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Postglacial ecotype formation under outcrossing and self-fertilization in *Arabidopis lyrata*

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Abstract

The formation of ecotypes has been invoked as an important driver of postglacial biodiversity, because many species colonized heterogeneous habitats and experienced divergent selection. Ecotype formation has been predominantly studied in outcrossing taxa, while far less attention has been paid to the implications of mating system shifts.

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Here we addressed whether substrate-related ecotypes exist in selfing and outcrossing populations of *Arabidopsis lyrata* subsp. *lyrata* and whether the genomic footprint differs between mating systems. The North American sub-species colonized both rocky and sandy habitats during postglacial range expansion, and shifted the mating system from predominantly outcrossing to predominantly selfing in a number of regions. We performed an association study on pooled whole-genome sequence data of 20 selfing or outcrossing populations, which suggested genes related to substrate adaptation. Motivated by enriched gene ontology terms, we compared root growth between plants from the two substrates in a common environment and found that plants originating from sand grew roots faster and produced more side-roots, independent of mating system. Furthermore, single nucleotide polymorphisms associated with substrate-related ecotypes were more clustered among selfing populations. Our study provides evidence for substrate-related ecotypes in *A. lyrata*, and divergence in the genomic footprint between mating systems. The latter is the likely result of selfing populations having experienced divergent selection on larger genomic regions due to higher genome-wide linkage disequilibrium.

Key words: Divergent natural selection, genome structure, ecological speciation, linkage disequilibrium, self-incompatibility, soil substrate.

Introduction

Ecological specialization as a result of divergent selection between environments has the potential to rapidly generate distinct ecotypes and eventually separate species (Schluter 2000; Nosil 2012; Seehausen *et al.* 2014). Ecotype formation has been

commonly reported in species that underwent postglacial range expansions following the last Pleistocene glaciation cycle, including species of vertebrates (Cutter & Gray 2016), invertebrates (Forbes *et al.* 2017) and plants (Baack *et al.* 2015). In plants, ecotypes often evolve as a consequence of the colonization of and subsequent adaptation to different substrates (e.g. Turner *et al.* 2010; Andrew *et al.* 2013; Arnold *et al.* 2016; Gould *et al.* 2017). Recent research focused on understanding the genomic underpinning of such substrate-related ecotypes, with an emphasis on outcrossing taxa (Turner *et al.* 2010; Andrew *et al.* 2013; Arnold *et al.* 2016; Gould *et al.* 2017). However, mating system is predicted to have a strong impact on the genetics of ecotype formation as well as the rate of evolution (Hartfield *et al.* 2017). To understand the potential impact of the mating system on the evolution of ecotypes, we made use of a unique system in which postglacial range expansion was associated both with independent shifts in the mating system and as we found here – adaptation to different substrates.

Theory and empirical studies suggest that at an early stage, ecotypes are often distinct by only few adaptive alleles (Feder *et al.* 2012b; Seehausen *et al.* 2014). These may rise to high frequency or even become fixed. In contrast, at neutral regions of the genome, gene flow may still be abundant and genetic divergence therefore limited (Feder *et al.* 2012b; Seehausen *et al.* 2014). Because gene flow can break up genomic regions of adaptive genetic differentiation, mechanisms that shield part of the genome from recombination are important to further stabilize ecotypes (Butlin 2005; Kirkpatrick & Barton 2006). Changes in the genome structure such as inversions or the rearrangements of chromosomes often represent such mechanisms (Kirkpatrick & Barton 2006; Demuth *et al.* 2014; Hooper & Price 2017; Lucek 2018). A less frequently studied mechanism that can also reduce gene flow is the shift in mating system from

obligate outcrossing to self-compatibility and selfing (Hartfield *et al.* 2017). Self-incompatibility is widespread among hermaphroditic flowering plants and prevents selfing, but self-compatibility has repeatedly evolved from outcrossing ancestors in many flowering plant families (Igic *et al.* 2008) as well as within species (Goodwillie *et al.* 2005; Willi & Määttänen 2010).

Selfing *per se* has a couple of implications for adaptation and thus the potential for ecotypes to evolve (Hartfield *et al.* 2017): Selfing decreases the drift-effective population size (Pollak 1987), and as a consequence leads to a reduction in genetic variation and an increase in the frequency of slightly deleterious alleles (Wright 1931), both of which may lower the adaptive potential within selfing populations. Another effect of selfing is that effective recombination declines and linkage disequilibrium generally increases across the genome (Nordborg 2000; Slatkin 2008). Because of increased linkage disequilibrium, directional selection in the area of a target region may affect a larger part of the genome for selfing than for outcrossing populations and may promote the buildup of regions under divergent selection (Gordo & Charlesworth 2001). Under polygenic adaptation, initial responses to selection can moreover occur more rapidly in selfing compared to outcrossing populations because of the initial conversion of dominance and epistatic variation into additive genetic variation (Cockerham 1984; Hartfield *et al.* 2017). Long-term responses to selection might, however, be compromised by a lack of available genetic variation (Noël *et al.* 2017; Hartfield *et al.* 2017).

