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CHROMOSOME NUMBERS OF THE EDELWEISS, *LEONTOPODIUM* (ASTERACEAE, COMPOSITAE – GNAPHALIEAE)

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The genus *Leontopodium* (Pers.) R.Br. (Asteraceae, Compositae) is economically important for both pharmaceutical and horticultural purposes. This importance, however, has not led to a good understanding of species coherence and the delimitation of species. One fundamental aspect of a good understanding of a species is how many chromosomes it has and any possible indication of polyploidy. Here we present somatic chromosome numbers for 16 *Leontopodium* species, of which six are new for science. The results indicate basic chromosome numbers of $x = 6$, 8, 9 and 11, with $x = 8$ being most frequent among the species examined. While obviously including several distantly related lineages, the $x = 8$ species have distributions that are concentrated in the centre of diversity of the genus in southwest China. We identified two 'species-pairs' (*Leontopodium dedekensii–L. sinense* and *L. souliei–L. calocephalum*) in which the tetraploid species has more vigorous growth, but is confined geographically to the centre of diversity. The diploid species ascend to generally higher elevations and extend more towards the Tibetan Plateau. In contrast, our data also suggest range expansions in other polyploid species, such as the hexaploid *Leontopodium ochroleucum* extending into the mountains of Central Asia. Deviations from $x = 8$ are found at the edges of the wide Eurasian distribution of the genus. These may relate to subsequent range expansions into the Himalayas, northern Asia, the Far East, and a far disjunctive expansion to the mountains of Europe. This implies an increased ability of these species to colonise mountain floras and adapt to different environmental conditions. Thus, formation of higher ploidy levels in general might be significant for a successful radiation process.

Keywords. Alpine flora, biogeography, polyploidy, radiation, Sino-Himalayan flora, speciation.

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Introduction

Speciation in plants is the result of a complex series of evolutionary processes, with biotic and abiotic selection pressures acting upon taxa. The genus *Leontopodium* (Pers.) R.Br. (Asteraceae, Compositae), the edelweiss, is a genus in which species delimitation is in dispute, notably due to only limited knowledge of variation in their natural habitats. Similarly our knowledge is rather poor on the variability of taxonomically important characters, such as head construction, differential seed set, and morphological changes in achene and flower structure depending on the position in the flower head. According to the detailed monograph by Handel-Mazzetti (1927), the genus contains 41 species. Since then, however, new species have been described and previously recognised species have been synonymised. Today, up to 58 species are recognised (Anderberg, 1991; Bayer *et al.*, 2007; Russell *et al.*, 2013). Dickoré, in a wider but as-yet-unpublished taxonomic work and as part of his contribution to this paper, suggests a total of about 35 species. We follow Handel-Mazzetti (1927), along with Dickoré's improvements, as was also followed by Blöch *et al.* (2010). *Leontopodium* is economically important for both pharmaceutical and horticultural purposes (Safer *et al.*, 2011b) and its distribution range from the Himalayan region to the Alps raises a number of questions concerning biogeographical patterns, distribution ranges, species delimitations, reproduction, etc. The highest number of *Leontopodium* species occurs in a relatively limited region, comprising the mountains of southwest China (Hengduan Shan) and the eastern Himalayas. Two species are found in Europe, with the most prominent representative of the genus being *Leontopodium alpinum* Cass.

A considerable amount of ecological (Erhardt, 1993; Vigneron *et al.*, 2005; Grabherr, 2009), medical and pharmaceutical research (Dobner *et al.*, 2003; Schwaiger *et al.*, 2004, 2005, 2006; Li *et al.*, 2006; Reisinger *et al.*, 2009; Safer *et al.*, 2011a) has focused on species of *Leontopodium*. Despite this high profile, however, molecular and phylogenetic studies on *Leontopodium* are scarce (Blöch, 2005; Blöch *et al.*, 2010; Safer *et al.*, 2011b). Although knowledge on chromosome numbers of *Leontopodium* species is fragmentary, several different numbers have been reported (see ICNA database; IPCN; Darlington & Janaki Ammal, 1945; Darlington & Wylie, 1955; Meng *et al.*, 2012; Russell *et al.*, 2013).

