

Recent autopolyploidization in a naturalized population of *Mimulus guttatus* (Phrymaceae)

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Polyploidization can trigger rapid changes in morphology, ecology and genomics even in the absence of associated hybridization. However, disentangling the immediate biological consequences of genome duplication from the evolutionary change that subsequently accumulates in polyploid lineages requires the identification and analysis of recently formed polyploids. We investigated the incidence of polyploidization in introduced populations of *Mimulus guttatus* in the UK and report the discovery of a new mixed diploid–autopolyploid population in the Shetland Isles. We conducted a genetic analysis of six Shetland populations to investigate whether tetraploid individuals may have originated from local diploid plants and compared the morphology of tetraploids and local diploids to assess the phenotypic consequences of genome duplication. Autotetraploids are genetically close to sympatric diploids, suggesting that they have originated locally. Phenotypically, whole genome duplication has resulted in clear differences between ploidies, with tetraploids showing delayed phenology and larger flowers, leaves and stems than diploids. Our results support the hypothesis that novel evolutionary lineages can rapidly originate via polyploidization. The newly discovered autopolyploidization event in a non-native *Mimulus* population provides an opportunity to investigate the early causes and consequences of polyploidization in the wild.

ADDITIONAL KEYWORDS: autotetraploid – neopolyploid – Shetland Isles – sympatric speciation – whole genome duplication.

INTRODUCTION

Polyploidization is a recurrent event in nature and one of the main drivers of evolution and diversification in angiosperms (Stebbins, 1971; Grant, 1981; Otto & Whitton, 2000; Otto, 2007; Soltis, Visger & Soltis, 2014a; Soltis *et al.*, 2016; Barker *et al.*, 2016a). A traditional view has been that the majority of polyploids are allopolyploids (i.e. products of polyploidization associated with hybridization) because they are easily recognized by their distinct phenotypic features and are usually classified as different species (Parisod, Holderegger & Brochmann, 2010; Soltis *et al.*, 2010). In

contrast, autopolyploids (i.e. within-species polyploids) often remain unrecognized as, superficially, they may morphologically resemble their diploid ancestors and even when identified there is reluctance in classifying them as a separate taxon, with the consequence that their prevalence in nature is probably underestimated (Lewis, 1967; Soltis *et al.*, 2007; Wood *et al.*, 2009; Parisod *et al.*, 2010; Husband, Baldwin & Suda, 2013; Barker *et al.*, 2016a). Estimating the frequency of autopolyploidization events in nature requires screening ploidy (or genome size) in plant populations (Ramsey & Schemske, 1998; Soltis *et al.*, 2007; Barker *et al.*, 2016a). Efficient estimation of genome size for the indirect assessment of ploidy level (i.e. cytotype) in large numbers of individuals was enabled by the development and widespread application of flow cytometry (Doležel, Greilhuber & Suda, 2007). Flow cytometry is

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a powerful tool to identify and characterize polyploidization events in natural populations and, because it does not rely on the presence of morphological differences between ploidies, it works equally well in allo- and auto-polyploids.

Genome duplication can have immediate consequences for the morphology, physiology, ecology and genomics of polyploids (Soltis *et al.*, 2014a). For instance, polyploids are usually more robust and have larger flowers, pollen grains, seeds and stomata than their diploid parents (Müntzing, 1936; Ramsey & Schemske, 2002; Husband *et al.*, 2013). These changes can affect ecological interactions with pollinators (Thompson & Merg, 2008), alter stomatal function (Li, Berlyn & Ashton, 1996), photosynthetic activity (Warner & Edwards, 1993) and drought tolerance (Ramsey, 2011), and/or increase the competitiveness of polyploids, thus favouring their successful establishment (Blossey & Nötzold, 1995; Bretagnolle, Thompson & Lumaret, 1995; Jakobs, Weber & Edwards, 2004; Stastny, Schaffner & Elle, 2005). However, identifying the immediate effects of polyploidization in the wild can be complicated as polyploid lineages continue accumulating evolutionary changes as they age (Levy & Feldman, 2004; Flagel & Wendel, 2009; Ramsey, 2011; Hegarty *et al.*, 2013). For this reason, neo-polyploidization events (i.e. recent natural occurrences of whole-genome duplication) can be particularly useful in understanding the early consequences of polyploidization (Ramsey & Schemske, 2002; Ainouche, Baume & Salmon, 2004; Abbott & Lowe, 2004; Parisod *et al.*, 2010; Soltis *et al.*, 2010, 2016; Zozomová-Lihová *et al.*, 2014; Vallejo-Marín *et al.*, 2015).

Introduced populations offer an excellent opportunity to study recent polyploidization events. By definition, introduced populations have become established beyond the native range of a species in the recent evolutionary past (usually < 500 years and often in the last 200 years; Stace & Crawley, 2015) and therefore polyploidization events occurring in the introduced range are evolutionarily young. Despite this potential, few studies have used flow cytometry to compare the distribution of ploidies in native and introduced plant populations of the same species (Schlaepfer *et al.*, 2008; Treier *et al.*, 2009; Ferrero *et al.*, 2015) and even fewer have attempted to determine if autopolyploids have originated in the introduced range.

Here we use introduced populations of *Mimulus guttatus* DC. [*Erythranthe guttata* (Fisch. ex DC.) G.L.Nesom, yellow monkey flower, Phrymaceae] to investigate the incidence and morphological consequences of recent autopolyploidization. *Mimulus* L. has served as a model system for ecological and evolutionary studies in the native range for > 60 years (Wu *et al.*, 2008), but has only recently begun to be used as a study system for ecology and evolution of

non-native populations (Truscott *et al.*, 2006; van Kleunen & Fischer, 2008; Puzey & Vallejo-Marín, 2014). Whole genome duplication has played a major role in the evolution of and speciation in *Mimulus* and there are several well-characterized examples of ancient and recent polyploidization (Vickery, 1995; Beardsley *et al.*, 2004; Sweigart, Martin & Willis, 2008; Buggs, 2008, 2012; Benedict *et al.*, 2012; Modliszewski & Willis, 2012), including recent allopolyploidization events in the introduced range (*M. peregrinus* Vall.-Marín; Vallejo-Marín, 2012; Vallejo-Marín *et al.*, 2015). In its native range (western North America), *M. guttatus* occurs mostly as a diploid ($N = 14$; Vickery, 1978), but tetraploid individuals ($N = 28$) can be found at an appreciable frequency (7/76 surveyed populations; Vickery *et al.*, 1968). Some of these tetraploid populations are allotetraploids resulting from hybridization with *M. nasutus* Greene (*M. sookensis* B.G.Benedict, Modlisz., Sweigart, N.H.Martin, Ganders & John H.Willis; Modliszewski & Willis, 2012) or other closely related taxa. Nevertheless, other tetraploids are hypothesized to be autopolyploids based on their morphological similarity with coexistent diploids (McArthur *et al.*, 1972). The incidence of these putative autotetraploids has been assessed in the native range using cytological observations. For instance, Mia, Mukherjee & Vickery (1964) reported a single autotetraploid population of *M. guttatus* (Arizona) in a cytological survey of 44 populations and Vickery *et al.* (1968) recorded an additional five populations of putative autotetraploids in New Mexico, Colorado and northern Mexico. Based on these findings, McArthur *et al.* (1972) suggested that autotetraploids are more common at the southern edge of the native distribution of *M. guttatus*. The distribution of these tetraploids suggests independent origins via recurrent autopolyploidization, but this hypothesis remains to be tested. The age of native autotetraploids is unknown, but chromosome pairing at meiosis ranges from exclusive bivalent associations (as is often observed in older autopolyploids; Mia *et al.*, 1964) to between three and nine tetravalent associations (suggesting more recent polyploidization; McArthur *et al.*, 1972). In the introduced range in the UK, most populations of *M. guttatus* are diploid (McArthur, 1974; Stace, 2010) and there is only one previous record of a putative autotetraploid individual ($N = 28$; Maude, 1940). Detailed information about the source material for this observation is, however, not available. Further studies of autotetraploid occurrence and formation in the introduced range are clearly needed to assess whether recent polyploidization may contribute to the composition and identity of invasive populations.

