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# Genetic basis of tristyly in tetraploid *Oxalis alpina* (Oxalidaceae)

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The inheritance of style-morphs was investigated in tetraploid populations of tristylous *Oxalis alpina* (Oxalidaceae) to determine if alleles controlling style-morphs are expressed at duplicated loci. In tetraploid populations, a dominant *S* allele leads to expression of the short-styled phenotype at the short/non-short locus and is epistatic to the *M* allele at the mid/long locus. The *M* allele results in expression of the mid-styled phenotype but only if the *S* allele is absent. Long-styled morphs are homozygous recessive at the short and mid loci. Test crosses of many tetraploid short-styled individuals resulted in segregations of short-, mid- and long-styled individuals which, because of linkage between the short and mid loci, can only occur with polyploidy and expression of alleles at duplicated loci. Segregation patterns from three crosses suggest the possibility of disomic inheritance via preferential pairing of chromosomes in tetraploid populations of *O. alpina*. Segregation patterns in the progeny of mid-styled individuals indicated that only a few individuals had more than one copy of the *M* allele, despite the potential for accumulation of *M* alleles via self-fertilization of partially self-compatible mid-styled morphs in some populations. © 2015 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2015, **179**, 308–318.

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## INTRODUCTION

Flowering plants have evolved a great diversity of breeding systems that promote outcrossing, including heterostylous systems in which differences in morphology are linked to self-incompatibility (Barrett & Shore, 2008). In distylous species, two self-incompatible floral morphs occur in populations, one with stigmas located in the short position and anthers in the long position and one with stigmas in the long position and anthers in the short position. In tristylous species, three self-incompatible floral morphs occur in populations (Weller *et al.*, 2007), with stigmas located in the short, mid or long position of each flower and anthers located in the remaining two positions. In both distyly and tristyly, pollinations leading to fertilization and seed production are normally outcrosses between floral morphs that have anthers and stigmas at the same level. Although the genetic basis for heterostyly in diploid populations of *Oxalis* L. has been characterized, the effects of polyploidy on the genetic control of style morphs in related species are not well understood. We used a crossing programme to investigate segregation patterns of style morphs in several tetraploid populations of *O. alpina* Rose ex R.Knuth.

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Heterostylous species were some of the earliest examples of application of Mendelian principles to inheritance patterns (Bateson & Gregory, 1905; Ernst, 1936a,b; Fisher, 1941; Fisher & Mather, 1943). Insights into the genetic mechanism controlling heterostyly (reviewed by Barrett & Shore, 2008) were possible because of the conspicuous floral morphology associated with heterostyly and the ease of carrying out controlled crosses. In distylous, diploid species a single locus determines style morphs and in most distylous species the genotype of the short-styled morph is *Ss* and the genotype of the long-styled morph is *ss* (Barrett & Shore, 2008). Distyly appears to be controlled by a supergene (Ernst, 1936a,b), with several tightly linked genes controlling different features of distyly. In tristylous, diploid species determination of floral morphs is controlled by two loci, the short/non-short locus (hereafter the *S* locus) and the mid/long locus (hereafter the *M* locus), which may also be supergenes although no direct evidence supports this idea (Barrett & Shore, 2008). Short-styled individuals have at least one copy of a dominant allele conferring the short-styled phenotype (hereafter the *S* allele). Typically, with the presence of a dominant *S* allele the *S* locus is epistatic to the *M* locus; in the absence of a dominant *S* allele, individuals in tristylous diploid populations are either mid-styled (*ssMm* or *ssMM*) or long-styled (*ssmm*; Table 1a). The *S* and *M* loci may be linked, as they are in several *Oxalis* species (Fyfe, 1950; Weller, 1976), or unlinked, as in tristylous species of *Lythrum* L. (Lythraceae; Fisher, 1941) and *Pontederia* L. (Pontederiaceae; Gettys & Wofford, 2008).

In *Oxalis* section *Ionoxalis* (Small) R.Knuth, previous crosses using tristylous, diploid species indicated that the *S* allele is dominant and epistatic to the *M* locus and that the *S* and *M* loci are linked (Table 1a; Weller, 1976). Evidence for linkage between the *S* and *M* loci included crosses between short- and long-styled morphs that yielded 1:1 segregations of short- and mid-styled morphs, probably indicating the presence of an allele conferring the mid-styled phenotype (hereafter the *M* allele) linked in repulsion (*trans* linkage) to the *S* allele.

The effects of polyploidy on inheritance will depend on many factors including the range of relatedness of the genomes (auto- vs. allopolyploidy; Soltis, Soltis & Tate, 2003), which leads to different expectations for pairing at meiosis and segregation patterns in offspring (Soltis & Soltis, 1993; Stift *et al.*, 2008). Predicted segregation patterns and thus ratios of style morphs in polyploid, tristylous species depend on how many copies of heterostyly alleles are present, whether duplicated genes retain their original function or evolve new functions, become silenced via epigenetic effects, lose function and become pseudogenes through the accumulation of mutations, or whether the chromosomes carrying these duplicated genes undergo selection for pairing behaviour (Soltis & Soltis, 2000; Adams, 2007; Tate *et al.*, 2009; Koh, Soltis & Soltis, 2010). Variation among populations along the spectrum between disomic and tetrasomic segregation patterns is likely, in view of differences in degree of chromosomal homology between species or populations within species (Stift *et al.*, 2008; Koh *et al.*, 2010), and the occurrence of disomic segregation patterns in autotetraploids via selection for diploidized meiotic pairing (Wendel, 2000). With the use of genomic approaches, the consequences of gene duplication arising via polyploidy have become increasingly clear (Soltis *et al.*, 2003; Adams, 2007; Tate *et al.*, 2009; Buggs *et al.*, 2012). Although differences in gene function may evolve with polyploidy, few studies have addressed how polyploidy might impact style-morph ratios and breeding system evolution in heterostylous species. Tetraploid populations of *O. alpina* provide an opportunity to determine how polyploidy may affect the genetic control of style-morph expression.

