



# Extreme copy number variation at a tRNA ligase gene affecting phenology and fitness in yellow monkeyflowers

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## Abstract

Copy number variation (CNV) is a major part of the genetic diversity segregating within populations, but remains poorly understood relative to single nucleotide variation. Here, we report on a tRNA ligase gene (Migut.NO2091; RLG1a) exhibiting unprecedented, and fitness-relevant, CNV within an annual population of the yellow monkeyflower *Mimulus guttatus*. RLG1a variation was associated with multiple traits in pooled population sequencing (PoolSeq) scans of phenotypic and phenological cohorts. Resequencing of inbred lines revealed intermediate-frequency three-copy variants of RLG1a (*trip+*; 5/35 = 14%), and *trip+* lines exhibited elevated RLG1a expression under multiple conditions. *trip+* carriers, in addition to being over-represented in late-flowering and large-flowered PoolSeq populations, flowered later under stressful conditions in a greenhouse experiment ( $p < 0.05$ ). In wild population samples, we discovered an additional rare RLG1a variant (*high+*) that carries 250–300 copies of RLG1a totalling ~5.7 Mb (20–40% of a chromosome). In the progeny of a *high+* carrier, Mendelian segregation of diagnostic alleles and qPCR-based copy counts indicate that *high+* is a single tandem array unlinked to the single-copy RLG1a locus. In the wild, *high+* carriers had highest fitness in two particularly dry and/or hot years (2015 and 2017; both  $p < 0.01$ ), while single-copy individuals were twice as fecund as either CNV type in a lush year (2016:  $p < 0.005$ ). Our results demonstrate fluctuating selection on CNVs affecting phenological traits in a wild population, suggest that plant tRNA ligases mediate stress-responsive life-history traits, and introduce a novel system for investigating the molecular mechanisms of gene amplification.

## KEYWORDS

balancing selection, flowering time, fluctuating selection, life history, standing variation, structural variant

## 1 | INTRODUCTION

The standing genetic variation for fitness-related traits is a key determinant of a population's evolutionary potential, providing the raw material for adaptive evolution when environments change

(Anderson, 2016; Barrett & Schluter, 2008; Hoffmann & Sgrò, 2011). However, the abundant genetically based fitness polymorphism seen within many populations is also paradoxical. At any given locus, efficient directional or stabilizing selection should winnow out all but the variant with highest geometric mean fitness. Balancing

selection, in which context-dependent effects of alleles on fitness maintain polymorphism, resolves this problem. Long-term protected polymorphism is robustly predicted under models of heterozygote advantage (overdominance) (Gillespie, 1984) and frequency-dependent selection (Clarke, 1979; Wright, 1939), as well sexual antagonism (Connallon & Clark, 2014) and gamete–zygote conflict (Fishman & Kelly, 2015; Immler, Arnqvist, & Otto, 2011). Empirical examples of such protected polymorphism, often involving multi-gene structural variants with suppressed recombination in heterozygotes, are increasingly common (Barson et al., 2015; Fishman & Kelly, 2015; Joron et al., 2011; Llaurens, Whibley, & Joron, 2017). However, these intrinsic mechanisms of balancing selection likely apply to only a small subset of traits and genes. Spatial and (especially) temporal shifts in selection are more controversial as sources of intra-population polymorphism, but their ubiquity creates abundant opportunities to contribute to the maintenance of functional genetic variation within natural populations (reviewed in Bell, 2010; Delph & Kelly, 2013). Furthermore, new models that incorporate biologically realistic factors that buffer against allele loss, such as switches in dominance (Posavi, Gelembiuk, Larget, & Lee, 2014; Wittmann, Bergland, Feldman, Schmidt, & Petrov, 2017), plasticity (Gulisija, Kim, & Plotkin, 2016) and the storage effect of unselected life stages (Ellner & Hairston, 1994; Svoldal, Rueffler, & Hermisson, 2015), suggest that environmental variation can maintain a large pool of polymorphism. However, although a few recent studies demonstrate fluctuating selection on intermediate-frequency genetic variants (Chakraborty & Fry, 2016; Kerwin et al., 2015; Lee, Fishman, Kelly, & Willis, 2016; Troth, Puzey, Kim, Willis, & Kelly, 2018; Wittmann et al., 2017), more work is needed to reveal whether and how environmental fluctuations contribute to functional polymorphism within natural populations.

Genic copy number variants (CNVs), including duplication and deletion of entire genes (Hastings, Lupski, Rosenberg, & Ira, 2009), are likely to be functionally important components of standing variation across diverse taxa (Chain et al., 2014; Marroni, Pinosio, & Morgante, 2014; Prunier et al., 2017; Salojärvi et al., 2017; Zarrei, MacDonald, Merico, & Scherer, 2015; Żmieńko, Samelak, Kozłowski, & Figlerowicz, 2014). Changes in copy number are a common form of mutation—in plants, segmental or tandem duplication events occur at a per-gene rate at least as high as the per-site nucleotide mutation rate (Lynch & Conery, 2000). Much of this variation appears to persist in populations; for example, diverse maize lines differ in copy number at nearly 35% of genes (Chia et al., 2012) and >15% of *Arabidopsis* genes are stabilized tandem duplicates (reviewed in Żmieńko et al., 2014). Initially, whole-gene duplications or deletions may directly alter expression (Stranger et al., 2007), though various forms of dosage compensation can buffer these immediate effects (Veitia, Bottani, & Birchler, 2013). Duplicates can develop novel or subdivided functions over time (Lynch & Conery, 2000), but they may also influence phenotypic variation within and among populations as CNVs on shorter timescales. In plants, CNVs contribute to rapid evolution under experimental thermal stress (DeBolt, 2010), underlie the contemporary evolution of glyphosate resistance in

weeds (Gaines et al., 2010), and have been implicated in phenological and edaphic (soil stress) adaptation across populations (Gordon et al., 2017; Hanikenne et al., 2013; Maron et al., 2013; Rosloski, Jali, Balasubramanian, Weigel, & Grbic, 2010). Over the longer term, CNV caused by reciprocal deletion of non-tandem duplicates is a common source of epistatic Dobzhansky–Muller incompatibilities (Bomblies et al., 2007; reviewed in Fishman & Sweigart, 2018). However, because CNVs are difficult to assay with reference-based short-read resequencing approaches (Hunter, Wright, & Bomblies, 2013; Tiffin & Ross-Ibarra, 2014), we are only beginning to understand their contribution to within-population variation, local adaptation and speciation.

Here, we identify and characterize copy number variants of a gene contributing to the abundant phenological and fitness variation within the Iron Mountain (IM) population (Central Cascades, OR, USA) of yellow monkeyflower (*Mimulus guttatus*, Phrymaceae). This high-elevation annual population exhibits extremely high levels of nucleotide diversity ( $\pi_{\text{synonymous}} = 0.033$ ; Puzey, Willis, & Kelly, 2017) and quantitative genetic variation (Bodbyl Roels & Kelly, 2011; Kelly & Willis, 2001; Scoville, Lee, Willis, & Kelly, 2009, 2011). The abundant variation in fitness-relevant traits (e.g., pollen viability, flower size and seeds/fruit) is largely due to intermediate-frequency polymorphism, as expected under local balancing selection rather than mutation–selection or migration–selection balance against unconditionally deleterious variants (Kelly, Koseva, & Mojica, 2013; Kelly & Willis, 2001). Spatial and temporal environmental variations are plausible sources of balancing selection at IM, where the depth of winter snowpack and (rare) rain events set the length and lushness of the summer growing season (Mojica, Lee, Willis, & Kelly, 2012; Troth et al., 2018). Furthermore, field measures of fitness in QTL introgression lines (Mojica & Kelly, 2010; Mojica et al., 2012) and a genomewide association mapping panel (Troth et al., 2018) demonstrate fluctuating selection on IM variants that affect flower size via developmental rate. In wetter years, large-flowered, relatively–slowly developing genotypes have high fecundity, whereas in dry years, they have a lower probability of living to flower (Mojica & Kelly, 2010; Mojica et al., 2012; Troth et al., 2018). Such antagonistic pleiotropy across environments is key to the models for the selective maintenance of standing variation by environmental fluctuations (Brown & Kelly, 2018; Wittmann et al., 2017), but the genes that underlie such trade-offs are almost completely unknown.