We tested here for substrate-related ecotypes in *Arabidopsis lyrata* subsp. *lyrata* by a comparison of outcrossing and selfing populations growing on sand and rock substrates. The species is a short-lived perennial and hermaphroditic plant, and it is

closely related to the model species *A. thaliana* (Hohmann *et al.* 2014). Following the retreat of the glaciers starting about ~20'000 years ago, *A. lyrata* underwent a range expansion in North America (Griffin & Willi 2014; Willi et al. 2018) and colonized distinct substrates that can be broadly categorized as rock and sand (Willi & Määttänen 2011; Figure 1, Table S1). Rocky substrates comprise for example bare mountaintops of the Appalachians, bare rocky shores of larger lakes and bare cliffs along rivers, while sandy sites include sand dunes on lakes, sand deposits along rivers and eroded sandstone. Along the postglacial range expansion, mating system shifts towards selfing occurred independently in several regions (Mable *et al.* 2005; Willi & Määttänen 2010), but particularly at the edges of the geographic distribution (Griffin & Willi 2014). The mating system of our studied populations was mainly inferred by multi-locus outcrossing rates based on progeny arrays; populations were considered as selfing if the outcrossing rate was ≤0.2 and as outcrossing if the rate was >0.8 (Willi & Määttänen 2010; Foxe *et al.* 2010; Griffin & Willi 2014). The trait of self-incompatibility seems quite stable throughout the life of a plant in *A. lyrata* (Willi & Määttänen 2010). While populations may have been well connected in the past, ongoing gene flow seems to be restricted even among populations separated by a few hundred meters (Willi & Määttänen 2010; Foxe *et al.* 2010; Griffin & Willi 2014; Tables S2 & S2).

Here we assessed whether populations growing on rock or sand showed a genomic signature consistent with separate ecotypes in *A. lyrata,* while also assessing the impact of mating system shifts onto the genomics of such adaptation. We first performed a genome-wide association study (GWAS) for substrate type separately for selfing and outcrossing populations. Similar to other cases of substrate-related ecotypes in *Arabidopsis*, we expected a genetic basis for adaptation as opposed to plasticity

(Alcázar *et al.* 2012; Flood & Hancock 2017). We then tested whether there is a common genomic basis for adaptation to different substrates, i.e. on the SNP, gene or gene ontology level between mating system. Given that populations occur on ecologically similar substrates, we expected an overlap in outliers or the genes affected by them. Based on the aforementioned theoretical predictions, we also expected genome-wide LD to be increased among selfing populations. As a consequence, substrate dependent divergent selection may act along a wider range of the genome (Gordo & Charlesworth 2001), resulting in a clustering of tightly linked GWAS outliers in regions under selection (Rincent et al. 2014) – a pattern that we subsequently tested for. Lastly, because gene ontology terms identified by our association study suggested phenotypic differentiation in root growth (see Results), we tested for phenotypic differentiation between individuals from either substrate by a common garden study on seed material from selfing and outcrossing populations.

Material & Methods

Sample collection & sequencing

We used a subset of a previously published genomic dataset of population-based pool-sequences (Pool-seq) from Willi *et al.* (2018; European Nucleotide Archive accession number PRJEB8335). In short, 20 *A. lyrata* populations growing on distinct substrates, broadly categorized as rock and sand (Willi & Määttänen 2011), were sampled during the reproductive season in 2007, 2011 or 2014 (Figure 1; Table S1). Whereas rock-dwelling populations were collected in rock crevices or on rocky ledges (often growing on moss and lichens), sand-dwelling populations occurred on the shores of lakes and

rivers, eroded sandstone ridges or inland sand deposits. At each site, 25 flowering individuals were collected over a surface area of comparable size (about 450 m^2). Census size was shown to be smaller in populations on rock compared to those on sand, but this difference did not translate into reduced genetic diversity and effective population size (Willi & Määttänen 2011). We included all (N=8) available selfing populations described in a previous study (Griffin & Willi 2014). Outcrossing populations (N=12) were selected such that we had pairs of populations from different substrates that were geographically and phylogenetically close (Willi *et al.* 2018) in order to increase the chance to detect SNPs associated with environment-dependent selection (Hoban *et al.* 2016). For each population DNA for all individuals was pooled into a single library, which was then paired-end sequenced for 100 bases (PE100) on four Illumina HiSeq2000 lanes, using one quarter of each lane (see Fracassetti *et al.* 2015 for details).

Data preparation

We first trimmed the raw sequences for each population using *trim-fastq.pl*, which is part of the PoPoolation 1.2.2 software package (Kofler *et al.* 2011a). We used a minimal base quality threshold of 20 and kept only reads ≥ 84bp. We subsequently mapped all retained reads against the *A. lyrata* reference genome v1.0 (Hu *et al.* 2011), which included the plastid as well as mitochondrial genomes of *A. thaliana* (Genbank accessions NC_000932 and NC_001284, respectively) using BWA-MEM 0.7.13 (Li 2013). We masked the centromeric regions as well as two regions on scaffold 2 (position ranges: 8'746'475-8'835'273 and 9'128'838-9'212'301), which share very high similarity with the *A. thaliana* chloroplast genome, suggesting an assembly error in the

A. lyrata reference genome. Only reads that mapped to scaffolds I-VIII, representing the eight chromosomes of *A. lyrata* were retained. We next used PICARD 2.1.1 (http://broadinstitute.github.io/picard) to remove duplicate reads and SAMTOOLS 1.3.1 (Li *et al.* 2009) to retain only properly paired reads with mapping quality over 20.

