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Speciation on a local geographic scale: the evolution of a rare rock outcrop specialist in *Mimulus*

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Speciation can occur on both large and small geographical scales. In plants, local speciation, where small populations split off from a large-ranged progenitor species, is thought to be the dominant mode, yet there are still few examples to verify speciation has occurred in this manner. A recently described morphological species in the yellow monkey flowers, *Mimulus filicifolius*, is an excellent candidate for local speciation because of its highly restricted geographical range. *Mimulus filicifolius* was formerly identified as a population of *M. laciniatus* due to similar lobed leaf morphology and rocky outcrop habitat. To investigate whether *M. filicifolius* is genetically divergent and reproductively isolated from *M. laciniatus*, we examined patterns of genetic diversity in ten nuclear and eight microsatellite loci, and hybrid fertility in *M. filicifolius* and its purported close relatives: *M. laciniatus*, *M. guttatus* and *M. nasutus*. We found that *M. filicifolius* is genetically divergent from the other species and strongly reproductively isolated from *M. laciniatus*. We conclude that *M. filicifolius* is an independent rock outcrop specialist despite being morphologically and ecologically similar to *M. laciniatus*, and that its small geographical range nested within other wide-ranging members of the *M. guttatus* species complex is consistent with local speciation.

1. Introduction

Most species are formed in allopatry, but allopatric speciation can occur on a variety of geographical scales. Speciation may happen on a broad scale such as when a species is split by a cataclysmic geographical event like the uplift of a mountain range or the rise of the Isthmus of Panama. The populations on either side of the divide will diverge, eventually forming new sister species [1–4]. Alternatively, speciation could occur on a local geographical scale with small populations splitting off and diverging from a large progenitor species' range [5–9]. In plants, it has been argued that speciation happens mostly in small populations [9–13]. One reason given for this is that plants' sessile lifestyle restricts gene flow geographically compared with animals [12,14,15]. Frequent adaptation to local conditions (reviewed in [16,17]) and the repeated evolution of self-fertilizing mating systems [10] also seem highly relevant to the pervasiveness of local speciation in plants. In fact, evidence for local speciation has recently been found in several plant groups within North America [18,19]. When plant populations adapt to local conditions this differential adaptation may lead to forms of reproductive isolation, and eventually speciation. Self-fertilization assures the reproductive success of colonizing plants in the absence of con-specific neighbours [20] and may maintain favourable combinations of locally adaptive alleles [21,22]. Self-fertilization is also an effective reproductive isolating barrier from a nearby outcrossing relative [23–26].

One of the best-studied examples of local speciation in plants is that of the species pair *Stephanomeria exigua* ssp. *coronaria* and *Stephanomeria malheurensis* [9,27–29]. *Stephanomeria exigua* ssp. *coronia* is a highly outcrossing species with a large geographical range extending from southern California through Oregon, whereas *S. malheurensis* occurs only at one location at the very northern

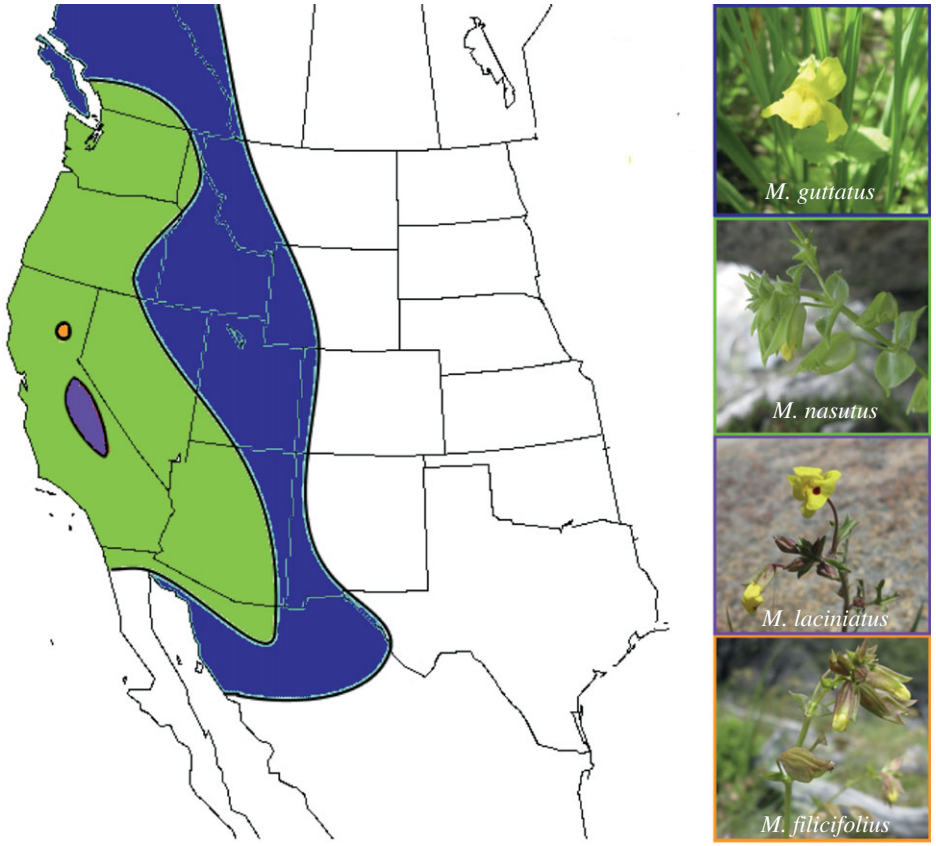

Figure 1. Geographical distributions of *M. guttatus* (indigo, green, purple and orange), *M. nasutus* (green, purple and orange), *M. laciniatus* (purple) and *M. filicifolius* (orange). *Mimulus guttatus*' distribution encompasses the ranges of the other three taxa. (Online version in colour.)

end of *S. exigua's* range [9]. The two species are highly similar morphologically, but using allozymes Gottlieb determined that the self-fertilizing *S. malheurensis* contains a distinct subset of the genetic variation present in the outcrossing ssp. *coronaria* indicating that *S. malheurensis* is a recent derivative of ssp. *coronia*. Gottlieb also found that the two species were reproductively isolated by differences in mating system, chromosomal rearrangements and Dobzhansky–Muller incompatibilities, despite genetic and geographical proximity [27]. In his 2003 review of local, or as he calls it progenitor-derivative, speciation Gottlieb [9] argues that this type of speciation is common in plants.

