Genetic relationships and interspecific hybridisations in the genus *Helleborus* and characterisation of the causal agent of hellebore leaf spot disease

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Abstract

The genus *Helleborus* comprises 22 species, which are allocated to six *Helleborus* sections. *Helleborus* species are distributed in different parts of Europe and East Asia. They show differences with regard to leaf and flower morphology, especially flower colour, and in susceptibility to hellebore leaf spot disease (*Coniothyrium hellebori*). Breeding programs aiming at these traits require the inclusion of a broader spectrum of *Helleborus* species in addition to the most popular species *H. niger* (Christmas Rose) and *H. x hybridus* (Lenten Rose).

As a prerequisite for interspecific hybridisations, the *Helleborus* plant material was characterised cytologically, via flow cytometry and DNA fingerprinting. Cytological analyses revealed the same chromosome number of 2n=32 for all analysed *Helleborus* species. Nuclear DNA contents of *Helleborus* species were estimated via flow cytometry and varied from 18.3 pg DNA/2C to 33.2 pg DNA/2C. Based on 1109 genome-wide distributed AFLP markers, genetic distances between species were calculated and a dendrogram was constructed to visualise genetic relationships. The phenogram reflected the taxonomic sub-division of the *Helleborus* genus into sections.

As a next step, crossing barriers between *Helleborus* species were localised as predominantly postzygotic. Therefore, embryo rescue techniques via ovule culture were established to overcome these barriers. Ovules were isolated from the maternal plants five to seven weeks after pollination and then cultured *in vitro*. Overall, 217 hybrid offspring were successfully obtained, whereof 14 were derived from parental species belonging to different *Helleborus* sections. Thereby, larger genetic distances in hybrids between parental species belonging to different sections than in hybrids between species within the same section were overcome.

In addition, the causal agent of the hellebore leaf spot disease was studied. A collection of 25 *C. hellebori* isolates was established from infected leaf material of different host species from various geographical locations. Their morphological characterisation by mycelial growth at different temperatures, conidial size and the induction of pycnidia revealed only marginal differences. All isolates were confirmed as causal agent of the disease by inoculation of *H. niger* plants. Using a molecular genetic approach based on RAPD markers, the genetic relationships were displayed in a phenogram, in which two *C. hellebori* groups were identified. A possible correlation of the isolates in these groups with the original *Helleborus* host species and morphological characteristics was found. This result indicates that more than one species may be associated with the disease.

Keywords: *Coniothyrium hellebori*, embryo rescue, genetic diversity, *Helleborus*

Zusammenfassung

Die Gattung *Helleborus* umfasst 22 Arten, die sechs Sektionen zugeordnet sind. *Helleborus* Arten sind natürlicherweise sowohl in verschiedenen Teilen Europas als auch in Ostasien verbreitet und unterscheiden sich hinsichtlich Blatt- und Blütenmorphologie, insbesondere Blütenfarbe, und weisen ein unterschiedliches Resistenzverhalten in Bezug auf den Erreger der Schwarzfleckenkrankheit (*Coniothyrium hellebori*) auf. Im Hinblick auf diese Merkmale ist die züchterische Weiterentwicklung der bekannten *Helleborus* Arten wie *Helleborus niger* (Christrose) und *H. x hybridus* (Lenzrose) nur durch Einbezug weiterer Arten möglich.

Als Grundlage für die Durchführung von Artkreuzungen wurden verschiedene *Helleborus* Arten cytologisch, durchflusscytometrisch und molekulargenetisch mittels einer PCR-basierten DNA Fingerprinting-Methode charakterisiert. Für alle untersuchten Arten konnte eine gemeinsame Chromosomenzahl von 2n=32 ermittelt werden. Die DNA-Gehalte des Kerngenoms variierten zwischen den Arten von 18.3 pg DNA/2C bis 33.2 pg DNA/2C. Basierend auf 1109 genomweit verteilten AFLP Markern wurden genetische Distanzen ermittelt, und es wurde ein Dendrogramm erstellt, worin die Cluster der *Helleborus* Arten die Einteilung der Gattung in sechs Sektionen widerspiegeln.

Für die Durchführung von Artkreuzungen wurden mittels blütenbiologischer Untersuchungen Kreuzungsbarrieren zwischen den *Helleborus* Arten als vorwiegend postzygotisch identifiziert. Aus diesem Grund wurde ein Embryo Rescue Verfahren entwickelt, bei dem Samenanlagen fünf bis sieben Wochen nach einer Bestäubung von der Mutterpflanze isoliert und *in vitro* kultiviert wurden. Damit wurden insgesamt 217 interspezifische Hybriden gewonnen, von denen 14 aus Kreuzungen zwischen Arten stammen, die unterschiedlichen *Helleborus* Sektionen zugeordnet sind. Dabei wurden größere genetische Distanzen zwischen den elterlichen Arten überwunden als bei Hybriden zwischen Arten der gleichen Sektion.

Neben der Pflanze *Helleborus* wurde der Fokus auf den Erreger der Schwarzfleckenkrankheit gelegt. Anhand einer Sammlung von 25 *C. hellebori* Isolaten deckten morphologische Vergleiche der Sporengröße, der Induktion von Pyknidien und des radialen Mycelwachstums nur marginale Unterschiede zwischen den Isolaten auf. Im Rahmen eines Pathogenitätstests an *H. niger* Pflanzen wurden die Isolate als Erreger der Krankheit identifiziert. Weiterhin wurden die *C. hellebori* Isolate molekulargenetisch mittels RAPD Markern untersucht, wobei die Isolate in zwei Cluster aufspalteten. Dabei war ein Zusammenhang mit der *Helleborus* Art der Wirtspflanze und morphologischen Parametern zu erkennen. Möglicherweise handelt es sich bei den zwei Gruppen um zwei Arten oder Unterarten des Erregers.

Schlagwörter: *Coniothyrium hellebori*, Embryo Rescue, genetische Diversität, *Helleborus*

Publications derived from this thesis

Journal research papers (peer-reviewed)

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Meiners, J., Winkelmann, T. (2011). Morphological and genetic analyses of hellebore leaf spot disease isolates (*Coniothyrium hellebori*) from different geographic origins. *Journal of Phytopathology*, submitted

Miscellaneous research papers

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Conference contributions

Talks

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Meiners, J., Oenings, P., Winkelmann, T. (2010). Entwicklung resistenter, homogener und ertragreicher Sorten von *Helleborus* spec.. BLE Innovationstage 2010, Berlin, Germany

Posters

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Abbreviations

%	Percent
(v/v)	Volume/volume
(w/v)	Weight/volume
°C	Degree Celsius
2C	DNA contained in a diploid nucleus
3’	Three prime end of a DNA fragment
5’	Five prime end of a DNA fragment
AFLP	Amplified fragment length polymorphism
BAP	6-Benzylaminopurine
bp	Base pair(s)
CV	Coefficient of variation
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
e.g.	*Exempli gratia* (lat.: for example)
et al.	*Et alii* (lat.: and others)
EtOH	Ethanol
GA3	Gibberellic acid 3
h	Hour
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
ITS	Internal transcribed spacer
L	Liter
M	Molar
matK	*Maturase K*
MD	Morphological dormancy
min	Minute
MPD	Morphophysiological dormancy
MS	Murashige and Skoog
MTT	3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide
no.	Number
PCR	Polymerase chain reaction
RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rounds per minute
sec	Seconds
std	Standard deviation
TAE	Tris Acetate EDTA
Taq	*Thermus aquaticus*
trn	*Transfer RNA*
trnL-F	Region consisting of *trnL* intron and *trnL-F* spacer
Tris	Tris(hydroxymethyl-)aminomethane
U	Units
UV	Ultraviolet
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
V	Volt
x	Crossed to (crosses are always indicated in the order: female x male)

1 General foreword

1.1 The genus *Helleborus*

Since very early in antiquity, the name *Helleborus* has been associated with plants of medical interest. These plants were already known to Theophrastus (372-287 BC) (Mathew, 1989). *Helleborus* species contain alkaloids, which are known to have cardiac effects (Mathew, 1989). The ancient Greeks described Black and White Hellebores, *Elleboros melas* and *Elleboros leucas*, but the species identity of these plants is not known (Mathew, 1989). The etymology of the name *Helleborus* is not clear. Different explanations have been proposed, and the most plausible originates from the Greek terms »*hellein* = killing« or »*ellos* = deer« and »*bora* = food« (Schiffner, 1890). The Greek term »*(h)elleborosus* = crazy« points to the fact that in antiquity, *Helleborus* was used to treat the mentally ill. All the parts of hellebores are poisonous because of the alkaloid and glycoside content. The roots are especially toxic and contain various poisonous substances.

1.1.1 Taxonomy

Hellebores are rhizomatous, herbaceous perennials that flower from early winter until late spring. Plants of the *Helleborus* species are distributed across parts of Europe and West Asia, with the exception of *H. thibetanus,* which is native to East Asia.

As a member of the *Ranunculaceae* family, hellebores bear a characteristic fruit, which consists of a cluster of follicles and multiovular carpels (McLewin & Mathew, 1995). The *Ranunculaceae* family has been divided into subfamilies and tribes, according to morphological or anatomical characteristics (Prantl, 1888; Hutchinson, 1923; Hoot, 1991; Wang & Ren, 2008) and cytological studies (Langlet, 1932; Gregory, 1941). Members of the *Ranunculaceae* are classified into two groups based on chromosome size and type. Genera with large, long and bent chromosomes belong to the *Ranunculus* group, while those with small and kidney-shaped chromosomes belong to the *Thalictrum* group (Gregory, 1941). In addition, serological studies (Jensen, 1968) and molecular data have been obtained for the *Ranunculaceae* family (Hoot, 1995; Ro et al., 1997; Wang et al., 2005). According to results from these studies, *Helleborus* has been classified in the subfamily *Ranunculoideae,* which comprises all genera with *Ranunculus*-type chromosomes (Ro et al., 1997), but the relationship of *Helleborus* to other genera in that subfamily is still debated.

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 that produce leaves and flowers and a less developed rhizome (Figure 1.1 A). 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 relatively few flowers per stem (Figure 1.1 B; McLewin & Mathew, 1995). The true leaves in the acaulescent species often develop as the flowers mature and fade. Therefore, bracts are often mistaken for leaves because of their size and divisions (Figure 1.1 B).

Figure 1.1: *Helleborus* species with different growth types.
A: caulescent *H. foetidus* (http://www.hellebores.org/foetidus.html), B: acaulescent *H. x hybridus* (http://www.hellebores.org/gallery.html), C: intermediate *H. vesicarius* (http://www.hellebores.org/vesicarius .html).

Three exceptional species do not fit into either of these two groups and represent intermediates between the caulescent and acaulescent *Helleborus*: *H. niger*, *H. thibetanus* and *H. vesicarius*.

H. niger's acaulescent traits include basal leaves and leafless flower stems, but it possesses small undivided bracts and often produces only one flower per stalk. It also hybridises easily with the caulescent species *H. argutifolius* and *H. lividus*, but not with the acaulescents (McLewin & Mathew, 1995). *H. thibetanus* can be considered as acaulescent by its basic appearance, but the rhizome and roots are atypical for this group. The inflorescence appears before the true leaves start to expand (McLewin & Mathew, 1995). It is also the only *Helleborus* species with hypogeal germination (McLewin & Mathew, 1999). In the early summer months, both *H. thibetanus* and *H. vesicarius* plants go dormant and disappear during the summer (McLewin & Mathew, 1999). The species *H. vesicarius* does also not fit into any

of these two groups (Figure 1.1 C). Although its leaf and stem structures are typical for the acaulescent *Helleborus* (McLewin & Mathew, 1999), its thick inflorescence stalks and pollen morphology are very similar to the caulescent group (Nowicke & Skvarla, 1983).

The classification of the *Helleborus* species into these two groups (acaulescent/caulescent) seemed useful for horticultural purposes (McLewin & Mathew, 1995). Based on this growth phenotype, hellebores can be easily divided into 'stemmed' and 'stemless' species by their outward appearance. This classification is easy if vegetative propagation is considered (Chapter 1.2). Nevertheless, this division is phylogenetically insufficient for the above-mentioned reasons. Mathew (1989) suggested a classification that divides the genus into six sections according to plant structure, the ability to hybridise, pollen morphology and seed characteristics (Table 1.1).

Table 1.1: Classification of the *Helleborus* genus into sections according to Mathew (1989).

Section	Species
Syncarpus	*H. vesicarius*
Griphopus	*H. foetidus*
Chenopus	*H. argutifolius* *H. lividus*
Helleborus	*H. niger*
Helleborastrum	*H. atrorubens* *H. cyclophyllus* *H. dumetorum* *H. multifidus* *H. odorus* *H. orientalis* *H. purpurascens* *H. torquatus* *H. viridis*
Dicarpon	*H. thibetanus*

The names of the *Helleborus* sections are not in italicized in the following thesis in order to prevent confusion with the genus name, subgenus name or with the species.

Werner and Ebel (1994) suggested the additional division of *Helleborus* into the two subgenera, *Helleborus* and *Helleborastrum*, according to the species' hypsophylls. The sections Griphopus, Chenopus and Helleborus belong to the subgenus *Helleborus,* and the subgenus *Helleborastrum* includes the sections Syncarpus, Dicarpon and Helleborastrum. Additionally, two new Italian *Helleborus* species, *H. abruzzicus* and *H. liguricus*, have been recently described (Thomsen, 2008), and some groups that were regarded as subspecies,

namely *H. multifidus* subsp. *bocconei*, *H. multifidus* subsp. *hercegovinus*, *H. multifidus* subsp. *istriacus* and *H. viridus* subsp. *occidentalis* are now considered to be distinct species and bear the name of the subspecies. In conclusion, 22 species and four hybrids between *H. argutifolius* x *H. lividus*, *H. niger* x *H. argutifolius*, *H. niger* x *H. lividus* and *H. niger* x *H. x sternii*, can be listed to date (Table 1.2).

Table 1.2: List of *Helleborus* species and hybrids according to their natural distribution, their growth type and their flower colour.

