Sorghum as a Novel Crop for Central Europe: Using a Broad Diversity Set to Dissect Temperate-Adaptation

André Schaffasz †, Steffen Windpassinger *,†, Wolfgang Friedt, Rod Snowdon and Benjamin Wittkop

Department of Plant Breeding, Research Center for BioSystems, Land Use and Nutrition (IFZ), Heinrich-Buff-Ring 26–32, 35392 Giessen, Germany; andre.schaffasz@agrar.uni-giessen.de (A.S.); wolfgang.friedt@agrar.uni-giessen.de (W.F.); rod.snowdon@agrar.uni-giessen.de (R.S.); benjamin.wittkop@agrar.uni-giessen.de (B.W.)
* Correspondence: steffen.m.windpassinger@agrar.uni-giessen.de; Tel.: +49-(0)641-99-37443; Fax: +49-(0)641-99-37429
† These authors contributed equally to this work.

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Abstract: Sorghum (Sorghum bicolor L. Moench) is a promising novel crop for Central Europe. However, enhancements in cold tolerance and early maturity are essential for a successful adaptation to cooler climates. We scored a broad sorghum diversity set (n = 338) for early chilling tolerance, high-latitude adaptation, and bioenergy related agronomical traits in multi-environment trials. Our results show a high phenotypic variation and medium to high heritabilities for most traits, indicating that a robust breeding progress is feasible. Several public accessions with a good adaptation to cooler climates were identified, which can serve as valuable base material for sorghum breeding in temperate areas. Genome-wide association studies reveal a polygenic (quantitative) character for most of the traits, confirming previous studies. Hence, for practical breeding, it will be difficult to conduct efficient marker-assisted selection for temperate-adaptation traits in genetically diverse material.

Keywords: sorghum breeding; cold tolerance; temperate-adaptation; sorghum germplasm resources; GWAS; bioenergy crops

1. Introduction

Sorghum (Sorghum bicolor L. Moench, 2n = 20) is one of the most important cereals of the world, ranking fifth in terms of global production. It is important as a stress-tolerant staple crop in Africa and India, and represents an important commodity for feed, export, and bioenergy in countries like the USA, Mexico, Argentina, and Australia. The availability of its complete genome sequence [1] holds the potential for a more efficient exploitation of its huge genetic diversity [2–4] to accelerate breeding progress and yield gains, which have substantially lagged behind other crops during the last decades [5].

In the context of climate change, the resilient and versatile sorghum [6] is expected to be of increasing importance also in temperate areas such as Central Europe. It may be a valuable alternative to maize as a biogas and fodder crop, owing to its higher drought tolerance, nutrient efficiency, and Diabrotica resistance. Breeding progress in chilling tolerance and early maturity is essential to make it competitive for farmers [7,8]. Being an originally tropical C₄ plant, the sensitivity of sorghum to temperatures below 15 °C [9] substantially delays the sowing time on the expense of growth period and yield potential. Chilling stress has detrimental effects on nearly all physiological and developmental processes in juvenile sorghum plants, affecting both heterotrophic (emergence) and autotrophic growth (photosynthesis and root development) [10,11]. Fortunately, a broad variation for chilling tolerance
among different germplasms has been observed [12–14], with tolerant accessions originating, for example, from China or tropical highlands. Although, selection for chilling tolerance among breeding lines is complicated because of unpredictable weather from year to year, resulting in too harsh or too mild conditions for a proper selection, as well as unstable correlations between controlled environment and field conditions [11,15].

Several studies have aimed at the identification of quantitative trait loci (QTL) for a better understanding of the physiological mechanisms behind chilling tolerance related traits, either in bi-parental populations [10,16,17] or diversity sets: 194 biomass lines from KWS company (Einbeck, Germany) [7,18–20]; 242 accessions of the ICRISAT (International Crops Research Institute for the Semi-Arid Tropics) sorghum mini core collection [21]; 136 sorghum accessions from cooler regions of the world [14]; and 300 sorghum accessions from the U.S. sorghum association panel [22]. Genomic regions influencing traits of interest have been concordantly confirmed in these studies.

Besides chilling tolerance, early flowering is a crucial trait for adaptation into higher latitudes, allowing for enough time to reach an adequate maturity before the first frosts in fall. Photoperiodism of sorghum is controlled by four major maturity loci, designated $Ma_1$, $Ma_2$, $Ma_3$, and $Ma_4$, where dominant alleles cause late flowering [23,24]. More recently, two additional, epistatic maturity genes were described ($Ma_5$ and $Ma_6$ [25]). Out of these loci, $Ma_1$ (cloned by the authors of [26]) has the largest impact [27], and a recessive mutation at this locus alone, which occurred independently in different parts of the world, is sufficient to confer some temperate-adaptedness [28]. However, among genotypes with the same configuration of $Ma$-alleles, flowering time is considered largely additive, with a small degree of dominance for early flowering observed in hybrid combinations [29], and several QTL were detected in studies on nested association mapping (NAM)-populations [30], bi-parental populations [31,32], and a diversity panel [33]. When the intended use of sorghum is whole-plant silage (e.g., for cattle feeding or biogas), it is important that at least 28% dry matter content is reached at harvest time, representing a major challenge in high-latitude environments with a short growing season and low insolation. Hence, the whole-plant dry matter fraction attained by the end of the growing period is a more valuable indicator for maturity than days to flowering, even if a relation between these two traits can be expected. To our best knowledge, no QTL study has aimed at this particular trait yet.

Plant height, in the case of sorghum mainly determined by four unlinked major dwarfing loci ($Dw_1$, $Dw_2$, $Dw_3$, $Dw_4$) [34] being incompletely dominant for tallness [35], is considered a good predictor for biomass yield [36–38]. Even though this concept has to be relativized with medium-height, early dual-purpose, or silage types, which are the preferable ideotype for biogas generation in Central Europe [8]. Nevertheless, in these dual-purpose types with a higher proportion of grains, stems still account for around 40% of total biomass [8]. Because these ideotype plants should not be too tall, enhancements of the stem as a major yield component can mainly be achieved via selection for its diameter. Zhao et al. [33] found this trait to be highly quantitative with several small-effect QTL. Regarding stem sugar content (expressed as brix), QTL have been detected in several studies on both bi-parental populations [32,36,39–42] and genome-wide diversity panels [38,43]. However, for the particular use of sorghum for biogas generation in temperate environments, juicy stems, going on the expense of dry matter content and yield, do not fit the described ideotype. However, some soluble sugars in a predominantly dry stem contributing to a high digestibility seem to be desirable, as long as no strong sink competition for seed yield is effected, and in a recent study, Shukla et al. showed that a high sugar concentration is not conditional on the plants being tall [44].

