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ORIGINAL PAPER

A first step toward the development of a barley NAM population and its utilization to detect QTLs conferring leaf rust seedling resistance

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Abstract
Key message **We suggest multi-parental nested association mapping as a valuable innovation in barley genetics, which increases the power to map quantitative trait loci and assists in extending genetic diversity of the elite barley gene pool.**
Abstract Plant genetic resources are a key asset to further improve crop species. The nested association mapping (NAM) approach was introduced to identify favorable genes in multi-parental populations. Here, we report toward the development of the first explorative barley NAM population and demonstrate its usefulness in a study on mapping quantitative trait loci (QTLs) for leaf rust resistance. The NAM population HEB-5 was developed from crossing and backcrossing five exotic barley donors with the elite barley cultivar 'Barke,' resulting in 295 NAM lines in generation BC_1S_1. HEB-5 was genetically characterized with 1,536 barley SNPs. Across HEB-5 and within the NAM families, no deviation from the expected genotype and allele frequencies was detected. Genetic similarity between 'Barke' and the NAM families ranged from 78.6 to 83.1 %, confirming the backcrossing step during population development. To explore its usefulness, a screen for leaf rust (*Puccinia hordei)* seedling resistance was conducted. Resistance QTLs were mapped to six barley chromosomes, applying a mixed model genome-wide association study. In total, four leaf rust QTLs were detected across HEB-5 and four QTLs within family HEB-F23. Favorable exotic QTL alleles reduced leaf rust symptoms on two chromosomes by 33.3 and 36.2 %, respectively. The located QTLs may represent new resistance loci or correspond to new alleles of known resistance genes. We conclude that the exploratory population HEB-5 can be applied to mapping and utilizing exotic QTL alleles of agronomic importance. The NAM concept will foster the evaluation of the genetic diversity, which is present in our primary barley gene pool.

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Introduction

Cultivated barley (*Hordeum vulgare* ssp. *vulgare*) is an important crop species, mainly used for animal feeding and beer production. Due to this fact, barley is used in breeding programs for more than 100 years. However, in most crops, also in barley, a loss of allelic diversity has been observed during domestication and the onset of modern breeding (Tanksley and McCouch 1997). Thus, barley breeders currently try to re-introduce favorable alleles using different breeding strategies. Additionally, barley is a model species for crop genetic research due to its comparatively easy to manage diploid genome. Recently, the barley gene space was sequenced (The International Barley Genome Sequencing Consortium 2012), laying the foundation for an accelerated avenue to gene discovery and elucidation of

gene function. However, 'classical' genetic tools as QTL mapping still provide effective preconditions for genetic mapping and map-based cloning. QTL mapping usually is applied in (i) biparental populations derived from crossing two genetically diverse parents (Collard et al. 2005) or (ii) genome-wide association studies (Mackay et al. 2009; Waugh et al. 2009; Tondelli et al. 2013). In the latter case, multiple unrelated genotypes with unknown kinship are the basis for detecting genomic regions where alleles are associated with the control of quantitative traits.

Nested association mapping (NAM) represents a further refined QTL mapping approach (Yu et al. 2008). In this case, multiple donor parents are crossed with one recipient parent resulting in a final NAM population, which carries multiple alleles at each investigated locus. The advantage of the NAM approach relies on the combination of linkage analysis with high-resolution (HR) association mapping (Yu et al. 2008). Yu and Buckler (2006) were the first to propose the implementation of the NAM approach for maize. The maize NAM population consists of 5,000 recombinant inbred lines (RILs) within 25 NAM families with 200 lines per family (Buckler et al. 2009). It was demonstrated that large differences in flowering time are not caused by a few genes with large effects, but by the cumulative effects of numerous QTLs (McMullen et al. 2009; Larsson et al. 2013). Furthermore, the maize NAM lines were investigated in regard to pathogen resistances (Kump et al. 2011; Poland et al. 2011), morphological traits (Tian et al. 2011), kernel composition (Cook et al. 2012), and stalk strength (Peiffer et al. 2013). Additionally, QTLs involved in hydroxamic acid synthesis were mapped for a subset of NAM lines (Butrón et al. 2010).

The NAM approach was also applied to sorghum. The sorghum NAM population consists of 56 NAM families with 30–90 NAM lines per family (Jordan et al. 2011). In contrast to the maize NAM population, the sorghum population structure was compromised to better detect QTLs across NAM families (Jordan et al. 2011). Hybrid performance regarding nodal root angle was analyzed and, within a subset of seven sorghum NAM families, nodal root angle QTLs were located. The detected QTLs confirmed already known QTL regions or represented new loci, so far unknown to control the investigated traits (Jordan et al. 2011; Mace et al. 2012). Additionally, synteny between sorghum and maize was detected in regions, where exotic flowering time alleles for both crops were detected (Mace et al. 2013). Also computer simulations were conducted to underline the power of NAM for QTL detection and to allocate optimal parental numbers (Stich et al. 2007, 2010; Stich 2009; Li et al. 2011). Stich (2009) compared different mating designs for developing maize and *Arabidopsis thaliana* NAM populations based on simulation data. He recommended creating NAM populations with a large number of parents. However, simulations for QTL detection may have limitations because they are forced to make assumptions about the sharing of QTL among individuals and their frequency distributions (Myles et al. 2009).

