DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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SPECIES STRUCTURE OF *NEOTINEA USTULATA*

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

I Tali K., Kull T. 2001. Highly variable flowering time in *Orchis ustulata*: consequences for population dynamics. Nordic Journal of Botany 21: 457–466. Copenhagen.

II Tali K. 2002. Dynamics of *Orchis ustulata* L. populations in Estonia. In: Kindlmann P., Willems J. and Whigham D. (eds). Trends and fluctuations and underlying mechanisms in terrestrial orchid populations. Bakhuys Publishers, The Netherlands, 33–42.

III Tali K., Foley M., Kull T. 2004. Biological flora of the British Isles No. 232 *Orchis ustulata* L. Journal of Ecology 92, 174–184.

IV Tali K., Fay M., Bateman R. Minimal genetic differentiation among early and late flowering populations of the declining orchid *Neotinea ustulata* across Europe. Submitted to the Biological Journal of the Linnean Society.

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The defendant's contribution to the respective papers is as follows: 80% (paper I), 100% (paper II), 70% (paper III), and 60% (paper IV).

This doctoral thesis (including the article in the press) is not a publication in the sense of International Code of Botanical Nomenclature.

1. INTRODUCTION

The terrestrial orchid *Neotinea ustulata* (L.) Bateman, Pridgeon & Chase (formerly *Orchis ustulata* L.) has recently been a species of interest because of its confounding taxonomic status: it has been split into two subspecies, differing remarkably in their flowering time, but only slightly in terms of morphological characteristics; it has also been recently moved from the genus *Orchis* to the genus *Neotinea* by Bateman and others (1997). Procházka (1977) described it as one of the least variable species of the genus *Orchis* and all of its deviations have been considered taxonomically unimportant expressions of individual variability. Later Gumprecht (1981) recognized that some populations bloom much later than the nominate race and have a slightly different morphology. This led Kümpel (1988) to describe a new variety (*Orchis ustulata* var. *aestivalis* Kümpel), which was elevated to the subspecies level by Kümpel & Mrkvicka (1990) as *Orchis ustulata* subsp. *aestivalis* (Kümpel) Kümpel & Mrkvicka. Other authors however, regard the morphological differences between the two subspecies/varieties as minimal and questionable (Reineke & Rietdorf 1991, Tali 1996, Jensen & Pedersen 1999) and/or consider flowering time as crucial for distinguishing between them.

To further confuse matters, in 1991 Reineke & Rietdorf argued that there are two different forms of late-flowering *N. ustulata* in Germany, one of which occurs simultaneously with early-flowering plants in all large populations. Their flower buds develop at the same time as those of the early-flowering plants, but expansion of inflorescence is delayed until mid-June when they develop rapidly, flower and bear fruit relatively quickly.

During the last decades, *Neotinea ustulata* 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 (Haraštova *et al* 2004), and by more than 90% in Bohemia (Procházka & Velísek 1983). It is one of the most rapidly decreasing plant species in Britain (80% decline) having disappeared from 210 of 265 formerly occupied 10 x 10 km squares (Preston *et al.* 2002). In most of Europe, there are laws that protect the species.

In general flowering time is a phenotypically plastic character, so I asked the question: is the flowering time of *N. ustulata* a genetically fixed character, suitable for distinguishing subspecies or even higher taxa?

A few studies have shown that traits associated with the time of flowering are genetically correlated to components of plant size, reproductive effort and frost tolerance (Ratchke & Lacey 1985). In some species flowering and germination times may represent one component of an integrated gene complex; in other species selection may act upon each trait individually. Hence the

question: what population dynamics characters discriminate late and early flowering taxa?

Most species are expected to flower during a limited season. Individuals that flower out of season will be reproductively isolated, thus the flowering time should be subject to natural selection. Recently cases of variable flowering time has been detected in several species, with no coinciding morphological variation (Winfield *et al*. 2003, Zopfi 1995, 1998). Together with these species, *N. ustulata* presents an excellent opportunity not only to investigate the biology, taxonomy and possibilities of protecting an endangered species, but also to address more fundamental questions concerning evolutionary processes and speciation.

If the species does exist in the form of two distinct taxa, then further research on their distribution and ecological demands will be necessary to prevent the extinction of either of them. Therefore my following questions are: how diverse is the species and does possible low diversity play a role in the recent regression in its distribution.

I also seek further answers to what evolutionary factor may cause the divergence of these taxa with no evident difference in their morphology, whether the late flowering plants diverged from the early flowering ones or vice versa and has this kind of divergence taken place once or repeatedly throughout the history of this species?

2. MATERIAL AND METHODS

2.1 The species

I will now present an outline of the biology and ecology of *Neotinea ustulata* (L.) Bateman, Pridgeon and Chase as most Floras describe it. For a more detailed review, see paper III. The difference between the two putative subspecies described by Kümpel and Mrkvicka (1990) is discussed further on in greater detail.

The species under study is a rather small, tuberous, perennial orchid. Two to six unspotted leaves form a bluish green wintergreen rosette. When flowering, its stem is 5–50 (usually 10–30) cm high; the flowers produce no nectar. Its flowers are sessile, opening from the base upwards. They are dark purple when unopened, hence its Latin name, since "*ustulo*" means "burnt to brown". The labellum is white or pale pink with papillose purple spots and is deeply trilobed; the middle lobe is dilated at the apex. The capsule is about 1 cm long and erect. Its seeds are very numerous and tiny.

N. ustulata occurs throughout much of Europe reaching its northern geographical limit in the Faroe Islands, the Swedish island of Gotland and Estonia. The species has also been found in the Ural Mountains and in West-Siberia (Baumann & Künkele 1982; Vakhrameeva *et al*. 1991); whilst its southern distributional limit passes through Spain and the Mediterranean coast of France. This species is in general decline and is protected throughout its range; it is sometimes abundant in mountains, but rare elsewhere and very rare in the Mediterranean region (Delforge 1995).

Neotinea ustulata favours sunny, open habitats in short, lightly grazed calcareous grasslands with only moderate competition, but in continental Europe it is also known to inhabit open woodlands offering plenty of light. The largest populations in Britain are usually found on old pastures, moderately grazed by rabbit or sheep, which have not been treated with artificial fertilizers, herbicides, or pesticides.

In continental Europe *N. ustulata* grows on limestone pastures or poor meadows, in light scrub on rather dry base-rich (also lime-free), mildly to moderately acid humus. Most populations occur in *Mesobromion* alliance, rarely also in *Cirsio-Brachypodion* or poor *Arrhenatherion* alliance (Oberdorfer 1994).

Kümpel (1988) described a late-flowering variety, var. *aestivalis,* in *Orchis ustulata* L.*,* with a holotype from Nordhausen (leg. Vocke 22.7.1879). As a result of further findings, Kümpel & Mrkvicka (1990) changed the rank of this taxon and distinguished between two subspecies of *O. ustulata* — ssp. *ustulata* L. and

ssp. *aestivalis* (Kümpel) Kümpel et Mrkvicka, demonstrating the remarkably different flowering times and small differences in plant height as well as conformation of the lateral sepals.

On the islands of Muhu and (eastern) Saaremaa in Estonia, populations flower from May until June and on the mainland and western Saaremaa from July until August, or September in some years.

Flowering time can also greatly depend on heights above sea level (Reineke & Rietdorf 1987).

2.2 Permanent study areas

Material for population dynamics analysis is collected from 5 populations in Estonia (Table 1 in I) where permanent plots have been established and all individual plants marked and mapped.

All vegetative and generative plants were marked with a numbered tag on permanent 1x1 m^2 plots as well as labelled on a map. Vegetative plants include juveniles, since it was impossible to distinguish between actual juvenile plants and small two-leafed vegetative plants that sprouted after some years of dormancy. The number of leaves, height of a flowering plant, length of an inflorescence and number of fruits were counted on each plant.

Early flowering populations are all on Muhu island (about 20 sq km): Aljava — a *Carici montanae-Seslerietum* meadow (*Scorzonera humilis* variant) on the seashore and ungrazed for the last 10 years so young junipers have begun to rise and old grass severely threatens the population; Lõetsa — a *Seslerio-Filipenduletum* pasture less than 1 km from the sea, covered with juniper bush and Kapi — a drier version of a *Seslerio-Filipenduletum* grassland in the middle of the island, covered with young pine forest. Here the surface is very rocky and covered with a thin layer of humus.

Late flowering populations are all on the mainland of Estonia: Sillukse — an overgrown *Seslerio-Filipenduletum* grassland some km-s from the sea, covered with pines and junipers, now almost destroyed by a limestone quarry and Jäneda — a *Melampyreo-Scorzoneretum* grassland. Formerly a village school's athletic field, it is now overgrown with pines and spruce. The area is far from the sea and any other known population of *N. ustulata*.

2.3 Replanting experiment

To test if locality influences flowering time, four plants were taken from the late-flowering Sillukse population and four plants from the early-flowering Aljava population, on June 10, 1995. They were then planted in a common garden on

Muhu Island, i.e. in a location where only the early-flowering plants have been found (about 1 km from the nearest local population). The phenology of these plants was monitored every year.

Another replanting had to be carried out because part of the Sillukse site was going to be destroyed due to the creation of a quarry. Sixty plants were dug out and re-planted near Pivarootsi village, 10 km away. The progress of these plants was also constantly monitored.

2.4 Soil analysis

Material for soil analysis was collected from five populations (Aljava, Kapi, Lõetsa, Sillukse and Jäneda). About half a kilo of material was dried at 80ºC and then ground up. The analysis was performed at the Estonian Agricultural University's Laboratory of Plant Biochemistry.

The pH was determined in 1 N KCL suspension. Other determined characteristics were: available potassium and phosphorus, nitrogen, magnesium, calcium and organic matter (loss on ignition).

2.5 DNA analysis

In the summer of 2002 flowers, buds and leaves were collected from six early- and three late-flowering populations in the UK and three early- and four late-flowering populations in Estonia. This was supplemented with a French sample drawn from the RBG Kew DNA bank (accession 12848) collected by Civeyrel and others in May 2001 near Nalzen (Table 1 in IV).

In addition to samples from permanent study areas, two additional late-flowering populations were sampled: one on the island of Saaremaa and the other on the mainland not far from the Sillukse population.

All samples were collected into silica gel bags (Chase & Hills, 1991). DNA was extracted from approximately 0.1 g of dried material using a modified 2x-CTAB (cetryltrimethyl-ammonium bromide) procedure (Doyle & Doyle, 1987), then purified on a QIA quick column and quantified using a spectrophotometer. AFLP (amplified fragment length polymorphism) analysis was performed according to the AFLP Plant Mapping Protocol of Applied Biosystems Inc., and a Genotyper 2.0 was used to analyze the resulting bands.

ITS (internal transcribed spacer) analysis was performed at the Jodrell Laboratory in Kew as described by Pridgeon *et al*. (1997).

2.6 Data analysis

2.6.1 Population dynamics analyses

In all populations, the plants were divided into three classes — dormant, vegetative and flowering plants (Table 2 in I). It is difficult to distinguish between juveniles and weaker vegetative plants; therefore all non-flowering rosettes counted were included in the vegetative group. Three height classes were used to compare the influence of fruiting (Table 5 in I). As plant heights differ among populations and from year to year, relative heights were used: for the small plants $H \leq 0.80$ of the population average, for medium sized plants $0.8 < H \leq 1.2$ and for large plants $H > 1.2$.

On the mapped plots, a marked plant was considered dormant for one year before the plant's first appearance, and for up to three years after its last appearance. A plant was considered dead if it had not appeared above ground for the last three years.

Clusters of plants with a diameter of 5 cm or less were considered to be genets.

The SAS mixed procedure (SAS Institute Inc., SAS/STAT Users Guide, Version 6, Fourth Edition, Volume 2, Cary, NC: SAS Institute Inc., 1996) was used to analyze differences in population dynamics between the subspecies and the populations. Annual status class percentages (flowering, vegetative, dormant) were used as characters, subspecies as a fixed factor, and site as a random factor. Site significance was tested by Wald's Z-test, and subspecies significance by the likelihood-ratio F-test.

Half-lives from the survivorship curves of *Neotinea ustulata* were calculated independently for each cohort in each population. Cohorts of even-aged plants were used only from within the population's third year study group; depletion curves were also calculated using multi-aged plants that were found in the first year in every population.

FoxPro version 2.6 was used to manage the data and calculate transition matrices.

2.6.2 DNA data processing

Bands for all individuals were scored as either present (1) or absent (0). The generated binary matrix was analyzed using the UPGMA (Unweighted Pair-Group Method using Arithmetic Averages) algorithm in the software package PAUP version 4.0d64 for Macintosh and the principal coordinates analysis (PCOA) was completed in the R Package for Multivariate Analysis version 4.0. F-statistics were calculated with the Tools For Population Genetic Analyses (*TFPGA*), a Windows (TM) program for the analysis of allozyme and molecular population genetic data.

3. RESULTS

3.1 Replanting experiment (I)

The flowering time of the late-flowering subspecies remained unchanged (late July-August) after the transfer to the common garden.
At least 34 plants out of 60 flowering ones from Sillukse quarry area, which were planted on a seashore meadow in 1996, were alive 4 years after replanting and 1 new, vegetatively propagated rosette appeared. The flowering time did not change.

3.2 Phenology (II)

The first rosettes of the early-flowering plants appeared in September-October and those of the late-flowering subspecies in October–November. In all populations a number of plants emerged in the spring and no significant relation between the time of appearance and the behaviour of the plant (flowering or remaining vegetative) could be detected. Depending on the year, the early-flowering plants showed buds from the end of May to the first week of June and the blooming never extended past June 24th. The start of flowering amongst late populations also depended on the year; normally it began in July but in some years in the last week of June and lasted until the middle of August, in one case until September 8th. The flowering was not simultaneous throughout the populations or within a single population.

3.3 Reproduction dynamics (I, II)

Figure 4 in paper II presents the maximum number of successive flowering events exhibited by the monitored plants. The number of plants that never flowered varied among populations; most plants flowered once during their lifetime.

Flowering was variable from year to year but flowering percentages were highest between 1994 and 1996, with the exception of a high-flowering percentage in 1997 in Jäneda. In 1995 there was a relatively large number of flowering shoots in the Aljava population. In Jäneda, there were no plants above ground in 1998, when July and August were unusually rainy and relatively cold (14.4^{0}C compared to the average of 16^{0}C, and 176 mm compared to the average of 87 mm). The summer of 1999 was exceptionally dry (28.3 mm compared to the normal 49.9 mm) and flowering was inhibited in most populations (Fig. 2).

Late-flowering populations exhibit a greater percentage of fruiting plants compared to the early-flowering ones.

Abundant flowering and fruiting did not trigger dormancy, slightly more non-fruiting plants went dormant the next year (Table 5 in I). Almost half of the fruiting plants flowered again the following year, and most of those that did stayed in the same size class as the previous year.

3.4 Morphometry of vegetative characteristics (I, II)

The heights of the individual plants varied from 5 cm to 50 cm. Late-flowering plants were on average, (over all years) 3.3 cm higher than early-flowering plants. The greatest difference in height recorded for the same plant in different years was 20 cm. Inflorescence height varied considerably throughout the years. In general, tall spikes were associated with wet (and warm) springs (Table 1 in II). Fluctuations were greater in plant measurements in different years than the differences between the populations or subspecies (Table 2 in II).

The number of vegetatively propagated shoots varies in different populations (Table 4 in I). The number of shoots in genets is higher amongst early-flowering populations that grow on gravel soil (Kapi, Lõetsa). In Jäneda, all genets had only one shoot.

3.5 Longevity (II)

The longevity of individual plants was, in general, low (Fig. 3 II). Half-lives of different cohorts of plants in the given populations vary from 0.6 to 3.0 years (average 1.75) but some plants survive much longer. Six of the 40 plants marked in 1993 in the late-flowering Sillukse population and three of the 24 plants in the early-flowering Lõetsa population were still present in 2000.

Plants that emerged, flowered only once and then disappeared had a large influence on the half-life calculations of a population. Once again, survivors showed a range of behaviours: the length of dormancy and the length of flowering varied a great deal from being vegetative throughout their lives to flowering throughout their lives. More than 70% of the survivors, i.e. the plants that lived longer than 1–2 years, exhibited dormancy during their lifetime.

3.6 Dynamics of the life-cycle and transitions between the classes (I, II)

The proportions of flowering, vegetative and dormant plants have varied during the observation period in all of the populations (Table 2 in I). The dormant stage

dominates in all populations but all possible transitions occur between dormant, vegetative and generative stages (Table 7 in I, Fig 6 in II). Due to the calculation method, the number of dormant plants is underestimated in the first two years of the study.

Dormancy, or plants spending a few years below ground without the appearance of any above ground organs, is a well-known phenomenon in terrestrial orchids (Tamm 1972; Hutchings 1987b; Wells & Cox 1991; Willems & Bik 1991) and is well expressed in *N. ustulata.* During 8 years only 6 out of 600 plants did not experience dormancy and all these were part of early-flowering populations.

The mean percentage of dormant plants in both late-flowering populations is higher than in any of the early-flowering populations studied. However, the SAS mixed procedure analysis showed no significant differences in stage class distribution between populations and between late and early-flowering subspecies, when subspecies was considered a fixed factor and site a random factor.

The largest number of plants have been dormant for one year, a smaller number for two years, and only 5.3% of all the monitored plants for three consecutive years. In most populations (with the exception of Kapi) the greatest number of formerly dormant plants flowered when they reappeared. Vegetative plants that appear after some years of dormancy are often small two-leafed rosettes. There are very few plants that have been above ground for six or seven consecutive years, and these exist only in early-flowering populations.

It is possible that the plants recorded as being dormant for 6 and 7 years may have been vegetative in the spring of 1997. The census was carried out too late that year and some vegetative plants may have passed unnoticed, since they tend to wither before the full flowering of the rest of population.

The frequencies of transition between the three stages are presented in Fig. 1 (I). In the early-flowering populations the ratio of vegetative plants is slightly higher and they behave differently from the late-flowering populations. Most of the vegetative plants in late-flowering populations become dormant or die and a few set flowers the following season. In early-flowering populations, more vegetative plants remain vegetative in the next season.

Flowering plants in late-flowering populations tend to go dormant, while in early-flowering populations successive flowering is more common.

3.7 Molecular characteristics (IV)

ITS sequences of one early- and one late-flowering Estonian plant were identical to each other as well as to that previously obtained from an Italian specimen of *N. ustulata* and published by Bateman *et al.* (2003).

The AFLP analysis yielded 89 interpretable bands (Table 2 in IV), 79% of these were polymorphic. Seven bands were found only in Estonian material and five bands only in UK material; a further ten bands occurred in very limited numbers in Italian, Georgian and/or French plants, but did not occur in any British or Estonian material.

The UPGMA dendrogram generated from this data (Fig. 2 in IV) shows no clear differentiation between early-flowering and late-flowering populations or between Estonian and English material.

The unrooted phylogram based only on Estonian material (Fig. 3A in IV) shows lower variance among populations than within populations and imperfect assortment into early- and late-flowering plants. Separately analysed English plants (Fig. 3B in IV) form two clusters of approximately equal size, one of them being an equal mixture of early-flowering and late-flowering plants, but the other dominantly early-flowering and including only one errant late-flowering plant.

When estimation of differentiation between populations was calculated separately for Estonia and England, $F_{ST} = 0.254$ for Estonian populations and $F_{ST} = 0.551$ for English populations; F_{ST} calculated over all populations was 0.509.

The PCoA plot of all samples (Fig. 4A in IV) shows little structure beyond the first co-ordinate, which accounts for 23.8% of the total variance. The Georgian and Italian plants appear to be more closely associated with the relatively compact Estonian cluster in the PCoA plot than in the UPGMA tree. Axes 2–4 all separate the two Georgian plants from the remainder and Axis 4 separates the two Italian plants from the remainder (ordinations not shown). As in the UPGMA tree, the French sample and one Estonian sample cluster with the English plants, which form two imperfect clusters.

Separate ordinations illustrate the contrasting distances between early- and late-flowering populations in Estonia and England. In Estonia (Fig. 4B in IV), early- and late-flowering populations show a slight tendency toward genetic divergence on the plot of axis 1 versus axis 2. In English populations (Fig. 4C in IV) the genetic structure is based less on early- versus late-flowering patterns and more on geographical location.

3.8 Soil analysis (III)

The chemical characteristics are shown in Table 1 in III. The pH in Aljava (4.9) was lower than amounts given by any previous author (Arditti 1992; Procházka & Velísek 1983). Organic matter was highest in Kapi. That was expected, since the site has practically no humus between the rocks and mostly litter was collected. Late flowering populations presented the lowest percentage of organic matter.

4. DISCUSSION

4.1 Replanting experiment (I)

It is a well-known phenomenon that plants of the same species may exhibit somewhat different sprouting or flowering times when growing in different habitats. Rasmussen (1995) reported that after some plants from wintergreen populations of *Orchis morio* in Öland and Denmark were moved to the Botanical Gardens in Copenhagen, the plants from Öland sprouted in the autumn, whereas the plants from Denmark did not unfold until the spring. In mountain areas, the flowering time of *Neotinea ustulata* greatly depends on heights above the sea level. In Estonia, as well as in Great Britain, no height factor is present.

Since the Burnt Orchid is an endangered and protected species in Estonia, extensive replanting experiments were not possible. Two series of replanting were done and these plants did not exhibit any changes in their phenology as a result. I assume that flowering time in *N. ustulata* is a genetic character that does not depend on site and year.

4.2 Phenology (II)

Reineke & Rietdorf (1987, 1991) claim that in all *N. ustulata* populations rosettes rise constantly from autumn to spring (including beneath the snow), and that both autumn-risen and spring-risen plants can flower. Plants of the late subspecies that flower until autumn and therefore do not have summer rest, begin to form rosettes in March-April (Kümpel & Mrkvicka 1990). In Estonia, the early-flowering plants begin to emerge in early September, but there may be a delay during dry autumns. In the late-flowering populations some plants emerge in late October, but most of them emerge in the spring.

