Annals of Botany **110**: 1047–1055, 2012
doi:10.1093/aob/mcs177, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

Cytotype distribution at a diploid–hexaploid contact zone in *Aster amellus* (Asteraceae)

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Received: 13 March 2012 Returned for revision: 28 May 2012 Accepted: 26 June 2012 Published electronically: 10 August 2012

• *Background and Aims* The present study aims to assess the diversity and distribution of cytotypes of *Aster amellus* in central and eastern Europe, contributing with data to improve understanding of the evolutionary dynamics of the contact zone between diploids and hexaploids of this polyploid complex.
• *Methods* Large-scale cytotype screening of 4720 individuals collected in 229 populations was performed using 4′,6-diamidino-2-phenylindole (DAPI) flow cytometry. Fine-scale cytotype screening was performed in the mixed-ploidy population. Reproductive variables, such as number of florets per flower head, seed set and seedling emergence, as well as ploidy level of seeds and seedlings were recorded in this population.
• *Key Results* The diploid–hexaploid contact zone is large and complex, reaching the Czech Republic in the west, Austria in the south, Poland in the north-east and Romania in the extreme east of the surveyed areas. Most populations presented only one cytotype, either diploid or hexaploid. In several areas of the contact zone both cytotypes were found to grow in parapatry. One mixed-ploidy population of diploids and hexaploids was detected for the first time, but no signs of hybridization were detected. In this population, diploids had a significantly lower reproductive success, and significantly higher production of intercytotype offspring, being in reproductive disadvantage in comparison with hexaploids.
• *Conclusions* The contact zone of diploid and hexaploid *A. amellus* in central and eastern Europe seems to be highly dynamic and diffuse, with both primary and secondary contacts being possible. The obtained results suggest the origin of hexaploids through diploids, overall supporting previous hypotheses that this species is autopolyploid. Data from the only mixed-ploidy population detected so far suggest that the minority cytotype exclusion is an important evolutionary mechanisms driving the prevalence of single-cytotype populations, and thus contributing to the current distributional patterns of the cytotypes of *A. amellus*.

Key words: *Aster amellus*, contact zone, cytotypes, diploids, DNA ploidy level, flow cytometry, hexaploids, hybridization, polyploid aggregate, spatial distribution, tetraploids.

INTRODUCTION

Polyploidy has played a key role in the evolution and diversification of the plant kingdom (Otto and Whitton, 2000; Soltis *et al.*, 2009). The most recent estimations suggest that up to 100 % of angiosperms have experienced one or more episodes of polyploidization during their evolutionary history (Grant, 1971; Masterson, 1994; Soltis, 2005; Wood *et al.*, 2009), and huge explosions in species diversity were shown to coincide with the timing of ancient genome duplications (De Bodt *et al.*, 2005). To understand the adaptive significance of this widespread phenomenon, many studies have addressed the ecological, physiological and genetic potential of polyploids (e.g. Pecinka *et al.*, 2006; Richardson and Hanks, 2011). However, only recently has some attention been paid to large- and small-scale distribution patterns and to the interactions among cytotypes, especially at contact zones (e.g. Buggs and Pannell, 2007; Kolař *et al.*, 2009; Trávníček *et al.*, 2011).

One of the first steps in the study of polyploidy in natural systems is the knowledge of the diversity and geographical distribution of cytotypes in nature. Such information is the basis for exploring the natural processes involved in the origin, establishment and evolution of polyploids. Recently, with the development of rapid and efficient tools for assessing nuclear DNA content and ploidy level in plants, large-scale cytotype screenings became possible enabling detailed studies of cytotype distribution patterns (Kron *et al.*, 2007). The emergent picture suggests that differences in ploidy are a common phenomenon not only among species but also within species and populations. Indeed, sympatric occurrence of several cytotypes is surprisingly common and has been repeatedly reported in numerous families (e.g. Asteraceae: Suda *et al.*, 2007*a*, *b*; Halverson *et al.*, 2008; Poaceae: Felber-Girard *et al.*, 1996; Keeler, 2004; Onagraceae: Husband and Schemske, 1998; Orchidaceae: Trávníček *et al.*, 2011).

In flowering plants, polyploids arise most frequently by the fusion of unreduced gametes, and may result either from the doubling of a single genome (autopolyploidy) or by the combination of two or more distinct, yet related, genomes (allopolyploidy) (Grant, 1971). The establishment and maintenance of newly developed cytotypes in mixed-ploidy populations will

only occur if an array of breeding barriers and/or ecological features promote assortative mating (Rieseberg and Willis, 2007), increasing its probability to reproduce successfully; otherwise, new cytotypes will be subjected to frequency-dependent selection and become extinct (minority cytotype exclusion; Levin, 1975; Rodriguez, 1996; Husband and Schemske, 2000). In this context, contact zones of different cytotypes provide natural laboratories for studying evolutionary transitions in flowering plants (Lexer and van Loo, 2006). Furthermore, cytotype screenings provide new insights on niche segregation and, thus, information on the occurrence of potential barriers to cytotype coexistence. Finally, by detecting minority cytotypes, insights on the frequency of polyploidy origin may be obtained (Segraves *et al.*, 1999).