Helleborus species	Natural distribution	Growth type	Ranges of flower colour
Subgenus *Helleborus*			
Section Griphopus			
H. foetidus	Western, Central and Southern Europe	caulescent	green and pendent
Section Chenopus			
H. argutifolius	Corsica, Sardinia	caulescent	green
H. lividus	Majorca, Cabrera	caulescent	cream, green often with pinkish markings
Section Helleborus			
H. niger	South-, East Alps to Northwestillyria	intermediate	white to pinkish
Subgenus *Helleborastrum*			
Section Syncarpus			
H. vesicarius	South Turkey, Syria	intermediate	green with a red/brown thick collar
Section Helleborastrum			
H. abruzzicus	Abruzzo (Italy)	acaulescent	yellow-green to light yellow
H. atrorubens	Slovenia, North Croatia	acaulescent	green to dark violet
H. bocconei	Sicily, Calabria	acaulescent	green to white-green
H. croaticus	Northeast Croatia	acaulescent	green to violet
H. cyclophyllus	Albania, Greece, Bulgaria	acaulescent	green to yellow-green
H. dumetorum	Austria, Hungary, Romania, Croatia	acaulescent	green
H. hercegovinus	Montenegro, Hercegovina	acaulescent	green to yellow-green
H. orientalis	Turkey, Caucasus, Ukraine	acaulescent	white-cream-pink, spotted or unspotted
H. istriacus	Northwest Croatia, Northeast Italy	acaulescent	green
H. liguricus	Liguria, Tuscany, Emilia Romagna (Italy)	acaulescent	whitish
H. multifidus	Croatia, Herzegovina, Albania	acaulescent	green
H. occidentalis	Belgium, Britain, France, Germany, Spain	acaulescent	green
H. odorus	Albania, Hungary, Italy, Romania, Bosnia	acaulescent	green to yellow-green
H. purpurascens	Romania, Hungary, Czech republic, Slovakia Poland, Ukraine	acaulescent	purple-brown-green
H. torquatus	Croatia, Serbia, Bosnia, Montenegro	acaulescent	green to violet
H. viridis	Austria, North Italy, Southern Germany, Switzerland, France	acaulescent	green
Section Dicarpon			
H. thibetanus	Western China	intermediate	white-pink, often veined
Hybrids	**Parents**		
H. x ballardiae	*H. niger* x *H. lividus*	intermediate	pinkish green to creamy green
H. x ericsmithii	*H. niger* x *H. x sternii*	intermediate	pale pink or white inside and darker and more often green- or pink-tinted on the reverse
H. x nigercors	*H. niger* x *H. argutifolius*	intermediate	white, white with green tints, or rather creamy
H. x sternii	*H. argutifolius* x *H. lividus*	caulescent	pink to red and green inside

The Lenten Rose *H. orientalis* was intensely used for hybridisation with other acaulescent species (Chapter 1.3) resulting in forms that are currently called 'garden hybrids' or 'orientalis hybrids'. Therefore, the garden hybrids are also called Lenten Rose, but the botanical name is *H. x hybridus*. *H. orientalis* is reserved for the original *H. orientalis* in its natural habitat.

1.1.2 Economical importance

Of the 22 species (Table 1.2), *H. niger* and *H. x hybridus* have attained the highest commercial interest, as indicated by increasing sales figures. According to the Dutch Flower Auctions Association (VBN) *Helleborus* is traded as a cut flower, an indoor plant and a bedding plant. In year 2004 1.4 million plants were sold for an average price of 1.95 €/plant, and in 2009, 2.7 million plants were sold for an average price of 2.1 €/plant at the Dutch Flower Auctions (Vakblad voor de Bloemisterij, 2010). Within five years the sales figures had nearly doubled with prices remaining constant. This underlines the rising importance of *Helleborus* as an ornamental crop. Hellebores are generally sold in November and December. *H. niger*, the Christmas Rose, is predominantly sold around Christmas. After Christmas, *H. niger* is replaced by the colourful Lenten Roses, *H. x hybridus*. In spring, hellebores compete against cheaper bedding plants (Vakblad voor de Bloemisterij, 2010). In addition to its ornamental use, *H. niger* is used in homeopathy, e.g. in cases of meningitis, and as an adjuvant in tumour therapy in anthroposophical medicine (Buessing & Schweizer, 1998).

1.2 Propagation and cultivation of *Helleborus*

Hellebores are propagated by rhizome division, by seed and with *in vitro* techniques.

Division is one method of vegetative propagation, and it has the advantage that all offspring are identical to the parent plant. Rhizomes are cut into pieces with shoots and roots attached. Acaulescent species can be readily propagated by division, whereas division of the caulescent species is very uncertain due to their less developed rhizomes. Thus, the number of offspring is limited. Overall, division is a very slow process due to previous stock production from the original plant.

Compared to division, seed propagation has the advantage that a large number of offspring can be produced by one flower from its multiovular carpels. Therefore, seed propagation is important for the production of *Helleborus* cultivars and in *Helleborus* breeding. For controlled seed propagation, cross-pollination by unrequested pollinators often has to be avoided, e.g., during the production of intraspecific F_1-hybrid cultivars. Then the flowers have

to be emasculated and isolated, if necessary. If the stigma is pollinated and fertilisation occurs, the carpels begin to swell. Depending on the environmental conditions, seed maturation in the plant takes 10 to 12 weeks. If the seeds are sown immediately after maturation, germination naturally occurs in the following autumn or winter. During commercial production, the time between sowing and germination takes at least 34 weeks due to strong seed dormancy. To release the seed from dormancy, warm and cold temperatures are applied.

When the seeds are released from the plant, the embryo is still underdeveloped, mostly in a heart-shaped stage, which is referred to as morphological dormancy (MD) (Baskin & Baskin, 2004; Finch-Savage & Leubner-Metzger, 2006). In addition to MD, morphophysiological dormancy (MPD) is expressed in *Helleborus* seeds, meaning that the seeds require a treatment to break the dormancy, e.g., the combination of warm and/or cold stratification (Baskin & Baskin, 2004). Eight classes of MPD, which are distinguished based on the level of physiological dormancy, have been described (Table 1.3). The levels of physiological dormancy are non-deep, intermediate and deep. If temperature changes are needed to break dormancy, as is the case for *Helleborus* seeds, the first phase of warm temperatures promotes embryo growth (Baskin & Baskin, 2004). Depending on the type of morphophysiological dormancy, a treatment with gibberellic acid might release dormancy (Table 1.3), but the period of time during which a gibberellic acid treatment could be effective for *Helleborus* seeds, is not known.

Table 1.3: Levels of morphophysiological dormancy (Table was adopted from Baskin & Baskin, 2004).

Table 3. Eight levels of morphophysiological dormancy (Baskin and Baskin, 1998; Walck *et al.*, 1999) and temperature, or temperature sequence, required to break them

	Temperature required[a]		
Type of MPD[b]	To break seed dormancy	At time of embryo growth	GA_3 overcomes dormancy
Non-deep simple	W or C	W	+[c]
Intermediate simple	W + C	W	+
Deep simple	W + C	W	+/−
Deep simple epicotyl	W + C	W	+/−
Deep simple double	C + W + C	W	?
Non-deep complex	C	C	+
Intermediate complex	C	C	+
Deep complex	C	C	−

[a]W, warm stratification; C, cold stratification.
[b]MPD, morphophysiological dormancy.
[c] +, yes; +/−, yes/no; −, no.

Following germination in autumn or early winter, *Helleborus* seedlings are transplanted around January. From May, the plants can be cultivated outdoors. From November to April liners can be used for further cultivation, or the plants can be sold to other producers for

continued cultivation. After germination, at least two complete growing seasons are required for most species and hybrids; some require even longer to produce the first flowers. *Helleborus* breeding (Chapter 1.3) is also strongly affected by these long periods between sowing and first flowering.

In vitro propagation of *Helleborus* combines the advantages of division and seed propagation, namely clonal and mass propagation. *Helleborus in vitro* cultures can either be established by *in vitro* sowing (Seyring, 2002), axillary buds (Dhooghe & van Labeke, 2007), rhizome buds or meristem tips (Poupet et al., 2006). The problem with rhizome buds is that they are often strongly contaminated because of their subterranean origin. Shoots from *in vitro* cultures initiated by seeds are often not contaminated, but *in vitro* sown seeds need a dormancy breaking treatment. Additionally, endophytes often cause problems during *in vitro* culture (Tisch, 2009). Nevertheless, the number of *Helleborus* plants produced *in vitro* in Germany has increased during the last few years from 0.5 million in 2006 to 2.5 million in 2009 (ADIVK, 2010). *In vitro* culture techniques often enable additional different breeding strategies, e.g., *in vitro* polyploidisation (Dhooghe et al., 2009), or support the conventional breeding progress, e.g., the application of embryo rescue techniques.

During cultivation of hellebores, black spots may arise at the cotyledons and primary leaves of *H. niger* seedlings. The reason is not totally clear, but phosphate excess is known to be involved in the generation of black spots (Richter, 2009). Stagnant moisture should be avoided during cultivation and at the final growth site; otherwise, the roots suffer from secondary pathogen infections. Hellebores grow naturally in semi-shade, but are not considered as shade-loving plants. Most *Helleborus* species are frost-hardy during European winters. In colder regions, the plants tend to stay dormant for a longer period of time. Problems with frost hardiness may arise for *H. lividus*, *H. multifidus*, *H. thibetanus*, *H. vesicarius* and *H. cyclophyllus* (Mathew, 1989).

1.3 Breeding of *Helleborus*

Breeding with *Helleborus* is mainly based on hybridisation and selection. Breeding of *Helleborus* started with the introduction of hellebores into cultivation in the mid 19th century in different countries of Europe (Mathew, 1989). From pollination to flowering it can take up to five years depending on the species. Therefore, *Helleborus* breeding is a long process.

Early cultivars in the mid 19th century were mainly selections of *H. orientalis* called ‘orientalis hybrids’. In Germany, the first of these hybrids was produced in the 1840s

(Mathew, 1989). After different *H. orientalis* genotypes were combined, the dark flower colours were introduced into the 'orientalis group' from *H. torquatus* or *H. atrorubens* (Table 1.4). There appears to be no breeding barrier between species of section Helleborastrum, and fertile offspring are produced (Mathew, 1989). Except for crossing experiments, hybridisation barriers have never been investigated systematically.

The two caulescent species of section Chenopus *H. argutifolius* and *H. lividus* hybridise easily when brought together in gardens. Fertile intermediates were first recorded in the 1940s and were named *H. x sternii* (Table 1.4). Interestingly, *H. niger* hybridises with the two species from section Chenopus to produce sterile intermediate offspring. The first hybrid *H. x nigercors*, an offspring from *H. niger* x *H. argutifolius,* was reported in the 1930s (Mathew, 1989). *H. x nigercors* has caulescent and acaulescent characteristics. It has short tough stems carrying the leaves and terminal clusters of flowers and additional basal leaves, which are truly intermediate. The flowers are more similar to *H. niger* than to *H. argutifolius*. They are produced on short stalks around the base of the plant. The hybrid between *H. niger* x *H. lividus* is called *H. x ballardiae* (Table 1.4).

For the species *H. vesicarius* and *H. thibetanus,* there is no information available regarding their hybridisation ability, and their genetic relationships to other species are still unclear. The situation with caulescent *H. foetidus* is similar, but unconfirmed hybrids with *H. viridis* or *H. argutifolius* have been reported (Table 1.4, Mathew, 1989). A hybrid between *H. niger* and *H. x hybridus* with rose flowers, called `Walberton's Rosemary`, was described recently (Rice, 2009), although *H. niger* does not hybridise readily with species from section Helleborastrum.

An overview of the existing *Helleborus* hybrids and those that have been reported but never confirmed is given in Table 1.4.

Table 1.4: *Helleborus* hybrids and reports of putative hybrids (Mathew, 1989).

	Name	Crossing combination	Combination of characteristics
Confirmed hybrids	Orientalis hybrids	intraspecific *H. orientalis* crosses	variation of sepal shape and colours
	Orientalis hybrids, garden hybrids	interspecific crosses between species within section Helleborastrum, in which *H. orientalis* is involved	variation of sepal shape and colours, especially dark colours, double flowers
	H. x sternii	*H. argutifolius* x *H. lividus*, also possible reciprocal: *H. lividus* x *H. argutifolius*	fertile intermediates referring to growth type and flowers between the parental species
	H. x ballardiae *H. x nigercors* *H. x ericsmithii*	*H. niger* x *H. lividus* *H. niger* x *H. argutifolius* *H. niger* x *H. x sternii*	sterile intermediates referring to growth type and flowers between the parental species
Unconfirmed putative hybrids	*H. x jourdanii*	*H. foetidus* x *H. viridis*	differences in growth type and flower morphology
	--	*H. foetidus* x *H. argutifolius*	sterile offspring, differences in growth type and flower morphology
	-- 'Walberton's Rosemary'	*H. niger* x *H. orientalis* *H. niger* x *H. x hybridus*	differences in growth type and leaf and flower morphology; especially sepal colour and shape
	--	*H. niger* x *H. viridis*	differences in growth type and leaf and flower morphology
	--	*H. niger* x *H. purpurascens*	differences in growth type and leaf and flower morphology

Karyotypic analyses have confirmed that *Helleborus* species are diploid 2n=2x=32 (D'Amato & Bianchi, 1989; Yuan & Yang, 2006; Castro & Rosselló, 2007). It is assumed that the chromosome number might have originated by polyploidy based on the most common basic chromosome number in the *Ranunculaceae* x=8.

The *Helleborus* species *H. x hybridus* and *H. niger* are economically important ornamentals. Several other species with minor impacts exhibit various interesting features, which should be combined with those of *H. x hybridus* or *H. niger,* e.g., scent is a characteristic of *H. odorus* and *H. liguricus*. In general, traits like sepal colour and sepal shape, flower size and number, nectaries, bracts, foliage and plant health are valuable for the horticultural improvement of *Helleborus* cultivars and could be combined by interspecific hybridisation. The most distinct morphological differences, which also have an ornamental value, are found for foliage and flower. These are described in the subsequent paragraphs.

Leaf and flower morphology

The morphology of flowers and leaves varies among species. Most of the descriptions of the following were taken from Mathew (1989) and from own observations. In general, *Helleborus* leaves are mostly pedately divided with different numbers of segments. In some species the leaves are weakly pedate, inclining to palmate or digitate. The texture is often leathery, and

the venation is prominent on the abaxial side. Most leaf characteristics are similar for species of the same section.

Leaflets of *H. vesicarius* are cut into several jagged teeth, which are wedge shaped at the base and coarsely toothed at the apex, the teeth and terminal lobe acute (Figure 1.2 A). Leaves of *H. foetidus* are long-petiolate and born at the stem. Leaf segments are narrowly lanceolate or elliptical with serrated and sometimes almost entire margins (Figure 1.2 C). The colour of petioles varies between green and purple-red. The unpleasant smell of *H. foetidus* is released by the foliage. Both *H. foetidus* and *H. vesicarius* leaves have leaf blades that are pedate with three primary divisions; two lateral leaflets are sometimes divided into two.

H. argutifolius and *H. lividus* have three-lobed leaves, all born on the stem. The central leaf is regularly elliptical, while the lateral ones are irregular with unequal sides (Figure 1.2 B). Leaflets are coarsely and sometimes spiny-toothed and rarely have entire margins (Figure 1.2 B). Petioles of *H. lividus* are suffused purple (Figure 1.2 B), whereas those of *H. argutifolius* are green. *H. niger* leaves are pedate with oblong or oblanceolate segments that are toothed towards the apex. Petioles are green or purple spotted (Figure 1.2 D).