Further complicating the discussion, Reineke & Rietdorf (1991) argued that in Germany there are two different forms of late-flowering *N. ustulata*, one of which co-occurs with early-flowering plants in all populations containing more than about 30 flowering individuals. The leaves of these widespread late-flowering plants emerge between September and November, together with those of the early-flowering plants, and their flower buds develop at the same time as those of the early-flowering plants. However, while the early-flowering plants wither at the end of the flowering period, plants of the later-flowering group remain green. Expansion of the inflorescences is delayed, but from mid-June they develop rapidly, flower and set fruit relatively quickly. This form is said to occasionally show flowering periods intermediate to those of the early-

flowering populations and bona fide late-flowering *aestivalis.* So there may be more than one late-flowering taxon within *N. ustulata s.l.*

4.3 Reproduction dynamics (I, II)

No plants flowered for longer than seven years in a row. Wells *et al.* (1998) state that *Orchis morio* can flower successively for 17 years. Wells suggested in 1981, that only those individuals occupying the most favourable sites flower for more than 5 years in succession and that is in accordance with our data (see Fig 4 in II). The flowering tends to be irregular in many terrestrial orchids; the number of times that individuals have flowered and the number of years between flowering events varies considerably (Farrell 1985; Hutchings 1987a; Inghe & Tamm 1988; Whigham & O'Neill 1991). Repeated flowering in consecutive years by a large number of individuals has been reported in only a few species, e.g., *Ophrys apifera* (Wells & Cox 1991), *Liparis lilifolia* (Whigham & O'Neill 1991) and *Orchis simia* (Willems & Bik 1991).

The number of flowering plants is the most stable in Kapi. Kapi is also the site of the highest number of vegetatively propagated genets. It is possible that when there is a very dense cluster of vegetative rosettes with one flowering shoot every year, it may be recorded as stable flowering / being vegetative in the database, while in fact different vegetative rosettes may be flowering in consecutive years.

Several authors have claimed that plants that flower and set fruit abundantly are least likely to flower the following year and are, as a rule, also smaller than the plants that did not set fruit. Calvo (1993) has shown that one year after high pollination treatment, the treated plants of the orchid *Tolumnia variegata* were significantly smaller and produced less flowers. Snow & Whigham (1989) pointed out that the plants of *Tipularia discolor* with many fruits were less likely to flower the following year, probably because they were smaller than those with fewer fruits. *Orchis mascula, Spiranthes spiralis and Tipularia discolor* have been observed as paying a price for flowering (Inghe & Tamm 1988; Whigham & O'Neill 1991 Willems & Dorland 2000). The setting of fruit also promoted dormancy according to Primack and Stacy (1998). Surprisingly, we found the opposite pattern for *Neotinea ustulata* (Table 5 in I). Definitely resources are not limiting the fruit set in this species, but rather the lack of pollinators. Compared to the fruit set in England's populations of *Neotinea ustulata* (J. Foley pers. comm.) the fruit set is much higher in Estonia.

Little is known about the pollinators of *Neotinea ustulata*. Different authors have named different kinds of insects (Reineke pers. comm; van der Pjil & Dodson 1969); Vöth (1984) documented a tachinid fly as a pollinator for *N.*

ustulata var. *ustulata.* A beetle, *Leptura livida*, is mentioned as a pollinator for *N. ustulata* var. *aestivalis* (Mrkvicka 1991; van der Cingel 1995).

Temperate tuberous orchid species can produce more than one new tuber per year and thus multiply vegetatively. The percentage of rosettes originating from vegetative multiplication is not high — usually less than 5% (Hutchings 1987a; Willems & Melser 1998). In early-flowering populations of *N. ustulata*, vegetative propagation is more common and more new vegetative rosettes appear near old plants. Still, this may result from differences between populations rather than differences between varieties, since no such clusters were found at Jäneda nor were they numerous in Sillukse. Among early-flowering populations, vegetative propagation is least pronounced in Aljava where, as in Jäneda, the soil is sandy and lacks stones and gravel.

4.4 Morphometry of vegetative characteristics (I, II)

The fluctuations in height among different years are larger than the differences between the populations or subspecies, though average heights are greater in late-flowering populations, obviously because the surrounding vegetation is generally higher in July-August.

Studying populations in Central Europe, Haraštova *et al.* (2004) used a more thorough morphometric approach to test supposed vegetative differences between early- and late-flowering populations. Most supposed differences failed detailed analysis, demonstrating that the main characters distinguishing later-flowering populations were taller stems and longer leaves exhibiting greater surface areas, accompanied by what we interpret as the developmental transition of one leaf from being basal and expanded to sheathing the stem.

The height of plants is usually a poor characteristic for solving taxonomical problems among closely related taxa. Therefore, I disagree with Kümpel & Mrkvicka 1990 in promoting these taxa into subspecies.

4.5 Longevity (II)

Orchids have often been referred to as species with a long life span. Tamm (1991) has shown that *Dactylorchis sambucina* may have an average life span of up to 40 years and *Listera ovata* even up to 160 years. More recently some authors have shown mean half-lives of less than 2 years in some species. Like these, *Neotinea ustulata* is a short-lived species with an average half-life of 1.75 years. That is considerably less than many orchid species like *Aceras anthropophorum, Ophrys apifera, Orchis militaris or Spiranthes spiralis* and comparable to such species as *Coeloglossum viride* or *Ophrys sphegodes* (Wells

1981; Wells & Cox 1991; Waite & Farrell 1998; Willems & Melser 1998; Hutchings 1987a,b).

Wet and warm springs influence flowering and rosette forming, but not the fate of cohorts. The establishment of new plants was not affected by the year or the size of a cohort.

4.6 Dynamics of the life-cycle and transitions between the classes (I, II)

Flowering irregularity is a common feature in most orchid populations (Wells 1981; Tamm 1972, 1991; Hutchings 1987b; Inghe & Tamm 1988, Mehrhoff 1989; Whigham & O'Neill 1991) In *N. ustulata* populations, all stages vary irregularly over years as well as throughout the populations.

Dormancy is the dominant stage in all the populations for *N. ustulata*. If we do not count the first monitoring year, when the number of dormant plants cannot be estimated, then only in the Aljava population have there been two years when the number of flowering plants exceeded the number of dormant plants. In both above ground stages transition to dormancy exceeds 50%, with the exception of Kapi, where only 45% of flowering and 50% of vegetative plants stay dormant the next year. Foley (1987, 1990) studied and documented the decreasing populations of *Neotinea ustulata* in England in the years 1982–1986, and noted that even in favourable years, more than 50% of the plants were in the dormant stage. Several other orchid species studied for this aspect showed smaller percentages of dormancy (Wells & Cox 1991; Waite & Farrell 1998)

The existence of dormant plants may easily lead to the underestimation of the size of the population. When conducting long term censuses, it is necessary to visit plots at least three times during the vegetative period, as plants which still possess aerial parts during the flowering season may only be a small proportion of those present at the start of the vegetative period (Hutchings 1991). This is especially important in late-flowering populations where practically all non-flowering plants have disappeared by the time the others flower.

Waite and Farrell (1998) reported that in the case of *Orchis militaris*, the dormant part of a population was 14% of all the plants present and the length of the apparent dormancy period was 1–8 years. This is longer than proposed for *Neotinea ustulata* by other authors (Foley 1992) and also observed during this study. Kull (2002) claims that the maximum length of dormancy in temperate orchids is five years, a number which coincides with my observation of *N. ustulata*.

Observations at five populations in Estonia during 1994–1999 did not reveal any relation between heavy fruiting and staying dormant. Dormancy is

often referred to as the "resting" phase of plants, though resting does not seem to be the main function here. Nearly half of the dormant plants emerged flowering the next year (Fig. 6 in II). Dormant plants may act as a reservoir of recruits in a population of otherwise short-lived species. The population in Jäneda recovered after several years of total decline years, during which the above ground orchid population had dwindled to very low numbers (Fig. 1 in II).

Long dormancy periods seem to be more common in late-flowering populations, although this phenomenon is not statistically significant from the present data set.

4.7 Molecular characteristics (IV)

Strongly differing flowering periods and the geographical distance between samples used in this study have not resulted in any ITS divergence between the analyzed individuals of *N. ustulata.* Karyological investigations also failed to distinguish between early- and late-flowering forms, both of which yielded a count of $2n = 42$ (Mrkvicka, 1991), which is plesiomorphic for the subtribe *Orchidinae* (Bateman *et al.*, 2003). This does not automatically indicate a lack of taxonomically meaningful genetic structure, although it strongly suggests that any divergence among taxa occurred recently.

The trees and ordinations based on AFLP data indicate a lack of long-term genetic discontinuities between any obvious groupings of populations. The majority of the variations form a linear pattern that is largely encapsulated by the first axis on the PCoA plot and reflects a west-east cline of geographical variation.

A recent genetic study of Czech populations of *N. ustulata* by Haraštova *et al.* (2004) was able to distinguish early- and late-flowering populations using RAPDs. In accordance to our study, considerable divergence was evident among populations within each phenological group. Moreover, the geographical distances between the clustered early-flowering and late-flowering populations were so great that genetic differentiation due to geographical distance cannot be separated effectively from genetic differentiation due to phenological separation.

Variation within populations is considerable relative to which exists between populations. These results indicate certain levels of gene flow among populations, at least until recently. In several areas of Europe, *N. ustulata* flowers continuously from April to August caused by a wide range in altitude extending to 1700 m OD (Ferlinghetti & Grünanger, 2001), so gene flow between early- and late-flowering populations appears likely in light of our AFLP data. Regrettably, the precise times of opening of the first receptive flower and of the atrophy of the last receptive flower are not documented anywhere.

It seems that the rapid decline of the species across Europe cannot be attributed to reduction in overall levels of genetic variation.

Bateman and others (2003) showed that *N. ustulata* is the most geographically widespread member of a set of morphologically similar Mediterranean species, being placed between *N. lactea*, *N. conica* and *N. tridentata* on the one hand and the *tridentata*-like *N. commutata* on the other. As these species tend to flower in May rather than in June-July at any given latitude, it can be assumed that early flowering is in this case a plesiomorphic character and that the late-flowering populations diverged from the early-flowering ones.

The next question to ask is whether this event has taken place once and then dispersed to achieve almost as wide a distribution (the monotopic hypothesis), or whether it has taken place at several locations (and maybe several times at each location) across Europe (the polytopic hypothesis).

The strong geographical signal evident in our AFLP data for both early- and late-flowering plants is more consistent with the polytopic hypothesis.

Theoretically the monotopic hypothesis could also hold, but then the subsequent introgression should have occurred across the range of both groups to create the dominantly geographical pattern we revealed. This seems highly unlikely as the population density of the species is rapidly diminishing and fragmenting.

Post-glacial migration tends to be reflected in relatively high genetic diversity along the three favoured northward migration routes through Europe (Iberia, Italy, Greece/Turkey), and relatively low diversity in the peripheral regions of the British Isles and Scandinavia. Thus it is not surprising that AFLP studies of orchids such as *Orchis simia* (Qamaruz-Zaman *et al.*, 1998) and the *O. mascula* group (Redmond *et al.*, in prep.) showed that genotypes present in the British Isles are a subset of those found in Continental Europe. Interestingly and unusually, this does not appear to be the case with *N. ustulata*, where similarly sized samples reveal much greater genetic diversity among populations in England than those in Estonia. This indicates that UK populations have experienced either (1) less introgression (unlikely, given the formerly extensive distribution of the species); (2) stronger selection pressure, perhaps in response to contrasting climates or modes of pollination (again unlikely, as this would affect only a few genes under the pressure of selection and so should not significantly influence a whole-genome diversity measure such as AFLP); or (3) a longer divergence period. It is obvious that in England, the late-flowering populations are a genetic subset of (and thus presumably derived from) the more widespread early-flowering populations.

Some morphological evidence also supports the polytopic hypothesis. Kümpel & Mrkvicka (1990) pointed out several characters supposedly distinguishing German and Austrian populations of subsp. *ustulata* from subsp. *aestivalis* in addition to contrasts in flowering period, seed set (*aestivalis*

reputedly being more successful), pollinator identity and timing of appearance of the leaves. These morphological characteristics included the colour and posture of basal leaves, the number of stem leaves, stem height, inflorescence length and apical shape, lateral sepal posture and labellum lobe angularity. Researchers studying *N. ustulata* elsewhere in Europe have challenged the diagnostic value of many of these characteristics.

4.8 Habitat differentiation (IV)

In Estonia early-flowering populations exist less frequently and are more geographically localized than late-flowering populations. Present day early-flowering populations in Estonia are all limited to the island of Muhu. They inhabit dry areas with a very shallow soil layer; generally areas that have never been used for anything other than pastures. No clear habitat differentiation is revealed by soil analysis (Table 1 in III). Late flowering populations presented the lowest percentage of organic matter and early flowering ones the highest, since the Muhu sites are much drier and rockier with little or practically no humus between the rocks. The soil layer in late-flowering populations is thicker in Estonia; they often grow on former or actively growing hayfields as well as old fields. I haven't been able to find any late- and early-flowering populations that grow near to each other, although there are some historical records of these from the Loode tammik (oak forest) on the island of Saaremaa.

By contrast, late-flowering populations are less frequent in England, where they have a more restricted distribution. There is no obvious habitat differentiation between English populations (Foley 1992). Moreover, in both Wiltshire to the west and Sussex to the east, some early- and late-flowering populations occur within 500 m of each other. Still, it has been repeated in literature (Foley 1992; Lang 2001) that in England *N. ustulata* is confided to ancient earthwork areas and is therefore influenced by human activity.

In the Czech Republic, late-flowering populations are again more frequent than early-flowering populations, but here a degree of geographical separation and some divergence of habitat preferences have been described (Haraštova *et al.* 2004).

Winfield *et al.* (2003) suggested that *Gentianella anglica* might be an early flowering form of *G. amarella* that has evolved and maintained as a consequence of former grassland management practices. Those two species are thought to be completely sexually isolated because of their distinct flowering times, which do not overlap. However, all populations of *G. anglica* occur nearby or occur simultaneously with populations of *G. amarella*.

In that case PCO Analysis of the AFLP data of *Gentianella anglica, G. amarella and G. uliginosa* clustered in the same group, when analyzed together with two other *Gentianella* species, namely *G. campestris* and *G. germanica*.

The difference in soil thickness gives reason to believe that the differentiation of the two subspecies originates from different management regimes of their habitats in the past. The typical practice of mowing or grazing in July would have removed all forms with intermediate flowering times and therefore driven selection in favour of the two extremes, early and late.

Today grazing areas in Estonia have diminished greatly and the plants have to cope with overshadowing and an old layer of grass rather than early haymaking. The early-flowering plants have withdrawn to the dry alvars on the islands, where the midsummer drought keeps the vegetation lower and makes it easier for the plants to compete.

Perhaps the most significant conclusion made through this study is the inferred multiple divergences of late-flowered lineages at different geographic locations across the geographic distribution of a widespread early-flowered species. This parallels the multiple origins of autogamous *Epipactis* species from within the allogamous *Epipactis helleborine* documented by Squirrell *et al.* (2002) yet was achieved through a shift in phenology rather than a reproductive syndrome. The Fruit-set reputedly differs considerably between early- and late-flowering plants (e.g. Kümpel & Mrkvicka 1990) and between different geographic regions. Long-term, Europe-wide monitoring of the relative reproductive success of these contrasting populations might prove unusually informative, especially given the climate changes that could easily favour one phenological mode over the other in contrasting geographical regions.

5. CONCLUSIONS

There are two distinct varieties of *N. ustulata* (L.) RM Bateman, Pridgeon & MW Chase, that flower at different times. Flowering time in *N. ustulata* is a genetic character that does not depend on the site and year. There may be even more than one late-flowering taxon within *N. ustulata s.l.*

Kümpel & Mrkvicka (1990), while elevating these taxa to subspecies level pointed out several characteristics supposedly distinguishing subsp. *ustulata* from subsp. *aestivalis*. These included the colour and posture of basal leaves, the number of stem leaves, stem height, inflorescence length and apical shape, lateral sepal posture and labellum lobe angularity. Researchers studying *N. ustulata* elsewhere in Europe have challenged the diagnostic value of many of these characteristics. The fluctuations in height among different years are larger than the differences between the populations or subspecies, though average height is greater in late-flowering populations, obviously because the surrounding vegetation is generally higher in July-August. The height of plants is usually a poor characteristic for solving taxonomical problems among closely related taxa.

Orchids have often been considered species with a long life span. *Neotinea ustulata* is a short-lived species with an average half life span of 1.75

Dormancy is the dominating stage in all the populations studied. The existence of dormant plants may easily lead to the underestimation of the actual numbers of this endangered and protected orchid.

Dormancy is often referred to as the “resting” phase of plants, though resting does not seem to be the main aim here, as observations in five populations in Estonia during 1994–1999 did not reveal any relation between heavy fruiting and staying dormant. Dormant plants may act as a reservoir of recruits in a population of otherwise short-lived species. The length of successive flowering and being dormant show very similar patterns — the largest number of plants are dormant or flowering one year at a time. In early-flowering populations vegetative propagation is more common and more new vegetative rosettes appear near old plants.

The chromosome number is the same for early- and late-flowered plants, both of which yielded a count of $2n = 42$. In addition, the different flowering periods and geographical distance have not resulted in any ITS divergence between the analysed individuals of *N. ustulata* in England and Estonia. The divergence among these taxa is most likely a recent occurance.

The trees and ordinations based on AFLP data indicate a lack of long-term genetic discontinuities between any obvious groupings of populations. The majority of variation forms a linear pattern that is largely encapsulated by the first axis on the PCoA plot and reflects a west-east cline of geographical variation.

Variation within populations is considerable, relative to that which exists between populations. These results indicate some level of gene flow among populations, at least until recently. Early flowering is a plesiomorphic characteristic for this species and the late-flowering populations most likely diverged from the early-flowering ones.

The strong geographical signal evident in our AFLP data for both early- and late-flowering plants is more consistent with the hypothesis that the late-flowering plants have diverged several times in history.

The differences in flowering times may have been evolved and maintained as a consequence of human grassland management practices over many centuries.

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KOKKUVÕTE

Tõmmu käpp, *Neotinea ustulata* (L.) Bateman, Pridgeon & Chase (varasema nimetusega *Orchis ustulata* L.), tekitas huvi eelkõige tänu õitseaegade omapärale — populatsioonid, mis morfoloogiliselt väga vähe või üldse mitte erinevad, on suuresti erineva fenoloogiaga. Kirjandusse ilmus sellesisuline tähelepanek möödunud sajandi 80ndate aastate alguses. Täheldati, et osa populatsioone õitseb märksa hiljem kui nominaatliik (Gumprecht 1981). Enne seda peeti tõmmu käppa üheks perekonna *Orchis* vähem varieeruvaks liigiks ja kõiki kirjeldatud kõrvalekaldeid taksonoomiliselt ebaolulisteks (Procházka 1977).

Kümpel kirjeldas 1988 aastal uue varieteedi (*Orchis ustulata* var. *aestivalis* Kümpel), mis hiljem alamliigiks ülendati (*Orchis ustulata* subsp. *aestivalis* (Kümpel) Kümpel & Mrkvicka) (Kümpel & Mrkvicka 1990). Samas ei leidnud mitmed teised autorid (Reineke & Rietdorf 1991, Jensen & Pedersen 1999) neil alamliikidel olulisi morfoloogilisi erinevusi.

Hiljem, uurides molekulaarsete meetoditega alamtriibuse *Orchidinae* fülogeneetilisi seoseid, eraldasid Bateman jt. (1997) tõmmu käpa perekonnast *Orchis* ja liitsid ta perekonnaga *Neotinea.*

Viimastel kümnenditel on *Neotinea ustulata* kadumas või juba hävinud Euroopa paljudest maadest (Davies et al. 1988, Foley 1992, Preston et al. 2002, Kreutz & Dekker 2000). Inglismaal osutus tõmmu käpp üheks kiiremini kaduvaks liigiks, olles hävinud 79% 10 x 10 km ruutudes, kus seda taime varem leida võis (Preston *et al.* 2002). Tšehhis on tema kasvukohad vähenenud piirkonniti 69–90% (Haraštova *et al* 2004).

Viimasel ajal on kirjanduses ilmunud andmeid mitmete erineva õitseaja kuid sarnase morfoloogiaga liikide kohta (Zopfi 1995, Zopfi 1998, Winfield *et al*. 2003). Analoogselt nendele liikidele pakub *N. ustulata* suurepärast võimalust mitte ainult ohustatud liigi bioloogia ja taksonoomia uurimiseks vaid ka evolutsiooniliste protsesside ja liigitekke paremaks mõistmiseks.

Seega keskendusin käesoleva töö tegemisel järgmistele küsimustele:

Kas tõmmu käpa õitseaeg on geneetiliselt fikseerunud tunnus, mis oleks sobilik alamliikide või muude taksonite eristamiseks?

Kas vara- ja hiljaõitsevate taksonite eristamiseks on ka teisi häid tunnuseid?

Kas liik on geneetiliselt mitmekesine ja kas see mõjutab liigi levikut?

Millised evolutsioonilised tegurid võisid põhjustada nende taksonite lahknemise?

Kas hilisema õitseajaga taimed on lahknenud varase õitseajaga populatsioonidest või vastupidi ning kas selline lahknemine on toimunud ajaloos vaid korra või erinevatel maadel ja eri aegadel korduvalt?

Vaatlesin oma töös ka tõmmu käpa kui liigi elutsüklilised parameetreid. Kui liik tõepoolest koosneb kahest alamliigist, on mõlema edaspidise vähenemise

vältimiseks vajalikud nende leviku ja kasvukohanõudluste laialdasemad uuringud.

Ligi kümneaastase uurimistöö käigus märgistatud taimedega selgus, et rohkem kui kuuluvus vara- või hiljaõitsevate taimede hulka, mõjutas taimede mõõtmeid kasvuaasta.

Olulisi erinevusi vara- ja hiljaõitsevate taimede populatsioonide struktuuris antud materjali põhjal ei leitud. Generatiivsete, vegetatiivsete ja soikeseisundis olevate taimede vaheliste üleminekute sagedusi võrreldes ilmnes erinevusi vegetatiivsete taimede üleminekul teise kasvufaasi. Varase õitseajaga populatsioonides jäid enamus vegetatiivseid taimi ka järgmisel aastal vegetatiivseteks, hilise õitseajaga populatsioonides on vegetatiivsed taimed järgmisel aastal suurema tõenäosusega soikesesundis või surnud.

