

Distribution of cytotypes and seasonal variation in the *Odontites vernus* group in central Europe

Rozšíření cytotypů a sezónní variabilita skupiny *Odontites vernus* ve střední Evropě

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Based on differences in chromosome number two cytotypes were reported in the *Odontites vernus* group in central Europe by earlier studies. These cytotypes were also considered to correspond to two seasonal ecotypes that differ in phenology, morphology and ecology. In this study, we conducted a broad screening of central European populations of the *O. vernus* group using flow cytometry and morphological analysis of characters underpinning the seasonal variation (number of internodes). We confirmed the existence of a widespread diploid ($2n = 2x = 18$) with a high but variable number of internodes and an early-flowering tetraploid ($2n = 4x = 40$) with a low number of internodes occurring on fallows or as an agricultural weed. In contrast to previous studies, we discovered an additional type, which is a distinctly late-flowering tetraploid ($2n = 4x = 40$) that has the highest number of internodes of all the three types. These plants were mostly recorded in disturbed places in thermophilous steppic grassland and sporadically also in meadows in moderately warm regions. Thus, the close association between seasonal and cytotypic variation was rejected in favour of a concept of a seasonally undifferentiated diploid type, which is an ecological generalist, and two seasonally and ecologically distinct tetraploid types. The reproductive isolation may be based mainly on incompatibility between the ploidy levels (diploid vs. tetraploid plants) and phenological differentiation in the time of reproduction (early vs. late tetraploids).

Key words: chromosome, flow cytometry, hemiparasite, phenology, reproductive barrier, sympatry

Introduction

The genus *Odontites* Ludw. (Rhinanthoid clade of *Orobanchaceae*; Bennett & Mathews 2006, Těšitel et al. 2010) is comprised of annual or perennial hemiparasitic herbaceous plants or dwarf-shrubs (Bolliger 1993, 1996). It is most diverse in terms of species and life-forms in the Western-Mediterranean area. In central Europe however, it is represented only by two groups of annual herbaceous plants, the *Odontites vernus* group and *O. luteus* (L.) Clairv., the only central-European representative of the *O. luteus* group.

The *O. vernus* group is one of the most common hemiparasitic plants in central Europe and occurs throughout the region (Hartl 1974, Kubát 2000). It is also by far the most widespread group within the genus *Odontites* as its geographical range extends from the western coast of Europe to the Himalayas and Siberia (Bolliger 1996). As suggested by “group”, it is assumed to represent a taxonomically complicated aggregate of several variable microspecies, which are difficult to identify. More taxa at different levels were described and partly accepted in the past (e.g. Schneider 1964, Hartl 1974), but only three

are now generally accepted (Webb & Camarasa 1972, Bolliger 1996). Thus, three taxa are usually recognized at the species level in the *O. vernus* group: *O. littoralis* (Fr.) Fr., *O. vernus* (Bellardi) Dumort. and *O. vulgaris* Moench, of which the first occurs along the coast of the Baltic Sea and does not occur in central Europe.

Odontites vernus and *O. vulgaris* are defined on the basis of ploidy level and seasonal variation. *Odontites vernus* is an early-flowering (late May–June) tetraploid species ($2n = 4x = 40$) while *O. vulgaris* is diploid ($2n = 2x = 18$ or 20) and begins flowering after mid-July (Snogerup 1983, Michalková 1998). Some authors, nonetheless, consider these taxa conspecific and delimit them as subspecies (*O. vernus* subsp. *vernus* and *O. vernus* subsp. *serotinus* Corb.; e.g. Hartl 1974, Kubát 2000) even though they do not cross-breed (Snogerup 1983). This treatment is based on a lack of reliable morphological characters for separating diploids and tetraploids, except those based on plant architecture.

The traits related to plant architecture (i.e. number of internodes, number of branches etc.) are very variable in the *O. vernus* group, which is a common feature of most annual hemiparasites of the Rhinanthoid clade of *Orobanchaceae* (e.g. Ronniger 1911, Zopfi 1993a, 1995, 1998a, b). Due to its close association with the phenology of the plants, it is usually termed seasonal variation (or seasonal polymorphism). There are numerous studies on the evolutionary source and genetic basis of this seasonal variation and its close association with environmental gradients on which a species occur and different types of grassland management (Zopfi 1993b, 1998a, Štech 1998, 2000, Těšitel 2005). Therefore, the seasonal types are usually called ecotypes as they are distinct genetic lineages within a species, adapted to particular environmental conditions (Begon et al. 1990). Indeed, similar ecotypes have also evolved within species or cytotypes of related genera such as *Rhinanthus* L. and *Euphrasia* L., and species of the latter are also very variable but the pattern of cytotypic variation is always orthogonal to the differentiation of ecotypes (Yeo 1954, 1972, Smejkal & Dvořáková 2000). In the *Odontites vernus* group, however, the seasonal variation in the ecotypes is reported to coincide with the distinction of the cytotypes. According to current taxonomic treatments (Bolliger 1996, Kubát 2000, Jäger & Werner 2005) the tetraploid *O. vernus* (s. str.) corresponds to an early-flowering ecotype and the diploid *O. vulgaris* to a late-flowering ecotype. These types also differ in their ecological ranges as *O. vernus* is predominantly an agricultural weed while *O. vulgaris* is a ruderal species occurring also in pastures and mesotrophic and flood-plain meadows in central Europe (Hartl 1974, Kubát 2000).

The current concept of the *O. vernus* group appears to be well established as all the definitions in the literature are very similar. Nevertheless, there are still several issues that need investigation. Despite the reproductive barrier between *O. vernus* and *O. vulgaris* (Snogerup 1983), plants intermediate in terms of the morphology of these two species are frequently reported (Schneider 1964, Hartl 1974, Bolliger 1996, Kubát 2000). Therefore, the delimitation of these two species can be assumed to be difficult if based only on morphology. This is crucial since the descriptions of the ecology and geographical distribution of the cytotypes and seasonal ecotypes, i.e. the concept of the group is mostly based on field samples and herbarium vouchers and implicitly assumes close relationships between morphology, phenology and ploidy level. The ploidy level is, however, rarely identified by means of karyology and if it is (e.g. Schneider 1964, Snogerup 1983), then only based on a few samples. However, the basis for any hypothesis on the evolution of polyploid taxa (e.g. Kolář et al. 2009, Trávníček et al. 2010), that is evidence of the distribution of

cytotypes of the *O. vernus* group, is lacking. In addition, chromosome numbers of $2n = 18$ (or $n = 9$) and $2n = 20$ (or $n = 10$) are reported for *O. vernus* without any further details of the importance of this cytotypic variation (the former e.g. Bolliger 1996, Michalková 1998, Dobeš & Vitek 2000 and references therein, and the latter e.g. Hartl 1974, Albers 1998 and references therein).

The aim of this study was to determine the geographical distribution of cytotypes in the context of the seasonal variation in the *O. vernus* group in central Europe. An extensive screening of the ploidy level of the populations was conducted using flow cytometry (FCM), which make it possible to rapidly and accurately identify the ploidy level (Suda & Pyšek 2010). The flow cytometric approach was supported by direct chromosome counts of selected populations. The cytotypic part of the study was furthermore complemented by a statistical analysis of plant architecture, in particular of the key traits that are used to delimit the seasonal ecotypes.

Materials and methods

Field sampling and morphological analysis

In total, 139 population samples (2–15 randomly selected individuals per population; one individual was analysed in exceptional cases due to either mixed populations or just one plant at a given site) of the *O. vernus* group were collected in the Czech Republic, Slovakia and Austria (Fig. 1, Appendix 1). Fresh specimens were transported to the laboratory for the flow cytometric analysis (see the next paragraph for details). In addition, the morphological variation in a subset of 74 populations (each of which contained at least three individuals) was analysed. The following morphological characters were recorded: number of vegetative internodes (internodes between cotyledons and the uppermost pair of branches), number of intercalary internodes (internodes between the uppermost pair of branches and lowermost flowers on the main stem; these are present due to abortion of lowermost flower buds) and the sum of these two values representing the total number of internodes. The internode numbers are the most important morphological character associated with seasonal variation in the Rhinanthoid *Orobanchaceae*, largely determining other morphological characters such as number of branches and branching pattern (Zopfi 1995, 1998b, Štech 1998, Těšitel 2005). The analysis of the morphological characters was not possible for all the populations that were analyzed using flow cytometry because some were sampled prior to flowering or late in the fruiting season, when it was difficult to obtain a reliable count of the number of internodes. Plants from all the samples were processed as standard herbarium vouchers and are kept in the herbarium of the Faculty of Science, University of South Bohemia (CBFS).

