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ORIGINAL PAPER

Breeding barriers at a diploid–hexaploid contact zone in *Aster amellus*

Sílvia Castro · Zuzana Münzbergová · Jana Raabová · João Loureiro

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Abstract Polyploidization is an important mechanism of sympatric speciation, but few studies have addressed breeding barriers between polyploids and their diploid progenitors in the field, and the available data have been mainly obtained from diploid-tetraploid contact zones. In contrast to diploid-tetraploid complexes, hybridization between diploid and hexaploid individuals may lead to viable fertile tetraploid offspring, and thus the interactions between these ploidy levels can be more complex. We investigated the breeding barriers operating between diploid and hexaploid individuals of *Aster amellus* at a contact zone in Central Europe to understand the absence of hybrids (i.e., tetraploids) and mixed populations. Phenological segregation, assortative mating mediated by pollinators and crossing ability were assessed under natural and controlled conditions in diploid and hexaploid populations growing in close proximity. The results revealed low levels of reproductive isolation (RI) due to flowering phenology (RI = 11–45%) and pollinator behavior (RI = 17%), so that pollen transfer between diploids and hexaploids is possible. In contrast, almost complete reproductive isolation was observed due to a series of post-pollination barriers that significantly reduced the production of offspring from inter-cytotype crosses (RI = 99.9%), even though some tetraploids were detected in seeds and seedlings. We conclude that the absence of tetraploids at the contact zone is probably due

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S. Castro · Z. Münzbergová
Institute of Botany, Academy of Sciences, Zámek 1, 252 43 Průhonice, Czech Republic

S. Castro (✉) · J. Loureiro
Center for Functional Ecology and Department of Life Sciences, University of Coimbra, PO Box 3046, 3001-401 Coimbra, Portugal
e-mail: scastro@bot.uc.pt

J. Raabová
Department of Botany, National Museum, Cirkusová 1740, 193 00 Praha 9, Czech Republic

S. Castro · Z. Münzbergová
Department of Botany, Faculty of Science, Charles University in Prague, Benátská 2, 128 01 Prague, Czech Republic

to a combination of several factors, including spatial segregation, strong post-pollination barriers (such as gametic isolation, low viability of tetraploid seeds and/or inability of tetraploid plants to reach the flowering stage), and to a lesser extent, temporal and behavioral segregation. Future studies should explore the fitness of tetraploids and the effect of different traits on the reproductive success and fitness of each cytotype. This will enable a fuller understanding of the dynamics and mechanisms acting in contact zones.

Keywords Flow cytometry · Phenology · Pollination experiment · Pollinator behavior · Polyploid aggregate · Tetraploids

Introduction

Polyploidy has long been recognized as a major mechanism of plant speciation and evolution (Stebbins 1950; Grant 1981; Masterson 1994; Soltis et al. 2009). This widespread phenomenon is observed in 47–100% of the flowering plants, appearing in many lineages (Soltis 2005; Wood et al. 2009) and being an ecologically and evolutionarily dynamic process (Soltis and Soltis 1993; Ramsey and Schemske 1998). As new evolutionary entities can arise by a single genomic event, polyploidy has been proposed as an important mechanism of sympatric speciation. It has been found to have potentially wide-ranging effects on gene regulation and developmental processes, leading to immediate shifts in morphology, breeding system and ecological tolerances (Ramsey and Schemske 1998; Otto and Whitton 2000; Adams and Wendel 2005). To understand the importance of polyploidization for sympatric speciation, the ecological and genetic conditions allowing the establishment and co-occurrence of new cytotypes in close proximity to its progenitors need to be addressed (Levin 1975; Fowler and Levin 1984; Felber 1991; Rodriguez 1996). Contact zones between different cytotypes are ideal natural laboratories for studying these processes (Thompson and Lumaret 1992; Lexer and van Loo 2006).

Under random mating, the establishment of a new cytotype will be subjected to strong frequency-dependent selection as most of the crosses will occur with the progenitor's cytotype, decreasing the chance of successful reproduction (minority cytotype exclusion; Levin 1975; Rodriguez 1996). Thus, the establishment of a new cytotype will only be possible when a set of breeding barriers and/or ecological features increase the probability of successful intra-cytotype mating (Rieseberg and Willis 2007; Paun et al. 2009). Ultimately, assortative mating will be involved in long-term maintenance of mixed-ploidy populations (Levin 1975; Husband and Schemske 2000).

A diverse array of breeding barriers can mediate assortative mating in natural populations, including heterogeneity in the spatial distribution of cytotypes (van Dijk et al. 1992; Husband and Schemske 1998; Kolař et al. 2009) due to different microhabitat preferences (Felber-Girard et al. 1996); temporal isolation due to shifts in flowering phenologies (Petit et al. 1997; Marques et al. 2007; Jersáková et al. 2010); mechanical isolation due to differences in floral morphology (reviewed in Grant 1994); segregation resulting from divergent behaviors and preferences of pollinators (Grant 1994; Thompson et al. 2004; Marques et al. 2007; Thompson and Merg 2008); gametic isolation (Husband et al. 2002; Mráz 2003; Brock 2004); and/or reduced fitness or sterility of the hybrids (Ramsey and Schemske 1998; Buggs and Pannell 2006; Prentis et al. 2007). All together, such breeding barriers will act in concert and govern the levels of pollen flow between cytotypes, thereby determining the cytotype composition of the offspring and population.

Although contact zones have received particular attention in recent years (e.g., Hardy et al. 2000; Husband et al. 2002; Husband and Sabara 2004; Lexer and van Loo 2006; Buggs 2006), so far, most of the studies have focused on diploid-tetraploid aggregates (Petit et al. 1999; Soltis and Soltis 1999, but see Buggs and Pannell 2006, 2007). Relative to such study systems, contact zones encompassing diploids and higher ploidies (e.g., hexaploids) are theoretically capable of presenting a wider combination of new cytotypes (e.g., from $3x$ up to $12x$ in diploid-hexaploid aggregates). In the case of inter-cytotype crossing, single hybridization events may lead to the occurrence of a new cytotype that is potentially fertile so long as the chromosomes of the parents are compatible (e.g., tetraploids in diploid-hexaploid aggregates). In this sense, species pairs with higher ploidies constitute novel and interesting systems for studying the mechanisms involved in the coexistence of different cytotypes, as well as in the appearance of new cytotypes.

