Annals of Botany **107**: 77–87, 2011
doi:10.1093/aob/mcq217, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY Founded 1887

Remarkable coexistence of multiple cytotypes of the *Gymnadenia conopsea* aggregate (the fragrant orchid): evidence from flow cytometry

Pavel Trávníček[1,2], Barbora Kubátová[3], Vladislav Čurn[3], Jana Rauchová[1,2], Eva Krajníková[3], Jana Jersáková[4] and Jan Suda[1,2,*]

[1]*Institute of Botany, Academy of Sciences of the Czech Republic, CZ-252 43 Průhonice, Czech Republic,* [2]*Department of Botany, Faculty of Science, Charles University in Prague, CZ-128 01 Prague, Czech Republic,* [3]*Biotechnological Centre, Faculty of Agriculture, University of South Bohemia, CZ-370 05 České Budějovice, Czech Republic and* [4]*Faculty of Science, University of South Bohemia and Institute of System Biology and Ecology, CZ-370 05 České Budějovice, Czech Republic*
* *For correspondence. E-mail* suda@natur.cuni.cz

Received: 20 June 2010 Returned for revision: 27 July 2010 Accepted: 4 October 2010 Published electronically: 7 November 2010

• *Background and Aims* One of the prerequisites for polyploid research in natural systems is knowledge of the geographical distribution of cytotypes. Here inter- and intrapopulational ploidy diversity was examined in the *Gymnadenia conopsea* aggregate in central Europe and potential explanations and evolutionary consequences of the observed spatial patterns investigated.
• *Methods* DAPI flow cytometry supplemented by confirmatory chromosome counts was used to determine ploidy in 3581 samples of the *G. conopsea* aggregate from 43 populations. The fine-scale spatial pattern of cytotype distribution (intra- and interploidy associations) was analysed with univariate and bivariate *K*-functions.
• *Key Results Gymnadenia* tissues undergo a progressively partial endoreplication, which accounts for about 60 % and 75 % of the total genome in *G. conopsea* and *G. densiflora*, respectively. Flow cytometric profiles are therefore species-specific and can be used as a marker for rapid and reliable species recognition. Two majority (4*x*, 8*x*) and three minority (6*x*, 10*x*, 12*x*) cytotypes were found, often in mixed-ploidy populations (harbouring up to all five different ploidy levels). The scarcity of the minority cytotypes (about 2·7 %) suggests the existence of strong pre- or postzygotic mating barriers. Spatial structure was observed in plots of populations with the highest cytotype variation, including clumping of individuals of the same ploidy and negative association between tetra- and octoploids.
• *Conclusions* The remarkable ploidy coexistence in the *G. conopsea* aggregate has reshaped our perception of intrapopulational ploidy diversity under natural conditions. This system offers unique opportunities for studying processes governing the formation and establishment of polyploids and assessing the evolutionary significance of the various pre- and postzygotic mating barriers that maintain this ploidy mixture.

Key words: Coexistence, contact zone, cytotype mixture, flow cytometry, *Gymnadenia conopsea*, hybridization, mating barriers, polyploidy, progressively partial endoreplication, spatial distribution, sympatry.

INTRODUCTION

Polyploidy, the presence of more than two complete genomes per cell, has long been recognized as an important evolutionary force in the plant kingdom. Since the pioneering studies conducted about a century ago (e.g. Lutz, 1907), the number of recognized polyploid plant species has increased dramatically, and recent genomic data suggest the near ubiquity of polyploidy in angiosperms (Soltis *et al.*, 2009). The evolutionary success of polyploids may be related to increased heterozygosity, reduced inbreeding depression, broader ecological amplitude and/or better colonizing ability relative to their diploid counterparts (Levin, 2002; Parisod *et al.*, 2010).

The last decade has seen significant progress in our understanding of the mechanisms and rates of polyploid formation in both autopolyploids (polyploids arising within or among populations of the same species) and allopolyploids (polyploids combining genomes of at least two parental species) (Ramsey and Schemske, 1998). Extensive use of molecular markers has markedly changed our perception of the dynamics of polyploidy under natural conditions and has revealed that genome duplication is not a rare, macroevolutionary step but a dynamic and ongoing process (Soltis *et al.*, 2004). In addition, early views on ploidy variation in several plant groups had to be revisited in light of recent findings that revealed much more prolific cytotype differentiation in wild populations than previously thought (Kron *et al.*, 2007). Although different ploidy levels may delineate different taxa (Suda *et al.*, 2007*a*; see also Soltis *et al.*, 2007), intraspecific ploidy variation is not a rare phenomenon (Suda *et al.*, 2007*b*; Duchoslav *et al.*, 2010; Li *et al.*, 2010; Marhold *et al.*, 2010). When multiple cytotypes occur within the same species, zones of ploidy overlap usually appear. Two kinds of contact zones are generally recognized depending on their history (Petit *et al.*, 1999): primary (when a new cytotype originates *in situ*) and secondary (when two formerly allopatric cytotypes meet). Transitional zones are of particular interest to evolutionary biologists as they allow mechanisms involved in the early stages of polyploid speciation to be studied and the selective forces operating in mixed-ploidy

© The Author 2010. Published by Oxford University Press on behalf of the Annals of Botany Company. All rights reserved.
For Permissions, please email: journals.permissions@oxfordjournals.org

Downloaded from https://academic.oup.com/aob/article/107/1/77/176429 by guest on 17 April 2024

populations to be assessed. Sympatric growth of different ploidies is mostly explained either by directional or balanced selection (Weiss *et al.*, 2002). Directional selection assumes that cytotype intermingling is only a transitional stage and that one cytotype will finally be outcompeted (the minority cytotype exclusion; Levin, 1975). Balanced selection assumes that long-term coexistence can be maintained due to assortative mating that reinforces isolation between related cytotypes. Assortative mating can be attained by (among other ways) divergent flowering time (Bretagnolle and Thompson, 1996; Petit *et al.*, 1997), microhabitat differentiation (Hülber *et al.*, 2009; Šafářová and Duchoslav, 2010), differences in floral morphology resulting in species-specific placement of pollen on the pollinator (Grant, 1994), apomixis, selfing and vegetative propagation (Kao, 2007). In addition, pollinator behaviour is another recently acknowledged component that may shape the dynamics of mixed-ploidy populations in animal-pollinated species (Husband and Schemske, 2000; Thompson and Merg, 2008).

Advances in our understanding of patterns and dynamics of genome duplication in natural conditions have been catalysed by the advent of flow cytometry (FCM), which has allowed us to gain detailed insight into ploidy variation at different spatial and temporal scales (Kron *et al.*, 2007; Suda *et al.*, 2007*a*). In contrast to other cytogenetic techniques, FCM can easily process large population samples and therefore provides a much more accurate picture of ploidy variation (Kolář *et al.*, 2009; Trávníček *et al.*, 2010). Consequently, the biogeographical and evolutionary processes that shape cytotype distribution patterns can be reliably assessed, as can the interactions, ecological preferences and pre- and postzygotic breeding barriers of individual cytotypes. In addition, FCM sampling is virtually non-destructive, thus paving the way for detailed studies of rare and endangered plants without the risk of population destruction.

Gymnadenia conopsea (the fragrant orchid; Orchidaceae) is a Eurasian taxon that shows considerable variation across its distributional range (Hultén and Fries, 1986). Four ploidy levels ($2n = 40$, 80, 100 and 120) have been found to occur in central Europe (Groll, 1965; Marhold *et al.*, 2005). In addition to its karyological heterogeneity, differences in plant morphology (Soliva and Widmer, 1999; Dworschak, 2001), flowering phenology (Lönn *et al.*, 2006; Jersáková *et al.*, 2010), genetic diversity (Campbell *et al.*, 2007) and habitat preference (Möseler, 1987; Gustafsson and Lönn, 2003) have also been reported. The species is self-compatible but pollinator-dependent for fruit set (Gustafsson, 2000). Attempts to classify the observed variation formally led to the description of several taxa at below-species ranks (e.g. Dworschak, 2001; Vöth and Sontag, 2006), but the taxonomic value of several of these is rather low. Two basic types are generally recognized in central Europe nowadays, referred to as *G. conopsea* and *G. densiflora*. Floral scent is considered the most important diagnostic characteristic, but differences in plant morphology (total height, number of flowers per inflorescence, number and width of leaves, flower size), time of flowering and ecological preferences have also been reported (Rose, 1988; Marhold *et al.*, 2005; Jersáková *et al.*, 2010). However, species determination may not always be straightforward, as shown, for example, by the incidence of morphotypes corresponding to 4*x* *G. conopsea* but flowering later in the season (Gustafsson and Lönn, 2003; Lönn *et al.*, 2006). Molecular studies have been used to justify the splitting of *G. conopsea* aggregate into several genetically distinct groups with restricted gene flow, although the genetic groups often showed only little correspondence with morphotypes (Scacchi and Angelis, 1989; Soliva and Widmer, 1999; but see Campbell *et al.*, 2007). A precise taxonomic treatment of the group may further be impeded by the fact that several morphotypes/phenotypes/evolutionary units often grow in sympatry (Soliva and Widmer, 1999; Gustafsson and Lönn, 2003; Marhold *et al.*, 2005; Lönn *et al.*, 2006).

