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Cytotaxonomical study of *Draba incana* L. from Iceland

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ABSTRACT: *Draba incana* L. (Brassicaceae) is an arctic-alpine species which is both common and widely distributed in Iceland. All specimens of *Draba* (and *Erophila*) in the ICEL and AMNH herbaria – a total of about 1,200 specimens – were examined, and no major changes in taxonomic identification were made. Living plants were collected in this study: nine from northeastern Iceland, and one from southern Iceland. The plants were maintained indoors in winter and outdoors in summer. A new morphological feature was discovered in this study: a creeping growth habit, not reported in any major floras examined. This habit was observed in plants on Hrútey island in Lake Mývatn. The creeping habit was confirmed in the transplanted samples grown indoors. Both groups of plants, i.e. those having a normal growth form with erect inflorescences and plants showing a creeping growth habit, were used in the cytotaxonomical investigation reported here. Chromosomes were prepared from shoot tips and whole seedlings combined, using the protoplast dropping method originally developed for birch (*Betula* spp.). The protocol was successfully modified for use with the tiny, rosette-forming plants of *Draba incana*. Both plant groups, the normal growth form and the creeping habit, showed the same tetraploid chromosome number 2n = 4x = 32. The chromosomes were found to be 2 – 5 µm in size, and were morphologically similar. The study not only confirms that *D. incana* in Iceland is a tetraploid species, but also confirms a stable chromosome number in morphologically variable material. Cytotaxonomical aspects of *Draba* are discussed.

KEYWORDS: Brassicaceae, chromosome, *Draba*, *Draba incana*, polyploid

INTRODUCTION

Draba L. (bitterwort, whitlowgrass) is the largest genus of the mustard family Brassicaceae, containing over 350 species worldwide although it is found primarily in the Northern Hemisphere (WARWICK et al. 2006). It is a conspicuous element of alpine, arctic and subarctic floras. The genus has numerous polyploid species and is morphologically complex (BROCHMANN et al. 2004, GRUNDT et al. 2004); morphological species limits are often based on minor differences of trichomes (AL-SHEHBAZ 1987, AKEROYD 1993). At present, eight species of the genus *Draba* are recognized in the Icelandic flora: *Draba arctogena, D. glabella, D. incana, D. lactea, D. nivalis, D. norvegica, D. oxycarpa* and *D. verna* (INGIMUNDARDÓTTIR et al. 2008, KRISTINSSON 2008).

Draba incana L. (twisted bitterwort, hoary whitlowgrass) is a lectotype of the genus *Draba* (AL-SHEHBAZ 1987). The species is amphi-Atlantic (LID & LID 2005): in Europe its distribution covers most of northern Europe, including Iceland, as well as the mountains of western and central Europe (HULTÉN & FRIES 1986, AKEROYD 1993), while in North America its distribution range is from Greenland to Manitoba in Canada and south to Michigan state in the USA (SCOGGAN 1978). *Draba incana* is considered the best defined among the eight species mentioned above. It is easy to recognize, being the only species of *Draba* in Iceland that has over ten leaves on the flowering stem, and is quite common, especially in lowland areas. LINNAEUS (1753) described *D. incana* and the name has been relatively stable since then, although synonyms, varieties and subspecies have occasionally been introduced (HEILBORN 1927, FERNALD 1934, GLEASON 1958, BÖCHER 1966). The chromosome number appears to be constant, i.e. material from both North America and Europe show the tetraploid number 2n = 4x = 32, with the basic number x = 8 (e.g. HEILBORN 1927, MULLIGAN 1970, BROCHMANN et al. 1992b). The species is also listed as being tetraploid with 2n = 32 in a study regarding the Icelandic flora (LÖVE & LÖVE 1956).