In this study, we focus on one gene contributing to the abundant variation in life-history phenotypes within Iron Mountain *M. guttatus*, and dissect its structural variation, phenotypic associations and fitness effects in the wild. Previously, a tRNA ligase (Migut.N02091, RLG1a) was identified as a significant genomewide single nucleotide polymorphism (SNP) outlier in PoolSeq scans for differentiation between early- and late-flowering cohorts at both IM and the nearby Quarry population (Monnahan & Kelly, 2017). This gene is one of two (unlinked) *Mimulus* homologs of *Arabidopsis* RLG1 (Englert & Beier, 2005), a multi-functional ligase recently implicated in regulation of both the growth hormone auxin and the stress-induced unfolded

protein response (Leitner et al., 2015; Nagashima, Iwata, Mishiba, & Koizumi, 2016). Here, CNV-focused analyses of that IM flowering time data set, a previously published flower size selection data set (Kelly et al., 2013), and four new PoolSeq populations with contrasting flowering cues and germination times in the field, reveal that RLG1a coverage varies strongly across phenotypic cohorts. By scanning for coverage variants in a whole-genome resequenced inbred line set (Puzey et al., 2017), we identify an intermediate-frequency (5/35 lines) CNV with two additional copies of RLG1a (*trip+*). We develop a diagnostic marker and use individual genotyping to confirm that *trip+* increases in frequency under artificial selection for large flower size and is over-represented in late-flowering plants in 2014 (a dry year). We then use RT-qPCR to test whether *trip+* plants exhibited constitutively elevated expression of RLG1a, consistent with a dosage effect (e.g., Gaines et al., 2010), and a conduct a common-garden growout to test for environment-specific life-history effects. We find that *trip+* carriers exhibit elevated RLG1a expression and delay flowering under experimentally water-stressed conditions relative to single-copy (*solo*) individuals, indicating that this RLG1a CNV is a stress-responsive contributor to the fast-slow axis of life-history/floral variation at IM.

However, because shifts in the frequency of a 3-copy CNV could not explain the >10x coverage differences between some PoolSeq population pairs, we hypothesized the existence of rare higher copy haplotypes shifting in frequency among wild-derived cohorts but absent from the inbred lines. In large wild-derived population samples, we identify two individuals carrying sequence variants common in the high-coverage PoolSeq cohorts; whole-genome resequencing of these plants (*high+* carriers) reveals that they carry ~300 ampliconic copies of RLG1. Individual genotyping confirms that *high+* alleles significantly decrease in frequency between early- and late-flowering cohorts in 2014 and thus account for much of the RLG1a coverage variation among pools. We then use genotyping and qPCR on the selfed progeny of one *high+* carrier to test whether *high+* "alleles" segregate as expected for a single tandemly duplicated chromosomal array (vs. the alternative hypotheses of transfer to an organelle, transmission as extra-chromosomal DNA, or dispersion across multiple chromosomes). We find that *high+* behaves as a presence-absence polymorphism at a single locus unlinked from the single-copy version of RLG1a. Finally, we examine the frequency and fitness effects of RLG1a CNV genotypic categories (*solo*, *trip+*, *high+*) over three years spanning extremes of fecundity and survival in the Iron Mountain population. We document significant (but variable) associations between CNV genotype and fitness in each year, with *high+* strongly favoured in two low survival and/or fecundity years, and both *high+* and *trip+* disfavoured relative to *solo* in an intervening lush year. As predicted from relative fitnesses in the preceding year, the frequency of each CNV fluctuates across the three seasons. Together, these analyses suggest that a series of copy number variants of a *Mimulus guttatus* tRNA ligase influence life-history traits that experience fluctuating selection across years and thus are likely maintained as polymorphisms by this form of balancing selection.

## 2 | METHODS

### 2.1 | Study system

The *Mimulus guttatus* (also known as *Erythranthe guttata*; Phrymaceae) species complex exhibits tremendous diversity in life history, mating system and other traits across Western North America. *Mimulus guttatus*, the most widespread and diverse species (and the likely progenitor of other complex members), is self-compatible but largely outcrossing (Sweigart, Karoly, Jones, & Willis, 1999). We focus here on the well-studied Iron Mountain (IM, Oregon) high-elevation annual population, from which the inbred line (IM62) used to construct the *Mimulus guttatus* v2.0 reference genome (www.Phytozome.jgi.doe.gov) was derived. Ecologically, the life cycle of the IM population is driven by variable yearly patterns of snow accumulation and melt-out. Germination occurs in both fall (November, with plants overwintering under snow as small rosettes) and in spring after melt-out (May–June, generally). The maximum summer growing season varies from five to twelve weeks depending on snowpack and other climate variables. This year-to-year variation in the length, timing and lushness of the growing season creates high temporal variance in mean survival and fecundity (Fishman & Kelly, 2015; Lee et al., 2016; Mojica et al., 2012; Monnahan, Colicchio, & Kelly, 2015; Troth et al., 2018).

### 2.2 | PoolSeq of phenotypic populations

We re-analysed PoolSeq data sets from two previous experiments, which examined allele frequency differences among bulk-sequenced phenotypic cohorts, as well as conducting two new PoolSeq experiments (Table 1).

#### 2.2.1 | Existing data sets

The High-Low-Ancestral set (Kelly et al., 2013) includes large- and small-flowered populations derived by nine generations of artificial selection with outbreeding (High and Low selection lines; pool  $N = 49$  and 78, respectively), starting from a large outbred IM-derived base population (Ancestral, pool  $N = 75$ ). A Control population maintained under the same conditions, but without selection, was also generated (Kelly et al., 2013); it was not sequenced, but we use it here for individual genotyping tests. The Early-Late flowering time set consisted of field-collected cohorts of early- and late-flowering plants from IM and nearby Quarry populations in both 2013 and 2014 (Monnahan & Kelly, 2017). Because the Quarry population is the product of recent admixture between IM-like annuals and perennial genotypes (Monnahan et al., 2015), we restrict our analyses here to the IM data sets.

#### 2.2.2 | Fall-Spring germination time study

In Fall 2012 and Spring 2013, we collected fall-germinating and spring-germinating seedlings from nine quadrats spaced across the

Data set	Components	Analyses	Source
Flower size selection expt	High vs. Low vs. Ancestral	PoolSeq, marker genotyping	Kelly et al. (2013)
Flowering time cohorts	IM13_Early vs. IM13_Late	PoolSeq	Monnahan and Kelly (2017)
	IM14_Early vs. IM14_Late	PoolSeq, marker genotyping	
Germination time cohorts	Fall vs. Spring (2012/2013)	PoolSeq	This study
Vernalization requirement	Bolt vs. Rosette	PoolSeq	This study
IM inbred lines (N = 35)	IM62, IM767, IM664, etc.	Whole-genome resequencing, <i>trip+</i> RT-qPCR (N = 11), marker design	Puzey et al. (2017) Lee et al. (2016)
Greenhouse populations	IM13P, IM15P (from outbred wild seeds)	Flowering time growout, screens for <i>high+</i> carriers	This study
Individual <i>high+</i> carriers	IM13P_627c, IM13P_644, IM15P_317	Whole-genome resequencing, <i>high+</i> segregation & qPCR	This study
Wild populations	IM2015, IM2016, IM2017	Fitness and frequency in field	This study

**TABLE 1** Plant materials (all *Mimulus guttatus* from Iron Mountain, Oregon, population) used in this study

IM site (N = 40 samples per quadrat collected 20/tube, except for one Spring quadrant with only 10 seedlings; total N = 320 and 290, respectively). After DNA extraction, tubes were combined proportionately (equimolar concentrations by sample size) into Fall and Spring pools for library preparation.

### 2.2.3 | Bolt–Rosette vernalization requirement study

In Spring 2012, we grew >600 independent (1 per maternal family) plants derived from 2011 field-collected seeds in University of Montana greenhouse under non-inductive daylengths (12-hr days) for 2 months and then transitioned to 16-hr days. These conditions induce flowering in IM *M. guttatus* that do not require vernalization (a cold treatment simulating winter) and inhibit it in those that do (Fishman, Sweigart, Kenney, & Campbell, 2014; Friedman & Willis, 2013). About 60% of plants had flowered after 60 days at 16-hr daylengths. Individuals were then assigned to Bolt (flowered without vernalization) and Rosette (vernalization-requiring, non-flowering) cohorts, and leaf and/or bud tissue collected in sets of 20 plants per extraction tube. After DNA extraction using a standard *Mimulus* protocol (Fishman & Kelly, 2015), 10 and 15 tubes per population were pooled in equimolar amounts (N = 300 and 200 plants per Bolt and Rosette pools, respectively).

### 2.3 | PoolSeq and inbred line resequencing and analysis

The High-Low-Ancestral flower size and Early-Late flowering time cohorts were sequenced as described previously (Kelly et al., 2013;

Monnahan & Kelly, 2017). The Fall-Spring and Bolt–Rosette population pairs were each sequenced with Illumina HiSeq 2500 (PE 100) at the University of Kansas Genomics Core, following the same library preparation protocol as previous pools (Monnahan & Kelly, 2017). All eleven PoolSeq populations were processed with the same bioinformatics pipeline (see Table 1 and Supporting Information for details).

For analyses of coverage differences among pooled populations, we restricted our analyses to sites in exons, as extremely high sequence diversity within *M. guttatus* hampers read-alignment outside of genes (Puzey et al., 2017). We calculated mean read depth at each annotated exon with Mosdepth (Pedersen & Quinlan, 2018) and then standardized exon read-depth values by the genomewide median for a given pool or sequenced individual. After excluding a subset of annotated genes that appeared to be chloroplast–nuclear transfers (or mis-assembled), and a set that appeared to be mis-annotated repetitive DNA (see supplemental methods in Supporting Information), we retained 25,019 genes with coverage data from one or more exons. To compare read depths among pools genomewide, we calculated the absolute value of the difference in standardized read depth between each pair of populations (e.g., High vs. Ancestral, Low vs. Ancestral, Bolt vs. Rosette, Spring vs. Fall, IM13\_Early vs. IM13\_Late, IM14\_Early vs. IM14\_Late). For comparisons of mean RLG1a coverage among pools, we restricted estimates to the final 23 exons (spanning ~9 kilobases) of Migut.N02091, as the first three exons showed high variance in coverage depending on whether or not unpaired reads were filtered out and we cannot definitively distinguish additional duplication of this portion of the gene from mis-mapping.

