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Minority cytotypes in European populations of the *Gymnadenia conopsea* complex (Orchidaceae) greatly increase intraspecific and intrapopulation diversity

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• *Background and Aims* Patterns of ploidy variation among and within populations can provide valuable insights into the evolutionary mechanisms shaping the dynamics of plant systems showing ploidy diversity. Whereas data on majority ploidies are, by definition, often sufficiently extensive, much less is known about the incidence and evolutionary role of minority cytotypes.
• *Methods* Ploidy and proportions of endoreplicated genome were determined using DAPI (4',6-diamidino-2-phenylindole) flow cytometry in 6150 *Gymnadenia* plants (fragrant orchids) collected from 141 populations in 17 European countries. All widely recognized European species, and several taxa of less certain taxonomic status were sampled within *Gymnadenia conopsea sensu lato*.
• *Key Results* Most *Gymnadenia* populations were taxonomically and/or ploidy heterogeneous. Two majority (2*x* and 4*x*) and three minority (3*x*, 5*x* and 6*x*) cytotypes were identified. Evolution largely proceeded at the diploid level, whereas tetraploids were much more geographically and taxonomically restricted. Although minority ploidies constituted <2 % of the individuals sampled, they were found in 35 % of populations across the entire area investigated. The amount of nuclear DNA, together with the level of progressively partial endoreplication, separated all *Gymnadenia* species currently widely recognized in Europe.
• *Conclusions* Despite their low frequency, minority cytotypes substantially increase intraspecific and intrapopulation ploidy diversity estimates for fragrant orchids. The cytogenetic structure of *Gymnadenia* populations is remarkably dynamic and shaped by multiple evolutionary mechanisms, including both the ongoing production of unreduced gametes and heteroploid hybridization. Overall, it is likely that the level of ploidy heterogeneity experienced by most plant species/populations is currently underestimated; intensive sampling is necessary to obtain a holistic picture.

Key words: Coexistence, contact zone, cytogeography, flow cytometry, fragrant orchid, *Gymnadenia*, Orchidaceae, hybridization, mixed-ploidy population, polyploidy, sympatry, unreduced gametes.

INTRODUCTION

Polyploidy (the multiplication of complete chromosome sets in somatic cells above the diploid state) is a prominent and recurring process in the evolution of eukaryotic organisms (Otto and Whitton, 2000). Although polyploidy has been documented in all major lineages of eukaryotes, land plants show the highest incidence of polyploidy (Jiao *et al.*, 2011). Karyological evidence suggests that at least 70 and 95 % of angiosperms and ferns, respectively, are polyploid (Masterson, 1994). Genomic data also support the near ubiquity of polyploidy, traces of

ancient whole-genome duplication having been detected in virtually all angiosperms (Soltis *et al.*, 2009). The success of polyploid plants can be related to different evolutionary transitions that may alter their genetic composition, phenotypic plasticity or ecological amplitude, and can ultimately lead to increased vigour and competitive superiority over diploid ancestors (Levin, 2002). Polyploid plants can combine genomes of two or more parental species (allopolyploids) or arise from the same parental species (autopolyploids). Whereas allopolyploids have long been assumed to prevail *in situ*, recent data suggest that the frequency of autopolyploids is much higher than previously considered and they play important evolutionary and ecological roles in natural populations (Soltis *et al.*, 2007; Parisod *et al.*, 2010). Autopolyploid derivates may originate through somatic chromosome doubling, but it is the formation of unreduced gametes that drives the dynamics of their genesis (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998).

Although genome duplication is often associated with speciation (Wood *et al.*, 2009), ploidy variation is also observed within traditionally delimited taxonomic species. This is especially true for autopolyploids, which more closely resemble their diploid/lower ploid progenitors than do allopolyploids and so are rarely recognized in formal classifications. For example, chromosomal data for the Californian flora indicate that approx. 13 % of the species listed are ploidy polymorphic and several of them possess more than two different cytotypes (Soltis *et al.*, 2007). Based on a broad survey of species, Wood *et al.* (2009) reported that 12–13 % of angiosperm species and 17 % of fern species are variable for ploidy. In general, ploidy heterogeneity within species is likely to have been underestimated and is predicted to continue to increase with more intensive sampling. Indeed, ploidy screening across large spatial scales and in a representative number of individuals per population, made possible by the advent of flow cytometry (FCM), has resulted in a substantial increase in the number of ploidy-heterogeneous plant species recognized and in the number of different cytotypes recorded per species (Kron *et al.*, 2007).

Fragrant orchids of the *Gymnadenia conopsea* aggregate constitute a highly ploidy-variable and taxonomically challenging species complex native to temperate Europe and Asia. Besides the karyological polymorphism (Marhold *et al.*, 2005; Trávníček *et al.*, 2011), members of the complex were also found to vary in morphology (Dworschak, 2002; Marhold *et al.*, 2005; Vöth and Sontag, 2006; R. Bateman *et al.*, unpubl. res.), floral scent biochemistry (Huber *et al.*, 2005; Jersáková *et al.*, 2010), flowering phenology (Soliva and Widmer, 1999; Gustafsson and Lönn, 2003) and preferred habitats (Dworschak, 2002). Investigations into phenotypic and genetic variation have often revealed strong genetic divergence among the recognized taxa but a lower level of morphological differentiation (e.g. Scacchi and de Angelis, 1989; Soliva and Widmer, 1999; Bateman *et al.*, 2003; Gustafsson and Lönn, 2003; Stark *et al.*, 2011; R. Bateman *et al.*, unpubl. res.). Taxonomic delimitation is further complicated by weak pre-zygotic and post-zygotic barriers (Jersáková *et al.*, 2010) that allow frequent formation of spontaneous hybrids at both intrageneric and intergeneric levels (e.g. Hedrén *et al.*, 2000; Lönn *et al.*, 2006).

