General introduction

1.1 Background

Pearl millet (*Pennisetum glaucum* (L.) R. Br., [syn. *Cenchrus americanus* (L.) Morrone]) is a highly cross-pollinated cereal (more than 85% out-crossing) diploid annual (*2n = 2x = 14*) with a large genome size (2450 Mbp). The crop was domesticated 4000 years ago (Brunken et al. 1977). There is dispute among scholars as to whether pearl millet has a single centre of origin, or more than one place of origin, the so-called "non-centres", which would have resulted from domestication processes occurring independently in several regions (Guarino 2012). Accordingly, the whole diffuse belt stretching from Senegal to western Sudan is considered as a center of origin (Harlan et al. 1971; Brunken et al. 1977; Marchais 1994; Oumar et al. 2008). It reached Eastern Africa and India about 3000 years ago and spread to Southern Africa about 2000 years ago (Tostain and Marchais 1993; Khairwal et al. 2007). Based on a number of floral and grain characteristics of pearl millet, Brunken et al. (1977) classified diverse genepools/basic races in which grain shape follows a geographic pattern. These four basic races are: *typhoides*, the most widely distributed; *nigritarum*, generally found in western Sudan to northern Nigeria; *globosum*, the most common race in Benin, Ghana, Niger, central Nigeria and Togo; and *leonis*, specific to Sierra Leone, but also grows in Senegal and Mauritania (Brunken et al. 1977). However, not all pearl millet accessions found in the world collection fit neatly into any of these four basic races.

Pearl millet is one of the most important cereal crops world-wide. It is a staple food crop for about 90 million people living in the semi-arid tropical regions of Africa and the Indian subcontinent (Gulia et al. 2007). Besides being gluten-free, pearl millets have higher nutritional value than other cereals, and are supplying 80 to 90% of the calories for many millions of poor people in semi arid regions (Govindaraj et al. 2009). The crop globally ranks sixth among cereals in terms of area cultivated, after wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), maize (*Zea mays* L.), barley (*Hordeum vulgare* L.) and sorghum [*Sorghum bicolor* (L.) Moench] (FAO 2010). It is grown annually on more than 29 million ha in the arid and semi-arid tropical regions of Asia, Africa and Latin America. According to the Food and Agriculture Organization of the United Nations (FAO 2013) the estimated world production of millets is about 29 million tons per year from about 31 million hectares. Pearl millet is also considered as important forage and stover crop in regions of the United States, Australia, and South America as its vegetative matter provides excellent forage which has low hydrocyanic acid content, and is rich in protein, calcium, phosphorous and other minerals (Chowdari et al. 1998; Gupta,

1975). The crop is mostly grown under harsh environmental conditions on infertile soils of low water-holding capacity, where other cereal crops are prone to fail (Manning et al. 2011). Therefore, the future importance of pearl millet is expected to increase under various climate scenarios (Lane and Jarvis 2007).

1.2 Pearl millet in Sudan

In Sudan, pearl millet is the second most important crop after sorghum in terms of area cultivated and total production. The total area planted in the country in 2011 was about 2.4 million ha with an annual production of about 0.63 million metric tons (FAO 2013), indicating an extremely low yield production. Pearl millet is the preferred staple food for the majority of inhabitants of Western Sudan (Darfur and Kordofan States). Here, pearl millet is mainly produced under rainfed conditions in traditional farming systems where drought is causing substantial yield reduction. In these areas, pearl millet is the most extensively grown crop, and therefore, a millet-based farming system prevails. Malnutrition in children is a serious problem in the region, where dietary deficiencies in iron, zinc, essential amino acids and ß-carotene are commonly observed. Further production constrains are downy mildew (*Sclerospora graminicola*), ergot (*Claviceps fusiformis*), smut (*Moesiziomyces penicillariae*), rust (*Puccinia substriata*), and the parasitic weed *Striga hermonthica* as well as bird attack. In addition there are civil war problems in different parts of the country, particularly in Western Sudan, which lead to huge losses of genetic resources there - many valuable landraces of pearl millet either have been lost or are under serious risk. Although there is a great expected genetic variability among Sudanese pearl millet landraces, the Sudanese pearl millet breeding program, founded in the early 1970s, exploited a narrow gene pool to develop the currently used improved cultivars (Bashir 2006). Expanding pearl millet production in Sudan is of overriding importance and has actually been negotiated to meet the growing demand for food which critically depends on the successful research activities in pearl millet variety improvement and hybrid development programs. The potential of making progress in selection of traits towards higher grain- yielding varieties lies with the high genotypic variability existing among the available pearl millet landraces.