The *Mimulus guttatus* species complex is a closely related group of morphologically and ecologically diverse species with numerous genetic resources including the completely sequenced and annotated genome of *M. guttatus* (www.phytozome.net [30]). Like *Stephanomeria*, it is an excellent system for the study of local geographical speciation, because it consists of the large-ranged outcrossing putative progenitor species *M. guttatus*, and many geographically restricted morphological species that are often self-fertilizing and adapted to specialized edaphic environments [30]. The evolutionary history of the species complex is largely unresolved owing to recent divergence and ongoing interspecific introgression [31]. Recently, a new morphological species has been described in the complex, *Mimulus filicifolius* [32]. *Mimulus filicifolius* was originally categorized as a geographically disjunct population of *M. guttatus*' close relative *Mimulus laciniatus*. *Mimulus laciniatus* is a highly self-fertilizing annual that occurs above 900 m in the central and southern Sierra Nevada mountains of California, USA (figure 1). *Mimulus laciniatus* and *M. filicifolius* do closely resemble each other since both species have highly lobed leaves, small flowers and are endemic to similar dry, exposed rock outcrop habitat. They are the only two species with dissected leaves in the entire genus *Mimulus*. *Mimulus filicifolius* was described as a new species owing primarily to its more finely divided bi-pinnately compound leaves [32]. It is restricted to a few populations in eastern Butte and western Plumas counties in the northern foothills of the Sierra Nevada 150 km away from any known *M. laciniatus* population (figure 1) [32]. However, it is not known whether *M. filicifolius* is genetically distinct and/or reproductively isolated from *M. laciniatus*, or whether it is simply a morphologically divergent variety.

To address this question, we examined patterns of genetic variation in four members of the *M. guttatus* species complex: *M. guttatus*, *M. nasutus*, *M. laciniatus* and *M. filicifolius*. *Mimulus guttatus* is a genetically diverse outcrossing species that occurs in moist seeps and streams across much of western North America (figure 1). *Mimulus nasutus* is a highly self-fertilizing species that also lives in seeps and streambeds primarily along the west coast of North America from British Columbia to northern Mexico. These two closely related species have overlapping geographical ranges that encompass the geographical range of both *M. laciniatus* and *M. filicifolius* (figure 1) [31]. We included *M. guttatus* and *M. nasutus* in this analysis to see whether *M. filicifolius* was more genetically similar to *Mimulus* species in close geographical proximity than to *M. laciniatus*. In this study, we use a combination of ecological measurements, molecular population genetics, flow cytometry (FCM) and interspecific crosses to address three main questions: (i) Does the recently described *M. filicifolius* differ from *M. laciniatus* in ecology, mating system or genome size as well as in morphology? (ii) Is *M. filicifolius* genetically distinct from *M. laciniatus*? (iii) Is *M. filicifolius* reproductively isolated from *M. laciniatus* or other members of the *M. guttatus* species complex?

2. Material and methods

(a) Habitat characterization and collections of four *Mimulus* species

Although *M. guttatus*, *M. nasutus*, *M. laciniatus* and *M. filicifolius* overlap a great deal in their geographical ranges, they seem to occupy very distinct habitats within those ranges. To quantitatively characterize each species' habitat, we measured three environmental variables (per cent soil moisture, soil saturation point and ground temperature) in four populations of *M. guttatus*, two of *M. nasutus*, seven of *M. laciniatus* and one of *M. filicifolius*. We measured environmental variables at only one *M. filicifolius* location (Bald Rock (BR), the type locality of the species where several thousand individuals occur) because it was the only site where plants could be located during the study period. We chose to measure these three variables because they should capture much of the abiotic environmental variation between rocky outcrop and grassy seep habitats. Soil moisture and soil saturation point were measured with a Decagon soil moisture probe, while ground temperature was measured using an infrared thermometer.

Environmental variables were measured across three to five transects per species habitat per population site. This means that if both *M. guttatus* and *M. laciniatus* occurred at a single site then three to five transects would be measured in the *M. laciniatus* habitat and another three to five would be measured in the *M. guttatus* habitat. Transects were approximately 1.5 m long. Measurements were taken adjacent to one or more plants at five regularly spaced intervals along each transect. We chose nine population sites that varied in elevation (approx. 3000–7000 ft) and geographical location: BR (*M. filicifolius*, 3020 ft), Sandy Bluff (*M. laciniatus* and *M. guttatus*, 3100 ft), Cedar Vista (*M. guttatus*, 3369 ft), Peterson Road (*M. laciniatus*, *M. nasutus* and *M. guttatus*, 4123 ft), Willow Creek (*M. laciniatus*, *M. nasutus* and *M. guttatus*, 3395 ft), Central Camp Road (*M. laciniatus*, 4182 ft), Dinky Creek (*M. laciniatus* and *M. guttatus*, 6075 ft), Shaver Lake (*M. laciniatus* and *M. guttatus*, 5231 ft) and Huntington Lake (*M. laciniatus*, 7122 ft). All measurements took place between 10 May and 31 May 2010. Each population (except BR) was surveyed on two to three separate occasions over the 21 days at different times of day. Given environmental variation among and within sites, measuring each site multiple times should produce a better estimate of the consistent differences between species environments. At this time of year, low-elevation sites were beginning to dry out, whereas mid- and high-elevation sites still contained ample amounts of snow melt. In order to test whether species habitats differed significantly in these three variables, we performed three factorial analyses of variance (ANOVAs) with each environmental variable as the dependent variable and species and site as the independent variables. In order to control for repeated measures of each population site over the three week period of our field study, we averaged our environmental measurements at each position of each transect across time. To examine which species differed significantly in each variable, we performed Tukey HSD tests on each of our ANOVAs.

(b) Comparing the mating system and genome size of *Mimulus filicifolius* and *Mimulus laciniatus*

We used the populations described in table 4 to characterize the mating system and genome size of the new rock outcrop species *M. filicifolius* for comparison with *M. laciniatus*. To characterize the mating system of *M. filicifolius*, we genotyped individuals in the BR population of *M. filicifolius* and five *M. laciniatus* populations at 11 co-dominant markers. Tissue for DNA extraction was collected from plants grown from field-collected seed in the greenhouse. We used three single-copy, nuclear-gene-intron-length markers [33–35] and eight microsatellite markers [36]. Marker sequences are listed in table S4 of Sexton *et al*. [37]. PCR products were analysed with an ABI 3730 DNA Analyzer and size polymorphisms were visually scored in GeneMarker (SoftGenetics LLC, State College, PA, USA). All markers were located on different linkage groups and therefore represent genetically independent loci. In order to compare heterozygosity and inbreeding levels between *M. filicifolius* and *M. laciniatus*, we estimated the observed heterozygosity (*Ho*), the expected heterozygosity (*He*) and the mean fixation index (F). We used the mean fixation index to calculate the rate of self-fertilization (S) within each population with the equation $S = 2F/(1 + F)$. We also tested for Hardy–Weinberg equilibrium within populations (sites). Population genetic estimates were averaged across loci for all populations (table 5). We used GenAlEx v. 6 [38] to calculate population genetics estimates and to test for Hardy–Weinberg equilibrium within populations. Allele frequencies for co-dominant markers are given in the electronic supplementary material, table S2.

