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Leaf shape evolution has a similar genetic architecture in three edaphic specialists within the *Mimulus guttatus* species complex

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• **Background and Aims** The genetic basis of leaf shape has long interested botanists because leaf shape varies extensively across the plant kingdom and this variation is probably adaptive. However, knowledge of the genetic architecture of leaf shape variation in natural populations remains limited. This study examined the genetic architecture of leaf shape diversification among three edaphic specialists in the *Mimulus guttatus* species complex. Lobed and narrow leaves have evolved from the entire, round leaves of *M. guttatus* in *M. laciniatus*, *M. nudatus* and a polymorphic serpentine *M. guttatus* population (M2L).
• **Methods** Bulk segregant analysis and next-generation sequencing were used to map quantitative trait loci (QTLs) that underlie leaf shape in an *M. laciniatus* × *M. guttatus* F_2 population. To determine whether the same QTLs contribute to leaf shape variation in *M. nudatus* and M2L, F_2s from *M. guttatus* × *M. nudatus* and lobed M2L × unlobed *M. guttatus* crosses were genotyped at QTLs from the bulk segregant analysis.
• **Key Results** Narrow and lobed leaf shapes in *M. laciniatus*, *M. nudatus* and *M. guttatus* are controlled by overlapping genetic regions. Several promising leaf shape candidate genes were found under each QTL.
• **Conclusions** The evolution of divergent leaf shape has taken place multiple times in the *M. guttatus* species complex and is associated with the occupation of dry, rocky environments. The genetic architecture of elongated and lobed leaves is similar across three species in this group. This may indicate that parallel genetic evolution from standing variation or new mutations is responsible for the putatively adaptive leaf shape variation in *Mimulus*.

Key words: Leaf shape, genetic architecture, parallel evolution, leaf boundary layer, *Mimulus guttatus* species complex, *M. guttatus*, *M. laciniatus*, *M. nudatus*, edaphic specialists, bulk segregant analysis, QTL mapping.

INTRODUCTION

An organism's form is intimately related to its physiological and biomechanical function. Given this close relationship, morphological variation has fascinated evolutionary biologists since Darwin. Within plants, leaf shape has been one of the best-studied morphological characters due to its extensive variation across the angiosperms. There is a tremendous diversity in leaf form within genera, and leaf shape polymorphisms often segregate within species and populations (Wyatt and Antonovics, 1981; Bright and Rausher, 2008; Jones *et al*., 2009). Convergence on similar leaf shapes is also frequently observed across genera and species, and this provides an excellent opportunity to study the genetics of parallel phenotypic evolution. Whether convergent phenotypes have the same genetic underpinnings can inform us about the predictability of evolution and the extent of genetic constraint (Williams, 1957; Cooley and Willis, 2009; Stern, 2013). Two main questions have arisen from the impressive diversification in leaf shape: is this variation adaptive and what is its genetic basis? Here we focus on the latter by examining multiple independent instances of leaf shape divergence within and between species in the *Mimulus guttatus* species complex.

The adaptive significance of leaf shape has long interested botanists and evolutionary biologists alike (Parkhurst *et al*., 1968; Vogel, 1968; Givnish, 1987; Nicotra *et al*., 2011). Leaves are the major photosynthetic organs of a plant and thus their shape affects an array of important physiological processes, and consequently plant fitness. Many functional consequences of leaf shape have been discussed in the literature, but two major themes arise: (1) its impact on hydraulic efficiency and leaf water potential (reviewed by Nicotra *et al*., 2011) and (2) its effect on leaf temperature through changes in the boundary layer (Vogel, 1970; Givnish, 1979; Gurevitch and Schuepp, 1990; Schuepp, 1993). Leaf hydraulic resistance (R_{leaf}) accounts for 30 % of the total resistance that water encounters on its route through the plant (Nicotra *et al*., 2011). This is because leaves contain a series of veins of decreasing size and hydraulic resistance increases exponentially with decreasing vein diameter. Thus, minor veins provide the majority of the resistance to water flowing through a plant (Zwieniecki *et al*., 2006).

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As resistance increases along the path of evaporation, water potential (ψ) becomes more negative making tissues with high resistance more prone to wilting and water stress (Yapp, 1912). Because lobed leaves have fewer minor veins than entire ones, they are expected to have increased hydraulic efficiency and thus be less prone to water stress, properties advantageous in dry environments (Thoday, 1931; Givnish, 1979).

Leaf shape also affects the thickness of the boundary layer, a region of immobile air adjacent to the leaf's surface, which in turn affects the rate of gas and heat exchange between the leaf and its environment (Schuepp, 1993). Boundary layer thickness increases with distance from a leaf's windward edge and consequently rounded, entire leaves have thicker boundary layers than elongated or dissected leaves (Vogel, 1970; Givnish, 1979; Schuepp, 1993; Nobel, 2005). A thin boundary layer increases the efficiency of convective heat exchange between the leaf and its environment. During the day leaves are heated above ambient temperature by direct solar radiation. This increase in leaf temperature can cause protein denaturation and decrease photosynthetic efficiency (Berry and Björkman, 1980; Crafts-Brander and Salvucci, 2000). A reduced leaf boundary layer could be advantageous in exposed, dry environments because leaves can stay cool without having to transpire as much as those with thicker boundary layers (Nobel, 2005).

As the temperature or moisture environment may vary within an individual's lifetime or with microenvironment, leaf shape within or among individuals can be highly plastic, particularly in response to density or seasonal cues (Vogel, 1968; Ghent, 1973; Tsukaya, 2005; Sack *et al*., 2006; K. Ferris and K. Toll, unpubl. res.). Although plasticity can make leaf shape a more complex trait to study genetically, substantial progress in understanding the molecular genetic mechanisms underlying leaf shape diversity has been made in the past several decades. Quantitative trait locus (QTL) mapping studies in tomato, eggplant, soybean and cotton have demonstrated that many loci of small effect often underlie leaf shape variation within domesticated species (Jiang *et al*., 2000; Yamanaka *et al*., 2001; Frary *et al*, 2003, 2004). Variation in leaf shape has been characterized to the level of individual genes in several species including *Arabidopsis thaliana*, *Cardamine hirsuta* and tomato. These studies have found that changes in the leaf margin such as serration, lobing or leaflet production are often due to similar genetic mechanisms across species, indicating that leaf shape evolution can be predictable at the molecular level (Bharathan *et al*., 1999; Koenig and Sinha, 2010; Scarpella *et al*., 2010; Nicotra *et al*., 2011). However, most of these studies were performed in crop and model species. Considerably less is known about the genetic architecture of leaf shape variation in natural populations (but see Kimura *et al*., 2008; Bright and Rausher, 2008). Are leaf shape differences in nature due to many loci of small effect, or a few of large effect? Do similar leaf shapes have similar genetic architectures across species?

