

Phenotypic and genetic differentiation among yellow monkeyflower populations from thermal and non-thermal soils in Yellowstone National Park

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Abstract In flowering plants, soil heterogeneity can generate divergent natural selection over fine spatial scales, and thus promote local adaptation in the absence of geographic barriers to gene flow. Here, we investigate phenotypic and genetic differentiation in one of the few flowering plants that thrives in both geothermal and non-thermal soils in Yellowstone National Park (YNP). Yellow monkeyflowers (*Mimulus guttatus*) growing at two geothermal (“thermal”) sites in YNP were distinct in growth form and phenology from paired populations growing nearby (<500 m distant) in non-thermal soils. In simulated thermal and non-thermal environments, thermal plants remained significantly divergent from non-thermal plants in vegetative, floral, mating system, and phenological traits. Plants from both thermal populations flowered closer to the ground, allocated relatively more to sexual

reproduction, were more likely to initiate flowering under short daylengths, and made smaller flowers that could efficiently self-fertilize without pollinators. These shared differences are consistent with local adaptation to life in the ephemeral window for growth and reproduction created by winter and spring snowmelt on hot soils. In contrast, habitat type (thermal vs. non-thermal) explained little of the genetic variation at neutral markers. Instead, we found that one thermal population (Agrostis Headquarters; AHQ-T) was strongly differentiated from all other populations (all $F_{ST} > 0.34$), which were only weakly differentiated from each other (all $F_{ST} < 0.07$). Phenotypic differentiation of thermal *M. guttatus*, but little population genetic evidence of long-term ecotypic divergence, encourages further investigations of the potential for fine-scale adaptation and reproductive isolation across the geothermal gradient in Yellowstone.

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Introduction

Characterizing patterns of phenotypic and genetic differentiation across complex landscapes is important for understanding both the evolutionary dynamics of populations and the process of ecological speciation. Local forces (drift and selection) dominate when the scale of environmental variation is large relative to the scale of gene flow, and ecotypic differentiation appears common in geographically widespread species (Clausen et al. 1940; Schluter 2000; Hereford 2009). However, along steep ecological gradients or in mosaic environments, there may

be no extrinsic barriers to genetic exchange between habitats with divergent selection pressures. This may favor the evolution of plasticity (Sultan and Spencer 2002) or maintain standing variation within a single interbreeding population (e.g., Mitchell-Olds et al. 2007). Local adaptation can occur even in the face of gene flow if selection is strong (Rieseberg et al. 2002; Kawecki and Ebert 2004), particularly if plastic or genetic responses to environmental heterogeneity also cause assortative mating (Levin 2006, 2010). Furthermore, theory shows that sympatric or parapatric speciation occurs more easily when either plasticity or adaptation creates barriers to successful mating between organisms from different habitats (Coyne and Orr 2004). Thus, systems in which no geographical barriers separate extreme habitats may provide unique insight into the joint evolution of phenotypic differentiation and reproductive isolation (Rasanen and Hendry 2008).

Heterogeneous soil environments (“edaphic mosaics”) are ideal for investigating plant evolution (e.g., Sambatti and Rice 2006; Ellis et al. 2007). Local adaptation to metal-contaminated or serpentine soils appears widespread, and may be accompanied by increased reproductive isolation (e.g., Antonovics and Bradshaw 1970; Macnair 1987; Brady et al. 2005). Soil temperature, which can vary across local spatial scales in geothermally influenced sites worldwide (e.g., Yellowstone National Park in North America, Valley of the Geysers in Kamchatka) may also exert strong selection on plant populations and elicit diverse evolutionary responses. Few flowering plants live in the hot soil around thermal vents in Yellowstone (Stout and Al-Niemi 2002), but two grasses—*Dichanthelium lanuginosum* (Marquez et al. 2007) and *Agrostis scabra* (Tercek and Whitbeck 2004)—exhibit patterns of phenotypic variation suggestive of local adaptation to hot soils. Further study of genetic and phenotypic variation across these geothermal mosaics provides a rich system for addressing fundamental questions about adaptation and speciation in the absence of geographical barriers to gene flow. Such mosaics also provide a unique opportunity to understand the genetic and developmental mechanisms of plant adaptation to high temperatures, which is of broad interest as global climates become more extreme.

Here, we focus on yellow monkeyflower (*M. guttatus*) populations at extremes of soil temperature gradients in Yellowstone National Park (YNP). *Mimulus guttatus* occurs in abundance in geothermal soils at several locations within YNP (Bunn and Zabinski 2003) and also occurs at nearby (<500 m away) non-thermal wetland sites and along the edges of thermally influenced streams. This broad distribution is not surprising given the extreme plasticity and adaptability of *M. guttatus*, which has long been a model system for ecological and evolutionary genetics (reviewed in Wu et al. 2008). In addition to

exhibiting substantial intra-specific physiological and life-history diversity, *M. guttatus* is the central member of a complex of ecologically distinct but cross-fertile taxa, including taxa adapted to serpentine soil and copper mine tailings (e.g., MacNair 1983). In addition, the *M. guttatus* species complex exhibits broad variation in mating system, with some taxa almost exclusively self-pollinating and populations of *M. guttatus* varying from predominantly outcrossing to mixed mating (e.g., Fenster and Ritland 1994). Recent work on the ecology (Hall and Willis 2006; Martin and Willis 2007; Fishman and Willis 2008; Lowry et al. 2008; Wu et al. 2008) and genetic architecture (Fishman et al. 2002; Hall et al. 2006; Lowry et al. 2009) of phenotypic diversification within the *M. guttatus* complex provides a rich comparative context for understanding variation in this species across geothermal soil mosaics in YNP. In addition, recently developed genomic resources for *M. guttatus* make divergence between thermal and non-thermal populations readily accessible to further genetic and molecular dissection.

Here, we characterize phenotypic and genetic differentiation between *M. guttatus* populations found at the extremes of the thermal soil gradient in YNP. We focus on two locations (Rabbit Creek and Agrostis Headquarters) with exemplar “thermal” (T) and “non-thermal” (NT) sites. The environmental differences between thermal and non-thermal extremes at both locations are driven by the interaction between localized geothermal activity and the severe climate of YNP, where monthly minimum temperature means are below freezing October–April and annual snowfall averages 2 m (<http://www.wrcc.dri.edu/cgi-bin/cliMAIN.pl?wyell>). Field observations over several seasons (M.H. Hendrick, personal observation) indicate that plants in both thermal populations emerge as seedlings in the late fall, overwinter as vegetative rosettes, and flower in early March through May/June (Fig. S1a). At thermal sites, the hot soils dry by mid-June causing water-loving *M. guttatus* senescence, with the plant acting as an obligate annual in the field (Fig. S1b). In contrast, plants at NT sites maintain underground rhizomes year-round, but are under snowpack from October through at least May. They grow and flower only in the summer and early fall amid dense wetland vegetation (Fig. S1c).