Setting aside the former genus *Nigritella*, recent classifications of *Gymnadenia* in Europe mostly recognize five major taxa at different taxonomic levels, depending on the author's preferred concept. Most recent British authors have followed Bateman *et al.* (2003) in recognizing all of these taxa as full species, whereas the most influential Continental monographers (e.g. Kreutz, 2004; Delforge, 2006) have treated most of these taxa as varieties only. In addition to the widespread *G. conopsea* (L.) R.Br. *sensu stricto* (*s.s.*), *G. densiflora* (Wahlenb.) A.Dietr. and *G. odoratissima* (L.) Rich., *G. frivaldii* Hampe ex Griseb. is a Balkan endemic only recently confirmed as assignable to *Gymnadenia* (Bateman *et al.*, 2006). Originally described from a type locality in Cumbria, *G. borealis* (Druce) R.M.Bateman, Pridgeon & M.W.Chase is regarded by some authors as being confined to Britain and Ireland, though morphologically identical plants also occur along the Scandinavian mountain chain (Strann and Bjerke, 2010). Several local morphotypes with a more questionable taxonomic status have also been described, including the compact, late-flowering *G. conopsea* var. *friesica* Schlechter from sand dunes on the Friesian Islands (Schlechter, 1919; Kreutz and Dekker, 2000) and the slender alpine ecotype referred to as *G. conopsea* var. *alpina* Rchb.f. ex Beck (1893). Robust plants from the Pyrenees that resemble the short-spurred *G. odoratissima* but have a spur about one-third longer than the ovary have been recognized as var. *pyrenaica* (Philippe) P.Delforge (2005). A substantially longer spur is also supposedly diagnostic of *G. odoratissima* subsp. *longicalcarata* C.E.Hermosilla & J.Sabando (1996) from northern Spain. Several additional taxa from the Bavarian Alps were recently described on the basis of morphological observations (Dworschak, 2002): *G. graminea* Dworschak, *G. conopsea* subsp. *serotina* (Schönh.) Dworschak, *G. splendida* Dworschak and *G. vernalis* Dworschak.

Our previous study (Trávníček *et al.*, 2011) provided new insights into ploidy variation but only at population and regional scales, being confined to the Czech Republic plus Slovakia. We found a surprisingly high proportion of mixed-ploidy populations, consisting of different combinations of two majority and three minority cytotypes. In addition, unique FCM profiles (i.e. different levels of progressively partial endoreplication; see Discussion for detailed explanation) were observed for *G. conopsea s.s.* and *G. densiflora*. The present study builds on our previous research, aiming to assess ploidy variation across much larger spatial scales and encompassing all major European *Gymnadenia* species. Patterns of ploidy variation, both among and within populations, can provide useful insights into the evolutionary mechanisms that shape the dynamics of these polyploid systems.

Specifically, we address the following questions. (1) Which patterns of progressively partial endoreplication can be found among the investigated plants? Is this variation geographically or taxonomically structured? (2) Where is the geographical centre of ploidy variation located? (3) How frequent are mixed-ploidy populations? Do different *Gymnadenia* taxa differ in this respect? (4) How common and how widespread are minority cytotypes? Do they preferentially occur in populations with a particular composition of majority ploidies?

MATERIALS AND METHODS

Field sampling

Plant samples were collected in 17 European countries between 2004 and 2011, spanning the geographical range

40 °57′N–59 °17′N and 06 °01′W–30 °30′E (for locality details, see Supplementary Data Table S1) and totalling 6150 individuals from 141 populations. The number of localities and individuals sampled for specific countries were as follows: Austria, 9/318; Belgium, 1/26; Bulgaria, 3/36; Estonia, 3/91; France, 20/958; Germany, 20/877; Italy, 10/594; Macedonia, 1/6; The Netherlands, 1/48; Poland, 2/58; Romania, 9/209; Russia, 7/130; Scotland, 19/600; Slovakia, 5/266; Spain, 1/13; Sweden, 15/1348; and Switzerland, 15/572. Although taxonomic revision of the *Gymnadenia conopsea* aggregate was beyond the scope of this study, we aimed to encompass most of the taxonomic and phenotypic diversity recognized in Europe. In addition to traditionally accepted species, we also sampled known localities for recently described taxa of questionable taxonomic status (e.g. Dworschak, 2002; Supplementary Data Table S1). Due to taxonomic uncertainties, some plants from France with distinct FCM profiles and morphology were not assigned to any particular taxon and instead are provisionally named 'French diploid' and 'French tetraploid'. The taxonomic composition of our data set is summarized in Table 1.

Whenever possible, leaf tissue from at least 50 individuals was collected at each locality (the actual number of samples per locality varied from one to 191; Supplementary Data Table S1). The number of samples chosen per locality reflected (1) population size; (2) taxonomic composition (more intensive sampling in mixed-species populations); and (3) morphological/phenological variation (more intensive sampling in populations showing high phenotypic variation or supporting multiple variants with contrasting flowering periods). Leaf tissue was wrapped in moist paper towels, placed in plastic bags and transported rapidly to the FCM laboratory. Because one or more *Gymnadenia* species rank among threatened plants in several European countries, we preferred images to herbarium specimens as vouchers. Plants were imaged at each locality (Supplementary Data Fig. S2), and herbarium specimens (kept in PRC or CBFS) were taken only from selected representative sites (Supplementary Data Table S1). Because the majority of diagnostic characters are located on floral parts, two flowers per plant were collected at each locality and stored in 70 % ethanol.