In the present study, we composed a broad diversity set ($n = 338$) for extensive phenotyping regarding crucial high-latitude adaptation traits of sorghum, such as early-stage chilling tolerance and juvenile development (under both controlled and field conditions), early maturity (flowering and whole-plant dry matter content), and suitable bioenergy related traits (plant height, stem diameter, and brix). Aiming at a contribution to temperate-adaptation breeding of sorghum, the goals of this study are (1) to assess the extent of variation for these traits in multiple-environment trials in Central
Europe, analyzing genotype x environment interactions, heritability, and trait correlations; (2) to identify suitable accessions with a good local adaptation for breeding; and (3) to find marker–trait associations by genome-wide association studies (GWAS) and compare them with the results of the aforementioned studies.

This is the first study analyzing both agronomical and cold tolerance traits on a broad, publicly available diversity set under Central European environmental conditions.

2. Materials and Methods

2.1. Germplasm

A *Sorghum bicolor* diversity set comprising 338 lines of different origin, subspecies, and use was utilized for the present study. The original germplasm was mainly received from the United States Department of Agriculture–Agricultural Research Service (USDA-ARS). A representative selection of sorghum conversion lines, obtained in the 1960s by repeated backcrossing of genetically diverse tropical accessions to a short, photoperiod-insensitive cultivar [45], accounts for 65% (*n* = 220) of the set. These conversion lines are short or medium statured and photoperiod-insensitive owing to the selection for recombinants with recessive *dw* and *ma* alleles, but otherwise closely related to their exotic progenitors. Hence, they represent a valuable and widely used base material for sorghum breeding in temperate areas [46,47]. The uniformity of the conversion lines used in this study was improved over four cycles before the start of the experiments. Some lines showed a greater heterogeneity for traits like earliness or plant height from the outset; in these cases, up to three sublines were selected per accession. However, only sublines whose genetic distance (Rogers distance) to each other was higher than the threshold of 6.8% (mean Rogers distance among respective sublines) were included in the diversity set. The remaining part of the diversity set consists of breeding lines for dual-purpose, forage, or sweet sorghum from different parts of the world, including *n* = 55 brown-midrib (*bmr*) lines. These *bmr*-lines are of special interest for biogas or cattle feeding purposes, owing to their reduced lignin content and higher digestibility, provided that their agronomical performance is satisfactory [48]. Considering the origins of the conversion line progenitors, our set contains accessions from a total of 26 countries. Furthermore, 88 lines of this set are also part of the U.S. sorghum diversity panel [49]. The composition of the diversity set, and all phenotypic data collected in this study, are shown in detail in Tables S1–S3, Supplementary material.

To exclude the influence of different production environments on seed traits, all seeds used for chilling stress experiments (both controlled environment and field trials) were taken from one seed lot, which was produced in a winter nursery in Puerto Vallarta, Mexico, under optimal conditions and harvested in April 2013. Further, seeds were treated with MaximXL™ (Syngenta, agent fludioxonil) at the label rate to avoid fungal infections distorting the results.

2.2. Phenotyping

Controlled Chilling Stress Experiment

Controlled chilling stress (13 °C day/10 °C night; day/night period set at 14 h/10 h to resemble actual Central European conditions in late spring) was applied on the diversity set in a climate chamber, using the experimental setting of a randomized complete block design (RCBD) with four replications. A total of 32 seeds were sown in approximately 2 cm depth in pots (12 × 12 × 12 cm, one pot per replication) filled with sterilized sand. Adequate plant nutrition was provided by applying Murashige & Skoog Basal Salt Mixture™ (Duchefa Biochemie B.V., Haarlem, The Netherlands) as fertilizer (4 × 100 mL of 0.25 × MS). The number of emerged seedlings was counted each day, and 28 days after sowing, the final emergence was scored. The emergence index (EI; low values indicate a fast and complete emergence) was calculated as described by Smith and Millet [50] using the following formula:

$$EI = \frac{\sum (E_j \times D_j)}{E}$$

(1)
where $E_j$ is the number of newly emerged plants on day $j$, $D_j$ is the days after planting, and $E$ is the final emergence. The experiment was finalized after 28 days. Leaf greenness was visually scored (1 white, complete chlorophyll degradation; 5 medium chlorosis; 9 dark green, no chlorosis), after which the seedlings were harvested and rinsed. The total shoot and root matter of each replication was dried for several days at 70 °C, weighed, and divided by the number of emerged plants to determine shoot (SDW) and root (RDW) dry weight per plant.

2.3. Field Experiments for Chilling Stress and Juvenile Development

To score the variation for emergence and juvenile development under field conditions, experiments were conducted at two sites in Germany during the years 2013 and 2014. Poel (PL), a small island near Wismar in the Baltic Sea, is characterized by a maritime climate with delayed warming in spring and light soils (loamy sand). Giessen (GI), located in the Lahn river valley in Hesse (Germany), has heavy clay soils and higher daily temperature amplitudes (Tables 1 and 2). In 2013, the trials were sown at the recommended (‘normal’) planting dates for sorghum and chilling stress was only light, while in 2014, sowing was approximately one month earlier, resulting in notably harsher growth conditions. At both sites, a RCBD with two replications was used. Entries were grown in single-row plots (2.5 × 0.5 m) in GI and double-row plots (2.5 × 1.0 m) in PL, with 0.5 m row spacing, 50 seeds per row, and 2 cm sowing-depth at both sites. Fertilizer and herbicide applications were executed following good agronomical practice. Approximately four weeks after sowing, final emergence was counted and 10 representative plants per plot were harvested by hand (around 1 cm above ground) and dried overnight at 105 °C to determine the shoot dry weight per plant (SDW).