We obtained genome size estimates of *M. filicifolius* from the BR population [32] and *M. laciniatus* from two populations approximately 60 km apart: Devil's Postpile National Monument (37.6238, −119.0854) and Grand Bluff (GB) in the Sierra National Forest (electronic supplementary material, table S1). For each population, approximately 30 seeds from a single maternal family of one field-collected plant were pooled for FCM analysis with three (much larger) seeds of an internal standard, *Solanum lycopersicum*, having a diploid (2C) genome size of 1.96 pg [39]. Approximate genome size was then estimated through comparison with the internal standard by the general relationship: genome size (bp) $= (0.978 \times 10^9) \times$ DNA content (pg) [40]. Additionally, *M. filicifolius* seeds were pooled with the GB population of *M. laciniatus* seeds and analysed using FCM to verify genome size differences through analysis of double peaks [41]. Sample and solution preparation and FCM analyses were performed as in McIntyre [42]. FCM data were visualized using Cyflogic data analysis tool (v. 1.2.1; http://www.cyflogic.com/).

(c) Is *Mimulus filicifolius* genetically distinct from *Mimulus laciniatus*?

In order to determine whether *M. filicifolius* is a genetically distinct species, we sampled populations across the geographical ranges of *M. guttatus*, *M. nasutus*, *M. laciniatus* and *M. filicifolius*. We collected between 15 and 50 individuals per population from eight *M. laciniatus*, five *M. guttatus* and one *M. filicifolius* population(s) across the Sierra Nevada (electronic supplementary material, table S1). All 30 *M. nasutus* and the other 47 *M. guttatus* populations used in this analysis are from previous collections [31,43]. Samples were derived from live plants that were propagated in the greenhouse and self-fertilized to produce seed. The progeny of these seed families were grown in the greenhouse under uniform conditions where tissue was collected for DNA extraction. Three of our *M. laciniatus* populations (GB, Black Point (BP) and Snow Trail (ST)) and the BR population of *M. filicifolius* were used in both sequencing and microsatellite analyses (tables 1 and 5). *Mimulus nasutus* and *M. guttatus* populations were only used in our sequencing analysis.

To examine patterns of genetic variation in these four species, portions of seven nuclear genes were amplified in populations from across the range of *M. guttatus*, *M. nasutus*, *M. laciniatus* and *M. filicifolius* by polymerase chain reaction (PCR). Genomic DNA was isolated from leaf and bud tissue using a modified CTAB extraction protocol [36]. Primers for the CYCLOIDEA-A (CYCA) locus were developed from a portion of the gene's first exon [31]. The other six loci, *Mg1–6*, were developed by Modliszewski & Willis [43] from exonic regions of single copy genes. They were chosen because they represent the average sequence divergence between *M. nasutus* SF and *M. guttatus* IM62 [43]. We used

Table 1. Results of a one-way analysis of variance (ANOVA) with each environmental variable as the dependent variable and species as the independent variable. We report the degrees of freedom (d.f.), sum of squares (Sum Sq), *F*-statistic (*F*) and *p*-value from each ANOVA.

environmental variable	model	d.f.	Sum Sq	*F*-value	*p*-value
% soil moisture	species	2	9954.66	86.8	<0.0001
	site	9	5969.41	11.57	<0.0001
	error	532	30504.9		
soil saturation point	species	2	1.37	80.59	<0.0001
	site	8	1.27	18.74	<0.0001
	error	433	3.68		
ground temperature	species	2	3724.98	46.29	<0.0001
	site	8	9783.19	30.39	<0.0001
	residual	456	27809		

previously published sequence data from 30 *M. nasutus* and 47 *M. guttatus* individuals [31,43] and added sequences from our eight *M. laciniatus*, five *M. guttatus* and single *M. filicifolius* populations (electronic supplementary material, table S3). PCR conditions, primers and all available sequence data can be found in Modliszewski & Willis [43].

Mimulus laciniatus is a highly self-fertilizing species, and therefore we expect the majority of its genome to be homozygous [44]. Consequently, a single individual from each *M. laciniatus* population was directly sequenced at each of the above nuclear loci. When chromatograms were examined, we did not find any double peaks that could be due to heterozygosity, confirming that *M. laciniatus* was homozygous at all loci examined. As *M. guttatus* is a genetically diverse outcrossing species, we expected it to be heterozygous at many of these loci. All *M. guttatus* individuals were first directly sequenced, and when chromatograms were examined we did see double peaks indicative of heterozygosity. Then PCR products were cloned into the pGEM-T Easy Vector system. Six colonies from each individual were PCR amplified and direct sequenced to identify both alleles at each locus and check for PCR-generated errors such as point mutations or recombination. DNA sequences from each of the seven loci were aligned in Sequencher (Gene Codes Corp., Anne Arbor, MI, USA). Chromatograms were used to identify and correct erroneous polymorphism. Ambiguous insertion/deletion polymorphisms were removed from the dataset by eye using MacClade (© 2011 Maddison & Maddison [45]). Basic polymorphism and divergence data such as the number of segregating sites (S), nucleotide diversity (π) and pairwise nucleotide diversity between species (dxy) were obtained using the program DNAsp [46].

To investigate the degree of genetic similarity across species, we performed principal components analysis (PCA) on our sequence data and created gene trees at each of our seven nuclear loci. In order to perform our PCAs, we used the R package adegenet 1.3-8 [47]. PCA is a distance-based method that clusters DNA sequences based on their genetic similarity. We performed two genetic PCA: one on the CYCA locus and one on a concatenated alignment combining the six *M. nasutus*-based EST loci (*Mg1–6*). The CYCA locus was left out of the concatenated analysis, because it did not contain enough overlapping individuals with the *Mg* alignment. The *M. guttatus* and *M. nasutus* population dataset for the CYCA locus largely does not overlap with that of the *Mg1–6* loci. This is because the CYCA *M. guttatus* and *M. nasutus* dataset was generated by Sweigart & Willis [31], while the *Mg1–6* sequences for these two species were generated by Modliszewski & Willis [43]. Including the CYCA data allows us to compare the patterns of genetic variation between two independent sets of populations from the same four species. We computed 95% confidence intervals (95% CI) for the mean genetic value of *M. guttatus*, *M. nasutus* and *M. laciniatus* along all three major principal component (PC) axes to determine their genetic similarity. As we only had a single sequence of *M. filicifolius* in our analysis, we could not compute confidence intervals for its PC values. In order to determine whether *M. filicifolius* was genetically distinct from the other three species, we compared its location along each PC axis to the 95% CIs of the other three species.

Gene trees were constructed at each locus using the general time reversible (GTR + I+ G) model of sequence evolution with a maximum-likelihood approach in Garli v. 2.0 [48]. Support for the resulting trees was determined using a 50% maximum-likelihood bootstrap (BS) criterion in Garli.

(d) Is there post-zygotic reproductive isolation between *Mimulus filicifolius* and the *Mimulus guttatus* species complex?

