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Establishment of polyploidy in natural populations of *Mimulus guttatus*

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Abstract

Premise of the study: Whole genome duplication (WGD) is a leading force of plant sympatric speciation. However, evolutionary mechanisms promoting establishment on neopolyploid mutants in nature remain elusive. In theory, polyploids can establish in case of assortative mating or fitness advantage. We study polyploid establishment in a unique natural system of *Mimulus guttatus* (Phrymaceae) in the Shetlands where a recently (< 100 years ago) locally formed autotetraploid still coexists with its diploid progenitor.

Methods: We cytotyped 679 adults and 766 seedlings and scored relevant reproductive traits in the field and performed controlled crossings to infer fitness differences and crossing barrier between ploidies.

Key results: Tetraploids grew in 25% of Shetland populations, mostly together with diploids. In spite of large spatiotemporal overlap of the ploidies in nature, triploids were absent both in field and in controlled crossings, suggesting strong post-zygotic isolation. Fitness cost of triploid block, however, was not manifested in the field where we observed no decrease in fertility in areas of close ploidy sympatry. This suggests an additional post-pollination prezygotic isolation mechanism is involved. Indeed, mothers experimentally pollinated by a mixture of pollen from diploid and tetraploid fathers set fertile progeny whose ploidy was identical with the mother.

Conclusions: In contrast to theory, neither pre-pollination segregation nor WGD-linked fitness benefit but prezygotic post-pollination barriers seems important for autotetraploid *Mimulus* establishment. Abundant presence of fertile tetraploids demonstrate the ability of novel autopolyploids to cope with both intrinsic and extrinsic challenges associated with WGD and to successfully establish in nature.

KEYWORDS

autopolyploidy, *Mimulus*, neopolyploid, post-pollination barrier, reproductive isolation, whole- genome duplication

43 INTRODUCTION

44 Polyploidy, which is the result of whole-genome-duplication, has long been recognized to be
45 a prominent evolutionary process for plant speciation and adaptation. It has been estimated
46 that up to 15 % of speciation events in flowering plants are associated with an increase in
47 ploidy (Wood et al., 2009). In addition, it has been demonstrated that polyploids have
48 established themselves repeatedly within species (Ramsey and Schemske, 1998; Soltis and
49 Soltis, 1999). Despite such a strong representation of polyploids, novel polyploid lineages
50 (e.g., neo-tetraploids) are expected to face numerous challenges associated with their survival
51 and establishment. The discrepancy between theoretical expectations on challenges during
52 polyploid establishment (outlined below) and ubiquity of natural polyploidy represents a
53 conundrum that calls for investigation of natural systems of incipient polyploid speciation.

54 Polyploidization within a species (autopolyploidization) could be regarded as severe
55 mutation with considerable negative effects on phenotypic, physiological, and life-history
56 traits including cell-cycle regulation (e.g., (Levin, 1983; Otto and Whitton, 2000; Wright et
57 al., 2015), meiosis (Doyle and Coate, 2019) and reproductive success (Porturas et al., 2019;
58 Clo and Kolář, 2021). As a result, neo-polyploids (exemplified by the most frequent neo-
59 tetraploids with four chromosome sets, hereafter) are faced with competitive disadvantage
60 with the well-established diploids, thereby making their establishment unlikely (Arrigo and
61 Barker, 2012; Clo, 2022). To add insult to injury, novel tetraploid lineages arise sporadically
62 within the diploid population, and, consequently, these rare tetraploids are expected to have
63 lower reproductive success due to the effect of minority cytotype exclusion (MCE) (Levin,
64 1975). Inter-cytotype hybridization is typically observed as a consequence of a lack of mating
65 opportunities among the (initially rare) autotetraploids, resulting in unfit or sterile triploid
66 progeny (Levin, 1975; Thompson and Lumaret, 1992; Husband, 2000; Otto and Whitton,

2000; Ramsey and Schemske, 2002; Hazzouri et al., 2008; Parisod et al., 2010; Fowler and Levin, 2016). In sum, the combination of intrinsic challenges of WGD and reproductive interference with their diploid progenitors (MCE) are expected to make establishment of novel autotetraploids highly unlikely (Levin, 1975).

The wealth of naturally established autopolyploid lineages within many species (Kolář et al., 2017), however, demonstrated these challenges may often be overcome. Although polyploidization results in an initial fitness disadvantage (Porturas et al., 2019; Clo and Kolář, 2021), novel polyploids can rapidly adapt to the challenges of genome doubling (Yant et al., 2013; Bohutínská et al., 2021a, b). A competitive advantage resulting from changes in plant traits in direct consequence of WGD or later adaptation may lead to niche differentiation and/or colonization of new environments, thereby reducing competition between the cytotypes (Stebbins, 1985; Baack, 2005). In the longer term, polyploids may display higher adaptability than their progenitors, especially during stressful environmental conditions (Oswald and Nuismer, 2011; Yao et al., 2019; Van Drunen and Friedman, 2022).

In addition, emergence of reproductive barriers between cytotypes, either as a direct consequence of WGD *per se* or subsequent reinforcement selection, may enable cytotype coexistence. Various prezygotic mechanisms have been demonstrated to strengthen assortative mating between cytotypes, and thus to counteract the negative fitness effects of MCE. Besides shift towards asexual reproduction (such as apomixis or vegetative spreading, Van Drunen and Husband, 2019; Van Drunen and Friedman, 2022) or self-fertilisation (Rausch and Morgan, 2005; Husband et al., 2008; Griswold, 2021), prezygotic barriers associated with phenological shift (Rezende et al., 2020), pollinator spectra (Segraves and Anneberg, 2016), pollen precedence (i.e. preference for pollen of the same cytotype, Baldwin and Husband, 2011) were reported from mixed-ploidy systems. However, the

91 importance of these barriers strongly varies between systems and there is not a general trend
 92 in traits associated with pre-pollination segregation (Porturas et al., 2019). The scarcity of
 93 studies of pollen-pollen and pollen-pistil interactions does not allow for general conclusions
 94 on post-pollination prezygotic barriers in natural systems. Additionally, if the triploids are
 95 partially fertile, they may backcross with diploids and tetraploids and contribute to recurrent
 96 polyploid formation in sympatry (triploid bridge, Husband, 2004; Suda and Herben, 2013).
 97 Finally, stochastic processes, such as demographic and environmental fluctuations, may
 98 favour the establishment of the novel tetraploid lineage (Otto and Whitton, 2000; Oswald and
 99 Nuismer, 2011; Bomblies and Madlung, 2014; Clo et al., 2022).

100 In sum, there is a dearth of natural examples from the critical phase of polyploid
 101 evolution - when a recently formed autotetraploid population that is in the phase of
 102 establishment, still coexists with its diploid progenitors. Here we leverage a unique example
 103 of such a system - less than 100 years old, spontaneously formed autotetraploid cytotype of
 104 *Mimulus guttatus* (syn. *Erythrante guttata*) in the Shetlands islands (Simón-Porcar et al.,
 105 2017). Diploid *Mimulus guttatus* was first introduced to the UK at the beginning of the 19th
 106 century from Alaska and since then rapidly spread especially in the northern UK (Stace,
 107 2010; Vallejo-Marin and Lye, 2013; Vallejo-Marín et al., 2021). A recent spontaneous
 108 genome duplication has been discovered by genetic clustering of an autotetraploid found in
 109 one Shetland population together with nearby diploids (Simón-Porcar et al., 2017). Local
 110 polyploidization in the Shetlands has been further supported by a global genetic analysis of
 111 native and introduced *M. guttatus* populations demonstrating monophyly of the Shetland
 112 populations regardless of ploidy (Vallejo-Marín et al., 2021). Provided that the oldest known
 113 records of *Mimulus guttatus* from Shetlands are from the early 1950's (five sites N and S of
 114 Lerwick, including the target QUA population, Anon., 1836, BSBI records) an origin of the
 115 tetraploid less than 100 years ago is likely. Given such a local and recent origin, *Mimulus*

116 autotetraploids provide a rare window into the early drivers and consequences of spontaneous
 117 whole genome duplication and its establishment in natural conditions.