In the present work, we used the *Aster amellus* aggregate as a case study to investigate breeding barriers involved in the maintenance of diploid-polyploid contact zones. This aggregate is an interesting model system because it comprises a contact zone between diploids and hexaploids, with several populations growing in close proximity, but no hybrids (i.e., tetraploids) have been detected so far (Mandáková and Münzbergová 2006). Furthermore, according to previous studies, the habitat requirements appear to be insufficient to lead to habitat segregation (Mandáková and Münzbergová 2008; Raabová et al. 2008) and no morphological differentiation has been detected between diploid and hexaploid plants (Mandáková and Münzbergová 2008). To understand the dynamics of this contact zone, we investigated the breeding barriers between the cytotypes, namely, temporal segregation, assortative mating mediated by pollinators and crossing ability by asking the following questions: (1) Does any asynchrony in floral phenology exist that may lead to temporal segregation of the cytotypes? (2) Are there any differences in pollinator assemblage and/or behavior that could lead to cytotype isolation? (3) Are the cytotypes able to cross with each other? To address these questions, we evaluated the following: (1) phenological differences under both natural and controlled conditions; (2) pollinator assemblages and behavior in natural populations and (3) hybrid formation both under open pollination in a simulated mixed-ploidy population and after controlled hand-pollinations. The study was conducted in nine natural populations where diploids and hexaploids grow in close proximity and also included two distinct habitats where hexaploid individuals grow (following Mandáková and Münzbergová 2006).

Materials and methods

Plant species

Aster amellus L. (Asteraceae) is a polymorphic perennial herb that can grow up to 40 cm high in open xerothermic habitats. In Europe, its distribution area ranges from northern France to Lithuania, reaching Italy and Macedonia in the south, and the Black Sea, Caucasus and west Siberia outside of Europe (Meusel and Jäger 1992). Within the whole area of distribution, three ploidy levels have been described: diploid ($2n = 2x = 18$), tetraploid ($2n = 4x = 36$), and hexaploid ($2n = 6x = 54$) (Merxmüller et al. 1976; Májovský 1978). Although it has been proposed that hexaploids had an allopolyploid origin (Májovský 1978), recent molecular approaches suggest that hexaploids instead are of autopolyploid origin (Mandáková and Münzbergová 2008). The three cytotypes are geographically separated, with diploids occurring in western and southern Europe,

hexaploids occurring in the continental part of Eurasia and eastern Europe and tetraploids being proposed to occur in the Caucasus region (Májovský 1978, and reviewed in Münzbergová et al. accepted). Recent detailed studies on cytotype distribution revealed a complex and diffuse contact zone between diploid and hexaploid cytotypes in Central Europe (Mandáková and Münzbergová 2006; Castro et al. unpublished data). Across the Central European contact zone, diploids and hexaploids frequently occur in close proximity, but no mixed-ploidy populations or hybrids (i.e., tetraploids) have been found so far (Mandáková and Münzbergová 2006).

Aster amellus cytotypes have received several taxonomic treatments (reviewed in Münzbergová et al. accepted). However, morphological distinction of cytotypes using diagnostic characters is extremely difficult and recent experiments in a common garden revealed no morphological differentiation between diploid and hexaploid plants (Mandáková and Münzbergová 2008). Due to these data, *A. amellus*, including all its cytotypes, is treated as a polymorphic species (following also Merxmüller et al. 1976).

The plant is described as self-incompatible (Kovanda 2005), and it is visited by a large spectrum of generalist insects (Mayor 2008). The flowering heads are gynomonoecious, being composed by an external row of female pink-purple ligules and central yellow tubular and hermaphroditic flowers. The average number of flower heads is 4.8 (SD = 1.5) per flowering stem and does not differ significantly between diploid and hexaploid plants (mean ± SD: 4.6 ± 0.4 in diploids and 5.2 ± 0.5 in hexaploids). The species flowers from mid-July to September and fruits develop from September to October (Kovanda 2005).

Study area

The present study was performed in a diploid-hexaploid contact zone occurring in northern Bohemia, Czech Republic, in an area delimited by the towns of Úštěk, Roudnice nad Labem and Litoměřice. In this area, diploid and hexaploid populations of *A. amellus* occur in close proximity (the nearest distance between the two cytotypes is 500 m), but no mixed-ploidy populations or hybrids have been found (Mandáková and Münzbergová 2006). A previous large-scale study on the total aboveground biomass at 27 sites showed that hexaploid populations occur in both low- and high-productivity habitats, whereas diploid populations are confined to low-productivity habitats (Mandáková and Münzbergová 2006; Münzbergová 2007a). Following these data, three diploid populations in low-productivity habitats, three hexaploid populations in low-productivity habitats and three hexaploid populations in high-productivity habitats were selected (Table 1). Seeds from each population were collected and plants were grown in a greenhouse for: simulation of a mixed-ploidy population, assessment of phenological patterns under controlled conditions and crossing experiments (for details see Münzbergová 2007b).

Ploidy level estimation

The ploidy level of individual plants was assessed using flow cytometry in several stages of the study. Flow cytometric analysis followed the chopping procedure of Galbraith et al. (1983) using Otto's buffers (Otto 1990; Doležel and Göhde 1995). Briefly, nuclei were released after chopping one seed or 0.5 cm^2 of fresh leaf tissue of *A. amellus* and 0.5 cm^2 of fresh leaf tissue of *Bellis perennis* (internal reference standard with 2C = 3.38 pg; Schonswetter et al. 2007) with a razor blade in a Petri dish containing 0.5 ml of Otto I buffer (0.1 M citric acid, 0.5% Tween 20). Afterwards, the nuclear suspension was filtered using a 42 μm nylon mesh and stained with a solution containing 1 ml of Otto II buffer

Table 1 Details of the studied populations of *Aster amellus* at diploid-hexaploid contact zone in the Czech Republic

ID	Population	Ploidy level	Habitat productivity	GPS coordinates	Altitude [m a.s.l.]	Slope [°]	Population size
1	Malíč	2*x*	Low	50° 32′ 24.2″ N 14° 05′ 16.1″ E	310	25	10,000
2	Holý vrch	2*x*	Low	50° 31′ 40.9″ N 14° 13′ 48.8″ E	260	20	1,400
3	Encovany	2*x*	Low	50° 31′ 45.8″ N 14° 15′ 33.3″ E	250	15	1,100
4	Malešov	6*x*	Low	50° 30′ 02.9″ N 14° 18′ 57.9″ E	200	10	1,050
5	Na Baušce	6*x*	Low	50° 29′ 36.1″ N 14° 18′ 40.7″ E	200	20	1,200
6	Sovice	6*x*	Low	50° 27′ 55.8″ N 14° 18′ 22.8″ E	190	25	1,100
7	Svařenice	6*x*	High	50° 29′ 45.7″ N 14° 18′ 09.9″ E	190	5	500
8	Vrutice I	6*x*	High	50° 30′ 14.9″ N 14° 17′ 56.8″ E	190	20	1,500
9	Vrutice II	6*x*	High	50° 30′ 14.9″ N 14° 17′ 51.1″ E	190	15	1,400

Habitat productivity was characterized following Mandáková and Münzbergová (2006). Population sizes are estimates based on field observations