In this study, we investigate the occurrence and phenotypic consequences of autopolyploidization in alien populations of *M. guttatus* by conducting the

first survey of relative genome size level (ploidy) in the UK. We address the following specific questions. (1) To what extent are introduced populations of *M. guttatus* formed exclusively of diploid individuals? (2) In cases where polyploids occur, are they genetically similar to nearby diploids (consistent with a local origin or co-dispersal)? (3) What is the fine-scale spatial distribution of diploid and polyploid individuals when they co-occur? (4) What are the morphological characteristics of polyploid individuals of *M. guttatus* compared to related diploids? We used flow cytometry to assess relative genome size as a proxy for ploidy in 29 introduced populations and we provide the first report of a neo-autopolyploidization event in the wild, found in a mixed diploid–tetraploid population of *M. guttatus* at the northern end of the range in the Shetland Isles.

MATERIAL AND METHODS

STUDY SPECIES

Mimulus guttatus DC. [= *Erythranthe guttata* (DC.) G.L. Nesom; Phrymaceae] is a species complex whose native range spreads across the west coast of North America from Mexico to Alaska (Grant, 1924; Wu *et al.*, 2008). The species complex shows remarkable variation in many biological aspects such as life form, mating system, drought and salt tolerance, and phenology (e.g. Willis, 1993; Hall & Willis, 2006; Lowry *et al.*, 2009; Wu *et al.*, 2010; Twyford & Friedman, 2015). *Mimulus guttatus* was first introduced to the UK at the beginning of the 19th century (Stace, 2010; Vallejo-Marín & Lye, 2013). The exact origin of UK populations is unknown but genomic analyses suggest a common source towards the northern portion of its range, possibly in Canada or Alaska (Puzey & Vallejo-Marín, 2014). Currently, *M. guttatus* is widespread across the UK, although it may be more common and form larger populations in the north (Preston, Pearman & Dines, 2002; V. I. Simón-Porcar, P. Pantoja & M. Vallejo-Marín, unpubl. data). In its introduced range, *M. guttatus* inhabits wet places by streams, rivers, ponds, roadside ditches and waterlogged ground.

POPULATION SAMPLING AND CYTOTYPE SCREENING

We surveyed 29 populations of *M. guttatus* in the UK in summer 2014 (Table 1) and collected plant cuttings from 265 individuals across these populations (two to 27 individuals per population, proportional to population size). Because previous work suggested that *Mimulus* polyploids may be more likely to occur at range extremes (McArthur *et al.*, 1972), we focused our sampling in northern Scotland, where *M. guttatus* is particularly abundant and can form populations of thousands of individuals, and in southern England, where it is rarer

and population sizes are generally smaller (tens to a few hundred plants; M. Vallejo-Marín, pers. obs.). Plant cuttings were transported from the 29 populations to the glasshouses at the University of Stirling and individual plants were grown in 9-cm-diameter pots filled with General Purpose compost (Sinclair, Lincoln, UK), kept on plastic trays with abundant water. Plants were occasionally sprayed with SB Plant Invigorator (Fargro Ltd, Littlehampton, UK) and Provado Ultimate Bug Killer (Bayer Garden, Cambridge, UK) to control for fungal and aphid infections.

To assess the ploidy level of each population, we collected fresh leaves from each plant in the greenhouse and analysed their relative genome size with flow cytometry. Flow cytometry has been widely applied for the indirect assessment of ploidy level (i.e. cytotype; e.g. Doležel *et al.*, 2007). Nuclear suspensions for flow cytometry were prepared following the protocol of Doležel, Binarova & Lucretti (1989). Approximately 100 mg leaf tissue of the target samples and an internal standard of diploid *M. guttatus* were gently chopped together for 30 s with a razor blade onto a Petri dish with 1 mL of Woody Plant Buffer (Loureiro *et al.*, 2007). The suspension was filtered through a 50-µm mesh CellTric disposable filter (Partec GmbH, Münster, Germany), stained with 20 µg/mL propidium iodide and 20 µg RNase was added. Samples were immediately analysed using a Guava EasyCyte Flow cytometer and GuavaSoft software (Millipore, Hayward, CA, USA), with a minimum of 5000 cells acquired or 10 min at low flow rate, whichever occurred first.

Samples were run in batches of five test individuals and one internal control using approximately equal amounts of each individual for a total of 100 mg of leaf tissue. The internal control consisted of a diploid individual of known genome size (*M. guttatus*, accession P114, Dunblane, UK; 2C = 0.84 pg), which was prepared with the test samples. Relative fluorescence of the G1 nuclei was quantified in the yellow fluorescence channel using histograms of relative fluorescence units (RFU) analysed in GuavaSoft 2.6 (Merck Millipore, Billerica, MA, USA). If any G1 fluorescence peaks other than the expected diploid one were detected, samples were analysed individually with the internal control. To estimate ploidy, we compared the RFU of diploid peaks (internal control) to the values of the test sample. We used the ratio of RFU of the test sample to the diploid peak as an estimate of ploidy (e.g. 4:2 indicates a tetraploid individual with twice the amount of DNA as the diploid control). Neither the diploid individual used as internal standard nor any of the individuals analysed showed marked G2 peaks due to endopolyploidy.

Additional sampling of one population was conducted in summer 2015, after detecting diploid and tetraploid

Table 1. Location and cytotype of introduced populations of *Mimulus guttatus* surveyed in the UK and sampling size (*N*) for flow cytometry and genetic analyses

Population code	Population	Latitude	Longitude	Altitude (m a.s.l.)	Cytotype	<i>N</i> for flow cytometry	<i>N</i> for genetic analysis
BKN	Balnakeil	58.576	−4.768	8	2x	27	—
BLA	Bailmore	58.488	−5.106	44	2x	16	—
BOD	Boddam	59.904	−1.303	55	2x	17	28
BOG	Bognor Regis	50.797	−0.698	6	2x	11	—
CAR	Carbisdale	57.924	−4.409	43	2x	4	—
CRO	Crowan	50.163	−5.293	129	2x	3	—
DAL	Dalmore	57.683	−4.265	6	2x	24	—
DAR	Dartmouth Devon	50.329	−3.575	69	2x	2	—
DEA	West Dean	50.905	−0.780	50	2x	4	—
DEE	Deerhill	57.592	−2.896	196	2x	13	—
EAS	East Prawle	50.216	−3.713	129	2x	2	—
POR	Portesii Beach	57.694	−2.926	7	2x	10	—
FUN	Funtington	50.863	−0.855	20	2x	4	—
GAR	Garve	57.615	−4.673	75	2x	6	—
HAM	Hamnavoe	60.503	−1.099	4	2x	7	19
HOU	Houghton	51.097	−1.508	33	2x	2	—
HUN	Hunston	50.811	−0.789	7	2x	3	—
KIN	Kinloss	57.631	−3.575	6	2x	13	—
MAR	Maryburgh	57.572	−4.427	3	2x	24	—
MOO	Moorswater	50.451	−4.486	54	2x	3	—
MUK	Mukle Roe Island	60.348	−1.414	8	2x	12	23
NIN	St Ninians Bay	59.978	−1.300	87	2x	10	15
QUA	Quarff	60.105	−1.227	25	2x 4x	14 (2014) 51 (2015)	14 (2014) 49 (2015)
SIN	Singleton	50.912	−0.753	60	2x	5	—
SOU	Manaton-Southcott	50.602	−3.768	259	2x	3	—
TOU	Toulton	51.074	−3.124	124	2x	5	—
UPL	Uplowman	50.938	−3.413	127	2x	4	—
WEI	Weisdale Fork	60.254	−1.290	6	2x	5	8
WHI	Whitehillock Farm	57.497	−2.931	223	2x	12	—

individuals in this locality (QUA, Shetland; Table 1). In this population, we mapped and sampled leaf tissue from an additional 51 individuals. Fresh leaf tissue was collected and immediately transported on ice to the University of Stirling for analysis in the flow cytometer as above. To determine chromosome number, we conducted a cytological analysis in representative individuals from both ploidies (accessions P218 and P224 for diploids and tetraploids, respectively). We isolated nuclei from flower buds fixed in 3:1 (v/v) ethanol/acetic acid solution and followed the protocol described by Higgins *et al.* (2014) to visualize chromosomes at mitotic metaphase using DAPI (4',6-diamidino-2-phenylindole).

