A comparative genomic analysis of the discovered haplotypes in the landrace-derived lines and a panel of 65 breeding lines, both genotyped with 600k SNPs, points to untapped beneficial variation for target traits in the landraces. The superior phenotypic performance of lines carrying favorable landrace haplotypes as compared to breeding lines with alternative haplotypes confirms these findings. Stability of haplotype effects across populations and environments as well as their limited effects on undesired traits indicate that our strategy has high potential for harnessing beneficial haplotype variation for quantitative traits from genetic resources.

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Harnessing the allelic diversity of genetic resources is considered essential for overcoming the challenges of climate change and for meeting future demands on crop production[1,2]. For most traits of agronomic importance, modern breeding material captures only a fraction of the available diversity within crop species[1]. In the case of maize (*Zea mays* L.), today's elite germplasm went through several bottlenecks, first by geographical dispersion from its center of origin[3,4], second through the selection of only a few key ancestors sampled from a small number of landraces to establish heterotic groups[5,6], and third through decades of advanced cycle breeding with high selection intensities[7,8]. For traits that were not targets of selection in the past, but are important today, like abiotic stress tolerance and resource-use efficiency[9], this might have resulted in the loss of favorable alleles during the breeding process. In addition, unfavorable alleles might have become fixed during the selection process due to drift and/or hitchhiking effects[10–12].

Impressive examples exist where introgression of alleles from genetic resources has improved mono- or oligogenic traits[13–15], but for broadening the genetic diversity of complex traits, such as yield or abiotic stress tolerance successful examples are scarce[2]. Up to date, the genomic characterization of genetic resources has been based predominantly on sampling individuals across a wide range of accessions, maximizing the level of diversity in the genetic material under study[2,16–20]. Such diverse samples are characterized by high variation in adaptive traits and strong population structure, leading to spurious associations and limited power for detecting associations with nonadaptive traits of agronomic importance[21,22]. Furthermore, alleles which are locally common, but globally rare likely remain undetected in broad, species-wide samples, whereas in a more targeted approach they might show sufficiently high frequencies for detection[22].

Here, we propose a genome-based strategy (Supplementary Fig. 1) for making native diversity of maize landraces accessible for improving quantitative traits, showing limited genetic variation in elite germplasm, such as cold tolerance and early plant development[23–25]. Capitalizing on low levels of linkage disequilibrium (LD), we map haplotype-trait associations at high resolution in ~1000 doubled-haploid (DH) lines derived from three European flint maize landraces. The genetic material has been preselected for adaptation to target environments to avoid confounding effects of strong adaptive alleles as suggested by Mayer et al.[26]. We assess promising haplotypes genotypically by quantifying their frequency in a diverse panel of 65 European flint breeding lines. Phenotypically, we evaluate the direction and magnitude of haplotype effects relative to a subset of breeding lines. Many of the discovered haplotypes show stable trait associations across populations and environments. In addition, most of them do not exhibit undesired trait associations, making them ideal for introgression into elite germplasm. We show that our strategy to sample comprehensively individuals from a limited set of preselected landraces is successful in linking molecular variation to meaningful phenotypes, and in identifying alleles for quantitative traits that will enrich the genetic diversity of our crops.

Results

Molecular variation in landraces and breeding lines. The genetic differentiation of 941 DH lines derived from three landraces (Kemater Landmais Gelb, KE; Lalin, LL; and Petkuser Ferdinand Rot, PE) and a diverse panel of 65 European breeding lines[27] based on principal coordinate analysis (PCoA) with 501,124 single-nucleotide polymorphism (SNP) markers is shown in Fig. 1a. The first principal coordinate explained 6.2% of the molecular variation and separated the landrace-derived and the breeding lines based on their geographical origin within Europe from northeast (Germany) to southwest (southern France, Spain). The second principal coordinate explained 5.4% of the variation and separated the two landraces KE and PE from the panel of breeding lines. Diversity parameters polymorphism information content (PIC), gene diversity (*H*), and minimum number of historical recombination events (nR) were higher in the set of breeding lines compared to the three landrace-derived DH libraries, irrespectively if they were calculated from SNP or haplotype information (Supplementary Table 1). This was expected, as the landraces represent self-contained populations, whereas the breeding line panel was derived from a large number

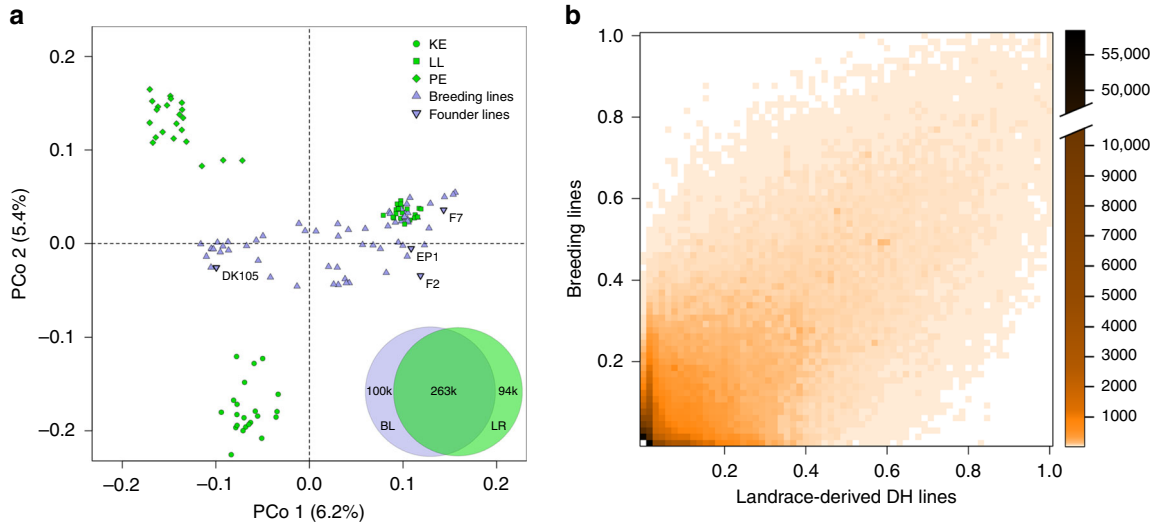

Fig. 1 Molecular inventories point to untapped variation in landraces. a Principal coordinate analysis based on pairwise modified Rogers' distances of 66 landrace-derived DH lines and 65 breeding lines (triangles), including four prominent founder lines (downward triangles). From each of three DH libraries, KE (circles), LL (squares), and PE (diamonds), 22 lines were sampled randomly. Axis labels show the percentage variance explained per principal coordinate. Venn diagram shows overlap of 456,911 haplotypes between 941 landrace-derived DH lines (LR) and 65 European breeding lines (BL). Haplotypes were constructed for nonoverlapping genomic windows of 10 SNPs. **b** Frequency of 456,911 haplotypes in DH lines (*x*-axis) and breeding lines (*y*-axis). Colors indicate the number of haplotypes within each cell of the heat map. Source data are provided as a Source data file.

of different source populations across Europe[27]. Combined across landraces, the DH lines almost reached the level of diversity of the breeding line panel.