Kuigi orhideesid on sageli peetud suhteliselt pikaealisteks taimedeks, on *Neotinea ustulata* taimede keskmine eluiga alla 2 aasta. Üksikud hea asukohaga taimed võivad siiski aastaid kasvada ja õitseda, eesti populatsioonides on seni teadaolev pikaealiseim taim 7 aastane.

Soikeseisund on kõigis populatsioonides valdav staadium. Seda seisundit peetakse sageli taimede puhkeajaks, kuid minu vaatluse andmetel ei selgunud mingit seost rikkaliku õitsemise ja viljumise ning soikesesundisse jäämise vahel, seega võib see faas pigem kujutada endast taimede reservi muidu lühiealises populatsioonis.

Levinuim soikeseisundi pikkus on üks aasta. Üksikud taimed suudavad vahepeal leherosetti moodustamata puhata ka neli-viis aastat.

Nii vara- kui hiljaõitsevatel taimedel on sama kromosoomiarv ja identne nrDNA ITS järjestus. AFLP andmestikul põhinevad ordinatsioonipuud viitavad pikaajalise isolatsiooni puudumisele vara- ja hiljaõitsevate taimede vahel. Populatsioonidesisene varieeruvus on suhteliselt suur võrreldes populatsioonidevahelise varieeruvusega. Nende taksonite lahknemine on tõenäoliselt toimunud võrdlemisi hiljuti ning tugev geograafiline mõju AFLP andmestikus nii vara- kui hiljaõitsevate taimede puhul viitab sellele, et lahknemine on toimunud erinevates maades ja eri aegadel korduvalt.

Kuna varasuvine õitsemine on selle liigi puhul plesiomorfne (algsem) tunnus, siis on hilise õitseajaga populatsioonide tekkimine varaõitsevatest tõenäolisem kui vastupidine variant.

Erinevused õitseaegades tõmmul käpal võivad olla pikaajalise rohumaade majandamise tagajärg.

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Highly variable flowering time in *Orchis ustulata*: consequences for population dynamics

Kadri Tali and Tiiu Kull

Two subspecies of *Orchis ustulata* differ considerably in their flowering time, but in only a few morphometric parameters; this makes the rank of these taxa problematic. The flowering time was not affected by replanting individuals to another habitat, including to the habitat of the other subspecies. In this study, populations from both subspecies were studied, measuring 464 marked genets during 5-6 years. Populations with different flowering times exhibited notable differences in their local distribution areas and the mean height of specimens. In the late-flowering populations the proportion of dormant plants is higher and the proportion of vegetatively propagated shoots lower than in the early-flowering ones. Going to (or staying) dormant is the biggest possibility in all stage groups. Flowering is more likely to be followed by dormancy than vegetative stage, but setting fruit does not affect the possibility. Vegetative propagation may play an important role in keeping the populations viable. Vegetative growth is more pronounced on stony soils.

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Introduction

Kümpel (1988) described a late-flowering variety, var. *aestivalis*, in *Orchis ustulata* L., with holotype from Nordhausen (leg. Vocke 22.7.1879). As a result of further findings, Kümpel & Mrkvicka (1990) changed the rank of this taxon and distinguished between two subspecies of *O. ustulata* — ssp. *ustulata* L. and ssp. *aestivalis* (Kümpel) Kümpel et Mrkvicka, demonstrating the remarkably different flowering times and small differences in plant height and the conformation of the lateral sepals. Namely, the tips of the lateral sepals are usually recurved in ssp. *aestivalis*. Van der Cingel (1995) also mentioned the more rounded ends of the lobes of the labellum for early-flowering plants. Further, Foley (1992) described a late-flowering colony in N. Wilts (S-England) where the plants are somewhat smaller than those of the "normal" variant found nearby. Nevertheless, these morphometrical differences have been found to be minimal and arguable (Reineke & Rietdorf 1991; Tali 1994; Jensen & Pedersen 1999; Lang 2001). Several authors (Foley 1990; Jenkinson 1995) have emphasized the need to resolve the question of these extraordinary taxa.

Orchis ustulata is a declining species throughout its total range (Davies *et al.* 1983; Foley 1992) and is an endangered and protected species in Estonia. However, there are several vital populations of *O. ustulata* in Estonia, which provide a good opportunity to measure demographic and other quantitative parameters on the population level. After 1950 plants of *O. ustulata* have been found in 31 localities in Estonia. Since the beginning of the 19th century, 81 sites have been recorded in total (Kuusk 1996).

In Estonia, the early populations flower from the end of May till the end of June, whereas in the late populations, flowering begins not earlier than at the beginning of July and lasts till the end of August (Tali 1994). Depending on the geographical location, the flowering time differs in different parts of Europe, but is still not later than August for ssp. *aestivalis* and the end of July for ssp. *ustulata* (in Switzerland 2000 m above the sea level) (Reineke & Rietdorf 1987). Vegetative phenology of the subspecies also differs. Most of the new rosettes of subsp. *ustulata* usually emerge in September, and are wintergreen. The plants of the subspecies *aestivalis* emerge much later – at the

end of October and in November, or also from March to July. After flowering, the plants rest below-ground for a couple of months. (Tali 1996).

O. ustulata has Eurosiberian distribution area, Estonia being close to its Northern limit. The species is frequent in some European mountain ranges, but is rare elsewhere and very rare in the Mediterranean. Subspecies *ustulata* seems to occur throughout the total range of the species. Subsp. *aestivalis* has been recorded from (at least): England, Germany, Estonia, France, Switzerland, Italy, Austria, Czech Republic, Slovakia, Rumania, Bulgaria and Denmark (Reineke & Rietdorf 1987; Kümpel & Mrkvicka 1990; Foley 1994; Jensen & Pedersen 1999; Lang 2001). However, in Estonia the subspecies have (at least contemporarily) quite a well distinguished distribution (Tali 1994). The late-flowering populations can be found on the mainland, on Hiiumaa Island, and less frequently on the southwestern coast of Saaremaa Island. The early-flowering populations of *O. ustulata* presently grow only on Muhu and Saaremaa islands (though there are some earlier records from the mainland, too). One can also observe a difference in population size and density. The monitored early-flowering populations occupy larger territories and are more numerous in specimens than the late-flowering ones. No distinct differences in habitat preference have been found between the early-flowering and late-flowering plants.

Phenological isolation is unusual in the case of plant subspecies that occupy proximate territories and are morphologically alike. Therefore, it is interesting to find out if the difference in the life cycle phenology is correlated with any population-level characteristics, like the proportions between the flowering, vegetative, and dormant specimens, or the frequency of fruiting.

Dormancy (an ability of the plant to spend one to several years below-ground without any aboveground organs) is a well-known phenomenon in terrestrial orchids (Wells 1967; Tamm 1972; Hutchings 1987a,b; Mehrhoff 1989; Wells & Cox 1991; Willems & Bik 1991; Kull & Tuulik 1994). The existence of dormant specimens may easily lead to underestimation of the size of the population. For instance, Foley (1987, 1990) studied and documented the decreasing

populations of *Orchis ustulata* in England in the years 1982–1986, and he noted that even in favourable years more than 50% of the plants were in the dormant stage. Therefore it is very important to follow individual plants and not just count shoots in a population (Hutchings 1990; Tamm 1991).

In this paper we analyse whether differences in the flowering time of *Orchis ustulata* are correlated with the demographic parameters of populations, with an emphasis on differences in the frequency of dormancy and on fruit set.

Material and methods

The material for the present study was collected mostly from five populations in Estonia (Table 1), of which the Aljava, Lõetsa and Kapi populations (all on Muhu Island) flower in May-June, and the Sillukse and Jäneda populations (on the mainland) flower in July-August.

In ten permanent 1x1 m^2 plots in each population all vegetative, generative and juvenile plants were marked (with a numbered stick) and mapped. Number of leaves, height of a flowering plant, length of an inflorescence were counted every year on each plant during the peak flowering period of the population, and number of fruits about a month later. The number of monitored genets varied from 63 to 108 in different populations (Table 1).

As the plants which still possess aerial parts during the flowering season may only be a small proportion of those present at the start of the vegetative period (Hutchings 1991), plots were visited at least three times during the vegetative period. This is especially important in late-flowering populations where practically all non-flowering plants have disappeared by the time the others flower.

In all populations the plants were divided into three classes – dormant, vegetative and flowering plants (Table 2). Three height classes were used to compare the influence of fruiting

(Table 5). As plant heights differ among populations and among years relative heights were used. For the small plants $H \leq 0.80$ of the population average, medium sized plants $0.8 < H \leq 1.2$ and big plants $H > 1.2$.

On the mapped plots, a marked plant was considered dormant for one year before the plant's first appearance, and for up to three years after it's last appearance. A plant was considered dead if it had not appeared aboveground for the last three years. Clusters of plants with a diameter of 5 cm or less were considered to be genets. This has been confirmed in a few cases when plants were dug up for replanting. It is difficult to distinguish between juvenile and weaker vegetative plants, therefore, all non-flowering rosettes counted were included in the vegetative group.

SAS mixed procedure was used to analyse the differences in population dynamics between the subspecies and the populations. Annual status class percentages (flowering, vegetative, dormant) were used as characters, subspecies as a fixed factor, and site as a random factor. Site significance was tested by Wald's Z-test, and subspecies significance by the likelihood-ratio F-test.

To check if the locality influences the flowering time, four plants were taken from the late-flowering Sillukse population and four plants from the early-flowering Aljava population, on June 10, 1995. These were planted in a common garden on Muhu Island, i.e. in a location where only the early-flowering plants have been found (about 1 km from the nearest local population). The phenology of these plants was monitored every year. Another replantation had to be carried out because part of the Sillukse site was going to be destroyed for the creation of a quarry. Sixty plants were dug out and planted near Pivarootsi village, 10 km away. The fate of these plants was also constantly followed.

Results

Morphometry of vegetative characteristics

The average heights of the plants are given in Table 3. The height of the individual plants varied from 6 to 44 cm. Late-flowering plants were, on average, (over all years) 3.3 cm higher than early-flowering plants. However, when the two phenologically different populations which are most similar in their habitat (Lõetsa and Sillukse) are compared on a year-to-year basis, then the difference in shoot height is remarkable — 10.3 cm (the means over all years being 16.6 cm in Lõetsa and 26.9 cm in Sillukse). The largest difference in height recorded in one and the same plant in different years was 20 cm.

The number of vegetatively propagated shoots is different in different populations (Table 4). The number of shoots in genets is higher in early-flowering populations that grow on gravel soils (Kapi, Lõetsa). In Jäneda, all plants had only one shoot.

Fruiting

Late flowering populations exhibit a greater percentage of fruiting plants compared to the early flowering ones. The T-test showed that this difference is significant (p=0,009). The mean percentage of fruiting specimens over 6-7 years and all plants in the early-flowering populations was 19.7, and in the late-flowering populations 37.1.

Though most of the fruiting plants bore one or two fruits, there were also a few plants with 10-20 fruits (with a maximum of 21 in the Lõetsa population on a single medium-height plant). Almost half of the fruiting plants flowered again in the following year, and most of those that did, remained in the same size class as they were in the previous year (Table 5).

Replanting experiment

The flowering time of the late-flowering subspecies remained unchanged (late July-August) after the transfer to the common garden. The flowering periods of the replanted specimens from the late-flowering population were 20.7-18.8 in 1996, 15.7-3.8 in 1997, and 20.7-15.8 in 1998. Four plants originally from Sillukse (one with an additional new vegetative ramet) have come up every year, but the early-flowering plants brought from the Aljava population probably did not survive the planting.

At least 34 plants, of 60 flowering ones from Sillukse quarry area, planted on a seashore meadow in 1996, were alive 4 years after replanting and 1 new, vegetatively propagated rosette appeared. 10 plants flowered and 9 were vegetative in 1997, and 11 flowered and 12 remained vegetative in 1998. In 1999, 14 plants emerged and 5 plants had flower buds, but as it was a very dry and hot summer (mean twenty-four-hours temperature in June was 17.2^0C while mean over seven years was 14.8^0C) none of the plants flowered. The flowering time did not change.

Dynamics of the life-cycle and transitions between the classes

The ratios of flowering, vegetative and dormant plants have been different through the years in all the populations (Table 2). The dormant stage dominates in all the populations. Due to the method of calculating the number of dormant plants, it may be slightly underestimated in the first two years of the study.

The mean percentage of dormant plants in both the late-flowering populations is higher than in any of the studied early-flowering populations. However, the SAS mixed procedure analysis showed no significance among differences in stage class distribution between populations and between late and early-flowering subspecies, if subspecies was considered a fixed factor and site a random factor.

Table 6 presents the ratio of plants that have been dormant for one, two or three consecutive years during the 6 years of monitoring. One and the same plant may occur on two rows of the table, if it had, for example, a one-year long and a two-year-long period of dormancy during these six years. The largest number of plants in all the populations has been dormant for one year (except at Kapi where the number of 3 years of dormancy is bigger and at Sillukse where more plants have rested for two years), a smaller number for two years, and only 5.3 % of all the monitored plants for three consecutive years. At Jäneda, the number of emerged plants has declined drastically and only two plants have emerged after two years of dormancy. In most populations (with the exception of Kapi) the most dormant plants were flowering when they reappeared. The Kapi population is exceptional also for its large percentage of 3-years dormant plants. Often vegetative plants that appear after some years of dormancy are small two-leaved rosettes. There are very few plants (2-5.4%) that have been above ground for six or seven consecutive years, and these exist in early-flowering populations only.

In the studied populations, all possible transitions occur between dormant, vegetative, and generative stages (Table 7).

The frequencies of transitions between the three stages are presented in Fig. 1. In every stage the plants going into dormancy form the largest proportion in all the populations. In the early-flowering populations the ratio of vegetative plants is slightly higher and they behave differently from those of the late-flowering populations. Most of the vegetative plants in late-flowering populations go into dormancy and a few set flower in the following season; in early-flowering populations more vegetative plants remain vegetative, and in the next season the number of V-V transitions is also relatively larger.

As seen from Table 5, transitions to dormancy were slightly less common among the fruiting plants (50.9%) than among the non-fruiting ones (63.9%).

Discussion

Phenology and behaviour of the populations

In all the populations the plants have been tallest in 1995-96. During 1997-98 the height growth of the plants was inhibited, and, in most populations, plants have grown taller again in 1999. Though one could expect the late-flowering plants of *Orchis ustulata* to grow higher, together with the rest of the vegetation, the average height is definitely bigger only in Sillukse; in Jäneda the average height of the plants is on about the same level as in Kapi and Aljava. The fluctuations in height among different years are larger than the differences between the populations or subspecies, though average heights are greater in late-flowering populations obviously because the surrounding vegetation is generally higher in July-August. The height of plants is usually a poor character for solving taxonomical problems among closely related taxa.

The years have been quite different in different populations – in 1993, in the Lõetsa population, very few plants flowered; in the Sillukse population, at the same time, flowering was at an average level. Still, the average trend of the heights of the plants follows the same patterns in the Sillukse and Lõetsa populations throughout the years.

In 1995 there were a surprisingly large number of flowering shoots in the Aljava population. Compared to the first year in all populations, the number of flowering shoots had increased in all populations except in Jäneda. In 1995 the beginning of the summer was very wet and warm and the weather conditions in 1996 also favoured flowering in almost all populations. This may cause a rise in the number of dormant plants in the following years. In Jäneda there were no plants above ground in 1998, when July and August were unusually rainy and relatively cold there (14.4^0C compared to the average of 16^0C, and 176 mm compared to the average of 87 mm). The summer of 1999 was exceptionally dry (28.3 mm compared to the normal 49.9 mm) and flowering was inhibited in most populations.

Little is known of the pollinators of *Orchis ustulata*, different authors have named different kinds of insects (Reineke pers. comm; van der Pjil & Dodson 1969); Vöth (1984) documented a tachinid fly as a pollinator for *O. ustulata* ssp. *ustulata.* A beetle *Leptura livida* is mentioned as a pollinator for *O. ustulata* ssp. *aestivalis* (Mrkvicka 1991; van der Cingel 1995). The lack of pollinators is considered to be the limiting factor of fruitset. Compared to the fruit set in England's populations of *Orchis ustulata* (J. Foley pers. comm.) the fruit set is much higher in Estonia.

Reineke & Rietdorf (1987, 1991) claim that in all *O. ustulata* populations rosettes rise constantly from autumn to spring (also under the snow), and that both autumn risen and spring risen plants can flower. Plants of the late subspecies that flowers till autumn and therefore does not have summer rest begin to form rosettes in March-April (Kümpel & Mrkvicka 1990). In Estonia the early-

flowering plants begin to emerge in early September, but in dry autumns there may be a delay. In the late-flowering populations some plants emerge in late October, but most of them in spring.

Rasmussen (1995) reported that after some plants from wintergreen populations of *Orchis morio* in Öland and Denmark were moved to the Botanical Gardens in Copenhagen the plants from Öland sprouted in autumn, whereas the plants from Denmark did not unfold until spring. In Estonia, two series of replantings were done and these plants did not exhibit any changes in their phenology as a result. Plants from Sillukse survived better when planted into the common garden on Muhu Island where originally only the early-flowering subspecies was found. The early-flowering plants from nearby did not survive, maybe because they were already flowering when planted, whereas the plants from Sillukse were only showing flower-buds. In the case of rescuing 60 plants from Sillukse quarry area, the new site was probably not the best choice for these plants, as the late-flowering plants tend to grow on deeper soils and in more shady conditions. However, this new site is rather well suited for early-flowering plants. Still, half the plants are alive after four years and their phenology has been very similar to that of the mother population. Difference in flowering time seems to be a genetic character that does not depend on site and year.

The difference in soil thickness may also give reason to believe that the differentiation of the two subspecies originates from different management regimes of their growing areas in the past. While the early flowering plants at least in Estonia inhabit areas that have never been used for anything else than pastures, the late flowering populations often grow on former or present day hayfields. In late summer of 2001 in Hiiumaa even on regularly mown lawn several very tall specimens sprouted.

Dynamics of the life-cycle and transitions between the classes

Gillman et al. (1993) have shown that the number of flowering plants fluctuates less than other stages in *Cirsium vulgare*. This is definitely not the case with *O. ustulata*. Here, all stages vary irregularly over years as well as over the populations. Dormancy is the dominating stage in all the populations. If we do not count the first monitoring year, only in Aljava population there have been two years when the number of flowering plants exceeded the number of dormant plants. In both the aboveground stages the transitions to dormancy far exceed 50%, with the exception of Kapi where only 45% of flowering and 50% of vegetative plants stay dormant the next year.

Long dormancies seem to be more common in late-flowering populations, although this phenomenon is not statistically significant from the present data set. The fact that in the late-flowering populations most vegetative plants become dormant, while in the early-flowering populations a large part of vegetative plants remain vegetative, is probably caused by the fact that in early-flowering populations vegetative propagation is more common and more new vegetative rosettes appear near old plants. In Jäneda no such clusters were found and these were not numerous in Sillukse either. Among early-flowering populations, vegetative propagation is least pronounced in Aljava, where the composition of soil is somewhat similar to that of Jäneda.

The finding that the number of flowering plants is the most stable in Kapi might be explained in a similar way: if there is a very dense cluster of vegetative rosettes with one flowering shoot every year, it may be recorded as stable flowering/being vegetative in the database, while, in fact, in consecutive years, different vegetative rosettes may be flowering. This gives us reason to believe that, though the young pines at the Kapi site are growing rapidly and after some years this forest may be too dense for *Orchis ustulata* to survive, the present population in Kapi is younger and more viable than the other four.

Calvo (1993) has shown that one year after high pollination treatment the treated plants of the orchid *Tolumnia variegata* were significantly smaller and produced fewer flowers. Snow & Whigham (1989) pointed out that the plants of *Tipularia discolor* with many fruits were less

likely to flower in the following year, probably because they were smaller than those with few fruits. Surprisingly, we found the opposite pattern for *Orchis ustulata* (Table 5).

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Dynamics of *Orchis ustulata* populations in Estonia

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Abstract
Six hundred *Orchis ustulata* L. plants have been monitored in Estonia since 1993. The plants are distributed in two late-flowering and three early-flowering populations. Late-flowering plants are in the subspecies *aestivalis* and early-flowering plants are in the subspecies *ustulata.* Dormancy was common in all populations and over 70% of the plants that survived for longer than 1-2 years did not appear aboveground in one or more seasons of their lifetime. In the late-flowering populations the proportion of dormant plants was higher and the proportion of vegetative plants lower than in the early-flowering populations. All possible transitions between dormant, generative and vegetative stages were expressed in all populations. This study demonstrates that *O. ustulata* is a short-lived species.

Key words: dormancy, half-life, late- and early-flowering subspecies, life history traits, successive flowering.

Introduction

Information on life-history patterns of terrestrial orchids is incomplete as there has been insufficient number of long-term studies following individual plants. Understanding flux, particularly in populations of rare and endangered species, is an essential requirement before sensible decisions can be taken concerning site management (Hutchings 1990). While population characteristics of *Orchis morio* L., *Orchis mascula* (L.) L. and *Orchis militaris* L. are fairly well documented (Vanhecke 1994) there is no observation series published for *Orchis ustulata* L., which is a declining species throughout the whole world (Davies *et al.* 1983, Foley 1992) and is an endangered and protected species in Estonia. Several populations of *O. ustulata* occur in Estonia, thus providing an opportunity to measure demographic and other quantitative parameters at the population level. This study was conducted to gain information about life history and population parameters of two subspecies of *O. ustulata* that occupy distinct habitats.

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Material and methods

The species

Orchis ustulata is a tuberous orchid. When flowering, its stem is 5-50 cm high, usually slender. Two to six unspotted leaves with a length of 2-10 cm and width of 0.5-2 cm form a bluish green wintergreen rosette. Flowers produce no nectar. Kümpel and Mrkvicka (1990) have described two subspecies of *O. ustulata*. *Orchis ustulata* ssp. *aestivalis* (KÜMPEL & MRKVICKA) flowers about 1-2 months later than *O. ustulata* ssp. *ustulata* (KÜMPEL & MRKVICKA). In Estonia the early flowering subspecies is in anthesis from May till June and the late flowering subspecies from July till August, or September in some years. Flower fragrance also differs between the subspecies: a weak citron smell in the ssp. *aestivalis* and a strong honey fragrance in ssp. *ustulata*.