Using a similar bioinformatics pipeline, we examined Migut.N02091 exon coverage in previously resequenced IM inbred lines (1 from Lee et al., 2016; 34 from Puzey et al., 2017) (Table 1). To

identify sequence variation among genomic copies in carriers of RLG1a CNV, we also performed metagenome de novo assembly on sequence reads mapping to Migut.N02091 in one inbred line with elevated coverage (IM664). After converting the SAM alignments for extracted reads to fastq format, we used the metagenome assembler MEGAHIT (Li, Liu, Luo, Sadakane, & Lam, 2015) with default settings. Assembled contigs were mapped back to the *Mimulus guttatus* v2.0 reference genome with minimap2 (Li, 2018) to identify haplotypes diagnostic of duplicated copies of the gene.

## 2.4 | RLG1a genetic marker design and genotyping

For analyses of individual genotype, we designed and amplified an exon-primed length-polymorphic marker (mN2091x16) spanning a 5-amino acid (5AA; 15 base) insertion-deletion polymorphism in Exon 16 of Migut.N02091, as well as the highly variable Exon 16–17 intron (Supporting Information Table S1). In inbred lines ( $N = 14$  tested) and wild-derived plants, we found multiple (diploid) amplicons corresponding to single-copy sequences (RLG1a\_ solo), plus two amplicons carrying the Exon 16 5AA deletion. A 426-bp fragment was exclusive to IM lines with ~3x coverage of Migut.N02091 (*trip+*) and a rare 428-bp amplicon was uniquely associated with very high-coverage individuals (*high+*) found in wild collections (IM13P, IM15P; Table 1). Both 5AA-deletion variants at mN2091x16 were scored as presence/absence polymorphisms, as the *high+* 428-bp allele was always “homozygous” due to out-competition of all other alleles in PCRs and the *trip+* 426-bp allele was always found with 2–3 additional amplicons also found in *solo* lines (see Results).

## 2.5 | Characterization of RLG1a *trip+*: vernalization, RLG1a expression and flowering time under stress

To test whether the patterns observed the PoolSeq results from shifts in RLG1a CNV frequencies, we genotyped individual plants from the flower size selection (High, Low, and Control) populations and from IM14 (Early and Late flowering) populations at mN2091x16 marker. We also performed two separate experiments to assess phenological phenotypes. We grew *solo* and *trip+* inbred lines (inferred from marker genotyping) under the same greenhouse regime as in the Bolt-Rosette PoolSeq experiment in Spring 2015 and sampled rosette leaf tissue one day prior to and six days after the transition from 12-hr daylength to 16-hr daylengths ( $n = 9$  *solo* lines and 2 *trip+* lines, respectively; 2–4 separate individuals per treatment per line; total  $N = 56$ ). Both *trip+* lines (IM664 and IM115) flowered within one month under this regime, consistent with a lack of vernalization requirement, as did three of the 10 *solo* lines. We extracted RNA, made cDNA and measured expression of RLG1a (mN2091q primers in Exon 26) relative to two previously developed controls (EF1a and UBQ5) (see Supporting Information for details). Differences in expression were determined from threshold cycle number (Ct) relative to the control ( $\Delta Ct = Ct_{mN2091} - Ct_{EF1a}$ ; averaged over 2 technical replicates each) and analysed using ANOVA with daylength (12 hr,

16 hr), RLG1a CNV category (*solo*, *trip+*), and line (nested within CNV) as main effects and the daylength  $\times$  CNV interaction effect.

As part of a separate study on differentiation in plant–soil–microbe interactions, we grew IM outbred plants (from 2013 wild seed) in the University of Montana greenhouse in Summer 2017 in two soil treatments: soil from the Oregon Dunes perennial *M. guttatus* site (DUN) (Hall & Willis, 2006) and from IM, each cut 50:50 with sand. Plants were top-watered daily and fertilized weekly with a low-phosphorus fertilizer. Both treatments mimicked field conditions and produced rapid-flowering phenotypes resembling wild IM plants. However, we infer the DUN soil to be more stressful because it produced a ~2-fold reduction in mean biomass and fruit number at harvest vs. the IM soil (data not shown). We recorded the number of days to first flower, extracted DNA from IM plants and genotyped individuals at the mN2091x16 marker. Flowering time was analysed with ANOVA with soil type and genotype (*trip+/solo*; three *high+* carriers were excluded from this analysis) and their interaction.

## 2.6 | Characterization of RLG1a *high+* variants: resequencing and qPCR

To characterize rare high-copy (*high+*) variants of RLG1a, we grew and PCR-genotyped plants from 2013 and 2015 seed collections (IM13P, IM15P;  $N = 120$  maternal families total, 1–4 progeny each). One *high+* individual from each year (IM13P\_627c and IM15P\_317d, respectively) was chosen for whole-genome sequencing. Library preparation, sequencing (HiSeq 4000, PE125) and alignment to the *M. guttatus* v2.0 reference are described in Supporting Information. Median-standardized exon coverage for Migut.N02091 was calculated as with the PoolSeq and line data sets.

In addition, we counted RLG1a genomic copy number by qPCR in two wild-collected (as seed) *high+* plants, IM13P\_644 and IM13P\_627c, as well as *trip+* and *solo* lines. As single-copy control, we used primers within Exon 12 of Migut.G00571 (isopropylmalate isomerase large subunit; mG571q in Supporting Information Table S1). RLG1a  $\Delta Ct$  values ( $Ct_{[mN2091]} - Ct_{[mG571]}$ ) were calculated for each sample, averaged cross two technical replicates, and copy number estimated using the equation:  $2^{(\Delta Ct_{solo} - \Delta Ct_{high+})}$ . To corroborate these estimates in IM13P\_644 (which had highest copy number), we created a standard curve by performing threefold serial dilutions of template DNA and measuring Ct for each dilution. We then used the linear fit of the standard curve to estimate mN2091q copy number relative to mG571q in the same plant.

## 2.7 | Characterization of RLG1a *high+* variants: genetic segregation

To segregate the high-copy allele, we selfed IM13P\_627c and grew the resultant progeny (which segregate as a pseudo- $F_2$  at loci heterozygous in the parent plant) in the University of Montana greenhouse in Spring 2018. All progeny ( $N = 192$ ) were genotyped with mN2091x16 to score *high+* presence/absence. For tests of location, we also genotyped a linkage-informative (heterozygous



in IM13P\_627c) marker <100 kb from Migut.N02091 (mN2089; Supporting Information Table S1) on a subset. To confirm segregation of the *high+* cluster as a single locus, we conducted genomic qPCR (as above) on a subset of progeny ( $N = 39$ ). If all copies are in tandem, *high+* carriers should fall into two distinct categories with a ~2-fold difference in copy number. Individual  $\Delta C_t$  values (calculated as above) were clustered using Normal Mixture methods, the cluster number with the lowest AIC/BIC value chosen and cluster means compared with ANOVA. Finally, to assess whether expression was elevated in high-copy carriers, we also conducted RT-qPCR on mRNA extracted from floral shoot tissue of a small set of progeny (segregating for 0, 1 or 2 *high+* alleles based on the genomic DNA qPCR), using EF1a as a control as in the *trip+* analyses. Additional methods in Supporting Information.

## 2.8 | Frequency and fitness in the wild

To evaluate RLG1a CNVs in the field, we genotyped and measured fitness of wild IM plants in 2015–2017. In 2015, we marked spring (cotyledons) and fall (rosette leaves) germinants in May, but almost all of the spring germinants died as seedlings and were lost before DNA could be collected. We marked a second set of flowering plants on two dates a week apart in mid-June; most of these died as well, but we obtained DNA from many non-survivors. Finally, we collected tissue (whole plant except for seeds) for DNA extraction and all seeds from survivors among the marked germinants and flowerers, as well as additional random survivors as in previous studies (Fishman & Kelly, 2015; Lee et al., 2016). Thus, we can estimate both survival from flowering to fruit and seedset among survivors, from which we can calculate lifetime fitness conditioned on surviving long enough to be collected. In 2016 and 2017, we obtained DNA and fruit/seed counts of plants that survived to fruit, as in previous studies. Individuals were genotyped at mN2091x16, and coded as *trip+*, *high+* or *solo*. We analysed log-transformed lifetime female fitness (survival  $\times$  seedset +1) from 2015 alone using standard least-squares

ANOVA. We analysed seedset and fruitset from 2016 and 2017 together, with GLM analyses (Poisson, log link) including year, RLG1a genotype and their interaction.

All statistical analyses of qPCR, genotypic and phenotypic data were performed in JMP 13 (SAS Institute, 1994).

