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Phenotypic and Genetic Analyses of Adaptation to Geothermal Soils in Yellow Monkeyflowers of Yellowstone National Park

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PHENOTYPIC AND GENETIC ANALYSES OF ADAPTATION TO GEOTHERMAL SOILS IN YELLOW MONKEYFLOWERS OF YELLOWSTONE NATIONAL PARK

By

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ABSTRACT

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Faculty Mentor: Lila Fishman

Microgeographic adaptation, which occurs on a spatial scale smaller than the dispersal distance of the evolving organisms, provides a fertile context for understanding the genetic processes that shape natural variation and contribute to biological diversity. In plants, mosaics of distinct soil conditions can select for microgeographic divergence in the face of gene flow, leading to major life history transitions and novel trait evolution. *Mimulus* (monkeyflowers) is an emerging model genus for ecological genomics, due to tremendous diversity, experimental tractability, and a wealth of genomic resources. In Yellowstone National Park, *Mimulus guttatus* occurs in both geothermal soils and nearby nonthermal bogs within a few meters of each other. Thermal populations have evolved a distinct suite of traits, including both novel adaptations (e.g. dwarfism) and shifts parallel to independent transitions elsewhere in the *M. guttatus* species complex (e.g. annuality, self-pollination). In this experiment, I generate a quantitative trait locus (QTL) mapping population of *M. guttatus* to investigate the genetic basis of adaptation to geothermal soils. Using a previously-gathered replicated PoolSeq data and targeted genetic mapping, we identify a 24-gene region on linkage group 6 that is a major QTL for several traits related to thermal adaptation, such as dwarfism and annuality. This finding contributes to our understanding of the mechanisms driving adaptation to extreme habitats, as well as the genetics of parallel trait evolution.

Phenotypic and Genetic Analyses of Adaptation to Geothermal Soils in Yellow Monkeyflowers of Yellowstone National Park

INTRODUCTION

Organisms have adapted to live in nearly every possible habitat on Earth through a process that has generated an impressive amount of phenotypic and genetic diversity. Evolution by natural selection continues to produce phenomenal organisms that exceed human-made technologies in terms of specialization, resiliency, and sustainability. Despite this, we still know very little about the evolutionary processes and genetic materials that are responsible for generating the myriad of complex biological systems, tools, and relationships that surround us. Understanding the genetic basis of phenotypic differences between closely related organisms is key to gaining insight into the underlying mechanisms that shape adaptive variation and contribute to worldwide biological diversity. In particular, microgeographic adaptation provides a fertile context for studying the genetic basis of adaptation, as acute environmental conditions can provide sufficient selective pressure for divergence in the face of gene flow, leading to major life history transitions and novel trait evolution (Lekberg 2012; Hendrick et al. 2016).

Quantitative trait locus (QTL) mapping studies often reveal the genetic architecture of traits within species and provide an understanding of relative effect sizes of genomic regions in their contribution to adaptive evolution (reviewed in Olson-Manning et al. 2012). QTL analyses provide insight into the genetic processes of evolution (e.g. many small steps through several minor genes vs. adaptive "leaps" via major genes), as well as an understanding of genetic barriers that maintain species differences after secondary contact (e.g. hybrid sterility) (Bradshaw et al. 1995; Fishman and Willis 2006). By comparing QTL maps in separate crosses between species that have undergone similar phenotypic transitions, we can also ascertain the genetic approaches used by different organisms to achieve the same evolutionary results (Patterson et al. 1995).

In my Honors Senior Research Project, I analyzed a QTL mapping population of yellow monkeyflowers (*Mimulus guttatus*) from Yellowstone National Park to investigate the genetic basis of adaptation to geothermal soils. In Yellowstone, *M. guttatus* occurs in both geothermal

soils and nearby nonthermal bogs. Thermal populations are derived from nonthermal populations and have evolved a distinct suite of traits, including both novel traits (e.g. dwarfism) and shifts parallel to independent transitions observed elsewhere in the plant community (e.g. self-pollination, annuality). For my project I grew *M. guttatus* hybrids and their thermal and nonthermal parental plants in a greenhouse common garden and measured over 15 different floral and vegetative traits for the purpose of QTL mapping. While I was not able to complete the full QTL map before graduating, I still gathered much genetic data from targeted QTL analyses. Using previously gathered PoolSeq data from three thermal/nonthermal population pairs of Yellowstone *M. guttatus* (Findley Finseth and Lila Fishman, unpublished data), our targeted analyses reveal a major 24-gene QTL on linkage group 6 (*out6*) that is associated with multiple floral and vegetative traits related to thermal adaptation. This data provides a glimpse of the genetic basis of adaptation to geothermal soils in *M. guttatus* and sets the stage for the full QTL map to come.

