Population genomics of selectively neutral genetic structure and herbicide resistance in UK populations of *Alopecurus myosuroides*.

Running title: Population structure in herbicide resistant *Alopecurus myosuroides*

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ABSTRACT

BACKGROUND: *Alopecurus myosuroides* (blackgrass) is a major weed in Europe with known resistance to multiple herbicide modes of action. In the UK, there is evidence that blackgrass has undergone a range expansion. In this paper, genotyping-by-sequencing and population-level herbicide resistance phenotypes are used to explore spatial patterns of selectively neutral genetic variation and resistance. We also perform a preliminary genome-wide association study and genomic prediction analysis to evaluate the potential of these approaches for investigating non-target site herbicide resistance.

RESULTS: Blackgrass was collected from 47 fields across the British Isles and up to eight plants per field population (N = 369) were genotyped by RAD-sequencing. 20,426 polymorphic loci were identified and used for population genetic analyses. Phenotypic assays revealed significant variation in herbicide resistance between populations. Population structure was weak (F_{ST} = 0.024-0.048), but spatial patterns were consistent with an ongoing westward and northward range expansion. We detected strong and consistent Wahlund effects (F_{IS} = 0.30). There were no spatial patterns of herbicide resistance or evidence for confounding with population structure. Using a combination of population-level GWAS and genomic prediction we found that the top 20, 200, and 2,000 GWAS loci had higher predictive abilities for fenoxaprop resistance compared to all markers.

CONCLUSION: There is likely extensive human-mediated gene flow between field populations of the weed, blackgrass at a national scale. The lack of confounding of adaptive and neutral genetic variation can enable future, more extensive GWAS analyses to identify the genetic architecture of evolved herbicide resistance.

Keywords: blackgrass, RAD-sequencing, population genetics, population structure, GWAS, metabolic resistance.

1 INTRODUCTION

Weedy plants are ideal models for studying plant adaptation in rapidly changing, human-influenced environments[1] and efforts to understand the environmental, genetic and management factors that shape the evolution of weeds are crucial for the design of sustainable weed management strategies.[2] Since the middle of the 20th Century, weed control in agroecosystems has become dominated by the use of synthetic herbicides, with notable success.[3,4] Now, however, the rapid and repeated evolution of resistance is reported in almost all cropping systems where herbicides have been used intensively for weed control.[5,6] Rapid adaptation to the intense selection pressures imposed by herbicides is a classic example of human-directed evolution and has been extensively studied by weed biologists and plant physiologists.[7]

The diploid, allogamous grass, *Alopecurus myosuroides* (blackgrass), is thought to be native to the eastern Mediterranean and to have subsequently spread throughout Europe and Western Asia.[8] It is a major weed of autumn-sown cereal crops in England,[9] France,[10] and Germany.[11] Its range as an agronomically damaging weed has recently expanded to include Denmark, Sweden, the Netherlands, Poland, and northern Italy. In the United Kingdom, a survey in 1988 identified blackgrass as the ninth most frequent weedy plant in cereal fields.[12] However, now it is evidently the most problematic UK agricultural weed and there is clear evidence of a northwards and westwards range expansion in the last 30-40 years.[9]

Blackgrass is also prone to the rapid evolution of resistance to herbicides, with populations from many European countries exhibiting resistance to multiple herbicide modes of action.[13] Two major classes of herbicide resistance mechanisms have been reported in blackgrass.[14] Target-site resistance (TSR) is usually endowed by single mutations in herbicide target genes and gives rise to 'specialist' resistance to a single herbicide mode of action. Non-target site resistance (NTSR) is thought to evolve via the coordinated up-regulation of a suite of metabolic and stress-

responsive pathways and gives rise to a more 'generalist' resistance phenotype, with cross-resistance to multiple herbicide modes of action.

The increasing prominence of blackgrass as a major agricultural weed and its ongoing range expansion within Europe and the United Kingdom, raises several interesting questions about 'wild-to-weed'[1] evolutionary dynamics in this species. Increasing weediness may be driven by rapid evolution of resistance to herbicides, which leads to weed control failures, increased population sizes and greater potential for blackgrass dispersal. However, there may also be agronomic and climatic drivers for blackgrass expansion. For example, in the UK, and in other parts of northern and western Europe, there has been a decline in cropping system diversity and an increase in the inter-annual proportion of autumn-sown cereal crops, which provide an optimal environment for blackgrass, an early autumn germinating weed,[15] to thrive as a competitive weed. At the same time, climate change, rising average temperatures and changing precipitation patterns may be expanding the geographical range over which blackgrass can succeed as an agricultural weed.

