

# Analysis of the taxonomic subdivision within the genus *Helleborus* by nuclear DNA content and genome-wide DNA markers

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## ABSTRACT

*Helleborus* is a genus of herbaceous perennials belonging to the family *Ranunculaceae*. Within this genus six sections with a total of 22 species are found. The largest section *Helleborastrum* contains 16 species for which genetic relationships are still unclear. This study represents the first genetic analysis in the genus *Helleborus*, including the two newly described species *H. liguricus* and *H. abruzzicus* based on multilocus amplified fragment length polymorphism (AFLP) markers with a genome-wide distribution in combination with nuclear DNA content data. Chromosome analyses of roots tips revealed a number of  $2n = 32$  for the selected species, which was congruent with previous observations. The nuclear DNA content of *Helleborus* was estimated by flow cytometry applying propidium iodide staining and varied between 18 and 33 pg/2C, depending on the species. For AFLP analyses, 19 out of the 22 *Helleborus* species were studied using 10 AFLP primer combinations, resulting in a total of 1109 polymorphic bands among all species including the outgroup. The genetic distances between species varied between 0.034 and 0.330. Based on genetic distances a phenogram using the Neighbor-joining cluster method with bootstrap analysis was calculated. The results support the previously suggested division of the genus into six sections and thereby approve AFLP data to be applicable for phenetic analyses. Moreover, this genetic information is significant for the development of future *Helleborus* breeding strategies.

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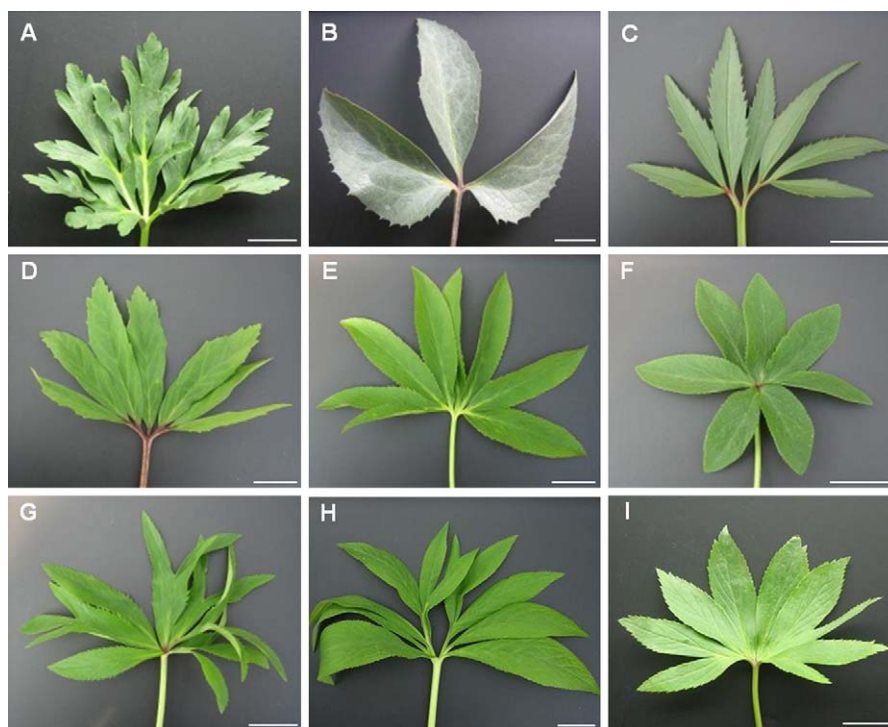
## 1. Introduction

*Helleborus* are rhizomatous, herbaceous perennials that flower from late winter until early spring. As members of the *Ranunculaceae*, they bear a characteristic fruit consisting of a cluster of follicles and multiovular carpels (McLewin and Mathew, 1995). Different approaches have been undertaken to better understand the classification and phylogeny of this plant family. This group has been divided into subfamilies and tribes either by morphological or anatomical characteristics (Prantl, 1888; Hutchinson, 1923; Hoot, 1991; Wang and Ren, 2008), cytological (Langlet, 1932; Gregory, 1941) or serological studies (Jensen, 1968) and recent molecular data (Hoot, 1995; Ro et al., 1997; Wang et al., 2005). As a result, within the *Ranunculaceae*, *Helleborus* belongs to the subfamily *Ranunculoideae*, which comprises all genera with *Ranunculus*-type chromosomes (Ro et al., 1997), but their relationship to other genera in that subfamily is still topic of debate.