Leaf rust (LR), caused by the biotrophic fungal pathogen *Puccinia hordei*, is an economically important barley foliar disease. Currently, at least 21 qualitative (also called 'major' or 'race-specific') resistance genes against *P. hordei,* named *Rph1* to *Rph21,* are known (Golegaonkar et al. 2009b; Hickey et al. 2011, 2012; Sandhu et al. 2012). All of the LR resistance genes, except of *Rph20,* are effective at seedling stage, and some are also expressed during adult plant stage (Golegaonkar et al. 2009b). *Rph* genes were detected on all seven barley chromosomes (cf. Chełkowski et al. 2003; Weerasena et al. 2004; Sandhu et al. 2012).

Pathogen resistance may also be inherited quantitatively. The underlying genes are regarded as durable due to the assumption that additive minor allelic effects result in race-nonspecific, basal resistance (cf. Schweizer and Stein 2011). Multiple studies were conducted to map QTLs, and alleles were associated with leaf rust resistance in different genomic regions (e.g., Qi et al. 1998, 1999, 2000; Kicherer et al. 2000; van Berloo et al. 2001; Backes et al. 2003; Kopahnke et al. 2004; von Korff et al. 2005; Kraakman et al. 2006; Marcel et al. 2007a, b; Schmalenbach et al. 2008; Cakir et al. 2011; Castro et al. 2012; González et al. 2012). The detection of LR resistance QTLs in seedlings is usually plant stage dependent (Wang et al. 2010; Castro et al. 2012). An advanced QTL approach, the 'Meta-QTL' approach, was applied by Schweizer and Stein (2011). The authors remapped QTLs detected in different populations for several fungal pathogens including *P. hordei* and detected hot spot regions on all chromosomes of the barley genome. Also, candidate gene studies (González et al. 2010; Chen et al. 2011), expression analyses (Millett et al. 2009; Chen et al. 2010a, b), and proteomic characterization of individual *Rph* genes (Bernardo et al. 2012) were reported to further understand the genetics of nonhost resistance against *P. hordei* (Jafary et al. 2006, 2008; Marcel et al. 2007a; Zellerhoff et al. 2010).

In the present paper, we report toward the development of the first explorative barley NAM population. We present data on mapping QTLs in HEB-5 conferring seedling resistance against leaf rust as a first application.

Materials and methods

Plant material

To develop the NAM population HEB-5 ('Halle Exotic Barley'), five *H. vulgare* ssp. *spontaneum* accessions

Table 1 Development of the barley NAM population HEB-5

NAM family/ population	Initial cross	Origin of HID	No. of F_1 plants	No. of BC_1 plants	No. of NAM lines in BC_1S_1
HEB-F06	HID-069 × 'Barke'	Turkey	13	20	60
HEB-F08	HID-099 × 'Barke'	Syria	14	20	71
HEB-F14	HID-140 × 'Barke'	Iraq	24	20	59
HEB-F15	HID-144 × 'Barke'	Iran	13	20	55
HEB-F23	HID-359 × 'Barke'	Israel	12	20	50
HEB-5			76	100	295

The origin of the *Hsp* parents is referenced in Badr et al. (2000) and Badr personal communication. HID-069, HID-144, and HID-359 were exclusively used as female parents, whereas HID-099 and HID-140 were used both as female and male parents. All F_1 plants were backcrossed once with 'Barke' as the female parent to ensure that the final cytoplasm originates from the *Hv* parent 'Barke'. Twenty BC_1 plants per family were used to generate 50–71 BC_1S_1 lines per family through one round of selfing

(*Hordeum* identity, HIDs) were selected from Badr et al. (2000) in order to represent independent *Hsp* origins of the Fertile Crescent (Table 1). The *Hsp* donors were crossed with the German spring barley (*H. vulgare* ssp. *vulgare*) cultivar 'Barke' (Fig. S1). The resulting F_1 plants were backcrossed with 'Barke' as the female parent. Subsequently, 20 BC_1 plants per NAM family were selfed once to produce the final BC_1S_1 generation of HEB-5 (Table 1). During the development of HEB-5, no artificial selection was carried out. For leaf rust testing, the HEB-5 lines were bulk propagated once resulting in $BC_1S_{1:2}$ offspring.

SNP genotyping

Genomic DNA extraction was conducted as described in Schmalenbach et al. (2011). Leaf material was collected from a pool of at least 10 $BC_1S_{1:2}$ offspring in order to represent the original BC_1S_1 HEB-5 line. SNP genotyping of the 295 NAM lines, the five donor HIDs, and the recurrent parent 'Barke' with the barley 1,536 Infinium BOPA1 SNP set (Close et al. 2009) was carried out at the Southern California Genotyping Consortium (SCGC) at the University of California, Los Angeles (http://scgc.genetics.ucla.edu/). The obtained raw data were transformed to genotype calls and subsequently manually supervised to correct for heterozygote calls using GenCall software (Illumina, San Diego, CA) at the Close lab (University of California, Riverside, CA). Mapping information of the BOPA1 SNPs was taken from Muñoz-Amatriaín et al. (2011).

Cultivation of NAM lines and leaf rust resistance test

The 295 NAM lines in $BC_1S_{1:2}$ plus the HID donor parents and 'Barke' were sown in 150 well trays and, after germination, vernalized for 4 weeks at 8 °C and 2 further weeks at 12 °C in a climate chamber at the experimental station of the Martin Luther University of Halle-Wittenberg. After vernalization, seedlings were transferred to 1.25-L pots, containing the standard plant cultivation substrate 'ED73' (Einheitserde- und Humuswerke Gebr. Patzer, Sinntal-Jossa, Germany) and cultivated under controlled glasshouse conditions. The average cultivation temperature was 19 °C with a minimum temperature of 10 °C at night and a maximum temperature of 32 °C during the day. Plants were grown under quartz metal halide lamps (Master HPI-T Plus 400 W, Philips, Amsterdam, The Netherlands) with 10–30 klx of light for 16 h per day and with automated shading. Watering, nitrogen, phosphorus and potassium fertilization, and pesticide treatment followed local glasshouse cultivation practices.