Using SAMTOOLS, we generated *mpileup* files for (i) outcrossing and selfing populations separately in order to increase the number of SNPs available for our subsequent analyses (see below) and (ii) all 20 populations combined (Figure 1, Table S1). We called SNPs using VARSCAN 2.4.1 (Koboldt *et al.* 2012), requiring a minimal read depth of 100 at a given position to make a call. Following Willi *et al.* (2018), we used a minimal variant allele frequency threshold of 0.03. From each VCF file we removed previously identified repeat sites in the *A. lyrata* genome (Fracassetti *et al.* 2015) with BEDTOOLS 2.26.0 (Quinlan & Hall 2010). We further filtered each VCF file with VCFTOOLS 0.1.14, removing indels and keeping only biallelic SNP positions that had a depth of 100-500, a minimal genotype quality of 28 and a minor allele frequency of 0.03, allowing a maximum of 25% missing data in each dataset. Lastly, SNPs with a strand bias of more than 90% were filtered out. This procedure resulted in three datasets comprising 500'877, 437'228 and 156'024 polymorphic SNPs for outcrossing, selfing or all populations combined, respectively.

Genome-wide association study

To identify SNPs associated with substrate-dependent segregation, we performed a genome-wide association study (GWAS) using BAYPASS 2.1 (Gautier 2015). BAYPASS extends the approach of Coop *et al.* (2010) and Günther & Coop (2013) by estimating

and accounting for the hierarchical structure of populations using the (scaled) covariance matrix of population allele frequencies (Gautier 2015). We ran BAYPASS separately for five SNP datasets, considering (i) only outcrossing populations, (ii) only selfing populations, (iii) all 20 populations combined, and this combined dataset was also separately analyzed for (iv) outcrossing and (v) selfing populations. The combined dataset was established to verify results produced by (i) and (ii). Substrate type was treated as a binary variable in the GWAS. We used the auxiliary covariate model with default parameters and 5000 burn-in iterations in the MCMC chain, followed by 25'000 iterations. To reduce artifacts due to potential variability between runs, we performed 10 independent BAYPASS runs for each SNP dataset. We then calculated the average Bayes Factor (BF), expressed in deciban units (dB), for each SNP as a quantification of the degree of relationship between substrate type and the standardized allele frequency. Following the suggestions of Gautier (2015), all SNPs were included for each dataset. Outlier SNPs were defined as being the 1% SNPs with the highest average BF across all runs (i.e. 5008, 4372, 1560 SNPs, respectively, for the datasets of outcrossing, selfing or all populations combined).

Outlier SNPs were analyzed for the number of genes they were positioned in. The number of overlapping outlier genes between the outcrossing and selfing datasets was tested for being lower or higher than expected by chance. This was done by a resampling analysis based on 10'000 iterations, where each time the same number of genes that were affected by outliers were drawn from the total pool of covered genes, calculating each time the overlap. We further employed a gene ontology (GO) enrichment analysis to identify the biological processes that genes containing at least one of the top 1% outlier SNPs were involved in. Enrichment analyses were restricted

to exon region and based on 10'000 randomization steps in R 3.3.1 (R Core Team 2016) using SNP2GO (Szkiba *et al.* 2014). We used the most recent annotation of *A. lyrata* (Rawat *et al.* 2015) and set a false discovery rate (FDR) < 0.05 for the GO enrichment tests.

Verification of outlier SNPs by population-structure analyses

Genetic differentiation between populations from different substrates is predicted to be higher at SNPs under putative substrate-associated selection than elsewhere in the genome (Nosil & Feder 2012), overcoming patterns of isolation-by-distance (IBD; Nosil *et al.* 2008). We tested this prediction by calculating pairwise distance matrices on locus-based F_{ST}-values between all populations using either all SNPs or the top 1% outliers of the genomic dataset comprising all 20 populations in POPOOLATION2 (Kofler *et al.* 2011b). We then tested for a pattern of IBD by correlating the genomic distance matrices with pairwise geographic distances (km) using a Mantel test. We further employed partial Mantel tests to assess the correlation between genetic distance and difference in substrates while controlling for geographic distance. To further assess if outliers were affected by demography, i.e. shifts in mating system, we performed partial Mantel tests between genetic distance and difference in mating system, while controlling for geographic distance. Significance levels were established using 100'000 permutation steps in the R package VEGAN 2.4-5 (Oksanen *et al*. 2017). We also calculated F_{ST}s between population pairs from different substrates using the large outcrossing and selfing datasets. Average F_{ST}s across all outlier SNPs were then compared with an F_{ST} distribution based on 1000 random resampling steps, where each

time the same number of SNPs as outlier SNPs was drawn from the pool of non-outlier SNPs.

To further confirm habitat-dependent genetic differentiation among our outlier SNPs, we performed a principal component (PC) analysis with PCADAPT (Luu *et al.* 2017), respectively, for outcrossing, selfing or all populations combined. In each case, we generated 25 genotypes for each population based on binomial random draws. To test for segregation between populations from rock and sand substrates along any of the two leading PC axes we next employed a linear model using the average PC scores per population.