Flow cytometry

DNA ploidy levels were determined using a Partec PA II flow cytometer (Partec GmbH., Münster, Germany) equipped with a mercury arc lamp. The samples were prepared following the simplified two-step protocol using Otto buffers (Doležel et al. 2007). About 0.25 cm² of leaf tissue (field-collected leaves, stored in plastic bags for a maximum of 1 week at 4°C) was chopped together with an appropriate volume of the internal standard

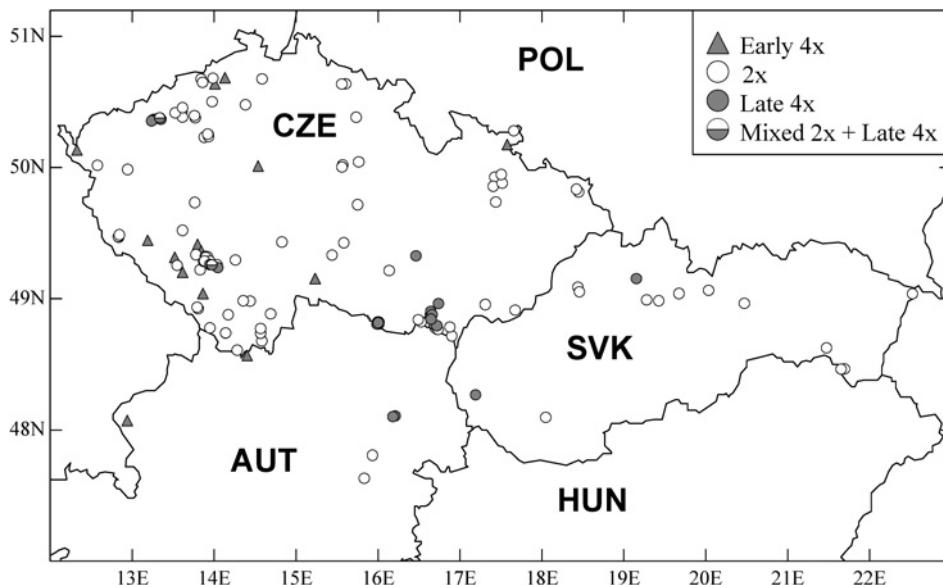


Fig. 1. – Localities where the samples used in the present study were collected. The populations are classified on the basis of cytotypic and seasonal variation. Diploid populations, early-flowering tetraploid populations and late-flowering tetraploid populations are depicted as white circles, grey triangles and grey circles, respectively. Borders of the following central-European countries are displayed: CZE – Czech Republic, AUT – Austria, SVK – Slovakia, HUN – Hungary and POL – Poland.

(*Glycine max* ‘Polanka’, $2C = 2.50$ pg; Doležel et al. 1994) using a sharp razor blade in a Petri dish containing 0.5 ml of ice-cold Otto I buffer (0.1M citric acid, 0.5% Tween-20). The suspension was filtered through a 42- μ m nylon mesh and incubated for ca 1 min. at room temperature. After incubation, 1 ml of the staining solution was added. The staining solution consisted of 1 ml of Otto II buffer (0.4M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$), 2-mercaptoethanol (2 μ l/ml) and the fluorochrome DAPI (4 μ g/ml). Samples were run on the flow cytometer after about one minute of staining and the fluorescence intensity of 3000–5000 particles was recorded. The *Odontites* material proved to be problematic due to the sensitivity of cytometry to the state of leaf tissue and fast deterioration of the nuclei in suspension (< 10 min after isolation). Histograms with coefficients of variation for the G_0/G_1 peaks of both the analysed *Odontites* sample and the standard of less than 4% were accepted. Pooled samples of up to five individuals could be used due to high-resolution of the histograms. However, in the samples from some populations there was a high number of nuclei with 4C DNA content. In such cases, each plant was separately reanalysed. Results of the ploidy level analysis were calibrated using the populations for which there was a chromosome count.

For estimating the size of the genome three populations of each cyto- and ecotype from different geographic areas of the Czech Republic were used. The same internal standard and the same method of sample preparation and staining as for the ploidy level analysis were used, only propidium iodide (50 μ g/ml) and RNase IIa (50 μ g/ml) were used instead of DAPI. The genome size was determined using a Partec CyFlow SL flow cytometer equipped with a 532 nm (green) diode-pumped solid-state laser (100 mW output). One

individual per sample was measured and the fluorescence intensity of 5000 particles was recorded. Histograms with coefficients of variation for the G_0/G_1 peaks of both the *Odontites* sample analysed and that of the standard below 4.5% were considered. In order to minimize random instrumental error each individual was analysed three times on three different days and the average value was used as the genome size. If the range in variation of the three measurements exceeded 2% of the average value, the most outlying measurement was discarded and the sample reanalysed.

Chromosome counts

To calibrate the results of the flow cytometry the chromosome numbers of the diploid Tatinná (population no. 24, Appendix 1) and tetraploid Drásov populations (population no. 125, Appendix 1) were determined. Chromosomes were counted in apical root meristems of seedlings that were germinated from seeds collected in the field. To induce germination, the seeds were placed on moist filter paper in a Petri dish and kept at 4°C until germination occurred (ca 6 weeks). The seedlings were pre-treated with a saturated water solution of p-dichlorobenzene for 3 hours at room temperature, fixed in a mixture of ethanol and acetic acid (3:1) for 24 hours at 4°C and stored in 70% ethanol at 4°C. They were macerated for about 3–5 min in a mixture of ethanol and hydrochloric acid (1:1). The apical part of the root was then cut and squashed using a cellophane square (Murín 1960) and stained for 1 h in a 10% Giemsa-Romanowski solution in 0.2M sodium phosphate buffer, pH 7.2. At least three samples (seeds originating from different individuals) per population were analysed and at least two mitoses per plant were studied.

Data analysis

Variability in the internode characters and its relationship to the cytotypic and phenological variation was analysed using linear mixed-effect models and analyses of variance. The counts of internodes were log-transformed prior to the statistical analyses. Distribution of variance in the number of internodes was analysed using a restricted maximal likelihood linear mixed-effect model with population and type included as random factors. Differences in the number of internodes among the types defined by phenology and cytometry were analysed using one-way analyses of variance (ANOVA), which were calculated based on the results for individual plants and population means. The ANOVAs were followed by Tukey's honest significance difference tests to test for differences among individual pairs of the types. All statistical analyses were performed in R, version 2.13.1 (R Development Core Team 2011); the mixed effect models were calculated in R package nlme, version 3.1–101 (Pinheiro et al. 2011).

Results

The FCM screening of *O. vernus* group plants collected across central Europe revealed two cytotypes (Tables 1, 2; Fig. 2), which direct microscopic inspection of mitotic metaphase chromosomes confirmed as diploid ($2n = 2x = 18$) and tetraploid ($2n = 4x = 40$) (Fig. 3). The diploids formed a single phenologically rather variable group of populations within which the earliest flowers (in southern-Moravian salt marsh populations 85, 86 and

Table 1. – DAPI-based relative fluorescence of samples analysed using flow cytometry. The relative fluorescence is the sample : *Glycine max* standard ratio of fluorescences.

Type	n	Mean relative fluorescence	SE
2x	227	0.442	0.001
Early 4x	63	0.848	0.001
Late 4x	40	0.860	0.001

Table 2. – Genome sizes (propidium iodide staining) and their variability across selected population samples of the *Odontites vernus* group. C-values and Cx-values are indicated in pg of DNA. See Appendix 1 for details of the populations.

Type	Population	n	2C-value	Mean 2C-value	SE	Variation (%)	Mean Cx-value
2x	Dětrichov (no. 93)	3	1.19	1.20	0.01	1.7	0.60
2x	Jihlava (no. 82)	3	1.19				
2x	Vrbno (no. 46)	3	1.21				
Early 4x	Františkovy Lázně (no. 1)	3	2.31	2.35	0.02	2.4	0.59
Early 4x	Hostovice (no. 2)	3	2.37				
Early 4x	Řepice (no. 10)	3	2.36				
Late 4x	Havraníky (no. 124)	3	2.37	2.36	0.01	0.5	0.59
Late 4x	Drásov (no. 125)	3	2.36				
Late 4x	Úhošťany (no. 117)	3	2.35				

87) were not recorded before late July (25 July). Individual populations of this type then continued to flower until late September (25 September; population 72). By contrast, two clearly phenologically defined seasonal types were found within the tetraploids. The early-flowering tetraploids flowered from late May (29 May; population 16) to early July (7 July; population 5) while the late-flowering population of the same cytotype flowered from the second half of August (17 August; population 129) to late September (20 September; population 133), i.e. later than most of the diploid populations. Although these may not be the complete flowering periods since some more phenologically extreme populations might occur in each of the types, it is clear there is hardly any overlap in the flowering periods of the early and late flowering types. In particular, the early and late tetraploids are clearly phenologically distinct with more than a one month gap between the last recorded flowering of the early type and the earliest recorded flowering of the late type population. The flow cytometry revealed a small but significant difference in the DAPI-based relative fluorescence (Table 1, Welch t test: $t = -7.175$, $df = 85.05$, $P < 0.0001$) but not in the (propidium iodide-based) genome size of the two seasonal types of tetraploids (Table 2). This small difference (about 1.5%) was nonetheless not recorded in a simultaneous FCM analysis of a mixed sample of early- and late-flowering tetraploids. Thus, based on the pattern of cytotypic and seasonal variation there are three distinct types within the *O. vernus* group in central Europe: diploids, early-flowering tetraploids (early tetraploids) and late-flowering tetraploids (late tetraploids).