Flow cytometry

Relative fluorescence intensities of plant samples were determined by DAPI (4',6-diamidino-2-phenylindole) FCM following the methodology detailed by Trávníček *et al.* (2011). Up to five individuals were processed together. Each plant was re-analysed separately in cases of mixed-ploidy samples or if the coefficient of variation of either the unknown sample or the internal standard peaks exceeded 5 %. *Pisum sativum* 'Ctirad' (2C = 9·09 pg) was selected as a primary reference standard, as it has a genome size close to, but not overlapping, that of most *Gymnadenia* samples. *Vicia faba* 'Inovec' served as a reference standard for measurements of *G. borealis*; the relative nuclear DNA amount of *Vicia* was calibrated against *Pisum* (3·14× greater; Suda *et al.*, 2007). Karyologically counted ($2n = 40$ and $2n = 80$) plants of *G. conopsea* from the Czech Republic were used as reference points when interpreting the FCM results. Some data, such as the incidence of individuals with putatively 50 somatic chromosomes among FCM-screened progeny of our experimental crosses (J. Jersáková *et al.*, unpubl. res.; see also Trávníček *et al.*, 2011) may indicate that $x = 10$ is the basic chromosome number in the *G. conopsea* aggregate. Nonetheless, in line with the generally accepted view (e.g. Marhold *et al.*, 2005; Stark *et al.*, 2011), we interpreted here plants with $2n = 40$ and $2n = 80$ as diploids and tetraploids, respectively, pending any stronger cytological evidence for $x = 10$.

Statistical analyses

Flow cytometry data were analysed using the SAS 8·1 statistical package (SAS Institute, Cary, NC, USA). Interspecific differences in relative fluorescence intensities and proportions of endoreplicated genome were tested by GLM (general linear model) because of unbalanced data design, and Tukey's procedure was applied to compare mean values.

Binomial multiple regression (LOGISTIC procedure in SAS) was used to test whether polyploids (i.e. $3x$–$6x$) or tetraploids specifically are linked to geographical parameters of sampled populations (latitude, longitude, altitude and their combinations; Manzaneda *et al.*, 2011). The presence/absence of polyploids or

TABLE 1. *Flow cytometric results for five major European* Gymnadenia *species and two undetermined taxa from France*

Species	Ploidy level	Relative fluorescence intensity against internal reference standard, *Pisum sativum* (mean ± s.d.)*	Proportion of replicated genome (mean ± s.d., %)*	No. of FCM analyses	No. of individuals
G. borealis	2*x*	0·956 ± 0·017^{c}	53·7 ± 1·7^{e}	139	599
G. conopsea (incl. subsp. *serotina* p.p., var. *alpina*, *G. graminea*, *G. splendida* p.p., *G. vernalis*)	2*x*	0·853 ± 0·021^{f}	58·1 ± 1·9^{c}	496	2114
	4*x*	1·588 ± 0·029^{b}	60·7 ± 2·3^{b}	161	528
G. densiflora (incl. *G. conopsea* subsp. *serotina* p.p., *G. conopsea* var. *friesica*, *G. splendida* p.p.)	2*x*	0·748 ± 0·014^{g}	74·4 ± 2·4^{a}	362	1538
G. frivaldii	2*x*	0·857 ± 0·031^{f}	50·8 ± 1·9^{e}	10	32
G. odoratissima	2*x*	0·906 ± 0·019^{e}	56·8 ± 1·8^{cd}	106	464
French diploid	2*x*	0·923 ± 0·018^{d}	56·2 ± 1·7^{d}	163	565
French tetraploid	4*x*	1·673 ± 0·026^{a}	60·6 ± 2·0^{b}	90	192

*Different letters indicate groups of taxa that are significantly different at $\alpha = 0{\cdot}05$.

tetraploids in populations fitted a binomial distribution, which was therefore used with the logit link function as parameters of the model.

RESULTS

Genome characteristics

Flow cytometric analysis of 6150 plants (Fig. 1) resulted in five distinct groups of fluorescence intensities, corresponding to diploids (5312 individuals; 86·4 %), triploids (94 individuals; 1·5 %), tetraploids (720 individuals; 11·7 %), pentaploids (17 individuals; 0·3 %) and hexaploids (seven individuals; 0·1 %). Table 1 shows FCM characteristics of the majority (2*x* and 4*x*) ploidies for five species and two undetermined *Gymnadenia* taxa. Two groups of tetraploids with significantly different relative nuclear DNA contents were found; one corresponded to *G. conopsea s.s.* (Trávníček *et al.*, 2011), whereas the other was not assigned to any species; it is provisionally referred to simply as 'French tetraploid'. Disregarding minority ploidies, all other species were diploid. Their mean relative fluorescence intensities (setting the value for the reference standard *P. sativum* to unity) varied 1·278-fold, ranging from 0·748 in *G. densiflora* to 0·956 in *G. borealis*. With the exception of *G. conopsea* vs. *G. frivaldii*, the remaining diploids possessed significantly different relative amounts of nuclear DNA (Table 1). The proportions of endoreplicated genome also differed significantly among several *Gymnadenia* taxa (Table 1). *Gymnadenia frivaldii* was the species with the lowest level of progressively partial endoreplication (50·8 % on average), whereas *G. densiflora* showed the highest level (74·4 % on average). Flow cytometric profiles (a combination of relative fluorescence values together with the proportion of endoreplicated genome) therefore offer a reliable method of distinguishing between all major *Gymnadenia* species recognized in the more accurate of the recent European classifications.

Cytogeography and population structure

Half of the *Gymnadenia* populations sampled (71 of 141) were deemed complex in terms of species composition, karyological variation or both (Table 2). Up to three different taxa and five different cytotypes coexisted at a single site. In total, we found 22 different species–majority ploidy combinations (Table 2), and the frequent occurrence of one or more minority cytotypes further increased the intrapopulation heterogeneity. Diploids and tetraploids were recorded in 133 and 25 populations, respectively; however, only 83 and four populations, respectively, were homogeneous for ploidy. The most common type of ploidy mixture involved sympatry of diploids and triploids, suggesting regular formation of unreduced gametes. Some form of ploidy variation was observed in 54 (38·3 %) populations; two, three and four different cytotypes coexisted in 40, ten and three populations, respectively. All five cytotypes grew together in population FR04 near Sainte-Maure-de-Touraine in France (Supplementary Data Table S1), which also maintained two coexisting taxa. In total, more than two taxa were observed in nearly one-third (41) of the populations analysed, the most common combination being 2*x G. conopsea*, 2*x G. densiflora* and 2*x G. odoratissima* (ten populations), followed by sympatry of the two former species (nine populations; Table 2).

