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MORPHOMETRIC AND GENETIC DIVERGENCE AMONG POPULATIONS OF *NEOTINEA USTULATA* (*ORCHIDACEAE*) WITH DIFFERENT FLOWERING PHENOLOGIES

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Abstract: The terrestrial orchid species *Neotinea ustulata* has recently been split into two subspecies, differing remarkably in their flowering time, but only slightly in morphological characteristics, which makes their taxonomic status uncertain. We have analyzed morphometric and genetic differences between the early- and late-flowering populations in Central Europe. Our results on morphology are ambiguous. Indirect gradient analysis has not shown a distinct separation of early- and late-flowering individuals in the ordination space. However, according to MANOVA, populations of early- and late-flowering plants can be distinguished by plant height, leaf length, numbers of basal (rosette) and stem leaves and even better by certain ratios of these numbers. All genetic analyses, on the other hand, are definite and consistently distinguish two groups. Random amplified polymorphic DNA (RAPD) markers have shown that the early- and late-flowering populations differ significantly from one another. Principal coordinate analysis (PCoA) based on presence/absence matrix of RAPD bands separated the two groups, implying that the difference in flowering phenology could form an effective barrier to gene exchange. Partitioning of genetic diversity in analysis of molecular variance (AMOVA) has shown that the genetic divergence between the two groups, early- and late-flowering populations, is somewhat greater (33%) than the genetic variability among populations within particular group (23%). Using the Mantel test, we found that genetic differentiation coefficients between populations closely correspond to their geographic distribution. After elimination of the effect of sample origin from the model, direct gradient analysis (RDA) has shown that the early- and late-flowering groups differ significantly in their RAPD spectra. To conclude, our results indicate the presence of two genetically and phenologically distinct taxa, but the weak morphological differentiation supports the taxonomic rank of variety rather than subspecies.

Keywords: Flowering phenology, Orchids, RAPD, Taxonomy, Variety

INTRODUCTION

During the last decades, *Neotinea ustulata* (L.) BATEMAN, PRIDGEON et CHASE has undergone a severe regression of its distribution range (DAVIES et al. 1988, FOLEY 1992, PRESTON et al. 2002) and completely disappeared from some parts of Europe (e.g., the Netherlands: KREUTZ & DEKKER 2000). In the Czech Republic, the number of its sites decreased by 69% in Moravia (ŠMITÁK & JATIOVÁ 1996), and by more than 90% in Bohemia (PROCHÁZKA & VELÍSEK 1983).

The position of *Neotinea ustulata* was recently reclassified using nuclear ITS sequences (PRIDGEON et al. 1997, BATEMAN et al. 1997). Previously, it was regarded as one of the least variable species of the genus *Orchis* and all described deviations were considered as taxonomically unimportant expressions of individual variability (PROCHÁZKA 1977). More recently, it has been recognized that some populations bloom much later than the nominate race and have a slightly different morphology (GUMPRECHT 1981). This led KÜMPEL (1988) to describe a new variety (*Orchis ustulata* var. *aestivalis* KÜMPEL), which was later elevated to subspecies level as *Orchis ustulata* subsp. *aestivalis* (KÜMPEL) KÜMPEL et MRKVICKA (KÜMPEL & MRKVICKA 1990) and subsequently transferred in the same rank into the genus *Neotinea* (*Neotinea ustulata* subsp. *aestivalis* (KÜMPEL) JACQUET et SCAPPAT., JACQUET & SCAPPATICCI 2003). Other authors, however, regard the morphological differences between the two subspecies/varieties as minimal and questionable (REINEKE & RIETDORF 1991, JENSEN & PEDERSEN 1999, TALI & KULL 2001) and/or consider flowering time as crucial for distinguishing between them (TALI 1996).

The recent emphasis on protecting world biological diversity has moved from species to genetic level – that is, to conservation of all variation in life, especially in local varieties. Thus, the correct assessment of how much *N. ustulata* is endangered by extinction due to the above-mentioned regression of its distribution range requires the taxonomic status of the intraspecific variation of this species to be resolved correctly. Research is needed to solve the contradiction of existence of one or two taxa in *N. ustulata* and to determine the most appropriate rank (FOLEY 1990, JENKINSON 1995). If the species really consists of two distinct taxa, then further research on their distribution and ecological demands will be necessary to prevent extinction of either of them.

Distinguishing between the subspecies/varieties used to be based solely on morphological characteristics. However, the recent development of a number of different genetic markers has provided a helpful tool to strengthen or refute the unclear classifications. Recently, random amplified polymorphic DNA (RAPD) markers are popular for determining orchid fingerprints (LIM et al. 1999), for distinguishing among orchid cultivars (DUBOUZET et al. 1997), and even for differentiating among orchid subspecies (*Ophrys bertolonii* agg.; CAPORALI et al. 2001). RAPDs are amplified by PCR using short oligonucleotide primers of randomly chosen sequence. Different RAPD patterns arise when genomic regions vary because of the presence/absence of complementary annealing sites (WILLIAMS et al. 1990). RAPDs can potentially provide a much higher number of marker loci and higher levels of polymorphism than allozymes (PARKER et al. 1998). RAPDs are also more sensitive than allozymes for detecting genetic structure at lower taxonomic levels (NYBOM & BARTISH 2000). RAPDs, however, can lack reproducibility (LEVI et al. 1993, PÉREZ et al. 1998). Even if conditions of PCR are carefully controlled, unpredictable patterns of inheritance are sometimes found, which can limit comparability among studies.

Here we report results of our analyses on morphometric and genetic differences of 13 populations of *N. ustulata* in Central Europe and on morphometric screening of extensive herbarium material. We determined the morphological characters that can best distinguish between the early- and late-flowering populations. We used genetic markers to test whether genetic similarity is larger within or between early- and late-flowering populations.

METHODS

Study species

Here we present an outline of the biology and ecology of the two putative subspecies treated by KÜMPEL & MRKVICKA (1990). The scientific names of orchids follow BATEMAN et al. (1997, 2003), and that of plant communities MORAVEC (1995).

Early-flowering *Neotinea ustulata* (L.) BATEMAN, PRIDGEON et CHASE (*Orchis ustulata* L. subsp. *ustulata*, *Neotinea ustulata* (L.) BATEMAN, PRIDGEON et CHASE subsp. *ustulata*) – a perennial tuberous herb usually 10–35 cm tall. Green bluish rosettes appear above ground in autumn (September) and assimilate throughout the winter, even under snow. Flowering stems appear in spring. The main flowering period ranges from early May to about mid-June, although the first individuals in the Mediterranean start to flower in mid-April and in higher elevations in Alps flowering may extend until late July (REINEKE & RIETDORF 1987). The inflorescences are dense, narrow, up to 8 cm long, composed of 15–60 small flowers. Sepals are 3–4.5 mm long, 1.5–2.5 mm broad and form a hood, the outer side of which (especially in buds) has a dark reddish purple colour – hence its Latin name, as "ustulo" means "burnt to brown". White, 3.5–8 mm long lip is covered with dark red spots and divided into three parts. The middle segment is extended into two lobes with small tip between them. Blunt, downward curved spur is about one-third of the length of the ovary.

The subspecies occurs from lowlands to alpine level (up to 2000 m a.s.l. in the Alps). As a distinct heliophyte preferring basic soil, it usually grows on dry or slightly humid meadows and pastures, shrubby slopes, forest edges, rarely in light open forests. It frequently shares sites with *Anacamptis morio*. Prevalent biotopes in Central Europe belong to the vegetation of xerotherm grasslands of alliance *Festucion valesiacae*, meadows, pastures and grasslands on nutrient-rich soils of alliance *Arrhenatherion*, acidophilous grasslands of alliance *Violion caninae*, thermophilous forest fringes of alliance *Geranion sanguinei* and thermophilous oak forest steppe of alliance *Quercion pubescenti-petraeae*.