In total, the landrace and breeding line panels comprised 356,724 and 363,290 haplotypes (Fig. 1a), constructed for nonoverlapping windows of ten SNPs, corresponding to an average of 7.12 and 7.25 haplotypes per window, respectively. As expected for genetic material originating from the same germplasm group (European flint maize), haplotype frequencies were positively correlated (Pearson's $r = 0.74$, $P < 2.2e{-}16$) between the two panels (Fig. 1b). Overall, 26.2% of the haplotypes of the landrace panel were not present in the breeding lines, indicating untapped haplotype variation. For those haplotypes, median and mean frequencies in the landrace panel were 0.005 and 0.039, respectively. Only 2.7% of those haplotypes occurred in all three landraces, whereas 82.8% occurred in only one landrace. Within the respective individual landraces their median and mean frequencies increased to 0.065 and 0.101, respectively. The landrace panel captured 72.4% of the haplotypes present in the panel of breeding lines.

Trait-associated genomic regions. A key question for the targeted utilization of genetic resources is, if molecular inventories of landrace-derived material are predictive of their potential to improve traits of agronomic importance. Using SNPs and haplotypes for genome-wide association scans (GWAS), we identified associations for all nine traits under study. Results were very similar for both types of genomic information, as exemplarily shown in Supplementary Fig. 2 for the trait tillering (TILL). As haplotypes are more informative than biallelic SNPs for the comparison with breeding lines, we focused on haplotypes in further analyses. Trait-associated genomic regions were defined based on LD between significant haplotypes ("Methods" section; Fig. 2 and Supplementary Data 1). As landraces were preselected for variation in early plant development[26,28], most associations (37–55) were detected for the traits early vigor (EV_V4/V6) and early plant height (PH_V4/V6). Haplotypes explained between 2 (female flowering time, FF) and 57% (lodging, LO) of the total genetic variance of the respective traits (Fig. 2). Despite the large sample size ($n = 899$), the proportion of genetic variance explained might be somewhat overestimated[29,30], and thus has to be interpreted with caution. Only few genomic regions were detected for flowering time, indicating that alleles with large effects were fixed during adaptation of the respective landraces to their geographical region, thus having little impact on GWAS for other traits.

Average r^2 decay distances ($r^2 < 0.2$) within the three DH libraries were 203 (LL), 484 (PE), and 973 kb (KE), and 201 kb for the combined set. This is consistent with previous results[26] and warrants high mapping resolution in the three DH libraries under study. For comparison, the diverse panel of 65 breeding lines across Europe exhibited an average r^2 decay distance of 107 kb. The lower LD level in the breeding line panel can be explained by admixture of many different source populations with varying linkage phases, which is generally undesired in GWAS. The median size of genomic regions associated with the nine traits under study was 92 kb, with a median number of three annotated genes per region (Supplementary Fig. 3), enabling prediction of candidate genes and functional analyses. Only for a few regions (<5%) resolution was not optimal, as they comprised >100 annotated genes. Mapping resolution in the three DH libraries is best demonstrated by an example of an already well-characterized locus: *teosinte branched 1* (*tb1*). The gene *tb1* played a major role in the transition from highly branched teosinte to maize with strongly reduced branch development[31]. In our study, a strong significant association for TILL was found in a genomic region on chromosome 1 comprising the *tb1* locus (size 1.3 Mb, including in total 22 genes; Supplementary Data 1 and Supplementary Fig. 2). In silico fine-mapping in the respective region ("Methods" section) identified a ten-SNP window, which overlapped perfectly with *tb1* and its regulatory upstream region.

Effect size and stability of trait-associated haplotypes. The potential of the identified landrace haplotypes for elite germplasm improvement depends on the size and direction of their effects on the traits of interest, their environmental stability and their dependence on the genetic background. In a given trait-associated genomic region, one window of ten SNPs comprising several haplotypes was selected. Significant haplotypes, hereafter referred to as focus haplotypes, entered into a multi-environment model (Supplementary Fig. 4) and were classified into favorable, unfavorable, or interacting based on the direction and stability of their effects in the different test environments (Supplementary Fig. 5). According to this categorization scheme, a high number of favorable haplotypes for early plant development traits were found in the DH libraries (Table 1 and Fig. 3a), representing potential candidates for introgression into elite germplasm. For the undesirable traits LO and TILL, many haplotypes had unfavorable effects. Overall, haplotypes identified for all nine traits showed moderate to high effect stability across environments, with similar patterns for favorable and unfavorable haplotypes (Fig. 3a, b).

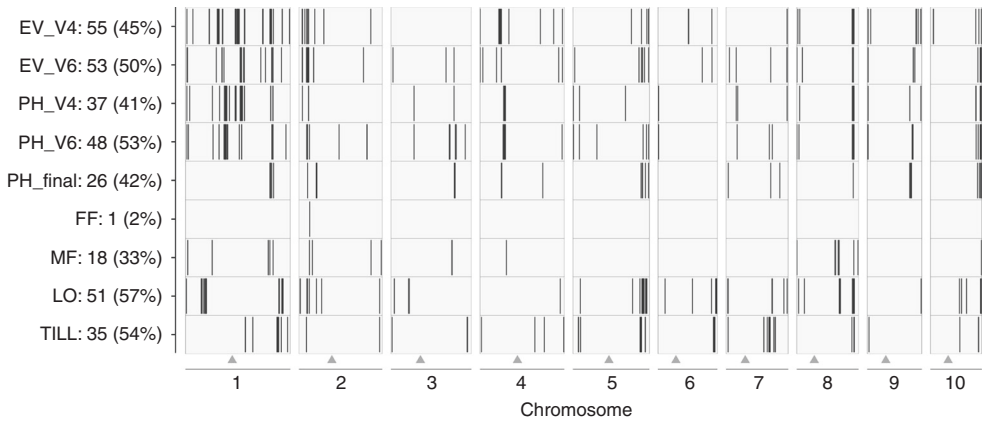

Fig. 2 Results from GWAS in DH libraries derived from maize landraces. Black vertical bars indicate the position of genomic regions significantly associated with nine traits (*y*-axis) in 899 landrace-derived DH lines. The *x*-axis shows the ten chromosomes of maize. Triangles mark the position of the centromere for each chromosome. The *y*-axis indicates the trait, the number of significant regions per trait, and the percentage genetic variance explained.