A number of previous studies have explored genetic variation, population structure and diversity of herbicide TSR alleles in *Alopecurus myosuroides*, generally concluding that: (1) genetic diversity within populations is high, (2) differentiation between populations is low, (3) evolution of resistance has not reduced genome-wide patterns of genetic diversity and (4) resistance evolves as multiple independent events, though with some localized dispersal of resistance alleles via gene flow.[10,16–18]

In this study, we explore patterns of genetic variation using 20,426 single-nucleotide polymorphisms (SNPs) and a sample of 369 blackgrass individuals from 47 fields across the British Isles to test if spatial patterns of population structure are consistent with general expectations under rapid range expansion (i.e. isolation by distance (IBD), allele frequency gradients along major vectors of migration and reduced diversity in more recently colonized areas[19–21]). We then quantify the extent to which population-level herbicide resistance is confounded with neutral population structure. Finally, and for the first time in blackgrass, we

perform a pilot-scale genome-wide association study (GWAS) and genomic prediction analysis to assess the potential of these approaches for dissecting the architecture of NTSR in the future.

2 MATERIALS AND METHODS

2.1 Populations and phenotypic data

2.1.1 Population collection

Alopecurus myosuroides (blackgrass) seed was collected from 206 winter-wheat fields from 128 farms across the British Isles (126 in England, one in each of Scotland and Ireland) during July and August 2014. These sampling locations were identified by seeking farmer participation in a large project which explored the epidemiological drivers of blackgrass population expansion across the British Isles. The population sample therefore represents a diverse collection of agricultural blackgrass populations, albeit that participating volunteers were likely biased towards those with larger and difficult to control blackgrass populations. Seed was collected from multiple locations in each field, using either a stratified sampling design where seed was collected from mature plants within a 10 metre radius at 10 locations throughout a field, or by randomly collecting seed from several random locations in each field. Seed from all collection sites was bulked to create a single seed population from each sampled field (referred to hereafter as 'populations'). For most populations, we generated data on herbicide resistance phenotype through glasshouse bioassays (see below), and for some populations we were able to gather data on historical (up to 10 years) field management histories.[9] All but one of the collected blackgrass populations demonstrated some evolved resistance to at least one of the three herbicide resistance modes of action tested. Using principal components analysis, we identified 47 populations for inclusion in this study (Figure S1) based on (i) contrasting relative frequencies and levels of resistance to three different herbicides (fenoxaprop-p-ethyl, mesosulfuron and cycloxydim), (ii) geographical locations, and (iii) herbicide selection histories. In addition, a naïve control population which has never been exposed to herbicide was included.

2.1.2 Herbicide phenotyping greenhouse assay

For 46 of the populations (all but P200), data were available for population-level sensitivity to three herbicides commonly used for blackgrass control in the UK; fenoxaprop-P-ethyl, cycloxydim (acetyl-coA carboxylase [ACCase] inhibitors), and mesosulfuron + iodosulfuron (acetolactate synthase [ALS] inhibitors).[9] Briefly, herbicides were applied to glasshouse-grown plants (n = 18 per population) at a dose known to provide an efficacy comparable with herbicide application in the field. For mesosulfuron + iodosulfuron, this dose was 0.75x the recommended field rate (14.4 g ha^{-1}). For fenoxaprop and cycloxydim, 1x of the field rate was applied (68.75 and 150 g ha^{-1}, respectively). Three weeks after herbicide application, sprayed and unsprayed plants were assessed for mortality, and above-ground leaf and shoot biomass was harvested, dried at 70˚C for 48 hours, and weighed.