Distribution of outlier SNPs across the genome and linkage disequilibrium

To test if the distribution of the top 1% outlier SNPs differed from the distribution of non-outliers, we calculated the distance between adjacent outlier SNPs for outcrossing and selfing populations as well as the combined dataset analyzed separately for outcrossing and selfing populations. We subsequently assigned the pairwise distances to two distance classes across 360'000 bps. In the absence of a genome-wide estimate of LD for *A. lyrata*, we chose distance classes to reflect estimates of twice the average LD across the genome of *A. thaliana*, i.e. 20kb (Kim *et al.* 2007) and 40kb (Nordborg *et al.* 2002), respectively. We then compared the distribution of frequencies per distance class for outcrossing and selfing populations with a *G*-test. We further contrasted the distribution of these distances with a random null distribution for each dataset by sampling 10'000 times the same number of SNPs as outliers from the pool of non-outlier positions and calculating the distance between adjacent SNPs. A

pattern of increased genomic clustering is suggested if the frequencies of SNPs in both the short and long distance classes exceed the frequencies observed for the random null distribution.

In a next step, we investigated the role of the mating system and linkage disequilibrium (LD). First, we tested whether LD differs between outcrossing and selfing populations. We calculated LD as r^2, i.e., the square of the correlation between alleles of SNP pairs within the paired sequence reads of each population using the *direct estimate* method of LDx (Feder *et al.* 2012a). Because we worked with Pool-seq data, the range for estimating LD was short, i.e. across paired reads. We only considered sites with an intersecting read depth greater than 5 and a minor allele frequency at either locus > 0.15 (Feder *et al.* 2012a; Tuttle *et al.* 2016). We subsequently calculated the average LD for each pairwise distance and compared populations differing in mating system using paired *t*-tests, applying a FDR.

Finally, we tested how LD differs between top 1% outlier SNPs and SNPs that were not found to be outliers in the GWAS analyses. Using LDx, we calculated LD between pairs of outlier SNPs and pairs of non-outlier SNPs for each population. We weighted the average LD by the respective distances in base pairs between SNPs. We then tested for a difference in LD between outliers/non-outliers and mating system for both GWAS of the larger separate datasets and the combined dataset split by mating system using linear models in R.

Root growth

Motivated by the GO term "*root morphology*" that was enriched among selfing populations and that occurred as annotation also among the outliers of the outcrossing dataset (see Results section), we experimentally tested for differences in root phenotype under common garden conditions. We had seeds available for 16 of the 20 populations studied, produced by the same individuals that were used for the genomic Pool-seq analysis (Table S1). We performed the experiment in two replicates. Two sterilized seeds of nine individuals per population were stratified in sterile water at 4°C under dark conditions for two weeks (sample size: 16 populations x 9 seed families x 2 replicates = 288). All seeds were randomly assigned to a position within one of 36 sterile, 12x12 cm agar plates per replicate. Four seeds were put on each plate along a horizontal line 2 cm from the top and 1.5 cm away from each other. We used a 1.83 ‰ Murashige and Skoog medium mixed with 1 % phyto agar and adjusted the pH to 5.7. To account for border effects, the positions next to borders were filled with additional seeds that were not analyzed. We placed all agar plates upright in a Sanyo plant growth chamber (Sanyo, Moriguchi, Japan) with 12h light:12h dark conditions and temperatures of 20°C (day) and 18°C (night), respectively. The experiment was conducted between the 11th of May and the 26th of June 2017. Every 3-4 days all agar plates were scanned and root length and the number of primary side roots of each plant measured with IMAGEJ 1.45s (Abràmoff *et al.* 2004). We stopped measuring when a plant reached a length of 80mm to account for potential border effects on the base. We selected the best fitting growth model that described the increase in root length and the number of side roots by fitting seven alternative models separately for each plant: 1) linear, 2) exponential, 3) power function, 4) three-parameter logistic, 5) two-parameter

logistic, 6) Gompertz and 7) von Bertalanffy. Models were fit in R following Paccard *et al.* (2014). The best-fitting model was Gompertz for both measurements based on Akaike information criterion (AIC) values (Figure S1). We then used linear mixed models to test if the maximum relative growth rate parameter of the Gompertz model (i.e. k_G, see Tjørve & Tjørve 2017) differed between individuals from different substrates and mating system using population and seed family as random effects.

Results

Genome-wide association study

The top 1% outlier SNPs identified by BAYPASS for the outcrossing ($N_{\text{Outcrossing}}$=5008) and selfing (N_{Selfing}=4372) datasets were spread across the genome (Figure 2a&b). Of these, only 24 outliers overlapped between the two datasets (Figure 2c). However, among all outlier SNPs of both datasets, 99 genes were affected in common by at least one outlier SNP (Figure 2d). This overlap was higher than expected by chance as suggested by our resampling analysis (p=0.0001). Of the 99 shared genes, 45 were annotated with 90 GO terms, including *root growth* (Table S4). Outlier SNPs were associated with 1247 and 582 GO terms for the outcrossing and selfing dataset, respectively. Of these, 506 GO terms overlapped between both datasets and included 9 terms linked to *root morphology* (Table S5). Twenty-three and 68 GO terms were significantly enriched for outcrossing and selfing populations, respectively (Figure 2e; Tables S6, S7). However, only one GO term –*chromatin remodeling* – was enriched in both datasets. For outcrossing populations, enriched GOs were mainly linked to *response to iron* and *RNA*

processing (Table S6), whereas for selfing populations, 13.4% of enriched GOs were linked to *root morphology* (Table S7).