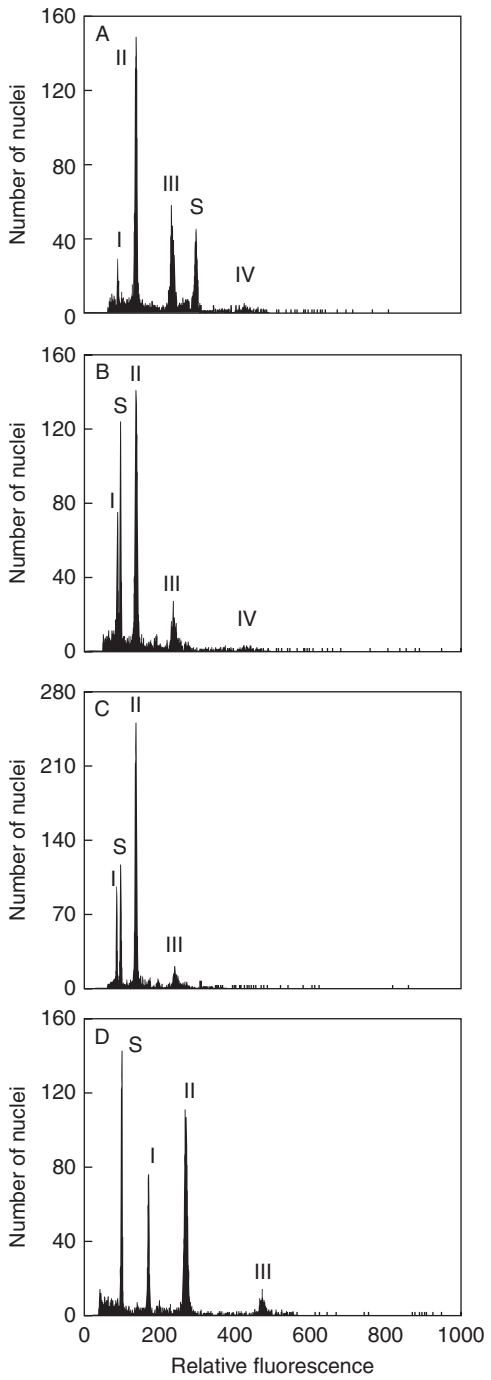

FIG. 1. Representative flow cytometric histograms of the studied *Gymnadenia* taxa (analysed together with the internal reference standard). Nuclei of both the sample and standard were isolated, stained with DAPI and simultaneously run on the flow cytometer. (A) Diploid *G. borealis* (loc. GB05) – ratios between individual *Gymnadenia* peaks 1 : 1·54 : 2·63 : 4·81; (B) diploid *G. odoratissima* (loc. IT05) – peak ratios 1 : 1·56 : 2·71 : 4·91; (C) French diploid (loc. FR04) – peak ratios 1 : 1·58 : 2·78; (D) French tetraploid (loc. FR 04) – peak ratios 1 : 1·58 : 2·78. I, II, III and IV, peaks of *Gymnadenia* nuclei undergoing different numbers of partial endoreplication cycles. S, internal standard: *Vicia faba* in (A), *Pisum sativum* in (B–D).

TABLE 2. *Taxonomic and ploidy composition of the 141* Gymnadenia *populations investigated*

Taxonomic composition (majority ploidies)	Total no. of populations with the given taxonomic composition	No. of populations for a given taxonomic composition harbouring minority cytotypes						
		3*x*	5*x*	6*x*	3*x* + 5*x*	5*x* + 6*x*	3*x* + 6*x*	3*x* + 5*x* + 6*x*
2C	34	9						
2D	21	6						
2O	2							
2Sp	8	3						
2B	19	1						
2F	2	1						
2C + 2D	9	3						
2C + 2O	6	2						
2C + 2Sp	2	2						
2D + 2Sp	1							
2O + 2Sp	1							
2C + 2D + 2O	10	6						
2C + 2D + 2Sp	1							
4C	7	1	1			1		
4Sp	1		1					
4C + 2C	6	3			1		1	
4C + 2D	4	1			1			
4C + 2C + 2D	2	1		1				
4C + 2C + 2O	1		1					
4C + 2D + 2O	1	1						
4Sp + 2Sp	2							1
4Sp + 2Sp + 2D	1	1						

2B, 2*x G. borealis*; 2C, 2*x G. conopsea*; 2D, 2*x G. densiflora*; 2F, 2*x G. frivaldii*; 2O, 2*x G. odoratissima*; 2Sp, undetermined diploid from France; 4C, 4*x G. conopsea*; 4Sp, undetermined tetraploid from France.

Diploids were recorded in all 17 countries (Fig. 2A), whereas tetraploids were restricted to just five of these countries: Austria, France, Germany, Romania and Switzerland (Fig. 2B). The multiple regression analysis showed a significant negative relationship between the incidence of polyploids and latitude ($\beta \pm$ s.d. $= -0{\cdot}294 \pm 0{\cdot}095$; d.f. 1,133; $P = 0{\cdot}0021$). Tetraploids were strongly negatively associated with latitude and also less strongly with altitude ($\beta \pm$ s.d. $= -0{\cdot}607 \pm 0{\cdot}188$; d.f. 1,133; $P = 0{\cdot}0013$ and $\beta \pm$ s.d. $= -0{\cdot}036 \pm 0{\cdot}018$; d.f. 1,133; $P = 0{\cdot}0433$, respectively).