2.2 Tissue sampling and DNA extraction

Seeds were germinated in Petri dishes with filter paper and wetted with 3-4mL of 0.02M potassium nitrate; excess liquid was removed. Dishes were placed in incubators (Sanyo, MLR-350) for seven days at 14/10-hour day/night cycles at 17°C and 11°C, respectively. Seedlings that had produced a shoot and radicle at day 7 were transplanted into larger pots and grown in a glasshouse set to maintain 16°C/10°C day/night temperatures with supplementary lighting provided over a 14-hour day length, until the five to six leaf stage. Eight individuals from each of the 47 populations were sampled. Leaf tissue was collected into 2-mL Eppendorf tubes and snap frozen in liquid nitrogen.

Samples were ground using a mortar and pestle with liquid nitrogen and extracted using the DNeasy Plant mini-prep kit (Qiagen, Valencia, CA, USA). Extracted DNA was run on a 1.5% agarose gel to assess DNA quality and DNA concentration was measured using the Qubit dsDNA high sensitivity assay kit (Thermo Fisher Scientific, Waltham, MA, USA). DNA was then normalized to a concentration of 20 ng/µL. For four populations (P163, P175, P186, and P191), it was only possible to extract sufficient DNA for seven individuals, meaning that a total of 372

individuals were genotyped. The 372 samples were distributed across four plates with two individuals from each population placed in each plate. Samples were randomly assigned positions within the plates.

2.3 RAD-sequencing and SNP calling

RAD-sequencing was performed by Floragenex (Floragenex, Inc., Portland, OR, USA) using standard methodology.[22] Libraries were created using the *PstI* restriction enzyme and all four plates were sequenced across all four runs of NextSeq 500 (Illumina, San Diego, CA, USA). A total of 1,024,832,390 reads were generated, with each individual covered by an average of 2.5 million reads (Supporting information Table S1). SNPs were called using the UNEAK pipeline implemented in TASSEL v 3.0.[23,24] The pipeline was run with default parameters, except for the UMapInfotoHapMapPlugin step, where the minimum minor allele frequency was 0.0025 and the minimum call rate was 0.8. This analysis resulted in 26,746 RAD-Seq tags, which were further filtered using VCFtools v 0.1.14.[25] Individuals and loci were filtered according to percent missing data and allele frequency (Supporting information Table S2). Three individuals with more that 45% missing data across all loci were removed, leaving 369 individuals for further analysis. Loci with a minor allele frequency (MAF) of less than 1% or more than 20% missing data were removed (n = 3,614), leaving 22,862 loci for further analysis. Linkage disequilibrium among SNPs was calculated using the *--geno-r2* option. Thirteen locus pairs had $r^2 > 0.8$, and the locus with the lower MAF was removed from the data set. Hardy-Weinberg Equilibrium (HWE) tests were performed independently in each population using the *–hardy* option. Loci that deviated significantly ($P < 0.01$) from HWE were removed (n = 2,423), leaving a final dataset of 369 individuals and 20,426 loci (Supporting information Table S2.).

2.4 Data analysis

2.4.1 Population structure and relatedness

Population structure was detected and quantified using an individual-based principal component analysis (PCA)[26] and an analysis of molecular variance (AMOVA),[27] respectively. The PCA was performed using the smartPCA program in the EIGENSOFT v 7.2.1 software.[26] This program was also used to calculate pair-wise F_{ST} values based on Hudson's estimator, which is robust to the effects of rare alleles.[28] Arlequin v. 3.5.2.2[29] was used to perform a locus-by-locus AMOVA, resulting in estimates of F-statistics calculated as 'ratios of averages' following the recommendations of Bhatia *et al*..[28] Identity By State (IBS) kinship matrices were calculated within each population to obtain measures of relatedness that are not affected by allele frequency differentiation.[30]

To investigate the relationship of population structure with geography, Spearman rank correlations between an individual's field location (i.e. latitude and longitude) and its principal component (PC) score for the first two PCs were calculated in R.[31] Finally, to test for IBD, a simple Mantel test[32] was performed for pair-wise F_{ST} and geographic distances. Geographic distance was calculated using the "geosphere" package v. 1.5-7[33] using the *distGeo* option to calculate the shortest distance on a ellipsoid. The Mantel tests were executed using the "vegan" package v. 2.4-6[34] in R.