The coexistence of different phenological variants (e.g. Lönn *et al.*, 2006) and indices for intrapopulational cytotype heterogeneity (Marhold *et al.*, 2005) have prompted the present research into ploidy variation in *G. conopsea*. Four basic questions were addressed: (1) What is the population cytotype structure of the *G. conopsea* aggregate in central Europe (based on representative sampling)? (2) How frequent are mixed-ploidy populations and which cytotypes are involved? (3) Which minority cytotypes can be found and under which conditions? (4) What is the fine-scale distribution pattern in mixed-ploidy populations? The present results form the basis for a detailed investigation of processes governing the formation, establishment and persistence of polyploids in populations of the *Gymnadenia conopsea* aggregate.

MATERIALS AND METHODS

Sampling design

Forty-three *Gymnadenia* populations from the Czech Republic and Slovakia were sampled during 2004–2009, spanning the geographic range 48°49′N to 50°44′N and 13°32′E to 19°23′E (Table 1 and Fig. 1; see Supplementary data, available online for locality and herbarium voucher details). When possible, leaf tissue from at least 50 individuals was collected at each locality. The sampling was designed to cover the entire range of morphological and phenological variation at each locality and to include plants from various microhabitats. If spatially separated habitats with different ecological conditions (e.g. dry upper slopes and waterlogged valley bottom) occurred at a given locality, it was then considered to constitute separate localities. Leaf tissue was wrapped in moist paper towels, kept in plastic bags and processed within 2 d.

A detailed fine-scale analysis of cytotype distribution was performed for the three localities where the highest ploidy variation was found (Table 1). Five plots were laid out in areas with abundant *Gymnadenia* plants, every individual was mapped, and leaf tissue was collected for FCM estimation of DNA ploidy level. The total number of cytotyped plants in the present study was 3581.

Flow cytometry

DNA ploidy levels were inferred from the relative fluorescence intensities of DAPI-stained nuclei using flow cytometry. Intact leaf tissue from each plant to be analysed (about 0·5 cm^2) was chopped together with an appropriate volume of the internal standard (*Pisum sativum* ‘Ctirad’, 2C = 9·09 pg;

Downloaded from https://academic.oup.com/aob/article/107/1/77/176429 by guest on 17 April 2024

TABLE 1. *Population cytotype structure in 43 central-European localities of* Gymnadenia conopsea *agg. (for locality details, see Supplementary data, available online)*

No.	Locality	No. of plants	DNA ploidy level					
			4*x* C	4*x* D	8*x*	6*x*	10*x*	12*x*
1	Milčice	73			72			1
2	Javorník	60	58			2		
3	Opolenec	68			68			
4	Chvalšiny	14			14			
5	Chrášťany	50		50				
6	Líský	50		50				
7	Houžetín	70		70				
8	Granátka	24		24				
9	Knobloška	63		62		1 (D)		
10	Polabská černava	50	1	49				
11	Bílé stráně	95	48	43		2 + 2 (D)		
12	Podloučky	50			50			
13	Tanvald	56	55			1		
14	Rokytnice nad Jizerou	50	48			2		
15	Janova hora	76	74			2		
16	Dolní Čepí	50		5	41		2	2
17	Jobova Lhota	50		5	44		1	
18	Kuřim	50		49		1 (D)		
19	Hustopeče	66			66			
20	Čertoryje	50	50					
21*	Zahrady pod Hájem	867	295	152	386	22 + 1 (D) + 5 (?)	4	2
22	Machová – wetland	60		58		2 (?)		
23	Machová	53	50		3			
24*	Jazevčí	475	328		137	4	4	2
25	Porážky	67	8	1	57	1		
26	Velká Javořina	62	56		1	5		
27	Cestiska	100	49	50		1		
28	Ježůvka	50	48			2		
29	Lúčky–Roveňky	50		50				
30	Bílé potoky	30	30					
31	Velké Karlovice	50	50					
32	Veterník	60			60			
33	Súlov	72	29	39	2	2		
34	Malé Lednice	37		37				
35	Turie	69	16		48	4	1	
36*	Porúbka	102			92	2	6	2
37	Nižné Kamence	30	29			1		
38	Rovná hora	50	12		36	2		
39	Vlkolínec – wetland	50		50				
40	Vlkolínec	50	2		47			1
41	Vlkolínec – valley	50			50			
42	Osádka	12	8	3	1			
43	Lúčky Kúpele	20		20				
Σ		3581	1343	867	1275	67	18	10

Populations in which the fine-scale spatial distribution was studied in detail are marked with an asterisk. 4*x* C = tetraploid *G. conopsea*; 4*x* D = tetraploid *G. densiflora*. Minority cytotypes (6*x*, 10*x* and 12*x*) were derived from *G. conopsea*, unless indicated otherwise. D = hexaploids derived from *G. densiflora*; (?), hexaploids of uncertain origin, possibly hybrids between *G. conopsea* and *G. densiflora*.

Doležel *et al.*, 1998) using a sharp razor blade in a Petri-dish containing 0·5 mL of ice-cold Otto I buffer (0·1 M citric acid, 0·5 % Tween 20; Otto, 1990). The crude suspension was filtered through a 42-μm nylon mesh and incubated for 10 min at room temperature. Isolated nuclei were stained with 1 mL of Otto II buffer (0·4 M $Na_2HPO_4.12H_2O$) supplemented with 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 4 $\mu g\ mL^{-1}$ and β-mercaptoethanol (2 $\mu L\ mL^{-1}$). After a few minutes, the relative fluorescence intensity of at least 3000 particles was recorded using a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) equipped with a mercury arc lamp as the source of UV excitation light. Histograms were evaluated using FloMax software, ver. 2·4d (Partec GmbH). Up to three *Gymnadenia* plants were analysed together during the large-scale ploidy screening. Each plant was re-analysed separately if mixed-ploidy samples were found or if the quality of the histograms was not sufficient (i.e. coefficient of variation of any peak above 5 %). Karyologically counted tetraploid ($2n = 40$) and octoploid ($2n = 80$) plants were used as reference points when inferring DNA ploidy levels.

Chromosome counts

Chromosomes were counted in the actively growing root tips of mature plants. Samples were pretreated with a saturated solution of *p*-dichlorbenzene (3 h, room temperature), fixed in

Downloaded from https://academic.oup.com/aob/article/107/1/77/176429 by guest on 17 April 2024