GENETIC ANALYSES OF DIPLOID AND TETRAPLOID POPULATIONS IN SHETLAND

To establish the genetic relationships between the newly discovered tetraploid population in Shetland

and other diploid populations in the archipelago, we genotyped 156 individuals from all six Shetland populations analysed with flow cytometry (five diploid and one mixed diploid–tetraploid; Fig. 1). We extracted DNA from dried leaves preserved in silica gel, with a modified CTAB protocol (Doyle & Doyle, 1990) and quantified DNA yield using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). Twelve loci (eight microsatellite and four intron-based markers; Kelly & Willis, 1998; Fishman *et al.*, 2001, 2014; Vallejo-Marín & Lye, 2013) were amplified in two multiplex reactions (Supplementary Information, Table S1). Multiplex reactions were done using the Qiagen Type-it Microsatellite PCR Kit (Qiagen, Crawley, UK), 2 µM of each fluorescent forward primer labelled with 6-FAM (Eurofins MWG Operon, Ebersberg, Germany), VIC, PET or NED (Applied Biosystems, Foster City, CA USA) dyes, 2 µM of each reverse primer and 0.5 µL of template DNA. PCR cycles consisted of a denaturing

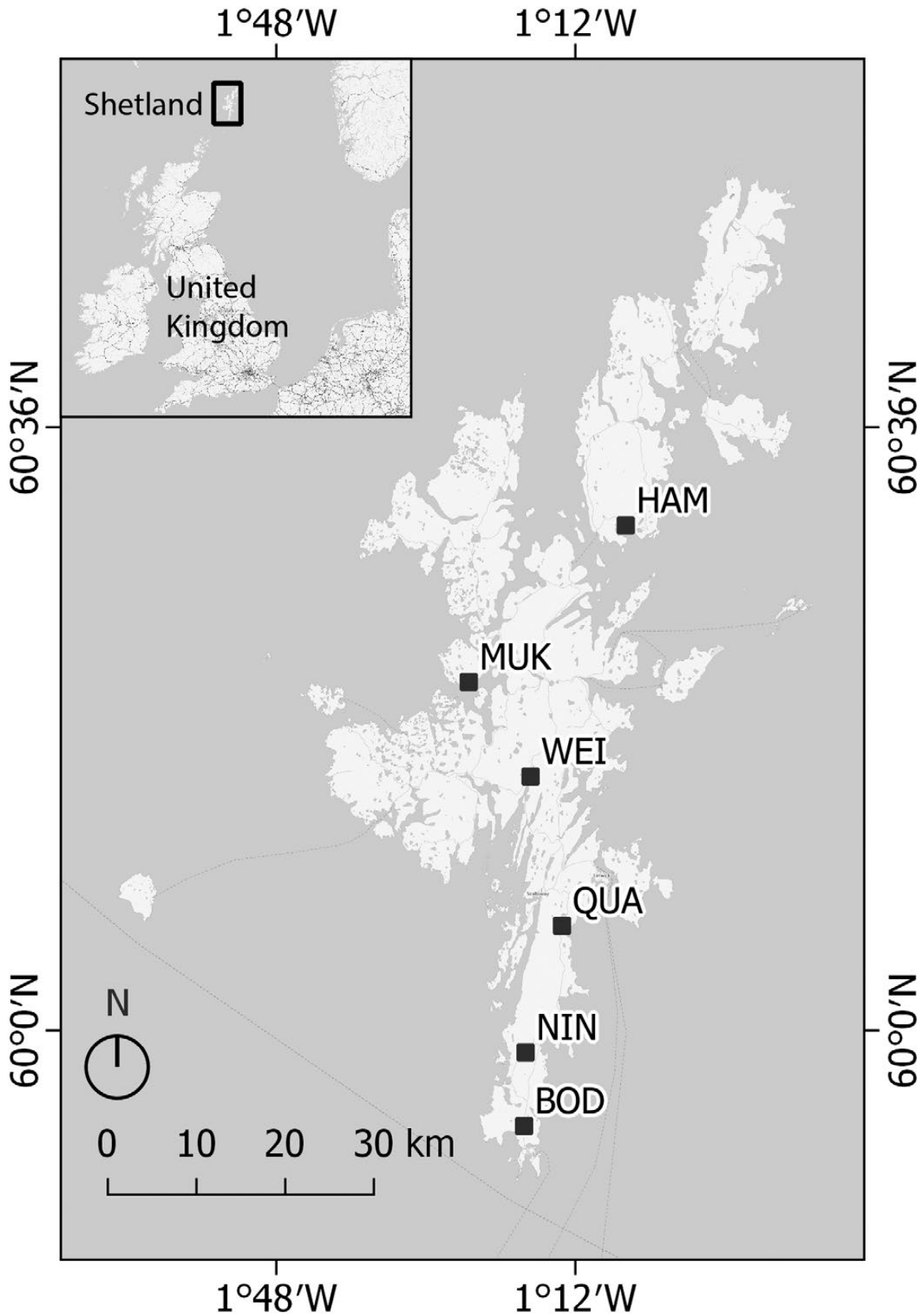


Figure 1. Map of the populations of *Mimulus guttatus* in the Shetland Isles included in the present study.

step of 5 min at 95 °C, followed by 30 cycles of 95 °C for 30 s, 55 °C for 180 s and 72 °C for 30 s, and a final elongation step of 30 min at 60 °C. We checked PCR products in a 2% agarose 1× Tris-borate-EDTA electrophoresis gel and sent them to DNA Sequencing and Services (Dundee, UK) for fragment analysis on an ABI 3730xl capillary sequencer with a GeneScan 500 LIZ internal size standard (Applied Biosystems). We analysed fluorescence profiles using Peak Scanner 2.0 (Applied Biosystems). Genotyping error per marker was estimated from five repeated individuals (3% of samples) as 0%.

Genotypes were grouped in seven ‘populations’ defined by locality and ploidy, pooling samples collected in 2014 and 2015 within cytotypes for the QUA population. We estimated genetic diversity of the co-dominant markers with GenAlEx 6.5 (Peakall & Smouse, 2006, 2012). For each population, we calculated the percentage of polymorphic loci, mean number of alleles (N_a), number of effective alleles (N_e), number of private alleles (P_A), unbiased diversity (u_h) and genotypic richness ($R = G - 1/N - 1$; where G is the number of multilocus genotypes and N is the number of genotyped individuals; Dorken & Eckert, 2001). Twenty-four multilocus genotypes in our database were repeated from two to 29 times, which can influence estimates of genetic differentiation among populations (Balloux, Lehmann & de Meeûs, 2003). All analyses of genetic differentiation and structure below were performed using the complete genotyping matrix and also a genotyping matrix with only unique multilocus genotypes ($N = 92$) for comparison.

Analysing genetic relationships between individuals of different ploidies may be problematic due to the difficulty in distinguishing the exact number of copies for a given allele in polyploids and to violations of assumptions of disomic or tetrasomic inheritance (Bruvo *et al.*, 2004; De Silva *et al.*, 2005; Dufresne *et al.*, 2014). One commonly used method to compare diploid and polyploid taxa is to analyse the presence and absence of alleles at each loci (‘allele phenotype data’; e.g. Kloda *et al.*, 2008; Vallejo-Marín & Lye, 2013; Zozomová-Lihová *et al.*, 2014) and here we used this approach. We scored presence/absence as a binary trait in 40 polymorphic alleles of the 12 loci amplified. This binary genotypic matrix was first used to compute a hierarchical AMOVA in GenAlEx 6.5. We nested populations within cytotypes and used the F fixation index analogue Φ as input distance calculation to explore the partition of genetic variance within and between populations and cytotypes (Meirmans, 2006; Peakall & Smouse, 2015). In contrast to F_{st} , Φ_{st} suppresses intra-individual variation (heterozygosity) and so it is appropriate for binary databases with different ploidies (Assoumane *et al.*, 2013; Teixeira, Rodríguez-Echeverría & Nabais, 2014). The statistical

significance of Φ -statistics was determined by 1000 permutations of the data.