The goal of our study was to compare phenotypic variation in vegetative and floral traits to genotypic variation of neutral markers across the pairs of thermal and non-thermal populations. For land management reasons, we were not able to conduct reciprocal planting experiments in YNP, so we used artificial environments simulating thermal and non-thermal growth conditions to assess the robustness of these differences to representative environmental variation. A comparison of phenotypic differentiation to variation at putatively neutral genetic markers

allows us to test for several possible scenarios. At one extreme, the populations may split into two distinct ecotypes, with the two thermal populations allied with each other and distinct from non-thermal populations. This scenario allows for the possibility of adaptation (as seen in many geographically isolated ecotypes), but makes it very difficult to distinguish selection from demographic processes as a source of divergence. At the other extreme, populations may cluster by geography. If thermal populations are both closely allied with paired adjacent non-thermal populations, but not with each other, drift would be an unlikely explanation for any shared differentiation. Of course, these are not the only possible outcomes, and a small number of thermal populations makes inference difficult, but such joint phenotypic and genetic investigations are a first step toward deeper genomic and ecological analyses of divergence over extremely local spatial scales.

Materials and methods

Study species

The common yellow monkeyflower, *M. guttatus* ($2n = 28$; Phrymaceae, formerly Scrophulariaceae), is a self-compatible North American wildflower widespread from the Aleutian Islands to Baja California, and from the Pacific coast to the east slope of the Rocky Mountains. *Mimulus guttatus* occurs in diverse habitats ranging from roadside ditches to alpine meadows and coastal dunes, but requires moist soils. Generally, populations found in seasonally moist habitats are annual and those in more wetland habitats are perennial, and widespread annual and perennial ecotypes have been characterized (Lowry and Willis 2010).

Sites and thermal environments

We selected two areas within YNP with both thermal (T) and non-thermal (NT) *M. guttatus*; Rabbit Creek (RC) in the Midway Geyser Basin ($44^{\circ}31'02''\text{N}$, $110^{\circ}49'21''\text{W}$, elevation 2,225 m) and Agrostis Headquarters (AHQ) in the Lone Star Geyser Basin ($44^{\circ}25'53''\text{N}$, $110^{\circ}48'48''\text{W}$, 2,371 m). The T and NT sites within each area were within 500 m of each other, and the distance between RC and AHQ is approximately 10 km. To establish locations for plant collection, we measured soil temperatures at 5 cm depth in five discrete patches with flowering *M. guttatus* at each thermal site (May–June) and one and three such patches at AHQ and RC non-thermal sites, respectively (late August). Thermal patches all had maximum soil temperatures $>32^{\circ}\text{C}$, whereas non-thermal sites all had maximum soil temperatures $<25^{\circ}\text{C}$ at the time of plant collection.

To characterize the vertical extent of thermal influence on air and soil temperatures, which determines the window for plant growth in thermal sites, we used a datalogger (CR23X micrologger; Campbell Scientific, Logan, UT, USA) equipped with copper-constantan thermocouples to record temperature at 20, 11, 5, 2, and 0 cm above the soil surface, and at 1, 3, 5, and 6 cm depth, at a single representative AHQ-T patch during the 2007 flowering season (8 May–8 June). To characterize patterns of temperature variation relevant to plant growth, we recorded temperature continuously (4-h intervals) at AHQ-T and AHQ-NT sites with paired dataloggers (DS1921G-F5 Thermochron iButton; Embedded Data Systems, Lawrenceburg, KY, USA) set at 5 cm above the soil surface (leaf level) and 3 cm below the soil surface (root level) in 2010. For AHQ-T, we present data from a representative *M. guttatus* patch from late April (peak flowering in that year) with a cold snap at midweek. We also recorded ambient air temperatures (1.5 m above soil surface) during that period, to represent spring conditions at heights and sites beyond thermal influence. We present parallel data from the AHQ-NT site during peak flowering in late July–early August, when both thermal sites were completely dry and their *M. guttatus* populations senescent (Fig. S1b).

Field measures of plant phenotypes

In 2007, we measured total plant height and height to the first flower ($n = 2\text{--}8$ plants per patch, depending on density). We measured plants during the flowering seasons at AHQ-T (May 8) and both NT sites (August 27), and after fruit set at RC-T (June 5), due to spring closure of that area for bear management. A two-way ANOVA was used to test for differences in plant traits due to temperature environment (T and NT) and location (RC and AHQ) using Minitab (Minitab, State College, PA, USA). Mean separations were performed by the least significant difference (lsd) method.

Common garden experiment 1: trait variation under simulated thermal and non-thermal conditions

Seed sources and growing conditions

Seeds were collected from AHQ-T and RC-T in June, and from AHQ-NT and RC-NT in August 2006. Seed capsules from at least 15 plants were pooled within each of the four sites. Seeds were germinated under mist in a 1:1 peat:perlite mixture at 23°C . After 4 weeks, seedlings were transplanted into 7.6-cm pots containing equal parts by volume of pasteurized loam soil:concrete sand:Canadian sphagnum peat moss with AquaGro 2000 G wetting agent (0.59 kg m^{-3}), and exposed to either spring thermal

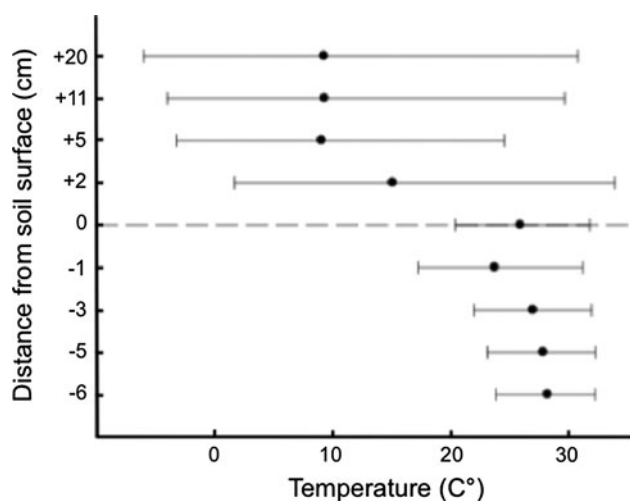


Fig. 1 Air and soil temperatures at AHQ-T from 8 May to 7 June 2007, dots mean, bars range ($n = 30$ days)

