Annals of Botany **115**: 263–273, 2015
doi:10.1093/aob/mcu244, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY Founded 1887

Are tetraploids more successful? Floral signals, reproductive success and floral isolation in mixed-ploidy populations of a terrestrial orchid

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Received: 6 August 2014 Returned for revision: 7 October 2014 Accepted: 22 October 2014 Published electronically: 13 January 2015

• **Background and Aims** Polyploidization, the doubling of chromosome sets, is common in angiosperms and has a range of evolutionary consequences. Newly formed polyploid lineages are reproductively isolated from their diploid progenitors due to triploid sterility, but also prone to extinction because compatible mating partners are rare. Models have suggested that assortative mating and increased reproductive fitness play a key role in the successful establishment and persistence of polyploids. However, little is known about these factors in natural mixed-ploidy populations. This study investigated floral traits that can affect pollinator attraction and efficiency, as well as reproductive success in diploid and tetraploid *Gymnadenia conopsea* (Orchidaceae) plants in two natural, mixed-ploidy populations.
• **Methods** Ploidy levels were determined using flow cytometry, and flowering phenology and herbivory were also assessed. Reproductive success was determined by counting fruits and viable seeds of marked plants. Pollinator-mediated floral isolation was measured using experimental arrays, with pollen flow tracked by means of staining pollinia with histological dye.
• **Key Results** Tetraploids had larger floral displays and different floral scent bouquets than diploids, but cytotypes differed only slightly in floral colour. Significant floral isolation was found between the two cytotypes. Flowering phenology of the two cytotypes greatly overlapped, and herbivory did not differ between cytotypes or was lower in tetraploids. In addition, tetraploids had higher reproductive success compared with diploids.
• **Conclusions** The results suggest that floral isolation and increased reproductive success of polyploids may help to explain their successful persistence in mixed-ploidy populations. These factors might even initiate transformation of populations from pure diploid to pure tetraploid.

Key words: Floral signals, floral scent, floral morphology, floral colour, pollination, reproductive success, polyploidization, mixed-ploidy populations, floral isolation, *Gymnadenia conopsea*, orchid, Orchidaceae, diploid, tetraploid.

INTRODUCTION

Polyploidization, the duplication of the genome, is strikingly prevalent in flowering plants, with all angiosperms having undergone at least one polyploidization (Jiao *et al.*, 2011). Polyploidization plays an important role in angiosperm speciation (Wood *et al.*, 2009) and is a core type of sympatric speciation (Coyne and Orr, 2004). As triploid hybrid seeds from crosses between diploid and tetraploid plants are less viable than parental plants or are non-viable, and as mature triploid plants are largely sterile due to problems in pairing/segregation of chromosomes during meiosis, newly formed polyploids are post-zygotically strongly reproductively isolated from their diploid progenitors (Ramsey and Schemske, 1998). This is true not only for allopolyploidization (polyploidization as a result of interspecific hybridization), but also for the less well recognized and less studied autopolyploidization (intraspecific genome duplication; Soltis *et al.*, 2007). A major problem of newly formed polyploids is that they initially occur at very low frequencies, and thus largely lack compatible mating partners, and hence polyploid lineages are prone to extinction as soon as they emerge (Levin, 1975). Nevertheless, polyploids are observed in nature, and plants with different ploidy (cytotypes) often grow sympatrically (Husband and Schemske, 1998; Burton and Husband, 1999; Halverson *et al.*, 2008; Mráz *et al.*, 2008; Trávníček *et al.*, 2011*a*, *b*). A recent study by Mayrose *et al.* (2011) suggests that the net diversification rate of recently formed polyploids is lower than that of their diploid relatives because of an increased extinction rate, but a thorough analysis of a more representative data set would probably decrease the estimate of the extinction rate (Soltis *et al.*, 2014); another study suggested that polyploidy led to an increase in species richness in multiple angiosperm families (Soltis *et al.*, 2009). This still unsolved discrepancy has motivated studies exploring mechanisms and factors that could explain the high prevalence of polyploids in angiosperms.

The evolutionary success of polyploids is divided into their formation, establishment and persistence (Thompson and Lumaret, 1992; Parisod *et al.*, 2010). The major mechanism of polyploid formation is the production of unreduced gametes (Harlan and deWet, 1975; Bretagnolle and Thompson, 1995;

Ramsey and Schemske, 1998). Newly formed polyploids are necessarily rare and, thus, for successful polyploid establishment, mechanisms counteracting minority cytotype exclusion are necessary. The production of unreduced gametes (Felber, 1991; Bretagnolle and Thompson, 1995; Felber and Bever, 1997; Ramsey and Schemske, 1998) and triploids, which are often not completely sterile (Felber and Bever, 1997; Ramsey and Schemske, 1998; Husband, 2004; Yamauchi *et al*., 2004), ensure recurrent formation of polyploids, thereby fostering their establishment. Several non-mutually exclusive mechanisms may promote within-cytotype matings and thus reduce the wastage of gametes to the non-compatible cytotype. First, self-pollination was one of the first factors suggested to slow down the extinction of newly formed polyploid lineages (Levin, 1975) and has been shown to be generally higher in polyploids than in diploids (Barringer, 2007). Secondly, cytotypes are often ecologically differentiated even on small scales (Felber-Girard *et al*., 1996; Raabová *et al*., 2008; Sonnleitner *et al*., 2010). Thirdly, assortative mating has been predicted to have the ability to make polyploid establishment a common scenario (Oswald and Nuismer, 2011*b*). Indeed, pollinator-mediated assortative mating seems to contribute considerably to the reproductive isolation between diploids and tetraploids (Segraves and Thompson, 1999; Husband and Sabara, 2003; Kennedy *et al*., 2006). In addition, a higher relative fitness can prevent the extinction of newly formed polyploid lineages (Felber, 1991; Rodríguez, 1996; Suda and Herben, 2013). Seed set in natural populations is generally higher in polyploids (Petit *et al*., 1997; Nuismer and Cunningham, 2005; Castro *et al*., 2012), but see Mráz *et al*. (2011). Such mechanisms, which enable the establishment of polyploid lineages, may also ensure their persistence. However, the relative importance of such mechanisms that foster polyploid establishment and persistence in natural, mixed-ploidy populations is still largely unknown (but see Husband and Sabara, 2003). In a study in *Chamerion angustifolium*, geographic isolation and pollinator fidelity were the strongest reproductive barriers of five pre-zygotic and two post-zygotic reproductive barriers investigated (Husband and Sabara, 2003). This highlights the importance of pre-zygotic barriers for the reproductive isolation between cytotypes. Hence, it has been suggested that assortative mating in conjunction with the relative fitness influences the change of tetraploid frequency (Husband and Sabara, 2003) and thus is likely to influence the evolutionary fate of polyploids. However, quantification of both assortative mating and reproductive success in natural populations in conjunction with the study of a wide range of floral traits possibly affecting pollinator attraction is still lacking.