The *Mimulus guttatus* species complex is an excellent system for studying the genetics and evolution of leaf shape diversity. This closely related group of highly inter-fertile wild flowers varies in leaf shape and occurs across a wide ecological spectrum. A wealth of genetic tools has been developed for this group, including the fully sequenced and annotated genome of *M. guttatus* (Wu *et al*., 2007; www.phytozome.org). The most striking differences in leaf shape in the complex occur between *M. guttatus*, *Mimulus nudatus*, *Mimulus laciniatus* and the newly described *Mimulus filicifolius*. *Mimulus guttatus* has rounded entire leaves and occurs in perennially moist streams and seeps (Fig. 1A, B). *Mimulus nudatus* has very narrow leaves and is endemic to dry, rocky serpentinitic soils that are toxic to most plant species (Fig. 1E, F). *Mimulus laciniatus* and *M. filicifolius* possess highly lobed leaves and occur on patches of moss in exposed granite outcrops that are subject to severe seasonal drought (Fig. 1C, D; Peterson *et al*., 2013; Ferris *et al*., 2014). In addition to the independent evolution of lobed and narrow leaf shapes in different species of the complex, we have recently discovered a population of *M. guttatus* (M2L) that is polymorphic for lobed leaf shape and occurs on a serpentinite outcrop in western California (Fig. 1E, F).

Due to its wide geographical range and high levels of genetic diversity, *M. guttatus* is believed to be the progenitor of other species in the complex (Sweigart and Willis, 2003; Modliszewski and Willis, 2012). Therefore, rounded entire leaves are inferred to be the ancestral state in the *M. guttatus* species complex while the lobed and narrow leaf margins of *M. laciniatus*, *M. nudatus*, *M. filicifolius* and M2L are inferred to be derived. As discussed above, narrow and lobed leaves have thinner boundary layers than the rounded entire leaves of *M. guttatus*, which should allow them to be cooled more effectively by convection (Givnish, 1979; Schuepp, 1993; Nobel, 2005). They also should have higher hydraulic efficiency. The association between leaf shapes that reduce the leaf boundary layer and increase hydraulic efficiency and the occupation of hot, dry rocky habitats in the *M. guttatus* species complex suggests that these changes in shape are driven by adaptation to stressful local habitats. The repeated independent evolution of modified leaf shape in this group allows us to examine whether similar genetic changes underlie potentially adaptive leaf shape diversity in these closely related species.

To investigate the genetics of leaf shape evolution in the *M. guttatus* species complex we used a QTL mapping approach. We created mapping populations by crossing *M. guttatus* to *M. laciniatus*, *M. guttatus* to *M. nudatus*, and a lobe-leaved individual from the M2L population to a round-leaved *M. guttatus*. We attempted to create a mapping population with *M. filicifolius*, but the F_1 hybrids in our crosses were sterile (Ferris *et al*., 2014). We used bulk segregant analysis (BSA) combined with next-generation sequencing (Magwene *et al*., 2011) to quickly map major QTLs for lobed leaf shape in our *M. laciniatus* cross. To look for parallel genetic evolution in our *M. nudatus* and M2L mapping populations we used single PCR-based markers located within our *M. laciniatus* QTL regions.

MATERIALS AND METHODS

Crossing design and phenotypic analysis

To investigate whether leaf shape diversity among these closely related species was generated through similar genetic pathways, we created QTL mapping populations using inbred lines of *M. guttatus*, *M. nudatus* and *M. laciniatus*. The *M. laciniatus* inbred line WLF47 was crossed to the *M. guttatus* inbred line IM62 to generate F_1s that were then self-fertilized to produce 650 F_2s. An F_2 mapping population of 108 individuals was

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FIG. 1. Photographs of (A) an *M. guttatus* leaf, (B) typical *M. guttatus* seep habitat, (C) *M. laciniatus* (left) and *M. filicifolius* (right) leaves, (D) typical *M. laciniatus* and *M. filicifolius* granite outcrop habitat, (E) *M. nudatus* (left) and M2L (right) leaves, and (F) typical *M. nudatus* and M2L (*M. guttatus*) serpentine outcrop habitat.

created for narrow leaf shape by crossing the inbred line DHRO of *M. nudatus* × MED of *M. guttatus* and self-fertilizing F_1s. A lobed leaf line from the M2L population was crossed to IM62 to produce F_1s that were self-fertilized to produce an F_2 mapping population of 416 individuals. All seeds were cold stratified for at least 1 week at 4 °C. *Mimulus laciniatus* parents and hybrids were stratified for 10 d. All plants except the M2L F_2s were grown in the Duke Biology Greenhouses in Fafard 4P potting mix in 2·5-inch pots under 16-h days. M2L × IM62 F_2s were grown at the University of Virginia greenhouses in Fafard 3B potting mix in 2·5-inch pots under 16-h days.

Each of these F_2 mapping populations was grown up alongside parents and F_1s in the greenhouse and phenotyped for leaf shape. The first or second true leaf was collected from each plant in a grow-out, taped to a piece of white paper and digitally scanned. The first and second true leaves do not systematically differ in leaf shape. Narrow leaf shape in the *M. nudatus* × *M. guttatus* cross was quantified by digitally measuring the length and width of each leaf and computing the length to width ratio for the parental and F_2 generations in the program ImageJ v1.47 (Rasband, 2012). Lobed leaf shape was quantified for both *M. laciniatus* and M2L crosses in ImageJ using a convex-hull analysis. In this analysis ImageJ creates a convex-hull shape by connecting the outermost points of each leaf (Supplementary Data Fig. S1). To determine the degree of leaf lobing the area of the actual leaf was subtracted from the area of the leaf's convex-hull and then divided by the area of the convex-hull to control for size. The petiole was removed from each leaf because petiole length segregated independently from lobing and proved to be a confounding factor when quantifying leaf shape. This measurement was then log transformed to normalize the variance in the parental generations. Histograms of

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leaf shape distributions were created in R (http://www.r-project.org/) and the degree of overlap between the parental and F_2 distributions was examined to estimate the genetic complexity. Substantial overlap should indicate genetic simplicity because the fewer loci involved in a trait the greater the variance in the F_2 compared with the difference between the parental phenotypes (Castle, 1921; reviewed by Lynch and Walsh, 1998). Broad sense heritability was calculated using the equation $H^2 = V_G/V_P$. V_G was calculated by subtracting the average variance in the parental lines (V_E) from the total variance in the F_2 (V_P; Falconer & MacKay, 1996).