In many tetraploid populations of *O. alpina* from the Sky Islands region of the south-western United States and northern Mexico, modifications of incompatibility have led to selection against mid-styled morphs (Weller *et al.*, 2007; Weber *et al.*, 2013) and, in combination with stochastic events (Pérez-Alquicira *et al.*, 2010), have resulted in loss of mid-styled morphs in some populations. In many tristylous populations, modifications of incompatibility relationships in short- and long-styled morphs favour increased exchange of alleles between these morphs at the expense of alleles from mid-styled morphs (genic selection; Weller *et al.*, 2007), favouring loss of the mid-styled morph. Mid-styled morphs in populations with modified incompatibility relationships are typically moderately to highly self-compatible (Weller *et al.*, 2007; Weber *et al.*, 2013), which might slow loss of the mid-styled morph if self-fertilization of mid-styled morphs leads to multiple copies of the *M* allele in mid-styled morphs of polyploid populations (and also in short-styled morphs via outcrossing). Alternatively, increased expression of inbreeding depression in progeny of partially self-compatible mid-styled morphs could lead to selection against the mid-styled morph (Weber *et al.*, 2013).

To understand how polyploidy and modifications of the heterostylous breeding system affect style-morph segregations, our first objective was to use tetraploid Sky Island populations in a crossing programme and characterize the genetic basis underlying style morphs in tetraploid populations. We assumed linkage between the *S* and *M* loci based on results from closely related diploid species (Weller, 1976). We predicted dominance of the *S* allele, and epistasis of this allele at

**Table 1.** Expected patterns of style morph segregations in crosses of diploid and polyploid species of *Oxalis* section *Ionoxalis*, assuming linkage between the short and mid loci and disomic (preferential pairing) vs. tetrasomic (non-preferential pairing) segregation patterns in polyploid species; predicted ratios following test crosses to homozygous recessive long-styled individuals (*ssmm* diploid, or *ssssmmmm* tetraploid) are shown, in addition to ratios expected following self-fertilization of short- and mid-styled morphs

(a) Expected segregation patterns in diploid species. All hypothesized segregation patterns were found in outcrosses of diploid species of *Oxalis* section *Ionoxalis* (Weller, 1976). These segregation patterns could also be found in polyploid species in which gene silencing has occurred.

| Phenotypes of plants used in crosses and selfs | Expected ratios in progeny | | | Genotypes of short- or mid-styled individuals used in test crosses to long-styled morphs or in self pollinations. |
|---|---|---|---|---|
| | S | M | L | |
| Short- × long-styled | 1 | 0 | 1 | $\frac{Sm}{sm}\times\frac{sm}{sm}$ or $\frac{SM}{sm}\times\frac{sm}{sm}$ |
| Short- × long-styled | 1 | 1 | 0 | $\frac{Sm}{sM}\times\frac{sm}{sm}$ or $\frac{SM}{sM}\times\frac{sm}{sm}$ |
| Mid- × long-styled | 0 | 1 | 1 | $\frac{sm}{sM}\times\frac{sm}{sm}$ |
| Mid- × long-styled | 0 | 1 | 0 | $\frac{sM}{sM}\times\frac{sm}{sm}$ |
| Short-styled self | 3 | 0 | 1 | $\frac{Sm}{sm}\times\frac{Sm}{sm}$ |
| Mid-styled self | 0 | 3 | 1 | $\frac{sm}{sM}\times\frac{sm}{sM}$ |

(b) Expected patterns of style morph segregations for a tetraploid species with tetrasomic inheritance. Linkage patterns are shown for each chromosome. Rare cases of short-styled individuals with more than one *M* allele are not shown. Examples of possible chromosomal combinations are given in Supporting Information B for several key crosses.

| Phenotypes of plants used in crosses and selfs | Expected ratios in progeny | | | Genotypes of short- or mid-styled individuals used in test crosses to long-styled morphs or in self pollinations. |
|---|---|---|---|---|
| | S | M | L | |
| Short- × long-styled | 1 | 0 | 1 | *Sm sm sm sm* × *sm sm sm sm* or *SM sm sm sm* × *sm sm sm sm* |
| Short- × long-styled | 3 | 2 | 1 | *Sm sM sm sm* × *sm sm sm sm* |
| Mid- × long-styled | 0 | 1 | 1 | *sM sm sm sm* × *sm sm sm sm* |
| Mid- × long-styled | 0 | 5 | 1 | *sM sM sm sm* × *sm sm sm sm* |
| Short-styled self | 3 | 0 | 1 | *Sm sm sm sm* × *Sm sm sm sm* |
| Short-styled self | 27 | 8 | 1 | *Sm sM sm sm* × *Sm sM sm sm* |
| Mid-styled self | 0 | 3 | 1 | *sM sm sm sm* × *sM sm sm sm* |
| Mid-styled self | 0 | 35 | 1 | *sM sM sm sm* × *sM sM sm sm* |

(c) Expected style morph segregations for a tetraploid species with disomic inheritance via diploidized meiotic pairing behaviour and absence of gene silencing. Rare cases of short-styled individuals with more than one *M* allele are not shown. Examples of possible chromosomal combinations are given in Supporting Information B for several key crosses.