conditions (T conditions) or summer non-thermal conditions (NT conditions). Spring thermal conditions were simulated by growing seedlings on a heat blanket (BH Thermal, Columbus, OH, USA) in a growth chamber set at 4 °C with 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation for 12 h day^{-1} . Soil temperature at the bottom of the pot averaged 27 °C and the surface temperature ranged from 8.9 to 13.6 °C, similar to field conditions (Fig. 1). To simulate the progression of spring, we increased the light intensity to 170 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and the day length to 16 h day^{-1} 7 weeks after transplanting. We also increased the air temperature to 10 °C, which generated an average surface temperature of 15.3 °C (11.3–20.1 °C, min–max). Three months after transplanting, all plants were transferred from the growth chamber to a greenhouse set at 23 °C/20 °C (day/night). With the heat blankets, temperatures at the bottom of the pots reached 50 °C and surface temperatures averaged 35 °C, simulating the geothermal environment of early June. Summer NT conditions were simulated in the same greenhouse with 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of supplementary PAR for 16 h day^{-1} . Plants grown under summer NT conditions were fertilized weekly with 15 ml of half-strength Hoagland solution starting 16 January, whereas plants grown under spring T conditions were fertilized bi-weekly for the first 2 months due to the slow growth and low nutrient demands, and thereafter weekly. Plants were watered as needed with tap water until capsules had begun to mature.

Measurement of traits and statistical analyses

Plants were harvested when seed capsules had begun to ripen seeds. At harvest, total height, height to the first flower, total number of flowers, and shoot dry weight (DW)

were recorded. Significant differences in plant traits due to location (RC and AHQ), habitat type (T and NT), and growth environment (T and NT conditions) were tested using a three-way ANOVA, with contrasts within and across growth environments to determine the significance of location and habitat type effects. To explicitly consider genotype \times environment interactions for traits potentially associated with fitness, we examined the habitat type \times growth environment interaction effects for flower number, plant height, and plant dry weight. These analyses were performed in JMP 7.0 (SAS Institute, Cary, NC, USA).

In addition to the individual plant measures, we also recorded the number of seedlings with pubescence (first pair of true leaves) in each population sample. These data were analyzed with a χ^2 test in JMP 7.0 (SAS Institute).

Common garden experiment 2: floral and mating system traits

Floral traits

To investigate floral trait divergence among populations, we grew progeny from field-collected seeds (2007 field survey) in a greenhouse at the University of Montana (UM) in Autumn 2008. A single plant represented each wild maternal family ($n = 9\text{--}13$ per population). Plants were grown in 6.3-cm pots filled with Sunshine Mix #1 and bottom-watered daily. After initial stratification (1 week at 4 °C), germination and thinning, plants were kept under long day conditions with light supplementation (16-h days) and moderate temperatures (27 °C day/12 °C night). Corolla width, corolla length and stigma–anther distance were measured on one flower per plant (Fishman et al. 2002; Fishman and Willis 2008). ANOVA was used to test the effects of location, habitat type, and the habitat \times location interaction (=population) as fixed variables.

Ability to self-fertilize

To directly assess differences in autonomous self-pollination ability, we grew a second set of plants ($n = 20$ per population) under similar conditions in late spring 2009. Paired flowers (at a single node) were subjected to two treatments: autonomous selfing (no manipulation) and supplemented selfing (hand-pollination with pollen from the same plant). Seed set in the autonomous treatment indicates the ability to produce seed in the absence of pollinators, whereas the supplemented treatment indicates maximum (resource- or ovule-limited) seed set. Using self-pollen in the supplemented treatment controls for effects of inbreeding depression on seed set. We used the ratio of autonomous seed set to supplemented set (index of autonomous selfing) as an estimate of the efficiency of

self-pollination under conditions of severe pollinator limitation. Because multiple pairs of flowers were used per plant ($n = 1-3$), we analyzed these data with a mixed model including location, habitat type, and habitat \times location interaction (=population) as fixed variables, and individual (nested within the habitat \times location interaction) as a random variable. The values of the index of autonomous selfing, which are similar to proportions but can include values greater than 1, were log-transformed for analysis.

Daylength sensitivity of flowering

Mimulus plants in thermal sites initiate flowering as early as February, but many *M. guttatus* accessions require >14-h days to flower (L. Fishman, personal observation), so we tested for differences in daylength sensitivity of flowering under greenhouse conditions. To mimic fall germination and overwintering of rosettes, we germinated seeds and transplanted seedlings to pots (as above) in November 2008. The rosettes were maintained in randomized arrays in the UM greenhouse under a 10-h daily light cycle (supplemental light during natural daylight hours) and cool temperatures ($\sim 18/10$ °C day/night) until 25 January 2009. At that time, the lights were shifted to a 12-h day and daytime temperatures increased to a maximum of 27 °C. Floral bud initiation under 12-h days was recorded until 20 March 2009, at which point light was supplemented to produce 16-h days.

Flowering was treated as a categorical response variable (floral initiation by 20 March vs. no floral initiation) because we were primarily interested in the ability to flower under spring light regimes (relatively low irradiance, ~ 12 -h days). The phenology data were analyzed with a logistic regression with habitat type, location, and their interaction ($n = 328$ individuals). Plants in this experiment represented multiple wild-collected maternal families, so we also calculated a single probability of early flowering for each family ($n = 44$ families) to account for pseudo-replication across families. This summary statistic was analyzed with a two-way ANOVA with habitat type, location, and their interaction.

All statistical analyses for the floral and mating system traits were performed in JMP 7.0 (SAS Institute).

Population genetics

Population genetic analyses were conducted on samples from the same four extreme sites (AHQ-T, AHQ-NT, RC-T, RC-NT). Samples were collected from multiple areas within each site as seeds in 2007 (one seed per wild maternal plant germinated prior to DNA extraction) and as leaf tissue in 2008.