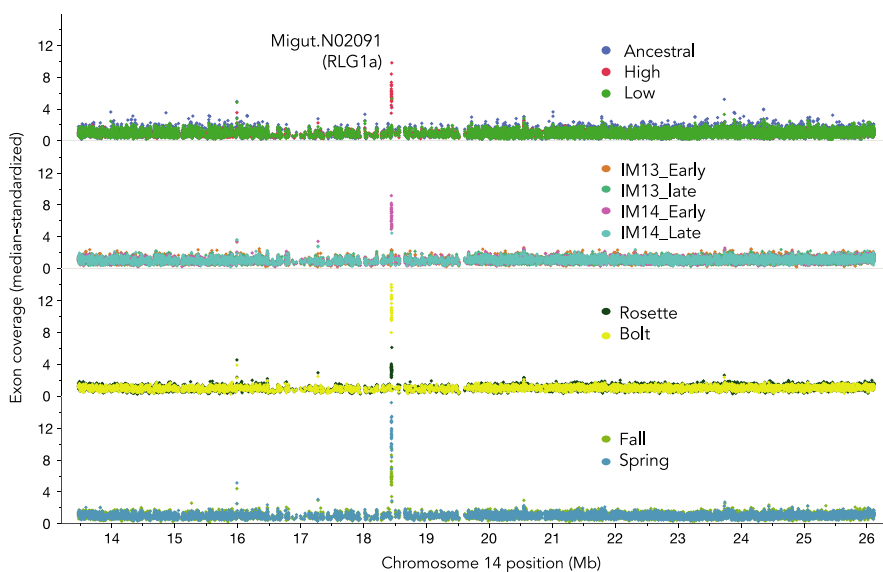
## 3 | RESULTS

### 3.1 | PoolSeq reveals RLG1a coverage variation associated with multiple life-history traits

A tRNA ligase gene, Migut.N02091 (RLG1a), was a strong read-depth outlier in four of the six PoolSeq comparisons (Figure 1), including the IM14\_Early vs. IM14\_Late comparison in which it was previously identified as a SNP outlier (Monnahan & Kelly, 2017). The 11 PoolSeq populations varied widely in genome-standardized coverage across Migut.N02091 (Exons 6–28), ranging from near-background in the Ancestral (mean = 1.18) to >10x in the Bolt and Spring germinant pools (means = 11.20 and 11.07, respectively). Like the Ancestral population for flower size selection, both IM13 flowering cohorts, IM14\_Late and the Low flower size selection population exhibited relatively low RLG1a coverage (1.2–1.67x background) indicating few individuals with high-copy genotypes in these pools. The High flower size selection population, Fall germinant, IM14\_Early and Rosette pools were intermediate in coverage (5.82, 5.99, 6.70 and 3.1, respectively),

### 3.2 | A common 3-copy (*trip+*) variant of RLG1a contributes to flower size and flowering time variation

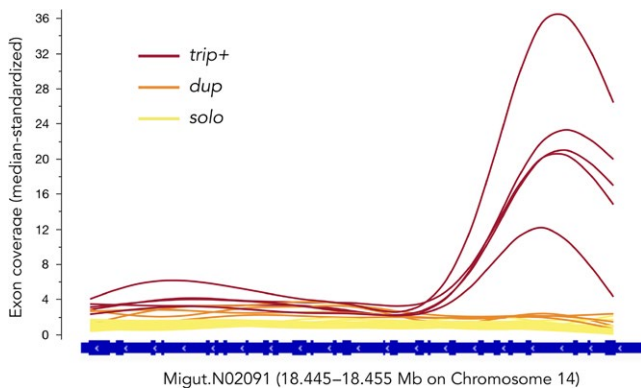
Differences in the standardized coverage of RLG1a between paired population pools (Figure 1) suggest shifts in the frequency of multi-copy haplotypes between cohorts/phenotypes. To identify haplotypes contributing to these PoolSeq patterns, we examined sequence variation and RLG1a exon coverage in a set of 35 whole-genome



**FIGURE 1** Median-standardized exon coverage (read depth) in pooled whole-genome resequencing of 11 *Mimulus guttatus* phenotypic populations (cohorts), grouped by experiment/data set (Table 1). Planned contrasts of cohorts from top to bottom: Flower size after artificial selection, flowering time in the field, vernalization requirement and germination time in the field. Values are shown for 13 Mb of Chromosome 14 flanking the focal gene Migut.N02091

resequenced inbred lines from IM. Four lines (IM664, IM115, IM922, IM239) exhibited a characteristic pattern of ~3x elevated coverage (and heterozygosity) across most of the gene (last 23 exons) plus extremely high coverage (26–47x background) of the first three exons (Figure 2). These lines were uniquely scored as heterozygous for a 15-bp (5 amino acid; 5AA) deletion in Exon 16 relative to the reference sequence plus three linked intronic SNPs. Metagenomic assembly of IM664 identified three haplotypes across this section of the gene; one of these contains the 5AA Exon 16 deletion and linked SNPs. One additional line (IM1145) exhibited a similar (but weaker) pattern of elevated coverage genewide (mean = 16.4x across first 3 exons) and was only heterozygous at the SNPs. We infer it to be a heterozygote of multi- and single-copy genotypes at the time of resequencing. Thus, 1/7 of the inbred lines carry an RLG1a genotype (henceforth *trip+*) with three distinct paralogs plus (apparently) additional duplications of the 5' end of the gene. All *trip+* lines tested ( $n = 4$ , including IM1145) carried a 426-bp mN2091x16 amplicon (with 5AA Exon 16 deletion), which is absent from single-copy (*solo*) lines ( $n = 9$ ). *trip+* lines (which are highly inbred and should be homozygous genewide) always had at least two other amplicons, which differed among *trip+* plants and overlapped in size with single-copy (*solo*) amplicons. Therefore, we score *trip+* as a dominant (presence/absence) marker. An additional four inbred lines (IM138, IM359, IM549, IM709) exhibited ~2x coverage (Figure 2) and dozens of heterozygous sites genewide. Together, these data suggest stacking of diverged duplicates rather than retained heterozygosity. Unfortunately, we were unable to identify diagnostic marker alleles for this putative RLG1a\_ *dup* genotype; therefore, we roll them into the single-copy (*solo*) genotypic class for analyses of outbred individuals.

Individual genotyping confirmed a substantial ( $\chi^2_{2df} = 11.2$ ,  $p < 0.005$ ) difference in *trip+* frequency among the flower size



**FIGURE 2** Smoothed exon coverage (standardized by chromosomal median) of Migut.N02091 (RLG1a) in 35 inbred lines of Iron Mountain *Mimulus guttatus*. Colours indicated inferred copy number category based on genewide coverage patterns and “heterozygosity.” The marker mN2091x16 spans exons 16–17, and the qPCR marker mN2091q is in Exon 26 (exons are numbered consecutively from right to left). The gene region containing the first three exons shows homology to the housekeeping gene EF1a and appears to be additionally amplified in *trip+* individuals

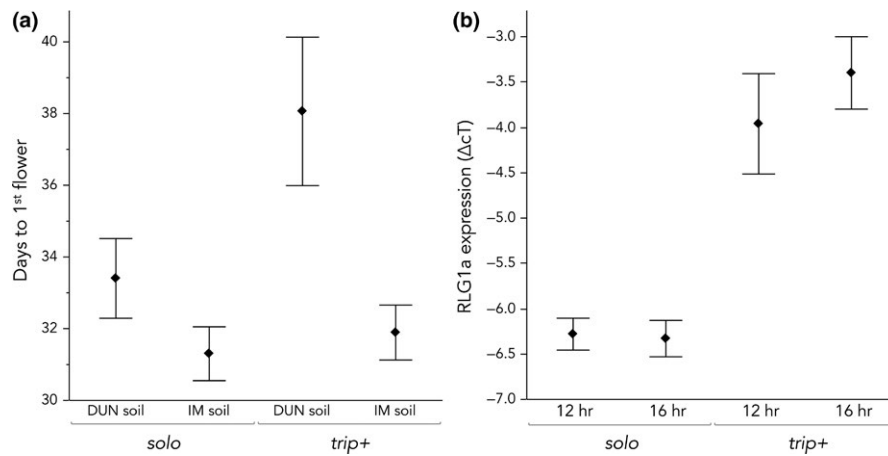
selection populations, with twice as many *trip+* plants in the High (36/72 = 50%) vs. Low (13/55 = 26%) and Control (7/29 = 24%) cohorts individually genotyped. Plants carrying *trip+* also exhibited late flowering in two independent data sets, one from the field and one under field-mimicking conditions in the greenhouse. First, individual genotyping of the 2014 field PoolSeq collections revealed twice the frequency of *trip+* plants (32%) in Late vs. Early (15%) flowering cohorts ( $\chi^2_{1df} = 6.3$ ,  $p = 0.012$ ,  $N = 179$ ). This may reflect a relatively slow transition to flowering by *trip+* plants under the drought-stress conditions experienced in normal-to-dry years at Iron Mountain. Consistent with this inference, *trip+* was associated with later flowering in plants grown under two stressful soil conditions in the greenhouse ( $F_{3, 75} = 6.6$ ,  $p = 0.03$  for genotype effect). Although the interaction with soil type was non-significant ( $p = 0.09$ ), the main genotypic effect was primarily due to delayed flowering of *trip+* plants (mean =  $38.1 \pm 1.3$  SE days,  $n = 17$ ) relative to non-carriers (mean =  $33.4 \pm 1.3$  SE days,  $n = 18$ ) in the sandier and more drought-stressed DUN soil, with no difference evident in the IM soil (Figure 3a).