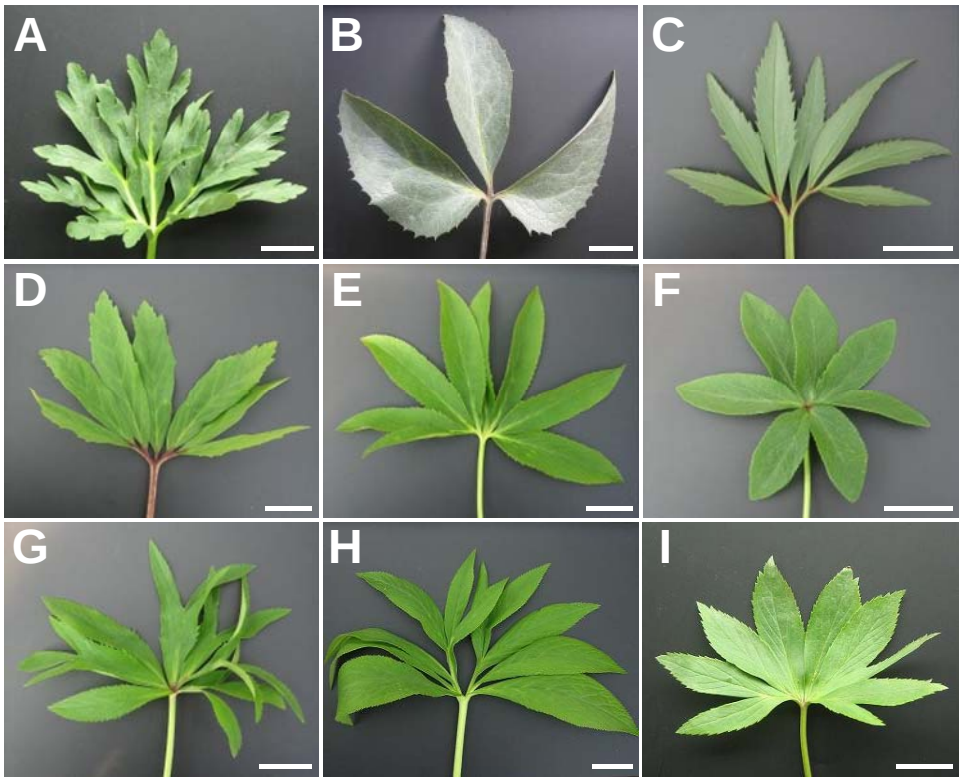


Figure 1.2: Leaf morphologies of adult plants from different *Helleborus* species.
A: *H. vesicarius*, B: *H. lividus*, C: *H. foetidus*, D: *H. niger*, E: *H. x hybridus*, F: *H. cyclophyllus*, G: *H. dumetorum*, H: *H. torquatus*, I: *H. thibetanus*. Bars represent 3 cm.

For all species of section Helleborastrum, it is difficult to determine species-specific leaf characteristics. The morphological variation is quite high even within a species, e.g., multiple divided leaves have been described for *H. abruzzicus* (up to 200 times), and *H. hercegovinus* leaves may be heavily dissected (45-100 times). Examples of leaves from the Helleborastrum species are shown in Figure 1.2 E-H. The leaves of *H. cyclophyllus*, *H. dumetorum* and *H. torquatus* in Figure 1.2 F-H represent the genotypes used in this study.

Leaves of *H. thibetanus* are pedate (Figure 1.2 I). The lobes are elliptical to oblanceolate and cuneate at the base with coarsely and sharply serrated margins.

In general, the flowering season of *Helleborus* species ranges from December until late spring. Some species start flowering earlier, while other species start flowering in April or May. Most of the flowering times of different species overlap. Flowering time depends on the conditions, especially the temperature, under which the plants are cultivated.

Helleborus flowers are quite variable in shape and flower colour. The largest variation in flower colour is found in section Helleborastrum (Table 1.2, Figure 1.3 D-I), especially in the *H. x hybridus* genotypes (Figure 1.3 G-I). Colours range from white over pink to dark purple and spotted. Double-flowering forms are also found.

While most of the *Helleborus* flowers are similar in shape, *H. foetidus* and *H. vesicarius* flowers differ from all the other species. The flowers of both species are globular or bell-shaped, pendent and smaller in size than other species (Figure 1.1 C, Figure 1.3 B). *H. foetidus* flowers are green, sometimes rimmed with brown or purple, while *H. vesicarius* flowers are green but are often stained purple or brownish at the apex.

The flower composition is similar for all species. *Helleborus* flowers are bisexual and protogynous, meaning that the stigma is receptive before the pollen is shed from the anthers. Flowers are usually cross-pollinated, although they are self-compatible. In nature, pollination is mediated by bees of the genera *Apis* (honey bees), *Bombus* (bumble bees) and *Anthophora* (flower bees), which are attracted by the flower scent and ultraviolet reflection of the sepals.

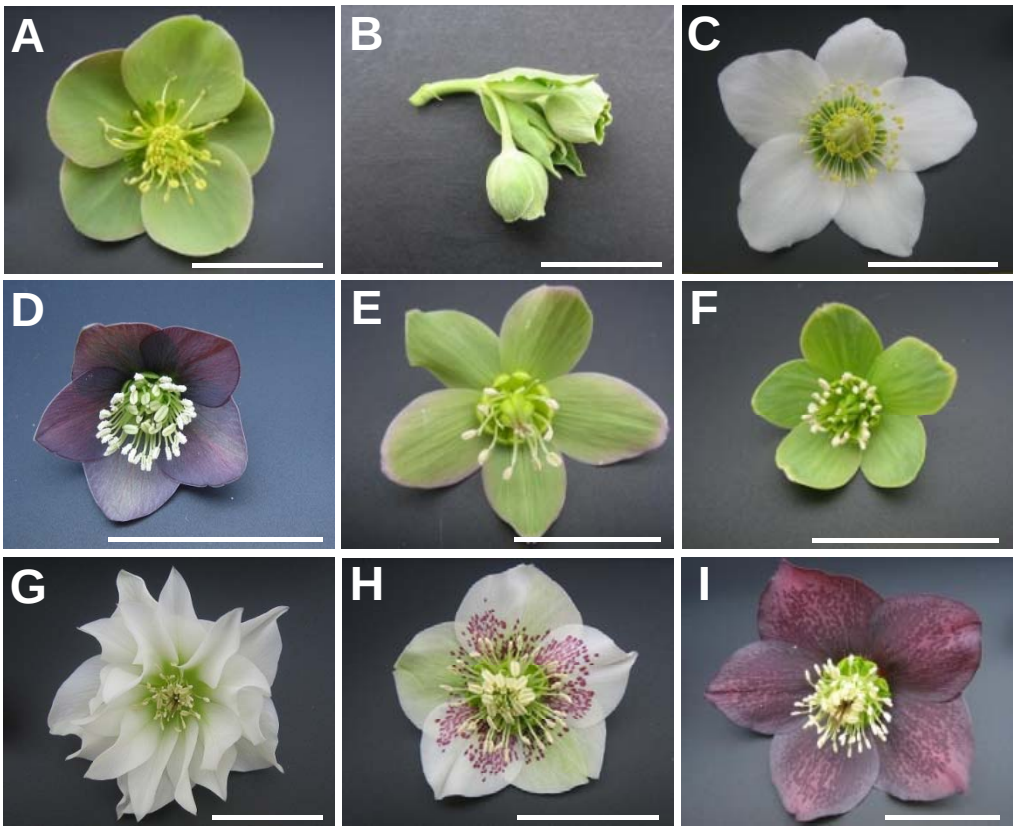


Figure 1.3: Variability in flower morphology of different *Helleborus* species and cultivars.
A: *H. argutifolius*, B: *H. foetidus*, C: *H. niger*, D: *H. atrorubens*, E: *H. dumetorum*, F: *H. purpurascens*, G: *H. x hybridus* `Mrs. Betty Ranicar´, H: *H. x hybridus* `Spring Promise Sue´, I: *H. x hybridus* `Spring Promise Rachel´. Bars represent 3 cm.

Flower assembly is now described from the outside to the centre. Flowers mostly consist of five large, usually overlapping perianth segments, the sepals, which vary in colour (Figure 1.3). The sepals turn green after anthesis and contribute to photosynthesis. The petals become funnel-shaped, shortly stalked nectaries that provide food for pollinators. Numerous stamens (the number is variable) are found next to the nectaries. The stamens are erect at first and then elongate and arch outwards as they mature (Figure 1.4 A). Anthers are elliptical or oblong and are yellow to cream in colour (Figure 1.4 A and E). Carpels are sessile, variable in number and disconnected or slightly fused at the base (Figure 1.4 B). The number of ovules in the ovary differs across species (Figure 1.4). In *H. vesicarius,* three to six ovules are found (Mathew, 1989), while 10 to 20 are present in ovaries of other species (Schiffner, 1890). Styles are straight or curved and differ in length, depending on the species. The stigmas are punctiform and adhere pollen, if they are receptive (Figure 1.4 C and F).

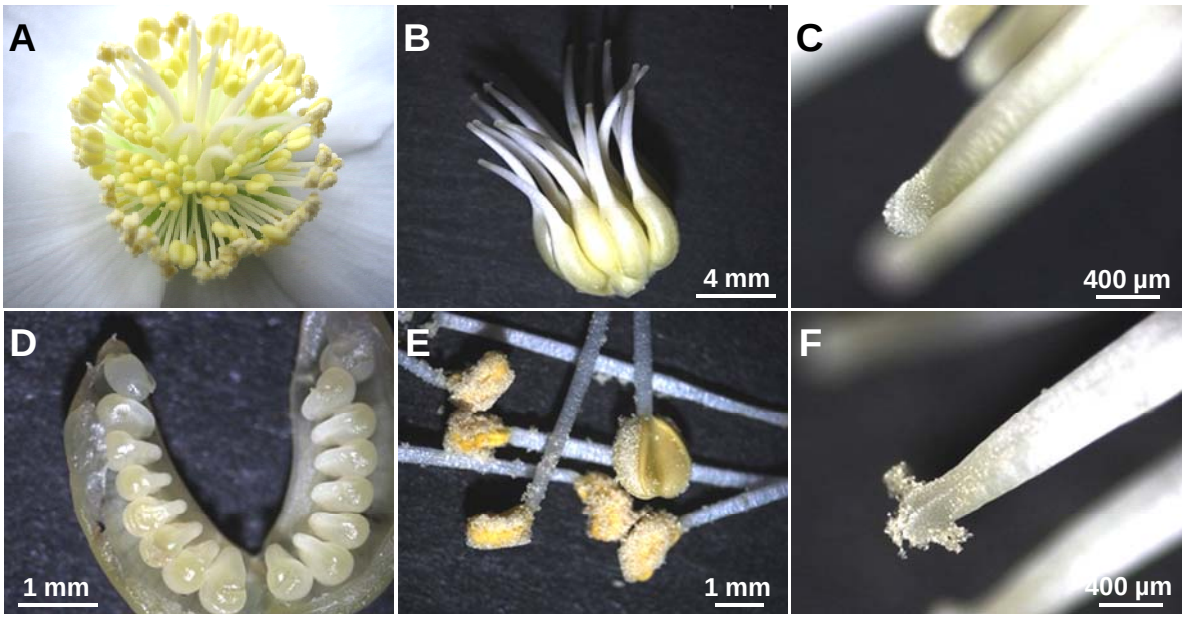


Figure 1.4: Close-up views of *H. niger* flower organs.
A: overview of carpels, stamens, green nectaries below the stamens and the white sepals, B: apocarpe carpels, C: stigma with papillae, D: inner view of an ovary with single ovules, E: stamens with freshly dehisced anthers, F: pollinated stigma.

1.4 Diseases and pests of *Helleborus*

Many diseases and pests affect *Helleborus* during cultivation or at the final growth site.

The larvae of fungus gnats cause severe damage to *Helleborus* roots during propagation or greenhouse cultivation if they are not recognised. Leaves and flowers can suffer from aphids, e.g., the hellebore aphid, *Macrosiphum hellebori,* (Mathew, 1989). Additionally, whiteflies, the hellebore leaf miner, *Phytomyza hellebori* (Mathew, 1989), and snails affect hellebore leaves. Thrips damage flowers and dramatically reduce the ornamental value of the plant. Lastly, nematodes can affect the roots and leaves.

There are also viral diseases associated with *Helleborus* plants, e.g., *Helleborus mosaic virus* belonging to the *Carlaviruses*, *Cucumber mosaic virus* and *Broad bean wilt virus* (Eastwell et al., 2009). Since the 1990s, a *Helleborus* virus disease referred to as 'black death' has gained importance. Helleborus net necrosis virus (Eastwell et al., 2009), also a *Carlavirus*, has been identified as the causal agent. The symptoms are black streaks that appear on the leaves, often following the veins, as well as on the flowers and stems. *Carlaviruses* are transmitted nonpersistently by aphids.

Fungal diseases, like *Ramularia* and downy mildew, often occur on the leaves as a primary infection. Additionally, the roots may be affected by *Pythium*, *Phytophthora* or *Rhizoctonia*,

causing rhizome and stalk decay as a secondary infection. Infection from these three pathogens can occur if growth conditions are not suitable, e.g., during container cultivation.

Helleborus leaves are affected by some leaf spot diseases. The most important of these is *Coniothyrium hellebori,* which causes hellebore leaf or black spot. The pathogen is less of a problem during propagation than at the final growth site in botanical and private gardens (Figure 1.5). The symptoms of *C. hellebori* are blackish-brown spots that often appear as concentric rings either on the leaf blade or at the margin. As the disease progresses, the spots grow and become irregular, dark brown or black, round or elliptical areas, until they infect leaf parts and whole leaves. Petioles and flowers are also affected but to a lesser extent than the leaves. Mild wet winters may encourage leaf spot disease. The symptoms become visible in early spring and increase in severity for the rest of the year, which dramatically reduces the ornamental value of the plants.

Figure 1.5: Symptoms of *C. hellebori* on hellebores.
A and B: Botanical garden in Munich, July 2008, C: *H. x hybridus* in the Hofgarten in Freising December 2007.

1.5 Collaboration project: "Developing resistant, homogenous and high-yielding cultivars of *Helleborus* species"

The studies in this thesis were performed as part of a collaboration entitled "Developing resistant, homogenous and high-yielding cultivars of *Helleborus* species", which was financially supported by the German Federal Ministry of Food, Agriculture and Consumer Protection administrated by the Federal Agency for Agriculture and Food [grant number 28-1-41.035-06].

The project was aimed at obtaining fundamental prerequisites for interspecific hybridisations within the genus *Helleborus* and to create new interspecific hybrids. Resistance against *C. hellebori* was the trait with top priority in addition to morphological plant characteristics. The collaboration between the company Heuger, Glandorf, Germany, a producer and breeder of hellebores, the Research Station for Horticulture at the Weihenstephan-Triesdorf University of Applied Sciences, Freising, Germany and the Leibniz Universitaet Hannover, Germany was divided into two sub-projects. Sub-project 1 was attended to the company Heuger, coordinator: Peter Oenings.

Sub-project 1: Development of a resistance test against *Coniothyrium hellebori* and development of hybrids and methods for their propagation.