118 Using *M. guttatus* as a case of incipient sympatric polyploid speciation, we addressed
 119 potential mechanisms promoting establishment of neo-autotetraploid plants in sympatry with
 120 their diploid progenitors. Specifically, we asked the following questions. 1) How large is the
 121 natural range of the neo-autotetraploid *M. guttatus* in the Shetlands? 2) How strong are the
 122 components of prezygotic isolation with its diploid progenitor (spatial arrangement, overlap
 123 in flowering, relative investment into clonality, pollen precedence)? 3) What is the relative
 124 fitness of neo-polyploids compared to diploids in natural conditions? 4) Is there a fitness cost
 125 of spatial coexistence of ploidies, in line with predictions of minority cytotype exclusion? 5)
 126 Are triploid individuals readily formed in mixed populations and/or controlled crosses and if
 127 so are they fertile, possibly contributing to interploidy gene flow and polyploid
 128 establishment?

129

130 **MATERIALS AND METHODS**

131 *Sampling*

132 Sampling was aimed at covering the entire Shetland Isles, in the UK where the tetraploids
 133 were previously recorded in a single mixed population (QUA, Simón-Porcar et al., 2017). In
 134 total, we surveyed 15 populations and sampled 679 adult individuals of *M. guttatus* across the
 135 Shetland Isles in August 2022 (Appendix S1). For a broad-scale screening of ploidy
 136 distribution, 15 populations were located based on available floristic data (Anon., 1836, BSBI
 137 records) and our own additional search in suitable habitats. At each site with *M. guttatus*,
 138 GPS coordinates were taken and, depending on the size of the population, 5 to 20 individuals
 139 were collected, spaced at regular intervals of at least 1 m, to avoid collecting clones. To

140 further explore the fine-scale distribution of diploid and tetraploid cytotypes in the natural
 141 mixed ploidy patches, 221 and 270 adult individuals were sampled from the two largest
 142 mixed-ploidy populations (BIG, Bigton; QUA, Quarff, respectively) in a systematic stratified
 143 design along natural transects. The plants grow in wet road ditches and along small streams,
 144 i.e. sites that are well set for sampling along a linear transect (Fig. 2). In each transect, we
 145 collected one individual every 1 m, unless we reached a distribution gap $> 1\text{m}$ when we
 146 sampled the first individual beyond such a gap.

147 For each sampled individual, reproductive and vegetative morphology were recorded
 148 to compare the phenotypic trait differences between diploid and tetraploid individuals in pure
 149 vs. mixed populations and transects. From all collected individuals, we inferred fitness and
 150 reproductive potential by recording the following traits: (1) number of flowers, (2) number of
 151 buds, (3) number of fruit capsules (both mature and immature), and (4) number of stolons
 152 branching at the base of each sampled stem. To be able to score both vegetative and
 153 reproductive traits on the same plants, we focused primarily on sampling flowering
 154 individuals. However, there were very few non-flowering individuals present in the transects
 155 in general, preventing sampling bias (pers. obs.). Prior statistical analysis, we omitted 35
 156 individuals that lacked some traits to ensure that a homogeneous set of individuals is
 157 compared across traits. For statistical analyses, we thus used phenotype data of 320 diploid
 158 and 324 tetraploid adult plants (a subset of 180 diploids and 309 tetraploids came from the
 159 two deeply sampled mixed-ploidy populations). Wherever possible, mature capsules were
 160 collected from these plants and ripe seeds were stored in dry paper bags. In addition, parts of
 161 the stem with leaves were collected from each individual, placed in plastic bags and stored at
 162 cold temperatures (no more than 1 week) until ploidy level was estimated using flow
 163 cytometry.

164 *Controlled crossings*

165 In order to check for the presence of triploid block and the effect of pollen
 166 competition/pollen-pistil interactions, we raised plants (18 h light at 24°C and 6 h dark at
 167 18°C, 70% relative humidity in controlled conditions) from mothers sampled in the two
 168 mixed-ploidy QUA and BIG populations and performed the following four treatments: (i)
 169 interploidy cross where a mother plant was pollinated by father of different ploidy (six $2x \times$
 170 $4x$ and four $4x \times 2x$ with maternal ploidy indicated first), (ii) mixed-cytotype cross where one
 171 mother was pollinated by two fathers (ten $2x \times [2x + 4x]$ and ten $4x \times [2x + 4x]$) (iii) control
 172 diploid cross ($2x \times 2x$, 13 crosses) and (iv) tetraploid control cross ($4x \times 4x$; three crosses as
 173 fewer tetraploids were available, they were less likely to flower in growth chambers, which
 174 may be due to their sensitivity to day length conditions, Simon Porcar et al., 2017). Young
 175 buds were emasculated and bagged one day prior to the pollination to avoid self-fertilization.
 176 Each pollination was ensured by pollen coming from four ripe anthers from a recently (1-2
 177 days ago) open flower with fresh pollen. For controls and interploidy crosses, four anthers of
 178 a single father were used; for mixed-cytotype treatment, we simultaneously deposited pollen
 179 from two anthers of a father of the same ploidy and two anthers of a father of different ploidy
 180 from the mother. Mature seeds were harvested after ~25 days after pollination and stored
 181 until germination in cold (+ 4 °C). On a subset of 23 individuals (11 diploid and 12 tetraploid)
 182 where an undamaged terminal flower was well-developed at the time of scoring, we also
 183 recorded corolla height (measured from the base of the calyx to the highest tip of the petals)
 184 to check for ploidy-related quantitative difference in flower size reported previously (Simón-
 185 Porcar et al., 2017).

186 *Experimental cultivations of seed progenies*

We sowed seeds of 69 mothers collected in the field from five populations as well as all 28 mothers from a crossing experiment to assess germination rates as a fitness proxy. For field-collected samples, we germinated seeds from the two mixed populations (BIG: 28, QUA: 28 mothers, representing both the areas of closest ploidy sympatry, i.e. mixed ploidy transects [subset of 27 mothers], and ploidy pure transects) and 3 diploid populations (COF: 3, AIT: 5, HOL: 5 mothers; seeds from the other ploidy pure populations were not available for germination). From the crossing experiment, we germinated progeny from all mothers that set any seed following the interploidy and mixed-cytotype treatments and from a subset of six control crosses.

Mimulus seeds were not cold-stratified prior to germination; to avoid possible infestation of cultivation spaces with thrips the seeds were treated at 37°C for 2 days, followed by incubation at –18°C for another 2 days. Twenty seeds per each mother (or all seeds if less seeds were available) were sown in each petri dish in the soil (multi-purpose potting soil) and cultivated in a controlled environment facility (18 h light, 50% far-red light + 50% white light, at 24°C and 6 h dark at 18°C, with sufficient watering) at the Department of Botany, Charles University. Seedlings were emerging by approximately four days after sowing (not shown) and germination success was thus scored 16-20 days after sowing when all germinable seeds were germinated and seedlings were clearly visible.

To assess frequency of triploid formation in natural populations we determined ploidy variation in progeny coming from areas of close ploidy sympatry. To do so, 766 seedlings from a subset of 40 mothers from the two mixed-ploidy populations (all 27 mothers available from mixed-ploidy transects BIG:T1 and QUA:T2 and 13 mothers from diploid pure transects BIG: T2, T3 and QUA:T13, T14) were further analysed for ploidy by flow cytometry (see below). The mother plants from the two mixed-ploidy transects represented sites where individuals of both ploidies were intermingled at the scale of metres (Fig. 2).

212 *Flow cytometry*

213 We assessed the ploidy level of all 679 adult individuals as well all 1302 germinated
 214 seedlings using flow cytometry (536 seedlings resulting from controlled crosses, 766
 215 seedlings from natural mixed-ploidy populations, respectively). We prepared the samples
 216 following the two-step procedure using Otto buffers (Doležel et al., 2007). We isolated the
 217 cell nuclei from fresh leaf tissues in the Otto I buffer (0.1 M citric acid, 0.5 % Tween-20;
 218 Otto, 1990) together with *Carex acutiformis* (2C = 0.82 pg; Lipnerova et al., 2013) as an
 219 internal standard. The solution was passed through a 42-µm nylon filter, stained with a
 220 staining solution consisting of Otto II buffer (0.4 M Na₂HPO₄·12H₂O), β-mercaptoethanol,
 221 and a fluorochrome (DAPI, 4',6-diamidino-2-phenylindole). The prepared samples were
 222 analysed in a Partec CyFlow ML (UV LED diode, fluorochrome – DAPI) flow cytometer,
 223 with a minimum of 1500 events analysed per sample. The histograms were evaluated with
 224 FloMax FCS 2.0 software (Partec, Münster, Germany). Only histograms with coefficients of
 225 variation (CVs) for the G0/G1 peak of the analysed *Mimulus* sample below five were
 226 considered. Low background debris, clear peaks and lack of prominent G2 or endopolyploid
 227 peaks in *Mimulus* enabled us to use pooled samples (up to 5-10 individuals) for ploidy
 228 screening. If a ploidy mixture was detected (multiple sample peaks), each adult sample was
 229 re-analyzed separately. This was not possible for young seedlings that were completely
 230 processed during the sample preparation. Lack of ploidy mixtures in progeny of one seed
 231 family, except for two analyses with a peak at position corresponding to a triploid among
 232 other tetraploid individuals (Appendix S2), enabled us to apply such a high-throughput
 233 screening approach also for ploidy characterisation in the progeny of all analysed seed
 234 families.