In order to ascertain whether post-zygotic reproductive isolation existed between the new species, *M. filicifolius*, and members of the *M. guttatus* species complex crosses were made between inbred lines of *M. laciniatus* and *M. filicifolius*, and *M. guttatus* and *M. filicifolius*. In each cross, F_1 hybrids were self-fertilized either by hand or automatically when the cross involved two self-fertilizing taxa. Hybrid fertility was assessed in each cross with *M. filicifolius* by counting the number of fruits containing viable seeds that developed post self-fertilization. In our *M. laciniatus* × *M. filicifolius* cross, we examined between four and six autonomously pollinated fruits per plant on 60 individual F_1s. In the *M. guttatus* × *M. filicifolius* cross, we attempted to pollinate 36 ovules by hand on six F_1 plants and checked three to four ovules per plant on 32 F_1s.

3. Results

(a) Do the geographically disjunct *Mimulus filicifolius* and *Mimulus laciniatus* have quantitatively similar niches?

To better understand whether the geographically disjunct rock outcrop specialists *M. filicifolius* and *M. laciniatus* have ecological niches that are more similar to each other than to nearby *M. guttatus* and *M. nasutus*, we characterized the habitat of each species. To test whether these species' habitats differed significantly in soil moisture and ground temperature, we

Table 2. Means and standard errors for per cent soil moisture (% sm), soil saturation point (sp) and ground temperature (gt) for species in our analysis.

species	mean % sm	s.e. % sm	mean sp	s.e. sp	mean gt (°C)	s.e. gt
M. filicifolius	18.4	1.96	34.8	1.81	22.5	0.92
M. laciniatus	12.4	0.47	23.4	0.63	19.3	0.65
M. guttatus	21.5	0.85	32.1	1.53	17.6	0.58
M. nasutus	25.2	1.92	44.3	2.76	16.6	1.23

Table 3. Results of Tukey HSD test of the significance of difference between species means for per cent soil moisture, soil saturation point and ground temperature.

species comparison	difference % soil moisture	difference soil sat. point	difference ground temp.
M. guttatus versus *M. filicifolius*	3.90	0.12	−4.93*
M. laciniatus versus *M. filicifolius*	−5.02**	−9.92***	−3.25
M. nasutus versus *M. filicifolius*	6.77**	9.5**	−5.95*
M. laciniatus versus *M. guttatus*	−8.92***	−10.04***	1.68
M. nasutus versus *M. guttatus*	2.86	9.38***	−1.02
M. nasutus versus *M. laciniatus*	11.79***	19.42***	−2.70

*$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table 4. Population location and genetic summary statistics for 11 loci for *Mimulus filicifolius* and *M. laciniatus* collection sites in the Sierra Nevada, California. Population genetic statistics include the number of samples per site (N), observed heterozygosity (H_o), expected heterozygosity (H_e), mean fixation index (F) and the rate of self-fertilization (S).

site	*N*	latitude	longitude	*F*	H_e	H_o	*S*
Mimulus filicifolius							
BR	28	39.6445	−121.3427	0.93	0.59	0.04	0.96373057
Mimulus laciniatus							
GB	33	37.0746	−119.2299	0.86	0.76	0.11	0.924731183
BP	46	37.2384	−119.2599	0.95	0.63	0.03	0.974358974
JM	41	37.5069	−119.3387	0.91	0.63	0.06	0.952879581
HS	43	37.8939	−119.849	0.92	0.73	0.07	0.958333333
ST	46	37.7663	−119.5421	0.88	0.73	0.09	0.936170213
mean	41.8	—	—	0.9	0.72	0.07	0.949294657

performed three factorial ANOVAs. We found that species differed significantly in per cent soil moisture, soil saturation point and ground temperature (table 1). Our post hoc comparisons using the Tukey HSD test indicate that *M. nasutus* and *M. guttatus* occur in habitats with similar ground temperatures (means = 16.6°C, 17.6°C) and levels of soil moisture (means = 25.2%, 22.3%, tables 2 and 3). *Mimulus laciniatus*' habitat is warmer than *M. guttatus* and *M. nasutus*' (mean = 19.3°C) and significantly drier than all three other species (mean = 13.4%, tables 2 and 3). *Mimulus filicifolius*' habitat is similar to *M. laciniatus*' in ground temperature (mean = 22.5°C) and is significantly warmer than *M. nasutus* and *M. guttatus*' and drier than *M. nasutus*' (mean = 18.4%, tables 2 and 3). Thus, we see that overall *M. filicifolius* and *M. laciniatus*' rock outcrops are significantly warmer and drier than *M. guttatus* and *M. nasutus*' seeps.

(b) Does *Mimulus filicifolius* differ from *Mimulus laciniatus* in mating system or genome size?

In our mating system characterization of *M. filicifolius* and *M. laciniatus*, we found that all genetic markers successfully amplified across populations and were highly polymorphic. Marker scores were consistent across repeatability tests. *Mimulus laciniatus* and *M. filicifolius* have similarly high levels of inbreeding and self-fertilization indicating that they have similar mating systems. As expected for self-fertilizing species, all loci in all *M. filicifolius* and *M. laciniatus* populations were not at Hardy–Weinberg equilibrium ($p < 0.001$). The fixation index (F) for *M. filicifolius* was 0.93 and the mean fixation index for the five *M. laciniatus* localities was 0.90 (table 4). We used the equation $S = 2F/(1 + F)$ to calculate S, the rate of self-fertilization, in each population [49]. One caveat is that

Table 5. Sequence polymorphism statistics for each locus and species: S stands for the number of segregating sites and pi is the estimate of 4Nu using average number of nucleotide differences per site.

locus	size (bp)	S *M. laciniatus*	S *M. nasutus*	S *M. guttatus*	S *M. filicifolius*	π *M. laciniatus*	π *M. nasutus*	π *M. guttatus*	π *M. filicifolius*
CYCA	425	3	13	23	n.a.	0.0033	0.0055	0.0102	n.a.
Mg1	362	3	2	2	n.a.	0.0021	0.0009	0.0009	n.a.
Mg2	364	3	0	12	n.a.	0.0028	0.0013	0.0079	n.a.
Mg3	370	2	2	10	n.a.	0.0016	0.0037	0.0096	n.a.
Mg4	408	2	2	7	n.a.	0.0017	0.0007	0.0022	n.a.
Mg5	456	6	9	28	n.a.	0.0048	0.0048	0.0063	n.a.
Mg6	495	11	0	25	n.a.	0.0059	0	0.0082	n.a.

this estimate of *S* assumes that it is at equilibrium and this may not in fact be the case. The rate of self-fertilization (*S*) for *M. filicifolius* was 0.96, which is similar to the average *S* of our *M. laciniatus* populations, 0.95 (table 4).