To test for leaf rust resistance in the HEB-5 population, five $BC_1S_{1:2}$ plants per NAM line, 'Barke,' and the five HIDs were grown in 150-well plates as indicated above. The plates were transferred to the Julius Kuehn Institute, where screening for leaf rust resistance took place. Individuals of the highly susceptible cultivar 'Großklappige Wintergerste' were placed surrounding the NAM lines on the plates to control for infection success. Before inoculation, plants were sprayed with a 0.01 % 'Tween 20' suspension to support adhesion of uredospores to the leaves. Subsequently, freshly propagated uredospores of *P. hordei* isolate 'I-80' were mixed with white clay (ratio 1:3) and the powder was attached with approximately 0.1 mg of uredospores per individual to the first leaf of each NAM line at Zadoks GS 11 (Zadoks et al. 1974). Isolate 'I-80' is capable to overcome common leaf rust resistance genes in the European barley gene pool, except for *Rph7*, *Rph15*, *Rph16*, and $Rph_{MBR1012}$ (König et al. 2012). Kopahnke et al. (2004) reported that 'I-80' is also avirulent against *Rph5*, *Rph6*, *Rph13*, and *Rph14*. The virulence behavior of 'I-80' against *Rph17–Rph21* is unknown so far. After inoculation, the plants were grown at 18 °C and 100 % relative humidity in a growth chamber for 24 h. After incubation, the plants were cultivated at 20 ± 3 °C with 50–70 % relative humidity in a climate chamber in the glasshouse. Ten days after inoculation, the seedlings were screened for LR symptoms according to Levine and Cherewick (1952).

Statistical analyses

All statistical analyses were performed with SAS Enterprise Guide 4.2 (SAS Institute 2008). Heritability (h^2) for LR was estimated as $h^2 = 100 \times V_G/(V_G + V_\epsilon/r)$, where

Table 2 Transformation of original leaf rust classes into quantitative scores of leaf rust symptoms

Original class[a]	Quantitative score
0cn	1
0–1; 1	2
0–2-; 2–	3
0–2	4
2	5
2+	6
2–3	7
3; 3–4	8
4	9

[a] According to Levine and Cherewick (1952)

V_G and V_ϵ are the variance components genotype (NAM line) and experimental error, respectively, with $r = 5$ replicates per NAM line. Variance components were calculated with 'proc VARCOMP'. Genetic similarity (GS) between the NAM lines, 'Barke,' and the HIDs were estimated with 'proc DISTANCE' based on simple matching of SNP markers. A principal component (PCo) analysis based on genetic similarities was conducted with 'proc PRINCOMP'. For illustrating genetic similarities, a cluster analysis ('proc CLUSTER') was carried out. Subsequently, 'proc GPLOT' and 'proc TREE' were applied for graphical presentation of the results.

In order to locate QTLs which explain the variation in leaf rust reaction, two mixed model analyses of variance were conducted. Prior to this, SNPs with a minor allele frequency (MAF) <0.05 were excluded from analysis to reduce false-positive associations (Tabangin et al. 2009). Also, according to Pillen et al. (2003), only homozygous *Hv* or *Hsp* genotypes at a marker locus were included in the calculation. This was carried out since repeated selfing of heterozygous BC_1S_1 lines will lead to a mixture of homozygous genotypes resulting in a false estimation of the true performance of heterozygous NAM lines. For mixed model analysis, the LR classes were transformed to quantitative scores as shown in Table 2. The first mixed model analysis was carried out with 'proc MIXED' across all five families of population HEB-5:

$$Y_{ijkl} = \mu + F_i + L_j + M_k + \text{PCo1} + \text{PCo2} + \varepsilon_{ijkl} \text{ [model 1]},$$

where μ is the general mean, F_i is the fixed effect across the $i = 5$ NAM families, L_j is the random effect across the $j = 295$ NAM lines, M_k is the fixed effect across the $k = 2$ homozygous genotypes, which can be distinguished at the SNP locus under investigation, PCo1 and PCo2 are the two principle components, used as fixed covariates, which explain more than 5 % of the genetic variation between the NAM lines, and ε_{ijkl} is the random error effect across the $l = 5$ replicates per NAM line. The principal components are included in the model to account for the genetic relatedness between the NAM lines.

In addition, the 2-factorial mixed model was carried within each NAM family in order to search for QTL effects, which are present within single families:

$$Y_{jkl} = \mu + L_j + M_k + \text{PCo1} + \text{PCo2} + \varepsilon_{jkl} \text{ [model 2]},$$

where the remaining parameters are in agreement with model 1.

Following the mixed model analysis, a false discovery rate (FDR) adjustment was conducted according to Benjamini and Hochberg (1995) with 'proc MULTTEST,' to account for multiple testing. Marker-trait associations were accepted with P(FDR) <0.05. Marker-trait associations showing the same effect were interpreted as a single putative QTL if linked markers were mapped ≤20 cM apart.