In orchids, pollinator-mediated assortative mating via floral isolation plays an important role in the reproductive isolation among species (Schiestl and Schlüter, 2009), and polyploidy may have contributed to the high diversification of orchids (Amich *et al*., 2007). The terrestrial orchid *Gymnadenia conopsea* (L.) R.Br. *sensu lato* (*s.l.*) probably contains two distinct species, namely *G. conopsea* (L.) R. Br. *sensu stricto* (*s.s.*) and *G. densiflora* (Wahlenb.) Dietrich (Scacchi and de Angelis, 1989; Marhold *et al*., 2005; Stark *et al*., 2011). While *G. densiflora* seems to be uniformly diploid, *G. conopsea s.s.* is known to vary in its ploidy level among and within populations, with the two major cytotypes being diploid and tetraploid (Marhold *et al*., 2005; Jersáková *et al*., 2010; Stark *et al*., 2011; Trávníček *et al*., 2011*b*, 2012). Polyploid *G. conopsea* plants are most likely of autopolyploid origin. The only possible hybridization leading to allotetraploids similar to *G. conopsea* would be between *G. conpsea* and *G. densiflora*. Scent differences are, however, more pronounced between *G. conopsea* and *G. densiflora* than between diploid and tetraploid *G. conopsea* plants; in addition, internal transcribed spacer (ITS) sequences differ between the two species but not between diploid and tetraploid *G. conopsea* plants (Jersáková *et al*., 2010). These orchids produce nectar in a long floral spur and have a functionally specialized pollination system, being mainly pollinated by long-tongued moths (van der Cingel, 1995; Vöth, 2000; Huber *et al*., 2005; Meekers *et al*., 2012). A recent study has shown that floral morphology, floral signals and flowering phenology differ not only between *G. conopsea* and *G. densiflora*, but also between diploids and tetraploids in *G. conopsea* (Jersáková *et al*., 2010). However, reproductive-isolation mechanisms between cytotypes within the species *G. conopsea* are not yet well understood. *Gymnadenia conopsea* is relatively common and forms large populations in suitable habitats, making it an ideal system to study evolutionary dynamics in autopolyploids and their diploid progenitors.

In this study, we aim to investigate factors, in particular assortative mating and reproductive success, that foster the persistence of polyploids in natural mixed-ploidy populations. We specifically asked the following questions. (1) What are the differences in floral traits between sympatric diploid and tetraploid *G. conopsea* cytotypes? (2) How strong is floral isolation between cytotypes? (3) Do the two cytotypes differ in flowering phenology, herbivory and fruiting success?

MATERIALS AND METHODS

Study species and populations

The terrestrial orchid *Gymnadenia conopsea* (L.) R.Br. *s.s.* has a wide distribution range throughout most of Europe and parts of Asia and mainly grows on calcareous soil, from dry meadows to marshes or fens to light forests, and from the lowland to alpine habitats (Hess *et al*., 1976). In Switzerland, where we conducted the study, it occurs throughout the country and grows locally in high numbers. The flowering season extends from the middle of May to the middle of August. *Gymnadenia conopsea* produces a single inflorescence with approx. 15–120 flowers. Floral colour ranges from pale pink or rarely white to dark purple. *Gymnadenia conopsea* has a functionally specialized pollination system with a long nectar-holding spur and mainly diurnal and nocturnal Lepidoptera as pollinators (van der Cingel, 1995; Vöth, 2000; Huber *et al*., 2005; Meekers *et al*., 2012). *Gymnadenia conopsea* is presumably largely outcrossing and, even though self-compatible, relies on pollinators to set fruits (Sletvold *et al*., 2012). We conducted this study in 2011 and 2012 in two Swiss *G. conopsea* populations: Döttingen (47 °34'N, 8 °16'E, 500 m a.s.l.) and Remigen (47 °31'N, 8 °09'E, 570 m a.s.l.). In June each year, when most plants fully flowered, we individually marked plants along paths through the populations. In 2011, we counted the number of plants within 2 m of a marked plant to estimate local plant density.

Ploidy-level analysis

From each marked plant, we collected 2–6 pollinaria. We used pollinaria because the usage of meiotically reduced plant tissue allows assessment of the ploidy level that contributes to the next generation. To analyse the relative ploidy levels, we used a Cell Lab Quanta^{TM} SC-MPL flow cytometer (Beckman Coulter, Fullerton, Canada) equipped with a mercury arc lamp. For sample preparation, we followed a two-step protocol (cf. Doležel *et al*., 2007), using Baranyi's (0·1 M citric acid, 0·5 % Triton X-100; Baranyi and Greilhuber, 1995) and Otto II solution [0·4 M $Na_2HPO_4{\cdot}7H_2O$ supplemented with 4 µg mL^{-1} DAPI (4', 6-diamidino-2-phenylindole); Doležel *et al*., 2007] and leaf material of *Phaseolus coccineus* as internal standard (IS). All pollinaria collected per individual were used, and the detailed sample preparation and analysis was as described by Xu *et al*. (2011), but runs were stopped after 8000 particles or 5 min. The ploidy of a few plants could not be determined because mould grew on the pollinaria. In the flow cytometric histograms, the pollinaria material (P) appeared in two peaks: the first peak represented meiotically reduced germ cells, and the second peak represented maternal tissue cells and unreduced germ cells. To assess the relative ploidy level of an individual, we divided the median of the first P peak by the median of the IS peak (named the 'P:IS ratio' hereafter). The mean ± s.d. count was 812·26 ± 432·07 nuclei for the first P peak and 1353·48 ± 772·39 nuclei for the IS peak, and the mean ± s.d. coefficient of variation (CV) was 4·54 ± 1·30 % for the first P peak and 6·30 ± 1·44 % for the IS peak. Overall, we found three different relative ploidy levels with a mean ± s.d. P:IS ratio of 2·56 ± 0·18, 3·63 ± 0·18 and 4·69 ± 0·29, respectively. As karyological counts have revealed the smallest *G. conopsea* cytotype to be diploid ($2n = 2x = 40$) (Trávníček *et al*., 2012), we assumed that the pollinaria with the lowest relative ploidy were haploid produced by diploid plants. Thus, we found three cytotypes: diploid (2*x*; lowest P:IS ratio), triploid (3*x*; medium P:IS ratio) and tetraploid (4*x*; highest P:IS ratio) plants (Table 1). Cytotypes grew intermixed especially in the population Döttingen (Supplementary Data Fig. S1).