1.3 General challenges in pearl millet breeding

The floral biology of pearl millet is unique among the other major cereals in that the hermaphrodite flowers are protogynous with fully emerged and unpollinated stigma normally remaining receptive for 3 – 4 days. Such a situation makes both crossing without emasculation and selfing convenient

operations in breeding of pearl millet. Several other features that make pearl millet an ideal organism for basic and applied research include a low chromosome number, short life cycle, easy selfing and crossing, plasticity for tillering, high number of seeds per panicle, good transplanting success and a low seed rate (Khairwal et al. 1999). The low productivity of unimproved pearl millet cultivars is generally related to a low harvest index; numerous biotic stresses including diseases, insect pests and striga parasitism, as well as abiotic stress factors such as heat, drought and low soil fertility (Rai et al. 1999; Haussmann et al. 2012). Among these, factors with high relative impact on yield loss are the primary criteria in research prioritization (Rai et al. 1999). However, the probability of success in genetic enhancement can be affected by a complex interplay of several factors, such as availability of genetic resources, inheritance and stability of the traits desired to be improved, simplicity and effectiveness of screening techniques (reliability and cost-effectiveness), access to appropriate test environments as well as the availability of technical manpower and material resources (Rai et al. 1999).

1.4 Pearl millet genetic diversity

Genetic variability plays a vital role in the improvement of target traits since it offers scope for natural and artificial selection to tailor genotypes to better suit diverse agro-ecological conditions. Thus the more genetic variability present in the base material, the better are the chances for improvement. Plant genetic resources are the component of genetic diversity that provide raw material for breeding new varieties of crops better able to cope with biotic and abiotic stresses. The exploitation of genetic resources for crop improvement is one of the most sustainable methods to conserve valuable genetic resources, and to simultaneously increase agricultural production and food security (Haussmann et al. 2004). Therefore, germplasm collection and conservation has become an integral component of pearl millet programs. Several national and international centers throughout the world have been established to conserve the collected pearl millet genetic resources. The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) has consolidated the single largest collection of pearl millet in the world. This collection comprises approximately 22,200 accessions from all over the world, of which about 3.0% are from Sudan (Upadhyaya et al. 2007).

Appropriate estimation and understanding of the pattern of genetic diversity can be extremely useful in crop breeding for diverse applications. Such genetic diversity studies may include assessment of genetic variability in cultivars (Cox et al. 1986), identifying contrasting parental materials to enhance heterozygosity or to optimize the genetic heterogeneity in a hybrid population and hence enhance yield stability in variable and changing climates (Haussmann et al. 2012) and transferring desirable genes

from native or exotic germplasm into the available genetic base through gene introgression (Gupta et al. 1996; Cai et al. 2001; Ali et al. 2010). Moreover, information on genetic distances among inbreds is important for the identification of an essentially derived variety as well as for legal protection of plant varieties or germplasm (Lombard et al. 2000; Nepolean et al. 2012) and for the identification of QTLs that control important agronomic traits (Dudley 1993).

Several methods were used to assess the genetic diversity among the breeding materials of cereal crops; among these techniques which relied on different types of data were: pedigree analysis (van Hintum and Haalman 1994; Mohammadi and Prasanna 2003), morphological characterization (Rai et al. 2009; Bar-Hen et al. 1995); assessment of biochemical data obtained by isozyme analysis (Hamrick and Godt, 1997; Tostain 1994); enzyme diversity analysis (Tostain 1992); storage proteins analysis (Wei-dong et al. 2006) and recently DNA-based markers studies which allow faster and more reliable genotype differentiation and genetic diversity assessment (Koebner et al. 2003; Mohammadi and Prasanna 2003).

Due to its high out-crossing rate pearl millet exhibits a tremendous amount of variation on both phenotypic and genotypic levels (Liu et al. 1994). The more complicated distribution of diversity in pearl millet, as well as the higher degree of marker polymorphisms, will make genetic diversity studies in this crop more complicated than in many other crops such as sorghum a closely related, self-pollinated cereal in which most of the genetic diversity is distributed between rather than within cultivars, and levels of marker polymorphism are relatively low. Thus, the breeding behavior of pearl millet, and the structure of genetic diversity within this species, has strong implications for the use of molecular markers in its diversity assessment (Hash and Bramel-Cox 2000).