Not only do these types differ in phenology, but also in number of internodes on their stems, i.e. characters associated with seasonal variation in hemiparasitic *Orobanchaceae*. Distribution of variance in the number of vegetative internodes, number of intercalary internodes and total number of internodes clearly indicates that the highest proportion of

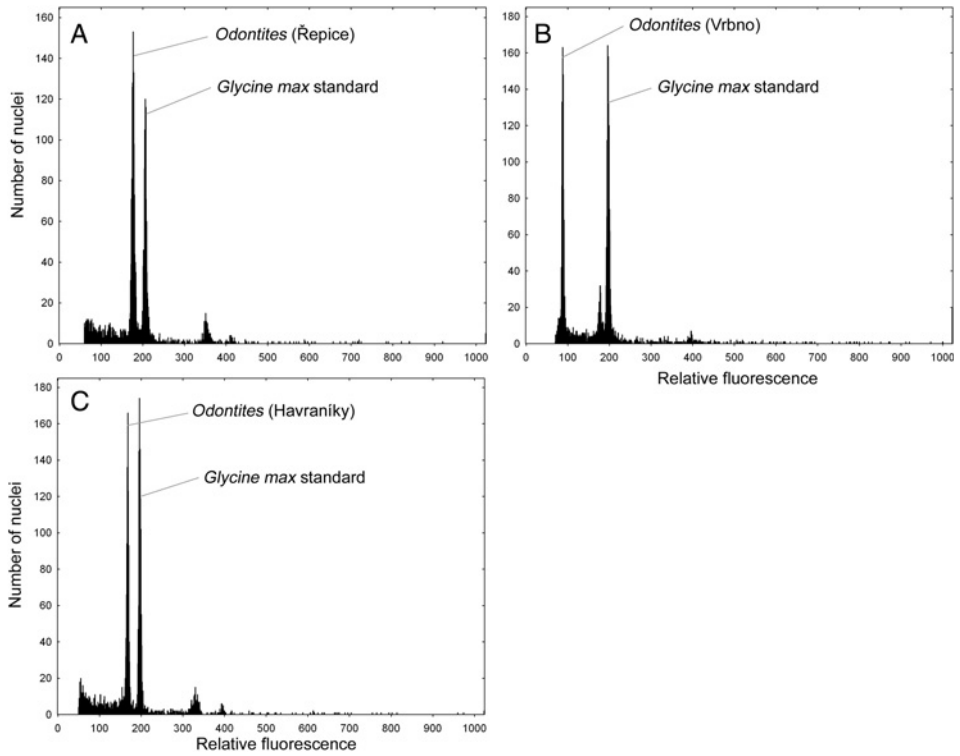


Fig. 2. – Flow cytometric profiles (DAPI staining) of three phenological and cytological types of the *Odontites vernus* group occurring in central Europe: (A) early-flowering tetraploid, (B) diploid and (C) late-flowering tetraploid.

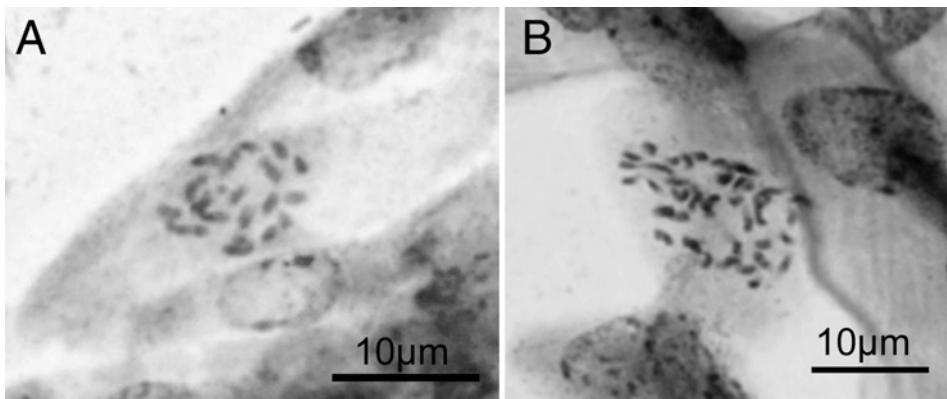


Fig. 3. – Photographs of mitotic metaphase chromosomes of (A) diploid ($2n = 18$) cytotype (population no. 24, Tatinná) and (B) tetraploid ($2n = 40$) cytotype (population no. 125, Drásov) of the *Odontites vernus* group taken using a light microscope.

Table 3. – Distribution of variance in the number of internodes in the *Odontites vernus* group inferred from the calculation of the variance components. Tests of statistical significance at the higher hierarchical levels of variation (types, populations) are displayed. Numbers of internodes were log-transformed prior to the analysis.

	% variance	Likelihood ratio	df	P
Number of vegetative internodes				
type	78.92	569.87	1	< 0.0001
among populations	10.38	414.90	1	< 0.0001
within populations	10.70			
Number of intercalary internodes				
type	54.60	246.57	1	<0.0001
among populations	13.55	175.19	1	<0.0001
within populations	31.85			
Total number of internodes				
type	85.06	765.50	1	<0.0001
among populations	6.86	364.62	1	<0.0001
within populations	8.08			

Table 4. – Summary of the analyses of variance testing the effect of the *Odontites vernus* types on the values of the seasonal characters at the level of individual specimens and population means. The type identity is used as a single predictor in all the one-way ANOVA models. The numbers of internodes were log-transformed prior to the analysis.

Morphological character	Level of testing	df	R ²	F	P
Number of vegetative internodes	individual plants	2, 822	0.508	303.31	< 10 ⁻⁶
	population means	2, 71	0.684	76.81	< 10 ⁻⁶
Number of intercalary internodes	individual plants	2, 822	0.270	46.11	< 10 ⁻⁶
	population means	2, 71	0.549	43.22	< 10 ⁻⁶
Total number of internodes	individual plants	2, 822	0.613	650.01	< 10 ⁻⁶
	population means	2, 71	0.776	122.85	< 10 ⁻⁶

the variation in all these characters occurs at the level of the types, while variation among populations within types displayed the lowest, albeit statistically significant component of variance (Table 3). Further tests of the effect of type on seasonal characters using analyses of variance yielded statistically significant results for all the characters at the level of both individual plants and population means (Table 4). Values for internode numbers were lowest in the early tetraploids (Fig. 4). This applies in particular to the number of intercalary internodes, which was mostly one and reached two only in rare cases compared to 2–5 most frequently present in plants of the late-flowering type (Fig. 4). In addition, the vegetative and total internode numbers also allowed a clear identification of this type in the whole dataset. Morphological differences between the diploids and late tetraploids were much less pronounced. The tetraploids however, had a significantly higher number of vegetative internodes, which was also reflected in the total internode count (Fig. 4). However, these two types did not differ significantly in the number of intercalary internodes.

The geographic distributions of the *Odontites vernus* group types (Fig. 1) are clearly different in the Czech Republic where the sampling was extensive enough to be representative. The diploids occur throughout the whole country as an abundant component of

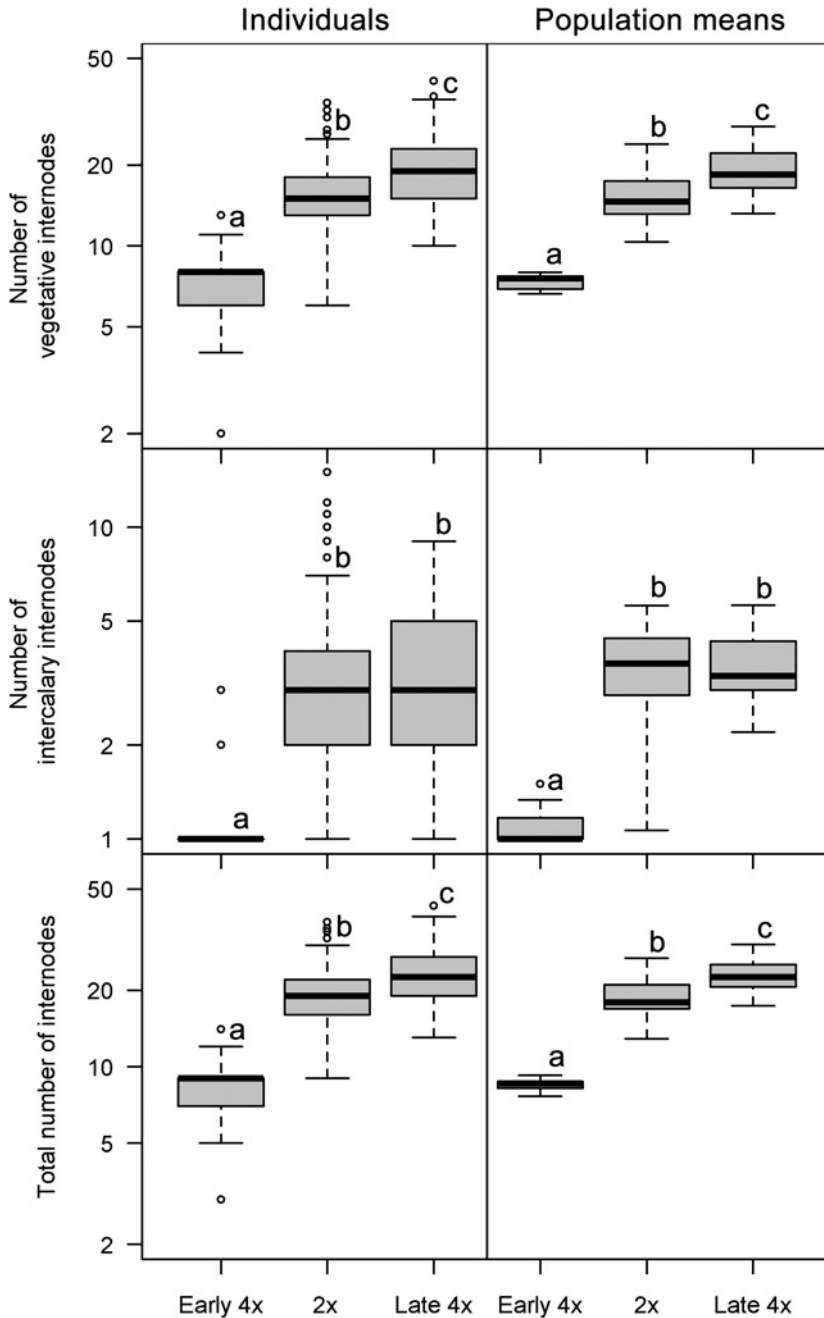


Fig. 4. – Box-and-whisker plots of the associations between the numbers of internodes and phenological and cytological types of the *Odontites vernus* group in central Europe at the level of individual plants and mean number of internodes per population. Note the logarithmic scales of the y-axes. Median, quartiles, non-outlier ranges and outliers are depicted by a bold line, boxes, whiskers and points, respectively. Different letters at the upper limit of the non-outlier range indicate a statistically significant ($P < 0.05$) difference inferred from post-hoc Tukey HSD multiple comparison tests applied to the ANOVA models summarized in Table 4.