Measurement of floral traits and phenology

When both cytotypes were in full flower, we measured display size, floral morphology, floral colour and floral scent of marked *G. conopsea* plants. We quantified these traits only for the two major cytotypes, the diploids and the tetraploids, because triploids were too rare to conduct multivariate statistical analyses.

We measured plant height (ground to uppermost flower) and inflorescence length [difference between plant height and stem length (ground to lowermost flower)] to the nearest centimetre using a measuring tape. In addition, we counted the total number of flowers per inflorescence. We measured plant height, inflorescence length and number of flowers, hereafter summarized under the term 'display size', of 195 plants in 2011 (Döttingen: $n_{2x} = 76$, $n_{4x} = 23$; Remigen: $n_{2x} = 65$, $n_{4x} = 31$) and of 107 plants in 2012 (Döttingen: $n_{2x} = 36$, $n_{4x} = 26$; Remigen: $n_{2x} = 19$, $n_{4x} = 26$).

To quantify floral morphology, we cut one fully open but still fresh flower per inflorescence from 107 plants (Döttingen: $n_{2x} = 36$, $n_{4x} = 26$; Remigen: $n_{2x} = 19$, $n_{4x} = 26$) in 2012. Due to time constraints during fieldwork, we had to store these flowers for a few days in a fridge at 4 °C. We then quantified flower size as width and length measures of the whole flower as well as of individual flower parts and flower shape as width to length ratios, resulting in a total of 25 floral morphology traits (Supplementary Data Table S1). All measurements were conducted with a 135-mm digital calliper (DigiMax, Hamm, Germany) to the nearest 10 nm. A few flowers that were rotten were excluded from analysis.

Morphological and physiological characteristics of the orchid pollinaria allow their precise positioning on pollinators and hence on conspecific stigmas (Johnson and Edwards, 2000). Thus, pollinator-induced cross-pollination will only be possible if the size of pollinaria, their positioning on pollinators or their bending characteristics [after removal from the flower, pollinaria undergo a bending movement into the correct position to touch the stigma (Johnson and Edwards, 2000)] are similar enough. To quantify pollinaria properties in *G. conopsea*, we imitated pollinaria removal by a pollinator in 52 plants (Döttingen: $n_{2x} = 16$, $n_{4x} = 11$; Remigen: $n_{2x} = 9$, $n_{4x} = 16$) in 2012 using a wooden toothpick. We measured pollinia and pollinaria size as well as bending distances, resulting in a total of six traits, to the nearest 10 nm with the digital calliper, and we measured bending time using a stop watch (Supplementary Data Table S1).

To quantify floral colour, we cut another flower from the same plants from which the flower was cut to measure floral morphology. A few flowers rotted and were excluded from analysis. To quantify floral colour, we used a spectrophotometer (AvaSpec-2048 Fiber Optic Spectrometer; Avantes, Eerbeek, The Netherlands) and a short-arc xenon lamp (AvaLight-XE Pulsed Xenon Lamp; Avantes). We acquired the relative spectral reflectance of the flower's labellum [percentage of a white reference tile (Avantes)] between 300 and 700 nm. For each labellum, we measured the relative reflectance three times and then calculated the mean of these three measurements at every nanometre. To investigate the pollinators' view of floral colour, we adapted the colour hexagon for bee colour vision (Chittka, 1992; Chittka and Kevan, 2005) to the colour vision of *Macroglossum stellatarum*. *Macroglossum stellatarum* is an important *G. conopsea* pollinator (Meekers *et al*., 2012) with a well-studied colour vision (Kelber, 1996, 1997; Kelber and Henique, 1999). We calculated the spectral sensitivity function of the three photoreceptors of *M. stellatarum* (Stavenga *et al*., 1993) using the maximum sensitivity

TABLE 1. *Number of* Gymnadenia conopsea *plants subjected to ploidy level analysis using flow cytometry and frequency of diploid (2x), triploid (3x) and tetraploid (4x) plants in the two study populations, Döttingen and Remigen, in 2011 and 2012*

Population	Year	*n*	Frequency (%)		
			2*x*	3*x*	4*x*
Döttingen	2011	100	76·00	1·00	23·00
	2012	110	69·09	5·45	25·45
Remigen	2011	98	66·33	2·04	31·63
	2012	49	46·94	0·00	53·06

values given by Briscoe and Chittka (2001). For each plant, we calculated the position (values of the colour hexagon *x*- and *y*-axis) of the labellum colour, the so-called colour locus, in the colour hexagon (Chittka and Kevan, 2005). For the calculation, we used standard daylight illumination (D65; CIE standard http://www.cis.rit.edu/mcsl/online/cie.php, accessed on 31 October 2012) converted to quantal units (Kelber *et al*., 2003) and a standard background estimated as the mean reflectance spectrum of leaves of 38 plant species growing in Europe (Arnold *et al*., 2010). To quantify colour contrast between diploid and tetraploid plants, we calculated distances between each pair of colour loci (Chittka, 1992).