Genomic DNA was extracted from fresh or silica gel-dried tissue using a CTAB-chloroform extraction protocol

(Fishman et al. 2002) modified for 96-well format, then diluted to a standard concentration ($\sim 2-10$ ng/ μ L). We used the polymerase chain reaction (PCR) to amplify length polymorphisms at seven nuclear markers: two microsatellite loci (AAT356, AAT217; Fishman et al. 2002) and five exon-primed intron-containing MgSTS (for *Mimulus guttatus* Sequence Tagged Site) markers (MgSTS.423, MgSTS.500, MgSTS.698, MgSTS.772, MgSTS.783). Information on these markers, including linkage map locations and primer sequences, can be found at <http://www.mimulusevolution.org>. Markers were chosen because they were known to be polymorphic within a large Oregon population of *M. guttatus* (Kelly and Willis 1998; Young Wha Lee, personal communication), are genetically unlinked (on different chromosomes or >50 cM apart; <http://www.mimulusevolution.org>), and reliably amplified samples from all Yellowstone populations. The 5'-fluorescent-labeled fragments were amplified using a standard touchdown (58–48 °C annealing temp) PCR protocol and electrophoretically separated using an ABI 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) with in-lane size standards. Genotypes were assigned automatically using Genemapper software (Applied Biosystems) and then verified individually by eye, with careful attention to differential amplification of alternative alleles.

A total of 120 individuals were included in the genetic analyses (mean n across markers = 55.4, 11.3, 37.0, and 17.8 for AHQ-T, AHQ-NT, RC-T and RC-NT, respectively). The variation in sample size parallels variation in the extent and census population size of the sampling sites. We used Genalex 6.0 (Peakall and Smouse 2006) to calculate basic diversity statistics for each marker and population and FSTAT (Goudet 1995) to generate multi-locus pairwise F_{ST} s among the four populations, with bootstrapping over loci to calculate confidence intervals for each pairwise F_{ST} value. For AMOVA using Genalex 6.0, populations were regrouped into regions by habitat type (T vs. NT), by location (AHQ vs. RC), and by AHQ-T versus all others. Finally, we used STRUCTURE software (Pritchard et al. 2000) to assess the most likely number of population clusters (k) and assign individuals to clusters based on their multi-locus genotypes. We ran 10 replicates at each level of k (1–4), using the admixture and correlated allele frequency models. The burn-in length was set at 10^5 steps, followed by 10^6 steps.

Results

Site conditions and field measures

During the May 2007 flowering season at AHQ-T, air temperatures were consistently above freezing only within

5 cm of the soil surface (Fig. 1), and soil temperatures exceeded 30 °C at rooting depth. In April 2010, our surveyed thermal patch was substantially hotter but also highly variable, illustrating the temporal and spatial range of thermal soil conditions. While *Mimulus* were flowering, temperatures at rooting depth (3 cm below surface) peaked to 45–50 °C and at plant height (5 cm above surface) to 38 °C (Fig. 2a). Temperatures at plant height never dropped below freezing, despite 3 days of subzero ambient temperatures. The ambient temperatures also represent conditions at the nearby (but inaccessible under snow at that time) AHQ-NT site. In contrast, air and soil temperatures during the summer flowering season at AHQ-NT were always well above freezing (Fig. 2b), consistent with long-term monthly recordings of minimum and maximum air temperature for June (1.3–18.3 °C), July (4.1–23.9 °C), and August (2.8–23.4 °C) at Old Faithful (<http://www.wrcc.dri.edu/cgi-bin/cliMAIN.pl?wy6845>). Together, these data demonstrate that thermal *Mimulus* must flower in a narrow layer of non-freezing air near the soil surface during spring, when soil temperatures are variable but generally hot, whereas plants in non-thermal sites experience permissible conditions for growth and reproduction only in mid-late summer.

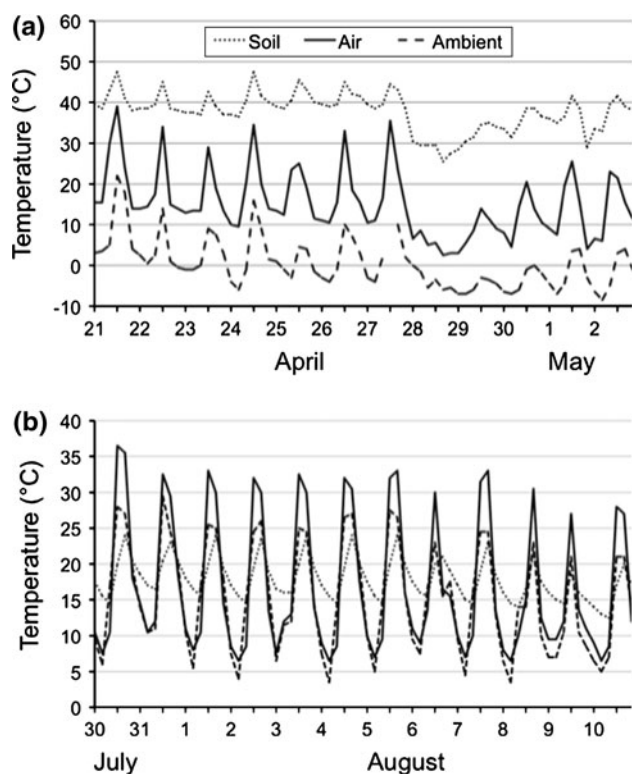


Fig. 2 Daily temperature profiles (°C) of soil at root depth (–2 cm; dotted), air at shoot height (2 cm; solid), and ambient air (1 m; dashed) in *Mimulus guttatus* plots during representative weeks of the 2010 flowering seasons. **a** AHQ-T in late April–May and **b** AHQ-NT in late July–early August. Note the difference in y-axis scale

Table 1 Mean \pm SE of total shoot height and height to the first flower of *Mimulus guttatus* growing in thermal (T) and non-thermal (NT) habitats within the Rabbit Creek (RC) and Agrostis Headquarters (AHQ) locations

Population	<i>n</i>	Total height (cm)	1st flower height (cm)
RC-T	22	8.9 \pm 0.7 b	4.8 \pm 0.4 b
RC-NT	11	32.3 \pm 2.2 a	22.7 \pm 1.3 a
AHQ-T	20	5.6 \pm 0.4 c	2.2 \pm 0.4 c
AHQ-NT	8	27.3 \pm 2.1 a	23.5 \pm 2.1 a