*Helleborus* species are distributed over different parts of Europe and West Asia, with the exception of one species, *H. thibetanus*, which is native to East Asia. Since the descriptions of Braun and Bouché (1861), two morphological groups have been distinguished in the genus according to caulogenesis; the *Caulescentes* and *Acaules*. *Caulescent Helleborus*, including *H. argutifolius*, *H. foetidus* and *H. lividus*, have tough stems supporting the leaves and flowers and a less developed rhizome. They grow a large terminal inflorescence with many flowers. Species belonging to the *acaulescent* group are characterised by underground rhizomes that produce shoots with basal leaves, leafless flower stems with leaf-like bracts and rather few flowers per stem (McLewin and Mathew, 1995). *H. thibetanus* can be considered as *acaulescent* by its basic appearance, but the rhizome and the roots are atypical for this group because the inflorescence appears before the true leaves start to expand (McLewin and Mathew, 1995), and it is the only hellebore with hypogeal germination (McLewin and Mathew, 1999). One characteristic shared by *H. thibetanus* and *H. vesicarius* is that, in the early summer months, the plant goes dormant and disappears during summer (McLewin and Mathew, 1999). *H. niger* represents an intermediary between the *caulescent* and *acaulescent Helleborus* (Fig. 1). Its *acaulescent* traits include basal leaves and leafless flower stems, but it possesses undivided bracts and often

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**Fig. 1.** Leaf morphologies of different *Helleborus* species. A: *H. vesicarius*; B: *H. lividus*; C: *H. foetidus*; D: *H. niger*; E: *H. × hybridus*; F: *H. cyclophyllus*; G: *H. dumetorum*; H: *H. torquatus*; I: *H. thibetanus*; bars represent 3 cm.

produces only one flower per stalk and hybridises easily with the caulescent species *H. argutifolius* and *H. lividus* but not with the acaulescent (McLewin and Mathew, 1995). The species *H. vesicarius* does not fit into any of these groups. Although its leaf and stem structures are typical for the acaulescent *Helleborus* (Fig. 1) (McLewin and Mathew, 1999), its thick inflorescence stalks and pollen morphology show great similarity to the caulescent group (Nowicke and Skvarla, 1983).

The classification of the *Helleborus* species into two groups seemed useful for horticultural purposes (McLewin and Mathew, 1995), but phylogenetically insufficient for the abovementioned reasons. Therefore, Mathew (1989) suggested a classification dividing the genus into six sections (Table 1) according to plant structure, the ability to hybridise, pollen morphology and seed characteristics. Werner and Ebel (1994) suggested the division of *Helleborus* into two subgenera, *Helleborus* and *Helleborastrum*, according to the species hypsophylls, with sections *Griphopus*, *Chenopus* and *Helleborus* belonging to the subgenus *Helleborus* and *Syncarpus*, *Dicarpon* and *Helleborastrum* to the subgenus *Helleborastrum*. Furthermore, two new Italian *Helleborus* species, *H. abruzzicus* and *H. liguricus*, have been described recently (Thomsen, 2008), and no molecular genetic data describing their relationship to other species has been reported thus far, so that to date, 22 species can be listed (Table 1). Out of these, *H. niger* and *H. × hybridus*, a group of hybrids that have *H. orientalis* as one parent in common, have attained the highest commercial interest as demonstrated by increasing market shares. Some traits of other species, for example flower colour, growth type, scent and disease tolerance, are valuable for the horticultural improvement of hellebores and could be introduced by interspecific hybridisation. To utilize this breeding tool, it is necessary to obtain additional information on genetic relationships within this genus.

To clarify relationships within the *Ranunculaceae* and within the genus itself, cytological studies of some representatives within the genus were carried out (Gregory, 1941; D'Amato and Bianchi, 1989; Yuan and Yang, 2006). These investigations revealed that all stud-

ied species have the same chromosome number of  $2n = 32$  (Gregory, 1941; D'Amato and Bianchi, 1989; Yuan and Yang, 2006; Bennett and Smith, 1976; Dobes et al., 1997; Castro and Rosselló, 2007). In another attempt to evaluate *Helleborus* taxonomy, the nuclear DNA contents of the *Helleborus* species were estimated (Zonneveld, 2001). The genome size (C-value) is known to correlate with the geographical distribution of plants (Bennett, 1976) and can be useful for the examination of phylogenetic dimensions (Bennett et al., 2000) and the taxonomic classification of species or the relationships between those in narrow taxonomic groups (Ohri, 1998). Therefore, it is a valuable method for the analysis or verification of plant taxonomy. Zonneveld's results were in agreement with the genus division recommended by Mathew (1989) and Werner and Ebel (1994).

The first phylogenetic evaluation of *Helleborus* on the basis of the chloroplast (*trnL-F* and *matK*) and ribosomal ITS DNA sequence data was done by Sun et al. (2001), which supported the genus' division into sections, but did not resolve the problematic nature of the section *Helleborastrum*. Close relationships between species, and intraspecific morphological variation (Servettaz et al., 1988), make it difficult to differentiate and estimate the relationship between them. An initial taxonomic differentiation of four species and eight populations from Italy was done by Fico et al. (2005) based on RAPD markers (randomly amplified polymorphic DNA), which revealed a clear discrimination between the species and populations of *H. bocconei*, *H. niger*, *H. odorus* and *H. viridis*.

Due to the contradictory results regarding the taxonomic classification of the genus *Helleborus* and the discovery of new species, in this study, we set out to gather novel information on genetic relationships within the genus by combining different analyses with molecular marker data. The objectives of this study were (i) to confirm the chromosome numbers of representative species of all sections, (ii) to confirm the nuclear DNA content of 18 species and three hybrids and determine this nuclear DNA content for three new species and one hybrid and (iii) to evaluate genetic relationships within the genus *Helleborus* by the use of the multilocus AFLP

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