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Pearl millet breeding in West Africa – Steps towards higher productivity and nutritional value

Dissertation

submitted in fulfillment of the requirements for the degree
„Doktor der Agrarwissenschaften“ (Dr. sc. agr. /Ph. D. in Agricultural Sciences)
to the Faculty of Agricultural Sciences

presented by

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Stuttgart-Hohenheim

2018

This thesis was accepted as a doctoral dissertation in fulfillment of the requirements for the degree "Doktor der Agrarwissenschaften" (Dr. sc. Agr./ Ph. D. in Agricultural Sciences) by the Faculty of Agricultural Sciences at the University of Hohenheim, on December 14th, 2017

Day of oral examination: January 19th, 2018

Examination Committee:

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Content

Abbreviations

AFLPs	Amplified fragment length polymorphism markers
CMS	Cytoplasmic male sterility
Fe	Iron
GBS	Genotyping-by-sequencing
GCA	General combining ability
G×E	Genotype-by-environment
KASP	Competitive allele-specific PCR based
LG	Linkage group
OPV	Open-pollinated varieties
PBPH	Panmictic better parent heterosis
PMPH	Panmictic mid-parent heterosis
QTL	Quantitative trait loci
RFLPs	Restriction fragment length polymorphism markers
SSRs	Simple sequence repeat markers
SNP	Single nucleotide polymorphism
SSA	Sub-Saharan Africa
WA	West Africa
WCA	West and Central Africa
Zn	Zinc

1 General introduction

In Sub-Saharan Africa (SSA) the birth rate of 4.9 children per woman in 2005-2010, which is more than the double the replacement level, is resulting in an enormous population growth – 2.5 percent per year (World Bank 2017). It is estimated that the SSA population will grow from 0.86 billion in 2010 to 1.96 billion in 2050 and 3.36 billion in 2100 (Bongaarts and Casterline 2013). Such a population growth will put enormous pressure on food security, which is already insufficient in SSA, with an annual deficit of 9 million tons. By 2025 this deficit will be more than tripled to 35 million tons (Cooper et al. 2008). A global increase in food consumption per person and a shift towards animal-based food products (increasing feed production), scarcity of agricultural inputs especially fresh water and phosphorus (P) fertilizer (Rosegrant et al. 2009; Kearney 2010; Van Boeckel et al. 2015), plus a rising demand for resources for bioenergy production will further increase the prices of agricultural products and exacerbate the situation in developing countries especially for the poor (Ivanic and Martin 2008). It is expected that 90% of the crop production growth globally (80% in developing countries) will be achieved by higher cropping intensity and yields, while land expansion will contribute only 10%. However most of such land lies in SSA and Latin America (FAO 2009). This intensification will need the improvement of various agronomic management practices (intercropping, crop rotation, livestock integration, fertilizer input, etc.) as well as the use of genetically improved crops (Garnett et al. 2013). Besides the demand for high-yielding varieties, the alarming prevalence of micronutrient deficiency in SSA calls for crops with enhanced nutritional value. Thus the breeders' task is to develop farmer-preferred cultivars with enhanced nutritional value that are adapted to specific agro-ecologies and a changing climate.

1. 1 Pearl millet – a food security crop

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is the most commonly grown type of millet and accounts for almost half of global millet production. It is the sixth most important cereal globally and more than 90 million poor people rely on this crop for food and income (Taylor 2016). These people live in dry areas of Africa and Asia, where cropping of other cereals is not productive due to the very harsh environment. In Sahelian West and Central Africa, pearl millet is produced on about 15 million hectares (FAOSTAT 2017), which shows the importance of this staple crop for this region.

Pearl millet is a diploid (2n=2x=14) and allogamous species, with an outcrossing rate of more than 75% (Burton 1974; Sandmeier 1993), so pearl millet landraces represent heterozygous, genetically heterogeneous open-pollinating populations. Pearl millet, being a C4 plant, has a very high photosynthetic efficiency and dry matter productivity, thus besides its use as a cereal crop, it is a valuable fodder crop. It is also termed as "nutricereal" because of its great nutritional characteristics: an excellent amino acid profile, complex carbohydrates and other phytochemicals with nutraceutical qualities (Burton et al. 1972; Hulse et al. 1980).

The first evidence of domesticated pearl millet was found in Sahelian West Africa in the Lower Tilmesi Valley in northern Mali, where it was already being cultivated 4500 years ago (Manning et al. 2011). Due to the long history of cultivation, Sahelian West Africa is considered the main center of genetic diversity (Oumar et al. 2008). Some centuries later, pearl millet spread to India, which is now the largest millet-producing country worldwide (10 million tons are produced on 9.3 million hectares annually) (FAOSTAT 2017).

1.2 Pearl millet production constraints and corresponding breeding targets in West Africa

Pearl millet is capable of growing in environments with very severe production constraints like high temperatures, soils with high salinity and low fertility, and high rainfall variability leading to strong and unpredictable drought stress (Bidinger and Hash 2004). Cropping in such harsh, rain-fed farming systems requires very high population buffering capacity, which can be achieved by varieties with high heterozygosity and genetic heterogeneity for adaptation traits (Haussmann et al. 2012). Climate change will aggravate climatic constraints (heat stress, drought stress, flooding, inter-annual rainfall variability) during the next few decades, hence the task of developing well-adapted and stable varieties will become even more challenging (Cooper et al. 2008). Further, pearl millet is mostly cultivated on fields with very low soil fertility, which is associated with poor soil resources, soil degradation, extensive management practices and a low level of external inputs (Bekunda et al. 1997; Somda et al. 2002). The lack of financial resources, high prices, risk aversion and insufficient infrastructure inhibits many West Africa (WA) smallholder farmers from using fertilizers. Low P input especially will become an increasing constraint, since resources of P fertilizer are scarce and non-renewable. Thus, increasing prices will make it unfeasible for poor pearl millet farmers to use such fertilizer. Pearl millet breeding in WA has therefore the task of developing varieties that are highly adapted to low input conditions.

Beside such abiotic constraints, pearl millet has to cope with several biotic stresses (diseases, insect pests, and weeds) like downy mildew (caused by *Scelerospora graminicola)*, head miner (*Heliocheilus albipunctella)* and the parasitic weed *Striga hermonthica*, which are also likely to get worse with changing climate patterns. The development of resistant cultivars is apparently the most effective strategy to minimize losses by biotic constraints, as the use of plant protection products is too costly for most pearl millet farmers and might have side effects on the environment, human health, and food safety.

WA farmers have very specific preferences in the characteristics of their pearl millet. For instance, grain yield potential is not necessarily the most preferred trait. Other traits like flowering time, panicle length, taste and high dual purpose suitability (grain and fodder production) can have similar importance (Blümmel et al. 2003; Omanya et al. 2007). Thus knowledge and consideration of region-specific farmer-preferred characteristics is crucial to develop improved varieties which will be adopted by the farmers. Such farmer preferences can be identified and achieved in participatory breeding programs (Christinck et al. 2005; Ceccarelli et al. 2009; Haussmann et al. 2012). As farmer preferences are highly region- and even social context-specific, there is no "one-size-fits-all" type of pearl millet that responds to the diversity of demands in the entire pearl millet growing area in WA.

The plurality of pearl millet production constraints in combination with specific farmer preferences and the challenge of food insecurity results in multiple breeding targets such as higher productivity, yield stability, adaptation to low input conditions, pest resistance, dual purpose cultivars, diverse farmer preferences and processing suitability. Due to the alarming prevalence of micronutrient deficiency in WA, which is described in the following paragraph, enhanced micronutrient density should be an additional goal.

1.3 Micronutrient deficiency in pearl millet growing areas

The burden of micronutrient malnutrition, which is caused by low uptake of essential micronutrients such as iron (Fe), zinc (Zn), vitamin A and iodine, is estimated to affect 2 billion people worldwide (Tulchinsky 2010). In low and middle-income countries such as pearl millet growing countries, deficiencies of more than one nutrient are common due to diets that are insufficiently diverse and dominated by staple carbohydrate sources that are poor in micronutrient bioavailability (Muthayya et al. 2013).

Young children and pregnant women are most vulnerable to micronutrient deficiencies because of their rapid growth and development (Black et al. 2013). The term *hidden hunger* describes the invisible nature of the problem, where affected people have enough food to

satisfy their energy needs, but suffer from indirect and slowly arising symptoms such as mental impairment or increasing rates of illness and death from infectious diseases. Very strong micronutrient deficiency causes clinical manifestations, and moderated deficiencies can have lifelong consequences for health, mental development and productivity (Black 2003; Ruel-Bergeron et al. 2015).

Based on the Hidden Hunger Index, which considers Fe, Zn and vitamin A deficiency, the problem of hidden hunger is very severe in almost all African countries. The index revealed that 19 out of the 20 countries where hidden hunger is most prevalent are located in Africa. The population in Niger, where pearl millet is grown as a staple crop, is the most severely affected population worldwide (Ruel-Bergeron et al. 2015).

1.4 Pearl millet breeding approaches in West Africa

1.4.1 The approach of biofortification breeding

Various approaches have been developed and applied to prevent micronutrient deficiencies. Biofortification aims to increase nutrient density and bioavailability by conventional breeding or genetic engineering, and has been identified as a sustainable and cost-effective approach (Bouis 1999). In particular, poor people living in remote areas with limited access to diverse diets, supplements, or commercially fortified foods might benefit through biofortification (Saltzman et al. 2013). The daily calorie intake of families in WA is predominantly based on pearl millet, thus pearl millet biofortification could be one step towards reducing hidden hunger in this region. In India, the biofortified pearl millet variety 'Dhanashakti' with enhanced Fe and Zn density is already on the market (Saltzman et al. 2013; Rai et al. 2014). The desired effect of biofortified pearl millet has been proven by studies that showed enhanced Fe and Zn absorption after biofortified pearl millet had been consumed (Kodkany et al. 2013b; Finkelstein et al. 2015; 2017).

The selection gain during a pearl millet biofortification breeding program depends on the genetic variation of grain micronutrient densities in the adapted germplasm. By the time of initiating the present PhD research this had not yet been studied in WA pearl millet material. Thus, such investigations were necessary, before breeding programs targeting micronutrient density enhancement could be started. In WA, pearl millet is usually decorticated before consumption, thus a nutritional enhancement of peeled grains should be targeted. However, the decortication process is difficult to standardize and labor intensive, which would result in high costs if screening methods were based on

decorticated grain. Hence, the association between whole and decorticated grain micronutrient densities needed to be evaluated.

1.4.2 Exploiting local germplasm diversity

West Africa, as the center of diversity for pearl millet, exhibits diverse and well-adapted germplasm. Mass selection by farmers combined with the continuous gene flow among domesticated types as well as between domesticated and wild types is the basis of highly variable landraces and the maintenance of diversity (Bezançon et al. 2009; Lewis 2010). Such landraces can be considered as a store of valuable genetic diversity, which could be utilized in breeding programs like biofortification breeding.

National breeders focus on their locally available germplasm and hesitate to include external materials in their programs, although landraces originating from other countries with similar agro-ecologies could provide a great chance to increase the selection gain. Characterization of a broad set of WA landraces plus public sharing of such data could facilitate an efficient use of pearl millet diversity in breeding programs. For instance, knowledge of the geographic distribution of certain traits can assist the search for a specific characteristic. Further, characterization of a diverse WA germplasm collection is of interest in order to understand the structure of diversity and to identify genetically distinct clusters, which might be useful in order to develop heterotic groups for use in hybrid breeding.

The establishment of a well-documented WA germplasm diversity is also of importance, as the material bred in India is not well adapted to WA conditions, so the advanced breeding progress (including hybrid breeding) in India cannot easily be transferred.

1.4.3 Population and hybrid breeding

State of pearl millet breeding in India and West Africa

The pearl millet seed sectors in India and WA differ tremendously. In India there are various improved open-pollinated varieties (OPV) as well as hybrids on the seed market, which were developed by private breeding companies or public research institutions (Pray et al. 2007). Development of hybrid cultivars in India started in the 1960s and the resulting hybrid varieties have now the biggest market share and are well-adopted by many Indian farmers (Matuschke and Qaim 2008; Munasib et al. 2015). However, especially in Rajasthan, the major pearl millet-producing state in India characterized by low-input and highly variable drought stress conditions and lower productivity levels, the adoption rate of improved hybrid varieties is relatively low (~50%)(Munasib et al. 2015).

The fast spread of pearl millet hybrids within large parts of India was achieved with commercially-viable single cross hybrids which had a yield superiority of 20-30% over OPVs (Andrews and Kumar 1992). Hence, the use of hybrids in combination with improved crop management led to an enormous pearl millet productivity increase from 305 kg ha^{-1} during 1951–1955 to 998 kg ha^{-1} during 2008–2012 (Dave 1986; Yadav and Rai 2013).

Pearl millet breeding in WA is mainly conducted by national breeding institutions, which are financed by the government and some external funds, and the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) located in Niger (where this study was implemented in collaboration with the University of Hohenheim). So far, there are only very few private seed companies in WA that produce and market seed. OPVs are the main variety type in WA, and the availability of improved OPVs is increasing in several regions of WA, especially where farmer-managed seed cooperatives have been developed and associated with participatory breeding projects. Pearl millet hybrid breeding started some years ago at national levels, but it is still very limited and no hybrid seed is yet officially available on the seed market.

Heterotic groups as basis for sustainable hybrid breeding

Hybrid breeding benefits from the effect of heterosis, which is known to be strongly associated with heterozygosity of the genotype (Robertson and Reeve 1952). A high degree of heterozygosity in a hybrid can be achieved by choosing hybrid parents with a high genetic distance. To apply this in practical hybrid breeding programs, the concept of heterotic pools has been developed and is now established in various hybrid crops (Melchinger and Gumber 1998). Thus, a fundamental step in each hybrid breeding program should be the identification of heterotic patterns seeking maximum exploitation of heterosis.

Heterotic groups can be defined by field trials determining the testcross performance among available germplasm. Since the evaluation of all possible crosses is unrealistic in practical breeding, Melchinger and Gumber (1998) proposed (i) to group genetically similar germplasm using molecular markers, (ii) to select representative accessions from each subgroup, (iii) to assess the performance of crosses among selected accessions, and finally, (iv) to organize heterotic groups based on hybrid performance or its components such as heterosis and *per se* performance.

Current pearl millet hybrid breeding programs in India use two pools; one includes female parents (B-lines) and the other male parents (restorer lines), while systematic heterotic grouping based on diversity and/or combining ability studies has not been explicitly implemented. Such allocation of germplasm to either the female or the male heterotic group bears the risk of some genetic intermixture/relatedness between parents (Gupta et al. 2015), which should be avoided in order to maximize heterozygosity and therefore hybrid performance.

Besides hybrid breeding, the concept of heterotic croups can also benefit OPV breeding since the enhanced heterozygosity caused by a cross between two genetically distinct groups will be, to a certain extent, maintained after several generations of random mating through open pollination.

Aspects of yield stability in different hybrid types

The ability of a variety to cope with climate variability is due to specific adaptation traits such as resistances to drought, high temperature and flooding, but is also influenced by its genetic structure. Heterozygous and/or heterogenous cultivars have generally a high buffering capacity and could therefore be a preferable option (Haussmann et al. 2012). Development of single cross hybrids, which are the most common hybrid type, seeks a maximum of intra-genotypic diversity, which can increase the adaptation range of a genotype (e.g. by potentially higher enzyme diversity). But a hybrid cultivar, produced by a single cross between inbreed lines, is also genetically uniform (homogeneous), which allows no population buffering capacity. Thus, despite the potentially high yield, pearl millet single-cross hybrids could be risky for WA smallholder farmers due to the high genetic vulnerability of these hybrids and hence their potentially lower yield stability. The use of population hybrids (based on a cross between two OPVs) and top-cross hybrids (based on a cross between an inbred line and an OPV) is generally less common in hybrid breeding, however there are lot of promising results on pearl millet top-cross hybrids (Mahalakshmi et al. 1992; Bidinger et al. 1994; Yadav et al. 2000; Bidinger et al. 2005). Those two hybrid types are genetically heterogenous and heterozygous, which appears to be preferable to achieve high yield stability, which has major importance for subsistence farmers in WA.

Cytoplasmic male sterility for hybrid seed production

Cytoplasmic male sterility (CMS) is a maternally-inherited trait characterized by the absence of functional pollen. This trait enables large scale intercrossing and is therefore a crucial

tool for economic hybrid seed production (Burton 1974). Restoration of male-fertility in the background of the male-sterility-inducing cytoplasm is possible by the presence of dominantly-inherited nuclear restorer genes, called *Rf* genes. Male parents carrying such genes facilitate the production of male-fertile hybrid plants (Schnable and Wise 1998).