2.4.2 Analysis of phenotypic data

For each of the six phenotypic traits (mortality and biomass for each herbicide, see *herbicide phenotyping greenhouse assay*), we used the "lme4" package in R v. 0.999375-42[35] to fit the following linear mixed model:

$$X_{ijklm} = \mu + Dos_i + Gh_j + Tr_k(Gh_j) + Pos_l(TrGh_{kj}) + Pop_m(PosTrGh_{lkj}) + Pop_m(PosTrGh_{lkj}) \times Dos_i + \varepsilon_{ijklm}$$

where X_{ijklm} is the phenotypic measurement for the m^{th} population at the l^{th} pot position within the k^{th} tray within the j^{th} glasshouse treated with the i^{th} herbicide dosage; μ is the overall mean, Dos_i

is the fixed effect of the i^{th} herbicide dosage; Gh_j is the random effect of the j^{th} glasshouse; $Tr_k(Gh_j)$ is the random effect of the k^{th} tray within the j^{th} glasshouse; $Pos_l(TrGh_{kj})$ is the random effect of the l^{th} pot position within the k^{th} tray within the j^{th} glasshouse; $Pop_m(PosTrGh_{lkj})$ is the random effect of the m^{th} population at the l^{th} pot position within the k^{th} tray within the j^{th} glasshouse; $Pop_m(PosTrGh_{lkj})\times Dos_i$ is the population-by-dosage interaction (random effect); and ε_{ijklm} is the experimental error. These models were used to estimate the respective variance components for all random effects using restricted maximum likelihood, and confidence intervals were constructed using likelihood profile (i.e., for variance components) and bootstrapping functions (i.e., for ratios of variance components, based on 100 samples) available within the "lme4" and "boot"[36,37] R packages. In addition, we extracted best linear unbiased predictor (BLUP) values for each population by phenotype by herbicide combination. These BLUP values were used to calculate population-level genetic correlations (i.e., as Pearson correlations of BLUPs). BLUP values were used as population-level herbicide resistance phenotypes in subsequent redundancy, genome-wide prediction and association analyses (see below). In addition, we used Spearman rank correlations to test for associations between phenotypic PCs (calculated using the *prcomp* function in R) and geographic coordinates (i.e. to enable comparisons with spatial patterns of neutral population structure, see above).

2.4.3 Partitioning variance with redundancy analysis

A redundancy analysis[38] was used to estimate how much of the variance for multivariate resistance phenotypes is explained by neutral population structure. For each model, significant PCs ($P < 0.05$, based on the Tracy-Widom test) from the smartPCA program were used to explain the variation in population-level phenotypic BLUPs (see *Analysis of phenotypic data*). Using *ordistep* in the "vegan" package and initially considering all significant PCs as explanatory variables, backward elimination was implemented at $\alpha = 0.05$. Using the PCs significant from the backward elimination, an RDA was then performed in "vegan". A model was run using both

biomass and mortality phenotypes as the response variables for each herbicide alone and all three herbicides collectively.

2.4.4 Population level GWAS and genomic prediction

To further assess the relationship between population-level herbicide resistance phenotypes (i.e. both mortality and biomass, see *Analysis of phenotypic data*) and SNP allele frequencies, we performed genome-wide association study (GWAS) and prediction analyses using standard methodology as in previous studies.[22] Briefly, we used the EMMAX[39] and G-BLUP/ridge regression[40] approaches as implemented in the "rrBLUP" R package[41] for population-level GWAS and prediction analyses, respectively. Instead of individual SNP genotypes, we used population-level allele frequencies in rrBLUP. To account for multiple testing in GWAS, we calculated false discovery rate q-values using the "qvalue" R package v. 1.34.0.[42] Genomic predictive ability was calculated as the correlation between predicted and observed phenotypic BLUP values based on 100 iterations of random 11-fold cross-validations (i.e., training set = 40 populations and prediction set = 4 populations, excluding two strongly differentiated populations, see Results). Finally, prediction analyses were also performed using the 20 (~ 0.1%), 200 (~ 1%), 2000 (~ 10%), and 20000 (~ 98%) markers with lowest *P*-values in GWAS analyses of the training set.