METHODS

Study system

The yellow monkeyflower, *Mimulus guttatus*, occurs in a variety of habitats across western North America, although its range is limited to environments with at least ephemerally moist soils. *M. guttatus* is the central member of a self-compatible and interfertile species complex that demonstrates great ecological diversity and a broad range of adaptive characteristics (Wu 2008; Hendrick 2016). Due to a wealth of genomic resources and experimentally favorable life history characteristics, *Mimulus* is a model genus for understanding the genomic and genetic basis of adaptation and speciation (Wu 2008).

In Yellowstone, *M. guttatus* inhabit a wide range of environments, from cool marshlands and riverbanks to geothermal vents and hot springs. At the Agrostis Headquarters (AHQ), and Rabbit Creek (RC) study sites, 2 ecotypes of *M. guttatus* can be found along an extreme environmental gradient of less than 500 meters created by geothermal soils (Hendrick 2016). Thermally adapted (AHQT) plants grow in soils that can reach temperatures of over 50°C during the growing season. AHQT plants are annuals (no rhizomes for over-wintering) that flower under short days (March-June), exhibit dwarfism, and have small, self-pollinating flowers. Non-thermal (AHQNT) plants grow in a nearby bog with little thermal influence. AHQNT plants are

tall, outcrossing perennials that flower under long days (July-September) and produce abundant rhizomes for over-wintering under snow/mud.

Greenhouse methodologies

For phenotypic analyses and QTL mapping, I grew previously-generated F2 hybrids (n=400) between AHQT and AHQNT inbred lines, along with parental controls (n=20 each). Seeds were germinated in sand-filled petri dishes, then transplanted into 2" pots filled with unfertilized potting soil. The plants were grown in the UM Diettert Gardens greenhouse under long-day conditions (16-hour days), with the parents randomized spatially throughout the F2s. I watered the plants four times per week and fertilized three times throughout the 14-week experiment. I sprayed all plants with Conserve® insecticide one time to control for thrips. Eighteen individuals of each parental line and 364 F2s survived to flowering and phenotyping.

Phenotyping

I measured multiple plant leaf, plant architecture, and floral traits on each plant. I measured the following leaf and plant architecture traits: leaf length of the second leaf pair; trichome number on the second leaf pair (measured by counting the number of trichomes that extended past the edge of the leaf); number of days to produce the first flower (calculated from germination date and flowering date); plant node number that produced the first flower; height of the node that produced the first flower (distance between axillary bud on basal leaves and base of pedicel); and rhizome number after production of the last flower. The following floral traits were measured on the first flower produced by each plant: corolla width; corolla length; style length (measured to base of stigmatic lobes); and stamen length. For later estimation of pollen viability, anthers were collected into 60 MicroL of lactophenol aniline blue stain. Pollen viability was then estimated by calculating the proportion of stained (viable) pollen grains in a random sample of 100 grains.

For each character, we calculated the mean and standard error of each class (AHQNT parent, AHQT parent, F2) in JMP 13. To examine phenotypic and genetic associations among traits, Pearson correlations (*r*) among traits in the F2 hybrids and each parental were also calculated in JMP 13. To visualize the distribution of trait values in the F2 hybrids, we generated histograms representative of both quantitative (e.g. pollen viability) and major-gene or Mendelian traits (e.g. rhizome number).

DNA extraction

After recording all phenotypic data, I collected floral bud and/or rhizome tip tissue samples from each individual and extracted DNA from all individuals using a CTAB-chloroform extraction protocol (Doyle and Doyle 1987) modified for use in 96-well format.

Multiplex shotgun genotyping

I quantified each F2 DNA sample using a Qubit and individually diluted each sample to a concentration of 5ng/µl. I then conducted multiplex shotgun genotyping (MSG) in order to gain genome-wide genotype data for all individuals (Andolfatto et al. 2011).