Analyses were repeated based on the smaller SNP dataset comprising all populations. Figure S2 shows the distribution of the top 1% outlier SNPs as well as the intersection between outlier SNPs, genes with outlier SNPs, and gene ontology terms affected by outliers between the GWAS performed on outcrossing, selfing or all populations together. Four GO terms linked to *RNA processing* were enriched when all populations were included in the GWAS (Table S8). When analyzing the SNPs of the combined dataset separately for outcrossing and selfing populations, the proportions of overlapping top 1% outliers SNPs and genes containing outliers were similar to the ones observed between the larger datasets (i.e. Figure 2d, e). For the combined dataset but with analyses split by mating system, outlier SNPs, genes with such SNPs, GOs and enriched GO terms were not a complete subset of those revealed with the respective larger datasets (Figure S3). An interesting difference were the intersection of enriched GO terms between outcrossing (Table S9) and selfing populations (Table S10); while the overlap was low in outcrossing populations (6%, relative to those unique to GWAS on the combined data set; Figure S3), 80% overlapped for selfing populations. Of the enriched GO terms for selfing populations, 23.3% were associated with *root morphology*.

Verification of outlier SNPs by population-structure analyses

For the SNP dataset including all 20 populations, Mantel tests suggested a pattern of isolation-by-distance (IBD) using all SNPs (r = 0.349, p < 0.001; Table S2) or only the top 1% outliers (r = 0.277, p = 0.006; Table S3). When controlling for geographic distance, partial Mantel tests implied a strong association between genetic differentiation and substrate type for outlier SNPs (r = 0.502, p < 0.001) but not for the remaining SNPs (r = 0.086, p = 0.060). Partial Mantel tests between genetic differentiation and mating system, again controlling for geographic distance, were significant for both outlier SNPs (r = 0.148, p = 0.035) and all other SNPs (r = 0.196, p = 0.006), suggesting higher differentiation when mating system differed. Using the large SNP datasets, pairwise outlier-based F_{ST} values between populations from different substrates were significantly higher than across the rest of the genome for outcrossing populations (Figure S4). But this was not true for selfing populations, potentially as a result of the overrepresentation of SNPs with high F_{ST}s (>0.95) in all pairwise comparisons involving selfing populations as opposed to comparisons between outcrossing ones (Figure S5).

The principal component analysis using only outlier SNPs confirmed the GWAS outliers by separating populations from different substrates along the first PC axes (outcrossing: $F_{1,10}$=49.29, p<0.001; selfing: $F_{1,6}$=18.52, p=0.005; combined dataset: $F_{1,18}$=33.59, p<0.001; Figures 3 & S5). This was however not true for the second PC axes (outcrossing: $F_{1,10}$=0.01, p=0.999; selfing $F_{1,6}$=0.14, p=0.717; combined dataset: $F_{1,18}$=0.27, p=0.689). For non-outlier SNPs, no association with substrate was found, suggesting that the SNPs associated with different substrates were part of the top 1% outliers (PC1: outcrossing: $F_{1,10}$=0.25, p=0.630; selfing $F_{1,6}$=2.81, p=0.145; combined

dataset: $F_{1,18}$=2.95, p=0.103; PC2: outcrossing: $F_{1,10}$=0.17, p=0.691; selfing $F_{1,6}$=0.54, p=0.492; combined dataset: $F_{1,18}$=0.63, p=0.439).

Distribution of outlier SNPs across the genome and linkage disequilibrium

When calculating the distances among outlier SNPs and assigning them to distance classes, SNPs were differently distributed between outcrossing and selfing populations (40kb windows – separate datasets G = 281.6, d.f. = 8, p < 0.001; 40kb windows – combined dataset G = 15.8, d.f. = 8, p = 0.046 – Figure S7; 20kb windows: G = 637.6, d.f. = 17, p < 0.001 – Figure S8). Consistent with a clustering of outlier SNPs we found an overrepresentation for SNPs at relative short and long distances for both outcrossing and selfing populations, but clustering was more pronounced among selfing compared to outcrossing populations.

Short-distance LD (i.e. between SNPs on the same paired reads) was generally high in all of our studied populations (Figure S9; mean r^2 – outcrossing: 0.604 ±0.034 SD; mean – r^2 selfing: 0.680 ±0.014 SD). LD was significantly higher (p<0.001 after FDR) in selfing compared to outcrossing populations in all but one comparison (MI6 vs. ON7, see Table S11), with LD being on average 11.9% (±5.9% SD) higher, but there was also considerable variance among population pairs.