In Estonia the subspecies also have different distributions. *O. ustulata* ssp. *ustulata* only occurs on Saaremaa and Muhu islands, and the late flowering subspecies occurs on Hiiumaa Island, in the western part of Saaremaa Island and some scattered localities are on the mainland. The early flowering subspecies tend to grow on thinner and drier soils, sometimes even in limestone cracks, while the late flowering subspecies prefers thick soils in areas dominated by *Pinus sylvestris* L. (Tali 1996).

Study site

The present study included five populations in Estonia. The Aljava, Lõetsa and Kapi populations (all on Muhu Island) contained only early flowering plants and the Sillukse and Jäneda populations (on the mainland) only late flowering populations.

In each population, 10 permanent 1×1 m plots were established to map all vegetative and generative plants. Height of each inflorescence and the number of leaves and fruits have been determined every year from 1994-2000. The number of monitored genets per population varied from 63 to 108.

For purposes of analysis, all the plants were considered to be dormant, vegetative or flowering. It is practically impossible to distinguish between juvenile and weaker vegetative plants; therefore, all non-flowering rosettes were included in the vegetative group. Half-lives were calculated from the survivorship curves of *O. ustulata* independently in each population. FoxPro version 2.6 was used to manage the life-history data of every specimen and calculate transition matrices. For statistical analysis SAS mixed procedure analysis was applied (SAS Institute Inc., SAS/STAT Users Guide, Version 6, Fourth Edition, Volume 2, 1996).

Results

The first rosettes of the early flowering plants appeared in September-October and those of the late flowering subspecies in October-November. In all populations number of plants emerged in spring and no significant relation between the time of appearance and the behaviour of the plant (flowering or remaining vegetative) could be detected.

In all populations, the number of plants was greatest at the beginning of the study and the number of plants that appeared aboveground, while variable from year to year, declined in all populations over the course of the study (Fig. 1). Flowering was variable from year to year but flowering percentages were highest between 1994 and 1996, with the exception of a high flowering percentage in 1997 in the Jäneda population (Fig. 2). In 1995 there were a relatively large number of flowering shoots in the Aljava population. In Jäneda there were no plants above ground in 1998, when July and August were unusually rainy and relatively cold (14.4ºC compared to the average of 16ºC, and 176 mm compared to the average of 87 mm). The summer of 1999 was exceptionally dry (28.3 mm compared to the normal 49.9 mm) and flowering was inhibited in most populations (Fig. 2).

The height of the flowering plants varied from 5 cm to 37 cm for the early flowering plants and from 10 cm (with one exception of a 3 cm flowering plant) to 50 cm for the late flowering plants. Inflorescence height varied considerably throughout the years. In general, tall spikes were associated with wet (and warm) springs (Table 1). Fluctuations in heights in different years were larger than the differences between the populations or subspecies (Table 2).

The longevity of individual plants was, in general, low (Fig. 3). Half-lives of different cohorts of plants in populations vary from 0.9 to 3.2 years but some plants survive much longer. Six of the 40 plants marked in 1993 in the late flowering

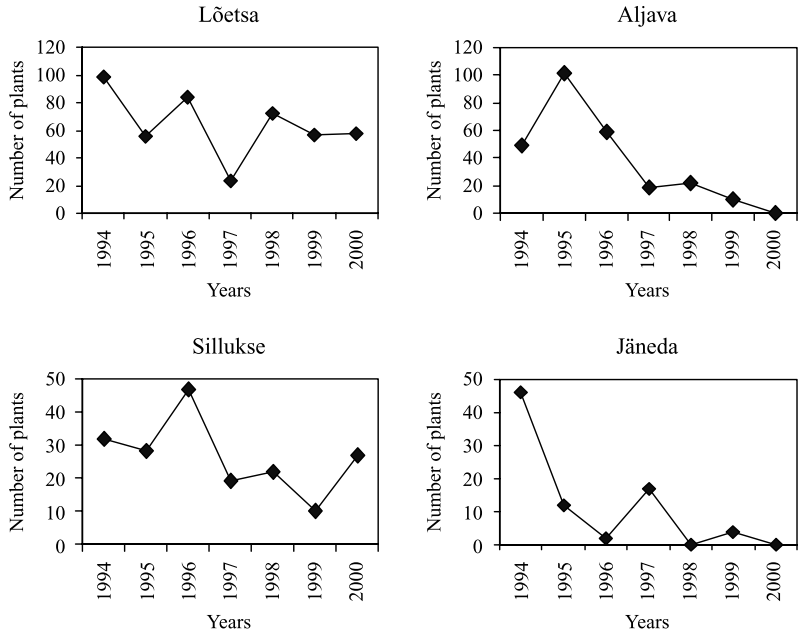

Figure 1. Number of plants of *Orchis ustulata* on plots in early flowering (Lõetsa, Aljava) and late flowering populations (Sillukse, Jäneda).

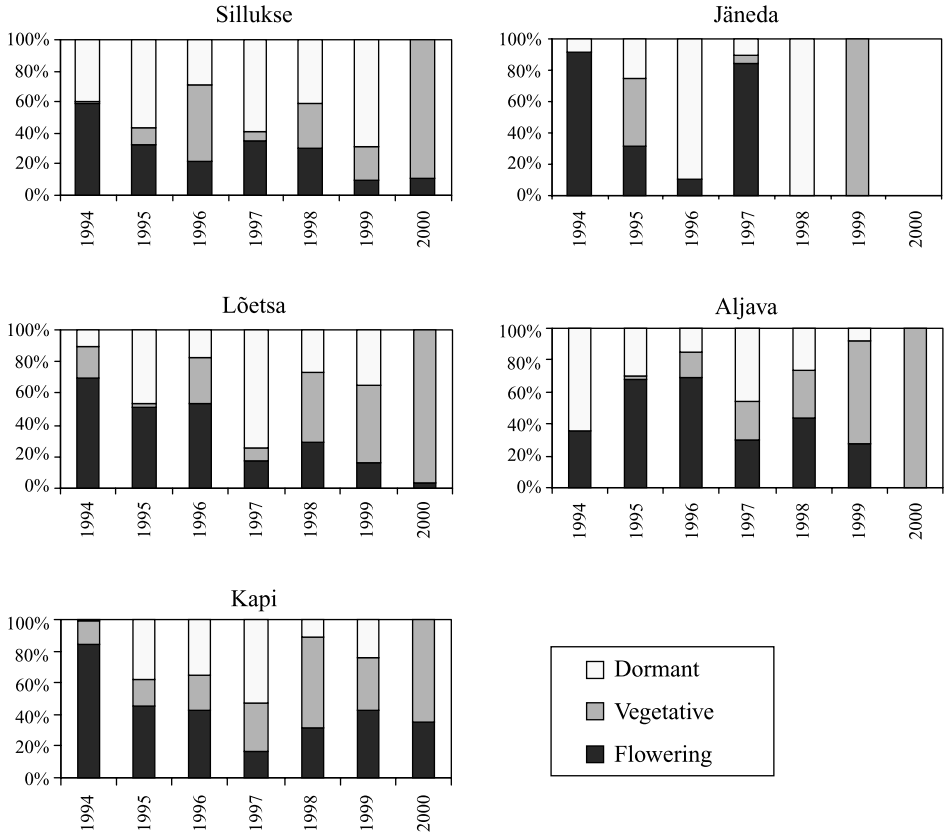

Figure 2. The percentages of dormant, vegetative and flowering plants of *Orchis ustulata* in five populations.

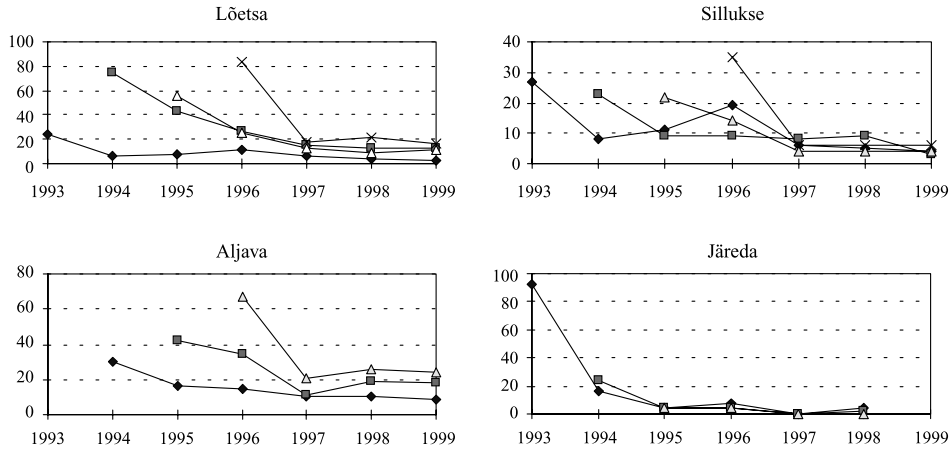

Figure 3. The longevity of different cohorts in four populations of *Orchis ustulata*.

Table 1. Temperature (C) and precipitation (mm) data from Virtsu (the closest weather station to all the early-flowering and Sillukse late-flowering populations) and Maarja (the closest weather station to Jäneda population). Source of data: Estonian Meteorological and Hydrological Institute.

	Virtsu				**Maarja**			
	Mean t (ºC) in winter (Dec-March)	Mean t (ºC) in the vegetation period	Mean precipitation in winter	Mean precipitation in the veg. period	Mean t (ºC) in winter (Dec-March)	Mean t (ºC) in the vegegation period	Mean precipitation in winter	Mean precipitation in the veg. period
1993	-0.30	12.62	45.20	48.70	-2.20	11.64	45.73	79.68
1994	-4.30	12.66	49.35	38.20	-5.78	12.30	41.40	57.76
1995	0.20	12.98	56.53	59.60	-2.00	12.10	44.58	71.42
1996	-5.80	11.68	22.23	38.28	-8.00	11.50	18.80	54.08
1997	-1.60	13.22	34.65	53.24	-3.90	12.22	42.60	46.42
1998	-1.30	12.20	40.00	56.94	-4.15	11.58	32.33	115.38
1999	-2.25	13.44	49.18	35.80	-4.35	13.10	46.18	39.00

Table 2. Heights of flowering plants in five populations (cm).

		1993	**1994**	**1995**	**1996**	**1997**	**1998**	**1999**	**2000**
Sillukse	average	25.51	26.37	33.90	27.69	24.71	25.00		21.33
	min	13.00	14.50	18.00	15.00	18.00	16.00		16.00
	max	36.00	40.00	50.00	45.00	34.00	35.00		29.00
	std	5.55	6.73	7.97	8.69	5.46	6.38		6.81
Lõetsa	average	13.23	16.76	20.28	18.08	14.19	15.62	13.64	11.00
	min	6.00	8.00	8.00	10.00	5.00	8.00	7.00	10.00
	max	23.00	34.00	41.00	32.00	25.00	27.00	20.00	12.00
	std	4.63	4.62	6.79	4.72	6.45	5.18	4.03	1.41
Jäneda	average		21.71	21.00	24.50	20.81			
	min		10.00	17.00	24.00	12.00			
	max		44.00	28.00	25.00	30.00			
	std		5.93	4.36	0.71	5.18			
Kapi	average		21.61	24.61	22.95		16.95	18.52	15.29
	min		13.00	9.00	8.00		5.00	9.00	11.00
	max		33.50	37.00	30.00		24.00	30.00	29.00
	std		4.01	6.51	4.03		4.40	4.69	6.65
Aljava	average		16.98	21.23	24.80	17.41	13.00	18.22	
	min		9.00	9.00	14.00	9.00	6.00	13.00	
	max		24.00	38.00	37.00	24.00	23.00	22.00	
	std		3.56	5.68	4.52	4.77	4.99	3.60	

Sillukse population and three of the 24 plants in the early flowering Lõetsa population were still present in 2000.

Plants that emerged, flowered only once and then disappeared had a large influence on the half-life calculations of a population. In 1995, 64 new plants emerged in the Aljava population and none of them ever appeared again. Only 2 plants of 13 plants that appeared in the Jäneda population in 1997, ever emerged again. Survivors again showed a range of behaviours: the length of dormancy and the length of flowering varied a great deal from being vegetative all their life to flowering all their life. More than 70% of the survivors, i.e. the plants that lived longer than 1-2 years exhibited dormancy during their lifetime.

Figure 4 presents the maximum number of successive flowering events exhibited by the monitored plants. The number of plants that never flowered varied among populations but in every population most plants flowered once during their lifetime. Dormancy, the observation that plants spend one to several years below-ground without the appearance of any aboveground organs, is a well-known phenomenon in terrestrial orchids (Tamm 1972, Hutchings 1987b, Wells and Cox 1991, Willems and Bik 1991) and is well expressed in *O. ustulata.* During 8 years only 6 of 600 plants did not experience dormancy and all these were in the early flowering populations**.**

The largest number of plants in most populations has been dormant for one year, a smaller number for two years, and only less than 10% of the plants for three or four consecutive years (Fig. 5). It is possible that the four plants recorded as dormant for 5 and 6 years may have been vegetative in spring of 1997; the census was too late that year and some vegetative plants may have passed unnoticed as they tend to wither before full flowering of the rest of population. For Figure 5, data were only compiled for plants that had been established for longer than 3 years were considered.

The percentage of flowering, vegetative, and dormant plants differ among populations during the observation period. Due to the method of calculating, the number of dormant plants is underestimated in the first two years of the study. Still the dormant stage dominates in all the populations (Fig. 2).

Among late flowering plants the proportion of dormant individuals was slightly larger than among the early flowering ones**.** The mean percentage of dormant plants in both the late-flowering populations was higher than in any of the early-flowering populations. However, the SAS mixed procedure analysis showed no significance among differences in stage class distribution between populations and between late and early-flowering subspecies, if subspecies was considered a fixed factor and site a random factor. Among early flowering plants the ratio of vegetative rosettes was higher. Proportion of plants in each life stage that either stayed in the same stage the next year or moved to one of the other two stages are shown in Figure 6. The biggest possibility in all populations is that a plant goes dormant the next year.

Most vegetative plants in late flowering populations are dormant next year while in early flowering populations more vegetative plants remain vegetative. Flowering plants in late flowering populations tend to go dormant while in early flowering populations successive flowering is more common.

In most populations, with the exception of Kapi, the biggest percentage of dormant plants were flowering when they reappeared. Often vegetative plants that appeared after some years of dormancy were small two-leaf-rosettes.

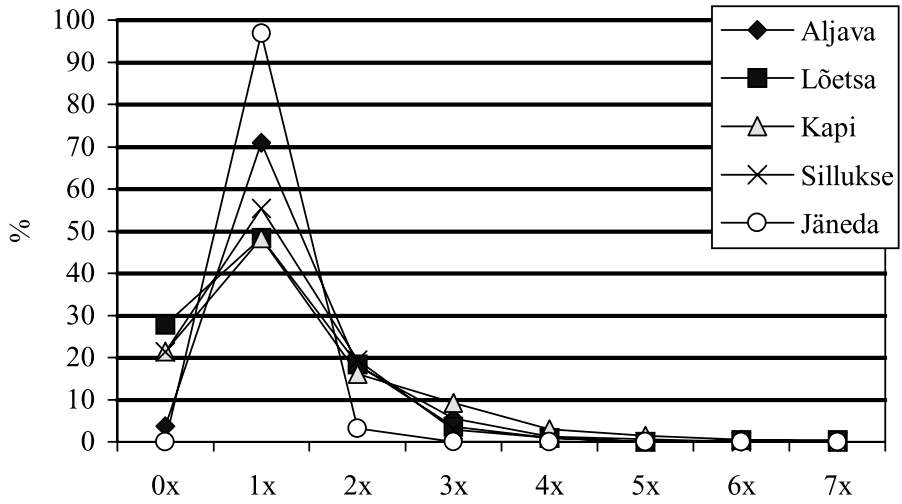

Figure 4. Lengths of successive flowering in five populations of *Orchis ustulata*.

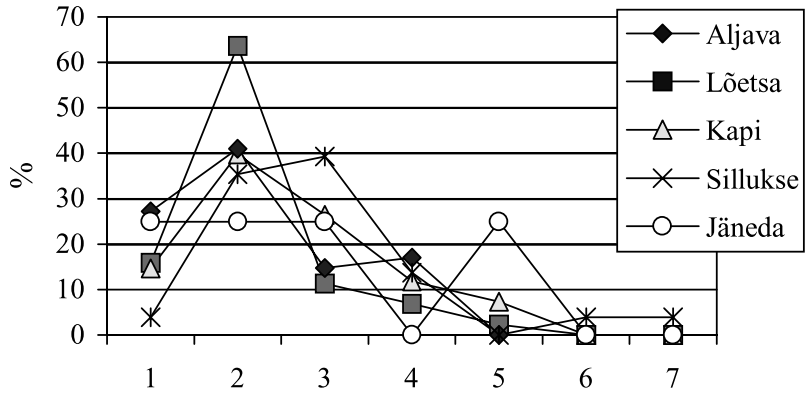

Figure 5. Lengths of successive dormancy in five populations of *Orchis ustulata*.

Abundant flowering and fruiting did not trigger dormancy. Slightly more non-fruiting plants went dormant the next year. Thirty-nine percent of fruiting plants compared to 26% of non-fruiting plants flowered again the next year.

Discussion

Wet and warm springs influence flowering and rosette forming, but not the fate of cohorts. The establishment of new plants was not affected by year or the size of a cohort. *Orchis ustulata* seems to be a short lived species and the half-life calculations

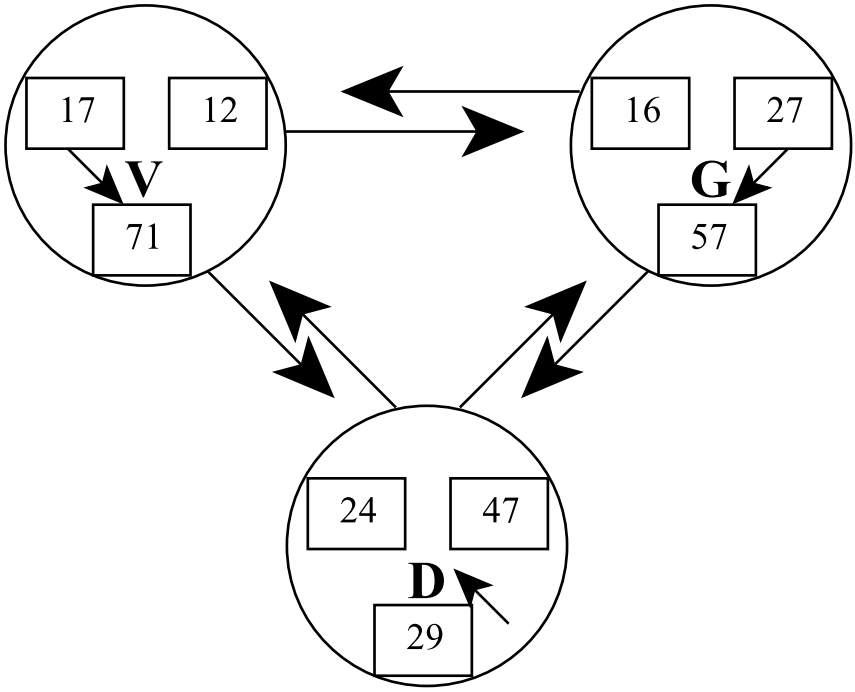

Figure 6. Average yearly transitions between 3 life-stages (V – vegetative, G – generative and D – dormant) in five populations of *Orchis ustulata* over 7 years. The numbers in the boxes indicate the percentage of plants in that life stage that move the next year to the life stage indicated by the arrow.

are in the same range as those reported for *Coeloglossum viride* (L.) Hartman (1.0-2.4) given by Willems and Melser (1998) while they are less than those reported for *O. militaris* (2.19-7.80) (Waite and Farrell 1998).

The existence of dormant plants may easily lead to the underestimation of the size of the population. Foley (1987 1990) studied and documented the decreasing populations of *O. ustulata* in England in the years 1982-1986, and noted that even in favourable years more than 50% of the plants were in the dormant stage. The proportion was not so large in Estonia, but the census time is still too short for precise evaluation of dormancy. When conducting long term censuses, it is necessary to visit plots at least three times during the vegetative period as plants which still possess aerial parts during the flowering season may only be a small proportion of those present at the start of the vegetative period (Hutchings 1991). This is especially important in late-flowering populations of *O. ustulata* where practically all non-flowering plants have disappeared by the time the others flower.

Waite and Farrell (1998) reported that in *O. militaris* the dormant part of a population was 14% of all the plants present and the length of apparent dormancy period was 1-8 years. This is longer than proposed for *O. ustulata* by several authors (Foley 1992, Davies *et al.* 1983) and also observed during this study. Shefferson *et*

al. (2001) point out, that the maximum length of dormancy in temperate orchids appear to be no more than five years.

The length of successive flowering and being dormant show very similar patterns – the largest number of plants being dormant or flowering one year successively. No plants flowered for longer than seven years in a row. Wells *et al.* (1998) state that *O. morio,* the closest relative species to *O. ustulata* according to Schlegel (1989), can flower successively for 17 years. The flowering tends to be irregular in many terrestrial orchids; the number of times that individuals have flowered and the number of years between flowering events vary considerably (Farrell 1985, Hutchings 1987a, Inghe and Tamm 1988, Whigham and O'Neill 1991).

According to the observations in these five populations going dormant is not related to cost of fruiting (Tali and Kull 2002). Dormancy is often referred to as "resting" phase of plants, though resting seems not to be the main aim here. Dormancy may act as a reservoir of recruits in a population of otherwise short-lived species.

Calvo (1993), Snow and Whigham (1989) and several other authors have claimed that plants that flower and set fruit abundantly are least likely to flower and were as a rule also smaller than the plants that did not set fruit. In *O. ustulata* this feature was not found.

Our data clearly demonstrate that proportions of life-history stages are slightly different in the subspecies. While the proximate causes require further investigation, we suggest that the differences are probably due to the differences in soil and light conditions in their respective habitats.