235 *Pollen staining*

To assess the pollen viability of both cytotypes, we randomly selected five diploid and five tetraploid flowering plants from the two mixed-ploidy populations (BIG and QUA). We harvested one mature flower bud (with already dehiscent anther) from each plant and stained it following Alexander's staining (Peterson et al., 2010). We removed two anthers from one bud and mounted them onto a microscope slide and stained with Alexander's stain diluted 1:1 with dH₂O. We captured images of a minimum of 100 pollen grains for each sample using an Olympus DP72 light microscope at ×20 magnification. Finally, we visually classified the pollen grains as either viable (> 80% of cytoplasm) or non-viable (< 20% of cytoplasm and non-stained).

Statistical analyses

The statistical tests have been performed with R (R Development Core Team, 2013) and the package "lme4" (Bates et al., 2014). We tested if diploid and tetraploid individuals differed significantly for the traits of interests (flowering proportion, reproductive investment, stolon number and germination rate, and size of the flowers) using mixed-effects generalized linear models. We used Poisson distributions and logarithmic link functions for the traits reproductive investment, stolon number, size of the flowers and pollen viability. We used binomial distribution with a logit link function for the flowering proportion and the germination rate. For the analyses on reproductive investment, stolon number, size of the flowers and pollen viability, we compared the two following models:

$$Y_j = \mu + \text{population}_j + \varepsilon_j \quad (\text{model 1}),$$

and

261 $Y_{ij} = \mu + \text{cytotype}_i + \text{population}_j + \varepsilon_{ij}$ (model 2).

262

263 In each models, Y represented the variable under study (reproductive investment,
 264 stolon number, flowering proportion or germination rate), μ was its mean, cytotype_i was the
 265 fixed effect of the i^{th} cytotype (diploid or tetraploid), population_j was the random effect of the
 266 j^{th} population, and ε_j or ε_{ij} were the residuals errors of models 1 and 2 respectively. For the
 267 germination rates, as we used several seeds from different mother plants, we also controlled
 268 for the among-mother variability. To do so, in addition to the random effect of population, we
 269 also added a random effect of a mother.

270 For all traits, the model 1 is always nested in model 2, and the significance of the
 271 cytotype effect was tested with Likelihood Ratio Tests (LRT) between the two models. We fit
 272 the above-mentioned models on the whole data set (containing the 15 sampled populations),
 273 and on the mixed-populations only (BIG and QUA). In addition, we tested if differentiating
 274 diploid and tetraploids within pure and mixed-populations increased the likelihood of the
 275 models, but it did not, and these results are only presented in the supporting information
 276 section (Appendix S3). In contrast, differentiation between pure and mixed transects (when
 277 dealing with mixed populations only) had a significant effect and such results are presented in
 278 the main text.

279 Differences in the germination rate of progeny from crossing experiments were tested
 280 using the same approach as described above for germination rate of cytotypes in the natural
 281 populations using treatment as a factor with three levels (control, interploidy and mixed-
 282 cytotype) and a random mother, accounting for the variability in germination success among
 283 offspring coming from different mother. Crosses yielding less than five seeds ($N = 5$) were
 284 excluded from the calculation to avoid imprecise estimates of germination rates.

285

RESULTS

Spatio-temporal distribution of cytotypes at a landscape and within-population scales

Among the 679 adult individuals sampled, we identified two clearly distinct relative genome size values corresponding to diploid and tetraploid cytotype (352/327, respectively), that have been previously karyologically validated (Simón-Porcar et al., 2017). Diploid individuals were distributed across three islands within the Shetlands archipelago, while tetraploid cytotypes were restricted to the southernmost part of the Shetland Mainland Island, from Lerwick southwards (Fig. 1).

At the within-population level, tetraploids mostly co-occurred with diploids: we sampled three ploidy-mixed and only one pure tetraploid population. In the remaining eleven populations we sampled only diploids. Within all three ploidy mixed populations, tetraploids were the dominant ploidy (57% - 69 %) and in each we found some areas with co-occurring diploid and tetraploid individuals, as well as ploidy-pure patches (Fig. 2). Strikingly, we found no adult triploid in spite of close spatial intermingling of diploids and tetraploids at a scale of less than a metre (Fig. 2).

We also detected a consistent trend in temporal change in ploidy frequency in favour of tetraploids in one mixed population, QUA, that has been partially sampled already seven years ago (Simón-Porcar et al., 2017). In transect T1, sampling from 2015 detected a mixture of diploid and tetraploid individuals (3/11 individuals respectively) while we found only tetraploids there (N = 124). A single tetraploid individual was sampled in 2022 in an area where seven diploids were sampled in 2015 (T5). In the other plant-rich transect, frequency of sampled tetraploids increased from 27% (11 diploids / 4 tetraploids) in 2015 to 47 % (45

diploids /40 tetraploids). The other resampled transects in the QUA population remained homogeneous with respect to ploidy (pure tetraploid in T3 and pure diploid in T13).

Differences in reproductive and fitness related traits

Within the mixed populations, we did not find any significant difference in the number of stolons (per plant) between both cytotypes (i.e. proxy of an investment into asexual reproduction; Table 1). In addition, the difference in the proportion of flowering units per plant (defined as the sum of flowers and flower buds divided by the sum of flowers, flower buds and fruits per plant) was also non-significant between diploids and tetraploids indicating large overlap in flowering, at least in the time of our sampling (Table 1). Further, we did not find a significant size difference between diploid and tetraploid flowers, when grown in standardised conditions of the cultivation chambers (corolla height, 41 and 45 mm on average, for diploids and tetraploids, respectively; $X^2 = 2.07$, $df = 1$, $p = 0.15$).

Tetraploid individuals sampled in the field had a significantly lower reproductive investment (total number of reproductive organs) and germination rate than their diploid counterparts within the two mixed ploidy populations (Table 1). It is, however, notable that reproductive investment and germination rate of tetraploids remains high, on average 74% and 95% of diploid values, respectively (Table 1). Similar differences in germination success was found even when additional scored individuals from cytotype pure populations were included (Appendix S3) but disappears when only strictly sympatric individuals from mixed-ploidy transects are taken into account where the germination rates of diploids and tetraploids are comparable and high (97% and 99%, respectively, Fig. 3). Finally, we did not find significant difference in the pollen viability between diploids and tetraploids, with an average viability of 60% for diploids and 77% for tetraploids ($X^2 = 0.10$, $df = 1$, $p = 0.74$).

Effect of sympatry on the differences in fitness proxies

Differentiating between diploids and tetraploids in pure and mixed populations did not improve the likelihood of the models for the reproductive investment (likelihood-ratio test, $X^2 = 4.38$, $df = 2$, $p = 0.11$) or the germination rate ($X^2 = 1.42$, $df = 2$, $p = 0.49$), meaning that cytotypes are performing similarly, on average, in pure and mixed-cytotype populations.

Differentiating between pure and mixed-ploidy transects within a mixed population gave an unexpected result that plants in mixed-transects generally performed better than in pure ones (Fig. 3). For the germination rate, the effect of ploidy was also significant ($X^2 = 52.9$, $df = 2$, $p < 0.001$), with seeds from tetraploid mothers sampled in pure transects germinated significantly less than seeds of both diploid and tetraploid mothers from mixed patches (Fig. 3), the latter two having similar values (germination rate ranging from 0.97 to 0.99). This result was mainly due to some mothers from the pure tetraploid transect performing badly, while other mother plants were in the range of diploids and tetraploids found in mixed transects (See Supplementary data table 1: sheet 3). For reproductive investments, differentiating between pure and mixed-ploidy transect within a mixed populations significantly improved the statistical model ($X^2 = 421.2$, $df = 2$, $p < 0.001$). On average, plants found in mixed-transect performed better than plants in pure transects (Fig. 3), and diploids performed better than tetraploids, even though there is large variation among diploid individuals from mixed patches (Fig. 3).