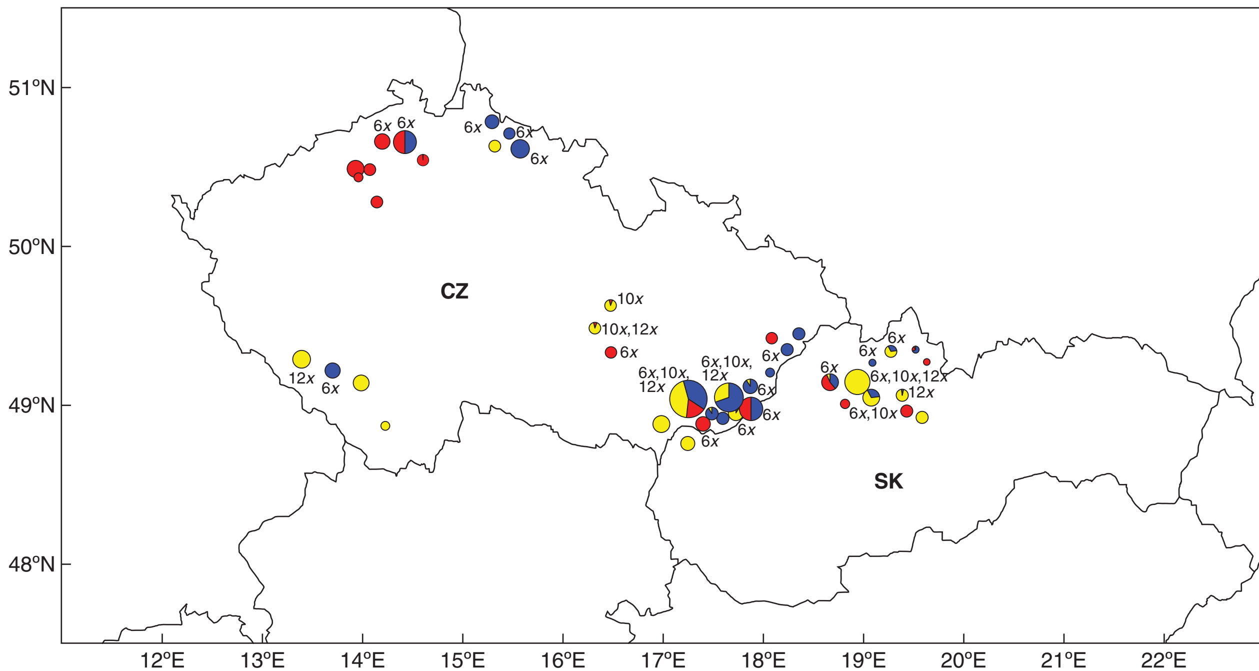

FIG. 1. Distribution of the cytotypes of *Gymnadenia conopsea* agg. in the area studied based on 3581 individuals from 43 localities. Chart size is proportional to the number of sampled individuals. Blue, 4*x* *G. conopsea*; red, 4*x* *G. densiflora*; yellow, 8*x* *G. conopsea*. Minority cytotypes are indicated with the numbers 6, 10 and 12.

a 3 : 1 mixture of ethanol and cold acetic acid (4 h, 4 °C), macerated in 1 : 1 hydrochloric acid : ethanol (30 s, room temperature) and immediately squashed in a drop of lactopropionic orceine. The number of chromosomes was determined in five to ten complete well-spread mitotic plates using a Carl-Zeiss Jena NU microscope equipped with an Olympus Camedia C-2000 Z camera. The basic chromosome number $x = 10$ was considered when interpreting chromosome counts in terms of ploidy levels.

Spatial analyses

To look for spatial patterns in the cytotype distribution, the mapped locations of the individuals were analysed using the *K*-function (Ripley, 1977) in the R-package 'spatstat' (Baddeley and Turner, 2005). With the *K*-function, the type (whether clumped, random or regular) and intensity of an individual distribution is determined by counting the number of neighbours within a circle of radius *r* of each individual in the study plot and comparing the mean number with the expected number derived from the unit density calculated on the basis of the plot area and the number of individuals in the plot. To depict the spatial patterns at various scales, the *K*-function was transformed to the *L*-function (Doležal *et al.*, 2006). On a graph of $L(r)$ vs. r, positive, zero and negative values of the $L(r)$ function indicate clumped, random and regular patterns, respectively, over a distances of *r*. Furthermore, pairwise inter-cytotype associations were examined with the bivariate $K_{12}(r)$-function (Cressie, 1993) and visualized with its derived $L_{12}(r)$-function. In this approach, positive, neutral and negative associations are assessed by counting only neighbours of the other cytotypes within a circle of radius *r* of each individual. The 95 % confidence interval was determined using a Monte Carlo simulation with 1000 replications. Distributions and associations were only determined for the three majority cytotypes (i.e. 4*x* and 8*x* *G. conopsea* and 4*x* *G. densiflora*).

RESULTS

Interpretation of flow histograms

Gymnadenia samples yield complex flow histograms with several peaks arranged in an endopolyploidy-like fashion (the average coefficient of variation for *Gymnadenia* peaks was 3·26 %). Up to four peaks of *Gymnadenia* nuclei (designated as 2C, 2C + P, 2C + 3P and 2C + 7P, in which P denotes the DNA content of the replicated part of the 2*n* nucleus; for more detailed explanation, see Discussion) were recognizable on the flow histograms (Fig. 2). Whereas the 2C + P and 2C + 3P peaks were present in most individuals, the 2C and 2C + 7P peaks were often not recognizable, making the inference of DNA ploidy level challenging. For instance, more than half of the tetraploid plants analysed lacked the first (2C) peak. In addition, interpretation of the results was further complicated by unusual peak ratios, which deviated markedly from genuine endopolyploidy (i.e. 1 : 2 : 4 : 8, etc.; (Table 2). Nevertheless, simultaneous evaluation of the ratios between individual *Gymnadenia* peaks and reference standard/sample peak ratios allowed DNA ploidy level to be reliably inferred. Figure 3 shows a two-dimensional scatterplot (based on ratios between 2C + P *Gymnadenia* peak/reference standard peak and 2C + 3P/2C + P peaks of *Gymnadenia*) with well-separated groups of measurements corresponding to different ploidy levels/different species.

Downloaded from https://academic.oup.com/aob/article/107/1/77/176429 by guest on 17 April 2024

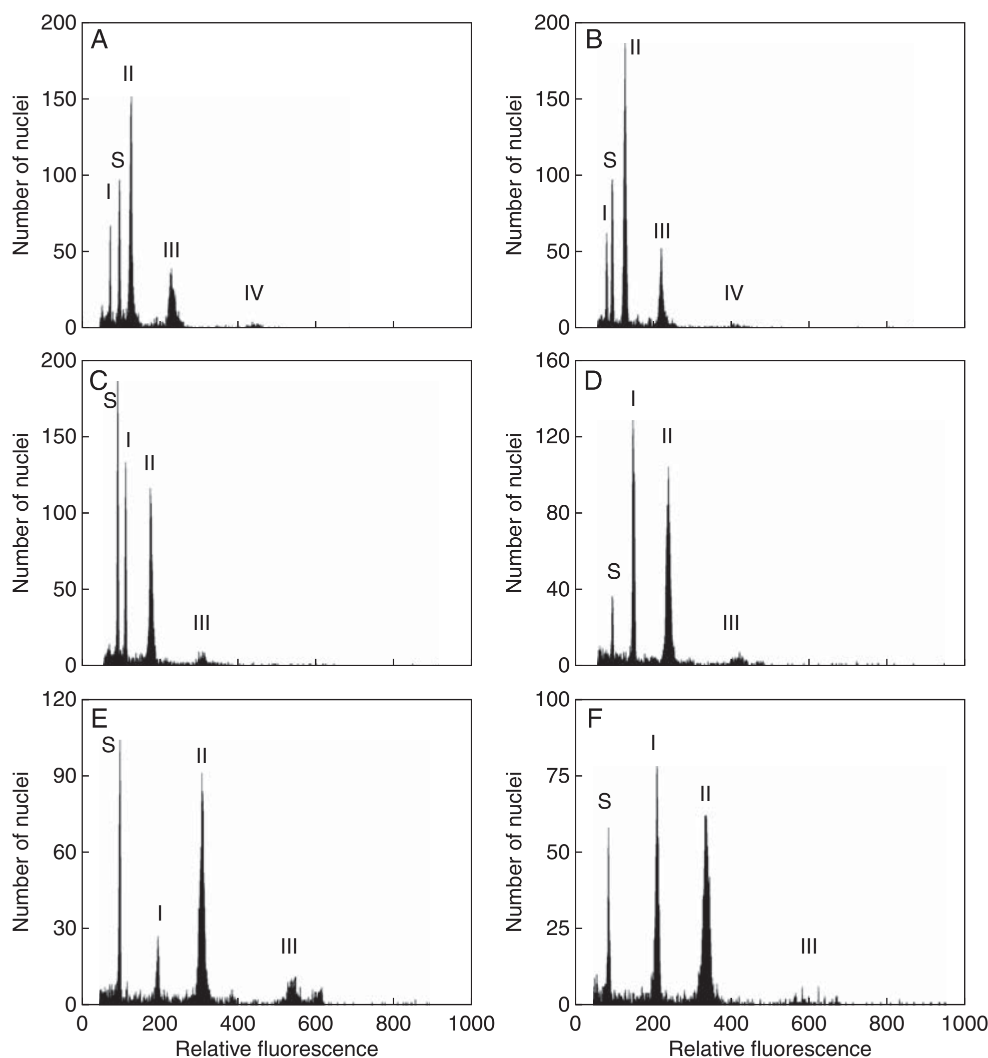

FIG. 2. Representative flow cytometric histograms of all ploidy levels found in *Gymnadenia conopsea* agg. (analysed together with the internal reference standard *Pisum sativum*). Nuclei of both the sample and standard were isolated, stained with DAPI and simultaneously run on the flow cytometer. (A) tetraploid *G. densiflora*; (B) tetraploid *G. conopsea*; (C) hexaploid *G. conopsea*; (D) octoploid *G. conopsea*; (E) decaploid *G. conopsea*; (F) dodecaploid *G. conopsea*. I, II, III and IV, peaks of *Gymnadenia* nuclei undergoing different numbers of partial endoreplication cycles (2C, 2C + P, 2C + 3P and 2C + 7P); S, internal standard *Pisum sativum*.