The patterns of spatial genetic structure of *M. guttatus* populations and cytotypes in Shetland were analysed in two ways. First, we produced a matrix of individual pairwise Dice genetic distance coefficients (Dice, 1945) with the *ade4* package (Dray & Dufour, 2007) in the *R* v.3.3.0 statistical software (R Core Development Team, 2016). Principal coordinate analysis (PCoA) and neighbor-joining clustering (NJ) were computed using this matrix with the package *ape* (Paradis, 2006) in *R*. In the second approach, we explored genetic structure in Shetland populations of *M. guttatus* with Bayesian clustering as implemented in Structure v.2.2.3 (Pritchard, Stephens & Donnelly, 2000). This software can also handle genotype ambiguities for co-dominant markers in polyploids working with binary matrices of presence/absence coding (Falush, Stephens & Pritchard, 2007). We ran ten independent replicates of $K = 1$ –6 with a burn-in period of 100 000 iterations followed by 1 000 000 Markov chain Monte Carlo (MCMC) repetitions for data collection. We provided prior information of sampling populations under an admixture model and assumed correlated allele frequencies. The STRUCTURE output data were analysed in STRUCTURE HARVESTER (Earl & von Holdt, 2012) to determine the optimal K value following Evanno, Regnaut & Goudet (2005). We aligned cluster assignments across different replicates of STRUCTURE on the optimal K value with CLUMPP1.1.2b (Jakobsson & Rosenberg, 2007) and visualized the results in DISTRUCT 1.1 (Rosenberg, 2004). To ensure that our binary data approach did not bias the results, we used the STRUCTURE algorithm that deals with genotypic ambiguity (Falush *et al.*, 2007) and repeated the previous analysis treating markers as co-dominant. Finally, we also ran STRUCTURE excluding tetraploid individuals, and compared the results with those obtained in the mixed ploidy analysis.

FINE-SCALE SPATIAL DISTRIBUTION OF DIPLOIDS AND TETRAPLOIDS

We explored the genetic spatial distribution in relation to ploidy of individuals sampled in the QUA population in 2015. We performed a spatial autocorrelation analysis in GenAlEx 6.5, computing the spatial autocorrelation coefficient (r) proposed by Smouse & Peakall (1999) for even distance classes, analysing each ploidy separately. The 95% confidence interval (CI) for each distance class was obtained by 999 random permutations of geographical locations of individuals and the 95% CI around mean r -values was estimated by bootstrapping pair-wise comparisons within each distance class (1000 replications).

We characterized the fine-scale spatial distribution of ploidy by mapping each individual in the QUA

population sampled in 2015. We used a GPS (Garmin Oregon 450; 3-m precision) and annotated the relative position of individuals in the field using an orthoimage of the area with manual field measures to distinguish individuals closer than 3 m. The final georeference of individuals was based on Google Earth (Google Inc., 2015). The location and ploidy of each mapped individual was plotted with QGIS v.2.14.3 (Quantum GIS Development Team, 2016). The QUA population consisted mainly of groups of individuals placed in four disjointed linear fragments (along roadside ditches) with a maximum distance between fragments of 700 m and a minimum distance of 12 m (Fig. 2). We analysed the spatial distribution of diploids and tetraploids in the QUA population to test for possible spatial segregation, which could indicate differentiation in their microhabitat and have implications for the formation of triploid hybrids and the coexistence of cytotypes. We first analysed the possible segregation of cytotypes among the four linear ditches by applying an exact Fisher test to a 2×4 contingency table in which the rows corresponded to ploidy and the columns to linear ditches. Secondly, we applied a chi-square test to a 2×2 nearest neighbour contingency table in which the rows corresponded to ploidy of focal plants and the columns to ploidy of nearest neighbours and calculated the *S* aggregation index of

Pielou (1961) for the population, which ranges from -1 (total mixture of cytotypes) to 1 (total aggregation of cytotypes).

MORPHOLOGY OF DIPLOID AND TETRAPLOID INDIVIDUALS

We conducted a common garden experiment to compare reproductive and vegetative morphology, phenology and survival of tetraploid individuals against diploid plants from the same locality (QUA). Seeds were collected in the field in 2015 from separate maternal plants (seed families) that were analysed using flow cytometry. We selected 22 diploid and 18 tetraploid maternal families and planted seeds in 9-cm pots filled with Seed Modular compost (Sinclair) on 16 October 2015. Pots were placed in plastic trays flooded with water and kept in a growth cabinet at 16-h/8-h and 24 °C/16 °C light/dark cycles with 70% relative humidity. For each family, we transplanted five seedlings chosen at random 14–21 days after planting, at which stage the seedlings were beginning to produce the first pair of true leaves (200 plants in total). Seedlings were grown in 9-cm-diameter pots with Seedling Modular compost and fertilized weekly with 10:30:20 N/P/K soluble fertiliser (Blossom Booster, Peters Professional, Scotts).

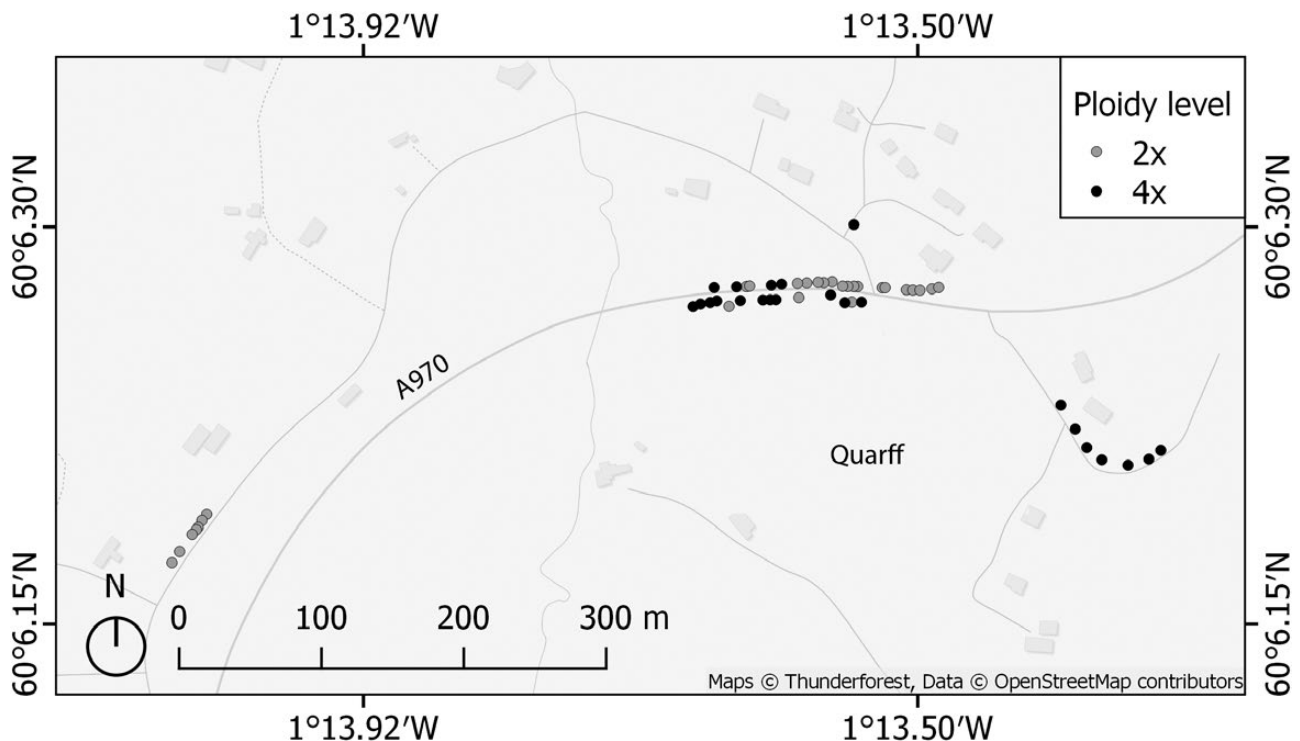


Figure 2. Fine-scale distribution of diploid and tetraploid individuals of *Mimulus guttatus* in the mixed-ploidy population QUA in the Shetland Isles.

For each transplanted individual we recorded (1) whether the plant had flowered by 16 December 2015 (8.5 weeks after planting) and, for those individuals that did flower, we also recorded the following traits: (2) days to flower; (3) node at which the first flower was produced; (4) length (including the petiole) of the largest leaf at flowering; (5) width of the largest leaf at flowering; (6) stem diameter (between the first and second nodes); and (7) plant height (from the soil surface to the highest meristem, measured to the nearest centimetre). For the first open flower of each individual we measured the following traits with digital callipers to the nearest 0.01 cm: (8) corolla width; (9) corolla height; (10) floral tube length (measured from the base of the calyx to the point where upper and lower petals fuse); (11) calyx length (measured from the base of the calyx to the tip of the upper and longest sepal); and (12) floral pedicel length. On 11–12 January 2016 when plants had begun senescing we recorded (13) total number of flowers and (14) survival. Phenotypic traits were analysed with generalized linear mixed-effects models implemented in *lme4* (Bates *et al.*, 2015) using maternal family as a random effect to account for the nested structure of the data. Maternal ploidy was analysed as a fixed effect. Probability of flowering was analysed with a binomial error distribution, number of flowers with a Poisson and the remaining variables with a Gaussian error distribution. Statistical significance of fixed effects was calculated with the package *lmerTest* (Kuznetsova, Brockhoff & Christensen, 2016). We also conducted a principal component analysis of the remaining eight morphological variables (variables 4–6 and 8–12; untransformed data) using the correlation matrix in package *princomp* in *R*. Significant differences between ploidies in the values of the scores of the first two principal components were assessed with linear mixed-effects models as above. Finally, we carried out a linear discriminant analysis (LDA) using maternal ploidy as a classifying variable using the package *MASS*. We tested the statistical significance of the marginal contribution of individual traits to the single canonical axis using the *discr.test* function (100 000 permutations) as described by Koutecký (2015) using the package *vegan* (Dixon, 2003). All morphological analyses were done in *R* v.3.3.0 (R Core Development Team, 2016).