The dependency of haplotype effects on the genomic background can be evaluated comparing effect significance and sign of the identified focus haplotypes between landraces. From the 48 haplotypes associated with PH_V6, 19 haplotypes were present in both KE and PE. Together, these 19 haplotypes showed 115 environment-specific haplotype-trait associations, of which 35 (30%) were significant for both landraces (Supplementary Fig. 6a). All of those 35 associations had equal effect signs for both landraces. Also for the 80 environment-specific associations significant for only one of the two landraces, a large majority (90%) had equal effect signs for both landraces. Similar patterns were observed for PH_V4 (Supplementary Fig. 6b).

Table 1 Number and percentage of favorable, unfavorable, and interacting focus haplotypes per trait.

Trait	Favorable, *n* (%)	Unfavorable, *n* (%)	Interacting, *n* (%)
EV_V4	16 (29%)	29 (53%)	10 (18%)
EV_V6	14 (26%)	26 (49%)	13 (25%)
PH_V4	15 (41%)	15 (41%)	7 (19%)
PH_V6	20 (42%)	22 (46%)	6 (13%)
LO	11 (22%)	35 (70%)	4 (8%)
TILL	11 (31%)	23 (66%)	1 (3%)

Haplotypes with consistent effect direction across environments were categorized as favorable or unfavorable. For EV_V4, EV_V6, PH_V4, and PH_V6 positive (negative) effects were defined as favorable (unfavorable). For LO and TILL negative (positive) effects were defined as favorable (unfavorable). Haplotypes with changing effect direction were categorized as interacting.

Haplotype congruency in landraces and breeding lines. The ultimate criterion for assessing the usefulness of landrace haplotypes for germplasm improvement is their frequency in breeding material. If favorable haplotypes are already present at high frequency in the genetic material to be improved, they are of no additional value. We assessed the frequencies of the identified trait-associated focus haplotypes in a panel of 65 breeding lines based on genotypic data. When tracking an ancestral haplotype potentially shared between landrace and breeding material, recombination might have broken up the respective haplotype, but the trait-associated causal mutation might still be present. Small window sizes (mean = 0.026 cM), low values of historical recombination events (mean = 1.20), and high levels of haplotype similarity (mean = 0.33) found in the panel of breeding lines

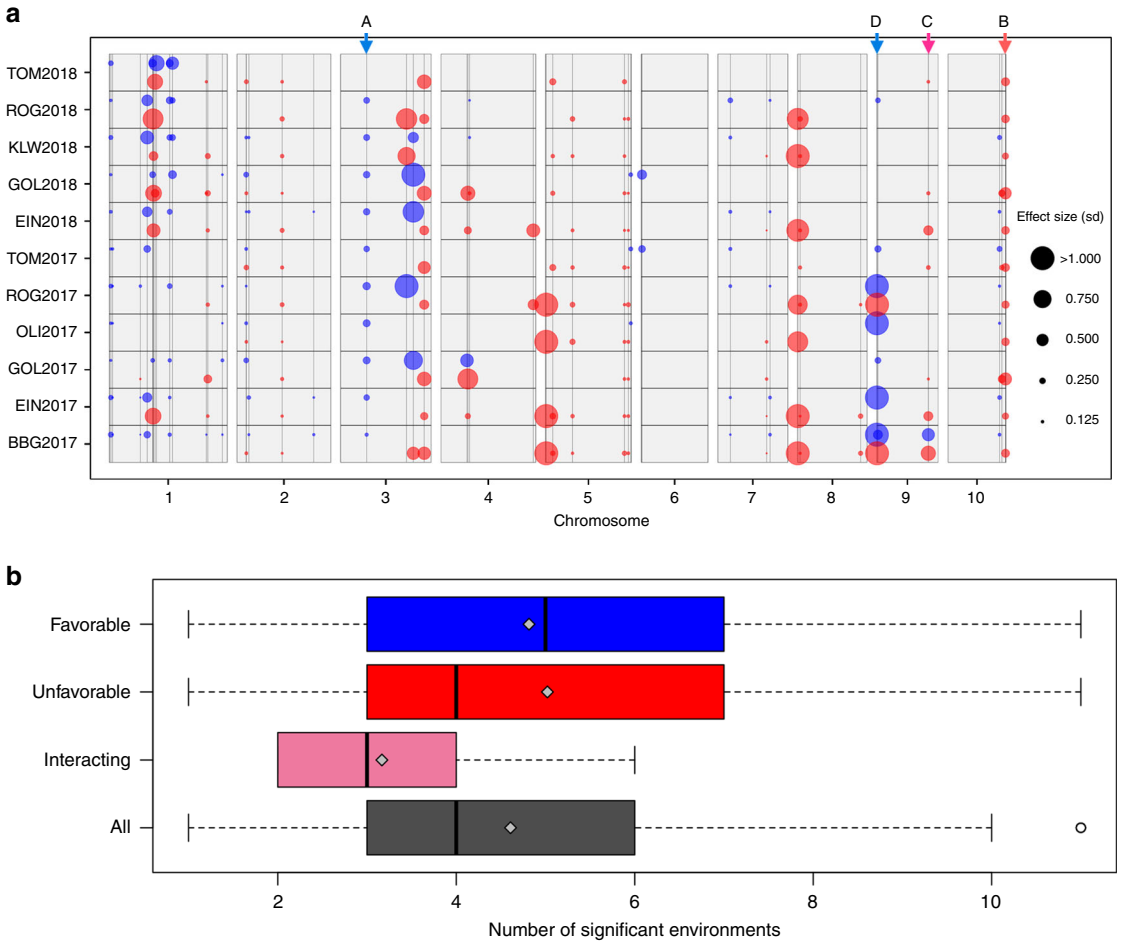

Fig. 3 Effect stability of focus haplotypes across environments. a Genomic position as well as effect size, and direction for 48 haplotypes associated with PH_V6 across 11 environments. Circles indicate significant haplotypes with effect sizes given in phenotypic standard deviations. Positive and negative effects are colored in blue and red, respectively. Arrows at the top indicate the positions of haplotypes described in Supplementary Fig. 5. **b** Number of environments in which favorable ($n = 65$), unfavorable ($n = 93$), interacting ($n = 36$), and all ($n = 194$) haplotypes had significant effects on four early plant development traits (EV_V4, EV_V6, PH_V4, and/or PH_V6). Boxplots show the upper and lower quartile, median (bold vertical bar), mean (gray diamond), and whiskers (dashed horizontal lines). Points outside the whiskers indicate values ±1.5 times the interquartile range. Source data are provided as a Source data file.