| Phenotypes of plants used in controlled pollinations | Expected ratios in progeny | | | Genotypes of short- or mid-styled individuals used in test crosses to long-styled morphs or in self pollinations. |
|---|---|---|---|---|
| | S | M | L | |
| Short- × long-styled | 1 | 0 | 1 | $\frac{Sm}{sm}\frac{sm}{sm}\times\frac{sm}{sm}\frac{sm}{sm}$ or $\frac{SM}{sm}\frac{sm}{sm}\times\frac{sm}{sm}\frac{sm}{sm}$ |
| Short- × long-styled | 1 | 1 | 0 | $\frac{Sm}{sM}\frac{sm}{sm}\times\frac{sm}{sm}\frac{sm}{sm}$ |
| Short- × long-styled | 2 | 1 | 1 | $\frac{Sm}{sm}\frac{sM}{sm}\times\frac{sm}{sm}\frac{sm}{sm}$ |
| Mid- × long-styled | 0 | 1 | 1 | $\frac{sM}{sm}\frac{sm}{sm}\times\frac{sm}{sm}\frac{sm}{sm}$ |
| Mid- × long-styled | 0 | 3 | 1 | $\frac{sM}{sm}\frac{sM}{sm}\times\frac{sm}{sm}\frac{sm}{sm}$ |
| Mid- × long-styled | 0 | 1 | 0 | $\frac{sM}{sM}\frac{sm}{sm}\times\frac{sm}{sm}\frac{sm}{sm}$ |
| Short-styled self | 3 | 0 | 1 | $\frac{Sm}{sm}\frac{sm}{sm}\times\frac{Sm}{sm}\frac{sm}{sm}$ |
| Short-styled self | 3 | 1 | 0 | $\frac{Sm}{sM}\frac{sm}{sm}\times\frac{Sm}{sM}\frac{sm}{sm}$ |
| Mid-styled self | 0 | 3 | 1 | $\frac{sM}{sm}\frac{sm}{sm}\times\frac{sM}{sm}\frac{sm}{sm}$ |
| Mid-styled self | 0 | 15 | 1 | $\frac{sM}{sM}\frac{sm}{sm}\times\frac{sM}{sM}\frac{sm}{sm}$ |

the mid/long locus, as in diploid species of *Oxalis* section *Ionoxalis*. Our second objective was to determine whether multiple copies of the *M* allele could be detected in mid-styled morphs, and the likelihood that potential accumulation of *M* alleles would affect segregation patterns and the frequency of mid-styled morphs in tetraploid populations of *O. alpina.* Accumulation of *M* alleles via polyploid segregation patterns could counter selection against the *M* alleles and mid-styled morphs occurring in some of these populations (Weller *et al.*, 2007; Weber *et al.*, 2013) and potentially delay the evolutionary transition from tristyly to distyly.

## MATERIALS AND METHODS

### Study system

Populations of *O. alpina* vary widely in ploidy (Weller & Denton, 1976) and phylogenetic studies of *Oxalis* section *Ionoxalis* indicate that *O. alpina* is not monophyletic when the entire range is considered (Gardner *et al.*, 2012). In this study, all populations used were from the Sky Islands of the Sonoran Desert, isolated mountain ranges in Arizona, New Mexico and the northern Mexican state of Sonora, and share a common ancestor (Pérez-Alquicira *et al.*, 2010). Individuals of *O. alpina* used for crosses were collected from seven populations throughout the Sky Islands [Supporting Information A; Weller *et al.* (2007); population numbers represent Weller or Weller and Sakai vouchers at University of California (UC) or the Smithsonian Institution (US) herbaria]. Populations in this geographical region with chromosome counts are uniformly tetraploid (Weller & Denton, 1976; Supporting Information A), and probably originated via autotetraploidy (O. V. Tsyusko, pers. comm.). Chromosome counts are not available for all populations, but crosses between known tetraploid populations and those without chromosome counts produce viable seed, indicating that all populations in this region are tetraploid as crosses between populations with different ploidies do not form viable seeds (Weller, 1978; S. G. Weller, unpubl. data). Flowering individuals were collected at intervals of at least 1 m to prevent sampling from the same clone; analyses of microsatellite variation have confirmed the absence of clonal growth at this scale (O. V. Tsyusko, pers. comm., 2009).

### Crossing programme

Our crossing programme characterized segregation patterns of style morphs in tetraploid populations to address our first objective, and the number of *M* alleles in mid-styled individuals to address our second objective. Crosses included legitimate and a few illegitimate but highly fecund outcrosses [legitimate crosses are those occurring between anthers and stigmas at the same level (Darwin, 1877); illegitimate crosses are those occurring between anthers and stigmas at different levels; see Weller *et al.* (2007) for details of incompatibility relationships in these populations]. In addition, we were able to infer parental genotypes from self-pollinations of some partially self-compatible individuals (especially mid-styled individuals) that produced sufficient progeny. Crosses were designated as female × male parent (e.g. cross S1 × L10 refers to short-styled individual 1 used as a maternal parent and long-styled individual 10 used as the paternal parent). To determine the frequency of the *M* allele in short- and mid-styled morphs, 5–21 short-styled individuals per population and 6–15 mid-styled individuals per population were used in outcrosses in each of the six populations (total across all six populations = 65 short-styled and 64 mid-styled individuals; a single highly self-fertile short-styled individual was used from the seventh population). Long-styled morphs, which were expected to be uniform at the loci determining style morph and contain only recessive *s* and *m* alleles, were used as parents in test crosses (Table 1). Six to 11 long-styled individuals were used per population (total for all populations = 56). Nine short-styled plants in three populations, 39 mid-styled plants in five populations and ten long-styled individuals from five populations were sufficiently self-compatible to provide progeny from self-pollinations.