As chromosome numbers can be useful for interpreting systematic and phylogenetic relationships (e.g. Loureiro *et al.*, 2007; Galbany-Casals & Romo, 2008), we have analysed 16 different species in *Leontopodium* and report the results here. However, it is not easy to extrapolate systematic or taxonomic conclusions from the results because it is not clear why, and if, intraspecific variability is so high in the genus. We therefore deliberately avoid a discussion on the biological implications of our study as the diversity of the existing studies does not yet allow for an ultimate and clear conclusion. The high diversity of chromosome base numbers, even within a species, leaves the impression that the base number itself is not a useful character for phylogenetic reconstructions as it might not carry any biologically relevant

information. Here we simply point to some possible implications of the variability in the ploidy levels.

Materials and Methods

The study was carried out on 16 species of *Leontopodium* (Table 1), comprising 15 Asian and one European species. Seed from wild populations was collected and cultivated under identical conditions at the Botanical Garden of Justus-Liebig-Universität Giessen, which has the world's most comprehensive collection of *Leontopodium* plants with more than 20 species. Figure 2 indicates the localities of the wild material. In most cases multiple accessions (in total 72) of each species were investigated; here we present the data from unambiguous counts on referenced material from the Botanical Garden, Giessen. We collected three individuals per accession in 2009. Voucher specimens are kept as living plant collections in the Botanical Garden, Giessen, as well as herbarium specimens that have been made from these same plants. The material, grown from seeds as indicated in Table 1, was examined and assigned to species by Bernhard Dickoré (Munich), Michael Jaeger and Sebastian Stille (both Giessen). Only material corresponding to the species descriptions was used for the analysis to avoid samples of unknown hybridogenic garden origin.

Root tips of the collected plants were harvested for chromosome counts between 4:45 and 6:00 a.m. This was the period in which *Leontopodium* root tips showed mitotic activity, similar to observations for other plants (Cota & Philbrick, 1994; Butorina & Do, 2008).

Excised root tips were immediately treated with 0.05% aqueous colchicine for 6–10 hours at room temperature. Root tissues were then fixed in 96% ethanol-glacial acetic acid (3:1, v/v) and stored at +4°C. Acetic acid (45%) was used to macerate the root tips at 70°C for 15 minutes. After repeated rinsings the root tips were stained with haematoxylin for 10 minutes at room temperature and rinsed again repeatedly. Immediately before microscopic preparation, samples were shaken at 110 rpm for 5–12 hours to allow for an even distribution of the chromosomes within the cells. The root tips were then squashed on microscope slides, mounted in glycerol with 0.1% paraformaldehyde (PFA), and the preparation was sealed with dental wax.

Root-tip preparations were observed with a Leitz Laborlux S microscope at 1000×. Cells in which chromosomes were counted were documented with a digital camera (Lumix FS3, Panasonic). If necessary, several pictures of a single cell were taken to account for chromosomes in successive image planes (Power Shot S5IS, Canon) (Fig. 1). For documentation the outlines of the chromosomes were marked on the digital images with Corel Draw Version X3. The small size of the contracted chromosomes, due to the method of colchicine treatment, often hindered the exact counting of mitotic chromosomes, especially for cells with high chromosome numbers. Only unambiguous chromosome counts were used for data acquisition.

On average, 10–20 root tips per accession were needed to find a sufficient number of good mitotic cells. Chromosome numbers were counted in four cells per accession

TABLE 1. Chromosome numbers (2*n*) of 16 *Leontopodium* species in sections *Nobilia* Hand.-Mazz. (N) and *Leontopodium* (L), with inferred basic chromosome number (*x*), ploidy level according to the inferred basic chromosome number, accession number in the Botanical Garden, Giessen, and the area of origin. Asterisks indicate collections from the wild rather than garden origin