Aster amellus (Asteraceae) is a polymorphic perennial herb growing 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 Europe (Meusel and Jäger, 1992; Münzbergová *et al.*, 2011). This species is considered to be polyploid, supposedly comprising three cytotypes: diploids ($2n = 2x = 18$ chromosomes), tetraploids ($2n = 4x = 36$ chromosomes) and hexaploids ($2n = 6x = 54$ chromosomes) (Meusel and Jäger, 1992). Based on morphological and cytological characters, as well as on distributional patterns, the aggregate received several taxonomic treatments (reviewed in Münzbergová *et al.*, 2011). Still, morphological distinction of cytotypes using the diagnostic characters is extremely difficult, and recent experiments in a common garden revealed no morphological differentiation between diploid and hexaploid individuals (Mandáková and Münzbergová, 2008). A recent study on cytotype distribution in the Czech Republic revealed a complex and diffuse secondary contact zone between diploid and hexaploid cytotypes (Mandáková and Münzbergová, 2006). These results emphasized the need to expand the screening area to other regions of Central Europe, to understand fully the structure, geographical pattern and dynamics of this contact zone.

Integrated in a broad research effort focused on the evolutionary dynamics of diploid and hexaploid cytotypes of *A. amellus*, the objectives of the present study were to assess the diversity and distribution patterns of cytotypes in Central Europe at regional and local scales, with special interest in the contact zone, and to evaluate the ability of the cytotypes to hybridize in natural populations. Addressing these questions provided background information to review the hypotheses for the origin of polyploids in this aggregate and insights on the ecological dynamics of the diploid–hexaploid contact zone.

MATERIALS AND METHODS

Large-scale cytotype screening

Large-scale cytotype screening was conducted using fresh leaves of *Aster amellus* L. directly collected in the field and seeds from natural populations provided by several Botanical Gardens (Supplementary Data Table S1). Fresh leaves were collected from late July to early September 2008 and 2009 in Austria, Croatia, Hungary, Poland, Romania, Slovakia and Slovenia. Seeds from natural populations of known origin were obtained through Botanical Gardens covering France, Germany, Italy, Poland, Romania, Russia, Slovakia, Switzerland and Turkey.

In the field, geographic co-ordinates were recorded and young leaves from up to 30 distinct plants were collected across the entire population, placed in hermetic plastic bags and maintained at 4 °C until flow cytometric (FCM) analyses. Herbarium vouchers were collected in all populations and deposited at the Herbarium of the Department of Botany, Charles University in Prague (PRC). Seeds provided by the Botanical Gardens were placed to germinate in pots in the growth chamber and seedlings were used for ploidy level estimation. When seedling emergence was unsuccessful, seeds were directly used for FCM analysis.

In total, 229 populations and 4720 individuals of *A. amellus* agg. (Münzbergová *et al.*, 2011) were screened for ploidy level (Supplementary Data Table S1). Previous cytotype distribution data of 87 populations from the Czech Republic (Mandáková and Münzbergová, 2006) were also added to the final map. An index of reproductive isolation between diploids and hexaploids due to geographical segregation was calculated as the percentage of single-ploidy populations (considering base ploidies only and adding the populations from Mandáková and Münzbergová, 2006).

Fine-scale cytotype screening

Fine-scale cytotype distribution was conducted in the only mixed-ploidy population that was detected (Strebersdorf, Austria). In this population, every adult plant (both flowering plants and vegetative rosettes) was labelled, its position recorded in a rectangular co-ordinate system, and its DNA ploidy level estimated by FCM. Moreover, 1–2 fruiting heads from all fructiferous plants were collected to assess reproductive variables and offspring ploidy level (at seed and seedling stages) for each cytotype. The following reproductive variables were assessed: number of florets per flower head, number of seeds per head and seed set (i.e. percentage of developed seeds from the total number of florets per flower head). One to three achenes from each fruiting head were randomly selected for DNA ploidy level estimation, while the remaining achenes were placed to germinate in 10 × 10 cm pots filled with garden substrate for assessing seedling emergence and ploidy level. The DNA ploidy level of all the obtained seedlings was subsequently analysed.

To analyse the spatial segregation between the two cytotypes, the corresponding bivariate *J*-function was estimated (Van Lieshout and Baddeley, 1999). This function was selected because it can be calculated without correcting for the edge effect (see Baddeley *et al.*, 2000), which is particularly important considering the dimensions of the mixed-ploidy population. The *J*-function was estimated as: $Jij\ (r) = [1 - Gij\ (r)]/[1\ Fj\ (r)]$, defined for all $r \geq 0$ with $Fj\ (r) \neq 0$, where the function $Gij\ (r)$ is the cumulative distribution of distances from a random plant of cytotype i to the nearest individual of cytotype j, and $Fj\ (r)$ is the distribution function of the distance from a fixed plant in space to the nearest individual of cytotype j in the pattern. If cytotype i is independent of cytotype j, then $Jij\ (t) = 1$. Deviations of the empirical estimate of $Jij\ (t)$ from 1 may suggest dependence between cytotypes,

with values of *Jij* >1 being interpreted as a 'negative association', and values of *Jij* <1 as a 'positive association' (Van Lieshout and Baddeley, 1999). The null hypothesis chosen was random labelling, and statistical analyses were conducted using the spatstat package (Baddeley and Turner, 2005) in the R environment.

Differences in the mean number of florets and seeds per flower head and seed set between cytotypes were analysed using a *t*-test. To achieve normality and homoscedasticity, the number of florets per flower head was transformed with $\log_{10}$, while seed set and seedling emergence were transformed with arcsine. Differences between expected and observed frequencies of tetraploids in the offspring (at both seed and seedling stages) were assessed using a *z*-test; the expected frequency of tetraploids was calculated assuming random mating as twice the product of the diploid and hexaploid frequencies.