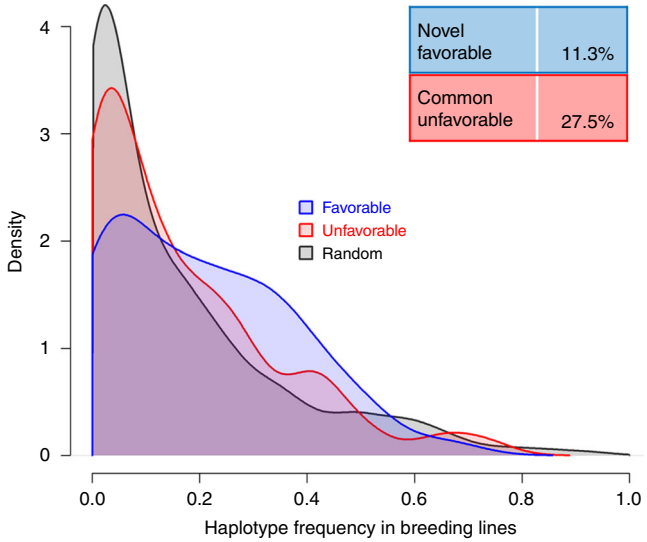

Fig. 4 Favorable and unfavorable landrace haplotypes in breeding lines. Density estimation for favorable ($n = 53$, blue), unfavorable ($n = 80$, red), and random ($n = 500$, gray) haplotypes in 65 breeding lines. Haplotypes significantly associated with four early plant development traits (EV_V4, EV_V6, PH_V4, and/or PH_V6) in landrace-derived DH libraries exhibiting a distance > 1 Mb and/or $r^2 < 0.8$ were considered. Six favorable haplotypes (11.3%) were absent in the breeding lines. Out of the 80 unfavorable haplotypes, 22 (27.5%) were common in the panel of breeding lines, i.e., exhibiting a frequency larger than the upper quartile (>0.231) of random haplotypes. Source data are provided as a Source data file.

pointed to a low probability of haplotypes being broken up by recombination.

Frequency distributions of favorable landrace haplotypes in the 65 breeding lines are given for early development traits (EV_V4, EV_V6, PH_V4, and PH_V6) in Fig. 4. As the haplotypes identified for each of the four single traits (Table 1) were partly from similar genomic regions, we only considered 53 favorable haplotypes with a minimum distance of 1 Mb and/or $r^2 < 0.8$. The frequency of favorable haplotypes (mean = 0.20) was significantly increased ($P < 0.01$) compared to randomly drawn haplotypes (mean = 0.16). Six favorable focus haplotypes (11%) were absent in the set of breeding lines, and thus have potential for elite germplasm improvement. The mean frequency of 80 unfavorable haplotypes associated with early plant development did not differ significantly ($P > 0.30$) from the frequency of random haplotypes. A substantial proportion of unfavorable haplotypes (27.5%) were common in the breeding lines (Fig. 4), suggesting that a targeted substitution with favorable haplotypes could lead to further germplasm improvement.

Linking haplotype variation to phenotypes. The potential of individual focus haplotypes to improve elite germplasm has to be evaluated phenotypically, comparing the performance of landrace-derived lines carrying these focus haplotypes and elite lines of the breeding pool one aims to improve. Using a subset of the breeding line panel ($n = 14$) phenotyped at six locations in 2017, we report exemplarily the results of such comparisons for two genomic regions on chromosomes 3 and 9, found to affect PH_V6 in the GWAS analysis (Fig. 5). On chromosome 3, the focus haplotype (haplotype A in Fig. 3a and Supplementary Fig. 5) was localized in a ten-SNP window, which explained 4.8% of the genetic variation for PH_V6 and comprised eight additional haplotypes in the DH lines. The focus haplotype had a frequency of 4.1% in the DH lines, outperformed six out of the eight alternative haplotypes significantly and was absent in the panel of breeding lines. A proportion of 93.8% of the 65 breeding lines carried one of the six haplotypes with significant negative effects relative to the focus haplotype (on average 0.61 standard deviations) in almost all environments. The remaining breeding lines (6.2%) carried a haplotype absent in the landrace panel, and thus without effect estimate. Averaged across environments, DH lines carrying the focus haplotype showed an increase of 6.06 cm over breeding lines, but the difference was not significant ($P > 0.056$; Fig. 5a). When looking at individual environments, however, significant differences ($P < 0.044$) were observed for locations OLI, EIN, and ROG (Supplementary Fig. 7a), which showed the lowest temperatures in the field[28], suggesting that the relative advantage of the identified haplotype might be temperature dependent.

On chromosome 9 in a genomic region of ~3 Mb, three independent focus haplotypes affected PH_V6 significantly (two favorably, one unfavorably). One of the three focus haplotypes (haplotype D in Fig. 3a and Supplementary Fig. 5) increased PH_V6 compared to the six alternative haplotypes in the respective window. The genetic variance explained by the haplotypes in this window was small (1.7%) most likely due to the low frequency (0.4%) of the focus haplotype in the DH lines. The focus haplotype was absent in the panel of 65 breeding lines. Instead, 95.4% of the breeding lines carried one of the six inferior haplotypes, while 4.6% carried haplotypes not present in the landrace panel. DH lines carrying the focus haplotype showed a significant increase of 15.1 cm compared to the breeding lines ($P < 0.009$). Similar as for the haplotype on chromosome 3, the difference was most pronounced in environments showing low temperature during early plant development (Supplementary Fig. 7b).

We also assessed genomic regions in more detail where the focus haplotype was unfavorable like, for example, the window comprising the *tb1* locus, which explained 13.1% of the genetic variance for TILL in the landrace panel. DH lines carrying the unfavorable focus haplotype showed a significant increase of 1.51 scores compared to the 14 phenotyped breeding lines not carrying the haplotype (Supplementary Fig. 8a; $P < 0.0001$). Here, the focus haplotype was carried by only two of the 65 breeding lines, but for other genomic regions associated with TILL frequencies were higher, e.g., 15.5% for a region on chromosome 5 explaining 6.6% of the genetic variance in the DH lines. In this case, DH lines carrying the focus haplotype showed a significant increase of 1.69 scores compared to 13 breeding lines not carrying the haplotype (Supplementary Fig. 8b; $P < 0.0004$). For a genomic region on chromosome 1 associated with EV_V4 (Supplementary Fig. 9), more than half of the 65 breeding lines carried the unfavorable focus haplotype, including six of the 14 phenotyped lines. The window in which the focus haplotype was located comprised four additional haplotypes and accounted for 5.1% of the genetic variance in the DH lines. We tested the effect of the focus haplotype in the 14 breeding lines and found a significant difference of 0.875 scores between lines with and without the focus haplotype ($P < 0.039$, Supplementary Fig. 9), indicating that a targeted substitution of the focus haplotype with one of the alternative haplotypes could lead to germplasm improvement.