The subspecies is distributed mainly in the Eurosiberian area, and rarely extends into the Mediterranean region. In the north it stretches from England and Denmark over southern Sweden to the eastern Baltic Republics. In the east it extends towards the western part of Siberia (river Ob) and Southern Caucasus (Azerbaidjan). In the south it ranges from the European part of Russia across the central part of the Balkan Peninsula, northern part of the Peloponnesus, central Italy to France, to Spain and northern part of Portugal.

Pollination of this species has been a mystery for a long time. In 1980–1983 VÖTH (1984) found *Echinomyia magnicornis* ZETT., a fly of the family *Tachinidae*, to be the regular pollinator. These middle-sized, dark-coloured, hairy flies are nectar feeders and their brood develops in caterpillars of nocturnal butterflies (family *Noctuidae*, *Lymantriidae*). The combination of brown-violet and white in the flower is supposed to optically attract the flies (VAN DER CINGEL 1995).

Late-flowering *Neotinea ustulata* (L.) BATEMAN, PRIDGEON et CHASE (*Orchis ustulata* subsp. *aestivalis* (KÜMPEL) KÜMPEL et MRKVICKA, *Neotinea ustulata* subsp. *aestivalis* (KÜMPEL) JACQUET et SCAPPAT.) – many orchidologists consider this taxonomically problematic subspecies to be a variety or ecotype of the nominate race, as there are few differences between the subspecies. Stems of late-flowering *N. ustulata* are taller (up to

80 cm), the leaves are longer and narrower, the stem has more leaves, inflorescence (especially in the juvenile stage) is sharply pointed and tips of lateral sepals are bent out, making the flower appear more open (KÜMPEL & MRKVICKA 1990). The last character has only low taxonomic importance, as we frequently observed lateral sepals bent out also in early-flowering populations (usually in old flowers) and the converse in late-flowering populations. The inflorescence of late-flowering *N. ustulata* has more, up to 120 flowers, 40 on average. Plants flower remarkably later in the season in comparison with early-flowering *N. ustulata*, from the end of June in the lowlands until mid-August in higher elevations.

The type of biotopes is similar to the nominate race, but they can very rarely occur together (TALI & KULL 2001) and their communication is unlikely because of their distinctly differing flowering times. Late-flowering *N. ustulata* prefers xerotherm vegetation of dry chalk grassland from lowland up to montane level in chalk and dolomite mountains (1500 m a.s.l.), very often in pastures. In Central Europe it inhabits orchid-rich meadows of the alliance *Bromion erecti*, dry grasslands of alliance *Festucion valesiacae*, extensive pastures of alliance *Violion caninae* and *Polygono-Trisetion*, or dry slopes of deciduous thickets of alliance *Prunion fruticosae*.

On the larger scale, the distribution area of late-flowering *N. ustulata* overlaps with that of the nominate race (e.g. in the United Kingdom), although regionally both subspecies may show a clear allopatric distribution (TALI 1996). Its distribution area extends from eastern France, across Switzerland, southern Germany and Lower Austria to the western part of the Carpathian Mountains (eastern part of the Czech Republic towards the central part of Slovakia). Late-flowering *N. ustulata* seems to be more abundant than the nominate race in the east. From Central Europe it stretches further southeast to northern Italy, Slovenia, Romania and Bulgaria. Other locations have been reported from Estonia (TALI 1996), Denmark (JENSEN & PEDERSEN 1999) and southern England (FOLEY 1992).

MRKVICKA (1991) observed a beetle, *Leptura livida* F. (*Cerambycidae*), exporting pollinia in the population of late-flowering *N. ustulata* in Lower Austria. He also reported several other non-pollinating insect visitors, mainly bees and flies. According to our observations, the main pollinator of late-flowering *N. ustulata* is the same as in the nominate race: *E. magnicornis* (J. JERSÁKOVÁ, unpubl. data).

Study sites

Morphometric and genetic data were collected in populations of *N. ustulata* at locations in Germany, and the Czech and Slovak Republics during the seasons 2000–2001 (Table 1, Fig. 1). We consider Central European populations flowering from May to mid-June as early-flowering populations and populations flowering from mid-June until August as late-flowering ones. Mid-June can be used to separate both groups well, because early-flowering populations are already fruiting and late-flowering ones start to open their first flowers. The populations in the Bílé Karpaty Mts. (Czech Republic) are assumed by botanists (I. JONGEPIEROVÁ and C.A.J. KREUTZ, pers. comm.) as an unclear and potentially intermediate type because they start to flower in early June. None of the studied populations are situated at high altitude (over 1000 m a.s.l.), which could delay its flowering time (Table 1).

Table 1. Description of study sites. The flowering time of populations is given from the first half of May until the second half of July. N – represents the number of individuals used in genetic analyses (GA), morphological analyses on live material (MA) and analysis of the number of flowers (NF). ▭ – early-flowering, ▬ – late-flowering; CZ – Czech Republic, SK – Slovak Republic, D – Germany.

Site	District	Country	Code	Co-ordinates	m a.s.l.	Habitat type	N for GA	N for MA	N for NF	Flowering time 1 2 1 2 1 2 5 5 6 6 7 7
Vědlice	Litoměřice	CZ	Ve	N 50°31′ E 14°20′	230	dry meadow	5	11	137	▭
Devínska Kobyla	Bratislava	CZ	DK	N 48°11′ E 16°59′	400	dry meadow	5	19	80	▭
Albrechtice	Sušice	CZ	Al	N 49°12′ E 13°34′	680	mesic meadow	5	-	84	▭
Čepice	Sušice	CZ	Ce	N 49°16′ E 13°36′	450	alluvial meadow	5	10	10	▭
Bílé Karpaty: Zahrady p. Hájem	Hodonín	CZ	B1	N 48°52′ E 17°31′	420	mesic meadow	-	-	35	▬
Bílé Karpaty: Čertoryje	Hodonín	CZ	B2	N 48°51′ E 17°24′	380	mesic meadow	-	-	28	▬
Bílé Karpaty: Drahy	Uh. Hradiště	CZ	B3	N 48°55′ E 17°38′	400	mesic meadow	4	-	23	▬
Ježůvka	Vsetín	CZ	V1	N 49°20′ E 18°00′	550	mesic meadow	3	-	60	▬
Hovězí	Vsetín	CZ	V2	N 49°18′ E 18°04′	450	mesic meadow	2	3	-	▬
Losový	Vsetín	CZ	V3	N 49°18′ E 18°05′	500	dry pasture	3	6	18	▬
Ludrová	Ružomberok	SK	R1	N 49°03′ E 19°18′	630	mesic pasture	3	-	32	▬
Biely Potok	Ružomberok	SK	R2	N 49°02′ E 19°18′	650	mesic pasture	3	3	42	▬
Rosenau	Mamming	D	Ro	N 48°39′ E 12°34′	370	alluvial meadow	4	-	-	▬

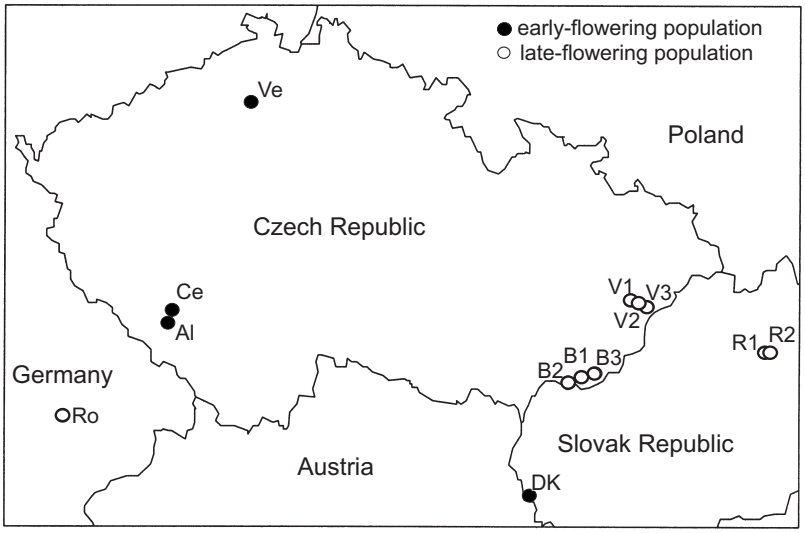

Fig. 1. Map showing distribution of the studied populations in the Czech Republic, Slovak Republic and Germany. For abbreviations of sites see Methods.