ruderal communities or mesotrophic meadows, often including those on floodplains. By contrast, the distribution and abundance of the other two types appeared more restricted. The distribution of the early tetraploids is scattered across the country (numerous sites located in southwestern Bohemia coincide with where the sampling effort was greatest). In addition, this type was much less frequent compared to the diploids and it always grew either as an agricultural weed or on fallows. The late tetraploids seemed to display an affinity for the warm yet hilly regions of the country occurring often in communities that could be classified as dry grasslands. At these localities, the populations were most often found at microsites that were at least slightly disturbed and displayed certain aspects of ruderal sites (such as edges of pathways). Occurrence in such habitats was most marked in southern Moravia. This type occasionally occurred in ruderal vegetation, especially edges of roads. Nevertheless we also detected clear exceptions to the general trend of late tetraploids growing in the warmest regions of the country. This is indicated by the occurrence of the late tetraploids in southern Bohemia (i.e. the region where the early tetraploids frequently occur) and in northern Slovakia (Fig. 1). Most of the populations analysed were comprised of plants of a single cytotype. Nevertheless, diploids and late tetraploids co-occurred at three sites, two in southern Bohemia (close to each other) and one in northern Bohemia.

Discussion

Cytotypic variation

The extensive screening of ploidy level in populations of the *Odontites vernus* group in central Europe by FCM suggests the need for a substantial update of the concept of the *O. vernus* group. For the first time, our study has demonstrated the existence of late tetraploid populations in this group. Not only is this a new addition to the flora of central Europe it is also a hitherto undescribed entity in this group at the global scale. It was also recorded in southern and western Europe during a preliminary screening of the *O. vernus* group on a continental scale (Appendix 2) suggesting its range extends substantially beyond the area of central Europe included in this study. Tetraploid *Odontites vernus* group plants morphologically indistinguishable from diploids have moreover been reported as common in Spain (Delgado et al. 2005, Rico 2009). Nevertheless, additional broad screening is certainly needed to determine the distribution of late tetraploids in Europe.

Apart from revealing the existence of late tetraploids, the FCM screening has supported other aspects of the current concept of the *O. vernus* group. All early-flowering plants were confirmed as tetraploid and all diploids as moderately late-flowering (complying with the diploid flowering period reported by other authors; Schneider 1964, Snogerup 1983, Kubát 2000, Jäger & Werner 2005, Fischer et al. 2008). In addition, the sites where both these types occur comply with the description of their ecology in local floras (Michalková 1997, Kubát 2000, Jäger & Werner 2005, Fischer et al. 2008) and monographs on mainly northern-European populations (Schneider 1964, Snogerup 1983). We did not detect any variation in the genome size of the diploids (even using the sensitive DAPI-staining) that could correspond to the reported existence of two chromosome numbers, $2n = 18$ and $2n = 20$ (cf. Schneider 1964 and Snogerup 1983). Therefore, all of the diploid populations analysed can be assumed to have $2n = 18$ chromosomes, as revealed by the direct count of the chromosomes of individuals from population 24 (Fig. 3), which is consistent with earlier reports of chromosome counts for *Odontites* in central Europe (Michalková 1997, Dobeš & Vitek 2000).

Seasonal variation

The analysis of the seasonal changes in morphology has supported the clear delimitation of early- and late-flowering types on the basis of the number of internodes. In particular, the number of intercalary internodes is a clear-cut way of identifying these types as suggested by earlier authors (Schneider 1964, Snogerup 1983, Bolliger 1996, Kubát 2000). In general, the overlap in the number of internodes between early and late types is very small. This applies especially to tetraploids within which the seasonal types are clearly separated on the basis of morphology. On the other hand, diploids and late tetraploids cannot be clearly delimited from each other based on the number of internodes (Fig. 4). Despite this lack of a clear separation, the tetraploids in the populations analysed had statistically significantly higher numbers of vegetative internodes than the diploids, which was also reflected by the counts of the total number of internodes.

The delimitation of the seasonal ecotypes was not associated with ploidy level in the *Odontites vernus* group, which is similar to the situation in species of the related genus *Euphrasia*, where seasonal ecotypes occur within cytotypes (Smejkal 1963, Yeo 1972, Karlsson 1976, Smejkal & Dvořáková 2000). In general, the tetraploids appear to be highly specialized in terms of seasonal characters and form clearly distinct ecotypes comparable, e.g. to some of the more extreme ecotypes present in *Euphrasia rostkoviana* Hayne in the Alps (Zopfi 1998b). The values for the seasonal morphological characters of diploids were more variable and positioned in between those of the tetraploid seasonal ecotypes. The diploids hence display a greater range of seasonal variation, which is associated with their broad ecological range, but lack any tendency to form distinct seasonal ecotypes. Very similar continuous patterns of seasonal variation are typical of related *Melampyrum pratense* L. and *M. sylvaticum* L., which also have broad ecological (Štech 1998) and wide altitudinal ranges (Těšitel 2005).

Geographical distribution and breeding barriers

The diploid type of the *O. vernus* group is an omnipresent species in the central-European landscape but the distribution of the tetraploid types is very different. The early tetraploids have a fairly scattered distribution (Fig. 1), but it is obvious they were more abundant in the past as indicated by numerous historical records of them occurring throughout the Czech Republic (Kubát 2000). Intensification of agriculture and improved weed control has, however, resulted in its decline in most of the country. By contrast, the late tetraploids occur frequently in regions with a warm and rather continental climate while being rather rare in colder areas. Even the southern-Bohemian late tetraploid populations located outside these warmest regions were found in an area with a rather mild climate. Interestingly, both the habitat preferences and geographical range of the late tetraploids are somewhat similar to those of *O. luteus*, which is a rather distantly related species that could be also considered as a distinct autumnal type as it flowers in August–September (Bolliger 1996, Kubát 2000).

In general, the geographical distributions of the *O. vernus* group in central Europe can be considered mostly sympatric (early tetraploids – diploids, late tetraploids – diploids) or sympatric-parapatric (early tetraploids – late tetraploids), but more data are needed to draw a more accurate picture of the distribution patterns. Due to this level of sympatry, the principal barriers preventing cross-breeding between the types are likely to be underpinned by incompatibility due to the different ploidy levels (Snogerup 1983) and habitat and phenological differentiation of the tetraploid types, which rarely share similar habitats and their flowering seasons do not overlap.

Wider perspectives

In this study, we have clearly demonstrated that there is a relationship between seasonal and cytotypic variation in the *Odontites vernus* group, and described the geographical distributions of the cytotypes and seasonal types in central Europe. Based on this, it appears necessary to update the taxonomic treatment of the group by including the existence of the late tetraploid type, which, moreover, might be a widespread element of the European flora. New questions have however, arisen about the evolutionary origin of the tetraploid ecotypes. Given the almost constant monoploid genome size (C_x -value) of all the types and lack of any co-occurring closely related species of *Odontites* with which they could hybridize (Bolliger 1996), it is likely that they originated via autopolyploidy (Trávníček et al. 2010). On the other hand, the different base chromosome numbers ($x = 9$ vs. $x = 10$ in diploids and tetraploids, respectively) and variability in this character in the diploids indicate the possibility that the evolution of the tetraploids involved a more complicated process. Both scenarios of ecotypic differentiation within tetraploids of either an independent origin of early and late tetraploids or a single polyploidization event and subsequent differentiation of the seasonal types are plausible explanations.

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Souhrn

Na základě karyologických dat byly v rámci agregátu *Odontites vernus* ze střední Evropy udávány dva cytotypy, diploidní ($2n = 2x = 18$ nebo 20) a tetraploidní ($2n = 4x = 40$). Podle dosavadní literatury souvisí s touto cytotypovou variabilitou i existence sezónních ekotypů. Diploidní typ by měl zahrnovat pozdně (červenec až srpen) kvetoucí rostliny s velkým počtem lodyžních článků, běžně rostoucí zejména na ruderalních stanovištích. Tetraploidní typ mají představovat naopak časně (konec května až červen) kvetoucí rostliny s malým počtem článků, vyskytující se především jako polní plevel nebo na úhorech. V rámci naší studie jsme pomocí průtokové cytometrie stanovili ploidní úroveň 139 populací z okruhu *Odontites vernus* agg. U vybraných populací jsme také analyzovali počty lodyžních článků, které těsně souvisejí s fenologií populací, a to počet vegetativních článků, počet interkalárních článků (článků mezi nejvyšše postavenými větvemi a nejnižšími květy) a celkový počet článků (součet dvou předešlých hodnot). Přítomnost diploidních ($2n = 2x = 18$) a tetraploidních ($2n = 4x = 40$) populací byla pomocí průtokové cytometrie potvrzena. V případě diploidního typu byl karyologicky potvrzen chromozómový počet 18 a v rámci cytometrických měření nebyla v rámci ploidií pozorována žádná výrazná variabilita. Na rozdíl od předchozích studií jsme však zaznamenali i tetraploidní, výrazně pozdně (druhá polovina srpna až září) kvetoucí populace s vůbec největším počtem článků. Tyto rostliny se nejčastěji vyskytují v teplých, kontinentálních částech střední Evropy na narušovaných místech v kontaktu se suchými trávníky nebo stepmi. Sporadicky však byly nalezeny i v oblastech mezofytika na Strakonicku. Naše výsledky ukazují, že místo dvou sezónně oddělených cytotypů se ve Střední Evropě vyskytují tři entity: dva sezónně vyhraněné ekotypy v rámci tetraploidů a diploidi, kteří vykazují značnou variabilitu ve fenologii i počtu článků a není možné mezi nimi rozlišit žádné jasné vyhraněné sezónní typy. Obdobný vztah mezi sezónní a cytotypovou variabilitou nalezneme např. i v příbuzném rodě *Euphrasia*. Jednotlivé typy jsou pravděpodobně reprodukčně dobře izolovány, jejich vzájemnému křížení brání buď odlišný počet chromozómů (diploidi vs. tetraploidi) nebo nepřekrývající se doba květu a rozdílné stanovištní nároky (časně vs. pozdně kvetoucí tetraploidi).