Table 1. Climate data of the field trial sites during the duration of the respective experiments for emergence and juvenile development.

| Site  | Year | Trial Dates (Sowing-Harvest) | Mean Temp. (°C) | Mean Max. Temp. (°C) | Mean Min. Temp. (°C) | Absolute Max. and Min. Temp. (°C) | Mean Soil Temp. (°C) | Min. Soil Temp. (°C) | Precipitations (mm) |
|-------|------|-----------------------------|------------------|----------------------|----------------------|----------------------------------|---------------------|-------------------|---------------------|
| Giessen (GI) | 2013 | 5 June–5 July | 17.4 | 23.3 | 11.5 | 34.5/5.1 | 20.5 | 17.7 | 44 |
|       | 2014 | 6 May–5 June | 13.8 | 19.8 | 7.8 | 28.5/2.3 | 17.3 | 14.2 | 68 |
| Poel (PL) | 2013 | 5 June–6 July | 16.5 | 20.7 | 12.3 | 31.7/8.3 | 18.8 | 15.6 | 79 |
|       | 2014 | 6 May–10 June | 15.1 | 19.4 | 10.8 | 30.3/6.7 | 16.1 | 10.4 | 93 |

Table 2. Geographical data of the different experimental locations.

| Site                  | Coordinates  | Altitude | Soil Type   | Day Length at Summer Solstice |
|-----------------------|--------------|----------|-------------|------------------------------|
| Poel (PL)             | 53°99′ N, 11°47′ E | 19 m    | Loamy sand  | 17 h 10 min                  |
| Rauischholzhausen (RH)| 50°46′ N, 8°53′ E  | 270 m   | Loam        | 16 h 31 min                  |
| Giessen (GI)          | 50°35′ N, 8°41′ E  | 158 m   | Clay        | 16 h 29 min                  |
| Gross-Gerau (GG)      | 49°55′ N, 8°29′ E  | 90 m    | Sand        | 16 h 22 min                  |

2.4. Field Experiments for Maturity and Bioenergy Related Agronomical Traits

Adaptation traits beyond juvenile development were scored in a total of seven environments. At Poel (2013 and 2014), the previously described experiments for juvenile development were continued over the whole vegetation period. Further, the diversity set was planted at the experimental fields of Gross-Gerau (GG; 2012, 2013 and 2014) and Rauischholzhausen (RH; 2013 and 2014) in an unreplicated RCBD with double-row plots (2.5 × 1.5 m, 0.75 m row spacing, and 50 seeds per row at both sites). Geographical and climate data of the locations are shown in Tables 1 and 2, respectively. Gross-Gerau, as the southernmost and warmest location, has the most favorable conditions for sorghum. It is located around 25 km SW of Frankfurt/M. in the Upper Rhine Valley, which enjoys the highest temperatures in Central Europe. In contrast, Rauischholzhausen, located in a low mountain range landscape of Central Hessen, has a rather cool, continentally-influenced climate and provides harsh conditions for sorghum.
Besides its already mentioned maritime climate, Poel is of special interest as the northernmost (54° N) location with the longest day length (17 h 10 min during summer solstice).

In contrast to juvenile development, the agronomical data described below (Table 3) were scored only in one plot. Flowering (days to flowering, DTF; expressed as days from sowing to flowering) was scored when 10% of the plot had commenced anthesis. Brix, which describes the percentage of soluble solids (i.e., sugars) in stalk juice, was measured with a hand-held refractometer (Arcarda, Reichelsheim, Germany; Type REF 111/112/113) on three plants per plot (considered as replicates) by squeezing the fourth node from the ground. The timing for the brix measurements was 40 days after anthesis, because sugar accumulation has been reported to reach its optimum at that stage [37]. Stem diameter (SD) was scored on the same three plants as used for brix around 20 cm above ground. Plant height (PH) was measured at the end of the growing season from the ground to the panicle tip on plot basis. Whole-plant dry matter content (DMC) was assessed at the end of the growing period in the first October decade. For this purpose, approximately 20 plants per entry were cut at 3 cm above ground, weighted, milled, and dried at 105 °C for several days and re-weighted.

Table 3. Overview of the field experiments scoring maturity and agronomical traits. DTF, days to flowering; PH, plant height; DMC, dry matter content; SD, stem diameter.

| Site               | Year | Traits Scored | Sowing Date | Climate Data from Sowing Until 10 October |
|--------------------|------|---------------|-------------|------------------------------------------|
| Poel (PL)          | 2013 | DTF, PH       | 5 June      | Mean Max. Temperature (°C) | Mean Min. Temperature (°C) | Precipitations (mm) |
|                    | 2014 | DTF, PH       | 6 May       | 21.0                                      | 12.5                        | 195                   |
| Rauischholzhausen (RH) | 2013 | DTF, PH, DMC  | 11 June     | 22.2                                      | 9.1                         | 240                   |
|                    | 2014 | DTF, PH, DMC  | 21 May      | 22.6                                      | 11.1                        | 424                   |
| Gross-Gerau (GG)   | 2012 | DTF, PH, Brix, SD, DMC | 29 May | 24.3                                      | 11.6                        | 338                   |
|                    | 2013 | DTF, PH, Brix, SD, DMC | 4 June | 24.4                                      | 12.1                        | 275                   |
|                    | 2014 | DTF, PH, Brix, SD, DMC | 20 May | 25.0                                      | 11.8                        | 330                   |

2.5. Statistical Analysis of Phenotypic Data

An analysis of variance (ANOVA) was performed for each trait using the following general linear model, in which genotypes and environments were considered as fixed and replicates as random effects:

\[ Y_{ijk} \sim \mu + G_i + E_j + GE_{ij} + R_{kj} + e, \]  

(2)

where \( \mu \) represents the population mean, \( G_i \) is the genotypic effect, \( E_j \) is the environmental effect, \( GE_{ij} \) is the genotype-by-environment interaction, \( R_{kj} \) is the replicate effect, and \( e \) is the residual effect.

To compare the levels of genotypic variance obtained in the different environments, ANOVA was also computed separately for each environment, using the following general linear model, where genotypes were considered as fixed and replicates as random effects:

\[ Y_{ij} \sim \mu + G_i + R_j + e. \]  

(3)

For the traits DTF, DMC, and PH, which were scored on a plot basis and thus lacked degrees of freedom in one experiment, the years at one specific location were taken as replicates and the locations as environments. For the other traits, one location in one year was considered an environment, and the controlled chilling stress experiment was also seen as a separate environment.