Morphometric analyses

In order to minimize the effect of intrinsic factors of a particular site on plant morphology, we gathered morphological data on plants from a large number of sites occurring in the Czech Republic and deposited in the main Czech herbaria (BRNM and BRNU in Brno, CB in České Budějovice, OLM in Olomouc, PR and PRC in Praha): 107 individuals from 41 early- and 129 individuals from 29 late-flowering populations, respectively (see Appendix). We measured plant height (PL), inflorescence length (IL), number of leaves in the basal rosette (RL), number of stem leaves (SL), and calculated the total number of leaves (TL = RL+SL). Further, we measured morphological variables, such as the number of flowers, position of the first stem leaf (distance between the first basal and stem leaves), mean length and width of basal and stem leaves, and total leaf area (sum of leaf length × width). These variables were not possible to measure on dry herbarium material and were obtained from live material (Table 1).

In order to find the gradient of the highest variability in the data, which would separate collected samples into groups corresponding to early- and late-flowering populations, we performed indirect gradient analysis (PCA, principal component analysis). We used the morphological data gathered from herbarium and live material (PL, IL, RL, SL) as dependent variables. The early- and late-flowering time was used as an environmental variable to interpret patterns extracted from all variation in the data. Data were scaled on interspecific correlations and centred by species.

We tested for the effect of flowering time on the set of morphological variables (PL, IL, RL, SL) using the module MANOVA in the program Statistica v. 5.5. We subsequently tested for the differences in morphological variables between early- and late-flowering plants, separately for herbarium and live material.

Since early- and late-flowering taxa are assumed to differ in the number of flowers per inflorescence, we analyzed the differences between early- and late-flowering populations (Table 1) in the number of flowers by means of nested design ANOVA in the module visual general linear model with subsequent *post-hoc* comparisons (Tukey HSD test for unequal N) in the program Statistica v. 5.5. We tested two factors: flowering time and population (random factor nested in the factor flowering time). The probability density function of the leaf index, LI, was calculated from the estimated mean, μ, and standard deviation, σ, of LI as:

$$f(LI) = \frac{e^{-(LI-\mu)^2/(2\sigma^2)}}{\sigma\sqrt{2\pi}} \quad .$$

Genetic analyses

We collected at random 42 leaf samples for DNA extraction from 11 populations (Table 1) during the 2001 season and stored them at −80 °C for five months. Total genomic DNA was isolated using the CTAB miniprep method (STEWART & VIA 1993) with these modifications: additional extraction of homogenate with 500 μl phenol-chloroform (1 : 1) and additional treatment of pellet in wash buffer (300 μl TE, 20 μl 7.5 M ammonium acetate and 600 μl 96% ethanol). Addition of PVPP (polyvinylpolypyrrolidone) to the homogenate, as recommended by LIM et al. (1997), improved neither quantity nor quality of the extracted DNA.

We screened DNA samples of two individuals per population with 45 arbitrary decamer primers (OPA, OPB, OPF and OPK kits obtained from Operon Technologies, Alameda, California, USA). Six primers were selected on the basis of their ability to amplify DNA, band intensity, the number of loci amplified, reproducibility of products and level of polymorphisms between the two subspecies and used them for analysis of the remaining individuals – OPA04, OPB11, OPB17, OPK01, OPK11, OPK17.

DNA amplifications were performed in 25 μl reaction volumes consisting of 20 pmol random 10-mer primer, 200 pmol of each dNTP (FINNZYME), 2.5 μl 10× polymerase buffer, 0.4 unit FINNZYME polymerase and ~ 25 ng of isolated DNA. Amplifications were carried out in a MJ Research PTC-100 thermocycler at 93 °C for 5 min (pretreatment), followed by 45 cycles at 92 °C for 1 min, 35 °C for 2 min and 72 °C for 3 min; final elongation step at 72 °C for 10 min. Reactions lacking DNA were considered as negative controls.

Fragments generated by amplification were separated according to size on a 1.5% agarose. Gels were run in TAE buffer, stained with ethidium bromide, and visualized by illumination with UV light. Software BioProfil Bio-1D++, v. 99 (Vilber Lourmat) was used for digital analysis of electrophoretic data. Only markers that were unambiguous, well amplified, and reproducible in replicate tests were scored. Genetic markers (bands) resulting from the RAPD amplifications were scored for each locus based on their presence (1) or absence (0).

To determine relationships between the individuals in eleven populations, the matrix of scores (1/0) was used to perform principal coordinate analysis based on Jaccard's coefficients (PCoA using SYN-TAX 5.0, PODANI 1994). In order to test for differences between the early- and late-flowering groups and between populations, we performed direct gradient analysis (RDA, redundancy analysis). The assignments of individuals to (1) flowering time (early- or

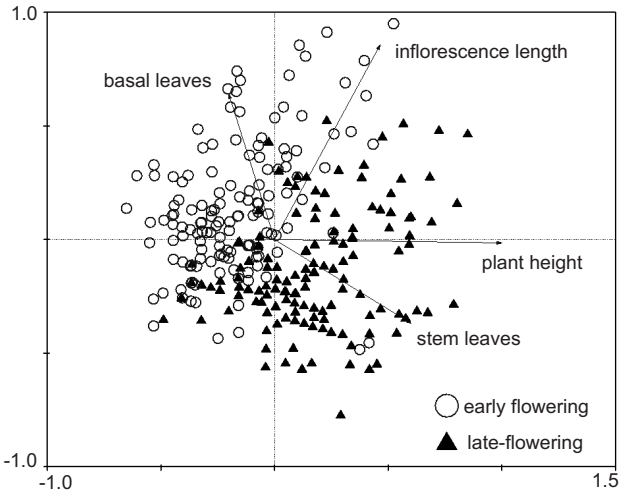

Fig. 2. Ordination diagram showing results of indirect gradient analysis (PCA, principal component analysis) on morphometric characteristics of *Neotinea ustulata*. The early- and late-flowering time was used as the environmental variable to interpret patterns extracted from all variation in the data. The gradient of largest variability in the data set coincides with the first horizontal ordination axis.

late-) and (2) population were used as the explanatory variables. In the next step, the obvious influence of population was eliminated from the model as a covariable.

Variation in RAPD patterns was analyzed by means of the analysis of molecular variance (AMOVA). AMOVA analysis was based on the pairwise squared Euclidean metrics defined by HUFF et al. (1993) as $E = n\,(1–2n_{xy}/2n)$, where $2n_{xy}$ is the number of markers shared by the two individuals, and n is the total number of polymorphic sites. A matrix of 55 pairwise linear genetic differentiation coefficients Φ_{PT} among 11 populations were calculated ($\Phi_{PT} = H_T\text{-}H_P/H_T$, where H_T is average heterozygosity among samples within the total area and H_P is average heterozygosity among subpopulations). Then we performed the Mantel test to determine whether the matrix of Φ_{PT} coefficients was correlated with the matrix of geographic distances (999 permutations). Both AMOVA and the Mantel test were performed using GenAlEx V5 software (Genetic Analysis in Excel; PEAKALL & SMOUSE 2001).

We carried out all gradient analyses using the program CANOCO for Windows v. 4.0 (TER BRAAK & ŠMILAUER 1998), and plotted ordination diagrams in the program CanoDraw for Windows.