Pairwise LD between outlier SNPs was significantly higher than between pairs of non-outlier SNPs for both the large (Δ_{LD} = 0.153, p < 0.001) and combined outcrossing dataset (Δ_{LD} = 0.202, p < 0.001; Figure 4). For selfing populations the pairwise LD between pairs of outlier and non-outlier SNPs was consistently high and did not significantly differ, neither for the large (Δ_{LD} = 0.026, p = 0.704) nor the combined

dataset (Δ_{LD} = 0.024, p = 0.091). In outcrossing populations, we found a pronounced increase in LD from pairs of non-outliers to pairs of outliers, also reflected in the significant interaction term of mating system-by-SNP type (large dataset: $F_{1,36}$= 13.9, p = 0.001; combined dataset: $F_{1,36}$= 106.8, p < 0.001).

Root growth

Following the removal of 102 individuals that failed to germinate and 32 individuals from eight agar plates that developed fungus, our experimental assay of root growth resulted in data for a total of 154 individuals (9.6 ± 4.8 SD per population). The maximum relative growth rate in root length and the number of primary side roots was higher in plants from sand than those from rock (root length: χ_1 = 7.64, p = 0.006; primary side roots: χ_1 = 4.71, p = 0.030; Figure 5). Individuals from sand grew roots faster and formed more side roots than individuals from rock under common-garden conditions. There was no significant interaction between mating system and substrate (Type II Wald χ^2-test: root growth: χ_1 = 0.04, p = 0.848; number of primary side roots: χ_1 = 0.16, p = 0.688) as well as no significant effect of mating system on the measured traits (root growth: χ_1 = 1.73, p = 0.188; number of primary side roots: χ_1 = 0.98, p = 0.322).

Discussion

Postglacial substrate-related ecotypes in A. lyrata

In plants, ecotype formation is frequently triggered by the colonization and subsequent adaptation to different substrates (e.g. Turner *et al.* 2010; Andrew *et al.* 2013; Arnold *et al.* 2016; Gould *et al.* 2017). Our genome-wide association study identified SNPs, genes and gene ontology terms directly or indirectly associated with divergent adaptation to rock and sand substrates in *A. lyrata*. The gene ontology term analysis suggested differences in *root morphology* between populations from different substrates. And indeed, phenotypic and potentially adaptive differences between plants from the two substrate types were confirmed under experimental conditions, which is consistent with the evolution of substrate-related ecotypes. Plants from sand substrates also showed faster root growth than those from rock (Figure 5).

Within the genus *Arabidopsis*, substrate-driven ecotype formation has so far rarely been found (Alcázar *et al.* 2012; Flood & Hancock 2017). The best example is adaptation to serpentine soils and heavy-metal tolerance in outcrossing *A. lyrata* (Turner *et al.* 2010; Arnold *et al.* 2016). The latter evolved over a similar time scale as our studied populations – since the end of the last glaciation cycle and range expansion since then – and adaptation involves only few genomic regions that are linked to iron transport. As for other flowering plants, differences in soil water availability may be another important aspect of substrate that can impose divergent selection because water availability represents a significant limiting factor for photosynthesis (Andrew *et al.* 2013; Gould *et al.* 2017). The postglacial range expansion and colonization of rocky and sandy substrates in North American *A. lyrata* may be associated with divergent selective regimes that are linked to water availability. Our finding that plants from

sandy substrates show faster root growth in length and a higher number of primary side roots is consistent with physiological predictions that sand dwelling plants should grow faster and deeper roots to increase their water-extraction capability (Jackson *et al.* 2000). Changes in root growth are likely to be polygenic with 399 genes in *A. lyrata* being annotated with GO terms linked to *root morphology* (Rawat *et al.* 2015). In addition to *root morphology*, GO terms were also associated with plant growth and stress responses in all datasets (Tables S4-S8). We suggest to further investigate differences in plant and/or leaf growth as well as to test for differences in responses to different stress factors, such as nematodes.

Even though we could identify ecotypes, our study also illustrates some of the difficulties of GWAS when studying populations with different mating systems. Average Bayes Factors (BF) were uplifted, i.e. were higher than zero for each dataset (Figures 2 & S2). Potential reasons for this include: the binary response variable for substrate, few populations studied, or because we dealt with a complex demographic history including mating system shifts and different glacial refugia, which may not be fully overcome by BAYPASS (Gautier 2015). We therefore used several additional verification approaches for outlier SNPs. Partial Mantel tests and principal component analyses both on outlier and non-outlier SNPs confirmed that genetic differentiation at outlier SNPs, but not at non-outliers, was strongly related with substrate (Figure 3). Furthermore, GWAS outliers showed increased genetic differentiation between outcrossing populations of the two substrate types compared to non-outliers loci (Figure S4). Other methods of finding outliers, such as those relying on divergence (F_{ST}) were unsuitable for our type of study because selfing populations had an excess of nearly fixed alleles across the

genome (Figure S4 & S5), which remains a key challenge in the study of adaptation in selfing taxa (Hartfield *et al.* 2017).

While our study is an example how GWAS and subsequent gene ontology analysis can suggest traits that can be studied phenotypically, GO terms related to *root morphology* were only enriched among selfing populations. Although several outlier SNPs overlapped with genes annotated with *root morphology* for outcrossing populations, these were not enriched (Tables S4-S10). This could represent a technical artifact of GO enrichment analyses that compare a set of outliers against the background of non-outliers to test for overrepresentations. Consequently, the more GOs are involved, the less likely it is for GOs to be enriched (Tipney & Hunter 2010). This is the case for our outcrossing populations where four times as many genes contained outlier SNPs than for selfing populations (Figure 2d). To further pinpoint causative genes, further in-depth studies using more nearby ecotype pairs are needed, ideally combining experimental work together with gene expression analyses.