Consistent with increased mRNA dosage due to increased copy number, two *trip+* lines exhibited highly elevated RLG1a expression relative to *solo* lines ( $n = 9$ ) under both short (12 hr) and long (16 hr, after 12 hr) daylength conditions ( $p < 0.00001$  for RLG1 genotype effect; Figure 3b). Daylength, non-vernalized flowering phenotype (Bolt/Rosette) and their interaction had no effect on RLG1a expression in leaves (all  $p > 0.15$ ), but line was highly significant beyond the RLG1a CNV effect ( $p < 0.002$ ). The RLG1a CNV effect was not a consequence of flowering phenotype; both *trip+* lines tested had the Bolt phenotype, but so did two of the *solo* lines. However, there was hint of interaction between daylength and CNV ( $p = 0.09$ ), with one of the *trip+* lines in particular (IM115) exhibiting increased expression after the shift to 16-hr days.

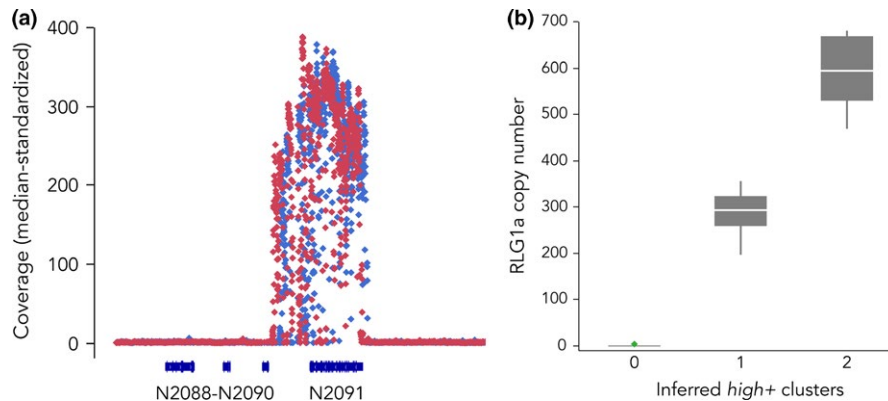
### 3.3 | A rare variant with ~300 RLG1a copies (high+) drives PoolSeq coverage patterns and segregates at a single nuclear locus

Even dramatic shifts in the frequency of a 3-copy *trip+* haplotype cannot explain the >10x genewide coverage of RLG1a in the Spring germinant, Bolt, and IM14\_Early cohorts. In genotyping individuals from the IM14 and flower size selection PoolSeq populations, we identified a second amplicon with the 5AA Exon 16 deletion (428 bp): This allele was rare in the former populations 3 each in High and Ancestral, 0 in Low; total  $N = 162$ ) and present in IM14\_Early (6/90 = 6.7%) but not IM14\_Late (0/95 = 0%). The skew in incidence of the 428-bp allele in IM14 was statistically significant, even when *trip+* (elevated in IM14\_Late; see above) and *solo* variants were coded as a single category (Pearson  $\chi^2 = 6.5$ ,  $p = 0.01$ ). Despite its rarity, the 428-bp amplicon (hereafter *high+*) was always “homozygous,” suggesting it out-competes other alleles during mN2091x16 PCR amplification.

None of the resequenced IM *M. guttatus* inbred lines ( $N = 35$ ) had RLG1a coverage greater than *trip+* or the indel variation



**FIGURE 3** Flowering time and expression of RLG1a genotypes (*solo*, *trip+*) under controlled conditions. (a) Time to first flower (mean  $\pm$  1 SE) for outbred plants grown in two field soils (DUN = drier; see Methods) in greenhouse. Analysed with ANOVA (overall  $r^2 = 0.21$ ,  $n = 79$ ). Soil type effect:  $p < 0.001$ ; RLG1a genotype effect:  $p = 0.03$ ; interaction:  $p = 0.09$ . (b) RLG1a expression (relative to EF1a control; mean  $\pm$  1 SE) in leaf tissue of *solo* ( $N = 9$ ) and *trip+* ( $N = 2$ ) inbred lines grown under two daylength conditions. Analysed with nested ANOVA (overall  $r^2 = 0.78$ ,  $n = 56$ ): Daylength effect:  $p = 0.58$ ; RLG1a CNV effect:  $p < 0.0001$ ; Line nested within genotype effect:  $p = 0.002$



**FIGURE 4** Identification and segregation of *high+* RLG1a genotype. (a) Median-standardized coverage (25-bp windows) of *high+* carriers IM\_627c and IM15P\_317d, showing amplification of Migut.N02091 and the Migut.N02090-2091 intergenic region. (b) Quantile box plots for RLG1a copy number in selfed progeny of IM13P\_627c segregating for 0 ( $n = 7$ ), 1 ( $n = 20$ ) or 2 ( $n = 12$ ) *high+* variants. RLG1a copy number was estimated as  $2^{\Delta Ct}$ , where  $\Delta Ct$  is the absolute difference between  $Ct_{mN2091q}$  and  $Ct_{mG571q}$  for an individual, averaged across two technical replicates

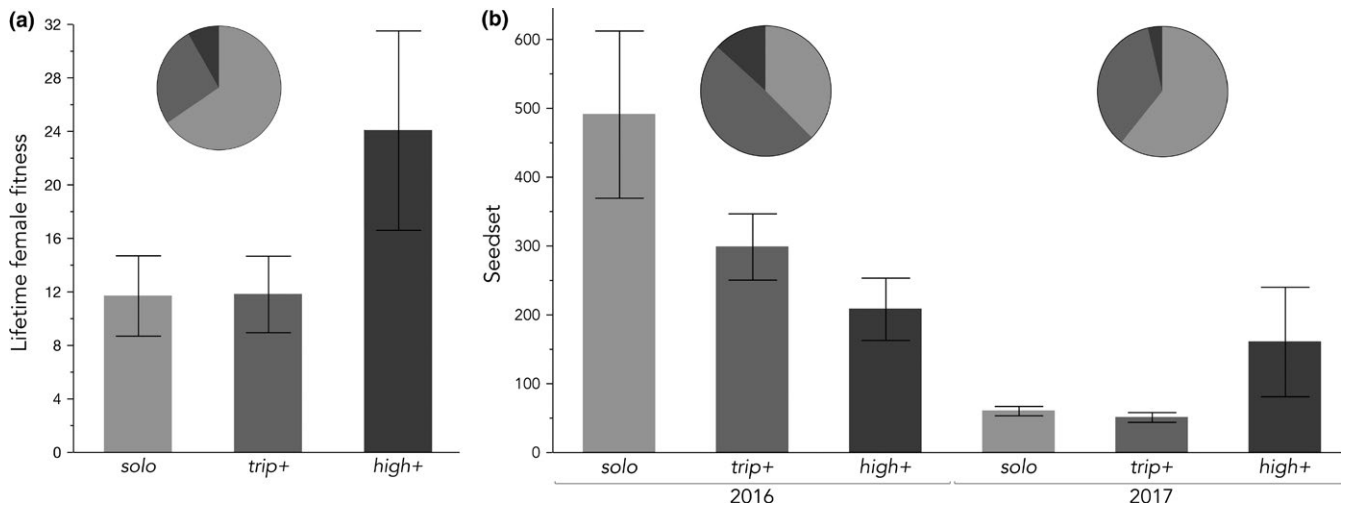
necessary to generate a 428-bp mN2091x16 allele. However, two wild-derived 428-bp carriers (IM13P\_627c and IM15P\_617d) exhibited a stack of  $\sim 275\times$  (median-standardized) coverage across Migut.N02091 (Figure 4a). The vast majority of stacked reads represented a single haplotype, with the Exon 16 5AA deletion present at  $>99\%$  (1,608/1,626 reads had no coverage at the central site of the deletion). Elevated coverage extended across the entire gene as well as the intergenic region between Migut.N02091 and Migut.N02090, spanning  $\sim 19$  kb. If all copies were arranged in tandem, this *high+* RLG1a haplotype would span at least 5.7 Mb, or  $\sim 22$ –46% of a *M. guttatus* chromosome in the current v2 assembly.

qPCR of genomic DNA confirmed a massive copy number expansion in *high+* carriers. In two wild-derived carriers (IM13P\_627c and IM13P\_644), mN2091q amplified 8.5 and 9.5 cycles earlier than the single-copy control gene, respectively. An internally controlled standard curve estimated copy number of the latter to be  $>500$  (502

and 513 in two technical replicates) and the single-cycle difference in  $\Delta Ct$  is consistent with IM13P\_627c having one *high+* "allele" and IM13P\_644 having two. In the PoolSeq populations, capture of fewer than 10 *high+* individuals (each with  $\sim 250$ –300 copies of RLG1a) can explain elevated coverage in IM14\_Early vs. IM14\_Late. Although we could not genotype individuals from the Fall-Spring and Bolt-Rosette experiments, commensurate shifts in frequency (e.g., *high+* carriers relatively common in Bolt and Spring, but absent from Rosette and other low coverage pools) plausibly explain their coverage differences.