To assess whether the newly described species *M. filicifolius* was divergent in overall genome size, we performed FCM analysis on a cohort of *M. laciniatus* populations and the BR population of *M. filicifolius*. The sampled *M. filicifolius* family had a mean genome size estimate of 0.65 pg or 315 Mb (CV = 1.41%), whereas the *M. laciniatus* samples averaged 0.72 pg or 360 Mb (CV = 2.04%) for the Devils Postpile population using the internal standard; and 0.85 pg or 367 Mb (CV = 0.79%) for the Grand Bluff population (estimated from double-peak analysis; electronic supplementary material, figure S1 and table S1). Our genome sizes are smaller than those estimated for close relatives *M. guttatus* and *M. nasutus* by Modliszewski & Willis [43]; however, this could be due to the fact that they used petunia as a genome size standard while we used tomato. From these estimates, genome size differs in the range of 10–14% between *M. filicifolius* and *M. laciniatus* and approximately 4% between the two *M. laciniatus* populations sampled (electronic supplementary material, figure S1).

(c) Determining whether *Mimulus filicifolius* is a genetically distinct species

The loci used in this analysis showed varying amounts of genetic diversity across *Mimulus* species. CYCA, *Mg2*, *Mg5* and *Mg6* had the greatest levels of informative genetic variation, whereas *Mg1* had the least (table 5). The greatest proportion of molecular variation at each locus was explained by polymorphism within *M. guttatus*. To determine whether *M. filicifolius* is genetically as well as morphologically divergent, we calculated pairwise nucleotide diversity (*dxy*) between each species, performed genetic PCA and created maximum-likelihood gene trees at CYCA and our six *Mg* nuclear loci [43]. We found several interesting patterns. As our measure of interspecific genetic divergence, we calculated the pairwise nucleotide diversity, *dxy*, between species in six one-way comparisons. At five out of seven loci, *dxy* was higher in the *M. filicifolius* versus *M. laciniatus* (*dxy* = 0.0180–0.0303) and *M. filicifolius* versus *M. nasutus* (*dxy* = 0.0146–0.0309) comparisons than when *M. filicifolius* was compared with *M. guttatus* (*dxy* = 0.0005–0.0178) or when *M. guttatus*, *M. nasutus* and *M. laciniatus* were compared with each other (table 6). This suggests that at the DNA sequence level *M. filicifolius* is more divergent from *M. laciniatus* than either of the other two taxa and that *M. filicifolius* is the least diverged from *M. guttatus*.

In our genetic PCA of the CYCA locus, PCs 1, 2 and 3 explained 30.7, 12.4 and 10.5% of the genetic variance, respectively, while in the concatenated *Mg* loci analysis PCs 1, 2 and 3 explained 9.6, 5.7 and 5.3% of the genetic variance. In both our PCAs, at the CYCA locus and the consensus sequence of the *Mg* loci, our *M. filicifolius* population is genetically divergent from *M. laciniatus* in terms of the first three PCs (figures 2 and 3). In fact, *M. filicifolius* does not cluster with any of the other three species, but instead remains genetically distinct from *M. laciniatus*, *M. nasutus* and *M. guttatus* in all analyses (figures 2 and 3). Our single *M. filicifolius* sample is outside the 95% confidence intervals (95% CI) of *M. guttatus*, *M. nasutus* and *M. laciniatus* at PCs 1, 2 and 3 (table 7). *Mimulus filicifolius* is particularly

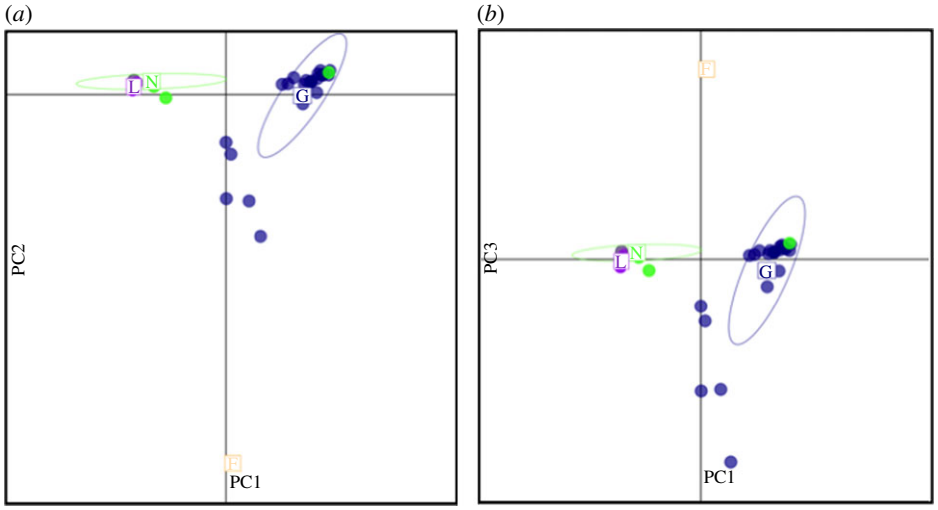

Figure 2. PCA of *M. guttatus* (indigo, G), *M. nasutus* (green, N), *M. laciniatus* (purple, L) and *M. filicifolius* (orange, F) genetic diversity at the CYCA locus. (*a*) A plot of genetic pc scores at the CYCA locus along axes PC1 and PC2. (*b*) A plot of genetic pc scores at the CYCA locus along axes PC1 and PC3. (Online version in colour.)

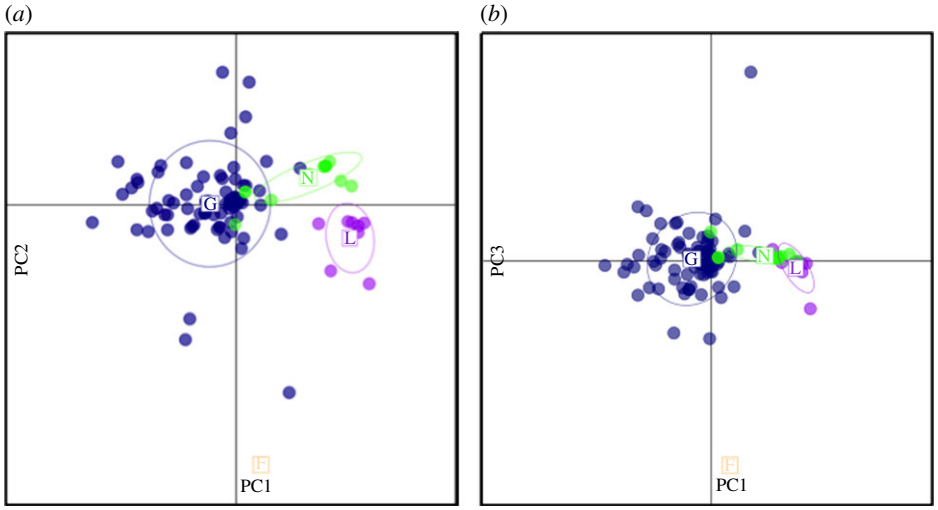

Figure 3. PCA of *M. guttatus* (indigo, G), *M. nasutus* (green, N), *M. laciniatus* (purple, L) and *M. filicifolius* (orange, F) genetic diversity on a concatenated alignment of six *M. nasutus*-based EST loci, *Mg1–6*. (*a*) A plot of genetic pc scores for the concatenated *Mg* loci along axes PC1 and PC2. (*b*) A plot of genetic pc scores for the concatenated *Mg* loci along axes PC1 and PC3. (Online version in colour.)