The heritability was calculated as proposed by Piepho & Möhring [51] using the following formula:

\[ H^2 = \frac{\sigma^2_G}{\sigma^2_G + \frac{1}{2} \sigma^d}. \]  

(4)
where $h^2$ represents broad-sense heritability, $\sigma^2_G$ is the genotypic variance calculated by a random effect model considering genotype and environment as random factors, and $\bar{d}$ is the average variance of the difference between two means.

The phenotypic stability of the best performing inbred lines for selected traits was analyzed by the weighted sum of the first and second principal component, using the R package agricolae [52].

### 2.6. Analysis of Genotypic Data

For all entries of the diversity set ($n = 338$), genomic DNA was isolated from young leaves using the extraction machine BioSprint 96 (Qiagen, Hilden, Germany). Genotyping was conducted with a *Sorghum bicolor* 3K-Illumina single nucleotide polymorphism (SNP) array comprising 2620 SNPs [53]. SNP-positions were converted from *Sorghum bicolor* version 1.4 (as used by the authors of [53]) to the most recent version v3.1.1 using http://ensembl.gramene.org and 2593 SNPs left. Data pre-processing and a genome-wide association analysis (GWAS) were performed using the statistical software R studio version 1.0.136 package GenABEL. SNPs with more than 10% missing data and a minor allele frequency <5% were excluded. After pre-processing, 1507 SNPs and 338 individuals remained. For the GWAS, we used a mixed model combined with principal component adjustment based on the first three principal components [54–56]. All SNPs with a $-\log_{10} (p$-value) $> 3$ were considered as significant, and Bonferroni correction was applied to find highly significant SNP-trait association.

Analysis of phylogenetic relatedness was conducted with TASSEL version 4.3 [57] using the neighbor-joining method [58], and Dendroscope 3.5.9 [59] was utilized to visualize the phylogram.

### 3. Results

#### 3.1. Phenotypic Variation for Chilling Tolerance and Juvenile Development

Highly significant differences among entries were observed for all traits in all environments (Tables 4 and 5). For the field trials, year effects were highly significant owing to the earlier sowings in 2014 compared with 2013. The experiment in GI 2014 had the harshest conditions with temperatures close to freezing on some nights (Table 1), resulting in a seven-fold reduction of SDW as compared with GI 2013. In contrast, at PL, there was no significant difference for SDW between 2014 and 2013, but average emergence was 20% less in 2014. Even though G × E interactions were also highly significant, their variance was notably lower than the total genotypic variance (Table 6). We observed heritabilities of 0.74 for emergence and 0.41 for SDW. The heritability for SDW is the lowest among the traits scored in the present study and underlines the strong G × E interaction.

#### Table 4. Genotypic variances (mean squares, MS) and descriptive statistical traits for the different field experiments scoring chilling tolerance and juvenile development. Genotypic variances were computed using a general linear model in which genotypes were considered as fixed and replicates as random effects (see Materials and Methods).

| Experiment | df | Emergence (%)        | Shoot Dry Weight (mg) (SDW) |
|------------|----|----------------------|-----------------------------|
|            |    | MS       | Error | Mean | Min | Max | MS       | Error | Mean | Min | Max |
| GI 2013    | 289| 382.3 *** | 191.2 | 56.3 | 22.5 | 87.5 | 15166.0 *** | 9097.4 | 215.1 | 43.4 | 1044.5 |
| PL 2013    | 288| 177.3 *** | 111.4 | 64.1 | 32.5 | 86.5 | 19299.4 *** | 9212.6 | 270.2 | 55 | 640 |
| GI 2014    | 337| 558.8 *** | 82.7 | 54.4 | 6 | 88 | 139.7 *** | 53.2 | 32.1 | 6.7 | 56.3 |
| PL 2014    | 337| 471.8 *** | 179.9 | 43.8 | 2.5 | 83 | 17210.5 *** | 10265.4 | 251.3 | 60 | 760 |

Significance level: *** 0.001.
Table 5. Genotypic variances (mean squares, MS) and descriptive statistical traits for the climate chamber experiment (df = 329). Genotypic variances were computed using a general linear model in which genotypes were considered as fixed and replicates as random effects (see Material and Methods).

| Trait                  | MS       | Error | Mean  | Min  | Max  |
|------------------------|----------|-------|-------|------|------|
| Emergence (%)          | 1222.4***| 135.2 | 80.2  | 17.2 | 100  |
| Emergence Index (EI)   | 18.9***  | 4.5   | 17.7  | 12.5 | 25.5 |
| Shoot dry weight (SDW) (mg) | 7.9***  | 0.9   | 5.6   | 2.1  | 9.2  |
| Root dry weight (RDW) (mg) | 6.3***  | 1.1   | 3.3   | 1.1  | 12.1 |
| Leaf Greenness         | 7.8***   | 0.6   | 5.2   | 1    | 8.3  |

Significance level: *** 0.001.

Table 6. Variances (mean squares) and heritability for emergence and SDW over all five environments. G, E, G × E, and error-variance were computed using a general linear model in which genotypes and environments were considered as fixed and replicates as random effects. For heritability estimation, a fully random model was applied (see Materials and Methods).

| Source      | df | Emergence     | Shoot Dry Weight |
|-------------|----|---------------|------------------|
| Genotype (G)| 337| 1209.2***     | 16921.4***       |
| Environment (E) | 4 | 176414.4***  | 12773831.5***    |
| G × E       | 1243 | 342.5***     | 9250.2***        |
| Error       | 2245 | 138.2        | 3915.1           |
| h²          | 0.74 | 0.41         |

Significance level: *** 0.001.

All collected phenotype data of the diversity set regarding chilling tolerance and juvenile development are shown in detail in Table S2, Supplementary materials.

Pearson’s correlations among traits and environments (Figure 1) were significant in most cases (57 out of 78), but generally only low to medium. Higher correlations between the two locations GI and PL (r = 0.70 *** for emergence and 0.49 *** for SDW) were observed under the early sowings of 2014. In contrast, under the less stressed conditions of 2013, these correlations were lower, and also the correlations between 2013 and 2014 at one location were relatively low. The correlation between the climate chamber and field experiments differed depending on the level of stress in the latter ones. Emergence and SDW of the climate chamber showed the highest correlations to GI 2014 (r = 0.44 *** and 0.49 ***), which had the most similar stress conditions, while the correlations to the other environments were lower, but still significant. RDW scored in the climate chamber displayed only weak correlations to field emergence and SDW. Also, leaf greenness evaluated after four weeks at constant 13/10 °C did not show a relation to the field trials, which had notably higher daily temperatures.