The extraordinary amplification of RLG1a in *high+* plants raises the possibility of transfer of nuclear DNA to an organellar genome (which occur in many copies per cell), rather than duplication within the nuclear genome. Furthermore, even if encoded in the nuclear genome, RLGA1 could be dispersed across many clusters genome-wide (Gaines et al., 2010). Finally, even if tandemly amplified as a single genetic locus, *high+* copies need not be located in tandem





**FIGURE 5** Fitness of RLG1a copy number variants in the field (means  $\pm$  1 SE) and frequencies of each genotype in survivors (pie charts). (a) Lifetime female fitness (survival  $\times$  seedset) in 2015, a severe drought year. (b) Seedset (fecundity) of plants that survived to fruit in 2016 and 2017. Note different y-axis scales

with the (*solo*) annotation of Migut.N02091 in the reference genome. To distinguish these possibilities from the working hypothesis that *solo* and *high+* versions of RLG1a are alternative alleles at the same locus, we examined segregation in a pseudo- $F_2$  made by selfing *high+* carrier IM13P\_627c. The proportion of *high+* carriers in the progeny (84% or 146/173) was slightly elevated over the Mendelian expectation of 75% for a dominant marker ( $\chi^2_{2df} = 4.58$ ,  $p = 0.03$ ), but also substantially lower than the two-locus expectation of 93.75% ( $\chi^2_{2df} = 7.57$ ,  $p = 0.006$ ). Genomic qPCR confirms the inference of a single *high+* locus: progeny  $\Delta\Delta$  Ct values ( $N = 39$ ) clustered into three distinct groups (normal mixtures analysis, AIC/BIC lowest for 3 clusters). *Solo* individuals had  $\Delta\Delta$ Ct values not different from 0 (mean =  $0.10 \pm 0.14$ ;  $n = 7$ ), whereas *high+* carriers sorted into hemizygous (mean  $\Delta\Delta$  Ct =  $8.195 \pm 0.08$ ;  $n = 20$ ) and homozygous (mean  $\Delta\Delta$  Ct =  $9.19 \pm 0.11$ ;  $n = 12$ ) classes with the expected twofold difference in inferred copy number ( $286.5 \pm 11.6$  SE and  $590.5 \pm 15.9$  SE, respectively; Figure 4b). Both marker and qPCR segregation ratios point to a single nuclear locus segregating 0, 1 or 2 *high+* RLG1a arrays consisting of  $\sim$ 300 copies of the gene.

To test whether the *high+* locus was genetically coincident with Migut.N02091 (i.e., a local tandem expansion on Chromosome 14), we genotyped 62 IM13P\_627c progeny at a tightly linked flanking marker (mN2089). In *solo* progeny ( $n = 8$ ), mN2091x16 segregated for two amplicons perfectly associated with segregating mN2089 genotype, indicating that Migut.N02091 is correctly located in the reference genome. However, mN2089 also exhibited Mendelian segregation within the remaining *high+* carriers ( $\chi^2_{2df} = 1.2$ ,  $p > 0.50$  vs. 1:2:1 expectation). Two segregating alleles at Migut.N02091 in *solo* progeny, plus no linkage between *high+* presence and m2089 genotype, demonstrate that RLG1a *high+* segregates as a presence/absence polymorphism at an unlinked locus. Thus, RLG1a exhibits multiple levels of structural variation, varying in the number of genetic loci as well as the number of copies per locus.

As a coarse assay of whether *high+* carriers exhibited constitutive expression of RLG1a in proportion to copy number, we conducted RT-qPCR on shoot tissue from greenhouse-grown siblings of the two sequenced *high+* plants (outbred, so segregating for all possible genotypes). Both *high+* ( $\Delta$ Ct =  $-2.87$ ,  $n = 3$ ) and *trip+* ( $\Delta$ Ct =  $-2.98$ ;  $n = 2$ ) plants had  $\sim$ 2-fold higher expression than their *solo* siblings ( $\Delta$ Ct =  $-3.90$ ;  $n = 2$ ), but any genotypic differences in expression between *solo* and multi-copy plants were not significant with this small sample size (ANOVA,  $p = 0.37$ ). Regardless, there was no evidence of 300-fold higher expression in *high+* plants.

### 3.4 | Copy number variants at RLG1a experience fluctuating selection across years

We examined genotype-fitness associations over three years (2015–2017) spanning the extremes of fecundity in the Iron Mountain population (seedset of plants surviving to fruitset =  $29.7 \pm 2.9$  SE,  $287.6 \pm 32.2$  SE, and  $63.8 \pm 5.5$  SE, respectively; all phenotypic  $N > 350$ ). In 2015, which was a record drought year in the Oregon Cascades (<https://wcc.sc.egov.usda.gov>) with an unusual April melt-out, we also monitored survival (<32% overall). We recovered tissue from only  $\sim$ 50% of marked seedlings ( $N = 243$  Spring/Fall germinant pairs) and all 59 Spring germinants recovered (as dead tissue for DNA extraction) died prior to flowering. Additional flowering plants marked one week apart in mid-June also mostly died prior to setting seed (54.6% mortality,  $N = 185$ ), with those that initiated flowering at the later time significantly more likely to survive (59.7% vs. 38.2%;  $\chi^2_{1df} = 7.7$ ,  $p < 0.01$ ). RLG1a genotype was significantly associated with survival to seedset ( $\chi^2_{2df} = 8.56$ ,  $p = 0.014$ ); 64.7% (11/17) of *high+* individuals marked as seedlings or flowerers set seed, whereas only 33.0% (33/100) and 29.8% (54/181) of *trip+* and *solo* plants did. The survival advantage of *high+* plants led to significantly higher log-transformed female fitness in monitored plants (ANOVA,  $p = 0.005$ ,  $N = 298$ ), as they made (on average)  $\sim$ 2x as many seeds (Figure 5a).

There was no significant fecundity effect in the set of random survivors collected at fruiting ( $p = 0.38$ ,  $N = 110$ ).

Fecundity varied significantly with RLG1a genotype in 2016 and 2017, but in opposite directions. In lush 2016, which had 5–10x the mean fecundity of flanking years, *solo* individuals matured 50% more fruits than plants with either CNV (12.4 vs. 7.7 and 8.6 for *trip+* and *high+* respectively) and, on average, produced twice as many seeds (Figure 5b). In 2017, a heat wave at the end of June/early July sharply ended an otherwise lush flowering season (L. Fishman, pers. obs.). In this truncated season, *high+* plants made (on average) twice as many fruits (4.7 vs. 2.4 and 2.2) and 3 times as many seeds as the other two genotypes. These inter-annual shifts result in significant (for fruits;  $p = 0.016$ ) and marginally non-significant (for seeds;  $p = 0.07$ ) year  $\times$  genotype interaction effects in the GLM, along with main effects of RLG1a genotype (both  $p < 0.005$ ). Along with the 2015 data (and the bias towards early flowering in 2014), shifts in its relative fecundity suggest that *high+* influences life history in ways that are beneficial in stressful years (and/or microsites), but deleterious when conditions are good.

Notably, the relative proportions of the CNVs changed as predicted from the previous year's fitness effects, consistent with real-time responses to fluctuating selection. Following disproportionate *high+* survival in 2015 (6.8% in fruiting plants), *high+* was maximally common (13%) in 2016 (when it had low seedset) and then dropped to 3.6% in 2017 (Figure 5). Together, these results suggest that RLG1a *high+* variants influence life-history traits whose fitness effects vary antagonistically from year to year.

## 4 | DISCUSSION

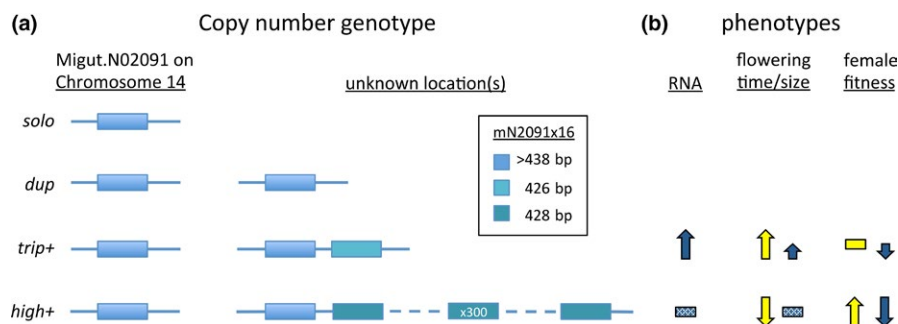
Despite the decreasing costs of genome sequencing, connecting phenotype to genotype remains an empirical challenge. Our original intention was to use simple PoolSeq genomewide association mapping experiments to gain insight into genetic basis of standing variation in floral and life-history traits: the focal Iron Mountain *M. guttatus* population is a place where selection should be maximally effective (and detectable) at the level of the individual nucleotide, yet also maintains tremendous diversity in fitness-relevant phenotypes. Our results provide a case study for our original

question about the processes maintaining polymorphism, but also highlight the challenging complexity of both genome architecture and phenotypic expression in wild populations.