RESULTS

PLOIDY OF INTRODUCED POPULATIONS

Our survey of relative genome size across introduced populations showed that the vast majority of

M. guttatus populations in the UK are diploid. However, we found one exception in the form of a mixed-ploidy population in the Shetland Isles (QUA), which included diploid (2x) and tetraploid (4x) plants (Table 1). Diploids and tetraploids were sampled in this population in a 4:10 ratio in 2014 ($N = 14$) and in a 27:24 ratio in 2015 ($N = 51$). The proportion of tetraploids was higher in 2014, although the difference was not statistically significant (71% vs. 47%; $\chi^2 = 1.73$, $P = 0.19$). The chromosome counts at mitotic metaphase confirmed the expected chromosome numbers of $2n = 28$ and $2n = 56$ for the diploid and tetraploid individuals, respectively (Fig. 3). Vouchers of a diploid and a tetraploid individual are deposited in the Herbarium of the Royal Botanic Garden, Edinburgh with barcodes E00808283 and E00808265, respectively (Supplementary Information, Fig. S1).

GENETIC ANALYSES OF SHETLAND POPULATIONS

Genetic variation of *M. guttatus* in Shetland was generally low. Diploid individuals sampled in the QUA population showed the highest values for most genetic diversity parameters, followed by tetraploid individuals which had high number of alleles and effective alleles and high unbiased diversity (Table 2). Genotypic richness in diploids and tetraploids of the QUA population was within the range observed in other Shetland populations. The AMOVA showed that 34% of the genetic variation occurred among populations ($\Phi_{PT_{5,155}} = 0.338$; $P < 0.001$). When excluding exact multilocus genotypes, genetic variation among populations decreased to 22% ($\Phi_{PT_{5,91}} = 0.224$; $P < 0.001$). We found no differences in genetic variation between diploid and tetraploid cytotypes ($\Phi_{RT_{1,141}} = -0.033$; $P > 0.9$).

The genetic analyses of Shetland individuals showed genetic differentiation along a north–south transect, as shown by the separation of two main groups along the first principal component of the PCoA (Fig. 4A) and in the Bayesian clustering computations with STRUCTURE (Fig. 4B; Supplementary Information, Fig. S2). One group was formed by northern populations, HAM, MUK and WEI, and a second group consisted of southern populations, BOD and NIN. The QUA population, which is geographically intermediate between these two groups, showed genetic membership to both northern and southern clusters, suggesting admixture at this point of contact (Fig. 4; Supplementary Information, Fig. S2). Genetically, the tetraploid individuals in the QUA population were mostly associated with the southern group, including other diploid individuals in the same locality (Fig. 4; Supplementary Information, Fig. S2). The NJ tree based on Dice genetic distances at the individual level showed that tetraploids are genetically closer to

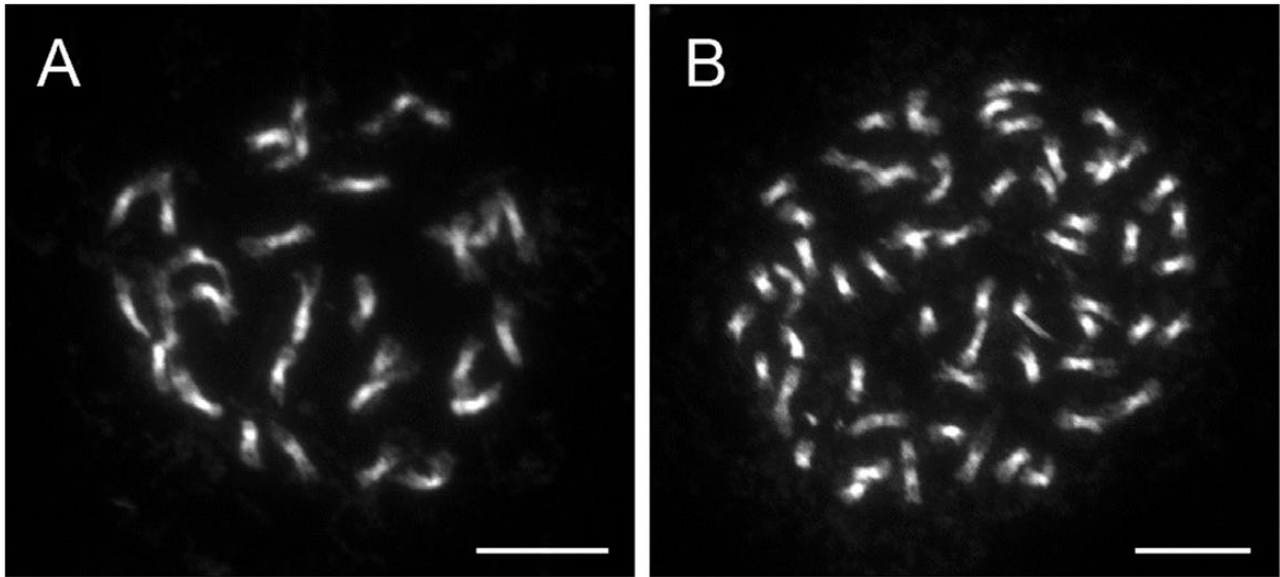


Figure 3. Chromosomes at mitotic metaphase of diploid (A, $2n = 28$) and tetraploid individuals (B, $2n = 56$) of the mixed-ploidy population of *Mimulus guttatus* in the Shetland Isles. Chromosomes have been stained with DAPI. Scale bars = 10 μm .

Table 2. Genetic diversity of 12 co-dominant neutral markers in six populations of *Mimulus guttatus* in the Shetland Isles, Scotland

Population	<i>N</i>	% <i>P</i>	<i>Na</i>	<i>Ne</i>	<i>PA</i>	<i>uh</i>	<i>R</i>
BOD	23.25 \pm 0.351	0.83	2 \pm 0.174	1.775 \pm 0.109	0	0.409 \pm 0.056	0.63
HAM	15.75 \pm 0.641	0.75	1.917 \pm 0.193	1.399 \pm 0.115	1	0.242 \pm 0.059	0.556
MUK	20.417 \pm 0.434	0.58	1.583 \pm 0.149	1.4 \pm 0.136	1	0.217 \pm 0.071	0.409
NIN	14.917 \pm 0.083	0.92	1.917 \pm 0.083	1.829 \pm 0.103	0	0.438 \pm 0.051	0.286
WEI	5.667 \pm 0.482	1	1.917 \pm 0.229	1.594 \pm 0.195	2	0.306 \pm 0.082	1
QUA (2x)	26.167 \pm 0.112	1	2.75 \pm 0.179	1.87 \pm 0.138	3	0.438 \pm 0.047	0.714
QUA (4x)	32.583 \pm 0.417	0.67	2.25 \pm 0.131	1.841 \pm 0.1	1	0.437 \pm 0.046	0.515

Population codes as in Table 1. Diploid (2x) and tetraploid (4x) cytotypes from the mixed-ploidy population (QUA) are presented separately. Abbreviations: average number of individuals successfully genotyped across all loci (*N*), percentage of polymorphic loci (%*P*), number of alleles (*Na*), number of effective alleles (*Ne*), number of private alleles (*PA*), unbiased diversity (*uh*), and genotypic richness (*R*). *Ne*, *uh* and *R* values are given for tetraploids, but note that unknown allele dosage in these individuals may bias these parameters. Means are shown \pm SD.

sympatric diploids in the QUA population than to other diploids from Shetland (Supplementary Information, Fig. S3).