Pollinations were carried out in June–September during the relatively short flowering period in a pollinator-free greenhouse using previously described methods (Weller *et al.*, 2007). Each pollinated flower was individually tagged, and capsules were collected approximately 14–15 days after pollination before the capsules opened. Controls (tagged flowers that were not manipulated) did not produce capsules, verifying the absence of contamination due to potential pollinating insects. Seeds were planted in community pots of ten seeds each, about 9 months after they were produced following a requisite dormant period, and seedlings were transplanted into individual pots about 2 months after germination and scored for floral morph as soon as flowering occurred, usually within 3–4 months following germination. Of the 4310 progeny scored for flowering, we detected only 13 (0.30%) apparent cases of contamination, although two of these cases may have resulted from crossing over.

### Crosses used to infer genotypes

Outcrosses of short- or mid-styled morphs with long-styled morphs (test crosses) and self-pollinations of partially or fully self-compatible individuals of all three style morphs were used to infer genotypes gov-

erning control of tristylous floral morphs. Progeny from crosses between short- and mid-styled morphs were not used to infer parental genotypes because predicted segregation patterns depending on different models of transmission differ only minimally in many cases and are therefore statistically difficult to distinguish from each other without very large numbers of progeny. Progeny from crosses of the same short- or mid-styled parent with different long-styled individuals were combined for analysis of segregation ratios of the style morphs. Reciprocal crosses were also combined because we did not detect heterogeneity in progeny morph ratios following reciprocal crosses. Progeny from different short- × long-styled crosses that segregated only short- and long-styled morphs were combined to test for potential distortion of the expected 1:1 ratio that might be detected only with larger samples. Similarly, progeny from different mid- × long-styled crosses were combined in those cases in which the mid-styled parent was inferred to have a single *M* allele. Chi-squared tests were used to test segregation patterns against expected patterns (Table 1) when there were 15 or more progeny of a single short- or mid-styled genotype and 1:1 ratios were expected. For all other segregations, a minimum of five expected individuals in each cell was required before calculating a $\chi^2$ test.

### Determination of expected segregation patterns

To characterize the genetic basis of style morphs in tetraploid populations (Objective 1), segregation patterns of style morphs were compared with those predicted for diploids (this hypothesis assumes loss of copies or gene silencing in tetraploids) and for tetraploids based on disomic and polysomic segregation patterns (Table 1; Supporting Information B). These same crosses were also used to determine whether multiple copies of *M* alleles could be detected within some tetraploid individuals of *O. alpina* (Objective 2). All primary data are given in Supporting Information C.

## RESULTS

### Crosses with short-styled plants

In test crosses to homozygous recessive, long-styled plants, short-styled individuals that yielded only short- and long-styled progeny usually produced them in 1:1 ratios (Table 2; Supplementary Material C), consistent with the occurrence of a single *S* allele. Unexpectedly, excesses of short-styled plants occurred in the Animas and White Mountains populations when progeny were combined across all short-styled parents. In five of six populations some short-styled plants segregated mid-styled progeny as well as short- and long-styled progeny (Table 2; Supporting Information C); segregation of mid-styled plants from these short- × long-styled crosses indicates that the *S* allele is epistatic to the *M* allele, as in diploid, tristylous species of *Oxalis* section *Ionoxalis*. These segregations also indicate the influence of polyploidy on segregation patterns (see Supporting Information B, test crosses 1 and 2, for diagrams and explanations of effects of duplicated loci on segregation patterns). In two populations (Mariquita and White, Table 2b, e) short-styled individuals with moderate self-compatibility yielded short- and long-styled progeny following self-fertilization in the 3:1 ratio predicted by disomic and polysomic modes of inheritance. Selfing of three short-styled individuals (S13, Mariquita; S2, White; S32, Azul, Table 2b, e, g.) resulted in short-and mid-styled progeny, again consistent with the occurrence of epistasis of the *S* allele in these populations. In test crosses, plant S2 also produced long-styled progeny in addition to short- and mid-style progeny, which was unexpected given the absence of long-styled morphs in progeny produced from self-fertilization, and was possibly related to sample size.

### Crosses with mid-styled plants

Mid-styled × long-styled test crosses usually resulted in equal numbers of mid- and long-styled plants; based on these results a single copy of the *M* allele was present in 48 of 49 mid-styled plants with sufficient progeny for $\chi^2$ analysis (Table 2; Supporting Information C). When selfed, most mid-styled plants produced the 3:1 segregation ratios predicted by disomic and polysomic modes of inheritance (Table 2; Supporting Information C). One mid-styled plant from the Animas Mts (plant M49, Table 2c) segregated only mid-styled progeny when crossed to long-styled individuals or when selfed (ignoring two short-styled progeny probably due to contamination), a result that could be explained by polysomic inheritance and the occurrence of three *M* alleles given the absence of long-styled progeny (with two *M* alleles, some long-styled progeny are expected in test crosses; Supporting Information B; test cross 4).