Not all species of the genus *Draba* are as well defined as *D. incana*, and numerous unidentified specimens exist in Icelandic herbaria. *Draba norvegica* is an example of a poorly defined polyploid species with very diverse morphological features. Based on isozyme studies, this highly variable polyploid species appears to have multiple and extremely complex species origins, from a minimum of 3 to probably 13 independent origins (BROCHMANN et al. 1992a). The Icelandic *D. oxycarpa* is another example of a problematic species. It was reported as *D. alpina* until the publication of Atlas Florae Europaeae in 1996 (JALAS et al. 1996). The two species are morphologically very similar but are believed to represent two different evolutionary lineages, *D. alpina* having 2n = 80 and *D. oxycarpa* 2n = 64 (BROCHMANN et al. 1993). Although *D. oxycarpa* is now considered part of the Icelandic flora, based on morphological features alone (JALAS et al. 1996, INGIMUNDARDÓTTIR et al. 2008, KRISTINSSON 2008) it does not rule out *D. alpina* being found there as well. LÖVE and LÖVE (1956) did

chromosome counts for alleged *D. alpina* and found 2n = 80. Since all the other species of *Draba* in Iceland have white flowers it is doubtful that any white-flowered species was counted instead of the yellow-flowered *D. alpina* or *D. oxycarpa*. However, the information on the specimens used for the counts is lacking and therefore no definite conclusion can be drawn from the findings of LÖVE and LÖVE (1956). Variation in ploidy levels within species is known in *Draba*, especially among polyploid species having multiple origins. Most arctic and alpine *Draba* species have the basic chromosome number x = 8 (e.g. LÖVE & LÖVE 1975), while the ploidy levels range from diploid (2n = 16) to 16-ploid with 2n = 128 (BROCHMANN 1993, WARWICK & AL-SHEHBAZ 2006). Many *Draba* species are reported with only one ploidy level although variation in ploidy levels within species is known in the genus, especially among polyploid species having multiple origins (LÖVE & LÖVE 1975, GRUNDT et al. 2005). Such variation is however unknown in *Draba oxycarpa* (BROCHMANN et al. 1993) and is therefore unlikely to explain the chromosome number 2n = 80 for alleged *D. alpina* in Iceland by LÖVE and LÖVE (1956).

Cytogenetic investigation has been used to answer some of the many questions regarding the phylogeny and relation of species within the genus *Draba* and undoubtedly will continue to be an important approach for answering such questions (BROCHMANN et al. 1993). Ploidy level information is of particular value in complex polyploid groups where reticulate evolution may have played an important role, as for instance in species groups within the genus *Draba* (KOCH & AL-SHEHBAZ 2002, GRUNDT et al. 2005). More than 70% of *Draba* species are polyploid taxa, and these estimates clearly point to the evolutionary importance of polyploidy in this genus as well as in other genera of Brassicaceae (MARHOLD & LIHOVÁ 2006). Recurrent (and recent) formation of polyploids, especially allopolyploids of hybrid origin, is known to be a significant factor behind the taxonomic complexity of the arctic flora (ABBOTT & BROCHMANN 2003, BROCHMANN et al. 2004, GRUNDT et al. 2006).

Existing knowledge about polyploidy and evolution of *Draba* in Iceland is extremely limited. Chromosome numbers, used in cytotaxonomy of *Draba*, have been listed or reported (LÖVE & LÖVE 1956), but no information exists about the plant materials or their origin. The objective of the current study was therefore to set out cytogenetic investigations of *Draba* in Iceland, using the chromosome preparation method of ANAMTHAWAT-JÓNSSON (2003) that was optimized in this study to suit *Draba* plants. The results reported here are from Icelandic samples of *D. incana.*

MATERIALS AND METHODS

Plant material and taxonomic identification

All specimens of *Draba* deposited in the herbaria of the Icelandic Institute of Natural History (AMNH in Akureyri and ICEL in Reykjavík) were studied.

Information on all specimens is accessible on GBIF (Global Biodiversity Information Facility) at www.gbif.net. This collection includes specimens that were formerly assigned to *Erophila* DC., but have recently been treated as *Draba verna* (KOCH & AL-SHEHBAZ 2002, INGIMUNDARDÓTTIR et al. 2008, KRISTINSSON 2008).