(0.4 M $Na_2HPO_4 \cdot 12H_2O$), 4 mg ml^{-1} of DAPI and 2 mg ml^{-1} of ß-mercaptoethanol. After 5 min of incubation, samples were analyzed in a Partec PA II flow cytometer (Partec GmbH, Münster, Germany). The fluorescence of at least 3,000 nuclei per sample was analyzed using FlowMax (v. 2.4, Partec GmbH). As a quality standard, for the fresh materials only histograms with a coefficient of variation (CV) below 4% were accepted, whereas for seeds CV values up to 6% were accepted, because this material typically produced histograms of lower quality. The DNA index was calculated for all the samples by dividing the relative fluorescence of the G_0/G_1 peak of *A. amellus* by the relative fluorescence of the G_0/G_1 peak of the standard species. Because in *A. amellus* there is a slight difference in DNA content between diploids and hexaploids (the mean ratio between the G_0/G_1 peak of hexaploids and diploids is 2.6; Mandáková and Münzbergová 2006), using the DNA index it is possible to distinguish tetraploids from different origins (i.e., tetraploids formed by the fusion of unreduced gametes from diploid parents, and tetraploids formed by the fusion of reduced gametes from diploid and hexaploid parents). Aneuploids were assigned to categories between the main ploidy levels (the categories were defined as mean DNA index of each ploidy level ± DNA index of one chromosome).

Temporal segregation of the cytotypes: flowering phenology

To assess differences in flowering phenology between cytotypes, the proportion of stems that were flowering was recorded at each of ten census dates in every population (natural conditions). Flowering phenology was recorded in ca. 100 individuals equally distributed across a linear transect spanning the whole population or on a spiral transect for small populations. The distances between individual plants depended on the population size and

ranged from 1 m (small populations) to a few meters (large populations). Each reproductive stem was classified according to one of the following stages: bud, flowering (when the ligules reached a horizontal position) and past flowering (when all the tubular florets had lost their yellowish color, as an indication of the lack of viable pollen). The observations were performed every 5–15 days, from 15 July to 20 October, and the data were collected on the same day for all the populations. Furthermore, the flowering phenology of 288 plants growing in a simulated mixed-ploidy population at the experimental garden was also recorded every 5–10 days during the same period. The index of flowering overlap was calculated following Husband and Schemske (2000) for each cytotype and habitat type in the field, as well as in the experimental garden. Index values are equal to 0% when flowering is completely asynchronous and equal to 100% when there is complete overlap. Reproductive isolation due to flowering phenology ($RI_{flowering}$) was calculated as: $RI_{flowering} = 100-$index of flowering overlap, yielding a value ranging between 0 and 100%.

Segregation of the cytotypes by pollinator assemblage and behavior

Pollinator assemblage and behavior were assessed by direct observations in six of the nine studied populations. The hexaploid populations 7 and 8 were excluded due to a low number of flowering individuals, as was population 5 because it was recently destroyed (Table 1). To assess pollinator assemblage, 4–6 randomly selected patches of ~ 2 m^2 were delimited and characterized with respect to the number of flowering plants and the number of flowering heads per plant. Visits were recorded during 10 min surveillance sessions at different hours of the day (from 0900 to 1800 h, GMT + 1). A total of 144 surveillance sessions evenly distributed per population were performed, corresponding to 24 h of net observation. During each session, the following variables were registered: visiting insect, number of flowering heads visited per plant and number of plants visited per patch. The flower visitation rate (number of flower heads visited per 10 min) was calculated for each insect species as the total number of visited flower heads per patch divided by the total number of surveillance sessions. The frequency of interactions was also calculated for each insect by multiplying the visitation rate by the mean number of insects per 10 min (insect abundance). Specimens of the floral visitors were collected for identification.

The behavior of pollinators when facing the two cytotypes was investigated using artificial arrays composed of three flowering stalks of each cytotype (each with a comparable number of flowering heads) displayed alternately in a circle 60 cm in diameter. The arrays were displayed in the six studied populations and monitored during 10-min surveillance sessions. A total of 126 surveillance sessions evenly distributed per population were performed, corresponding to 21 h of net observation. During each session, both the insect species and the sequence of cytotypes visited were registered. Indices of floral preference and floral constancy were calculated for the main pollinator species, excluding visits with less than three visited stalks. Floral preference was calculated as the proportion of visits to diploid plants, i.e., the ratio between the number of visits to the diploid cytotype and the total number of visits. Values of 0.5 indicate no preference by the pollinator, whereas values of 0 and 1 reveal a preference for hexaploids and diploids, respectively. Floral constancy was calculated as the ratio between the number of conspecific movements and the total number of movements during the visit. The value of 0 indicates alternating foraging behavior, 0.5 indicates random foraging behavior and 1 indicates constancy in foraging behavior within a cytotype. Overall reproductive isolation caused by pollinator fidelity ($RI_{pollinator}$) was calculated as: $RI_{pollinator} = [1-$(total no. of between cytotype

movements)/(total no. of movements)]*100, yielding a value ranging between 0 and 100%. Isolation due to pollinator behavior was also calculated for each cytotype as the percentage of conspecific movements.

Crossing ability under open pollination in a simulated mixed-ploidy population

A set of 810 flowering plants of the nine populations under study were displayed in a rectangle flower bed of 10 × 81 pots. The positions of pots in the array were determined by assigning a random number to each pot. In this way, we created a mixed-ploidy population at the experimental garden and let it undergo open pollination during the flowering period. The experimental garden (in the Institute of Botany, Průhonice) is located at the same altitude and ~64 km away from the most distant of the nine selected populations, enabling us to perform the experiment under climatic conditions similar to those of the natural populations. Furthermore, preliminary observations showed that the generalist pollinators present at the experimental garden were similar to those observed in the field. As diploid and hexaploid individuals do not occur in natural mixed populations, the simulated mixed population was composed of 33% diploids and 67% hexaploids, reflecting the proportion of diploid and hexaploid populations in the study area. When seeds were mature, fruiting heads were collected, and the number of florets and developed seeds per fruiting head were recorded. For each target plant, the ploidy level of the eight neighboring plants was also recorded to see if the proportion of each cytotype among the immediate neighbors affected the seed production and ploidy composition of the offspring. From each fruiting head, 20 seeds were selected for direct measurement of ploidy level and the remaining seeds were placed to germinate in 10 × 10 cm pots filled with garden substrate for ploidy level assessment in the germinated offspring.