Triploid formation in natural and experimental conditions

To assess the rates of spontaneous formation of triploids in natural populations, we cytotyped the progeny of 40 maternal families sampled *in situ* in the two ploidy mixed populations. Ploidy of all of the 766 cytotyped seedlings (Appendix S4) corresponded with that of their mother (510 diploids and 256 tetraploids).

We further performed controlled crosses to exclude possible effects of pre-pollination barriers and measure the rates of triploid formation. The effect of crossing treatment was highly significant ($X^2 = 210$, $df = 2$, $p < 0.001$). When pollinating only by an individual of the opposite ploidy, the proportion of viable seeds was markedly reduced (1.7%, i.e. 5 out of 300 seeds germinated) as compared to homoploid controls exhibiting 83% germination rate on average. When, however, a mother plant was pollinated by an even mixture of pollen from individuals of the opposite and its own ploidy, its fertility was largely restored, with germination rates of 89.6% and 54.3% on average, for diploid and tetraploid mothers respectively. Ploidy of the progeny largely corresponded to that of their mother in all treatments, with the exception of two cases (mothers) when an individual with genome size corresponding to a triploid was found in a mixture of other tetraploid progeny (relative genome size value of 0.73 corresponding to an expected 3:4 ratio, Appendix S2). Interestingly, these were the two mothers exhibiting the lowest germination in the mixed-ploidy treatment.

DISCUSSION

Here we leveraged a unique ‘natural experiment’ of a recent, spontaneously establishing autotetraploid cytotype of *Mimulus guttatus* in Shetland Islands (Simón-Porcar et al., 2017) to explore evolutionary drivers of incipient polyploid speciation. We discovered that since its formation < 100 years ago, the novel tetraploid cytotype already spread over the entire

southern part of the Shetland Mainland island and formed multiple populations, mostly still mixed with its diploid progenitor. Here we discuss possible drivers of its successful establishment and coexistence with diploids.

Interploidy reproductive barriers preventing triploid formation

In spite of strong barriers imposed by whole genome duplication, hybridisation among ploidies is frequently documented by field and experimental studies, especially in cases of autopolyploidy (Husband and Sabara, 2004; Kolář et al., 2017; Sutherland and Galloway, 2017; Morgan et al., 2021). Strikingly, we found no single triploid individual in our field survey of nearly 1400 adult plants and seedlings, suggesting a strong interploidy reproductive barrier. Large spatio-temporal overlap between diploids and tetraploids in field suggests that pre-pollination prezygotic barriers are not likely to be the major cause of such separation, which echoes and strengthens results from recent meta-analyses (Porturas et al., 2019; Clo and Kolář, 2021). Among the traits studied in controlled conditions, an increase in flower size found in cultivated tetraploids in a previous study (Simón-Porcar et al., 2017) might contribute to prezygotic isolation, however such a pattern is less evident in our study. Differences in floral morphology may affect pollinator spectra and promote assortative mating in some species (Segraves and Thompson, 1999; Kennedy et al., 2006; Thompson and Merg, 2008; Roccaforte et al., 2015; Laport et al., 2021). However, the lack of evidence for a ploidy-driven shift in pollinator preferences found in other systems (Jersáková et al., 2010; Castro et al., 2011, 2020) demonstrates the need for an experimental test of such an assumption. Evolution of the reproductive and/or mating system might play a role (Griswold, 2021; Van Drunen and Friedman, 2022), but we found no significant differences in vegetative reproduction among cytotypes. As *M. guttatus* is also able to self-fertilise, it would

be interesting to quantify the average selfing rate in diploid and tetraploid plants in order to see if it increases with the ploidy level.

In contrast to weak evidence for pre-pollination barriers, our experimental crossings demonstrated a strong postzygotic barrier in triploid formation. Formation of shrivelled, mostly non-germinable seeds in interploidy crosses suggests the presence of strong triploid block in our system (Fig. 4) which is in line with observation in other *Mimulus* species (Coughlan et al., 2020). These findings are also consistent with the rarity of triploids observed in interploidy hybridizations of *M. guttatus* (Meeus et al., 2020). Additionally, the interspecific crosses between different diploid and tetraploid *Mimulus* species exhibited a similar pattern of reduced viability of interploidy hybrids, although with some degree of permeability in the hybridisations when the tetraploid acted as a father (Vallejo-Marín et al., 2016). Thus, while the strength of the triploid block may vary between interspecific and intraspecific cross, triploid block serves as a potent mechanism for reproductive isolation between *Mimulus* diploids and tetraploids overall. Within natural populations of *M. guttatus*, however, other prezygotic post-pollination barriers are likely to precede triploid block, as is discussed below.

Possible pathways in Mimulus establishment

Successful establishment of a novel polyploid cytotype is expected under scenarios of either (i) neo-polyploid superiority, (ii) its escape to a novel niche or area or (iii) stable coexistence with its diploid progenitor (Ramsey and Schemske, 2002; Husband and Sabara, 2004; Gaynor et al., 2023). The first two explanations are unlikely in the case of *M. guttatus*. First, tetraploids exhibited either similar vegetative (stolon production) and generative (germination) fitness proxies as sympatric diploids, or were even less fit (reproductive

investment; Table 1). In fact, germination of tetraploid's progeny was even lower than diploid's in seed families sampled from cytotype-pure patches, although we rather consider this to be an effect of accidental sampling of a fraction of markedly unfit mothers (large CIs of tetraploid individuals on Fig. 3), perhaps linked with ongoing spread to novel yet less suitable microsites. Second, there were no signs of expansion of tetraploids beyond the area occupied by diploids (Fig. 1). We also had not observed any obvious niche differentiation: both ploidies grew in wet road ditches with no obvious preference for either position along the stream (Fig. 2) nor overall character of the water habitat (Appendix S5). Yet, our limited temporal comparison in the QUA population over seven years provided a subtle indication for tetraploid spread (Simón-Porcar et al., 2017) which is in line with presumed past expansion that resulted in the current tetraploid's range spanning over 20 km. Further experiments on competitive exclusion and overall temporal dynamics of natural populations may help to explain whether stochasticity or so far undetected tetraploid advantage underlies the tetraploid *M. guttatus* expansion.

Lastly, stable coexistence of ploidies may be possible if reproductive interactions between ploidies are minimised. Interploidy hybridization usually results in unfit triploids, causing frequency-dependent selection against the rarer cytotype (minority cytotype exclusion, Levin, 1975). Strong triploid block, demonstrated by unsuccessful interploidy crosses in *M. guttatus* (Fig. 4), results in wasting gametes on unviable progeny and thus promotes minority cytotype exclusion. However, this is not the case in the field as we found no fitness cost of living in cytotype sympatry, as measured by germination rates (Fig. 3, Table 1, Appendix S3). In light of utmost weak pre-pollinator barriers (discussed above), this suggests an existence of a strong prezygotic post-pollination mechanism preventing formation of the unviable triploid hybrids. Indeed, our crossing experiment demonstrated that when a mixture of pollen from diploid and tetraploid fathers lands on a stigma, germinability

of the progeny is mostly restored to the levels of pure cytotype controls for all diploid and also some tetraploid mothers. Interestingly, a subset of tetraploid mothers exhibited reduced germination and even a rare triploid progeny (Fig. 4). This may reflect higher siring success of pollen of diploid fathers, that fully pollinated diploid mothers while it left a fraction of unviable interploidy hybrid progeny on tetraploid mothers. Differences in siring success have been observed in mixed-ploidy *Chamerion* crosses, where neo tetraploids exhibited lowest siring success while established tetraploids were the most successful (Husband et al., 2002; Baldwin and Husband, 2011). The young *Mimulus* tetraploids, although seemingly with already restored pollen fertility, may still face additional early post-WGD problems and thus resembling rather the *Chamerion* neo-tetraploids. Regardless of the exact mechanism, the variable outcome of tetraploid mothers in the mixed-pollination treatment suggests there might be natural variation in the strength of the prezygotic post-pollination barriers among newly formed autopolyploids.