Introducing landrace alleles into elite germplasm for a target trait comes at the risk of undesired effects on other traits due to pleiotropy or linkage. We tested the identified focus haplotypes for each of the early plant development traits in bivariate models for significant effects on other traits (PH_final, FF, MF, LO, and TILL). Of the 53 favorable haplotypes referred to in Fig. 4, 20 had a significant effect on at least one out of the five other traits. Thereof, only three haplotypes increased LO or TILL, whereas four haplotypes slightly decreased LO or TILL. Fourteen

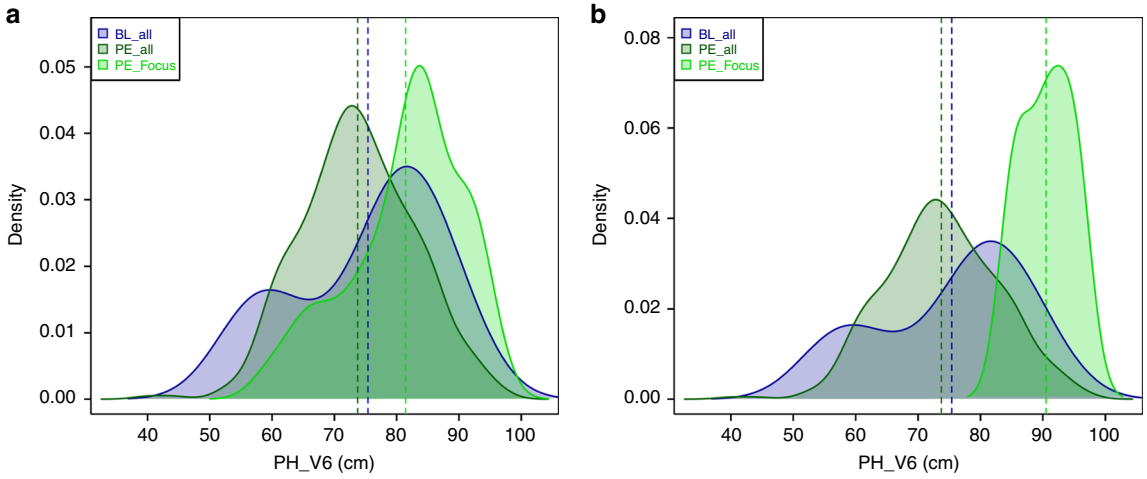

Fig. 5 Effect of favorable haplotypes not present in breeding lines on early plant development. Estimated densities of phenotypic values (BLUEs across locations in 2017) for PH_V6 for 14 breeding lines (BL_all), 402 DH lines of landrace PE (PE_all), as well as for DH lines of PE carrying **a** a focus haplotype on chromosome 3 (haplotype A in Fig. 3a; PE_Focus, 38 lines) and **b** a focus haplotype on chromosome 9 (haplotype D in Fig. 3a; PE_Focus; based on three data points only). Vertical lines indicate the mean of each group. The difference in means between BL_all and PE_all was not significant ($P > 0.514$; permutation test, two-sided). Source data are provided as a Source data file.

haplotypes increased PH_final and/or led to earlier flowering, whereas one haplotype slightly delayed FF. For some of those haplotypes the effect on traits other than early plant development was substantial (e.g., haplotype J in Supplementary Fig. 10a increasing LO). An enrichment of such haplotypes in the breeding germplasm is therefore not advisable. In contrast, haplotypes which explained more of the genetic variance for early plant development than for other traits (e.g., haplotypes E or G in Supplementary Fig. 10a) can still be used for improving germplasm for early plant development resulting in only slightly altered flowering time and/or PH_final. Of the 80 focus haplotypes unfavorable for early plant development (Fig. 4), 48 were significant for at least one other trait. Thereof, 14 haplotypes decreased TILL, while 40 decreased PH_final and/or delayed flowering. However, most of them had only moderate effects on these traits (Supplementary Fig. 10b). Therefore, in many cases selection against those haplotypes can still be recommended.

Discussion

The importance of genetic variation for selection and genetic improvement of crops is undisputed. Genetic resources of domesticated species, such as landraces, are a valuable source of diversity for broadening the genetic base of elite germplasm[1]. However, efficient strategies for utilizing this native diversity for the improvement of quantitative traits are lacking. Here, we developed a strategy to discover beneficial haplotypes for quantitative traits in maize landraces (Supplementary Fig. 1). The combination of comprehensive molecular inventories and meaningful phenotypes collected in landrace-derived DH libraries in multi-environment trials allowed detection of haplotype-trait associations for quantitative traits with limited genetic variation in elite material. Even though the DH libraries were derived from only three preselected populations, 26% of landrace haplotypes were absent in the panel of breeding lines, representing the allelic diversity of multiple diverse source populations[27]. While most of these haplotypes can be expected to be neutral[32] or disadvantageous, some might represent beneficial novel variation.

Landraces represent self-contained populations adapted to their geographical origin[33]. By focusing on diversity within rather than across landraces, confounding effects of strong adaptive alleles are avoided. Consequently, individual trait-associated haplotypes are expected to have moderate to small effects only. Our results meet these expectations. The majority of haplotype-trait associations detected in the DH libraries explained <5% of the genetic variance for all traits under study, including flowering time. However, as shown for the haplotype affecting PH_V6 on chromosome 9 (Fig. 5b), the genetic variance explained in GWAS is not only a function of effect size, but also of haplotype frequency. As DH and breeding lines were sampled from the same germplasm group (European flint maize), haplotype frequencies were positively correlated between the two panels (Fig. 1b). This exemplifies one of the key challenges when searching for untapped variation for quantitative traits, as haplotypes absent in the breeding material tend to have low frequencies also in landraces with shared historical ancestry. Focusing on a set of landraces preselected for variation in target traits increases the chances that they harbor alleles at frequencies large enough to be detected in GWAS. The success of this strategy was reflected in the high number of significant haplotype-trait associations found for target traits early vigor and early plant height.