QTL mapping using BSA and next-generation sequencing

To map lobed leaf shape in the *M. laciniatus* × *M. guttatus* F_2 population we used a BSA procedure where allele frequencies at single nucleotide polymorphism (SNP) markers in selected pools of individuals are estimated via Illumina sequencing (Magwene *et al.*, 2011; Friedman and Willis, 2013). We created two pools of 100 F_2s representing the extremes of the phenotypic distribution, a highly lobed pool and un-lobed pool. Equal amounts of leaf and bud tissue were collected from each individual. DNA was extracted from each F_2 using a modified CTAB protocol (Kelly and Willis, 1998). DNA from each individual was then combined to form two pooled samples, one lobed and one unlobed. Each pooled sample was sequenced in one lane of an Illumina Genome Analyzer IIx machine using 75-bp single end reads at the Duke University Sequencing and Analysis Core Resource.

BSA has long been used to roughly map QTLs of moderate to large effect (Michelmore *et al.*, 1991). With the advent of next generation sequencing it is possible to quickly and cost effectively generate dense SNP markers across the entire genome. Performing BSA with dense SNP markers makes it possible to quickly map QTLs at a finer scale (Magwene *et al.*, 2011). To map QTLs involved in the lobed leaf shape of *M. laciniatus* we used a custom pipeline developed by Friedman *et al.* (2015). First sequence read files from each pooled sample were aligned to the IM62 *M. guttatus* reference genome (www.phytozome.net) using BWA (Li and Durbin, 2010), and then SAMtools (Li *et al.*, 2009) was used to create an mpileup file. When calling SNPs we ignored sites with <4× coverage and more than two alleles segregating or where either allele was present in only one read. SNPs were called either 'IM62' (*M. guttatus*) or 'other' based on the reference genome sequence.

We calculated differences in allele frequency between the lobed and unlobed pools (~5× coverage on average in each pool) using a sliding window procedure. We first binned groups of SNPs into intervals containing a minimum of 250 reads with assigned paternity in each bulk (Friedman *et al.*, 2015). For sliding windows of ten intervals and a step size of one interval, we then calculated the difference in IM62 allele frequency between the un-lobed and lobed pools across the genome. For SNP intervals unlinked to leaf shape variants, no difference in allele frequency between leaf shape pools is predicted. In contrast, markers that are closely linked to QTLs should differ noticeably in allele frequency. To detect significant QTL regions we performed a non-parametric test of allelic frequency that accounts for sampling effects of read coverage and bulk size (Magwene *et al.*, 2011). A modified G-statistic was calculated for individual intervals and then smoothed for each sliding window (G'). *Z*-scores and *P*-values were calculated based on an empirical estimate of an underlying log-normal distribution of the observed data (Magwene *et al.*, 2011). *P*-values were corrected for multiple testing at a false discovery rate of 0·05 using the 'fdrtool' package (Strimmer, 2008) in R version 3.0.2 (http://www.r-project.org/). QTLs were further verified by additional PCR-based marker analysis (see below).

Single marker QTL analysis

BSA does not allow us to make inferences about the phenotypic effects of individual QTLs. To verify our bulk segregant QTLs and determine the effect size and dominance interactions at individual *M. laciniatus* leaf shape QTLs, we genotyped 300 random IM62 × WLF47 F_2s at three genic markers under each QTL. We screened parental inbred lines for polymorphism at exon-primed intron crossing (EPIC) markers derived from expressed sequence tags (ESTs) (Fishman *et al.*, 2008). Polymorphism at each marker was determined by variation in PCR fragment length, which is usually due to insertion/deletion (indel) variation in introns. Primers for these markers can be found on the Mimulus Evolution website (http://www.mimulusevolution.org). PCR products were analysed by capillary electrophoresis and fragment analysis on an ABI 3730 × l DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Fragment size was scored in the program GeneMarker (Soft Genetics, State College, PA, USA.).

We verified bulk segregant QTLs by testing marker–phenotype associations using a one-way analysis of variance (ANOVA) in JMP v9 (SAS, Cary, NC, USA) at each marker with marker genotype as the independent variable and leaf shape as the dependent variable. Effect size was estimated at each QTL in two ways: (1) by calculating the proportion of the segregating variation in leaf shape (R^2) explained by the most significant marker and (2) by calculating the proportion of the difference between the parents explained by each locus. We calculated the second measure of effect size by dividing the difference in leaf shape between the homozygotes at each locus by the difference between the parental means. Genotypic values for each QTL were calculated as the mean leaf shape value for each genotype. Additive (a) and dominance (d) effects were calculated for each QTL from the midpoint between the genotypic values of the homozygotes. The degree of dominance was calculated as d/a (Conner and Hartl, 2004). To test for epistasis between QTLs we looked for significant interaction terms in a multifactor ANOVA in JMP.