- Provision of *Helleborus* species
- Development of an evaluation of resistance in *Helleborus* species
- Diallelic crosses of all available *Helleborus* species
- Development of methods for hybrid propagation
- Evaluation of hybrid resistance against *C. hellebori* and hybrid performance

Sub-project 2 was conducted at the Research Station for Horticulture Weihenstephan (01.11.2007-31.05.2009) and at the Leibniz Universitaet Hannover, Institute for Floriculture and Woody Plant Sciences (01.06.2009-31.10.2010), coordinator: Prof. Dr. Traud Winkelmann, project agent: Julia Meiners.

Sub-project 2: Genetic diversity, crossability and hybridisations in the genus *Helleborus.*

- Cytological and flow cytometric analysis of *Helleborus* species
- Molecular genetic analysis of the genus *Helleborus*
- Molecular genetic description of different *C. hellebori* origins
- Viability and storage of pollen
- Analysis of pollen germination *in situ* and determination of crossing barriers
- Development of methods to overcome crossing barriers (embryo rescue)
- Identification of hybrids

1.6 Thesis objectives

Within both breeder and consumer groups, the demand for new *Helleborus* phenotypes of various flower colours and morphologies, scents, foliage, variation in growth type and disease tolerance and resistance has increased. The combination of these traits cannot be achieved with crosses of one *Helleborus* species. Large variations in flower colour are available in species of the section Helleborastrum. Scent is a characteristic of *H. odorus* and *H. liguricus*. Growth types with large terminal inflorescences are found in the sections Chenopus and Griphopus. Furthermore, some hybrids that already exist are less susceptible to hellebore leaf spot disease, the most important fungal pathogen at the final growth site. Therefore, interspecific hybridisation of *Helleborus* species is the method of choice for combining these traits.

The three main objectives of this thesis and sub-project 2 were as follows:

1. To obtain interspecific hybrids, fundamental knowledge of the relatedness of *Helleborus* species should be acquired with:

    - Cytological analyses that aimed at determining the chromosome number of species, which is an important indicator for the success of certain crossing combinations.
    - Determination of the nuclear DNA content of the available *Helleborus* species via flow cytometry in order to analyse taxonomic differences and to identify parental species, whose offspring could be identified by flow cytometry.
    - Molecular genetic analyses of the *Helleborus* plant material on the basis of multilocus AFLP markers in order to calculate genetic distances and to analyse the sectional taxonomic sub-division of the *Helleborus* genus.

2. Interspecific hybridisations often suffer from pre- or postzygotic crossing barriers. Therefore, crossing barriers needed to be identified and methods to overcome these barriers should be established by:

    - Tests of pollen viability of the *Helleborus* species and of stored *Helleborus* pollen.
    - Evaluation of pre- and postzygotic hybridisation barriers by tracking the pollen tube growth *in situ*.
    - Developing an embryo rescue technique to overcome postzygotic hybridisation barriers.
    - Developing a procedure to identify obtained putative hybrids.

3. Because disease tolerance against *C. hellebori* is a major concern, biological insights into this pathogen are essential and should be obtained by:

    - Comparing morphological characteristics of a collection of *C. hellebori* isolates from different geographical locations.
    - Developing a method to produce large amounts of spore material for inoculations.
    - Testing the pathogenicity to analyse whether the isolates are causing the disease.
    - Performing molecular genetic analyses of the relationships between *C. hellebori* isolates using RAPD markers.

The thesis is comprised of three chapters, which are attended to the three main objectives and are planned for subsequent publication, and conclusions and outlook.

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

2.1 Introduction

Information about chromosome numbers of plant species provides important knowledge referring to taxonomy and breeding. In hybrids between chromosomal divergent species meiotic chromosome pairing may be disturbed. Equal chromosome numbers of species facilitate hybridisation because these dysfunctions in meiosis seem to be improbable. Cytological studies of the *Ranunculaceae* including some representatives within the genus *Helleborus* were carried out to estimate chromosome numbers and in some cases karyotypes (Gregory, 1941; D'Amato & Bianchi, 1989; Yuan & Yang, 2006). These investigations revealed that all studied *Helleborus* species have the same chromosome number of 2n=32 (Gregory, 1941; D'Amato & Bianchi, 1989; Yuan & Yang, 2006; Bennett & Smith, 1976; Dobes et al., 1997; Castro & Rosselló, 2007).

Chromosome sizes of plants are closely correlated with their nuclear DNA contents, which can be determined by flow cytometry; a method that determines the nuclear DNA content of the sample by simultaneous measurement with an internal reference standard of known DNA content. The genome size (C-value) is known to correlate with the geographical distribution of plants, in terms of karyotypic and cytogenetic characters including polyploidy and chromosome size, (Bennett, 1976) and can be useful for the examination of phylogenetic dimensions (Bennett et al., 2000) as well as the taxonomic classification of species in narrow taxonomic groups (Ohri, 1998). The 2C DNA amount of herbaceous higher plants shows at least a 500-fold range, from less than half a picogram (pg) to more than 250 pg (Bennett & Smith, 1976). Nuclear DNA contents of the *Helleborus* species have already been determined to evaluate the *Helleborus* taxonomy (Zonneveld, 2001). Zonneveld's results were consistent with the genus division recommended by Mathew (1989) and Werner & Ebel (1994) (Chapter 1.1.1).

The first phylogenetic evaluation of *Helleborus* based on the chloroplast (*trnL-F* and *matK*) and ribosomal ITS DNA sequence data was done by Sun et al. (2001). This analysis also supported the genus' division into sections, but did not resolve the problematic nature of the section Helleborastrum (Figure 2.1). Additionally, bootstrap support in their analysis was low for the relevant branching differing between sections (Figure 2.1). Close relationships

between species and intraspecific morphological variation (Servettaz et al., 1988) make it difficult to detect the relationship between them.

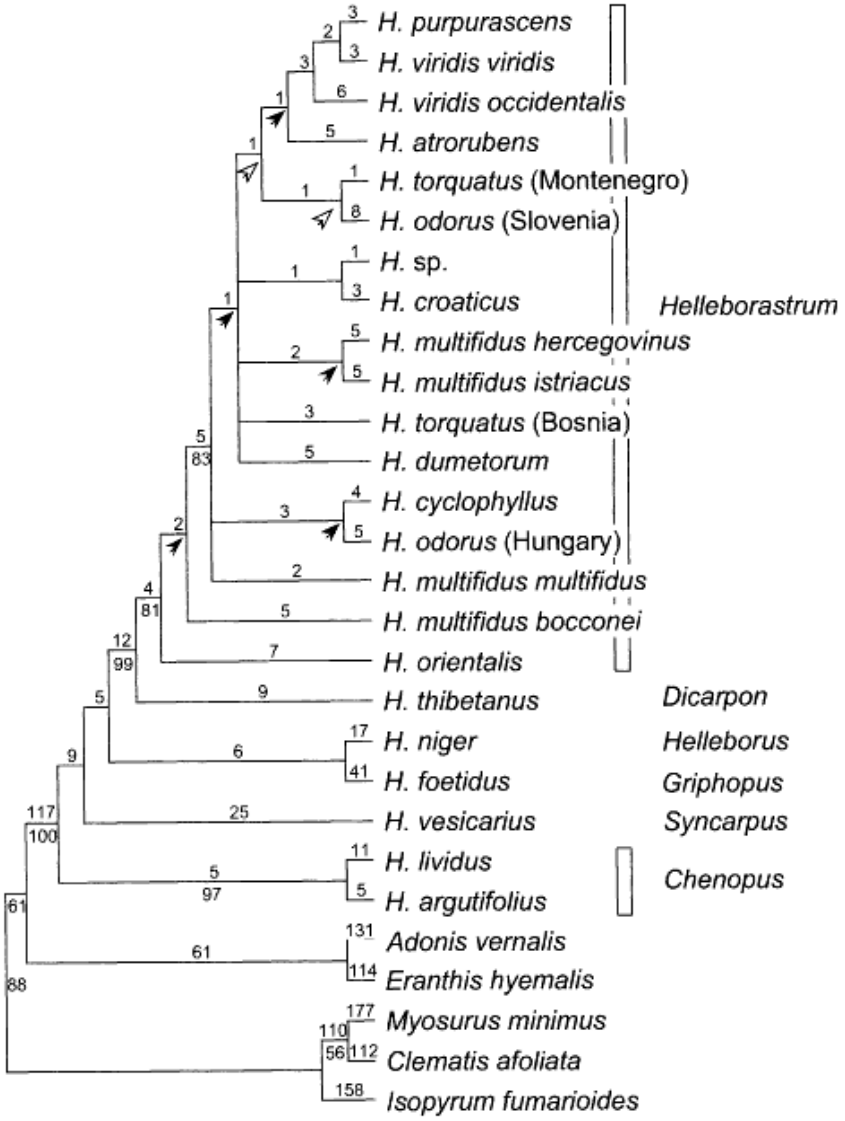


Fig. 2. One of 134 most-parsimonious trees obtained with successive weighting based on combined data (*trnL-F, matK,* ITS), Fitch length 1310, CI = 0.80, RI = 0.70 (weighted TL = 861.92057, CI = 0.96, RI = 0.92). Fitch branch lengths are shown above and bootstrap percentages below the branches. Branches not present in the strict consensus of either the equally weighted or SW trees are indicated with solid arrows. Branches not present in the strict consensus of the equally weighted trees are indicated with open arrows. The vertical bars show the position of the two sections *Helleborastrum* and *Chenopus* that contain more than one species.

Figure 2.1: Cladistic tree of the genus *Helleborus* generated by combining molecular data (*trnL-F*, *matK*, ITS).
Figure was entirely adopted from Sun et al. (2001).

An initial taxonomic differentiation of four *Helleborus* species and eight populations from Italy belonging to section Helleborastrum was performed by Fico et al. (2005). A clear discrimination between the species and populations of *H. bocconei*, *H. niger*, *H. odorus* and *H. viridis* was revealed on the basis of RAPD markers (Random Amplified Polymorphic DNA). 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 up to now.

Due to the contradictory results regarding the taxonomic classification of the genus *Helleborus* and the discovery of new species, the objectives in this study aimed at gathering novel information on the 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 determine the nuclear DNA content of 21 species and four hybrids and elaborate the potential of flow cytometry for the identification of interspecific hybrids, (iii) to evaluate genetic relationships within the genus *Helleborus* by the use of the multilocus AFLP technique (Vos et al., 1995). The integrative approach of this study is the basis for the establishment of future breeding strategies, especially with regard to interspecific hybridisations.

2.2 Materials and Methods

2.2.1 Plant material

In total, 21 out of 22 described *Helleborus* species, 11 genotypes of *H. niger*, 10 genotypes of *H. x hybridus*, one true *H. orientalis* and four *Helleborus* hybrids were used in this study (Table 2.1). At the beginning of the study, no plant material of *H. occidentalis,* formerly regarded as subspecies of *H. viridis,* was available; therefore, it was not integrated. Plants of most of these species were provided by the company Heuger, Glandorf, Germany. In addition, leaf material of six species for AFLP analysis was kindly provided by Will McLewin, Phedar Nursery, Bunkers Hill, Romiley, Stockport, UK. As a member of the *Ranunculaceae* family, *Pulsatilla vulgaris* 'Violet' was used as the outgroup within the calculated phenogram. Chromosome counts were performed for representative species of five *Helleborus* sections.

2.2.2 Cytological analysis

Two to three hours past sunrise, young root tips measuring 0.5-1 cm in length were harvested from the different *Helleborus* species and pretreated with 2 mM 8-hydroxyquinoline solution at room temperature for 4 hours. Next, the solution was replaced by fixative solution (three parts ethanol: one part glacial acetic acid) and stored overnight at 4 °C. Root maceration was carried out in 1 N HCl at 60 °C for 10 minutes. The maceration solution was replaced by 1 % Aceto-Orcein in 45 % acetic acid and chromosomes were stained for 40 minutes. Then, the staining solution was decanted and the remnants of the dye were washed off with deionised water. For microscopic analysis, approximately 1 mm of the root tip was transferred to a microscope slide. The root tip was covered with a cover slip and squeezed into a drop of 45 % acetic acid with a preparation needle. The preparations were observed under a bright field microscope (Axio Scope.A1, Zeiss, Oberkochen, Germany) at 1000 x magnification.

Table 2.1: *Helleborus* species, genotypes and hybrids analysed in the present study.

Helleborus species	Provided by	Available information
Section Syncarpus		
H. vesicarius	Heuger	
Section Griphopus		
H. foetidus	Heuger	
Section Chenopus		
H. argutifolius	Heuger	
H. lividus	Heuger	
Section Helleborus		
H. niger 1210[a]	Heuger	Open pollinated[b]
H. niger 1010	Heuger	Clone[c]
H. niger 1020	Heuger	Clone
H. niger 1220	Heuger	Open pollinated
H. niger 1030	Heuger	Clone
H. niger 1040	Heuger	Clone
H. niger 1050	Heuger	Clone
H. niger 1110	Heuger	F_1 hybrid cultivar
H. niger 1060	Heuger	Clone
H. niger 1120	Heuger	F_1 hybrid cultivar
H. niger 1070	Heuger	Clone
Section Helleborastrum		
H. abruzzicus	Heuger, McLewin	
H. atrorubens	Heuger	
H. bocconei	Heuger	
H. croaticus	Heuger	
H. cyclophyllus	Heuger	
H. dumetorum	Heuger	
H. hercegovinus	McLewin	
H. orientalis	McLewin	
H. x hybridus 1060	Heuger	Clone
H. x hybridus 1230	Heuger	Open pollinated
H. x hybridus 1070	Heuger	Clone
H. x hybridus 1080	Heuger	Clone
H. x hybridus 1090	Heuger	Clone
H. x hybridus 1240	Heuger	Open pollinated
H. x hybridus 10100	Heuger	Clone
H. x hybridus A	Heuger	
H. x hybridus B	Heuger	
H. x hybridus C	Heuger	
H. istriacus	Heuger	
H. liguricus	Heuger, McLewin	
H. multifidus	Heuger	
H. odorus	Heuger	
H. odorus 0613	McLewin	Unknown sample[d], from Serbia
H. purpurascens	Heuger	
H. purpurascens 0814	McLewin	Unknown sample, from Romania
H. torquatus	Heuger	
H. viridis	Heuger	
Section Dicarpon		
H. thibetanus	Heuger	
Hybrids		
H. x ballardiae	Heuger	
H. x ericsmithii	Heuger	
H. x nigercors	Heuger	
H. x sternii	Heuger	

[a]numbers represent different genotypes; from *H. niger* 1120 four sibling plants were used for AFLP analysis
[b]maternal parent was pollinated with a pollen mixture of different pollen donors
[c]clonally propagated
[d]at the beginning of the study, the species was only known by Will McLewin

2.2.3 Flow cytometric analysis of nuclear DNA content

For isolation of nuclei, 0.6 cm² of fresh young leaf tissue from adult plants was chopped together with leaf tissue of either *Secale cereale* subsp. *cereale* (16.01 pg/2C) (Gatersleben gene bank accession no. R 737) or *Vicia faba* 'Tinova' (26.21 pg/2C) (Gatersleben gene bank accession no. FAB 602) as internal reference standards. These internal standards were kindly provided by Dr. Joerg Fuchs, IPK Gatersleben, Germany. Sample tissues were chopped with a razor blade in a plastic petri dish in 0.5 ml extraction buffer (CyStain PI Absolute P; Partec, Muenster, Germany) and incubated for at least 30 sec. The nuclei suspension was filtered through a 30 µm CellTrics filter (Partec) into a sample tube. Two ml staining solution (CyStain PI Absolute P; Partec) containing propidium iodide (PI) and RNase were added to the nuclei extract. Samples were incubated for at least 60 minutes on ice in the dark. The fluorescence of the nuclei was then measured using the CyFlow Ploidy analyser (Partec). Fluorescence was excited at 532 nm using a Nd-YAG laser. The 2C DNA content of the sample was calculated as the mean sample peak divided by the mean standard peak and multiplied by the known amount of DNA of the standard. At least three replicates and at least 5000 nuclei were measured for each genotype. All peaks analysed revealed a coefficient of variation of less than 5 %. For all species, the DNA content was measured using *S. cereale* as the internal standard; additionally, *V. faba* was used as an internal reference for *H. argutifolius*, *H. lividus*, *H. dumetorum* and *H. thibetanus*.