Section	Species	2*n*	*x*	Ploidy level	Accession no.	Area of origin of the voucher
L	*L. calocephalum* (Franch.) Beauverd	32	8	4*x*	08-13* (J56)	China, Sichuan, Min Shan
N	*L. dedekensii* (Bureau & Franch.) Beauverd	16	8	2*x*	07-563* (J44)	China, Yardong valley, Yü Chu tributary
L	*L. discolor* Beauverd	24	6	4*x*	07-365 (J16)	Russian Federation, Sakhalin, Tymorsky Distr.
N	*L. franchetii* Beauverd	32	8	4*x*	07-560* (J50)	China, NW Yunnan, Zhongdian
N	*L. haplophylloides* Hand.-Mazz.	38	?	?	07-526* (J11)	China, Sichuan, Shaluli Shan
L	*L. himalayanum* DC.	18	9	2*x*	07-572* (J53)	China, Yunnan, Beima Shan
L	*L. jacotianum* Beauverd	24	6	4*x*	07-575* (J55)	India, Uttarakhand, Garhwal
L	*L. kurilense* Takeda	18	9	2*x*	08-86 (J49)	Cultivated, origin unknown: Botanical Garden Graz, Austria
L	*L. leontopodinum* (DC.) Hand.-Mazz.	40	?	?	2001- (J32)	Tian Shan, Terskiy Ala Tau
L	*L. leontopodioides* (Willd.) Beauverd	22	11	2*x*	07-565* (J60)	China, Qinghai, Huang He
L	*L. nivale* (Ten.) Huet ex Hand.-Mazz.	24	6	4*x*	07-557* (J69)	Italy, Gran Sasso
L	*L. ochroleucum* Beauverd	48	8	6*x*	08-14* (J67)	Tajikistan, Gorno-Badakhshan, Koi-Tezek Pass
L	*L. pusillum* (Beauverd) Hand.-Mazz.	32	8	4*x*	07-556* (J20)	China, Xizang/Tibet, Bamda
N	*L. sinense* Hemsl.	32	8	4*x*	03-303 (J02)	China, Yunnan, Hengduan Shan
L	*L. souliei* (Hook.f.) C.B.Clarke ex Hemsl.	16	8	2*x*	07-532* (J14)	China, Sichuan, Litang
N	*L. stracheyi* (Hook.f.) C.B.Clarke ex Hemsl.	32	8	4*x*	08-49* (J42)	China, Xizang/Tibet, Zogang