As pollination by multiple fathers is very likely to occur in dense mixed-ploidy populations such as those of Shetland's *M. guttatus* (Fig. 2), differences in siring success due to ploidy-altered pollen competition and/or pollen-pistil interaction may be the prime mechanism reducing reproductive interactions between *Mimulus* cytotypes and hence enabling their coexistence in nature. Prezygotic post-pollination barriers are considered the key reproductive barriers for interspecific hybridization (Carney et al., 1996; Diaz and Macnair, 1999). Such a barrier has been documented from few mixed-ploidy systems so far (likely due to the need of laborious crossing experiments), however it had an important effect on pollination success and progeny ploidy in all cases (Baldwin and Husband, 2011; Koutecký et al., 2011; Castro et al., 2020). Importantly for tetraploid's establishment, overall high germinability of progeny of tetraploid mothers from mixed-ploidy transects indicates that the likely difference in siring success observed in our experiment may be mitigated in

484 nature, perhaps by excessive pollen loads or at least partial non-random pollen transfer. To
 485 which extent pollen of different fathers of the same ploidy vs. selfing is involved in this
 486 process in natural *Mimulus* populations is unclear as our treatment included emasculated
 487 mothers. Theoretically, spontaneous selfing (Willis, 1993; Zimmer et al., 2023) or selfing
 488 induced by presence of the foreign pollen (mentor effect, Koutecký et al., 2011) may take
 489 part in nature.

490 Tetraploid establishment and its coexistence with diploids may be also enhanced by
 491 repeated tetraploid formation that depends on presence of fertile triploids and/or involvement
 492 of unreduced gametes of the diploid. Crosses involving (semi)fertile triploid individuals may
 493 contribute to formation of novel tetraploid individuals via backcrossing to diploids and
 494 tetraploids (so-called triploid bridge, (Husband, 2004). Even in absence of interploidy
 495 hybridization, triploids may be formed via merger of reduced and unreduced gametes (UGs)
 496 of a diploid. The lack of triploids in both mixed and pure diploid populations, however,
 497 demonstrates that neither triploid bridge, nor unreduced gamete formation significantly
 498 contribute to tetraploid persistence in mixed-ploidy *M. guttatus* populations. In fact, among
 499 the 536 cytotyped seedlings in crossing experiment, we found a single case when unreduced
 500 female gamete of a diploid was likely involved in the origin of a tetraploid progeny on a
 501 diploid mother pollinated by a tetraploid father (Table 2). No triploid individual was found in
 502 pure diploid populations or transects, further illustrating the low potential for unreduced
 503 gamete formation and triploid bridge (Kolář et al., 2017) in natural *M. guttatus*. UGs are
 504 expected to be an essential component of neo tetraploid formation (Ramsey and Schemske,
 505 1998, 2002) and we cannot exclude presence of genetically (UG producer) or
 506 environmentally increased UG formation (e.g. following a temperature shock, Mason et al.,
 507 2011) might have played a significant role at the time of tetraploid origin. Our findings,
 508 however, suggest a rather negligible role of UGs in the current diploid-tetraploid coexistence.

CONCLUSIONS

The presence of established populations of autotetraploid *M. guttatus* in the Shetland Islands provides unique evidence for rapid establishment of polyploidy under natural conditions. We found that rapid success of spontaneous whole genome duplication in *M. guttatus* likely stems from a combination of fitness- and reproduction-related traits. Neotetraploids were able to keep (or quickly restore) viability and fertility and assure pollination by their own ploidy, thus mitigating the strong fitness challenges of triploid block when growing in diploid-tetraploid sympatry. To which extent these traits result from genome doubling *per se* or from post-WGD adaptation, remains to be tested e.g. by interploidy crosses comparing natural and synthetic polyploids. Further experimental and population genomic studies are also needed to uncover genetic underpinning of these key traits for incipient polyploid speciation in this leading plant evolutionary model.

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AUTHOR CONTRIBUTIONS

S.S., and F.K. designed the methodology with help of materials provided by M.V.M. S.S., J.C., and F.K. carried out the fieldwork. S.S. conducted the plant cultivation and germination experiments. S.S. and J.C. carried out data analysis. S.S., J.C., and F.K. wrote the manuscript. S.S., J.C., and F.K. reviewed and edited subsequent versions of the manuscript, with contributions from M.V.M. F.K. conceived the ideas and supervised the study. All authors gave final approval for publication.

DATA AVAILABILITY

All data for this study are available as supplementary data.

SUPPORTING INFORMATION

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

Supplementary data table 1.

APPENDIX S1. Population and locality details of 15 sampled populations.

APPENDIX S2. Flow cytometry analysis of *Mimulus guttatus*.

APPENDIX S3. Trait differences between diploid and tetraploid *M. guttatus* based on the analysis of all scored individuals from total sampled populations.

APPENDIX S4. Natural progeny array in two mixed-ploidy populations: BIG and QUA.

APPENDIX S5. Natural growth habitat of *Mimulus guttatus*.

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TABLES

Table 1. Reproductive and fitness trait differences between diploid and tetraploid *Mimulus guttatus* based on the analysis of all field-scored individuals from the two large mixed populations BIG and QUA. Average and standard errors of diploid and tetraploid trait values and results of likelihood ratio tests are presented for each trait.

Trait	N (dip/tet) ^c	Diploid (s.e.)	Tetra (s.e.)	X ²	d.f.	p

Stolons	180/309	3.11 (1.09)	3.25 (1.06)	0.70	1	0.40
Proportion flowering ^a	180/309	0.23 (0.04)	0.19 (0.04)	2.86	1	0.09
Reproducti ve investment b	180/309	22.90 (1.41)	16.86 (1.02)	236.91	1	$< 10^{-16}$
Seed germinatio n	540/580 ^d	0.97 (0.01)	0.92 (0.02)	14.51	1	0.0001

756

757 Notes:

758 ^aThe sum of flowers and flower buds divided by the sum of flowers, flower buds and fruits
759 per plant

760 ^bThe sum of buds, flowers and fruits per plant

761 ^c*N (dip/tet): number of diploid and tetraploid individuals considered respectively for scoring*
762 *each trait*

763 ^d*N of seedlings germinated*

764

765 **Table 2.** Ploidy of progeny of different treatments in the controlled crossings of *Mimulus*
766 *guttatus*

767

	Crossing treatment
--	--------------------

Offspring ploidy	Control (2x x 2x)	Control (4x x 4x)	Interploid y (2x x 4x)	Interploid y (4x x 2x)	Mixed- cytotype (2x x [2x x 4x])	Mixed-cytotype (4x x [2x x 4x])
N ^a	15	15	1	4	270	231
2x (%) ^b	100	-	-	-	100	-
3x (%) ^b	-	-	-	-	-	0.9
4x (%) ^b	-	100	100	100	-	99.1

768

769 Notes:

770 ^aThe number of seedlings cytotyped (all germinated seeds were cytotyped in the interploidy
771 crossing treatment)

772 ^bProportion of respective cytotypes in each treatment (in %)

773

774 **FIGURE LEGENDS**

775 **Fig. 1** Distribution of diploid (red) and tetraploid (blue) *M. guttatus* in Shetland islands at the
776 scale of populations. In addition to 15 populations sampled by us, 4 diploid populations found
777 by Simon Porcar et al., 2017 are also depicted (BOD, HAM, NIN, WEI). The population
778 label for NIN is not visible on the map because it overlaps with the BIG population label. Pie-
779 charts denote the proportion of individuals of a particular cytotype in each population in our
780 sampling. Note that additional exclusively diploid *M. guttatus* populations were sampled in
781 mainland Scotland (Simon Porcar et al., 2017).

782

Fig. 2 Distribution of diploid (red) and tetraploid (blue) *M. guttatus* in the two mixed-ploidy populations from Shetland islands BIG (A) and QUA (B). Dots show the position of adult individuals sampled in regular min. 1m intervals around naturally occurring linear transects (water channels), the inset shows an illustrative photo of one such transect (T1 of population QUA).

Fig. 3 Performance of diploids and tetraploids in pure and mixed-ploidy transects of the mixed-ploidy populations (BIG and QUA) assessed by germination rate and reproductive investment of individuals (i.e., sum of buds, flowers, and fruits per plant). Diploid (red) and tetraploid (blue) individuals are classified according to pure and mixed-ploidy patches. Points and error bars respectively stand for the estimated mean and 95% confidence intervals. Upper limit of the confidence interval for the reproductive investment of diploids in mixed transects has been cut for the sake of visibility.

Fig. 4 Germination proportion of F1 hybrids across six crossing treatments, following hand pollinations of emasculated *Mimulus guttatus* plants. Ploidy of the mother is indicated first and reflected by the colour of boxplot. Photographs of mature seeds, showing phenotypes across the respective crossing treatments. Bars, 2.5 μ m.