RESULTS

Morphometric analyses

The multivariate method of indirect gradient analysis applied to selected morphometric characteristics has not shown a distinct separation of early- and late-flowering individuals in the ordination space; some plants from certain early- and late-flowering populations show an intermediate morphology. The variable plant height was parallel to the highest gradient of variability in the data set (first ordination axis). The early- and late-flowering time, which was

Table 2. MANOVA statistics showing the differences in mean values of morphometric variables in comparisons between early- and late-flowering populations. The first part of the table applies to the data set obtained from herbarium plant material, the second part to live plant material (see Methods). (n – number of measured individuals; *** – significant at $P < 0.001$; ** – significant at $P < 0.01$; * – significant at $P < 0.05$; ns – non-significant – $P > 0.05$)

Herbarium material

Morphometric variable	Early-flowering (n = 107)	Late-flowering (n = 129)	
Plant height (cm)	21.8	29.9	***
Inflorescence length (cm)	4.0	4.0	ns
No. of leaves	5.7	5.6	ns
No. of basal leaves	3.7	2.7	***
No. of stem leaves	1.9	2.9	***

Live material

Morphometric variable	Early-flowering (n = 40)	Late-flowering (n = 12)	
Plant height (cm)	20.7	33.3	***
Inflorescence length (cm)	5.4	5.8	ns
No. of flowers	40.6	49.0	ns
No. of leaves	6.1	5.4	ns
No. of basal leaves	4.4	2.9	***
No. of stem leaves	1.7	2.5	**
Total leaf area (mm^2)	3731.5	3931.1	ns
Basal leaf length (mm)	63.3	90.7	***
Basal leaf width (mm)	14.3	12.9	ns
Stem leaf length (mm)	51.7	63.3	*
Stem leaf width (mm)	10.3	9.5	ns
Position of 1st stem leaf (mm)	30.5	57.5	***

used to interpret patterns extracted from all variation in data, has shown that early-flowering individuals were characterized by a larger number of basal (rosette) leaves, and late-flowering individuals by a large number of stem leaves (Fig. 2).

The multivariate test (MANOVA) has shown significant differences in several morphological variables (PL, IL, RL, SL) for early- and late-flowering groups; Wilks $\text{Lambda}_{(4, 279)} = 0.44$, $P < 0.001$. The groups differed significantly in plant height, number of basal and stem leaves, basal and stem leaf length and position of the first stem leaf (Table 2). The other variables – inflorescence length, total number of leaves, number of flowers, total leaf area and leaf width – did not significantly differ between the early- and late-flowering groups ($P > 0.05$).

The arrows for basal and stem leaves in the ordination diagram pointed in opposite directions, which indicates their negative correlation ($r = –0.23$, $P < 0.05$, $n = 284$; Fig. 2). It is therefore not surprising that the ratio of number of stem leaves to the number of basal leaves ("leaf index", LI) distinguished between the groups even better than the number of basal leaves or the number of stem leaves individually (d.f. = 1; $F = 260.5$; $P < 0.001$). The values of LI were normally distributed; therefore the probability density functions (PDFs) of LI for

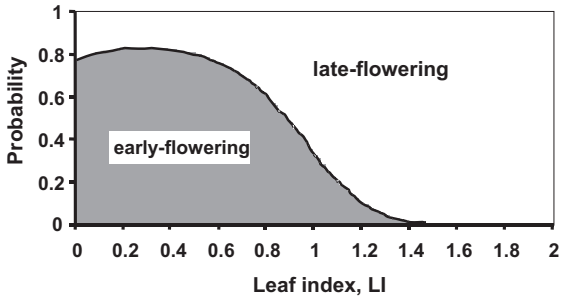

Fig. 3. Probability that an individual belongs to early-flowering plants (dark region) and that it belongs to late-flowering plants (white region) as a function of the leaf index, LI (the ratio of number of stem leaves to the number of basal leaves).

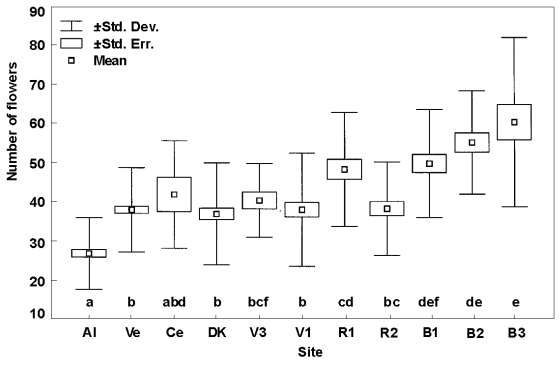

Fig. 4. Variability in the number of flowers recorded in 11 populations of *Neotinea ustulata*. The results of *post-hoc* comparisons between the sites are displayed as letters under each box-and-whisker plot. Different letters indicate significant differences at $P < 0.05$ (Tukey HSD test). Early-flowering populations: Al, Ve, Ce, DK; late-flowering populations: V3, V1, R1, R2, B1, B2, B3 (for abbreviations of sites see Methods).

each of the two groups were calculated from the mean and standard deviation of LI for early and late flowering plants, and Fig. 3 shows the ratio of the PDF for early-flowering plants to the sum of both PDFs. This figure therefore indicates the probability that an individual with a certain LI belongs to one of the different-flowering groups (ROSS 2004). Thus the largest probability (~ 0.83) that a plant belongs to early-flowering group exists for individuals with LI ~ 0.3 and individuals with large LI (> 1.2) are almost certainly late-flowering plants. Interestingly, the probability that an individual belongs to early-flowering group declines for very small values of LI (~ 0). This is because the variability of LI in early-flowering plants is much smaller than that in late-flowering plants. Thus, individuals with very small LI (< 0.3) are more likely to belong to the more variable late-flowering group than to the less variable early-flowering group.

Comparison of the number of flowers in 4 early- and 7 late-flowering populations, respectively, revealed significant differences in the number of flowers between populations within each different-flowering group (d.f. = 9; $F = 15.6$; $P < 0.001$) and in the number of flowers between different-flowering groups (d.f. = 1; $F = 5.8$; $P < 0.04$). However, according to the subsequent post-hoc comparisons, the number of flowers in some early-flowering populations did not differ from that in late-flowering populations (Fig. 4).

Genetic analyses

By means of six primers, we generated 90 RAPD markers in *N. ustulata*, ranging from 10 to 21 bands per primer (Table 3). Out of the 90 markers, as many as 88 (97.8%) were polymorphic at the species level.

Table 3. Primers used and the number of RAPD markers obtained.

Primers	Size (bp) min-max	Number of bands polymorphic	Unique/monomorphic within early	late	*N. ustulata*	Total
OPA04	160–980	19	1/1	2/0	1/0	19
OPB11	275–1595	21	2/2	1/0	2/0	21
OPB17	370–1390	15	0/1	1/0	4/0	15
OPK01	505–1970	11	0/0	0/0	2/0	11
OPK11	995–2945	8	1/3	1/0	4/2	10
OPK17	345–1465	14	0/1	1/0	2/0	14
		88	4/8	6/0	15/2	90

Principal coordinate analysis divided the 42 analyzed individuals into two separate groups, which differ in their flowering time (see Fig. 5). The analysis has also shown a geographic distribution pattern of the fingerprints (compare PCoA diagram with the distribution of the populations: map in Fig. 1). Multivariate method of direct gradient analysis (RDA) has demonstrated that the early- and late-flowering groups differ significantly from one another ($F = 4.48$, $P < 0.001$) in their RAPD spectra. Using forward selection we have found that the variable Flowering time has a slightly higher explanatory power in our RAPD data than the variable Population (24.7% > 24%, respectively).

The spectra of bands (RAPD markers) among 42 individuals from 11 populations were very variable. Out of 90 bands, only 15 common bands (as little as two monomorphic) were found in all populations of *N. ustulata* (Table 3). Early-flowering populations had four fixed bands (three of them monomorphic!), which could differentiate them from late-flowering ones. In contrast, late-flowering populations had six unique bands and were genetically more variable (Fig. 5); there were 76 polymorphic bands out of 90 in this group. Within the late-flowering group, the Rosenau population differed the most from the remaining populations (RDA, forward selection – 10.1%, $P < 0.001$). This population had 16 population-specific (unique) markers, from two to four bands per primer, and it was the most variable one with its 41 polymorphic bands. A population from the Bílé Karpaty Mts. with partly intermediate flowering time (Table 1) shared more bands with late-flowering populations than with early-flowering ones (five and two, respectively).

