Patterns of nucleotide variation and reproductive isolation between a *Mimulus* allotetraploid and its progenitor species

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Abstract

Here we report our characterization of a widespread, highly selfing *Mimulus* allotetraploid formed by interspecific hybridization between *M. nasutus* and *M. guttatus*. Nucleotide variation at two nuclear loci (mCYCA and mAP3) within and among tetraploid populations resolves two haplotype clusters for each locus: one shares near identity with sequences from *M. nasutus* and the other group shares substantial variation with *M. guttatus*. With respect to the two loci studied, each allotetraploid individual is a ‘fixed heterozygote’ carrying sequences from both clusters. Moreover, mCYCA variation is consistent with at least two evolutionary origins for the *Mimulus* allotetraploid. We show that the allotetraploid is strongly reproductively isolated from *M. nasutus* and *M. guttatus*; interploidy crosses produce almost no viable seeds. By extension, we infer strong triploid block and argue that *Mimulus* allotetraploid formation might proceed in one step via the union of unreduced gametes in an *M. nasutus–M. guttatus* F1 hybrid. We also discuss the potential roles of mating system and flowering asynchrony in allotetraploid establishment.

Keywords: genome duplication, hybridization, *Mimulus*, polyploidy, reproductive isolation, speciation

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Introduction

Polyploidy is a common feature of many flowering plants (Grant 1981; Masterson 1994; Otto & Whitton 2000), but its evolutionary significance is controversial. In one extreme view, polyploidy has been described as an evolutionary dead-end, contributing little to phenotypic diversification (Stebbins 1950). On the other hand, it has been suggested that polyploidy might be a creative force in evolution (Ohno 1970; Levin 1983). Currently, there is renewed enthusiasm for the idea that genome-doubling promotes adaptive evolutionary change (e.g. Adams & Wendel 2005; Comai 2005), as recent genomic analyses have uncovered ancient polyploidization events in diverse taxa, including angiosperms (Bowers et al. 2003; Blanc & Wolfe 2004; Jaillon et al. 2007), vertebrates (Hughes & Hughes 1993; Jaillon et al. 2004) and yeast (Wolfe & Shields 1997; Kellis et al. 2004). In any case, there is little doubt that polyploidy is a major mechanism of angiosperm speciation; an immediate effect of genome duplication is strong reproductive isolation between the nascent polyploid and its progenitor species. Otto & Whitton (2000) estimate that 2–4% of speciation events in angiosperms involve polyploidization. However, because they exclude species polymorphic for chromosome number, this figure likely represents a lower bound on polyploid speciation in flowering plants (their method discounts most autoploids, which have not been named as separate species, e.g. Soltis et al. 2007).

Polyploidization in flowering plants is a dynamic process. Spontaneous polyploid formation is ongoing in many taxa (Ramsey & Schemske 1998), and it is not uncommon for established polyploid species to have multiple origins (Soltis & Soltis 1999). Although the rate of polyploid formation (estimated on the order of 10–5 per generation for autoploids by Ramsey & Schemske 1998) clearly exceeds the rate of establishment, closely related species often do differ in ploidy (Lewin 1980). The likelihood that a new polyploid persists long enough to become established as a new species depends on its ability to overcome minority
cytotype exclusion (i.e. natural selection against the rare cytotype, Levin 1975) and ecological competition with its progenitor species. The former can be achieved by any form of prezygotic reproductive isolation (e.g. a shift in mating system or flowering phenology), and the latter by invasion of a novel ecological habitat, which might be facilitated by a suite of physiological changes that often accompany polyploidization (Levin 1983, 2002). To understand the dynamics of polyploidy, including the formation, establishment, and long-term evolutionary contribution of polyploids, we need to study natural populations of closely related species formed by polyploidization.

The well-studied *Mimulus* species within the section *Simiolus*, a group of morphologically diverse wildflowers (reviewed in Vickery 1978), provide such a system. This section of the genus primarily consists of four closely related, but generally recognized species complexes that all contain polyploids and aneuploids relative to the basal chromosome count of *n* = 14, including the *M. guttatus* complex (*n* = 13, 14, 15, 16, 28), *M. tiltingii* complex (*n* = 14, 15, 24, 28), *M. luteus* complex (*n* = 30, 31, 32), and *M. glabratus* complex (*n* = 14, 15, 30, 31, 45, 46) (Vickery 1966, 1978). Evolutionary investigations of polyploidy in the *M. guttatus* complex seem especially promising given the recent development of genomic resources for this group and ongoing sequencing of the *M. guttatus* genome (Wu et al. 2007).