3 RESULTS

3.1 Population structure

An AMOVA was used to assess variation for three levels - among populations, within populations, and within individuals. From this analysis, variation among populations accounted for 2.4% of the total variation (i.e. F_{ST} = 0.024), while the variance components within populations and within individuals were much higher, accounting for 28.9% (F_{IS} = 0.289) and 68.7% of the total variation, respectively (Table 1). As expected, based on the known effects of

SNPs with low-frequency alleles,[28] F_{ST} based on Hudson's estimator was slightly higher (average = 0.048 across all pairs of populations, Supporting information Fig. S2).

Two populations (P128 and P163) were strongly differentiated from the other populations (Figs. 1a, S2), presumably because of substantially higher relatedness among the sampled individuals (Supporting information Fig. S2). This high-level of relatedness may be a sampling artifact, whereby low population sizes necessitated sampling of seeds from a restricted set of individuals from a single or few locations within these fields. The following results are therefore reported without P128 and P163; although results do not change substantially with their inclusion.

After removing populations P128 and P163, the individuals from the remaining 45 populations were distributed more continuously along the first two principal components (PC1 and PC2) of population structure, but apart from one population (P148) *a priori* populations could not be discriminated (Fig. 1b). However, population genetic variation was structured geographically. As expected from our *a priori* demographic model, which assumes a recent northwards and westwards range expansion for blackgrass in the UK, SNP PC1 was significantly correlated with both latitude (Spearman rho = 0.46, $P < 0.001$; Fig. 2a) and longitude (Spearman rho = -0.17, $P = 0.001$; Fig 2b), whereas PC2 was significantly correlated with latitude (Spearman rho = -0.13, $P = 0.017$; Supporting information Fig. S3a), but not longitude (Spearman rho = -0.048, $P = 0.36$; Supporting information Fig. S3b). Furthermore, both observed and expected heterozygosity were positively correlated with longitude (Spearman rho = 0.40, $P = 0.0072$ and Spearman rho = 0.45, $P = 0.0022$, respectively; Fig. 3), though no linear relationship was detected with latitude (Spearman rho = 0.034, $P = 0.82$ and Spearman rho = 0.021, $P = 0.89$, respectively). Finally, pair-wise F_{ST} and geographic distances were significantly correlated (Mantel r = 0.50, *P*-value < 0.001; Supporting information Fig. 4a), consistent with IBD models.

Upon the removal of the two geographically disjunct populations (i.e. the Irish and Scottish populations, n = 43), PC1 was still significantly correlated with both latitude (Spearman rho = 0.45, $P < 0.001$; Supporting information Fig. S4a) and longitude (Spearman rho = -0.13, $P =$

0.021; Supporting information Fig. S4b). While this was no longer the case with PC2, English populations exhibited a similarly strong IBD pattern (Mantel r = 0.41, $P < 0.001$; Supporting information Fig. S5).

3.2 Phenotypic variation for resistance to herbicides

Phenotypic data for six quantitative traits (i.e., mortality and biomass for each of three herbicides) were analyzed using a linear mixed model to assess the relative importance of population effects, herbicide dosage and their interaction. The population-by-dosage interaction was consistently significant (95% confidence intervals did not include zero) and accounted for at least 50% and as much as 84% of the phenotypic variance, after removing the effects of glasshouse, tray and pot position (Supporting information Table S3, S4). Furthermore, the main population effects were consistently very weak and genetic correlations between BLUPs with herbicide treatment versus without were close to zero (Supporting information Table S5). Finally, biomass and mortality BLUPs under the herbicide treatment were strongly but not perfectly correlated (r ~ 0.9), suggesting that both measures of population level herbicide resistance could be useful in downstream analyses. Taken together, these results suggested that the large population differences were almost exclusively due to the interaction with herbicide treatment, reflecting differences in the herbicide resistance status of populations. However, there were no significant correlations between individual resistance phenotypes (or their first two PCs) and geographic coordinates.

3.3 Partitioning of variance and Redundancy analysis

A redundancy analysis was used to quantify the extent to which variation for population resistance phenotypes (i.e. BLUPs for biomass and mortality for each herbicide) can be explained by presumably neutral population genetic structure. Using backward elimination, PCs representing population structure were selected if they significantly explained variation in the resistance phenotypes (Table 2). For all six phenotypes in combination, population structure (PC

6; Supporting information. Fig. S6) explained 6.5% of the variation. For cycloxydim biomass and mortality, 29% of the variation was explained (PC 6, PC 9, and PC16), whilst 16% of the variation was explained for mesosulfuron + iodosulfuron biomass and mortality (PC 6 and PC 14). From the backward elimination, no PCs were found to significantly explain variation for fenoxaprop. Similar patterns were detected using univariate regression analyses for each resistance phenotype (data not shown).