From initial PoolSeq scans, we identified a tRNA ligase (RLG1a) as a coverage outlier associated with intra-population variation in flowering time, germination time, flower size and vernalization requirement. Elevated RLG1a exon coverage in some populations and inbred lines pointed to CNV, and we identified an intermediate-frequency (>14%) variant with at least three copies of the gene (*trip+*) (Figure 6a). Individual genotyping confirmed an association of *trip+* variants with a "slow" life-history (late flowering under stress and large flower size), and expression analyses suggest that elevated RLG1a dosage in *trip+* plants may contribute to its effects (Figure 6b). However, even complete fixation of a 3x variant could not account for observed 10x coverage in three of our population pools, implying the existence of even higher copy number variants. By targeting rare wild individuals carrying a marker allele sharing a diagnostic exonic indel with *trip+*, we identified a second CNV (*high+*) that carries an unprecedented 250–300 haploid copies of RLG1a in a single (presumably tandem) cluster segregating as a presence/absence polymorphism at a nuclear locus unlinked from the single-copy RLG1a (Figure 6a). The presence of even a small number of *high+* individuals in a pool was sufficient to grossly bias initial PoolSeq scans, but individual genotyping confirmed significant shifts in CNV frequency in the flower size and 2014 flowering time data sets. Finally, using field fitness measures from three years with very different climatic conditions, we found that *high+* experiences fluctuating selection, increasing survival and/or seed production in two drought years, but associated with low fitness relative to non-carriers in a lush year (Figure 6b). Taken together, our discovery and analysis of the *trip+* and *high+* variants provides insight into the origins of CNV, suggests new candidate functions for an essential but poorly understood plant gene, and supports a role for climatic fluctuation in the maintenance of standing variation for fitness traits.

### 4.1 | An embarrassment of riches: detection and dissection of multiple layers of genic CNV

This study of just a single gene, within a single population of monkeyflowers, illustrates both the richness of copy number variation



**FIGURE 6** Proposed models for (a) the structure of RLG1a copy number variation (CNV) in *Mimulus guttatus* and (b) the phenotypic effects of *trip+* and *high+* CNVs relative to *solo* and *dup* genotypes. Yellow/light = drought-stress conditions; blue/dark = lush conditions. Solid effects had statistical support in at least one field or greenhouse experiment; stippling indicates sample size was too low for inference

in natural populations and the significant challenges it creates for connecting genotype and phenotype (Hoban et al., 2016). Using genomic resequencing, qPCR and genetic markers, we define and describe three distinct classes of RLG1a genotype, spanning at least two loci (Figure 6). One locus, Migut.NO2091 (RLG1a\_1) on LG14, segregates diverse single-copy variants. A second unlinked locus (RLG1a\_2) segregates for the ~300-copy *high+* variant. The *trip+* variant(s), in which one of two inferred copies share a diagnostic 5AA deletion in Exon 16 with *trip+*, is likely also an allele at this locus and there are apparently also duplicated (*dup*) individuals with one copy at each locus. Thus, the history of these variants includes (at minimum) duplication/insertion of a gene into new chromosomal location, local tandem duplication and sequence divergence, and massively ampliconic tandem duplication, spanning the described mechanisms of CNV generation (Hastings et al., 2009). Although more work will be necessary to fully describe the evolutionary history of this extensive copy number variation, we can propose testable hypotheses about its origins.

The tRNA ligase RLG1 is considered essential (Yang, Guo, Hou, Gong, & Liu, 2017) and is the sole member of its gene family in plants (Englert & Beier, 2005). Except for three Brassicaceae and *M. guttatus*, all 37 Eudicot genomes in Phytozome (www.Phytozome.org, accessed 07/15/2018) encode just a single copy of this large gene (26 coding exons, >1200AA in *M. guttatus*). Thus, RLG1 appears highly conserved for copy number despite the multiple rounds of whole-genome duplication across Eudicot lineages. *Mimulus* appears different, even beyond the remarkable *high+* amplification. In addition to our focal locus Migut.NO2091, a second tRNA ligase homolog (Migut.D02182; RLG1b) is found on Chromosome 4. As annotated, these two genes share 82.6% amino acid identity, suggesting a recent (most likely within *Mimulus*) duplication. Retention of two copies of RLG1 in an ancestral *Mimulus* genome may have been key to further copy number diversification within *M. guttatus*. Although the functional biology of RLG1 is still poorly understood (see next section), relaxation of the constraints imposed by a single gene performing multiple essential functions (Englert & Beier, 2005) may have allowed for neo- and/or sub-functionalization (Lynch & Conery, 2000) that then facilitated intraspecific CNV.

The origin of copy number variants at Iron Mountain likely involved whole-gene duplication and deletion deeper in the history of the *M. guttatus* species complex. Segregation analyses show that the *high+* haplotype is present as an unlinked locus (RLG1a\_2 with 250–300 copies in *high+* and putatively two copies in *trip+*; Figure 6a). This non-tandem duplication is likely recent, as good agreement between estimates of copy number from qPCR amplification of a highly conserved segment and resequence read-coverage indicates high sequence identity among copies at the two loci. The diagnostic 5AA indel in Exon 16 (captured by our marker mN2091x16) provides some intriguing clues as to how this may have happened. All IM individuals genotyped (including *dup*, *trip+* and *high+*, as well as the reference genome and other *solo* genotypes) have at least one copy with the longer sequence at this position, suggesting that it is ancestral. However,

genome alignments from *M. nasutus* and other “Southern Clade” members of the *M. guttatus* complex (Brandvain, Kenney, Flagel, Coop, & Sweigart, 2014) reveal that the deletion found in *trip+* (as one of several copies) and *high+* (as 99% of hundreds) is the common *single-copy* variant elsewhere in the range (L. Fishman, unpublished data). Barring recurrent mutation or gene conversion, this suggests that the IM reference single copy (insertion) and the Southern clade single copy (deletion) are both derived (via reciprocal gene loss) from a duplicated ancestor similar to putative *dup* IM lines (Figure 6a). Such reciprocal deletion of duplicates has recently been implicated in hybrid lethality in *M. guttatus* species complex (Zuellig & Sweigart, 2018), and is a common mechanism of plant hybrid incompatibility (Fishman & Sweigart, 2018), so may have implications beyond phenotypic differences of the alternative genotypes. Testing this scenario against alternatives will require genetic mapping and physical deconvolution (e.g., with long-range sequencing technologies) of *high+*, *trip+* and *dup* RLG1a haplotypes, as well as molecular evolution analyses of genotypes from throughout the species range.

Finally, how did the massive expansion of RLG1a gene copies in *high+* haplotypes occur? The largest genic CNV known in plants is the target gene for glyphosate herbicides, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which expands to 5–160 copies in *Amaranthus palmeri* (Gaines et al., 2010). Expansion of EPSPS confers herbicide resistance via gene dosage effects (i.e., increased expression) and is thus strongly favoured in sprayed crop fields. *Amaranthus* EPSPS clusters are dispersed throughout the genome (Gaines et al., 2010), and cytogenetics reveals that EPSPS clusters form extra-chromosomal circular DNA (eccDNA) molecules rather than being integrated into linear chromosomes (Koo et al., 2018). Rolling circle amplification in eccDNA was previously implicated in the origin of the *sobo* satellite repeat in *Solanum bulbocastanum*, in which a 360-kb (nongenic) monomer amplified to 4.7 Mb in a single (hemizygous) chromosomal location (Tek, Song, Macas, & Jiang, 2005). RLG1a *high+* segregates as the presence/absence of a tandem array, and thus may have originated via rapid amplification in eccDNA and re-insertion (without copy number intermediates) into the nuclear genome (Charlesworth, Sniegowski, & Stephan, 1994). More work is necessary to evaluate this scenario, and to test the effects of RLG1a2 expansion on the recombination and segregation of linked loci.

This study highlights how much CNVs complicate genome scans for adaptive differentiation, despite being a large proportion of potentially functional variants. Especially in pooled population sequencing (PoolSeq) experiments, CNV generates substantial complications. For example, Migut.NO2091 outlier sites were censored (for excess coverage) in SNP scans of the High-Low flower size selection experiment (Kelly et al., 2013), but it appeared to be strong SNP outlier in the IM flowering time study (Monnahan & Kelly, 2017). Sites with low coverage are rightly dropped from most analytical pipelines (DePristo et al., 2011), so gene deletions relative to a reference genome (particularly within clusters of paralogs where cross-mapping may occur) are difficult to identify and such loci may be lost from

scans. Particularly in PoolSeq studies, the coverage stacks created by duplication can generate false positives via statistical (overconfidence) and biological bias (mis-representation of allele frequencies). Summary statistics of differentiation between pooled populations reflect sample size (coverage) as well as allele frequencies (Kelly et al., 2013; Kofler et al., 2011; Magwene, Willis, & Kelly, 2011), so even identical duplicates can inflate statistical significance. At the same time, stacking of multiple paralogs with shared SNP/indel differentiation (like *trip+* and *high+* vs. *solo*) exaggerates differences among pools. Without the 2-bp indel that happens to distinguish *trip+* and *high+* diagnostic PCR amplicons (and follow-up targeted resequencing, crossing, and qPCR), we would have parsimoniously inferred the existence of a 5-copy variant much more differentiated in frequency among pools. Distortion of PoolSeq SNP scans by CNV adds to the existing arguments for using large sample sizes (>100 individuals) in PoolSeq experiments (Lynch, Bost, Wilson, Maruki, & Harrison, 2014). In addition, validation of results from genome scans (using both individual and pooled sequencing approaches) with independent genotyping is an important step in investigating the genetic basis of adaptive traits.