Results

Development of the barley NAM population HEB-5

In the present paper, we report on progress toward the development of an explorative barley NAM population *HEB-5*. For this, we crossed five *H. vulgare* ssp. *spontaneum* accessions (HIDs) with the German elite cultivar 'Barke'. In total, 76 F_1 seeds were obtained, although the number of fertile F_1 offspring ranged from 12 to 24 across the five NAM families (see Table 1). Subsequently, the F_1 individuals were backcrossed once again with 'Barke'. Out of this set, 20 BC_1 individuals for each NAM family were chosen randomly for selfing. In total, 295 resulting BC_1S_1 plants were randomly selected to define population HEB-5. The number of lines per NAM family ranged from 50 to 71 lines. In order to propagate seeds of the 295 NAM lines, one round of bulk propagation was carried out producing at least 10 $BC_1S_{1:2}$ seeds per NAM line (Table 1).

SNP genotyping of HEB-5

In generation BC_1S_1, HEB-5 lines derived from five NAM families were genotyped with the BOPA1 SNP set described in Muñoz-Amatriaín et al. (2011). Out of 1,536 SNPs, 1,211 informative SNPs (excluding 19 unmapped SNPs) were used to differentiate the 295 NAM lines. The SNP coverage across the seven barley chromosomes varied. Between 135 (7H) and 230 (5H), SNPs were genotyped per chromosome. An overview describing the genome coverage is given in Table S1. Mapped SNPs resulted in an average marker distance varying from 0.8 to 1.2 cM across the seven barley chromosomes. The biggest

gap between two adjacent SNPs was detected on chromosome 4H (11.2 cM).

Frequencies of the three genotype classes *HvHv*, *HvHsp*, and *HspHsp* and the two allele classes *Hv* and *Hsp*, respectively, were calculated for each NAM line (Table S2, Table S3). In addition, *Hsp* allele frequency distributions for HEB-5 and the five NAM families are shown in Fig. S2. The average genotype and allele frequencies for each NAM family and HEB-5 were compared to the expected genotype frequencies (i.e., *HvHv* = 62.5 %, *HvHsp* = 25.0 %, *HspHsp* = 12.5 %) and allele frequencies (i.e., *Hv* = 75 %, *Hsp* = 25 %), respectively (Table S4, Table S5). No NAM family or HEB-5 deviated significantly from the expected genotype or allele frequency across chromosomes. However, five significant deviations ($P < 0.05$) of genotype frequency were observed on chromosomes for HEB-F08 (1H), HEB-F14 (5H), and HEB-F23 (1H, 6H, 7H), respectively. For HEB-F08 and HEB-F14, more than expected *HspHsp* genotypes and for HEB-F23 less than expected *HspHsp* genotypes were observed. These observations corresponded to a higher *Hsp* allele frequency of HEB-F14 on chromosome 5H and a lower *Hsp* allele frequency of HEB-F23 on chromosomes 1H, 6H, and 7H (Table S4, Table S5).

Genetic similarity (GS) and principal component (PCo) analysis

The genetic similarity (GS) estimation of HEB-5, based on simple matching, is shown in Table 3. GS between 'Barke' and the five HIDs ranged from 33.0 (HID-359) to 49.7 % (HID-099). Presumably, due to the backcrossing step, the GS estimates increased to an average of 77.9 % between 'Barke' and HEB-5. Detailed GS estimates between NAM lines, Barke, and HIDs are given in Table S6. The highest individual GS estimate was observed between HID-099 and the NAM line HEB-F08-021 (98.5 %). On the contrary, the lowest GS estimate was observed between HID-359 and the NAM line HEB-F14-130 (26.9 %).

Table 3 Mean genetic similarity between 'Barke' and HIDs and NAM families

Comparison[a]	(%)
'Barke'-HEB-5	77.9
'Barke'-HID-069	44.8
'Barke'-HEB-F06	78.1
'Barke'-HID-099	49.7
'Barke'-HEB-F08	80.4
'Barke'-HID-140	44.7
'Barke'-HEB-F14	76.4
'Barke'-HID-144	44.7
'Barke'-HEB-F15	78.7
'Barke'-HID-359	33.0
'Barke'-HEB-F23	81.1

[a] Comparison between 'Barke' and HIDs, and 'Barke' and the five NAM families, and the NAM population HEB-5, respectively. Genetic similarity was calculated based on simple matching analysis with 1,230 informative SNPs

A principal component (PCo) analysis, based on the GS estimates between the NAM lines and the HEB-5 parents, was calculated to further illustrate the genetic relatedness across HEB-5 and within the NAM families. The first two principal components (PCos) calculated for NAM lines accounted for 67.5 and 5.2 % of the total variance. Figure S3 illustrates that most NAM lines cluster close to 'Barke,' whereas the *Hsp* donor parents were located more distantly. An exception is line HEB-F08-021, which is placed next to HID-099, confirming the GS results given before. The first two PCo coordinates were included in the mixed model analyses to account for the genetic relatedness between the NAM lines.

Table 4 Leaf rust performance of HEB-5 parents, NAM lines and NAM families

NAM family/population[a]	*Hv*/*Hsp* donors	*N*[b]	Mean[c]	Min[d]	Max[d]	SD[e]	CV (%)[f]
'Barke'	*Hv* donor for all NAM families	37	4.1	3	7	1.5	36.1
HID-069	*Hsp* donor for HEB-F06	7	3.1	2	5	0.9	28.6
HEB-F06		286	2.9	1	6	1.0	33.0
HID-099	*Hsp* donor for HEB-F08	6	4.5	1	6	2.1	46.1
HEB-F08		325	4.5	1	7	1.6	35.9
HID-140	*Hsp* donor for HEB-F14	6	4.8	3	6	1.5	30.5
HEB-F14		279	4.9	1	8	1.6	32.2
HID-144	*Hsp* donor for HEB-F15	8	6.0	3	7	1.3	21.8
HEB-F15		266	5.2	1	8	1.9	35.7
HID-359	*Hsp* donor for HEB-F23	10	2.4	1	6	2.1	86.1
HEB-F23		237	4.3	1	8	1.6	36.5
HEB-5		1,393	4.3	1	8	1.7	40.0