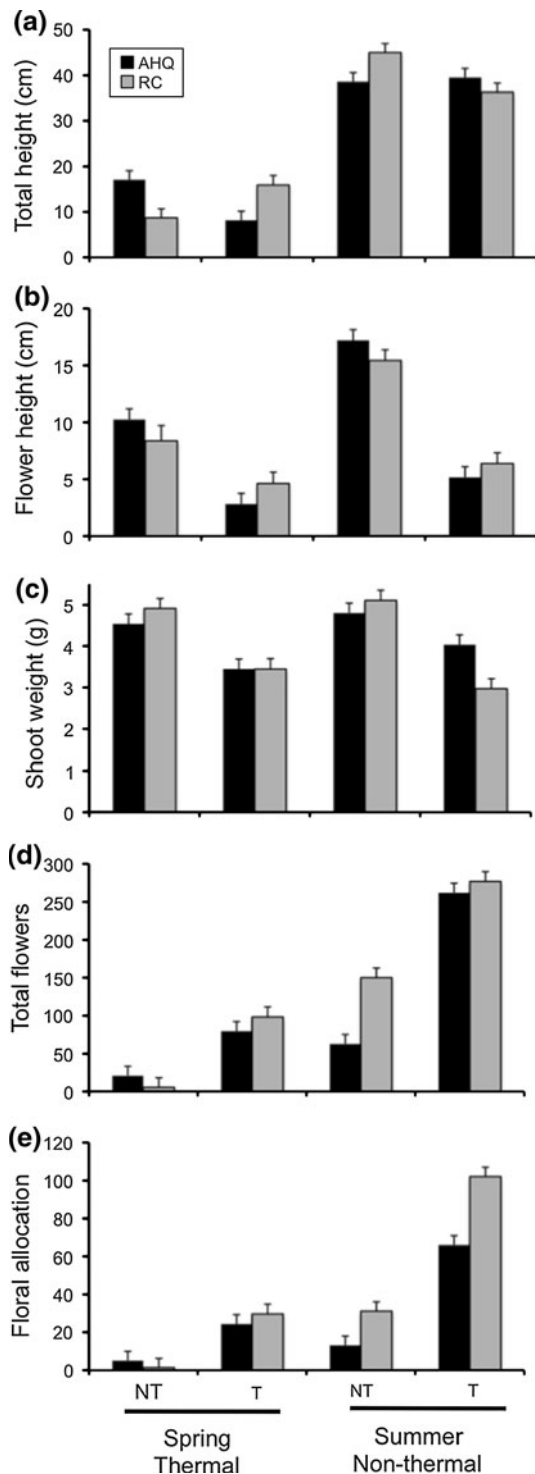
Means with different letters are significantly different ($P < 0.05$)

In the field, T plants were significantly shorter than NT plants ($F_{1,57} = 277$, $P < 0.001$; Table 1) and set their first flower closer to the ground ($F_{1,57} = 509$, $P < 0.001$; Table 1). This difference was more pronounced at AHQ, resulting in a significant habitat type \times location interaction ($F_{1,57} = 8.66$, $P = 0.005$). There was also a significant effect of location, with AHQ plants significantly shorter than RC plants, on average ($F_{1,57} = 9.95$, $P = 0.003$). The location effect could be due in part to the earlier sampling of AHQ plants in the field, but all plants had finished flowering (and therefore increasing in height) at the time of measurement.

Common garden experiment 1: heritable and plastic variation in plant architecture

The common garden experiments under simulated spring thermal and summer non-thermal growth conditions revealed both plastic and heritable components to plant architecture (Fig. 3). With the exception of total plant height, which showed strong effects of the experimental conditions ($F_{1,134} = 375.3$, $P < 0.0001$; Fig. 3a) but no significant effect of habitat type ($P = 0.10$), all plant architecture traits were differentiated between thermal and non-thermal populations. As in the field, experimental plants grown under spring thermal conditions set their first flower significantly closer to the soil surface relative to plants in summer non-thermal conditions ($F_{1,125} = 30.9$, $P < 0.001$; Fig. 3b). However, irrespective of growing conditions, plants from both T populations set their first flower significantly closer to the ground compared with NT plants ($F_{1,125} = 159.0$, $P < 0.001$; Fig. 3b), indicating a large genetic component to this trait. Shoot dry weight was not significantly affected by growth conditions ($F_{1,134} = 0.68$, $P = 0.41$), but plants from NT populations were significantly heavier than T plants across both treatments ($F_{1,134} = 63.92$, $P < 0.001$; Fig. 3c). This difference was more pronounced at AHQ, resulting in a significant habitat type \times location interaction ($F_{1,134} = 6.53$, $P = 0.012$).

Growth conditions strongly influenced flower number ($F_{1,134} = 230.7$, $P < 0.001$), but T populations produced



significantly more flowers across both treatments ($F_{1,134} = 175.8, P < 0.001$; Fig. 3d). This translated into a highly significant difference in allocation between T and NT populations ($F_{1,134} = 150.0, P < 0.001$; Fig. 3e), with 3- to 25-fold higher allocation to flowers per gram shoot biomass for thermal plants when compared to non-thermal plants from the same location in the same treatment. Under

◀ **Fig. 3** Plant architecture traits of *Mimulus guttatus* grown in common garden environments simulating either spring thermal conditions or summer non-thermal conditions. **a** Total plant height (cm), **b** height at the first flower (cm), **c** above-ground dry weight (g), **d** flower number at harvest, and **e** floral allocation (number of flowers per g dry weight). Bars show least squared mean + 1SE from a full factorial ANOVA ($n = 18$) with growth conditions (spring thermal vs. summer non-thermal), thermal (T) versus non-thermal (NT) habitats, and location (AHQ vs. RC) as fixed effects. All traits, other than aboveground dry weight, were significantly influenced by growth conditions ($P < 0.001$). Contrast of habitat type (T vs. NT) were highly significant ($P < 0.001$) for all traits, other than total height

the simulated thermal conditions, this allocation difference may in part be due to differences between T and NT plants in the daylength sensitivity of flowering (see below). However, because these differences were also highly significant under simulated NT conditions ($P < 0.0001$ for T vs. NT least squared means contrasts for both RC and AHQ locations), they also suggest evolved differences in allocation to reproductive versus vegetative biomass.

We observed significant habitat type \times growth condition interactions for height to first flower and total flower number ($P < 0.001$), but not for total height or dry weight (all $P > 0.10$). There was also a significant 3-way growth condition \times location \times habitat type interaction ($P < 0.001$) for plant height. However, in each case with a significant interaction involving growth condition, the pattern appeared driven by greater plasticity of the thermal plants rather than by reduced performance in foreign conditions (i.e., T plants produced even more flowers than NT plants under the simulated non-thermal conditions; Fig. 4d).

Populations also differed in the occurrence of pubescence on young leaves (Pearson $\chi^2 = 63, P < 0.001$). Only 0–3 plants from AHQ-NT, RC-T and RC-NT ($n = 36$ each, across the two treatments) were pubescent at this stage. In contrast, 53 % (9/17) and 94 % (17/18) of AHQ-T plants were pubescent when grown under simulated summer non-thermal and spring thermal conditions, respectively. This suggests genetic differentiation for trichome production between AHQ-T and the other populations.