To determine whether divergent leaf shapes in *M. nudatus*, the M2L *M. guttatus* population and *M. laciniatus* have a similar genetic basis we performed single marker analysis in M2L and *M. nudatus* F_2 populations in our known *M. laciniatus* leaf shape QTL regions. A caveat of this approach is that because we did not use markers spanning the entire *Mimulus* genome we cannot detect QTLs involved in leaf shape in *M. nudatus* and M2L that are not shared with *M. laciniatus*. We genotyped 108 *M. nudatus* × *M. guttatus* and 384 M2L × IM62 F_2s at three polymorphic markers in the genomic region beneath each *M. laciniatus* QTL. One-way ANOVAs were performed in JMP to

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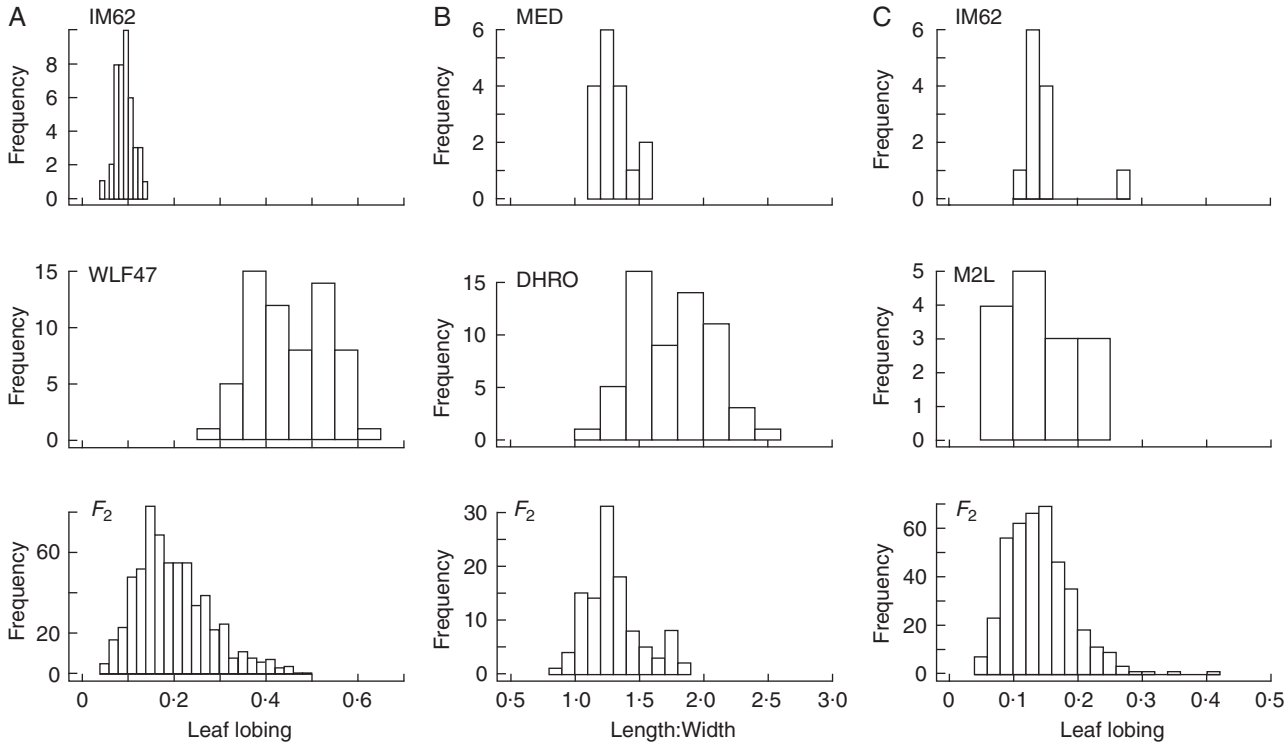

FIG. 2. Leaf shape distributions for the three mapping crosses. (A) Leaf shape measured with convex hull analysis on the IM62 *M. guttatus* inbred line, the WLF47 *M. laciniatus* inbred line and an IM62×WLF47 F_2 population. (B) Leaf shape measured using the length to width ratio in the MED *M. guttatus* inbred line, the DHRO *M. nudatus* inbred line and an MED × DHRO F_2 population. (C) Leaf shape measured with convex hull analysis on the IM62 inbred line, the M2L inbred line and an IM62 × M2L F_2 population.

determine whether there was a statistically significant association between marker genotype and leaf shape phenotype in each cross. Effect size, genotypic value, and additive and dominance effects of, and epistasis between, each QTL were determined as described above in our *M. laciniatus* single marker analysis.

Candidate gene analysis

To identify candidate genes for leaf shape beneath our QTLs, we downloaded all predicted coding sequences in each significant region using the *M. guttatus* v2.0 genome annotation (Phytozome v10.0) and then determined their closest homologue in *Arabidopsis thaliana* (TAIR10_pep_20101214_updated annotation) by blastx. Based upon known gene function of *A. thaliana* and tomato homologues, we selected the top candidates for leaf lobing and elongation from the larger list. Using our bulk segregant data, DNA sequences of the coding region of each of these candidates were then analysed using Integrative Genomics Viewer (Robinson *et al.*, 2011). Non-synonymous SNPs were identified between our lobed and unlobed DNA sequence pools in regions with 3 × coverage or greater.

RESULTS

Analysis of phenotypic variation in leaf shape

The patterns of phenotypic variation observed in our parental and F_2 hybrid generations permit inferences about the underlying genetic architecture of a trait. In our *M. laciniatus* cross the phenotypic distributions of leaf lobing in our parental and F_2 generations indicate that leaf phenotype of *M. laciniatus* ($V_E = 0{\cdot}0067$) is more environmentally variable than that of *M. guttatus* ($V_E = 0{\cdot}0003$, Fig. 2). Phenotypic variation in an inbred line like WLF47 must be due entirely to environmental variance (V_E) as all individuals are genetically identical (Falconer and MacKay, 1996). Another pattern is that the F_2 leaf shape distribution overlaps substantially with both parental distributions (Fig. 2). This suggests that leaf shape is a genetically simple trait in *M. laciniatus*. The broad sense heritability of lobed leaf shape in our *M. laciniatus* × *M. guttatus* cross is 76·6 %. In our *M. nudatus* cross we see similar patterns with *M. nudatus* leaf shape being more phenotypically variable ($V_E = 0{\cdot}0857$) than that of *M. guttatus* ($V_E = 0{\cdot}0164$) and the F_2 distribution largely overlapping both parental distributions (Fig. 2). The broad sense heritability of narrow leaf shape in our *M. nudatus* × *M. guttatus* cross is 23 %. The genetics of the M2L cross are more difficult to describe from the given phenotypic distribution because the original parental line from M2L died out and could not be phenotyped in large numbers. Another, less strikingly lobed M2L line was grown with the F_2 grow-out. The subtle lobing of the M2L line chosen for the grow out combined with the serrated leaf margins of the un-lobed parent, IM62, weakened our ability to quantify differences in parental lobing (Fig. 2). However, we again see greater environmental variance in the lobed parent, M2L ($V_E = 0{\cdot}0022$), than in the round leaved parent, IM62 ($V_E = 0{\cdot}0014$). The broad sense heritability of lobed leaf shape in our M2L × IM62 cross is 21 %.