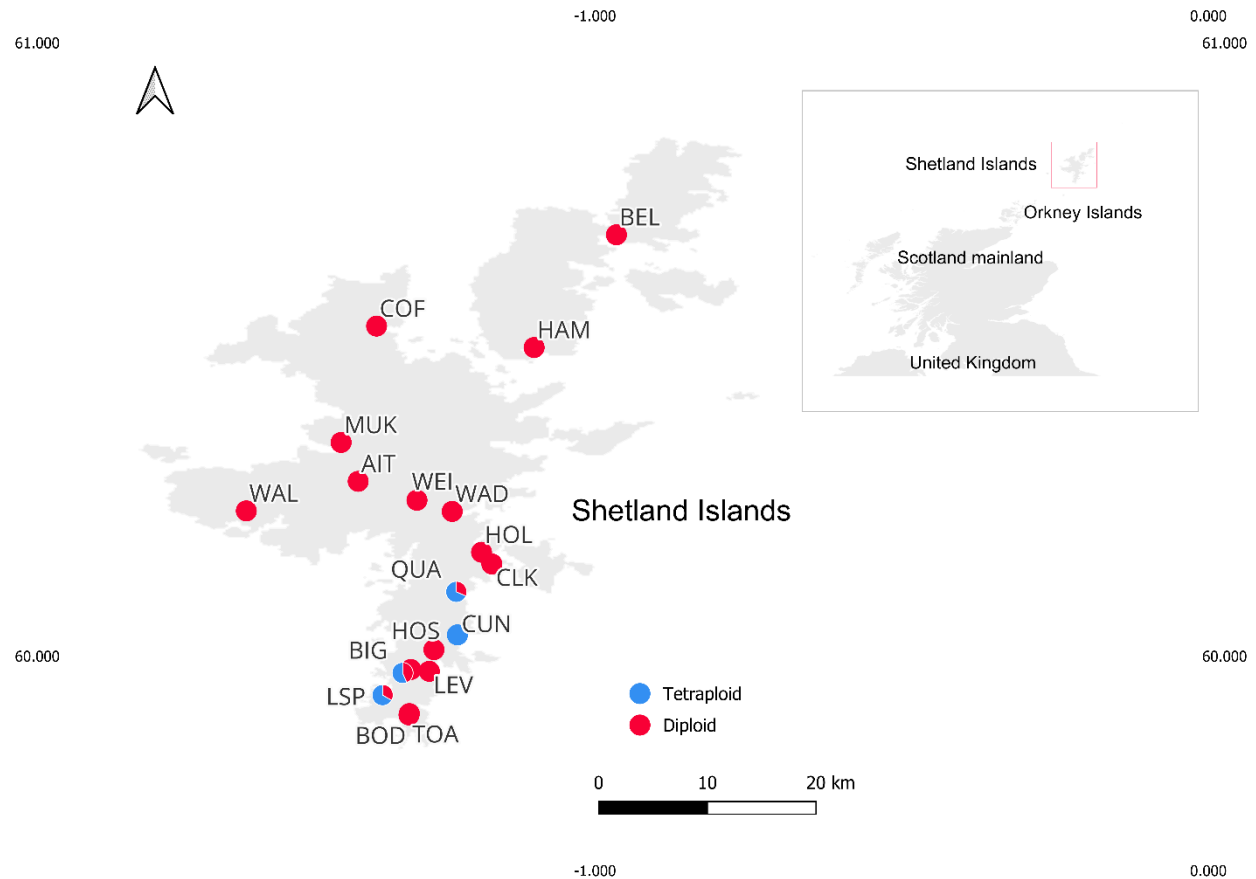


Fig. 1 Distribution of diploid (red) and tetraploid (blue) *M. guttatus* in Shetland islands at the scale of populations. In addition to 15 populations sampled by us, 4 diploid populations found by Simon Porcar et al. (2017) are also depicted (BOD, HAM, NIN, WEI). The population label for NIN is not visible on the map because it overlaps with the BIG population label. Pie-charts denote the proportion of individuals of a particular cytotype in each population in our sampling. Note that additional exclusively diploid *M. guttatus* populations were sampled in mainland Scotland (Simon Porcar et al. 2017).

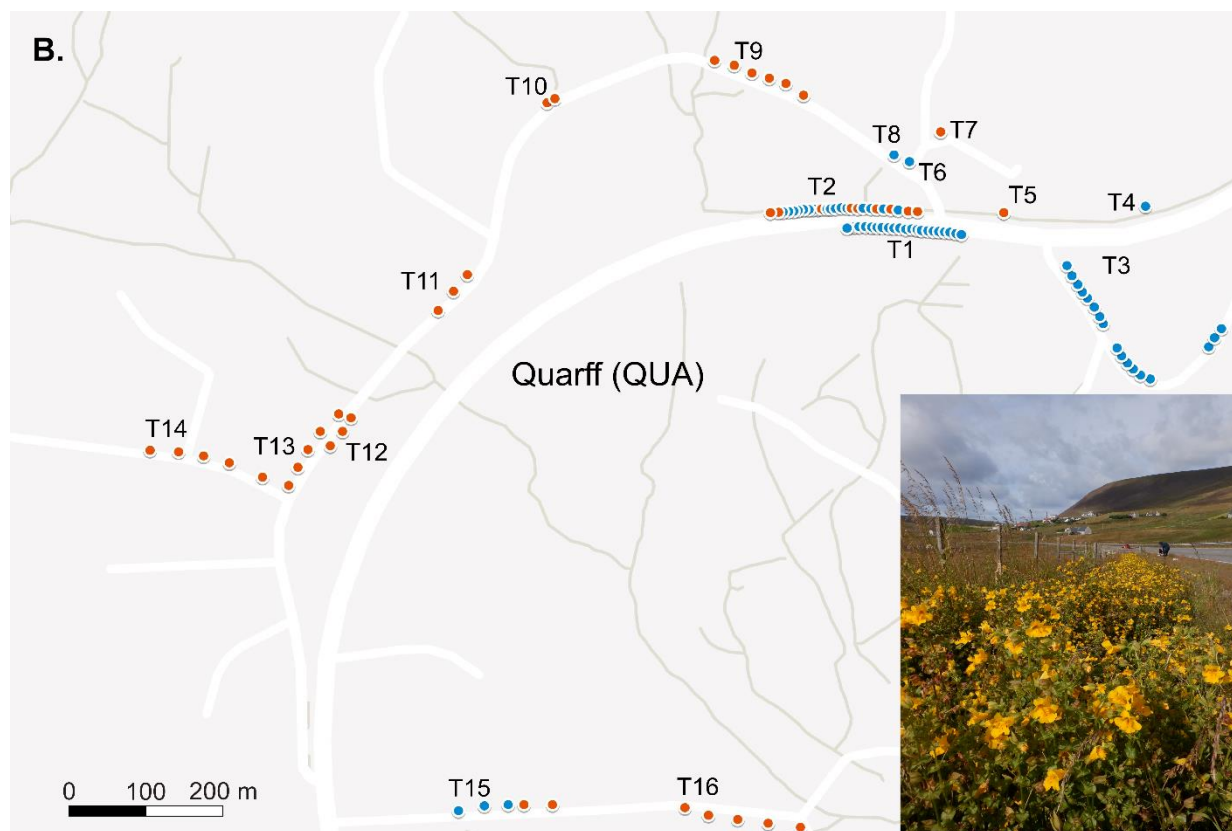


Fig. 2 Distribution of diploid (red) and tetraploid (blue) *M. guttatus* in the two mixed-ploidy populations from Shetland islands BIG (A) and QUA (B). Dots show the position of adult individuals sampled in regular min. 1m intervals around naturally occurring linear transects (water channels), the inset shows an illustrative photo of one such transect (T1 of population QUA).