1.5 Molecular marker technology

Assessment of genetic diversity based on morphological characteristics might not provide an accurate classification of the genetic divergence among the genetic materials, due to the restricted number of morphological traits evaluated, environmental influences and development-specific trait expression. Molecular genetic markers, based on DNA sequence polymorphism, have been applied successfully to accelerate and refine assessment of genetic diversity among genotypes in a wide range of plant species (Chowdari et al. 1998; Poland and Rife 2012; Chen et al. 2013).

Several types of DNA-based markers are available for assessing the genetic variation and relatedness among cultivars of various crops. They are generally classified into different groups based on mode of

transmission, mode of gene action and method of analysis; group i) hybridization based markers in which the DNA profiles are visualized by hybridizing the restriction endonuclease digested DNA fragment to a labelled probe (DNA fragment of known sequence), group ii) PCR based markers involve in vitro amplification of particular DNA sequences with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme (Sharma et al. 2008). Among these different types, restriction fragment length polymorphism (RFLP) (Liu et al. 1994), random amplified polymorphic DNA (RAPD) (Chandra-Shekara et al. 2007), amplified fragment length polymorphism (AFLP) (vom Brocke et al. 2003), SSRs or microsatellites (Qi et al. 2004; Mariac et al. 2006; Senthilvel et al. 2008), expressed sequence tag (EST) (Senthilvel 2008), inter-simple sequence repeat (ISSR) (Yadav et al. 2007), single nucleotide polymorphism (SNP) (Sehgal et al. 2012), and Diversity array technology (DArT) (Jaccoud et al. 2001; Supryia et al. 2011) have been applied on pearl millet. These markers do vary in their principles and technical requirements, polymorphism detection level as well as in their total cost and time required. Most of these markers are PCR-based markers (e.g. RAPD, AFLP, ISSR, EST and SSR). SSRs are co-dominant markers with multi-allelism, genome specificity, even distribution, high polymorphism level and easy detection and have become markers widely used for a broad spectrum from genetic mapping, QTL analysis, to population and evolutionary studies in many plants, including pearl millet (Qi et al. 2004; Senthilvel et al. 2008; Supryia et al. 2011). The use of fluorescent primers in combination with automatic capillary or gel-based DNA sequencers has been adapted in most advanced laboratories and SSRs are excellent markers for fluorescent techniques, multiplexing and high-throughput analysis (Koumi et al. 2004; Guichoux et al. 2011).

1.6 Pattern of genotype by environment interaction

Global climate change is likely to increase the occurrence and severity of rainfall-related stresses such as drought and flooding (Tuberosa 2012). Unpredictably variable rainfall across different sites and years in a target area may cause genotype by environment (G×E) interactions that need to be considered by the plant breeders in order to identify the respective best genotypes for different locations and to identify genotypes that produce stable performance over years and therefore contribute to food security of smallholder farmers. G×E interactions complicate the process of selecting genotypes with superior performance but also offer the opportunity of exploiting specific adaptation (Ebdon and Gauch 2002). Consequently, multi-environment trials (METs) are widely used by plant breeders to evaluate the relative performance of genotypes in different types of target environment (Delacy et al.

1996). Moreover, there are two types of GE, namely quantitative and qualitative. Quantitative interaction is the change in the magnitude of differences among genotypes in different test environments without any rank changes. Change in rank orders, or crossover interaction, is a qualitative type of interaction and is the most important in plant breeding because it prevents prediction of genotype performance on different locations (genotype by location interaction – GL), during different years (genotype by year interaction – GY) or on both (genotype by location by year interaction – GYL), (Baker 1988). GL (repeatable or non-repeatable) is the varied genotypic response on different locations. Repeatable GL interaction is when genotypes are ranked the same on each site from year to year and can be exploited by growing specifically adapted germplasm or minimized by growing widely adapted material. However, where the GL interaction variance component is smaller than the genotypic variance, the advantage of breeding for specific adaptation is reduced. The repeatability or non-repeatability of GL interaction is determined by multi-location trials repeated in time. GY is the varied response of genotypes across years and is not exploitable as it is not possible to predict future climatic conditions (Annicchiarico 2002; Pswarayi et al. 2008).

Furthermore, for target regions where major climatic characteristics show high year-to-year variation, a wide adaptation strategy is frequently caused by, and has to cope with, large GY interaction effects within locations. Even for carefully chosen selection sites, environments in individual years may frequently misrepresent the target population of environments, thereby failing to adequately reproduce the mean responses of genotypes across the region. This may result in low selection gains, especially when using just a few selection environments (Cooper et al., 1996; Annicchiarico, 2002).