To quantify floral scent bouquets, we collected floral scent of intact inflorescences of 189 plants (Döttingen: $n_{2x} = 73$, $n_{4x} = 21$; Remigen: $n_{2x} = 65$, $n_{4x} = 30$) in 2011, using headspace sorption. We performed scent collections between 0900 and 1800 h (GMT + 1) on days without rain. Because of the relatively long time needed for scent collection, it was not possible to collect scent at the same time for all plants. We enclosed each inflorescence in an oven bag (Nalophan®) tied up with short pieces of florist wire. We placed a small absorbent glass tube (hereafter called the filter), which was filled with approx. 20 mg of 80/100 mesh Tenax® powder (Supelco, Bellefonte, PA, USA), inside the bag. For 30 min at a rate of 150 mL min^{-1}, a battery-operated vacuum pump (PAS-500 Micro Air Sampler; Spectrex, Redwood City, CA, USA) pulled air out of the bag through the filter to trap the floral volatiles on the Tenax® adsorbent. To control for background contaminants, we collected ambient air from 2–3 empty bags per population. After scent collection, we wrapped PTFE (Teflon®) thread sealing tape around the ends of the filters and enclosed the whole filters in aluminium foil or small glass vials. We stored the filters in a −30 °C freezer until analysis. For sample analysis, we used an Agilent GC 6890N gas chromatograph [(GC) Agilent Technologies, Palo Alto, CA, USA] connected to an Agilent MSD 5975 mass selective detector (Agilent Technologies). The GC was equipped with an HP-5 column (0·25 mm diameter, 0·32 µm film thickness, 30 m length) and helium was used as carrier gas at a flow rate of 1·9 mL min^{-1}. Sample injection was carried out by a thermal desorption system [(TDS) TDS3; Gerstel, Mühlheim an der Ruhr, Germany] with a cold injection system [(CIS) CIS4; Gerstel]. We programmed the TDS temperature to rise from 30 °C (0·5 min hold) to 240 °C (1 min hold) at 60 °C min^{-1} for thermal desorption; the CIS temperature was −150 °C during thermodesorption and rose from −150 °C (0·5 min hold) to 150 °C at 16 °C s^{-1} and from 150 °C to 250 °C (0·5 min hold) at 12 °C s^{-1} for injection. The GC oven temperature was programmed to rise from 50 °C to 230 °C at 8 °C min^{-1}. To identify compounds, the mass spectra obtained were compared with those from the NIST spectral reference database (NIST 05) implemented in the ChemStation Enhanced Data Analysis program (G1701EA E.02.02 MSD Productivity ChemStation Software; Agilent Technologies, Germany). To verify compound identification and to quantify absolute amounts of compounds, we analysed synthetic standards in one to two concentrations to obtain calibration curves using the peak area of a compound-specific qualifier ion. Based on these calibration curves, the peak areas of the compound-specific qualifier ions in the *G. conopsea* samples were converted into nanograms. We manually double-checked all samples and compounds and, if necessary, manually integrated them. For each compound, we calculated the absolute amount in nanograms per litre of air sampled per inflorescence. We included a compound as floral scent compound when their median concentration in the air controls was <80 % of their mean concentration in the plant samples of the corresponding population. Furthermore, we considered only compounds with a mean of 0·5 ng L^{-1} air sampled per inflorescence, to exclude trace compounds. These criteria revealed 25 floral scent compounds. For each of these compounds, we calculated the amount in nanograms per litre of air sampled per flower by dividing the amount per inflorescence by the number of open flowers for further analysis.

To estimate flowering phenology, we recorded the total number of flowers, the number of withered flowers, the number of open flowers and the number of buds for each plant for which we quantified display size (see above). We then calculated the proportion of withered flowers, the proportion of open flowers and the proportion of buds by dividing the number of withered flowers, the number of open flowers and the number of buds, respectively, by the total number of flowers.

Herbivory

The only flower-related herbivory in our *G. conopsea* populations was aphid infestation. Aphid load on inflorescences on a scale from 0 (no aphids) to 5 (many aphids) was used as a measure of aphid infestation. We recorded the aphid load along with measuring display size (see above).

Reproductive success

Between mid-July and the end of August, when plants had fully developed fruits, we counted the number of fruits on all marked plants. In orchids, ovaries only swell when seeds develop, making fruits easily recognizable and countable. Several plants or labels were missing because of browsing mammalian herbivores. We calculated relative fruiting success as the number of fruits of an individual divided by the population mean, and the percentage fruit set as the number of fruits divided by the total number of flowers of an individual. We cut the inflorescence with developed fruits of ten diploids, six triploids and eight tetraploids in Döttingen in 2012. From each of these inflorescences, we randomly selected 1–3 fruits and counted viable (well-developed embryo) and non-viable (no or shrunken embryo) seeds of a random sub-set of the thousands of seeds encapsulated in a fruit (mean ± s.d. = 423·37 ± 28·53 seeds per fruit). We counted seeds at a ×64 magnification under a SZH stereo microscope (Olympus Optical Co., Ltd) equipped with a SZH-ILLD Brightfield/Darkfield Transmitted Light Illumination Base (Olympus Optical Co., Ltd). For each individual, we calculated the mean proportion of viable seeds.

Floral isolation experiment

To measure floral isolation between diploids and tetraploids, we set up three experimental arrays (two in Döttingen, one in Remigen). Each array consisted of ten diploid and ten tetraploid

cut plants set up in two rows with an interplant distance of 20 cm within and between rows. To track pollen flow, we stained pollinia in a sub-set of 6–13 flowers per inflorescence with histological dye (Peakall, 1989) using one colour per inflorescence, alternating between orange (Peakall, 1989) and pink (Johnson *et al*., 2004) in diploids and between violet (van der Niet *et al*., 2011) and green (Peakall, 1989) in tetraploids. All dyes were dissolved in H_2O and mixed with 1·33 μL mL^{-1} Tween-20 (van der Niet *et al*., 2011). The staining of pollinia does not affect pollinaria removal, bending time, and deposition by pollinators (Peakall, 1989). The proportion of open flowers of the experimental plants did not differ between diploids (mean ± s.e. = 39·52 ± 2·40 %) and tetraploids (44·50 ± 2·52 %; two-sample *t*-test: $t_{58} = -1{\cdot}432$, $P = 0{\cdot}158$). Cut plants were put in 15 mL BD Falcon™ conical tubes filled with water, which were placed in the ground at least 20 m apart from naturally growing *G. conopsea* plants; the distance between the two arrays in Döttingen was approx. 100 m. After four sunny days, plants were removed and the number of stigmas pollinated by stained massulae (pollen clumps) was counted separately for each colour using a hand lens and a binocular microscope. To estimate the proportion of pollinator-mediated autogamy/geitonogamy, we divided the number of flowers with self-massulae on the stigmas by the total number of flowers with stained massulae on the stigmas. As pollinators usually move from one plant to a directly neighbouring plant (K. Gross, pers. obs.), pollinations by massulae of the same colour as the focal plant were most likely of autogamous/geitonogamous origin and not cross-pollinations from the four other plants with the same colour within the array. Such unexpected cross-pollinations would have led to a slight overestimation of autogamy/geitonogamy, which, however, would have been similar for both cytotypes. The floral isolation index was calculated for each experimental array as 1 − (observed/expected intercytotype pollen flow)/(observed/expected intracytotype pollen flow) (Martin and Willis, 2007; Lowry *et al*., 2008) with the expected intercytotype and intracytotype pollen flow being (intercytotype + intracytotype pollen flow)/2.