The first mentioned CMS system in pearl millet was A_1, which was found in the Tift23A germplasm (Burton 1974) and used during the first decades of hybrid breeding in India. An alternative source to this solely-used A_1 CMS system was desired to avoid cytoplasmic uniformity which can give rise to vulnerability (Yadav et al. 1993). Various sources were evaluated (Appadurai et al. 1982; Aken'ova 1982; Hanna 1989) but only the A_4 and A_5 CMS systems were determined to be commercially feasible (Rai et al. 2001; Rai et al. 2009).

Seeking a stable CMS system in pearl millet for WA conditions requires investigations on appropriate CMS systems and corresponding restorer germplasm followed by introgression in the target genotypes. The A_4 and A_5 CMS systems appear to be suitable for high-temperature environments and hence interesting for WA hybrid breeding. The frequency of maintainer lines for both of these systems is high in WA pearl millet germplasm while the frequency of restorer types is highly limited (Issoufa 2010). Thus a transfer of *Rf* genes available in Indian germplasm will be needed. Gene mapping of *Rf* loci would facilitate efficient transfer of the desired alleles into potential male hybrid parents well adapted to WA conditions.

1.4.4 Use of modern genetic tools

Identification of quantitative trait loci (QTL) requires genetic linkage maps, which cover the entire genome with a preferably high marker density and distribution. In pearl millet, various marker types have been developed during the last 30 years. The first markers were restriction fragment length polymorphism markers (RFLPs). The continuous improvement of marker technology resulted in higher map quality. However, maps based on RFLPs, amplified fragment length polymorphism markers (AFLPs), and simple sequence repeat markers (SSRs) contained marker gaps greater than 20 cM at the distal ends (Supriya et al. 2011; Rajaram et al. 2013; Ambawat et al. 2016).

Linkage maps based on single nucleotide polymorphism (SNP) marker technology are becoming common in many crops because of the low cost of high throughput sequencing methods (Ganal et al. 2011; Kumar et al. 2012). The high abundance of SNPs in the genome enables the possibility of creating much denser linkage maps compared to other marker types. SNP-based genetic maps are highly useful for disclosing the structure and

organization of the genome and identifying the genetic basis of loci linked to a trait with excellent resolution (Krawczak 1999; Mammadov et al. 2012). Genotyping-by-sequencing (GBS) is one powerful tool to create SNP datasets. Its methodology is based on complexity reduction by restriction enzymes, which cuts the DNA at specific sites into fragments. Subsequently, barcodes are ligated to each fragment, which facilitate multiplexing of pooled samples in a single sequencing lane.

The power and usefulness of GBS has been demonstrated in various crops like maize, barley, sorghum, grapes, etc. (Elshire et al. 2011; Nelson et al. 2011). Moumouni et al. (2015) and Punnuri et al. (2016) studied the applicability of GBS in pearl millet and developed dense and reasonably uniform linkage maps. Thus, association studies or QTL mapping using SNP based genetic maps seems promising (Morris et al. 2013). In pearl millet breeding programs, such mapping studies could enable an efficient introgression of traits like fertility restoration into the target WA or Indian germplasm.

1.5 Objectives of this study

The goal of my thesis was to set the scientific basis for more efficient pearl millet breeding in WA with a specific focus on achieving higher productivity and nutritional value. The specific objectives were:

1. To characterize a broad set of WA pearl millet accessions and to investigate their diversity and geographic patterns based on their phenotypes.
2. To identify the potential and strategies for increasing the micronutrient level in WA pearl millet.
3. To evaluate the performance of population hybrids and to derive initial strategies of pearl millet hybrid breeding in WA based on combining ability and heterotic patterns among geographically close versus distant pearl millet populations.
4. To identify the male-fertility restoration locus for the A4 cytoplasmic-genic male-sterility system in WA pearl millet using a GBS based linkage map.

Achieving these objectives is expected to enable a more efficient use of locally adapted pearl millet genetic diversity in WA breeding programs, to open a door for biofortification breeding in WA pearl millets, and to guide and facilitate future pearl millet hybrid breeding in WA including heterotic grouping and fast development of female and male hybrid parents.

2 Agro-morphological characterization of West and Central African pearl millet accessions

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Crop Science (2015), Volume 55, pages 737-748, doi: 10.2135/cropsci2014.06.0450

The original publication is available at:
https://dl.sciencesocieties.org/publications/cs/abstracts/55/2/737

Abstract

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] was domesticated in Sahelian West Africa. This highly outcrossing crop is one of the most important staple cereals in the semiarid tropics, adapted to very harsh rain-fed conditions. Agro-morphological characterization of local germplasm is very important to better understand existing diversity, ease targeted genetic broadening of breeding populations, and potentially link this knowledge to genotypic information. The objectives of our study were to (i) characterize West and Central African (WCA) pearl millet accessions based on their agro-morphological traits; (ii) evaluate the possibility to group accessions based on their agro-morphological characteristics; (iii) determine geographic patterns of phenotypic differentiation; and (iv) derive conclusions for pearl millet improvement in WCA. A total of 360 early-tomedium maturity accessions were phenotyped for 12 agro-morphological traits at six environments in WCA. Wide ranges of all observed traits indicated a high diversity of the tested accessions. Principal component analysis revealed very large diversity within individual countries, especially within Mali and Burkina Faso. Some limited grouping of accessions from Niger, Senegal, Cameroon, Morocco, and Mauritania was observed for individual principal component axes. Geographical differentiation and country differences were detected for several traits. The results and data presented in our study reflect WCA pearl millets' tremendous diversity and adaptability to a wide range of environments and give a sound basis for breeders to select and utilize this germplasm to serve the manifold needs of WCA pearl millet farmers.

3 Micronutrient density and stability in West African pearl millet - potential for biofortification

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Crop Science (2014), Volume 54, pages 1709–1720 doi: 10.2135/cropsci2013.11.0744

The original publication is available at:
https://dl.sciencesocieties.org/publications/cs/articles/54/4/1709

Abstract

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is one of the most important cereals in West and Central Africa (WCA). Human populations in WCA are strongly affected by micronutrient deficiencies. Biofortification, the development of pearl millet varieties with enhanced micronutrient levels, is recognized as a suitable approach to reducing this widespread health problem. To assess the potential of biofortification of WCA pearl millet germplasm, we studied quantitative-genetic parameters of eight mineral densities in whole and decorticated grains, their stability over environments, and the correlations among minerals and agromorphological traits. The study included 72 WCA pearl millet genotypes grown in three environments in Niger, contrasting in soil fertilization. Significant genotypic effects, moderate estimates of heritability, and genetic variation for mineral densities, especially for Fe and Zn, indicate a high potential for biofortification of WCA pearl millet. However, screening of additional landraces or introgression of favorable alleles from highly nutrient-dense Indian germplasm could expedite achievement of higher densities. Genotype-by-environment interaction effects were significant for Fe and Zn grain densities, showing the importance of multienvironmental evaluation for identifying stable genotypes. Identified genotypes with relatively stable Fe and Zn grain densities appear suitable for use in future WCA pearl millet biofortification breeding programs.

4 Combining ability patterns among West African pearl millet landraces and prospects for pearl millet hybrid breeding

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Field Crops Research (2016), Volume 195, pages 9–20, doi: 10.1016/j.fcr.2016.04.035

The original publication is available at:
http://www.sciencedirect.com/science/article/pii/S0378429016301319

Abstract

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is an important hybrid crop in India. However, to date limited pearl millet hybrid development has been undertaken in West Africa (WA), which is the center of pearl millet origin and diversity and where this crop is most important outside India. Using a diverse set of WA pearl millet germplasm, objectives of this study were to determine the superiority of population hybrids over open-pollinated varieties for agro-morphological and agronomic traits in WA pearl millet germplasm; and (ii) to derive strategies for pearl millet hybrid breeding in WA, based on quantitative-genetic parameters, combining ability and heterotic patterns among geographically close versus distant pearl millet populations. A 10 × 10 factorial mating design was performed with four parental OPVs from each of five WA countries. The 100 population hybrids and their parents were tested for 14 traits at six locations in one year, thereby using contrasting locations to indirectly sample the rainfall variability inherent to WA pearl millet production environments. Grain yield showed an average panmictic mid-parent heterosis (PMpH) of 16.7%, ranging from −26 to 73%. The mean grain yield of hybrids based on inter-country crosses did not differ significantly from intra-country crosses. Geographic distance between parents was positively correlated with hybrid grain yield ($r = 0.31$), but not with PMpH . Some crosses between accessions from Niger/Nigeria and Senegal were outstanding. Predictability of population hybrid performance for grain yield was moderate based on midparent values ($r = 0.43$) and slightly better based on general combining ability (GCA) ($r = 0.56$). Overall, pearl millet hybrid breeding in WA seems very promising, but there do not seem to be clear "natural" heterotic groups among WA pearl millet landraces. Such heterotic groups as the basis of sustainable hybrid breeding need rather to be created systematically, by building on existing combining ability patterns and aiming to maximize combining ability between the groups.

5 Mapping a male-fertility restoration locus for the A_4 cytoplasmic-genic male-sterility system in pearl millet using a genotyping-by-sequencing based linkage map

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Submitted to BMC plant biology

Abstract

Background: Pearl millet (*Pennisetum glaucum* (L.) R. Br., syn. *Cenchrus americanus* (L.) R. Br) is an important cereal and fodder crop in hot and arid environments. There is great potential to improve pearl millet production through hybrid breeding. Cytoplasmic male sterility (CMS) and the corresponding nuclear fertility restoration / sterility maintenance genes (*Rf*s) are essential tools for economic hybrid seed production in pearl millet. Mapping the *Rf* genes of the A_4 CMS system in pearl millet would enable more efficient introgression of both dominant male-fertility restoration alleles (*Rf*) and their recessive male-sterility maintenance counterparts (*rf*).

Results: A high density linkage map based on single nucleotide polymorphism (SNP) markers was generated using an F_2 mapping population and genotyping-by-sequencing (GBS). The parents of this cross were 'ICMA 02777' and 'ICMR 08888', which segregate for the A_4 *Rf* locus. The linkage map consists of 460 SNP markers distributed mostly evenly and has a total length of 462 cM. The segregation ratio of male-fertile and male-sterile plants (3:1) based on pollen production (presence/absence) indicated monogenic dominant inheritance of male-fertility restoration. Correspondingly, a major quantitative trait locus (QTL) for pollen production was found on linkage group 2, with cross-validation showing a very high QTL occurrence (97%). The major QTL was confirmed using selfed seed set as phenotypic trait, though with a lower precision. However, these QTL explained only 14.5% and 9.9% of the phenotypic variance of pollen production and selfed seed set, respectively, which was below expectation. Two functional KASP markers were developed for the identified locus.

Conclusion: This study identified a major QTL for male-fertility restoration using a GBS-based linkage map and developed KASP markers which support high-throughput screening of the haploblock. This is a first step toward marker-assisted selection of A_4 male-fertility restoration and male-sterility maintenance in pearl millet.

Background

Pearl millet (*Pennisetum glaucum* (L.) R. Br., syn. *Cenchrus americanus* (L.) R. Br), a highly nutritious, drought- and salinity-tolerant cereal crop, is grown predominantly by poor farmers in semi-arid regions of West Africa and South Asia, where its yield levels are generally low due to limited water availability, high temperatures, and low soil fertility. Pearl millet is a naturally outcrossing species and benefits greatly from exploitation of heterosis (Presterl and Weltzien, 2003); hybrid breeding programs for this crop are already well established in India and are in the early stages of development in West Africa.

Cytoplasmic male sterility (CMS) is characterized by anthers failing to produce functional pollen while stigma develops normally. CMS occurs when recessively-inherited nuclear genes interact with a male-sterility-inducing cytoplasm. CMS is thus inherited maternally and facilitates large-scale hybrid seed production by preventing self-pollination. CMS systems are utilized in pearl millet and many other hybrid crops for which grain or fruit is an economically important component of the harvest (Burton, 1977; Wise and Pring, 2002). Male-fertility can be restored in the background of the male-sterility-inducing cytoplasm by dominantly-inherited nuclear restorer genes, termed *Rf* genes. These genes counteract the effects of the sterility-inducing genes in the cytoplasm (meaning mitochondria and/or chloroplasts) and allow the production of male-fertile hybrid plants (Schnable and Wise, 1998) .

In pearl millet, the first reported CMS system (A_1) was based on the Tift 23A_1 cytoplasm (Burton, 1958). Subsequently, the A_2, A_3 & A_β systems were found as alternatives (Burton and Athwal, 1967; Appadurai et al., 1982); however, these systems all proved to be less stable than the A_1 CMS system, so the A_1 system alone was used in hybrid pearl millet breeding in India for several decades. To avoid cytoplasmic uniformity, which can cause the vulnerability to disease and insect pest epidemics (Yadav et al., 1993), alternative CMS sources to the A_1 system were sought for cytoplasmic diversification in hybrid pearl millet. Several sources were studied (Appadurai et al., 1982; Aken'ova, 1982, 1985; Marchais and Pernes, 1985; Hanna, 1989; Sujata et al., 1994), but only the A_m=A_4 and A_5 CMS systems were identified as commercially viable (Rai et al., 2001, 2009). Other CMS systems did not satisfy the required attributes like complete male sterility of A-lines, high degree of male-fertility restoration of their hybrids and the stability of these traits across environments.

Although hybrid breeding for pearl millet is well established in India, it is just developing in West Africa. Current activities include identifying promising hybrid parents, determining appropriate CMS system(s), and introgression of appropriate male-sterility

maintenance/male-fertility restoration alleles into locally-adapted germplasm. A_4 and A_5 CMS systems appear to offer more stable male-sterility than A_1 in the hotter production environments of West Africa, which agrees with higher rates of pollen shed in supposedly male-sterile plants under higher temperature conditions in India. Currently, the A_4 and A_5 *Rf* genes are most readily available in germplasm adapted to Indian conditions (Gupta et al., 2012), and the frequency of maintainer alleles for both of these systems in West African pearl millet germplasm appears to be high (Issoufa, 2010). Genetic mapping of *Rf* loci for the A_4 and A_5 CMS systems would enable more efficient transfer of these fertility restoration alleles into one or more potential male heterotic pools adapted to West African conditions. In addition, such mapping could facilitate further diversification of potential hybrid seed parents by making it easier to track and manipulate male-sterility genes as diverse germplasm is integrated into breeding programs.

Over the past three decades many different types of markers were developed and used for genetic mapping and/or diversity assessment in pearl millet, including restriction fragment length polymorphism markers (RFLPs), amplified fragment length polymorphism markers (AFLPs), simple sequence repeat markers (SSRs), diversity arrays technology markers (DArT™s), and single-nucleotide polymorphisms (SNPs). The quality of genetic maps improved by increasing marker density and coverage, but many maps based on RFLPs, AFLPs, and SSRs are still not satisfactory due to marker clustering in peri-centromeric regions and extremely high rates of recombination in peri-telomeric regions, which causes gaps greater than 20 cM (Senthilvel et al., 2008; Supriya et al., 2011; Rajaram et al., 2013; Ambawat et al., 2016).

SNP markers, which are very common throughout the genome, are now commonly used in many crops. The low costs of high-throughput sequencing methods facilitate the development of high density linkage maps based on SNP markers. Genotyping-by-sequencing (GBS) is one sequencing technique that is able to generate such genome-wide SNP datasets (Elshire et al., 2011). In the first step of the GBS method, genome complexity is reduced using restriction enzymes, which cut the genomic DNA selectively. In the next step, 'barcoded' DNA adapters are ligated to each fragment to enable sequencing of many samples in one sequencing lane. GBS has already proven its success in several crops like maize, barley, sorghum, and grapes. (Elshire et al., 2011; Nelson et al., 2011). Moumouni et al. (2015) and Punnuri et al. (2016) have shown that GBS can develop reasonably uniform and dense genetic linkage maps in pearl millet. Such genetic maps can

be used in association or linkage studies to identify QTL, and occasionally SNPs (Morris et al., 2013), controlling traits of interest.

The objectives of this study were (1) to construct a genome-wide linkage map based on GBS-derived SNP markers in a pearl millet F_2 mapping population, and (2) to map one or more major *Rf* loci governing male-fertility restoration and male-sterility maintenance in the A_4 CMS system of pearl millet.