In general, stability parameters are employed to describe the adaptation behavior of genotypes in diverse environmental conditions. Most of the models used in the stability studies (e.g. regression coefficient (*bi*) (Finlay and Wilkinson 1963) and deviation from regression (S^2_{di}) (Eberhart and Russell 1966)) are based heavily on the assumption that a positive linear correlation exists between the improved growing conditions and performances of genotypes. Two major concepts of yield stability have been distinguished in relation to G×E interaction; 1) the stable genotype maintains constant yield across different environments (“static” stability concept); 2) the response of a stable genotype to environments is parallel to the mean response of all genotypes in the trial (“dynamic” stability concept) (Becker and Léon 1988). Understanding the patterns of G×E interactions in a target region can help the breeder to define the appropriate selection and testing strategies, and to select genotypes with specific adaptation to different mega-environments within the target area, and/or yield stability over years.

1.7 Nutrition deficiency and biofortification

Nutrition security means access to the adequate utilization and absorption of nutrients in food, in order to be able to live a healthy and active life (FAO 2009). Micronutrient malnutrition, the so-called "hidden hunger", is increasingly being recognized as a serious public health problem affecting more than half of the population worldwide, with preschool children and women in the developing countries being most vulnerable (UN SCN 2004). Most widespread are dietary deficiencies in iron (Fe), zinc (Zn) and vitamin A (ß-carotene). These nutritional deficiency problems have enormous socioeconomic impacts at the individual, community and national levels (Darnton-Hill et al. 2005; Stein 2010). They have an immense impact on the health of the population (with high social and public costs), learning ability (with a vast loss of human potential) and productivity (with greatly reduced work capacity). Malnutrition in children is a serious problem in Sudan, especially in regions where dietary deficiencies in iron, zinc, essential amino acids and ß-carotene are commonly observed.

Pharmaceutical supplementation, industrial food fortification, and agricultural approaches of dietary diversification and biofortification have been suggested to address these problems. Biofortification of staple crops is the most cost-effective, sustainable, consumer acceptable, pro-rural and pro-poor intervention (Rai et al. 2013). Therefore, nutrient density traits must be transferred to high-yielding cultivars. These efforts are called 'biofortification' because they refer to the bioavailable micronutrient content of food crops enhanced through genetic improvement. Biofortification, is a new approach that relies on conventional plant breeding and modern biotechnology to increase the micronutrient density of staple crops and holds great promise for improving the nutritional status and health of poor populations in both rural and urban areas of the developing world (Graham et al. 2001; Pinstrup-Andersen 2000; Underwood, 2000; Bouis, 2003). For biofortification to be successful, three important things must be considered; the possibility of breeding to increase the micronutrient density in staple food to a level that will have a significant impact on nutritional status, the bioavailability and absorption of the extra nutrients bred when consumed under controlled conditions and the acceptance and adoption of the biofortified varieties by farmers and consumers (Howarth et al. 2011). To have a high adoption and maximum impact, high-yielding genotypes with excellent, farmer-preferred grain quality are needed (Graham et al. 2001).

Crop improvement activities related to biofortification focus, first, on exploring the available genetic diversity for micronutrients such as Fe, Zn, and ß-carotene (provitamin A) to identify parental genotypes that can be used in crosses, genetic studies, molecular marker development, and parent-

building. Similarly, existing varieties, pre-varieties in the release pipeline, or promising germplasm should be identified for "fast-tracking." Fast-tracking means releasing, commercializing, or introducing genotypes that combine the target micronutrient density with the required agronomic and end-use traits so they can be quickly delivered to producers and have immediate impact on micronutrient-deficient populations (Pfeiffer and McClafferty 2007).

1.8 Research objectives

Any successful breeding program relies on using promising sources of characterized germplasm. Accordingly, the overall goal of the present research investigation was to enhance pearl millet diversification and breeding efficiency for increased yield and grain nutritional stability in Sudan. In particular, the objectives of this research study were to:

i. characterize pearl millet germplasm from Sudan for agro-morphological traits and macro- and micronutrient concentrations (including Fe, Zn and β-carotene),
ii. study the relationship between agro-morphological and grain nutrient traits and the geographic distribution of pearl millet phenotypic diversity in Sudan,
iii. determine the level of molecular genetic diversity and structure of studied pearl millet germplasm across a range of collection regions and define potential distinct groups with the use of SSR markers,
iv. analyze patterns of G×E interaction for grain yield, Fe and Zn concentrations of the studied pearl millet accessions across different test sites in Sudan; and
v. identify stable and superior genotypes for grain yield and high grain Fe and Zn concentrations for the target regions in Sudan and for future pearl millet improvement programs in Sudan and other pearl millet growing regions.

1.9 References

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