Two distinct patterns of fluorescence intensity were observed when tetraploid plants tentatively identified as *G. conopsea* and *G. densiflora* were measured. Whereas the average peak ratios for the former species were 1 : 1·59 : 2·74 : 5·09, the latter species showed average ratios of 1 : 1·75 : 3·24 : 6·23 (see Table 2). These data indicate that FCM profiles in *Gymnadenia* are species-specific and can be used as a taxonomic marker. The peak ratios of the higher ploidy levels mostly matched those of tetraploid *G. conopsea* (Table 2), suggesting that the higher polyploids were largely derived from this species.

Chromosome counts

The number of chromosomes was determined in 11 plants representing all ploidy levels detected using FCM. The following chromosome numbers were obtained: $2n = 4x = 40$ (one sample), $2n = 6x = 60$ (four samples), $2n = 8x = 80$ (three samples), $2n = 10x = 100$ (two samples) and $2n = 12x = 120$ (one sample).

Ploidy variation and population cytotype structure

Five different ploidy levels (4*x*, 6*x*, 8*x*, 10*x* and 12*x*) were detected among 3581 plants analysed (Fig. 2 and Table 1), with hexaploids being recorded for the first time. Tetraploid and octoploid cytotypes clearly predominated and accounted for 61·7 % (37·5 % corresponding to *G. conopsea* and 24·2 % corresponding to *G. densiflora*) and 35·6 % of all plants, respectively. The frequency of the minority ploidy levels (6*x*, 10*x* and 12*x*) varied from about 1·9 % for hexaploids (67 individuals) to about 0·5 % for decaploids (18 individuals) and about 0·3 % for dodecaploids (ten individuals). Although the minority cytotypes accounted for only 2·7 % of all samples,

Downloaded from https://academic.oup.com/aob/article/107/1/77/176429 by guest on 17 April 2024

TABLE 2. *Flow cytometric profiles of five different ploidy levels of* Gymnadenia conopsea *agg. showing progressively partial endoreplication*

DNA ploidy level	Peak ratios against internal reference standard (*Pisum sativum*)								Ratios between individual *Gymnadenia* peaks		
	2C		2C + P		2C + 3P		2C + 7P		2C + P/2C	2C + 3P/2C + P	2C + 7P/2C + 3P
	Mean ± s.d.	*n*	Mean ± s.d.	*n*	Mean ± s.d.	*n*	Mean ± s.d.	*n*	Mean ± s.d.	Mean ± s.d.	Mean ± s.d.
4*x* D	0·747 ± 0·017	94	1·301 ± 0·027	151	2·419 ± 0·058	151	4·656 ± 0·136	144	1·747 ± 0·030	1·859 ± 0·021	1·925 ± 0·023
4*x* C	0·839 ± 0·023	125	1·327 ± 0·035	317	2·303 ± 0·059	318	4·269 ± 0·117	275	1·585 ± 0·024	1·736 ± 0·017	1·853 ± 0·023
6*x*	1·236 ± 0·042	43	1·985 ± 0·044	60	3·479 ± 0·100	59	6·376 ± 0·218	8	1·607 ± 0·043	1·753 ± 0·046	1·853 ± 0·028
8*x*	1·587 ± 0·036	339	2·542 ± 0·058	340	4·439 ± 0·116	335	8·147 ± 0·208	37	1·601 ± 0·019	1·746 ± 0·020	1·841 ± 0·017
10*x*	1·995 ± 0·034	18	3·161 ± 0·065	18	5·517 ± 0·129	17	–	–	1·584 ± 0·017	1·740 ± 0·021	–
12*x*	2·380 ± 0·048	9	3·798 ± 0·083	10	6·588 ± 0·257	6	–	–	1·595 ± 0·011	1·731 ± 0·030	–

2C denotes the DNA content of the 2*n* nucleus; P denotes the DNA content of the replicated portion of the 2*n* nucleus (see the text for explanation). The lower number of observations for the 2C and 2C + 7P peaks is due to the fact that these peaks were not recognizable in some individuals. Note the different peak ratios in the tetraploid species. 4*x* C = tetraploid *G. conopsea*; 4*x* D = tetraploid *G. densiflora*.

they were found in more than half of the populations studied (23 out of 43). Tetraploids corresponding to *G. conopsea* and *G. densiflora* occurred at 22 and 20 localities, respectively, whereas hexaploids occurred at 19 localities, octoploids at 20 localities, and both decaploids and dodecaploids occurred at six localities.

Ploidy mixing was common and nearly 60 % of the *Gymnadenia* populations analysed (25 out of 43) harboured two or more cytotypes (Table 1). Two different ploidies co-occurred at 13 localities (= 30 %), three ploidies at seven localities (= 16 %) and four ploidies at three localities (= 7 %). All five cytotypes grew in sympatry at two localities: no. 21 (south-east Moravia, Velká nad Veličkou–Zahrady pod Hájem) and no. 24 (south-east Moravia, Javorník–Jazevčí). Whereas tetra- and octoploid plants formed some cytotype-uniform populations (three of 4*x G. conopsea*, six of 8*x G. conopsea*, eight of 4*x G. densiflora*), minority cytotypes only occurred together with individuals of one or both majority ploidies (Table 1). Disregarding single-cytotype populations, 16 different cytotype/species combinations were observed, the most common of which was a sympatric occurrence of 4*x G. conopsea* and hexaploid individuals, which was encountered at six localities.

In addition to ploidy co-occurrence, *G. conopsea* and *G. densiflora* plants also co-occurred occasionally (seven localities). Their relative abundance varied from rather equal proportions (e.g. locations 11, 27 and 33) to a marked predominance of one or the other species (e.g. locations 10 and 25). Sometimes, *G. conopsea* and *G. densiflora* grew in spatially isolated microhabitats within the same macrolocality (see locations 22 + 23 and 39 + 40).

Fine-scale cytotype distribution

To get greater insight into ploidy distribution at fine spatial scales, detailed cytotype screening was performed in five plots laid out in the three most ploidy-diverse localities (three plots at location 21 and one plot each at locations 24 and 36). Figure 4A shows the cytotype distribution in plot I at location 21 (Zahrady pod Hájem); the distribution patterns in the remaining four plots are shown in the Supplementary data.

Intra- and intercytotype associations were examined in plots with two or three coexisting majority ploidy levels/species. In all cytotypes, individuals of the same ploidy clumped together, especially at distances larger than 50 cm (Fig. 4B and Supplementary data). Octoploid *G. conopsea* showed either negative (location 21 – plot I, location 24) or no association (location 21 – plots II and III) with tetraploid *G. conopsea*, and negative (location 21 – plot III) or no association (location 21 – plots I and II) with tetraploid *G. densiflora* (Fig. 4C and Supplementary data). Both tetraploid species were positively associated in two plots (location 21 – plots I and III) or showed neutral associations (location 21 – plot II).