Living plants of *D. incana* (ten in total) were collected between June and August 2005 from Eyjafjörður district (below 200 m a.s.l.) and Lake Mývatn (300 m a.s.l.) in northern Iceland, with a single plant from the farm Vatnsendi (20 m a.s.l.) in southern Iceland (Fig. 1). They were then grown in pots during the winter months, in a growth chamber at 27 °C and with UV-enriched light; the pots were placed outdoors in the summer. Taxonomic identification followed Norsk Flora (LID & LID 2005) and other publications (BJARNASON 1994, KRISTINSSON 1986, MULLIGAN 1976). Morphological characters for identification of *D. incana* included the number of leaves on a flowering stem; hair type and density on leaf, stem and silicula; and shape of leaf and silicula.

Seeds were collected from the plants and stored in paper bags in a dry, dark

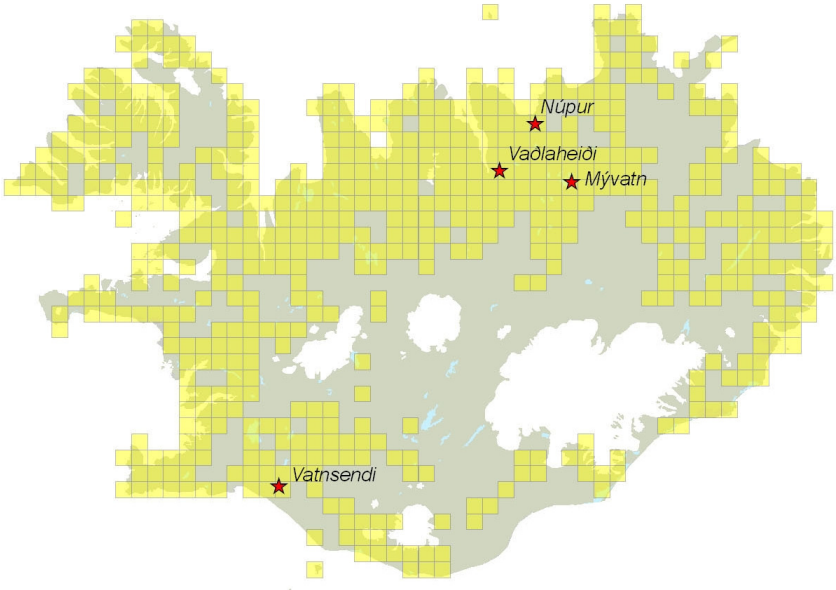

FIGURE 1. Distribution of *Draba incana* in Iceland (□) and the sampling sites (★). Based on databases of the Icelandic Institute of Natural History accessible via "Plöntuvefsjá" (http://vefsja.ni.is/website/plontuvefsja/). Map: Lovísa Ásbjörnsdóttir.

place. Three methods of sowing were used to obtain seedlings: one was the use of moist filter paper under UV-enriched light; the second was the use of sterilized agar in closed Petri dishes under UV-enriched light; and the third in-

volved sowing the seeds directly on sterilized soil in dark conditions. The temperature for germination was around 25 °C.

Chromosome preparation and analysis

The chromosome preparation procedure followed ANAMTHAWAT-JÓNSSON (2003), a protoplast dropping method developed for chromosome isolation from leaf buds of tree species such as birch (THÓRSSON et al. 2007) and oak (CHOKCHAICHAMNANKIT et al. 2007). The following steps were performed:

Tissue collection and fixation: Leaf buds and one-week-old seedlings of *Draba* were collected and placed in iced water (4 °C) for 24 h to arrest metaphases (no more than 5 pieces per 20 ml tube of iced water). Afterwards the samples were transferred to a cold but freshly prepared fixative (one part glacial acetic acid and three parts absolute ethanol) and treated in this solution for about 2 h at room temperature before being stored at -20 °C until the next step in this protocol.

Protoplast enzyme digestion: Fixed samples used in each digestion included 5-10 buds or whole seedlings. The buds were first trimmed to remove most of the differentiated leaf tissues but the meristem was left behind. The fixed material was then rinsed in distilled water before digesting in 100 µl of the protoplast enzyme mixture TG1 for 2 – 3 h at room temperature. Ten ml of the TG1 enzyme mixture contained 0.5 g (500 units) of Cellulase Onozuga R10 (102321, Merck, Germany) and 0.5 ml (280 units) of Pectinase (P4716, Sigma, USA) in a buffer containing 75 mM KCl and 7.5 mM EDTA, pH 4.