Results

Phenotypic variation in the mapping population

All F_1 hybrid individuals produced from the ICMA 02777 × ICMR 08888 cross were fully male-fertile, as were the selfed progeny of ICMB 02777 and ICMR 08888. The parental plant ICMA 02777 used in the cross was fully male-sterile, as were the progeny when it was crossed to its maintainer, ICMB 02777. The observation that all F_1 plants were fully male fertile suggests dominant inheritance of male-fertility restoration in the pearl millet A_4 CMS system. A total of 138 plants in the F_2 population produced pollen (and hence were male fertile) and 50 plants did not produce pollen (male sterile) (Fig. 1), which fits well the 3:1 segregation ratio of a single dominant gene (χ^2=0.26, p= 0.614). The distribution of phenotypes for selfed seed set percentage also revealed two major classes (no seed set and medium to good seed set) plus an additional low-frequency intermediate class with low to medium seed set (Fig. 1). Plant height was almost normally distributed and exhibited high variation in the F_2 population, ranging from 38 cm to 270 cm, with an average plant height of 163 cm. This high variation for plant height, and its slightly bi-modal distribution, suggested that the F_2 population was segregating for a recessive dwarfing gene, as well as many loci of small effect governing this trait.

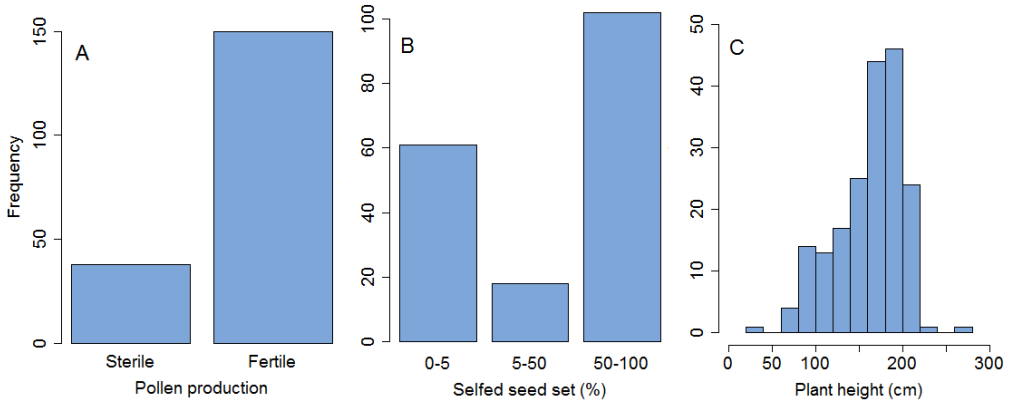

Fig. 1: The distribution of phenotype scores for (A) pollen production, (B) selfed seed set and (C) plant height

Genetic map construction based on polymorphic markers

A total of 449.5 million reads were generated by sequencing the 196 samples; 2 samples were subsequently excluded due to low sequencing quality. The 194 high-quality samples had on average 2.31 million reads (range 0.33-6.81 million). The two samples of the parental line ICMB 02777 had a total of 4,990,691 reads and the four samples of ICMR0888 had a total of 14,680,021 reads.

A total of 160,000 raw SNPs were called using the pearl millet reference genome version 1.1 sequence (Varshney et al., 2017) (kindly provided by the Pearl Millet Genome Sequencing Consortium). Filtering for high quality polymorphic SNPs reduced the number of SNPs to 2416, which were used in the first step of the map construction. The MSTmap algorithm grouped all SNPs in 7 linkage groups (LGs), except 73 outlying SNPs, which were excluded. The grouping of LGs agreed with the grouping of the reference genome sequence. The 2343 SNPs included many redundant markers which were filtered out. The final genetic map was based on 460 SNPs and had an overall length of 462.2 cM Markers are favorably distributed (Fig. 2, Supplemental table 1), with an average inter-marker spacing of 1.0 cM and a maximum spacing of 11.1 cM (Table 1). The length of the LGs ranged from 39.7 cM (LG 4) to 90.4 cM (LG 5). While this manuscript was under review, the final pearl millet reference genome was published (Varshney et al., 2017)(The physical location and genetic context of all SNPs in this map are included in Supplemental File 3.)

Table 1. Statistics of the pearl millet linkage map

Linkage Group	Markers	Length (cM)	Average spacing	Maximal spacing
1	93	87	0.9	7.8
2	95	82.3	0.9	5.5
3	47	54.2	1.2	6.6
4	54	61.7	1.2	9.3
5	91	90.4	1.0	8.2
6	39	39.7	1.0	4.4
7	41	46.9	1.2	11.1
overall	460	462.2	1.0	11.1

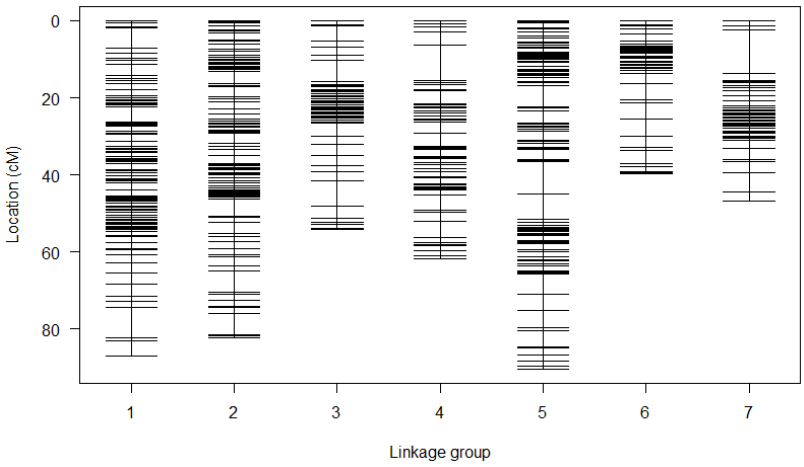

Fig. 2 F_2 genetic linkage map of pearl millet obtained using genotyping-by-sequencing (GBS) single-nucleotide polymorphism (SNP) markers. Each vertical bar represents one linkage group with black horizontal lines showing the SNP locations on each linkage group.

Identification of male-fertility restoration and plant height QTLs

The SNP-based genetic linkage map was used in multiple regression analyses to identify QTL for the traits male-fertility restoration (determined by both pollen production and selfed seed set) and plant height. One marker interval on LG 2 was significantly associated with both pollen production and selfed seed set. For pollen production, the QTL explained 14.5% of the observed phenotypic variance, while it explained only 9.9% of the observed phenotypic variance for selfed seed set (Table 2). For plant height, one QTL was identified on LG 4, which explained 24.5% of the observed phenotypic variance.

The QTL frequency analysis showed that the QTL position for pollen production was found in 97% of the cross-validation runs, and the QTL detected for selfed seed set in 38% of the runs. The QTL for plant height on LG 4 was found in 69% of the runs.

We verified the QTL analysis with a single marker regression model implemented in R/qtl to confirm the multiple regression model used within PLABMQTL. Both algorithms identified the QTL at the same positions, and had very similar proportions of phenotypic variances explained.

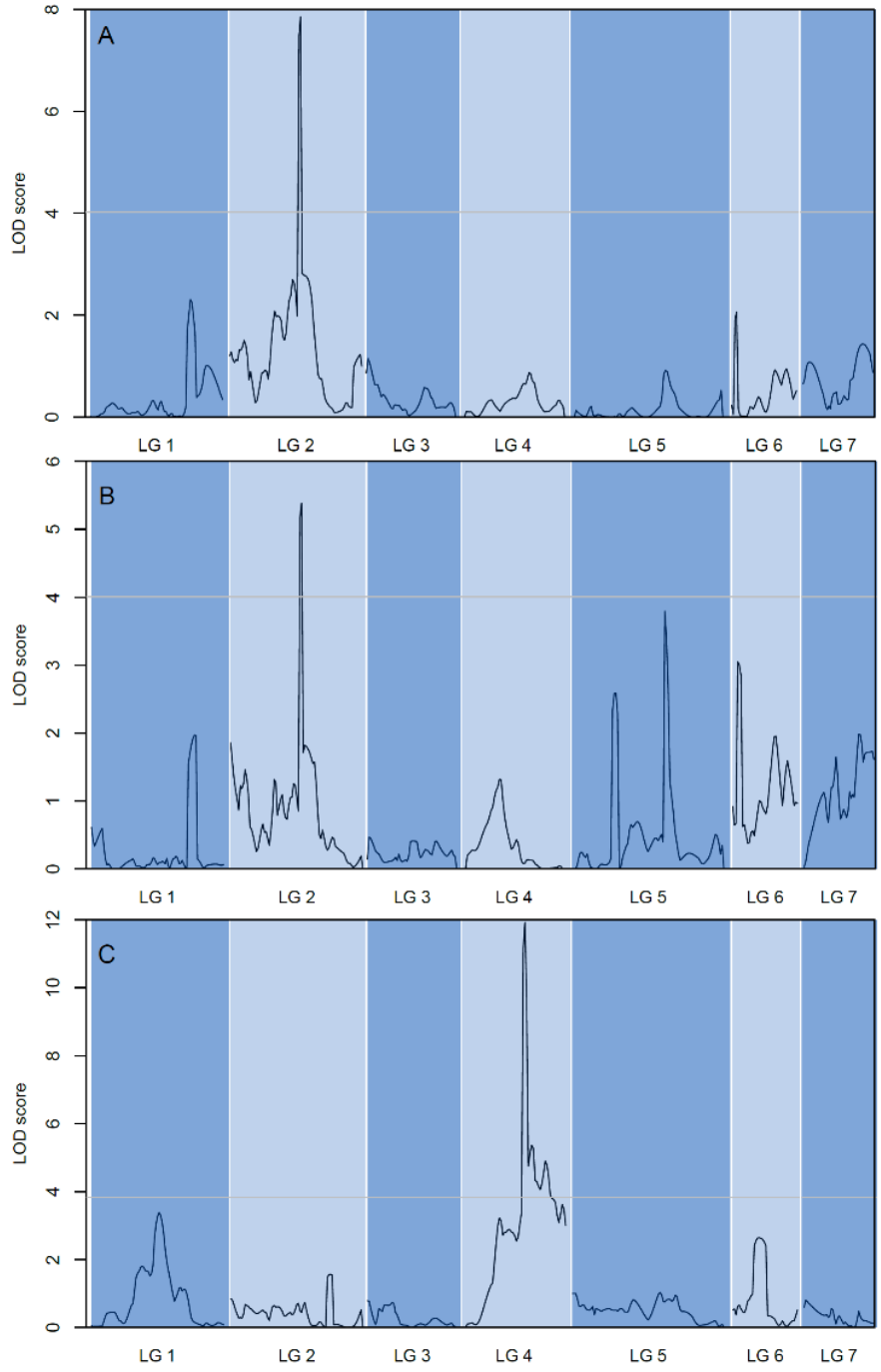

Fig. 3. LOD curves from QTL mapping; male-fertility restoration / male-sterility maintenance for pearl millet's A_4 CMS system using F_2 pollen production (A) and selfed seed set (B) phenotype data and for plant height (C)

Table 2. Quantitative trait loci for pollen production and selfed seed set (traits indicating male-fertility restoration of the A_4 CMS system) and plant height.

	LG[1]	Position (cM)	Support Interval (cM)	LOD	Left SNP[2]	Right SNP[2]	R^2_{adj}	Genetic Effect		Cross-Validation Frequency (%)
								Additive	Dominant	
Pollen Production[2]	2	44	42-45	7.86	S2_110825781	S2_195649011	14.48	0.381	0.437	97
Selfed Seed Set	2	44	42-45	5.39	S2_110825781	S2_195649011	9.98	0.349	0.382	38
Plant Height	4	38	37-40	11.52	S4_48328719	S4_70429741	24.54	26.686	27.697	69

[2] Pollen Production was scored as 0=no pollen production, 1= pollen production; Selfed Seed Set was scored as 1 = up to 5% seed set, 2= 5 to 50% seed set, 3 = more than 50% seed set when plants were self-pollinated; Plant Height was measured in cm

[1] LG, Linkage group; 1-LOD Support Interval; LOD, logarithm of odds; R^2_{adj}, adjusted percentage of observed phenotypic variance; Frequency, QTL frequency determined by cross-validation

[2] The genomic context of all SNPs in the linkage map, along with their physical location in the current pearl millet reference genome (NCBI Assembly (Varshney et al., 2017)), are presented in Supplemental File 3.

Conversion of flanking SNPs to KASP assays

In order to make the two flanking SNP markers of the QTL usable for applied marker-assisted selection, they were converted into single marker assays. To enable a cheap, fast and high-throughput screening, we chose to convert them into allele-specific PCR based (KASP) markers. For both SNPs (S2_110825781 and S2_195649011) the KASP assay was successful and showed three genotypic classes (Fig. 8A). There were two haplotypes that showed a very high frequency for fertile individuals, while one haplotype had approximately equal frequency of sterile and fertile individuals (Figure 4B, Supplemental table 2). We genotyped all members of the F_2 population and verified the functionality of the detected QTL and obtained a very similar R^2 as observed with the original genotype data.

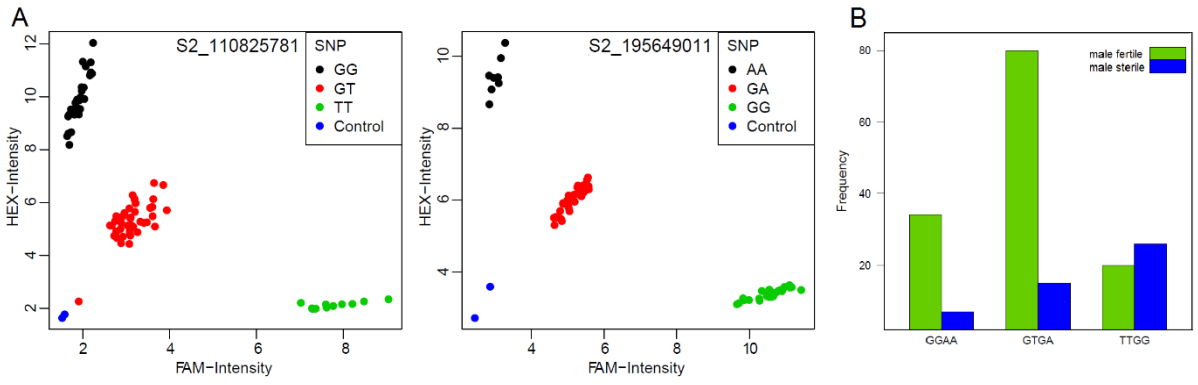

Fig. 4. **A** Development of functional markers for the SNPs S2_110825781 and S2_195649011 and **B** Bar plot of male fertile and male sterile plants of the three haplotypes from these two markers.

Discussion

Comparison to existing genetic linkage maps based on GBS and other markers

High-throughput sequencing technologies and the development of user-friendly software packages for sequencing analysis have advanced the options for marker detection tremendously. SNP-based linkage maps are already used in many crops, especially in those where the reference genome sequence is available. In pearl millet, two GBS-based linkage maps have been recently published. Moumouni et al. (2015) published a map based on a small F_2 population, without using a reference sequence (using the UNEAK pipeline (Lu et al., 2013) in TASSEL), while Punnuri et al. (2016) used a mapping population based on recombinant inbred lines (RIL) with the same draft reference genome sequence that we used in our study (The final pearl millet reference genome was published (Varshney et al., 2017) while this manuscript was in review.). The total map lengths of Moumouni et al. (2015) and Punnuri et al. (2016) were 717 cM and 641 cM, respectively, both substantially longer than our map (462 cM). Sehgal et al. (2012) published a consensus function map based on gene-based SNPs, CISPs and EST-SSRs which was 815.3 cM. However, there are also previous genetic maps with similar or shorter total map lengths compared to ours: Qi et al. (2004) published an 473 cM long map based on a F_2 pearl millet population and 242 SSR and RFLP markers, and the original pearl millet map of Liu et al. (1994) spanned only 303 cM. The total length of a linkage map is influenced by several factors including the recombination rate of the mapping population and the relatedness of the parents. Thus precise comparison of map lengths from different studies is not meaningful so long map lengths are within the same approximate range, as is the case for our map.

Both our analysis and Punnuri et al. (2016) numbered the LGs according to the concensus map of Rajaram et al. (2013). However, the relative lengths of the LGs were quite different in our map and those reported by Punnuri et al. (2016). Especially LG 3 and LG 6, which were relatively short in our map (54.2 cM and 39.7 cM, respectively), were quite long in the map of Punnuri et al. (2016) (175 cM and 112 cM). The relative lengths of the LGs in the consensus map of Rajaram et al. (2013) were much closer to the LGs relative lengths of our map than to those on the map of Punnuri et al. (2016).