Common garden experiment 2: floral, mating system, and phenological traits

Under common greenhouse conditions, T and NT populations also differed significantly in floral morphology, mating system, and phenology (Fig. 4). On average, T plants had decreased floral dimensions consistent with the evolution of increased autonomous self-fertilization under conditions of pollen limitation. There were no consistent differences in corolla length between T and NT populations ($P = 0.78$), but plants from both thermal populations had lower corolla widths ($P < 0.01$), resulting in flowers with relatively

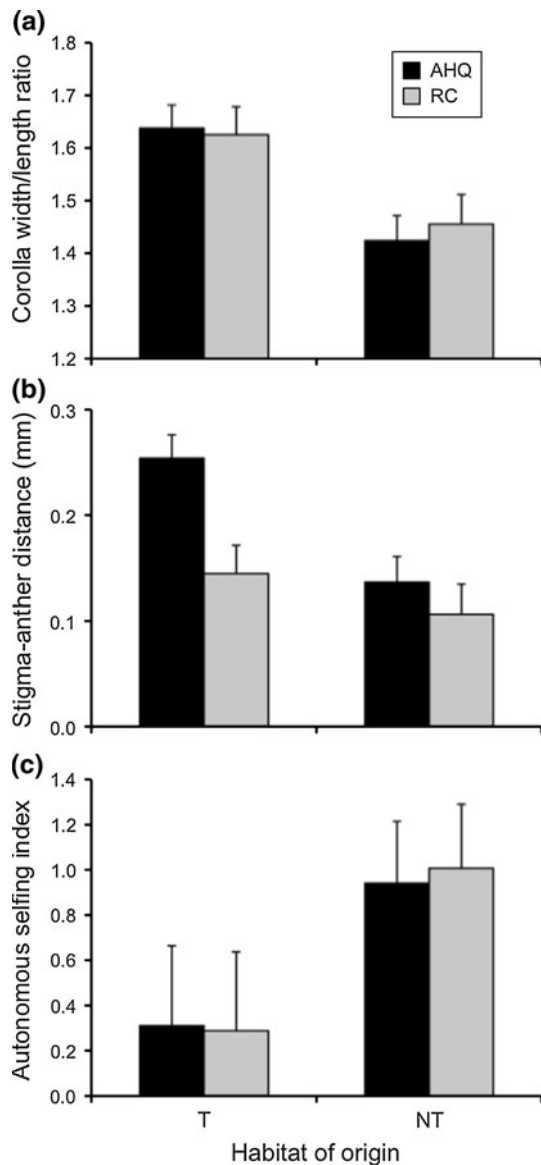


Fig. 4 Floral and mating system traits of thermal (*T*) and non-thermal (*NT*) *Mimulus guttatus* from Yellowstone National Park grown in a greenhouse common garden. **a** Corolla width–length ratio, **b** stigma–anther separation (mm), and **c** index of autonomous selfing (unmanipulated seedset/supplementally pollinated seedset in paired flowers). All *T* versus *NT* contrasts were significant ($P < 0.01$). Bars show least squared mean + 1SE from ANOVA with maternal plant as a nested random variable ($n = 8$ –13 per population)

narrow width/length ratios (Fig. 4a). Overall, stigma–anther separation was also significantly lower in the *T* populations than in *NT* populations ($P < 0.005$); however, this was due to the low stigma–anther separation of both thermal populations relative to the AHQ-*NT* plants, as RC-*NT* plants also had relatively low stigma–anther distances (Fig. 4b).

Consistent with the differences in floral morphology, plants from *T* populations had relatively high levels of autonomous selfing (Fig. 4c). In fact, thermal plants set as many seeds by selfing in the greenhouse as they did when

supplementally pollinated (mean autonomous selfing index $\geq 100\%$). In contrast, the mean autonomous selfing index for non-thermal plants was $\sim 30\%$. This difference produced a significant ($P < 0.005$) habitat type effect, with no effect of location or the location \times habitat interaction ($P > 0.23$). On average, thermal plants (means = 123 and 124, respectively, for RC-*T* and AHQ-*T*) set significantly ($P < 0.01$) fewer seeds per flower in the supplemental pollination treatment than non-thermal plants (means = 339 and 154 for RC-*NT* and AHQ-*NT*, respectively), but the overall pattern appears primarily driven by the high maximum seed set of RC-*NT*.

Thermal and non-thermal populations also exhibited strong differentiation in flowering cues, as indicated by both the nominal logistic regression of individuals and the ANOVA of family means (both $P < 0.001$ for *NT* vs. *T* effect). Whereas 25% (RC) and 40% (AHQ) of plants from thermal families initiated flowering under March (12-h) daylength conditions, no non-thermal plants ($n = 88$) flowered until after the lights were shifted to long days. Further study will be necessary to determine whether genetic variation for flowering requirements exists within or among the thermal populations, as our greenhouse experiment had to be truncated when daylengths naturally exceeded 12 h. However, flowering appeared to cluster across thermal families ($P < 0.001$ for a family effect when added as a nested nonrandom variable in the logistic regression), which suggests that there may be heritable or maternally influenced variation within thermal populations in the ability to flower rapidly under <12-h day lengths.

Population genetic diversity and structure

The microsatellite and gene-based markers exhibited substantial polymorphism across the populations sampled, providing adequate resolution for analyses of genetic structure within YNP (Table S1). Each of the markers had genotypic ratios deviating from expectations under Hardy–Weinberg equilibrium (HWE) within at least one population, and one (MgSTS.698) deviated significantly from HWE expectations in all four populations. However, because *M. guttatus* is self-compatible and may autonomously self at high rates in at least some YNP populations, these deviations most likely reflect inbreeding rather than common null alleles at individual markers.

With the exception of AHQ-*T*, all populations exhibited similarly moderate levels of genetic diversity ($H_o = 0.33$ –0.41; $H_e = 0.44$ –0.54) (Table S1). AHQ-*T* had significantly lower observed ($H_o = 0.17$) and expected ($H_e = 0.25$) heterozygosities, suggesting an overall reduction in genetic diversity despite its large census and sample size. Inbreeding coefficients ranged from 0.20 (AHQ-*NT*) to 0.27 (AHQ-*T*), but did not differ significantly among populations.

