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ORIGINAL PAPER

## Differentiation in morphology and flowering phenology between two *Campanula thyrsoides* L. subspecies

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**Abstract** Subspecies are usually characterised by sets of morphological discontinuities. By means of common garden experiments, we investigated genetic differentiation in morphological and phenological traits in two geographically disjunct subspecies of *Campanula thyrsoides* L., i.e. subsp. *thyrsoides* (= *C.\* thyrsoides*) occurring in the European Alps and Jura Mountains, and subsp. *carniolica* (= *C.\* carniolica*) occurring in the Southeastern Alps and the Dinaric Arc. Nine out of 16 investigated traits were significantly different between *C.\* thyrsoides* and *C.\* carniolica*. For *C.\* carniolica* inflorescence length was 1.4×, and above-ground biomass 2.7× higher, while flower density was significantly lower. *Campanula\* carniolica* also showed delayed flowering and flower development from bottom to top as compared to *C.\* thyrsoides* which flowered from top to bottom. The inflorescence growth was indeterminate and flowering took several weeks in *C.\* carniolica*, whereas *C.\* thyrsoides* showed determinate flowering, rapidly opening all flowers within a few days. This differentiation in flowering phenology is likely to be adaptive. The submediterranean climate favours indeterminate flowering in *C.\* carniolica*, allowing ongoing

growth of the inflorescence throughout the long summer until environmental conditions worsen, whereas determinate and early flowering in *C.\* thyrsoides* is favourable in the short growing season in the high Alps where seed production must be secured before temperature drops. Glacial survival in refugia with different climates (alpine vs. submediterranean) may have caused this regional differentiation.

**Keywords** *Campanula thyrsoides* subsp. *carniolica* · Common garden · Determinate flowering · European Alps · Glacial history

### Introduction

Subspecies are usually characterized by sets of morphological discontinuities but incomplete reproductive isolation (Stuessy 1990). The European Alps are notably rich in endemics, whether at species or subspecies level (Aeschimann et al. 2004), suggesting that speciation rate is high as compared to the surrounding lowland. Glacial history has likely played a major role in taxon differentiation within the European Alpine flora, due to lineage divergence during survival in isolated refugia (Comes and Kadereit 2003; Tribsch and Schönschwetter 2003; Paun et al. 2008). Additionally, the spatial heterogeneity of the Alps in terms of topography, geology and regional climate may have caused local or regional adaptation of the various lineages (Alvarez et al. 2009; Stöcklin et al. 2009; Scheepens et al. 2010; Winkler et al. 2010). The last decade experienced a wave of studies investigating differentiation of Alpine species with a focus on glacial history as the driver of neutral genetic differentiation within and among taxa (Schönschwetter et al. 2005; Gugerli et al. 2008). While strong genetic barriers

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between intraspecific groups of populations have been widely detected, these molecular studies usually do not extend to differentiation at the phenotypic level and thus may overemphasize the role of neutral processes in differentiation of related taxa (but see Paun et al. 2008; Winkler et al. 2010). In this study on *Campanula thyrsooides*, we hypothesise the presence of glacial history-related phenotypic differentiation between the subspecies in traits observed in two common gardens and we discuss whether these patterns could be due to adaptation.

*Campanula thyrsooides* L. (Campanulaceae) is a monocarpic, yellow-flowering bell flower occurring predominantly on calcareous grassland slopes from 1,600 to 2,200 m a.s.l. in the European Alps (Kuss et al. 2007). Seeds have a low dispersal capacity and populations are rare and isolated but sometimes harbour high numbers of individuals, most of them as rosettes and some conspicuously flowering (Kuss et al. 2007). Within-population genetic diversity is high and among-population differentiation is substantial (Kuss et al. 2008a; Ægisdóttir et al. 2009). Its distribution ranges across the European Alps and the Jura Mts. with patchy occurrences in the Dinaric Arc (Kuss et al. 2007), but its occurrence is sparse in the Dolomites and Tyrol. To the west of this distribution gap the subspecies *Campanula thyrsooides* subsp. *thyrsooides* (*C.\* thyrsooides*) occurs, and to the southeast of this gap, in the Southeastern Alps and the Dinaric Arc, the majority of populations can be classified to the other subspecies, *Campanula thyrsooides* subsp. *carniolica* (*C.\* carniolica*). It is unknown if the two subspecies are interfertile and produce viable offsprings.

The Southeastern Alpine subspecies *C.\* carniolica* was first described as a variety by Sündermann (1925) who observed it to be taller than *C.\* thyrsooides* due to an elongated inflorescence. Flower density was reported to be more lax in the lower part of the inflorescence as compared to *C.\* thyrsooides*, and its bracts were found to be up to double the length of those of *C.\* thyrsooides*. The time of flowering was observed to be delayed for *C.\* carniolica* as compared to *C.\* thyrsooides*, and Jäger (2000) observed that *C.\* thyrsooides* flowered in July and August and *C.\* carniolica* in the first half of August, thus partly overlapping in flowering. The varieties were ranked as subspecies by Podlech (1964) based on the marked difference in their geographical distribution in addition to the morphological and phenological characteristics described above: in contrast to the alpine to subalpine *C.\* thyrsooides*, *C.\* carniolica* occurs at submontane to montane elevations (400–1,800 m a.s.l.) and is confined to the Carnic area in the broad sense, which includes parts of Italian Carnia and Slovenia (Carnian and Julian Alps) as well as Austrian Carinthia (Jäger 2000; Kuss et al. 2007 and references therein). It has been speculated that these two subspecies are altitudinal vicariants (Tomažič 1941) though the elongated inflorescence of *C.\* carniolica*

may also be the result of adaptation to the submediterranean climate (Kuss et al. 2007).