The *M. guttatus* complex is a group of closely related, largely interfertile species with mating systems ranging from complete outcrossing to obligate self-fertilization, with its centre of diversity in western North America (Vickery 1978). The extreme species in terms of selfing rate are represented by the bee-pollinated, predominantly selfing *M. guttatus*. *M. guttatus* is highly polymorphic, exhibiting tremendous phenotypic diversity (Pennell 1951) and extensive molecular variation (Sweigart & Willis 2003). Consistent with its mating system, *M. nasutus* has much lower species-wide nucleotide diversity, which is almost entirely partitioned among populations (Sweigart & Willis 2003). Most populations of the named species in the *M. guttatus* complex are diploid (*n* = 14; Vickery 1964, 1973, 1978) but cytological studies have occasionally revealed tetraploid (*n* = 28) populations (Mia et al. 1964; Calder & Taylor 1965; Vickery et al. 1968; McArthur et al. 1972; Benedict 1986). Most of these reported tetraploids are morphologically similar to the geographically widespread (Alaska to Mexico, Pacific Coast to the Rockies) *M. guttatus*, and their locations suggest multiple independent origins. In Oregon, Colorado, Nevada, and Mexico, apparently isolated tetraploid populations have been found that are indistinguishable from nearby *M. guttatus*, leading to speculation that they originated by autopolyploidy (Mia et al. 1964; Vickery et al. 1968). At the northern edge of the *M. guttatus* range, phenotypically differentiated tetraploid populations are suspected to have arisen by allopolyploidy (perhaps via hybridization between divergent *M. guttatus* ecotypes, or between *M. guttatus* and the high-altitude species, *M. tiltingii*, Vickery et al. 1968). For example, tetraploids located on the Queen Charlotte Islands in southwestern Canada (recognized as *M. guttatus* ssp. *haidensis*) differ from nearly diploid *M. guttatus* in leaf morphology and altitudinal range (Calder & Taylor 1965). Finally, several populations of small-flowered, self-fertilizing tetraploids that resemble *M. nasutus* were discovered on Vancouver Island and the Gulf Islands in southwestern Canada, as well as in southwestern Oregon (Benedict 1986). Unlike the other *M. guttatus*-like polyploids, this species was found growing sympatrically with both diploid *M. guttatus* and *M. nasutus* individuals (Benedict 1986). Based on fixed heterozygosity at allozyme loci, Benedict (1986) suggested an allopolyploid origin for these selfing tetraploids.

In the present study, we focus on this highly selfing *M. nasutus*-like polyploid. To understand its origin, establishment, and evolutionary dynamics, we examined patterns of nuclear DNA content and nucleotide variation among collections of the tetraploid and other members of the *M. guttatus* complex, and estimated the degree of reproductive isolation between tetraploids and sympatric diploid species. Our findings indicate that the selfing tetraploid is widespread and common, from northern California to British Columbia, typically co-occurring with diploid *M. guttatus* and occasionally also with diploid *M. nasutus*. Patterns of molecular variation provide evidence that this species is an allotetraploid, formed at least twice by hybridization between the diploids *M. nasutus* and *M. guttatus*. Moreover, our crossing experiments show that these allotetraploids are nearly completely reproductively isolated from their typically interfertile diploid progenitors. In the light of our findings, we discuss the likely mechanism of *Mimulus* allopolyploid formation and the potential role of mating system in allotetraploid establishment.

**Materials and methods**

**Sampling and propagation of species and populations**

In a study originally designed to examine postzygotic barriers between diploid *Mimulus guttatus* and *Mimulus nasutus*, we found several anomalous Oregon populations that were nearly completely isolated from all other populations (i.e. intercrossing produced no F1 hybrids). This finding was quite unexpected because, on the basis of morphology, we had previously identified these populations as *M. nasutus*. Like *M. nasutus*, individuals from these atypical populations are obligate selfers: they lack floral nectaries characteristic of the outcrossing *M. guttatus* and produce a full component of seeds in a pollinator-free greenhouse...
(A.L. Sweigart, N.H. Martin, unpublished results). Because of these unexpected crossing results, we decided to investigate the possibility of polyploidy in *Mimulus* populations from Oregon’s Willamette Valley to northern California (Table 1), a geographical region that was underrepresented in earlier cytological analyses (Vickery 1964, 1973, 1978). Shortly thereafter, we encountered a master’s thesis by Benedict (1986) describing a highly selfing tetraploid in southwestern Canada, and we also obtained samples from this region (Table 1). In addition, our analyses included samples from populations of diploid *M. guttatus* and *M. nasutus* (Table 1), as well as previously published sequences (Sweigart & Willis 2003; also see below).

Seed samples were collected by several different researchers and on multiple collecting trips from 15 to 30 different plants at each population. Samples for this study originated either as field-collected seeds or as plants that were propagated and selfed in the greenhouse to produce seeds. Some lines have been maintained in the greenhouse for several generations of autonomous self-fertilization. Seeds were stored in airtight containers and kept refrigerated until use. All plants were grown using similar conditions for both molecular analysis and crossability studies. Individual seeds were planted in 2.25-in pots filled with soil-less potting mix, watered, and stratified in a dark cold room (4 °C) for 1 week. Pots were then moved to a controlled environmental chamber with constant light and temperature (16 °C) for 1 week to promote germination. After germination, plants were moved to the Duke University greenhouses for subsequent growth. Greenhouse conditions included 18-h days at 24 °C with supplemental high-pressure sodium lights and 6-h nights at 16 °C.