Statistical analysis

All statistical analyses were conducted in IBM SPSS Statistics 20.0.0 (IBM Corp. 2011, Armonk, NY, USA). To achieve normal distribution and improve homogeneity of variances, all data apart from floral morphology, floral colour, number of fruits in 2012 and floral isolation index were log, square root or arcsine or arcsine square root (for proportion data) transformed. We analysed differences between cytotypes separately for the trait groups display size, floral morphology, pollinaria properties, floral colour and floral scent because we did not measure all trait groups in all marked plants and in both years. To reduce the number of variables and to account for correlations among traits, we conducted principal component analyses (PCAs) on traits standardized to a mean of 0 and an s.d. of 1, separately for floral morphology, pollinaria properties and floral scent. We extracted principal components (PCs) with an eigenvalue >1, after varimax rotation (Supplementary Data Table S2). Separately for the three trait groups, we conducted a stepwise discriminant function analysis (DFA) with the extracted PCs as independent variables and the cytotype/population identity as grouping variable (Table S3). We used the stepwise procedure to assess variables most relevant for differences among groups. For entering and removing new variables, the Wilks' lambda method was used, which, at each step, enters the variable that minimizes the overall Wilks' lambda. The probability of *F* was used as the criterion for entering and removing variables; a variable was entered into the model if the significance level of its *F*-value was < 0·05 and was removed if the significance level was >0·10. To quantify which differences were explained by individual discriminant functions (DFs), we conducted general linear models (GLMs) with the DF as dependent variable, cytotype as fixed factor and population as random factor. Similar GLMs were used to analyse differences in individual traits, aphid load and fruiting success between cytotypes and populations. To assess differences in flowering phenology, we compared the proportion of open flowers, the proportion of withered flowers and the proportion of buds between diploids and tetraploids with two-sample *t*-tests separately for each date of collection and population. Correlations between floral scent compounds and inflorescence length or the number of flowers were analysed using Pearson correlations. To analyse differences in the proportions of viable seeds, we conducted a one-way analysis of variance (ANOVA). We analysed pairwise differences between diploids, tetraploids and triploids with Tukey's post-hoc tests. The relationship between density and reproductive success was analysed with linear regression analyses. To compare the proportion of autogamy/geitonogamy between diploid ant tetraploid plants, we used a two-sample *t*-test. Similarly, we analysed differences between intracytotype and intercytotype pollen flow with two-sample *t*-tests. To assess differences between observed and expected values of floral isolation and colour distances, we used one-sample *t*-tests.

RESULTS

Cytotype differences in floral traits and phenology

Most floral traits differed significantly between cytotypes, but to a lesser extent between populations (Fig. 1; Tables 2 and 3; Supplementary Data Table S4). Tetraploid plants had a significantly larger display size (Fig. 1A). Floral morphology also differed between cytotypes but not between populations (Fig. 1B; Tables 2 and 3). Most 'flower size traits' were significantly larger in tetraploids, but 'floral shape traits' did not differ between cytotypes (Table S4). Most floral morphology traits did not differ significantly between populations (Table S4). Pollinaria properties also differed between cytotypes, but to a lesser extent between populations (Fig. 1C; Tables 2 and 3). In univariate comparisons, some properties of pollinaria were significantly enhanced in tetraploids, but rarely differed between populations (Table S4). Among floral signals, floral colour differed only slightly (Fig. 1D), but floral scent differed more strongly (Fig. 1E) between cytotypes. The *y*:*x* ratio of the floral colour loci (a measure of the position of the colour point in the colour hexagon) differed significantly between cytotypes, but not between populations; however, distances between floral colour loci of diploids and tetraploids were significantly shorter than 0·1 colour hexagon units in both populations (Fig. 1D). Such distances are most probably too short for pollinators to be