Table 6. Average pairwise sequence divergence (*dxy*) between *M. guttatus, M. nasutus, M. laciniatus* and *M. filicifolius* at each of our seven nuclear loci (CYCA and *Mg1–6*).

	dxy						
species comparison	CYCA	*Mg1*	*Mg2*	*Mg3*	*Mg4*	*Mg5*	*Mg6*
M. guttatus versus *M. nasutus*	0.0258	0.0005	0.0050	0.0172	0.0027	0.0044	0.0054
M. guttatus versus *M. laciniatus*	0.0275	0.0008	0.0117	0.0160	0.0047	0.0074	0.0063
M. nasutus versus *M. laciniatus*	0.0086	0.0014	0.0179	0.0021	0.0014	0.0136	0.0119
M. guttatus versus *M. filicifolius*	0.0278	0.0005	0.0059	0.0214	0.0069	0.0138	0.0144
M. nasutus versus *M. filicifolius*	0.0309	0.0146	0.0221	0.0019	0.0027	0.0218	0.0202
M. laciniatus versus *M. filicifolius*	0.0303	0.0180	0.0207	0.0046	0.0053	0.0213	0.0213

divergent from *M. laciniatus* by being more than 3 s.d. away from the mean *M. laciniatus* value for PCs 1, 2 and 3.

As another method of determining whether *M. filicifolius* was a distinct genetic species, we estimated gene trees at each of our nuclear loci. These trees are meant to indicate the overall genetic similarity of populations of *M. guttatus, M. nasutus, M. laciniatus* and *M. filicifolius*. BS support values were low (many nodes had less than 50% BS support) across

Table 7. Genetic PCA summary statistics. For both the concatenated *Mg* loci (*Mg1–6*) and CYCA datasets the mean, standard deviation and 95% confidence interval (95% CI) were calculated for PC1, PC2 and PC3 in each species.

		PC1			PC2			PC3		
locus	species	mean	s.d.	95% CI	mean	s.d.	95% CI	mean	s.d.	95% CI
Mg1–6	*M. guttatus*	−1.84	2.86	[−2.84, −1.21]	0.08	2.98	[−0.59, 0.74]	0.2	2.97	[−0.46, 0.86]
	M. nasutus	5.1	2.6	[3.71, 6.48]	1.92	1.22	[1.27, 2.57]	0.53	0.64	[0.19, 0.87]
	M. laciniatus	8.05	1.22	[7.03, 9.07]	−2.34	1.74	[−3.79, −0.88]	−0.66	1.67	[−2.06, 0.74]
	M. filicifolius	1.76	—	—	−18.35	—	—	−19.42	—	—
CYCA	*M. guttatus*	4.72	1.84	[4.06, 5.37]	−0.06	2.57	[−0.97, 0.84]	−0.79	3.59	[−2.06, 0.48]
	M. nasutus	−5.4	0.6	[−5.65, −5.16]	0.76	0.3	[0.64, 0.88]	0.42	0.37	[0.27, 0.57]
	M. laciniatus	−5.67	0.12	[−5.81, −5.52]	0.49	0.28	[0.14, 0.83]	−0.14	0.52	[−0.79, 0.51]
	M. filicifolius	0.42	—	—	−22.67	—	—	13.58	—	—

loci. This is most probably the result of using a branching diagram to represent the relationships among populations of closely related species between which there is ongoing introgression [31]. However, despite these low support values, we saw a similar pattern in our gene trees to that in our PCAs. At five out of seven loci *M. filicifolius* did not cluster with *M. laciniatus* sequences (figures 4, 5 and electronic supplementary material, S2–S6). Neither did *M. filicifolius* cluster consistently with *M. guttatus* or *M. nasutus* sequences, but instead occurred in an unsupported location at the majority of loci. Thus, it is not clear from these analyses what species *M. filicifolius* is most closely related to, but it is genetically distinct from *M. laciniatus* despite its similar habitat, level of self-fertilization and lobed leaf shape.

(d) Is *Mimulus filicifolius* reproductively isolated?

To ascertain whether post-zygotic reproductive isolation existed between *M. filicifolius* and other members of the species complex, we crossed this species to *M. laciniatus* and *M. guttatus*. We found that both crosses produced viable F_1 hybrids. However, in our *M. laciniatus* × *M. filicifolius* F_1s we found no fruit with viable seeds after checking hundreds of autonomously self-pollinated ovules. This was unexpected since the species have similar sized flowers and both self-fertilize automatically before flowers even open. *Mimulus laciniatus* × *M. filicifolius* F_1 flowers had no obvious morphological defects. Many fruits developed, but they were empty. Similarly, when *M. guttatus* × *M. filicifolius* F_1s were self-fertilized barely any seed was produced by either hand or autonomous self-pollination (1 out of 36 hand pollinations, 0 out of more than 100 autonomous pollinations). There were only three seeds present in the single fruit produced by hand pollination. For comparison, when three *M. laciniatus* × *M. guttatus* F_1s were self-fertilized in a separate quantitative trait locus mapping experiment a large viable F_2 population was created (thousands of seeds, 700 were planted and germinated successfully). This is evidence of a strong hybrid sterility barrier between *M. filicifolius* and both *M. guttatus* and *M. laciniatus*. Hybrid sterility is further evidence that *M. filicifolius* is a genetically divergent *Mimulus* species and provides a strong reproductive isolating barrier from nearby *Mimulus* taxa.

4. Discussion

(a) *Mimulus filicifolius* and *Mimulus laciniatus* have similar ecology, mating system and genome size

Ecological divergence between closely related species contributes directly to reproductive isolation and may be particularly important during local speciation, as it is in sympatric speciation, given the close geographical location of a newly budded species to its progenitor. This divergence also allows for the coexistence of allopatric species by reducing competition when they come back into secondary contact during range expansion [8,50]. To assess the amount of ecological divergence among four taxa in the *M. guttatus* species complex whose ranges largely overlap, we measured several important ecological variables on a fine environmental scale across populations in the Sierra Nevada of California. Both *M. laciniatus* and *M. filicifolius* occupy rock outcrop habitats. *Mimulus guttatus* and *M. nasutus* occur in seeps and streams, often adjacent to the rocky outcrops of *M. laciniatus* populations. We found that when we empirically characterize each species environment *M. guttatus* and *M. nasutus* occupy similar habitats that are cool and wet, whereas both *M. laciniatus* and *M. filicifolius* occur in relatively hot and dry habitats (tables 2 and 3). *Mimulus laciniatus* and *M. filicifolius*' ranges are geographically isolated from one another but overlap with *M. guttatus* and *M. nasutus*.