### Flow cytometry

To confirm polyploidy and estimate nuclear DNA content, we performed flow cytometry on samples of *M. guttatus*, *M. nasutus*, and putative tetraploids following Ramsey (2007). In brief, for each sample, 50 mg of leaf tissue was chopped in 2 mL of buffer (3.6 g HEPES, 2 mL of a 0.5 M solution of EDTA, 6.0 g KCl, 1.2 g NaCl, 102.7 g sucrose, 2 mL Triton X-100, 1 mL β-mercaptoethanol and 0.1 g spermine in 1.0 L distilled water). The resulting slurry was passed through a syringe filter and centrifuged at 10,000 × g for 1 min. The nuclei pellet was resuspended in chopping buffer containing 2% propidium iodide (PI) and 0.1% RNase (QIAGEN). As an external control, we added 4 μL of chicken erythrocyte nuclei (CENs; Biosure) to each sample. All samples were run on a BD FACSCalibur flow cytometer (BD Biosciences) located in the Cell Sorting Core Facility at the University of Rochester Medical Center. We analysed all samples for relative fluorescence (FL2-A), which was summarized as a frequency histogram using the CELLQUEST software package (version 5.2.1, Becton-Dickerson and Company). For each sample, an estimate of nuclear DNA content was calculated as the product of the CENs external control 2C value (2.5 pg) and the ratio between *Mimulus* and CENs relative fluorescence. For each *Mimulus* individual, nuclear DNA content was estimated by the average of several replicate samples that were independently collected, prepared, and analysed.

### Molecular analyses

Genomic DNA was isolated from bud tissue using a modified hexadecyl trimethyl-ammonium bromide

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Table 1 Geographical locations of the *Mimulus* populations included in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Population code</th>
<th>Location*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. guttatus</em></td>
<td>IM†‡</td>
<td>Iron Mountain, Highway 20, Linn County, Oregon</td>
</tr>
<tr>
<td></td>
<td>GTR‡</td>
<td>Highway 128 and Berryessa-Knoxville Road, Napa County, California</td>
</tr>
<tr>
<td><em>M. nasutus</em></td>
<td>SF†‡</td>
<td>Sherar’s Falls, Tygh Valley, Wasco County, Oregon</td>
</tr>
<tr>
<td></td>
<td>NDR2†</td>
<td>2 miles south of Dos Rios, Mendocino County, California</td>
</tr>
<tr>
<td>Allotetraploid</td>
<td>FAN</td>
<td>Finlayson Arm Road, Vancouver Island, B.C.</td>
</tr>
<tr>
<td></td>
<td>OHN</td>
<td>Observatory Hill, Victoria, B.C.</td>
</tr>
<tr>
<td></td>
<td>PSG</td>
<td>Mount Pisgah, near Springfield, Lane County, Oregon</td>
</tr>
<tr>
<td></td>
<td>SPB</td>
<td>Spencer’s Butte, near Eugene, Lane County, Oregon</td>
</tr>
<tr>
<td></td>
<td>DEX†‡</td>
<td>Dexter Reservoir, Lane County, Oregon</td>
</tr>
<tr>
<td></td>
<td>LSN†‡</td>
<td>Lowell, Lane County, Oregon</td>
</tr>
<tr>
<td></td>
<td>ROG†‡</td>
<td>Rogue River, near Grave Creek Falls, Josephine County, Oregon</td>
</tr>
<tr>
<td></td>
<td>NDR‡</td>
<td>Dos Rios-Laytonville Road, 4 miles west of Dos Rios, Mendocino County, California</td>
</tr>
</tbody>
</table>