Crossing ability under controlled pollinations

To assess the ability of the two cytotypes to produce hybrids, hand-pollination experiments under controlled conditions were conducted with plants from the nine populations under study. Due to the display of the florets in a compact capitulum (which opens gradually and centripetally) and the development of the pistil within the anther tube from the early stages of floret development, the emasculation procedure as described by Gadella (1984) was very difficult to apply to *A. amellus* without completely damaging the stigmatic surface (see results of the preliminary experiment provided in Online Appendix A). Therefore, pollination experiments were performed without emasculation. On the one hand, using this approach, we were not able to control the origin of all the pollen grains deposited in the stigma (as self-pollen is always present). On the other hand, it allowed a perfect mimicry of inter-cytotype crosses under natural conditions (in which self-pollen will always be present), and together with the analysis of the ploidy level of the offspring, it enabled us to understand the mating processes occurring in natural populations. The pollination experiments were conducted in August during the flowering peak. All the flower heads involved in the pollination experiments were covered with a bag of fine nylon mesh before floret anthesis to exclude pollinators, and the bags were maintained until fruiting. Each flower head served both as pollen donor and as pollen recipient (e.g., Reinartz and Les 1994) and hand-pollinations were performed by gently rubbing the two flower heads together two to five times over the following 5 days to guarantee that all the florets were successfully pollinated (following Raabová et al. 2009). The pollination treatments for each cytotype were the following: (a) self-pollination, i.e., a flower head pollinated with another flower

head from the same plant; (b) xenogamous pollination within each population, (c) xenogamous pollination between populations of the same cytotype and (d) xenogamous pollination between different cytotypes. Due to the highly variable phenology of the plants, we could not guarantee that each plant received all the treatments. However, each treatment was only applied to one flower head per plant. Per treatment, 44–214 flower heads belonging to 266 distinct plants were used. When the seeds were mature, fruiting heads were collected and the number of both florets and developed seeds per flower head were recorded. Seeds were placed to germinate in 10 × 10 cm pots filled with garden substrate and the ploidy level of the offspring was determined for all the seedlings (up to 11 seedlings per cross). The following variables of reproductive success were calculated for each treatment and cytotype: proportion of developed seeds per flower head, proportion of germinated seeds per flower head and proportion of germinated seeds with the ploidy level of the mother (proportion of germinated seeds per flower head*proportion of individuals with the ploidy of the mother). This latter variable allows one to assess the success of self-pollen in inter-cytotype crosses and thus to determine whether self-pollination was more successful after pure selfing or after inter-cytotype pollination (mentor effect; Richards 1986). Reproductive isolation due to post-pollination processes ($RI_{post\text{-}pollination}$) was calculated as: $RI_{post\text{-}pollination} = [1-(\text{total no. of hybrids in seedling stage})/(\text{total no. of flowers from inter-cytotype crosses})]*100$, yielding a value ranging from 0 to 100%.

Statistical analysis

Differences in pollinator assemblage between populations and ploidy levels were explored using detrended correspondence analysis (DCA). The significance of these differences was tested using a permutation test with 500 permutations implemented in canonical correspondence analysis (CCA) in Canoco (ter Braak and Šmilauer 1998). Floral preference and floral constancy were used as two measures of pollinator behavior, and the deviations from random behavior are usually expressed only as a magnitude of deviation from the randomly expected value (0.5; Dafni et al. 2005). In cases of small sample size, which is the case in almost all studies on pollinator behavior, one can observe large deviations from the expected proportion just due to random processes. To see what deviations were actually significantly different from the random expectation considering the sample size, a permutation test was developed in MATLAB® (The MathWorks, Inc., Natick, MA, USA; the script is available on request) that used 1,000 random permutations of the data in each case. For each visit of every pollinator, a cytotype was randomly selected in the simulation (assuming 1:1 proportion of the two cytotypes as they were present in the arrays) and the number of visits to each cytotype and the number of changes between cytotypes were counted. In this way, it was possible to say which deviations from the expected value (0.5) for floral preference and floral constancy have true biological meaning and which ones are just random consequences of small sample size. Because we obtained one P value for each individual insect, P values are given as the mean of all the individuals of each insect species to allow easy presentation in the table.

Differences in the proportion of developed seeds per flower head between cytotypes growing in an artificial mixed-ploidy population were analyzed using a t test, with proportion of developed seeds transformed with an arcsine function. Pearson and Spearman (depending on the distribution of the dependent variable) correlations were used to analyze whether the proportion of diploids in the eight neighbor plants in the artificial mixed-ploidy population had any effect on: the proportion of developed seeds (Pearson correlation), the

proportion of seeds with the ploidy level of the mother (Spearman correlation) and the proportion of seeds from inter-cytotype crosses (i.e., tetraploids, pentaploids, and heptaploids) (Spearman correlation) separately for each cytotype (all variables were arcsine transformed).

To test differences between expected and observed frequencies of tetraploids in the offspring, the expected frequency of tetraploids was calculated assuming random mating as two times the product of the diploid and hexaploid frequencies. The observed frequency of tetraploids at seed and seedling stages (excluding tetraploids formed by the fusion of unreduced gametes from diploid parents; see details in "Ploidy level estimation" section above) were then compared with the expected frequencies for the simulated mixed-ploidy population using a χ^2 test.

Differences in the reproductive success (proportion of developed seeds per flower head, proportion of germinated seeds per flower head and proportion of germinated seeds with the ploidy level of the mother) after controlled pollinations were analyzed using Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's method for pair-wise multiple comparisons (Zar 1984). Dependent variables were arcsine transformed before the analyses.

Results

Temporal segregation of the cytotypes: flowering phenology

Diploid plants began and finished flowering earlier than hexaploid plants, both in the field and in the experimental garden (although in the latter it was less pronounced; Fig. 1), suggesting that part of the phenological differences may have a genetic basis. The differences in flowering phenology were higher in the field than in the experimental garden (mean overlaps of 68 and 88%, respectively; Fig. 1). Under natural conditions, hexaploid plants in high-productivity habitats flowered later than plants in low-productivity habitats (both diploid and hexaploid) (Fig. 1a), suggesting that part of the phenological differences in the field are also due to habitat. Under controlled conditions, the differences in flowering phenology were less evident with a high percentage of overlap between cytotypes and habitats (83–93%; Fig. 1b). Reproductive isolation between diploids and hexaploids due to flower phenology varied from 11 to 45%. Despite this, the overlap was still large enough to allow inter-cytotype crosses (Fig. 1).