The large sample of landrace-derived DH lines employed in this study enabled mapping of haplotypes with moderate effect size and comparably low frequency, but as is known for GWAS studies, some of these significant trait associations might be spurious[34]. Here, the sequential determination of significance (Supplementary Fig. 4) should have minimized the proportion of false positives[35]. In addition, the haplotype-based approach enabled tracking of ancestral alleles between landrace-derived and breeding material, and the phenotypic comparison between the two groups supported the usefulness of identified haplotypes for germplasm improvement. Nevertheless, the construction of haplotypes in landrace-derived material warrants further research. Different methods for haplotype construction exist, generating population-specific haplotype blocks based on LD[36,37] or linkage[38]. Here, we used fixed window sizes, as it is advantageous in comparing haplotype frequencies across datasets varying in their extent of LD. The choice of window size depends on the available marker density and affects the number of haplotypes per window as well as the risk of haplotypes being broken up by recombination. Thus, defining the haplotype inventories of landraces and comparing them to elite germplasm is not trivial. Comprehensive sampling of individuals or lines from a limited number of landraces mitigates difficulties in haplotype

construction and at the same time warrants sufficient statistical power and mapping resolution in GWAS through absence of pronounced population structure, rapid decay of LD, and consistency of linkage phases[26]. Here, we put this strategy into practice and showed its potential in identifying favorable alleles not present in breeding lines for improving quantitative traits.

For early development traits, overall performance did not differ significantly between the DH libraries and the subset of phenotyped breeding lines, but DH lines carrying specific focus haplotypes not present in breeding lines outperformed the set of breeding lines significantly in environments favoring trait differentiation. This is a first step toward identifying haplotypes from genetic resources for germplasm improvement, but the final proof of concept will have to come from crosses of landrace-derived material with elite material. As landraces represent open-pollinated populations, background dependency of the identified trait-associated haplotypes should not be as pronounced as in mapping populations tracing back to few genetic founders, such as multi- or biparental crosses. In our study, the vast majority of trait-associated haplotypes occurring in landraces KE and PE had equal effect signs across landraces and environments, supporting this hypothesis. In addition, for cases where it was possible to contrast different haplotypes in the breeding lines (Supplementary Fig. 9), the effect of the focus haplotype in the breeding lines was consistent with the effect in the DH lines. If the selected landraces and the target germplasm to be improved share historical ancestry, we expect only minor genetic background effects when introducing favorable haplotypes discovered in landraces into elite material.

After identification of trait associations, fine-mapping of the respective genomic regions and functional characterization of candidate genes is a logical next step. With a limited number of annotated genes per trait-associated genomic region, high mapping resolution was obtained in this study. The envisaged functional validation of relevant haplotypes opens many options for utilization: targeted allele mining from genetic resources, unlocking diversity trapped in disadvantageous or incompatible haplotypes, broadening the genetic diversity at relevant loci in elite germplasm, and improvement of unfavorable haplotypes through gene editing[39]. In addition to targeted haplotype management, genome-wide approaches will also profit from functional knowledge. Pre-breeding programs[2] might be accelerated through the use of genome-based prediction[40,41]. It has been shown that the prediction accuracy is increased if known trait associations are included as fixed effects in prediction models[42]. As our results indicate high stability of haplotype effects across environments and genetic background, as well as limited haplotype-induced correlations between traits, the prospects of the germplasm improvement through the use of landrace-derived material are promising.

By successfully linking molecular inventories of landraces to meaningful phenotypes and identifying beneficial variation for quantitative traits of agronomic importance, the results of this study represent a first step toward the long-term goal of accessing native biodiversity in an informed and targeted way. The strategy proposed in this study and demonstrated experimentally with the European flint germplasm can be extended to other maize germplasm groups and even to other allogamous crop species. The key to an efficient use of genetic resources is to understand how genomic information of gene bank accessions can be translated into plant performance[43]. We envision a future where haplotypes characterized for their genomic structure, allele content and functional relevance can be freely moved between populations. Our goal is to create plants with novel combinations of alleles that will lead to varieties with novel combinations of traits, thus securing sustainable crop production in a changing world.

Methods

Plant materials. We generated >1000 DH lines derived from three European maize landraces: Kemater Landmais Gelb (KE), Lalin (LL) and Petkuser Ferdinand Rot (PE)[28]. The landraces were preselected for phenotypic variation in cold-related traits assessed in field trials and population genetic analyses[26]. The set of breeding lines used in this study was selected from a broad panel of 68 flint lines[27]. The initial dataset included two US sweetcorn lines, IL14H and P39, which we excluded from our analyses. The remaining 66 lines, released between ~1950 and 2010, were selected to represent the genetic diversity of the European flint elite breeding germplasm. The panel also includes prominent founder lines like EP1, F2, F7, and DK105 (ref. [44]).

Genotypic data. In total, 1015 landrace-derived DH lines were genotyped with the 600k Affymetrix® Axiom® Maize Array[45]. After stringent quality filtering[28], 941 lines (KE = 501, LL = 31, and PE = 409), and 501,124 markers mapped to B73 AGPv4 (ref. [46]) remained for genetic analyses. Calls indicating heterozygosity (0.19%) were set to missing as in DH lines they can be assumed to result from technical artefacts, and all missing values were imputed separately for each landrace using Beagle version 5.0 (ref. [47]) with default settings. From the set of 66 breeding lines, 64 lines were genotyped with the same 600k array[27], whereas for two lines (EZ5 and F64) overlapping SNP positions (85%) were extracted from the HapMap data[48], which is based on whole-genome sequences. For making the 600k genotyping data comparable to the HapMap data, all alleles were coded according to the B73 AGPv4 (ref. [46]) forward strand. The breeding line data were filtered for the 501,124 high-quality markers of the set of DH lines. Applying the same quality filter criteria as for the DH panel (heterozygous calls < 5%; callrate > 90%, except for EZ5 and F64 with callrate >84%), one breeding line (FV66) was removed due to an increased number of heterozygous calls. For the remaining 65 lines, calls indicating heterozygosity (0.31%) were set to missing and missing values imputed using Beagle version 5.0 (ref. [47]) with default settings. For the combined set of landrace-derived DH lines and breeding lines, PCoA[49] was conducted based on modified Rogers' distances[50], using the R-package ape version 5.3 (ref. [51]). Pairwise r^2 (ref. [52]) between SNPs within 1 Mb distance was calculated for the DH libraries (within and across the three landraces) and the panel of breeding lines, respectively. Average LD decay distance ($r^2 < 0.2$) was estimated using nonlinear regression[53]. If not denoted otherwise, analyses were done using R version 3.6.0 (ref. [54]). For plotting of results, R-packages ggplot2 version 3.2.0 (ref. [55]), plot3D version 1.3, and VennDiagram version 1.6.20 were used.