3.2. Phenotypic Variation for Maturity and Bioenergy Related Agronomical Traits

The present diversity set showed a high variation for maturity and bioenergy related traits as well (Tables 7–9). Highly significant differences for days to flowering (DTF) were observed at GG and PL, but surprisingly not at RH, which may be attributed to the conditions there being quite different in 2013 compared with 2014. The late sowing at RH in 2013, followed by adverse conditions (drought and an unusual cold spell by end of June), apparently induced a different reaction in time to flowering among the genotypes as compared with 2014, where the sowing was earlier and conditions for sorghum more favorable. As expected, the flowering in the cooler environments of RH and PL was substantially delayed compared with the warmer environment of GG (98 and 102 DTF vs. 78 DTF on average, respectively). Moreover, the number of inbred lines not reaching anthesis increased in the cooler environments (11% at RH and 6% at PL vs. 2% at GG). Concordantly, the dry matter content at harvest (DMC) was significantly lower at RH compared with GG (24.6% vs. 29.3% on average).
The maturity traits DTF and DMC were negatively correlated as expected (Figure 2), but the magnitude of their relation differed between GG (\( r = -0.56 \) *** and RH (\( r = -0.29 \) ***).

All collected phenotype data of the diversity set regarding maturity and bioenergy related traits are shown in detail in Table S3, Supplementary material.

The maturity traits DTF and DMC were negatively correlated as expected (Figure 2), but the magnitude of their relation differed between GG (\( r = -0.56 \) *** and RH (\( r = -0.29 \) ***).
Table 7. Genotypic variances (mean squares, MS) and descriptive statistical traits for days to flowering, dry matter content, and plant height, separately for the different environments. Genotypic variances were computed using a general linear model in which genotypes were considered as fixed and replicates as random effects (see Material and Methods).

| Environment | Days to Flowering (DTF) | Dry Matter Content (%) (DMC) | Plant Height (cm) |
|-------------|-------------------------|-----------------------------|------------------|
|             | df | MS | Error | Mean | Min | Max | df | MS | Error | Mean | Min | Max | df | MS | Error | Mean | Min | Max |
| GG          | 329| 211.7 *** | 119.6 | 77.1 | 51 | 111 | 334 | 30.8 *** | 6.4 | 29.2 | 18.9 | 45.6 | 329 | 3459.9 *** | 352.5 | 136 | 43 |
| RH          | 298| 205.5 | 239.4 | 96 | 65 | 129 | 295 | 13.8 *** | 6.0 | 24.7 | 16.6 | 37.5 | 298 | 3895.7 *** | 505.6 | 136 | 42 |
| PL          | 317| 192.2 *** | 72.3 | 100 | 67 | 130 | - | - | - | - | - | - | 329 | 4265.0 *** | 507.8 | 142 | 60 |

Significance level: *** 0.001.

Table 8. Genotypic variances (mean squares, MS) and descriptive statistical traits for brix and stem diameter, separately for the different environments. Genotypic variances were computed using a general linear model in which genotypes were considered as fixed and replicates as random effects (see Material and Methods).

| Environment | Brix (%) | Stem Diameter (cm) (SD) |
|-------------|----------|-------------------------|
|             | df | MS | Error | Mean | Min | Max | df | MS | Error | Mean | Min | Max |
| GG 2012     | 321| 52.4 *** | 4.0 | 12.4 | 3.3 | 21.3 | 321 | 0.42 *** | 0.04 | 1.75 | 0.7 | 2.77 |
| GG 2013     | 333| 41.7 *** | 3.3 | 13.5 | 3.3 | 21.3 | 374 | 0.42 *** | 0.04 | 1.63 | 0.77 | 2.7 |
| GG 2014     | 337| 45.8 *** | 3.2 | 13.7 | 4 | 22.3 | 378 | 0.39 *** | 0.03 | 1.63 | 0.63 | 3.13 |

Significance level: *** 0.001.

Table 9. Variances (mean squares, MS) and heritability for agronomical traits over all environments. G, E, G x E, and error-variance were computed using a general linear model in which genotypes and environments were considered as fixed and replicates as random effects.

| Source | DTF | Dry Matter Content | Plant Height | Brix | Stem Diameter |
|--------|-----|--------------------|--------------|------|---------------|
|        | df | ms | df | ms | df | ms | df | ms | df | ms | df | ms | df | ms |
| Genotype (G) | 334 | 397.9 *** | 337 | 37.5 *** | 334 | 9307.7 *** | 337 | 91.4 *** | 337 | 0.93 *** |
| Environment (E) | 2 | 110119.4 *** | 1 | 7489.0 *** | 2 | 22511.8 *** | 2 | 412.1 *** | 2 | 5.47 *** |
| G x E | 610 | 89.0 | 337 | 7.0 | 610 | 533.4 ** | 654 | 23.4 *** | 654 | 0.14 *** |
| Error | 614 | 141.4 | 863 | 6.4 | 614 | 454.5 | 1987 | 3.5 | 1987 | 0.04 | 0.80 | 0.83 | 0.96 | 0.72 | 0.82 |

Significance level: *** 0.001; ** 0.01.
Late maturity was associated with higher brix values ($r = 0.53^{***}$ for DTF and $r = -0.30^{***}$ for DMC), SD, and tallness. While brix was correlated with tallness ($r = 0.55^{***}$), no relation to SD was observed.

3.3. Adaptation to Cooler Climates

To identify useful lines with a good adaptation to Central Europe, the 5% best performing genotypes (based on their mean over all environments) for emergence and juvenile SDW under field conditions, and the 5% inbred lines with the earliest anthesis, were further dissected for their stability, expressed by the weighted sum of the first and second principal component. The inbred line PI602736 showed the highest field emergence on average (77.6%), but SC614 and SC1201 had a similar mean emergence (73.6% and 73.8%, respectively) combined with a higher stability, as shown by the lower sum of PC1 and PC2 (Figure 3a). For SDW, SC702 represents the most interesting accession, having the second highest mean value (341.5 mg) and best stability. SC52 had a similar mean SDW (343.6 mg), but was less stable; however, it was one of the best performers at GI 2014 (the field experiment with most severe cold stress). Furthermore, this inbred line was among the top 5% for both emergence and SDW, together with SC748 and PI599701. For DTF, SC609, SC942, and SC1103 showed the earliest flowering on average. While SC942 and SC1103 are fodder types with a *bicolor × sudanense* background, SC609 is a grain type of the race *guinea* originating from China. Even though SC1214 (*caudatum-guinea* grain type) flowered two days later on average than the previously mentioned genotypes, it showed the best stability for earliness. Interestingly, SC352 and SC614 combined early flowering with high SDW and emergence, respectively.