3.4 Population level GWAS and Genomic Prediction

After accounting for multiple testing, population level GWAS found no significant associations between herbicide resistance phenotypes and SNP allele frequencies. However, genomic prediction based on the 0.1-10% of markers with lowest GWAS *P*-values tended to be substantially more accurate than using randomly selected markers (Fig. 4). Remarkably, the top 20, 200, and 2,000 GWAS loci for fenoxaprop biomass all had higher predictive abilities compared to using nearly all markers.

4 DISCUSSION

We have shown that the herbicide resistance-prone, outcrossing, agricultural weed, *A. myosuroides* (blackgrass), exhibits low genetic differentiation amongst UK field-collected populations, but with some geographical structuring of genetic variation and evidence for latitudinal and longitudinal clines in genetic diversity, and isolation by distance. Our results are also suggestive of significant blackgrass sub-population structure within agricultural fields. The 47 populations used in this study exhibit phenotypic variation for resistance to three herbicides commonly used for blackgrass control. In this study system, there appears to be relatively little confounding between neutral population genetic structure and herbicide resistance, presenting a generally favourable situation for the design of GWAS type studies to unravel the genetic basis and genetic architecture of herbicide resistance traits. Although no significant associations between SNPs and resistance phenotype were found using a population level GWAS, a genomic

prediction analysis suggested that a subset of SNPs (0.01-10%) tended to have a higher predictive ability for resistance phenotype than all of the SNPs collectively.

4.1 UK blackgrass exhibits low genetic differentiation, but with clear spatial patterns of range expansion

Aside from two strongly differentiated populations, whose differentiation appeared to arise from a high degree of relatedness between individuals, PCA did not detect strong discontinuities among *a priori* (geographically sampled) populations. Genetic differentiation amongst blackgrass populations in this study (average F_{ST} = 0.024 – 0.048 across two estimators chosen for their robustness to the effects of rare alleles) was much lower than the average (0.16) for an annual, outcrossing, monocotyledonous species.[43] Notwithstanding this, our results were notably similar to those previously reported for blackgrass populations in France, the UK, Germany, and Israel and for a similar, weedy grass species in North America. Menchari *et al.*[18] used AFLPs to investigate the population structure of 36 French blackgrass populations, and found similarly low levels of population differentiation (F_{ST} = 0.023), concluding that this was indicative of the recent expansion of the species as a weed in France. An allozyme study of 19 populations, collected from the UK, France, Germany, and Israel, calculated mean (seven allozyme loci) F_{ST} values of 0.023.[16] In populations of the outcrossing grass weed, *Lolium perenne ssp. multiflorum*, sampled from vineyards in northwest California, only 3.5% of the total observed genetic variation was distributed amongst populations.[44]

In contrast to previous studies of blackgrass populations from France,[10,18] the genetic variation in UK populations was found to be geographically structured. Our results were consistent with all three expectations from our *a priori* demographic model of recent range expansion. Firstly, we identified a clear and robust signature of IBD. Secondly, there were significant latitudinal and longitudinal clines in the first principal component of population structure. Finally, there was also a positive correlation between heterozygosity (both observed and expected) and longitude, with western (i.e. more recently colonized) populations having

lower heterozygosities (n = 44; rho = 0.41, *P*-value = 0.0055). Taken together, our analysis of the structure of genetic diversity within the UK blackgrass population is consistent with the relatively recent demographic history of a species introduced to the UK from a few hundreds to a maximum of one to two thousand years ago with frequent human-mediated long-distance dispersal associated with agricultural practices. Low genetic differentiation is also consistent with large effective population sizes as changing farming systems and the herbicide resistance epidemic increase population sizes and persistence in UK agroecosystems.[9] At the same time, agronomic factors and, potentially, climate change are facilitating a westwards and northwards range expansion for the species, which is entirely consistent with the patterns of geographical structure that we observe in this study. It is believed that blackgrass seed can be dispersed through agricultural machinery and crop seed, but the evolutionary effects of this long-distance seed dispersal need to be assessed.