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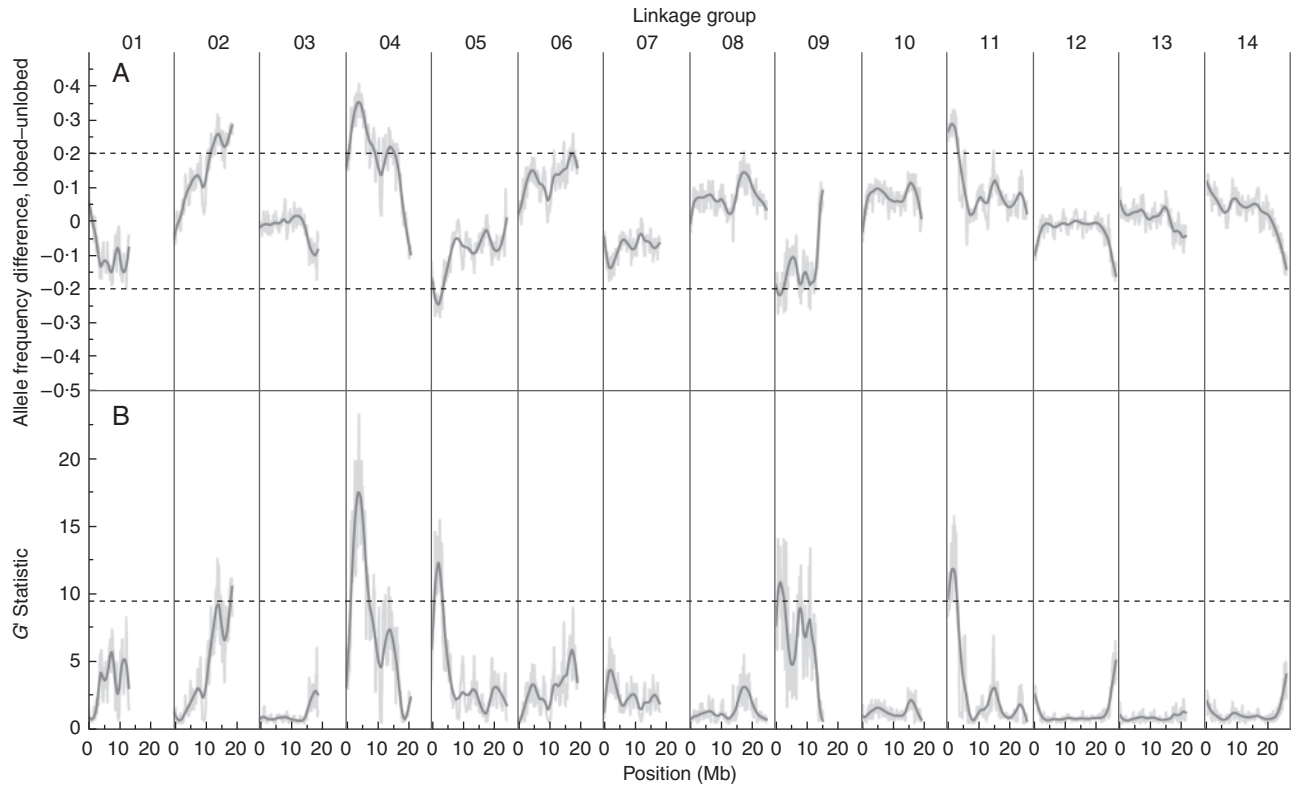

FIG. 3. Leaf shape QTL results from BSA in an *M. laciniatus* × *M. guttatus* F_2 population. (A) IM62 allele frequency difference between the lobed and unlobed bulk segregant pools. The dotted line indicates an allele frequency difference greater than 20 %. (B) A modified G' statistic for lobed leaf shape; dotted lines indicate false discovery rate corrected P-values of <0·05.

TABLE 1. *Marker and QTL data*

Experimental cross	Marker	Linkage group	Position (bp)	F ratio	R^2	Proportion of mean parental diff	Mean phenotype lobed/narrow homozygote	Mean phenotype heterozygote	Mean phenotype round homozygote	Direction	a	D	d/a
M. laciniatus × *M. guttatus*	MgSTS192	2	17 592 566	10·37***	0·077	20 %	–0·694	–0·706	–0·82	+	0·063	0·051	0·8095
	MgSTS262	4	5 222 698	10·99***	0·11	22 %	–0·644	–0·699	–0·798	+	0·077	0·022	0·2857
	MgSTS644	11	778 805	2·6*	0·02	10 %	–0·68	–0·717	–0·749	+	0·0345	–0·0025	0·0725
M. nudatus × *M. guttatus*	MgSTS184	2	3 093 681	3·99*	0·043	31 %	1·356	1·305	1·21	+	0·073	–0·022	0·3014
	MgSTS267	4	3 048 720	9·511**	0·102	51 %	1·469	1·316	1·227	+	0·121	0·032	0·2645
M2L × IM62	MgSTS530	2	18 312 396	3·35*	0·019	NA	0·134	0·143	0·153	–	0·0095	0·0005	0·0526
	MgSTS306	4	3 482 954	3·32*	0·02	NA	0·143	0·149	0·132	+	0·0055	–0·0115	2·0909
	MgSTS26	11	339 314	3·23*	0·019	NA	0·15	0·143	0·129	+	0·0105	–0·0035	0·3333

For each experimental cross we have listed the marker name, base pair position and linkage group location, the F-ratio and R^2 from logistic regressions of marker genotype on leaf phenotype, the proportion of mean parental difference (calculated by dividing the difference between the mean homozygote phenotypic values by the difference between the mean parental phenotypic values), the mean phenotype for each genotypic class in each cross at each marker, the direction of QTL effects, the additive (a) and dominance (D) effects, and the degree of dominance (d/a). *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$.