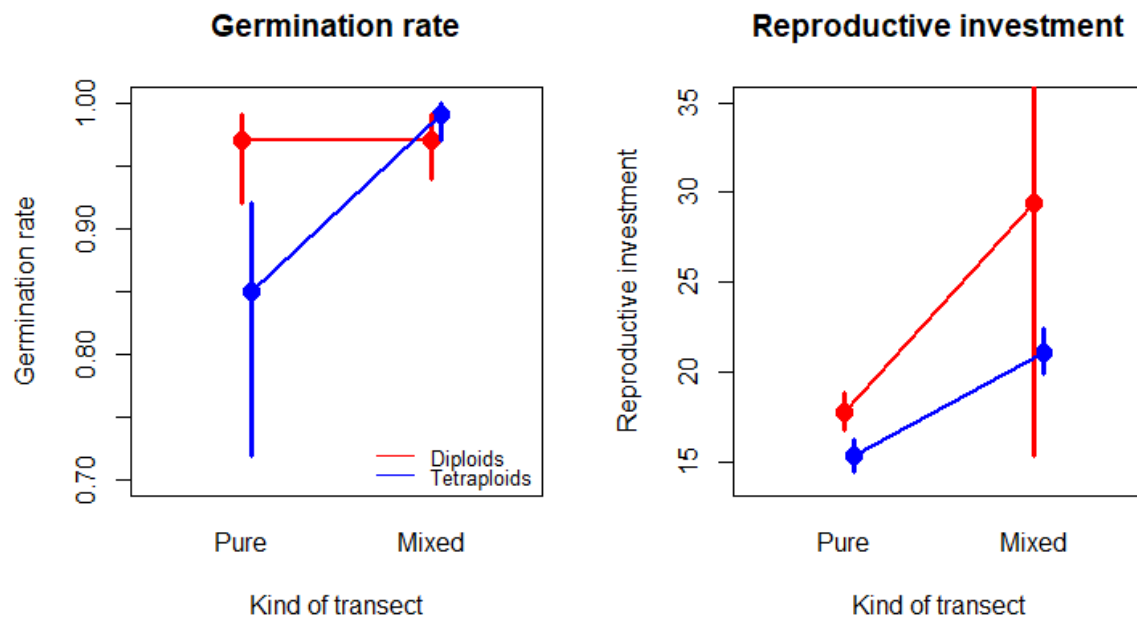


Fig. 3 Performance of diploids and tetraploids in pure and mixed-ploidy transects of the mixed-ploidy populations (BIG and QUA) assessed by germination rate and reproductive investment of individuals (i.e., sum of buds, flowers, and fruits per plant). Diploid (red) and tetraploid (blue) individuals are classified according to pure and mixed-ploidy patches. Points and error bars respectively stand for the estimated mean and 95% confidence intervals. Upper limit of the confidence interval for the reproductive investment of diploids in mixed transects has been cut for the sake of visibility.

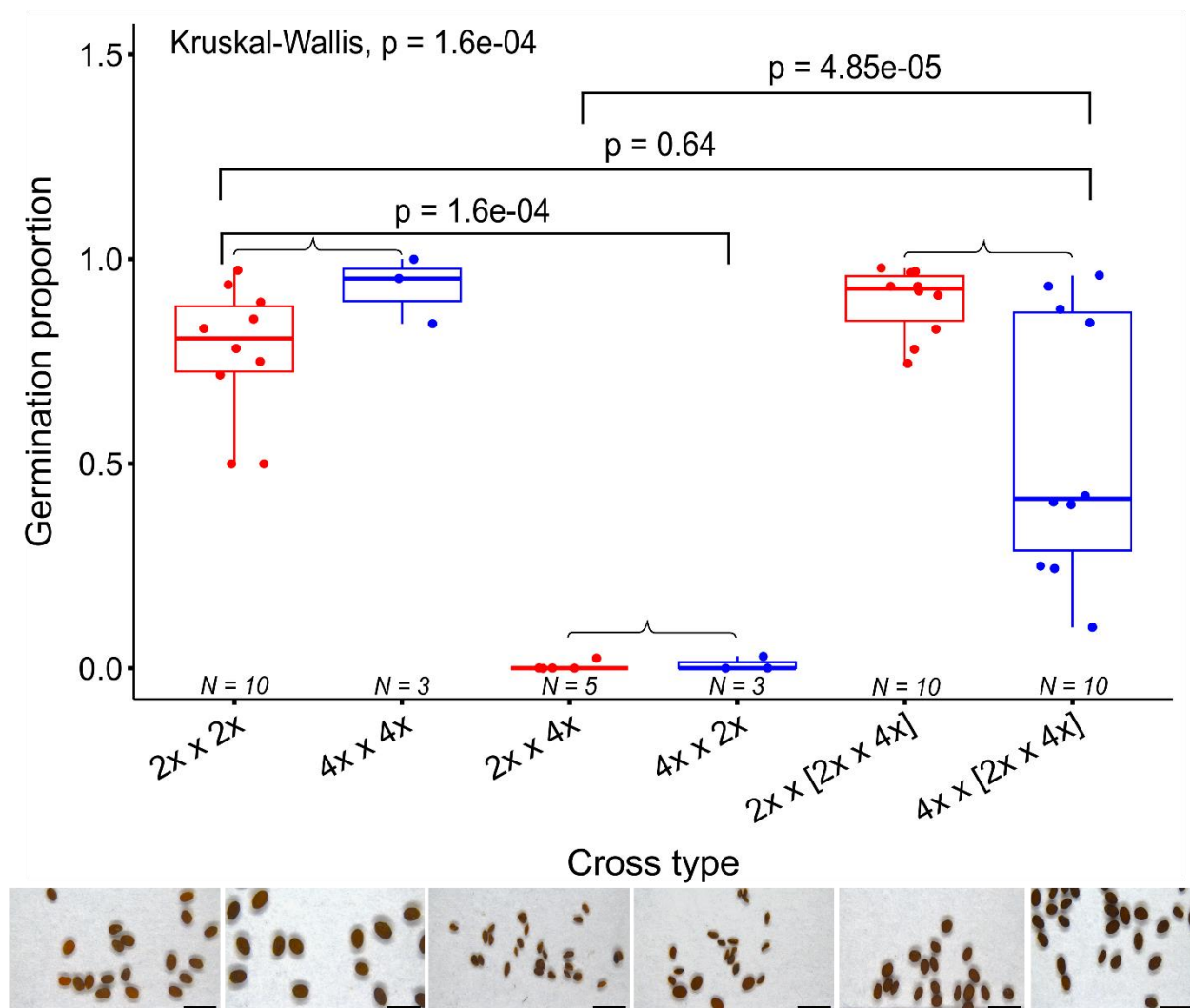


Fig. 4 Germination proportion of F1 hybrids across six crossing treatments, following hand pollinations of emasculated *Mimulus guttatus* plants. Ploidy of the mother is indicated first and reflected by the colour of boxplot. Photographs of mature seeds, showing phenotypes across the respective crossing treatments. Bars, 2.5 μ m.

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Appendix S1. Population and locality details of 15 sampled populations. Diploids and tetraploids are indicated as 2x and 4x, respectively.

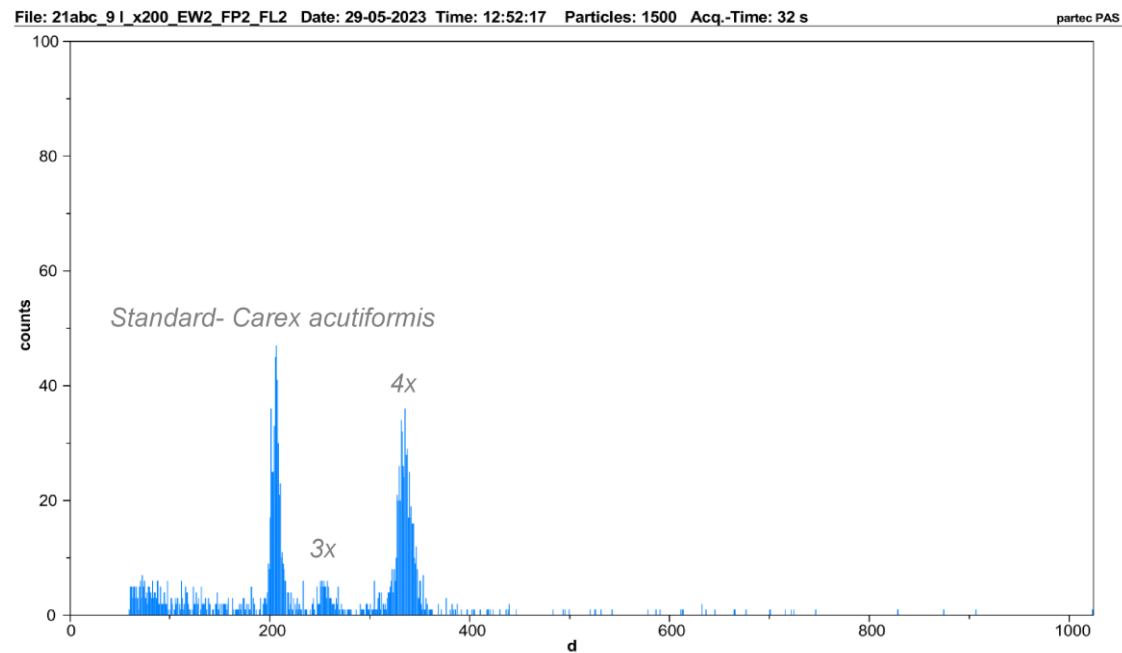
Population codes	Locality	Ploidy	Habitat	Coordinates	Size of population	Number of individuals	Germination rate
MG001	Bigton (BIG)	mix	road ditches	from 59.9723953N, 1.3134361W to 59.9714583N, 1.3161667W and to 59.9723911N, 1.3169819W	large pop	96/125 (2x/4x)	2x = 0.98, 4x = 0.94
MG002	Quarff (QUA)	mix	road ditches	from 60.1046414N, 1.2258031W to 60.1045264N, 1.2277233W and to 60.1037911N, 1.2216081W and to 60.1037911N, 1.2216081W	large pop	85/185 (2x/4x)	2x = 0.95, 4x = 0.88
MG003	Muckle Roe (MUK)	2x	small stream in a peat bog, grazed	from 60.3481949N, 1.4136468W to 60.3486051N, 1.4140797W	small pop., brought from elsewhere	20	NA
MG004	Colla Firth (COF)	2x	streams in grazed heathland	from 60.5379577N, 1.3557960W to 60.5369568N, 1.3558025W	medium pop	20	2x = 0.98
MG005	Aith (AIT)	2x	road ditches	from 60.2848069N, 1.3857821W to 60.2838605N, 1.3814918W	large pop	20	2x = 0.99