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Appendix 1. – List of the central-European localities of the *Odontites vernus* group sampled for this study. The format of the data is: Locality number – Country; Town (district): localization; coordinates (WGS 84); altitude; collector; collection date; number of individuals analysed using flow cytometry / number of individuals for which internodes were counted. Abbreviations of countries: AT – Austria, CZ – Czech Republic, SK – Slovakia. Abbreviations of collectors: GT – G. Tuleu, JT – J. Těšitel, MŠ – M. Štech, PK – P. Koutecký, TB – T. Baďurová.

Early flowering tetraploids. **1** CZ; Františkovy Lázně (distr. Cheb): NW of the town, crop-field edge ca 0.6 km SEE of the village of Antonínova Výchina; 50°08'08"N, 12°19'41"E; 470 m a.s.l.; PK & TB; 25 Jun 2010; 6/-. **2** CZ; Hostovice (distr. Ústí nad Labem): fallows (ca 5 years old) ca 0.4 km SW of the village, along the road to Újezd; 50°38'25"N, 14°00'48"E; 270 m a.s.l.; PK & TB; 20 Jun 2010; 18/17. **3** CZ; Povrly (distr. Ústí nad Labem): ca 2.5 km NW of the village, an old fallow ca 0.3 km W of the village of Mašovice; 50°41'07"N, 14°08'09"E; 400 m a.s.l.; PK & TB; 20 Jun 2010; 8/7. **4** CZ; Praha-Újezd (distr. Praha): crop-field ca 0.4 km SWW of the centre of the village; 50°00'43"N, 14°32'28"E; 280 m a.s.l.; A. Čurnová; 17 Jun 2011; 5/-. **5** CZ; Slatina (by Chudenice, distr. Klatovy): fallow ca 0.2 km E of the E edge of the village; 49°26'46"N, 13°11'31"E; 470 m a.s.l.; PK; 07 Jul 2009; 2/-. **6** CZ; Letovy (distr. Klatovy): edge of a barley field ca 1 km SW of the village; 49°18'57"N, 13°31'20"E; 540 m a.s.l.; GT; 17 Jun 2009; 10/7. **7** CZ; Rozsedly (distr. Klatovy): edge of a wheat field 0.8 km SE of the village; 49°12'04"N, 13°37'09"E; 575 m a.s.l.; GT; 17 Jun 2009; 10/6. **8** CZ; Vrbno (distr. Strakonice): abandoned field on the S edge of the forest 1 km N of the village; 49°24'51"N, 13°48'04"E; 481 m a.s.l.; PK & MŠ; 21 Jun 2009; 7/7. **9** CZ; Bratronice (distr. Strakonice): crop-field edge ca 0.4 km SW of the village; 49°21'49"N, 13°50'47"E; 535 m a.s.l.; PK; 22 Jun 2011; 2/-. **10** CZ; Řepice (distr. Strakonice): edge of a wheat field next to a Scotch Pine stand between Řepice and Rovná ca 0.6 km NE of the village; 49°16'58"N, 13°56'34"E; 430 m a.s.l.; GT; 17 Jun 2009; 10/11. **11** CZ; Domanice (distr. Strakonice): edge of a rape field ca 0.65 km SE of the village; 49°17'45"N, 13°55'52"E; 475 m a.s.l.; GT; 17 Jun 2009; 10/9. **12** CZ; Šumavské Hoštice (distr. Prachatice): edge of a wheat field near the football pitch in the NW part of the village; 49°02'22"N,

13°51'59"E; 820 m a.s.l.; GT; 14 Jun 2009; 10/9. **13 CZ**; Malý Jeníkov (distr. Jindřichův Hradec): in the middle of an abandoned field overgrown with clover ca 0.35 km N of the village; 49°09'12"N, 15°14'10"E; 629 m a.s.l.; P. Kúr; 01 Jul 2009; 5/4. **14 CZ**; Město Albrechtice (distr. Bruntál): NW corner of the crop-field (wheat) ca 0.85 km NNE of the railway station; 50°10'33"N, 17°34'49"E; 390 m a.s.l.; Z. Mruzíková; 25 Jul 2011 [fruiting plants, the ploidy level determined using seeds]; 5/- . **15 AT**; Dorfbm (Oberösterreich): crop-field edge ca 500 m NE of the centre of the village, by Heratinger See; 48°04'20"N, 12°56'36"E; 440 m a.s.l.; M. Lepší; 21 Jul 2010 [fruiting plants collected after the flowering period]; 1/- . **16 AT**; Eibenstein (Oberösterreich): crop-field edge ca 1.2 km NNW of the village; 48°34'13"N, 14°24'20"E; 650 m a.s.l.; MŠ; 29 May 2011; 3/- .

Diploids. **17 CZ**; Lázně Kynžvart (distr. Cheb): surrounding of the Malý Publák brook ca 3.5 km ENE of the town; 50°00'60"N, 12°34'45"E; 530 m a.s.l.; J. Laburdová; 17 Sep 2010; 5/- . **18 CZ**; Dobrá Voda (distr. Karlovy Vary): road edge ca 1 km WSW of the centre of the village; 49°59'02"N, 12°56'57"E; 650 m a.s.l.; J. Laburdová; 16 Sep 2010; 15/- . **19 CZ**; Kadaňská Jeseň (distr. Chomutov): meadow at the N edge of the village; 50°22'04"N, 13°16'01"E; 350 m a.s.l.; TB; 01 Sep 2010; 5/5. **20 CZ**; Kadaň (distr. Chomutov): road edge by the road crossing SW of the power station Tušimice, ca 5 km E of the town; 50°22'31"N, 13°19'26"E; 311 m a.s.l.; TB; 01 Sep 2010; 2/- . **21 CZ**; Škrle (distr. Chomutov): dry meadow at the edge of Slanisko u Škrle nature reserve, W of the village; 50°25'01"N, 13°31'47"E; 250 m a.s.l.; GT; 03 Oct 2009; 5/- . **22 CZ**; Nemilkov (distr. Most): NW bank of a small pond in the village; 50°27'20"N, 13°37'10"E; 245 m a.s.l.; GT; 03 Oct 2009; 6/- . **23 CZ**; Moravěves (distr. Most): on the W edge of the southern pond in the village; 50°25'25"N, 13°36'44"E; 289 m a.s.l.; GT; 03 Oct 2009; 5/5. **24 CZ**; Tatinná (distr. Louny): ca 0.75 km ENE of the bridge over the Chomutovka river in the village, road edge; 50°23'03"N, 13°37'02"E; 242 m a.s.l.; GT; 03 Oct 2009; 5/1. **25 CZ**; Lenešice (distr. Louny): forest road edge on the N slope of Lenešický chlum hill, ca 2.3 km N of the centre of the village; 50°23'47"N, 13°45'50"E; 280 m a.s.l.; TB; 06 Sep 2011; 3/- . **26 CZ**; Lenešice (distr. Louny): road edge ca 300 m N of the railway stop, NE of the village; 50°22'43"N, 13°46'25"E; 185 m a.s.l.; TB; 06 Sep 2011; 8/- . **27 CZ**; Proboštov (distr. Teplice): meadows ca 0.5 km NNE of the edge of the village; 50°40'31"N, 13°50'41"E; 270 m a.s.l.; J. Karásek; 04 Oct 2009; 9/- . **28 CZ**; Teplice (distr. Teplice): meadows E of the town, ca 1.2 km N of the summit of Doubravka hill; 50°38'58"N, 13°51'47"E; 230 m a.s.l.; J. Karásek; 04 Oct 2009; 10/- . **29 CZ**; Teplice (distr. Teplice): road edge E of the town, on the E foothills of Písečný vrch hill; 50°38'16"N, 13°50'54"E; 250 m a.s.l.; J. Karásek; 04 Oct 2009; 5/- . **30 CZ**; Ústí nad Labem (distr. Ústí nad Labem): meadows and fallows S of the town part Všebořice, ca 0.8 km NNW of the summit of Střížovický vrch hill; 50°40'50"N, 13°59'23"E; 320 m a.s.l.; PK & TB; 20 Jun 2010 [young non-flowering plants]; 10/- . **31 CZ**; Sutom (distr. Litoměřice): edge of a road through a meadow ca 0.4 km ESE of the church; 50°30'07"N, 13°58'49"E; 420 m a.s.l.; GT; 23 Aug 2009; 15/13. **32 CZ**; Srbeč (distr. Rakovník): road edge, ca 0.65 km N of the church; 50°13'50"N, 13°52'59"E; 330 m a.s.l.; TB; 31 Aug 2010; 12/12. **33 CZ**; Líský (distr. Kladno): forest clearing ca 0.6 km NNW of the centre of the village; 50°15'14"N, 13°55'30"E; 395 m a.s.l.; TB; 31 Aug 2010; 20/19. **34 CZ**; Líský (distr. Kladno): road edge ca 0.6 km NNW of the centre of the village; 50°15'16"N, 13°55'32"E; 390 m a.s.l.; TB; 31 Aug 2010; 5/- . **35 CZ**; Líský (distr. Kladno): crop-field edge ca 0.5 km NE of the centre of the village; 50°15'08"N, 13°55'54"E; 360 m a.s.l.; TB; 31 Aug 2010; 6/6. **36 CZ**; Pozdeň (distr. Kladno): ca 0.7 km SW of the centre of the village, along the road Pozdeň–Strbeč; 50°14'05"N, 13°56'08"E; 423 m a.s.l.; TB; 31 Aug 2010; 2/- . **37 CZ**; Radouň (distr. Litoměřice): grassy road at the S edge of the Radouň nature reserve, ca 0.6 km W of the centre of the village; 50°28'44"N, 14°23'11"E; 220 m a.s.l.; PK & TB; 19 Jun 2010 [young non-flowering plants]; 3/- . **38 CZ**; Česká Lípa (distr. Česká Lípa): pastures on the left bank of the Ploučnice river ca 0.7 km SE of the bridge on the road Žizníkov–Dobranov, ca 4 km E of the railway station Česká Lípa; 50°40'36"N, 14°35'17"E; 252 m a.s.l.; F. Kolář; 20 Aug 2009; 10/9. **39 CZ**; Starý Pařezov (distr. Domažlice): meadows and road edges W of the village; 49°28'16"N, 12°50'08"E; 410 m a.s.l.; TB; 08 Sep 2010; 20/18. **40 CZ**; Nový Pařezov (distr. Domažlice): ruderal vegetation on an abandoned building site; 49°28'07"N, 12°50'20"E; 440 m a.s.l.; TB; 08 Sep 2010; 27/18. **41 CZ**; Otov (distr. Domažlice): road edge at the E border of the village; 49°29'10"N, 12°50'48"E; 410 m a.s.l.; TB; 08 Sep 2010; 16/13. **42 CZ**; Dobršíň (distr. Klatovy): ca 1 km SW of the village, an abandoned area of the former factory; 49°15'16"N, 13°33'11"E; 463 m a.s.l.; GT; 25 Aug 2009; 12/11. **43 CZ**; Vrčeň (distr. Plzeň-jih): edge of the road to the village of Sedlišťe ca 1.35 km N of the church in Vrčeň; 49°31'09"N, 13°37'09"E; 432 m a.s.l.; GT; 29 Aug 2009; 8/7. **44 CZ**; Strašice (distr. Rokycany): E part of the village; 49°43'57"N, 13°45'59"E; 515 m a.s.l.; J. Karásek; 15 Aug 2009; 10/9. **45 CZ**; Mečichov (distr. Strakonice): dam of the Bejchlín fishpond, ca 2 km SWW of the village; 49°20'07"N, 13°46'56"E; 423 m a.s.l.; R. Paulič; 11 Sep 2010; 10/- . **46 CZ**; Vrbno (distr. Strakonice): road edge 1 km N of the village; 49°24'52"N, 13°48'15"E; 470 m a.s.l.; TB; 09 Aug 2010; 13/- . **47 CZ**; Švejarova Lhota (distr. Strakonice): road edge in the village; 49°13'10"N, 13°49'60"E; 601 m a.s.l.; GT; 25 Aug 2009; 10/7. **48 CZ**; Dražejov (distr. Strakonice): grassy field road ca 1 km NNE of the village; 49°17'03"N, 13°52'26"E; 445 m a.s.l.; R. Paulič; 11 Sep 2010; 10/- . **49 CZ**; Droužetice (distr. Strakonice): road edge, ca 0.9 km WSW of the centre of the village; 49°17'11"N, 13°53'11"E; 450 m a.s.l.; TB; 22 Sep 2010; 5/- . **50 CZ**; Droužetice (distr. Strakonice):