SPATIAL DISTRIBUTION OF CYTOTYPES IN THE MIXED-PLOIDY POPULATION

Genetic spatial autocorrelation in the mixed-ploidy population, QUA, in 2015 was only observed for the first two distance classes (up to 20 m) and only for diploid individuals when including all genotypes ($r > 0.133$; $P < 0.05$). There was no spatial autocorrelation for tetraploid or diploid individuals when excluding exact multilocus genotypes (Supplementary Information, Fig. S4). The analyses of the fine-scale distribution of individuals revealed spatial segregation of cytotypes

in the QUA population. Diploids and tetraploids were significantly separated among the four ditches (exact Fisher test; $P < 0.001$) and segregation of cytotypes based on the nearest neighbour contingency table was also significant ($P < 0.01$). The segregation index of Pielou was calculated as $S = 0.667$, indicating that diploids and tetraploids were located in close vicinity to individuals of the same cytotype.

PHENOTYPE OF DIPLOID AND TETRAPLOID *M. GUTTATUS* IN THE MIXED-PLOIDY POPULATION

Transplanted individuals of both maternal ploidies had high survivorship, with only one plant dying before 12.5 weeks (from a diploid maternal family). The proportion of individuals that flowered by 8.5 weeks

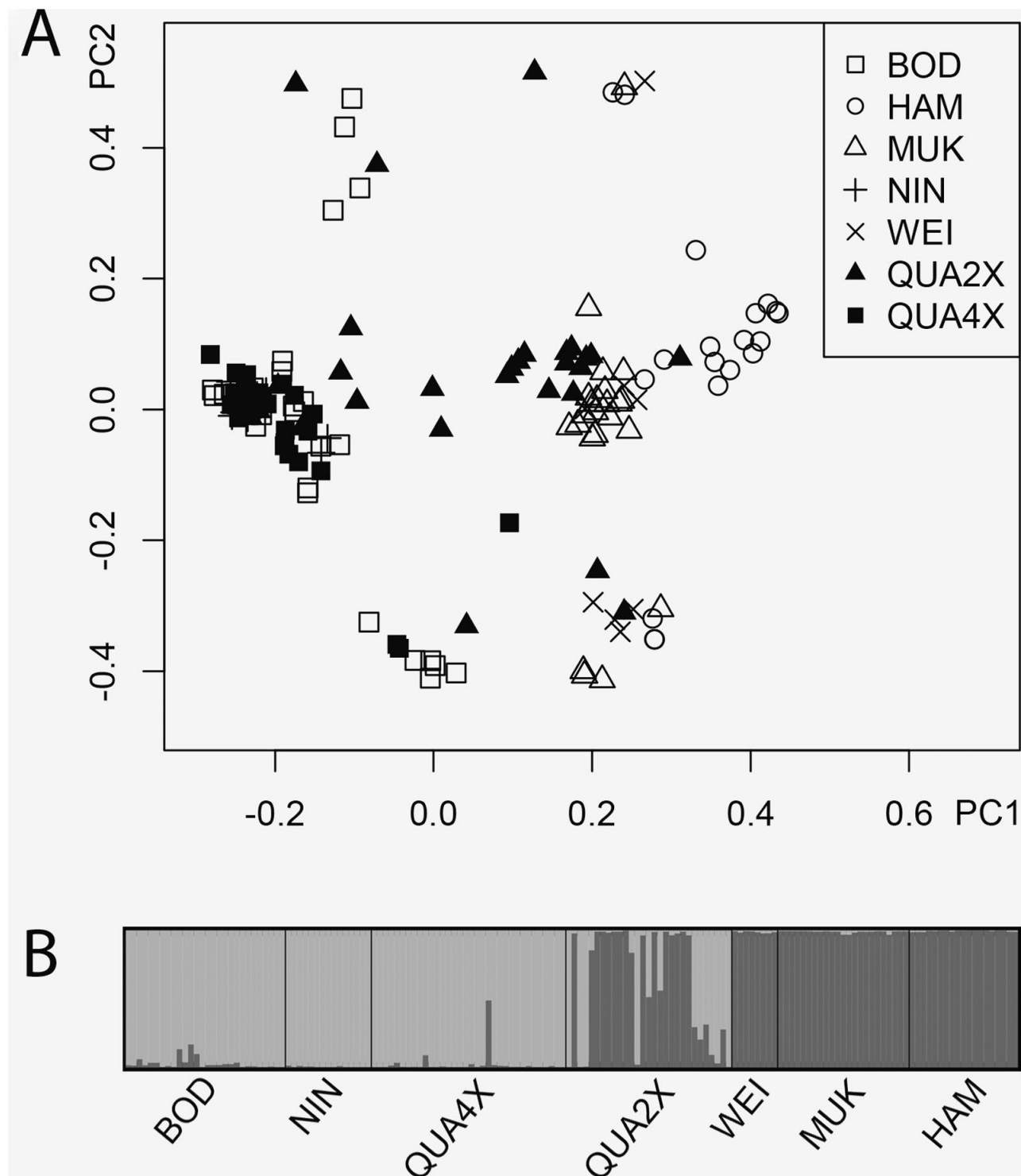


Figure 4. Genetic relationships between cytotypes of QUA and other populations of *Mimulus guttatus* in the Shetland Isles. (A) PCoA based on Dice genetic distance between all sampled individuals. (B) Bayesian inference of the two genetic clusters estimated with Structure for the populations of *M. guttatus* in the Shetland Isles. Within each column (individual), the membership coefficient (Q) to each cluster is indicated with different shades.

differed significantly between ploidies, with diploids much more likely to flower than tetraploids (93.3% vs. 32.6%, respectively, $P < 0.001$). Among those individuals that flowered, diploids flowered earlier on average than tetraploids, whether this was measured from the day on which seeds were planted (41.82 ± 4.31 vs. 50.93 ± 9.45 days, mean \pm SE; $P < 0.01$) or from the day when they were transplanted (26.05 ± 2.68 vs. 33.89 ± 6.29 days; $P < 0.01$). There was no difference between ploidy in the node number at which the first flower was produced, suggesting that delayed flowering in tetraploids was not accompanied by a shift in which node becomes reproductive (average node for first flower: 6.54 ± 0.67 vs. 6.68 ± 1.24 , for diploid and

tetraploid families, respectively; $P = 0.67$). The total number of flowers differed significantly between ploidies with diploids producing nearly twice as many flowers as tetraploids (43.25 ± 4.59 vs. 21.53 ± 3.93 ; $P < 0.001$).

Diploid and tetraploid families also differed in most other vegetative and reproductive traits (Fig. 5). Tetraploid families had larger corollas, longer floral pedicels, larger leaves and thicker stems than diploids (Fig. 5). A statistical analysis of the first two principal components (summarizing 44% and 18% of the variation in the traits shown in Fig. 5) clearly indicated significant differences between ploidies with tetraploid families having larger values for both principal