*Population locations are listed from north to south for each species.
†Denotes populations included in flow cytometry analyses.
‡Denotes populations included in the complete-diallel crossing experiment.
chboroform extraction protocol (Kelly & Willis 1998). Portions of the *Mimulus CYCLOIDEA-A* (*mCYCA*) and APETALA3 (*mAP3*) genes were amplified in polyploid samples via the polymerase chain reaction (PCR) from genomic DNA. Oligonucleotide primers for each gene had previously been used to amplify fragments in *M. guttatus*, *M. nasutus*, and other members of the complex (*mCYCA*: GenBank Accessions AY319524–AY319644, *mAP3*: GenBank Accessions AY319656–AY319799; see Sweigart & Willis 2003 for details on these samples). Our previous analyses could not reject a neutral explanation for the pattern of molecular variation at *mCYCA* and *mAP3* in diploid *M. guttatus* species (Sweigart & Willis 2003). Fragment lengths are approximately 570 bp and 590 bp for *mCYCA* and *mAP3*, respectively, but vary due to indel polymorphism. The *mCYCA* fragment consists entirely of coding sequence from the first exon, whereas the *mAP3* fragment spans four exons and three introns. Standard PCR conditions were used (90 s at 94 °C, followed by 30 cycles of 40 s at 92 °C, 1 min at 52 °C, and 40 s at 72 °C). Each PCR was performed using 10 ng of genomic DNA as template and supplemented with 3 mM MgCl2. PCR products were gel-purified using the QIAquick Gel Extraction Kit (QIAGEN) and cloned into pCR2.1-TOPO using the TOPO TA Cloning Kit (Invitrogen). Plasmid DNA from single colonies was isolated as template for sequencing using the QIAprep (Invitrogen). Plasmid DNA from single colonies was isolated as template for sequencing using the QIAprep Gel Extraction Kit (QIAGEN) and cloned into pCR2.1-TOPO using the TOPO TA Cloning Kit (Invitrogen). Plasmid DNA from single colonies was isolated as template for sequencing using the QIAprep Miniprep Kit (QIAGEN). For tetraploids and heterozygous diploids, a minimum of eight clones was sequenced to identify both haplotypes and to eliminate sequence variation caused by PCR-generated errors (due to misincorporation and/or PCR-mediated recombination). All sequencing reactions were performed using 30 ng DNA template, 10 μM M13 forward primer, and 2 μL BigDye Terminator Mix on an ABI 3700 DNA sequencer (Perkin Elmer).

To characterize the evolutionary origin of the *Mimulus* tetraploids, we conducted a phylogenetic analysis of *mCYCA* and *mAP3* nucleotide sequences, including both previously published sequences from populations and species of the *M. guttatus* complex (Sweigart & Willis 2003) and newly obtained tetraploid sequences (*mCYCA*: GenBank Accessions EU362996–EU363012, *mAP3*: GenBank Accessions EU363013–EU363028). Due to considerable indel polymorphism, *mAP3* introns were unalignable and were excluded from our analyses. Exons were aligned by eye in macclade 4.08. Phylogenetic analyses were conducted using the neighbour-joining algorithm in *paup* 4.0b10 (Swofford 2002). Support for individual nodes was evaluated via 100 bootstrapping replicates.

To obtain a more detailed picture of molecular variation within and among tetraploid individuals, we analysed a data set containing only tetraploid samples. Because this data set had fewer indel polymorphisms, we were able to align the entire amplicon by eye in macclade. Indel polymorphism was coded as a fifth character state and included in phylogenetic analyses using maximum parsimony. *paup* 4.0b10 (Swofford 2002) generated phylogenetic trees under maximum parsimony using 100 heuristic searches with tree-bisection–reconnection branch swapping and simple stepwise taxon additions. Support for individual nodes was measured by 1000 bootstrap replicates with 10 random additions per replicate.

**Crossing design and germination tests**

In order to examine postzygotic isolation (i.e. whether or not viable F1 seeds could be produced) between *Mimulus* populations, we performed crosses at two different levels: (i) between allopatric populations of tetraploid plants, and (ii) between tetraploid populations and diploid species *M. nasutus* and *M. guttatus*. For comparison, we also included intraspecific and interspecific crosses of *M. nasutus* and *M. guttatus*. Three populations of tetraploids and two populations each of *M. nasutus* and *M. guttatus* were included in the crossing study (Table 1). Each of the crosses was made between maternal families of up to 30 plants (instead of between individuals). When the plants began flowering, we emasculated several flowers per plant by removing the corollas of *M. guttatus* prior to pollen dehiscence (stamens are attached to corollas at the base of the flower), and by dissecting out anthers of *M. nasutus* and tetraploid plants with small fine-tipped forceps prior to pollen maturation.

We used a complete diallel crossing design, with all populations and species included in the study crossed with each other reciprocally. We used randomly chosen flowers to generate five to 15 independent, unrelated crosses for each off-diagonal cell of the diallel, so that crosses were reciprocal at the level of populations but not at the individual plant level. For the intrapopulational crosses, we crossed unrelated plants (i.e. nonsiblings) to produce seven to 14 independent outbred families per population. For all crosses, the maternal parent is indicated first.

Nearly all experimental crosses produced a large number of F1 ‘seeds’. However, these seeds ranged from round and apparently normal (presumably viable) to shriveled and dust-like (presumably lacking endosperm and inviable). Because of substantial variability in the physical appearance of seeds, it was difficult to visually determine their viability. Instead, we measured seed germination success of the F1 (interspecific, intraspecific, and interploidy) and parental intrapopulational crosses in a common garden experiment at the Duke University Biology Department greenhouse. We planted 15 seeds from each of the five to 15 crosses per cell of the diallel, with seeds from each cross planted in separate pots (in the rare case that fewer than 15 seeds per cross matured, all available seeds were planted). We placed the pots in a completely randomized design in flats, stratified them in a cold darkroom (4 °C) for 1 week,
placed them on greenhouse benches, and misted them regularly to prevent the potting soil from drying. After ~20 days in the greenhouse, we counted the number of seedlings that germinated per pot, and calculated the proportion of planted seeds that germinated per pot.