Estimation of DNA ploidy levels using flow cytometry

All 4720 individuals were subjected to DNA ploidy level (terminology following Suda *et al.*, 2006) estimation using FCM. Nuclei were released after co-chopping 0·5 cm^2 of fresh leaf tissue or a seed of *A. amellus* together with 0·5 cm^2 of fresh leaf tissue of *Bellis perennis* L. (internal reference standard with 2C = 3·38 pg; Schönswetter *et al.*, 2007) with a sharp razor blade in a plastic Petri dish containing 0·5 mL of Otto I buffer (0·1 M citric acid, 0·5 % Tween-20; Otto, 1992; Doležel *et al.*, 2007). Afterwards, the nuclear suspension was filtered using a 42 μm nylon mesh and stained with a solution containing 1 mL of Otto II buffer (0·4 M $Na_2HPO_4{\cdot}12\ H_2O$) supplemented with 4 mg mL^{-1} of the fluorochrome 4',6-diamidino-2-phenylindole (DAPI) and 2 mg mL^{-1} of the antioxidant β-mercaptoethanol. Five minutes after staining, the relative fluorescence intensity of at least 3000 nuclei was analysed in a Partec PA II flow cytometer (Partec GmbH., Münster, Germany), equipped with a mercury lamp for UV excitation using the FloMax software (Partec GmbH., Görlitz, Germany). The resulting histograms were evaluated and the DNA ploidy level of the individuals determined on the basis of the sample/standard ratio, i.e. DNA index. Considering that the selected tissues of *A. amellus* were not endopolyploid and have low mitotic activity, the pooled samples strategy was used for fresh leaves. In this case, up to six individuals were analysed simultaneously by adding equal amounts of plant material of each individual to the sample together with the internal standard. As a quality control, for fresh leaves, only histograms with a coefficient of variation (CV) of G_0/G_1 peaks below 4·0 % were accepted. For seeds, CV values up to 6 % were accepted as they generally produced histograms with lower quality. In cases where extra peaks were detected or if the CV exceeded the defined criteria, all the plants from the pooled samples were re-analysed separately and their ploidy level was confirmed.

Chromosome counts

Because hexaploid individuals do not have a simple multiplication of the diploid genome (2*x* : 6*x* ratio of 2·5 instead of 3; Mandáková and Münzbergová, 2006), conventional karyological analyses of 12 individuals representing different DNA indexes were performed to confirm the FCM results. These plants have been previously collected and grown in the greenhouse and served as the source of root tips and leaves for karyological and FCM analyses, respectively. Karyological analyses followed the methodology described in Mandáková and Münzbergová (2006). In this way it was possible to correlate the DNA index with the number of chromosomes and assign it to a specific cytotype (diploid, triploid, hexaploid, heptaploid or aneuploids).

RESULTS

Large-scale cytotype screening

In the study area, the DNA ploidy level of 4720 individuals from 229 populations was estimated (Supplementary Data Table S1). Most of the populations (89·6 %) were cytogenetically uniform, comprising either diploid ($2n = 2x = 18$ chromosomes; 53·4 % of all the populations) or hexaploid ($2n = 6x = 54$ chromosomes; 36·2 % of all the populations) individuals. Mixed populations comprising two or more cytotypes were detected in 10·4 % of the cases. Among these, one mixed-ploidy population with diploid and hexaploid individuals was detected for the first time (Fig. 1). The remaining mixed populations presented a large number of individuals with one of the main ploidy levels (2*x* or 6*x*) and few individuals with a different cytotype (triploids in diploid populations and heptaploids, octoploids and/or nonaploids in hexaploid populations) (Table 1) (Supplementary Data Table S1). Also,

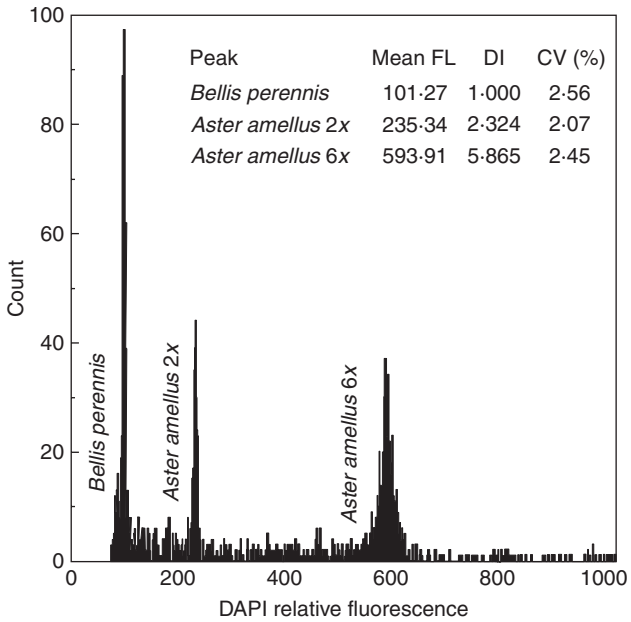

FIG. 1. Relative fluorescence histogram of DAPI-stained nuclei isolated from fresh leaf tissues of diploid and hexaploid *Aster amellus* from the mixed-ploidy (2*x*, 6*x*) population (Strebersdorf, Austria) and of the internal reference standard (*Bellis perennis* L.). Nuclei from all plants were isolated, stained and analysed simultaneously. The G_0/G_1 peak ratio between diploid and hexaploid individuals is 2·5.