DISCUSSION

A detailed screen of cytotype variation was performed in 43 populations of the *G. conopsea* aggregate in central Europe. Flow cytometric analyses of 3581 individuals and 11 confirmatory chromosome counts revealed the occurrence of two

Downloaded from https://academic.oup.com/aob/article/107/1/77/176429 by guest on 17 April 2024

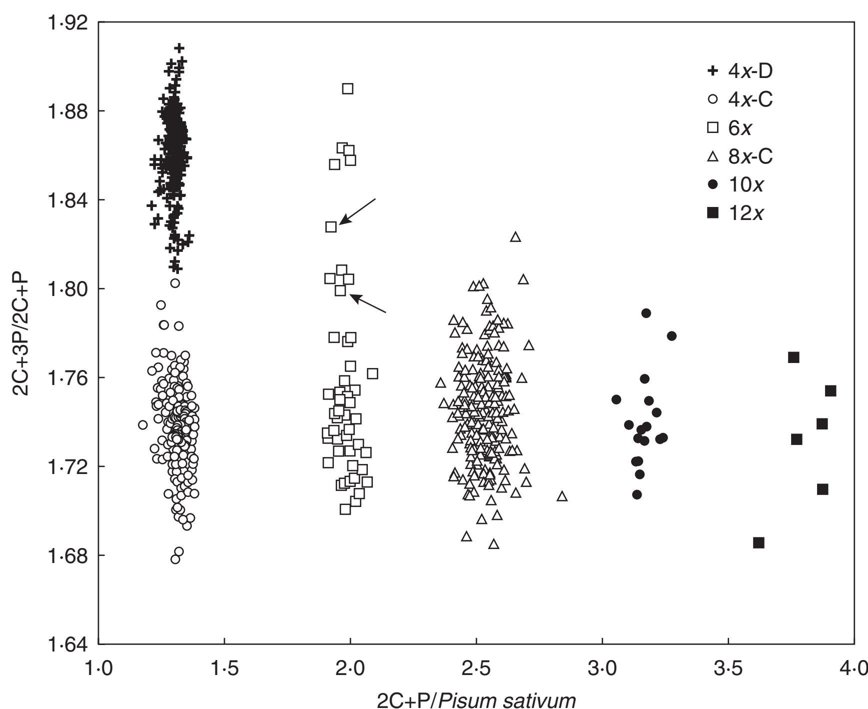

FIG. 3. Two-parametric scatterplot of the flow cytometric data. The *x*- and *y*-axes show the ratio between the 2C + P peak of *Gymnadenia* and the internal reference standard (*Pisum sativum*) and the ratio between 2C + 3P and 2C + P peaks of *Gymnadenia*, respectively. Arrows point to hexaploids originating in pure populations of *G. densiflora* (locations 9 and 18). 4*x*-D, tetraploid *G. densiflora*; 4*x*-C and 8*x*-C, tetraploid and octoploid cytotypes of *G. conopsea*, respectively.

majority (4*x*, 8*x*) and three minority (6*x*, 10*x*, 12*x*) ploidy levels, often in mixed-ploidy populations. With respect to the number of co-occurring cytotypes, the *G. conopsea* aggregate represents the most marked example of ploidy coexistence ever recorded.

Flow cytometric profiles and ploidy estimation

Fluorescence histograms of nuclei isolated from *Gymnadenia* leaves consist of several peaks and superficially resemble the FCM profile of endopolyploid tissues. However, unlike what would be observed for genuine endoreplication (Barow and Jovtchev, 2007), the peak ratios differ from integer multiples of two and are distinctly lower (Table 2; see also Suda *et al.*, 2007*a*). Moreover, the ratios between consecutive *Gymnadenia* peaks are not identical but increase progressively (e.g. the average ratios between the two neighbouring peaks in tetraploid *G. conopsea* varied from 1·59 to 1·85; Table 2). Despite the fact that the observed histograms are unusual (we have never seen a similar pattern in any other plant group except orchids), we are convinced that the present FCM measurements are reliable and not negatively influenced, for instance, by the presence of interfering secondary metabolites (Loureiro *et al.*, 2006). First, highly comparable peak ratios were obtained using DNA fluorochromes with different modes of binding (AT-selective DAPI, intercalating propidium iodide), different isolation buffers (Otto, LB01) and different reference species (*Pisum sativum*, *Vicia faba*, no internal standard) (data not presented). It should also be noted that reasonably low coefficients of variation were achieved regardless of the protocol modification. In addition, analyses of different plant tissues (leaves of various ages, stems, sepals, young capsules, roots, protocorms) resulted in identical peak ratios, suggesting that the FCM profile observed is systemic. Moreover, the peak ratios remained unchanged with simultaneous analyses of samples with different FCM profiles (e.g. different ploidy levels, *G. conopsea* + *G. densiflora*).

An analogous situation was previously reported in another orchid, *Vanilla planifolia* (Bory *et al.*, 2008). The authors observed peak ratios ranging from 1·43 to 1·82, and named this phenomenon 'progressively partial endoreplication'. They suggested that individual peaks of such samples should not be designated as 2C, 4C, 8C, 16C, etc. but rather 2C, 2C + P, 2C + 3P, 2C + 7P, etc., where P is the DNA content of the replicated part of the 2*n* nucleus (Bory *et al.*, 2008). This theory is in full accord with the present FCM measurements. As in *Vanilla*, perfect linearity was found between the DNA content (relative fluorescence intensity) of individual *Gymnadenia* peaks and the number of endoreplication cycles, suggesting that the same genome part (or chromosome batch) is amplified at each cycle. About 60 % and 75 % of the genome is replicated at each cycle in *G. conopsea* and *G. densiflora*, respectively (Table 2). The actual portion of the replicated genome and the mechanism(s) behind progressively partial endoreplication (e.g. whether genuine partial replication or rather elimination of some DNA after whole genome duplication) remain unknown. Potential links between progressively partial endoreplication and mycorrhizal infection, which was found to cause nuclear hypertrophy (Barroso and Pais, 1990), also need to be investigated.

Downloaded from https://academic.oup.com/aob/article/107/1/77/176429 by guest on 17 April 2024