meadow ca 750 m WSW of the centre of the village; 49°17'14"N, 13°53'16"E; 456 m a.s.l.; GT; 25 Aug 2009; 9/5. **51 CZ**; Chrástovice (distr. Strakonice): road edge by the Chválovec fishpond, ca 1.2 km S of the centre of the village; 49°19'19"N, 13°53'52"E; 470 m a.s.l.; TB; 22 Sep 2010; 2/-.

52 CZ; Podolí (by Radomyšl, distr. Strakonice): road edge and forest edge ca 0.5 km E of the village; 49°18'44"N, 13°54'09"E; 450 m a.s.l.; TB; 22 Sep 2010; 1/-.

53 CZ; Radomyšl (distr. Strakonice): road edge between fishponds Velká Cihelna and Malá Cihelna, W of the town; 49°19'03"N, 13°55'16"E; 455 m a.s.l.; TB; 22 Sep 2010; 2/-.

54 CZ; Rovná (distr. Strakonice): ruderal vegetation on the W edge of the village, S of the fishpond Rovenský; 49°17'12"N, 13°56'59"E; 410 m a.s.l.; TB; 22 Sep 2010; 3/-.

55 CZ; Hájská (distr. Strakonice): ruderal vegetation and road edges ca 300 m N of the village; 49°15'36"N, 13°56'57"E; 390 m a.s.l.; MŠ; 25 Aug 2011; 18/-.

56 CZ; Sudomeř (distr. Strakonice): meadow inside the backwater of the Otava river ca 1 km NW of the centre of the village; 49°15'28"N, 14°02'23"E; 377 m a.s.l.; GT; 25 Aug 2009; 10/6.

57 CZ; Březí (distr. Písek): forest road ca 1.8 km SSE of the chapel in the centre of the village; 49°17'42"N, 14°15'49"E; 452 m a.s.l.; P. Kúr; 02 Aug 2009; 16/16.

58 CZ; Lenora (distr. Prachatic): road edge N of the village, ca 400 m N of the railway stop; 48°56'02"N, 13°47'50"E; 780 m a.s.l.; Š. Svobodová; 24 Aug 2011; 20/18.

59 CZ; Lenora (distr. Prachatic): road edge E of the village, ca 0.5 km SSE of the railway stop; 48°55'20"N, 13°48'35"E; 760 m a.s.l.; P. Kúr; 24 Aug 2011; 20/-.

60 CZ; Nová Pec (distr. Prachatic): road crossing ca 1.6 km S of the railway station; 48°46'31"N, 13°57'09"E; 760 m a.s.l.; JT; 10 Sep 2009; 10/9.

61 CZ; Prachatic (distr. Prachatic): ruderal lawn in the town; 49°00'46"N, 14°00'02"E; 560 m a.s.l.; A. Jírová; 12 Sep 2010; 1/-.

62 CZ; Chvalšiny (distr. Český Krumlov): edge of a field by the road to Lhenice ca 3.3 km NW of the church in the village; 48°52'35"N, 14°10'45"E; 601 m a.s.l.; GT; 15 Aug 2009; 10/8.

63 CZ; Muckov (distr. Český Krumlov): old quarry E of the village; 48°44'16"N, 14°08'37"E; 792 m a.s.l.; J. Košnar; 30 Aug 2009; 3/-.

64 CZ; Světlík (distr. Český Krumlov): road edge in the village; 48°43'51"N, 14°12'46"E; 780 m a.s.l.; Š. Svobodová; 12 Sep 2010; 10/-.

65 CZ; Vyšší Brod (distr. Český Krumlov): forest road ca 2 km SW of the town square; 48°36'29"N, 14°17'12"E; 720 m a.s.l.; PK; 26 Aug 2010; 20/19.

66 CZ; Křenovice (distr. České Budějovice): garden ca 1 km E of the village centre; 48°59'01"N, 14°21'32"E; 393 m a.s.l.; MŠ; 30 Jul 2009; 10/9.

67 CZ; České Budějovice (distr. České Budějovice): ruderal lawn by the M. Horákové Street near Dubenská bus stop in the W part of the town; 48°58'51"N, 14°26'27"E; 395 m a.s.l.; GT; 28 Jul 2009; 26/26.

68 CZ; Nová Ves (by České Budějovice, distr. České Budějovice): meadow ca 0.7 km SW of the railway station; 48°55'02"N, 14°31'19"E; 445 m a.s.l.; M. Lepší; 02 Aug 2011; 5/-.

69 CZ; Jílovice (distr. České Budějovice): forest road edge ca 1.1 km NW of the railway stop Jílovice, SWW of the village; 48°52'59"N, 14°41'34"E; 470 m a.s.l.; MŠ; 20 Aug 2011; 2/-.

70 CZ; Trhové Sviny (distr. České Budějovice): ruderal lawn in the town; 48°50'29"N, 14°38'09"E; 470 m a.s.l.; TB; 05 Sep 2010; 5/-.

71 CZ; Soběnov (distr. Český Krumlov): forest road edge ca 500 m E of the summit of Kohout hill, NE of the village; 48°46'18"N, 14°34'14"E; 720 m a.s.l.; TB; 20 Aug 2011; 5/-.

72 CZ; Benešov nad Černou (distr. Český Krumlov): meadows ca 4.2 km WNW of the town, ca 0.7 km N of the village of Ličov; 48°44'11"N, 14°34'15"E; 590 m a.s.l.; PK; 25 Sep 2009; 1/-.

73 CZ; Malonty (distr. Český Krumlov): depression in the middle of the field ca 1 km SE of the village, direction Bělá; 48°40'43"N, 14°35'07"E; 718 m a.s.l.; GT; 15 Aug 2009; 10/8.