[a] Name of the HEB-5 parents, HEB-5 and of each NAM family, respectively

[b] Number of leaf rust scoring observations

[c] Average leaf rust score

[d] Minimum and maximum leaf rust score, respectively

[e] Standard deviation

[f] Coefficient of variation = standard deviation/mean

Leaf rust (LR) seedling resistance of HEB-5 parents and NAM lines

In Table 4, the parameters mean, minimum, maximum, standard deviation (SD), and coefficient of variation (CV) specify the observed leaf rust seedling reaction among the tested barley lines. Scores are given for the HEB-5 parents 'Barke' and the five HIDs, as well as for the HEB-5 population and the five NAM families. Distributions of LR scores for HEB-5 and the five NAM families are illustrated in Fig. S4. The three exotic accessions HID-099, HID-140, and HID-144 showed higher LR scores, i.e., more susceptibility, than cultivar 'Barke'. On the contrary, lower LR scores were observed for HID-069 and, in particular, for HID-359. LR scores for three NAM families were in the same range as their corresponding HIDs. However, for two families, HEB-F15 and HEB-F23, scores differed markedly from their corresponding HIDs. HEB-F15 had lower LR scores than HID-144, and the LR scores observed for HEB-F23 were higher than for HID-359. The mean LR score observed for HEB-5 was slightly higher than for 'Barke'. Furthermore, Table 4 shows that in all five families low and high LR scores were observed. This results in CVs ranging from 21.8 % (HID-144) to 86.1 % (HID-359). On the other hand, the CV for HEB-F23 is in the same range as CVs calculated for the five NAM families and HEB-5. Heritability (h^2) of LR was estimated with 92.4° %.

Mapping of leaf rust (LR) QTLs in HEB-5 and individual NAM families

Mixed model analyses were conducted across HEB-5 (model 1) and separately within each NAM family (model 2) in order to detect SNPs, which are significantly associated as QTLs with leaf rust (LR) seedling resistance.

Table 5 gives an overview of significant SNP × LR associations. Only SNPs with the lowest P(FDR) value are shown if a QTL was detected for more than one SNP. All significant associations are shown in Table S7. The *P*(FDR) values calculated for each SNP across the barley chromosomes are shown in Fig. S5. Five significant [*P*(FDR) < 0.05] SNP associations were detected across HEB-5 and 22 significant associations within NAM family HEB-F23. The remaining NAM families did not reveal any significant SNP × LR association. Associations detected with model 1 and model 2 were summarized to four QTLs, for each of the two models (Table 5, Fig. S5, Fig. S6). QTLs were detected on all barley chromosomes except of chromosome 6H.

At six QTLs, the *Hsp* allele was associated with an increase of LR scores and in two cases with a reduction. The strongest increase in LR symptoms was detected on chromosome 2H and the strongest decrease on chromosome 7H, both within HEB-F23. On chromosome 4H, two QTLs, one detected for HEB-5 and one detected within HEB-F23, partly overlapped at 61.56 cM (Table 5; Table S7; Fig. S6). For two HEB-5 QTLs, QLr.HEB-5-1H and QLr.HEB-5-5H.b, the direction of the QTL effect was also visible within at least one additional NAM family; however, those effects were not significant (data not shown). For QLr.HEB-5-5H.b, a MAF constraint prevented to test the significant SNP within individual NAM families (data not shown).

Discussion

We developed HEB-5, a first explorative multi-parental NAM population in barley in order to enhance the study of genetic diversity of wild barley. Originally, the cultivar 'Barke' was crossed with five exotic *H. vulgare* ssp. *spontaneum* accessions, then backcrossed and subsequently selfed to achieve a BC_1S_1 population. So far, HEB-5 consists of five NAM families and contains 295 NAM lines in total (Table 1).

The HEB-5 lines were subsequently genotyped with the BOPA1 SNP chip, and leaf rust seedling resistance was evaluated as a first example to study phenotypic diversity with a barley NAM population. Subsequently, a mixed model association study was conducted to map leaf rust resistance genes across HEB-5 and within single NAM families. Four QTLs across HEB-5 and four QTLs within NAM family F23 were detected to control LR resistance. We compared the detected QTLs with mapped LR resistance (*Rph*) genes, QTLs, and Meta-QTLs, respectively, and found potentially corresponding regions for all QTLs except for QTLs on chromosome 1H. In the following, the development and the genetic constitution of HEB-5 and the location of LR seedling resistance QTLs will be discussed.

Population design of HEB-5

Utilizing multi-parental populations for association mapping inevitably requires increasing the total population size compared to utilizing a single biparental population. Mapping populations resulting from biparental crosses usually comprise less than 200 lines in barley. For instance, the Oregon Wolfe Barley (OWB) population consists of 82 DH lines (Chutimanitsakun et al. 2011), a size potentially leading to underestimation of QTL number and overestimation of QTL effects (Vales et al. 2005). These authors tested different population sizes and concluded that large QTL effects are detected in small populations, but small effects can only be detected with increasing population size. Beavis (1998) regarded even 200 individuals as too few. Wang et al. (2012) concluded that for genome-wide association studies (GWAS) in a barley core collection, more than 384 individuals are necessary to detect QTLs consistently. However, for

Table 5 Significant SNP × leaf rust associations detected across the NAM population HEB-5 and within single NAM families, respectively