MG006	Walls (WAL)	2x	Small stream in a peat bog, grazed	from 60.2366042N, 1.5681484W to 60.2364666N, 1.5686608W	small pop.	14	NA
MG007	Wadbister (WAD)	2x	road ditches	from 60.2357211N, 1.2325493W to 60.2367924N, 1.2312943W	large pop, huge plants	20	NA
MG009	Bellmont (BEL)	2x	pasture	60.6867911N, 0.9647625W	small pop.	5	NA
MG010	Holmsgarth (HOL)	2x	road ditches	from 60.1692097N, 1.1846928W to ca 60.1698917N, 1.1769628W	medium pop	20	2x = 1
MG011	Hoswick (HOS)	2x	shallow road ditch	from 60.0104034N, 1.2622911W to 60.0100039N, 1.2638332W	medium pop	10	NA
MG012	Loch Spiggie (LSP)	mix	deep fertile road ditch	from 59.9362697N, 1.3460089W to 59.9357725N, 1.3459472W and to 59.9360614N, 1.3451533W	medium pop	3/6 (2x/4x)	NA
MG013	Toab (TOA)	2x	shallow road ditch	from 59.9061363N, 1.3024016W to 59.9069081N, 1.3022109W	medium – large pop	20	NA

MG014	Levenwick (LEV)	2x	small streams in pasture	59.9750929N, 1.2697913W and 59.9761619N, 1.2709078W	large metapopulation	10	NA
MG015	Cunningsburgh (CUN)	4x	along stream, deep water	from 60.0344402N, 1.2240132W to 60.0351452N, 1.2243041W	large pop, continues upstream	10	NA
MG016	Clickmin Loch (CLK)	2x	small springs above lake shore	from 60.1500768N, 1.1679034W to 60.1512427N, 1.1681606W	small pop.	10	NA

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Appendix S2. Flow cytometry analysis of *Mimulus guttatus*. Histogram of a mix-ploidy cross (4x x [2x x 4x]) using FloMax software, with ploidy indicated for the respective peaks.



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Appendix S3. Trait differences between diploid and tetraploid *M. guttatus* based on the analysis of all scored individuals from total sampled popula

Trait	N (dip/tet) ^b	Diploid (s.e.)	Tetra (s.e.)	X ²	d.f.	p
Reproductive investment ^a	320/324	16.12 (1.21)	11.88 (1.02)	239.89	1	< 10 ⁻¹⁶
Germination	800/580 ^c	0.98 (0.001)	0.94 (0.001)	17.47	1	3 · 10 ⁻¹⁵

Notes:

^a *The sum of buds, flowers and fruits per plant*

^b *N (dip/tet): number of diploid and tetraploid individuals considered respectively for scoring each trait*

^c *N of seedlings germinated*

tions.

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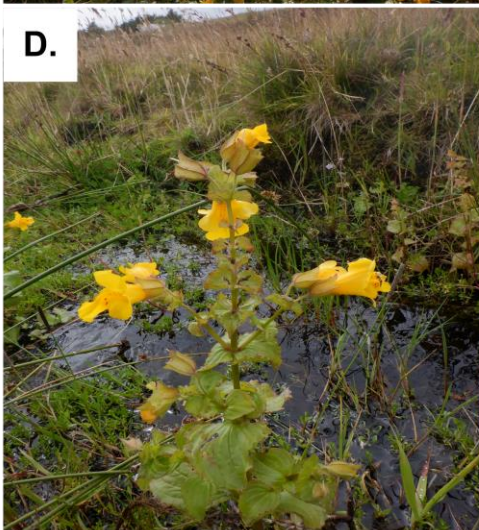
Appendix S4. Natural progeny array in two mixed-ploidy populations: BIG and QUA.

Ploidy of the progenies in pure diploid and mixed transects of the two mixed-ploidy populations is presented here.

Locality	Population codes	Ploidy of population	Ploidy of transect (type of patch)	Transect_ID	No of mothers	No of seedlings cytotyped	Ploidy of seedlings
BIG	MG001	mix	2x (mix)	T1	7	138	2x
BIG	MG001	mix	4x (mix)	T1	7	137	4x
BIG	MG001	mix	2x (pure)	T2, 3	7	127	2x
QUA	MG002	mix	2x (pure)	T13, 14	6	113	2x
QUA	MG002	mix	2x (mix)	T2	7	132	2x
QUA	MG002	mix	4x (mix)	T2	6	119	4x

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Appendix S5. Natural growth habitat of *Mimulus guttatus*. *M. guttatus* inhabits streams, roadside ditches, and waterlogged ground. (A-C) Pure diploid population, (D) Diploid *M. guttatus* growing in the water stream, (E-F) Pure and mix-ploidy transect in a mix-ploidy population (QUA), respectively.





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Dear Editors,

We are pleased to submit our manuscript titled ‘Establishment of polyploidy in natural populations of *Mimulus guttatus*’ for consideration in the American Journal of Botany. Our study focuses on elucidating the key evolutionary mechanisms important for the successful establishment of neo-polyploid lineages. We intend this submission as an invited contribution to the special issue ‘*Twice as Nice: New Techniques and Discoveries in Polyploid Biology*’.

- *What are the questions addressed or hypotheses tested?*

We conducted an empirical investigation to assess the variations in fitness and reproductive traits between neo-polyploids and their diploid progenitors. Additionally, we explored potential reproductive barriers that contribute to the successful establishment and coexistence of neo-polyploids alongside their progenitor counterparts.

- *What is the major contribution of your paper to your discipline?*

Polyploids have been demonstrated to occur recurrently in nature, however, the evolutionary mechanisms promoting their establishment in natural populations remain poorly known. In the present study, we investigate a unique system of a plant species in which recently formed local autotetraploids still coexist with their diploid progenitors. Thus, our study provides a rare insight into the critical early phases of polyploidization in natural populations, thereby advancing our understanding of the consequences of whole genome duplication.

- *How is this contribution of interest to a broad audience?*

Polyploidy can lead to the emergence of new species, which is a fundamental process in evolution. By investigating the natural establishment of polyploids, we gain insights into the mechanisms of speciation leading force of plant speciation. Additionally, successfully established polyploids can confer a survival advantage in challenging environments, offering a window into the ways organisms adapt to shifting environmental conditions.

Thank you for considering this manuscript for the *American Journal of Botany*.

Yours Faithfully

A handwritten signature in blue ink, appearing to read 'Filip Kolar', with a long horizontal flourish extending to the right.

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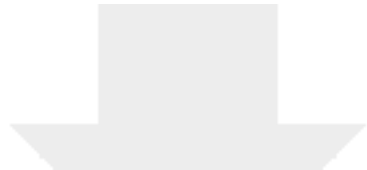
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