Segregation of the cytotypes by pollinator assemblage and behavior

Flower heads of *A. amellus* are regularly visited by a diverse assemblage of insects from the orders Diptera, Hymenoptera and Lepidoptera and sporadically by a few Coleoptera and Heteroptera (Online Appendix B). Among Diptera (45.0% of all visits), Syrphidae species were the most common visitors, with several species presenting the highest visitation rates and frequencies of interaction among all the observed insects (e.g., *Sphaerophoria stripta* and *Eristalis tenax* with 18.8 and 8.3% of all visits; Appendix B, Tables B1–B3). Within the Hymenoptera (43.2% of all visits), several species of Apidae, Megachilidae, Halictidae, Anthophoridae and sometimes Formicidae were observed visiting *A. amellus*; overall, insects from this order presented slightly lower visitation rates and frequencies of interaction than those from Diptera (Appendix B, Tables B2 and B3). Lepidoptera were less abundant than Diptera or Hymenoptera (9.5% of all visits), but still up to eight lepidopteran species were observed in the studied populations (Appendix B, Tables B1–B3). Pollinators were

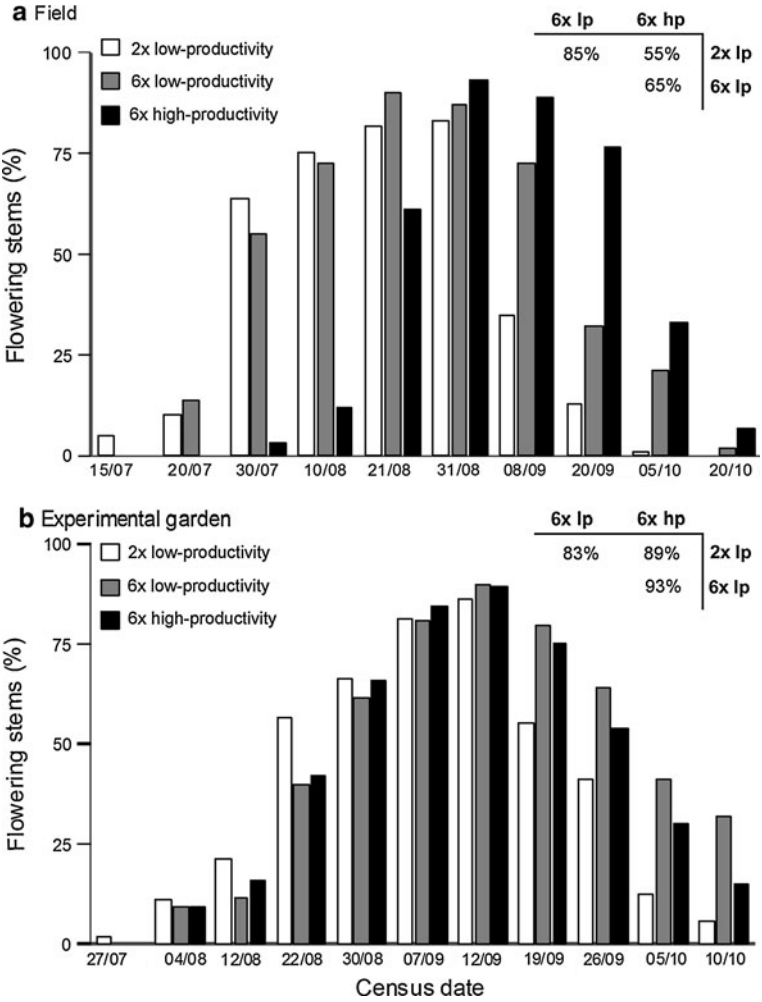

Fig. 1 Flowering phenology of diploid populations from low-productivity habitats (*open bars*), hexaploid populations from low-productivity habitats (*gray bars*) and hexaploid populations from high-productivity habitats (*black bars*) over the entire flowering period in **a** the field and **b** the experimental garden. The inserts provide percentages of overlap in flowering phenology between all the pairs in **a** the field and **b** the experimental garden; the percentage of flowering overlap was calculated following Husband and Schemske (2000); *lp* low-productivity habitat; *hp* high-productivity habitat

generally abundant, resulting in relatively high visitation rates and frequencies of interaction in all the studied populations (Appendix B, Tables B2 and B3).

The pollinator assemblage was found to vary significantly between the two cytotypes and between the different study populations ($P < 0.05$). However, whereas population explained 11% of the observed variance, cytotype only explained 2.8% of the variance, showing that the differences in pollinator assemblage between cytotypes are better explained by other characteristics of the populations than by ploidy level. This can also be seen from the large overlap in pollinator assemblage between diploid and hexaploid populations as revealed by the DCA analysis (Fig. 2).

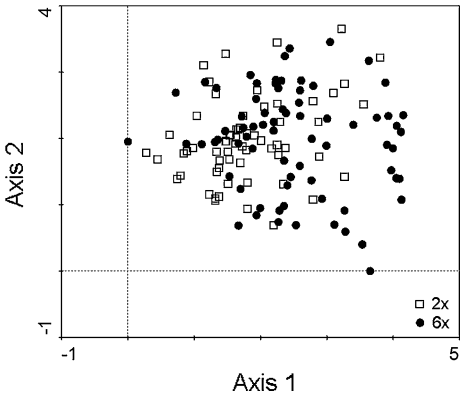

Fig. 2 Pollinator assemblages in the diploid and hexaploid populations: detrended correspondence analysis performed with flower heads visited by each insect species for all the observation sessions in diploid (2*x*, *open square*) and hexaploid (6*x*, *filled circle*) populations

Table 2 Pollinator behavior: preference and constancy indices of the main pollinators of diploid and hexaploid *Aster amellus*

Species	n	Preference index			Constancy index		
		Mean	SE	*P* value	Mean	SE	*P* value
Eristalis arbustorum (Syrphidae)	10	0.466	0.040	0.968	0.266	0.074	0.603
Eristalis tenax (Syrphidae)	18	0.487	0.032	0.950	0.166	0.056	0.456
Eupeodes corolla (Syrphidae)	8	0.529	0.045	1.000	0.000	0.000	0.364
Helophilus pendulus (Syrphidae)	8	0.552	0.061	0.956	0.208	0.103	0.650
Sphaerophoria scripta (Syrphidae)	30	0.478	0.029	0.966	0.111	0.034	0.525
Bombus sp. (Apidae)	10	0.618	0.038	0.963	0.092	0.069	0.541
Dasypoda hirtipes (Melittidae)	10	0.450	0.037	1.000	0.100	0.055	0.440
Heriades truncorum (Megachilidae)	7	0.395	0.043	0.911	0.262	0.095	0.660
Hoplosmia spinulosa (Megachilidae)	14	0.480	0.031	1.000	0.160	0.065	0.452
Megachile centuncularis (Megachilidae)	14	0.564	0.026	1.000	0.143	0.057	0.454
Halictus simplex (Halictidae)	6	0.375	0.042	0.936	0.056	0.056	0.471
Halictus sp. (Halictidae)	17	0.526	0.032	0.942	0.218	0.062	0.590
Nomada fucata (Anthophoridae)	5	0.527	0.087	0.873	0.250	0.112	0.670
Lepidoptera	12	0.469	0.029	0.986	0.228	0.055	0.506

Preference index: =0.5—no preference, <0.5—preference for hexaploids, >0.5—preference for diploids. Constancy index: 0—alternating foraging, 0.5—random foraging, 1—constant foraging within a cytotype. Preference and constancy indices are given as mean and standard error of the mean (SE). Total number of visits is also provided (n). *P* values are given as mean of all the individuals of each taxon (for details see "Materials and methods")

The use of artificial arrays composed of diploid and hexaploid flowering stalks allowed the collection of information on the behavior of 14 *A. amellus* pollinators (all Lepidoptera were included in one group) when facing both cytotypes. The results revealed that pollinators of *A. amellus* had no preference for a specific cytotype (Table 2). No significant deviations from 0.5 (the value indicating no preference) were obtained for floral preference during any observation. The constancy indices indicate that pollinators' behavior varied between alternating and random visits. However, the statistical test revealed that most of the observations did not differ significantly from random behavior (0.5; Table 2), with only

occasional observations revealing an alternating behavior. The overall reproductive isolation caused by pollinators was 17%. Considering the cytotypes separately, overall pollinator isolation was 8% for diploids and 9% for hexaploids.