Our results, along with increasing evidence that CNV contributes to fitness-relevant variation in diverse systems (Dolatabadian, Patel, Edwards, & Batley, 2017; Girirajan, Campbell, & Eichler, 2011; Maron et al., 2013; Mickelbart, Hasegawa, & Bailey-Serres, 2015; Prunier et al., 2017), suggest that explicit CNV surveys should be a routine part of genomewide scans for adaptive loci. A number of new pipelines combine coverage, read-pair information and other data to infer structural variants from short-read data (Layer, Chiang, Quinlan, & Hall, 2014; Mohiyuddin et al., 2015) and could fruitfully be used to catalog CNV in, for example, the Iron Mountain inbred line set. The detection of putative 2x-covered *dup* individuals in that set (Figure 2) is promising, though poor alignment in intergenic regions in diverse *M. guttatus* limits the utility of read-pair information as corroborative evidence. Without such a reference catalog of variants, however, whole-gene CNVs are likely to often remain a component of the missing heritability (Manolio et al., 2009).

#### 4.2 | One piece of the puzzle: a tRNA ligase and standing variation for life-history traits

The genic CNVs identified in this study are undoubtedly a tiny sample of the variants affecting life-history phenotypes in IM *M. guttatus* (Troth et al., 2018), but RLG1a is the first (and hopefully worst!) gene to be dissected mechanistically as a direct contributor to fitness phenotypes in this model population. Importantly, despite not knowing the chromosomal locations of the CNVs or full individual genotypes, we can be reasonably confident that RLG1a copy number per se and/or sequence variation within the characterized CNVs underlies the observed phenotypic and fitness associations. In IM *M. guttatus*, linkage disequilibrium (LD) falls off sharply at distances >1,000 bp (with the exception of several inversions and/or recent selective sweeps; Puzey et al., 2017). Thus, *trip+* and *high+* are unlikely to be neutral markers for functionally distinct

loci tightly linked to RLG1a2; in addition, a preliminary scan for associated sites (i.e., scanning for differences in nucleotide diversity within vs. between *solo* and *trip+* inbred line sets; data not shown) did not reveal any strong or extensive LD with other genomic regions. Thus, we discuss the phenotypic and fitness results in the context of RLG1 function.

Why a tRNA ligase CNV? Plant tRNA ligases share little sequence similarity with tRNA ligases in other taxa, but retain the core function of splicing the two intron-containing nuclear-encoded tRNAs (tRNA<sup>Tyr</sup> and elongator tRNA<sup>Met</sup>) (Englert & Beier, 2005). The single-copy *Arabidopsis* RLG1 is expressed in most tissues and is embryolethal when knocked out (Yang et al., 2017). RLG1 has also been implicated in the joint regulation of the key plant hormone auxin and the endoplasmic reticulum-based unfolded protein response (UPR) in *Arabidopsis* (Leitner et al., 2015; Nagashima et al., 2016). One arm of the UPR is regulated by cytoplasmic splicing of bZIP60 transcription factor mRNAs; RLG1 performs the key ligation step (Nagashima et al., 2016). Via its regulation of the UPR, RLG1 may play a central role in plant reactivity and resistance to abiotic stressors such as heat and drought, as well as hormonal control of trade-offs between vegetative growth and reproduction (Deng, Srivastava, & Howell, 2013). In addition, the bZIP60 branch of the UPR is constitutively upregulated in pollen (which is notoriously vulnerable to heat stress) (Deng et al., 2016) and integral to plant responses to viral infection (Zhang, Chen, Brandizzi, Verchot, & Wang, 2015). Even beyond its remarkable copy number diversity, *M. guttatus* (with both RLG1a and RLG1b in everyone) provides a rare opportunity to investigate the multiple functions of plant tRNA ligases without the constraint of an essential single copy.

Our phenotype and fitness results suggest that RLG1a CNVs contribute to *stress-responsive* variation in the timing of reproduction, along a spectrum from highly reactive to stress (fast) to relatively nonchalant (slow). Interestingly, carriers of the *trip+* variant appear “slow” (large flowers, later reproduction in field and under drought stress), whereas *high+* carriers appear “fast” (early flowering in 2014, highest survival and/or seedset in drought years). Further analyses of sequence variants and expression patterns will be necessary to determine the physiological mechanisms underlying this pattern. Importantly, the fecundity effects of RLG1a CNV that we see in the field are caused by differences in fruit number rather than seedset/fruit (which did not significantly differ among years or RLG1a genotypes). Thus, fluctuating fitness effects depend on life-history plasticity/stress tolerance traits that affect the timing of flowering and senescence. This parallels work showing fluctuating selection on “fast/small” vs. “slow/large” alleles using QTL introgression lines (Mojica & Kelly, 2010; Mojica et al., 2012) and a large genomewide association mapping set (Troth et al., 2018) planted at a nearby site, but highlights genotype × environment interactions for phenotype as well as fitness. For example, alternative RLG1a genotypes may differ in whether they accelerate bolting when sensing short-term heat stress via the UPR, with the fitness consequences depending on whether heat stress accurately predicts imminent dry-down and death in a given year and

microsite. In addition to mediating stress-responsive cues, RLG1a CNVs could also affect stress tolerance; elevated expression of RLG1a (as in *trip+* lines; Figure 3) may increase the efficiency of UPR-upregulation and thus protect against heat or cold damage that shortens lifespan. Unmeasured effects on male fitness (e.g., via heat tolerance of pollen) may also contribute to the maintenance of alternative RLG1a CNVs. Exploring these intriguing possibilities in the appropriate environmental contexts will require long-term monitoring of natural populations, as well as experimental manipulation of candidate stress regimes.

Temporally fluctuating selection is an appealing but controversial mechanism to maintain fitness variation, as simple models cannot indefinitely maintain multiple alleles when their fitness effects are not perfectly balanced (reviewed in Delph & Kelly, 2013). However, plasticity (which we observe) and shifts in dominance (which are implied by *high+/solo* fitness reversals; Figure 4) can enlarge the parameter space over which temporal variation prevents allele loss (Gulisija et al., 2016; Posavi et al., 2014; Wittmann et al., 2017). In addition, dormant life stages (e.g., a seedbank) and spatial variation within a population (e.g., unpredictable soil depth) can slow the dynamics of displacement and thus help maintain standing variation over observable timescales (Ellner & Hairston, 1994; Svardal et al., 2015). Although we cannot be sure that *high+* will not either fix or be driven from the Iron Mountain population in the long term (especially as it may be a relatively new variant not at equilibrium), the observed shifts in relative fitness from year to year plausibly contribute to its maintenance at low to intermediate frequency. This parallels the classic *Linanthus parryae* system, in which temporal and spatial variations in drought stress contribute to the maintenance of a flower colour polymorphism (Schemske & Bierzychudek, 2001, 2007). In a model inspired by that system, variants with geometric mean fitness  $<1$  (i.e., ones that should be deterministically lost otherwise) can be maintained if they have highest fitness in “good” years and a seedbank spreads that advantage over time (Turelli, Schemske, & Bierzychudek, 2001). Like other variants contributing to life-history trade-offs in the IM *M. guttatus* population (Troth et al., 2018), RLG1a CNVs experience similar dynamics, mediated (in part) by stress-responsive phenological traits. Our results add to the growing evidence that temporally fluctuating selection contributes to the abundant quantitative genetic variation seen in many short-lived taxa (Siepielski, DiBattista, & Carlson, 2009; Troth et al., 2018).

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## DATA ACCESSIBILITY

Whole-genome resequence data sets (fastqs or bams): NCBI Sequence Read Archive: Inbred lines—SAMN05852485–SAMN05852522, SAMN04517335; Iron Mountain flowering time pools—PRJNA336318; other pools—SAMN10169032–SAMN10169038; *high+* accessions—SAMN10134433, SAMN10134434. Genotype, phenotype and/or qPCR data sets: <https://doi.org/10.5061/dryad.m1j24tf>.

## AUTHOR CONTRIBUTION

L.F. and J.K.K. designed experiments; P.M., L.F. and J.K.K. collected PoolSeq samples; P.M., F.R.F., J.K.K. and T.N. constructed genomic libraries and analysed sequence data; L.F., F.R.F., T.N. and K.A. conducted qPCR experiments; L.F., M.M. and E.M.-W. collected phenotypic data and PCR-genotyped wild and greenhouse-grown plants; L.F. wrote paper with input from all authors.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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