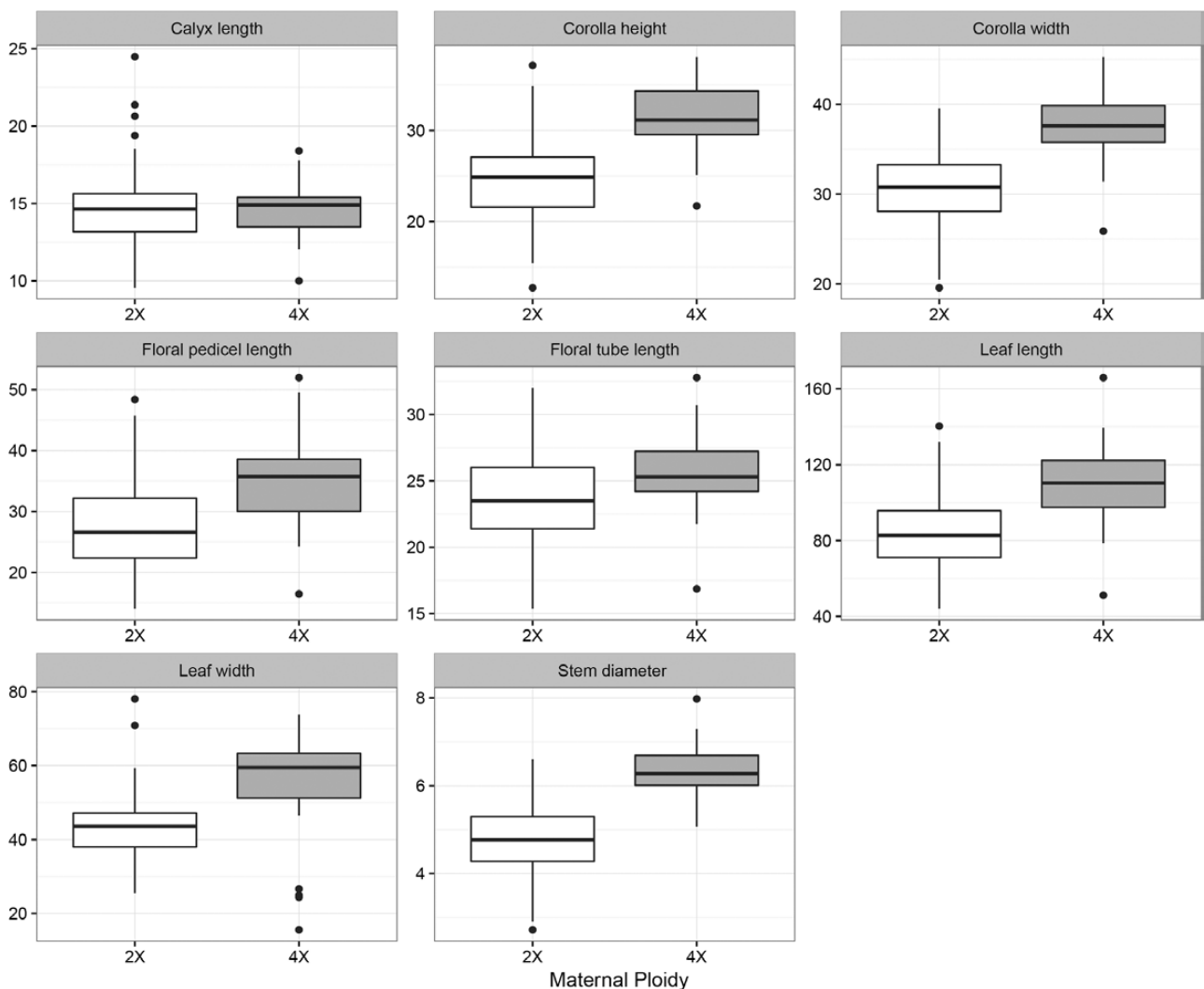


Figure 5. Boxplots showing morphological differences in eight floral and vegetative traits between diploid and tetraploid families of *Mimulus guttatus* in the mixed-ploidy population QUA in the Shetland Isles. The box indicates the 25th–75th interquartile range (IQR); the line inside the box is the median; upper (lower) whisker is the largest (smallest) observation less than or equal (greater than or equal) to the 75th quantile + 1.5×IQR (25th quantile – 1.5×IQR); open symbols are outliers (data beyond the whiskers). All measurements are in millimetres.

components ($P < 0.001$) (Supplementary Information, Fig. S5). The first principal component (PC1) was positively correlated with all variables, thus representing overall floral and vegetative size (Supplementary Information, Fig. S5 and Table S2). Higher values of the second principal component were associated with larger leaves, thicker stems and longer pedicels, but shorter calices and floral tubes (Supplementary Information, Table S2). The LDA correctly predicted the maternal ploidy of 94% of the individuals (96% of diploids and 90% of tetraploids; Supplementary Information, Fig. S6). Analysis of the contribution of individual traits to the single discriminant axis showed that corolla height, calyx length, pedicel length, leaf length and stem thickness significantly contributed to distinguish diploids and tetraploids (Supplementary Information, Table S3). Together, our results indicate a clear morphological and phenological distinction between diploid and tetraploid *Mimulus guttatus*.

DISCUSSION

Our survey of genome size in UK populations of *M. guttatus* identified one case of autopolyploidization in the Shetland Isles. Genetic analyses are consistent with the hypothesis that autopolyploids have evolved from local diploids in the UK in the last 200 years. Confirmed cases of recent neopolyploidization (< 200 years old) are rare (Vallejo-Marín *et al.*, 2015; Soltis *et al.*, 2016) and, to our knowledge, our report is the first documented case of a newly formed autopolyploid in the non-native range. The neo-autotetraploid *M. guttatus* is larger, more robust and less prone to flower than local diploids, a result that is in line with theoretical expectations of the immediate effects of genome doubling (Ramsey & Schemske, 2002). Diploids and tetraploids were present in similar proportions and showed spatial segregation in the mixed-ploidy population. No triploids were observed in the mixed population. Together, our results suggest a recent and successful establishment of phenotypically differentiated tetraploids among diploid ancestors, supporting the hypothesis that novel evolutionary lineages can rapidly originate via polyploidization in the wild. The evolution of polyploid lineages with distinct morphological and phenological properties from their immediate ancestors is especially important in invasive populations that have to rapidly adapt to novel environments.

INCIDENCE OF AUTOPOLYPOIDS AND ABSENCE OF TRIPLOIDS IN NATIVE AND INTRODUCED RANGES

Our study in the introduced range of *M. guttatus* found autopolyploids in a single population (3% or 1/29 of

sampled populations), which also contained diploid individuals. Autopolyploids in the native range seem to be more abundant (9% or 7/76 populations, mostly restricted to the southern range limit; Vickery *et al.*, 1968), but comparisons of the incidence of autopolyploids in the native and introduced range should be done with caution. The identity of autopolyploids in the native range is based on phenotypic characteristics, and chromosome pairing behaviour and genetic markers would help to confirm undeniably the nature of these putative autopolyploids. Conducting more extensive surveys of ploidy across the native range (e.g. Modliszewski & Willis, 2012) and throughout the introduced range in the UK will be required to understand the incidence and distribution of autopolyploid *M. guttatus* fully.

Mixed diploid–tetraploid populations can potentially form triploid (and often sterile) individuals when reduced gametes of diploid and tetraploid individuals fuse (Vallejo-Marín & Hiscock, 2016). We did not observe any triploids in the mixed population in the introduced range. Similarly, triploids are also absent in the two documented cases of mixed diploid–autopolyploid populations in the native range (McArthur *et al.*, 1972). Lack of triploids in mixed-ploidy populations may result from either pre- or post-zygotic reproductive barriers (Husband & Sabara, 2004). For instance, the difference in flowering time we observed between diploid and tetraploid genotypes may reduce, although not completely prevent, the opportunities for inter-cytotype mating. Moreover, low viability and fertility of triploids (Ramsey & Schemske, 1998; Vallejo-Marín & Hiscock, 2016) and minority cytotype exclusion (Levin, 1975) may prevent triploid persistence. Nevertheless, other (interspecific) *Mimulus* triploids can overcome viability barriers and persist even in the face of near sexual sterility, at least in the short term (Vallejo-Marín & Lye, 2013; Vallejo-Marín & Hiscock, 2016). The reason for the absence of triploid *M. guttatus* in native and introduced mixed populations remains to be established.

WHOLE GENOME DUPLICATION AND MORPHOLOGICAL DIFFERENTIATION OF CYTOTYPES

An immediate consequence of polyploidization is an increase in cell size (Müntzing, 1936; Stebbins, 1971). This is known as the ‘gigas’ effect and can result in, typically, polyploids being larger and more robust, with larger flowers, pollen grains and seeds (Müntzing, 1936; Stebbins, 1971; Garbutt & Bazzaz, 1983; Bretagnolle *et al.*, 1995; Ramsey & Schemske, 2002; Ramsey, 2007; Knight & Beaulieu, 2008; Finigan, Tanurdzic & Martienssen, 2012; Husband *et al.*, 2013). The largest differences in phenotype are expected

immediately after polyploidization, with subsequent evolution ameliorating the consequences of genome duplication for cell size (Otto & Whitton, 2000). Our results indicate that tetraploid individuals of *M. guttatus* have fewer but larger flowers, larger leaves, thicker stem, and take longer to flower than sympatric diploids. These results are consistent with a recent polyploidization event in the introduced range.

Although the morphological and physiological effects of polyploidization have been documented previously, the ecological consequences of these phenotypic changes are still poorly understood (Soltis *et al.*, 2010). Genome duplication can simultaneously affect multiple traits including plant morphology, development, flower shape (Taylor & Smith, 1979; Segraves & Thompson, 1999) and plant chemistry (Gross & Schiestl, 2015) (reviewed by Soltis *et al.*, 2014b). These multifactorial changes have the potential to rapidly generate considerable phenotypic novelty and facilitate the successful establishment of neopolyploids. Rapid adaptation may be particularly important in non-native populations and in species faced with global change. We speculate that, in these scenarios, polyploidy represents a valuable source of variation, some of which may contribute to rapid evolutionary change and adaptation.