Hypotonic treatment: The digested tissues were minced gently in a microtube using a pipette tip to break up large pieces and to release protoplasts into suspension. The suspension was filtered through a nylon mesh to remove tissue debris and then treated with hypotonic solution (1.5 ml of cold 75 mM KCl) for 6 – 9 min at room temperature. The suspension was then centrifuged at ca. 7,000 rpm for 5 min to collect the cell (protoplast) precipitate, while the supernatant was discarded. The precipitate was resuspended in 1 ml of fresh fixative.

Protoplast dropping: The protoplasts were cleaned 3 times by centrifugation with fresh fixative before dropping from a height of 10-20 cm onto ice-cold, wet, acid-cleaned microscope slides, one drop per slide. The final protoplast suspension was in 20 – 50 µl of fresh fixative, depending on the cell density. The chromosome slides were air-dried briefly.

Microscopic examination: The chromosome spreads were stained with fluorochrome DAPI (4, 6-diamidino-2-phenylindole) and examined under 1000x magnification in an epifluorescence microscope Nikon Eclipse 800 using the UV filter sets. Chromosome number was determined directly through the microscope and was confirmed after images were captured with a Nikon DXM 1200F digital camera using the maximum

resolution of 12.5 megapixels.

RESULTS AND DISCUSSION

Morphological assessment

Approximately 1,200 specimens belonging to the genus *Draba* in the ICEL and AMNH herbaria were examined, of which 405 belonged to *D. incana* and only a few had been misidentified. *Draba incana* is relatively common in Iceland (Fig. 1) and is usually found in dry grassland, hillsides and on gravelly soil. In the field, plants of *D. incana* were easily recognizable by erect flowering stems with many leaves densely covered with greyish-white hair, the colour of which is reflected in the Icelandic vernacular name of this species, *grávorblóm*. The twisted silicule (dry fruit) is also typical of this species.

Draba incana is biennial to perennial, with erect, simple or branched, leafy, hairy flowering stems (LID & LID 2005). The plants examined in the field had this characteristic growth form, except at one site, Hrútey island in Lake Mývatn (specimen VR-47268 (ICEL)), where many plants had an unusual growth form. This was a creeping habit (Fig. 2), not mentioned in any of the literature used in this study (FERNALD 1934, STEFÁNSSON 1948, GLEASON 1958, MULLIGAN 1976, LÖVE 1977, SCOGGAN 1978, BÖCHER et al. 1978, KRISTINSSON 1986, BJARNASON 1994, AKEROYD 1993, LID & LID 2005).

The creeping habit was first thought to be due to some growth conditions,

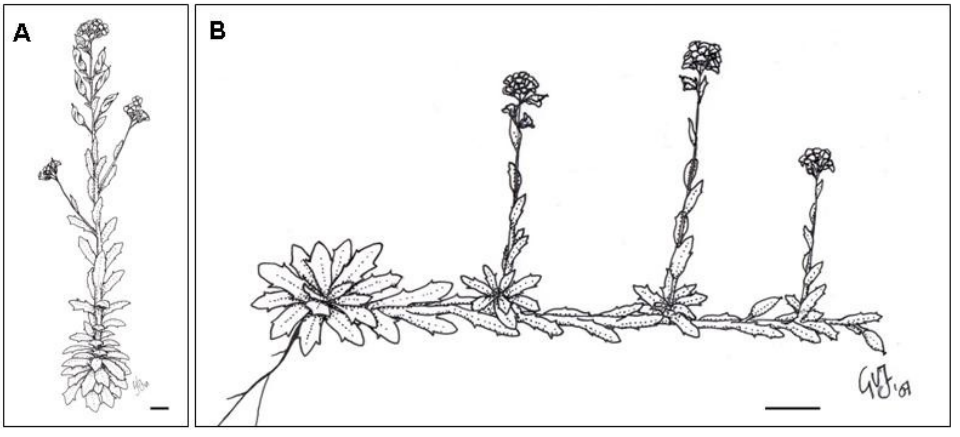

FIGURE 2. Drawings of *Draba incana* (by GVI), showing the plant with normal growth form (A) from Vatnsendi and the plant with creeping habit (B) from Hrútey,

because the field specimens in this study came from an area that seemed heavily grazed by waterfowl. But after the plants were transferred indoors and grown in pots, new flowering stems showed the same tendency to grow sideways and form rosettes and inflorescences at pedicel axils. The *D. incana* plants

typically became more glabrous, looser and larger when grown indoors (in a growth chamber maintained at 27 °C and with UV-enriched light). The creeping habit of *D. incana* is thus clearly not influenced by environmental conditions, and could be a genetic trait of a new variety.