2.2.4 DNA extraction and AFLP analysis

For DNA extraction, young leaves were collected, freeze-dried and ground to powder using a bead mill. Total plant DNA was isolated in two replicates from each genotype based on the CTAB procedure described by Saghai-Maroof et al. (1984). The DNA concentration was estimated in comparison to known DNA concentrations of λ-DNA and standard plant DNA in a 0.8 % agarose gel. The procedure for the AFLP markers (amplified fragment length polymorphism) was performed as described by Vos et al. (1995) with minor modifications (Hartl et al., 1999; Schmolke et al., 2005). Genomic DNA (250 ng) was digested with the restriction enzymes *Pst*I (2.5 U) and *Mse*I (1 U). The *Pst*I adapter (2.5 pmol) and the *Mse*I adapter (25 pmol) were ligated to the restriction fragments with 1 U of T4-DNA ligase. Restriction digestion and adapter ligation were performed in one step for 3 h at 37 °C followed by an overnight incubation at 16 °C. For preamplification, primers homologues to the adapters and the restriction sites, containing two selective nucleotides were used. Selective amplification was carried out using 10 *Pst*I / *Mse*I primer combinations (Table 2.3) with three

selective nucleotides on the 3'-end of either primer. The 5'-end of the *Pst*I primers was labelled with fluorescein, and the standard list for AFLP primer nomenclature (http://wheat.pw.usda.gov/ggpages/keygeneAFLPs.html) was used. All reactions were performed with two independent DNA extractions to ensure pattern reproducibility. Only reproducible bands were included in subsequent analyses. The PCR fragments were separated using 5 % denaturing polyacrylamide gels under standard sequencing conditions. The gels were scanned with a Typhoon 9200 fluorescence scanner (GE Healthcare Europe, Freiburg) for fragment detection.

2.2.5 Data analysis

The banding patterns were evaluated by visual inspection and transformed into a 0/1 matrix for each DNA fragment. Genetic distances and phenograms were calculated using the PHYLIP 3.69 software package (http://evolution.genetics.washington.edu/phylip.html). The pairwise distances between the analysed genotypes were calculated using the similarity index of Nei and Li (1979). For the construction of the phenograms, three different distance methods available in the PHYLIP software were used: Fitch and Margoliash, UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and Neighbour-joining. Relative branch support was assessed by bootstrap analysis (Felsenstein, 1985) with 1000 replicates. Majority rule consensus trees were constructed with a reduced number of *H. niger* and *H. x hybridus* genotypes to focus on sectional divisions. Consensus trees were based on 1000 trees for all three distance methods. The phenograms were visualised with the TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

2.3 Results

2.3.1 Cytological analysis

Chromosomes were counted and their lengths were measured for the representative species of all six *Helleborus* sections, except for the section Syncarpus, because no mitotically active root tips were available for *H. vesicarius*. All other species of the monospecific sections, namely *H. foetidus*, *H. niger* and *H. thibetanus*, and *H. argutifolius* and *H. lividus* in the section Chenopus, were analysed (Figure 2.2).

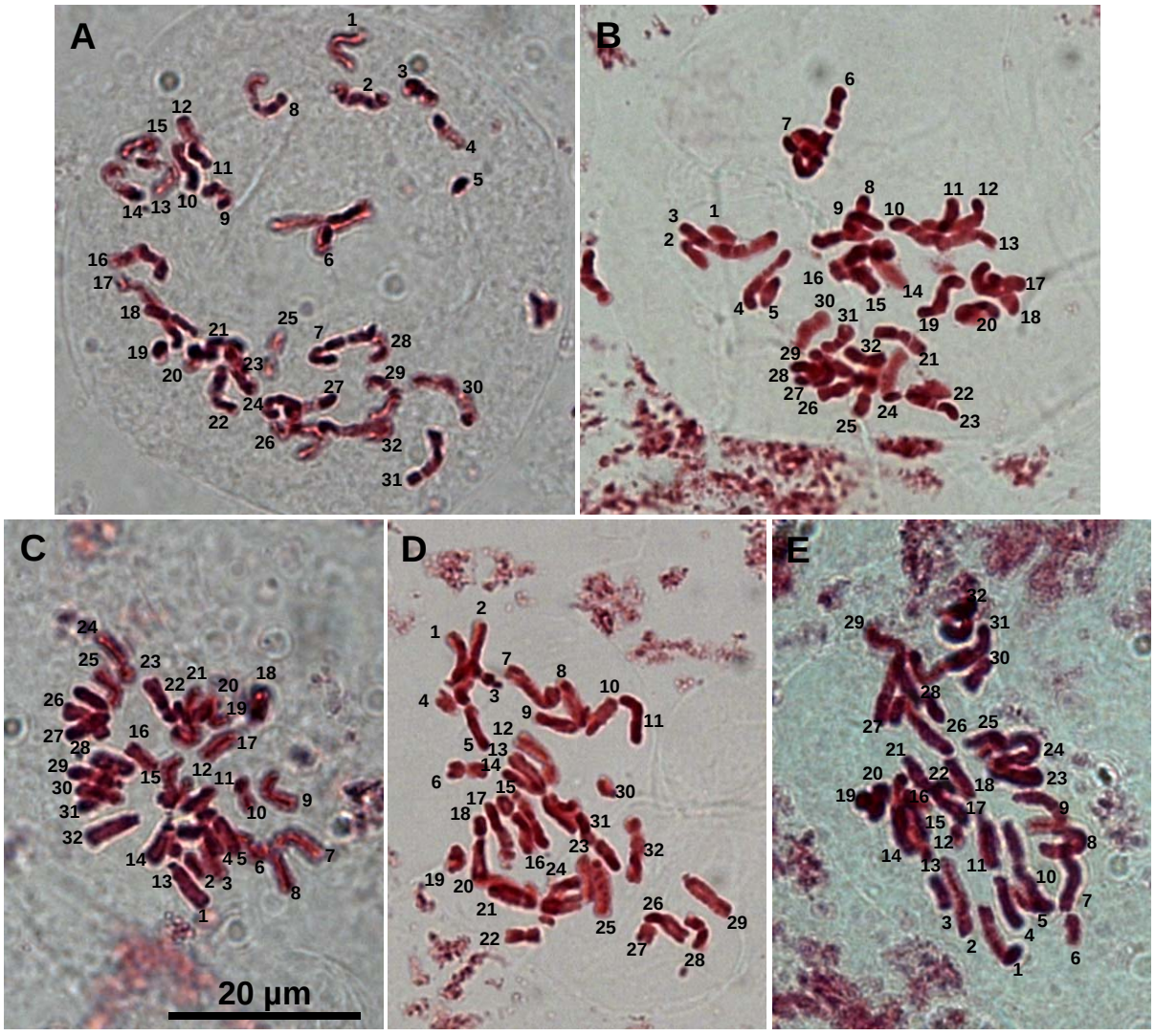


Figure 2.2: Chromosome counts from root tips of five *Helleborus* species stained with Aceto-Orcein.
A: *H. argutifolius*, B: *H. foetidus*, C: *H. niger*, D: *H. odorus*, E: *H. thibetanus.* Numbers in the sub-figures represent chromosome counts.

For the Helleborastrum section, chromosomes were counted for *H. atrorubens*, *H. cyclophyllus*, *H. dumetorum*, *H. x hybridus*, *H. multifidus* and *H. odorus*. For all species, a chromosome number of 2n=32 was determined (Table 2.2). Within the section

Helleborastrum, the chromosomes differed in length from 2 µm to 13 µm. In the sections Chenopus (2-9 µm) and Griphopus (3-9 µm), chromosomes were slightly shorter. Chromosome sizes of *H. niger* varied from 2 µm to 13 µm, while those of *H. thibetanus* were 3 µm to 17 µm long.

2.3.2 Estimation of the nuclear DNA content

The nuclear DNA content was estimated for 21 *Helleborus* species and four hybrids, with *S. cereale* as internal reference standard (Table 2.2). One histogram of a measurement of *H. niger* nuclei together with *S. cereale* is demonstrated in Figure 2.3 A. In addition, the nuclear DNA content was measured with *V. faba* as a reference standard for *H. argutifolius*, *H. lividus*, *H. dumetorum* and *H. thibetanus* (Table 2.2). To determine intraspecific differences, three measurements of DNA amount, corresponding to three biological replicates of three genotypes each, were performed for *H. niger* and *H. x hybridus*.

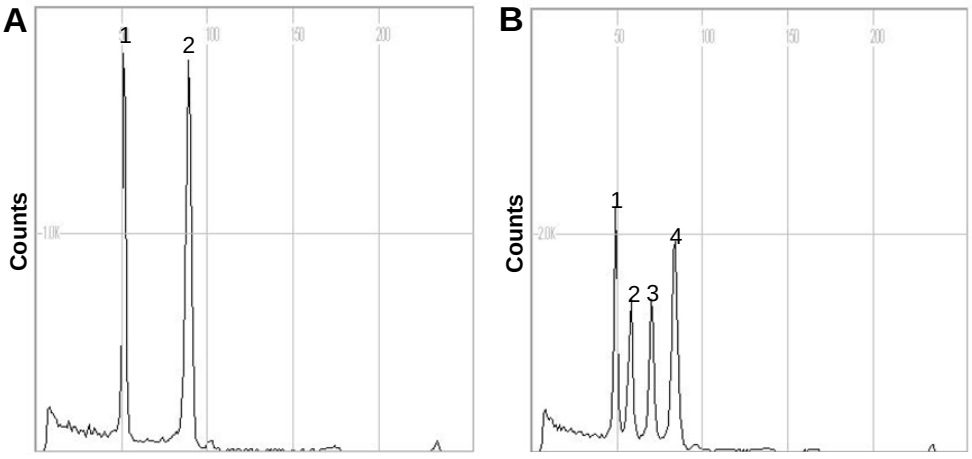
Over all species, the nuclear DNA content varied between 18.3 pg/2C and 33.2 pg/2C, with standard deviations of 0.05 to 0.53. *H. argutifolius* (18.3 pg/2C) and its sister species *H. lividus* (19.4 pg/2C) in the section Chenopus had the lowest DNA amounts in the genus (Table 2.2). The differences in nuclear DNA content between the measurements using the two different reference standards were 0.1 pg for *H. argutifolius* and 0.2 pg for *H. lividus*. *H. foetidus,* representing the section Griphopus, had the second lowest DNA content with 22.0 pg/2C. Hence, these three species were clearly separated from the other species. The nuclear DNA content of *H. niger* varied from 26.6 pg/2C to 27.0 pg/2C for three genotypes, with an average of 26.8 pg/2C. All species from the section Helleborastrum and *H. vesicarius* had similar DNA amounts that ranged from 27.0 pg/2C to 31.3 pg/2C (Table 2.2), whereas *H. x hybridus* contained 28.5 pg/2C to 29.0 pg/2C (28.7 pg/2C in average) of nuclear DNA. The highest nuclear DNA content was observed in *H. thibetanus,* with 33.2 pg/2C on average, although a difference of 1 pg between the calculations based on the two internal reference standards, *S. cereale* and *V. faba*, was recorded. For the first time the nuclear DNA contents of *H. abruzzicus* with 27 pg/2C, *H. liguricus* with 29.9 pg/2C and *H. croaticus* with 30.2 pg/2C were determined.

As expected, the nuclear DNA contents of the four hybrids, *H. x ballardiae*, *H. x ericsmithii*, *H. x nigercors* and *H. x sternii,* represented the average DNA amounts of their parental species (Chapter 1.1.1, Table 1.2; shown for *H. x nigercors* in Figure 2.3 B).

Table 2.2: Nuclear DNA contents and chromosome numbers determined in this study in comparison to previously published data.
Determinations for the first time are coloured grey.

Helleborus species	Internal reference standard	Nuclear DNA content [pg/2C]		Nuclear DNA content [pg/2C] (Zonneveld, 2001)[a]		Chromosome numbers	
		mean	std	mean	std	Determined/ verified in this study	Previously published by[b]
Section Syncarpus							
H. vesicarius	*S. cereale*	27.2	0.05	28.3	1.10		
Section Griphopus							
H. foetidus	*S. cereale*	22.0	0.14	23.3 23.4	0.31 0.47	32	1, 3
Section Chenopus							
H. argutifolius	*S. cereale* *V. faba*	18.3 18.2	0.50 0.32	18.9	0.54	32	
H. lividus	*S. cereale* *V. faba*	19.5 19.3	0.3 0.17	19.0	0.52	32	3, 6
Section Helleborus							
H. niger 1070	*S. cereale*	27.0	0.34	28.0	1		
H. niger 1070	*S. cereale*	26.6	0.30	28.3	0.7	32	1, 2, 3
H. niger 1070	*S. cereale*	26.9	0.15	29.4	0.61		
	Average	**26.8**	**0.21**				
Section Helleborastrum							
H. abruzzicus	*S. cereale*	27.0	0.44				
H. atrorubens	*S. cereale*	29.1	0.22	29.6 30.8	1.19 0.97	32	
H. bocconei	*S. cereale*	30.5	0.27	30.8	1.01		3
H. croaticus	*S. cereale*	30.2	0.43				
H. cyclophyllus	*S. cereale*	28.6	0.22	29.9	0.74	32	
H. dumetorum	*S. cereale* *V. faba*	29.6 29.7	0.27 0.25	32.4	0.61	32	1, 4
H. hercegovinus	*S. cereale*	27.4	0.12	29.6	0.92		
H. x hybridus A	*S. cereale*	28.5	0.32	[c]29.7-30.6	0.37-0.79	32	1
H. x hybridus B	*S. cereale*	28.7	0.1				
H. x hybridus C	*S. cereale*	29.0	0.41				
	Average	**28.7**	**0.29**				
H. istriacus	*S. cereale*	29.2	0.35	30.1	1.10		
H. liguricus	*S. cereale*	29.9	0.22				
H. multifidus	*S. cereale*	29.1	0.26	30.2	0.02	32	1, 3
H. odorus	*S. cereale*	28.6	0.04	30.2 30.7	0.85 0.95	32	1, 3
H. purpurascens	*S. cereale*	31.3	0.04	30.5	1.21		
H. torquatus	*S. cereale*	28.2	0.22	29.8 30.1	1.16 1.06		
H. viridis	*S. cereale*	28.7	0.37	30.4	1.26		
Section Dicarpon							
H. thibetanus	*S. cereale* *V. faba*	32.7 33.7	0.13 0.53	35.7	1.23	32	5
Hybrids							
H. x ballardiae	*S. cereale*	23.5	0.34	23.8			
H. x ericsmithii	*S. cereale*	22.8	0.18				
H. x nigercors	*S. cereale*	23.2	0.22	22.5-23.1	0.76	32	
H. x sternii	*S. cereale*	18.2	0.20	19.1	0.12		

[a]three different samples were measured twice for each clone; other genotypes were used compared to this study and if more than one genotype was measured for one species, the results are shown for all of them
[b]1: Gregory (1941), 2: Bennett & Smith (1976), 3: D'Amato & Bianchi (1989), 4: Dobes et al., (1997), 5: Yuan & Yang (2006), 6: Castro & Rosselló (2007)
[c]range of seven different *H. orientalis* genotypes



Peak no.	Counts	Mean	CV
1	4419	53.6	1.8
2	6271	91.3	1.8

Peak no.	Counts	Mean	CV
1	5607	51.47	2.9
2	5072	59.46	3.0
3	5009	72.24	2.5
4	7854	86.00	2.0

Figure 2.3: Relative DNA contents of nuclei from leaf tissue of *S. cereale* and *Helleborus*.
A: 1. *S. cereale*, 2. *H. niger*, B: 1: *S. cereale*, 2: *H. argutifolius*, 3. *H. x nigercors*, 4. *H. niger*. All nuclei were isolated from leaf tissue and stained simultaneously.