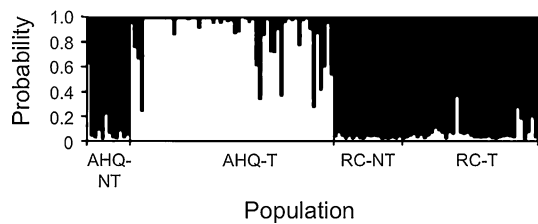


Fig. 5 Population structure of Yellowstone National Park *Mimulus guttatus* estimated from STRUCTURE for $k = 2$ clusters. Each vertical bar represents an individual whose estimated proportion of membership to either cluster (y-axis) is indicated by the two different colors. Tick marks separate populations

Pairwise F_{ST} analyses indicated strong genetic differentiation between AHQ-T and all other populations ($F_{ST} = 0.34, 0.39,$ and 0.43 vs. RC-T, AHQ-NT, and RC-NT, respectively) and relatively low differentiation for all other comparisons (F_{ST} range 0.04 – 0.07). All pairwise F_{ST} estimates were significantly different from zero ($P < 0.01$) and all values involving AHQ-T were significantly higher than the others. A significant but small amount of the total genetic variance (6 %; $P < 0.01$) was distributed between regions in AMOVA when regions were defined to group thermal versus non-thermal populations. Geographic location (AHQ vs. RC) explained more variation (15 %; $P < 0.01$) in the corresponding AMOVA. However, regional variance was highest (28 %; $P < 0.001$) when regions were defined as AHQ-T versus all other population groups. Together, these data suggest that there is only weak genetic structure among most Yellowstone *Mimulus* populations (including the phenotypically differentiated RC-T and RC-NT populations), but strong isolation of AHQ-T from both the other thermal population and the nearby non-thermal population.

In STRUCTURE, there was a strong increase in the likelihood of $k = 2$ clusters (mean $\ln L = -1422$) relative to $k = 1$ (mean $\ln L = -1743$) and little improvement with increasing numbers of clusters, consistent with the pairwise F_{ST} and AMOVA results. At $k = 2$, most individuals were completely assigned to either AHQ-T or to the cluster of all other populations (Fig. 5). However, a substantial number of AHQ-T samples, as well as a few individuals from RC-T and AHQ-NT, had partial membership in both clusters, potentially due to ongoing gene flow.

Discussion

Potential for local adaptation of thermal populations

The thermal vent areas supporting *Mimulus* populations at AHQ-T and RC-T are harsh environments; during the flowering season, temperatures were sometimes as high as

50 °C in the root zone of the shallow soil layer (Fig. S1). Extreme heat, combined with low summer water availability (Fig. S1b), clearly limits the plant community at these sites (e.g., Stout and Al-Niemi 2002) and may also exert strong selection. However, the geothermal influence creates a unique opportunity for *M. guttatus*, which requires saturated soil for growth and flowering. At the extreme thermal *Mimulus* patches sampled in this study, there is no source of surface soil moisture other than precipitation. Instead, plants grow and reproduce during winter and spring, when hot soils and heavy snow interact to create a narrow layer of warm air over wet, but snow-free, soil.

The shared shifts in growth form, phenology, and mating system we see in the two thermal *Mimulus* populations, relative to nearby non-thermals, are precisely those we would predict from their restricted and ephemeral environment. Thermal *M. guttatus* plants in the field are extraordinarily short, with leaves and flowers appressed to the ground (Fig. S1a). This “thermal” growth form is partly plastic, as both T and NT plants were smaller under simulated spring thermal conditions (Fig. 3). However, it also has a strong genetic component, with T plants setting their first flower at about half the height of non-thermals under all conditions (Fig. 3b). Plants in thermal sites must produce flowers very close to the soil surface to avoid freezing ambient air temperatures (Figs. 1, 2), and must also ripen seeds prior to the drought and high soil temperatures of summer. Plants from both thermal populations also make more flowers (Fig. 3c), and thus allocate a higher proportion of their biomass to flowers despite having lower overall biomass (Fig. 3b). This difference, which persisted even under 16-h days in the simulated summer non-thermal conditions, is consistent with an evolved shift from perennial to annual life history. However, further work, including reciprocal transplants in the field, will be necessary to fully disentangle the genetic and plastic components of growth form differences and their fitness consequences in each habitat.

Flowering under short day lengths enables early spring reproduction by thermal plants. None of the NT accessions from YNP produced flowers under a short-day (<12-h) light regime, whereas substantial fractions of the AHQ-T and RC-T samples did (25 and 40 %, respectively). This difference is consistent with the observation that flowering at both thermal sites begins as early as February and peaks by late May, whereas flowering at NT areas does not begin until late June or July. The environmentally imposed annual life-history of plants at T sites places a very high premium on spring reproduction, and may therefore select for the loss of the photoperiod requirement seen in non-thermal plants. Conversely, non-thermal genotypes migrating into thermal sites would have a much shorter

window for flowering by waiting to initiate buds until at least late March (when daylengths naturally exceed 12 h).

Thermal plants are far more efficient at self-fertilization than non-thermals (100 vs. 30 % autonomous seed set; Fig. 4c). This shift is plausibly adaptive, as other flying insects are not available as pollinators during most of the flowering season at thermal sites (M. Hendrick, personal observation). Thermal plants achieve such high rates of self-fertilization with relatively low stigma–anther distances and corolla width–length ratios (Fig. 4), but these differences are subtle compared to the extreme floral reduction of obligate selfers in the complex such as *Mimulus nasutus*, *M. micranthus*, and *M. laciniatus* (Fenster and Ritland 1994). Interestingly, the two floral traits that are most divergent between thermal and non-thermal YNP plants also exhibited the strongest negative selection gradients in a pollinator exclusion experiment with an outcrossing annual population of *M. guttatus* (Fishman and Willis 2008). This suggests that a narrow corolla tube, as well as the more obvious lack of stigma–anther separation, may be functionally associated with increased self-pollination ability and among the first traits to respond to selection imposed by pollinator loss.

Finally, one of the thermal populations (AHQ-T) differed strikingly from all others in the incidence of glandular trichomes (pubescence) on the upper leaf surfaces of young plants, especially under simulated spring thermal conditions. Trichomes are important as a constitutive and induced defense against herbivory (Hanley et al. 2007), as well as drought, UV radiation, and other forms of abiotic stress in plants (e.g., Roy et al. 1999). In *M. guttatus*, the density of glandular trichomes varies genetically among populations (Holeski et al. 2010), and also increases plastically in response to simulated herbivory (Holeski 2007). Pubescence may simply have drifted to high frequency at AHQT, as the trait is not shared between the two thermal populations and low genetic diversity at AHQ-T is consistent with a history of genetic drift. However, the well-documented costs (e.g., Sletvold et al. 2010) and benefits (e.g., Roy et al. 1999) of trichome production in other polymorphic species suggest that it may be an adaptation for snow, wind, or UV protection of growing rosettes overwintering at this relatively exposed site.