![Figure 3. Mean values and stability, depicted as weighted sum of PC1 and PC2, of the top 5% (n = 19) (a) performing genotypes for field emergence; (b) juvenile shoot dry weight (SDW); and (c) days to flowering (DTF).](image-url)

Figure 3. Cont.
3.4. Population Structure of the Diversity Panel

The neighbor-joining method [58] shows that the present diversity set clusters into five phylogenetic groups, which are principally based on geographic origin and morphotype (Figure 4). Only group 2, which is determined rather by the ideotype (biomass and sweet sorghum types) than by race and origin, does not fit into this scheme.

3.5. Genome Wide Association Study for Early Cold Tolerance Traits

After removing SNPs with more than 10% missing data and a minor allele frequency <5%, 1507 SNPs remained for the association studies. For the field trials, only in the particular environment of GI 2014 (which had the hardest stress conditions), significant marker-trait associations were found (two for emergence and one for SDW). In contrast, under controlled conditions, we found in total 12 significant SNP-trait associations for the traits RDW, emergence (%), emergence index, and leaf greenness (Table S4, Supplementary material). The following Manhattan plots depict marker-trait associations for SDW (GI 14), EM (GI 14), SDW (climate chamber, CC), EM (CC), RDW (CC), EI (CC), and LG (Figure 5).
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Figure 5. Cont.
Figure 5. Manhattan plots showing p values and marker-trait associations for different chilling-tolerance related traits. Blue horizontal line indicate threshold for significant marker–trait associations ($p < 1 \times 10^{-3}$). CC, climate chamber; EM, emergence; EI, emergence index; LG, leaf greenness.

3.6. Genome-Wide Association Studies for Agronomical Traits

In total, we found 46 significant SNP–trait associations, all with minor effects on phenotypic variation (Table S4, Supplementary material). For PH, SNP UGSDII_09970 (SB-06) explained 8.7% of the phenotypic variation, being the marker with the highest impact among all traits. For the other agronomical traits, the phenotypic variation explained by the specific SNP–trait association was less than 8%. Significant SNP–trait associations for PH were found on four different chromosomes (Table S4, Supplementary material). Figures 6 and 7 show the marker–trait associations for PH and for SD and brix, respectively, as Manhattan plots. For the remaining traits, the marker–trait associations are depicted in the Supplementary material (Figures S1–S3).
Figure 6. Manhattan plot showing $p$-values and marker–trait associations for PH. Red and blue horizontal lines indicate thresholds for significant marker–trait associations after Bonferroni correction ($p < 3.32 \times 10^{-5}, 0.05/N$) and weak associations ($p < 1 \times 10^{-3}$). The known plant height gene $dw2$ is marked in the plot.

4. Discussion

4.1. Phenotypic Variation for Chilling Tolerance and Juvenile Development

The observed heritabilities of $h^2 = 0.74$ for emergence and 0.41 for SDW are in line with those published by Fiedler et al [7]. The heritability for SDW is the lowest among the traits scored in the present study and underlines the high amount of G × E interaction. However, taking into account that this trait actually represents (early) biomass yield, the observed heritability of 0.41 is not so surprising, as for yield itself, only medium heritabilities are usually reported, too.
RDW scored in the climate chamber displayed only weak correlations to field emergence and SDW, which would not justify its laborious assessment in a practical breeding program. Even though leaf greenness scored under controlled cold stress did not show a relation to field performance either, we do consider this trait an efficient selection tool for cold tolerance of autotrophic growth (see Section 4.5).

4.2. Phenotypic Variation for Maturity and Bioenergy Related Agronomical Traits

While high heritability for plant height (0.96) could be expected, stem diameter (0.82) showed a high heritability, confirming the results of Zhao et al. [33], who reported a heritability of 0.88 for stem circumference. Both maturity traits DTF and DMC showed a comparable heritability (0.80 and 0.83, respectively). The heritability for brix in our study (0.72) is in a similar range as observed in another diversity panel (0.81) by Burks et al. [38].

The traits DTF and DMC were negatively correlated, but the magnitude of their relation differed among GG and RH, probably as a result of a different panicle: stem ratio. At the warmer location of GG, the ratio of panicles/grains on whole-plant dry matter was higher, owing to a better seed set and a more advanced seed maturity. Hence, the high correlation between DTF and whole-plant DMC observed at GG was not surprising, as a high correlation between DTF and seed DMC under Central European conditions was already reported by Windpassinger et al [8]. In contrast, at RH, the relative importance of grains on whole-plant dry matter was low because of the late flowering, and for stem and leaves, only a low correlation between DTF and component-specific DMC was reported [8].

The observed correlation between brix and tallness (r = 0.55 ***) coincides with results reported by Shiringani et al [41]. However, a recent study [44] showed that the tallness of sweet sorghum cultivars is likely to be the result of selection for total sugar yield (which is obviously higher in tall plants) rather than physiologically or genetically determined. Concordantly, the positive correlation between brix and DTF (r = 0.53 ****) observed here could also be the result of the selection for tall and late-flowering sweet sorghums. Surprisingly, the negative correlation between brix and DMC (r = −0.30 ***) was relatively low, facilitating the breeding for cultivars with a high stem digestibility, but still an adequate maturity. Stem diameter was not associated with brix, being in concordance with previous studies [36,41], but showed a low negative correlation (r = −0.25 ***) with plant height, which was also described by the authors of [41], but not found in the study of [36]. Further, SD was weakly correlated with DTF and DMC, with early maturity going on the expense of stem thickness, as was to be expected.