### Crosses with long-styled plants

Seven long-styled individuals from five populations were relatively self-compatible and produced progeny ($N = 99$) following self-pollination. With the exception of two plants, all progeny were long-styled, consistent with the prediction that long-styled morphs are homozygous recessive at both the *M* and the *S* loci. The occurrence of two short-styled progeny presumably resulted from contamination with pollen from

**Table 2.** Summary of style morph frequencies in progeny following controlled crosses in seven tetraploid populations of *Oxalis alpina*, with expected style-morph segregations, depending on whether inheritance is disomic (diploid case), tetrasomic (all possible chromosome pairing equally likely) or tetraploid and disomic (preferential pairing via diploidized meiotic pairing behaviour); see Supporting Information C for details of all crosses

| Progeny | | | | | | Possible models of inheritance | | |
|---|---|---|---|---|---|---|---|---|
| | S | M | L | $\chi^2$ (1:1 ratio) | $\chi^2$ (3:1 ratio; two *M* alleles, disomic inheritance) | Disomic, (through gene silencing) | Polysomic, tetraploid | Disomic, tetraploid |
| **(a) Sierra La Purica (968)** | | | | | | | | |
| Short-styled parent (test cross) | | | | | | | | |
| Sum (five crosses) | 109 | 0 | 129 | 1.681 | | 1:1, yes | 1:1, yes | 1:1, yes |
| Mid-styled parent (test cross) | | | | | | | | |
| Sum (six crosses) | 0 | 91 | 76 | 1.347 | 37.46*** | 1:1, yes | 1:1, yes | 1:1, yes |
| **(b) Sierra La Mariquita (960)** | | | | | | | | |
| Short-styled parent ( test crosses) | | | | | | | | |
| Sum (four crosses) | 41 | 0 | 31 | 1.389 | | 1:1, yes | 1:1, yes | 1:1, yes |
| Sum (two crosses) | 19 | 13 | 4 | | | **1:1, no** | 3:2:1, yes ($\chi^2$ = 0.806) | 2:1:1, yes ($\chi^2$ = 4.556) |
| Short-styled parent (self) | | | | | | | | |
| S13 × S13 (mid-styled morphs also segregated following test cross) | 5 | 2 | 0 | | | 3S:1M, yes | 27:8:1, yes | 12:3:1, yes |
| Sum (two crosses) | 19 | | 9 | | 0.762 | 3:1, yes | 3:1, yes | 3:1, yes |
| Mid-styled parent (test cross) | | | | | | | | |
| Sum (eight crosses) | 0 | 67 | 64 | 0.0687 | 39.76 | 1:1, yes | 1:1, yes | 1:1, yes |
| Mid-styled parent (self) | | | | | | | | |
| Sum (nine maternal genotypes) | 0 | 31 | 15 | | 1.4202 | 3:1, yes | 3:1, yes | 3:1, yes |
| Long-styled parent (self) | | | | | | | | |
| Sum (two maternal genotypes) | 0 | 0 | 37 | | | 0:0:1, yes | 0:0:1, yes | 0:0:1, yes |
| **(c) Animas Mts (973)** | | | | | | | | |
| Short-styled parent (test crosses) | | | | | | | | |
| Sum (15 crosses; individual crosses did not show excesses of short-styled progeny) | 202 | 1† | 154 | 6.472* | | **1:1, no (S excess)** | **1:1, no (S excess)** | **1:1, no (S excess)** |
| Sum (five crosses) | 66 | 37 | 23 | | | **1:1, no** | 3:2:1, yes ($\chi^2$ = 0.929) | 2:1:1, yes ($\chi^2$ = 3.397) |
| S143 | 16 | 10 | 13 | | | **1:1, no** | **3:2:1, no ($\chi^2$ = 7.821)** | 2:1:1, yes ($\chi^2$ = 1.718) |
| Mid-styled parent (test cross) | | | | | | | | |
| Sum (14 crosses) | 1†* | 174 | 193 | 0.984 | 148.98*** | 1:1, yes | 1:1, yes | 1:1, yes |
| M49 (test cross) | 0 | 41 | 0 | | | yes (2 *M* alleles) | yes (3 *M* alleles) | Possible (two *M* alleles in different chromosome pairs) |
| Mid-styled parent (self) | | | | | | | | |
| Sum (five crosses) | 0 | 52 | 21 | | 0.553 | 3:1, yes | 3:1, yes | 3:1, yes |
| M49 × M49 (self) | 2† | 14 | 0 | | | yes (2 *M* alleles) | Yes (3 *M* alleles) | Possible (two *M* alleles in same chromosome pair) |
| Long-styled parent (self) | | | | | | | | |
| Sum (three maternal parents) | 2 | 0 | 43 | | | 0:0:1, yes | 0:0:1, yes | 0:0:1, yes |
| **(d) Chiricahua Mts (727)** | | | | | | | | |
| Short-styled parent (test cross) | | | | | | | | |
| Sum (eight crosses) | 78 | 0 | 68 | 0.685 | | 1:1, yes | 1:1, yes | 1:1, yes |
| S26 | 13 | 10 | 4 | | | **1:1, no** | 3:2:1, yes ($\chi^2$ = 0.185) | 2:1:1,yes ($\chi^2$ = 2.704) |
| Mid-styled parent (test cross) | | | | | | | | |
| Sum (11 crosses) | 0 | 126 | 132 | 0.140 | 94.19*** | 1:1, yes | 1:1. yes | 1:1, yes |
| Mid-styled parent (self) | | | | | | | | |
| Sum (ten genotypes) | 1† | 84 | 25 | | 0.248 | 3:1, yes | 3:1, yes | 3:1, yes |
| Long-styled parent (self) | | | | | | | | |
| (one maternal parent) | 0 | 0 | 37 | | | 0:0:1, yes | 0:0:1, yes | 0:0:1, yes |
| **(e) White Mts (713)** | | | | | | | | |
| Short-styled parent (test cross) | | | | | | | | |
| Sum (seven genotypes; one individual cross with an excess of short-styled morphs) | 137 | 0 | 93 | 8.417 | | **1:1, no** | **1:1, no** | **1:1, no** |
| S2 | 23 | 8 | 7 | | | **no** | 3:2:1, yes ($\chi^2$ = 2.632) | 2:1:1, yes ($\chi^2$ = 1.737) |
| S15 | 14 | 15 | 13 | | | **no** | **3:2:1, no ($\chi^2$ = 7.548)** | 2:1:1, yes ($\chi^2$ = 4.857) |
| Short-styled parent (self) | | | | | | | | |
| Sum (four genotypes) | 41 | 0 | 18 | | 0.955 | 3:1, yes | 3:1, yes | 3:1, yes |