The genetic architecture of leaf shape in M. laciniatus

Using a combination of BSA and SNP markers generated through Illumina sequencing we mapped five putative leaf shape QTLs in our *M. laciniatus* × *M. guttatus* F_2 population (Fig. 3). We were able to confirm three of these QTLs with single marker analysis in a random selection of F_2s (Table 1). Markers within bulk segregant QTL intervals on linkage groups (LGs) 5 and 9 were not significantly associated with leaf shape variation (P-values: 0·232, 0·302) and are thus not included in our remaining analyses. The size of and number of genes located within each significant QTL region can be found in supplementary Table S1. The largest effect QTL is located on LG4 ($R^2 = 11$ %), the second largest effect on LG2 ($R^2 = 7·7$ %), and a QTL of smaller effect on LG11 ($R^2 = 2$ %). The combined effects of these three loci account for less than one-third of the broad sense heritability in this cross ($H^2 = 76·6$ %). However, all three loci explain 52 % (LG4 = 22 %, LG2 = 20 %, LG11 = 10 %) of the difference in

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mean parental leaf shape. QTLs on LG2 and LG4 cover wide genomic regions (~300 kb – 4·5 Mb, Supplementary Data Table S1) and have multiple significant peaks. This could be due either to multiple loci underlying each QTL or to poor resolution of the physical map of these two chromosomes. We found no evidence of epistasis between any of these three genomic regions. The *M. laciniatus* allele is partially dominant at QTLs on LG2 ($d/a = 0{\cdot}809$) and LG4 ($d/a = 0{\cdot}287$, Table 1). The heterozygotes at the LG11 QTL have intermediate leaf lobing, indicating that this locus is additive ($d/a = 0{\cdot}072$).

Parallel evolution at the QTL level

We found evidence of parallel leaf shape evolution at the QTL level in both our *M. nudatus* and M2L mapping populations. Markers located within *M. laciniatus* QTLs on LGs 2 and 4 were significantly associated with narrow leaf shape in the *M. nudatus* × *M. guttatus* F_2 and markers beneath all three QTLs (LG2, LG4, LG11) were significantly associated with lobed leaf shape in the M2L F_2. *Mimulus nudatus* leaf shape QTLs were of small and moderate effect (LG2 = 4%, LG4 = 10%, Table 1) and together these two loci account for over half of the broad sense heritability ($H^2 = 22$ %). All EPIC markers located near the QTL peak on LG2 displayed significant segregation distortion in our *M. nudatus* × *M. guttatus* F_2. This distortion is why the marker we used to estimate effect size on LG2, MgSTS184, is outside the main QTL region (Table 1). Together the QTLs on LG2 and LG4 account for 82 % of the difference between the mean parental leaf shape values (LG2 = 31 %, LG4 = 51 %). Elongated leaves are partially dominant to round leaves at both *M. nudatus* QTLs ($d/a = 0{\cdot}301$ (LG2), $d/a = 0{\cdot}264$ (LG4)). We did not detect evidence of epistasis between *M. nudatus* leaf shape QTLs. The R^2 values for the QTLs in the M2L × IM62 F_2 population were of equal and small effect (LG2 = 2 %, LG4 = 2 %, LG11 = 2 %). In total these three QTLs account for less than one-third of the broad sense heritability in M2L leaf shape. We were unable to calculate the proportion of the mean parental difference for each of these loci due to loss of the true M2L parental line. The M2L leaf lobing QTL on LG2 was negative, meaning that homozygotes with the IM62 (un-lobed parent) allele were more lobed than homozygotes with the M2L (lobed parent) allele. Lobed leaf shape displayed partial dominance at LG11 ($d/a = 0{\cdot}333$) and overdominance at LG4 ($d/a = 2{\cdot}09$), but acted additively at LG2 ($d/a = 0{\cdot}053$). There was no evidence of epistasis between any of the M2L leaf shape QTL.

Candidate genes for leaf shape diversity in the M. guttatus *species complex*

All annotated genes located within each QTL interval defined by our BSA are reported in Supplementary Data Table S2. Here, we discuss several promising candidate genes that co-localize with our leaf shape QTLs and are homologous to genes relevant to leaf shape and development in other species. We also note when *M. laciniatus* and *M. guttatus* differ by non-synonymous SNPs that could alter protein structure and potentially leaf morphology. We acknowledge that further fine mapping and functional tests will be necessary to determine which of these candidates or other genes in our QTL intervals contribute to leaf shape diversity in *M. laciniatus*, *M. nudatus* and *M. guttatus*.

Within the LG2 QTL region, there are homologues of two genes involved in leaf development in *A. thaliana*: *BYPASS 1* (*BPS1*) (Van Norman *et al.*, 2004) and *BETA AMYLASE 2* (*BAM2*) (DeYoung *et al.*, 2006). The QTL region on LG4 contains strong candidates for variation in leaf lobing in the genes *KNOX ARABIDOPSIS THALIANA MIENOX* (KNATM) (Kimura *et al.*, 2008), *LIGHT SENSITIVE HYPOCOTYL 3* (LSH3) (Ichihashi *et al.*, 2014) and *LEAFY* (LFY) (Koenig and Sinha, 2010) as well as a good candidate for leaf elongation, *ROTUNDIFOLIA9* (ROT9) (Hay and Tsiantis, 2010). Beneath the QTL on LG11 there are homologues of two genes involved in leaf development: *YABBY 5* (YAB5) (Bartholmes *et al.*, 2012) and *KNOTTED-1 LIKE HOMEOBOX DOMAIN 3* (KNAT3) (Serikawa *et al.*, 1997).

To further analyse these candidates we compared the coding sequences of each of the above genes between the lobed and unlobed DNA pools from the *M. laciniatus* × *M. guttatus* F_2 population to identify non-synonymous SNPs. Only two candidate genes contained non-synonmous SNPs. We found one SNP (A to G, Scaffold 4: 6351 371 bp) in the second exon of *KNATM* that changed an isoleucine in the unlobed pool to a methionine in the lobed leaf pool (Supplementary Data Table S3). A non-synonymous SNP (G to A, Scaffold 2: 18 192 146 bp) was identified in Exon 5 of *BAM2* that changes a glycine in the unlobed pool to an arginine in the lobed leaf pool (Table S3). These are both derived changes in *M. laciniatus*.