4.3. Lines with a Good Adaptation to Cooler Climates

The accessions previously highlighted combine a good and stable early cold tolerance with early maturity, hence representing valuable base material for the breeding of sorghum towards a better adaptation in cool temperate areas. These results show that among the sorghum conversion lines, interesting sources for cold tolerance can also be found. In contrast to Chinese kaoliang types, which are the commonly described donors for cold tolerance, albeit their generally poor agronomic performance (e.g., low disease tolerance and yield potential) [60,61], the conversion lines are more amenable base material for utilization in temperate breeding programs. Further, some brown-midrib lines also excelled in cold tolerance and early flowering, showing that the desirable bmr-trait is no penalty for temperate-adaptation.

4.4. Population Structure of the Diversity Panel

The phylogenetic structure of the present diversity set (Figure 4) confirms the results of [62,63]. Nonetheless, group 2, consisting mainly of biomass and sweet sorghum types, does not fit into this scheme, as it is based on the ideotype (crop type) and not on morphotype (race) or origin. This seems somehow surprising, as the authors of [64] reported that sweet sorghum lines cluster with grain sorghums of similar racial origin, suggesting a polyphyletic origin of sweet sorghum, which is also supported by the high diversity found among sweet sorghum accessions [43]. Also, in the present
Thus, the clustering of sweet- and biomass-types in group 2 might be explained by separate breeding programs and their geographic origin in Southern Africa, which was one of the main sources for sorghum germplasm introductions into the USA [65].

4.5. Genome-Wide Association Studies for Juvenile Chilling Tolerance

In temperate Europe and other high-latitude areas of the world, early season cold stress is one of the major obstacles for sorghum production. Enhancements of emergence and early vigor under suboptimal temperature conditions can facilitate earlier sowings and increase the yield potential. Several publications describe different methods for the dissection of early season cold tolerance [7,10,11,17]. The present study analyzed the broadest diversity set \( n = 338 \) so far for cold tolerance traits under both field and controlled environment conditions via GWAS. In contrast to QTL-studies on bi-parental populations, GWAS captures a much higher amount of genetic diversity, also considering ancient recombination events.

Our results (Table S4) confirm juvenile cold tolerance of sorghum being a highly quantitative trait with multiple physiological pathways involved, as already shown in previous studies [19,22,53]. Most of the QTL described in these studies lie nearby genes involved in the anthocyanin and carbohydrate metabolism.

Even though in our experiments, only a weak correlation between RDW under controlled stress conditions and field experiments was observed, a proper root development also under adverse soil- and temperature conditions is essential for a satisfying establishment; subsequent water and mineral uptake; and, ultimately, a high yield [66]. A lethal decline in leaf relative water content caused by a chilling induced reduction in root conductance was observed in maize and rice roots [67,68]. Hund et al. [69,70] reported that root architecture in maize was also influenced by early season chilling stress. Bekele et al. [10] highlighted the importance of root development and structure on early sorghum cold tolerance, and described SB-01 and SB-06 as a source of genomic regions influencing several root traits.

Three SNPs, inter alia SNP UGSS_03534 located on SB-04, showed a significant SNP–trait interaction for leaf greenness. Photosynthesis is considered one of the most chilling-susceptible processes in plants [71]. Prolonged chilling stress induces chlorosis, which can aggravate into complete and irreversible etiolation due to chlorophyll degradation among susceptible genotypes (see Figure 8). Hence, the ability of sorghum to maintain leaf greenness under cold indicates stress tolerance of the photosynthetic apparatus and, furthermore, a successful switch from heterotrophic to autotrophic growth. Also, for maize, leaf greenness was reported as an important and easily scorable trait [72]. The high variation for cold tolerance of the photosynthetic apparatus observed in the present study by a simple scoring method coincides with the results of [73], who conducted extensive analyses on different photosynthetic parameters like carbon assimilation, transpiration rate, and stomatal conductance.

![Figure 8. Differential reaction of the inbred lines to constant 13/10 °C for four weeks shows the high variation for cold tolerance in the diversity set studied.](image-url)
Emergence as a product of successful germination and sufficient seedling vigor is also a crucial trait for the estimation of cold tolerance in different plant species. Under controlled stress conditions (13/10 °C), we localized only one SNP–trait interaction on SB-10. In addition to (final) emergence, we also analyzed the trait emergence index (EI), which considers also the speed of emergence. Three out of four significant SNPs for EI were localized on SB-04.

To summarize, early chilling tolerance is a highly quantitative trait that is influenced by many small-effect genes, most likely governing multiple physiological pathways. We identified several significant SNPs for diverse cold-tolerance related traits. However, we are aware that the relatively low marker coverage of the used 3K-SNP Chip implies some limitations. With a higher marker density, more significant marker–trait associations might have been detected, and candidate gene analysis could be conducted to get more insights into the physiology behind cold tolerance in sorghum. Nevertheless, association studies on sorghum cold tolerance using a much higher marker density showed the highly quantitative character of this trait. For instance, the authors of [21] performed GWAS using approximately 162,000 SNPs and found only one marker locus significantly associated with sorghum low temperature germination and none with vigor. For practical breeding, the highly quantitative character of cold tolerance traits strongly limits the possibilities of marker-assisted selection. As sorghum cold tolerance traits have been shown to be heterotic and with a generally low correlation between per se and hybrid performance [11], for applied sorghum hybrid breeding QTL-studies which focus on combining ability instead of per se performance or genomic prediction will be more promising.

4.6. Genome-Wide Association Studies for Bioenergy Related Agronomical Traits

The present diversity set shows a high variation for the five agronomical traits plant height (PH), stem diameter (SD), brix, days to flowering (DTF), and dry matter content (DMC) (see Section 4.2). Our detected associations for PH on SB-06 and SB-07 (Table S4) most probably correspond to the well-known sorghum plant height genes \( dw2 \) and \( dw3 \) [27,74,75].

For DTF, we found different significant genomic regions (Table S4). However, the SNP–trait associations are not consistent over the three locations. For DTF at the southernmost and warmest site, Gross-Gerau (GG), we found four significant SNP-trait associations. At PL, the northernmost location with the longest days in summer, we found more SNPs for DTF than in GG. Considering that most of the accessions in our diversity panel probably have the same configuration of \( ma \)-alleles, allowing for flowering even under extreme long-day conditions, the quantitative character of DTF with environment-specific QTL found in this study is not surprising and confirms previous results. Mace et al. [76] identified 40 small-effect QTL influencing sorghum flowering time in Australia. Adequate flowering time is a critical, site-specific adaptation trait with a high impact on yield. The marker–trait associations identified for PL are of special interest because they provide insights how an originally tropical short-day plant can achieve flowering even under cool temperatures and extreme day lengths.