Mating system shift & the evolution of ecotypes

A shift to selfing is generally associated with increased genome-wide linkage disequilibrium (LD), that may be enhanced by past bottlenecks (Wright *et al.* 2013; Hartfield *et al.* 2017). Across the genome, LD may further vary punctually, e.g. through background or directional selection (Hartfield *et al.* 2017). We concordantly found that average genome-wide short-range LD was significantly elevated in selfing compared to outcrossing populations (Figure S9, Table S11). Independent of mating system, LD decayed only over the first fifty base pairs before stabilizing at a relatively high level

(Figure S9). This is consistent with findings from the close selfing relative *A. thaliana* (Nordborg *et al.* 2002).

Ecotypes are often ephemeral because isolating genomic mechanisms that prevent gene flow, recombination and thus the breakup of selected genomic regions are missing (Nosil *et al.* 2009; Nosil 2012; Seehausen *et al.* 2014). As selfing should reduce intraspecific gene flow (Willi & Määttänen 2011; Wright *et al.* 2013; Hartfield & Glémin 2016), it may act as such an isolating mechanism and could help to stabilize ecotypes, allowing to maintain genomic regions of increased differentiation (Hu 2015). The analyses of GWAS outliers revealed significant clustering of outlier SNPs in populations of both mating systems when compared to a random null distribution and clustering was more pronounced among selfing populations (Figures 2 & S2). A clustering of putative outlier SNPs found by GWAS can be the result of tight linkage in regions under selection (Rincent *et al.* 2014). In line, we found that pairs of physically nearby outlier SNPs of outcrossing populations showed a sharp increase in average LD compared to pairs of non-outlier SNPs. In contrast, LD between pairs of outlier and between pairs of non-outlier SNPs was similarly high in selfing populations (Figure 4). The results of generally high LD and clustering of outliers in selfing populations (Figure 5) suggests that this reproductive mode may promote the buildup of regions under divergent selection (Gordo & Charlesworth 2001; Feder *et al.* 2012b).

The theoretical prediction of reduced response to selection in selfing populations seems less supported. Even though selfing has been considered as constraining adaptive evolution (Noël *et al.* 2017), we found that selfing and outcrossing did not differ in root characters related to rock or sand. This suggests that genetic variation to respond to selection must have been sufficient in the selfing populations, and that the response to

directional selection was not too constrained by genetic drift. We can presume that directional selection must have been strong because for the same populations studied, we found good evidence for decreased efficacy of purifying selection; selfing and long-term small outcrossing populations had increased mutational load that translated in reduced population performance under common garden conditions (Willi 2013 Evolution; Willi et al. 2013 Heredity; Willi et al. 2018). Selfing does not seem to preclude considerable adaptive change to environmental heterogeneity.

Conclusions

Our genome-wide association analysis provided evidence for habitat dependent differentiation in *A. lyrata* between two broadly defined substrate categories, i.e. rock and sand, in both selfing and outcrossing populations. Gene ontology analysis on outlier SNPs motivated a common garden study, which confirmed that populations from rock and sand differed, with seedlings from sandy substrates showing faster root growth (Figure 5). The colonization of different substrates during the range expansion of *A. lyrata* seems thus to have triggered substrate-related adaptation, independent of mating system. SNPs associated with substrate-related differentiation were more clustered across the genome for selfing populations (Figures 2 & S2). This seems to be the result of selection on some SNPs underlying the distinct ecotypes combined with increased genome-wide LD due to a selfing reproductive mode. Hence, the switch to selfing may initially boost the evolution of distinct ecotypes by increasing genomic regions of divergence (Via 2009; Feder *et al.* 2012b; Hartfield *et al.* 2017). Finally, selfing is a widespread phenomenon among plants (Igic *et al.* 2008). Similar processes may therefore be at play in other taxa that underwent postglacial range expansions and

established in different environments (Grundt *et al.* 2006; Birky & Barraclough 2009; Foxe *et al.* 2009; Hu 2015).

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Data Accessibility Statement

We used a subset of a previously published genomic dataset of population based pooled (Pool-seq) sequences (Willi *et al.* 2018; European Nucleotide Archive accession number PRJEB8335). VCF files, R scripts and the phenotypic data are deposited on DRYAD (doi:XXXXXX).

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Figure Legends:

Figure 1: Map of the *Arabidopsis lyrata* populations studied. The color of outer circles indicates the predominant mating system, outcrossing in green and selfing in orange, while the color of the inner circle indicates the substrate, sand in red and rock in blue (see Table S1 for detailed information). Population abbreviations use the official abbreviation for each US state and Canadian province followed by a sampling index number. The actual distances between populations ON5, ON6 and ON7 are inflated on the map (see Table S2 for distances between populations).