Jäger (2000) suggested that the geographic partitioning might be a result of glacial history, the two subspecies having survived glaciated periods in different refugia on the fringes of the Alps. A microsatellite study investigating neutral genetic differentiation among 51 populations of *C. thyrsooides* across the Alps increased the understanding of the effects of glacial history on intraspecific differentiation (32 Swiss populations in Ægisdóttir et al. 2009; 51 populations in Kuss et al., unpublished results). The among-population genetic structure showed four spatial clusters across the Alps, which corresponded to the general phylogeographic pattern observed in several widespread Alpine species (Schönswetter et al. 2005; Alvarez et al. 2009; Thiel-Egenter et al. 2009). The most eastern cluster of these belonged to *C.\* carniolica* and was clearly separated from the other three to the west, belonging to *C.\* thyrsooides*. Analysis of molecular variance (AMOVA) showed that differentiation between the subspecies *C.\* carniolica* and *C.\* thyrsooides* (i.e. the single eastern versus the three western clusters taken together) explained 8.4% of the variation. This was higher than differentiation between clusters within *C.\* thyrsooides* and it was even higher than the partitioning of variation among all four distinct groups of populations, i.e. 6.7%. The genetic structure therefore supports the subspecies division of Podlech (1964) and is in line with Jäger's (2000) suggestion that glacial survival in separate refugia is at the origin of the subspecies division.

Morphological differences allow to identify the two subspecies in the field, but a study on their quantitative differences in various traits, however, has never been conducted. Botanists have sometimes found that presumed subspecies phenotypes are merely due to environmental differences, so it is important to be aware of environmental effects on phenotypic expression when taxonomy is concerned (Sultan 2000). To make sure that a quantitative analysis of morphological and phenological differences reflects genetic differences and not the influence of the environment, the subspecies can be grown together in a common garden (Clausen et al. 1948). The uniform environmental conditions in a common garden eliminate variation due to the environmental component, leaving only genetic differences (or rather genotype × environment expressions based on a single environment) between the subspecies to be observed (Connor and Hartl 2004).

In this study, we have chosen traits that are used to differentiate between the subspecies (inflorescence height, number of flowers per inflorescence length, flowering phenology) as well as traits that have not been investigated so far (e.g., leaf size, number of leaves, number of flowers, above-ground biomass). Specific leaf area (SLA) and leaf thickness have been measured because they are known to be

differentiated between the subspecies (Scheepens et al. 2010). Since *C.\* carniolica* generally occurs on roadsides and on rock outcrops, as opposed to *C.\* thyrsoides* which mainly occurs in managed grasslands, it is likely that grazing regimes differ between the subspecies, which may have led to adaptive differentiation in response to grazing between the subspecies. Therefore, we assess the response to simulated herbivory in *C.\* thyrsoides* and *C.\* carniolica* observed in one of the two common garden experiments. Additionally, the common garden results are compared with field data to see if patterns of quantitative differentiation match between the common garden and the field. In summary, we ask (1) whether the subspecies *C.\* thyrsoides* and *C.\* carniolica* show glacial history-related differentiation in quantitative traits and (2) whether patterns of differentiation could be explained as adaptation to the subspecies' respective environments.

## Methods

### Experimental design

Two common garden experiments were performed in the Swiss Alps in order to obtain data on 16 quantitative traits. The seed sources of these experiments were populations which have been investigated as part of a phylogeographic study and for which spatial genetic information is available (Ægisdóttir et al. 2009; Kuss et al., unpublished results). The first experiment was located in Davos (1,530 m a.s.l.) and herbivory was simulated by clipping half of the plants 8 weeks after transplantation. This experiment included seed-derived plants from 17 populations of *C.\* thyrsoides* and 4 populations of *C.\* carniolica*. Six seed families per population and eight individuals per seed family made a total of 963 individuals at the beginning of the experiment (45 individuals were missing from the start).

The second experiment near Chur included three common gardens at different elevations along the slope of Mt Calanda, Switzerland (600, 1,235, 1,850 m a.s.l.). This experiment included seed-derived plants from 12 populations of *C.\* thyrsoides* and 6 populations of *C.\* carniolica*. A range of 2–7 (median 7) seed families per population and 2–12 (median 6) individuals per seed family added up to 600 individuals. For both experiments, seeds were germinated in the greenhouse during autumn 2007 and plants were transplanted to the common gardens of Davos and Chur in late spring and early summer 2008, respectively. In total, 15 out of 24 sampled populations were represented in both experiments. Blocks were initially incorporated in both common gardens, but these were never significant and therefore not considered in subsequent analyses. Locations, altitudes and sample size of populations are given in Online Resource 1.

More detailed descriptions of the design of the Chur experiment are given by Scheepens et al. (2010).