**Table 2.** *Continued*

| Progeny | | | | | | Possible models of inheritance | | |
|---|---|---|---|---|---|---|---|---|
| | S | M | L | $\chi^2$ (1:1 ratio) | $\chi^2$ (3:1 ratio; two *M* alleles, disomic inheritance) | Disomic, (through gene silencing) | Polysomic, tetraploid | Disomic, tetraploid |
| S2 × S2 (preferential pairing assumes that *S* and *M* alleles are on different homoeologous pairs, as suggested by test cross) | 14 | 11 | | | 4.81 | **3S:1M, no** ($\chi^2$ = 4.81, assumes *trans* linkage of *S* and *M* alleles) | **27:8:1, no** ($\chi^2$ = 4.81, combining mid- and long-styled morphs) | **12:3:1, no** ($\chi^2$ = 4.81, combining mid- and long-styled morphs) |
| Mid-styled parent (test cross) | | | | | | | | |
| Sum (ten genotypes) | 0 | 203 | 227 | 1.340 | 177.1*** | 1:1, yes | 1:1, yes | 1:1, yes |
| Mid-styled parent (self) | | | | | | | | |
| Sum (eight genotypes) | 0 | 129 | 55 | | 2.348 | 3:1, yes | 3:1, yes | 3:1, yes |
| Long-styled parent (self) | | | | | | | | |
| Sum (two genotypes) | 0 | 0 | 37 | | | 0:0:1, yes | 0:0:1, yes | 0:0:1, yes |
| **(f) Pinos Altos (971)** | | | | | | | | |
| Short-styled parent (test cross) | | | | | | | | |
| Sum (13 genotypes) | 202 | 1† | 193 | 0.205 | | 1:1, yes | 1:1, yes | 1:1, yes |
| S14 | 5 | 1 | 12 | | | **no** | **3:2:1, no** ($\chi^2$ = 32.94) | **2:1:1, no** ($\chi^2$ = 17.0) |
| S15 | 5 | 3 | 3 | | | **no** | 3:2:1, yes ($\chi^2$ = 0.909) | 2:1:1, yes ($\chi^2$ = 0.091) |
| Mid-styled parent (test cross) | | | | | | | | |
| Sum (14 crosses) | 0 | 205 | 208 | 0.022 | 141.70*** | 1:1, yes | 1:1, yes | 1:1, yes |
| Mid-styled parent (self) | | | | | | | | |
| Sum (six genotypes) | 0 | 82 | 35 | | 1.505 | 3:1, yes | 3:1, yes | 3:1, yes |
| Long-styled parent (self) | | | | | | | | |
| Sum (two genotypes) | 1† | 0 | 29 | | | 0:0:1, yes | 0:0:1, yes | 0:0:1, yes |
| **(g) Sierra Azul** | | | | | | | | |
| Short-styled parent S32 (self) (assuming one *M* allele, *S* and *M* alleles are on different homoeologous pairs) | 14 | 5 | 0 | 3:1, expected value for mid-styled morphs is <5 | | 27:8:1, expected value for combined mid- and long-styled morphs is <5 | | 12:3:1, expected value for combined mid- and long-styled morphs is <5 |

Linkage between the *S* and *M* loci is assumed in all models of inheritance. In all outcrosses, short- and mid-styled morphs were crossed to homozygous recessive long-styled individuals. Reciprocal crosses using the same short- or mid-styled parents are combined. Within cross categories in each population, segregations from different parents but with identical style morphs combinations were grouped if the individual crosses showed the same pattern of inheritance and crosses did not differentiate among different modes of segregation. Progeny of short-styled morphs were grouped by presence or absence of mid-styled morphs. Progeny of mid-styled morphs were grouped by presence of one or more *M* alleles. In a few cases, progeny sizes were large enough to test models of inheritance for individual parents used in test crosses and self-pollinations. Segregations consistent with patterns from Table 1 are shown under 'Possible modes of inheritance;' 'yes' indicates a model of inheritance is possible; 'no' in bold type indicates that the observed segregation pattern was inconsistent with the proposed genetic model, either through the presence of an unexpected style morph or because of a skewed representation of style morphs that were predicted to occur. For segregations following mid- × long-styled crosses, ratios were tested against the 1:1 ratio expected for parents with a single *M* allele, and 3:1 ratios expected for preferential pairing (disomic inheritance) when two *M* alleles are present (no tests of 5:1 ratios expected for tetrasomic inheritance were carried out as all ratios were close to 1:1). Progeny produced from self-pollinations of short-styled morphs were tested against a 3:1 ratio, unless mid-styled morphs segregated. Progeny produced from self-pollinations of mid-styled morphs were tested against a 3:1 ratio, except for those mid-styled morphs that segregated no long-styled morphs. Progeny from self-pollinations using two possible anther whorls were combined. A † indicates presumed contamination in the style morph columns. Asterisks in columns showing $\chi^2$ values indicate significance level (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

short-styled individuals carrying the dominant *S* allele (Table 2, Supporting Information C).