TABLE 1. *Summary of* Aster amellus *cytotype distribution across Europe (data from the populations collected in this study, only)*

Type of populations	No. and percentage of populations	No. of populations (individuals) with a particular ploidy level							Distribution area
		2*x*	3*x*	6*x*	7*x*	8*x*	9*x*	An.	
Pure 2*x*	122 (53·4 %)	122 (1867)	–	–	–	–	–	–	Whole investigated area, except RU
2*x* + mc	9 (3·9 %)	9 (237)	7 (8)	–	–	–	–	2 (2)	AT, DE, HR, PL, SI, SK
2*x* + 6*x*	1 (0·4 %)	1 (12)	–	1 (51)	–	–	–	–	AT
Pure 6*x*	83 (36·2 %)	–	–	83 (2168)	–	–	–	–	AT, HU, PL, RO, RU, SI, SK
6*x* + mc	14 (6·1 %)	–	–	14 (351)	7 (13)	3 (3)	1 (1)	5 (7)	AT, HU, SK

Cytotypes: 2*x*, diploid; 3*x*, triploid; 6*x*, hexaploid; 7*x*, heptaploid; 8*x*, octoploid; 9*x*, nonaploid; An., aneuploid; mc, minority cytotype.
Country codes: AT, Austria; DE, Germany; HR, Hungary; PL, Poland; RO, Romania; RU, Russia; SI, Slovenia; SK, Slovakia.

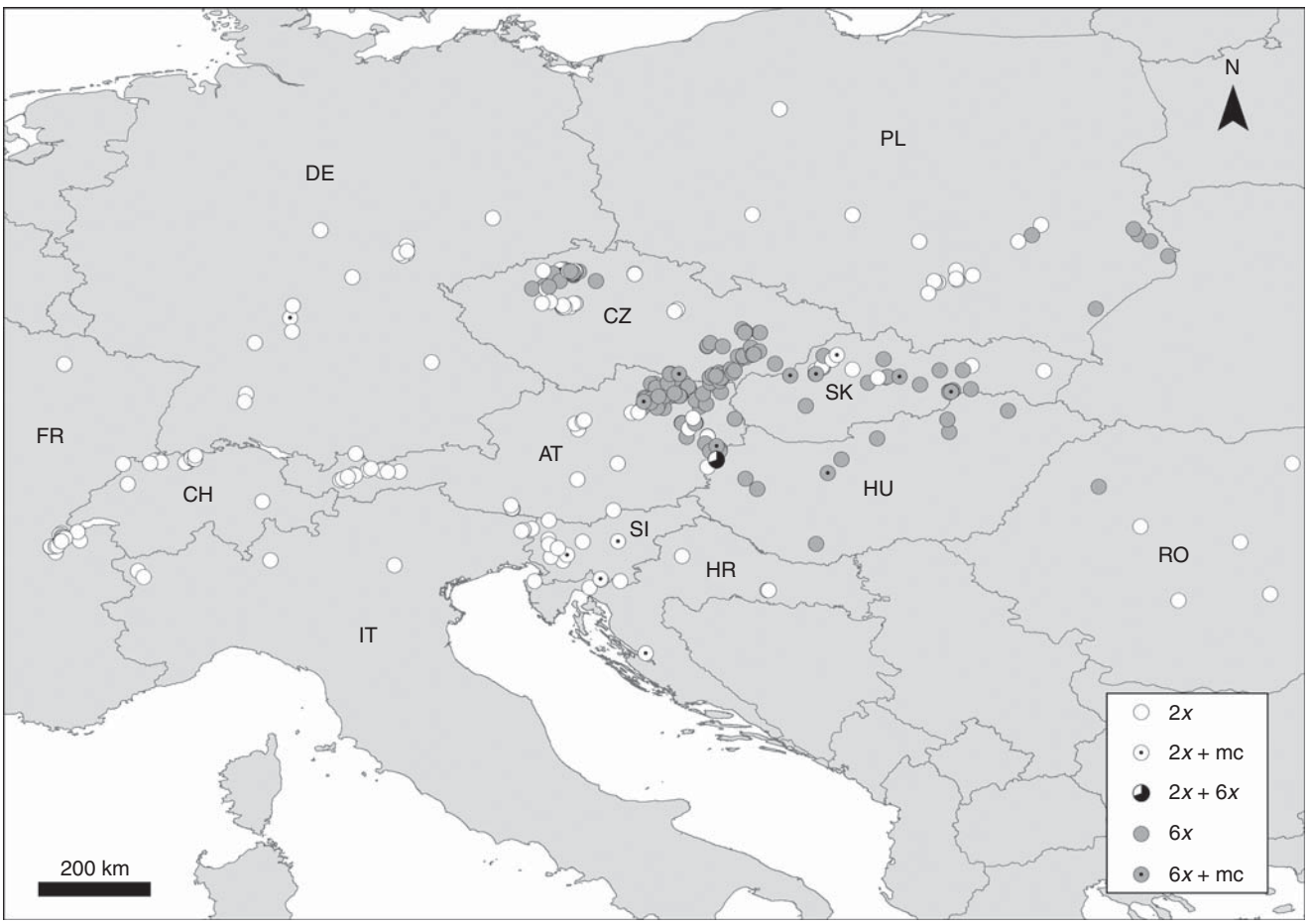

FIG. 2. Large-scale distribution of *Aster amellus* agg. cytotypes in Europe. The inset shows the symbols used to characterize the cytotype composition of each population (2*x*, diploid; 6*x*, hexaploid; mc, minority cytotypes). Country codes: AT, Austria; CH, Switzerland; CZ, Czech Republic; FR, France; DE, Germany; HR, Croatia; HU, Hungary; IT, Italy; PL, Poland; RO, Romania; SI, Slovenia; SK, Slovakia. The populations from Russia and Turkey (6*x* and 2*x*, respectively) are not displayed on the map. The populations from the Czech Republic were obtained from Mandáková and Münzbergová (2006).

in some populations (of both diploids and hexaploids), a few aneuploids were detected. Various degrees of aneuploidy were also confirmed by chromosome counting (e.g. $2n = 59$, 68 or 83 chromosomes).