### Common garden measurements

Eight weeks after transplantation to Davos, survival was recorded and leaf thickness was measured (Teclock SM-112 dial thickness gauge, Okaya, Japan). Number of leaves was counted at the end of the growing season 2008. Length and width of longest leaf were measured at the end of the growing season 2008 as well as at the start of the growing season 2009. Reproductive traits were quantified halfway of the second growing season 2009, i.e. number of inflorescences, total inflorescence length, maximum inflorescence height, total number of flowers on all inflorescences, maximum number of flowers on an inflorescence, and number of flowers per inflorescence length (concerning the flower-bearing part of the inflorescence). Finally, when a plant had finished flowering in 2009, aboveground biomass was harvested, dried for 72 h at 60°C, and weighed. Only data from unclipped plants were used (477 individuals), except for data on the response to the herbivory simulation.

Data on SLA and leaf hair density were obtained from the Chur experiment. Details on the measurement of SLA are described in Scheepens et al. (2010). Leaf hair density was measured per individual as the mean number of leaf hairs on five randomly chosen areas of 0.25 cm<sup>2</sup> on the topside of leaves. For SLA and leaf hair density, mean values for both subspecies were calculated via population means, which in turn were based on seed family means. For SLA, population averages were calculated for each elevational site, which were subsequently averaged over all three sites. Leaf hair density data were analysed without respect to altitudinal origin.

The response of selected traits to simulated herbivory was expressed as the proportional difference of clipped plants to control plants at the seed family level and averaged at the population level. A value of 1 thereby indicates absence of response in clipped plants relative to the control plants (i.e. full recovery after clipping). The three elevational sites in Chur were used to investigate the response of SLA to the elevation treatment, which was measured as the coefficient of variation at the population level and based on seed family averages.

### Field measurements

The common garden results for inflorescence height, maximum number of flowers per inflorescence as well as number of flowers per inflorescence length were compared with those from field data obtained in 2006 from populations of both *C.\* thyrsoides* and *C.\* carniolica* in Slovenia, Italy and Austria. Percentage of withered flowers was estimated in

these natural populations in order to investigate phenological differences between the subspecies. Here, the number of withered flowers was divided by the total number of flowers (including buds).

### Data analysis

Subspecies comparisons were performed for all traits for both common garden and field data using non-parametric Mann–Whitney *U* tests, which do not rely on normality of the data and are more robust to outliers than Student's *t* tests (Quinn and Keough 2002). We did not use any correction for multiple testing (e.g., sequential Bonferroni correction; Holm 1979), since most traits showed conspicuous though moderately significant differences between the subspecies, which would be eliminated by a multiple-testing correction (Moran 2003). All comparisons were based on population means, which in turn were based on seed family means for the common garden data. The coefficient of variation was calculated for each trait among populations within each subspecies, in the common garden as well as in the field, to investigate whether the two subspecies differed in variability and whether common garden and field sites differed in variability.

## Results

### Common garden measurements

Nine out of 16 traits were significantly different between *C.\* thyrsoides* and *C.\* carniolica* (Table 1). Post-plantation survival did not differ significantly between the subspecies. Leaves were on average 10% thicker and SLA was on average 14% lower in *C.\* carniolica*. In fact, SLA was lower at all experimental elevations for *C.\* carniolica* as compared to *C.\* thyrsoides* (data not shown, see Scheepens et al. 2010). Leaf hair density was 53% higher in *C.\* carniolica* compared to *C.\* thyrsoides*, but this difference was not significant. Rosettes of *C.\* carniolica* had 25% fewer leaves. During the following year, flowering *C.\* carniolica* plants had few rosette leaves or none at all, whereas flowering *C.\* thyrsoides* plants showed a full (although withering) rosette (J.F. Scheepens, personal observation from both experiments). In both late and early season (i.e. measurements from the first and the second season, respectively), full-grown leaves were remarkably longer and wider in *C.\* carniolica*, although width was not significantly different between the subspecies. The number of inflorescences did not differ statistically, but tended to be higher in *C.\* carniolica*. The cumulative lengths of inflorescences was 1.8 times higher and the maximum inflorescence length was 1.4 times higher in *C.\* carniolica*.

The total number of flowers on all inflorescences as well as the maximum number of flowers on an inflorescence did not differ significantly between the subspecies, but tended to be lower in *C.\* carniolica*. The number of flowers per length of inflorescence, however, was significantly lower for *C.\* carniolica*. Finally, above-ground biomass was about 2.7 times higher in *C.\* carniolica*.

Variation in trait values was considerably higher in *C.\* carniolica* compared to *C.\* thyrsoides* (>5%; Table 1) for the following traits: leaf width at end of season, total number of flowers on all inflorescences and number of flowers per inflorescence length. By contrast, considerably lower variation (<5%) was found for leaf hair density, number of inflorescences and aboveground biomass.

Response to simulated herbivory was indifferent between subspecies for most of the selected traits: number of leaves, leaf length and width in both seasons, total inflorescence length and maximum inflorescence height showed comparable variability in the two subspecies (Table 2). *Campanula\* carniolica* showed a significantly larger reduction in number of inflorescences after clipping (−15%;  $P = 0.04$ ) and a marginally significant larger reduction in aboveground biomass after clipping (−21%;  $P = 0.07$ ). *Campanula\* carniolica* showed a less strong reduction in the number of flowers due to clipping as compared to *C.\* thyrsoides*, but this difference was not significant (+11%;  $P = 0.26$ ). The response of SLA to elevational treatments did not differ between the subspecies (mean  $\pm$  se of population-level coefficient of variation (%): *C.\* thyrsoides*  $12.4 \pm 1.0$ ; *C.\* carniolica*  $12.3 \pm 1.6$ ;  $P = 0.89$ ).