Marker genotyping

Pooled population sequencing of three Yellowstone population pairs (thermal/nonthermal) revealed several prominent regions of shared genomic divergence (Findley Finseth and Lila Fishman, unpublished data). One 24-gene outlier region on Linkage Group 6 (hereafter *out6*) contains > 50% of the shared highly differentiated SNPs (> 50% difference in allele frequency between T and NT pairs) and is the largest contiguous outlier region. To test whether it was also associated with trait differentiation in the F2s, we targeted several gene-based exon-primed, indel-flanking markers to *out6* and nearby regions. After testing them in parental lines to verify informativeness, I genotyped four markers (F1832, L1029, F2118, F2137) in the full F2 dataset. We ran two markers within the *out6* region—F2118 and F2137—which span 20 of the 24 genes in the region. We also genotyped two nearby markers—F1832 and L1029—which are located more toward the center of Linkage Group 6. Markers were amplified with a standard touchdown polymerase chain reaction (PCR) protocol and sized on ABI 3130 automated sequencers with an in-lane size standard. Markers were visualized with 5' fluorescent (6-FAM or HEX) labels, either by direct labelling the forward (F) primer or by M13 tailing of the F-primer plus addition of a labelled M13 primer in the amplification reaction. Genotypes were scored in GENEMAPPER software (Applied Biosystems, Waltham, MA) with visual verification and double-checking of all putative crossovers in mapping populations. For comparability, the 202 F2 individuals with genotype data at all four markers were included in the final genotype-phenotype association analyses.

RESULTS

Phenotypes: means, variances, correlations, and distributions

The parental lines were highly differentiated for nine out of fourteen floral and vegetative traits (Table 1; Figure 1). Among the most highly differentiated traits are trichome number and rhizome number, as thermal parents completely lack rhizomes and nonthermal parents completely lack trichomes on the second leaf pair. Trichome traits, height traits (height to first flower; average internode length), and stigma-anther separation exhibited phenotypic additivity, with F2 hybrids being intermediate, on average. Several length traits (leaf length, corolla length, stamen length, and style length) exhibited possible heterosis and transgressive segregation, as the F2 hybrids were larger, on average, than either parent.

We examined phenotypic correlations between traits in the thermal parents, nonthermal parents, and F2 hybrids (Table 2). In each parental set, most floral traits were phenotypically correlated with one another, and there were significant correlations between plant height at the first flower and internode length ($r > 0.75$ for both parents), but no significant correlations between plant height and node number on which the first flower emerged. These associations are strictly environmental/developmental rather than genetic, as the parental replicates are genetically identical. All flower size traits in the F2s were highly correlated ($r > 0.55$; p-value < 0.0001), and height to first flower was strongly correlated with both internode length and flower node number, although the former was higher ($r = 0.69$; $r = 0.58$). Interestingly, flowering time shows significantly negative correlation with rhizome number ($r = -0.66$) and internode length ($r = -0.33$) in the F2s.

In F2 hybrids, trichome number and rhizome number segregate as Mendelian traits, with approximately 25% of F2s lacking either structure (Figure 2a, 2b). In contrast, most other floral and vegetative traits, such as stigma-anther separation, exhibited quantitative distributions (Figure 2c). However, the distribution of F2 stigma-anther separation is slightly skewed to the right. This skew is likely a result of pollen sterility in the F2 hybrids, as about one-third of F2s were completely male-sterile and a smaller proportion showed an intermediate level (20-80%) of viability (Figure 2d). Indeed, sterile anthers were observed to be greatly shrunken, and most of the largest stigma-anther distances occurred in sterile individuals (Figure 2c, 2d—see shaded bars).

Genome-wide genotyping

Dual-indexed MSG libraries were prepared (N = 364; 144 by me) and their quality confirmed using qPCR. The samples were sequenced at HudsonAlpha Institute for Biotechnology Genome

Service Lab (on 2 lanes of Illumina HiSeq2500). The sequence data will be used for genome-wide QTL mapping, but those analyses are not complete for inclusion here.

Targeted QTL analysis

Consistent with the inference that the *out6* region is involved in thermal adaptation, we found that it is indeed a major QTL for multiple traits. Flowering time, rhizome number, node number of the first flower, and internode length were all found to have strong associations with genotype in this region. No flower size traits showed any association with this region. However, pollen viability shows a significant signal from only one flanking marker (F1832), suggesting a region toward the center of the chromosome plays a larger role in this trait.