DISCUSSION

In this study we have investigated the genetic basis of divergent leaf morphology in three closely related edaphic specialists in the *Mimulus guttatus* species complex. We found that divergent leaf shapes are more environmentally variable than *M. guttatus*' ancestral round leaf. Using QTL mapping, we determined that the genetic architecture of leaf shape is quantitative in all three species. Most interestingly, we discovered a large degree of parallelism between the genetic architecture of lobed leaves in *M. laciniatus*, narrow leaves in *M. nudatus* and lobed leaves in the M2L population of *M. guttatus* at the QTL level. We discuss these results in the context of the ecological and genetic literature on the evolution of leaf morphology.

Members of the M. guttatus *species complex with divergent leaf shapes occupy harsh habitats*

Most members of the *M. guttatus* species complex, such as the putative progenitor *M. guttatus*, occur in perennially moist habitats (Wu *et al.*, 2007). However, several species have colonized and adapted to relatively dry, harsh habitats. *Mimulus laciniatus* and *M. filicifolius* occur in granite outcrops in the Sierra Nevada of California. These outcrops have thin rocky soils that are significantly drier than adjacent seeps and streams where *M. guttatus* occurs (Ferris *et al.*, 2014). The onset of severe summer drought causes granite outcrops to completely dry out a month earlier than nearby *M. guttatus* habitat (Peterson *et al.*, 2013). *Mimulus nudatus* is endemic to serpentinitic soils in Lake and Napa county, Califiornia, and the lobed M2L population of

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M. guttatus occurs on a serpentinite outcrop in Tehama county, California. Serpentinitic soils have a high heavy metal content and an abnormal calcium to magnesium ratio. These characteristics make serpentine toxic to the majority of plant species (Macnair and Gardner, 1998). Serpentinitic soils are also nutrient poor and fast draining compared with the typical *M. guttatus* habitat, making them hot and dry in a manner similar to granite outcrops. Each of these edaphic specialists has independently evolved a divergent leaf shape (lobed or elongated) that should reduce the leaf hydraulic resistance and boundary layer compared with the rounded, entire leaves of the mesic *M. guttatus*. This suggests that leaf shape is involved in adaptation to dry, harsh environments in the *M. guttatus* species complex.

Genetic architecture of leaf shape in the M. guttatus *species complex*

Despite substantial plasticity in leaf shape we were able to detect several QTLs of moderate to small effect in each of our crosses (*M. laciniatus* × *M. guttatus*, *M. nudatus* × *M. guttatus*, and M2L × IM62). Given the overlap between our parental and F_2 phenotypic distributions we initially predicted that leaf shape would be genetically simple. Instead, we found that leaf shape is highly quantitative in the *M. guttatus* species complex. There was substantial missing heritability in each cross after the combined effects of the QTLs were accounted for, with less than one-third of the heritability explained in the *M. laciniatus* and M2L F_2 populations.

There are two factors that probably contribute to this missing heritability. First, because our BSA could only coarsely resolve the QTL regions, recombination events between our EPIC markers and the causal mutation(s) could have significantly reduced the detected value of R^2 (Falconer and MacKay, 1996). Alternatively, the low proportion of leaf shape variation explained by our identified QTLs may be due to the contribution of additional small effect QTLs undetectable by BSA.

Our finding that leaf shape variation has a complex genetic basis differs from recent work in other natural systems such as the ivy-leaved morning glory (Campitelli and Stinchcome, 2013) or between closely related species in the Solanaceae (Kimura *et al*., 2008) and Brassicaceae (Hay and Tsiantis, 2006), where differences in leaf dissection were due to single loci. Kimura *et al*. (2008) found that the increase in leaf complexity between two wild Galapagos tomato species, *Solanum cheesmaniea* and *Solanum galapagense*, was due entirely to a single mutation in the promoter region of the KNOX gene *PETROSELINUM*. However, the genetic complexity we find in *Mimulus* agrees with the body of literature from crop species where the genetic architecture of leaf shape variation is often highly quantitative (Jiang *et al*., 2000; Yamanaka *et al*., 2001; Frary *et al*., 2003, 2004). The complex nature of leaf shape genetic architecture in the *M. guttatus* species complex makes it even more interesting that divergent leaf shapes, and specifically lobed leaves, seem to have evolved more than once in closely related species.

Evidence of parallel evolution at the QTL level

The shared genetic architecture of leaf shape variation in our *M. laciniatus*, *M. nudatus* and the *M. guttatus* M2L population crosses provides evidence of parallel leaf shape evolution at the QTL level. QTL regions on LG2 and 4 were significantly associated with variation in leaf shape in all three of our F_2 mapping populations. This overlap was initially unexpected because the narrow leaf shape of *M. nudatus* is phenotypically distinct from the lobed leaf shape of *M. laciniatus* and M2L. However, upon closer consideration of our convex hull leaf shape metric, it seems that we may be capturing leaf elongation in addition to lobing in the crosses where lobing segregates. Both *M. laciniatus* and the lobed form of M2L have leaves that are more elongated than typical *M. guttatus*. An explanation for this genetic correlation may be that the LG2 or LG4 QTL regions contain two (or more) separate loci that affect leaf elongation and lobing independently, a particularly plausible hypothesis for the LG4 QTL given the variety of candidate genes involved in leaf development found within that region. Alternatively, elongation and lobing variation may in fact be controlled by one pleiotropic locus. Further functional studies will be necessary to determine whether QTL overlap is due to pleiotropy or multiple linked genes with independent phenotypic affects.

All three of *M. laciniatus*' leaf shape QTLs were significantly associated with leaf shape variation in the M2L population of *M. guttatus*. The M2L population is polymorphic for lobed leaf shape. The fact that lobed leaves of M2L and *M. laciniatus* have overlapping genetic architectures raises the possibility that *M. laciniatus* leaf shape was derived from segregating variation in *M. guttatus*. This may explain why lobed leaf shape has evolved multiple times in the *M. guttatus* species complex in *M. laciniatus* and *M. filicifolius* (Ferris *et al*., 2014). Similar scenarios have occurred in other systems such as the evolution of stickleback armour and reduced pigmentation in cavefish (Stern, 2013). In stickleback fish the freshwater form has evolved reduced lateral plate size multiple times during the independent colonization of rivers through selection on segregating variation in the ancestral marine population at the EDA locus (Colosimo *et al*., 2005). Similarly, segregating lobe-leaved variants could have been repeatedly selected upon when the ancestor of the *M. guttatus* species complex colonized dry, rocky habitats such as granite outcrops and serpentinitic soils.