### Field measurements

The field data showed similar differences between the subspecies as did the common garden experiments, but the field and common garden data differed quantitatively (Tables 1, 3). In natural populations, the inflorescence height was 1.7 times higher in *C.\* carniolica* as compared to *C.\* thyrsoides*. Comparably, in the common garden of Davos the maximum inflorescence height was 1.4 times higher in *C.\* carniolica* (Table 1). Whereas the relative differences were similar between the subspecies, the average heights of 24 and 40 cm for *C.\* thyrsoides* and *C.\* carniolica* in the field were smaller than in the common garden, where average heights of 39 and 54 cm were observed, respectively. The number of flowers did not differ between the two subspecies in the natural populations, in accordance with the results from the common garden. The number of flowers per inflorescence length was 30% lower in *C.\* carniolica* both in the field and the common garden, but the absolute values in the field were smaller than in the common garden. The percentage of withered flowers was only measured in the

**Table 1** Mean, standard error (se), and coefficient of variation (cv) for 16 traits of two subspecies of *Campanula thyrsoides* L., as well as quantitative differences between subspecies (%) and significance levels (*P*) based on Mann–Whitney *U* tests

	<i>Campanula* thyrsoides</i>			<i>Campanula* carniolica</i>			%	<i>P</i>
	Mean	se	cv (%)	Mean	se	cv (%)		
Survival (proportion)	0.88	0.05	22.2	0.77	0.09	22.5	–12.5	0.33
SLA (cm <sup>2</sup> g <sup>-1</sup> )	160.9	2.4	5.1	137.8	3.9	6.9	–14.3	<0.001
Leaf thickness (mm)	0.486	0.006	5.1	0.534	0.021	7.9	9.9	<0.05
Leaf hair density (cm <sup>-2</sup> )	15.2	3.1	70.3	23.3	4.7	49.6	53.3	0.18
Number of leaves	131.2	6.8	21.2	97.5	10.7	22.0	–25.7	<0.05
Leaf length end of season (cm)	7.20	0.24	13.8	9.92	0.87	17.5	37.8	<0.01
Leaf width end of season (cm)	2.65	0.08	12.5	3.31	0.37	22.6	24.9	0.08
Leaf length start of season (cm)	11.3	0.4	14.6	14.1	1.0	13.9	24.8	<0.01
Leaf width start of season (cm)	1.58	0.05	12.7	1.92	0.16	16.8	21.5	0.07
Number of inflorescences	3.80	0.40	43.2	4.69	0.31	14.0	23.4	0.41
Total inflorescence length (cm)	176	14	31.7	317	52	32.6	80.1	<0.05
Maximum inflorescence height (cm)	39.2	1.3	14.0	54.3	3.8	14.2	38.5	<0.01
Total number of flowers on all inflorescences	348	25	29.8	276	70	50.8	–20.7	0.32
Maximum number of flowers on inflorescence	102.8	4.3	17.1	86.0	9.2	21.6	–16.3	0.17
Number of flowers per inflorescence length (cm <sup>-1</sup> )	6.67	0.13	8.2	4.96	0.44	17.9	–25.6	<0.001
Above-ground biomass (g)	25.7	2.5	40.4	69.5	5.0	14.4	170.4	<0.001

**Table 2** Response to simulated herbivory of two subspecies of *Campanula thyrsoides* L. Given are mean, standard error (se) and coefficient of variation (cv) in ten traits, as well as quantitative differences between subspecies (%) and significance levels (*P*) based on Mann–Whitney *U* tests

	<i>Campanula* thyrsoides</i>			<i>Campanula* carniolica</i>			%	<i>P</i>
	Mean	se	cv (%)	Mean	se	cv (%)		
Number of leaves	0.82	0.02	9.7	0.83	0.03	7.8	0.78	1.00
Leaf length end of season	0.85	0.01	6.6	0.83	0.03	7.4	–3.20	0.44
Leaf width end of season	0.95	0.02	5.2	0.91	0.03	3.5	–4.48	0.34
Leaf length start of season	0.84	0.01	8.7	0.81	0.01	6.9	–3.70	0.18
Leaf width start of season	0.99	0.01	5.4	0.98	0.03	5.2	–1.16	0.56
Number of inflorescences	0.91	0.03	13.7	0.77	0.02	5.0	–14.79	<0.05
Total inflorescence length	0.88	0.04	18.8	0.86	0.06	14.8	–2.86	1.00
Maximum inflorescence height	0.99	0.02	9.5	0.95	0.04	9.5	–4.05	0.75
Total number of flowers	0.79	0.04	19.3	0.87	0.06	14.0	10.85	0.26
Above-ground biomass	0.84	0.07	34.2	0.66	0.02	5.4	–21.10	0.07

field and was significantly lower for *C.\* carniolica* than for *C.\* thyrsoides*, since populations from the latter finished flowering already at the time of measurement (Table 3).

### Phenology

In the common garden in Davos, *C.\* carniolica* showed a delayed flowering as compared to *C.\* thyrsoides*. At the start of the second season (1 June 2009) on average 89% of surviving *C.\* thyrsoides* plants had already started bolting, whereas *C.\* carniolica* plants showed no signs of bolting yet. On 27 July 2009, 93% of *C.\* thyrsoides* plants had finished flowering and were in their seed ripening stage and

ready to be harvested, whereas 83% of *C.\* carniolica* plants were now fully flowering and 7% ripening. Harvesting of *C.\* carniolica* took place on 20 October 2009 when snow and frost were abundant. However, *C.\* carniolica* plants were still flowering or had just started ripening, indicating that they were not adapted to the short growing season at this elevation.