The strongest signal for flowering time, rhizome number, node number, and internode length came equally from the central markers F2118 and L1029, suggesting that the QTL is coincident with *out6* rather than elsewhere on the chromosome (Figure 3). F2118 and L1029 are perfectly linked (same genotypes in the 202 F2s analyzed), so I use L1029 to refer to these two markers in further discussion.

The largest QTL in this region is for flowering time, for which L1029 explains 43% of the F2 variance (FDR LogWorth = 22.44). This marker explains 33% of F2 variance in rhizome number (FDR LogWorth=16.38), 22% of F2 variance in node number (FDR LogWorth=10.02), and 8% of F2 variance in internode length (FDR LogWorth=3.35). The marker F1832 (distal flanking marker) explains 7.5% of F2 variance in pollen viability, although no other markers in this region have a significant association with this trait (Figure 3).

DISCUSSION

Studying the phenotypic and genetic differences between closely related organisms is one of the most effective tools for understanding the molecular mechanisms of adaptation. This is especially useful in organisms that have undergone morphological or phenological transitions similar to those of other species, as it allows for comparisons of the genetic processes involved in parallel evolution (Colosimo et al. 2005). Here, I investigated the genetic basis of adaptation to geothermal soils in *M. guttatus* from Yellowstone National Park by phenotyping a QTL mapping population of F2 hybrids (N=364) and implementing targeted QTL analyses to determine genotype-phenotype associations. While this particular case of local adaptation to thermal habitats is rather unique within the plant community, this system exhibits many traits and

transitions that are common in plants (e.g. self-pollination, annuality, hybrid sterility) and have been well-studied in *Mimulus* (Fishman et al. 2002; Fishman and Willis 2006; Lowry and Willis 2010). It is already known that Yellowstone *M. guttatus* lack the chromosomal inversion discovered by Lowry and Willis (2010) that differentiates widespread annual and perennial *M. guttatus*, and my results reveal a major QTL elsewhere in the genome that likely plays a role in this independent derivation of annuality. Additionally, I found that *out6* is a major QTL for several other floral and vegetative traits that relate to this unique adaptation to an extreme environment.

Phenotypes: F2 correlations and distributions

Inferences about the functional bases of traits

In the F2 hybrids (N=364), nearly all floral traits are strongly correlated, with *P* values well below α (Table 2). Interestingly, stigma-anther separation is strongly correlated with stamen length, but shows no correlation with style length, which suggests a functional basis for a trait that is greatly involved in selfing ability. However, this trait may also be confounded by the large amount of hybrid sterility observed in F2 hybrids. While flowering time shows a significant negative correlation with rhizome number and internode length, it is negatively correlated with node number of the first flower. This is interesting because developmental traits such as rhizome number and node number are often thought to increase with the amount of time spent prior to flowering. While this is the case with node number, rhizome production appears to have a different functional basis.

Hybrid incompatibilities

Nearly one-third of F2 hybrids were found to be completely sterile (Figure 2). Hybrid sterility has been well-studied in interspecific crosses in *Mimulus* (Fishman and Willis 2006; Barr and Fishman 2011; Stathos and Fishman 2014), and hybrid incompatibilities have been observed in intraspecific crosses in the *M. guttatus* species complex as well (Martin and Willis 2010). While the sterility observed in this experiment may be a result of deleterious recessive alleles retained from the formation of our inbred parental lines (i.e., inbreeding depression) rather than hybrid sterility per se, the cause is still unclear and will be of particular interest in the full-genome QTL analyses.

Genotype-phenotype associations in *out6* region

A 24-gene region on linkage group 6 (*out6*) was previously found to be a locus of shared genomic divergence between three thermal/nonthermal population pairs of Yellowstone *M. guttatus*, and contains approximately 50% of the shared SNPs among these population pairs (Findley Finseth and Lila Fishman, unpublished PoolSeq data). Using gene-based markers located in and nearby the *out6* region, I found that it is a major QTL for four traits potentially related to adaptation to geothermal soils: flowering time, rhizome number, node number of the first flower, and internode length. L1029 was most strongly associated with the all of these traits.