## DISCUSSION

### OBJECTIVE 1: INHERITANCE OF STYLE MORPHS IN TETRAPLOID POPULATIONS OF *O. ALPINA*

Segregations and style-morph ratios following controlled crosses in tetraploid populations of *O. alpina* demonstrate that the genetic basis of style morphs is similar in many respects in diploid and polyploid species of *Oxalis* section *Ionoxalis*. The similarity among these species in the genetic system controlling style morphs is consistent with the origin of most North American species of section *Ionoxalis* from a South American ancestor in the same section (Gardner *et al.*, 2012). As in diploid species of North American section *Ionoxalis*, in polyploid populations the *S* allele is dominant and epistatic to the *M* allele and the mid-styled phenotype occurs only in the

absence of the *S* allele (Weller, 1976). In both diploid and tetraploid populations, most short-styled morphs cannot self or cross with other short-styled individuals to produce progeny with more than a single copy of the *S* allele because strong self-incompatibility prevents matings between two short-styled plants (the cause for excesses of the short-styled morph in test crosses from several populations is unknown). The occurrence of considerable self-compatibility in some individuals of all three style morphs in tetraploid populations of *O. alpina* facilitates understanding of dominance and epistasis; segregation of mid-styled morphs following selfing of short-styled individuals is direct evidence for epistasis of the *S* allele, and segregation of only long-styled progeny following self-pollinations of the long-styled morph is conclusive evidence that this morph is homozygous and recessive at the *S* and *M* loci (genotype *ssssmmmm*). Segregations following self-pollinations in tetraploid populations were consistent with those expected based on results from outcrossing in these populations, suggesting that the inbreeding depression detected in these populations (Weber *et al.*, 2013) has not altered predicted segregation patterns.

Several crosses clearly demonstrated the influence of polyploidy on segregation patterns. In most populations test crosses of some short-styled individuals segregated short-, mid- and long-styled individuals, which because of linkage between the short and mid loci would not occur for the diploid case unless crossing over had occurred, and then few mid-styled morphs would occur (Weller, 1976). These segregation patterns from short-styled individuals suggest that silencing (or loss) of heterostyly genes has not occurred. With loss or gene silencing, segregation of only short- and mid-styled progeny would occur for some test crosses of short-styled individuals, as in diploid species where the two alleles are linked in the *trans* position (Table 1a). Segregations of only short- and mid-styled individuals could also occur in tetraploid species with disomic inheritance if the *S* and *M* alleles were linked in the *trans* position, although we found no evidence for any individuals with this linkage pattern (Supporting Information C). Consistent with earlier results for diploid species (Weller, 1976) we found little evidence for crossing over between the *S* and *M* loci, although two mid-styled progeny in progeny of test crosses of short-styled individuals from the Animas and Pinos Altos populations may have resulted from crossing over between *S* and *M* alleles linked in the *cis* position. The occurrence of multiple alleles at the heterostyly loci is consistent with the ability to detect more than two alleles in individuals in ten of 12 microsatellite markers isolated from the Animas Mts and tested for amplification in eight Sky Island populations of *O. alpina* (Tsyusko *et al.*, 2007). Segregation patterns consistent with expression of multiple copies of alleles at the heterostyly locus were also found in artificially synthesized tetraploids of *Turnera ulmifolia* L. (Passifloraceae; Shore & Barrett, 1985).

Aside from other *Oxalis* species in section *Ionoxalis*, control of tristyly in tetraploid populations of *O. alpina* is most similar to the pattern found for *O. valdiviensis* Barnéoud (Fyfe, 1950). Although *O. valdiviensis* is diploid, the two species are otherwise similar in possessing a two-locus system governing expression of style morphs, an *S* locus that is epistatic to the *M* locus and linkage between the *S* and *M* loci. In contrast to *O. alpina* and related diploid species, considerable crossing over in *O. valdiviensis* is necessary to explain the segregation of long-styled progeny from short-styled morphs with the *M* allele linked in repulsion (table 1 in Fyfe, 1950). Genetic control of tristyly diverges significantly in *O. articulate* Savgny, in which the *S* allele is recessive to the *L* allele (Fyfe, 1956; Bennett, Leach & Goodwin, 1986), and in *O. tuberosa* Molina, in which short-styled morphs consistently appeared in small numbers in the progeny of mid- × long-styled crosses, complicating interpretation of the genetic basis of style morph expression for this species (Trognitz & Hermann, 2001). Features of the genetic system controlling expression of tristyly in *O. alpina* are shared by species in Lythraceae and Pontederiaceae, in which *S* and *M* loci control expression of style morphs, the *S* locus is epistatic to the *M* locus, the loci may be linked or unlinked, and polyploidy occurs in some species (Fisher & Mather, 1943; Barrett, Morgan & Husband, 1989; Eckert & Barrett, 1993; Gettys & Wofford, 2008).

### Objective 2: influence of polyploidy on segregation patterns of *M* alleles and loss of the mid-styled morph during the evolutionary transition from tristyly to distyly

Fisher (1941) developed equations to calculate the equilibrium frequencies of the mid-styled morphs with more than a single copy of the *M* allele in disomic, tetrasomic and hexasomic cases. In populations with equal morph representation and tetrasomic inheritance, an individual with two *M* alleles was predicted to occur at a frequency of 0.0352 relative to a frequency of 0.00241 for individuals with three *M* alleles (Fisher, 1941). In our study, one of 49 families (plant M49, Animas; Table 2c) produced only mid-styled offspring, a pattern expected with polysomic inheritance when the mid-styled parent had three or more *M* alleles. Fisher (1941) predicted these individuals to be quite rare; among the 49 mid-styled families the predicted number of individuals with three *M* alleles is 0.12, indicating that the *O. alpina* individual that segregated only mid-styled progeny is unlikely to have had