Inflorescences of *C.\* carniolica* flowered from the bottom to the top, whereas *C.\* thyrsoides* flowered from the top to the bottom. More specifically, flowering in *C.\* carniolica* started where the lowest flowers were still closely connected to the inflorescence stem, thus without pronounced secondary stem formation. Below this point secondary stems

**Table 3** Locations and means of four traits in two subspecies of *Campanula thyrsooides* L. from field data

Population (number of individuals)	Subsp.	North	East	Elevation (m a.s.l.)	Inflorescence height (cm)	Number of flowers	Flowers per cm inflorescence	% flowers withered
Rüfikopf (20)	<i>thyr</i>	47°12'00"	10°10'08"	2,307	25.6 ± 1.2	69.4 ± 4.9	4.6 ± 0.2	100
Elmer Kreuzspitze (7)	<i>thyr</i>	47°20'41"	10°34'34"	1,874	22.3 ± 0.9	43.8 ± 5.9	4.7 ± 0.5	100
Rinnen (13)	<i>thyr</i>	47°24'23"	10°42'46"	1,215	23.3 ± 1.2	28.7 ± 2.7	3.4 ± 0.2	100
Hintertux (15)	<i>thyr</i>	47°06'46"	11°39'05"	2,011	25.3 ± 2.3	58.9 ± 6.3	4.9 ± 0.4	89
				mean ± se	24.1 ± 0.3	50.2 ± 8.9	4.4 ± 0.3	97.3 ± 2.8
				cv (%)	6	35	15	6
Passo di Monte Croce Carnico (3)	<i>carn</i>	46°36'02"	12°57'05"	1,629	33.7 ± 1.8	47.7 ± 6.4	2.8 ± 0.4	72
Sella Nevea <sup>a, b</sup> (7)	<i>carn</i>	46°23'35"	13°27'46"	932	34.3 ± 4.0	45.0 ± 6.1	3.6 ± 0.3	47
Plesa (4)	<i>carn</i>	46°17'55"	13°58'54"	950	38.5 ± 9.8	49.5 ± 12.1	4.6 ± 0.2	57
Nemski Rovt <sup>a, b</sup> (11)	<i>carn</i>	46°23'35"	13°58'89"	663	23.1 ± 3.3	46.0 ± 4.2	3.2 ± 0.2	63
Postojna (19)	<i>carn</i>	45°49'37"	14°14'25"	515	27.1 ± 1.8	32.7 ± 2.2	2.5 ± 0.2	34
Sklendrovec <sup>b</sup> (22)	<i>carn</i>	46°06'22"	14°56'56"	339	49.6 ± 2.2	57.6 ± 3.2	2.8 ± 0.1	93
Brodnice <sup>a, b</sup> (16)	<i>carn</i>	46°06'24"	15°16'53"	283	56.3 ± 4.3	100.9 ± 21.2	3.0 ± 0.4	90
Vitanje <sup>a, b</sup> (18)	<i>carn</i>	46°22'27"	15°17'16"	422	55.4 ± 3.2	57.9 ± 4.3	2.5 ± 0.3	75
				mean ± se	39.8 ± 4.5	54.7 ± 7.2	3.1 ± 0.2	66.6 ± 7.2
				cv (%)	32	37	22	31
				% difference	65	9	-30	-31
				<i>P</i> ( <i>thyr.</i> vs. <i>carn.</i> )	<0.05	0.93	<0.05	<0.05

Given are population-level mean ± se or percentage, subspecies mean ± se and coefficient of variation (cv), as well as quantitative differences between subspecies (%) and significance levels (*P*) based on Mann–Whitney *U* tests. Subsp. *thyr*: *C. thyrsooides* subsp. *thyrsooides*; *carn*: *C. thyrsooides* subsp. *carniolica*

<sup>a</sup> Population also used in the common garden experiment in Davos; <sup>b</sup> Population also used in the common garden experiment near Chur (see Scheepens et al. 2010)

usually occurred, each with multiple flowers which opened later as well. Flowering was indeterminate in *C.\* carniolica*, so new buds were continuously being formed at the apex, and anthesis from bottom to top took several weeks. Consequently, fruits in different stages of ripening could be found below the open flowers. This is in contrast to *C.\* thyrsooides* which showed determinate flowering from the top, rapidly opening all flowers within a few days.

Figure 1 shows the most pronounced differences in the habitus of the two subspecies, including for *C.\* carniolica* (i) the taller inflorescence, (ii) the smaller number of rosette leaves in the flowering individual, (iii) the lax flower positioning, (iv) the thinner inflorescence and (v) the indeterminate flowering phenology.