Traits involved in adaptation to thermal-soil habitats

L1029 explains 33% of F2 variance for rhizome number (Figure 3). At this marker, thermal homozygotes make more rhizomes than heterozygotes and homozygous thermal plants (Figure 4c), which is consistent with the observation that nonthermal plants make more rhizomes than thermal plants, and may contribute to the annual-perennial differences in these plants. This marker also explains 22% of F2 variance for first flower node number, as thermal homozygotes consistently flower over one node higher than heterozygotes and nonthermal homozygotes (Figure 4b).

"Wrong-way" QTL for flowering time

Days to first flower shows the strongest association with this region, as L1029 explains 43% of the F2 variance for flowering time (Figure 3). Interestingly, the direction of association within this region is the opposite of what is expressed genome-wide in the parents and F2 hybrids. F2s that are homozygous for the thermal allele at this marker flower over 10 days later than heterozygous and homozygous nonthermal individuals (Figure 4a), while parental means for thermal and nonthermal plants were essentially equal. This suggests that there are other major QTLs elsewhere in the genome that suppress the activity of *out6* with respect to flowering time.

L1029 as a candidate marker for plant growth and flowering time traits

L1029 is a marker located within the gene Migut.0L1029, which is a homolog of the *Arabidopsis thaliana* gene CLAVATA2, and is known to affect flowering and meristem activity (Kayes and Clark 1998). Migut.0L1029 is physically mapped to Linkage Group (LG12) in the *M. guttatus*

reference genome (Hellsten et al. 2013). However, both patterns of synteny and my genetic mapping data suggest that this gene is located in the *out6* region on LG6. Synteny patterns around CLAVATA2 suggest that Migut.0L1029 is located between markers F1832 and F2118 and is perfectly linked to F2118. Thus, it is a strong candidate gene for underlying the QTL and contributing to local adaptation in Yellowstone *Mimulus*.

CONCLUSION

Studying the phenotypic and genetic differences between closely related organisms is key to gaining insight into the underlying mechanisms that shape adaptive variation and contribute to worldwide biological diversity. By conducting targeted QTL analyses on F2 hybrids between thermal and nonthermal *M. guttatus*, I found strong associations between an outlier region in population genomic analyses and several floral and vegetative traits related to thermal adaptation. This study provides us with a glimpse into the genetic mechanisms underlying this unique case of microgeographic divergence, and I look forward to the results of the complete genome-wide QTL map.

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FIGURES

Trait	AHQNT (N=18)	AHQT (N=17)	AHQF2 (N=364)
Leaf length (mm)	4.35±0.73	5.03±0.25	6.00±0.10
Trichome number	0.00±0.00	50.53±2.98	32.98±1.23
Trichome density	0.00±0.00	10.10±0.43	5.67±0.21
Rhizome number	5.39±0.60	0.00±0.00	6.74±0.26
Flowering time (days)	80.20±2.32	80.76±1.29	78.96±0.58
Height of first flower (mm)	84.87±5.08	27.47±2.76	49.62±0.98
Node of first flower	7.80±0.22	5.00±0.22	6.04±0.08
Average internode length (mm)	10.91±0.61	5.63±0.66	8.31±0.14
Corolla width (mm)	22.37±0.48	18.50±0.41	21.79±0.15
Corolla length (mm)	12.80±0.19	12.47±0.21	14.03±0.07
Style length (mm)	16.37±0.20	14.33±0.34	16.52±0.06
Stamen length (mm)	14.67±0.16	13.83±0.41	15.42±0.08
Stigma-anther distance (mm)	1.70±0.13	0.50±0.15	1.10±0.05
Pollen viability (%)	0.93±0.01	0.94±0.01	0.54±0.02

Table 1: Phenotypic means (±1 SE) for all parental *M. guttatus* nonthermal (AHQNT) and *M. guttatus* thermal (AHQT) plants, and F_2 hybrids in common garden