The overall distribution pattern of cytotypes is depicted in Fig. 2. The populations from Russia and Turkey, which are not displayed in the figure, were pure hexaploid and pure diploid populations, respectively. The results of the population from Turkey were surprising considering that the plants were identified as *A. amellus* subsp. *ibericus*, supposedly tetraploids. Similarly to what was observed by Mandáková and Münzbergová (2006), the secondary contact zone is rather complex, not being restricted to the Czech Republic, but expanding to Austria in the south, Hungary (despite the fact that the only Hungarian diploid population was found near the border with Austria) and Romania in the south-east,

Slovakia in the east, and Poland in the north-east. Diploid populations were found throughout the whole studied area, while hexaploid populations were only found in central and eastern Europe. Indeed, no hexaploid populations were found west of the Czech Republic (Fig. 2). As reported for the Czech Republic (Mandáková and Münzbergová, 2006), diploid and hexaploid individuals are found growing in populations in close proximity in Austria and Slovakia. Nevertheless, they co-occur in the same population only in one case (Strebersdorf, Austria) (Fig. 3) and the reproductive

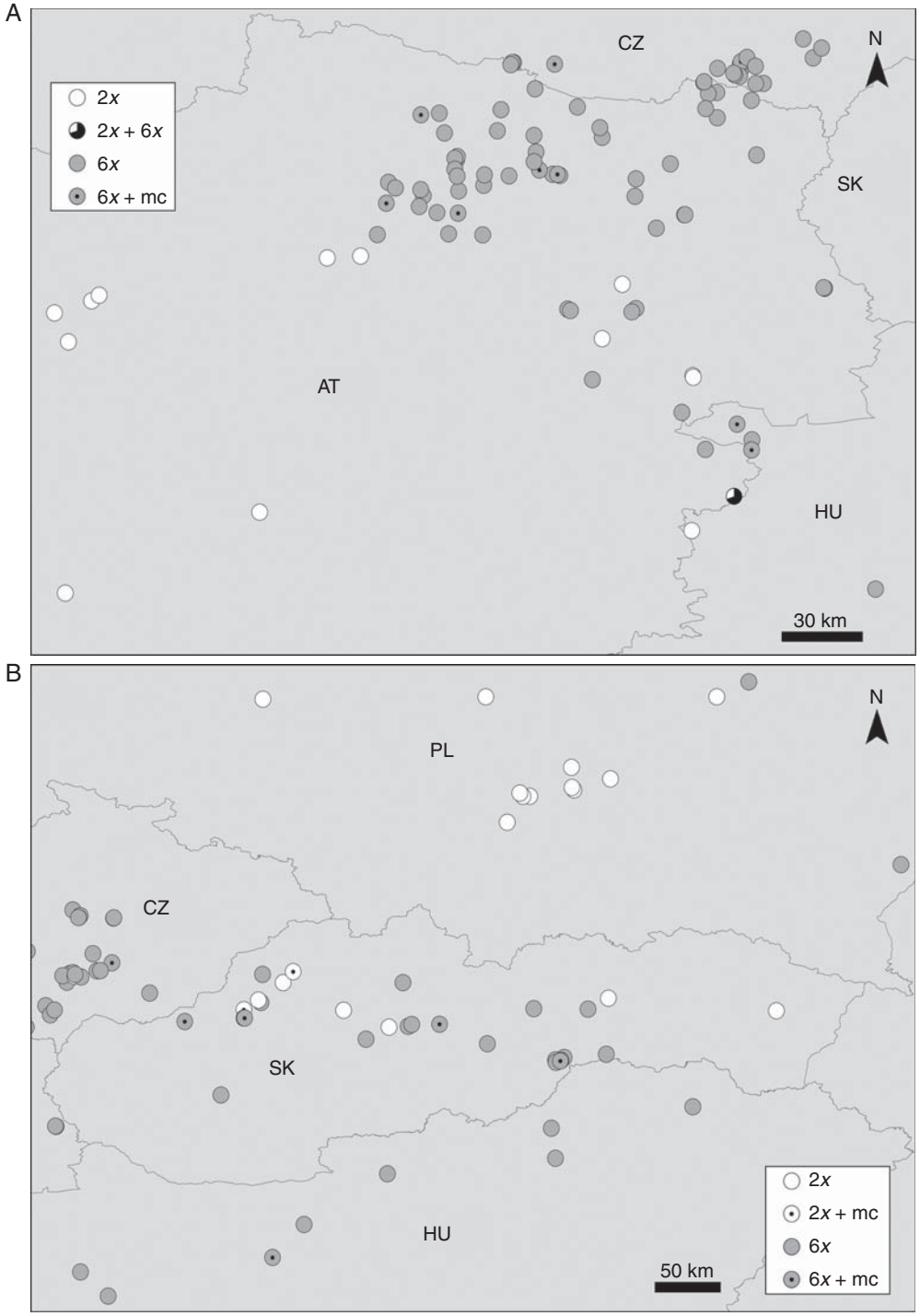

FIG. 3. Distribution of *Aster amellus* agg. cytotypes at the regional scale: a detailed distribution in the secondary contact zone in north east Austria (A) and Slovakia (B). The insets show the symbols used to characterize the cytotype composition of each population (2*x*, diploid; 6*x*, hexaploid; mc, minority cytotypes). In (A) the symbol of the mixed-ploidy population reflects the relative proportion of cytotypes (2*x*, white; 6*x*, black). Country codes: AT, Austria; CZ, Czech Republic; HU, Hungary; PL, Poland; SK, Slovakia.