A mixed-model analysis of variance (ANOVA) was used to analyse the germination data, with germination success of individual ‘pots’ being the unit of replication. Germination success was not transformed since both transformed and untransformed results did not significantly alter the interpretation of the model, and untransformed mean-squares are easier to interpret. The following fixed factors were included in the model: ‘Paternal Ploidy’ (whether the pollen parent was diploid or tetraploid; diploid species-assignments to *M. guttatus* or *M. nasutus* were not included in this model) and ‘Maternal Ploidy’ (whether the seed parent was diploid or tetraploid). ‘Paternal Population’ (i.e. the population from which the pollen donor originated – a factor fully nested within the factor Paternal Ploidy) and ‘Maternal Population’ (i.e. the population from which the seed parent originated – a factor fully nested within Maternal Ploidy) were assigned as random factors, and an interaction effect between the terms Maternal Ploidy and Paternal Ploidy was also included in the model. All analyses, including planned linear contrasts to explore significant differences were performed in jmp version 4.0.4.

### Results

**Estimation of nuclear DNA content**

Our estimates of nuclear DNA content via flow cytometry provide evidence for tetraploid populations in the *Mimulus guttatus* complex (Table 2). The 2C DNA content of several putative polyploid samples (DEX, LSN, ROG) is nearly double that of *Mimulus* diploids, consistent with whole genome duplication. Our results also indicate that the 2C DNA content of diploid *M. guttatus* is ~89% that of diploid *M. guttatus*. It should be noted, however, that our estimate of DNA content for each diploid *Mimulus* species is based on a single individual, and therefore should be considered preliminary.

In several of the tetraploid samples, flow cytometry detected a relatively weak and small peak (i.e. a lower relative fluorescence), in addition to the prominent peak generated by tetraploid nuclei. Further investigation is needed to determine whether this second peak was generated by a contaminant, an endosymbiont, or an unknown cytological mechanism.

### Patterns of nucleotide variation in diploid and tetraploid *Mimulus*

In a previous study, we found a complex pattern of molecular variation among diploids of the *M. guttatus* complex (Sweigart & Willis 2003). We showed that *M. guttatus* is extremely polymorphic throughout its range (θSil = 0.090 and 0.063 for the nuclear genes mCYCA and mAP3, respectively) and shares substantial variation with its close relatives (we found no nucleotide differences distinguishing species). In contrast, there is a high degree of genetic similarity among populations of *M. nasutus*, which carry only a subset of the nucleotide diversity found in *M. guttatus* (θSil = 0.013 and 0 for mCYCA and mAP3, respectively).

In the current study, we amplified two distinct haplotypes at each of the nuclear genes mCYCA and mAP3 in each of the tetraploids. Considering that these *Mimulus* tetraploids are extreme selfers, it is notable that each individual is a ‘fixed heterozygote’. To compare tetraploid variation to that of other *M. guttatus* complex species, we constructed distance trees using the neighbour-joining algorithm (Saitou & Nei 1987) for sequences of mCYCA (Fig. 1a) and mAP3 (Fig. 1b). For both genes, all tetraploids carry one haplotype that shares near identity with sequences from *M. nasutus*. In addition, all tetraploids carry a second, divergent haplotype that cannot be resolved from *M. guttatus* or other members of the complex. We interpret this pattern as evidence that these *Mimulus* polyploids are allotetraploids formed by interspecific hybridization between *M. nasutus* and *M. guttatus* (also see discussion).

To explore the pattern of molecular variation within and among *Mimulus* allotetraploids, we also grouped tetraploid haplotypes using maximum parsimony (Fig. 2). Narrowing our focus to include only tetraploid sequences allowed us to include indel variation, which is substantial but unalignable in the large, multispecies data set. For both loci, *M. nasutus*-like haplotypes form a single well-supported group that is strongly differentiated from all other haplotypes. For mCYCA, we observed two subgroups among the divergent ‘*M. guttatus*-like’ tetraploid haplotypes (Fig. 2a), each distinguished by a unique set of shared variation with *M. guttatus* (Fig. 1a). One of these subgroups

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**Table 2** Estimates of 2C nuclear DNA content in *Mimulus* taxa using flow cytometry

<table>
<thead>
<tr>
<th>Species</th>
<th>Individual</th>
<th>N*</th>
<th>Mean nuclear DNA content (pg)†</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. nasutus</em></td>
<td>SF5</td>
<td>9</td>
<td>0.622 (0.013)</td>
</tr>
<tr>
<td><em>M. guttatus</em></td>
<td>IM12</td>
<td>2</td>
<td>0.699‡</td>
</tr>
<tr>
<td>Allotetraploid</td>
<td>DEX2</td>
<td>6</td>
<td>1.206 (0.011)</td>
</tr>
<tr>
<td></td>
<td>LSN</td>
<td>2</td>
<td>1.205‡</td>
</tr>
<tr>
<td></td>
<td>ROG22</td>
<td>7</td>
<td>1.189 (0.019)</td>
</tr>
</tbody>
</table>