Trait	Leaf L	Trich. #	Rhiz. #	FT	Fl H	Fl Node	Node L	Cor W	Cor L	Style L	Stam L	S-A dis.	Pollen
Leaf length		0.00 **0.70**	0.50 0.00	**-0.63** -0.44	0.48 0.00	-0.12 0.31	**0.57** -0.15	0.53 **0.62**	0.43 0.20	0.45 0.53	0.49 **0.57**	0.09 -0.41	0.43 -0.49
Trichome number	**0.29**		0.00 0.00	0.00 -0.46	0.00 0.26	0.00 0.12	0.00 0.20	0.00 0.50	0.00 0.33	0.00 0.40	0.00 0.45	0.00 -0.38	0.00 -0.20
Rhizome number	**0.28**	-0.01		**-0.66** 0.00	0.45 0.00	-0.04 0.00	0.51 0.00	0.24 0.00	0.23 0.00	0.03 0.00	0.44 0.00	-0.49 0.00	0.34 0.00
Flowering time	**-0.43**	-0.04	**-0.60**		-0.54 -0.35	0.34 -0.46	**-0.75** -0.01	-0.50 -0.24	**-0.61** -0.15	-0.54 -0.50	**-0.70** -0.37	0.04 -0.15	**-0.63** -0.15
First flower H	**0.14**	-0.10	0.08	**0.14**		0.36 0.26	**0.88** **0.76**	**0.75** 0.14	0.44 0.40	0.50 0.19	0.54 0.16	0.10 -0.01	0.20 0.09
Flower node	0.03	-0.06	**-0.32**	**0.57**	**0.58**		-0.12 -0.39	0.27 0.24	-0.13 -0.14	-0.04 0.38	-0.07 0.17	0.02 0.48	-0.25 0.26
Internode length	**0.17**	-0.07	**0.39**	**-0.33**	**0.69**	**-0.15**		**0.65** -0.06	0.51 0.43	0.52 -0.06	**0.58** 0.05	0.07 -0.30	0.33 -0.18
Corolla width	**0.28**	0.07	**0.16**	**-0.20**	**0.36**	0.07	**0.40**		**0.72** 0.49	**0.75** **0.78**	**0.81** **0.81**	0.15 -0.50	0.39 -0.09
Corolla length	**0.25**	**0.11**	0.09	**-0.14**	**0.26**	0.06	**0.29**	**0.65**		**0.69** 0.22	**0.84** 0.30	0.02 -0.37	**0.59** -0.21
Style length	**0.25**	0.09	0.08	-0.09	**0.42**	**0.19**	**0.35**	**0.62**	**0.67**		**0.77** **0.96**	**0.60** -0.40	0.29 0.04
Stamen length	**0.26**	**0.16**	0.01	**-0.14**	**0.24**	**0.12**	**0.21**	**0.57**	**0.75**	**0.80**		-0.06 **-0.65**	**0.61** -0.14
S-A distance	-0.10	**-0.15**	0.08	**0.11**	**0.15**	0.05	**0.11**	**-0.13**	**-0.36**	0.00	**-0.60**		-0.28 **0.56**
Pollen viability	**0.12**	0.02	-0.08	**-0.13**	-0.01	-0.02	0.00	**0.23**	**0.14**	**0.19**	**0.37**	**-0.36**	

Table 2: Correlations between phenotypic traits estimated by Pairwise method. F2 hybrids (N=307) are displayed in the lower left. Nonthermal (N=13; top value) and thermal (N=14; bottom value) parental plants are displayed in the upper right. Units for floral and vegetative traits are the same as for Table 1. Non-bolded values are not statistically significant; **bolded values** represent a p-value=0.05>0.0001; **bolded and underlined values** represent a p-value <0.0001.

***Figure 1**. Thermal (left) and nonthermal (right) parental plants grown in a greenhouse common garden under 16-hour days.*