The life-history differences between non-thermal and thermal populations in YNP are parallel to other perennial-to-annual transitions in *Mimulus*. Elsewhere in *M. guttatus*, widespread perennial and annual ecotypes are defined by the presence/absence of an inverted region of LG8 co-mapping with a major QTL affecting multiple floral, vegetative, and phenological traits (DIV1; Lowry et al. 2009; Lowry and Willis 2010). Perennials, which occur along the Pacific coast and at permanently moist sites inland, are

generally characterized by slower growth rates, delayed flowering, and relatively high allocation to vegetative reproduction. The AHQ-NT population is known to carry the perennial version of the DIV1 inversion (Lowry and Willis 2010). Additional crosses will be necessary to determine whether thermal populations carry the “annual” DIV1 inversion type, indicating long-distance migration of annuals from outside the park, or the “perennial” type, indicating a local, independent, derivation of annuality.

Life-history differences are also seen between thermal and non-thermal taxa of *Agrostis* that occur at our geothermally influenced sites. The thermal endemic *Agrostis rossiae* and a thermal ecotype of *A. scabra* are both relatively (and genetically) short-statured, annual, and early flowering compared to non-thermal *A. scabra* and other *Agrostis*. Tercek and Whitbeck (2004) argue that the life-history differences between thermal and non-thermal *Agrostis* represent an adaptive strategy of stress avoidance, and thermal *Mimulus* exhibit a similar strategy. We caution, however, that the apparent stress-avoiding life-history of thermal *M. guttatus* in no way rules out additional adaptation in the form of physiological thermotolerance. Soils in the rooting region of flowering thermal populations regularly exceeded temperatures considered to be heat shock in studies of the model plant *Arabidopsis* (Queitsch et al. 2000). An early study (Delmer 1974) reported no evidence of thermal adaptation in leaf protein biochemistry or growth performance in tissue culture of *M. guttatus* collected along hot creeks in Yellowstone (not dry thermal vent areas, as in this study). However, as evidenced by its evolution of heavy metal tolerance at mine-waste sites (Macnair et al. 1993), *M. guttatus* is clearly capable of physiological adaptation to novel extreme conditions. Additional experiments will be necessary to determine whether thermal and non-thermal populations are differentiated in their biochemistry and physiology under extreme soil conditions.

Finally, these phenotypic data guide future field experiments investigating fitness trade-offs in reciprocal environments. The common gardens under simulated conditions suggest that thermal genotypes have evolved trait differences (reduced height to flowering, loss of day-length sensitivity, increased allocation to flowering, increased self-pollination ability) that uniquely suit them to conditions at thermal sites, and non-thermal genotypes lacking these traits may be expected to experience fitness costs in thermal areas. In contrast, thermal plants outperformed non-thermals (in terms of flower number) in both simulated thermal and non-thermal environments, potentially suggesting little trade-off associated with any adaptation. However, their early allocation to sexual reproduction may reduce survival and lifetime reproduction in sites where perenniality is possible, and short internodes may render them competitively inferior to

non-thermal *M. guttatus* in densely vegetated non-thermal field sites.

Patterns of population genetic structure

Despite shared phenotypic divergence, the two thermal *M. guttatus* populations did not cluster genetically by soil temperature category (T vs. NT). This is in contrast to the co-occurring grass *Agrostis*, in which genetic analyses (as well as morphology) suggest that thermally adapted *A. rossiae* and *A. scabra* ecotypes represent a single taxon, which also contains thermally restricted species from Lassen and Kamchatka and excludes all non-thermal species (Tercek et al. 2003). However, *Mimulus* populations also did not cluster by geography, as might be expected if thermal populations were independently derived within Yellowstone and exchanging genes primarily with nearby non-thermal populations. Instead, individuals clustered into two groups corresponding to AHQ-T versus all others in all genetic analyses (Fig. 5). In addition, inspection of individual genotypes indicates that AHQ-T was generally near fixation for alleles common or at intermediate frequency in the other Yellowstone populations, and no alleles were private to AHQ-T.

Several factors likely contribute to the apparent genetic isolation of AHQ-T. First, the AHQ area has a relatively rugged topography and thus there is little intermediate habitat between thermal and non-thermal extremes, which are essentially non-overlapping in phenology. In contrast, a year-round hot stream (Rabbit Creek) links RC-T vent areas with RC-NT and other non-thermal sites near the Firehole River. Abundant *M. guttatus* (not sampled) found in this intermediate habitat flower in both spring and summer and may provide a bridge to pollen flow between extremes.

Second, AHQ-T is less genetically diverse than the other populations. Values of both H_e and H_o were about 50 % those of other populations, despite a relatively large census size (several orders of magnitude larger than AHQ-NT) and population sample. This low diversity directly contributes to high F_{ST} in pairwise comparisons involving AHQ-T (Charlesworth et al. 1997) and likely reflects a history of inbreeding as well as reproductive isolation from other populations. A population-level inbreeding coefficient (F) < 0.30 for AHQ-T suggests predominant outcrossing (Table S1), but the low values for both H_e and H_o in this population make this estimate uninformative about individual outcrossing rates. Given the strong contrast with the surrounding montane forest habitat (which can maintain deep snow cover until mid-June; L. Fishman, personal observation), the spring flowering plants at AHQ-T are likely to have few opportunities for bee visitation and to predominantly self-fertilize in all but the most benign years. In comparison, the less extreme environmental

contrast between thermal areas at RC-T and the surrounding landscape may promote relatively high outcrossing rates despite evidence of floral adaptation for self-pollination. Distinguishing these demographic alternatives, and placing shared phenotypic divergence of individual thermal populations in the context of their distinct evolutionary histories, will require explicit landscape genetics approaches including sampling of additional extreme and intermediate populations.

Conclusions

Mimulus guttatus populations from thermal areas in YNP are similarly and strikingly divergent from non-thermal populations nearby, consistent with local adaptation to life in the ephemeral window for growth and reproduction created by snowmelt on hot soils. These findings, in concert with only weak genetic differentiation between thermals and non-thermals at one site, set the stage for whole genome association mapping of loci underlying divergent traits and genome scans to identify “outlier” loci potentially under divergent selection (Sjol et al. 2010). Finally, substantial trait differentiation of both thermal *M. guttatus* populations, despite a lack of taxonomic distinctness, argues for particular attention to conservation of the genetic and biological diversity at these sites.

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