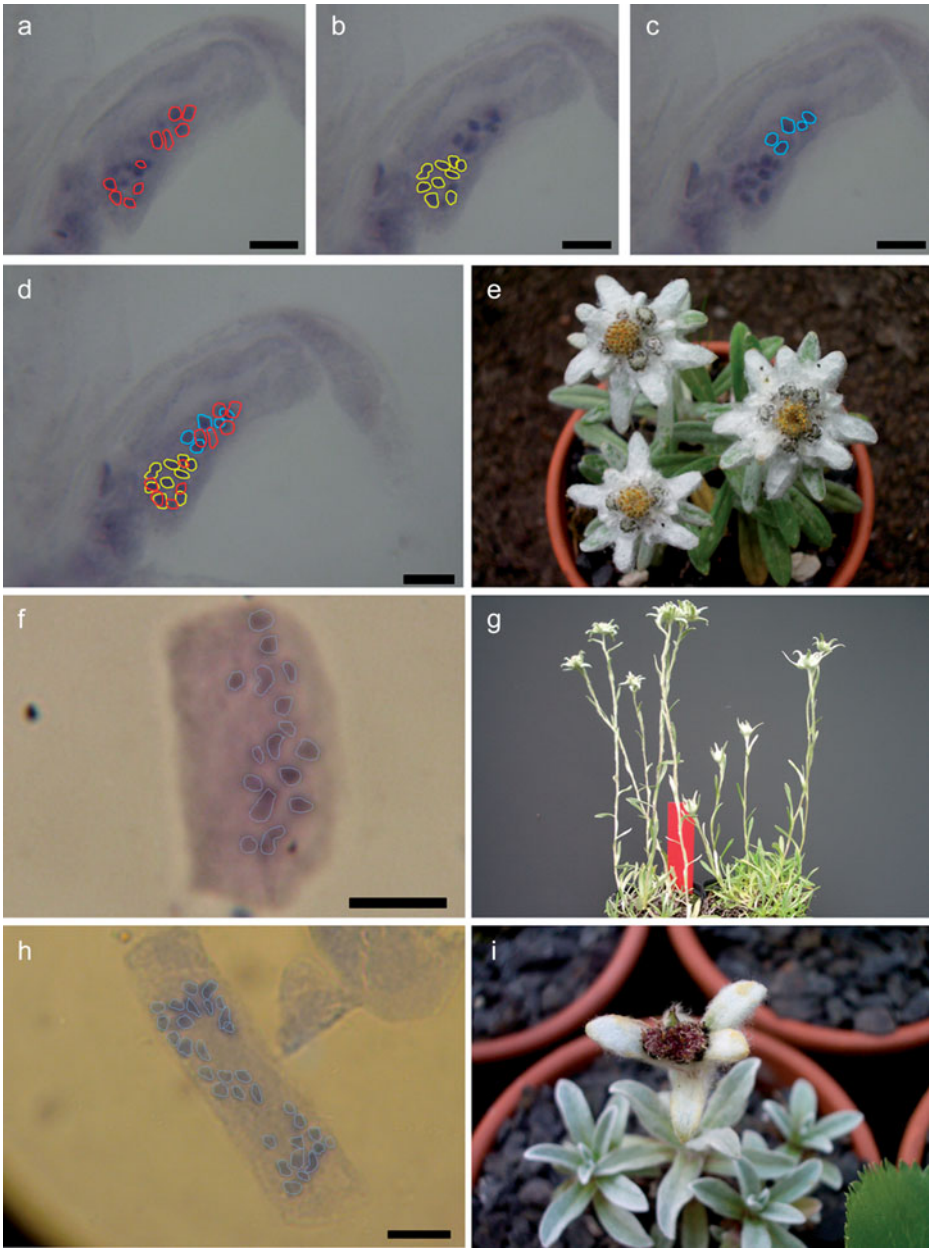

FIG. 1. **a**, **b**, **c**, Outlines of chromosomes in consecutive focus layers in a root-tip cell of *Leontopodium nivale*; **d**, all 24 chromosomes marked in a, b, c shown in one picture of a cell of *L. nivale*; **e**, *L. nivale* growing in BG Giessen; **f**, 16 chromosomes marked in a cell of *L. souliei*; **g**, *L. souliei* growing in BG Giessen; **h**, 32 chromosomes marked in a cell of *L. pusillum*; **i**, *L. pusillum* growing in BG Giessen. Scale bar = 5 µm.

on average, except for *Leontopodium franchetii* and *L. sinense*, where only single mitotic cells were available. All accepted individual counts were obtained independently and cross-confirmed by Stille and Ehlers.

Results

Examples of chromosome counts and investigated species of *Leontopodium* are shown in Fig. 1. Chromosomes of *Leontopodium* were found to be small, with an average length of 1 μm (Fig. 1). Table 1 presents chromosome numbers for all 16 investigated species along with accession number and provenance. Figure 2 illustrates the distribution area.

For most of the species examined the inferred basic chromosome number was $x = 8$. Diploid counts with $2n = 2x = 16$ were found in two species, *Leontopodium dedekensii* and *L. souliei*. Polyploid counts were also recorded, with tetraploid ($2n = 4x = 32$) numbers in five species and a hexaploid number ($2n = 6x = 48$) in *Leontopodium ochroleucum* (Table 1).

Three other inferred basic chromosome numbers were obtained exclusively for species of *Leontopodium* sect. *Leontopodium* (Table 1). In *Leontopodium discolor*, *L. jacotianum* and the European species *L. nivale* chromosome counts of $2n = 4x = 24$ were detected and interpreted as tetraploids with a basic chromosome number of $x = 6$. However, this could also be interpreted as a diploid level of $2n = 2x = 24$ with a basic chromosome number of $x = 12$. For *Leontopodium kurilense* and *L. himalayanum* a diploid number of $2n = 2x = 18$ was found, indicating a basic chromosome number of $x = 9$. For *Leontopodium leontopodioides* a basic chromosome number of $x = 11$ with a diploid number of $2n = 2x = 22$ was most likely.