Our findings thus partially agree with the theory that closely related species should differ ecologically [8,50], since we find that *M. laciniatus* occupies a significantly hotter and drier habitat than either of its close sympatric relatives *M. nasutus* or *M. guttatus* and is thus ecologically isolated from them even though all three species occur within metres of each other. In fact, *M. laciniatus* has been shown to have a significant fitness advantage over *M. guttatus* in its local habitat [51]. *Mimulus filicifolius* occupies a significantly hotter environment than the sympatric *M. nasutus* and *M. guttatus* and its habitat is drier on average, although not significantly so. By contrast, *M. filicifolius* and *M. laciniatus* are the most genetically and geographically distant species in our analysis, but they are ecologically similar. Perhaps these two species have been able to adapt to similar environments because they are freed from

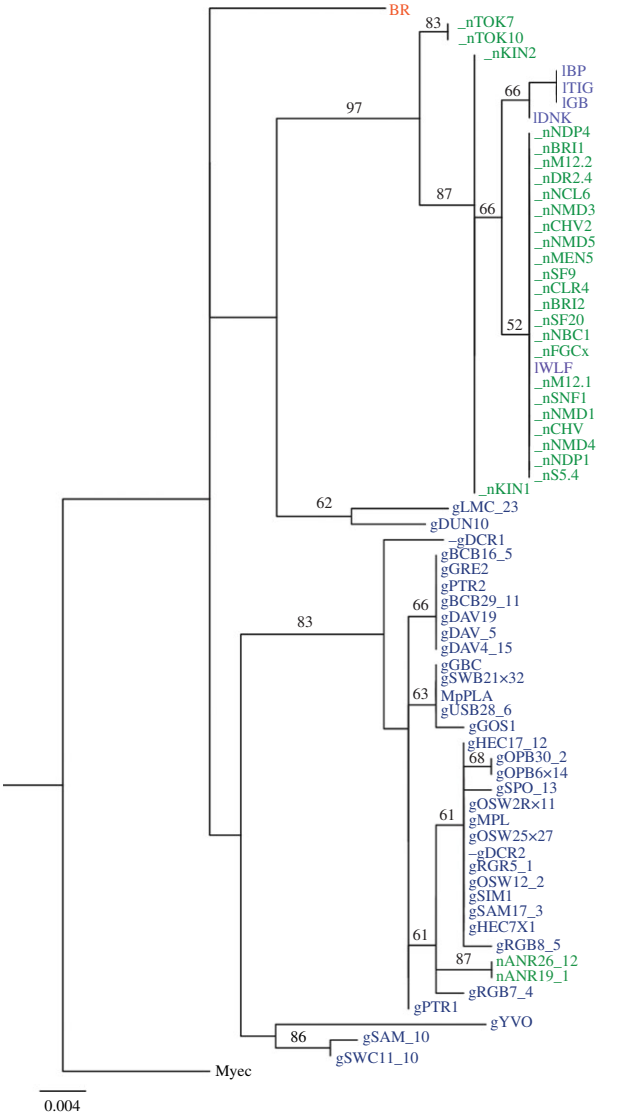

Figure 4. Maximum-likelihood gene tree at the CYCA locus of *M. guttatus* (indigo, g), *M. nasutus* (green, n), *M. laciniatus* (purple, l) and *M. filicifolius* (orange, BR) populations. Myec refers to the outgroup *M. yecorensis*. (Online version in colour.)

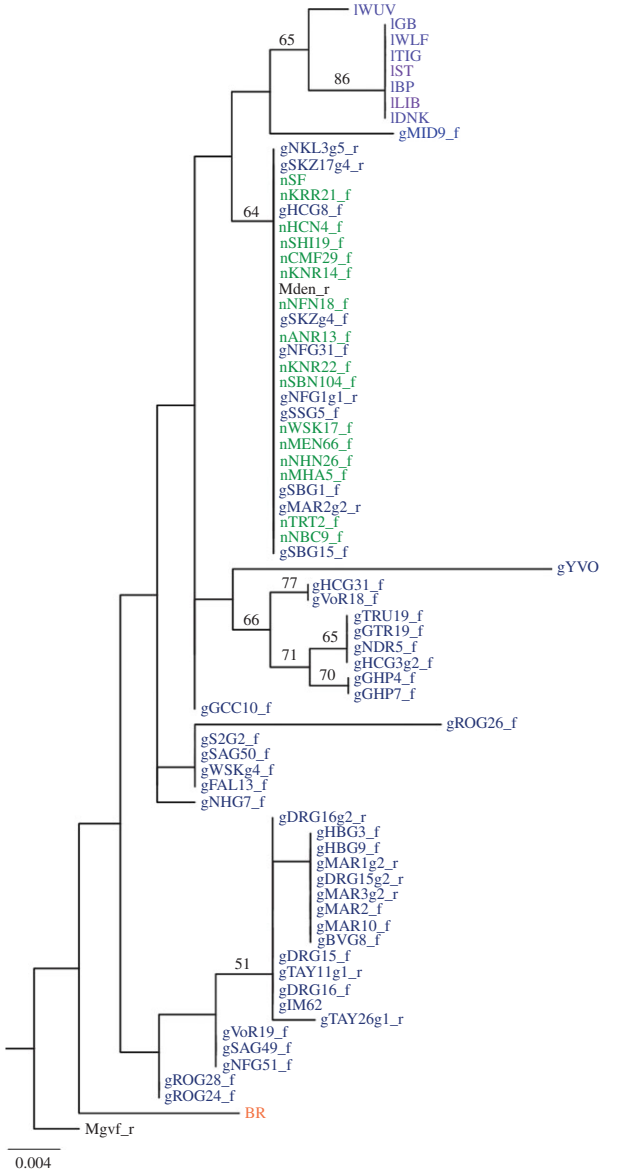

Figure 5. Maximum-likelihood gene tree at the *Mg2* locus of *M. guttatus* (indigo, g), *M. nasutus* (green, n), *M. laciniatus* (purple, l) and *M. filicifolius* (orange, BR) populations. Mgvf refers to the outgroup *M. glabratus*. (Online version in colour.)

competition and interbreeding by their geographical separation and other reproductive isolating barriers [8,50]. However, we also found that *M. guttatus* and *M. nasutus* have highly overlapping environmental niches, and these species are closely related with highly overlapping geographical ranges.

In order to further characterize the new morphological species *M. filicifolius* and assess potential sources of reproductive isolation, we compared its mating system and genome size to that of *M. laciniatus*. Both species have highly self-fertilizing mating systems and similar genome sizes. The repeated evolution across taxa of a self-fertilizing mating system from a primarily outcrossing one may allow frequent speciation in small, ecologically marginal populations. Self-fertilization allows plants to colonize a new habitat with a very small number of individuals, theoretically just one [18]. An observed pattern in plant species distributions is that self-fertilizing taxa occur in ecologically or geographically marginal habitat compared with their outcrossing relatives [52]. The evolution of self-fertilization is also an effective reproductive isolating barrier from proximate outcrossing species [23,26], because gene flow is greatly reduced between self-fertilizing populations and species [24,25,32,53]. Both *M. laciniatus* and *M. filicifolius* possess small flowers and are highly self-fertilizing with very similar levels of inbreeding. *Mimulus filicifolius*' ability to self-fertilize may have played an important role in its successful colonization of a marginal rock outcrop habitat and subsequent genetic divergence from its progenitor species [18].