74 CZ; Chýnov (distr. Tábor): abandoned quarry on Pacova hora hill, ca 2.2 km NE of the railway station in the town; 49°25'54"N, 14°49'53"E; 530 m a.s.l.; PK; 05 Sep 2010; 18/16.

75 CZ; Valteřice (distr. Semily): meadow ca 1.2 km NNE of the village; 50°38'16"N, 15°33'44"E; 700 m a.s.l.; TB; 13 Aug 2011; 18/8.

76 CZ; Vrchlabí (distr. Trutnov): road edge ca 30 m S of the bridge over the Labe river in the N part of the town; 50°38'17"N, 15°36'25"E; 500 m a.s.l.; TB; 13 Aug 2011; 18/-.

77 CZ; Bílé Poličany (distr. Trutnov): S of the village, meadow at the N edge of the village of Lhotka; 50°22'59"N, 15°44'13"E; 290 m a.s.l.; J. Lepš; 29 Aug 2009; 3/1.

78 CZ; Brloh (distr. Pardubice): meadow ca 600 m NE of the village; 50°00'14"N, 15°33'52"E; 230 m a.s.l.; TB; 25 Aug 2010; 20/17.

79 CZ; Přelouč (distr. Pardubice): road edge along the road Benešovice–Přelouč S of the town; 50°01'11"N, 15°34'24"E; 235 m a.s.l.; TB; 25 Aug 2010; 16/11.

80 CZ; Pardubice (distr. Pardubice): meadow at the bank of the Labe river in the town by the bridge of the road no. 324; 50°02'33"N, 15°46'07"E; 220 m a.s.l.; JT; 30 Aug 2009; 6/4.

81 CZ; Podmoklany (distr. Havlíčkův Brod): meadow by the hamlet of Branišov; 49°42'57"N, 15°45'17"E; 470 m a.s.l.; JT; 30 Aug 2009; 10/9.

82 CZ; Jihlava-Lesnov (distr. Jihlava): ruderal meadow N of the main road crossing at the S end of the village; 49°25'32"N, 15°35'15"E; 526 m a.s.l.; PK; 02 Aug 2009; 18/18.

83 CZ; Spělov (distr. Jihlava): road edge in the village; 49°19'56"N, 15°26'37"E; 530 m a.s.l.; L. Ekrt; 28 Aug 2010; 5/5.

84 CZ; Náměštl nad Oslavou (distr. Třebíč): grassy road along the N bank of the fishpond Rathan, W of the town; 49°12'49"N, 16°08'18"E; 390 m a.s.l.; V. Joza; 08 Jul 2011 [young non-flowering plants]; 2/-.

85 CZ; Novosedly (distr. Břeclav): salt marsh in the N part of the village; 48°50'24"N, 16°29'46"E; 176 m a.s.l.; JT; 26 Jul 2009; 10/10.

86 CZ; Dobré Pole (distr. Břeclav): salt marsh at the edge of the football pitch in the village; 48°49'20"N, 16°31'56"E; 183 m a.s.l.; JT; 26 Jul 2009; 12/12.

87 CZ; Sedlec (distr. Břeclav): Slanisko u Nesytu salt marsh, ca 0.5 km SE of the village; 48°46'32"N, 16°42'12"E; 180 m a.s.l.; JT; 26 Jul 2009; 12/12.

88 CZ; Sedlec (distr. Břeclav): edge of a field by the S corner of Nesyt fishpond ca 3.3 km ESE of the village; 48°45'54"N, 16°44'15"E; 178 m a.s.l.; JT; 08 Sep 2009;

10/10. **89 CZ**; Břeclav (distr. Břeclav): floodplain of the Dyje river N of the town; 48°46'58"N, 16°52'51"E; 160 m a.s.l.; JT; 08 Sep 2009; 3/3. **90 CZ**; Břeclav (distr. Břeclav): ca 1.4 km SE of Pohansko castle, SSE of the town; 48°43'00"N, 16°54'17"E; 154 m a.s.l.; JT; 16 Aug 2009; 10/8. **91 CZ**; Bzenec (distr. Hodonín): meadows in the floodplain of the Morava river ca 4 km SEE of the main railway station; 48°57'13"N, 17°18'45"E; 167 m a.s.l.; JT; 08 Sep 2009; 10/1. **92 CZ**; Strání (distr. Uherské Hradiště): road edge ca 2.2 km NW of the village, by the beginning of the local road to Korytná; 48°54'46"N, 17°40'38"E; 530 m a.s.l.; PK; 24 Aug 2009; 14/- . **93 CZ**; Dětřichov nad Bystřicí (distr. Bruntál): forest road edge ca 2.8 km NNE of the village; 49°51'24"N, 17°24'33"E; 630 m a.s.l.; PK; 22 Aug 2010; 15/15. **94 CZ**; Valšov (distr. Bruntál): edge of a road through a meadow ca 1 km SW of the railway station; 49°55'39"N, 17°25'43"E; 557 m a.s.l.; PK; 15 Aug 2009; 15/12. **95 CZ**; Rázová (distr. Bruntál): road edge on the E bank of Slezská Harta river dam ca 2.3 km NW of the village; 49°56'48"N, 17°30'32"E; 510 m a.s.l.; V. Koutecká; 06 Sep 2010; 5/- . **96 CZ**; Roudno (distr. Bruntál): meadows S of Velký Roudný hill, ca 1.3 km SE of the S end of the village; 49°52'55"N, 17°31'09"E; 660 m a.s.l.; PK; 21 Aug 2010; 18/18. **97 CZ**; Domašov nad Bystřicí (distr. Olomouc): road edge in the S part of the village; 49°44'12"N, 17°26'29"E; 495 m a.s.l.; PK; 15 Aug 2009; 10/7. **98 CZ**; Hlinka (distr. Bruntál): ruderal vegetation between road and crop-fields ca 0.7 km W of the church; 50°16'50"N, 17°39'40"E; 270 m a.s.l.; Z. Mruzíková; 04 Sep 2010; 15/- . **99 CZ**; Orlová-Zimní Důl (distr. Karviná): grassy ruderal vegetation on reclaimed coal-mining deposits; 49°49'58"N, 18°25'30"E; 260 m a.s.l.; V. Koutecká; 14 Sep 2010; 6/- . **100 CZ**; Horní Suchá (distr. Karviná): exposed bottom of a lake by the former coal mine Dukla, N of the town, ca 2.6 km NNW of the railway station Havířov-Suchá; 49°48'48"N, 18°27'06"E; 265 m a.s.l.; V. Koutecká; 14 Sep 2010; 15/15. **101 AT**; Semmering (Niederösterreich): road edge ca 0.5 km E of the mountain saddle; 47°37'54"N, 15°50'09"E; 920 m a.s.l.; MŠ; 18 Sep 2009; 12/- . **102 AT**; Puchberg am Schneeberg (Niederösterreich): road edge ca 3.5 km NE of the Puchberg railway station; 47°48'19"N, 15°56'14"E; 640 m a.s.l.; MŠ; 18 Sep 2009; 7/4. **103 SK**; Tvrdosovce (distr. Nové Zámky): salt marsh by flooded pits at the N border of W part of the village, ca 0.9 km NEE of the railway station; 48°05'45"N, 18°03'06"E; 109 m a.s.l.; PK; 28 Aug 2009; 10/10. **104 SK**; Dolný Moštenec (distr. Považská Bystrica): between Dolný Moštenec and Považská Bystrica, road edge; 49°05'13"N, 18°26'39"E; 350 m a.s.l.; J. Lepš; 19 Sep 2009; 4/1. **105 SK**; Poskalie (distr. Považská Bystrica): former football pitch ca 1 km NNE of the village; 49°03'08"N, 18°27'39"E; 380 m a.s.l.; J. Lepš; 19 Sep 2009; 3/1. **106 SK**; Podsuchá (distr. Ružomberok): road edge at the S border of the village, by the main road to Bánska Bystrica; 48°59'26"N, 19°16'56"E; 565 m a.s.l.; PK; 24 Aug 2009; 10/10. **107 SK**; Partizánska Lupča (distr. Liptovský Mikuláš): road edge in the Lupčianska dolina valley, ca 9 km S of the village; 48°59'01"N, 19°25'48"E; 750 m a.s.l.; PK; 25 Aug 2009; 10/- . **108 SK**; Liptovský Ján (distr. Liptovský Mikuláš): meadow N of the main parking place by the spa S of the village; 49°02'17"N, 19°40'33"E; 650 m a.s.l.; PK; 25 Aug 2009; 10/2. **109 SK**; Liptovský Ján (distr. Liptovský Mikuláš): road margin in „Stanišovská Poľana“ meadows, ca 4.7 km S of the village; 49°00'21"N, 19°41'24"E; 870 m a.s.l.; PK; 26 Aug 2009; 10/6. **110 SK**; Štrba (distr. Poprad): edge of the road through a mesophilous meadow, ca 3 km E of the village; 49°03'49"N, 20°02'20"E; 906 m a.s.l.; PK; 13 Jul 2009 [young plants, non-flowering or at the stage of first flower buds]; 10/1. **111 SK**; Spišské Tomášovce (distr. Spišská Nová Ves): ruderal grassland at the S edge of the village, next to the railway station; 48°57'51"N, 20°28'26"E; 520 m a.s.l.; PK; 04 Nov 2011 [fruiting plants, the ploidy level analysed from seeds]; 5/- . **112 SK**; Slanec (distr. Košice-okolie): path through a meadow ca 1 km S of the village centre; 48°37'37"N, 21°28'41"E; 325 m a.s.l.; MŠ; 09 Jul 2009 [young non-flowering plants]; 2/- . **113 SK**; Čerhov (distr. Trebišov): vineyards on the W slope of the Černá hora hill (239.7), ca 0.9–1.1 km E of the church in the village; 48°27'50"N, 21°39'18"E; 140–160 m a.s.l.; PK; 09 Jul 2009 [young non-flowering plants]; 10/1. **114 SK**; Veľká Trňa (distr. Trebišov): dry meadows and abandoned vineyards by the carriage-road ca 1.05 km ESE to 0.65 km SE from the church in the E part of the village; 48°27'58"N, 21°41'38"E; 260–330 m a.s.l.; PK; 09 Jul 2009 [young non-flowering plants]; 2/- . **115 SK**; Nová Sedlica (distr. Snina): edge of the road through mesophilous meadow, ca 1 km SE from the village; 49°02'13"N, 22°31'33"E; 564 m a.s.l.; PK; 12 Jul 2009 [young plants, non-flowering or at the stage of first flower buds]; 10/3.