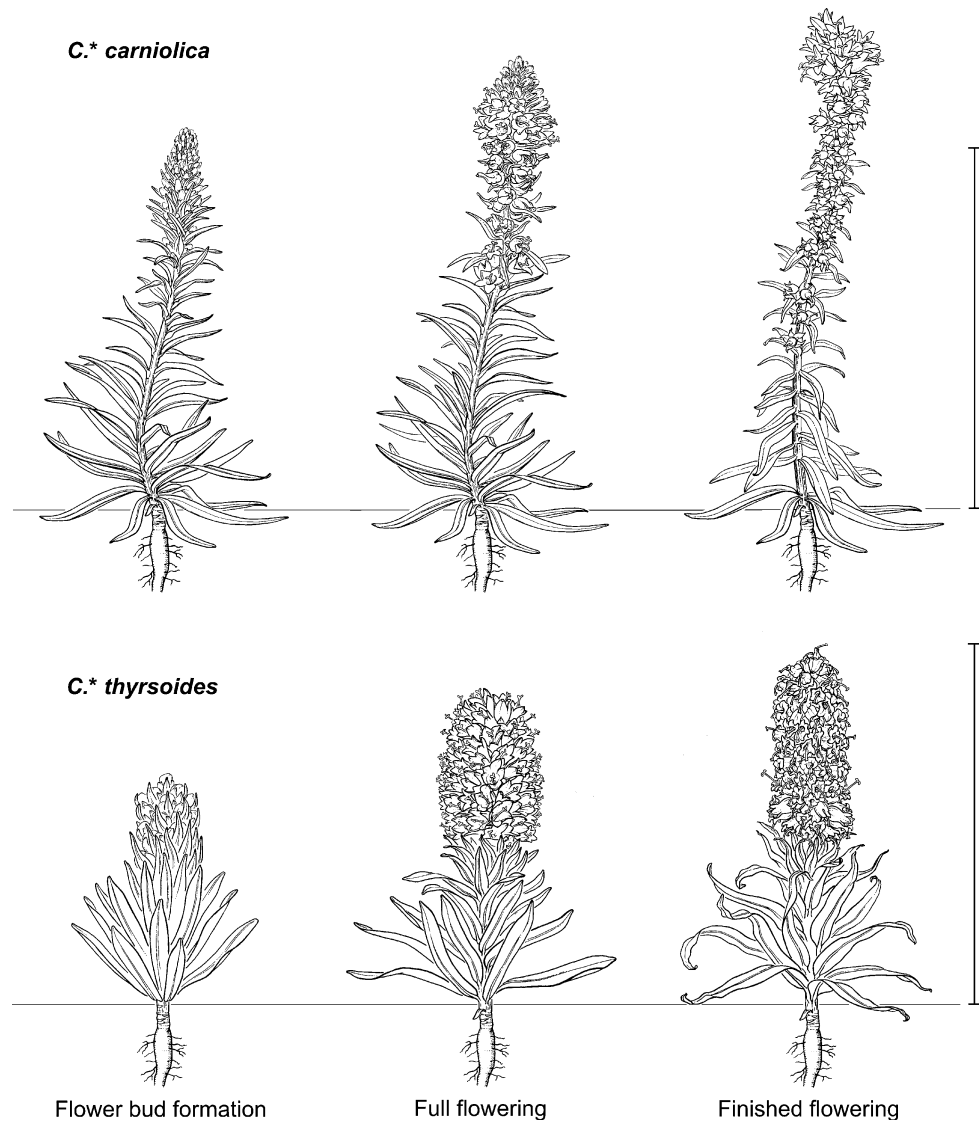
## Discussion

The common garden data support the subspecies division in *Campanula thyrsooides* since clear differences were observed between the two subspecies in the majority of the examined traits, among them traits used to distinguish the subspecies taxonomically such as inflorescence height (Table 1). Although we did not correct for multiple-testing, obtaining

*P* < 0.05 for nine out of 16 traits by chance has a probability of  $<1.6 \times 10^{-8}$  based on a Bernoulli process (Moran 2003). The morphological and phenological differences observed in the field were also observed in the common garden indicating genetic differences: *C.\* carniolica* showed a taller inflorescence height, a more lax flower positioning and delayed flowering (Tables 1, 3). In addition, the similar number of flowers in the two subspecies was observed in the common garden as well as in the field (Tables 1, 3). Although flower number was similar between subspecies, the advantage of a taller inflorescence in *C.\* carniolica* may lie in farther seed dispersal (Tackenberg et al. 2003; Tackenberg and Stöcklin 2008). Lower SLA and higher leaf thickness in *C.\* carniolica* have been explained as adaptation to drought in the submediterranean climate (Scheepens et al. 2010). Length and width of leaves showed that start-of-season leaves were more elongate than end-of-season leaves, which is in accordance with observations by Jäger (2000) who described the two-phased rosette growth of *C. thyrsooides*.

Maternal effects have often been reported to affect a variety of traits, but maternal effects are usually pronounced at the seedling stage while decreasing over time (e.g., Ouborg et al. 1991; Schmid and Dolt 1994). Since plantlets

**Fig. 1** Habitus of *Campanula thyrsoides* subsp. *carniolica* and subsp. *thyrsoides* in three phenological phases of flowering. Scale bars have the same length, indicating the relative size of the two subspecies. Drawing by Atelier Guido Köhler, Basel



were circa 6 months old at transplantation and since most traits were measured 2.5 months or more after transplantation, we assume that maternal effects are negligible, but cannot verify this. Initial maternal effects can be propagated through unequal intra- and interspecific competition, but a weeded common garden with ample space between experimental plants, as in our study, limits the enhancement of initial phenotypic differences. Genotype  $\times$  environment effects were likely to be small in our study, as traits that were measured in both common gardens (comparison not shown) and in the field showed similar patterns.