4.2 Spatial scale of blackgrass random mating population

We detected persistent heterozygote deficiency relative to Hardy-Weinberg Equilibrium (HWE) expectations in all populations and across the vast majority of SNP markers. A very similar pattern (F_{IS} = 0.176) was reported previously based on an allozyme study of 19 blackgrass populations from the UK, France, Germany, and Israel.[16] While there are different possible explanations for these deviations from HWE,[45,46] unaccounted population subdivision resulting in 'Wahlund effects'[47,48] appear to be the most plausible. This is because positive F_{IS} values were estimated for the vast majority of SNPs (77%) and in all populations, while the correlation between F_{IS} and F_{ST} was weak (r = 0.19), consistent with simulated Wahlund effects shared across all populations.[46]

Population substructure is commonly detected in plants with life histories ranging from nearly obligate self-pollination, such as *Arabidopsis thaliana*[49] to nearly or completely obligate out-crossing, with frequent long-distance seed and pollen dispersal, such as in pines [50] and poplars.[51] Furthermore, both pollen and seed dispersal are believed to occur predominantly over

very short distances (< 1 m) in blackgrass,[52] and computer simulations demonstrated that the resulting genetic patchiness and nearest-neighbour pollination can drive F_{IS} to the values observed in our study in as few as 20-30 generations, even in the absence of self-fertilization.[53] Thus, the precise spatial scale of a random-mating population in blackgrass remains unknown (i.e. because our sampling scheme did not allow analyses of fine-scale genetic structure), but our results suggest that this scale is likely to be much smaller than hundreds of metres (i.e. the typical dimensions of the fields we sampled). In any case, the finding that genetic structure within fields appears to be an order of magnitude stronger than that at broader spatial scales will inform the design and sampling approaches in future genetic studies.

4.3 Population genetic structure of herbicide resistance

In addition to depicting patterns of putatively neutral population structure using a genome-wide set of SNPs, we performed a preliminary assessment of the extent of confounding[54] that these patterns would cause in GWAS aimed at dissecting the genomic architecture of quantitative (non-target site-based) herbicide resistance.[55] Although we detected some associations between population structure PCs and resistance phenotypes, these correlations consistently involved minor axes of population structure (i.e. not PC1-4) and explained relatively small proportions of the variation for resistance (i.e. only 6.5% in the RDA across all traits). This is in stark contrast with broadly distributed model plants,[56–61] major crops[62–65] and a cosmopolitan agricultural weed, *Capsella bursa-pastoris*,[66] in which geographic, climatic variables, adaptive, and/or growth traits and primary axes of population structure tend to be moderately to strongly inter-correlated. Thus, the general pitfalls of association mapping may be largely mitigated in UK blackgrass populations.

Consistent with this hypothesis, the highest genomic predictive abilities for resistance to fenoxaprop (i.e. the least stratified phenotype) were achieved based on very small numbers of loci (i.e. as few as 20) selected using GWAS in the training population. Furthermore, it was notable that when the top SNPs from the GWAS analysis for fenoxaprop resistance were mapped

to reference genomes for related graminaceous species, the most significantly associated SNP was a cytochrome P450 (*P450 72A15, Brachypodium distachyon*). The large cytochrome P450 gene family performs a range of plant defense-related roles in plants and has been widely implicated in evolution of non-target site herbicide resistance,[7] including the sub-class 72.[67] This analysis also identified several other genes previously associated with drug and herbicide metabolism amongst the top 200 SNPs.

4.4 Designing GWAS studies to determine genetics of herbicide resistance in blackgrass.

Despite these encouraging anecdotal observations, the feasibility of GWAS approaches for dissecting NTSR in blackgrass is largely unclear and will depend on several currently unknown factors. Firstly, this study was based on very few data points as it used population-level estimates of resistance phenotype (n = 44), and therefore focused entirely on variation among populations as have the majority of GWASs in plants so far.[68] However, within-population and even within-family variation in herbicide resistance may be extensive within these wild populations which are undergoing contemporary and ongoing adaptation to herbicides, and future GWAS efforts should be based on individual rather than population-level phenotypes. Given the weak differentiation but strong Wahlund effects we observed at the field level, the optimal design for constructing a homogeneous GWAS population (i.e. without cryptic relatedness and with as little differentiation as possible) may involve sampling individuals spaced at least several hundred meters apart, even if this means a relatively large overall sampling area.