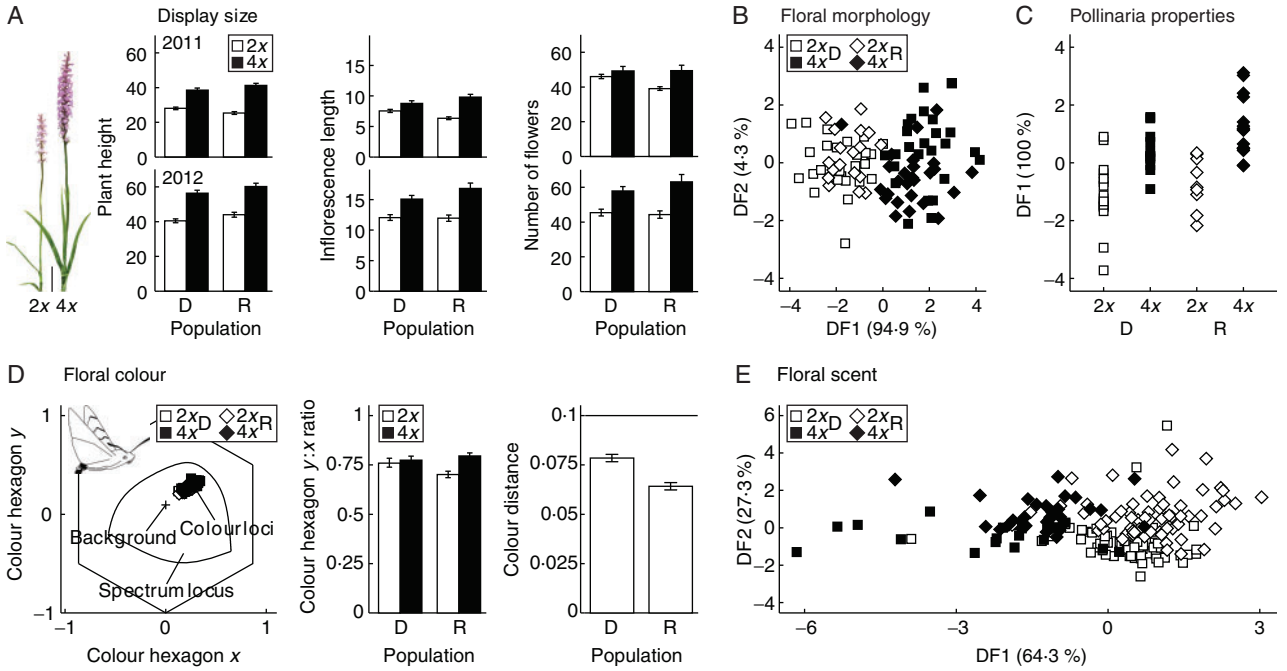

FIG. 1. Differences in floral traits between diploid (2*x*) and tetraploid (4*x*) *Gymnadenia conopsea* plants in the populations Döttingen (D) and Remigen (R). (A) Picture of a diploid and tetraploid *G. conopsea* plant scaled to their mean height (scale bar = 5 cm) and graphs (mean ± s.e.) of display size traits (cytotype differences for all traits, $P \leq 0{\cdot}001$; population differences, $P < 0{\cdot}050$ only for plant height in 2012 and inflorescence length and number of flowers in 2011). Multivariate comparisons of (B) floral morphology and (C) pollinaria properties by stepwise discriminant function analyses (DFAs; for statistics, see Tables 2 and 3). (D) Colour differences among cytotypes displayed through loci in the colour hexagon (left) adapted to the colour vision of the pollinator *Macroglossum stellatarum* (insert), mean (± s.e.) colour hexagon *y*:*x* ratio (middle; cytotype difference, $P = 0{\cdot}019$; population difference, $P = 0{\cdot}474$), and colour distance between diploid and tetraploid colour loci in relation to the presumed detection threshold (horizontal line) (right; always $P < 0{\cdot}001$). (E) Multivariate comparison of floral scent bouquets through stepwise DFA (for statistics, see Tables 2 and 3). For all DFA plots, percentages of variance explained by specific DFs are indicated in parentheses.

TABLE 2. *Statistics of stepwise discriminant function analyses (DFAs) with cytotype population identity as grouping variable for the three trait groups floral morphology, pollinaria properties and floral scent*

Trait group	Test of functions	Wilks' λ	χ^2	d.f.	*P*
Floral morphology	DF1–DF3	0·231	134·980	12	**<0·001**
	DF2–DF3	0·869	12·868	6	**0·045**
	DF3	0·979	1·971	2	0·373
Pollinaria properties	DF1	0·495	34·070	3	**<0·001**
Floral scent	DF1–DF3	0·272	238·845	15	**<0·001**
	DF2–DF3	0·584	98·544	8	**<0·001**
	DF3	0·869	25·702	3	**<0·001**

Significant separations by discriminant functions (DFs; for details, see Supplementary Data Table S3) are highlighted in bold.

TABLE 3. *General linear model (GLM) statistics of cytotype and population differences in the discriminant functions (DFs) resulting from stepwise discriminant function analyses (DFAs; for details, see Supplementary Data Table S3)*

Trait	Cytotype		Population	
	*F**	*P*	*F**	*P*
Floral morphology				
DF1	237·00	**<0·001**	0·48	0·490
DF2	0·10	0·753	2·70	0·104
DF3	0·04	0·840	1·52	0·221
Pollinaria properties				
DF1	35·73	**<0·001**	4·51	**0·039**
Floral scent				
DF1	202·10	**<0·001**	18·07	**<0·001**
DF2	3·04	0·082	82·32	**<0·001**
DF3	0·36	0·549	0·04	0·838

Significant differences are highlighted in bold.
*Degrees of freedom: floral morphology, 1,94; pollinaria properties, 1,49; floral scent, 1,186.

able to discriminate them (Chittka *et al*., 2001). Multivariate comparisons of floral scent bouquets showed that cytotypes differed in their scent bouquet (Fig. 1E; Tables 2 and 3). Populations showed significant but weaker differences in scent bouquet (Fig. 1E; Tables 2 and 3). While total scent amount and total amount of aromatics were significantly lower in tetraploids, other scent compounds were significantly more abundant in tetraploids (Table S4). The emission of several floral scent compounds correlated negatively with inflorescence length and, to a lesser extent, with the number of flowers (Table S5). The amounts of some scent compounds also differed significantly between populations (Table S4).

Differences in the proportion of open flowers, the proportion of withered flowers and the proportion of buds between diploids and tetraploids were not always significant, and these differences tended to be less pronounced at later flowering dates,

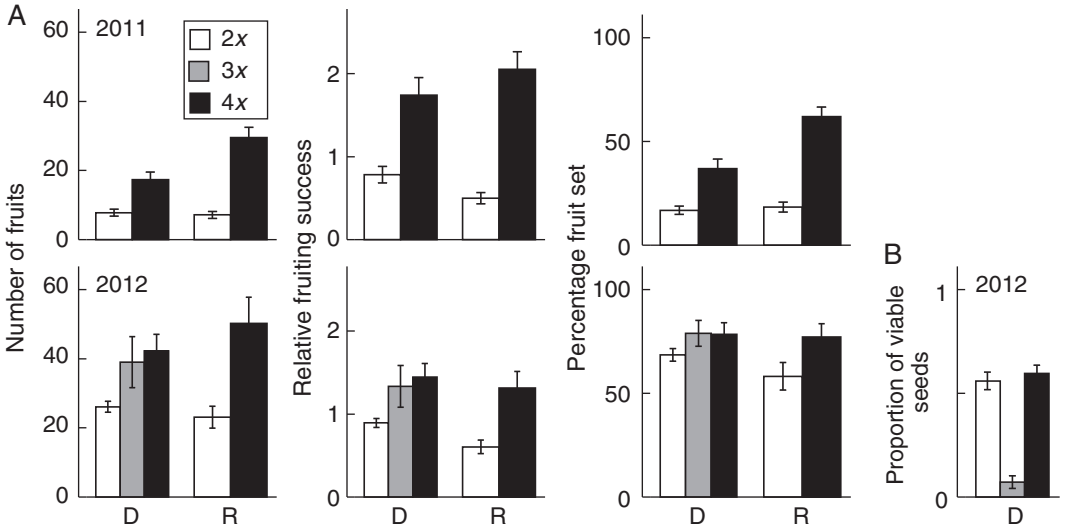

FIG. 2. Differences (mean ± s.e.) in reproductive success between diploid (2*x*), triploid (3*x*) and tetraploid (4*x*) *Gymnadenia conopsea* plants in the populations Döttingen (D) and Remigen (R) in 2011 (upper) and 2012 (lower). (A) Three measures of fruiting success (differences between 2*x* and 4*x*, always $P < 0{\cdot}05$; differences between 2*x* and 3*x* as well as between 3*x* and 4*x*, always $P > 0{\cdot}05$; population differences, $P < 0{\cdot}05$ only for relative fruiting success in 2012). (B) Proportion of viable seeds (difference between 2*x* and 4*x*, $P = 0{\cdot}785$; difference between 2*x* and 3*x* as well as between 3*x* and 4*x*, $P < 0{\cdot}001$).

especially for the proportion of withered flowers and the proportion of buds (Supplementary Data Fig. S2). This indicates that the flowering phenology of diploids and tetraploids greatly overlapped.

Cytotype differences in herbivory

The extent of aphid infestation was significantly higher in diploids than in tetraploids in 2011, but not in 2012 (Supplementary Data Fig. S3). Similarly, there was a difference in the aphid load between populations in 2011, but not in 2012 (Fig. S3).