2.3.3 Genetic relationships based on AFLPs

To evaluate the genetic relationships within the genus, 19 out of 22 *Helleborus* species were analysed using 10 AFLP primer combinations. The numbers of scored marker fragments per primer pair are shown in Table 2.3. In total, 1109 marker fragments were produced across all *Helleborus* genotypes and the outgroup. Of these 0.3 % were monomorphic for the genus *Helleborus.* Analysis of the section Helleborastrum revealed 38.1 % shared fragments, whereas only 5.8 % were shared by the 13 genotypes of the monospecific section Helleborus (Table 2.3).

Table 2.3: Scored AFLP marker fragments, levels of polymorphism and the number of section-specific fragments.

No.	AFLP primer pairs	No. of scored markers	Polymorphic fragments within the genus *Helleborus* [%]	No. of specific fragments for section Helleborus	No. of specific fragments for section Helleborastrum
1	S12 M50	144	99.3	5	69
2	S13 M54	143	100	2	58
3	S15 M60	122	100	0	53
4	S16 M62	99	100	7	30
5	S18 M52	140	100	10	53
6	S19 M51	70	100	6	18
7	S20 M53	55	100	8	16
8	S22 M56	85	100	6	36
9	S26 M57	96	100	8	51
10	S26 M62	155	98.7	12	38
		∑ 1109	Ø 99.8	∑ 64 (5.8%)	∑ 422 (38.1%)

The maximal genetic distance between two species was 0.330, observed between *H. lividus* and *H. liguricus,* and the minimum value, 0.034, was observed between *H. cyclophyllus* and *H. torquatus* (Table 2.4).

Table 2.4: Pairwise genetic distances between 19 *Helleborus* species and *Pulsatilla vulgaris* 'Violet' based on Nei and Li coefficients.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	*P. vulgaris*	0																			
2	*H. vesicarius*	0.409	0																		
3	*H. foetidus*	0.374	0.296	0																	
4	*H. argutifolius*	0.407	0.303	0.241	0																
5	*H. lividus*	0.367	0.319	0.259	0.069	0															
6	*H. niger* 1030	0.389	0.295	0.27	0.187	0.206	0														
7	*H. abruzzicus*	0.356	0.25	0.249	0.263	0.261	0.231	0													
8	*H. atrorubens*	0.339	0.225	0.264	0.251	0.25	0.237	0.105	0												
9	*H. croaticus*	0.392	0.25	0.261	0.286	0.272	0.236	0.092	0.097	0											
10	*H. cyclophyllus*	0.33	0.243	0.265	0.257	0.276	0.228	0.108	0.088	0.112	0										
11	*H. dumetorum*	0.326	0.267	0.293	0.255	0.263	0.23	0.114	0.113	0.115	0.12	0									
12	*H. hercegovinus*	0.373	0.259	0.296	0.308	0.327	0.249	0.113	0.124	0.12	0.122	0.153	0								
13	*H. liguricus*	0.32	0.268	0.303	0.319	0.33	0.25	0.131	0.142	0.142	0.157	0.16	0.126	0							
14	*H. multifidus*	0.364	0.26	0.267	0.301	0.302	0.243	0.078	0.091	0.097	0.105	0.121	0.125	0.107	0						
15	*H. odorus*	0.347	0.239	0.267	0.288	0.302	0.248	0.105	0.1	0.115	0.091	0.123	0.113	0.16	0.112	0					
16	*H. orientalis*	0.406	0.227	0.27	0.267	0.291	0.243	0.131	0.105	0.118	0.1	0.123	0.14	0.173	0.124	0.112	0				
17	*H. purpurascens*	0.372	0.243	0.264	0.272	0.286	0.262	0.13	0.124	0.141	0.137	0.093	0.169	0.195	0.132	0.138	0.153	0			
18	*H. torquatus*	0.352	0.236	0.256	0.244	0.269	0.231	0.108	0.095	0.106	0.034	0.116	0.122	0.153	0.099	0.095	0.106	0.116	0		
19	*H. viridis*	0.374	0.227	0.268	0.296	0.296	0.235	0.108	0.092	0.112	0.112	0.107	0.13	0.137	0.095	0.099	0.113	0.146	0.114	0	
20	*H. thibetanus*	0.394	0.251	0.268	0.259	0.31	0.235	0.16	0.161	0.161	0.163	0.177	0.182	0.182	0.17	0.17	0.174	0.196	0.173	0.171	0

In the section Helleborus, 4 of the 13 genotypes tested, namely *H. niger* 1120 a-d, represented sister plants of a F_1 hybrid cultivar (Figure 2.4) and showed relatively low genetic distances of 0.004 to 0.008. Over all other *H. niger* genotypes, the genetic distance varied from 0.009 to 0.064. In the section Chenopus, the genetic distance was slightly higher, showing a value of 0.069 between the two species *H. argutifolius* and *H. lividus* (Table 2.4). Within the section Helleborastrum, the genetic distances ranged from only 0.034 between *H. cyclophyllus* and *H. torquatus* to 0.195 between *H. liguricus* and *H. purpurascens* (Table 2.4). The genetic distance between the true species *H. orientalis* and *H. x hybridus* varied from 0.069 to 0.078, whereas for the *H. x hybridus* genotypes it ranged from 0.020 to 0.070. The low genetic distances observed between species within Helleborastrum demonstrate the close genetic relationships within this section. The monospecific section Griphopus is represented by *H. foetidus,* with genetic distances of 0.241 with respect to *H. argutifolius* and up to 0.303 with respect to *H. liguricus,* both of which belong to the section Helleborastrum (Table 2.4)

Based on the Nei and Li coefficients, three phenograms were calculated using the following cluster methods: Fitch and Margoliash, UPGMA and Neighbour-joining. In each case, the dendrograms differentiated two major clades with minor clusters representing the six sections. In the case of the Fitch and Margoliash model, the first clade comprised the sections

Chenopus and Helleborus, and the second contained Griphopus, Syncarpus, Dicarpon and Helleborastrum. Phenograms calculated using the UPGMA and Neighbour-joining models demonstrated that the clades had three sections each (Chenopus, Helleborus and Griphopus in one clade and Dicarpon, Helleborastrum and Syncarpus in the second) (Figure 2.4). The two newly described species *H. abruzzicus* and *H. liguricus* were grouped into a small subcluster together with *H. croaticus* within the section Helleborastrum (Figure 2.5). *H. abruzzicus* was placed next to *H. multifidus*, while *H. liguricus* was placed next to *H. hercegovinus.*

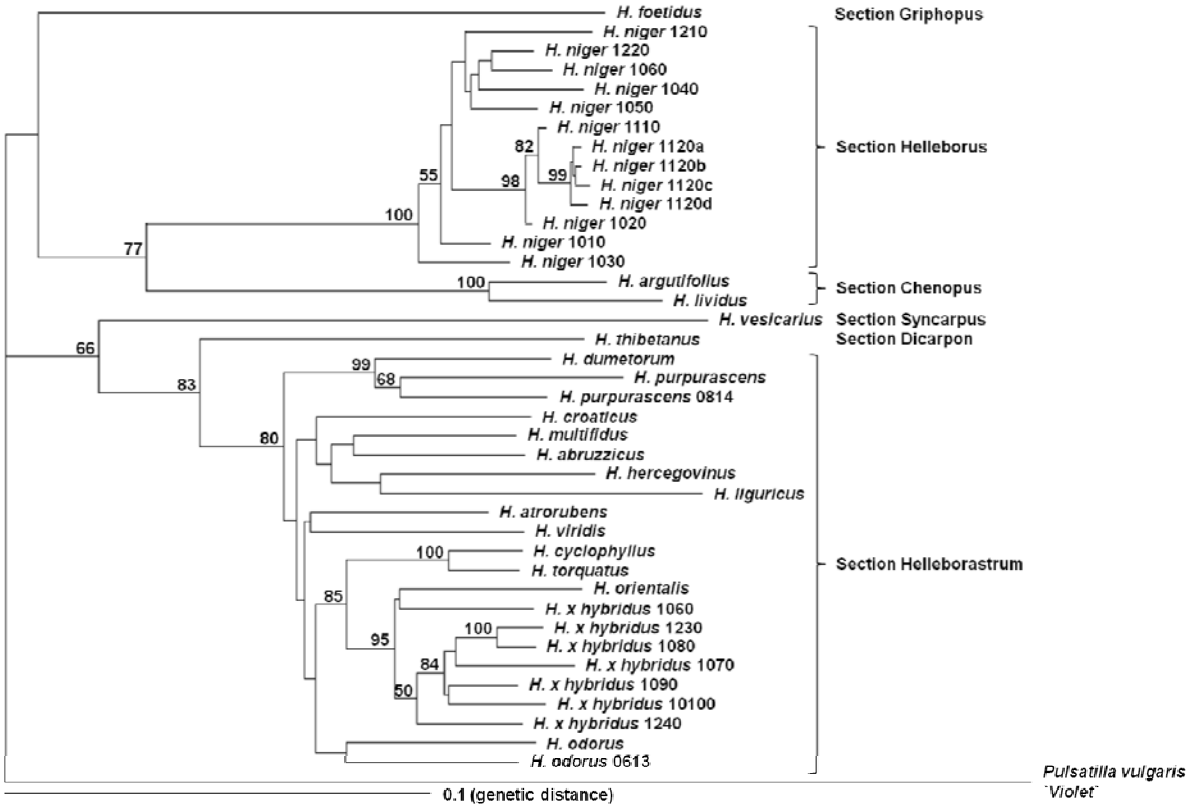


Figure 2.4: Neighbour-joining phenogram based on Nei and Li similarity indices computed from 1109 AFLP markers for 40 *Helleborus* species and genotypes and *Pulsatilla vulgaris* 'Violet' as the outgroup.
The bootstrap percentages >50 % are shown above the branches. From *H. niger* 1120 four sibling plants (a-d) were used for AFLP analysis. The six *Helleborus* sections are noted at the right margin.

Additionally, majority rule consensus trees based on 1000 trees were computed for all three distance algorithms: Fitch and Margoliash, UPGMA and Neighbour-joining. All consensus trees displayed two clades and *H. foetidus* in a separate position. The Neighbour-joining majority rule consensus tree is shown in Figure 2.5. The consensus tree displayed the sectional divisions in basic overview. Furthermore the consensus tree also supports the position of sections Dicarpon, Syncarpus and Chenopus and reflects the close relationships between the species in section Helleborastrum (Figure 2.5).

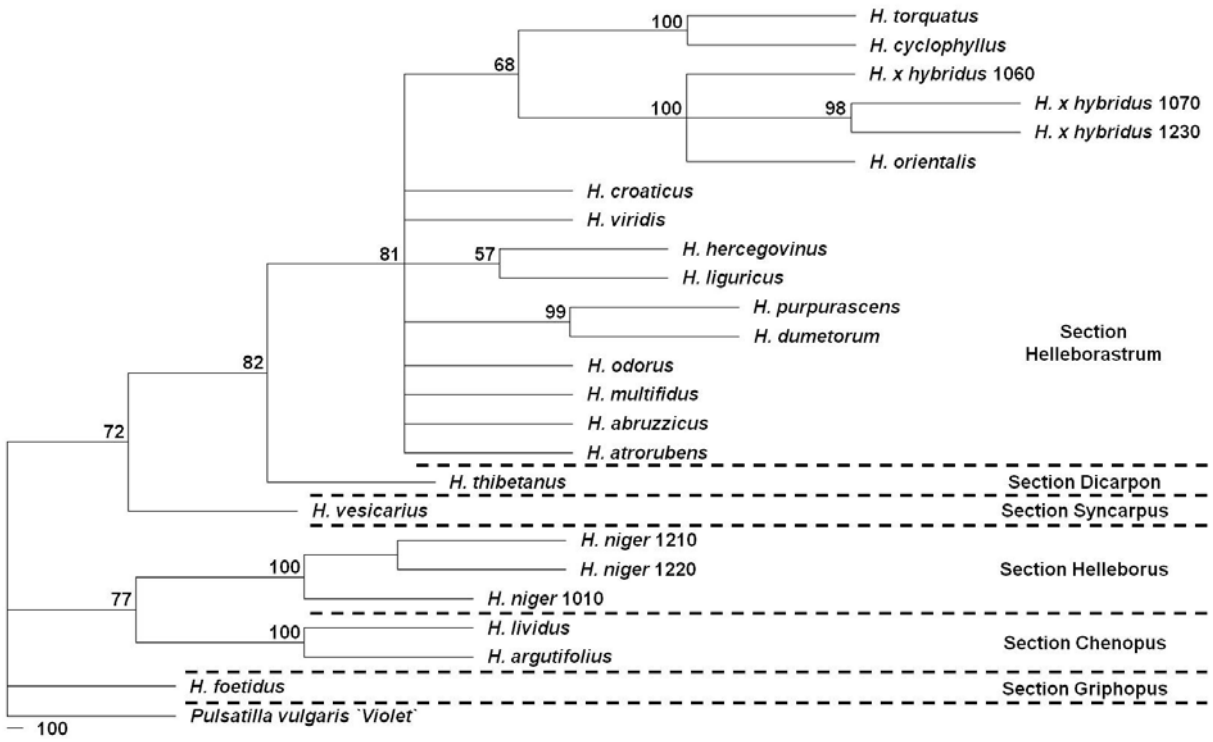


Figure 2.5: Majority rule consensus tree of 1000 Neighbour-joining trees based on Nei and Li similarity indices computed from 1109 AFLP markers for 19 *Helleborus* species and *Pulsatilla vulgaris* 'Violet' as the outgroup.
The bootstrap percentages >50 % are shown above the branches. The six *Helleborus* sections are noted at the right margin and separated by broken lines.