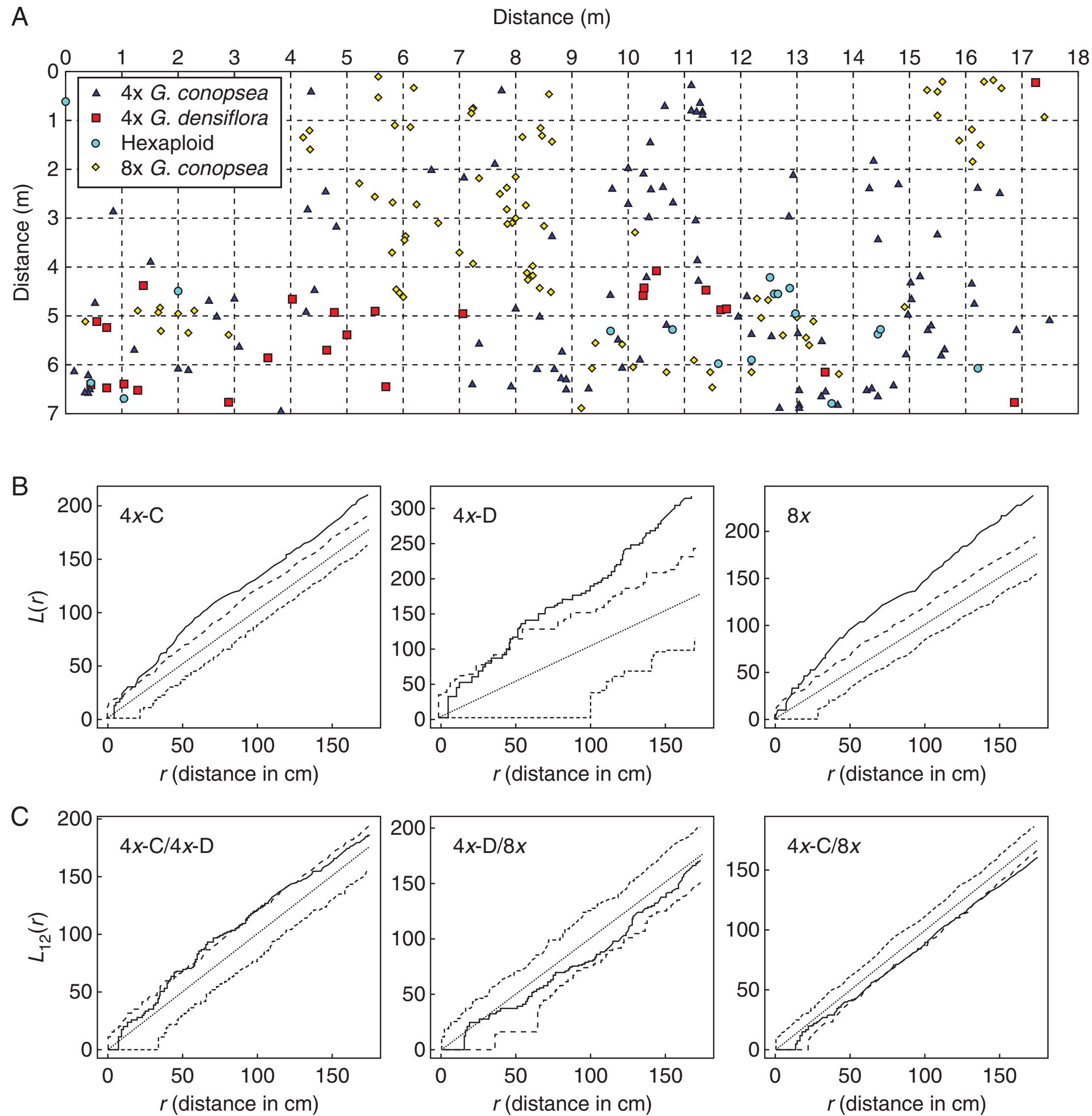

FIG. 4. (A) Cytotype distribution in plot I at location 21 (south-east Moravia, Velká nad Veličkou–Zahrady pod Hájem): 4*x* *G. conopsea*, 4*x* *G. densiflora*, hexaploid and 8*x* *G. conopsea* as indicated. (B) Spatial pattern of cytotype distribution – values of the $L(r)$-function are shown by a thick continuous line, dashed lines denote the 95 % confidence interval. Values larger than the upper confidence limit indicate significant intracytotype aggregation at the particular distance of r. (C) Pairwise inter-cytotype associations – values of the $L_{12}(r)$-function are shown by a thick continuous line, dashed lines denote the 95 % confidence interval. Values larger than the upper confidence limit indicate positive association and values smaller than the lower confidence limit indicate negative association. See Supplementary data, available online, for spatial patterns of cytotypes [and corresponding $L(r)$- and $L_{12}(r)$-functions] in other plots.

In addition to the occurrence of progressively partial endoreplication, the estimation of ploidy in *Gymnadenia* using FCM is further hindered by different proportions of nuclei undergoing different numbers of endoreplication cycles. Unreplicated nuclei (2C phase) often constitute only a minority fraction and can be unrecognizable on a flow histogram (most often in tetraploid plants). Nevertheless, such cases can be easily identified on the basis of cycle-specific fluorescence intensities of individual nuclei classes (by comparing the ratios between individual pairs of *Gymnadenia* peaks; see Table 2). Progressively partial endoreplication therefore allows reliable recognition of the presence or absence of unreplicated nuclei (and thus unambiguous ploidy inference), in contrast to genuine endopolyploidy, in which this task may pose a serious problem (Barow and Jovtchev, 2007). The reliability of the present ploidy estimates was confirmed by conventional karyological counts and using two-dimensional scatterplot on FCM data, which resulted in six well-separated clusters of samples corresponding to the different ploidy levels/species (Fig. 3).

The observed differences in FCM profiles between *G. conopsea* and *G. densiflora* (about 12 % smaller genome size and a higher proportion of endoreplicated genome in the latter species) indicates that *G. densiflora* is a well-defined evolutionary unit that merits classification as a separate taxon. Considering its ecological, phenological and phenotypic differences, the rank of species seems to be appropriate (see Marhold

Downloaded from https://academic.oup.com/aob/article/107/1/77/176429 by guest on 17 April 2024

et al., 2005). Flow cytometry can be utilized as a convenient, cheap and fast analytical tool for reliable species recognition.

Ploidy diversity and the origin of higher polyploids

Substantial karyological diversity (five different even ploidy levels ranging from *4x* to 12*x*) was found in populations of the *G. conopsea* aggregate in central Europe; individuals of odd ploidy levels seem to be lacking under natural conditions. Comparable intraspecific ploidy variation is only rarely seen in other sexually reproducing plants with monocentric chromosomes: *Ixeris nakazonei* with six cytotypes (Denda and Yokota, 2004), *Senecio carniolicus* with five cytotypes (Suda *et al.*, 2007*b*) and *Cardamine yezoensis* with six cytotypes (Marhold *et al.*, 2010) are some exceptions. Hexaploids of *G. conopsea* are reported here for the first time, documenting the value of flow cytometry for detecting rare evolutionary events and cryptic diversity *in situ*. Quite surprisingly, these previously unknown hexaploids were the most common minority cytotype in the present study (they accounted for about 1·9 % of the samples analysed), whereas the much rarer deca- and dodecaploids (with a cumulative frequency below 0·8 %) had already been recorded in the past using conventional karyological methods (Groll, 1965; Marhold *et al.*, 2005). It should be noted that the latter authors considered the basic chromosome number in *Gymnadenia* to be $x = 20$. Although the published karyological evidence does not contradict their opinion, we believe that the correct basic chromosome number is $x = 10$ (as suggested by Fuchs and Ziegenspeck, 1924), because our preliminary ploidy estimates in experimental *Gymnadenia* crosses revealed a few plants with fluorescence intensities corresponding to putative pentaploids with 50 somatic chromosomes.

The present FCM data (the pattern of progressively partial endoreplication in particular) suggest that *G. conopsea s.s.* shows high ploidy variation whereas *G. densiflora* is karyologically much less variable and encompasses only tetraploid and rare hexaploid plants. Octoploids of *G. conopsea* are most likely of autopolyploid origin. In addition to our FCM evidence, further support comes from the very similar composition of flower scent and the results of preliminary genetic analyses (Jersáková *et al.*, 2010). Two evolutionary pathways were most likely involved in the genesis of hexaploids as indicated by the cytotype composition of populations harbouring hexaploid plants: (1) fusion of reduced and unreduced gametes of a tetraploid (only tetraploids of *G. conopsea* and *G. densiflora* coexisted with 6*x* individuals in seven and three populations, respectively), and (2) hybridization between 4*x* and 8*x* cytotypes (both majority cytotypes were observed in five populations; Table 1). High polyploids (10*x*, 12*x*) can originate by several different evolutionary pathways that involve either reduced or unreduced gametes of majority cytotypes and hexaploids.

Population cytotype structure and ploidy coexistence

Populations of the *G. conopsea* aggregate were found to be very diverse with respect to cytotype composition. Because these orchids are declining in many European countries (e.g. Holub and Procházka, 2000), this finding has important conservation implications. Not only the total population size but also intrapopulational cytotype variation needs to be considered when conservation priorities are being set because population size does not always correlate with ploidy diversity. Whereas some large *Gymnadenia* populations in the present study appeared to be ploidy-uniform (e.g. nos. 19 and 30), other populations with few surviving individuals showed high karyological diversity, one example being locality no. 16 (Dolní Čepí), which harbours four different cytotypes.

Cytotype distribution at large spatial scale (Fig. 1) is more or less random, and only slight associations between ploidy and geographical position and/or altitude were found (e.g. the lack of *G. densiflora* in south-west Bohemia and its prevalence in north-west Bohemia). Populations with multiple cytotypes are more common in the area studied than their single-cytotype counterparts. Although the number of known mixed-ploidy populations has been increasing steadily in recent years (largely as a result of more representative sampling and easy and convenient cytotyping using flow cytometry; Kron *et al.*, 2007), usually only two or rarely three coexisting cytotypes have been reported (Šafářová and Duchoslav, 2010, and references therein). *Gymnadenia* therefore represents an exceptional case of high intrapopulational cytotype diversity, with >10 % of investigated populations harbouring four or five different cytotypes (Table 1). In the light of these findings it may be surprising that the majority of previous studies (e.g. Gustafsson, 2000; Gustafsson and Sjögren-Gulve, 2002; Gustafsson and Lönn, 2003; Huber *et al.*, 2005; Lönn *et al.*, 2006; Campbell *et al.*, 2007) neglected the karyological variation, and only recently have ploidy data been incorporated into phenotypic, ecological and/or genetic investigations on the *G. conopsea* aggregate (Marhold *et al.*, 2005; Jersáková *et al.*, 2010).

Despite being quite rare, the minority cytotypes (6*x*, 10*x*, 12*x*) significantly contributed to the ploidy mixture. Minority ploidies altogether occurred in 23 populations but in most populations were only represented by one (nine populations) or two (seven populations) individuals. It is therefore likely that without detailed ploidy screening using FCM many such cases would remain undetected. The occurrence of minority cytotypes in geographically distant populations suggests that they originated recurrently, and it illustrates the high dynamics of genome duplication and the complex inter-ploidy reproductive interactions that occur under natural conditions. However, no conclusion about the evolutionary stability of mixed-ploidy populations (whether in equilibrium or only temporary) can be drawn at the current stage of the investigation. In addition, possible links between the history of the localities and their ploidy diversity remain to be established.