Alternatively M2L and *M. laciniatus* may have evolved lobed leaves independently, but through similar genetic mechanisms. This could be evidence of mutational bias or evolutionary constraint via negative pleiotropy. There are examples of this type of parallel evolution throughout the literature, including the evolution of red flowers in morning glories, lactase persistence in humans and cardenolide resistance across multiple species of insects (reviewed by Stern, 2013). We cannot distinguish between selection on segregating leaf shape variation versus selection on independent mutational events in the *M. guttatus* species complex because (1) we have yet to identify the causal loci and mutations underlying the different leaf shapes, and (2) we do not understand the genetic basis of *M. filicifolius*' lobed leaves due to post-zygotic reproductive isolation between it and other members of the species complex (Ferris *et al*., 2014). Nevertheless, we have demonstrated that the evolution of narrow and lobed leaves involved the same genetic regions in three members of the *M. guttatus* species complex, and this is a critical step toward describing the evolutionary process that has resulted in this striking, repeated pattern of morphological adaptation.

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Candidates for the genetic basis of leaf shape diversity in the M. guttatus *species complex*

We found several promising leaf shape candidate genes within each of our three QTL regions. On LG2, homologues of the *A. thaliana genes BPS1* and *BAM2* were identified within the QTL region. *BPS1* is involved in leaf development and regulation of the shoot apical meristem (SAM) (Van Norman *et al*., 2004). *BAM1*, *BAM2* and *BAM3* are required for proper development of leaf vasculature, shape, size and symmetry, and they are also critical for male gametophyte and ovule development (DeYoung *et al*., 2006). In exon 5 of *BAM2* we found an SNP in the coding sequence that changed a glycine in the unlobed bulk segregant pool to an arginine in the lobed pool. Glycine is a neutral non-polar amino acid, while arginine is basic and polar. Consequently, this change in amino acid chemistry probably alters the tertiary structure of the *BAM2* protein in *M. laciniatus*.

Our first leaf shape candidate in the LG4 region is a homologue of *ROT9*. *ROT9* is in the same gene family as, and is functionally related to, *ROT3* and *ROT4*, which are involved in leaf elongation through cell expansion and proliferation in *A. thaliana* (Hay and Tsiantis, 2010). Either loss of function mutations in or overexpression of *ROT4* causes leaves to be short and wide (Tsukaya, 2006). *ROT9* appears to be a particularly good candidate gene for the elongated leaf phenotype in *M. nudatus*.

There are several strong candidates on LG4 involved in natural variation in leaf lobing. The first is the KNOX gene *KNATM*, which is a homologue of *PETROSELINUM* (PTS) in tomato. The up-regulation of *PTS* is responsible for divergence in leaf complexity between two wild species of tomato (Kimura *et al*., 2008). KNOX genes regulate the SAM and from experiments in several compound-leaved species it has been determined that they create the indeterminate growth environment necessary for leaflet initiation (reviewed by Koenig and Sinha, 2010). We identified a non-synonymous SNP in the 2nd exon of *KNATM* that changes an isoleucine in the unlobed leaf pool to a methionine in the lobed pool. Both of these amino acids are neutral and non-polar, but it is still possible that this change alters the *KNATM* protein structure and function. LSH3 is also located under our LG4 QTL and directly regulates *PTS* expression in tomato, making it another excellent candidate for divergence in leaf lobing (Ichihashi *et al*., 2014). *LFY* (LG4) is a critical gene in the transition from vegetative to floral tissue, and has also been shown to be necessary for compound leaflet development in several legume species (Koenig and Sinha, 2010). *LFY* knock-out mutants cause a complete reversion from compound to simple leaves in *Medicago truncatula* (Wang *et al*., 2008).

There are also several good leaf shape candidates beneath our smaller effect QTL on LG11. *KNAT3* is another member of the KNOX gene family that regulates the SAM and is expressed in the developing leaf (Serikawa *et al*., 1997). YAB5 is a member of the YABBY transcription factor family that is important in root and shoot development in flowering plants (Bartholmes *et al*., 2012). YAB5 is expressed in developing leaf and floral organs and is necessary for the establishment of marginal leaf domains, the development of the leaf lamina, maintenance of leaf polarity and distribution of auxin maxima in *A. thaliana* (Sarojam *et al*., 2010). These auxin maxima drive the formation of lateral organs from the SAM and vasculature, serrations, lobes and leaflets in the developing leaf (Koenig and Sinha, 2010; Scarpella, 2010). These candidate genes and SNPs should provide a strong foundation for future functional work on the molecular genetic basis of leaf shape diversification in the *M. guttatus* species complex.

CONCLUSIONS

Leaf shape diversity is extensive across and within angiosperm species. This is fascinating because of the effect of shape on leaf hydraulic resistance and the boundary layer, which subsequently affect plant physiology and fitness. Similar leaf shapes have evolved many times independently across species. We found that leaf shape is a quantitative trait in the *M. guttatus* species complex, and despite that complexity, we determined that the independent evolution of narrow and lobed leaves in three edaphic specialists has a similar genetic architecture. Thus, our investigation of the genetic basis of divergent leaf shape in the *M. guttatus* species complex has illustrated a degree of genetic parallelism in the evolution of a putatively adaptive trait.

SUPPLEMENTARY DATA

Supplementary Data are available online at www.aob.oxfordjournals.org and consist of the following. Fig. S1: binary scan of the area of a lobed *M. laciniatus* × *M. guttatus* F_2 leaf, illustration of the convex hull area of an F_2 leaf, and the convex hull measurement of a round *M. guttatus*. Table S1: length of and number of genes in each significant *M. laciniatus* × *M. guttatus* QTL region. Table S2: complete list of annotated *Mimulus* genes located under each of the three significant bulk segregant QTL regions including *Mimulus* ID number, *A. thaliana* orthologue and predicted gene function. Table S3: amino acid sequences from the *M. guttatus* and *M. laciniatus* homologues of the leaf shape candidate genes *BAM2* (Exon 5) and *KNATM* (Exon 2).

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