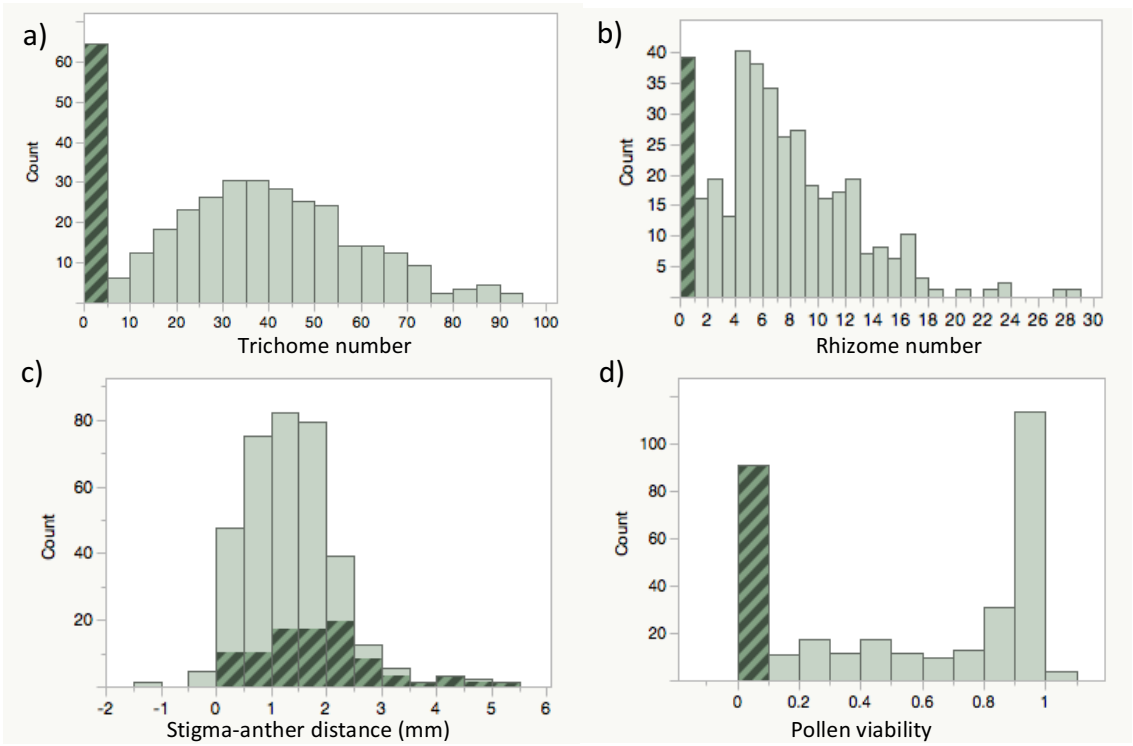

***Figure 2**. Distributions of (a) trichome number, (b) rhizome number, (c) stigma-anther separation, and (d) pollen viability in F2 hybrid plants (N=364). Shaded bars in (a) and (b) are arbitrary; shaded bars in (c) and (d) represent the same individuals.*

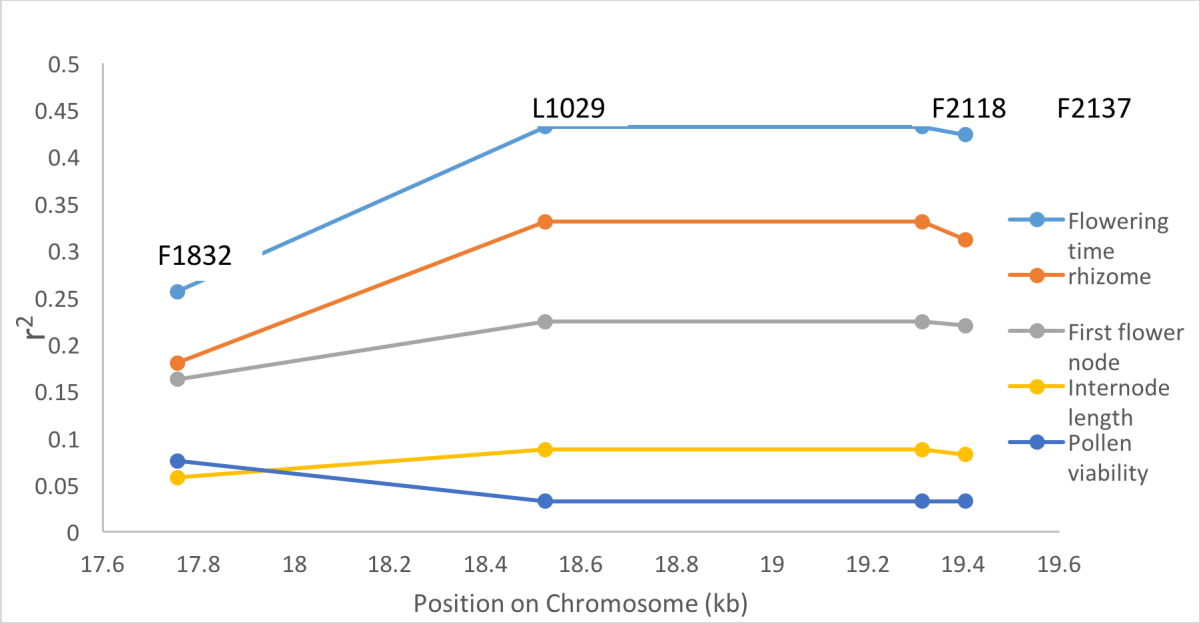

Figure 3. *Associations between markers in the out6 region and traits related to thermal adaptation for individuals with complete genotypic data (N=202). R^2 values on the y-axis represent the amount of F2 variance for each trait that is explained by a given genetic locus.*