In the most ploidy-diverse populations, the spatial distribution of the cytotypes was examined at a microgeographical scale to address whether geographic segregation contributes to inter-cytotype reproductive isolation. Theoretical studies suggest that long-term sympatric growth of cytotypes can only be maintained if the different ploidy levels have strong pre- or postzygotic reproductive isolation mechanisms (Levin, 1975; Rodriguez, 1996). Unlike other studies that tested the aggregation of cytotypes using various randomization analyses (Halverson *et al.*, 2008; Šafářová and Duchoslav, 2010; Trávníček *et al.*, 2010), a more sophisticated statistical approach was employed here.

Downloaded from https://academic.oup.com/aob/article/107/1/77/176429 by guest on 17 April 2024

It involved univariate and bivariate *K*-functions (Ripley, 1977; Cressie, 1993), which provide detailed information about the type of distribution and inter-cytotype associations, respectively, over particular distances (Fig. 4 and Supplementary data). In all of the mixed-ploidy *Gymnadenia* populations, a more or less distinct spatial structure was identified (at least at the scales we examined): (*a*) individuals of the same ploidy level always clumped together, and (*b*) octoploids and tetraploids tended to be negatively associated. Whether this non-random ploidy distribution is a consequence of microhabitat differentiation (e.g. soil patchiness; Diez, 2007), the spatial structure of symbiotic mycorrhizal fungi (Batty *et al.*, 2001) or some other factor remains to be determined.

Spatial segregation of different cytotypes (either due to ecological differentiation or environmentally independent processes such as founder effects or limited seed dispersal) seems to be the most common prezygotic reproductive barrier, and it was observed in ten out of 16 papers in which detailed intrapopulational ploidy distributions were examined in detail (see Šafářová and Duchoslav, 2010). Several authors counted spatial separation among the key factors for long-term ploidy coexistence (Husband and Sabara, 2004; Hülber *et al.*, 2009). However, the neutral inter-cytotype associations that we found in some plots (Fig. 4C and Supplementary data) leads us to presume that the relative contribution of geographical segregation to the total reproductive isolation of the *Gymnadenia* populations studied is rather low. Previous investigations into pre-mating barriers operating in mixed-ploidy *Gymnadenia* populations revealed a lack of assortative behaviour of pollinators and only partial temporal segregation between 4*x* *G. densiflora* and 8*x* *G. conopsea*, but a marked shift in flowering phenology between 4*x* and 8*x* cytotypes of *G. conopsea* (Jersáková *et al.*, 2010). Taken together, these data suggest that beside (partial) phenological separation alternative evolutionary mechanisms are most likely involved in the maintenance of mixed-ploidy populations of *Gymnadenia*.

Conclusions and future prospects

The *Gymnadenia conopsea* aggregate presents a remarkable example of high intraspecific ploidy variation (common 4*x* and 8*x* individuals, and rare 6*x*, 10*x* and 12*x* individuals) coupled with the frequent sympatric occurrence of several (up to five) different cytotypes. The scarcity of minority cytotypes suggests the existence of strong pre- or postzygotic mating barriers, the nature of which remains to be determined. Relative to other thoroughly investigated polyploid complexes (*Chamerion angustifolium* in particular; Husband and Sabara, 2004), *Gymnadenia* is a more complicated system (e.g. greater ploidy variation, coexistence of more cytotypes and dependence on mycorrhizal symbiosis) that can provide novel insight into the mechanisms and dynamics of polyploid speciation under natural conditions. The data presented here will set the stage for forthcoming studies aiming to understand the population processes governing the formation and establishment of polyploids and to assess the evolutionary significance of the various pre- and postzygotic reproductive barriers that maintain this remarkable ploidy mixture.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following: locality details for 43 *Gymnadenia* populations from the Czech Republic and the Slovak Republic; cytotype distribution at fine spatial scales; and pictures of the three main *Gymnadenia* types.

ACKNOWLEDGEMENTS

We thank the Czech authorities for nature conservation for issuing collecting permits, I. Jongepierová for assistance with the location of plant populations and Filip Kolář for help with statistical analyses of spatial distribution. This work was supported by the Czech Sciences Foundation (grant number 206/09/0843). Further support was provided by the Academy of Sciences of the Czech Republic (grant number AV0Z60050516) and the Ministry of Education, Youth and Sports of the Czech Republic (grant numbers MSM0021620828, MSM6007665806 and MSM6007665801).

LITERATURE CITED

Baddeley A, Turner R. 2005. Spatstat: an R package for analyzing spatial point patterns. *Journal of Statistical Software* **12**: 1–42.

Barow M, Jovtchev G. 2007. Endopolyploidy in plants and its analysis by flow cytometry. In: Doležel J, Greilhuber J, Suda J. eds. *Flow cytometry with plant cells. Analysis of genes, chromosomes and genomes*. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA, 349–372.

Barroso J, Pais MS. 1990. Nuclear features in infected roots of *Ophrys lutea* Cav. *New Phytologist* **115**: 93–98.

Batty AL, Dixon KW, Brundrett M, Sivasithamparam K. 2001. Constraints to symbiotic germination of terrestrial orchid seed in a Mediterranean bushland. *New Phytologist* **152**: 511–520.

Bory S, Catrice O, Brown S, *et al*. 2008. Natural polyploidy in *Vanilla planifolia* (Orchidaceae). *Genome* **51**: 816–826.

Bretagnolle F, Thompson JD. 1996. An experimental study of ecological differences in winter growth between sympatric diploid and autotetraploid *Dactylis glomerata*. *Journal of Ecology* **84**: 343–351.

Campbell VV, Rowe G, Beebee TJC, Hutchings MJ. 2007. Genetic differentiation amongst fragrant orchids (*Gymnadenia conopsea s.l.*) in the British Isles. *Botanical Journal of the Linnean Society* **155**: 349–360.

Cressie NAC. 1993. *Statistics for spatial data*. New York, NY: J. Wiley and Sons.

Denda T, Yokota M. 2004. Cytogeography of *Ixeris nakazonei* (Asteraceae, Lactuceae) in the Ryukyu Archipelago of Japan and Taiwan. *Journal of Plant Research* **117**: 3–11.

Diez JM. 2007. Hierarchical patterns of symbiotic orchid germination linked to adult proximity and environmental gradients. *Journal of Ecology* **95**: 159–170.

Doležel J, Bartoš J. 2005. Plant DNA flow cytometry and estimation of nuclear genome size. *Annals of Botany* **95**: 99–110.

Doležel J, Greilhuber J, Lucretti S, *et al*. 1998. Plant genome size estimation by flow cytometry: inter-laboratory comparison. *Annals Botany* **82** (Suppl. A): 17–26.

Doležal J, Šrůtek M, Hara T, Sumida A, Penttilä T. 2006. Neighborhood interactions influencing tree population dynamics in nonpyrogenous boreal forest in northern Finland. *Plant Ecology* **185**: 135–150.

Duchoslav M, Šafářová L, Krahulec F. 2010. Complex distribution patterns, ecology and coexistence of ploidy levels of *Allium oleraceum* (Alliaceae) in the Czech Republic. *Annals of Botany* **105**: 719–735.

Dworschak W. 2001. Versuch einer Gliederung der verschiedenen Erscheinungsformen von *Gymnadenia conopsea* in Südbayern. *Berichte der Arbeitskreise Heimische Orchideen* **18**: 193–207.

Fuchs A, Zeigenspeck H. 1924. Aus der Monographie des *Orchis traunsteineri* Saut. IV. Chromosomen einiger Orchideen. *Botanisches Archiv* **5**: 457–470.

Downloaded from https://academic.oup.com/aob/article/107/1/77/176429 by guest on 17 April 2024

Grant V. 1994. Modes and origins of mechanical and ethological isolation in angiosperms. *Proceedings of the National Academy of Sciences, USA* **91**: 3–10.

Groll M. 1965. Fruchtansatz, Bestäubung und Merkmalsanalyse bei diploiden und polyploiden Sippen von *Dactylorchis* (*Orchis*) *maculata* und *Gymnadenia conopsea*. *Österreichische Botanische Zeitschrift* **112**: 657–700.

Gustafsson S. 2000. Patterns of genetic variation in the fragrant orchid (*Gymnadenia conopsea*). *Molecular Ecology* **9**: 1863–1872.