GENETIC RELATIONSHIPS BETWEEN DIPLOID AND TETRAPLOID *M. GUTTATUS* IN SHETLAND

Our genetic results are consistent with the hypothesis of a single neopolyploidization event of *M. guttatus* in the admixed QUA population in Shetland. In other taxa, the hypothesis of a primary contact zone is seen as the most plausible explanation for isolated mixed-ploidy populations (Trávníček *et al.*, 2011), although genetic evidence is scarce (but see, e.g. Halverson *et al.*, 2008). The alternative hypothesis to this local origin scenario is that tetraploids have been formed in a different (unsampled) population with a similar composition to QUA and migrated secondarily into the QUA population, admixing with diploid individuals. We think this latter hypothesis is unlikely because secondary contacts of cytotypes are usually associated with taxa displaying more complex patterns of broader contact zones and/or multiple cytotypes (Hardy *et al.*, 2000; Weiss *et al.*, 2002; Stuessy, Weiss-Schneeweiss & Keil, 2004; Mandáková & Münzbergová, 2008; Balao *et al.*, 2009; Duchoslav, Šafářová & Krahulec, 2010; Castro *et al.*, 2012; Kolář *et al.*, 2012; Zozomová-Lihová *et al.*, 2015). We thus believe that the most likely explanation for the tetraploid *M. guttatus* discovered in QUA is a local origin in Shetland, probably within the QUA population. Future sampling of additional populations of *M. guttatus*, combined with genetic analyses using markers across the genome (e.g.

genotype-by-sequencing, RADseq or whole genome resequencing) have the potential to refine the hypothesis for the origin and incidence of autopolyploid populations further.

ENVIRONMENTAL FACTORS INFLUENCING AUTOPOLYPLOID FORMATION

Unreduced gametes are considered a major route for the formation of polyploids, including autopolyploids (Mason & Pires, 2015). The rate of production of unreduced gametes can be relatively high (Ramsey & Schemske, 1998), and it can be increased by environmental factors (Bretagnolle & Thompson, 1995; Parisod *et al.*, 2010; De Storme & Geelen, 2013, 2014; De Storme & Mason, 2014; Mason & Pires, 2015). For instance, water and nutritional stress may increase the production of unreduced gametes (Stebbins, 1971; Favarger, 1984; Parisod *et al.*, 2010) and cause polyploids to be particularly common in habitats affected by edaphic disturbance (Ramsey & Schemske, 1998). Another abiotic factor that has been related with the production of unreduced gametes is temperature stress (Negri & Lemmi, 1998; Ramsey, 2007; Mason *et al.*, 2011). For instance, low temperatures may increase the rate of production of unreduced gametes (Ramsey, 2007; Mason *et al.*, 2011; De Storme, Copenhaver & Geelen, 2012), which may underlie the positive association of polyploid occurrence with cold climates (Aleza *et al.*, 2011; Liu *et al.*, 2011) and, consequently, with high latitudes (Grant, 1981; Soltis, 1984; Stebbins, 1984; Suda *et al.*, 2009; Husband *et al.*, 2013; but see Stebbins, 1950, 1971, 1985; Ehrendorfer, 1980; Levin, 2002; Brochmann *et al.*, 2004). In this regard, it may not be coincidental that the mixed-ploidy population found in our survey in the UK was located at the northern end of the introduced range (60°N). In the native range of *M. guttatus*, polyploids are also found at range limits (McArthur *et al.*, 1972). Whether climatic or other stresses are involved in the production of *M. guttatus* polyploids at range limits remains to be determined. Both broader surveys of ploidy across the native and introduced ranges of plant species, including populations at intermediate latitudes in the UK, and further mechanistic studies of the causes of unreduced gamete formation, will contribute to our understanding of the global patterns of polyploid occurrence.

CONCLUSIONS

The long-term evolutionary advantages of polyploidy are still unclear (Comai, 2005; Soltis *et al.*, 2016), although evidence continues to accumulate suggesting that polyploidization is an important driver of plant evolution at both short- and long-term evolutionary

timescales (Soltis & Rieseberg, 1986; Otto & Whitton, 2000; Parisod *et al.*, 2010; Soltis *et al.*, 2014b; Vallejo-Marín & Hiscock, 2016; Barker, Husband & Pires, 2016b). Autopolyploids are particularly puzzling given that, unlike allopolyploids, they lack some of the hypothesized benefits brought by hybridization, such as fixed heterozygosity. It has been suggested that autopolyploids may represent a neutral process (Meyers & Levin, 2006) or an 'evolutionary dead-end' (Stebbins, 1950). Our results indicate that, even in the absence of interspecific hybridization, genome duplication can occur rapidly and trigger phenotypic changes. The potential of polyploidization to rapidly generate phenotypic novelty could facilitate the establishment of non-native populations in new environments (Verlaque, Aboucaya & Fridlender, 2002; Mandák *et al.*, 2003; Mandák, Pysěk & Bímová, 2004; Pandit, Tan & Bisht, 2006; Pandit, Pocock & Kunin, 2011; Suda *et al.*, 2010). However, further work is needed to firmly establish an association between polyploidy and range expansion (Te Beest *et al.*, 2012). Although we show that introduced *M. guttatus* in the UK is mostly diploid, future ploidy screenings of introduced populations, including those in Shetland, may help establish whether autotetraploids can spread beyond their place of origin. Nevertheless, the discovery of recent polyploids formed on short time scales is a reminder that, in addition to the historical contribution of polyploidy to plant evolution, genome duplication holds enormous potential as a source of variation allowing populations to deal with environmental change.

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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.

Figure S1. Vouchers of diploid (A) and tetraploid (B) individuals from the QUA population deposited at the Royal Botanic Garden, Edinburgh. RBGE barcode: E00808283 (diploid) and E00808265 (tetraploid).

Figure S2. Genetic relationships between cytotypes of QUA and other populations of *Mimulus guttatus* in the Shetland Isles based on Bayesian inference of the two genetic clusters estimated by Structure with genotypes coded as co-dominant data. Within each column (individual), the membership coefficient (Q) to each cluster is indicated with different colour. (A) Analysis with diploid and tetraploid individuals, using the algorithm that deals with genotypic ambiguity (Falush *et al.*, 2007); (B) analysis excluding tetraploids and using the common approach.

Figure S3. Neighbor joining tree based on Dice genetic distances between individuals, showing genetic relationships between cytotypes of QUA and other populations of *Mimulus guttatus* in Shetland.

Figure S4. Autocorrelograms for diploid (a) and tetraploid (b) individuals of *Mimulus guttatus* sampled in QUA (Shetland) in 2015. Blue line indicates r value and dotted lines upper and lower 95% confidence limits.

Figure S5. Bidimensional principal component analysis (PCA) plot of individuals of the diploid and tetraploid families of *Mimulus guttatus* from a population in the Shetland Isles (QUA) based on analysis of eight floral and vegetative variables. Diploids are represented by grey dots and tetraploids black dots.

Figure S6. Linear discriminant analysis of floral and vegetative traits from individuals derived from either diploid ($2x$) or tetraploid ($4x$) maternal families of *Mimulus guttatus* from the QUA population in the Shetland Isles. The histogram depicts values of the single linear discriminant axis (LD1). Per cent of correct classifications: 96% (diploids), 90% (tetraploids).

Table S1. Co-dominant genetic markers used for genetic analysis of *Mimulus guttatus* populations in Shetland. Marker type: MnSTS, *Mimulus nasutus* sequence-tagged sites (AAT motif); MgSTS, *M. guttatus* sequence-tagged sites (= intron-based length polymorphism markers). Linkage group refers to the *M. guttatus* genome.

Table S2. Principal component analysis (PCA) of eight floral and vegetative variables in diploid and tetraploid families of *Mimulus guttatus* from a population in the Shetland Isles (QUA). PC1–8: Eigenvectors (loadings) of eight principal components.

Table S3. Statistical significance of the contribution of individual traits to the single canonical axis distinguishing the floral and vegetative phenotype of diploid and tetraploid individuals of *Mimulus guttatus* in a population in the Shetland Isles (QUA). Significance was tested using the *discr.test* function from Koutecký (2015) ('unique contributions'). df, degrees of freedom; P -value, calculated using 100 000 permutations.

SHARED DATA

Data have been archived in the Stirling Online Repository for Research Data (DataSTORRE) under URL: <http://hdl.handle.net/11667/96>.