isolation between diploids and hexaploids due to geographical segregation is 98·7 %. In Austria there is a prevalence of hexaploid populations in the north-east, with diploid populations occurring in the centre and the south (Fig. 3A). In Slovakia, hexaploid populations are also in the majority, but diploid populations are found along the longitudinal range of the country and intermingled with hexaploid populations (Fig. 3B).

Fine-scale cytotype screening

A detailed study of cytotype distribution was performed in the only population exhibiting both diploid and hexaploid individuals (Fig. 4; Table 2). The population is a small sized patch of 26 × 5 m in a *Pinus sylvestris* forest border near a secondary road in Strebersdorf (Austria). The mixed-ploidy population comprised 34 vegetative plants and 29 reproductive plants (in 2009). Independently of the ontogenic stage, there was a prevalence of hexaploids (81·0 % overall) over diploids (19·0 % overall). Diploid individuals occurred in only half of the population, but hexaploid individuals grew intermingled with diploid plants (Fig. 4) and no spatial segregation was detected [no significant deviations of J*ij* (r) from 1). Despite of the close proximity and lack of physical barriers, no hybrids (i.e. tetraploid individuals) were detected among the adult plants (Table 2). No differences were observed between cytotypes for the number of florets per flower head, but significant differences were detected in the reproductive success measured as seed set and seedling emergence (Table 3), with hexaploids producing more seeds per head and having a higher percentage of seedling emergence than diploids (Table 3).

The analysis of the ploidy level of seeds and seedlings (germinated from collected seeds in the mixed-ploidy population) enabled us to obtain a more complete picture of the ploidy composition and of the dynamics in the mixed-ploidy population (Table 2). For seeds the pattern was complex, with diploid individuals giving origin mostly to diploids (68·0 %), but also to a substantial number of tetraploids (28·0 %) and one pentaploid (4·0 %). Also, the majority of the hexaploid mothers produced hexaploid seeds (73·8 %), but several tetraploid (7·7 %) and aneuploid (17·7 %) seeds were also detected. Surprisingly, one of the seeds obtained from hexaploid individuals was triploid (0·8 %), suggesting the possible occurrence of apomixis. Still, despite the fact that some tetraploids were detected at the seed stage, all the seedlings presented the ploidy of the mother (except two aneuploids produced by diploid mothers). The observed frequencies of tetraploids at both seed and seedling stages were significantly lower than expected ($z = 5{\cdot}77$, $P < 0{\cdot}001$ and $z = 8{\cdot}69$, $P < 0{\cdot}001$, re-

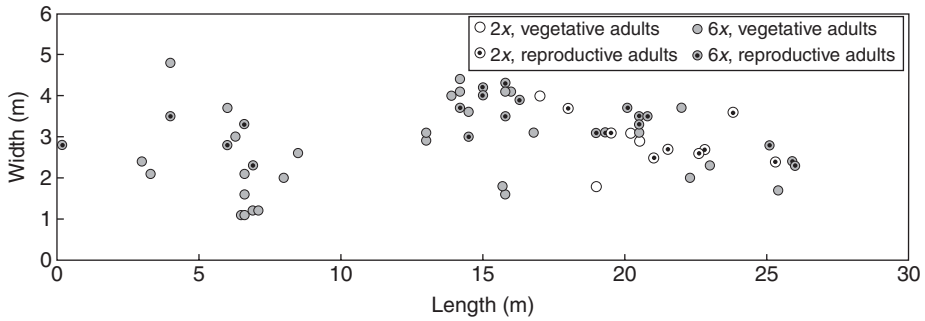

FIG. 4. Fine-scale distribution of *Aster amellus* cytotypes in the mixed-ploidy (2*x*, 6*x*) population (Strebersdorf, Austria). All adult plants (both reproductive and vegetative plants) were mapped.

TABLE 2. *Ploidy level of* Aster amellus *adult plants (reproductive and vegetative plants), seeds and seedlings from the mixed-ploidy population (2*x *+ 6*x*, Strebersdorf, Austria)*

Ontogenic stage	Ploidy level of the mother plants	No. and percentage (%) of samples with a given ploidy level						Total no. of analysed samples	Total no. of analysed mother plants
		2*x*	3*x*	4*x*	5*x*	6*x*	An.		
Vegetative plants	Unknown	4 (11·8 %)	–	–	–	30 (88·2 %)	–	34	–
Reproductive plants	Unknown	8 (27·6 %)	–	–	–	21 (72·4 %)	–	29	–
Seeds	2*x*	17 (68·0 %)	–	7 (28·0 %)	1 (4·0 %)	–	–	26	4
	6*x*	–	1 (0·8 %)	10 (7·7 %)	–	96 (73·8 %)	23 (17·7 %)	130	15
Seedlings	2*x*	15 (88·2 %)	–	–	–	–	2 (11·8 %)	17	5
	6*x*	–	–	–	–	305 (100·0 %)	–	305	18

Cytotypes: 2*x*, diploid; 3*x*, triploid; 4*x*, tetraploid; 5*x*, pentaploid, 6*x*, hexaploid; An., aneuploid. Percentages provide the fraction of each cytotype within the ontogenic stage.