Markers amplified by the primer OPK11 provided the simplest and best differentiation between early- and late-flowering plants: early-flowering individuals had a nearly unique monomorphic marker (band about 995 bp in size; see Fig. 6). Note that one individual from late-flowering population Rosenau had this band as well.

Variation in RAPD banding patterns between early- and late-flowering groups was highly significant (AMOVA; $P < 0.001$), as well as variation between populations within groups and between individuals within each of the 11 populations (Table 4). Of the total genetic diversity, only 33% was attributable to divergence between early- and late-flowering populations, 23% to population divergences within groups, and 44% to individual differences within a population. Results of the Mantel test have shown that geographic distances are significantly positively correlated with the genetic distances between populations ($r = 0.544$; $P < 0.01$).

Table 4. Summary of analysis of molecular variance (AMOVA). Genetic variability based on 90 RAPD markers for 42 individuals of *Neotinea ustulata* from 11 populations was analyzed. Variability was partitioned into three levels: (1) between early- and late-flowering populations, (2) among populations within groups and (3) among individuals within populations. Levels of significance are based on 999 permutation steps; *** – significant at $P < 0.001$.

		Variance component		
Level of variation	d.f.	absolute	%	*P*
Between groups (early versus late)	1	5.026	33	***
Among populations	9	3.604	23	***
Within populations	31	6.768	44	***
Total			100	

DISCUSSION

Morphological differentiation

Multivariate analyses of morphological characteristics indicate that the early- and late-flowering plants can be distinguished by the number of basal or stem leaves (Fig. 2) and even better by the ratio of these two (Fig. 3). However, these and other characteristics like plant height and size of leaves can be frequently influenced by environmental factors, such as height of the surrounding vegetation (FOLEY 1987), hydrology of the site (SCHÖDELBAUEROVÁ 2002) or weather (TALI 1996, TALI & KULL 2001). For instance, plants of the early-flowering population at the site Devínska Kobyla with very low and sparse vegetation reached the average height of 17 cm, compared to the site Vědlice with tall herb vegetation, where they reached the average height of 25 cm. Also TALI & KULL (2001) concluded that late-flowering populations are higher on average, but that fluctuations in the height between different years (which probably reflect different weather conditions) are larger than the differences between the populations.

In both herbarium and living material we found that the late-flowering plants, even if they very frequently inhabit low grassland communities and thus should be short, are on average 10 cm taller than the early-flowering plants (Table 2). Transplantation experiments by TALI & KULL (2001) further indicated that even if populations bloom later, when the surrounding vegetation is generally higher, which could account for its larger height, it keeps its height even when transplanted into different conditions. Together, these observations indicate that the observed difference in plant height is an inherited characteristic of the late-flowering plants and not a consequence of phenotypic plasticity.

Genetic differentiation

Morphometric analyses, when combined with genetic methods rather than used alone, proved to be a powerful tool in solving intraspecific taxonomy problems in *Cardamine amara* s.l. (LIHOVÁ et al. 2000) and *Cardamine acris* (PERNÝ et al. 2004), taxonomical status of *Dactylorhiza lapponica* (BATEMAN 2001) and *Sedum integrifolium* subsp. *leedyi* (OLFELT et al. 2001) and the question of hybrid origin of *Armeria villosa* subsp. *carratracensis* (NIETO

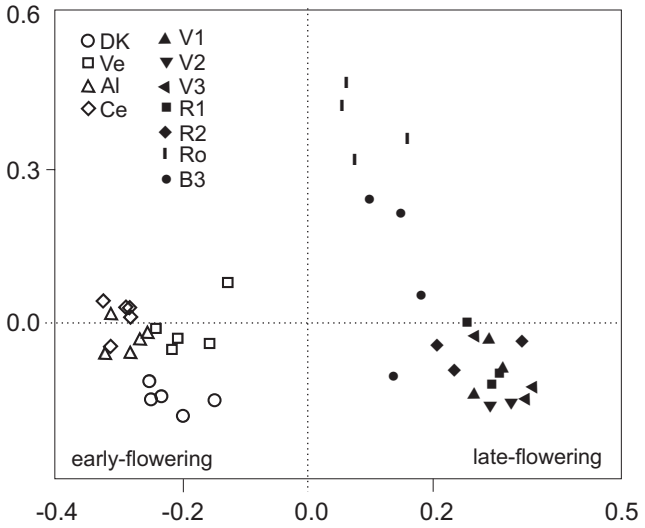

Fig. 5. Diagram showing results of principal coordinate analysis (PCoA) of 42 individuals of *Neotinea ustulata* from 11 populations based on 90 RAPD markers generated by six primers (OPA04, OPB11, OPB17, OPK01, OPK11 and OPK17). Early-flowering populations: Al, Ve, Ce, DK; late-flowering populations: V1, V2, V3, R1, R2, B3, Ro (for abbreviations of sites see Methods).

FELINER et al. 2002). SOLIVA & WIDMER (1999) used a combination of genetic and morphometric approaches to solve differences between sympatric populations of early-flowering *Gymnadenia conopsea* subsp. *conopsea* and late-flowering *Gymnadenia conopsea* subsp. *densiflora*. Allozyme markers clearly distinguished subspecies into two genetically distinct, monophyletic groups and thus indicated that the difference in flowering phenology represented an effective barrier to gene flow. Although most morphological characteristics significantly differed between the populations, they separated the *Gymnadenia* subspecies poorly. On the contrary, further studies have shown that both subspecies may be clearly separated from each other both morphologically (MARHOLD et al. 2005, BATEMAN & DENHOLM, unpubl.) and molecularly (ITS sequences; BATEMAN & HOLLINGSWORTH, unpubl.).

Results of our RAPD analyses in *N. ustulata* are consistent with those of the morphometric analyses. The coordinate analysis of the RAPD data has divided the populations into two groups, which can be predicted from their flowering phenology and regional distribution. RAPD's could therefore be a helpful tool for determining to which group, early- or late-flowering, problematic populations with either intermediate flowering time (REINEKE & RIETDORF 1987; the Bílé Karpaty Mts. population in this study), or with intermediate morphometric characteristics (JENSEN & PEDERSEN 1999) belong.

Several RAPD markers were shared by early- and late-flowering populations (e.g. Fig. 6, where the band 995 bp differentiates early-flowering – monomorphic within this group! – from late-flowering populations, but is also present in one Rosenau individual). This could indicate that the early- and late-flowering plants are able to hybridize, but this seems not to happen frequently at present due to strongly different flowering times. However, because the genetic distances between populations correspond with their geographical distribution, it

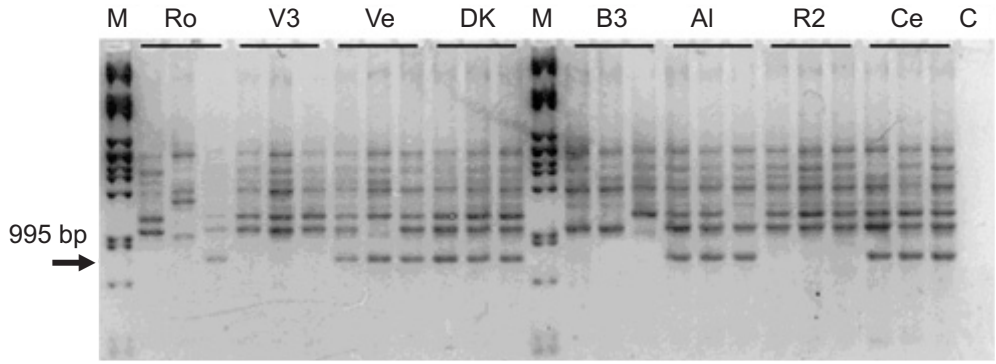

Fig. 6. RAPD profiles of individuals in early-flowering populations – Ve, DK, Al, Ce; and in late-flowering populations – Ro, V3, B3, R2 using primer OPK11. The fragment differentiating the populations (except of one individual from Ro population) is marked by an arrow. M – weight marker (λ DNA digested PstI), C – control sample without DNA. For abbreviations of sites see Methods.

seems that the observed gene similarity could reflect some hybridization between the early- and late-flowering groups in the past, when their flowering times differed less than now and/or until recent habitat fragmentation occurred. Finally, the shared markers could also be remnants of a common gene pool once belonging to an ancestor of both flowering forms.