2.4 Discussion

2.4.1 One common chromosome number for all *Helleborus* species

Chromosome numbers are a dynamic characteristic in eukaryotic evolution. Therefore, the analysis of chromosomes by counting and karyotyping offer the opportunity to investigate plant families, genera and species for phylogenetic and breeding purposes. All species examined in this study had a chromosome number of 2n=32, which was consistent with data previously described in the literature (Gregory, 1941; D'Amato & Bianchi, 1989; Yuan & Yang, 2006; Bennett & Smith, 1976; Dobes et al., 1997; Castro & Rosselló, 2007). Overall, the longest chromosomes, ranging from 3 µm to 17 µm, were determined for *H. thibetanus*. The 2n=32 chromosome number that is observed in *Helleborus* species indicates that it could have originated by polyploidy, based on the most common chromosome number observed (x=8) in the *Ranunculaceae* (Yuan & Yang, 2006). Karyotype analyses for some *Helleborus* species like *H. lividus* (D'Amato & Bianchi, 1989; Castro & Rosselló, 2007), *H. foetidus*, *H. niger*, *H. bocconei*, *H. multifidus*, *H. odorus* (D'Amato & Bianchi, 1989) and *H. thibetanus* (Yuan & Yang, 2006) showed that the karyotypes of the European species were different from that of the East Asian species *H. thibetanus*. This may indicate evolutionary specialization and supports the division into a separate section, Dicarpon, as suggested by Mathew (1989). The question whether *Helleborus* is tetraploid or diploid could be resolved by meiotic analyses. However, because of their good fertility and commercial seed propagation, they are more likely to exist in an amphidiploid rather than an autotetraploid state.

2.4.2 DNA contents in *Helleborus* support the classification

The term C-value, which refers to the DNA content of an unreplicated haploid chromosome complement (n) of an individual (Swift, 1950), has been established for the description of genome size. Although nuclear DNA amounts and phylogeny do not correlate overall, a comparison of C-values offers an opportunity to explain the phylogenetic relationships and systematics of narrow taxonomic groups, such as species within a genus (Ohri, 1998). Examples include taxa of the genera *Petunia* (Mishiba et al., 2000), *Lupinus* (Naganowska et al., 2003) and *Alstroemeria* (Buitendijk et al., 1997).

In the genus *Helleborus,* the nuclear DNA contents ranged from 18.3 pg/2C in *H. argutifolius* to 33.2 pg/2C in *H. thibetanus,* which is similar to the data obtained by Zonneveld (2001), who reported genome sizes of 19 pg/2C and 35.7 pg/2C, respectively. Presently, nuclear DNA contents in terms of mass in pg are reported, which correspond to 17920 to 32470 Mb.

Nuclear DNA contents correlated with chromosome lengths in this study: the section Chenopus with *H. argutifolius* possessed the shortest chromosomes and the lowest nuclear DNA amount, whereas *H. thibetanus* had the longest chromosomes and the largest genome size. Interestingly, the largest genome size was observed for the species *H. thibetanus*, section Dicarpon, the only species native to East Asia. However, no accurate conclusions regarding the chromosome lengths can be drawn with respect to genome size because the phase of maximal chromosome condensation was not observed in all preparations. Lower but distinguishable nuclear DNA contents were estimated for species of the sections Chenopus (18/19 pg/2C) and Griphopus (22 pg/2C), which were considered to be caulescent species, in agreement with Zonneveld (2001). There is no clear differentiation possible between the genome sizes of the other three sections. *H. vesicarius* lies between the two sections Helleborus and Helleborastrum in terms of genome size, perhaps indicating an intermediate position.

In general, Zonneveld (2001) revealed higher nuclear DNA contents in comparison to those that were determined in this study (Chapter 2.3.2, Table 2.2). Additionally, standard deviations of his measurements, (three different samples were measured twice for each clone) were higher in almost all cases (0.02-1.3), than in this study (0.05-0.53), although more repetitions were conducted in his experiments. The highest difference between the results obtained in this study and those of Zonneveld (2001) was found for *H. dumetorum,* with an 8.6 % deviation (29.6 pg/2C vs. 32.4 pg/2C), which seems low in comparison to the variation observed in other plant species (Ohri, 1998).

Divergent measurements may be the result of instrumental-technical, inter-laboratory, material-dependent, or intraspecific variation (Greilhuber, 2005). Differences in the age of the leaf material and the genotypes used may cause material-dependent variation. Additionally, the internal reference standard influences the determination of nuclear DNA contents. In this study, *S. cereale* and *V. faba* were used as internal reference standards and were compared for measurements of the nuclear DNA content in *H. argutifolius*, *H. lividus*, *H. dumetorum* and *H. thibetanus* (Table 2.2); in three cases, the differences were below 0.2 pg, while for *H. thibetanus* it was 1 pg. Zonneveld (2001) used *Agave americana* (15.9 pg) as internal standard. Therefore, the differences in the nuclear DNA contents between this study and Zonneveld (2001) may be the result of instrumental-technical and inter-laboratory variation in combination with intraspecific variation. Genome size differences between genotypes of the same species may be a result of repetitive DNA elements (Kubis et al., 1998) or

retrotransposons (Sanmiguel & Bennetzen, 1998), but this has not been confirmed in the genus *Helleborus* up to date. Furthermore, variation is correlated with environmental factors, as shown in *Helianthus* (Price et al., 1998) and maize (Poggio et al., 1998).

In this study, flow cytometry offered a good method to detect interspecific hybrids like *H. x ericsmithii* or *H. x nigercors*. Furthermore, hybrids between the sections Chenopus or Griphopus and all other sections and hybrids with *H. thibetanus* as one parent could be identified by flow cytometry in future experiments due to large differences in their nuclear DNA contents.

2.4.3 First implementation of genome wide DNA markers in *Helleborus* classification

Within the genus *Helleborus,* a phylogenetic analysis was carried out by Sun et al. (2001), based on the chloroplast markers *trnL-F* and *matK* and the ribosomal ITS DNA sequence. Sun et al. (2001) used a parsimony algorithm that does not include distance estimation between taxa and calculated a consensus tree that combined all of the sequence information under the Fitch criterion (Figure 2.1). There are two major disadvantages of using chloroplast markers and ITS sequences: the first is that for most species, chloroplast genes are maternally inherited and ITS sequences represent only one or two loci in the genome. The second is that both sequence types provide only limited information in terms of sequence length and conservation between taxa. Therefore, the aim in this study was to apply the AFLP technology in an attempt to determine genetic relationships within the genus *Helleborus*. AFLP markers are considered to be randomly distributed DNA fragments from the whole nuclear genome with high information contents. The AFLP technique has been successfully used to evaluate genetic relationships or diversity in several plant taxa like *Prunus* (Depypere et al., 2009), *Alstroemeria* (Han et al., 2000), *Dahlia* (Wegner & Debener, 2008) and *Ranunculaceae* (Després et al., 2003).

The phenogram obtained in this study using AFLP markers (Figure 2.4) revealed very similar classifications, as compared to the report of Sun et al. (2001), but the bootstrap support in their analysis was low for the relevant branching differences between sections, which may be due to the above-mentioned disadvantages. In addition, this study grouped two samples with initially unknown origin (*H. odorus* 0613 and *H. purpurascens* 0814) next to the species they were assumed to belong to (McLewin, personal communication). Sun et al. (2001) were able to distinguish between the six *Helleborus* sections, although *H. foetidus* was considered to be a sister group to *H. niger* without any bootstrap support. This is in contrast to this study,

which showed similarities between the average genetic distances between *H. foetidus* and the genotypes of section Helleborus (0.271) and all genotypes of section Helleborastrum (0.268).

The Dicarpon section with *H. thibetanus* was placed next to the Helleborastrum section in both phenograms, but was supported by a high bootstrap value of 83 % in this study, in contrast to Sun et al. (2001), where its position was in doubt. Similarly, the position of *H. vesicarius* between the Chenopus and Griphopus sections was weakly supported (Sun et al., 2001), whereas its position in this study was confirmed by a bootstrap value of 66 %. However, the average genetic distance obtained for *H. vesicarius* in this study with respect to the section Helleborus (0.288) was higher than the genetic distance obtained with respect to Dicarpon (0.251) and species of the section Helleborastrum (0.248). Therefore, the placement next to Dicarpon and Helleborastrum seems more probable. The genetic distance between Syncarpus and Griphopus (0.296) or Chenopus (0.303) was even higher. The problematic classification within the section Helleborastrum is obvious in both analyses.

The genetic distances as estimated in this study were lower between certain species than within other species. This was shown for the genetic distance between *H. torquatus* and *H. cyclophyllus* (0.034) and between the two genotypes of *H. odorus* (0.085) and *H. purpurascens* (0.086), respectively. Here, it has to be considered that the genotypes of these two species probably come from different origins. The only possible way to clarify the ambiguous results observed in the section Helleborastrum is to increase the number of individuals per species and to collect them from different origins as Sun et al. (2001) already suggested.

Since then, some groups within section Helleborastrum that were regarded as subspecies, namely *H. multifidus* subsp. *bocconei*, *H. multifidus* subsp. *hercegovinus*, *H. multifidus* subsp. *istriacus* and *H. viridus* subsp. *occidentalis* were regarded to be distinct species and bear the name of the subspecies. Additionally, *H. croaticus* and *H. torquatus* had been treated as synonyms but were subsequently considered to be distinct. A successful investigation of the distinction between *Helleborus* species of section Helleborastrum and *H. niger* based on RAPD markers has been carried out by Fico et al. (2005) (Chapter 2.1).

Similar to RAPD markers, AFLP markers are distributed over the whole genome but have a much higher reproducibility. However, in contrast to markers based on plastid DNA or ribosomal sequences that sometimes fail to provide phylogenetic information (Després et al., 2003), AFLP analysis would be a reliable technology for species discrimination and the

evaluation of genetic relationships within genera. For the analyses performed in this study, AFLP has proven to be sufficiently powerful. The phenogram obtained here supports the genus classification suggested by Mathew (1989) and the division of the genus into two subgenera as proposed by Werner and Ebel (1994). The two newly described species, *H. abruzzicus* and *H. liguricus,* have been characterised according to their nuclear DNA content and phylogenetic position for the first time in this study. This is important for future breeding purposes because their characteristics, including the multiple divided leaves (up to 200 times) and large yellowish flowers seen in *H. abruzzicus* and the large pale whitish flowers and strong pleasant fragrances of *H. liguricus,* make them valuable candidates for hybridisation programs.

The phenogram resulting from the molecular marker analysis in combination with the cytological and genome size information are the basis for the development of future breeding strategies and may be useful for the prediction of hybridisation success within the genus. In most cases, only one plant per species was available in this study, which would not be enough to display phylogenetic relationships. Therefore, several plants per species from different origins would be necessary for future investigations. However, with regard to interspecific hybridisation in this study, one plant per species was significant enough because exactly the same plants, which were used for AFLP analyses, were taken for the interspecific hybridisation experiments. Whether the differences in genetic distances are helpful for predicting the success of interspecific hybridisations in *Helleborus* will be debated later (Chapter 3.4.4).

3 Interspecific hybridisations within the genus *Helleborus*

3.1 Introduction

Interspecific hybridisations have been carried out in many crop plants to increase genetic variation and to confer traits such as phenotypic characters or disease resistance from other species into well-established cultivars. In particular, interspecific hybridisation has been successfully used as a breeding tool in ornamental plants, including *Alstroemeria* (Buitendijk et al., 1995), *Lilium* (Van Tuyl et al., 2000), *Delphinium* (Honda et al., 2003) and *Rhododendron* (Eeckhaut et al., 2007). Interspecific hybrids are the offspring of crosses between two different species belonging to the same genus. Natural interspecific hybrids may arise between species that are native to the same place and that overlap in flowering time. However, pre- and postzygotic barriers often hinder the development of interspecific hybrids.

Prezygotic barriers include all mechanisms that act to prevent fertilisation after a flower is pollinated. Such mechanisms can occur at different stages, such as lack of stigma receptivity or pollen viability, failure of pollen to adhere the stigma surface, abnormal pollen tube growth and inhibition of pollen tube growth before reaching the ovule. Techniques to overcome prezygotic barriers include the cut-style and grafted-style methods (Van Tuyl et al., 2000) and *in vitro* fertilisation. Furthermore, environmental conditions, including high temperatures and hormones (Sood et al., 1982), as well as compounds that influence stigma receptivity, such as specific proteins and exudates (Martin, 1970) and lipids (Wolters-Arts et al., 1998) can be applied.

Postzygotic barriers act after successful fertilisation and reduce the viability or fertility of the hybrid zygote (Rieseberg & Carney, 1998). According to the Dobzhansky-Muller model, postzygotic reproductive barriers result from deleterious interactions between functionally divergent genes from the hybridising species (Dobzhansky, 1937). The terms hybrid inviability, hybrid sterility and hybrid breakdown describe postzygotic barriers at different developmental stages (Rieseberg & Carney, 1998). Hybrid inviability acts after successful fertilisation during embryo development, resulting in embryo abortion. Postzygotic barriers during embryo development can be circumvented by embryo rescue techniques, in which either embryos, ovaries or ovules are rescued before embryo abortion and cultured *in vitro* until seedlings develop (Winkelmann et al., 2010). To overcome hybrid sterility, polyploidisation techniques can be applied (Van Tuyl & Lim, 2003). To develop an appropriate method to overcome crossing barriers, the barriers must be identified as either

pre- or postzygotic (or both). Plants chosen for use in interspecific hybridisation must be evaluated for these two types of crossing barriers.

The viability of the pollen donor plants can be determined to ensure that the pollen is capable of functioning in the sense of effecting fertilisation. A variety of pollen viability assays are available, including *in vivo* germination, *in vitro* germination and several staining techniques (Dafni & Firmage, 2000). Using aniline blue staining, the initial interaction between the pollen and the stigma can be monitored and the pollen tube growth can be visualised *in situ* until the pollen tube grows into the micropyle of the ovule immediately before fertilisation (Kho & Baer, 1968). Aniline blue staining reveals callose structures, which appear during pollen tube formation, in the walls of pollen tubes.

Within the genus *Helleborus,* crossing barriers have not been identified and localised to date. However, observations based on existing *Helleborus* hybrids have been made (Chapter 1.3, Table 1.4). According to Mathew (1989), various fertile hybrids exist within section Helleborastrum. The intersectional hybrids *H. x ballardiae* and *H. x nigercors* (hybrids between *H. niger* and the stemmed species *H. lividus* and *H. argutifolius*, respectively) produce intermediate but sterile phenotypes (Mathew, 1989). Traits such as flower colour, growth type, scent and disease tolerance are valuable characteristics of *Helleborus* species. These traits could be introduced into *H. niger* or *H. x hybridus* cultivars by interspecific hybridisation, resulting in new phenotypes. Combinations of *H. foetidus*, *H. thibetanus* or *H. vesicarius* with other species would also be interesting due to their growth types and their relationships to species from other sections (Chapter 2.3.3).