Pop/Fam[a]	QTL name[b]	Chr[c]	Range[d]	SNP locus[e]	N[f]	LSMEANS (*Hv*)	LSMEANS (*Hsp*)	Difference[g] (in %)	*P*(FDR) value[h]	Corresponding R genes[i]	Corresponding Meta-QTLs/QTLs[j]
HEB-5	QLr.HEB-5-1H	1H	142.55	4592_118	1,192	4.5	3.0	**−33.3**	0.017		
HEB-F23	QLr.HEB-F23-2H	2H	61.49–75.95	6911_866	163	3.5	5.6	60.0	0.030	*Rph14*[k], *Rph15*[l], *Rph16*[m], *Rph17*[n]	MQTL4[p] (Rphq18[q])
HEB-F23	QLr.HEB-F23-3H	3H	98.05–104.15	4019_302	143	3.6	5.8	61.1	0.010		MQTL7C[p] (Rphq20[q])
HEB-5	QLr.HEB-5-4H	4H	56.22–61.56	3042_1225	1,050	4.2	5.3	26.2	0.017		MQTL9[p] (Rphq19[q])
HEB-F23	QLr.HEB-F23-4H	4H	61.56–78.50	7069_1149	178	3.7	5.9	59.5	0.027		MQTL9[p] (Rphq19[q])
HEB-5	QLr.HEB-5-5H	5H	7.26	1582_63	1,126	4.3	5.7	32.6	0.046		Rphq4[q]
HEB-5	QLr.HEB-5-5H.b	5H	166.65	552_188	1,184	4.3	5.7	32.6	0.049		MQTL13[p] (Rphq7[q]), MQTL14[p] (Rphq16[q])
HEB-F23	QLr.HEB-F23-7H	7H	85.28–89.78	1674_468	174	4.7	3.0	**−36.2**	0.036	*Rph19*[o]	MQTL18[p] (Rphq1[q])

Bold values indicate *Hsp* genotypes reducing leaf rust symptoms

For QTLs consisting of multiple QTLs, only the SNP with the lowest P(FDR) value is given

[a] Name of NAM population (HEB-5) and family (HEB-F06, -F08, -F14, -F23), respectively

[b] QTL name is indicated by the letter 'Q,' trait, population, and chromosome

[c] Chromosomal location of the SNP

[d] The range of the QTL in centiMorgan (cM) is indicated from the position of the first to the last significant SNP, based on Muñoz-Amatriaín et al. (2011)

[e] SNP name based on Muñoz-Amatriaín et al. (2011), showing the lowest P(FDR) value within the QTL range

[f] Number of observations at SNP locus used for calculation

[g] Relative score difference between homozygous *Hsp* and *Hv* genotypes, calculated as: [LSMEANS(*Hsp*) − LSMEANS(*Hv*)] * 100/LSMEANS(*Hv*)

[h] False discovery rate (FDR) adjustment of raw p values according to Benjamini and Hochberg (1995)

[i] References for *Rph* genes located on the same chromosome (or chromosome arm) as the detected QTL: [k]Golegaonkar et al. (2009a), [l]Weerasena et al. (2004), [m]Ivandic et al. (1998), [n]Pickering et al. (1998), [o]Park and Karakousis (2002)

[j] References for Meta-QTLs and QTLs located in the same genomic region as the detected QTL: [p]Schweizer and Stein (2011), [q]Marcel et al. (2007a)

multi-parental barley populations, the definition of the optimal population size to detect most of the QTLs has not been estimated so far. It is obvious that one has to keep time and costs for phenotyping and genotyping in a reasonable relation to the number of QTLs detected. Nevertheless, Stich (2009) demonstrated through computer simulation studies that for maize and *Arabidopsis*, diallel and factorial designs had greater power to detect QTLs than recombinant inbred line (RIL) designs with 25 and 19 donors, respectively. However, we are confident that under practical considerations, the latter multi-donor RIL design may be more advantageous due to the ease of population development and the ease of comparing donor allele effects against a single recipient parent.

Recently, a maize NAM population and a sorghum NAM population were reported. The genetic architecture (number of NAM families and NAM lines) is different compared to HEB-5 (five NAM families, 295 NAM lines). Buckler et al. (2009) investigated a maize NAM population consisting of 5,000 NAM lines (25 NAM families with 200 lines per family) in regard to three flowering time (FT) traits. Across the maize NAM population, the number of lines provided sufficient power to detect QTLs. Additionally, FT QTLs were detected for NAM subsets across at least three to four NAM families. However, no FT QTL was detected within single NAM families. Also, for the two maize foliar diseases, southern leaf blight and northern leaf blight resistance QTLs were detected across the maize NAM population and within each NAM family separately (Kump et al. 2011; Poland et al. 2011). Also, 23 maize NAM families were analyzed in regard to starch, protein, and oil content. QTLs within individual NAM families were detected for at least 22 NAM families per trait (Cook et al. 2012). Resistance QTLs against several corn borers were evaluated for a subset of 281 maize NAM lines (eight NAM families). However, QTL stability was detected only across two NAM families (Butrón et al. 2010) indicating that the donor accessions of the NAM population may contribute different effective resistance QTL alleles. The maize data also indicate that in most maize NAM studies, finding QTLs across families is improved compared to finding QTLs within individual NAM families.

In sorghum, Jordan et al. (2011) developed a NAM population, consisting of 56 NAM families with 30–90 lines per family. The sorghum population structure may reduce the chance to locate QTLs in individual NAM families in favor of increasing the chance to locate QTLs across the NAM population due to the increased number of donor accessions used. A subsample of the sorghum NAM population, consisting of seven families with 31–59 NAM lines per family, was used to locate QTLs for plant height, FT and grain yield (Jordan et al. 2011), and stay-green effects (Mace et al. 2012). In these cases, the population size of 339 lines was sufficient to detect effects of exotic donor alleles in multiple individuals (Jordan et al. 2011).