#### Common garden versus field measurements

The common garden and field data showed that observations from experimental and natural settings can diverge. This is most evident from the number of inflorescences: common

garden-grown individuals were predominantly multi-stemmed (90% in Davos, data not shown) in contrast to predominantly single-stemmed individuals under natural field conditions (88%, data not shown). It was most likely the benign conditions in the common garden, due to weeding and nutritious soil, which caused the growth of multiple inflorescences. Another notable difference between plants in the common garden and wild individuals is that the latter are reported to flower once after 3–16 years with an average of about 10 years (Kuss et al. 2008b), whereas the vast majority (93%) of the surviving experimental plants in Davos flowered already in their second year. Comparing the common garden results with the field observations also showed smaller inflorescence height, number of flowers and flowers per inflorescence length in the field. To conclude, on the one hand common gardens are an ideal method to detect and compare genetic differences between subspecies, whereas

on the other hand the obtained values cannot be used as descriptors to identify the subspecies in the field.

#### Coefficient of variation

Knowledge on intraspecific variability in plant traits is important as it could inform us about differences in niche breadth among taxa (Rotundo and Aguiar 2008; Milla et al. 2009; Albert et al. 2010). We found that coefficients of variation were remarkably similar for both subspecies in most traits, notably in those traits that differed significantly in their average values. Exceptions were number of flowers per inflorescence length and aboveground biomass, which differed both in their mean and coefficient of variation. This result indicates that variability in most traits among populations did not differ between the subspecies and may suggest that niche breadths of the two subspecies are similar. This is an interesting result, suggesting that trait variability is inherently constant (relative to the average) whereas average trait values can shift according to environmental conditions.

Another exception to the general pattern was the number of flowers in the common garden, which showed a much higher coefficient of variation for *C.\* carniolica*. This high variability could be due to the uncommon environment for this subspecies. The number of flowers at harvesting was probably largely dependent on time of flowering in this indeterminately flowering subspecies, with early flowering individuals yielding more flowers at harvesting than late-flowering individuals, resulting in high variability. In line with this argumentation, the coefficient of variation for the number of flowers of individuals in the field was similar for both subspecies.

Coefficients of variation showed a large range across traits, indicating that some traits (e.g., maximum inflorescence height) were more stable across populations and would therefore be more reliable as taxonomic indicators than others (e.g., biomass). Stronger environmental influences would be expected in the field, leading to increased variability. However, field measurements did not systematically show a higher coefficient of variation than the common garden measurements for the same traits.

#### Herbivory

Compensatory growth following herbivory is a general phenomenon in plants, but its extent can differ inter- and intraspecifically (Strauss and Agrawal 1999). This study showed that compensation in *C. thyrsooides* was generally strong and that the response to the herbivory simulation did not differ between the subspecies for most traits (Table 2), suggesting either (a) that any contrasting herbivory pressure

between the habitats of the two subspecies did not lead to genetic differentiation in these traits or (b) that the two habitats did not differ in herbivory pressure. Suzuki (2008) similarly found no differences in plasticity in response to clipping in a common garden experiment among three populations of the annual *Persicaria longisetata* with different long-term deer grazing histories, and Rotundo and Aguiar (2008) likewise observed similar tolerance to defoliation among three populations of *Poa ligularis* differing in recent sheep grazing histories, although other studies do report differences among varieties or closely related species in response to herbivory simulation (Welter and Stegall 1993; Westberg et al. 2010). It may be that herbivory thresholds, above which plant performance is differentially affected in the subspecies, have not been reached in our experiment (Strauss and Agrawal 1999).

#### Phenology

Differences in phenology between the subspecies may be hypothesised based on the contrasting environments they inhabit. As stated in the introduction, Sündermann (1925) reported *C.\* carniolica* to flower later than *C.\* thyrsooides*, and Jäger (2000) found that *C.\* thyrsooides* reached full flowering between July and August whereas *C.\* carniolica* reached full flowering in the first half of August. In line with these observations, *C.\* carniolica* flowered later than *C.\* thyrsooides* in our common gardens. However, the phenology as recorded from the common garden experiment must not necessarily represent the phenology under natural conditions, since the delayed flowering of *C.\* carniolica* may be the result of a genotype × environment interaction. From the literature it is known that, together with photoperiod and moisture, temperature is a major cue to flowering, usually observed in common garden experiments using plants from different latitudes (Rathcke and Lacey 1985; Weber and Schmid 1998; Olsson and Ågren 2002). If there would be a temperature threshold as cue to flowering in *C.\* carniolica*, this should then have occurred much later in the season at the high elevation of the common garden in Davos. The field observations were in agreement with the observed delayed flowering in the common garden, showing 97% of flowers of *C.\* thyrsooides* plants withered and 67% of flowers of *C.\* carniolica* withered (Table 3). It should be noted that three of the four *C.\* thyrsooides* populations were censused 1 month later than all other populations in the field study. However, the Hintertux population of *C.\* thyrsooides* was measured at the same time as the *C.\* carniolica* populations and had also finished flowering (89% withered), thereby contrasting with *C.\* carniolica* populations. Thus, the subspecies differ in phenology, with *C.\* carniolica* showing delayed flowering.



Strictly speaking, that *C.\* thyrsoides* plants in natural populations were already ripening when *C.\* carniolica* was still flowering in Slovenia indicates an earlier end of flowering in *C.\* thyrsoides* and not per se advanced flowering initiation. Nevertheless, delayed start of flowering at lower elevation for *C.\* carniolica* fits well with the phenology of alpine versus lowland populations, reflecting the different geographical distributions of the two subspecies. Advanced flowering at higher elevations could be explained as adaptation to the short growing season during which the plant needs to fulfill its life-cycle (Kudo 1993; Olsson and Ågren 2002; Sandring et al. 2007). Thus, the delayed flowering of *C.\* carniolica* in Slovenia's relatively low mountains could be explained as adaptation to the submediterranean climate of this area, but more evidence is needed to strengthen this claim. We speculate that this phenological mismatch between the two subspecies as observed in a common environment as well as in the field could essentially entail reproductive isolation, which is a key driver of speciation (Coyne and Orr 2004).