The two existing GBS-based linkage maps have all higher marker densities than previous maps based on other marker types. The map of Punnuri et al. (2016) showed a higher density than our map, which in turn is denser than the map of Moumouni et al. (2015). The higher density reported by Punnuri et al. (2016) was expected because they used a RIL mapping population, which has a higher recombination rate (effectively double that of an

F_2 population of similar size and parentage). However, we can still classify our map as mostly dense, uniformly- and well-saturated, because there was only one gap with more than 10 cM between adjacent markers. The integrated EST-SSR + DArT marker-based pearl millet linkage map reported by Ambawat et al. (2016) spanned 740 cM (Haldane), with an average adjacent-marker distance of 2.7 cM for a RIL population of 140 individuals from a cross of inbred lines that are expected to segregate for not only the *d2* dwarfing gene, but also for male-fertility restoration and male-sterility maintenance for both the A_1 and A_4 CMS systems of pearl millet. Testcrossing that RIL population to iso-nuclear seed parents $81A_1$ and $81A_4$ would permit independent confirmation of our results for A_4, as well as demonstrating the relationship, if any, between fertility restoration / sterility maintenance loci for these two commercially exploited pearl millet CMS systems. The superior genomic coverage of the map of Ambawat et al. (2016) in peri-telomeric regions of most linkage groups could also help identify modifiers of any major fertility restoration / sterility maintenance loci detected for either of these two CMS systems. The utility of genic markers detected using the *PstI* endonuclease for ensuring marker coverage in such regions was demonstrated by Ambawat et al. (2016) when they were able to map a major gene for rust resistance that had previously proven "un-mappable" as its position was more distal than any RFLP or SSR marker at the top of LG 1.

Inheritance of male-fertility restoration

In crops where seed or fruit comprise the economic harvest, the restoration of male fertility in F_1 hybrids is usually an important prerequisite for an economically viable hybrid cultivar that are harvested prior to flowering (such as beets, carrots, leeks and onions), are examples of crops in which hybrid cultivars need not have restored male fertility as are parthenocarpic cucumbers. Similarly, many forages and most ornamentals need not have restored male fertility because seed set is not required for their use in agriculture.

Gupta et al. (2012) showed that male-fertility restoration in the A_4 CMS system of pearl millet followed a monogenic dominant pattern of inheritance using phenotyping procedures similar to those we have used. Our observations are in line with this result because we also found a 3:1 (male-fertile : male-sterile) segregation pattern in the F_2 population. However, the assumption of single gene-control based on the phenotypic data does not seem certain yet because the results of our mapping study indicated that there could also be minor genes.

Previous studies on the A_4 CMS system in pearl millet have demonstrated its stable male sterility and reliable male fertility restoration across Indian environments. A number of seed parent pairs (male-sterile A-lines and their iso-nuclear B-line maintainers) based on the A_4 CMS system are now available to pearl millet breeders in South Asia and sub-Saharan Africa, as well as in the Americas. Our phenotypic data suggest that a substantial portion of this stability may be due to simple inheritance of male-sterility maintenance and male-fertility restoration in this system (compared to 1-, 2- and 3-gene male-fertility restoration found for A_1; CT Hash unpublished). In this case, one can reasonably expect similar stability for both sterility and restoration in West African environments.

Detection of male-fertility restoration and plant height loci

The QTL analysis of this study identified a major fertility restoration / sterility maintenance locus of the A_4 CMS system on LG 2. Assuming single-gene control, we expected that the identified locus would explain a relatively high percentage of observed phenotypic variation. However, the estimated R^2_{adj} values were only 14.5% and 9.9% for pollen production and selfed seed set, respectively, which were significantly below our expectations. This discrepancy might be affected by some minor or modifying R_f genes that could not be detected in this QTL analysis. The assumption of modifying genes is supported both by the observed frequency distribution (Fig. 1B) and by the LOD score curve for selfed seed set (Fig. 3B), with the latter showing some peaks that are just below the LOD threshold (e.g. on LG 1, LG 5 and LG 6). Such non-significant loci might be associated with minor R_f genes, although especially those detected on LG 5 are more likely to be associated with protogynous period or stigma receptivity given that they were detected only for selfed seed set and not for pollen production.

Cross-validation strengthened the evidence for high accuracy of this major R_f gene position, as cross-validation runs identified this same QTL for pollen production score and selfed seed set score 97% and 38% of the time, respectively. The lower R^2_{adj} and QTL frequency of selfed seed set compared to pollen production might be caused by those plants with low to intermediate (5-50%) seed set. Low seed set in fertile plants can be caused by several factors, such as partial male-fertility, a combination of short stigma receptivity with long protogynous period (time between first stigma emergence and initiation of anthesis on the same panicle), heat stress (as our screening was done in the hot season) and/or insect-feeding damage to stigmas. Male-sterile plants can show higher-than-expected seed set due to pollen contamination inside the selfing bag due to poor closure of

the base of the bag, bag entry by pollen-bearing insects, or by the glued corners of the selfing bag opening during sprinkler irrigation or rainfall. In contrast, classification of anthers as sterile and fertile was more distinct, thus we can assume a smaller error rate for pollen production as compared to selfed seed set.

Based on the developed KASP markers for the QTL detected by pollen production, we saw that fertile individuals could be predicted with reasonable accuracy, while sterile genotypes would not be well predicted (Fig. 4B). This indicates that at this stage our KASP markers would be appropriate to select for restorer types, but not for maintainers. This finding is certainly linked with the relative low R^2 value, and should be validated in future studies, to develop KASP markers that are also suitable to select maintainer lines.

Plant height was analyzed as a reference trait because our mapping population segregated for a dwarfing gene (d_2); this gene was previously mapped to LG 4 by Azhaguvel et al. (2003) and Parvathaneni et al. (2013). Since we also identified one major height QTL on LG 4 (R^2_{adj} = 24.5%), we can assume that this locus is associated with d_2. Finding this QTL on the same linkage group was used as a cross-check for the correctness of our other QTL analyses. However, Azhaguvel et al. (2003) estimated that the d_2 locus on LG4 explained 64% of observed phenotypic variance, which is much higher than the R^2adj. value we estimated (24.54%). The most likely reason for this is that the population used by Azhaguvel et al. (2003) was derived from a cross of two non-allelic semi-dwarf lines, and so had a substantially smaller proportion of tall plants than did our F_2 population. They found additionally on LG 1 the locus of the d_1 dwarfing gene. In our LOD curve for plant height there is one peak on LG1 (Fig. 7C) which is just below the LOD threshold, that is presumably associated with the d_1 locus originally mapped by Azhaguvel et al. (2003).

Importance for future breeding programs

Our study and that of Punnuri et al. (2016) have shown that GBS-SNP-based linkage maps, based on F_2 or RIL mapping populations, are suitable for QTL detection in pearl millet due to high marker saturation. GBS is currently the most informative and cost-effective marker type, but it should be noted that the high marker number achieved by GBS cannot entirely be exploited in an F_2 mapping population due to its lower recombination rate (and therefore higher marker redundancy) compared to RILs. Validation of our results using a RIL population is required in order to verify the existence of further minor genes modifying the fertility restoration in the A4 cytoplasm.

This study identified a major male-fertility restoration / male-sterility maintenance gene for the A_4 CMS system of pearl millet, which is a crucial step in understanding the genetic basis of this economically important trait. Knowledge of the gene location will offer pearl millet breeders more efficient strategies to develop male parents carrying the major *Rf* allele. Introgression of the restoration allele by integrated conventional and marker-assisted selection will save time, compared to introgression based on purely phenotypic selection. The resources saved by using an integrated approach can then be used to develop a higher number of strongly-restoring hybrid male parents for the A_4 CMS system or allocated to other parts of the breeding program. Especially in West Africa, where hybrid breeding is just starting and where restorer genotypes are relatively uncommon in local landrace and improved open-pollinated genotypes (Issoufa, 2010), more efficient introgression of restorer genes will be highly beneficial.

Similarly, this study is a first step towards efficient introgression of the major A_4 maintainer allele (*rf*) into seed parent genepools. Efficient introgression will allow heterotic pools in pearl millet to be built up independently of the maintainer/restorer characteristics of specific germplasm, thus allowing breeders to focus on genetic diversity, combining ability, and agro-morphological traits.

Conclusions

The phenotypic data of this study and that by Gupta et al. (2012) indicate a monogenic inheritance of the A_4 male-fertility restoration / male-sterility maintenance. Such inheritance is desired in hybrid breeding, as it is relatively simple to introgress and is usually little influenced by the environment. However the unexpected low variance explained by our mapped QTL suggests the presence of minor or modifying genes. Future studies using RIL populations, should investigate whether the fertility restoration of the A_4 system is influenced by only one major gene, by several additional minor genes, or by more than one major gene depending upon the genetic backgrounds of the parents. This could explain the relatively low portion of the observed phenotypic variance explained by the QTL in the present study. Beside this verification, the developed KASP markers can be used for high-throughput screening of the desired haploblock in applied pearl millet hybrid breeding, thereby facilitating development of pearl millet hybrid parents.

Materials and Methods

Plant material

An F_2 mapping population of 190 plants was developed for this study. Plants were segregating for A_4 male-fertility restoration as the primary target trait and d_2 dwarf plant height as the secondary target trait. This F_2 mapping population was produced at the ICRISAT Sahelian Center, in Niamey, Niger, by selfing F_1 plants derived from a single plant × plant cross of inbred lines ICMA 02777 × ICMR 08888. The 190 plantes were created by advancing three sub-populations of 70 F_2 plants, each sub-population being derived by selfing a single F_1 plant. A portion of the hills sown (3 seeds per hill, thinned to 1 plant per hill immediately before tissue sampling), failed to establish and so could not be phenotyped. The A_4-cytoplasm male-sterile line ICMA 02777 was derived from ICMB 02777 by backcrossing its nuclear genome to 81A_4 cytoplasm source and is homozygous for semi-dwarf plant height at the d_2 dwarfing gene locus. The pedigree of ICMB 02777 is HHVBC-II HS-9-1-1-2-7-1, in which HHVBC-II is the second High Head Volume B-Composite bred at ICRISAT-Patancheru, and has a substantial portion of its genetic background derived from *Iniadi* landrace germplasm from Togo. The restorer line ICMR 08888 was bred at ICRISAT-Patancheru by selfing within improved synthetic variety ICMS 7704, which is genetically tall at the d_2 locus. ICMR 08888 has the pedigree ICMS 7704-S1-52-3-1-2-1-2-1-6-B-B, indicating that this inbred is derived from the 52nd S1 progeny of ICMS 7704 that was evaluated, and that seven generations of single-plant selection with selfing were followed by two generations of advance of bulks of seed from two or more selfed plants. Seed parent pair ICMA 02777/ICMB 02777 and restorer line ICMR 08888 were both developed at ICRISAT-Patancheru. Although they are relatively long-duration for Indian dryland conditions, their lifecycles are generally too short for most pearl millet producing regions in West and Central Africa.

Phenotyping

The F_2 population of 190 plants plus their parental lines were raised under irrigated conditions at the ICRISAT research station in Sadoré (Niger) during the dry season of 2014 (sowing in March). The crop was grown as single plants per hill under irrigation with recommended fertilization. At the five-leaf stage a single leaf was collected from each plant into a labelled coffee filter, with the stapled and labelled coffee filters placed in zip-lock plastic bags containing silica gel desiccant, and the plastic bags then stored with additional desiccant in an air-conditioned seed store until they could be shipped for DNA isolation

and genotyping. At the boot-leaf stage, two emerging panicles per plant were covered with semi-transparent parchment paper bags closed with a paper clip to enforce self-pollination, with later-appearing panicles being left uncovered to facilitate observation of anther structure, pollen shed, and open-pollinated seed set. During the pollen shedding period, anthers of plants were classified as male-fertile (bearing pollen-producing anthers) or male-sterile (bearing only shrunken anthers with no pollen). In the following this trait will be called pollen production. It could be scored on 188 F_2 plants.

At maturity, two panicles of each F_2 plant were scored for selfed seed set as an additional phenotype to assess the target trait male-fertility restoration; the scoring system was: 1 = up to 5% selfed seed set from the total number of flower buds (male sterile), 2 = 5 to 50% selfed seed set (partially male -fertile); 3 = more than 50% selfed seed set (male fertile). Selfed seed set could be scored on 181 plants because of selfing bag losses from some plants due to strong winds.

The F_2 population segregated for the *d_2* dwarfing gene, which has already been mapped in previous studies (Azhaguvel et al., 2003; Parvathaneni et al., 2013) and was intended as a reference trait to verify he quality of our linkage map and F_2 population. We recorded plant height (cm) on all 190 F_2 plants.

For pollen production we tested a 3:1 segregation ratio of male-fertile:male-sterile plants using a χ^2-test.

DNA extraction, genotyping-by-sequencing and SNP calling

Genomic DNA was extracted from dried young leafs of individual F_2 plants and their parental lines using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Quality and quantity check of extracted DNA was performed using *Hind*III digestion and gel analysis. Fifty µl aliquots of each of 196 DNA samples (190 F_2 individuals, two female and four male parental samples; we choose four male parental samples due to two free wells and limited amount of female DNA) containing >10 ng μL^{-1} per sample were sent in four 96-deep well plates to the Genomic Diversity Facility at Cornell University in Ithaca, New York, for GBS analysis. The remaining space in the plates was filled with further pearl millet samples from our project. Each 96-well plate contained one randomly positioned blank.

GBS libraries were prepared and analyzed at the Genomic Diversity Facility at Cornell University according to Elshire et al. (Elshire et al., 2011), using the restriction enzyme *Pst*I and sequenced at 96-plex level on the Illumina HiSeq2000 with single-end read sequencing.

The raw GBS data files (FASTQ) were processed to SNP calls using the GBS version 2 pipeline of Tassel 5 (Version 5.2.28) (Glaubitz et al., 2014). The sequenced tags were aligned to the pearl millet reference genomic sequence provided by the Pearl Millet Genome Sequencing Consortium (Varshney et al., 2017), using the Burrows-Wheeler Alignment Tool (BWA) (Li and Durbin, 2009).

Quality check and genetic map construction

High-quality SNPs were selected by using TASSEL 5. SNPs with more than 20% missing data, a minor allele frequency below 40%, or those which were heterozygous in one or both parents were filtered out. Genotypes (plants) showing >50% missing data were removed. After this filtering, the remaining 2445 SNPs were imputed using the FSFHap algorithm (Swarts et al., 2014) implemented in TASSEL 5.

Chi-square tests were performed on each marker for 1:2:1 (A:H:B) expected genotypic segregation ratios to assess the amount of segregation distortion. Only 29 SNPs showed significant segregation distortion at the 5% level after a Bonferroni correction for multiple tests. These SNPs were discarded.

The genetic map was constructed using the MSTmap algorithm (Wu et al., 2008) implemented in the R package ASMap (R Core Team, 2014; Taylor and Butler, 2015). A total of 73 SNP markers were designated to outlying linkage groups (LG) with a very low number of SNPs and were discarded. The numbering of LGs was based on the genome sequence, which corresponds to the numbering of the consensus map published by Rajaram et al. (2013). The map length was re-estimated using the Lander-Green algorithm within the software package R/qtl, and choosing the Haldane function. The genetic map with its 2343 markers contained many redundant markers (caused by co-segregation) which were excluded, thus the final linkage map was based on 460 markers.

QTL mapping

QTL analysis was performed with the software PLABMQTL (Utz, 2012) using composite interval mapping based on multiple regression (Haley and Knott, 1992). The QTL mapping model included additive and dominance effects, and cofactors were chosen by stepwise regression.

The critical logarithm of odds (LOD) scores were determined empirically according to Churchill and Doerge (994) using 1000 permutation runs and $\alpha = 0.05$. The LOD thresholds were for pollen production = 4.02, for selfed seed set = 4.01, and for plant

height = 3.83. The adjusted proportion of the phenotypic variance explained by the individual QTL (R^2_{adj}) was calculated. To assess the quality of results of QTL detection, the occurrence of the QTL (QTL frequency) within a 1-LOD support interval was determined by conducting 1000 five-fold cross-validation runs (Utz, 2012). Due to the non-normal distribution of phenotypes, we also fitted a logistic regression to the data using the glm() function in R. The results were almost identical to the original results, thus they were not considered further.