Crossing ability under open pollination in a simulated mixed-ploidy population

No significant differences were observed in the proportion of developed seeds between diploid ($70.5 \pm 5.8\%$) and hexaploid plants ($71.9 \pm 3.4\%$; $t = 0.367$, $df = 76$, $P = 0.714$) growing in the artificial mixed-ploidy population. No correlation was found between the proportion of seeds produced by either cytotype and the proportion of diploid neighbors ($P > 0.05$). Furthermore, the proportion of diploid neighbors affected neither the proportion of seeds with the ploidy level of the mother, nor the proportion of seeds from inter-cytotype crosses ($P > 0.05$).

Diploid plants produced mostly diploid offspring (80.0 and 89.6% at seed and at seedling stages, respectively) and rarely other ploidy levels at both stages (Fig. 3a). Hexaploid plants produced high proportions of aneuploids at the seed stage (79.8% of the seeds), most of them with DNA indices between hexaploid and pentaploid (70.3%), while only few hexaploids and pentaploids were produced (15.7% and 4.6%, respectively); at the seedling stage most of the offspring was hexaploid (96.6%; Fig. 3b).

Tetraploids were formed in significantly lower proportions than expected for a mixed-ploidy population, comprising 33% of diploids and 67% of hexaploids under random mating ($\chi^2 = 728.3$, $df = 1$, $P < 0.001$ and $\chi^2 = 347.8$, $df = 1$, $P < 0.001$ at seed and seedling stage, respectively). Tetraploids were only formed from diploid mothers and resulted from two distinct processes: by the fusion of unreduced gametes from both diploid parents (4 seeds and 0 seedlings, corresponding to 1.3 and 0.0% of the total number of seeds and seedlings, respectively) and by the fusion of reduced gametes from a diploid and

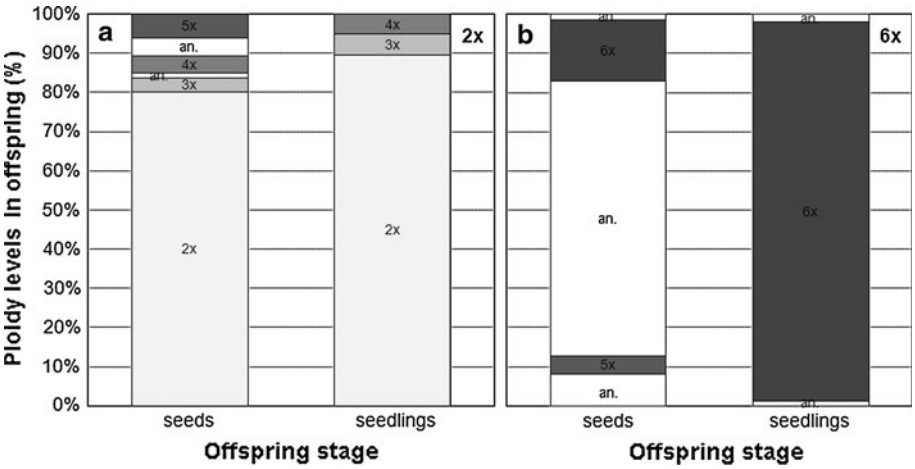

Fig. 3 Ploidy level of the offspring at seed and seedling stages in **a** diploid and **b** hexaploid plants obtained in a mixed-ploidy population with open pollination composed of 33% diploids and 67% hexaploids. Ploidy levels are provided within bars as follows: 2*x*—diploids, 3*x*—triploids, 4*x*—tetraploids, 5*x*—pentaploids, 6*x*—hexaploids, an.—aneuploids; aneuploids were classified between the main ploidy levels based on their DNA indices (See "Materials and methods" for further details)

a hexaploid parent (9 seeds and 4 seedlings, corresponding to 3.0 and 5.2% of the total number of seeds and seedlings, respectively).

Triploids and pentaploids were observed in very low proportions and resulted from the fusion of one reduced and one unreduced gamete (Fig. 3). Aneuploids were also produced, especially by hexaploid mothers (Fig. 3). The high proportion of aneuploids with a DNA index between hexaploid and pentaploid (70.3%) produced by hexaploid mothers may indicate abnormalities in meiosis.

Crossing ability under controlled pollinations

The proportion of seeds produced varied significantly among pollination treatments ($H = 79.77$, $df = 7$, $P < 0.001$; Fig. 4a–b). As expected, self-pollination generated the lowest seed yield. Although not statistically significant, the success of self-pollinations was higher in hexaploids than in diploids (seed germination per flower head ± SD: 2.1 ± 5.6% and 0.1 ± 0.4%, respectively; $P = 0.067$). In diploids, the remaining pollination treatments generated similar seed production (Fig. 4a). In hexaploids, pollinations between populations resulted in significantly higher number of developed seeds than pollinations within populations. Surprisingly, the inter-cytotype crosses yielded results similar to those observed after self-pollination (Fig. 4b). The proportion of germinated seeds also varied significantly among pollination treatments ($H = 109.94$, $df = 7$, $P < 0.001$) and showed a trend similar to that of the developed seeds (Fig. 4a–b). Self-pollination produced seeds in both cytotypes, but seed germination was relatively low (Fig. 4a–b).

As expected, crosses between diploids produced diploid offspring (100%; Fig. 4c). Crosses between hexaploids produced mostly hexaploid offspring (83%) and a few aneuploids (13%) and heptaploids (4%; Fig. 4d). The results of the inter-cytotype crosses were unexpected: a surprisingly low proportion of tetraploids (24%) was obtained from diploid mothers ($2x \times 6x$; Fig. 4c), and none were obtained from hexaploid mothers ($6x \times 2x$; Fig. 4d). Instead, most of the offspring from the inter-cytotype crosses had the ploidy level of the mother plant (Fig. 4c–d), revealing that most of them resulted from self-pollination rather than from inter-cytotype pollination. However, the results do not exclude pseudogamous apomixes and this fenemoena, if present, might also explain the large numbers of homoploid offspring formed by inter-cytotype crosses. Reproductive isolation due to post-pollination processes was 99.9% among all the plants and 99.9 and 100% for diploid and hexaploid plants, separately.