Cytotype differences in reproductive success

Reproductive success was always significantly higher in tetraploids, but in most cases did not differ between populations (Fig. 2). In both years, tetraploids produced more fruits and had a higher relative fruiting success and percentage fruit set than diploids (Fig. 2A). Fruiting success did not differ between triploids and either diploids or tetraploids (Fig. 2A). However, triploids had significantly lower proportions of viable seeds than diploids and tetraploids, but there was no difference in viable seeds between diploids and tetraploids (Fig. 2B). Relative fruiting success and percentage fruit set increased significantly with local density only in diploids in Remigen. In tetraploids and in Döttingen, there was no significant association between density and reproductive success (Supplementary Data Table S6).

Floral isolation

Significantly more stigmas were pollinated by colour-stained pollinia in tetraploids than in diploids, indicating that tetraploids were visited more or had a higher pollination efficiency (mean ± s.e.; tetraploids, 2·50 ± 0·39; diploids, 0·73 ± 0·29; $t_{58} = -4{\cdot}656$, $P < 0{\cdot}001$). The proportion of autogamy/geitonogamy was similar between the cytotypes (mean ± s.e. diploids, 0·48 ± 0·15; tetraploids, 0·51 ± 0·08; $t_{32} = 0{\cdot}202$, $P = 0{\cdot}841$). There was more pollen flow within than among cytotypes, but this difference was only significant for tetraploids (Table 4). The mean ± s.e. floral isolation index was 0·76 ± 0·08, which was significantly higher than 0, i.e. random visitation ($t_2 = 9{\cdot}268$, $P = 0{\cdot}011$).

TABLE 4. *Mean ± s.e. and statistics for differences between intracytotype and intercytotype pollen flow for diploid (2x) and tetraploid (4x)* Gymnadenia conopsea *plants and for both cytotypes combined (overall)*

Ploidy level	Mean ± s.e.		Statistics		
	Intracytotype pollen flow	Intercytotype pollen flow	d.f.	*t*	*P*
2*x*	0·63 ± 0·49	0·10 ± 0·00	2	1·086	0·391
4*x*	1·97 ± 0·23	0·53 ± 0·30	2	4·300	**0·050**
Overall	1·30 ± 0·33	0·32 ± 0·15	2	4·005	0·057

Significant differences determined using two-sample *t*-tests are highlighted in bold.

DISCUSSION

Polyploidy is a common phenomenon in plants, and autopolyploids often co-occur with their diploid progenitors (Thompson *et al.*, 1997; Husband and Schemske, 1998; Burton and Husband, 1999; Halverson *et al.*, 2008; Trávníček *et al.*, 2011*b*). However, factors influencing the evolutionary fate of polyploids in such natural mixed-ploidy populations have remained largely unknown. In our study, we confirmed consistent

phenotypic differences between diploid and tetraploid orchid individuals, not only in plant and flower morphology, but also in floral scent. Most probably as a consequence of these differences, tetraploids achieve mostly within-cytotype pollen flow and have higher pollination success. Assuming similar figures of survival for both cytotypes, higher reproductive success and assortative mating will probably allow polyploids to persist in mixed-ploidy populations or even displace their diploid progenitors.

It is well known that (auto)tetraploid plants are larger and bear larger flowers than their diploid progenitors (Levin, 1983; Segraves and Thompson, 1999; Husband and Schemske, 2000; Hodálová *et al*., 2010; Münzbergová *et al*., 2013), with rare exceptions (Ståhlberg, 2009), but few studies have investigated how floral signals differ between cytotypes. Floral colour, when measured as binary character or as percentage reflectance, differs between cytotypes in *Centaurea stoebe* (Mráz *et al*., 2011) and *Heuchera grossulariifolia* (Segraves and Thompson, 1999), but has been reported to be very similar in different cytotypes in a previous study in *G. conopsea* (Jersáková *et al*., 2010). In our study, we showed that although colour differed statistically among cytotypes, this difference is unlikely to be detectable for pollinators. Floral colour distances between cytotypes were <0·1 colour hexagon unit, suggesting a lack of colour discrimination (Chittka *et al*., 2001). Floral scent has, until now, only been studied once in diploids and polyploids of the same plant species (Jersáková *et al*., 2010), despite its importance for pollinator attraction and floral isolation (Raguso, 2008; Schiestl and Schlüter, 2009). In our study, we analysed a large number of plants and found that floral scent bouquets differed between cytotypes, which is consistent with an earlier study in *G. conopsea* (Jersáková *et al*., 2010). In addition to analysing the floral bouquet, we also assessed differences in individual compounds. Unexpectedly, individual scent compounds were not always emitted in higher amounts in tetraploids, but the amount of several compounds as well as the total amount of scent were lower in tetraploids. This lower scent emission is likely to be caused by a trade-off between inflorescence size and floral scent production. In tetraploids, but not in diploids, a negative correlation between size and floral scent was evident for the sum of aromatic compounds and the individual compound phenylacetaldehyde in particular. Interestingly, an earlier study showed that the amount of phenylacetaldehyde is important in mediating floral isolation between two *Silene* species (Waelti *et al*., 2008). Thus, a trade-off between display size and key floral volatiles may indirectly impact pollinator attraction and floral constancy of pollinators by altering the combination of visual and key olfactory signals. In our study, the observed phenotypic differences could be the direct consequence of the genome duplication itself (Griesbach and Kamo, 1996; Ramsey and Schemske, 2002; Oswald and Nuismer, 2011*a*; Ramsey, 2011) or secondarily caused by divergent natural selection (Nuismer and Cunningham, 2005).