For dry matter content (DMC), we found more significant SNP–trait associations under the cooler environment of RH than at the warmer location GG (six vs. one). SNP UGSDL_29686 is of special interest, as it is not only significant for RH alone, but remains significant also when considering the mean values of both locations, RH and GG. Gene expression scenarios for this genomic region in Phytozome v12.1 showed a high expression during anthesis and grain maturity. These results confirm the strong relation between DMC and DTF, as was to be expected. However, for the breeding of bioenergy sorghum, correlation breakers showing late flowering going along with a high yield potential, but still sufficient DMC due to lower moisture content in stem and leaves, would be of special interest. Unfortunately, we could not detect SNPs for DMC, which are detached from flowering time regulation.

For stem diameter (SD), we found three significant SNP–trait associations on SB-06 and -08, with two of them even passing the high-significance threshold. The literature on the genetic architecture underlying SD is limited to only a few publications. Shirinigani et al. [41] identified 13 QTL for SD in a recombinant inbred line (RIL) population, clearly indicating it as a quantitative trait. Zou et al. [77]
described, in a bi-parental population, three QTL for SD under long day conditions on SB-04 and -06, all with minor impacts of the phenotypic variation. Furthermore, the authors of [33] detected 36 QTL in a diversity set and found six significant marker trait associations on five different chromosomes with GWAS. Their experiments were carried out in Iowa, which has higher average temperatures in summer and a more intense sunlight than Central Europe, owing to the lower latitude (~41° vs. ~50°) and continental climate. However, the SNP UGSDL_29686 identified in the present study is located on SB-06, most probably close to the SNP described by the authors of [33], indicating its environmental stability. On the whole, our results coincide with the previous studies regarding the quantitative character of this important bioenergy trait. Nonetheless, the two highly significant SNPs found in our broad diversity set deserve further interest and fine-mapping for marker development.

For brix, we found three significant SNP-trait associations on SB-01, -06, and -07. These associations have a minor phenotypic impact of below 6%. The polygenic character of brix found in our experiments coincides with several studies [32,36,39]. *S. bicolor* is closely related to sugarcane, and sweet sorghum is widely used for biofuel and sugar-syrup production. These sweet types are characterized by a juicy stem with a high amount of soluble sugars. However, beyond these classical sweet sorghums, our scorings show that other sorghum crop types such as dual-purpose types can have also sugar in their stems, even though their juice volume is lower. Several mapping studies detected QTL for brix on nearly all sorghum chromosomes. In our study, the SNP UGSDII_09970, which is highly significant for PH, matches also for brix (Table S4). Owing to the phenotypic correlation of plant height and brix, the identification of a common QTL is not surprising. Sweet sorghum types are usually rather tall, and in our diversity panel they are also notably taller than the grain type conversion lines. However, as described by the authors of [44] and already previously outlined, the tallness of sweet sorghum seems to be rather the result of both intended or natural selection than physiological causes.

Most of the QTL cited and discussed for the aforementioned traits were identified in specific, bi-parental populations. Using a diversity panel as in our study, both the number of significant marker-trait associations and their impact tend to decrease. As already outlined, the relatively low SNP coverage implies some limitations to the present study. Nevertheless, the principal findings regarding the quantitative character of most cold tolerance and agronomical traits are in line with other studies, and novel genomic regions influencing these traits under the particular high-latitude conditions of Central Europe could be identified.

5. Conclusions

For a successful implementation as a novel bioenergy and fodder crop in temperate Central Europe, sorghum needs significant enhancements in cold tolerance and early maturity. Further, depending on the intended crop type or ideotype, an adequate plant architecture and composition, expressed by traits such as plant height, stem diameter, and brix, are essential. Our study evaluated the broadest diversity set so far for these traits under both Central European field conditions and controlled environments, identifying novel, publicly available sources for cold tolerance and temperate-adaptation. The high amount of both phenotypic and genetic variation, along with satisfying heritabilities in most cases, underlines that a robust breeding progress for these complex quantitative traits is feasible. The genome-wide association studies (GWAS) conducted in this study confirm a highly quantitative character for most bioenergy and cold tolerance related traits. Interestingly, novel genomic regions influencing sorghum cold tolerance and adaptation could be identified, which should be further analyzed in fine-mapping approaches. However, for practical breeding, the highly quantitative trait characteristics imply serious limitations for marker-assisted selection in genetically diverse material.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/9/535/s1, Table S1: List of all sorghum accessions composing the present diversity set (*n* = 338), including origin, SC- and PI-Numbers when available, Table S2: Mean values of the cold tolerance and bioenergy related traits, Table S3: Mean values of the cold tolerance and bioenergy related traits, Table S4: Significant SNP-trait associations for the considered traits, Figure S1: Manhattan plot showing *p* values and marker–trait associations for PH_GG, PH_RH and PH_PL. Blue horizontal line indicate threshold for significant marker–trait associations (*p* < 1 × 10⁻³).
Figure S2: Manhattan plot showing p values and marker–trait associations for DMC, DMC_GG and DMC_RH. Blue horizontal line indicate threshold for significant marker–trait associations ($p < 1 \times 10^{-3}$). Figure S3: Manhattan plot showing p values and marker–trait associations for DTF, DTF_GG, DTF_RH and DTF_PL. Blue horizontal line indicate threshold for significant marker–trait associations ($p < 1 \times 10^{-3}$).

**Author Contributions:** A.S. and S.W. contributed equally to this manuscript and are both listed as first authors in consequence. B.W. devised the study. W.F. and R.S. received the funding. S.W. planned and oversaw the field trials and climate chamber experiments. A.S. conducted the data analysis. A.S. and S.W. interpreted the results and wrote the manuscript. B.W. edited the manuscript.

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**Abbreviations**

- CC: climate chamber
- DTF: days to flowering
- DMC: dry matter content
- EI: emergence index
- EM: emergence
- GWAS: genome wide association study
- LG: leaf greenness
- PH: plant height
- QTL: quantitative trait loci
- RCBD: randomized complete block design
- RDW: root dry weight
- RIL: recombinant inbred line
- SD: stem diameter
- SDW: shoot dry weight
- SNP: single nucleotide polymorphism

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