Calculation of the number of offspring with the ploidy level of the mother can reveal whether self-pollination is more successful after pure selfing or after inter-cytotype pollination (mentor effect; Richards 1986). The results revealed no significant differences for either cytotype (Fig. 4a–b), indicating that there is no mentor effect operating in *A. amellus*.

Discussion

Across Central Europe, diploid and hexaploid *A. amellus* occur in close proximity in a complex and diffuse contact zone (Mandáková and Münzbergová 2006). The lack of hybrids between the two cytotypes (i.e., tetraploids) raises questions regarding the reproductive mechanisms involved in the maintenance of this contact zone. In this study, phenological patterns, pollinator behavior and the ability to hybridize were assessed using populations of diploid and hexaploid plants growing in close proximity. The results

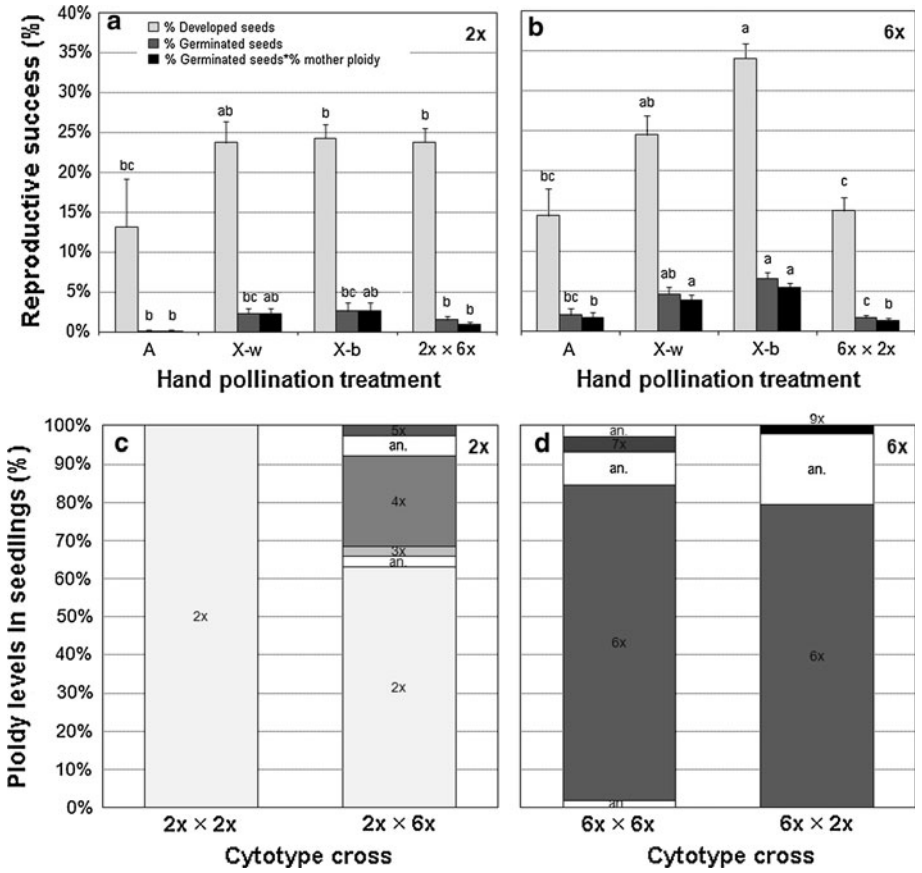

Fig. 4 Reproductive ability of the cytotypes assessed after controlled hand pollination experiments within and between cytotypes, showing proportion of developed seeds per flower head, proportion of germinated seeds per flower head and proportion of germinated seeds with the ploidy level of the mother are provided for **a** diploid and **b** hexaploid individuals. Values are given as means and the standard error of the mean. Hand-pollination treatments: *A* self-pollination, *X-w* xenogamous pollination within population, *X-b* xenogamous pollination between populations of the same cytotype, $2x \times 6x$ and $6x \times 2x$—pollination between diploid and hexaploid individuals. Ploidy levels of the seedlings after within ($2x \times 2x$ and $6x \times 6x$) and between cytotype crosses ($2x \times 6x$ and $6x \times 2x$) are also provided for **c** diploid and **d** hexaploid individuals (in cytotype crosses, the first ploidy corresponds to the ploidy of the mother and the second corresponds to the ploidy of the father). Values are given as proportions from the total number of analyzed individuals. *Different letters* reveal statistically significant differences between the four hand pollination treatments for each variable according to a Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's method for pair-wise multiple comparisons

revealed variable levels of reproductive isolation due to flowering phenology (RI = 11–45%) and low levels due to pollinator behavior (RI = 17%). Instead, almost complete reproductive isolation was achieved by a series of post-pollination barriers that significantly reduced the production of offspring from inter-cytotype crosses (RI = 99.9%), which, together with spatial segregation (Mandáková and Münzbergová 2006), may explain the lack of hybrids in natural populations.

Temporal segregation of the cytotypes: flowering phenology

The flowering period of the two cytotypes of *A. amellus* largely overlapped, showing that the two cytotypes have the opportunity to hybridize along most of its flowering period. Previous studies comparing phenological differences between diploids and polyploids have shown diverse patterns, from total flowering divergence (Petit et al. 1997) to different degrees of segregation (Lumaret et al. 1987; Bretagnolle and Thompson 1996; Husband and Sabara 2004) and nearly complete overlap (Jersáková et al. 2010). Phenological shifts in polyploid complexes may result directly from genetic differences generated by the process of polyploidization (Stebbins 1950), from subsequent selection processes acting over flowering time variation (van Dijk and Bijlsma 1994; Nuismer and Cunningham 2005) and/or micro-environmental differences that influence plant growth (Lumaret et al. 1987). In *A. amellus*, phenological patterns seem to be driven both by genetic and environmental factors, with no obvious selection towards phenological differentiation (see pollinator behavior below). Given the large overlap in phenology between diploid and hexaploid *A. amellus*, the phenological differences by themselves cannot prevent hybridization of the two cytotypes and only lead to minor levels of reproductive segregation.

Segregation of the cytotypes by pollinator assemblage and behavior

As in most Asteraceae, *A. amellus* is an enthomophilous species pollinated by a wide and diverse array of generalist insects (Torres and Galetto 2002; Mayor 2008; and results herein). Diploid and hexaploid populations of *A. amellus* shared most of their pollinators. Still, the pollinator assemblages were quite variable, with most of the variation being observed between localities rather than between cytotypes. Such spatial differences in insect assemblage and abundance were observed in many other systems and may arise from a diverse array of factors, such as variation in food resources, traits of available plants and/or microclimatic aspects (e.g., Herrera 1988, 2002; Gómez and Zamora 1999).