Gustafsson S, Lönn M. 2003. Genetic differentiation and habitat preference of flowering-time variants within *Gymnadenia conopsea*. *Heredity* **91**: 284–292.

Gustafsson S, Sjögren-Gulve P. 2002. Genetic diversity in the rare orchid, *Gymnadenia odoratissima* and a comparison with the more common congener, *G. conopsea*. *Conservation Genetics* **3**: 225–234.

Halverson K, Heard SB, Nason JD, Stireman JO. 2008. Origins, distribution, and local co-occurrence of polyploid cytotypes in *Solidago altissima* (Asteraceae). *American Journal of Botany* **95**: 50–58.

Holub J, Procházka F. 2000. Red list of the flora of the Czech Republic (state in the year 2000). *Preslia* **72**: 187–230.

Huber FK, Kaiser R, Sauter W, Schiestl FP. 2005. Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae). *Oecologia* **142**: 564–575.

Hülber K, Sonnleitner M, Flatscher R, *et al*. 2009. Ecological segregation drives fine scale cytotype distribution of *Senecio carniolicus* (Asteraceae) in the Eastern Alps. *Preslia* **81**: 309–319.

Hultén E, Fries M. 1986. *Atlas of north European vascular plants north of the Tropic of Cancer I.* Königstein: Koeltz Scientific Books.

Husband BC, Sabara HA. 2004. Reproductive isolation between autotetraploids and their diploid progenitors in fireweed, *Chamerion angustifolium* (Onagraceae). *New Phytologist* **161**: 703–713.

Husband BC, Schemske DW. 2000. Ecological mechanisms of reproductive isolation and coexistence of diploid and tetraploid *Chamerion angustifolium*. *Journal of Ecology* **88**: 689–701.

Jersáková J, Castro S, Sonk N, *et al*. 2010. Absence of pollinator-mediated premating barriers in mixed-ploidy populations of *Gymnadenia conopsea s.l.* (Orchidaceae). *Evolutionary Ecology* **24**: 1199–1218.

Kao RH. 2007. Asexuality and the coexistence of cytotypes. *New Phytologist* **175**: 764–772.

Kolář F, Štech M, Trávníček P, *et al*. 2009. Towards resolving the *Knautia arvensis* agg. (Dipsacaceae) puzzle: primary and secondary contact zones and ploidy segregation at landscape and microgeographic scales. *Annals of Botany* **103**: 963–974.

Kron P, Suda J, Husband BC. 2007. Applications of flow cytometry to evolutionary and population biology. *Annual Review of Ecology, Evolution, and Systematics* **38**: 847–876.

Levin DA. 1975. Minority cytotype exclusion in local plant populations. *Taxon* **24**: 35–43.

Levin DA. 2002. *The role of chromosomal change in plant evolution*. New York, NY: Oxford University Press.

Li D, Liu Y, Zhong C, Huang H. 2010. Morphological and cytotype variation of wild kiwifruit (*Actinidia chinensis* complex) along an altitudinal and longitudinal gradient in central-west China. *Botanical Journal of the Linnean Society* **164**: 72–83.

Lönn M, Alexandersson R, Gustafsson S. 2006. Hybrids and fruit set in a mixed flowering-time population of *Gymnadenia conopsea* (Orchidaceae). *Hereditas* **143**: 222–228.

Loureiro J, Rodriguez E, Doležel J, Santos C. 2006. Flow cytometric and microscopic analysis of the effect of tannic acid on plant nuclei and estimation of DNA content. *Annals of Botany* **98**: 515–527.

Lutz AM. 1907. A preliminary note on the chromosomes of *Oenothera lamarckiana* and one of its mutants, *O. gigas*. *Science* **26**: 151–152.

Marhold K, Jongepierová I, Krahulcová A, Kučera J. 2005. Morphological and karyological differentiation of *Gymnadenia densiflora* and *G. conopsea* in the Czech Republic and Slovakia. *Preslia* **77**: 159–176.

Marhold K, Kudoh H, Pak JH, Watanabe K, Španiel S, Lihová J. 2010. Cytotype diversity and genome size variation in eastern Asian polyploid *Cardamine* (*Brassicaceae*) species. *Annals of Botany* **105**: 249–264.

Möseler BM. 1987. Zur morphologischen, phänologischen und standörtlichen Charakterisierung von *Gymnadenia conopsea* (L.) R. Br. ssp. *densiflora*. *Floristische Rundbriefe* **21**: 8–21.

Otto F. 1990. DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In: Crisssman HA, Darzynkiewicz Z. eds. *Methods in cell biology*, Vol. 33. New York, NY: Academic Press, 105–110.

Parisod C, Alix K, Just J, *et al*. 2010. Impact of transposable elements on the organization and function of allopolyploid genomes. *New Phytologist* **186**: 37–45.

Petit C, Lesbros P, Ge XJ, Thompson JD. 1997. Variation in flowering phenology and selfing rate across a contact zone between diploid and tetraploid *Arrhenatherum elatius* (Poaceae). *Heredity* **79**: 31–40.

Petit C, Bretagnolle F, Felber F. 1999. Evolutionary consequences of diploid-polyploid hybrid zones in wild species. *Trends in Ecology and Evolution* **14**: 306–311.

Ramsey JD, Schemske W. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics* **29**: 467–501.

Ripley BD. 1977. Modelling spatial patterns. *Journal of the Royal Statistical Society, Series B* **39**: 172–212.

Rodriguez DJ. 1996. A model for the establishment of polyploidy in plants. *The American Naturalist* **147**: 33–46.

Rose F. 1988. Gymnadenia. *In*: Rich TCG, Jermy AC. eds. *Plant crib.* Botanical Society of the British Isles, 1–2.

Šafářová L, Duchoslav M. 2010. Cytotype distribution in mixed populations of polyploid *Allium oleraceum* measured at a microgeographic scale. *Preslia* **82**: 107–126.

Scacchi R, De Angelis G. 1989. Isoenzyme polymorphism in *Gymnadenia conopsea* and its inferences for systematics within this species. *Biochemical Systematics and Ecology* **17**: 25–33.

Soliva M, Widmer A. 1999. Genetic and floral divergence among sympatric populations of *Gymnadenia conopsea* s. l. (Orchidaceae) with different flowering phenology. *International Journal of Plant Sciences* **160**: 897–905.

Soltis DE, Soltis PS, Pires JC, Kovařík A, Tate JA, Mavrodiev E. 2004. Recent and recurrent polyploidy in *Tragopogon* (Asteraceae): cytogenetic, genomic and genetic comparisons. *Biological Journal of the Linnean Society* **82**: 485–501.

Soltis DE, Soltis PS, Schemske DW, *et al*. 2007. Autopolyploidy in angiosperms: have we grossly underestimated the number of species? *Taxon* **56**: 13–30.

Soltis DE, Albert VA, Leebens-Mack J, *et al*. 2009. Polyploidy and angiosperm diversification. *American Journal of Botany* **96**: 336–348.

Suda J, Kron P, Husband BC, Trávníček P. 2007*a*. Flow cytometry and ploidy: applications in plant systematics, ecology and evolutionary biology. In: Doležel J, Greilhuber J, Suda J. eds. *Flow cytometry with plant cells. Analysis of genes, chromosomes and genomes*. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA, 103–130.

Suda J, Weiß-Schneeweiß H, Tribsch A, Schneeweiß G, Trávníček P, Schönswetter P. 2007*b*. Complex distribution patterns of di-, tetra- and hexaploid cytotypes in the European high mountain plant *Senecio carniolicus* Willd. (Asteraceae). *American Journal of Botany* **94**: 1391–1401.

Thompson JN, Merg K. 2008. Evolution of polyploidy and the diversification of plant–pollinator interactions. *Ecology* **89**: 2197–2206.

Trávníček P, Eliášová E, Suda J. 2010. The distribution of cytotypes of *Vicia cracca* in Central Europe: the changes that have occurred over the last four decades. *Preslia* **82**: 149–163.

Vöth W, Sontag S. 2006. Die intraspezifischen Varietäten der *Gymnadenia conopsea* (L.) R. Br. *Journal Europäischer Orchideen* **38**: 581–624.

Weiss H, Dobes C, Schneeweiss GM, Greimler J. 2002. Occurrence of tetraploid and hexaploid cytotypes between and within populations in *Dianthus* sect. *Plumaria* (Caryophyllaceae). *New Phytologist* **156**: 85–94.

Downloaded from https://academic.oup.com/aob/article/107/1/77/176429 by guest on 17 April 2024