TABLE 3. *Summary of reproductive variables of diploid and hexaploid* Aster amellus *growing in the mixed-ploidy population (2x + 6x, Strebersdorf, Austria)*

Cytotype	*n*	No. of florets per flower head	No. of seeds per flower head	Seed set (%)	Seedling emergence (%)
Diploids (2*x*)	8	89·5 ± 11·6	22·6 ± 11·6	22·1 ± 7·4	24·6 ± 10·4
Hexaploids (6*x*)	20	85·5 ± 4·5	35·1 ± 4·5	41·7 ± 5·8	63·8 ± 7·2
Statistical test		$t = 0{\cdot}285$ $P = 0{\cdot}778$	$t = -1{\cdot}226$ $P = 0{\cdot}231$	$t = -1{\cdot}911$ $P = 0{\cdot}034$	$t = -2{\cdot}787$ $P = 0{\cdot}005$

Values are given as mean and s.e.m.; sample size (*n*) is also provided. Seed set was calculated as the percentage of developed seeds from the total number of florets per flower head.

spectively) for a mixed-ploidy population composed of 19·0 and 81·0 % of diploid and hexaploid reproductive plants, respectively.

DISCUSSION

The current study shows that the contact zone between diploids and hexaploids of *A. amellus* is much larger and more complex than previously envisaged (Mandáková and Münzbergová, 2006), as it reaches Austria in the south, Poland in the north-east and Romania in the extreme east of the surveyed areas. In several regions, diploids and hexaploids were found to grow in close proximity, but not in sympatry. One small mixed-ploidy population bearing the two basic ploidy levels (diploids and hexaploids) was detected for the first time in nature, but no established hybrids (tetraploids) were detected.

The cytotype distribution patterns revealed that diploid populations are scattered all over the surveyed area, while hexaploid populations are longitudinally restricted, occurring exclusively east of Germany. These results are in accordance with the little available information on chromosome counts (reviewed in Münzbergová *et al.*, 2011). Májovský (1978) hypothesized on the origin of the *A. amellus* agg. The author speculated that the diploid *A. amellus* was the relict member of the group, which during the harsh climate conditions of the Tertiary, in refuges in the Carpathian basin and through hybridization with a tetraploid type (theoretically, *A. ibericus*), gave rise to the hexaploids (after the doubling of chromosomes of the triploid hybrid; allopolyploid origin; Ramsey and Schemske, 1998). Indeed, the cytotype distribution patterns could support this hypothesis, with further expansion of the hexaploids to the north-west and (probable) extinction of the tetraploid progenitor. Still, not all pieces of this complex puzzle fit perfectly together: first, the hexaploid occurs much further north-west than expected based on this hypothesis; secondly, the hexaploid population detected in Russia is unexpected; thirdly, the supposed tetraploid *A. ibericus* (syn. *A. amellus* subsp. *ibericus*) might actually be hexaploid (as suggested by the population analysed from Turkey). Thus, another so far neglected but equally likely hypothesis can be anticipated. Hexaploids may have originated during the harsh climate conditions of the Tertiary through diploids, after the fusion of a reduced and an unreduced gamete, forming a triploid that subsequently suffered genome duplication (autopolyploid origin; Ramsey and Schemske, 1998). Indeed, several studies have shown that unreduced gamete formation is one of the main processes involved in the origin of polyploid plants (e.g. Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998; Lavia *et al.*, 2011). Despite autopolyploids being difficult to identify, several lines of evidence, such as isozymes and plant morphology, suggest that hexaploid individuals of *A. amellus* are of autopolyploid origin (Mandáková and Münzbergová, 2008), supporting the latter hypothesis. In the future, phylogenetic studies should be developed to test definitely autopolyploid vs. allopolyploid origin and single vs. multiple origins of the hexaploid populations (e.g. Soltis and Soltis, 1993; Halverson *et al.*, 2008; Mason-Gamer, 2008).

Contact zones between cytotypes are natural laboratories to study the evolution of reproductive interactions among ploidy levels (Thompson and Lumaret, 1992; Petit *et al.*, 1999; Lexer and van Loo, 2006). Two types of contact zones can be defined according to the evolutionary theory behind the emergence of cytotypes (Petit *et al.*, 1999). The primary contact zone is the result of the emergence of a (higher) polyploid within a diploid/lower polyploid population, whereas a secondary contact zone results from the contact between allopatric cytotypes after migration (Petit *et al.*, 1999). The present patterns of *A. amellus* cytotype distribution (Mandáková and Münzbergová, 2006; this study), as well as the absence of hybrids in natural conditions (Castro *et al.*, 2011; this study) and the different evolutionary histories of the two cytotypes (Mandáková and Münzbergová, 2008), support the hypothesis of a secondary contact zone (Thompson and Lumaret, 1992). Indeed, most of the contact zones involving diploids and autopolyploids are thought to result from secondary contact (e.g. Felber-Girard *et al.*, 1996; van Dijk and Bakx-Schotman, 1997; Kolař *et al.*, 2009), showing a parapatric distribution pattern and a low level of hybrid formation (Soltis and Soltis, 1993; Petit *et al.*, 1997; Kolař *et al.*, 2009). The *A. amellus* secondary contact zone may have arisen through range expansion after the Pleistocene and might be currently maintained by minority cytotype exclusion (Levin, 1975; see also discussion of the mixed-ploidy population below). Still, the complex contact zone revealed in this study, with intermingled parapatric populations of diploid and hexaploid plants and one mixed-ploidy population, suggests a more complex scenario.