Despite the heterogeneity in pollinator assemblages among populations of *A. amellus*, the most common floral visitors revealed no preference for a specific cytotype and displayed random behavior in the mixed-ploidy arrays, leading to low levels of reproductive isolation (17%). Taxa segregation due to pollinator behavior has been extensively studied (see review in Grant 1994); however, only a few studies have addressed polyploid complexes (Kennedy et al. 2006; Thompson and Merg 2008; Jersáková et al. 2010). Strong differentiation in the visits to different cytotypes was observed in *Chamerion angustifolium* as a result of both floral preference and floral constancy (Kennedy et al. 2006); strong differentiation was also observed in *Heuchera grossulariifolia* as a result of different sets of pollinators visiting each cytotype (Thompson and Merg 2008). In both cases, pollinator behavior lead to assortative pollination and contributed to high rates of reproductive isolation. Similar to what was observed in *Gymnadenia conopsea* (Jersáková et al. 2010), neither preference nor constancy was observed in *A. amellus*. Also as in *G. conopsea*, pollinator behavior mediated disassortative pollination between diploid and hexaploid *A. amellus*, and thus it is excluded as a breeding barrier to inter-cytotype hybridization.

Crossing ability

The crossing experiments revealed that *A. amellus* is able to set seeds after self-pollination, being partially self-compatible (in contrast to what was described by Kovanda 2005). However, seed germination after self-pollination was found to be lower than after cross

pollination, and thus the plant is essentially an outcrosser. The success of self-pollination was higher in the hexaploids than in the diploids. Several studies support the hypothesis that polyploids have, on average, higher rates of self-fertilization than their diploid relatives (Barringer 2007; and references therein). Selfing is particularly important for polyploid establishment, as it constitutes a mechanism of reproductive assurance for newly arisen cytotypes (Rausch and Morgan 2005; Rieseberg and Willis 2007).

In the *A. amellus* mixed-ploidy population with open pollination, tetraploids were obtained in significantly lower proportions than expected for a population under random mating. As neither temporal segregation nor segregation by a pollinator's behavior was observed, and as mechanical isolation can be excluded (because of the generalist architecture of the flower heads and of the morphological similarity between diploids and hexaploids), this observation constitutes the first evidence for the occurrence of post-pollination barriers to the development of inter-cytotype offspring. This result was reinforced after controlled pollination experiments.

In those experiments, the reproductive success of inter-cytotype crosses was lower than of intra-cytotype crosses. Additionally, the offspring produced after inter-cytotype crosses were mostly composed of individuals with the same ploidy level of the mother plant, and as in the mixed-ploidy population with open pollination, tetraploids were rarely formed. These results indicate that the offspring of inter-cytotype crosses result mainly from self-pollination. In *A. amellus*, self-pollination rates were not increased by mixed loads of self and foreign pollen (i.e., pollen from a different cytotype), which contrasts with other Asteraceae where the mentor effect has been observed (e.g., Desrochers and Rieseberg 1998; Mráz 2003; Brock 2004). This can be easily explained by the fact that *A. amellus* is partially self-compatible. Overall, the pollination experiments confirmed the existence of post-pollination barriers that contribute to the reproductive isolation of diploid and hexaploid *A. amellus*. Among the post-pollination barriers, those that may be operating in *A. amellus* include gametic barriers that probably occur both before and after fertilization, such as pollen-pistil interactions (e.g., pollen tube attrition; Cruzan 1989) and/or genomic imprinting (e.g., the ratio of maternal-to-paternal genomes in endosperm tissue; Grossniklaus et al. 2001). These barriers diminish seed production and inter-cytotype hybrid production, reinforcing cytotype reproductive isolation.

Gametic barriers have been shown to be very important to the reproductive isolation of several polyploid complexes (e.g., Husband et al. 2002). Differences in mating systems between cytotypes result in differences in reproductive success and have major implications for cytotype maintenance in mixed-ploidy populations and at contact zones (e.g., Husband et al. 2002; Rausch and Morgan 2005; Buggs and Pannell 2006). For example, asymmetric hybridization patterns were shown to favor tetraploid *Chamaerion angustifolium* (Husband et al. 2002) and diploid *Mercurialis annua* (Buggs and Pannell 2006), significantly influencing the dynamics of the contact zone. Within *A. amellus*, diploid and hexaploid individuals apparently differ in several aspects of their mating systems, and further comparative studies should explore how different mating traits (e.g., selfing and inter-cytotype rates) affect the maintenance of mixed-ploidy populations with different proportions of diploid and hexaploid individuals.

An interesting result of the mixed-ploidy open pollination experiment was the high proportion of aneuploids with a DNA index between hexaploid and a pentaploid produced by hexaploid mothers. The aneuploids were observed mainly at the seed stage, being in very low proportions in the germinated offspring. These results indicate that abnormalities in meiosis may be frequent in hexaploid plants. Indeed, a previous study of male and female gamete development revealed meiotic abnormalities in polyploid cultivars of

A. amellus (involving hexaploid cultivars), whereas diploid plants had normal gamete development (Annen 1945). A higher seed abortion rate in the hexaploids when compared with diploid plants was also observed by Münzbergová (2007a), which may be correlated with high production of unviable aneuploids. The production of such high proportion of fully developed but unviable seeds in hexaploids may constitute a major energetic cost, considering that only a few aneuploids are able to germinate. On the other hand, it may be extremely important from the perspective of genome evolution in this species, in case the aneuploids that germinate are able to successfully reproduce. Such knowledge may contribute to our understanding of the mechanisms involved in the genome downsizing observed in *A. amellus*. However, the significance of the aneuploids still remains to be tested.

Concluding remarks

This study revealed that reproductive isolation between diploid and hexaploid *A. amellus* is not determined by phenological divergence or selective pollinator behavior. Instead, inter-cytotype hybridization is reduced by a set of post-pollination barriers acting before and/or after fertilization, such as pollen-pistil interactions, and/or genomic imprinting and endosperm formation. The strength of such gametic barriers in each cytotype can have long-term fitness consequences and thus affect the fate of the populations at the contact zones. In the case of *A. amellus*, diploids may be at a disadvantage in the case of single individuals growing among hexaploids, as they have lower self-pollination rates and higher production of inter-cytotype offspring. However, when growing in mixed-ploidy populations with more diploid individuals, they may gain an advantage due to their higher reproductive success (mainly as a result of the higher production of unviable aneuploids by hexaploids). This study also indicates that hybrids (i.e., tetraploids) may arise in the contact zone. Their long-term survival and reproductive ability, however, remains to be tested. Our results indicate that the absence of tetraploids in the field is due to a combination of several factors that include spatial segregation of diploids and hexaploids (Mandáková and Münzbergová 2006), strong post-pollination barriers and (to a lesser extent) temporal isolation and segregation due to pollinator behavior. Future studies should explore how different selfing and inter-cytotype crossing rates affect the reproductive success and fitness of each cytotype (including tetraploids). This information will reveal whether cytotypes have similar or different probabilities of being maintained in mixed-ploidy populations and will provide further clues to understand the dynamics of the contact zone and the mechanisms responsible for the lack of mixed-ploidy populations.

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