Causes of seasonal dimorphism

It is not rare that a plant species has two subspecies that are largely reproductively isolated by flowering time (BORG 1972, LENNARTSSON 1997). This seasonal dimorphism is sometimes attributed to the method of haymaking in Central Europe: disruptive selection favours plants that fruit either before the first cut or after the crop has been taken away. Thus, management practices can act as selective pressures leading to quick separation of early- and late-flowering populations, with the subsequent effect on reproductive isolation. This may be the case of *N. ustulata*, as most of sites with appearance of this species were and still are managed by mowing or grazing. The reported half-life of cohorts of *N. ustulata* plants varied from 0.9 to 3.2 years (TALI 2002), what is low compared to other orchid species (~10–15 years, FARREL 1985, WILLEMS 2002). As the hypothesis of seasonal dimorphism was established on biennial model species *Rhinanthus* and *Gentianella*, the disruptive speciation of *N. ustulata* might have taken a similar time. Some non-anthropogenic factors, such as drought, or natural grazing (LENNARTSSON 1997) may also have a similar impact to a formation of seasonal forms, but they are difficult to identify.

Conclusions and forthcoming issues

We concluded that the taxonomic rank of varieties seems to be more suitable than the rank of subspecies, because the early- and late-flowering populations differ only in a few morphological characteristics, which can sometimes vary more within than between populations (TALI & KULL 2001), inhabit similar biotopes, have the same number of

chromosomes (n=42, MRKVICKA 1991), share the same pollinators, and have distribution areas that overlap. Although their genetic similarity is higher within than between early- and late-flowering populations, the difference (10%) is not sufficient for taxonomic rank of subspecies. Genetic distances among populations, however, strongly follow their overlapping geographical distribution. Further these taxa almost never grow together (no such site exists in the Czech Republic and Slovakia). They may have very distinct regional allopatric distributions (TALI 1996). Their flowering time is so different that they can hardly naturally hybridize and their genetic similarity is higher within than between early- and late-flowering populations. This would support the taxonomic rank of a subspecies. We have to be aware that the situation elsewhere may be completely different from that in Central Europe; e.g. in the British Isles, where the gene flow was observed among populations of *N. ustulata*, as the flowering time and geographic distribution of populations overlap (BATEMAN, pers. comm.). Therefore it would be valuable to conduct a more extensive morphological and genetic study over the whole distribution area. However, from the conservation point of view, the finding that there are two genetically and phenologically distinct taxa in N. *ustulata* is more important than the question of whether they are regarded as subspecies or varieties.

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APPENDIX

Vouchers of *Neotinea ustulata* used in morphometric analyses deposited in Prague (PR, PRC), Brno (BRNM, BRNU), České Budějovice (CB) and Olomouc (OLM) herbaria. The values in parentheses represent measured morphometric characteristics on individual plants (plant height, inflorescence length, number of leaves, number of rosette leaves + number of stem leaves). All specimens are from the Czech Republic.

Early-flowering *Neotinea ustulata* (*Neotinea ustulata* subsp. *ustulata*)

Bohemia: Distr. Kutná Hora: Dlouhá louka village near the village of Kácov (15.V.1923 K. ŽEBERA PRC; 26-4-6-4+2)

Bohemia: Distr. Beroun: Lochovice: Netolice: meadow below the Žebrácká road (21.V.1897 HAMPL PRC; 23-3.5-6-4+2; 21-3.5-5-5+1)

Bohemia: Distr. Beroun: Lochovice: Netolice: meadow below the Žebrácká road, (28.V.1897 HAMPL PRC; 25.5-4-6-4+2; 22-2.5-6-3+3)

Bohemia: Distr. České Budějovice: Litvínovice: meadow near the village of Litvínovice (12.V.1890 ANONYMOUS PR; 33-8-9-6+3)

Bohemia: Distr. České Budějovice: Rančice: small meadow on the bank of Vltava river at the inflow of Rančický brook into Třebonínský brook (28.V.1944 R. KURKA CB; 25-5.5-5-3+2)

Bohemia: Distr. České Budějovice: Rančice: the inflow of Rančický brook into Vltava river (27.V.1951 J. VANĚČEK CB; 19.5-4.5-3-2+1)

Bohemia: Distr. Chrudim: Golčův Jeníkov: meadow behind the village of Moravany (V.1904 K. DOMIN PRC; 19-4.5-8-6+3)

Bohemia: Distr. Děčín: Habartice (Ebersdorf) village near the town of Benešov nad Ploučnicí (Bensen) (21.V.1923 BORESCH PR; 22-3-5-3+2; 20-3-5-3+2)

Bohemia: Distr. Děčín: on the slopes of the Buková hora hill (Zinkenštejn), 683 m a.s.l. (25.V.1924 V. KRAJINA PRC; 27-5-6-3+3; 17.5-3-5-3+2)

Bohemia: Distr. Havlíčkův Brod: Olešná: marlstone slope at the peat bog near the village of Radostín (4.VI.1895 J. VITOUŠEK BRNU; 23-5-6-4+2)

Bohemia: Distr. Klatovy: Milčice: meadows on the slopes of the Sedlo hill (2.VI.1965 J. VANĚČEK CB; 26-4.5-4-2+2; 25-5-5-3+2)

Bohemia: Distr. Klatovy: region Horažďovicko: village of Svaté Pole (21.V.1961 J. VANĚČEK CB; 14-4-2-2+0; 13-2-3-2+1; 16-2.5-4-2+2)

Bohemia: Distr. Kolín: Doubravčany: behind the mill in Třešňovka (15.V.1926 s.coll. PRC; 17-3-4-4+0)

Bohemia: Distr. Litoměřice: edge of the pine forest "Velké háje", E of the village of Tetčiněves , SE of the Úštěk city, marlstone, ca. 310 m a.s.l. (20.V.1950 V. NĚMEČEK PR; 20-4-4-2+2)

Bohemia: Distr. Mladá Boleslav: on the rocky slope S of the Mladá Boleslav city above Červené Kolo, 250 m a.s.l. (30.V.1940 J. ŠOUREK PR; 17-2-7-5+2)

Bohemia: Distr. Mladá Boleslav: on the southern slope above the road from the village of Čejetice (Neuberg) to the Bezděčín village, 1.5 km below the Mladá Boleslav city, sparsely distributed, sandstone, ca. 210 m a.s.l. (21.V.1945 Z. MEJDR PRC; 18.5-4-5-3+2)

Bohemia: Distr. Plzeň: meadow on the left bank of the Úhlava river between the villages of Doudlevce and Hradiště (24.V.1899 T. MALOCH BRNU; 31-6-7-5+3; 23-4.5-5-4+1)

Bohemia: Distr. Praha: at the Vltava river near the village of Závist (in the vicinity of Zbraslav city) (V.1862 A.E. REUSS PRC; 24-7.5-4-2+2; 25-5.5-5-4+1; 15-4-4-3+1)

Bohemia: Distr. Praha: village of Hodkovičky (27.V.1880 F. ROSICKÝ PR; 24-5-8-5+3)

Bohemia: Distr. Praha: meadows behind the village of Závist (near the Zbraslav city) (17.V.1862 D. O. NICKERL PR; 22-3,5-6-5+1; 20-3.5-6-5+1; 24-4.5-6-4+2)

Bohemia: Distr. Praha: Stará Boleslav (2.VI.1873 F. ROSICKÝ PR; 15-4-6-4+2; 23-4-8-4+4)