Considering that previous studies have shown that the gene pools of the diploids and hexaploids are not isolated (Mandáková and Münzbergová, 2008) and that there is no hybridization between cytotypes in natural populations (Castro *et al.*, 2011; this study), gene flow can only occur through the recurrent formation of polyploids (Soltis and Soltis, 1993). The neopolyploid will establish when a set of breeding

barriers and/or ecological features promotes assortative mating, increasing its reproductive success (Rieseberg and Willis, 2007; see also discussion of the mixed-ploidy population below). Further support for this hypothesis is the detection of triploid plants in several populations of the diploid cytotype (Mandáková and Münzbergová, 2008; this study) and the detection of multiple chloroplast haplotypes that are shared across cytotypes, suggesting a multiple origin of hexaploids at the contact zone (S. Castro *et al.*, unpubl. res.). As both hypotheses are not mutually exclusive, it seems plausible to assume that both scenarios may be currently ongoing, and thus that the polyploid *A. amellus* presents a complex contact zone comprised of both primary and secondary contacts. The occurrence of primary and secondary contact zones has been suggested for several other polyploid complexes, such as *Knautia arvensis* agg. (Kolař *et al.*, 2009), *Dianthus* spp. (Weiss *et al.*, 2002) and *Melampodium* spp. (Stuessy *et al.*, 2004). To understand fully the origin of polyploid *A. amellus* individuals and the genetic patterns across the distribution range of this species, genetic analyses are currently being developed.

The discovery of the first mixed-ploidy population provided further clues on the ecological dynamics of the diploid–hexaploid contact zone. Partial reproductive barriers between cytotypes have often been reported (e.g. van Dijk *et al.*, 1992; Petit *et al.*, 1997; Husband and Schemske, 1998). These can result from pre-zygotic (e.g. habitat differentiation, phenological divergence) or post-zygotic (e.g. triploid block effect, hybrid sterility) isolation mechanisms (Petit *et al.*, 1999). In *A. amellus*, temporal isolation and segregation due to ecological preferences and pollinator behaviour seem to contribute little to cytotype isolation, whereas geographic and post-pollination barriers lead to complete isolation between diploids and hexaploids (Mandáková and Münzbergová, 2006; Castro *et al.*, 2011; this study). Within the natural mixed-ploidy population, the detection of a proportion of tetraploids lower than expected at the seed stage and their complete absence in seedlings and adult plants suggested that post-pollination barriers are operating both before and after fertilization, as already described by Castro *et al.* (2011) from common garden experiments. As suggested by those authors, the most important barriers that may contribute to such results are pollen–pistil interactions and/or genomic imprinting and endosperm formation.

The reproductive success of each cytotype is crucial for their maintenance within mixed-ploidy populations (Levin, 1975; Felber, 1991; Rodriguez, 1996). In a simulated mixed-ploidy population of *A. amellus*, both cytotypes presented similar reproductive success (Castro *et al.*, 2011). However, in natural conditions, the scenario was completely different, with diploids having significantly lower reproductive success, and significantly higher production of intercytotype (non-viable) offspring than hexaploids. This reproductive disadvantage of diploid individuals may have major long-term impacts on population structure, eventually leading to the exclusion of this cytotype as a result of frequency-dependent selection [minority cytotype exclusion theory (Levin, 1975; Rodriguez, 1996)] and, ultimately, resulting in another single-cytotype population. Hexaploids were also shown to have slightly higher selfing rates than diploids (Castro *et al.*, 2011). This feature will further increase the probability of successful establishment of a newly formed hexaploid or of a newly arrived hexaploid individual in a diploid population. Although only one mixed-ploidy population was found, these results suggest that the contact zone may be more dynamic than expected, and a long-term survey focused on the evolution of cytotype composition at different reproductive stages was initiated for this population.

In conclusion, the results of this study show that the contact zone is not restricted to the Czech Republic but expands to the east in several directions. In the surveyed area most populations presented only one cytotype; in the contact zone, several diploid and hexaploid populations are parapatric; and only one mixed-ploidy population bearing diploids and hexaploids was detected, but there were no signs of cytotype hybridization in the field. Regarding the prevailing theories for the origin of *A. amellus* cytotypes, the results obtained so far suggest an autopolyploid origin, and a highly dynamic and a complex contact zone potentially with primary and secondary contacts, with the minority cytotype exclusion being one of the driving forces operating in some populations.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Table S1: details of the populations of *Aster amellus* agg. studied.

ACKNOWLEDGEMENTS

The authors thank all the Botanical Gardens that provided seeds and information on the wild localities (see details in Supplementary Data); to numerous researchers for providing valuable chorological information on this species, particularly to Harald Niklfeld ('Mapping the Flora of Austria – Database'); to several researchers who kindly collected samples, namely Romain Mayor, Boštjan Surina, Viktor Kerényi-Nagy and Attila Mesterházy; to Mária Šurinová and Kateřina Seifertová for technical support; to Vlasta Jarolímová for assessing the chromosome numbers; and to Lucía DeSoto for discussion in spatial analysis. This work was supported by the Portuguese Foundation for Science and Technology and European Social Fund (FCT/BPD/41200/2007 to S.C.), by the Grant Agency of the Czech Republic (P506/10/P188 to S.C. and P504/10/0456 to Z.M.) and partially by the Institute of Botany of the Academy of Sciences of the Czech Republic (AV0Z60050516 to Z.M.).

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