Chromosome number of *Draba incana*

Tissue samples for chromosome isolation (buds and seedlings combined) were pooled into two groups: plants with creeping habit in one group and normal plants (with erect stems) in the other. The chromosome isolation was repeated several times and numerous cells at metaphase and other stages of mitosis were examined. At least four well-spread metaphases from normal plants and three metaphases from the plants with creeping habit were used for chromosome counting. Both groups of *D. incana* plants had the tetraploid chromosome number 2n = 32 (Fig. 3). No variation in chromosome number was found within groups. This is the first illustrated evidence that shows that *D. incana* in Iceland is a tetraploid species, supporting the indication by LÖVE and LÖVE (1956). This also shows for the first time that regardless of morphological variation, *D. incana* from Iceland has the same chromosome number and ploidy level as that reported for European and American material of this species (HEILBORN 1927, MULLIGAN 1970, LÖVE & LÖVE 1975, LÖVE 1982, BROCHMANN et al. 1992b, AKEROYD 1993, BROCHMANN 1993).

Variation in chromosome number can exist within a polyploid *Draba* species, and even within a population. *Draba lactea* from Alaska, for example, includes both tetraploid and hexaploid plants within the same population (GRUNDT et al. 2005), while molecular analysis has shown that both types evolved within the same lineage, via interspecific hybridization and polyploidy (BROCHMANN et al. 2004, GRUNDT et al. 2004). Such a process is common in the circumarctic regions (BROCHMANN et al. 2004). On the other hand, low-ploid *Draba* species, such as *D. nivalis* and *D. incana* in Iceland, should be more genomically and chromosomally stable. This is the case for the diploid (2n = 16) *D. nivalis* from Alaska, Canada, Greenland and northern Scandinavia (GRUNDT et al. 2005). The Icelandic *D. incana* is reported here to be a stable tetraploid (2n = 32), even among different morphological types.

Chromosome preparation method

Cytogenetic investigations at the population level are essential to the understanding of evolutionary processes involved in the speciation and dynamics of arctic species such as species of *Draba*. Most population-based cytogenetics rely on flow cytometry (rapid screening of ploidy levels: JOHNSTON et al. 2005; DOLEŽEL et al. 2007) and Feulgen densitometry (to obtain absolute DNA contents or C-values: GREILHUBER 2008). The C-value information in particular is recognized as a good taxonomic marker (OHRI 1998); various plant tissues collected directly in the field can be used (GREILHUBER 2008), but normally root-tip chromosome preparations have to be made in the same way as for

direct chromosome counting. In the case of *Draba*, the availability of such tissues (i.e. root tips) is extremely limited, mainly due to its small size and slow growth. Ploidy determination using flow cytometry facilitates the screening of a large number of plants rapidly and relatively inexpensively but does not provide exact chromosome numbers. A new protocol (ANAMTHAWAT-JÓNSSON 2003) for isolating chromosomes from actively growing leaf buds or shoot tips was therefore adopted for use with *Draba* in this study.