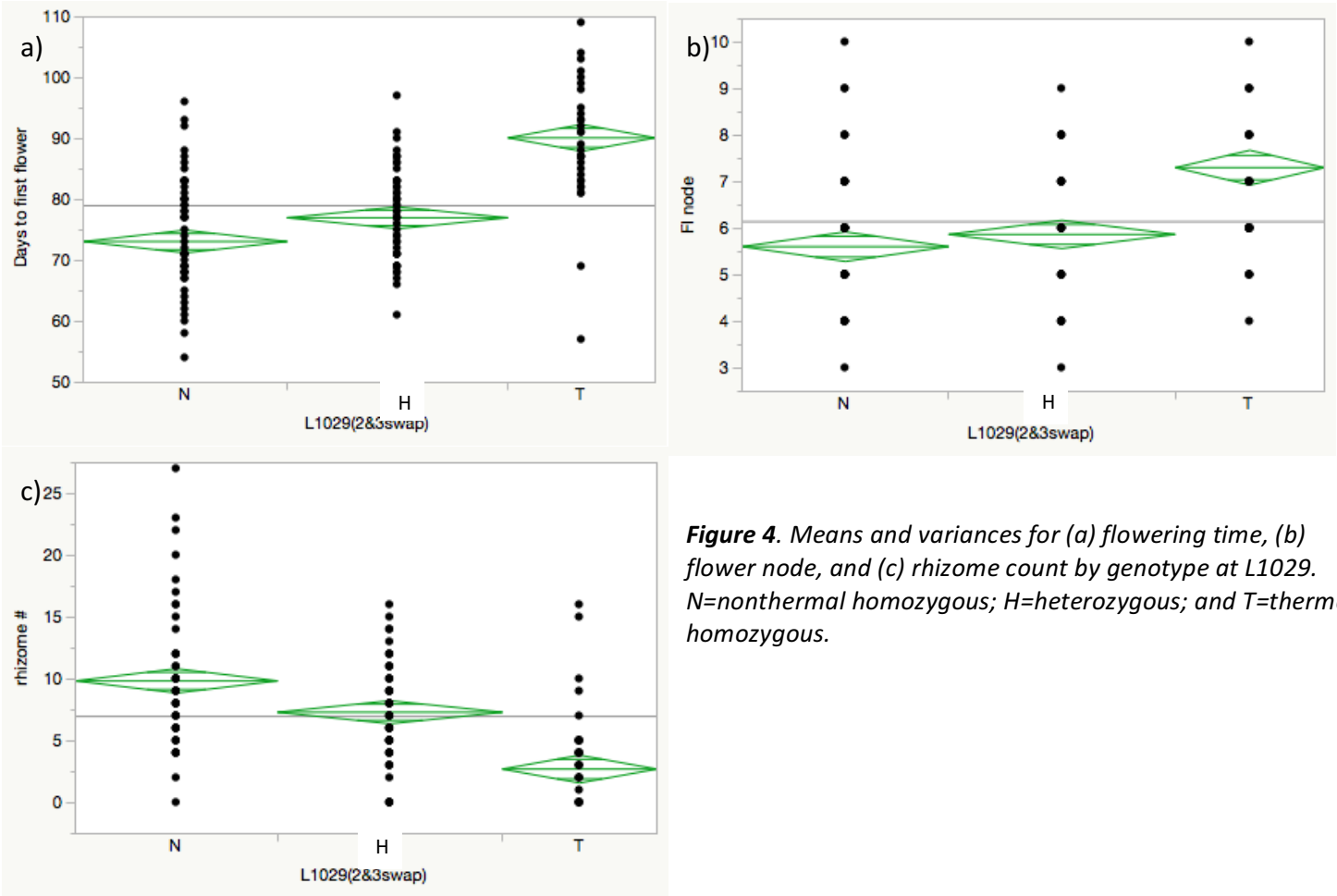

Figure 4. *Means and variances for (a) flowering time, (b) flower node, and (c) rhizome count by genotype at L1029. N=nonthermal homozygous; H=heterozygous; and T=thermal homozygous.*