Bohemia: Distr. Praha: village of Závist (18.V.1884 K. FIEDLER PR; 23-3.5-9-5+4; 19-3-7-4+3; 14-2-5-3+2; 28-6-7-5+2; 18-3-5-4+1; 24-4.5-8-5+3)

Bohemia: Distr. Praha-East: Brandýs: meadow near the village of Nový Vestec, close to the junction of the Jizera and Labe rivers, ca. 169 m a.s.l. (7.VI.1939 V. JIRÁSEK PRC; 35-9-7-5+2; 35-9-8-4+4)

Bohemia: Distr. Praha-est: meadow in the valley of Sázava river, below "Hláska" near the village of Senohraby, ca. 310 m a.s.l. (10.V.1930 J. DOSTÁL PRC; 24-3-7-5+2; 20-3.5-6-4+2; 17.5-2-5-3+2)
Bohemia: Distr. Příbram: meadow near the Sejcká Lhota village in the vicinity of thetown of Nový Knín (2.VI.1933 J. VÁCHA PRC; 36-7.5-7-4+3; 32-7-5-4+1)
Bohemia: Distr. Rakovník: Nové Strašecí: meadow Klíčavy, 0.5 km E from Klíčava game lodge, ca. 10 plants, 320 m a.s.l. (12.VI.1941 J. NETUŠIL PRC; 38-10-9-6+3)
Bohemia: Distr. Rakovník: region Křivoklátsko: dry meadow near the village of Zbečno (next to the camping site Sokol Praha III, together with *Orchis morio*) (20.V.1951 SKALICKÝ PR; 25-3.5-3-2+1)
Bohemia: Distr. Rakovník: Týřovice: meadow in the valley of Berounka river in the surrounding of Tejřov castle (19.V.1872 K. POLÁK Flora Bohemica PRC; 19-5.5-7-5+2; 16-2-4-3+1)
Bohemia: Distr. Strakonice: in dry sandy meadow at the W end of the forested slope "Kalvárie", near the Otava river W of the town of Strakonice, ca. 390 m a.s.l. (14.V.1945 J. MORAVEC PR; 15-3-6-5+1; 13-1.5-4-2+2)
Bohemia: Distr. Strakonice: in the dry meadow near Podskalí, on the left bank of the Otava river, S of the town of Strakonice, 394 m a.s.l. (28.V.1947 J. MORAVEC PR; 19-4-6-4+2; 13-4-5-5+0; 16-3-7-5+2)
Bohemia: Distr. Strakonice: in the dry meadow near the game reserve, on the right bank of Otava river, S of the town of Strakonice, abundant at some places, 394 m a.s.l. (26.V.1947 J. MORAVEC PR; 22-4-5-4+1; 24-4.5-5-4+1; 22-7-5-5+0; 27-5-8-6+2; 27-5-8-5+3; 20-3.5-4-3+1)
Bohemia: Distr. Strakonice: in the meadow near the Otava river ca. 1 km S of the town of Strakonice, rarely, ca. 394 m a.s.l. (25.V.1947 J. MORAVEC PR; 18-3.5-5-4+1; 16-4-6-5+1; 18-3,5-5-3+2)
Bohemia: Distr. Tábor: Radětice: bottom part of the grassland on the left bank of Smutná river V of the mill "Na Prádle", V of the village of Radětice, sparsely distributed, ca. 380 m a.s.l. (24.V.1964 J. KAISLER CB; 20.5-3-6-4+2; 24-3.5-5-3+2)
Bohemia: Distr. Tábor: Soběslav: meadow near the village of Doubí (25.V.1899 K. STEJSKAL PRC; 24-4-5-3+2)
Bohemia: Distr. Turnov: near Podrázec in the vicinity of the village of Úpice (26.V.1911 V. STUDNIČKA PRC; 32-5-5-3+2)
Bohemia: Distr. Ústí nad Labem: Malečov: Babiny village near the town of Litoměřice (in reality the place is closer to the Ústí nad Labem city), in the meadow between villages of Němčí and Babiny, 571 m a.s.l. (27.V.1925 F. HEJNÝ PRC; 23-3-7-4+3; 21-3-6-4+2; 24-3.5-4-3+1; 20-1.5-5-3+2)
Bohemia: Distr. Ústí nad Labem: Povrly: Mašovice (Meischlowitz) (24.V.1899 J. SCHUBERT PR; 24-6-6-4+2; 27-8-7-5+2; 28-6-7-4+3)
Bohemia: Distr. Ústí nad Labem: Povrly: Mašovice (30.V.1902 J. SCHUBERT PR; 16-3-8-5+3; 16-3-5-3+2)
Bohemia: Distr. Ústí nad Orlicí: at the edge of Pávkov forest in the village of Kerhartice, 1 plant (31.V.1942 TROJNA PRC; 21.5-3.5-5-3+2)
Bohemia: Distr. Ústí nad Orlicí: in the Končiny village (near Sloupnice village) in the vicinity of the town of Litomyšl (26.V.1910 J. OBR ÁLEK PRC; 23-5.5-7-5+2; 23-3.5-4-2+2; 25-4-6-4+2; 24-4-6-3+3; 15-3-7-5+2; 15-3-5-3+2; 21-4-4-3+1; 20-4-6-5+1)
Bohemia: meadows behind the village of Chalupice (31.V.1885 E. BINDER PR; 18-4-5-3+2; 21-4-6-4+2; 16-3-5-3+2)
Moravia: Distr. Hodonín: Bzenec: little hills near the village of Čejč (19.V.1881 J. BUBELA PRC; 16-3-7-5+2; 17.5-2-6-4+2; 17-2.5-5-3+2)
Moravia: Distr. Olomouc: southern slope of the Grygovské hills near the town of Olomouc (21.V.1914 A. KLÁPA BRNM; 20-3-6-4+2; 17-2-5-3+2)
Moravia: Distr. Uherské Hradiště: Uherský Brod: Nivnice: slopy meadows on "Králov" (30.V.1926 S. STANĚK BRNM; 18-4-4-3+1; 18-3-4-3+1; 21-5-6-4+2)
Moravia: Distr. Znojmo: Čížov: meadows near burg Hardegg (8.VI.1921. coll. J. ŠMARDA PR; 22-5-4-3+1; 27-6-5-4+1)
Moravia: Winterberg by the village of Skalice (20.V.1927 P. SILLINGER PR; 22-2-7-3+4; 22-2-7-3+4)

Late-flowering *Neotinea ustulata* (*Neotinea ustulata* subsp. *aestivalis*):

Bohemia: Distr. Beroun: Doutnáč hill near the village of Karlštejn (21.VI.1895, ex herb. C. TOOL PR; 46-9-6-3+3)