KASP-marker development

The two flanking SNPs of the major QTL on LG 2 were converted into KASP assays. SNP S2_11085781 was converted into KASP assay PM_S2_11085781, which comprised the two allele specific primers PM_S2_11085781_T (5'-FAM-TailSeqGGAACCATCGCAACATCGTAAGA-3') and PM_S2_11085781_G (5'-HEX-TailSeq-GGAACCATCGCAACATCGTAAGC-3') and the common primer PM_S2_11085781_Com (5'-GGGTTGAAGACCAGAGGATAGTCTGC-3'). SNP S2_19564901 was converted into KASP assay PM_S2_19564901, which comprised the two allele specific primers PM_S2_19564901_G (5'- FAM-TailSeqCTCGTTGGTCAGAATGGACATCAG-3') and PM_S2_19564901_A (5'-HEX-TailSeq-CTCGTTGGTCAGAATGGACATCAA-3') and the common primer PM_S2_19564901_Com (5'- ACGCAACATTCCCTAAGCGAAGTT-3'). For both KASP assays the FAM-allele corresponds to the sterile parent and the HEX allele to the fertile parent. Both assays were run as 6µl PCR reactions, with a standard KASP 61-55°C touchdown PCR program (http://www.lgcgroup.com/products/kasp-genotyping-chemistry/kasp-technical-resources/) on a Roche LightCycler®480II instrument.

Abbreviations

CMS, cytoplasmic male sterility; GBS, genotyping-by-sequencing; LG, Linkage Group; LOD, logarithm of odds; QTL, quantitative trait locus; SNP, single nucleotide polymorphism

Funding

The German Ministry for Economic Cooperation and Development (BMZ) supported the field research presented here (GIZ project numbers 13.1432.7-001.00), and the McKnight Foundation Collaborative Crop Research Program provided discretionary research funds to

B.I.G. Haussmann, used to support A. Pucher. J. Wallace was supported by the University of Georgia.

Acknowledgement

Further, we thank H. F. Utz and H. P. Mauer for helpful discussion and support and R. K. Varshney and S. Kale for providing us the current version of the pearl millet reference genome. We acknowledge critical comments of four anonymous reviewers on earlier versions of the manuscript.

Availability of data and materials

The datasets generated or analyzed during the current study are available from the corresponding author on reasonable request.

Authors contributions

All authors have confirmed their contribution, read, commented and approved this manuscript. AP, CTH and BIGH conceived and designed the experiments. AP, CTH and WL performed the experiments. AP, JGW, WL and SH analyzed the data. AP and CTH wrote the first draft of the manuscript

Competing interests

The authors declare that they have no competing interests

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6 General Discussion

Achieving the goal of more productive, stable and nutritious pearl millet varieties for WA farmers requires the consideration of various approaches in plant breeding like utilization of genetic diversity, biofortification breeding, maximizing heterosis through hybrid breeding and understanding genotype-by-environment interactions in the target region. Sustainable hybrid breeding needs to be based on the concept of heterotic groups, and appropriate mechanisms for economic seed production need to be developed. The present study contributes important new knowledge for achieving these tasks and therefore facilitates major breeding steps towards higher productivity and nutritional value of pearl millet in WA.

6.1 Pearl millet diversity in WA – a gold mine for breeders

Detailed knowledge of the existing pearl millet genetic diversity is in many ways essential for breeding, but is also crucial in order to maintain this genetic diversity for future uses. WA is one of the centers of origin of pearl millet (Oumar et al. 2008; Manning et al. 2011) and is home to a great amount of genetic variation with wide ranges for agro-morphological traits. These properties have been observed in the present study based on 360 early-to-medium maturing West and Central African accessions, which were grown at six experimental sites in WA (Chapter 2). The high agro-morphological diversity underlined pearl millet's adaptability to various environments and farmer preferences. It is fundamentally important to tap and use such diversity so that efficient breeding for specific contexts and matching the farmers' needs can be done. The entire data-set of our multi-environment characterization study is publicly accessible, and available to WA pearl millet breeders to encourage the use of external genetic material for their programs.

Identification of geographic patterns of specific traits showed in which regions certain pearl millet types are grown. The amount of rainfall and length of rainy season are important factors, which influence the presence of specific traits. For example, early maturing genotypes are found in the northern pearl millet growing areas in WA, where rainy seasons are shorter (Chapter 2, Stich 2010; Vigouroux et al. 2011). A further example is downy mildew susceptibility, which is moderately associated with latitude. Higher precipitation and humidity in the South causes higher downy mildew pressure and thereby better adaptation due to the development of resistances against this pest (Chapter 2). Characteristics of accessions also vary between the countries of origin. For instance, Senegalese accessions

have generally a lower thousand kernel weight, but higher volumetric weight than accessions from other countries. Breeders could use this information and enhance the thousand kernel weight in Senegalese accessions by introducing germplasm from countries like Mali or Burkina Faso. However, farmer preferences need to be considered before changing yield components like thousand kernel weight. The geographic pattern of panicle length visualizes the influence of farmer preferences quite nicely (Chapter 2). It shows that farmers in Burkina Faso and Mali prefer shorter panicles, while in Niger and Senegal farmers select for long panicles, which are better for transporting in bundles on the head. In Mali and Burkina Faso, the habit of making bundles for transport is less common.

Only very low correlations were observed between the geographic distance of accessions origin and the phenotypic distance between accessions in our study ($r = 0.18$, $p < 0.001$; Chapter 2), which is in line with the low correlation between genetic distance and geographic distance observed by Mariac et al. (2006). The differing farmer preferences for pearl millet characteristics as well as the number of varieties grown within a village might be one cause of such an unexpectedly low correlation. For example, in Niger, farmers usually grow one early and one later maturing variety, and a single village grows on average six different varieties, which shows the diversity of varieties within a small area (Chantereau et al. 2010). Farmers are determined to maintain the diversity of seeds within villages by seed management and some isolation during flowering (Bezançon et al. 2009).

6.1.1 Trait correlations for suitable use of genetic diversity

Trait correlations can be helpful information for a breeder when establishing selection strategies. Trade-offs between various traits need to be understood in order to strategically use diversity in breeding and to avoid unwanted, correlated shifts in certain traits. We found in our West and Central African pearl millet collection a strong association between flowering time and biomass ($r=0.68$; Chapter 2), which reveals a very low selection potential for early and high biomass varieties. In our study, the correlation between grain yield and flowering time appeared to be dependent on the environment and especially on the rainfall distribution, which observation is in line with that of Bidinger et al. (1987), who determined that terminal drought stress limits the grain yield performance of late-flowering accessions. Due to the inter-annual rainfall variability in WA, the relationship between grain yield and flowering time can be assumed to change across years at individual sites (Haussmann et al. 2012). Therefore, it seems to be a good strategy of WA farmers to maintain variability in flowering time to cope with rainfall variability. Further we found a

relatively strong correlation between grain yield and seedling vigor (r =0.63), which agrees with the study of Manga and Yadav (1995). Based on this result, early seed establishment might be a useful indirect trait during pre-selection, as it can be phenotyped early, fast and cost-effective by simple rating. However, our study showed a low heritability for seedling vigor, which reduces the efficiency of indirect selection considerably. If the heritability could be improved by experimental conditions, selection for seedling vigor could be efficient to improve grain yield.

6.1.2 Understanding diversity patterns to develop heterotic grouping strategies

Heterozygosity is a crucial factor affecting the yield potential of pearl millet, which can be influenced by breeding open-pollinated, synthetic, or hybrid varieties. The superiority of pearl millet hybrid varieties over open-pollinated varieties has been shown in some studies (Burton and Powell 1968; Ouendeba et al. 1993; Bidinger et al. 1994; Yadav et al. 2000) and will be discussed later on in more detail. In our study we observed only little evidence of geographically distinct groups' based on the phenotypic data of twelve traits (Chapter 2), which could be potential heterotic groups for hybrid breeding. Nevertheless, our findings are an important step towards better understanding the pearl millet diversity in WA in order to develop a heterotic grouping strategy. Stich et al. (2010) and Lewis (2010), who studied the diversity of inbred lines based on genetic data, also did not find any distinct groups. Our finding of one meta-population is not surprising, if we consider pearl millet's history with its center of origin and domestication in Mali and subsequent spreading of the crop to other areas with continuing gene flow between cultivars and wild relatives in WA. Since it seems that clearly distinct groups do not exist "naturally", phenotypic or geographic distance among individuals could be used to design combining ability studies which could then help to develop heterotic groups based on test cross performance and combining ability.

6.2 Good scope for pearl millet biofortification in West Africa

The aim of reducing hidden hunger by enhancing the grain Fe and Zn density in WA pearl millet is influenced by several factors and can be optimized by suitable breeding strategies.

One important aspect is attempting to achieve a sufficiently high genetic variation of the target nutrients within the available breeding material. A wide set of WA accessions tested in three environments in Niger showed that the genetic variation of grain Fe and Zn density is sufficiently high to expect that enhancement can be achieved by selection

(Chapter 3). However, studies on Indian and Sudanese pearl millet showed higher ranges and maximum values for grain Fe and Zn density than were found in our study (Velu et al. 2007; Govindaraj et al. 2013; Bashir et al. 2013). In particular, studies on Indian material indicated that there were some genotypes with a considerably higher Fe density (maximum of 77 mg kg^{-1} in ICTP 8303-Fe; Saltzman et al. 2013) in comparison to the best WA genotype (48.7 mg kg^{-1}) identified in our study (Chapter 3). Testing of this Indian high Fe material in WA environments is recommended to verify whether the high Fe densities in ICTP 8303-Fe are also found in WA environments. If such varieties prove to have a high and stable grain Fe density in WA environments as well, introgression of such material into WA germplasm would be highly adjuvant.

Beside the genetic variation, a sufficiently high heritability is an important factor if adequate selection gain is to be achieved. We observed for several whole- and decorticated-grain mineral densities, including Fe and Zn, that there were moderate to high heritabilities indicating the viability of biofortification breeding in Niger. Some studies on the heritability of grain Fe and Zn density showed higher heritabilities for Fe (Chapter 3, Baxter et al. 2012; Velu et al. 2012), while others reported higher heritabilities in the case of Zn (Gómez-Becerra et al. 2009; Bashir et al. 2013). Irrespective of which micronutrient is more heritable, biofortification breeding would be most effective if both grain Fe and Zn density will be enhanced. A positive correlation between these two minerals ($r = 0.73$) has been found in our and other studies on pearl millet (Chapter 3, Velu et al. 2007; Govindaraj et al. 2009; Gupta et al. 2009; Bashir et al. 2013), as well as in other crops (Husted et al. 2004; Gomez-Becerra et al. 2010b; Gomez-Becerra et al. 2010a; Velu et al. 2012), which makes it simple to enhance both minerals simultaneously. The combination of a high correlation between grain Zn and Fe density and the higher heritability of Fe compared to Zn found in WA pearl millet in Nigerien environments (Chapter 3) means that, in such environments, the breeder could focus on selection for high grain Fe density alone because this will, simultaneously, increase the Zn density.

The success of biofortification will also depend on how selection for high grain Fe and Zn densities influences other essential grain mineral densities and agro-morphological traits. Various studies, including this study, have shown that Fe and Zn are in most cases positively correlated with other micronutrient densities in pearl millet (Velu et al. 2007; Govindaraj et al. 2009; Bashir et al. 2014) and other crops (Stangoulis et al. 2006; Garcia-Oliveira et al. 2009; Gomez-Becerra et al. 2010b; Gomez-Becerra et al. 2010a; Velu et al. 2012). This indicates that selection for target nutrients will have, in most cases, a positive or

neutral effect on other grain micronutrient densities. The association between grain yield and Fe (Zn) density is crucial since grain yield is generally the most important agro-morphological trait. Our study and most other studies have shown no significant correlation between grain yield and Fe (Zn) density (Chapter 3, Govindaraj et al. 2009; Gupta et al. 2009), which reveals that biofortification breeding has no negative effect on gain yield.

A further aspect which needs consideration before starting a breeding program is the screening method used to identify the micronutrient densities. Usually, pearl millet meals are prepared with decorticated grains, so the nutrient density of decorticated grains can be considered as the target trait. However, since decortication is labor-intensive and difficult to standardize, it needs to be assessed if analysis of decorticated grain is worthwhile. High and significant correlations between whole and decorticated grain nutrient densities as well as similar heritability (Chapter 3) indicates that the screening of whole grains is feasible.

The environment plays another important role in biofortification breeding programs. While the total quantity of a certain element in the soil is often only weakly related to the micronutrient density in the plant, soil properties like redox potential, pH, organic matter content, and nutrient interaction as well as environmental conditions like temperature, water availability, and light strongly influence the availability of micronutrients (Mortvedt et al. 1991). A significant environmental effect on the grain mineral density was observed for several minerals (Mg, P, K, Mn, Fe, and Zn) in our study, where pearl millet was grown in three environments in Niger (Chapter 3). Soil fertilization especially seems to have a positive impact on the grain mineral density which we determined based on a one-location testing for pearl millet. Similar observations have been made for other cereals like wheat (Shi et al. 2010; Cakmak et al. 2010; Kutman et al. 2011) and maize (Ciampitti and Vyn 2013). This positive implication of fertilization (in addition to the strong impact on grain yield) could be used as an effective tool for agronomic biofortification, complementary to biofortification by breeding. However, fertilizer availability for small-scale farmers in Sahelian Africa is very limited due to the high cost and poor infrastructure in the region. Thus agronomic biofortification might be difficult to achieve.

One additional factor influencing the selection efficiency in biofortificaition breeding is the repeatability of a trial. If this parameter is insufficient, approaches like indirect selection to improve the selection efficiency can be useful. We observed low repeatability for pearl millet grain Fe density under one low-input condition, which is in line with a study on grain legumes (Høgh-Jensen et al. 2006). Although low-input conditions probably best represent

the on-farm situations of poor WA farmers, direct selection under low-input environments on-station seems inefficient in comparison to moderate- or high-input environments based on our findings. However, since our study is based on one location only, a further study comparing repeatabilities and heritabilities of high and low-input environments is required to identify an efficient selection strategy for pearl millet biofortification.

The success and impact of a biofortified variety depends on the bioavailability of Fe and Zn in the grain, which is not only determined by the amount of nutrients in the grain. Compounds like the antinutrient phytate (inositol-hexa-phosphate) are also important factors for bioavailability. Phytate is known to inhibit the bioavailability of minerals for the consumers (Nolan et al. 1987), but at the same time it has a crucial role in the grain, because it is the primary storage form of both phosphate and inositol in plant seeds (Kumar et al. 2010). Beside the negative effect on human health, consumption of phytate can have positive effects like prevention of several cancer types, and can be used therapeutically against diabetes mellitus, atherosclerosis and coronary heart disease and can reduce kidney stone formation (Kumar et al. 2010). However, knowledge of the appropriate dosage for eliciting beneficial effects in humans, as well as information on the amount of phytate necessary to fulfill the physiological functions in the grain is limited. Thus investigations on the role of phytate in pearl millet grain will be crucial in assessing whether selection for low phytate content or low phytate-Fe (Zn) molar ratio would be worthwhile to increase the micronutrient bioavailability, or whether adverse effects would predominate.

Studies on Fe and Zn biofortified pearl millet found that consumers, like young women or children, did absorb higher amounts of these micronutrients and could even exceed the daily requirement by eating biofortified pearl millet meals (Cercamondi et al. 2013; Kodkany et al. 2013a; Finkelstein et al. 2015). Such findings underline the great potential of pearl millet biofortification.

Future breeding programs should establish constant evaluation of the Fe and Zn density in the breeding material, which will, in the long run, increase the micronutrient levels in most varieties. This is especially true of pearl millet where breeding is still strongly dependent on locally adapted landraces whose Fe and Zn densities are highly variable. Our study identified genotypes with promising grain nutrient density and stability as well as good grain yield, which can be used as base materials in future biofortification breeding programs for WA (Chapter 3). With the first releases of a biofortified pearl millet variety in WA, nutritional information on each variety should be provided by means of labelling to

raise consumer awareness of the importance of micronutrient levels, and thus establish and develop quality standards.