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Orchis ustulata L.

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Section *Orchis.* A native perennial herb with 1–2(–3) ovoid, subglobose or ellipsoid tubers positioned 3–6 cm underground; 5–10 unbranched fleshy roots of diameter 0.8–2 mm. Stem (5–)10–20(–50) cm, erect, usually slender, with basal sheaths. Leaves two to six, unspotted, oblong-acuminate to broadly lanceolate (rarely linear), 2–10(–15) cm × 0.5–2(–3) cm, forming a bluish-green rosette, sometimes with 1–2 bract-like leaves on the stem. Bracts small, membranous, reddish, slightly shorter than the ovary. Spike compact, ovoid, subcylindrical, dense, 1–10 cm long, elongating after anthesis. Flowers sessile, opening from the base upwards, dark purple when unopened. Spur cylindrical, directed downwards, 1/4–1/2 of the length of the ovary. 'Sepals' oval-lanceolate, purple, 3.5–4.5 × 1.5–2.5 mm, laterals asymmetric, 3–3.5 mm long. 'Petals' linear, subspathulate, keeled, 3–3.5 mm long. Labellum 4–8 mm, longer than wide, white or pale pink with papillose purple spots narrowing into 2 long ridges framing the spur entrance, deeply trilobed; the middle lobe dilated at the apex, itself normally bilobed, rarely entire; the lateral lobes oblong, obtuse. Stigma with strongly enlarged flaps on both sides of the rostellum, the column noticeably shortened. Retinacles are more or less reduced, with two pollinia. Gynostegium (ovary) short, resupinate. The flower scent varies between the early and late-flowering plants: strongly honey-like in the former and weak citron-like in the latter. No nectar is produced. Capsule is about 1 cm long, erect. Seeds are very numerous and tiny (*c.* 0.4 × 0.15 mm).

Variants

Most authors do not distinguish taxa below subspecies level within *Orchis ustulata* and *Flora Europaea* neither mentions forms nor varieties (Tutin *et al*. 1980). However, white-flowered plants are often found (Foley 1990; Reineke & Rietdorf 1991). According to Procházka (1980) various forms exist which differ in morphology or size such as f. *ustulata*, f. *grandiflora* Gaud. 1825 (= f. *major* Weisb. 1891), f. *leopoliensis* Zapal. 1906, f. *emarginata* Zapal. 1906, f. *elongata* Zapal. 1906, f. *integriloba* Sabr. 1906), as well as others which differ in colour, such as lusus *albiflora* Thielens 1873, lusus *virescens* Caspary 1884, lusus *daphneolens* Beauv. 1905 and lusus *rubriflora* Vetter ex Keller et Soó 1931.

Kümpel & Mrkvicka (1990) have described the subspecies *aestivalis* that was previously considered to be an ecological form. This differs in its phenology, flowering about 1–2 months later than the nominal subspecies. Jenkinson (1995) claims that the British late-flowering form is not identical to ssp. *aestivalis* and should be regarded merely as a late-flowering variant. According to Reineke & Rietdorf (1991) there are two different forms of the late-flowering *O. ustulata* in Germany, one of them occurring together with early flowering plants in all the larger (more than 30 member) populations. Latest molecular research does not confirm existence of subspecies within Britain and Estonian material (K. Tali, M. Fay & R.M. Bateman, unpublished). In this paper, on the basis of their phenology, the early flowering populations are treated as var. *ustulata* and the late-flowering ones as var. *aestivalis*.

I. Geographical and altitudinal distribution

In the British Isles, *Orchis ustulata* (Burnt Orchid) was once locally frequent in suitable calcareous habitats throughout much of England as far north as Northumberland, but has subsequently declined drastically and is now extremely local. It is not recorded for Scotland or Ireland and only very locally from Wales.

Many of the best sites for *O. ustulata* are now protected as nature reserves or are on Ministry of Defence land, or at least have some conservation status. Unfortunately, others have none and, especially where these comprise small populations, they will come under considerable threat from man's usage. Changing agriculture techniques, the increase in ploughing during World War II, the reduction in rabbit grazing following the onset of myxomatosis, and damage from man-made incursions seem to have been the main causes of loss.

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*Abbreviated references are used for standard works: see *Journal of Ecology* (1975) **63**: 335–344. Nomenclature of vascular plants follows Stace (1997).

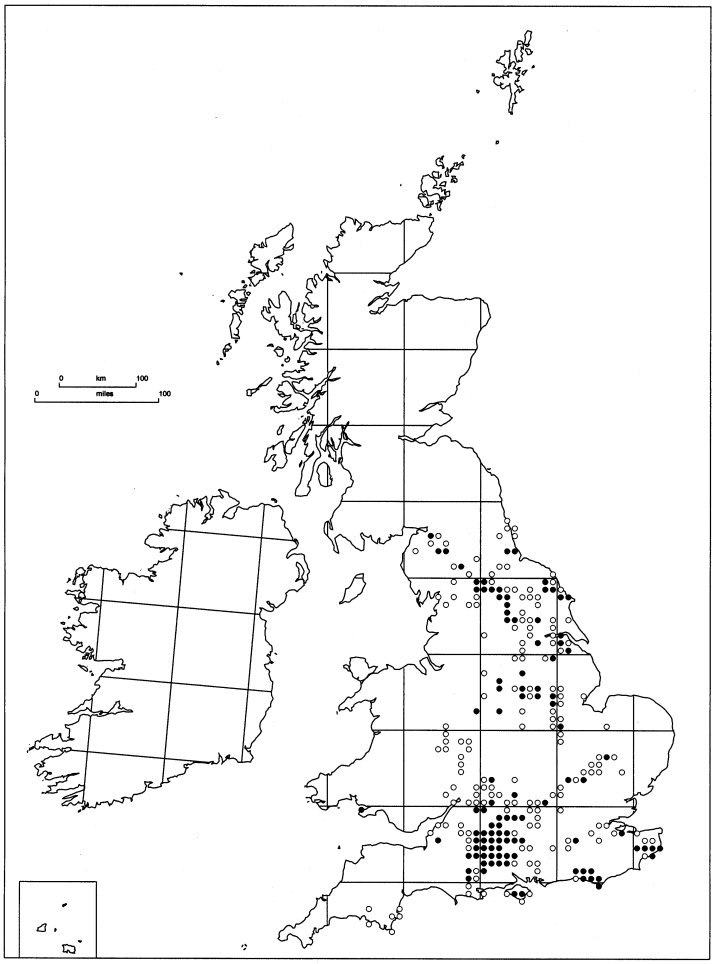

Fig. 1 The distribution of *Orchis ustulata* L. in the British Isles. Each symbol represents at least one record in a 10-km square of the National Grid. (○) Pre-1950, (●) 1950 onwards. Mapped by H. R. Arnold, Biological Records Centre, Centre for Ecology and Hydrology, mainly from records collected by members of the Botanical Society of the British Isles.

Of the formerly recorded 435 separate British populations only 75 have definitely survived (Fig. 1). Most of these are small, only 10 populations regularly comprising more than 200 flowering plants (Foley 1987, 1990). Nevertheless, a few populations are very large, for example that at Parsonage Down, Wiltshire, is estimated to contain 30 000 plants.

As well as in England, *O. ustulata* occurs throughout much of Europe, reaching its northern geographical limit in the Faroes, Gotland and Estonia, the southern shore of Lake Ladoga and the rivers of Vjatka and Kama. The species has been found also in the Urals and on the West-Siberian plains (Baumann & Künkele 1982; Vakhrameeva *et al*. 1991) whilst its southern distributional limit passes through Spain and the Mediterranean coast of France. It also occurs in Italy north of Rome, on almost the whole of the Balkan Peninsula and probably in the southern Ukraine up to the Volga River (Füller 1983) as well as in the Caucasus (Vakhrameeva *et al*. 1991) (Fig. 2). This species is in general decline and is protected throughout its range; it is sometimes abundant in mountains, but rare elsewhere and very rare in the Mediterranean region (Delforge 1995).

In the mountains and in subalpine meadows of Europe, *O. ustulata* populations have been found up to 2400 m altitude (Delforge 1995) but in Britain this is essentially a lowland plant occurring from almost sea level up to nearly 300 m in Derbyshire (Alt. Range Br. Pl.).

II. Habitat

(a) Climatic and topographical limitations

Orchis ustulata is classified as a European temperate species (Preston & Hill 1997). It appears to be favoured by regions where the summers are humid and warm. It can withstand quite cold conditions but probably not repeated freezing and melting, as its rosettes are wintergreen. Very dry conditions appreciably restrict flowering. In some localities plants are also found in damper transition zones.

Orchis ustulata is favoured by sunny, open habitats in short, lightly grazed calcareous grassland with only moderate competition, but in continental Europe it can also inhabit light, open woodland. The largest populations

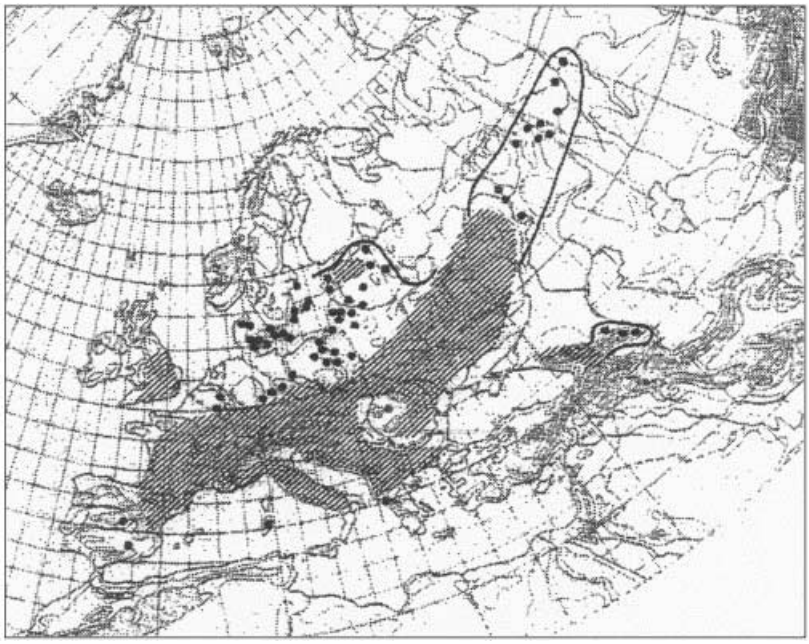

Fig. 2 The distribution of *Orchis ustulata* in the world (Hultén & Fries 1986).

Table 1 Soil chemical analyses for five localities in Estonia

Locality	pH	N (%)	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Organic matter (%)
Aljava	4.9	0.369	14.81	75.9	1636	282.0	7.84
Lõetsa	7	0.405	23.54	263.2	4014	368.2	8.66
Kapi	7.3	0.516	16.64	128.2	4418	360.3	10.76
Sillukse	7.3	0.261	18.40	106.9	3391	330.0	5.40
Jäneda	7.6	0.359	19.59	25.1	6001	69.7	6.70

in Britain are usually on old moderately rabbit- or sheep-grazed pastures, which have never been treated with artificial fertilizers, herbicides or pesticides.

(B) SUBSTRATUM

In Britain, *Orchis ustulata* usually grows best on well-aerated humus-rich soils, and is mainly a plant of calcareous substrates (chalk, and oolitic, Carboniferous, and Magnesian limestones). In Sweden, Estonia and elsewhere it can also be found on stony alvar where the soil is sparse as well as in limestone cracks which also have a very shallow soil (e.g. locality Kapi in Table 1). The plant has also been found on sand and gravels (Davies *et al*. 1983). The pH of sites it occupies range from 5.2 to 7.3 (Arditti 1992) and 6.0 to 8.5 (Procházka & Velísek 1983). Soil analyses from sites of five different populations in Estonia are shown in Table 1.

III. Communities

In Britain, fairly constant associates of *Orchis ustulata* include *Anthyllis vulneraria*, *Centaurea nigra*, *Conopodium majus*, *Gentianella* spp., *Hippocrepis comosa*, *Lotus corniculatus*, *Polygala* spp., *Primula veris*, *Rhinanthus minor* and *Sanguisorba officinalis*. Orchids such as *Dactylorhiza fuchsii*, *Gymnadenia conopsea*, *O. mascula*, *O. morio* and *Platanthera bifolia*, are frequent associates (Foley 1990). *Orchis ustulata* is also associated with *Juniperus communis* on the southern Downs (Lang 1980). The majority of populations occur in short-grazed pasture, usually with a southerly or westerly aspect, and often on a moderate slope. This is the *Festuca ovina–Avenula pratensis* (*Helictotrichon pratense*) community (CG2 of the NVC classification; Rodwell 1992). Some of the largest British populations occur where there is a well-documented history of traditional grazing and farming over past centuries. Having remained undisturbed for long periods, the banks of ancient prehistoric earth-works are also favoured. Whilst ploughing and agricultural improvement are usually anathema to the plant, there are instances where it has recolonized disturbed ground relatively quickly. One such instance is a Hampshire population at Martin Down where it reappeared about 30 years after the cessation of ploughing, presumably from a nearby seed source rather than from dormant vegetative stock. One small population on a golf course in northern England is apparently ungrazed but the habitat is kept open by the passage of golfers. Another atypical habitat is in lush meadowland near Eastoft, Linconshire, where grazing is absent; plants here are perhaps more robust and so compete adequately

with the taller surrounding vegetation. *Orchis morio* is also present in good numbers at this site. Abnormally tall plants are also known at one of the Wiltshire populations.

The late-flowering British populations are nearly always well separated from those of the early flowering variety and no precise localities are known where both occur; also the associated species are basically the same for both varieties. In one part of Wiltshire, there are several early flowering populations in close proximity to each other whilst in seemingly identical habitats nearby there are none. However, somewhat later in the year the late-flowering form is found at several of these 'uninhabited' sites; this is a phenomenon that is difficult to explain, although it is possible that this form originally occurred as an ecotype and has evolved into its present variety through different land management over a considerable period of time. The late-flowering variety also appears to occupy multi-aspect sites, and not necessarily those facing only south or west. The plants are also taller but this is probably a response to the higher competing vegetation present at this time of year. However in Britain, the subtle morphological differences between the two forms (i.e. labellum shape and markings, and degree of opening of the flowers) are not as consistent or marked as found in some German populations. Indeed some British populations are known in which flowers on the same plant exhibit both morphologies (D. C. Lang, personal communication). More than 20 late-flowering British populations are known, the majority of these being in East Sussex, with a few also in Wiltshire and Hampshire. This form is unknown anywhere north of the Thames valley.

In continental Europe, *Orchis ustulata* grows on limestone pastures or poor meadows, in light scrub on rather dry base-rich (also lime-free) mildly to moderately acid humus. There the majority of populations occur in the Mesobromion alliance, rarely also in Cirsio-Brachypodion or poor Arrhenatherion (Oberdorfer 1994). The accompanying species according to Oberdorfer (1992) include *Brachypodium pinnatum*, *Briza media*, *Carex caryophyllea*, *Centaurea jacea*, *C. scabiosa*, *Dactylis glomerata*, *Euphorbia cyparissias*, *Galium verum*, *Salvia pratensis* and *Scabiosa columbaria.*

In Estonia, as in Sweden, the early flowering plants grow on former limestone alvar (with a shallow soil layer) pastures and meadows, some of which are now overgrown with thick juniper and/or young pines. Late-flowering plants often inhabit localities with deeper soil. In these countries, associated species of *O. ustulata* are similar to those in Britain, the most frequent being *Antennaria dioica*, *Anthyllis vulneraria*, *Briza media*, *Campanula rotundifolia*, *Carex flacca*, *C. tomentosa (C. filiformis)*, *Festuca rubra*, *Filipendula vulgaris*, *Helictotrichon pratense*, *Plantago media*, *Sesleria caerulea*, *Stellaria graminea* and *Trifolium aureum*. Apart from these, *Cirsium acaule*, *Fragaria vesca*, *Potentilla reptans* and *Veronica verna* are characteristic of communities including the early flowering populations.

IV. Response to biotic factors

In Britain plants occur only in open, unshaded habitats, invariably with a sunny aspect, but in continental Europe (Estonia) plants have been found under the direct shade of trees. In some cases these plants have flowered for six consecutive years. There also, young plants of *O. ustulata* are established in the lighter, open places near to path sides or in forest clearings, and young seedlings can often be found in ground disturbed by wild boar, such conditions aiding their establishment. Compared to the tubers of other orchids, those of *O. ustulata* are small and so less attractive to mammals; nevertheless some of the continental populations have sometimes suffered major damage from uprooting and grazing. In Britain this is unlikely to be a problem although the inevitable presence of rabbits in the closely grazed pastures where *O. ustulata* often occurs may occasionally result in plants becoming dislodged. Flower or leaf damage by slugs or insects has been observed, but this appears to have no adverse effect, the plants developing normally the following year.

V. Response to environment

(a) Gregariousness

In England individual flowering plants usually grow in fairly close proximity to each other forming discrete, local populations; but in a few clearly favourable British localities the populations can be very extensive. Also in Estonia, the situation where several groups of plants or subpopulations are sparsely scattered over a suitable area of several square kilometres is common.

Occasionally, plants that appear to be vegetatively propagated form groups of 2–5. Usually only 1–2 spikes in such a group flower at the same time, but 4–6 flowering spikes are not rare. In one population in northern England, there were 10 plants, probably a clone, flowering within a radius of a few cm but no other plants could be seen within 100 m. Fruit set for plants in such groups in continental Europe was not found to be higher than for singly growing plants (Tali 1996).

(b) Performance in various habitats

Evidence is available where individual plants have been marked and monitored in Estonia. The figures given in Table 2 reflect the situation in permanent plots (ten 1 m^2 squares at each locality). As new plants are usually not established in the same plots the actual decline is not so great in populations. A severe decline was caused by quarrying, which destroyed over half of the Sillukse population. The noticeable height difference between late-flowering and early flowering plants appeared to be due to the differing height of the surrounding vegetation at their respective flowering seasons. Variation in the average height of any population depends on the conditions applicable that year and is fairly similar for

Table 2 Number of flowering *Orchis ustulata* plants for permanent plots in five different Estonian populations. H = average height (cm) of flowering plants; Average = average height (cm) of flowering plants in a population during the study; SD = standard deviation

		1993		1994		1995		1996		1997		1998		1999		2000		Average
		No.	H	No.	H	No.	H	No.	H	No.	H	No.	H	No.	H	No.	H	H
Late	Sillukse	40	25.5	31	26.3	20	33.9	13	27.7	14	24.7	7	25.0	0		3	21.3	26.4
	SD		5.5			6.7		7.9		8.7		5.5		6.4			6.8	
	Jäneda			46	21.7	5	21.0	2	24.5	16	20.8	0		0		2	26.0	22.8
	SD				5.9		4.4		0.7		5.2						0	
Early	Lõetsa	24	13.2	77	16.8	47	20.3	54	18.1	16	14.2	29	15.6	14	13.6	2	11.0	15.3
	SD		4.6		4.6		6.8		4.7		6.4		5.2		4.0		1.4	
	Kapi			100	21.6	47	24.6	42	22.9	0		41	16.9	21	18.5	7	15.3	20.0
	SD				4.0		6.5		4.0				4.4		4.7		6.6	
	Aljava			48	16.9	103	21.2	54	24.8	17	17.4	21	13.0	9	18.2	0		18.6
	SD				3.5		5.7		4.5		4.8		5.0		3.6			

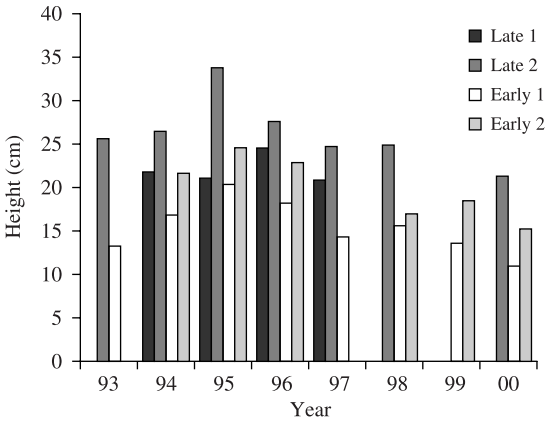

Fig. 3 Comparison of the height of flowering plants in two late-flowering and two early-flowering populations in Estonia.

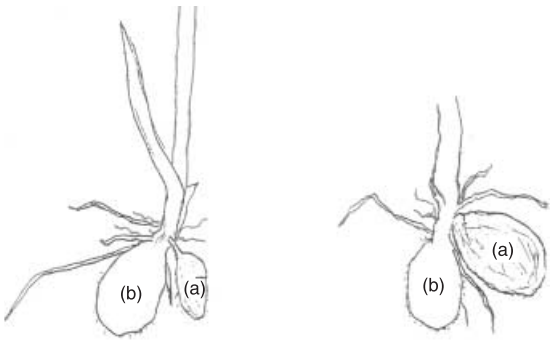

Fig. 4 Tubers of *Orchis ustulata* during the flowering season: (a) old tuber; (b) new tuber.

each population (Fig. 3). The inflorescence varies greatly in length and comprises 10–60 flowers, with approximately 6 flowers per cm of inflorescence.

(C) EFFECT OF FROST, DROUGHT, ETC.

This plant is favoured by a warm, humid climate and is more affected by moisture than by temperature. The rosettes withstand frosts well, the leaves showing only minor damage in spring (monthly mean temperatures of −11° to −12 °C are not uncommon in Estonia). In extremely dry years, plants seem to flower less with the rosettes perishing before flowering time.

VI. Structure and physiology

(A) MORPHOLOGY

At flowering time each plant has two underground tubers, one of which has produced the current year's growth and subsequently become wrinkled and brown, the other ready to produce the following season's growth from its tip; this tuber is new, white and fleshy (Fig. 4). Stomata occur only on the lower (abaxial) surface of the leaf at a density of 80–90 mm^{-2}. According to Mrkvicka (1994) seed size of the early flowering variety is 0.29–0.44 × 0.13–0.17 mm whilst that of the late-flowering variety is 0.35–0.50 × 0.12–0.15 mm. The seed morphology of the two is also different, the early flowering variety having smaller seeds with thicker walls and a bigger embryo (Mrkvicka 1994). The seeds of the late flowering plants from Estonia seen by scanning electron microscopy showed appreciable variation in size and shape (Fig. 5).