Figure 2: The genomic signature of substrate-related *A. lyrata* ecotypes. Manhatten plots depict the distribution of average Bayes Factors of ten GWAS runs on the independent variable of substrate type for (a) 500'877 biallelic SNPs of outcrossing populations and (b) 437'228 SNPs of selfing populations. The 1% SNPs with the highest Bayes Factor are highlighted in color. Chromosomes are indicated by alternating black and grey shading. Venn diagrams show (c) the overlap in the 1% outlier SNPs of GWAS runs for outcrossing populations, selfing populations and all populations combined (based on combined SNP dataset), (d) the number of genes affected by outliers and (e) the number

of gene ontology (GO) terms affected by outliers. For GOs the numbers in brackets represent the number of significantly enriched GO terms.

Figure 3: Principal component (PC) analyses using either the top 1% outlier SNPs identified by GWAS (a & c) or non-outlier SNPs (b & d), separately for outcrossing (a & b) and selfing (c & d) populations. The color of the inner circle indicates substrate type, rock in blue and sand in red; the color of the outer circle indicates the mating system, outcrossing in green and selfing in orange. 25 individuals were simulated for each population based on the population-based Pool-seq data using a binomial random distribution. The two leading PC axes are presented.

Figure 4: Average linkage disequilibrium (LD), weighted for the distance between SNP pairs, between pairs of outlier or non-outlier SNPs, respectively, for outcrossing and selfing populations from rock (blue) or sand (red) substrate. (a) Analyses for the large SNP datasets, (b) analyses for the combined dataset split by mating system. *P* values are given for the difference in LD between outlier and non-outlier SNPs within mating system.

Figure 5: Boxplots summarizing phenotypic differences between individuals from rock (blue) and sand (red) substrate in a common-garden lab experiment. Shown are the maximum relative Gompertz growth rate for (a) length of the main root and (b) the number of primary side roots. Sample sizes (N) are indicated.

Figure S1: Boxplots summarizing estimates of Akaike's Information Criterion (AIC) for each experimental individual and the seven fitted growth models for (a) length of the main root and (b) the number of primary side roots.

Figure S2: The genomic signature of substrate-related *A. lyrata* ecotypes based on the dataset of 156'024 polymorphic SNPs shared by all populations. Manhatten plots depict the distribution of average Bayes Factor of ten GWAS runs on the independent variable of substrate type when (a) all populations were included, or (b) only outcrossing or (c) only selfing populations. The 1% SNPs with the highest Bayes Factor are highlighted in color. Chromosomes are indicated by alternating black and grey shading. Venn diagrams show (d) the overlap in the 1% outlier SNPs of GWAS on the three datasets, (e) the number of genes affected by outliers and (f) the number of gene ontology (GO) terms affected by outliers. For GOs the numbers in brackets represent the number of significantly enriched GO terms.

Figure S3: Venn diagrams showing the overlap between GWAS results of the large SNP datasets of outcrossing and selfing populations separately and the combined but smaller SNP dataset. Overlap is shown for (from left to right): the number of 1% outlier SNPs, the number of genes affected by outliers, and the number of gene ontology (GO) terms affected by outliers. For GOs the numbers in brackets represent the number of significantly enriched GO terms.

Figure S4: Pairwise F_{ST}s between populations on rock and sand that are either (a) outcrossing or (b) selfing using only the top 1% GWAS outliers (green dots). Black boxplots depict observed F_{ST}s between each population pair based on 1000 resampling events picking randomly each time the same number of SNPs as the top 1% outliers from all non-outlier SNPs in the dataset. In all but one cases (ON1 vs. ON11) F_{ST}s was outside of the boxplot distribution, suggesting significant differentiation from a random genomic background.

Figure S5: Matrix of distribution plots of pairwise F_{ST}-values between rock and sand populations that are either (a) outcrossing or (b) selfing.

Figure S6: Principal component (PC) analyses using (a) the top 1% GWAS outliers or (b) the non-outlier SNPs. Analyses were performed on the combined SNP dataset for all 20 populations, irrespective of mating system. The mating system and substrate type of populations is indicated.

Figure S7: The clustering of GWAS outliers as calculated by the distance between adjacent outlier positions. The distribution of these distances is shown for the separate (a-c) and combined (d-f) datasets for outcrossing (a & d) and selfing (b & e) populations in 40kb bins. The blue polygon depicts the range of a null distribution obtained from 10'000 resampling replicates. The deviation of the observed values for each distance class from the null distribution is further shown (c & f). The latter differed significantly between outcrossing (green) and selfing populations (orange) for both the separately

analyzed datasets (G = 281.6, d.f. = 8, p < 0.001) and the analyses on the combined dataset (G = 15.8, d.f. = 8, p = 0.046).

Figure S8: The clustering of GWAS outliers as calculated by the distance between adjacent outlier positions in 20kb bins, for (a) outcrossing and (b) selfing populations, and (c) the deviation of the observed values for each distance class from the null distribution. GWAS was here performed on the large and separate SNP datasets. The blue polygon (a, b) depicts the range of a null distribution obtained from 10'000 resampling replicates. The deviation between observed and expected (under a null distribution) values differed significantly between outcrossing (green) and selfing populations (orange) (G = 637.6, d.f. = 17, p < 0.001).

Figure S9: Average linkage disequilibrium (LD [r^2]) for up to 200 bp distance between SNPs for (a) outcrossing and (b) selfing populations. LD in populations on rock is highlighted in blue and that of populations on sand in red.