Minority cytotypes

Minority ploidies constituted <2 % of all samples, but they were present in more than one-third (50 of 141) of our study populations, distributed across the area investigated (Table 2, Fig. 2C). Triploids, pentaploids and hexaploids occurred in 45, seven and four populations, respectively. Although it is difficult to determine the taxonomic identity of minority cytotypes in multispecies populations, our data indicate that they were formed in all widely recognized taxa (Table 2). Most triploids were recorded in otherwise exclusively diploid populations (33 populations), although in 11 populations they co-occurred with diploids and tetraploids. Significantly higher proportions of triploid individuals occurred in mixed 2*x*–4*x* populations than in otherwise uniform 2*x* populations (Mann–Whitney U-test: 6·9 % vs 3·2 %, $n = 44$, $P = 0{\cdot}0037$ and 7·1 % vs 2·2 %, $n = 39$, $P < 0{\cdot}001$, as assessed for, respectively, all populations and only populations yielding >30 analysed individuals). These observations suggest that, in addition to the formation of unreduced gametes, interploidy hybridization was also involved in the genesis of triploids. This inference can also be reached from the proportion of populations of different ploidy composition that harboured triploids; although triploids were present in 64·7 % of 2*x*–4*x* populations, this proportion fell to 28·4 % if only 2*x* populations were considered. Higher polyploids (5*x* and 6*x*) were always associated with tetraploids, and in six out of nine of these populations, diploids were also present.

DISCUSSION

This study represents by far the most comprehensive investigation of ploidy variation in the *G. conopsea* complex in terms of taxonomic coverage, geographical scale and the number of cytotyped plants.

Genome characteristics

Somatic tissues of at least some orchids are known to undergo ‘progressively partial endoreplication’, a phenomenon that was first described in *Vanilla planifolia* by Bory *et al.* (2008). Unlike conventional whole-genome endoreplication, which has been documented in plant species from a range of families (Barow, 2006), only part of the genome is duplicated during progressively partial endoreplication. Consequently, the ratio between the first and second peaks in FCM histograms is substantially less than 2:1. Previously (Trávníček *et al.*, 2011), we observed differences in the proportion of endoreplicated genome between the two *Gymnadenia* species native to the

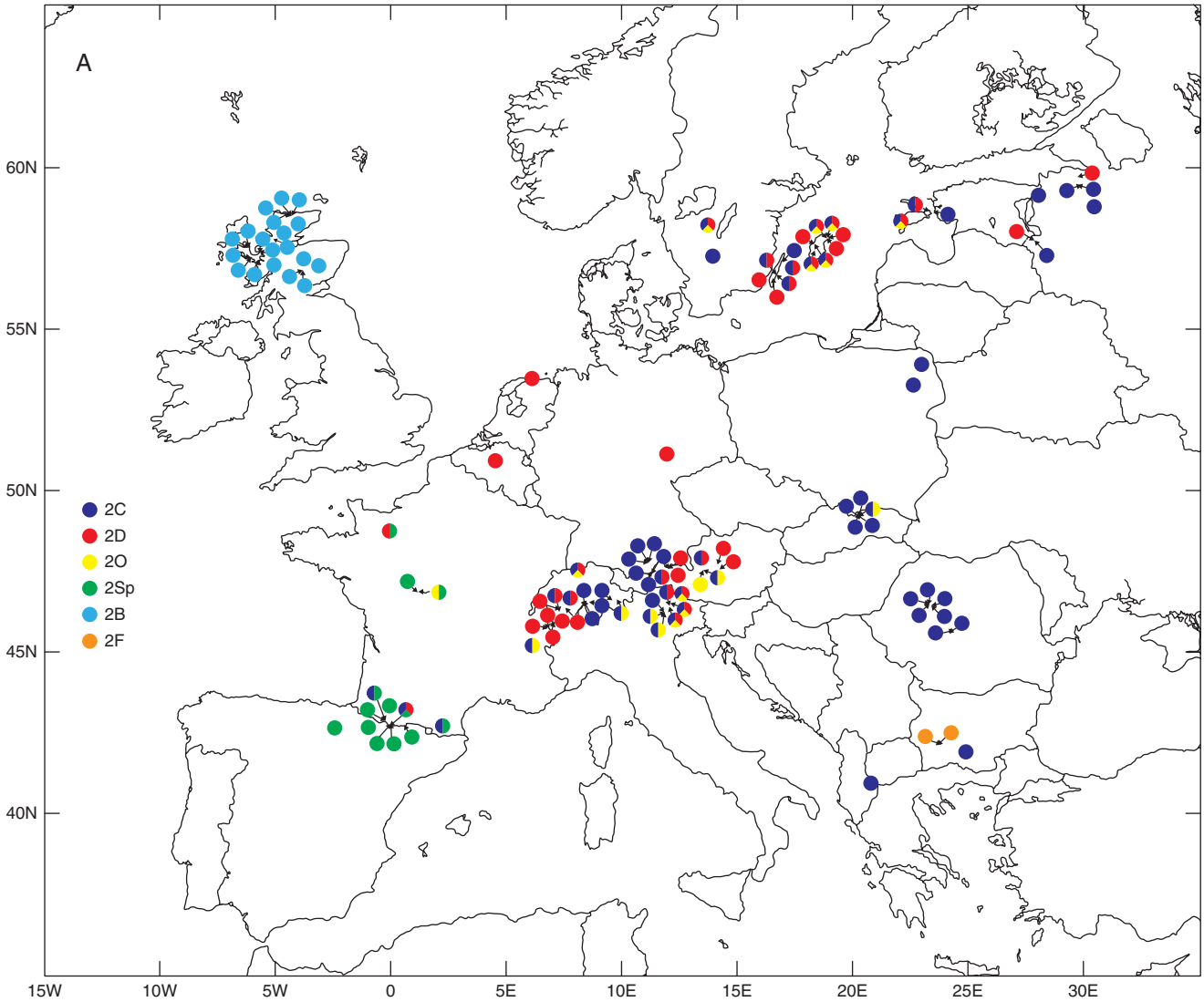

FIG. 2. Ploidy variation and taxonomic composition of 141 studied populations of the *Gymnadenia conopsea* complex in Europe. (A) Diploid populations (either ploidy-uniform or with the presence of minority cytotypes). Intrapopulation taxonomic heterogeneity is indicated by mixed colours. (B) Tetraploid (squares) and mixed 2*x*–4*x* (triangles) populations. Intrapopulation taxonomic heterogeneity is indicated by mixed colours. (C) Populations harbouring minority cytotypes (3*x*, blue; 5*x*, yellow; 6*x*, red). The presence of both majority ploidies (2*x* and 4*x*) is illustrated by a circle, whereas triangles illustrate exclusive di- or tetraploid populations. Co-occurrence of different minority cytotypes is indicated by mixed colours. Arrows indicate populations in which an additional cytotype (most probably diploid) is predicted (sympatry of $3x + 4x$ or $4x + 5x$). Taxa abbreviations in (A) and (B): 2B, 2*x G. borealis*; 2C, 2*x G. conopsea*; 2D, 2*x G. densiflora*; 2F, 2*x G. frivaldii*; 2O, 2*x G. odoratissima*; 2Sp, undetermined diploid from France; 4C, 4*x G. conopsea*; 4Sp, undetermined tetraploid from France.