It could be hypothesised that the indeterminate flowering in *C.\* carniolica* versus the determinate flowering in *C.\* thyrsoides* is due to adaptation to climate. The submediterranean climate could favour indeterminate flowering in *C.\* carniolica*, because this would allow a longer flowering period throughout the long summer until environmental conditions would deteriorate, whereas determinate and fast flowering would be more favourable in the short growing season in the high Alps where seed production must be secured before temperatures drop.

Our common garden data also showed that *C.\* thyrsoides* populations reached full flowering simultaneously. The same was true for *C.\* carniolica* populations, which flowered simultaneously but later than *C.\* thyrsoides*. If it is true that natural populations of *C.\* thyrsoides* have a wide range in peak flowering, as is suggested by Jäger's (2000) broad range in flowering time, our data would then suggest a plastic response of flowering to climatic factors, where the common environment in Davos led to synchronous flowering. It could therefore be hypothesised that, although flowering phenology is genetically differentiated between the subspecies, climatic factors influence the timing of flowering within subspecies. This possibility is supported by results from the altitudinal experiment in Chur, where flowering phenology within subspecies was more strongly affected by elevation than by population identity (data not shown). This plasticity in flowering time would be advantageous in the heterogeneous environment of the Alps, enabling dispersed individuals to adapt plastically to their environment. To conclude, phenology is genetically differentiated between subspecies and phenology is phenotypically plastic within *C.\* thyrsoides*; both phenomena are potentially adaptive.

## Glacial history

Glacial history, besides adaptation to climate, is a likely candidate for allopatric differentiation between the two subspecies (Tribsch and Schönswetter 2003; Schönswetter et al. 2005). Considering the present geographical distributions of the two subspecies, differentiation during glacial survival is even likely to have widened the niche breadth of the species. A general Eastern Alpine refugium southwest of Vienna, either in the most-eastern part of the European Alps or in northwestern Slovenia (both calcareous bedrock), or in the lowland between these two regions (siliceous bedrock), has been proposed based on floristic (Merxmüller 1952, 1953, 1954) and genetic data (Schönswetter et al. 2005). Considering the present-day occurrence of *C.\* carniolica*, the region of northwestern Slovenia is a potential in situ refugium where precursors of *C.\* carniolica* could have survived. According to the microsatellite data (Kuss et al., unpublished results), at least three other refugia north of the Alps are candidates for the three remaining major phylogeographic groups forming *C.\* thyrsoides*. Jäger (2000) explained the differentiation between the two subspecies as the result of isolated glacial survival of *C.\* thyrsoides* in one or more colder refugia, to which the subspecies adapted with short, determinate flowering. It thus seems that Jäger (2000) considers *C.\* carniolica* to be closer to the ancestral species and *C.\* thyrsoides* as the strongly adapted subspecies, deduced from the ancestral species by survival in a high-elevation refugium. This proposition is in line with genetic data which shows that the differentiation between the two subspecies is likely older than the differentiation within *C.\* thyrsoides*, which suggests survival in climatically different refugia during the last glacial oscillation (Kuss et al., unpublished results). This glacial survival in contrasting habitats may therefore have resulted in the present allopatric distribution of the two subspecies.

Allopatric subspeciation can result from neutral processes or selection, and both usually go hand in hand (Jolivet and Bernasconi 2007; Schönswetter and Schneeweiss 2009, but see García-Verdugo et al. 2010). Traits that are strongly related to fitness are expected to adapt over time to differences in the environment. We speculate that the allopatric subspeciation of *C. thyrsoides* was initiated by glacial history, as this caused survival in separate refugia and subsequent recolonization of different regions of the Alps, whereas selection processes meanwhile caused differentiation in fitness-related traits between the respective habitats. In this study we reported genetic differentiation in several traits and we argued that some of these traits, such as flowering phenology, were likely due to adaptation to the environment. It is, however, not possible to conclude from our observations to what extent the differentiation is due to adaptation and drift. To discern between neutral genetic drift

and past selection pressures on the measured traits,  $Q_{ST}-F_{ST}$  comparisons could be conducted (McKay and Latta 2002).

## Conclusion

The subspecies status of *Campanula thyrsoides* subsp. *thyrsoides* and subsp. *carniolica* is corroborated by the differentiation in morphological and phenological traits observed in the common garden. From an evolutionary point of view, a quantitative description of trait variation is a precondition for an understanding of the evolutionary processes that caused the differentiation and the possible adaptive nature of the differences. The most conspicuous observation in this light is the difference in flowering behaviour between the two subspecies, *C.\* thyrsoides* showing determinate and *C.\* carniolica* indeterminate flowering. Determinate flowering in *C.\* thyrsoides* is likely to be adaptive in the short flowering season in the high Alps, whereas indeterminate flowering may allow *C.\* carniolica* to maximise fitness in the long submediterranean summers of Slovenia. This study presented an example where evolution of traits fits with the view that glacial history caused adaptive evolution through long-term survival in contrasting climates in refugia and/or during recolonisation.

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