Modelling studies suggest that higher relative reproductive success and assortative mating are key factors for tetraploids to become successfully established and persist in mixed-ploidy populations (Rodríguez, 1996; Oswald and Nuismer, 2011*b*; Suda and Herben, 2013). Higher reproductive fitness of tetraploids compared with diploids enables tetraploids to become more common (Felber, 1991; Rodríguez, 1996; Rausch and Morgan, 2005; Suda and Herben, 2013). In our study, fruiting success was considerably higher in tetraploid than in diploid plants, which is consistent with earlier studies (Petit *et al*., 1997; Nuismer and Cunningham, 2005; Castro *et al*., 2012), but see Mráz *et al*. (2011). Assortative mating (i.e. intracytotype pollen flow) reduces the loss of male gametes to non-compatible cytotypes and ensures the inflow of compatible pollen (Levin, 1975), as matings between diploids and tetraploids usually lead to sterile offspring. In line with previous findings (Burton and Husband, 2000; Baack, 2005; Borges *et al*., 2012), post-zygotic isolation was strong between *G. conopsea* cytotypes, with the few occurring triploid hybrids being largely sterile. We observed assortative mating both in terms of pollinator-mediated autogamy/geitonogamy and through assortative pollinator visitation, collectively leading to considerable floral isolation between the two cytotypes. The strength of the here documented floral isolation is comparable with that between *C. angustifolium* cytotypes (Husband and Schemske, 2000; Husband and Sabara, 2003; Kennedy *et al*., 2006), but much stronger than that between *Aster amellus* cytotypes (Castro *et al*., 2011). Floral isolation might be caused by a switch to new pollinators after polyploidization as in *H. grossulariifolia* (Segraves and Thompson, 1999; Thompson and Merg, 2008). More probably, however, floral isolation in *G. conopsea* was caused by the pollinators' preference for floral signals of tetraploid plants, as shown in *C. angustifolium* (Husband and Schemske, 2000; Kennedy *et al*., 2006). In our plot arrays, tetraploids had higher pollination success than diploids, despite both cytotypes being in full flower and surviving during the plot experiment equally well. Morphological isolation and differences in pollination efficiency between the two cytotypes seem to be weak or absent, because differences in traits mediating morphological isolation (pollinaria and stigma properties) were generally less pronounced than differences in other floral traits. Moreover, spur length differences were <2 mm and thus also unlikely to cause mechanical isolation or differences in pollination efficiency (Jersáková *et al*., 2010). Tetraploids also did not show more pollinator-mediated autogamy/geitonogamy than diploids. Thus, even though detailed pollinator observations and behavioural experiments would be necessary to assess pollinator preferences, our study implies that the increased display size and/or changes in the floral scent bouquet in tetraploid *G. conopsea* plants most probably lead to higher reproductive success, as well as assortative pollen flow mostly within cytotypes.

Floral isolation and higher reproductive success in tetraploids suggests that tetraploids can displace diploids, leading to transformation of *G. conopsea* populations from pure diploid to pure tetraploid. Indeed, we found one population with only tetraploid and no diploid plants in Switzerland (K. Gross, unpubl. data), where such a transformation may have taken place. Several factors, however, can slow down or even prohibit such a transformation. First, high inbreeding depression may restrict tetraploid persistence and diploid displacement (Rausch and Morgan, 2005). In *G. conopsea*, inbreeding depression can be strong (Sletvold *et al*., 2012), but inbreeding depression has been shown to be generally lower in polyploids than in diploids (Barringer and Geber, 2008). Secondly, as an alternative to transformation, cytotypes may diverge into two species, mediated by an adaptive shift in flowering phenology (Husband and

Schemske, 2000; Thompson and Merg, 2008; Castro *et al*., 2011) or (micro-)habitat (Felber-Girard *et al*., 1996; Schönswetter *et al*., 2007; Raabová *et al*., 2008; Sonnleitner *et al*., 2010). Indeed, spatial clustering of cytotypes and flowering time shifts were found in populations with different cytotypes of *G. conopsea* in the Czech Republic (Jersáková *et al*., 2010; Trávníček *et al*., 2011*b*), suggesting adaptation to different flowering time niches and/or microhabitat. In our study, even though a slight spatial clustering of cytotypes was recognizable, different cytotypes were clearly still within flying distances of foraging pollinators (K. Gross, pers. obs.), and flowering phenology was only slightly advanced in diploids compared with tetraploids. Thus, whether polyploidization leads to transformation or divergence seems to be population or region specific. Finally, the success of tetraploids may be compromised by lower survival, for example mediated by lower competitive abilities or seedling establishment, selective herbivore grazing or mowing, or lower resistance against pathogens. However, competitive abilities (Levin, 1983), germination rate and seedling survival (Burton and Husband, 2000; Mráz *et al*., 2012) do not differ between cytotypes in other plant species. Several studies investigated the effect of ploidy on herbivore attack (Thompson *et al*., 1997; Nuismer and Thompson, 2001; Thompson *et al*., 2004; Halverson *et al*., 2008; Arvanitis *et al*., 2010). The only observed herbivores in our *G. conopsea* populations were aphids, but aphid load did not differ between cytotypes or was even lower in tetraploids. Collectively, factors acting on survival may have little effect on the process of cytotype displacement in *G. conopsea*, but more studies on differential survival of cytotypes are needed to confirm this.

Our data suggest that tetraploids are very successful in contemporary populations; though this may not necessarily be the case during establishment, when teraploids occur in very low frequencies. In theory, low frequency may decrease floral isolation and reproductive success, but a study in *C. angustifolium* showed that the proportion of intracytotype pollinator visits did not depend on cytotype frequency in diploids and was highest at low tetraploid frequencies in tetraploids (Husband, 2000). In support of this, our study shows that reproductive success of tetraploids was not generally density dependent, and, thus, even rare and/or isolated tetraploids had high reproductive success. Thus, floral isolation and reproductive success might also be high for tetraploids during the establishment phase, but only if the trait differences causing high reproductive success are directly caused by polyploidization. Thus, future experimental studies should investigate which of the here documented differences in floral traits are directly caused by polyploidization, and quantify reproductive success as well as floral isolation at low tetraploid frequencies, to learn more about these key factors during polyploidy establishment.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: cytotype distribution in the two study populations. Figure S2: cytotype differences in flowering phenology. Figure S3: cytotype differences in aphid load. Table S1: description of floral morphology traits and pollinaria properties. Table S2: factor loadings on principal components. Table S3: factor loadings on discriminant functions. Table S4: cytotype and population differences in floral morphology, pollinaria properties and floral scent compounds. Table S5: Pearson correlation coefficients of floral scent compounds and inflorescence length and number of flowers. Table S6: regression coefficients for local density on fruiting success.

ACKNOWLEDGEMENTS

We thank M. Sun for help in the field, E. C. Connor for help in the field and with scent analysis, C. Sailer for introduction to and kind support with flow cytometry, K. E. M. Sedeek for the preparation of the histological dyes, and M. Streinzer for helpful comments on floral colour analysis. Two anonymous reviewers provided helpful comments on the manuscript. The 'Departement Bau, Verkehr und Umwelt; Abteilung Landschaft und Gewässer; Sektion Natur und Landschaft' from Kanton Aargau, Switzerland provided collection permissions. We are also grateful to U. Somalvico from creaNatira for organizing the fencing of our plants in Remigen to protect them from cows. Financial support for this study was provided by the Swiss National Science Foundation grant 31003A-125340 to F.P.S.

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