*Number of replicate flow cytometry runs.
†Values in parentheses represent standard errors.
‡Sample size insufficient to calculate a standard error.
Fig. 1 Neighbour-joining trees of (a) mCYCA and (b) mAP3 sequences from diploid and allotetraploid Mimulus. Taxon names are coded in the following way (in this order): (i) a lower-case letter prefix represents the species; g, M. guttatus, n, M. nasutus; t, tetraploid; nud, M. nudatus; pla, M. platycalyx; til, M. tilingii; (ii) three upper-case letters indicate the population location code; (iii) a number designates a particular maternal family; and (iv) ‘a1’ and ‘a2’ signify different alleles. M. guttatus-like tetraploid haplotypes are indicated by blue colouration. M. nasutus-like tetraploid haplotypes are indicated by red colouration. Tetraploid samples from Oregon and California are highlighted by darker shades of red and blue, whereas tetraploid samples from Vancouver Island are shown by lighter shades of each colour. All nodes with less than 50% bootstrap support are collapsed; the other bootstrap values are indicated next to relevant nodes. YECB is the outgroup Mimulus yecorensis.
CHARACTERIZATION OF A *MIMULUS* ALLOTETRAPLOID

![Diagram of genetic relationships and substitutions between *M. guttatus* and *M. nasutus*]

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0.001 substitutions/site

Fig. 1 Continued

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A distance of 500 km in western Oregon and California. The second subgroup is formed by two tetraploids from two populations on Vancouver Island. In contrast, for mAP3, there is little variation among M. guttatus-like tetraploid haplotypes (Fig. 2b), despite high nucleotide diversity at mAP3 in diploid M. guttatus and extensive indel polymorphism.
Reproductive isolation between the tetraploid Mimulus and its diploid progenitors, M. guttatus and M. nasutus

There is strong reproductive isolation between tetraploid and diploid Mimulus species: diploid × diploid crosses and tetraploid × tetraploid crosses have high germination success (84.9% ± 1.2%, 85.7% ± 1.5%, respectively, Fig. 3), whereas diploid × tetraploid and tetraploid × diploid crosses have extremely low germination success (1.08% ± 1.35%, and 1.88% ± 1.34%, respectively, Fig. 3). The mixed-model ANOVA comparing the germination success of crosses was highly significant ($F_{13,483} = 605.26, MS = 6.70, P < 0.0001$) and explained a large amount of the total variance ($r^2 = 94.10$). The only significant effect in the model was the interaction of Maternal Ploidy × Paternal Ploidy ($F_{1,13} = 7606.88, P < 0.0001$), explained by the extreme difference in germination success between intraploidy and interploidy crosses. Linear contrasts showed no significant differences in germination success between diploid × diploid crosses and tetraploid × tetraploid crosses ($F = 0.17, P = 0.68$), nor between reciprocal crosses of tetraploids and diploids (i.e. diploid × tetraploid vs. tetraploid × diploid crosses, $F = 0.19, P = 0.66$). The small number of seeds that germinated from interploidy crosses produced sterile plants with various morphological abnormalities.

Discussion

In this study, we report our characterization and analysis of a small-flowered, highly selfing tetraploid species in the Mimulus guttatus complex that has natural populations ranging from southern Canada to northern California. These populations are typically found sympatrically with diploid M. guttatus, and in the northern and southern parts of the range all three species may co-occur. Our analysis of molecular variation shows that these tetraploids are ‘fixed heterozygotes’ for the two nuclear loci examined, consistent with allopolyploidy. All tetraploids carry a haplotype at each locus that clusters unambiguously with sequences from Mimulus nasutus, implicating the diploid species as an ancestor. However, assigning ancestry for the second, divergent haplotype at each locus is not as straightforward. Although these haplotypes share substantial similarity with sequences from M. guttatus, we did not find any exact matches to haplotypes sampled from that diploid species.

This result is perhaps not surprising given high rates of recombination and extensive polymorphism within M. guttatus (Sweigart & Willis 2003). Nevertheless, M. guttatus seems the most likely candidate to have given rise to the allotetraploid. Relative to other members of the complex, M. guttatus is the most widely distributed, and its range overlaps extensively with that of the widespread M. nasutus. Indeed, all other species of the M. guttatus complex have much more localized distributions, with many restricted to central California (Vickery 1978). Therefore, we argue that these Mimulus polyploids are allotetraploids, likely formed by hybridization between M. nasutus and M. guttatus. In this study, we have characterized patterns of nucleotide variation and reproductive isolation among species to investigate the mechanism of Mimulus polyploid formation and to infer the number of evolutionary origins. Our results also provide insight into the dynamics of Mimulus polyploid establishment. As we discuss below, we believe this model system has enormous potential to address many outstanding questions in polyploid evolution.