Late flowering tetraploids. **116 CZ**; Úhošťany (distr. Chomutov): grassy road ca 1.3 km W of the church, at the S foothill of Úhošť hill; 50°21'19"N, 13°14'26"E; 450 m a.s.l.; PK & TB; 22 Jun 2010 [young non-flowering plants]; 1/- . **117 CZ**; Úhošťany (distr. Chomutov): grassy road on the E slope of Úhošť hill, ca 1.2 km NNW of the village; 50°21'47"N, 13°15'08"E; 470 m a.s.l.; PK & TB; 01 Sep 2010; 23/19. **118 CZ**; Kadaň (distr. Chomutov): old fallow on Běšický chochol hill ca 6 km E of the town; 50°22'04"N, 13°21'15"E; 340 m a.s.l.; PK & TB; 24 Jun 2010 [young non-flowering plants]; 20/- . **119 CZ**; Mladějovice (distr. Strakonice): road edge and grassy patch between the road and a crop-field ca 0.7 km N of the village; 49°14'09"N, 14°02'59"E; 400 m a.s.l.; PK; 29 Aug 2010; 15/15. **120 CZ**; Hnanice (distr. Znojmo): road edge ca 1 km NNE of the church, at the S edge of dry grassland Havranické vřesoviště; 48°48'20"N, 15°59'30"E; 300 m a.s.l.; PK; 07 Sep 2010; 3/- . **121 CZ**; Havraníky (distr. Znojmo): grassy road ca 1.3 km NW of the church, at the N edge of dry grassland Havranické vřesoviště; 48°49'16"N, 15°59'55"E; 340 m a.s.l.; PK; 07 Sep 2010; 3/- . **122 CZ**; Havraníky (distr. Znojmo): dry grassland

Havranické vřesoviště, at the N edge of the village; 48°48'55"N, 16°00'20"E; 320 m a.s.l.; PK; 07 Sep 2010; 4/-.

123 CZ; Popice (distr. Znojmo): dry grassland Havranické vřesoviště, between the village and the chapel SW of the village; 48°49'13"N, 16°00'46"E; 290 m a.s.l.; PK; 07 Sep 2010; 15/15.

124 CZ; Havraníky (distr. Znojmo): dry grassland Skalky S of the village; 48°48'16"N, 16°00'35"E; 300 m a.s.l.; PK; 07 Sep 2010; 18/13.

125 CZ; Drásov (distr. Brno-venkov): road edge at the crossing of the road to Hradčany and former railway, ca 1 km SSW of the church in the village; 49°19'37"N, 16°28'00"E; 270 m a.s.l.; PK; 25 Aug 2010; 18/1.

126 CZ; Hustopeče (distr. Břeclav): road edge close to a steppe ca 2.4 km N of the town square; 48°57'42"N, 16°44'25"E; 224 m a.s.l.; JT; 08 Sep 2009; 10/8.

127 CZ; Strachotín (distr. Břeclav): road edge at the dam ca 0.5 km SSW of the church in the village; 48°54'08"N, 16°38'51"E; 174 m a.s.l.; JT; 08 Sep 2009; 10/9.

128 CZ; Pavlov (distr. Břeclav): fallow at the foothills of Děvín hill ca 0.75 km NW of the church; 48°52'42"N, 16°39'52"E; 280 m a.s.l.; MŠ; 08 Sep 2010; 14/12.

129 CZ; Horní Věstonice (distr. Břeclav): dry meadow ca 1.15 km SEE of the church in the village; 48°52'14"N, 16°38'22"E; 330 m a.s.l.; J. Karásek; 17 Aug 2009; 10/7.

130 CZ; Klentnice (distr. Břeclav): crop field edge ca 200 m E of the village; 48°50'35"N, 16°38'57"E; 280 m a.s.l.; JT; 02 Sep 2011; 5/-.

131 CZ; Sedlec (distr. Břeclav): road edge next to a steppe on Kienberg hill, ca 2.5 km NNW of the church in the village; 48°48'03"N, 16°41'13"E; 210 m a.s.l.; JT; 08 Sep 2009; 14/8.

132 CZ; Sedlec (distr. Břeclav): Studánkový vrch hill, ca 2.3 km NE of the church in the village; 48°47'35"N, 16°43'16"E; 245 m a.s.l.; JT; 08 Sep 2009; 11/10.

133 AT; Kaltenleutgeben (Niederösterreich): forest road edge ca 0.65 km NNW of the summit of Höllenstein hill, S of the village; 48°06'07"N, 16°11'07"E; 570 m a.s.l.; PK; 20 Sep 2010; 10/-.

134 AT; Geißhübl (Niederösterreich): forest road edge ca 1.3 km NW of the W edge of the village; 48°06'24"N, 16°12'49"E; 510 m a.s.l.; PK; 20 Sep 2010; 13/10.

135 SK; Svätý Jur (distr. Pezinok): road edge ca 2.1 km NW of the centre of the village; 48°16'08"N, 17°11'40"E; 320 m a.s.l.; MŠ; 07 Sep 2010; 12/10.

136 SK; Stankovany (distr. Ružomberok): road edge at the E border of the Močiar nature reserve, ca 1.5 km NW of the village; 49°09'07"N, 19°09'23"E; 440 m a.s.l.; PK; 06 Nov 2011 [fruiting plants, the ploidy level determined using seeds]; 5/-.

Mixed populations of diploids and late-flowering tetraploids. **137 CZ**; Kadaň (distr. Chomutov): grassy roads and road edges between the Tušimice power plant and N edge of adjacent fly ash deposits; 50°22'44"N, 13°20'31"E; 295 m a.s.l.; TB; 01 Sep 2010; 20/20.

138 CZ; Modlešovice (distr. Strakonice): road edge ca 630 m NNW of the railway stop Modlešovice, N of the village; 49°15'30"N, 13°58'05"E; 380 m a.s.l.; MŠ; 25 Aug 2011; 40/31.

139 CZ; Modlešovice (distr. Strakonice): road edge ca 100 m N of the railway stop, N of the village; 49°15'26"N, 13°58'33"E; 380 m a.s.l.; MŠ; 25 Aug 2011; 15/14.

Appendix 2. – List of the localities of the *Odontites vernus* group sampled for this study that were not in central Europe. See Appendix 1 for the format of the data. Abbreviations of countries: BA – Bosnia and Herzegovina, BG – Bulgaria, DK – Denmark, ES – Spain, FR – France, HR – Croatia, RS – Serbia, SE – Sweden.

Diploids. **140 BA**; Mljetvine: roadside in the village, 43°31'52"N, 18°04'45"E; 360 m a.s.l.; MŠ; 26 Aug 2010; 4/-.

141 BA; Sarajevo: pasture edge ca 1.7 km NE of the summit of Trebević mountain; 43°49'51"N, 18°28'23"E; 1150 m a.s.l.; MŠ; 26 Aug 2010; 5/-.

142 HR; Raduč: shrubs along the road ca 2.6 km NW of the village; 44°24'55"N, 15°33'59"E; 620 m a.s.l.; MŠ; 24 Aug 2010; 5/-.

143 RS; Jelašnica: fallow on a plateau N of the Jelašnica Gorge, ca 1.2 km SE of the village; 43°17'00"N, 22°04'02"E; 500 m a.s.l.; MŠ; 26 Jul 2010; 5/-.

144 SE; Öland, Solberga: alvar next to the road Solberga–Degerhamn, 1 km E of the village; 56°20'54"N, 16°28'53"E; 20 m a.s.l.; F. Kolář; 18 Aug 2010; 2/-.

Late-flowering tetraploids. **145 BG**; Dobrostan: edge of the road at the W edge of the village; 41°54'14"N, 24°55'10"E; 1280 m a.s.l.; MŠ; 24 Jul 2010; 10/-.

146 DK; Glatved; Glatved Strand, steep sandy slope next to the sea, 2.5 km ESE of the village, 56°18'26"N, 10°51'58"E; 10 m a.s.l.; F. Kolář; 19 Aug 2010; 2/-.

147 FR; L'Ile-Bouchard: on the top of a hill N of the village; 47°09'14"N, 00°26'19"E; 85 m a.s.l.; GT; 21 Sep 2009; 5/-.

148 ES; La Vall Fosca: edge of road by the Sallent river dam; 42°30'25"N, 00°59'24"E; 1770 m a.s.l.; PK; 30 Aug 2011; 5/-.

149 ES; Santa Pau: edge of road in the N foothills of Volca Bocanegra hill, ca 1.4 km SSW of the town; 42°08'29"N, 02°33'18"E; 550 m a.s.l.; PK; 1 Sep 2011; 5/-.