This particular protocol was originally developed for birch (*Betula* L.), and to use with *Draba* some modifications had to be made, as *Draba* is very small, forms rosettes and has very few shoot tips per plant. A shorter digestion time and milder hypotonic treatment was used, due to soft tissues and small cells. For the same reason the use of whole, one-week-old seedlings seemed worth a try. Seedlings turned out to be easy to obtain. Each plant produced numerous seeds as expected, partly because the species is a self-compatible inbreeder (MULLIGAN 1970). Sowing the seeds directly on soil, and maintaining them in the dark, proved much more successful and easier than using moist filter paper or sterilized agar to germinate seeds under light. The use of whole seedlings provided meristematic tissues (with cell divisions) from both shoot and root tips without the risk of losing these cells by trying to dissect the tiny seedlings for meristems. The only disadvantage of using seedlings was that they are not genetically the same as their mother plants, and therefore gene flow across species barriers could affect chromosome complement of the progeny, like in the case of *Betula* where triploid progeny can easily be formed by a diploid seed parent (ANAMTHAWAT-JÓNSSON & TÓMASSON 1999). Little is known about interspecific hybridization in *D. incana*, but it is not likely to be common. At least among the diploid *Draba* species, regular inbreeding by self-pollination ensures sexual reproduction in a severe arctic environment where pollinating insects are scarce (BROCHMANN et al. 2004). Nevertheless, triploid plants have been detected among diploid *D. nivalis* and related diploid species from Alaska and Canada (GRUNDT et al. 2005).

Leaf and shoot meristematic cells should be as chromosomally stable as those in root tips, at least in species from which thousands of metaphases from leaf buds have been examined (e.g. THÓRSSON et al. 2007). This also seems to be the case with *Draba*. Furthermore, chromosomes from shoot or leaf meristems tend to be more elongated (less condensed) than chromosomes normally isolated from root tips (ANAMTHAWAT-JÓNSSON 2003), and would hence be suitable for making reliable karyotypes and gene mapping in further cyto-genetic studies. Chromosomes of *D. incana* in the present study were about 2 – 5 µm in length (Fig. 3); compared to 1.5 – 3.5 µm in a number of species of *Brassica* L. (Brassicaceae) where chromosomes were prepared from root tips (e.g. OLIN-FATIH 1994, KOO et al. 2004). Ribosomal gene mapping has indeed been carried out on *Brassica* chromosomes prepared from whole seedlings, like in the present study; the metaphase chromosomes obtained were significantly

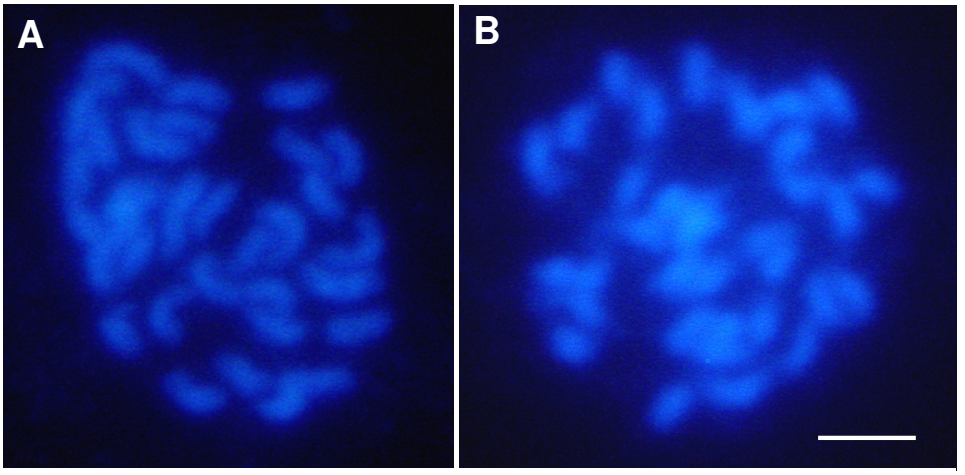

FIGURE 3. Microscopic images of mitotic chromosomes of *Draba incana* showing a tetraploid chromosome number 2n = 4x = 32, from plants with normal growth form (A) and plants with creeping habit (B). The scale bar represents 5 μm.

more extended, particularly where the ribosomal genes were expressed, making the ribosomal gene mapping more accurate (HASTEROK et al. 2001). A high-resolution karyotype of a *Brassica* species was made on meiotic pachytene chromosomes – which could be more than 15-fold longer than mitotic metaphase chromosomes of the same species – and enabled mapping of multiple genes simultaneously (KOO et al. 2004). Mapping of repetitive sequences, ribosomal genes in particular, can be used to reveal evolutionary relationships among taxonomically related polyploid plant species, in particular with non-commercial, wild species of Brassicaceae such as *Draba*.

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