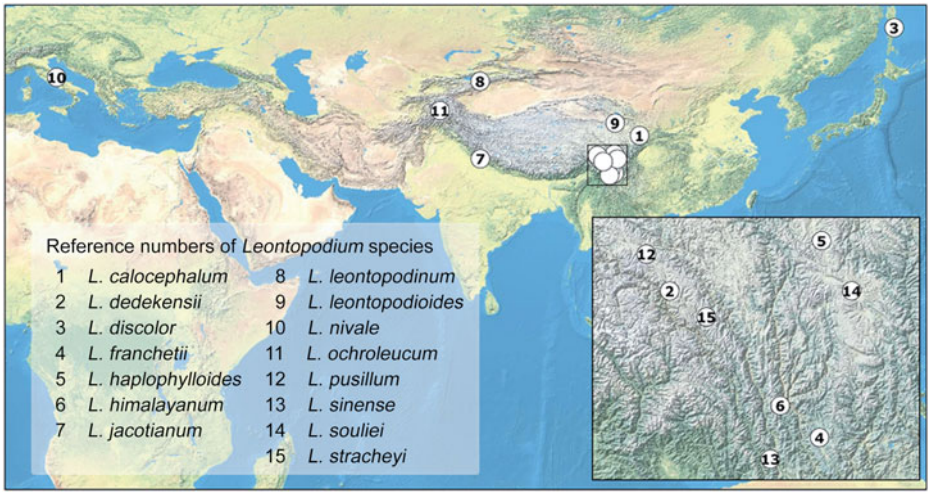

FIG. 2. Geographical distribution of the investigated species and accessions. *Leontopodium kurilense* is missing as it is cultivated in Graz. Its natural range would normally be NE Japan.

The total chromosome numbers counted for *Leontopodium haplophylloides* (*L.* sect. *Nobilia*) and *L. leontopodinum* (*L.* sect. *Leontopodium*) were 38 and 40, respectively, and do not match the basic chromosome numbers determined for the other species.

Overall, in the species examined, diploid and tetraploid chromosome numbers were most frequent. Where the basic chromosome number is $x = 8$ we suggest that polyploid series exist for species which are very closely related, for example at the diploid to tetraploid level for *Leontopodium dedekensii* and *L. sinense* ($2n = 2x = 16$ and $2n = 4x = 32$, respectively) and at the diploid to the hexaploid level for *L. souliei*, *L. calocephalum* and *L. ochroleucum* ($2n = 2x = 16$, $2n = 4x = 32$ and $2n = 6x = 48$, respectively).

Discussion

Here we present chromosome numbers for 16 species of the genus *Leontopodium*, including the first published accounts for six of these species (*L. calocephalum*, *L. franchetii*, *L. haplophylloides*, *L. leontopodioides*, *L. nivale* and *L. stracheyi*). A basic chromosome number of $x = 7$, as suggested by several authors (Darlington & Wylie, 1955; Arano, 1956; Kadereit & Jeffrey, 2007), or $x = 12$ or 13 (Russell *et al.*, 2013) or $x = 14$ (Meng *et al.*, 2012) could not be corroborated for the genus *Leontopodium*. Instead, our study revealed the basic chromosome numbers $x = 6$, 8, 9 and 11. However, different basic chromosome numbers within a genus are not unusual, as shown for other Gnaphalieae genera, for example *Anaphalis* DC., *Antennaria* Gaertn., *Gnaphalium* L. and *Helichrysum* Mill. (for a comprehensive list see table 36.1 in Ward *et al.*, 2009). For 10 of the 16 analysed species, which all belong to *Leontopodium* sect. *Leontopodium* and *L.* sect. *Nobilia*, previous chromosome counts were available, showing enormous variation (see Russell *et al.*, 2013). It has so far not been possible to check voucher material (should it even exist) for the earlier chromosome studies to check for possible misidentifications. With this caveat, however, we compare our results to the literature data. *Leontopodium dedekensii* was found to have $2n = 16$, whereas Russell *et al.* (2013) reported $2n = 26$. With $2n = 73$ (Nishikawa, 1985) and $2n = 104$ (Arano, 1963), very high chromosome numbers have been reported for *Leontopodium discolor* (as *L. hayachinense* var. *miyabeanum*, a synonym of *L. discolor* according to Blöch (2005), and as *L. hayachinense*, a synonym of *L. discolor* according to Dickoré, in prep.). Arano (1963), however, also counted $2n = 26$ for *Leontopodium discolor* under another synonym, *L. fauriei* (according to Dickoré, in prep.), a basic chromosome number similar to the count of $2n = 24$ recorded in our study. Russell *et al.* (2013) reported $2n = 24$ for *Leontopodium himalayanum* but we found $2n = 18$. For *Leontopodium jacotianum* the chromosome number of $2n = 24$ by Khatoon & Ali (1988) was confirmed. A comparison of the basic chromosome numbers of *Leontopodium kurilense* ($2n = 18$) and *L. leontopodinum* ($2n = 40$) with former counts suggests intraspecific variation. Counts for *Leontopodium leontopodinum* of $2n = 24$ (ICNA Database), $2n = 49$ (Sokolovskaja & Strelkova, 1938 – under the name *L. campestre*, a synonym of *L. leontopodinum* according to Dickoré, in prep.) and