Czech Republic and Slovakia, *G. conopsea* (mean value 58·5 %) and *G. densiflora* (mean value 74·7 %). The present study confirmed the validity of interspecific differences between *G. conopsea* and *G. densiflora* across Europe (Table 1) and revealed new species-specific profiles for *G. borealis* (53·7 % of endoreplicated genome) and *G. frivaldii* (50·8 % of endoreplicated genome). With the exception of *G. frivaldii*, there is a negative relationship between the proportion of endoreplicated genome and the total amount of nuclear DNA (Table 1). It is therefore possible that the level of endoreplication has an adaptive role and contributes to shaping, either directly or indirectly, optimal genome size and/or cell size (Gregory, 2005).

Genome characteristics of the less well known taxa (e.g. Dworschak, 2002) were indistinguishable from those of the major *Gymnadenia* species. Because their morphological delineation also remains ambiguous, we have provisionally synonymized *G. conopsea* var. *alpina*, *G. graminea* and *G. vernalis* with the nominate variety of *G. conopsea* and *G. conopsea* var. *friesica* with *G. densiflora*. On the basis of FCM results, individuals corresponding to *G. conopsea* subsp. *serotina* and *G. splendida sensu* Dworschak (2002) were classified as either *G. conopsea* or *G. densiflora* (Table 1).

Cytogeography and population structure

The results provided new insights into cytotype variation at different spatial scales, from transcontinental to intrapopulational. Five different ploidies (2*x*, 3*x*, 4*x*, 5*x*, and 6*x*) were