(b) Patterns of genetic variation in the *Mimulus guttatus* species complex

The *M. guttatus* species complex is a closely related group of wildflowers consisting of the wide-ranging and outcrossing *M. guttatus* and many smaller ranged morphological species that are often self-fertilizing. Owing to the recent nature of speciation and ongoing interspecific introgression in this group, the phylogeny of the species complex remains largely unresolved. However, because of its wide geographical range and high levels of intraspecific genetic diversity it is likely that *M. guttatus* is the progenitor of the other self-fertilizing species with restricted ranges. This makes the *M. guttatus* species

complex excellent for the study of the genetics of recent and local geographical speciation. In fact, a recent study by Grossenbacher *et al.* [19] suggests that local speciation has occurred frequently throughout the genus *Mimulus*.

Mimulus filicifolius has lately been described as a new species of *Mimulus* based on subtle divergence in morphological characters from a member of the *M. guttatus* species complex, *M. laciniatus* [32]. Populations of this new species were formerly identified as *M. laciniatus* because of similar leaf shape, flower size and rock outcrop habitat. This new species occurs only in a few locations in Butte and Plumas counties, CA, USA, making it a good candidate for speciation on a local geographical scale. However, it was not previously known whether this species was genetically divergent as well as phenotypically distinct from *M. laciniatus* and thus whether it was truly an independent species. We found evidence in our population genetic estimates, PCAs and gene trees that *M. filicifolius* is genetically divergent from *M. laciniatus*. We found the greatest amount of pairwise nucleotide divergence (*dxy*) in our comparisons between *M. filicifolius* and *M. laciniatus*, and *M. filicifolius* and *M. nasutus* suggesting that these species were the most diverged in terms of DNA sequence. We also discovered that *M. filicifolius* is the least divergent from *M. guttatus*, but that these two taxa are still more diverged than *M. guttatus*, *M. nasutus* and *M. laciniatus*. This lower level of divergence between *M. filicifolius* and *M. guttatus* suggests that either these two taxa are exchanging more genes or that they share a more recent common ancestor. However, the existence of strong post-zygotic reproductive isolation between *M. filicifolius* and *M. guttatus* casts doubt on the former hypothesis.

In both our genetic PCAs, *M. filicifolius* was significantly genetically differentiated from the other three *Mimulus* taxa. In addition, *M. filicifolius* was even more genetically distant from the average *M. laciniatus* individual than it was from either *M. guttatus* or *M. nasutus*. We saw a similar pattern in our maximum-likelihood trees, with *M. filicifolius* failing to cluster with *M. laciniatus* sequences at the majority of loci. These patterns of genetic variation indicate that *M. filicifolius* is indeed genetically distinct from *M. laciniatus*. However, because we were able to only include four species in this analysis, it is not possible to determine its closest relative from our current data. The narrow geographical range of *M. filicifolius* compared with other members of the *M. guttatus* species complex from which it might be derived is consistent with it being a product of local speciation. It is also possible that historically *M. filicifolius* had a much larger range and that over time that range contracted to its present size. However, we do not think this likely since members of the *M. guttatus* species complex have diverged very recently [54], which makes it improbable that enough time has passed since speciation for a substantial range contraction to have occurred.

Given our data, there are two main evolutionary scenarios that could have given rise to *M. filicifolius*. The first is that this new species originated from *M. laciniatus* and then subsequently diverged owing to geographical isolation over a long period of time. This geographical isolation could have originated from either a long distance dispersal event from *M. laciniatus*' current range or a contraction of a larger historical range. The alternative scenario is that *M. filicifolius* arose as a completely independent lobed-leaved rock outcrop specialist from some other species like the wide-ranging *M. guttatus*. Our population genetic divergence data tentatively suggest that this scenario is the most likely (table 6). This latter hypothesis is exciting since it would indicate that there has been parallel evolution of lobed leaf shape and rock outcrop specialization in *Mimulus*. The correlation between the independent evolution of a trait and occupation of a similar environment is considered evidence of adaptation [55]. Lobed leaves are in fact hypothesized to be adaptive in hot, dry environments like *M. laciniatus* and *M. filicifolius*' rock outcrop habitat [56,57].

(c) Hybrid sterility and potential chromosomal divergence

In our above analyses, we found that *M. filicifolius* is genetically as well as phenotypically divergent from *M. laciniatus* and that it has a highly self-fertilizing mating system. Self-fertilization acts as a pre-zygotic reproductive isolating barrier against nearby outcrossing and self-fertilizing species alike [25]. However, a single reproductive barrier often confers only partial reproductive isolation. It may therefore be necessary for a species to have more than one type of isolating barrier to be completely reproductively isolated from its relatives. Our discovery of a strong post-zygotic reproductive isolating barrier between *M. filicifolius* and *M. laciniatus* indicates that *M. filicifolius* is truly an independent biological species [58].

Hybrid sterility has two major causes: chromosomal rearrangements and Dobzhansky–Muller incompatibilities [58]. We do not currently have data that can conclusively tell us whether Dobzhansky–Muller incompatibilities or chromosomal rearrangements are to blame for *M. filicifolius*' hybrid sterility. We did find a small difference in genome size between *M. filicifolius* and *M. laciniatus* in our FCM analysis. However, the reduction in *M. filicifolius*' genome size was not far outside the variation in genome size observed within a single *Mimulus* species [41], indicating that it is unlikely there are chromosomal differences due to large deletions or aneuploidy.

(d) Conclusion

Whether speciation occurs primarily on a large or a small geographical scale has been debated for many years in the speciation literature. It is a difficult debate to settle since speciation is a historical process. However, looking at the current ranges of recently separated species can give us some information about the most probable geographical context of their divergence [19]. Over his career, L. D. Gottlieb produced a very strong case for the local speciation of *Stephanomeria malheurensis* from its large-ranged progenitor *Stephanomeria exigua* ssp. *coronaria* (reviewed in [9]). Like Gottlieb's study of *S. malheurensis*, we have found a new species, *M. filicifolius*, which is highly geographically restricted and reproductively isolated from its relative *M. laciniatus* due to both pre- and post-zygotic reproductive isolating factors despite these two species sharing highly similar habitats and morphologies. Thus, our findings are consistent with local geographical speciation of a new rock outcrop endemic in the yellow monkey flowers, and suggestive of the independent evolution of a second lobed-leaved rock outcrop specialist in the genus *Mimulus*.

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