(B) MYCORRHIZA

Orchis ustulata forms mycorrhiza with fungi of the genus *Rhizoctonia*. The degree of mycorrhizal infection for *O. ustulata* is high, i.e. 6 on a 6-point scale as given by Sadovsky (1965); however, according to Rasmussen (1995) the extent of mycorrhizal colonization is only 1 on a 3-point scale. This needs further clarification.

A mycorrhizal fungus isolated from pelotons in a mature root of *O. ustulata* from East Sussex, England, and subsequently grown in axenic culture, was identified using molecular methods as being a *Ceratobasidium*

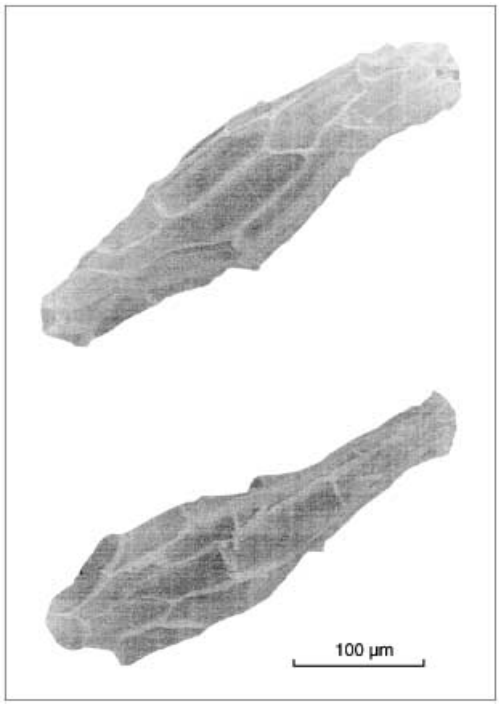

Fig. 5 Scanning electron micrograph of the seeds of *O. ustulata* (taken with Tesla BS 301 at 17 kV, sputter coated with gold). Scale bar = 100 µm.

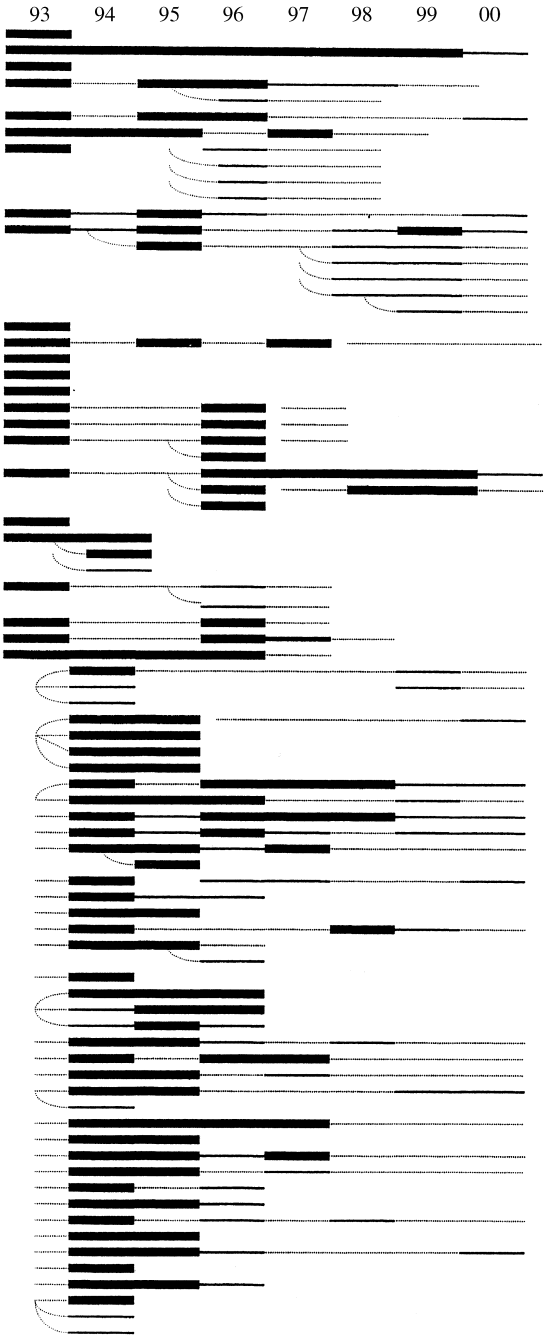

Fig. 6 Behaviour of plants in the early flowering Lõetsa population (Estonia) 1993–2000. Solid line – plant is flowering; normal line – plant is vegetative; dotted line – plant is dormant.

species (M. Bidartondo & D. J. Read, personal communication). Fungi of this genus have been shown to be capable of inducing germination in several other *Orchis* species (Muir 1989).

(c) PERENNATION: REPRODUCTION

The plant is a geophyte, perennating by the tuber formed during the previous spring. Dormancy is very common and a plant is able to survive without forming any above-ground parts for 1–3(–4) years (Fig. 6). Owing to dormancy, calculation of depletion curves is complicated and inaccurate. The species is relatively short-lived (Tali 2002). Among 464 plants, individually monitored over a 6–8 years period in five different populations in continental Europe, only four flowered for seven consecutive years; most plants flowered for 1–4 years and then either died or remained in a vegetative or dormant state for several years. Patterns of transition between the flowering, dormant and vegetative stages show that the transition to dormancy both from vegetative and generative stages is frequent and occurs more often in the late-flowering variety (Tali & Kull 2001). The early flowering variety shows a greater tendency for vegetative–vegetative and generative–generative transitions (Fig. 7).

Sexual reproduction dominates. Some studies have indicated that vegetative reproduction is possible by a secondary rhizome (Foley 1994) or that tubers may be produced from more than one of the basal buds, both in culture and under good conditions in nature (Rasmussen 1995). In four populations in Estonia, 5%–28% of the plants formed clusters of 2–6 specimens whereas one inland locality (Jäneda) lacked such clones altogether.

(d) CHROMOSOMES

$2n = 42$ (Tutin *et al.* 1980), $2n = 20, 40$ (Procházka 1980).

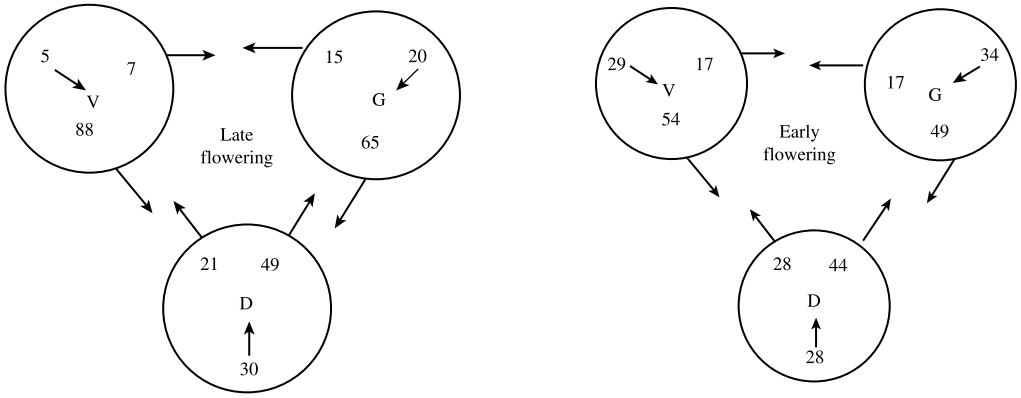

Fig. 7 Transitions (%) between three stages (V – vegetative, G – generative, and D – dormant) calculated from five populations in Estonia.

(E) PHYSIOLOGICAL DATA

No data.

(F) BIOCHEMICAL DATA

Tubers yield salep (containing starch and mucilage) and were once used as an aphrodisiac in ethnomedicine in a way similar to the tubers of other species of *Orchis*. A study of floral pigments showed that the plant differs clearly from other *Orchis* species in containing mecocyanin, but lacks cyanin and orchicyanins (Pridgeon *et al*. 1997).

(G) GENETIC DATA

Work has been conducted on this aspect in continental Europe. Enzyme systems phosphoglucomutase, malate dehydrogenase, leucine dehydrogenase, superoxide dismutase (SOD) and phosphoglucoisomerase (PGI) were analysed in six Estonian populations and one Gotland (Sweden) population to detect differences in allele frequencies between var. *ustulata* and var. *aestivalis*. No unique alleles were found; all alleles were detected in both varieties. The first three systems were monomorphic. No significant differences occurred in PGI diversity; the frequencies of the more rapid allele of SOD were higher in the late-flowering Sillukse and Pilguse populations and in the early flowering Gotland populations than in three geographically closer, early flowering populations from Muhu Island, Estonia (K. Tali & T. Paaver, unpublished).

From dendrograms based on the degree of heterozygosity for 10 enzyme loci, Schlegel *et al*. (1989) concluded that *O. ustulata* as well as *O. morio* are more distantly related to the other *Orchis* species and should therefore be considered to be members of another genus. Based on the nuclear ribosomal DNA internally transcribed spacers ITS1 and ITS2, it is evident that *O. ustulata* forms a well distinguished clade with *O. tridentata*, *O. lactea* and *Neotinea maculata* (Pridgeon *et al*. 1997), and in 1997 the species was described as *Neotinea ustulata* (L.) R. M. Bateman, Pridgeon, & M.W. Chase, based on ITS trees (Bateman *et al*. 1997).

VII. Phenology

Kümpel & Mrkvicka (1990) studied the phenology of the two varieties of *O. ustulata* in several plots in Austria at altitudes from 300 m to 1070 m a.s.l. (see Table 3). Rosettes of both varieties are produced in the autumn. In Britain, flowering occurs from about the second or third week of May until mid-June for var. *ustulata* and from early July to August for var. *aestivalis*. Plants without inflorescence primordia (i.e. vegetative plants) largely disappear by flowering time, when the leaves of flowering plants also start to decay. Capsules ripen in late June to July (var. *ustulata*) and August onwards (var. *aestivalis*). Stems bearing capsules can be encountered the following year.

Based on observations in continental populations, Kümpel & Mrkvicka (1990) claim that plants of the late-flowering variety begin to emerge in March to April whilst early flowering plants are wintergreen.

Table 3 Phenology of two varieties of *O. ustulata* in plots in Austria (from Kümpel & Mrkvicka 1990)

	var. *ustulata* (early)	var. *aestivalis* (late)
First leaves	September–November	October–December
Rosette formation	November–February	March–April
Beginning of flowering	April	End of May–June
Full flowering	May (June)	July–August
Fruiting	Middle/end of July	End of August–September
Withering	Middle/end of July	End of August–September

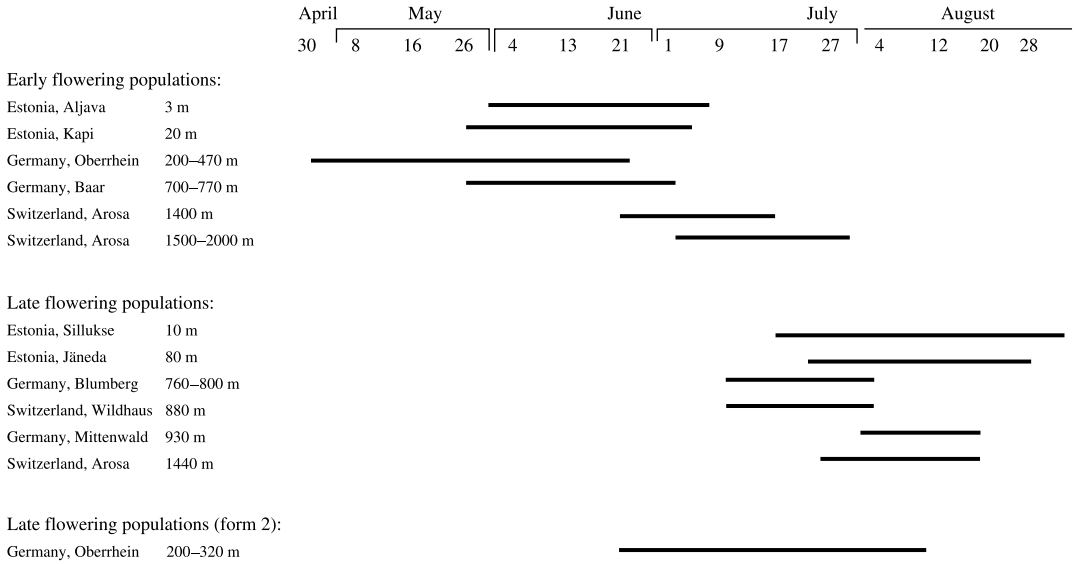

Fig. 8 Flowering times of different populations of *Orchis ustulata* (data for German and Swiss populations from Reineke & Rietdorf 1991).

Reineke & Rietdorf (1987, 1991) are of the opinion that in all *O. ustulata* populations new rosettes emerge continuously during autumn and winter, even under snow cover.

In 1993, in Estonia, measurements were made on one late-flowering and one early flowering population during anthesis. The early flowering var. *ustulata* started flowering on 20 May, continuing for about 30 days; the average length of the inflorescence at the start was 3.6 cm whilst at the end it was 4.2 cm. The late-flowering var. *aestivalis* started flowering at the beginning of July and flowered for about 50 days; the average length of the inflorescence increased from 2.1 cm at the start to 6.8 cm at the end.

Another form of late-flowering *O. ustulata* was described from Germany by Reineke & Rietdorf (1991). These plants grew together with early flowering ones and emerged in the period September to November; their flower buds developed at the same time as those of the early flowering plants. When the early flowering plants withered at the end of the flowering period, the plants of this late-flowering form remained green, like vegetative plants. The flower spike started to grow in mid-June and developed more rapidly than the spike of the early flowering variety. These lengthened, flowered and set fruit quickly. For this form, intermediate flowering has also been recorded. Differences in flowering times can also be related to the altitude of the various populations (Fig. 8).

VIII. Floral and seed characters

(A) FLORAL BIOLOGY

In *Orchis ustulata* nectar is not produced. Differences in scent (sweet for var. *ustulata* and rather unpleasant for var. *aestivalis*) suggest the existence of different pollinators for the early flowering and late-flowering plants. The uppermost buds usually fail to open.

The stigma of *Orchis ustulata* has strongly enlarged flaps on both sides of the rostellum and a noticeably shortened column, through which the bursicles and viscidia approach the labellum; also the spur entrance is narrowed (van der Cingel 1995). Data on pollen vectors for this species are scarce. Vöth (1984) and Mrkvicka (1991) have recorded a tachinid fly (*Echinomyia magnicornis* Zett.) for var. *aestivalis*. The number of *E. magnicornis* individuals counted by Vöth is appreciable: 9 approaches and 49 visits per small population of 11 plants during a 4-h period on a single day. Seven individuals out of 13 carried 26 pollinia. Jürgen Böhm has observed the same insect visiting *O. ustulata* several times in a German population (personal communication). The beetle *Leptura livida* Fabricius (Cerambycidae) has been recorded as a pollinator of var. *ustulata* (van der Cingel 1995). Although *O. ustulata* has been referred to as a butterfly flower (e.g. Pfl. Exk.; Baumann & Künkele 1982) conclusive evidence for this appears to be lacking.

(B) HYBRIDS

Rothmaler (1976) stated that *O. ustulata* produces hybrids with *O. simia* and *O. tridentata* but van der Pijl & Dodson (1969) argued that *O. ustulata* can form hybrids only with *Anacamptis pyramidalis*. According to Baumann & Künkele (1982) they can also occur with *O. morio* and *O. militaris,* whilst Davies *et al*. (1983) recorded hybridization with *O. coriophora* and R. M. Bateman with *Neotinea lactea* (personal communication). British populations, where *O. ustulata* and *O. morio* grow in close proximity, have frequently been examined for the presence of hybrids but none has so far been recorded.

Table 4 Flowering and fruiting data for five populations in Estonia, 1993–2000

	Early Aljava	Early Kapi	Early Lõetsa	Late Sillukse	Late Jäneda	Total or mean
Total number of flowering plants	266	323	274	141	69	1073
Number of fruiting plants	21	78	51	60	14	224
Number of capsules	62	306	173	176	49	766
Fruiting percentage	7.89	24.1	18.6	42.6	20.3	20.9
Mean number of capsules per flowering plant	2.95	3.92	3.39	2.93	3.5	3.4

(C) SEED PRODUCTION AND DISPERSAL

The overall average percentage of fruiting, calculated over an 8-year period for all flowering plants in five Estonian populations, was 20.9% (Table 4). Seed set is relatively infrequent in Great Britain and is not likely to exceed this figure. The number of seeds estimated in three capsules varied between 2000 and 4000 per capsule. The seed is very tiny and so can be dispersed hundreds of kilometres by the wind.

(D) VIABILITY OF SEEDS: GERMINATION

According to Sadovsky (1965) the germination of *Orchis ustulata* seed in cultivation is almost impossible. Vermeulen in the 1940s and Eiberg in the 1960s failed to germinate fresh seeds of *O. ustulata* in water (+0.05% Tween 80 detergent) (Rasmussen 1995). However, some gardeners (http://gardenbed.com) claim that ripe seed may germinate if surface-sown in the glasshouse.

(E) SEEDLING MORPHOLOGY

Orchis ustulata is characterized by normal embryo sac development and by the presence of T-tetrads. The embryos have a suspensor. Embryo size is 88–120 × 60–90 μm for the late-flowering and 120–160 × 100–130 μm for the early flowering *O. ustulata* (Mrkvicka 1994).

Stojanow (1916) observed seedlings of *O. ustulata* at a distance of 5–10 cm below the surface in thick humus. The mycorrhizome may grow in the soil for more than 10 years with one segment often (but not always) added each year (Fuchs & Ziegenspeck 1927; Baumann & Künkele 1982). After this initial stage, the root, then the leaf, and finally the tuber are formed; the plant's development has been illustrated by Summerhayes (1951; fig. 1, p. 3). *Orchis ustulata* is thought to have the longest seedling phase in the Orchidaceae, and up to 16 years may pass from germination before it first flowers (Davies *et al*. 1983). Summerhayes (1951) has questioned such estimates of the duration of the seedling stages as being exaggerated. There is at least one site in Estonia where plants grow on a field that was ploughed 6 years previously. Cultivated plants have been claimed to reach flowering within 3 years (Möller 1985), probably owing to the more stable and favourable growth conditions. The initial protocorm/mycorrhizome may attain a length of 20–30 mm before the first root is produced; this length is remarkable compared with other species with the similar life history of root-aerial shoot-tuber (Rasmussen 1995).

IX. Herbivory and disease

(A) ANIMAL FEEDERS OR PARASITES

Grazing animals (cattle and sheep) do not avoid *Orchis ustulata* plants and these are certainly not immune to attack by rabbits; cultivated plants are especially susceptible to slug and snail predation. In continental Europe, wild boar also feed on the tubers whilst several carnivorous bugs (Hemiptera) such as *Phymata crassipes* Fabricius have been observed on plants, apparently mimicking the remains of old flowers, but may not be feeding.

(B) & (C) PLANT PARASITES AND DISEASES

No data.

X. History

Linné described *Orchis ustulata* in his *Species Plantarum* in 1753. Before this, however, the species was already known by the 16th century, being described by Fuchs in 1543 (Jacquet 1994). Synonyms include: *Orchis amoena* Crantz 1769, *O. columnae* F. W. Schmidt 1791, *O. parviflora* Willd. 1805, *Himantoglossum parviflorum* Spreng. 1826 (Procházka 1980) and *Neotinea ustulata* (Bateman *et al*. 1997).

A Mr Stonehouse made the earliest known localized British record for *O. ustulata* by 1650 at 'Scasby-lease', near Doncaster, Yorkshire (How 1650). That this should have been the first is surprising, since in those days the plant would presumably have been much more frequent in the large populations on the southern Downs.

XI. Conservation

Although formerly widespread in the chalk and limestone regions of England, *Orchis ustulata* has suffered one of the severest declines of all wild orchids during the last 50 years and is rare there now (Foley 1994). It may be the most rapidly decreasing plant in Britain (80% decline) having been lost from 210 of 265 formerly occupied 10 × 10 km squares (Preston *et al*. 2002).

In the Netherlands, it has not been recorded since the 1980s and is extinct, or probably so, in the St. Petersburg region of Russia. It is categorized as 'vulnerable' in Estonia and Latvia, 'endangered' in Lithuania, Poland and Denmark (Ingelög *et al*. 1993), 'critically endangered' in Czechia (Holub & Procházka 2000), and given in Red List category 2 for Germany (category 3 for Bayern). The species is protected by law in most of Europe.

Acknowledgements

We are grateful to H. Arnold (Biological Records Centre) for supplying the map showing the British distribution of *Orchis ustulata*, to M. Rahi (Institute of Zoology and Botany) for producing the photos from electron microscopy and to M. Bidartondo and D. J. Read for the newest information about the mycorrhizal fungus found in *O. ustulata*. We also thank A. J. Willis (University of Sheffield) for helpful advice and for commenting on the manuscript, the Associate Editors and Professor M. J. Hutchings. We appreciate the financial support of ESF grant 4833.

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Little genetic differentiation across Europe between early-flowering and late-flowering populations of the rapidly declining orchid *Neotinea ustulata*

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Amplified fragment length polymorphism (AFLP) is used to characterise genetic diversity of the endangered burnt orchid, *Neotinea* (formerly *Orchis*) *ustulata*. Fingerprinting of Estonian and British populations revealed surprisingly little genetic differentiation between populations but larger amounts of diversity within populations, especially in Britain; the resulting mean F_{ST} value of 0.51 is unusually high for an orchid species. Much of the variation follows a west–east cline across Europe, whereas the much-discussed early-flowering and late-flowering taxa of *Neotinea ustulata* are considered insufficiently distinct to be viewed as separate subspecies. The later flowering *N. ustulata* var. *aestivalis* probably evolved independently on two or three occasions, each time diverging from the earlier flowering nominate race. The identity of the genes underpinning phenology in the species, and the potential selective advantages of phenological divergence, merit further study. Overall genetic diversity within populations is sufficiently high to render this an unlikely cause of their recent, precipitous decline.