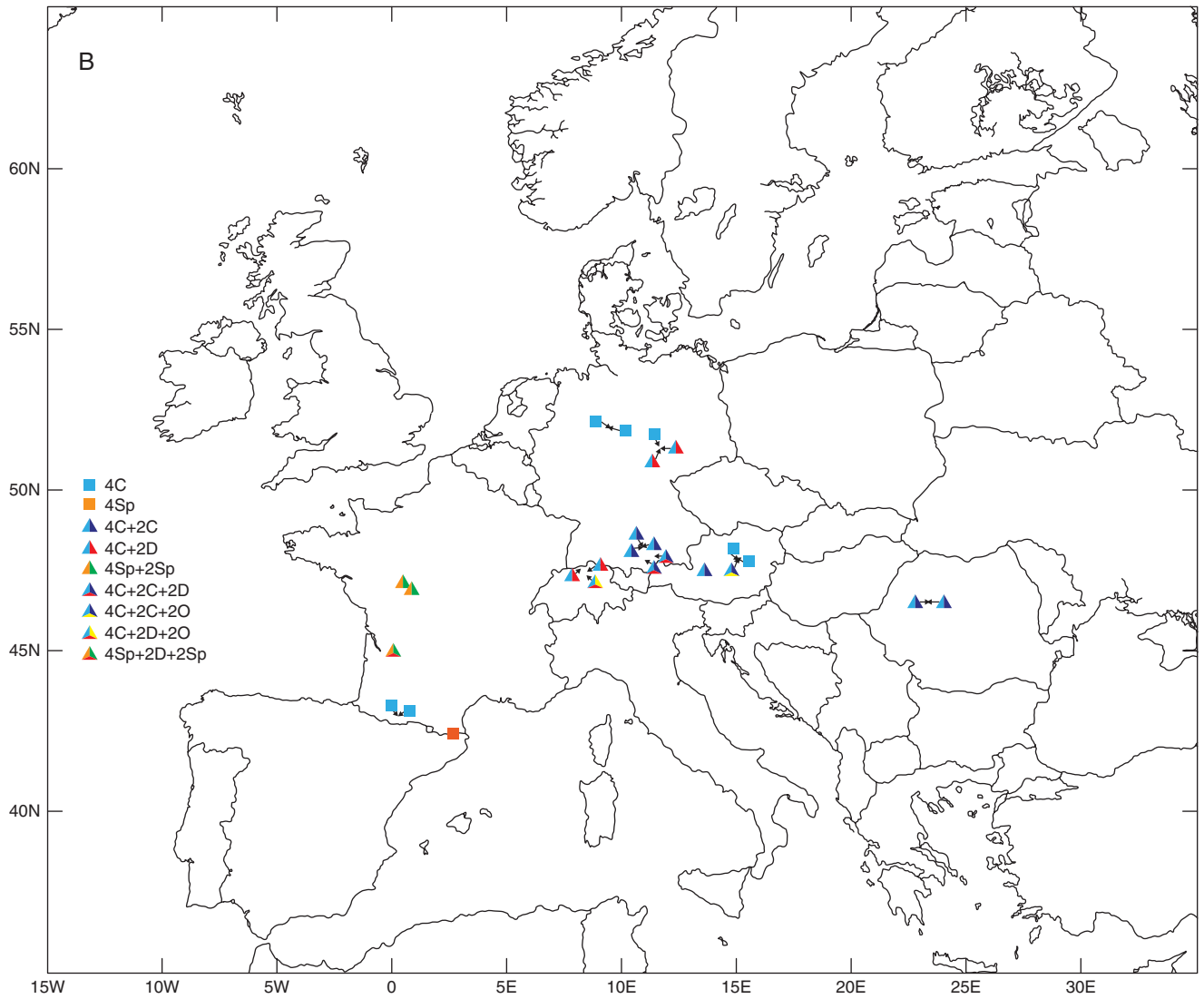

Fig. 2 *Continued*

found among the present samples, reflecting our previous smaller scale study confined to the Czech Republic and Slovakia (Trávníček *et al.*, 2011). (Note that previously we referred to these cytotypes as tetraploid, hexaploid, octoploid, etc.; Trávníček *et al.*, 2011.) The evolution of the *G. conopsea* complex proceeded mostly at the diploid level, which was detected in all five recognized species plus one undetermined taxon (Table 1, Fig. 2A, Supplementary Data Fig. S1A). Tetraploids were more restricted, both taxonomically and spatially. Although polyploidy is generally more frequent at higher latitudes (Brochmann *et al.*, 2004), the binomial multiple regression provided evidence that tetraploids (and polyploids in general) in *Gymnadenia* tended to occur in southern parts of the investigated area. The most common category of tetraploids corresponded to *G. conopsea*; it extends latitudinally from its centre of distribution in Central Europe at least as far as France and Romania (Fig. 2B, Supplementary Data Fig. S1B). France is also the home of tetraploids that possess slightly larger amounts of nuclear DNA and were not assigned by us to a particular pre-existing species. Potentially, they may correspond to *G. conopsea* var. *pyrenaica* (a full species according to Bournérias and Prat, 2005), but for the present we refrain from any taxonomic conclusion. Most published records of tetraploid fragrant orchids have been made in Austria (Groll, 1965; Mrkvička, 1993; Marhold *et al.*, 2005; Stark *et al.*, 2011), Germany (Wegener, 1966; Stark *et al.*, 2011) and the Czech Republic and Slovakia (Marhold *et al.*, 2005; Trávníček *et al.*, 2011). More recently, Stark *et al.* (2011) observed tetraploids at one locality in France, Heusser (1938) having earlier reported this cytotype from Switzerland. Our new discoveries from two sites in Romania (Fig. 2B, Supplementary Data Fig. S1B), and published counts from the Caucasus (Sokolovskaya and Strelkova, 1940) and Armenia (Torosyan, 1990), demonstrate that tetraploids extend from Central to Eastern Europe and further into Asia Minor. In contrast, they appear to be absent from northern Europe, as we did not find any tetraploid plants among samples from Sweden, Estonia or Russia.