From NAM reports in maize and sorghum, it is noticeable that the potential to detect QTLs strongly depends on the heritability of the trait investigated. Hence, the optimal size and composition of a NAM population should be adjusted to the particular target trait under study. We consider the explorative barley NAM population HEB-5 as comparatively small, in particular, in regard to the number of donor accessions included. Although the current architecture of HEB-5 already proved to be sufficient to detect leaf rust resistance genes (see below), increasing the number of wild barley donor accessions may lead to a further enhancement of the power to detect QTLs. This may be true because of the increased population size and because of the increased genetic diversity which can be interrogated, if the donor accessions are carefully selected. We, thus, consider HEB-5 only as an explorative step toward the development of a full barley NAM population.

SNP genotyping of HEB-5

The high-density genome coverage of 1.0 cM per SNP (Table S1) in HEB-5 is similar to genome coverages described for other populations (e.g., Schmalenbach et al. 2011). A number of SNPs were excluded due to monomorphic behavior or due to a minor allele frequency threshold of MAF <0.05. We plan to close the present genome gaps (Table S1) and to increase the SNP resolution by genotyping HEB-5 lines with the Infinium iSELECT 9 k barley SNP chip (Comadran et al. 2012) and, eventually, by exome capture sequencing (Mascher et al. 2013).

For population HEB-5, χ^2 p values were calculated to test whether genotype or allele frequencies across the genome and per chromosomes deviated from the expected segregation in BC_1S_1. No significant deviation was detected across population HEB-5. These finding corroborates that during the development of HEB-5, no obvious selection, neither naturally nor unintentionally, may have been imposed on the NAM population. The chances to study genetic diversity in HEB-5, resulting in the location of genetic effects are, thus, ideal. Nevertheless, genotype frequencies and allele frequencies of particular chromosomes deviated from the expectation in NAM families F08, F14, and F23 (Table S4, Table S5).

Genetic similarity (GS) and principal component (PCo) analysis

GS between parents (‘Barke,’ HIDs) and between NAM lines were estimated. GS scores between ‘Barke’ and the HEB-5 donors varied between 33.0 % and 49.7 % (Table S6). In contrast, GS between the maize elite parent and the ‘exotic’ donors (landraces) in the maize NAM population was estimated much higher with approximately 80.0 % (Liu et al. 2003). Unfortunately, no GS scores were

published for the sorghum NAM population (Mace et al. 2008; Jordan et al. 2011). We assume that the low level of genetic similarity between 'Barke' and the HEB-5 donors increases the chances to find DNA polymorphisms and, more important, to detect QTLs.

The average GS between 'Barke' and HEB-5 lines was 77.9 % (Table 3). This finding indicates the lack of significant natural or unintentional human selection during development of the NAM population. In addition, one outlier was identified based on calculating GS estimates. The highest GS was detected between donor accession HID-099 and HEB-F08-021 (GS = 98.6 %, Fig S2, Table S6). We assume that this genotype may be a variant of HID-099. The line was, thus, excluded from further analysis.

It is known that GS estimates can suffer from ascertainment bias, which may occur if genotype data are not obtained from a random sample of SNPs in the population of interest or, if only a small number of genotypes were used for SNP discovery (Heslot et al. 2013). BOPA1 SNPs were selected from comparing elite barley sequence variations, which potentially lead to an overestimation of GS between exotic donors (Russell et al. 2011). Ascertainment bias was demonstrated for BOPA1 while comparing elite and exotic barley (Moragues et al. 2010). Thus, the true GS between the five exotic donors, 'Barke,' and the HEB-5 individuals might be slightly lower than estimated with BOPA1. SNPs, which are derived from genotyping-by-sequencing (GBS), are expected to be uniformly distributed across the genome (Davey et al. 2011). They were, thus, suggested to reduce ascertainment bias in biparental populations (Poland et al. 2012; Poland and Rife 2012; Heslot et al. 2013). For HEB-5, we also suggest to apply exome capture sequence analysis and subsequent haplotype calling (Mascher et al. 2013) to reduce possible ascertainment bias effects.

Detection of QTLs for seedling leaf rust resistance and comparison with already located R genes and QTLs

We scored seedling leaf rust resistance to demonstrate the power to detect QTLs across the multi-parental barley NAM population HEB-5 and within individual NAM families. Variation in LR scores was found among HEB-5 parents as well as within HEB-5 and the five NAM families. LR scores were observed in a medium range from 2.4 to 6.0 (Table 4; Fig. S4). According to McMullen et al. (2009), low resistance presumes that minor QTLs will be detected by association mapping. LR resistance QTLs were detected on all barley chromosomes except of 6H. Four QTLs were detected across HEB-5 and four QTLs within one NAM family—HEB-F23. However, only one QTL was common between HEB-5 and HEB-F23. In the following, the QTLs are discussed and the chromosomal position of the new QTLs is compared with QTLs and R genes already detected in previous experiments. Table 5 shows known Meta-QTLs (MQTLs), QTLs, and R genes if located in the same chromosomal region as QTLs detected in the present study.

Detection of QTLs often depends on the pathogen isolate and the mapping population used (González et al. 2012). Two QTLs were mapped to centromeric regions on the chromosomes 2H and 4H (Fig S6). We presume that these regions are in accordance with specific resistance hot spot regions reported by Schweizer and Stein (2011). The exact centromeric location of the MQTL map published by Schweizer and Stein (2011) is unknown; however, we assume that QLr.HEB-F23-2H lies in the hot spot region MQTL4, where resistances against *Blumeria* spp. and *P. hordei* are mapped.