6.3 Pearl millet hybrid breeding to boost productivity in West Africa

6.3.1 Superiority of population hybrids over open-pollinated varieties

Our study on population hybrids was performed using a 10 × 10 factorial mating design with four parental open-pollinated varieties (OPVs) from each of five WA countries. The potential of pearl millet hybrids to increase the pearl millet productivity in WA agro-ecologies has been shown by our study, finding an average panmictic midparent heterosis (PMPH) of 17% and a maximum PMPH of 73.2% (Chapter 4) (PMPH describes the performance difference between a population hybrid and the mean of its parental populations (Lamkey and Edwards (1999)). Earlier studies on population hybrids or top-cross hybrids based on African and Indian germplasm showed a similar magnitude of heterosis (Ouendeba et al. 1993; Presterl and Weltzien 2003; Bidinger et al. 2003).

Maize, one of the most successful and comprehensively studied hybrid crops, has been shown to exploit similar or slightly higher heterosis in population hybrids (Reif et al. 2003; Carena 2005) in comparison to pearl millet, which indicates a similar potential for pearl millet hybrids.

The benefit of population hybrids over their best parental population can be determined by panmictic better parent heterosis (PBPH). In our study, the best and second best yielding hybrids showed 5.3% (PE03012×AON514) and 37.3% (PE02935×Souna3) PBPH, respectively, indicating a good yield increase especially for the latter one (Chapter 4). However, PE03012 as the best parental population of this study, yielded only 5% less than the best hybrid. In such cases, performance tests of the $F_1(=Syn_0)$ and $F_2(=Syn_1)$ generations of the best population hybrid should be performed to assess the hybrid superiority over certified seed and farmers-saved seed of the best OPVs. Such tests could elucidate whether investment in population hybrids might be cost-effective and less risky for farmers.

Beside the PBPH, the commercial heterosis (superiority over the control variety) is an important indicator to determine the economic benefit of hybrids for a farmer. We observed in our study for the two best hybrids a commercial heterosis of 25% based on one control variety (Chapter 4), indicating the benefit through heterosis although a sound conclusion cannot be drawn with only one control variety. Several studies including ours have shown that the complex trait grain yield exploits higher PMPH and PBPH compared

to less complex agro-morphological or yield-component traits (excluding downy mildew resistance in our study) in pearl millet (Chapter 4, Bidinger et al. 2003; Yadav 2006) and other crops (Niehaus and Pickett 1966; Lippman and Zamir 2007; Longin et al. 2013).

6.3.2 Aspects of heterotic grouping in West African pearl millets

One objective of our study was to identify heterotic patterns of WA landraces. Based on grain yield of the 100 testcrosses, it was obvious, that hybrid combinations, especially those with one parent from Senegal and one from Niger were generally performing very well (Chapter 4). This fact was causing significantly positive correlation (r=0.3) between hybrid performance and geographic distance of parental populations' origin. However, a significant relationship between heterosis (PMPH) and geographic distance could not be verified. Hence, putative heterotic groups cannot be based merely on the geographic origin of the parental populations. This result was confirmed by the observation that yields of intra-country crosses did not differ significantly from yields of inter-country crosses. Additionally, hybrid performance and PMPH were not associated with morphological distance (based on 11 traits) as an indicator or genetic distance, which could suggest that genetic distance and heterosis are not, or only slightly, associated in the tested material. Using the genetic distance as an initial basis for heterotic grouping has been suggested in several previous studies (Melchinger and Gumber 1998; Wu et al. 1999; Reif et al. 2003), but other studies identified this approach as unsuitable (Edmands 2002; Yu et al. 2005). The latter view was also supported by the study of Chowdari et al. (1998), who found no association between genetic distance and hybrid performance, midparent heterosis, or better parent heterosis using Indian pearl millet inbred lines. Hence, the question 'How does the genetic distance between hybrid parents influence the effect of heterosis?' still needs further investigation. The answer will show whether heterotic grouping will be based entirely on combining ability studies or if genetic diversity studies could provide a useful support in this progress. The currently running project "Bringing the benefits of heterosis to smallholder sorghum and pearl millet farmers in WA" led by ICRISAT-Niger in cooperation with the University of Hohenheim is addressing this question in a multi-location multi-year trial, using a diallel mating design.

Although there was no clear combining ability pattern for certain country combinations, crosses among Senegalese and Nigerian or Nigerien populations were in several cases outstanding (Chapter 4). Arranging potential parental material from these two geographically distant regions into a pair of genepools, and subsequent reciprocal recurrent

selection to improve the general combining ability (GCA) to the opposite heterotic pool, appears to be promising. In the longer term, a systematic approach to develop heterotic groups based on combining ability pattern trials seems to be most promising. This knowledge will also be useful for Indian hybrid breeding, which so far has not been based on systematically developed heterotic groups, and which might benefit from diversification using advanced African materials.

6.3.3 Development of high yielding and nutritionally valuable hybrids

An effective hybrid breeding program requires a strategy to predict hybrid performance from parental performance since with increasing number of parents, the number of possible hybrids is huge and all combinations cannot be tested. Predicting hybrid performance based on the effect of GCA has been identified to be more efficient than midparent values (Chapter 4; Gowda et al. 2012; Guo et al. 2013), because dominance effects are considered when using the GCA (Xu and Zhu 1999). We observed for grain yield a relatively high effect of specific combining ability (SCA) compared to the GCA effect, which demonstrates a high influence of dominance in comparison to additive effects. This is unfavorable for hybrid prediction and would lead to a two-step selection procedure, where pre-selection of parents is based on GCA and the second step will identify the best hybrids using factorial crosses.

One reachable goal in the long term would be a biofortified hybrid variety with high yield and quality stability. This would require high Fe and Zn densities in both parental pools, as the micronutrient contents are predominantly influenced by additive gene actions. An increased micronutrient content in hybrids through heterosis is not expected, as shown by Velu et al. (2011) in pearl millet. Continuous monitoring and selection for high Fe and Zn content in the male and female heterotic group could be one option to enhance the nutritional value steadily. Depending on the grain Fe and Zn level in the parental pools, introgression of germplasm with already enhanced micronutrient content and subsequent recurrent selection within each pool might be required to achieve hybrids with effectual nutrient levels.

6.3.4 Marker-assisted development of male-sterile female and corresponding fertility-restoring male parents

Although the CMS has been used in Indian pearl millet breeding for many years, no male-fertility restoration locus for the A_1, A_4 or A_5 CMS system has been identified to facilitate marker-assisted screening for effective introgression.

Advanced high-throughput sequencing technologies enable the development of SNP-based genetic linkage maps, which have been used in many crops. In pearl millet, our map is the third successfully developed GBS-SNP-based linkage map after those published by Moumouni et al. (2015) and Punnuri et al. (2016). The first published map was created without a reference genome using the UNEAK pipeline in TASSEL (Lu et al. 2013), while Punnuri et al. (2016) and ourselves were able to generate SNP markers based on the reference genome sequence (kindly provided by the Pearl Millet Genome Sequencing Consortium), which has by now been accepted for publication. The map of Punnuri et al. (2016) was based on a RIL mapping population and had therefore a higher density than the map of Moumouni et al. (2015) and our map (Chapter 5), while all the three GBS-based linkage maps had higher marker densities compared to those in previous maps based on other marker types. Our map showed only one gap (slightly) above 10 cM between adjacently-mapped SNP markers, thus we can classify our map as dense, uniform, and well saturated.

Restoration of male fertility in F_1 hybrids is crucial for hybrid cultivars where seed or fruit comprise the economic harvest. Earlier studies on the A_4 CMS system in pearl millet have shown its suitability through stable sterility and reliable male fertility restoration across Indian pearl millet growing regions. Our study indicates that a major proportion of this stability may be relatable to a simple inheritance of male-sterility maintenance and male-fertility restoration in this system. It suggests that similar stability of the CMS system can be expected in WA pearl millet growing environments. Based on the 1 : 3 (sterile : fertile) segregation ratios observed within F_2 populations by Gupta et al. (2012) and in our study (Chapter 5), monogenic dominant inheritance could be assumed for male-fertility restoration of the A_4 CMS system. Through this assumption of a single gene control, we expected to find one major QTL for fertility restoration / sterility maintenance explaining a high percentage of phenotypic variation. This was only partially fulfilled as we identified one major locus, but the estimated R^2_{adj} values reached only 14.5% for pollen production and 9.9% for selfed seed set (those two traits were used to rate the fertility status of the plant). The difference from expectation might be caused by some modifying or minor R_f

genes that were not identified by our QTL analysis. This was underlined by some loci that did not reach the LOD threshold to be detected as a significant QTL, but might be associated with minor R_f genes. The QTL position on linkage group (LG) 2 was detected by both traits, pollen production and selfed seed set score, which proves our result. A very high accuracy of the R_f gene position was verified by cross-validation, which found the same locus on LG 2 in 97% of the runs for pollen production.

The QTL identified by selfed seed set explained smaller phenotypic variance compared to pollen production, which might be explained by some plants that showed intermediate seed set caused by partial male-fertility (Chapter 5). This can be caused by heat stress, insect-feeding damage, pollen contamination inside the selfing bag and/or combination of a long protogynous period and a short stigma receptivity. In comparison, scoring of pollen production by classification of anthers as sterile or fertile was more distinct. Hence, we assume that phenotyping of pollen production was done with a smaller error rate than selfed seed set scoring.

Beside fertility restoration, the mapping population of our study segregated for a dwarfing gene (d_2), whose location had been mapped earlier to LG 4 by Azhaguvel et al. (2003) and Parvathaneni et al. (2013). This gave us the opportunity to verify our linkage map by analyzing plant height as a reference trait. Like the previous studies, we could identify one major QTL on LG 4 (R^2_{adj} = 24.5%), which is most likely linked with d_2 (Chapter 5). Locating the dwarfing gene at the same LG as previous studies was a useful validation for the accuracy of our QTL analysis. Azhaguvel et al. (2003) found additionally the d_1 dwarfing gene locus on LG 1, which was not significant in our study. However, we observed one LOD peak on LG 1 just below the threshold, which is probably associated with the d_1 locus mapped earlier.

Based on our results and those of Punnuri et al. (2016) we can state that highly-saturated GBS-SNP-based linkage maps represent a qualified option for QTL mapping in pearl millet, albeit the huge marker number achieved by GBS cannot be used to full capacity in F_2 populations because of the low recombination rate and thereby high marker redundancy.

The identification of the R_f gene for the A_4 CMS system in pearl millet enabled a considerable increase in knowledge about the genetic basis of this important trait and will facilitate breeders with the opportunity of marker-assisted selection to introgress the restoration gene into the male parental pool. This will be much more time-efficient than backcrossing programs based on phenotypic data. Therefore, it will be of great support to the slowly developing hybrid breeding in WA where restorer genotypes are very rare in

landraces or improved varieties. Likewise, our information can be used to introgress the major maintainer allele (*rf*) for the A_4 CMS system into the female parent genepool. Independently of maintainer/restorer characteristics of the target germplasm, heterotic pools could be developed based on combining ability patterns and traits and other specific traits required for seed parents and restorers.

Due to the discrepancy between our phenotypic data (suggesting monogenic inheritance) and QTL analysis (with the major QTL explaining only a low portion of the phenotypic variance), further studies should verify the inheritance of the fertility restoration in the A_4 system. In parallel with those investigations, functional competitive allele-specific PCR based (KASP) markers, which were developed within this study, can facilitate high-throughput screening and thereby speed up the development of both maintainer and restorer pearl millet hybrid parents.

6.3.5 Criticisms and benefits of hybrid breeding in West Africa

Hybrid breeding itself is often criticized in the media especially when poor smallholder farmers are involved. It has the reputation of causing farmers to become quickly dependent on breeders and seed producers because they cannot regrow their harvest, which is particularly serious if farmers are not able to buy the more expensive hybrid seed in the next season. Further, genetically uniform hybrids would be less stable under extremely variable and changing environmental conditions, such as those that occur in WA, compared to population varieties. With the start of hybrid breeding, other breeding strategies could be downsized due to resource allocation, thus in the case of pearl millet, the development of new population varieties could get less attention, which reduces the selection gain and output of those breeding programs. With a wide-spread distribution of hybrid varieties, the genetic diversity originally maintained by landraces and OPVs might be reduced. Additionally, hybrid seed production depends on CMS systems, which creates a problem if the narrow genetic base of the CMS cytoplasm increases the vulnerability of plants to diseases and insect pest epidemics.

All those criticisms of hybrid breeding, which have particular relevance in WA, should be thoroughly considered, but should also be assessed in relation to the benefits of high grain yield superiority (Ouendeba et al. 1993; Bidinger et al. 2005). As shown in some studies on pearl millet and sorghum, high-yielding hybrid varieties could also be stable across several environments (Yahaya et al. 2006; Gupta and Narayan 2013; Rattunde et al. 2013). However, the concern over low stability due to lower buffering capacity of genetically

uniform single-cross hybrids is still relevant, especially because it is not well studied for WA environments. An approach to reduce this risk might be the development and release of top-cross or population hybrids, which exhibit the advantage of high heterozygosity, but without extreme genetic uniformity and therefore have higher buffering capacity than single-cross hybrids. This is explained in more detail in the next paragraph.

The risk of farmers' dependency on breeders and seed producers, when starting to grow hybrids, has special relevance when private breeding companies dominate the seed sector, since they try to maximize the return on their investment. In WA the pearl millet breeding sector is predominantly managed by national and international institutions, while there are only very few private breeding companies. Against this background, the aim of breeding institutions like ICRISAT is to strengthen farmers' seed cooperatives by introducing the technology of hybrid seed production. Farmers who are able to produce hybrid seeds can combine income generation (from selling hybrid seed harvested from the female plant) with food security objectives (using the harvest from male plants as food for the family) The feasibility and success of hybrid seed production by farmer- managed seed cooperatives has been proven for sorghum in Mali where hybrid seed produced by famers doubled annually during the past six years (Kante et al. 2017). Further, a study on sorghum hybrid seed showed that the risk that Malian farmers will not recoup the value of their investment in purchased seed is small, since only 0.05 Mg ha^{-1} yield superiority is required (Kante et al. 2017).

Criticisms of CMS systems which state that they narrow the genetic base of the cytoplasm and thereby increase the vulnerability of plants to pests is justified if hybrids are based on only one CMS system. In pearl millet the A_1, A_4, and A_5 CMS systems have been identified as potential options so far, thus a certain variability will be possible. Further, an increasing vulnerability will be unlikely if farmers continue to grow various OPV, which would maintain the cytoplasm diversity within WA pearl millet.

6.4 Genotype-by-environment interaction and implications for pearl millet breeding

Yield stability has enormous importance especially under varying environmental conditions in WA. Such varying conditions were also visible in our study where the locations Cinzana, Bambey and Gampela received very different amounts of rainfall within the study year compared to the average rainfall at these sites (Chapter 2). Genotype-by-environment (G×E) interaction is therefore a crucial parameter in pearl millet breeding for WA.

Landraces as well as population hybrids showed in our study a very high G×E interaction for downy mildew susceptibility (Chapter 2, 4), which is also in line with the study of Kountche et al. (2013). It can be explained as a result of the varying pathogen population virulence in different environments and the development of resistance by landraces specific to the local pathogen. It emphasizes the importance of pathogen variability for virulence and site-specific selection for pearl millet downy mildew resistance across test locations.

The complex trait grain yield, which is influenced by various factors, is also highly dependent on G×E interaction in landraces as well as hybrids (Chapter 2, 4). Population hybrids and their parental populations showed a similar extent of G×E interaction and grain yield stability over a wide range of environments (Chapter 4), thus we can assume that the slightly higher heterozygosity in the population hybrids did not increase the genotypic buffering capacity measurably.

The pearl millet grain mineral density is also affected by the G×E interaction (Chapter 3; Gupta et al. 2009), indicating that genotypes respond differently to different environments, and that biofortification breeding needs to consider this circumstance.

The high levels of G×E interaction found for several traits underlines the importance of selecting for yield stability. Identification and subsequent selection for repeatable mega-environments could be one approach to find stable genotypes. Mega-environments are defined as not necessarily contiguous areas with similar climate, moisture regimes, soil types, growth habit, consumer performance, prevalent diseases and insect pests (Rajaram 1994), and are used to allocate resources in breeding programs and to increase the heritabilities within relative well-defined environments. While the development of mega-envrionments requires multi-year and multi-location trials and continuous monitoring of environmental patterns, it would enable site-specific breeding and higher selection gains for the target region. Five locations were the same in our characterization study (Chapter 2) and our study on combining ability patterns (Chapter 5). In both studies, genotypes responded very differently with respect to grain yield at Cinzana in comparison to the other locations, which would indicate that Cinzana should be considered to be within a different mega-environment than the other locations. However, this presumption concerning a mega-environment considers only grain yield whereas it would be ideal if breeders could develop mega-environments suitable for all important target traits which are substantially influenced by G×E interactions. This would be of especial interest when breeding programs aim to combine target traits like resistance, micronutrient content, and yield potential. But most likely, it is hardly possible to establish such a mega-environment, as

different traits are differently influenced by the environment. It therefore seems more realistic to develop mega-environments with respect to certain traits.