Phenotypic data. In total, 958 DH lines were phenotyped for 25 traits in replicated field trials[28]. Briefly, line per se performance was evaluated in five locations across Germany, and in two locations in northern Spain in 2017 and 2018, resulting in up to 11 environments (location by year combinations) per trait. In each environment, up to ten separate 10 × 10 lattice designs with two replicates per DH line were used. In addition to the DH lines, 15 breeding lines (duplicate entries) and the original landraces (quadruplicate entries) were included as checks in 2017. Fourteen checks comprised important lines of the European flint breeding pool and were included in the set of 65 genotyped breeding lines. In 2018, only four breeding lines were used as checks (three flint lines). A subset of nine traits was analyzed in this study (Supplementary Table 2), related to early plant development, maturity, as well as agronomic characteristics. After stringent quality filtering based on genotypic data[28], phenotypic data of 899 DH lines (KE = 471, LL = 26, and PE = 402) remained for further analyses. Best linear unbiased estimates (BLUEs) for each DH line and check were calculated across environments using a mixed linear model, with genotypes as fixed and environment as well as design factors as random effects[28]. Analogously, BLUEs were calculated within each environment using the same model without environment-related model terms.

Haplotype construction. For both, the landrace-derived DH lines, as well as the breeding lines, haplotypes were defined as a given nucleotide sequence within nonoverlapping windows of ten SNPs (Supplementary Fig. 4a), using the R-package zoo version 1.8-6 (ref. [56]). For the 600k chip, the density of SNPs along the chromosomes follows the average recombination rate[45]. Therefore, using a fixed number of SNPs per window leads to similar window sizes as defined based on genetic map units. The median physical window size was 13.5 kb (mean = 37.8 kb), corresponding to 0.006 cM (mean = 0.026 cM) according to a genetic map generated from a F_2 mapping population of a cross of EP1 × PH207 (ref. [44]). Within each window, haplotypes were coded as presence/absence markers, yielding genotype scores 0 and 2, respectively. To evaluate the potential of untapped variation in landraces for elite germplasm improvement, we compared haplotype frequencies between the landrace-derived DH lines and the panel of 65 breeding lines.

Diversity measures. PIC[57] and H[58] were calculated based on SNPs (PIC_{SNP} and H_{SNP}) as well as on haplotypes (PIC_{hap} and H_{hap}) constructed as described above. nR[59] was calculated within the genomic windows used for haplotype construction. For all five parameters mean values across SNPs or ten-SNP windows, respectively, were calculated for each DH library individually and for the combined set of 941 DH lines, as well as for the set of 65 breeding lines.

Identification of trait-associated haplotypes. For GWAS in the DH lines, haplotypes which were present less than three times in the panel of 899 phenotyped DH lines were excluded from the analysis. For haplotypes with $r^2 = 1$, only one was retained, resulting in 154,104 haplotypes used for GWAS (Supplementary Fig. 4a), with on average 5.73 haplotypes per window. The identification of trait-associated haplotypes was conducted in two steps following Millet et al.[35], (i) identification of candidate haplotypes in GWAS (Supplementary Fig. 4b) and (ii) backward elimination in a multi-locus multi-environment model (Supplementary Fig. 4c). GWAS were conducted for single environments, as well as across environments using the corresponding environment-specific and across-environment BLUEs as response variable in the model, respectively. A univariate linear mixed model, implemented in GEMMA version 0.98.1 (ref. [60]), was used:

$$\mathbf{y} = \mathbf{W}\alpha + \mathbf{x}\beta + \mathbf{Z}\mathbf{u} + \mathbf{e}, \quad (1)$$

where $\mathbf{y}$ is the n-dimensional vector of phenotypic values (BLUEs), with n being the number of lines; $\boldsymbol{\alpha}$ is a three-dimensional vector of fixed effects (intercept and landrace effects of KE and LL); β is the fixed effect of the tested haplotype; $\mathbf{x}$ is the vector of corresponding genotype scores coded as 0 and 2; $\mathbf{u}$ is the n-dimensional vector of random genotypic effects, with $\mathbf{u} \sim N(0, \mathbf{K}\sigma_g^2)$; and $\mathbf{e}$ is the n-dimensional vector of random residual effects, with $\mathbf{e} \sim N(0, \mathbf{I}_n\sigma^2)$. $\mathbf{K}$ denotes the $(n \times n)$ genomic relationship matrix based on SNP markers according to Astle and Balding[61], calculated using the R-package synbreed version 0.12-9 (ref. [62]). $\mathbf{I}_n$ denotes the $(n \times n)$ identity matrix. σ_g^2 and σ^2 refer to the genetic and residual variance pertaining to the model defined in Eq. (1), respectively. Matrices $\mathbf{W}$ $(n \times 3)$ and $\mathbf{Z}$ $(n \times n)$ assign phenotypic values to fixed and random effects, respectively. Significance of haplotype-trait associations was assessed for each single-environment as well as for the across-environment GWAS based on the likelihood ratio test, as implemented in GEMMA, using a 15% false discovery rate[63]. Haplotypes with a physical distance of <1 Mb and in high LD ($r^2 \geq 0.8$) were considered to mark the same genomic region. The corresponding trait-associated genomic region was described by the start and end positions of the first and last haplotype fulfilling the defined criteria, respectively. To represent genomic regions equally in subsequent analyses, only the most significant haplotype, the focus haplotype, was retained per region in the respective GWAS, resulting in a set of candidate haplotypes.

In the multi-locus, multi-environment mixed linear model, we conducted a backward elimination of those candidate haplotypes as suggested by Millet et al.[35], using the ASReml-R package version 3.0 (ref [64]):

$$y_{ijk} = \mu + \omega_i + \delta_j + \sum_{q \in \mathbf{Q}} x_{kq}\beta_q^i + u_k + e_{ijk}, \quad (2)$$

where y_{ijk} is the phenotypic value (BLUE) of line k belonging to landrace j tested in environment i; μ is the common intercept; ω_i is the fixed effect of environment i; δ_j is the fixed effect of landrace j; x_{kq} is the genotype score (0 or 2) of line k for haplotype q; β_q^i is the fixed effect of haplotype q in environment i, comprising the haplotype main and haplotype by environment interaction effect, i.e.,

$$\beta_q^i = \beta_q + (\beta \times \omega_i)_q, \quad (3)$$

u_k is the random genotypic effect of line k, and e_{ijk} is the random residual error with environment-specific residual error variance. $\mathbf{Q}$ represents the final set of haplotypes obtained through step-wise backward elimination based on the Wald test for β_q^i (ref. [65]). At each step, significance of each haplotype was tested when it was the last one entering the model and the least significant haplotype was removed if $P \geq 0.01$. The proportion of genetic variance explained by the set of trait-associated haplotypes was estimated by calculating the reduction in genetic variance between models including and excluding the haplotype effects, following Millet et al.[35]. For evaluating effect stability across landraces for the final set of haplotypes $\mathbf{Q}$, we extended Eq. (2) by changing the term $\sum_{q \in \mathbf{Q}} x_{kq}\beta_q^i$ to $\sum_{q \in \mathbf{Q}} x_{kq}\beta_q^{ij}$, with

$$\beta_q^{ij} = \beta_q + (\beta \times \omega_i \times \delta_j)_q. \quad (4)$$

For comparison, GWAS was also performed with 175,810 SNPs (minor allele counts ≥ 3 and $r^2 \neq 1$), analogously as described above.