Regarding chromosome 4H, two QTLs were detected across HEB-5 and within HEB-F23, respectively, which partly overlap (Table 5; Fig. S6; Table S7). These QTLs are, thus, potentially identical, demonstrating that allelic resistance effects present in HEB-5 can be traced back to the original donor accession used to develop the NAM family. The centromeric region of chromosome 4H is again a hot spot for different plant resistance genes (cf. Schweizer and Stein 2011). The leaf rust resistance QTL Rphq19 (Marcel et al. 2007a) was confirmed in the MQTL study of Schweizer and Stein (2011) and mapped to the QTL region MQTL9. In that genomic region, resistances against other fungi, for instance *Blumeria graminis,* were located. We assume that the QTLs detected in the present study correspond to the hot spot region MQTL9.

Three further QTLs (QLr.HEB-F23-3H, QLr.HEB-5-5H, QLr.HEB-F23-7H) were mapped to QTL regions identified as hot spot regions (Schweizer and Stein 2011), and one QTL (QLr.HEB-5-5H.b) was mapped to the same region, where resistance was exclusively detected against *P. hordei* (Marcel et al. 2007a; Schweizer and Stein 2011). Table 5 lists the potentially corresponding MQTL regions on the long arms of chromosomes 3H, 5H, and 7H and, additionally, the QTL region on the short arm of chromosome 5H.

Three of four QTLs detected across HEB-5 did not overlap with QTLs detected within individual NAM families. However, nonsignificant associations within two individual NAM families were found. These effects had the same direction within at least one NAM family. Concerning the third QTL, comparison of the direction of the effect was not possible, because SNPs representing the specific locus were excluded within individual families (data not shown). These results indicate that although some LR effects may already be visible in individual NAM families, they only become significant if analyzed across the total NAM population.

The QTLs cited so far were detected with various isolates. However, some previously mapped QTLs were detected with the LR isolate 'I-80,' which was also used in

our study. For instance, two QTLs were detected on chromosome 2H, where *Hsp* alleles were associated with LR resistance (Backes et al. 2003; Kopahnke et al. 2004). Taking into account that the QTL detected on chromosome 2H in Backes et al. (2003) was mapped to the telomeric region, we assume that this QTL is different from QLr.HEB-F23-2H, which was mapped to the centromeric region in the present study (Fig. S6). Regarding the QTL detected by Kopahnke et al. (2004), a correspondence may be possible. However, since the markers used in both studies are different (SSRs versus SNPs), a remapping of informative markers may be necessary to come to a final conclusion.

According to González et al. (2012), *Rph* genes and QTLs seem to rely on different types of genes due to map positions. However, some *Rph* genes that are potentially detectable with 'I-80' were mapped to the same chromosome arms where we detected QTLs (Table 5). For instance, *Rph14* (Golegaonkar et al. 2009a), *Rph15* (Weerasena et al. 2004), *Rph16* (Ivandic et al. 1998), and *Rph17* (Pickering et al. 1998) are mapped to the short arm of chromosome 2H. Thus, an overlap with the detected QTLs may be possible. *Rph19*, which was mapped to chromosome 7HL (Park and Karakousis 2002), may overlap with the QTL we detected on 7HL. However, fine mapping of the cited *Rph* genes is needed in HEB-5 to confirm this hypothesis. Also, it remains open if the QTLs detected in HEB-5 and the cited QTLs represent the same resistance alleles. To test this hypothesis, a gene postulation assay may be conducted, using a battery of contrasting LR isolates, to test whether the resistance QTLs in HEB-5 represent new LR resistance alleles or not (Dreiseitl and Steffenson 2000).

Conclusion

The explorative BC_1S_1 population HEB-5 was established as a first step toward the final development of a 26-parental barley NAM population. In total, eight QTLs controlling suppression of leaf rust occurrence were recorded studying LR seedling resistance in HEB-5. Our results confirm that the NAM approach allows detecting new wild barley QTLs for LR, demonstrating its potential value to utilize wild barley diversity in barley genetics. One QTL, QLr.HEB-5-1H, may be of particular interest. The *Hsp* allele at this locus was associated with a 33.3° % reduction in LR. The QTL may be further explored and utilized in barley-resistance breeding.

The barley NAM population is still under development. In the future, HEB-5 will be further expanded in regard to number of NAM families and number of NAM lines. The final barley NAM population HEB-25 will comprise 25 NAM families and approximately 1,500 NAM lines in BC_1S_3 generation. HEB-25 will be genetically characterized with the Infinium iSELECT 9 k barley chip, covering 7,864 barley SNPs (Comadran et al. 2012). Eventually, we plan to further increase the marker resolution by exome capture sequence analysis (Mascher et al. 2013). We assume that the final HEB population will provide a higher power to detect effects of exotic QTL alleles. We plan to phenotypically characterize the final HEB-25 population in regard to morphological traits, agronomic traits, and disease resistances against various barley pathogens. Following this strategy, new exotic barley alleles, potentially useful, may be detected and utilized in barley breeding with increased efficiency. In addition, HEB-25 may be very useful to support forward genetics studies since its size and the number of generations used to develop the population will foster the identification of informative recombination events in a target region, separating linked genes from the target gene that needs to be identified by map-based cloning.

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Conflict of interest We declare that we have no conflict of interests in regard to the present study.

Ethical standards We declare that we followed all relevant ethical standards while carrying out the present study.

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