There are two potential cytological mechanisms for allotetraploid formation in Mimulus: (i) somatic doubling, and (ii) gametic nonreduction during micro- and mega-sporogenesis. In the first mechanism, somatic doubling might occur in the meristem of an F1 hybrid sporophyte, or even in the hybrid zygote or young embryo. In the second mechanism, tetraploid formation might be induced by the union of two unreduced (2n) gametes. Although our data cannot distinguish between these two routes to Mimulus allotetraploidy, gametic nonreduction is considered the major mechanism of plant polyploid formation (Ramsey & Schemske 1998, and references therein). If, indeed, Mimulus allotetraploid formation occurred via this mechanism, it might have proceeded in ‘one step’ (i.e. a single generation, see Ramsey & Schemske 1998) by F1 selfing or backcrossing. To evaluate the likelihood of the one-step pathway in Mimulus, future experiments should measure the rate of spontaneous allotetraploid formation following self-fertilization of an M. nasutus–M. guttatus F1 hybrid. The alternative, ‘two step’ pathway to allotetraploid formation requires a triploid bridge (i.e. a triploid intermediate formed when an unreduced 2n gamete from one diploid parent unites with a regular n gamete from the other diploid

![Figure 3](image-url) Proportion of seeds that germinated in the crossing experiment. Data are presented as least square means (as calculated in the ANOVA model presented in the text) across all crosstypes (d, diploid; t, tetraploid). Error bars represent one standard error.
parent). However, this scenario might be less likely in *Mimulus* due to nearly complete triploid block (i.e. a lack of viable seeds resulting from diploid–tetraploid and tetraploid–diploid crosses, Fig. 3). Using the results from our crossing experiments with *M. nasutus*, *M. guttatus*, and the allotetraploid, we calculate the index of overall block to be 0.99 (this index subtracts from one the relative seed viability of interploidy to intraploidy crosses; see Ramsey & Schemske 1998, p. 480). Strong, bidirectional triploid block has been observed in several other plant taxa (Ramsey & Schemske 1998, WebTable 2), and is likely caused by an imbalance between ploidy levels of the embryo, endosperm and/or maternal tissue.

A first step toward understanding the dynamics of polyploid establishment and persistence is to estimate the number of polyploid origins in natural populations, which might be considerably lower than predicted by the rate of formation. Indeed, the frequency of hybridization between *M. nasutus* and *M. guttatus* in natural populations is sufficiently high to expect ongoing allotetraploid formation: ~1% of the seeds produced by *M. nasutus* in a population sympatric with *M. guttatus* are of hybrid origin (Martin & Willis 2007). Although the overall frequency of hybridization is somewhat lower than 1% due to several factors including asymmetric barriers (i.e. very few hybrids are formed from the reciprocal cross between *M. guttatus* and *M. nasutus*), it is still on the order required to equal the rate of autopolyplid formation calculated by Ramsey & Schemske (1998).

To estimate the number of evolutionary origins of the *Mimulus* allotetraploid, we examined patterns of nucleotide variation within and among populations. Because *M. guttatus* is highly polymorphic, we should have the power to detect each independent origin of polyploidy by its unique set of *M. guttatus*-like haplotypes. Indeed, we can infer at least two evolutionary origins among the sampled allotetraploid *Mimulus* by the two distinct subgroups of *M. guttatus*-like mCYCA haplotypes (see Figs 1a and 2a). One of these groups is geographically widespread, comprised of five populations in Oregon and northern California. The other group is defined by two populations originally identified by Benedict (1986) on Vancouver Island. Interestingly, Benedict (1986) also suggested that these northern allotetraploids might be independently derived from two tetraploid populations she studied in southwestern Oregon (located near our ROG collection). Although Benedict referred to allotetraploids sampled from both locations by the same name (*Mimulus queue*), she did detect modest differentiation in allozymes and morphology between the two groups (Benedict 1986).

Surprisingly, molecular variation at mAP3 provides no additional support for a scenario of two origins, as *M. guttatus*-like haplotypes show little variation among tetraploid populations (Figs 1b and 2b). The region we amplified from mAP3 includes three introns that are unalignable in the full *M. guttatus* data set, due to tremendous indel and nucleotide polymorphism. Therefore, it seems unlikely that our sample of *M. guttatus*-like mAP3 tetraploid haplotypes is the product of two different *M. guttatus* ancestors. Instead, it is possible that introgression between independently evolved tetraploid groups has homogenized certain loci, like mAP3. Indeed, Benedict (1986) reported cross-fertility between Oregon and Vancouver Island populations. Alternatively, it is possible that all *Mimulus* allotetraploids share a single evolutionary origin, but that subsequent backcrossing to *M. guttatus* populations has resulted in variation at some loci (e.g. mCYCA). Occasional backcrossing might be expected to occur, despite strong triploid block, when a 2n tetraploid gamete unites with an unreduced 2n *M. guttatus* gamete. The apparent contradiction between patterns of variation at mCYCA and mAP3 highlights the need for a multilocus approach to reconstruct the evolutionary history of the *Mimulus* allotetraploids.