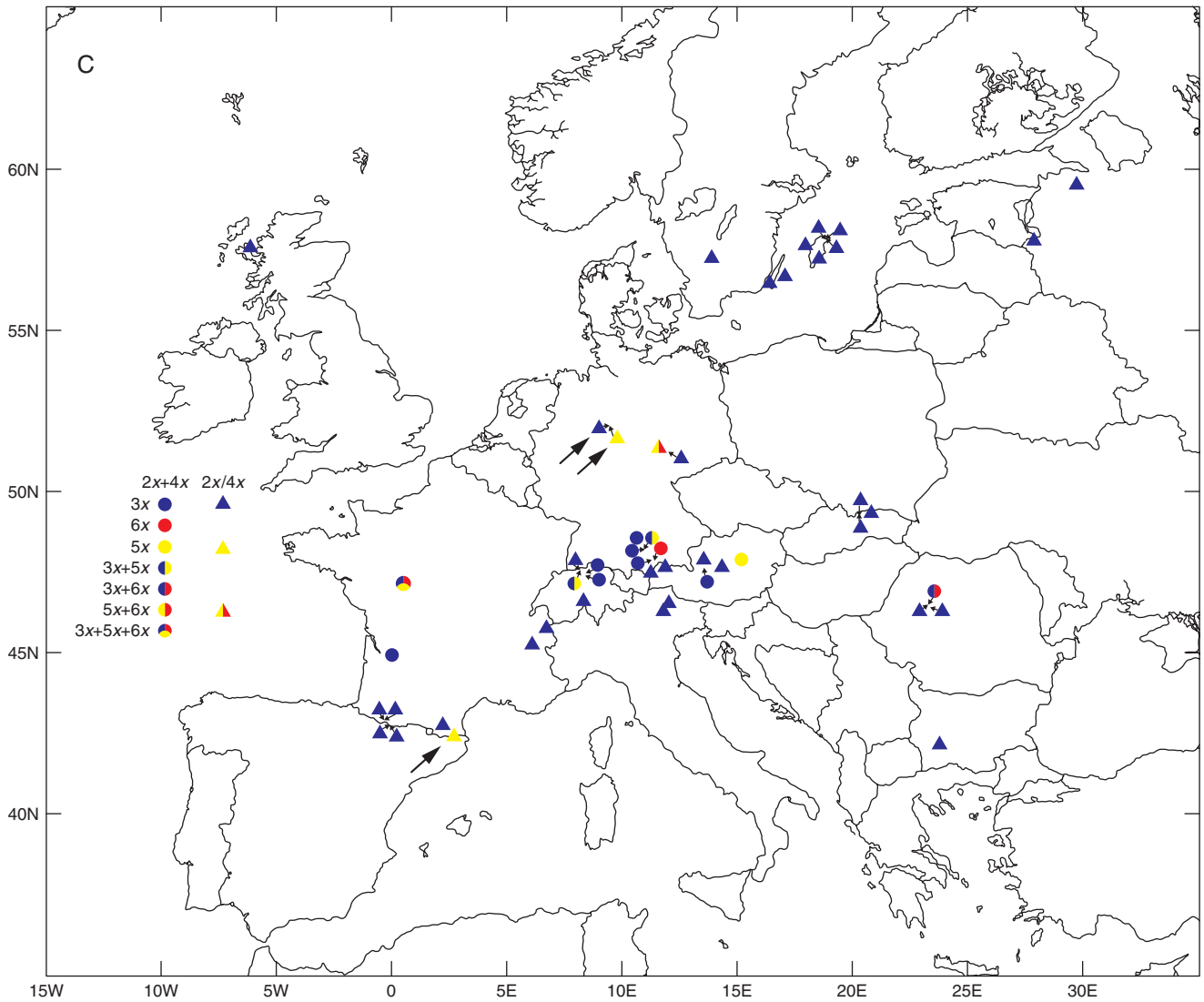

Fig. 2 *Continued*

Several species can co-occur at the sample locality, in particular when different microhabitats are present; we observed two and three different *Gymnadenia* taxa at 28 and 13 sites, respectively (Table 2). Mixed-species populations clearly prevailed in *G. odoratissima* (90·5 %) and *G. densiflora* (58·0 %), and were also common in *G. conopsea* (43·4 %). The coexistence of multiple species opens up obvious possibilities for interspecific hybridization. We occasionally observed morphotypes intermediate between 2*x* *G. conopsea* and 2*x* *G. odoratissima* (e.g. localities IT04, IT06; Supplementary Data Table S1). In addition, a few plants from mixed populations of *G. conopsea* and *G. densiflora* yielded unusual FCM profiles that might indicate hybridization (e.g. population AT07 from the Dachstein Mts.; Supplementary Data Table S1). Such individuals were excluded from the present study and will be subjected to further investigation using detailed molecular techniques. Although only species-uniform populations of *G. borealis* and *G. frivaldii* were recorded in our study, we regard this outcome as an artefact of sampling; only two populations were available for *G. frivaldii* and all of our numerous collections of *G. borealis* originated from Scotland. Mixed sites of *G. borealis* and *G. conopsea* have been reported from more southern parts of the UK (Campbell *et al.*, 2007). However, the only mixed-species populations in Britain and Ireland detected by one of us during 35 years of fieldwork involved *G. borealis* plus *G. densiflora* in central Scotland and *G. borealis* plus *G. conopsea s.s.* in western Ireland (R. Bateman *et al.*, unpubl. res.).

Minority cytotypes

Large-scale population screenings, made possible by FCM, have changed our perception of intraspecific and intrapopulation ploidy heterogeneity (Kron *et al.*, 2007; Suda *et al.*, 2007). Previously overlooked minority cytotypes (often occurring at frequencies <1 %), such as odd ploidy levels or high polyploids, have recently been discovered in several plant species; these include *Parasenecio auriculata* (0·4 % triploids;

Nakagawa, 2006), *Vicia cracca* (0·1 % triploids; Trávníček *et al.*, 2010), *Actinidia chinensis* (0·6 % pentaploids; Li *et al.*, 2010), *Pilosella officinarum* (0·3 % heptaploids; Mráz *et al.*, 2008) and *Senecio carniolicus* (0·1, 0·7, 0·1 and 0·1 % tri-, penta-, hepta- and nonaploids, respectively; Sonnleitner *et al.*, 2010).

Three minority cytotypes (3*x*, 5*x* and 6*x*) with a cumulative frequency of approx. 2·7 % have also been found in the *G. conopsea* complex in the Czech Republic and Slovakia (Trávníček *et al.*, 2011). The substantial extension of the investigated area and much more intensive sampling in the present study did not lead to the discovery of further minority cytotypes. However, although the minority ploidies accounted for only 1·9 % of all samples (118 out of 6150 individuals), they markedly increased estimates of both intraspecific and intrapopulation variation. Without minority cytotypes, only one species (*G. conopsea*) and 17 out of 141 populations (approx. 12 %) would be categorized as mixed ploidy. In reality, however, ploidy variation (mostly caused by the incidence of minority cytotypes) occurred in all recognized taxa and in 54 (approx. 38 %) study populations (Table 2). The number of populations with sympatric $2x + 3x$ cytotypes was almost double the number of populations where the two majority ploidies ($2x + 4x$) co-occurred (33 vs. 17). In addition, rare triploids also occupied much wider ranges in Europe than their more common tetraploid counterparts (cf. Fig. 2B, C; and Supplementary Data Fig. S1B, C).

A recent survey of ploidy diversity in natural plant populations (Husband *et al.*, 2012) revealed that although mixed-ploidy sites occur commonly in some species (e.g. Burton and Husband, 1999; Sonnleitner *et al.*, 2010), this pattern largely reflects the coexistence of two or more majority ploidies. *Gymnadenia* is thus far unique in that it is the incidence of rare minority cytotypes that largely drives intrapopulation ploidy variation. One of the few plant systems known to possess a similar population structure is the daisy *Aster amellus* (Mandáková and Münzbergová, 2006), which, however, maintains a much lower proportion of populations that show sympatry of a majority and a minority cytotype.

Conclusions

Although several chromosomal counts have been published for the *G. conopsea* aggregate (e.g. Marhold *et al.*, 2005, and references therein), only large data sets such as that presented here, requiring a sampling scheme that is both extensive (many sites throughout the distribution range) and intensive (many plants per site), can generate a genuinely holistic picture of ploidy variation of complex systems and thereby provide deeper insights into the population dynamics of the studied systems. We have shown that most *Gymnadenia* populations exhibit considerable cytogenetic (and, to a lesser degree, taxonomic) heterogeneity, which should be considered in any future research to avoid biases introduced by pooling data from coexisting but nonetheless cytogenetically distinct populations. We suggest that ongoing production of unreduced gametes in the majority (2*x* and 4*x*) cytotypes, together with their hybridization in contact zones, led to the establishment of the minority ploidies (3*x*, 5*x* and 6*x*). All of the minority cytotypes occur only at low frequencies. We assume that they most probably always originate *de novo* and that their reproductive potential is limited. Nonetheless, minority cytotypes substantially increase intraspecific and intrapopulation ploidy diversity estimates for fragrant orchids. Our ongoing research aims to explore, using morphometric, molecular and experimental approaches, the evolutionary history of populations with ploidy heterogeneity and mechanisms maintaining co-occurring mixtures of cytotypes.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following: Table S1: locality details and taxonomic/ploidy composition of 141 *Gymnadenia* populations from 17 European countries. Figure S1: distribution of *Gymnadenia* cytotypes in Europe based on a combination of present and our previous (Trávníček *et al.*, 2011) data. Figure S2: images of the investigated taxa of *Gymnadenia*.

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