Secondly, the extent of genome-wide linkage disequilibrium in blackgrass is currently unknown, but ongoing linkage mapping and genome sequencing efforts[69] will soon make it possible to estimate the marker density required for adequate genome coverage. We have recently determined that the blackgrass genome size is approximately 3.4 Gb (data not shown) and this large genome size likely means that the ~ 20,000 markers that we used tagged a very small fraction of the genome. Other genotyping approaches will likely be needed in the future.

Finally, and most importantly, the success of GWAS will depend on the actual genomic architecture of NTSR. The level of complexity of NTSR traits remains unknown. An increasing number of transcriptomics studies to explore differences in gene expression between NTSR and herbicide sensitive weed populations are being undertaken. Often, these studies identify hundreds to thousands of differentially expressed genes,[70,71] though confounding with population structure means that the rate of false positives is likely high. Where functional validation of a small number of putative over-expressed NTSR loci has been carried out, herbicide resistance phenotypes have been demonstrated,[72,73] suggesting that effect sizes are relatively large and that NTSR traits may be oligogenic rather than truly polygenic. Regardless, few studies in herbicide resistance have attempted to determine the genetic basis of NTSR and GWAS based approaches, enabled by increasing access to the genomes of weedy plants[69] offer great potential to unravel the genomic architecture of quantitative herbicide resistance traits.[74]

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DATA AVAILABILITY

The raw Illumina reads for the RAD-seq data have been uploaded to the NCBI Short Read Archive (BioProject ID PRJNA668070).

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Table 1. Analysis of molecular variance and F-statistics averaged over 20,408 SNP loci for 47 blackgrass populations across the UK.

Source of Variation	*df*	Sum of Squares	Variance components	Percentage Variation	F-statistic averaged over loci
Among populations	46	120926.87	39.40	2.40%	F_{ST} 0.024
Among individuals within populations	322	583429.47	475.70	28.93%*	F_{IS} 0.30
Within individuals	369	376329.00	1128.96	68.67%*	F_{IT} 0.31
Total	737	1080685.34	1644.065		

* $P < 0.05$

Table 2. The percentage of variance explained and adjusted R^2 for RDAs using population structure to explain the variation of population level phenotypic BLUPs for mortality and biomass of 44 blackgrass populations. All six combinations of phenotypes and herbicides (fenoxaprop, mesosulfuron + iodosulfuron, and cycloxydim) were assessed together as "All Herbicides". The RDA for fenoxaprop was not reported as no PCs were significant in the backward elimination selection process. Model significance from an ANOVA is denoted by asterisks.

Model	Percentage of variance explained	Adjusted R^2
All Herbicides ~ PC6	8.68%*	0.065
Mesosulfuron + iodosulfuron ~ PC6 + PC14	20.22%*	0.16
Cycloxydim ~ PC6 + PC9 + PC16	34.00%**	0.29

* $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

FIGURE LEGENDS

Figure 1. (A) PC1 and PC2 from the smartPCA for 47 UK blackgrass populations using 20,426 SNPs. The percent variance explained is in parenthesis. The two outlier populations (P128 and P163) are colored in red and blue. (B) PC1 and PC2 from the smartPCA after the removal of the two outlier populations (n = 45).

Figure 2. Spearman rank correlations of PC1 from the smartPCA (A) with latitude and (B) longitude using 45 blackgrass populations. (C) The average PC1 score for each population using inverse distance weighted interpolation across a map of the UK (n = 45).

Figure 3. Spearman rank correlations of (A) observed heterozygosity and latitude and (B) longitude using 45 blackgrass populations. (C) The observed heterozygosity for each population using inverse distance weighted interpolation across a map of the UK (n = 45). Population observed heterozygosity increases from blue to red, with populations with a higher observed heterozygosity in red.

Figure 4. Genomic predictive ability (i.e. correlation of predicted and observed phenotypic values) for different sets of markers selected at random (black circles) and based on GWAS *P*-values (red triangles) in the training set of populations. Error bars correspond to standard deviations across 10 random cross-validations.