three *M* alleles. An alternate hypothesis is the occurrence of two *M* alleles and disomic inheritance with the *M* alleles on the same chromosome pair. Few other crosses are capable of differentiating between disomic and tetrasomic inheritance because segregation patterns are so similar for the two modes of inheritance that large numbers of progeny are necessary [compare, for example, tetrasomic (Table 2b) vs. disomic (Table 2c) segregations for the short- × long-styled crosses where short-styled parents contain a single *M* allele and all three morphs segregate]. Two short-styled individuals, however, had segregation patterns consistent with disomic inheritance but not tetrasomic inheritance (Animas: plant S143; White: plant S15; Table 2c, e, respectively). Test crosses of short-styled individuals that segregated all three style morphs summed within populations provided no further insights as progeny sizes were still too small to differentiate between similar expected segregation patterns. Self-pollinations of two short-styled individuals (White: plant S2; Azul: plant S32; Supporting Information C) segregated only short- and mid-styled individuals. The plant from the White Mts (S2) produced long-styled plants in a test cross, indicating that in the case of preferential pairing, the *S* and *M* alleles were located on different homoeologous pairs. Absence of long-styled plants following selfing probably resulted from the small sample size. For the plant from Sierra Azul (S32) no test cross was carried out and therefore no model of inheritance could be rejected. Although segregation patterns in progeny of *O. alpina* clearly indicate that more than two copies of the heterostyly loci are functioning, in most cases they cannot distinguish between tetrasomic and disomic patterns of inheritance. Microsatellite markers (O. V. Tsyusko, pers. comm.; Weber *et al.*, 2013) suggest an autotetraploid origin of *O. alpina* populations in the Sky Island region, consistent with the absence of strong support for disomic segregations patterns at the heterostyly loci.

In polyploid populations, increased self-compatibility of the mid-styled morph, characteristic of populations of *O. alpina* with modified tristylous incompatibility (Weller *et al.*, 2007; Weber *et al.*, 2013), could result in mid-styled individuals with multiple copies of the *M* allele. Selection against the *M* allele via genic selection (Weller *et al.*, 2007) or selection against mid-styled genotypes (Weber *et al.*, 2013) would be less effective. Despite the potential for accumulation of *M* alleles in polyploid populations via higher levels of selfing, we found no evidence for increased frequencies of the *M* allele. Few mid-styled individuals had more than a single *M* allele, and mid-styled individuals in populations with the highest levels of mid-styled self-compatibility (e.g. Chiricahua and Pinos Altos Mts; Weller *et al.*, 2007) were no more likely to have multiple copies of the *M* allele than other populations. Expression of inbreeding depression in the progeny of these mid-styled individuals (Weber *et al.*, 2013) may have reduced *M* allele frequency, countering any effect of selfing on frequency of the *M* allele. Overall, polyploidy seems to have had little effect in delaying evolution of distyly in Sky Island populations of *O. alpina*, which appears to have evolved on several occasions based on crossing programmes (Weller, 1978) and phylogeographical studies (Pérez-Alquicira *et al.*, 2010).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**File S1.** A, populations of *Oxalis alpina* in the Sky Island region of the Sonoran Desert used for analysis of the genetic basis of style morphs. Style morph frequencies are based on field surveys from Weller *et al.* 2007. All vouchers are Weller and Sakai unless noted. Asterisks indicate that a gametic chromosome count was obtained (Weller & Denton, 1976); counts were $N = 14$ in all cases. Geographical coordinates are from Pérez-Alquicira *et al.* (2010). B, calculation of expected segregation patterns at tristyly loci based on disomic and polysomic inheritance. C, style-morph frequencies in progeny following controlled crosses in seven populations of *Oxalis alpina*, with expected style-morph segregations, depending on whether inheritance is disomic (diploid case), tetrasomic (all possible chromosome pairing equally likely), or polyploid and disomic (preferential pairing of two

sets of homologous chromosomes). Linkage between the *S* and *M* loci is assumed in all models of inheritance. In all outcrosses, short- and mid-styled morphs were crossed to homozygous recessive long-styled individuals. Reciprocal crosses using the same short- or mid-styled parents are combined. Progeny of short-styled morphs were grouped by presence or absence of mid-styled morphs. Chi-squared values for segregations following cross pollinations were calculated when 15 or more progeny were scored for style morph; segregations consistent with segregation patterns (from Table 1) are shown under 'Possible modes of inheritance'; a 'yes' indicates the model of inheritance is possible, although in many crosses the number of progeny was too small to calculate $\chi 2$ values or differentiate among models. A 'no' in bold type indicates that the observed segregation pattern was inconsistent with the proposed genetic model, either through presence of an unexpected style morph or skewed representation of style morphs that were predicted to occur. Expected segregations are shown for all crosses. Style morphs in progeny are the same as those in the parental cross, unless otherwise noted. For segregations following short- × long-styled crosses, expected 1:1 ratios under the diploid model with linkage could consist either of short- and long-styled progeny, or short- and mid-styled progeny, depending on pattern of linkage. For segregations following mid- × long-styled crosses, ratios were tested against the 1:1 ratio expected for parents with a single *M* allele, and 3:1 ratios expected for preferential pairing (allopolyploidy) when two *M* alleles are present. Progeny produced from self-pollinations of short-styled morphs were tested against a 3:1 ratio, unless mid-styled morphs segregated. Progeny produced from self-pollinations of mid-styled morphs were tested against a 3:1 ratio, except for those mid-styled morphs that segregated no long-styled morphs. Asterisks in columns showing $\chi^2$ values indicate significance level ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Progeny from self-pollinations using two possible anther whorls were combined. A † in a style morph column indicates presumed contamination.