A genome-wide investigation of molecular variation might also help us to address the effect of polyploidy on *Mimulus* phenotypic evolution. For example, what is the genomic basis for the morphological resemblance of the *Mimulus* allotetraploid to only one of its diploid ancestors (*M. nasutus*)? We observe fixed heterozygosity at mCYCA and mAP3, but what are the fates of other homeologous loci? At key floral morphology genes, is the *M. guttatus* homeolog silenced? Alternatively, are both homeologs functional, but homeoygous for *M. nasutus* alleles? When reproductive isolation is incomplete, introgression from diploid progenitors might be a source of genetic variation for polyploid species. In this case, however, gene flow between the *Mimulus* allotetraploid and *M. nasutus* is probably very limited due to allopatry, mating system (i.e. cross-fertilization between selfing populations is likely to be very rare), and triploid block. Another possibility is that early in their evolutionary history, allotetraploid populations became fixed for *M. nasutus* alleles at key loci. We know very little about meiotic inheritance in *Mimulus* allotetraploids, although our finding of fixed heterozygosity at mCYCA and mAP3 is consistent with disomic inheritance. Given the relatively high fertility of F1 hybrids between *M. nasutus* and *M. guttatus* (Sweigart et al. 2006; Martin & Willis 2007), it seems unlikely that major structural differences would have initially prevented tetrasomic inheritance in the allotetraploid. Moreover, there might have been strong selection for selfing alleles from *M. nasutus* as a mechanism for reproductive assurance (see below). In future studies, we hope to compare meiotic segregation in the *Mimulus* allotetraploid with that of spontaneous or induced neo-tetraploids.

For a polyploid lineage to become established, nascent polyploids must overcome an initial mating disadvantage, known as minority cytotype exclusion (Levin 1975). Because neopolyploids are often postzygotically isolated from their
diploid progenitors (as in *Mimulus*), which initially outnumber them, they might have very low fitness due to crossing with incompatible mates. Any form of premating isolation that prevents diploid pollen from landing on tetraploid stigmas and vice versa can diminish the strength of frequency-dependent selection against the rare cytotype (Stebbins 1950; Fowler & Levin 1984). A shift in mating system from outcrossing to self-fertilization, such as in the *Mimulus* allotetraploid, can act as an effective prezygotic barrier because it reduces the probability of pollen exchange between nascent species. Indeed, in locales where diploid species *M. nasutus* and *M. guttatus* occur in sympathy, the selfing ‘syndrome’ of *M. nasutus* completely prevents the export of its pollen onto *M. guttatus* stigmas and reduces the import of *M. guttatus* pollen onto its own stigmas by 98–99% (over that expected if mating were occurring at random, Martin & Willis 2007). Moreover, in sympatric populations of the allotetraploid and *M. guttatus*, species are at least partially reproductively isolated by differences in microhabitat associations and flowering phenology (i.e. the tetraploids grow in drier habitats and flower earlier, N.H. Martin personal observation). It seems possible that mating system differences and/or flowering asynchrony may have enhanced the establishment of the *Mimulus* allotetraploid.

Even if strong premating isolation allows a neopolyploid species to escape minority cytotype exclusion, it still faces the challenge of ecological competition with its diploid ancestors. Commonly, a host of physiological changes accompany polyploidization that may allow the neopolyploid to exploit a new ecological niche (Levin 2002). In Oregon, it is common for the *Mimulus* allotetraploid to occur exclusively (or in sympathy with *M. guttatus*) in habitats that appear ideal for *M. nasutus* (based on observations of similar habitats in California). The fact that we find few diploid *M. nasutus* populations in this region suggests that habitats may have subtle ecological differences, which are potentially exploited by the allotetraploid. At present, we cannot determine whether current species’ ranges reflect allotetraploid displacement of diploid *M. nasutus* or simply differential expansion of the selfing taxa. We know of two locales (ROG in southwestern Oregon: this study, and Nonoose Hill on Vancouver Island: Benedict 1986) where the allotetraploid grows in sympatry with both *M. guttatus* and *M. nasutus*. Indeed, natural populations of *M. guttatus*, *M. nasutus*, and the allotetraploid (in nearly all combinations of sympatry and allopatry) will facilitate field-based experiments to characterize the dynamics of polyploid formation and adaptation.

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**References**


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This study was conducted while Andrea Sweigart and Noland Martin were graduate students in John Willis’ laboratory at Duke University. Researchers in the Willis lab study the genetic basis of natural variation, adaption and speciation in Mimulus. Andrea Sweigart is currently a postdoctoral researcher at the University of Rochester, Rochester, NY, studying the genetics of speciation. Noland Martin is now a faculty member at Texas State University studying the ecological genetics of speciation and introgressive hybridization.
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