Beside the breeding strategies to select stable genotypes based on multi-location and multi-year trials, yield stability can be influenced by the variety type. In the highly variable and stressful WA Sahel, development of population hybrids or top-cross hybrids would be preferable due to their higher genetic heterogeneity than genetically uniform single-cross hybrids (Haussmann et al. 2012). Although seed production of top-cross hybrids might be a little more practical due to the required CMS system, which is more easily introgressed in a female inbred line, both types of hybrid should be worthwhile exploring. As CMS will not be available in WA pearl millet populations in the short term, one approach could be to use the promising population hybrids found in our study (Chapter 4) by multiplying those hybrids through random mating. Although the benefit of heterosis and thereby the grain yield will be slightly reduced in the progenies, the reduction will be much less in comparison to single-cross hybrids. After one generation of random mating, the Hardy-Weinberg equilibrium will be reached and the new population will remain superior to the initial parental populations.

6.5 Conclusions and recommendations for pearl millet breeding in West Africa

Applied pearl millet breeding in WA has to cope with various challenges and needs forward-looking strategies to meet the already existing and upcoming pressure on pearl millet production. This study investigated several aspects considering pearl millet variety development, which will be of direct relevance in breeding programs. The following conclusions, with possible implementations, can be drawn:

- Phenotypic characterization of a broad set of landraces identified large diversity within countries, and geographical differentiation for several traits
- The tremendous WA pearl millet diversity and adaptability to a wide range of environments should be used more intensively by breeders, and the germplasm exchange within similar agro-ecologies needs to be strengthened
- Phenotypic observations identified high genetic admixture among WA pearl millets, thus heterotic grouping based on "naturally" distinct groups is not feasible, and groupings based on geographic distance or country of origin do not seem to be appropriate either

- Biofortification breeding programs in WA using local as well as Indian material have great potential to reduce hidden hunger
- Selection for high grain iron and zinc density can be done simultaneously without a negative effect on grain yield or other micronutrients
- Constant monitoring and selection for high grain iron and zinc density within breeding programs could be a valuable long-term approach to reach high micronutrient levels in new varieties
- Considerable superiority of population hybrids over their parental populations illustrates that exploitation of heterosis in future WA breeding programs can be very rewarding
- Sustainable hybrid breeding will require several combining ability studies, which will develop heterotic groups step by step in a systematic manner
- Hybrids based on crosses between Senegalese and Nigerien or Senegalese and Nigerian landraces were in several cases outstanding, thus initial heterotic grouping could be done based on this information
- Phenotypic observations indicated that the A_4 male-fertility restoration is a monogenic dominantly inherited trait which is generally relatively simple for introgression and therefore desired in hybrid breeding
- The major QTL for A_4 male-fertility restoration was located on LG 2, which can be used for high-throughput screening to speed up the development of maintainer and restorer pearl millet hybrid parents
- GBS appears to be an appropriate tool to develop high quality genetic linkage maps and perform linkage studies in pearl millet

6.6 Outlook and perspectives

Population growth and its inevitable consequences for the WA population will require innovative strategies to reduce and avoid food insecurity and hidden hunger. Many sectors need to get involved, while the development of sustainable and productive agriculture will be a key component in meeting the upcoming challenges. However, successful agriculture is complex as it depends on numerous features such as education, political security, agricultural development plans at national level, gender equality, rights of access to land tenure, water availability, soil fertility (e.g. land degradation, P scarcity), adaptation to a changing climate, crop management systems, and the use of modern technologies. Many of

those aspects are directly or indirectly linked with plant breeding, thus interdisciplinary thinking will be required to develop varieties for the future.

6.6.1 Keeping genetic diversity for a changing climate

Beside the goal of bringing pearl millet hybrid varieties to WA farmers, it is at the same time important to keep a high genetic diversity including population varieties in the region. A diverse variety portfolio on the seed market offering well-adapted landraces, improved OPVs and hybrids (including top-cross and single-crosses) would have several benefits. It would help to maintain the genetic diversity, and enable a constant adaptation to a changing climate and other environmental factors, which is especially important in the highly unpredictable and stressful pearl millet growing environments. Such diversity will help farmers to manage their risks by choosing different varieties and will maintain the possibility of regrowing their seeds, which is of special importance as subsistence farmers have little monetary income.

Bezançon et al. (2009) has showed that pearl millet diversity in Niger has been maintained by farmer management (in-situ) during the study period between 1976 and 2003. However, the goal of a higher adaptation of improved varieties will most likely imply a slow but steady reduction of local landraces. Ex-situ conservation of genetic diversity should additionally sustain the opportunity to adapt to a changing climate. A global increase in temperature and frequency in drought periods might force some agricultural regions to change from crops like maize to pearl millet (Eyshi Rezaei et al. 2013). If the pearl millet growing area is going to expand through such crop changes, breeding for local adaptation will benefit from well-documented diversity, like the publicly available data from this study.

6.6.2 Preparation for agro-ecological intensification

The increasing pressure on food production especially in Sub-Saharan Africa (SSA) in combination with a very low availability of agricultural resources is devastating. Arable soil, P and water are most likely the key limiting resources for agricultural production in this region.

Intensification of agriculture based on inorganic fertilizers and its expansion are the primary causes of the calamitous extent of soil degradation in SSA (Tully et al. 2015). Effective solutions to reduce this problem will need to cut across agricultural, environmental and socioeconomic objectives. As one agricultural factor, plant breeding will play an important role, especially if approaches imply a change in crop management systems. Intercropping is one opportunity, which is known to reduced runoff and erosion

(Zougmore et al. 2000), and would benefit from adapted varieties. Thus breeding might increase the farmers' interest and adoption of intercropping due to the higher productivity of such systems.

Continuing soil fertility depletion will also exacerbate the problem of P scarcity. P is an essential mineral and is considered as probably the most limiting mineral nutrient for crop production worldwide because P fertilizer extraction requires rock phosphate which is a finite resource (Kochian 2012). Poor subsistence farmers are hardest struck by the already rising prices of P fertilizer, thus alternative strategies which are less dependable on external inputs will be required. Gemenet et al. (2015; 2016) showed that genetic improvement of pearl millet enabling it to grow under P-limited environments can be one efficient approach and suggested direct selection under low-P conditions. This recommendation for the breeding environment should be considered and, where applicable, be implemented in future breeding programs.

6.6.3 Future role of modern tools in pearl millet breeding

The use of modern breeding tools, like next-generation sequencing methods, holds great potential for efficient plant breeding especially in the contexts of climate change adaptation and low input agriculture. Gemenet et al. (2015) reported the potential of using marker-assisted selection in pearl millet breeding targeting low-P environments. They also showed that tolerance to low-P is a polygenic inherited trait with many contributing polymorphisms, which implies that QTL mapping can only succeed to a limited extent.

In contrast to linkage mapping, which ignores genes with small effects, genome-wide selection estimates all marker effects in all loci through the entire genome simultaneously, which enables successful prediction of more complex traits (Riedelsheimer et al. 2012; Technow et al. 2013). Whole-genome prediction is based on a prediction model, which is developed in a training population, and which is later used to predict phenotypes in the target population (Meuwissen et al. 2001). Apart from the opportunity to predict complex traits based on their genotype, it is also possible to use genome-wide selection to identify promising hybrids, as has been demonstrated in maize. Prediction of hybrid performance is even successful without testing the combining ability of the parents in the field (Technow et al. 2012). Such approaches might in the long term also be interesting for pearl millet hybrid breeding.

Genome-wide selection, in particular, will profit from next-generation sequencing methods such as GBS, since it requires a very high marker density on the entire genome to achieve

high prediction accuracy. However, the prediction accuracy of genome-wide selection is also highly dependent on the quality of the phenotypic data measured in the training population to develop the prediction model. Our study showed that the repeatabilities and heritabilities achieved in our WA field trial are often moderate (Chapter 2, 4), which could, for instance, be caused by inhomogeneous conditions at the experimental sites or phenotyping procedures. Hence improved trial conditions and implementation will be required to achieve a maximum heterosis and therefore prediction accuracy by genomic data.

6.6.4 Capacity building – at farmers and breeders level

Education is a fundamental requirement to reach goals in agriculture. But the education level in SSA is alarmingly low. In Niger, only 61% of school-aged children attend primary school and the literacy rate stays at 15.5%. This undesirable situation is also reflected in the education index, which is the lowest worldwide (UN 2016). In particular subsistence farmers in rural areas have only very little access to information on promising and more sustainable agricultural management systems, thus the establishment of innovative measures is difficult and slow (Spielman et al. 2008). These circumstances also have a direct influence on plant breeding because non-informed farmers have only little impulse to adopt improved varieties. Therefore it will be critical to improve information transfer to farmers and communication habits to change traditional habits and preferences.

For instance, grain of biofortified pearl millet varieties might not match the preferred taste or grain color, but if farmers gain knowledge about micronutrient deficiency, such preferences could change and lead to higher adoption rates of biofortified varieties. A resulting reduction in micronutrient deficiency, especially in iron, will increase the health and cognitive capability of the malnourished population. Therefore, education will, in turn, profit from biofortification through higher mental performance.

Further, the establishment of farmer schools teaching, for example, how to produce well-adapted hybrid seeds, would create new perspectives for young farmers and lead to a more successful use of hybrid varieties. Channels for broad-based information transfer could be learning DVDs or smart phone applications, which could aim at generating a generally higher adaptation rate.

Besides the capacity-building of farmers, the target region requires practical plant breeders, who are sufficiently skilled to achieve the high number of breeding targets necessary to develop cultivars acceptable to farmers that are more nutritious and well-adapted to

changing conditions. Such people should also be familiar with the local seed market. The urgent need for breeders is illustrated by the fact that SSA countries have on average only five breeders per country to cover all crops, climatic zones and purposes (Walker et al. 2014). Such a low capacity in the field of plant breeding will not be able to realize the necessary and theoretically possible genetic improvements that may help to combat food insecurity. Two projects funded by the Bill and Melinda Gates foundation are targeting this issue and aim to convey core competences by using modern tools like genomics, molecular markers, electronic data collection, data management and breeding pipeline optimization (Suza et al. 2016). However, more investment is required to ensure sustainable human capacity development, and to lay the foundations that will realize the potential benefits of the proposed breeding approaches.

7 Summary

The enormous human population growth in West Africa (WA) in combination with serious climatic and ecological production constraints poses a very problematic condition for future food security. The alarming status of micronutrient deficiency in the WA region exacerbates this situation and calls for effective strategies to combat malnutrition. For smallholder farmers in the Sahel, particularly those who cannot afford or do not have access to irrigation and sufficient quantities of fertilizer, improved, adapted and nutritious crop varieties derived from plant breeding could be a major contributor to enhancing agricultural productivity and reducing malnutrition.

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is the sixth most important cereal globally and a staple crop in Sahelian WA. Due to its high tolerance to drought, heat, as well as to saline and sandy soils with low soil fertility, it is capable of growing under very harsh environments, where cropping of other cereals is not productive. Despite the vital role for food security in WA, pearl millet has received only little attention in research, which offers enormous potential through agricultural technologies like breeding. Development and implementation of multiple complementary pearl millet breeding approaches will be crucial to exploit the potential of this fantastic crop.

The main goal of this study was to discuss and establish the scientific basis for more efficient pearl millet breeding in WA with a specific focus on achieving higher productivity and nutritional value. In order to accomplish this goal, the following objectives were defined: (I) to characterize a broad set of WA pearl millet accessions and to investigate their diversity and geographic patterns based on their phenotype; (II) to identify the potential and strategies to increase the micronutrient level in WA pearl millet; (III) to evaluate the performance of population hybrids and to derive initial strategies of pearl millet hybrid breeding in WA based on combining ability and heterotic patterns; and (IV) to identify molecular markers for the male-fertility restoration locus (*Rf*) for the A_4 cytoplasmic-genic male-sterility (CMS) system in pearl millet using a genotyping-by-sequencing (GBS) based linkage map.

Three different large-scale multi-location trials were conducted in WA to evaluate the WA pearl millet genetic diversity for agro-morphological and grain quality traits, and to determine the potential hybrid superiority over open-pollinated cultivars. Additionally, a linkage study was performed using a F_2 mapping population, segregating for A_4 male-

fertility restoration. The major results and conclusions of these experiments are summarized in the following:

Characterization of a broad collection of 360 WA pearl millet landraces at six environments in WA identified wide ranges for 12 agro-morphological traits such as yield, flowering time, panicle length, etc., indicating tremendous diversity of the tested landraces. Principal component analysis revealed very large diversity within individual countries, especially within Mali and Burkina Faso, and a high genetic admixture among WA pearl millet landraces. The high admixture and absence of "naturally" distinct groups, indicates that straightforward heterotic grouping based on morphological distance, geographic distance or country of origin is not possible; rather heterotic groups need to be systematically created by the breeders. Geographical differentiation and country differences were detected for several traits, indicating the high degree of adaptation of WA pearl millets to specific environments and regional farmer preferences. The published data of this study gives national breeders a sound basis to select and utilize this germplasm and to serve the manifold needs of WA pearl millet farmers.

In the second trial, pearl millet grain iron and zinc densities showed significant genetic variation in a set of 72 WA landraces evaluated at three environments and moderate-to-high heritability (h^2=0.70 for iron, h^2=0.53 for zinc), which emphasizes a high potential for biofortification breeding. Identified landraces with moderately high and stable micronutrient densities appear suitable for use in future WA pearl millet biofortification breeding programs, while screening of additional landraces or introgression of favorable alleles from highly nutrient-dense Indian germplasm could expedite achievement of higher micronutrient densities. Due to significant positive correlations among grain iron, zinc and other mineral densities and non-significant correlations between grain yield and mineral densities, selection for high grain iron and zinc density can be performed simultaneously without a negative effect on grain yield or contents of other micronutrients.

The third trial evaluated 100 population hybrids and their 20 parental populations (with four parental open-pollinated varieties from each of five WA countries) at six environments and showed hybrid superiority of, on average, 16.7% compared to their parental populations (ranging from -26 to 73%), reflecting the great potential of hybrid breeding to increase pearl millet productivity. The mean grain yield of hybrids based on inter-country crosses did not differ significantly from intra-country crosses, which supports the result of our first trial, showing high genetic admixture between countries. Geographic distance between parents was positively correlated with hybrid grain yield (r = 0.31), but

not with panmictic midparent heterosis, indicating that heterotic grouping based on geographic distance is not expedient. However, some crosses between accessions from Niger/Nigeria and Senegal were outstanding, thus initial heterotic pools could be based on this information. In the long term, sustainable pearl millet hybrid breeding will require several combining ability studies, whose results can be used to develop heterotic groups in a systematic manner.

All three multi-location trials showed for numerous traits (grain yield, downy mildew, grain iron and zinc density, etc.) high levels of genotype-by-environment interaction effects, which underlines the importance to select for stable varieties. Identification and subsequent selection for repeatable mega-environments could be a suitable approach towards finding stable genotypes, but this requires multi-year and multi-location trials and continuous monitoring of environmental patterns.

Within the fourth trial, a high-density linkage map based on single nucleotide polymorphism (SNP) markers produced by GBS was generated using a F_2 mapping population, which segregated for fertility restoration of the A_4 CMS system. A major *Rf* locus was found on linkage group 2, which was verified by cross-validation showing a very high quantitative trait locus (QTL) occurrence (97%). The QTL explained 14.5% of the phenotypic variance, which was below expectation because the segregation ratio of male-fertile and male-sterile plants (3:1) indicated monogenic dominant inheritance for male-fertility restoration. The two functional KASP markers developed for the identified locus will support high-throughput screening for the *Rf* locus and will facilitate the development of male parental pools exhibiting the fertility restoration, which is an essential step to enable economic pearl millet hybrid seed production. Given the fact that fertility restoration in the A_4 cytoplasm is a rare trait in WA pearl millet germplasm, the marker identification is a big step toward marker-assisted breeding of A_4 male-fertility restoration and male-sterility maintenance in pearl millet, which will greatly facilitate pearl millet hybrid breeding in WA.