$2n$ = 52 (Vir Jee & Kachroo, 1985), and especially counts reported for *L. kurilense* with $2n$ = 26 (Sakai, 1934), $2n$ = 48 (Zhukova, 1980) and $2n$ = 52 (ICNA Database), are much higher than the chromosome numbers presented here. The count for *Leontopodium ochroleucum* ($2n$ = 48) is within the range of former counts ($2n$ = c.50, Krogulevich, 1976; $2n$ = 49, Krogulevich, 1978; $2n$ = 52, Krasnikova *et al.*, 1984, but see ICNA Database: $2n$ = 24). A large discrepancy was found for *Leontopodium pusillum*. Meng *et al.* (2012) counted 72 chromosomes whilst here we present the number of $2n$ = 32. The situation for *Leontopodium sinense* remains uncertain. Our counts revealed $2n$ = 32, Meng *et al.* (2012) reported $2n$ = 28, Russell *et al.* (2013) $2n$ = 48. As for *Leontopodium pusillum* our results for *L. souliei* differ greatly from those of Meng *et al.* (2012). Whereas Meng *et al.* (2012) indicated $2n$ = 52, we counted $2n$ = 16.

For some of the species examined, a correlation between chromosome number and morphological attributes might be assumed, such as a difference in growth height being due to duplication of ploidy level (Briggs & Walters, 1997). In *Leontopodium* sect. *Nobilia*, *L. sinense* was characterised by having twice as many chromosomes as *L. dedekensii* ($2n = 4x = 32$ and $2n = 2x = 16$, respectively) and is twice as tall; the species are very similar in other morphological characteristics. A similar situation occurs between species of *Leontopodium* sect. *Leontopodium*, with *L. souliei* ($2n = 2x = 16$) and the larger *L. calocephalum* ($2n = 4x = 32$).

Our data suggest that species in *Leontopodium* with the highest chromosome numbers and highest ploidy levels originate within the Himalaya distributional area (Fig. 2). Whereas *Leontopodium haplophylloides* ($2n$ = 38) and the tetraploids *L. franchetii*, *L. sinense* and *L. calocephalum* (all $2n = 4x = 32$) occur mainly towards the eastern end of the range of the genus, *L. ochroleucum* ($2n$ = 48) and *L. leontopodinum* ($2n$ = 40) are both limited to the western end. For the European species *Leontopodium alpinum* chromosome numbers of up to $2n$ = 48 and 52 have been reported (Siljak, 1977; Murin & Paclova, 1979), thus corroborating a high ploidy level west of the main distributional area of *Leontopodium*. This argues for the ability of species with higher ploidy levels to colonise new habitats more successfully and better adapt to areas with different environmental conditions, as has previously been suggested by, for example, Stebbins (1950) and Pellicer *et al.* (2007). Polyploidy could thus be a crucial factor in adaptation to a changing environment and in determining future distributions. Intraspecific variation in chromosome numbers of Asteraceae as a general phenomenon was pointed out by Suda *et al.* (2007) but seems to be particularly pronounced in *Leontopodium*. Forthcoming research will determine if this is due to natural intraspecific variability or whether it is a sign of taxonomic uncertainty.

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