Bohemia: Distr. Chrudim: rarely in the meadow near the village of Dřenice (10.VIII.1889 J. ZITKO PR; 19-3-4-2+2; 17-3-5-3+2)
Bohemia: Distr. Děčín: mountain meadow near the toen of Děčín (16.VI.1884 L. ČELAKOVSKÝ PR; 25-6-7-5+2; 22-3-7-5+2; 26-6-7-4+3)
Bohemia: Distr. Trutnov: meadows near the village of Vrchlabí (Hohenelbe) – Lánov village (Langenau), 500-600 m a.s.l. (26.VII.1898 V. VON CYPERS PR; 24-4-4-1+3; 18-3-4-2+2)
Bohemia: Distr. Trutnov: near the village of Vrchlabí (Hohenelbe) – Lánov village (Langenau) (26.VII.1896 V. VON CYPERS PR; 20-3-6-3+3)
Bohemia: Distr. Trutnov: village of Vrchlabí (Hohenelbe) – Lánov village (Langenau), dry mountain meadows, chalk (16.VII.1887 V. VON CYPERS PR; 17-2.5-5-3+2; 14-1.5-6-3+3)
Bohemia: Distr. Trutnov: Vrchlabí, Ober-Langenau (Horní Lánov village) (26.VII.1902 F. BRANDRUP PR; 28-5-7-5+2)
Moravia: Distr. Břeclav: forest meadow ca. 0.9 km SW of the village of Kurdějov (15.VII.1965 F. KVAPILÍK OLM; 45-7.5-5-2+3 OLM)
Moravia: Distr. Brno-venkov: at the semi-steppe meadow near the Ochozká cave in the Hádecké valley (14.VII.1926 F. ŠVESTKA BRNM; 27-6-5-3+2; 35-4-4-2+2; 29-4.5-6-3+3; 58-10-8-4+4)
Moravia: Distr. Brno-venkov: Náměšť: on the Ketkovický castle (26.VII.1913 R. DVOŘÁK BRNM; 40-6-5-2+3)
Moravia: Distr. Hodonín: at the base of the Špidlák hill near the village of Čejč, 214 m a.s.l. (26.VI.1934 F. WEBER OLM; 28-3.5-4-2+2; 57-9-8-4+4)
Moravia: Distr. Hodonín: Strá nice: meadows in the Mandátské valley near the village of Radějov 300 m a.s.l. (14.VII.1951 F. ČERNOCH BRNM; 52-9-9-3+6; 30-4-6-3+3; 29-4-5-2+3; 37-8-5-3+2; 39-7-8-4+4)
Moravia: Distr. Hodonín: Uherský Ostroh: Suchov: shrubby meadow in the valley of Kazivec brook (23.VI.1924 S. STANĚK BRNM; 9-4-8-3+5; 21-2.5-4-2+2; 25-3-5-3+2; 29-1.5-4-2+2; 27-3-5-2+3; 51-4.5-8-4+4; 28-3-6-3+3; 29-5-5-2+3; 30-6-5-3+2; 30-4-5-3+2; 34-5.5-6-3+3; 25-2.5-5-2+3; 33-1.5-5-1+4; 28-2-4-2+2; 34-5.5-4-2+2; 27-3-5-3+2)
Moravia: Distr. Hodonín: Bílé Karpaty Mts.: forest-steppe meadows above the Lučina NE of the village of Radějov (by Strážnice village) (27.VI.1972 B. ŠULA OLM; 27-4-5-2+3)
Moravia: Distr. Hodonín: Bílé Karpaty Mts.: in the meadow near the Javorník village (3.VII.1941 V. SKŘIVÁNEK PRC; 27-3.5-5-2+3; 30-2.5-6-3+3; 33-5-6-3+3; 32-6.5-3-1+2)
Moravia: Distr. Hodonín: Bílé Karpaty Mts.: meadows in the Machová nature reserve ca. 3 km S of the village of Javorník, on the N and NW slope of the ground elevation 597, 450 m a.s.l. (8.VII.1927 P. SILLINGER PRC; 45-4-8-4+4; 38-6-8-4+4; 40-3-7-4+3; 37-2-6-3+3)
Moravia: Distr. Hodonín: Bílé Karpaty Mts.: meadows in Radějovské valley, ca. 300 m a.s.l. (2.VII.1927 P. SILLINGER PRC; 35-6.5-4-3+1; 36-3.5-7-3+4; 32-2.5-6-3+3; 39-4-5-3+2; 28-5-7-4+3)
Moravia: Distr. Hodonín: Bílé Karpaty Mts.: meadows on the Jasenová hill near the village of Blatnička, 410 m a.s.l. (1.VII.1927 P. SILLINGER PRC; 16-2-5-3+2; 34-2-7-3+4; 30-1.5-6-3+3; 22-3-6-3+3; 30-3.5-7-4+3; 23-3-6-3+3; 29-3-5-2+3; 32-3-5-3+2; 28-4-4-2+2; 39-3-6-3+3)
Moravia: Distr. Hodonín: Bílé Karpaty Mts.: meadows on the Jasenová hill above the village of Blatnička (4.VII.1930 F. WEBER OLM; 31-6-5-3+2; 28-3-7-3+4; 22-3-5-3+2; 42-5-5-1+4)
Moravia: Distr. Hodonín: Bílé Karpaty Mts.: meadows on the Holý hill, 700 m a.s.l. (23.VII.1927 P. SILLINGER PRC; 34-5-7-3+4)
Moravia: Distr. Hodonín: Bílé Karpaty Mts.: site "Retinka" near the village of Kněždub (29.VI.1930 F. WEBER OLM; 28-3-6-3+3; 25-3-6-3+3; 24-2.5-5-3+2; 25-4-4-2+2)
Moravia: Distr. Hodonín: Bílé Karpaty Mts.: steep meadows in Radějovské valley (2.VII.1928 P. SILLINGER PR; 39-5.5-4-2+2; 40-6-7-3+4; 38-7-4-2+2; 22-4-5-3+2)
Moravia: Distr. Hodonín: Bílé Karpaty Mts.: Uherský Ostroh: Blatnička: steep meadows "Horní louky" (close to the Jasenová hill) (22.VI.1924. coll. S. STANĚK BRNM; 41-4-7-3+4; 32-5-6-3+3; 32-4-8-4+4; 37-6-5-3+2; 33-4-7-3+4; 37-4-8-3+5; 25-4-6-3+3; 30-6-4-2+2; 26-3-6-2+4; 30-5-7-4+3; 27-3-5-2+3; 25-1.5-5-2+3; 25-2-5-2+3; 27-3-8-4+4; 26-3-4-2+2; 26-3-5-3+2; 33-5-5-2+3; 29-3-5-2+3; 31-3-6-3+3; 27-2-6-2+4; 34-3-6-2+4)
Moravia: Distr. Šumperk: at the Sázava river between the Ráječek and Leština villages near the town of Zábřeh (21.VI.1936 E. HEJNÝ PRC; 20-3-4-2+2; 18-2.5-3-1+2)

Moravia: Distr. Uherské Hradiště: Olšovec village near the village of Březová: steep meadows "Jamy" below the Lopeník village (19.VI.1924 S. STANĚK BRNM; 30-6-4-2+2; 33-5-4-1+3; 34-5-8-4+4; 29-4.5-5-2+3; 31-3.5-6-2+4; 28-3.5-7-3+4; 33-5-5-2+3; 31-3-5-2+3; 29-3-6-3+3; 31-4-8-4+4)

Moravia: Distr. Uherské Hradiště: Uherský Ostroh: Horní Němčí: Úlehle (17.VI.1926 S. STANĚK BRNM; 30-3.5-5-2+3; 30-5-4-2+2; 26-3.5-6-3+3)

Moravia: Distr. Zlín: steep meadow near Vápenky in the vicinity of the village of Valašské Klobouky (29.VI.1924 S. STANĚK BRNM; 39-9-7-3+4; 42-8-5-2+3; 40-7-6-2+4; 36-6-8-4+4)

Moravia: Distr. Zlín: Valašské Klobouky: Nedašova Lhota: steep meadow on the Vlahovec (30.VI.1924 S. STANĚK BRNM; 33-3-8-3+5; 40-6-8-4+4; 31-8-5-3+2; 30-3.5-5-2+3; 25-3-5-2+3)

Moravia: Distr. Zlín: Valašské Klobouky: Vrbětice: steep meadows in "Rubaný háj" (28.VI.1924 S. STANĚK BRNM; 36-2.5-6-2+4; 30-2.5-6-3+3; 36-3-7-3+4)

Moravia: Distr. Znojmo: Hardeggská meadow, slope above the right side of the road leading to the custom office 2.5 km J of the village of Čížov, 350 m a.s.l. (20.VII.1992 M. CHYTRÝ BRNU; 22-4-5-2+3)

Moravia: Moravské Lieskové: steep meadow below Beztinné near Želíbabka (11.VI.1925 S. STANĚK BRNM; 36-3-5-2+3; 35-2.5-7-3+4)

Moravia: Bílé Karpaty Mts.: valley of the Trnovský brook (25.VI.1932 F. WEBER, Flora Moravica PR; 30-3-7-3+4; 29-4-7-3+4; 22-3-4-2+2; 28-3-5-2+3; 25-3-4-2+2; 28-6-7-4+3; 24-5-5-3+2)