We can conclude that WA pearl millet breeding has the potential to increase the pearl millet productivity and nutritional value by utilizing the enormous pearl millet diversity in hybrid and biofortification breeding programs. Nevertheless, substantial progress through improved cultivars will only be achieved and reach millions of WA smallholder farmers if local seed systems can be strengthened and agronomic as well as socio-economic conditions can be improved.

8 Zusammenfassung

Die Bevölkerung in West Afrika (WA) wächst rasant, und gleichzeitig verschlechtern sich die Bedingungen für eine ausreichende Nahrungsmittelproduktion durch Klimawandel und weitere Umweltfaktoren. Ernährungssicherung in WA wird so zu einer gewaltigen Herausforderung. Häufig vorkommender Mikronährstoffmangel verschärft die desolate Situation der WA Bevölkerung und erfordert effektive Strategien zur Bekämpfung von Mangelernährung. Besonders Kleinbauern in der Sahelzone, die schlechten Zugang zu Bewässerung und ausreichend Dünger haben, könnten von verbesserten und lokal angepassten Getreidesorten mit einem erhöhten Nährwert profitieren.

Perlhirse (*Pennisetum glaucum* (L.) R. Br.) ist weltweit das sechst wichtigste Getreide und Hauptnahrungsmittel in der Sahelzone von WA. Die hohe Trocken- und Hitzeresistenz, sowie die Fähigkeit auf salzigen und sandigen Böden mit geringer Fruchtbarkeit zu wachsen, macht den Anbau von Perlhirse dort möglich, wo auf Grund der widrigen Umstände kein anderes Getreide Ertrag bringen würde. Trotz der enormen Bedeutung für die Ernährungssicherung wurde Perlhirse bisher relativ wenig erforscht, sodass die Anwendung von agrarischen Methoden wie Pflanzenzüchtung sehr Erfolgsversprechend ist. Die Entwicklung von verschiedenen und sich ergänzenden Ansätzen in der Perlhirsezüchtung ist erforderlich um das Potential dieses beeindruckenden Getreides auszuschöpfen.

Hauptziel dieser Studie war die Entwicklung einer wissenschaftlichen Grundlage für effiziente Perlhirsezüchtung in WA, wobei besonders die Ertragssteigerung und eine Verbesserung des Nährwertes im Focus standen. Um solch eine Grundlage zu schaffen, wurden folgende Ziele genauer definiert: (I) Charakterisierung einer umfassenden Auswahl an Perlhirse-Akzessionen aus WA und Bestimmung ihrer Diversität und geographischen Strukturen basierend auf phänotypischen Daten; (II) Identifizierung des Potenzials und Entwicklung von Strategien, um den Mikronährstoffgehalt in WA Perlhirse zu erhöhen; (III) Evaluierung der Ertragsleistung von Populationshybriden und Ableitung erster Strategien für die Perlhirsehybridzüchtung in WA basierend auf Kombinationsfähigkeit und heterotischer Strukturen; und (IV) Identifikation von molekularen Markern für den Locus, welcher für die Wiederherstellung der männlichen Fertilität (*Rf*) im System der A_4 cytoplasmatisch-genetischen männlichen Sterilität (CMS) verantwortlich ist. Hierzu dient eine genetische Karte, die mit Hilfe von ‚genotyping-by-sequencing' (GBS) Techniken generiert wurde.

In drei unabhängig voneinander, groß angelegten und mehr-ortigen Feldversuchen in WA wurde die genetische Diversität für agro-morphologische Merkmale und Kornqualität untersucht und das Ertragspotential der Hybriden gegenüber offen abblühenden Sorten bestimmt. Zusätzlich wurde eine Kopplungsanalyse mit einer F_2-Population durchgeführt. Diese Population spaltete für das Merkmal der männlichen Fertilität bzw. Sterilität auf, die durch das A_4 CMS System ausgelöst wurde. Die wichtigsten Ergebnisse und Schlussfolgerungen dieser Versuche werden im Folgenden zusammengefasst.

Bei der Charakterisierung der umfassenden Auswahl von 360 WA Perlhirse-Landrassen an sechs Standorten wurden weite Variationsbreiten bei 12 agro-morphologischen Merkmalen, wie z.B. Ertrag, Blühzeitpunkt, Rispenlänge, etc., festgestellt, was auf eine enorme Diversität bei den getesteten Landrassen hinweist. Eine Hauptkomponentenanalyse zeigte, dass eine sehr große Diversität innerhalb der WA Länder besteht, insbesondere in Mali und Burkina Faso, und dass WA Landrassen aus verschiedensten Ländern genetisch stark vermischt sind. Diese starke Durchmischung und das Fehlen von eindeutigen Gruppierungen deutet darauf hin, dass die Bildung von heterotischen Gruppen, basierend auf morphologischer oder geographischer Distanz oder anhand des Herkunftslandes, nicht möglich ist. Somit müssen heterotische Gruppen systematisch mit Hilfe von Studien zur Kombinationsfähigkeit entwickelt werden. Einzeln betrachtet, konnten für einige Merkmale geographische Unterschiede festgestellt werden, was die regionalen Präferenzen der Bauern und die hohe Anpassungsfähigkeit von Perlhirse an spezifische Umwelten verdeutlicht. Die veröffentlichten Daten dieser Studie werden nationalen Züchtern die Möglichkeit geben, das getestete Hirse-Material in ihre Zuchtprogramme einzuflechten, um den vielfältigen Bedürfnissen der WA Bauern gerecht zu werden.

Im zweiten Feldversuch wurde der Mikronährstoffgehalt von 72 WA Perlhirse-Landrassen in drei Umwelten untersucht. Hier wiesen der Eisen- und Zinkgehalt im Korn eine signifikante genetische Variation und eine mäßig bis hohe Heritabilität (h^2=0.70 für Eisen, h^2=0.53 für Zink) auf, was auf gute Voraussetzungen für Biofortifikationszüchtung schließen lässt. Es wurden Landrassen identifiziert, die einen hohen und stabilen Eisen- und Zinkgehalt zeigten und somit geeignet sind für Biofortifikationszuchtprograme für WA Perlhirse. Mikronährstoffanalysen und Selektion von weiteren Landrassen oder das Einkreuzen von günstigen Allelen aus nährstoffreichem indischem Material könnte eine weitere Erhöhung des Mikronährstoffgehaltes möglich machen. Auf Grund einer signifikant positiven Korrelationen zwischen Eisen- und Zinkgehalt im Korn und einer nicht-signifikanten Korrelation zwischen Kornertrag und Mikronährstoffgehalt, können bei

der Selektion auf hohen Eisen- und Zinkgehalt beide Nährstoffe erhöht werden, ohne dass negative Effekte für Kornertrag zu erwarten sind.

Im dritten Feldversuch wurde die Ertragsleistung von 100 Populationshybriden und ihren 20 Elternpopulationen (je 4 offen-abblühende Populationen aus 5 verschiedenen WA Ländern) an sechs Standorten getestet. Die Hybriden zeigten durchschnittlich einen Mehrertrag von 16.7% (mit einer Spanne von -26% bis 73%) im Vergleich zu ihren Eltern, was ein enormes Potential durch Hybridzüchtung verdeutlicht. Der durchschnittliche Ertrag von Hybriden, deren Eltern aus verschiedenen Ländern stammen, war nicht signifikant höher als der Ertrag von Hybriden, deren Eltern aus demselben Land stammen. Dies bestätigt das Ergebnis der starken genetischen Durchmischung der Landrassen zwischen Ländern, welches im ersten Versuch beschrieben wurde. Geographische Distanz zwischen Hybrideltern war positiv korreliert mit Kornertrag ($r = 0.31$), aber nicht mit sogenannter „panmictic mid-parent heterosis", was erneut zeigt, dass heterotische Gruppen nicht anhand der geographischen Distanz gebildet werden sollten. Allerdings waren einige Kreuzungen zwischen Akzessionen aus Niger/Nigeria und Senegal herausragend gut, sodass erste Zuordnungen zur mütterlichen oder väterlichen Gruppe mit dieser Information entschieden werden könnten. Auf lange Sicht wird es für eine nachhaltige Perlhirse-Hybridzüchtung erforderlich sein, dass heterotische Gruppen systematisch anhand von Studien zur Kombinationsfähigkeit aufgebaut werden.

In den drei genannten mehr-ortigen Versuchen wurde für einige Merkmale (wie z.B. Ertrag und Eisen- und Zinkgehalt im Korn) ein hoher Effekt der Genotyp × Umwelt-Interaktion identifiziert, was deutlich macht, dass eine Selektion auf Stabilität äußerst wichtig ist. Ein geeigneter Ansatz, um stabile Genotypen zu finden, ist die Identifizierung von wiederholbaren Mega-Umwelten, wobei hierzu mehrjährige und mehr-ortige Versuche sowie kontinuierliches Überprüfen von Umweltbedingungen nötig sind.

Im vierten Versuch wurde für eine F_2-Population, welche für das Merkmal der Fertilitätsrestauration des A_4 CMS-Systems aufspaltet, eine dichte genetische Karte basierend auf Einzelnukleotid-Polymorphismus (SNP) Markern, welche mit GBS generiert wurden, erstellt. Der Hauptlocus des *Rf* Gens wurde in der zweiten Kopplungsgruppe gefunden. Bei der Kreuzvalidierung wurde für den sogenannten quantitativen trait locus (QTL) eine sehr hohe Wiederfindungsrate (97%) ermittelt, was die Lokalität des Allels verifiziert. Der identifizierte QTL beschrieb 14.5% der phänotypischen Varianz, was deutlich unter dem Erwartungswert lag, da das Aufspaltungsverhältnis von männlich-fertilen und männlich-sterilen Pflanzen 3:1 betrug, was auf monogen dominante

Merkmalsvererbung schließen lässt. Es wurden zwei funktionale KASP-Marker für den identifizierten Lokus entwickelt, die zukünftig die Selektion von Genotypen, die das Gen für die Fertilitätsrestauration tragen, vereinfachen. Dadurch wird die Entwicklung von männlichen Hybrideltern beschleunigt und eine ökonomische Hybridsaatgutproduktion durch das funktionale CMS-System ermöglicht. Da die Fertilitätsrestauration im A_4 Cytoplasma sehr selten vorkommt, ist die Identifikation des QTLs ein sehr wichtiger Schritt in Richtung Marker-gestützter Selektion, wovon die Hybridzüchtung in WA sehr profitieren wird.

Zusammenfassend können wir sagen, dass die Perlhirsezüchtung in WA das Potential hat, die Produktivität und den Nährwert dieses Getreides zu erhöhen, indem die enorme Perlhirsediversität in Hybrid- und Biofortifikationszuchtprogrammen genutzt wird. So kann Perlhirse einen großen Beitrag im Kampf gegen Hunger und Mangelernährung leisten. Nichtsdestotrotz werden verbesserte Sorten nur den gewünschten Erfolg bringen und Millionen von WA Kleinbauern erreichen, wenn der lokale Saatgutsektor gestärkt wird und agronomische sowie sozioökonomische Bedingungen verbessert werden.

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10 Acknowledgements

Thank you, apl. Prof. Bettina Haussmann for giving me the chance to write this thesis within your working group, where you created a wonderful working atmosphere. Your way of mentoring was at any time supportive, helpful, open and very friendly. I could always contact you when I had scientific or personal questions, needed your opinion or liked to discuss my work. I could be sure to get an immediate response even though you have so many other obligations. This was incredible. Countless times, I thought about my situation and were more than happy to have so much freedom and trust for my work. This gave me a lot of motivation and appreciation. I am very happy to continue the work with you in the near future, although I am living in Berlin.

Tom Hash, I thank you for your great support during the process of my PhD. You taught me so many things about pearl millet, practically on the field as well as theoretically. I am impressed about your detailed knowledge on this crop, which also improved my papers considerably. Beside your very helpful advices during my time at the research station in Sadoré, you and your lovely wife Deanna made my live in Niamey very enjoyable.

I also thank Prof. Benjamin Stich and Prof. Folkard Asch for their interest in my work and their willingness to read and examine this thesis.

I greatly acknowledge the financial support of the German Federal Ministry of Economic Cooperation and Development (BMZ) and the McKnight Foundation.

I thank the whole pearl millet breeding team at ICRISAT –Niger, namely Prakash Gangashetty, Issa, Hama Adamou, Tahirou Boye, Ada Abarchi, Lankoande Djingri and Kadidia Daouda, for their great work and effort, and their kind welcome. Further, I like to thanks Tondi Akalilou for his pleasant support during my stays at the TVC in Niamey.

Many thanks to all the collaborating millet breeders in West Africa, namely Ousmane Sy, Ignatius I. Angarawai, Jada Gondah, Roger Zangre, Mahamadi Ouedraogo, Moussa D. Sanogo and S. Boureima. Your help made it possible to finalize the field trials and collect the data presented in this thesis.

I thank you Melina Bozkurt for your great support in the laboratory in Hohenheim. It was super that we could discuss initial challenges and got the analysis quickly done.

For the great support during the progress of GBS data analyzing, I thank Jason Wallace and the working group of Rajeev Varshney.

I thank all the friends in Niger, especially Sapna Jarial, for her very friendly hospitality at her house. I *really* enjoyed our discussions, adventurous drives through Niamey and your open personality. Maren Ralf and Miriam Eberle, meeting you was lucky coincidence. Without you, my free time in Niamey would not have been filled with so many lovely and amusing activities.

Wonderful colleges and friends in our working group made my time at the Institute in Hohenheim very enjoyable and productive. Many thanks to Willmar Leiser, Elfadil Bashir, Felix Sattler and Moctar Kante for all the interesting, funny and constructive discussions which we had in our office. Such breaks were often essential to find new ways and ideas.

Many thanks to the Phd students in our institute and all the friends around the campus, who made my time in Hohenheim very enjoyable and inspiring. Especially, I thank Juliane Böhm, Alisa Sieber and Sen Han.

Special thanks to my wonderful parents, who raised me in a fantastic way. You always gave me constant support and valuable advices but also enormous freedom to find my way in live. During my first time in Niger, terroristic attacks caused a very uncertain security situation, which created you many worries and sleepless nights. Your strong appeal to get home earlier was certainly justified.

Last but not least, I want to thank you, Johannes Pucher. You supported me at any time since we know each other, encouraged me to start the PhD, listened to everything I wanted to talk about and tried to push me forward when I was stuck with an issue. I am very grateful that you really tried to understand my topic, so we could have very fruitful discussions.

11 Curriculum Vitae

Personal Data

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Date of birth	15.03.1987
Place of birth	Lippstadt, Germany
Nationality	German
Marietal status	Maried, 1 child (15.05.2016)

Education

Since 10/2012	PhD at the University of Hohenheim / Institute of Plant Breeding, Seed Science and Population Genetics under the supervision of apl. Prof. Dr. Bettina I.G. Haussmann
10/2010 – 10/2012	Master of Science at the University of Hohenheim, Germany Major in Plant Breeding and Seed Science. Theses: "Genomic prediction of northern corn leaf blight resistance in maize with combined or separated training sets for heterotic groups" (published in 2013 in the Journal G3) Two year scholarship from Du Pont Pioneer
10/2007 – 04/2011	Bachelor of Science at the University of Hohenheim, Germany Major in plant production Theses: "Monitoring nutrient flow from uplands to lowlands through the irrigation channel by turbidity sensor calibration in the Chieng Khoi watershed, North - West Vietnam"
2006 – 2007 Olsberg, Germany	Abitur graduation (major: natural science) at the Berufskolleg
2003 – 2006	Technical diploma (Fachabitur) and apprenticeship as Chemical-Technical-Assistant (CTA) at the Berufskolleg Olsberg, Germany
1997 – 2003	School at Realschule Anröchte, Germany. Degree: Mittlere Reife
1993 – 1997	Primary school at Grundschule Mellrich, Germany

Experience

2007	Summer Au Pair in Washington DC, USA (three month)

2009	Internship at the research institute Plant and Food Research in Palmerston North, New Zealand (two month)
2010	Fieldwork in the Son La province in Vietnam to perform the Bachelor thesis (two month)
2011	Internship at the Du Pont Pioneer breeding station in Eschbach, Germany (four weeks)
2012	Tropical Excursion to Costa Rica with the University of Hohenheim (four weeks)
2013 – 15	Fieldwork in West Africa to perform PhD study • several stays at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) research station at Niamey, Niger (in total six month) • at the Institut Sénégalais de Recherches Agricoles (ISRA) research Station in Bambey, Senegal (four weeks)
2014 – 15	Managing board of the graduate student council (Promovierendenkonvent)