ADDITIONAL KEYWORDS: amplified fragment length polymorphism – introgression – polytopy – speciation

INTRODUCTION

The Burnt Orchid, *Neotinea* (formerly *Orchis*) *ustulata* (L.) RM Bateman, Pridgeon & MW Chase, is a small-flowered but charismatic tuberous terrestrial orchid that is distributed widely across central, north-central and Mediterranean Europe into Eurasia. It is nowhere frequent and often forms small, scattered

populations. Moreover, *N. ustulata* is rapidly declining in numbers throughout its distribution area, causing widespread (but as yet uncoordinated) conservation concerns in many European countries. Toward the eastern limit of its distribution, in Estonia, it is legally protected and has become a model organism for the study of plant conservation biology (Tali & Kull, 2001). At the western limit of its distribution, in the UK, a recent detailed survey of the flora identified *N. ustulata* as the most rapidly declining of all 1324 native vascular plants (Preston, Pearman & Dines, 2002a; Preston *et al.*, 2002b), its diminution being ascribed primarily to ploughing of grasslands, changes in grazing regime and habitat destruction through urbanisation (e.g. Foley, 1992, 1994).

In Britain, *N. ustulata* usually grows on well-aerated humus-rich soils of calcareous substrates, especially chalk. A particularly strong correlation is evident with ancient earthworks (Table 1). In Estonia, Sweden and elsewhere it also occurs on stony alvar or in limestone cracks and occasionally on sands and gravels (Tali, 1996; Tali *et al*., 2004).

Neotinea ustulata has also been the focus of recent taxonomic controversy. In Austria and Germany Kümpel (1988) distinguished two varieties of *Orchis ustulata*: the early-flowering var. *ustulata* L. and the late-flowering var. *aestivalis* Kümpel, which was subsequently raised to subspecies status (still under *Orchis*) by Kümpel & Mrkvicka (1990). This taxon has since been reported from the UK, France, Denmark, Germany, Switzerland, Italy, Czech Republic, Slovak Republic, Estonia, Slovenia, Romania and Bulgaria (Tali & Kull, 2001; Haratova *et al*., 2004). Subsequent debates have focused on whether *aestivalis* is a mere ecotype, probably unworthy of taxonomic recognition (e.g. Foley, 1994; Jensen & Pedersen, 1999; Delforge, 2001; Tali & Kull, 2001), or whether it merits at least subspecific status (e.g. Kümpel & Mrkvicka, 1990), and if so whether late-flowering populations across Europe are all ascribable to *aestivalis* or whether they should be distributed among two or more distinct late-flowering taxa (e.g. Reineke & Rietdorf, 1991; Jenkinson, 1995; Ettlinger, 1997; Lang, 2001). Representative early- and late-flowering plants from English populations are illustrated in Figure 1.

Our initial aim in this study was to determine whether each of these two putative taxa is genetically cohesive and, if so, to what degree the two taxa are genetically distinct. Further objectives were to infer whether *aestivalis* originated from *ustulata s.s*. or vice versa, and whether this event took place once, followed by presumed post-glacial migration to generate the current distribution, or whether separate divergences occurred in our two main sampled areas, the UK and Estonia. In addition, knowledge of genetic diversity within populations of *N. ustulata* should prove useful for determining in situ conservation priorities and for designing sampling strategies for possible ex situ propagation of this vulnerable species.

To investigate genetic diversity in these populations we focused on amplified fragment length polymorphism (AFLP™) genetic fingerprinting, supported by sequencing of the ITS region of nrDNA (e.g. Baldwin *et al.*, 1995). AFLP

should be treated as dominant markers, as homozygotes and heterozygotes cannot readily be distinguished (Qamaruz-Zaman *et al.* 1998b; Hollingsworth & Bateman *in* Bateman, 2001). This is because the method simultaneously and inextricably detects two types of polymorphisms: (1) the loss of a band marks a substitution in the restriction site or in the selective nucleotide of the primer, and (2) a different size band marks an insertion or deletion within the restriction fragment. Nonetheless, AFLP is a highly reproducible method of obtaining genetic fingerprints from small amounts of DNA (Vos *et al*., 1995; Jones *et al.*, 1998). The large number of bands gives a realistic and reproducible measure of variation across the entire genome, thus providing a good estimate of the overall level of genetic variation. Also, the AFLP method provides 10–100 times more markers, and thus is more sensitive than, most other fingerprinting techniques; small genetic differences can easily be detected (Matthes, Daly & Edwards, 1998).

Acknowledging these strengths, the AFLP method is increasingly used to investigate genetic variation within and between populations of vascular plant species. Recent botanical studies, many addressing the conservation challenges posed by regionally or globally rare species, have included orchids such as *Orchis s.s.* (Qamaruz-Zaman *et al*., 1998a), *Dactylorhiza* (Hedrén *et al.*, 2001), *Cleistes* (Smith *et al*., 2004) and *Spiranthes* (Forrest *et al.*, 2004), and genera of other angiosperm families such as *Astragalus* (Travis, *et al*.,1996), *Populus* (Winfield *et al*., 1998; Fay *et al*., 1999), *Pedicularis* (Schmidt & Jensen, 2000), *Medusagyne* and *Rothmannia* (Fay *et al*., 2000), *Gentianella* (Winfield *et al*., 2003) and *Phylica* (Richardson *et al*., 2003).

MATERIALS AND METHODS

ANALYTICAL METHODS

Intensive field collection was performed in the summer of 2002. Material was collected from six early- and three late-flowering populations in the UK and three early- and four late-flowering populations in Estonia, and was supplemented with representative samples from France, Italy and Georgia (Table 1).

All samples were collected and stored in silica gel (Chase & Hills, 1991). Whenever possible, flowers were collected rather than vegetative material, to facilitate DNA extraction and to provide additional voucher specimens. DNA was extracted from approximately 0.1 g of dried material using a modified 2x-CTAB (cetryltrimethyl-ammonium bromide) procedure (Doyle & Doyle, 1987), purified on QIAquick column and quantified using a spectrophotometer.

The nuclear ribosomal ITS1–5.8S–ITS2 assembly (totalling *ca* 670 bp) was amplified using the primers and methods of Baldwin *et al.* (1995). The PCR

program consisted of 25 cycles, each with 1 min denaturation at 97°C, I min annealing at 54°C and an extension of 3 min at 72°C, with a final extension of 7 min at 72°C. Amplified double-stranded DNA fragments were then purified using Promega Wizard PCR minicolumns and sequenced on an ABI 377 automated sequencer. Two sequencing reactions were performed for each completed sequence, one with each of the two PCR primers, and these generated complete overlapping single-strand sequences for the entire ITS assembly.

AFLP analysis was performed according to the AFLP™ Plant Mapping Protocol of Applied Biosystems Inc. Genome size can have a marked effect on the quality of AFLP (Fay & Cowan, 2001), and *N. ustulata* and its relatives have relatively large genomes. Sample DNA was restricted with the endonucleases *Eco*RI and *Mse*I and ligated to appropriate double-stranded adapters according to the manufacturer's protocols. Two steps of amplification followed: a pre-selective amplification that used primers, each with one additional base, was followed by a selective amplification using primers each with three or four additional bases, thereby further reducing the number of fragments. For the second amplification, 12 different primer combinations were tested, of which three were chosen for the full study: –ctac/-act, –ctac/-acg and –ctac/-acc. Amplification reactions were separated on a 5.0% polyacrylamide gel using an ABI 377 automated sequencer, and Genescan 3.1 and Genotyper 2.0 (Applied Biosystems) were used to analyse the resulting bands.

DATA ANALYSIS

Bands were scored as either present (1) or absent (0) for all individuals. They were exported from Genotyper as a binary matrix that was analysed using two methods. The first employed the UPGMA (Unweighted Pair-Group Method using Arithmetic Averages) algorithm in the software package PAUP version 4.0d64 for Macintosh (Swofford, 1998) and the second applied principal coordinates analysis (PCoA) in the R Package for Multivariate Analysis version 4.0 (Casgrain & Legendre, 1999) using Jaccard's coefficient, which is especially effective when applied to binary data matrices and excludes shared zeros. Gene diversity statistics were calculated using the shareware program TFPGA (Tools for Population Genetic Analyses), version 1.3 (Miller, 1997).

RESULTS

ITS SEQUENCING

ITS sequences of one early- and one late-flowering Estonian plant (Lõetsa and Pilguse populations, respectively) were identical to each other and to that previously obtained from an Italian specimen of *N. ustulata* and published by Bateman *et al.* (2003).

AFLP DATA

The three primer combinations yielded 89 interpretable bands (Table 2), ranging in size from 55 to 300 base pairs. Of these, 79% were polymorphic. Seven bands were found only in Estonian material and five bands only in UK material; a further ten bands occurred in the very limited numbers of Italian, Georgian and/or French plants analysed but did not occur in any British or Estonian populations.

The UPGMA dendrogram generated from these data (Fig. 2) shows no clear differentiation between early-flowering and late-flowering populations or between Estonian and English material. The two easternmost samples, from Georgia, cluster together and are most distinct from the other plants. The remaining samples form two groups. One group consists entirely of English samples, mostly from early-flowering populations but with two late-flowering samples interspersed near the base. The other group has at its base the two samples from Italy, followed by a pair of early-flowering English plants. The remainder of the group is an approximately equal mixture of alternating clusters of early- and late-flowering plants. Most of the remaining English samples form a cluster, together with one outlying Estonian sample and the single French sample.

The unrooted phylogram based only on Estonian material (Fig. 3A) shows lower variance among populations than within populations, and again there is imperfect assortment into early- and late-flowering plants. When analysed separately (Fig. 3B), the English plants once again form two clusters of approximately equal size, one cluster an equal mixture of early-flowering and late-flowering plants, but the other dominantly early flowering and including only one errant late-flowering plant. As in the UPGMA tree, clustering of samples according to source population is far from perfect.

The general patterns evident in the UPGMA trees (Figs 2, 3) are also revealed by the PCoA (Fig. 4A–C). The PCoA plot of all samples (Fig. 4A) shows little structure beyond the first coordinate, which accounts for 23.8% of the total variance; the second coordinate encompasses only 5.9% and lower order coordinates each account for less than 5%. The Georgian and Italian plants appear more closely associated with the relatively compact Estonian

cluster in the PCoA plot than in the UPGMA tree. Axes 2–4 all separate the two Georgian plants from the remainder, and Axis 4 separates the two Italian plants from the remainder (ordinations not shown). As in the UPGMA tree, the French sample and one Estonian sample cluster with the English plants, which form two imperfect clusters. One of these clusters comprises plants from the westernmost (Yarnbury, Odstock) and northernmost (Middleton) early-flowering English populations, which are linked to the other English populations (both early- and late-flowering) and the single French plant by the genetically variable, late-flowering population from Willingdon, Sussex.

Separate ordinations illustrate the contrasting distances between early- and late-flowering populations in Estonia and England. In Estonia (Fig. 4B), early- and late-flowering populations show a slight tendency toward genetic divergence on the plot of axis 1 versus axis 2. In English populations (Fig. 4C) the genetic structure is based less on early versus late flowering patterns and more on geographical location.

When estimation of differentiation between populations was calculated separately for Estonia and England, $F_{ST} = 0.254$ for Estonian populations and $F_{ST} = 0.551$ for English populations; F_{ST} calculated over all populations was 0.509.

DISCUSSION

NO DIVERGENCE IN ITS AND KARYOTYPE

In a thoroughly sampled molecular phylogenetic survey of the tribe Orchideae using ITS sequences, Bateman *et al.* (2003) found that substantial divergences between analysed accessions inevitably indicated species-level divergence, but noted that the converse was not the case; some species pairs judged *bona fide* on morphological evidence reliably yielded identical ITS sequences (examples included *Platanthera bifolia* versus *P. chlorantha*, *Gymnadenia conopsea s.s.* versus *G. odoratissima* and, in tribe Neottieae, *Epipactis helleborine* versus *E. purpurata*). Thus, the complete lack of ITS divergence between individuals of *N. ustulata* showing strongly contrasting flowering periods and geographical origins documented in the present study does not automatically indicate lack of taxonomically meaningful genetic structure, although it strongly suggests that any divergence among taxa occurred recently. Karyological investigations similarly failed to distinguish between early- and late-flowered forms, both of which yielded a count of $2n = 42$ (Mrkvicka, 1991) that is plesiomorphic for the subtribe Orchidinae (Bateman *et al.*, 2003).

GENE FLOW OCCURS BETWEEN ADJACENT POPULATIONS

Despite the fact that most of our samples were obtained from two extremes of the range of *N. ustulata*, our trees and ordinations based on AFLP data strongly indicate a lack of long-term genetic discontinuities between any obvious groupings of populations. The majority of the variation forms a linear pattern that is largely encapsulated by the first axis on the PCoA plot and reflects a west–east cline of geographical variation. Variation within populations is considerable relative to that between populations, often precluding grouping of individuals from the same population. These results are consistent with appreciable levels of gene flow among populations, suggesting that the recent rapid decline of the species across Europe and its consequent fragmentation into mostly small, isolated populations has not yet deleteriously affected diversity within populations and that, at least until recently, gene flow has occurred among populations in the same geographical region. It also suggests that the rapid decline of the species across Europe cannot be attributed to reduction in overall levels of genetic variation.

These conclusions are of particular relevance to any attempt to infer the cause(s) of the apparent differentiation across Europe between early- and late-flowering populations. In England, late-flowering populations are less frequent and have a more restricted distribution than early-flowering populations, but there is no obvious habitat differentiation (Foley, 1992). Moreover, in both Wiltshire to the west and Sussex to the east, some early- and late-flowering populations occur within 500 m of each other. In Estonia, by contrast, early-flowering populations are less frequent and more geographically localised than late-flowering populations, but again there is no obvious habitat differentiation (Tali & Kull, 2001). South of Estonia, in the Czech Republic, late-flowering populations are again more frequent than early-flowering populations, but here a degree of geographical separation and some divergence of habitat preferences has been described. Interestingly, a recent genetic study of Czech populations of *N. ustulata* by Haratova *et al.* (2004) was able to distinguish early- and late-flowering populations using RAPDs, but again considerable divergence was evident among populations within each phenological group. Moreover, the geographical distances between the clustered early-flowering and late-flowering populations were so great that, in our view, genetic differentiation due to geographical separation cannot be separated effectively from genetic differentiation due to phenological separation.

Thus, it appears unlikely that early-flowering populations and late-flowering populations are fully reproductively isolated anywhere in the distribution of *N. ustulata*.

LATE-FLOWERING POPULATIONS ARE PROBABLY DIVERGENT

In inferring whether the early-flowering populations diverged from the late-flowering populations or vice versa, the strongest (albeit still circumstantial) evidence is deduced from phylogenetic comparison. ITS data show that *N. ustulata* is the most geographically widespread member of a set of morphologically similar Mediterranean species, being placed between *N. lactea*, *N. conica* and *N. tridentata* on the one hand and the *tridentata*-like *N. commutata* on the other (Bateman *et al*., 2003). At any given latitude, these species coincide phenologically far more closely with the early-flowering populations of *N. ustulata* than with the late, suggesting that the latter diverged from the former.

LATE-FLOWERING LINEAGES PROBABLY HAD MULTIPLE ORIGINS: GENETIC EVIDENCE

This inference leads on to the question of where and how many times late-flowering lineages diverged from early-flowering populations. The present European flora is generally regarded as the product of dominantly northward migrations of species from North Africa, and to a lesser degree from some more northerly refugia, following the amelioration of global climate and concomitant ablation of the Pleistocene ice sheets (e.g. Hewitt, 1996). Among flowering plants, orchids are especially well-equipped to migrate rapidly, given that they produce vast quantities of minute, dust-like seeds that are easily transported in air currents (e.g. Rasmussen, 1995) and thus are capable of surmounting traditional physical barriers to migrations such as the Alps. (By contrast, the aggregation of pollen grains into relatively heavy pollinia limits pollen distribution to the physical endurance of the insect vector, resulting in lower pollen–seed flow ratios in orchids than in any other group of plants: Squirrell *et al.*, 2001.)

Thus, there exist two obvious competing hypotheses. The monotopic hypothesis states that the late-flowering genotype diverged from a single early-flowered population and then dispersed to achieve almost as wide a distribution. Alternatively, the polytopic hypothesis states that the early-flowering populations widely colonised post-glacial Europe before late-flowering populations diverged from early-flowering populations at several locations across Europe. (Ettlinger, 1997, p. 132 effectively combined both hypotheses by speculatively invoking multiple “waves of immigration from different areas of the Continent in antiquity.”) The strong geographical signal evident in our AFLP data for both early- and late-flowering plants accords more clearly with the polytopic hypothesis. It is also theoretically consistent with the monotopic hypothesis, but only if subsequent introgression has occurred across the range of both phenological groups to create the dominantly geographical patterns seen today. This scenario seems unlikely at a time when the population density of the species as a whole is rapidly diminishing and fragmenting.

Post-glacial migration tends to be reflected in relatively high genetic diversity along the three favoured northward migration routes through Europe (Iberia, Italy, Greece/Turkey) and relatively low diversity in the peripheral regions of the British Isles and Scandinavia. Thus, it is not surprising that in AFLP studies of orchids such as *Orchis simia* (Qamaruz-Zaman *et al.*, 1998a) and the *O. mascula* group (Redmond *et al.*, in prep.) showed that genotypes present in the British Isles are a subset of those found in Continental Europe. Interestingly, and unusually, this does not appear to be the case in *N. ustulata*, where similarly sized samples reveal much greater genetic diversity among populations in England than those in Estonia. This indicates that UK populations have experienced either (1) less introgression (unlikely, given the formerly extensive distribution of the species), (2) stronger selection pressure, perhaps in response to contrasting climates or modes of pollination (again unlikely, as this would affect only a few genes under selection pressure and so should not significantly influence a whole-genome diversity measure such as AFLP), or (3) a longer divergence period. What *is* clear is that, in England, the late-flowering populations are a genetic subset of (and thus presumably derived from) the more widespread early-flowering populations.

Squirrell *et al.* (2002) recently demonstrated that multiple lineages of autogamous *Epipactis* species evolved from the widespread and ecologically tolerant allogamous *Epipactis helleborine*, inferring that these lineages occurred because of independent losses of the rostellum, a reduced infertile stamen (staminode) specialised to minimise self-pollination via crumbling of the paired pollinia onto the stigmatic surface below. The iterative loss of the rostellum is believed to represent repeated mutations disabling what is assumed to be a simple genetic system controlling rostellum development (Bateman *et al.*, 2004).

It is tempting to invoke a similar mechanism to explain iterative origination of the late-flowering shift in many *N. ustulata* populations, given that transplant experiments have demonstrated that the differences in phenology among Estonian *N. ustulata* populations are genetically fixed (Tali & Kull, 2001). There is increasing evidence that many instances of speciation are driven by simple but phenotypically expressed mutations (Bateman & DiMichele, 2002), and first principles suggest that the accumulation of the much larger number of mutations, most not phenotypically expressed, that is necessary to cause substantial divergence in measures of overall genetic disparity such as AFLP occurs subsequently, over a considerable period of time (Bateman, 1999).

LATE-FLOWERING LINEAGES PROBABLY REPRESENT MULTIPLE ORIGINS: MORPHOLOGICAL EVIDENCE

The inferred independent evolution of the autogamous lineages of *Epipactis* is supported by the fact that they are morphologically distinct, albeit only subtly.

This observation encouraged us to re-examine the published evidence for the morphological cohesion of the so-called *Neotinea ustulata* subsp. *aestivalis*.

Kümpel & Mrkvicka (1990) tabulated several characters supposedly distinguishing German and Austrian populations of subsp. *ustulata* from subsp. *aestivalis* in addition to contrasts in flowering period, seed set (*aestivalis* reputedly being more successful), pollinator identity and timing of appearance of the leaves (September versus either November or spring: Tali & Kull, 2001). These morphological characters included colour and posture of basal leaves, number of stem leaves, stem height, inflorescence length and apical shape, lateral sepal posture, labellum lobe angularity, and scent ("strong honey" versus "weak lemon"). The diagnostic value of many of these characters has been challenged by researchers studying *N. ustulata* elsewhere in Europe, noting especially that the taller stem and more elongate inflorescence of *aestivalis* may simply reflect ecophenotypic modification by the increased height of the surrounding vegetation and are also modified by climatic differences between years (e.g. Tali & Kull, 2001).

In England, the later flowering populations are reputedly characterised primarily by having hoods of a purple hue that rarely fades and labella that bear larger reddish spots, sometimes associated with a pale rose flush (Ettlinger, 1997; Lang, 2001). Arguments that these plants have a narrower "waist" to their labella (e.g. Jenkinson, 1995) are not supported by our photographic evidence (Fig. 1: see also Ettlinger, 1998; Lang, 2001).

Studying populations in and around the Czech Republic, Haratova *et al.* (2004) used a more thorough morphometric approach to test supposed vegetative differences between early- and late-flowering populations. Most supposed differences failed detailed analysis, demonstrating that the main characters distinguishing later flowering populations were taller stems and longer leaves exhibiting greater surface areas, accompanied by what we interpret as a developmental transition of one leaf from being basal and expanded to sheathing the stem.

Further complicating the discussion, Reineke & Rietdorf (1991) argued that in Germany there are two different forms of late-flowering *N. ustulata*, one of which co-occurs with early-flowering plants in all populations containing more than about 30 flowering individuals. Leaves of these widespread late-flowering plants emerge between September and November, together with those of the early-flowering plants, and their flower buds develop at the same time as those of the early-flowering plants. However, when the early-flowering plants wither at the end of the flowering period, plants of the later flowering form remain green; expansion of the inflorescences is delayed but from mid-June they develop rapidly, flowering and setting fruit relatively quickly. This form is said to occasionally show flowering periods intermediate to those of the early-flowering populations and bona fide late-flowering *aestivalis*.

Neotinea ustulata has been the subject of several persistent myths or exaggerations. For example, many authors repeat flawed early studies by stating that it takes seeds 10–15 years to develop into flowering-sized plants, an assertion readily refuted by observations in cultivation (R. Manuel, pers. comm., 2003). Also, various published discussions regarding supposed pollinators reflect inadequate data; specifically, single observations of pollination of an early-flowering plant in Austria by a tachinid fly (Vöth, 1984) and of a late-flowering plant, also in Austria, by a lepturid beetle (Mrkvicka, 1991).