Favorable and unfavorable haplotypes and their effect stability. The number of environments in which a haplotype was significant was estimated by generating 95% confidence intervals (CI = effect estimate ± 1.96 × standard error) based on Eq. (2), following Millet et al.[35]. A CI not including 0 indicated significance of the haplotype in a given environment. Haplotypes with constant effect sign across significant environments were classified as favorable or unfavorable. For EV_V4, EV_V6, PH_V4, and PH_V6 positive (negative) effects were defined as favorable (unfavorable). For LO and TILL negative (positive) effects were defined as favorable (unfavorable). No classification was made for PH_final, FF, and MF, as breeding goals vary for these traits. Haplotypes with changing sign of significant effects in different environments were classified as interacting.

Haplotypes associated with multiple traits. We tested if haplotypes identified for early plant development also had an effect on other traits using a bivariate model, similar to Stich et al.[66]:

$$y_{tijk} = \mu_t + \omega_{ti} + \delta_{tj} + x_k\beta_t + u_{tk} + e_{tijk}, \quad (5)$$

where y_{tijk} is the phenotypic value (BLUE) for trait t of line k belonging to landrace j tested in environment i; μ_t is the intercept for trait t; ω_{ti} is the fixed effect of environment i for trait t; δ_{tj} is the fixed effect of landrace j for trait t; x_k is the genotype score (0 or 2) of line k for the tested haplotype; β_t is the fixed effect of the haplotype for trait t; u_{tk} is the random genotypic effect of line k for trait t, with $\mathbf{u} \sim N(0, \mathbf{G} \otimes \mathbf{K})$; and e_{tijk} is the residual with $\mathbf{e} \sim N(0, \mathbf{E} \otimes \mathbf{I}_n)$. $\mathbf{G}$ and $\mathbf{E}$ correspond to the $(t \times t)$ genetic and error variance–covariance matrices among traits pertaining to the model defined in Eq. (5), respectively, and $\otimes$ denotes the Kronecker product. Haplotypes for which the 95% CIs for both β_t did not include 0 were considered significant for both traits. The proportion of genetic variance explained per trait by significant haplotypes was estimated by calculating the respective reduction in $\mathbf{G}$ between models including and excluding the haplotype.

Haplotype comparison between landraces and breeding lines. We assessed frequency distributions of identified trait-associated favorable and unfavorable landrace haplotypes in the panel of 65 breeding lines, and compared them with 500 haplotypes randomly drawn out of the set of haplotypes occurring at least three times in the landrace panel. Significance for differences in means between the frequencies of favorable and random haplotypes, as well as unfavorable and random haplotypes was tested with the Mann–Whitney test (two-sided). When tracking potentially shared ancestral haplotypes between populations, the probability of a haplotype being broken up by recombination depends on the haplotype length, the recombination rate in the respective genomic region and the time span back to the most recent common ancestor. To evaluate to what extent recombination might have occurred in the haplotypes constructed in this study, we considered the physical and genetic length of each haplotype, as well as haplotype similarity ($1 - H_{\text{hap}}$) and nR within the respective genomic windows.

To evaluate the effect of the selected focus haplotype relative to the alternative haplotypes in a given ten-SNP window, we followed the approach of Bustos-Korts et al.[67], extending Eq. (2) to:

$$y_{ijk} = \mu + \omega_i + \delta_j + \sum_{q \in \mathbf{Q}'} x_{kq}\beta_q^i + x_{kh}\beta_h^i + u_k + e_{ijk}, \quad (6)$$

where $\mathbf{Q}'$ represents the set of haplotypes $\mathbf{Q}$ as described above without the respective focus haplotype of the window tested, x_{kh} identifies the haplotype (categorical variable) in the window tested carried by line k, and β_h^i represents the effect of the respective haplotype relative to the focus haplotype. Similar as above, significance of haplotype effects relative to the focus haplotype was determined by constructing 95% CIs. We further estimated the proportion of genetic variance explained by the given window by calculating the reduction in genetic variance between the null model (without $\sum_{q \in \mathbf{Q}'} x_{kq}\beta_q^i + x_{kh}\beta_h^i$) and the model with the $x_{kh}\beta_h^i$ term.

To evaluate to what extent haplotypes with favorable or unfavorable effects in landraces also have favorable or unfavorable effects in elite material, respectively, we compared performance levels between the landrace-derived DH lines and the 14 breeding lines used as checks. As phenotypic data for the 14 breeding lines were only available for 2017, only the six environments from 2017 were considered. For some traits, differences in means between the landraces were observed[28], thus comparisons were conducted for each landrace separately. Significance for differences in means between the respective landrace and the 14 checks was tested based on 10,000 permutations (two-sided test). In addition to a comparison of the overall performance level between all lines of the respective landrace and the 14 breeding lines, we compared means between groups of lines carrying a particular haplotype and lines not carrying the haplotype.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

A reporting summary for this article is available as a Supplementary Information file. The datasets generated and analyzed during the current study are available from the corresponding author upon request. Seeds from all genotypes used in the study are available through material transfer agreements. The genotypic data of 941 DH lines and the phenotypic data of 899 DH lines and 14 breeding lines are available in figshare (https://doi.org/10.6084/m9.figshare.12137142). The 600k data of 63 breeding lines can be accessed at figshare (https://doi.org/10.6084/m9.figshare.3427040.v1), while for two lines genotypic data based on whole-genome sequences were downloaded from CyVerse Data Store (http://cbsusrv04.tc.cornell.edu/users/panzea/download.aspx?filegroupid=34). The Source data underlying Figs. 1 and 3–5, as well as Supplementary Figs. 2, 3, 5, and 7–10 are provided as a Source data file. Source data are provided with this paper.

Code availability

All custom codes used in the article can be found at Github (https://github.com/Manfred-Mayer/GWAS_DHs_landraces).

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Author contributions

C.-C.S., M.O., and M.M. conceived the study; C.-C.S., M.O., and A.E.M. acquired funding for the study; M.M., A.C.H., E.B., T.P., M.O., A.E.M., and C.-C.S. generated phenotypic and genotypic data; A.C.H. contributed to analyses of phenotypic data; E.G.-S. contributed to haplotype construction; M.M. performed analyses and drafted the manuscript; C.-C.S. edited the manuscript; all authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

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