This leads us to question the rigour of the phenological observations said to demonstrate a genuine discontinuity between early- and late-flowering populations (e.g. Haratova *et al*., 2004), as histograms based on detailed records in the southern Alpine Italian province of Bergamo suggest that *N. ustulata* flowers continuously from April to August (admittedly, these records are complicated by delayed flowering caused by an unusually wide altitudinal range extending to 1700 m OD: Ferlingetti & Grünanger, 2001). Observations that document the precise times of opening of the first receptive flower and of the atrophy of the last receptive flower in each population are needed, so that the true potential for gene flow between early- and late-flowering populations can be assessed. The frequent assertions of lack of gene flow between early- and late-flowering populations appear unlikely in the light of our AFLP data, and it has become apparent that even limited gene flow is sufficient to undermine the genetic integrity of such groups (see, for example, the recent reversals in supposed divergent speciation of two lineages of Darwin's finches on the Galapagos islands following a recent minor hybridisation event: Grant & Grant, 2002).

CONCLUSIONS

In their synopsis of assessments of F_{ST} in 70 populations of 60 orchid species (most terrestrial), Forrest *et al.* (2004, table 5) reported a range of 0.01–0.92, with most species falling within the range 0.03–0.35 and yielding a mean F_{ST} of 0.19. Thus, the value of 0.51 for *N. ustulata* over all populations is relatively high, perhaps reflecting the relatively short average life-span of this species (Tali, 2002) and the considerable geographic distance separating populations.

There are precedents for strong geographical control over genetic variation within species. For example, after analysing the genetic diversity and geographic variation of the American chestnut, *Castanea dentate*, using RAPDs and allozymes, Huang, Dane & Kubisiak (1998) reported a strong positive correlation between genetic distance and geographic distance, particularly among populations along the north-south axis of the Appalachian mountains. The genetic distances between populations from Alabama and New York approximated 0.2, a similar

genetic distance to that separating the *N. ustulata* populations from Georgia and the UK analysed by us. The big question regarding how such geographically related and apparently continuous genetic variation is best partitioned taxonomically has not yet been satisfactorily addressed.

Perhaps the most significant conclusion of this study is the inferred multiple divergence of late-flowered lineages at different geographic locations across the geographic distribution of a widespread early-flowered species, paralleling the multiple origins of autogamous *Epipactis* species from within the allogamous *Epipactis helleborine* (Squirrell *et al.*, 2002; Bateman *et al.*, 2004) but achieved via shift in phenology rather than in reproductive syndrome. In *N. ustulata*, fruit-set reputedly differs appreciably between early- and late-flowering plants (e.g. Kümpel & Mrkvicka, 1990) and between different geographic regions (e.g. Tali & Kull, 2001). Long-term, Europe-wide monitoring of the relative reproductive success of these contrasting populations might prove highly informative, especially in the face of climate changes that could easily favour one phenological mode over the other in contrasting geographical regions.

TAXONOMIC NOTE

Although the late-flowering taxon *aestivalis* was originally described as a variety (Kümpel, 1988), a rank consistent with our own current taxonomic view, the combination was made under the formerly broader, polyphyletic genus *Orchis* L. rather than under the recircumscribed, monophyletic *Neotinea* Reichb.f. (e.g. Bateman *et al.*, 2003). We therefore make the requisite combination here:

Neotinea ustulata (L.) RM Bateman, Pridgeon & MW Chase var. ***aestivalis*** (Kümpel) Tali, MF Fay & RM Bateman, **comb. nov.**
Basionym: *Orchis ustulata* L. var. *aestivalis* Kümpel, Hausskn. 4: 23 (1988).
Synonym: *Orchis ustulata* L. subsp. *aestivalis* (Kümpel) Kümpel & Mrkvicka, Mitt.-Bl. Arbeitskr. Heim. Orchideen Baden-Württ. 22: ?315 (1990).

Contrary to the statements of some authors (e.g. Dusak & Pernot, 2002), this taxon was never formalised as *Neotinea ustulata* subsp. *aestivalis* (Kümpel) Bateman, Pridgeon & Chase. If further genetic studies allow clear differentiation of contrasting late-flowering morphs, revealing independent origins in different parts of Eurasia, it would be most appropriate if each late-flowered group were to be given a separate diagnosis at varietal level.

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FIGURES

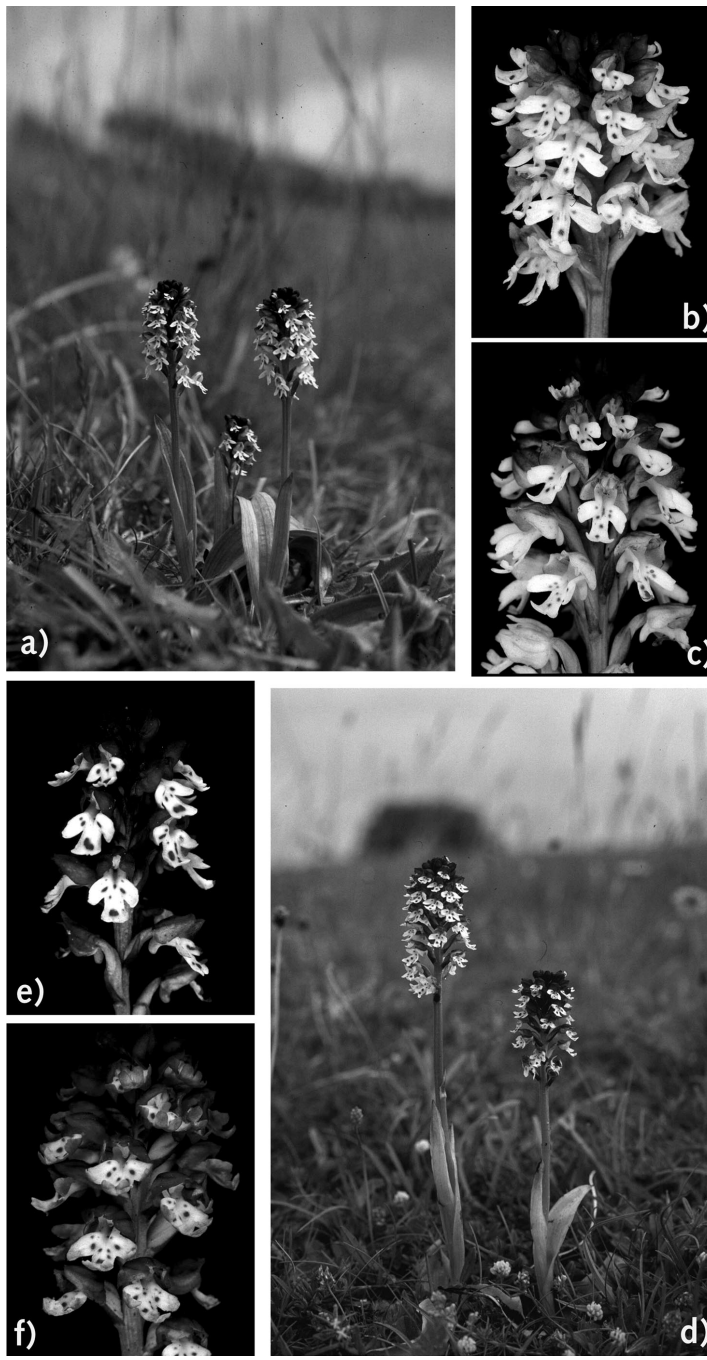


Figure 1. Illustrations of typical plants of early-flowering and late-flowering forms of *Neotinea ustulata* from England. A–C from Knocking Hoe, Bedfordshire; D–F from Ladle Hill, Hampshire.

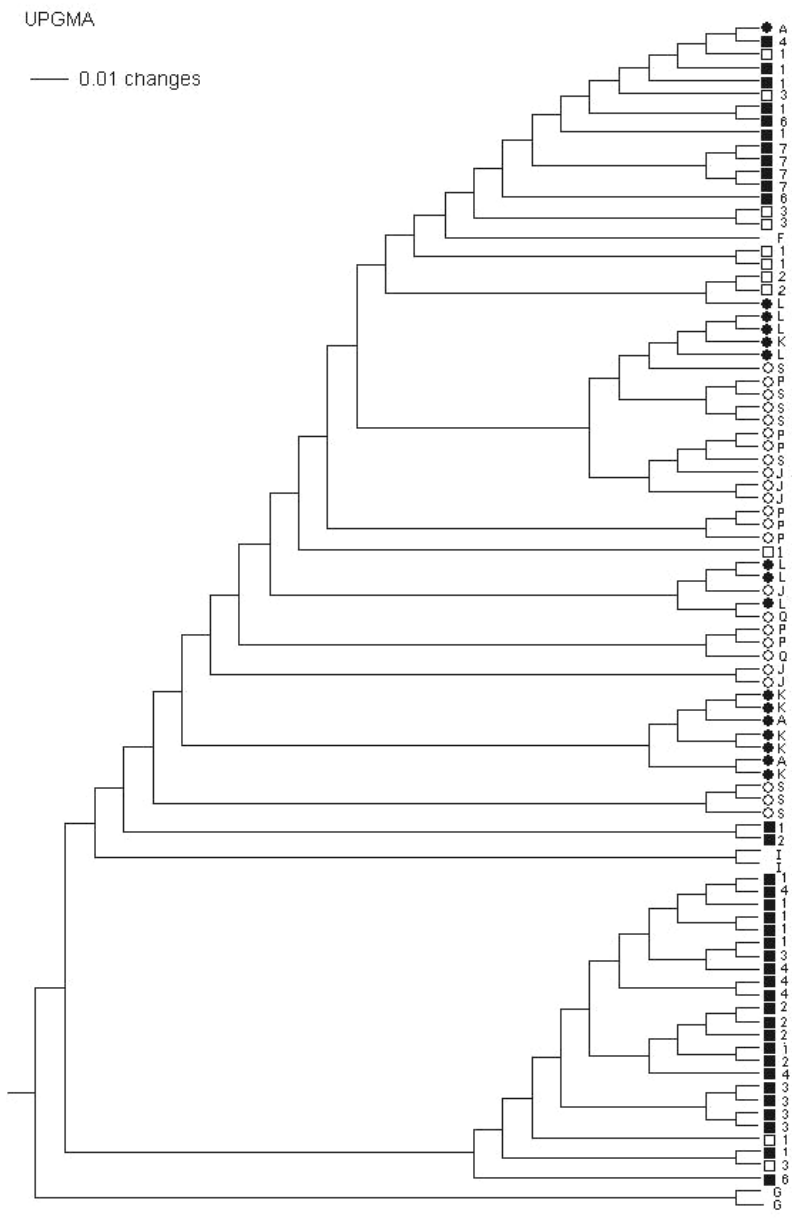


Figure 2. Rooted UPGMA tree including all samples. ■ – England, early flowering; □ – Estonian,early flowering; ● – England, late flowering; ○ – Estonian, late flowering. Letters and numbers denote populations in Estonia and England, respectively; the remaining five samples are: F – French, I – Italian, G – Georgian.

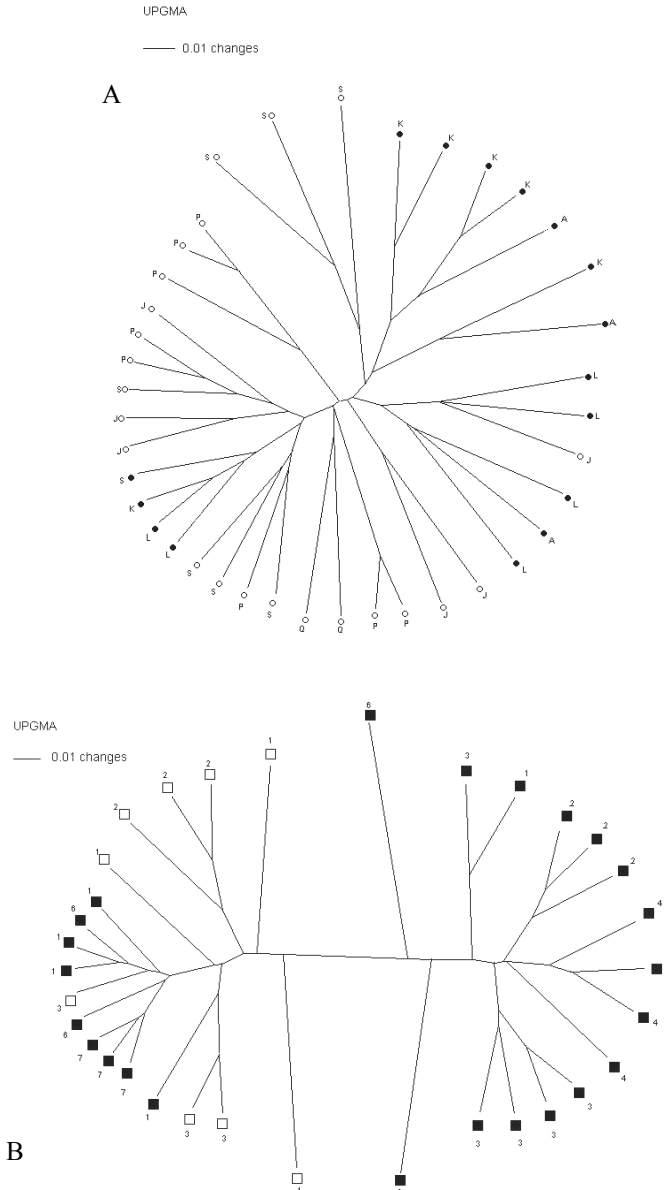
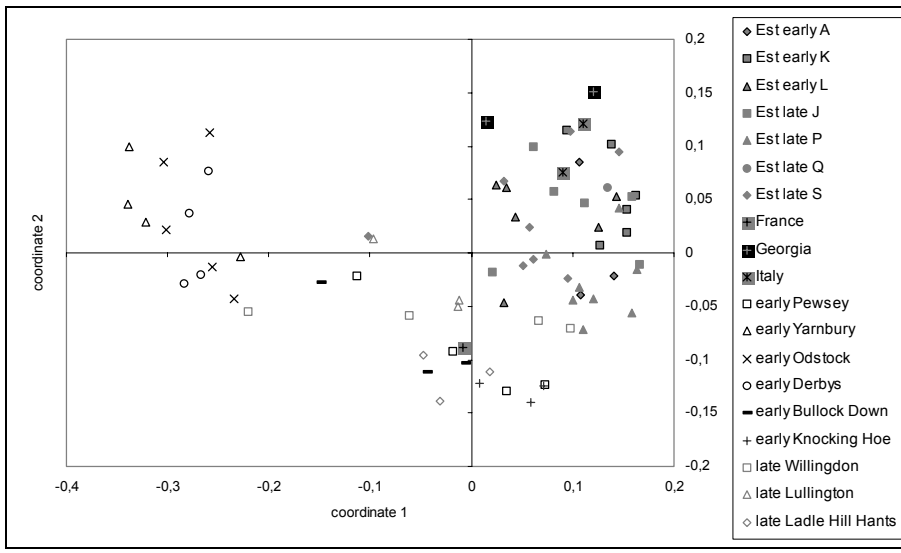


Figure 3. (A) Unrooted phylogram of the Estonian material. ○ – late-flowering populations; ● – early-flowering populations. Letters denote samples from different populations; (B) Unrooted phylogram of the English material. □ – late-flowering populations; ■ – early-flowering populations. Numbers denote samples from different populations.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Est late S
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Italy
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early Bullock Down
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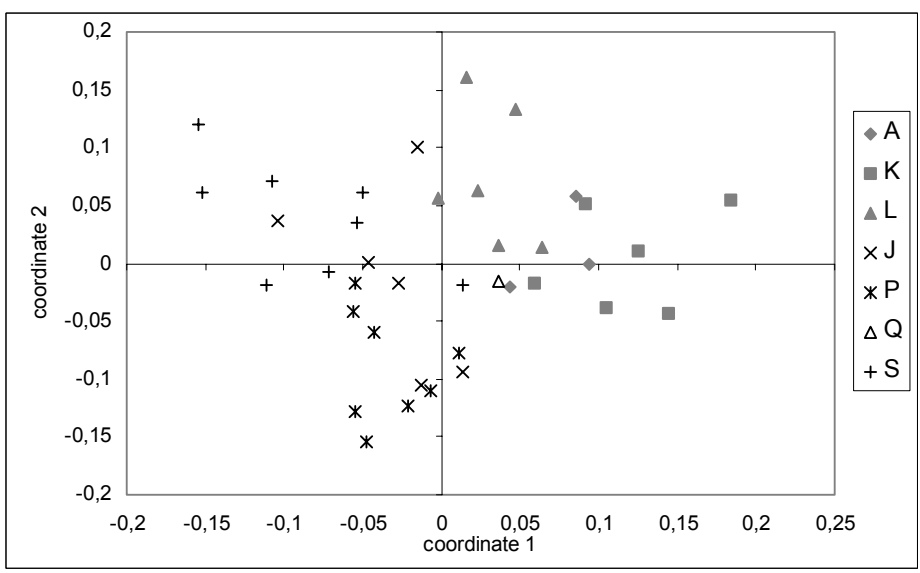
late Lullington
late Ladle Hill Hants

A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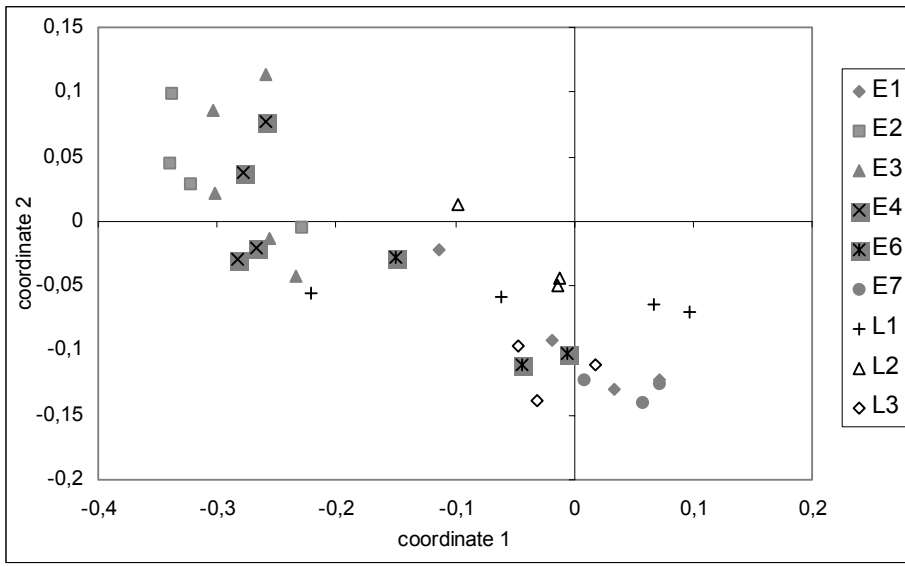

C

Figure 4. Principal coordinates plots of the first and second axes for the full data matrix (A), plus subsets of Estonian samples only (B) and English samples only (C).

Table 1. Details of sampled sites. Footnotes: [1] population included *ca* 5% albino plants; [2] considerable variation documented in floral morphology (Fig. 1); [3] population included a single plant of *N. ustulata* × *lactea*.

Popn Ref.	No. of samples	Taxon	Locality	Estimated peak flowering	No. of fl. plants	Geology	Aspect/habitat (human influence)
ENGLAND							
E1	12	ustulata	Walkers Hill, Pewsey Downs, Alton, N Wiltshire	22.05	100	Chalk	SE-facing short turf (lynchets, nearby hill fort)
E2	5	ustulata	Yarnbury Castle, Winterbourne Stoke, S Wiltshire	26.05	200	Chalk	SE-facing short turf (hill fort)
E3	5	ustulata	Odstock Down, Odstock, SW Salisbury, S Wiltshire	18.05	30	Chalk	W-facing short turf
E4[1]	5	ustulata	Above Hopton Quarry, Middleton, Derbyshire	04.06	500	Carboniferous limestone	SW-facing short turf
E6	6	ustulata	Bullock Down, W Beachy Head, E Sussex	26.05	10	Chalk	NW-facing short meadow
E7	4	ustulata	Knocking Hoe, Shillington, Bedfordshire	01.06	100	Chalk	NE-facing short turf (lynchets)
L1	4	aestivalis	Combe Hill, Willingdon, E Sussex			Chalk	
L2	4	aestivalis	Lullington Hill, Litlington, E Sussex			Chalk	
L3[2]	3	aestivalis	Ladle Hill, Old Burghclere, N Hampshire	15.07	180	Chalk	S+E-facing short turf (hill fort)
ESTONIA							
A	3	ustulata	Aljava, Muhu island	06.06	3	Sandy	open seaside grassland
K	6	ustulata	Kapi, Muhu island	06.06	80	Limestone	rocky pine/ juniper scrub
L	7	ustulata	Lõetsa, Muhu island	06.06	20	Limestone	juniper scrub
J	6	aestivalis	Jäneda, Lääne-Viru county	25.07	30	Sandy	young pine forest
P	8	aestivalis	Pilguse, Saaremaa island	20.07	25	Limestone	open scrub
Q	2	aestivalis	Kõmsi, Läänemaa county	20.07	2	Limestone	open scrub
S	9	aestivalis	Sillukse, Läänemaa county	20.07	25	Limestone	pine forest

Table 1. (continued)

ITALY							
I1[3]	1	ustulata	Mt San Angelo–Pulsano Abbey, W Mattinata, Gargano	22.04	50	Limestone	S-facing rocky turf (terraces)
I2	1	ustulata	SE San Salvatore turnoff, NW Manfredonia, Gargano	18.04	10	Limestone	W-facing rocky open scrub (terraces)
FRANCE							
F	1		Below Nalzen		–	–	–
GEORGIA							
G	2	ustulata	East of Tbilisi		–	–	–

Table 2. Numbers of variable and fixed bands used for the analysis generated from each primer combination.

MseI	CTAC	CTAC	CTAC
EcoRI	ACT	ACG	ACC
Number of variable bands	34	20	15
Number of fixed bands	13	4	3
Total	47	24	18