To conduct extensive hybridisation studies between *Helleborus* species, detailed information about crossing barriers must be gathered, and techniques to overcome these barriers must be developed. Therefore, the objectives of the following analyses were (i) to evaluate the pollen viability of different *Helleborus* species and develop a method for pollen storage over several months to synchronise the flowering times of different species, (ii) to identify the pre- and postzygotic crossing barriers for different interspecific combinations, (iii) to develop an embryo rescue method to overcome postzygotic barriers and (iv) to identify putative hybrids by flow cytometry and molecular markers.

3.2 Materials and Methods

3.2.1 Plant material

Plants of *H. argutifolius*, *H. atrorubens*, *H. croaticus*, *H. cyclophyllus*, *H. dumetorum, H. foetidus*, *H. x hybridus*, *H. lividus*, *H. multifidus*, *H. niger*, *H. odorus*, *H. purpurascens*, *H. torquatus*, *H. x ballardiae*, *H. x ericsmithii* and *H. x sternii* were cultivated in the greenhouse from December 2007 until April 2010. For most of these species, only one plant was available. For *H. x hybridus* and *H. niger*, many different genotypes were used in the experiments. All plants were provided by the company Heuger, Glandorf, Germany. From May to late October, plants were grown outside in a shaded area. During the winter months, the plants were kept at 12 °C (in 2007/2008 and 2009/2010) or at 5 °C (in 2008/2009) in the greenhouse. Plants were fertilised biweekly and plant protection measures were carried out when necessary according to standard horticultural practices. During the flowering season from November to April in the years 2007/2008, 2008/2009 and 2009/2010, experiments were carried out to determine pollen viability, pollen tube growth and the efficacy of embryo rescue techniques.

3.2.2 Viability analysis of fresh and stored pollen

For pollen viability analyses, freshly dehisced anthers were removed from open flowers of *Helleborus* plants. Anthers were dried in open Petri dishes at room temperature for 24 hours. Pollen viability was determined directly after removal of the anthers and after 24 hours of desiccation via *in vitro* germination and staining with MTT.

In vitro pollen germination was evaluated on a pollen germination medium following the method of Fast Plants (1979) during the flowering season in the winter of 2007/2008. For comparison, pollen from the same samples was also used for viability staining (see below). For the *in vitro* germination medium, two solutions were prepared: (i) 41 g sucrose was dissolved in 100 ml distilled water and (ii) 125 mg $Ca(NO_3)_2$, 60 mg H_3BO_3, 30 mg KNO_3 and 65 mg $MgSO_4$ x 7 H_2O were dissolved in 300 ml distilled water. The two stock solutions were combined, 1.1 g Gelrite (Duchefa, the Netherlands) was added and the medium was sterilised by autoclaving at 121 °C for 20 minutes (autoclave: Tuttnauer 3870 ELV, Biomedis, Gießen, Germany) and poured into 6 cm diameter Petri dishes. Pollen grains were washed out from freshly harvested anthers or anthers dried at room temperature with distilled water and transferred to the medium. After 12 hours of incubation at room temperature, at least 200

pollen grains were evaluated for germination under a bright field microscope, and the germination percentage was determined.

In addition to *in vitro* germination, the pollen viability of different *Helleborus* species and hybrids was determined by staining with thiazolyl blue (MTT) during all three flowering seasons. Pollen was stained in 20 µl thiazolyl blue solution (1 % MTT in a 5 % sucrose solution) for 10 minutes at room temperature. MTT is reduced into a coloured substance in the presence of dehydrogenase. Viable pollen appeared in various shades from rose to dark pink, whereas dead pollen was yellow, grey, black or unstained and deformed. Stained pollen was evaluated under a bright field microscope at 200 x magnification. To ensure that only viable pollen grains were stained, anthers of *H. niger* were incubated at 100 °C for 24 hours to kill the pollen and evaluated as a negative control.

To make it possible to hybridise *Helleborus* species with slightly different flowering times, suitable conditions for pollen storage were identified during the 2008/2009 flowering season. At least five dried anthers of each tested species were stored in reaction tubes, which were placed in closed glass containers with dried $CaCl_2$ and sealed with parafilm. The glass containers were stored at 20 °C for one or two months and at 4 °C or -20 °C for one, two or six months. In addition, pollen that had been stored at -20 °C for one year was used for staining and *in vivo* pollination experiments.

During the 2009/2010 flowering season, larger numbers of anthers than in 2008/2009 were collected from several *H. argutifolius*, *H. x hybridus* and *H. niger* plants. These anthers were stored in Petri dishes at -20 °C for nine months until their pollen viability was tested by MTT staining.

3.2.3 Crossing procedure for *Helleborus*

Plants of all *Helleborus* species listed above (Chapter 3.2.1) were used for crossing experiments. While flower buds were still closed, anthers were carefully removed using forceps (Figure 3.1). Emasculated buds were covered with paper bags to avoid cross-pollination. Two to three days after emasculation, when the buds began to open, the flowers were pollinated with fresh pollen. Each pollinated flower was again covered with a paper bag, which was closed with a paper clip, for approximately 10 days. Pollinated flowers were used either for the observation of pollen tube growth at 12, 24, 48, 72, 96 or 144 hours after pollination (Chapter 3.2.4) or for embryo rescue experiments (Chapter 3.2.5). In the latter case, carpels were isolated from three weeks after pollination onward. Table 3.1 summarises

the crosses that were carried out during the three years and the corresponding experiments that performed.

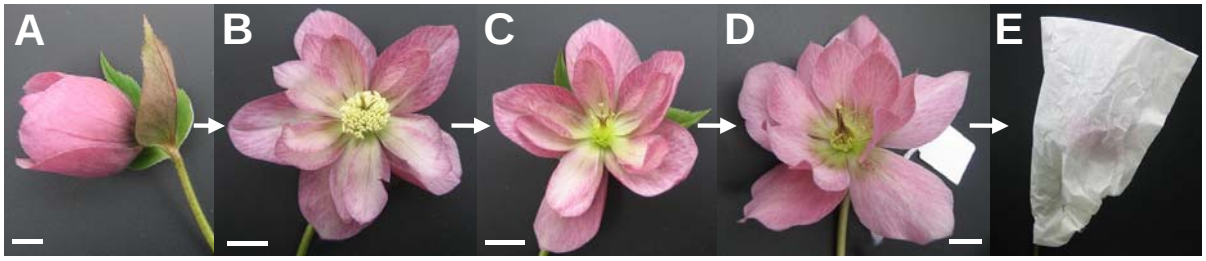


Figure 3.1: Crossing procedure for *Helleborus* flowers.
Pictures show flowers of *H. x hybridus*. A: closed flower bud, B: interior view of the flower bud before emasculation, C: emasculated bud, afterwards covered with a paper bag for 2-3 days (no picture shown) D: pollinated flower 2-3 days after emasculation, E: pollinated flower covered with a paper bag for approximately 10 days. Bars represent 1 cm.

Table 3.1: Crosses during three flowering seasons (2007/2008, 2008/2009, 2009/2010) that were used for analysis of pollen tube growth and embryo rescue experiments.
The first four crossing combinations are intraspecific and the following interspecific crosses are arranged in alphabetical order of first the maternal and second the paternal species. The tested experimental variants are shown for each flowering season and crossing combination.

♀	♂	Pollen tube growth [hours after pollination]			Embryo rescue [weeks after pollination]		
		2007/2008	2008/2009	2009/2010	2007/2008	2008/2009	2009/2010
H. argutifolius	*H. argutifolius*	--	--	--	3-9	--	--
H. foetidus	*H. foetidus*	12/24/48/72	--	--	3-9	--	--
H. x hybridus	*H. x hybridus*	12/24/48/72	--	72/96/144	3-10	--	--
H. niger	*H. niger*	12/24/48/72	--	72/96/144	3-10	--	--
H. argutifolius	*H. atrorubens*	--	--	--	--	5-7	--
H. argutifolius	*H. foetidus*	--	--	--	3-8	5-7	--
H. argutifolius	*H. x hybridus*	--	48/96	--	--	5-7	--
H. argutifolius	*H. lividus*	--	48/96	--	--	5-7	--
H. argutifolius	*H. multifidus*	--	48/96	--	--	5-7	--
H. argutifolius	*H. niger*	12/24/48/72	48/96	--	3-9	5-7	--
H. argutifolius	*H. purpurascens*	--	--	--	--	5	--
H. argutifolius	*H. torquatus*	--	48/96	--	--	5-7	--
H. atrorubens	*H. croaticus*	--	--	--	--	5-6	--
H. atrorubens	*H. x hybridus*	--	48/96	--	--	5-7	--
H. atrorubens	*H. niger*	--	48/96	--	--	5-7	--
H. atrorubens	*H. odorus*	--	--	--	--	5-7	--
H. atrorubens	*H. purpurascens*	--	--	--	--	5-6	--
H. croaticus	*H. multifidus*	--	--	--	--	5-7	--
H. croaticus	*H. niger*	--	--	--	--	5-7	--
H. croaticus	*H. odorus*	--	--	--	--	5-7	--
H. cyclophyllus	*H. multifidus*	--	--	--	--	5-7	--
H. cyclophyllus	*H. niger*	--	--	--	6	--	--
H. foetidus	*H. argutifolius*	12/24/48/72	--	--	3-10	--	--
H. foetidus	*H. x hybridus*	12/24/48/72	48/96	72/96/144	3-10	--	6
H. foetidus	*H. niger*	12/24/48/72	48/96	72/96/144	3-9	--	6
H. foetidus	*H. odorus*	--	--	72/96/144	--	--	6
H. x hybridus	*H. argutifolius*	12/24/48/72	48/96	--	3-8	5-7	--
H. x hybridus	*H. atrorubens*	--	48/96	--	--	5-7	--
H. x hybridus	*H. croaticus*	--	48/96	--	--	5-7	--
H. x hybridus	*H. cyclophyllus*	--	48/96	--	--	5-7	--
H. x hybridus	*H. dumetorum*	--	48/96	--	--	5-7	--

Table 3.1 (continued)

♀	♂	Pollen tube growth [hours after pollination]			Embryo rescue [weeks after pollination]		
		2007/2008	2008/2009	2009/2010	2007/2008	2008/2009	2009/2010
H. x hybridus	*H. foetidus*	12/24/48/72	--	72/96/144	3-9	5-7	--
H. x hybridus	*H. lividus*	--	48/96	--	--	--	--
H. x hybridus	*H. multifidus*	--	48/96	--	--	5-7	--
H. x hybridus	*H. niger*	12/24/48/72	48/96	72/96/144	3-11	5-7	--
H. x hybridus	*H. odorus*	--	48/96	--	--	5-7	--
H. x hybridus	*H. purpurascens*	--	48/96	--	--	5-7	--
H. x hybridus	*H. torquatus*	--	48/96	--	--	5-7	--
H. lividus	*H. argutifolius*	--	48/96	--	--	5-7	--
H. lividus	*H. x hybridus*	--	48/96	--	--	--	--
H. lividus	*H. niger*	--	48/96	--	--	--	--
H. lividus	*H. purpurascens*	--	--	--	--	5-7	--
H. multifidus	*H. argutifolius*	--	--	--	--	5-7	--
H. multifidus	*H. x hybridus*	--	48/96	--	--	5-7	--
H. multifidus	*H. niger*	--	48/96	--	3-5	5-7	--
H. multifidus	*H. odorus*	--	--	--	--	5-6	--
H. multifidus	*H. purpurascens*	--	--	--	--	5-6	--
H. niger	*H. argutifolius*	12/24/48/72	--	--	3-11	5-7	--
H. niger	*H. atrorubens*	--	48/96	--	3-5	5-7	--
H. niger	*H. croaticus*	--	48/96	--	--	5-7	--
H. niger	*H. cyclophyllus*	--	48/96	--	3-9	5-7	--
H. niger	*H. dumetorum*	--	48/96	--	--	5-7	--
H. niger	*H. foetidus*	12/24/48/72	48/96	72/96/144	3-11	5-7	6
H. niger	*H. x hybridus*	12/24/48/72	24/48/72/96	72/96/144	3-10	5-7	--
H. niger	*H. lividus*	--	48/96	--	3-10	5-7	--
H. niger	*H. multifidus*	--	48/96	--	3-9	5-7	--
H. niger	*H. odorus*	--	24/48/72/96	72/96/144	5-9	5-7	6
H. niger	*H. purpurascens*	--	48/96	--	--	5-7	--
H. niger	*H. torquatus*	--	48/96	--	3-10	5-7	--
H. odorus	*H. croaticus*	--	--	--	--	5-7	--
H. odorus	*H. multifidus*	--	--	--	--	5-7	--
H. odorus	*H. niger*	--	48/96	--	--	5-7	--
H. odorus	*H. torquatus*	--	--	--	--	5-7	--
H. purpurascens	*H. croaticus*	--	--	--	--	5-7	--
H. purpurascens	*H. x hybridus*	--	48/96	--	--	--	--
H. purpurascens	*H. niger*	--	48/96	--	3-4	5-7	--
H. torquatus	*H. croaticus*	--	--	--	--	5-7	--
H. torquatus	*H. niger*	--	48/96	--	3-4	--	--

3.2.4 Analysis of pollen tube growth *in situ*

Pollen tube growth was observed during the flowering season in each of the three years. During the winter of 2007/2008, carpels from selected intraspecific and interspecific crosses were removed 12, 24, 48 and 72 hours after pollination (Table 3.1). In the following flowering season (2008/2009), carpels were collected 48 and 96 hours after pollination; in 2009/2010, they were harvested 72, 96 and 144 hours after pollination. Carpels were fixed in two parts 96 % ethanol : one part lactic acid (approximately 90 %) for at least 24 hours. Fixed carpels were rinsed three times in deionised water and stained in aniline blue staining solution (100 mg aniline blue (Serva) dissolved together with 767.6 mg K_3PO_4 x H_2O (Riedel de

Häen) in 100 ml deionised H_2O) for at least 30 min. Stained carpels were transferred to a microscope slide and bisected with a scalpel. Divided carpels were covered with a cover slip, squeezed and observed under a fluorescence microscope (Axio Scope.A1 (Zeiss, Oberkochen, Germany), absorption at 470 nm and emission at 525 nm). Photographs were taken with an AxioCam MR3 and edited with the Axiovision software. Style length differed between species; therefore, pollen tube growth was not evaluated on the basis of absolute length, but relative to the style length. Pollen tubes appeared glaucous to turquoise, and its growth was classified according to the following criteria (Figure 3.2): (1) pollen visibly germinated but not growing into the stigmatic tissue, (2) pollen tube growing into the first half of the style, (3) pollen tube growing into the second half of the style and (4) pollen tube reaching the ovules.

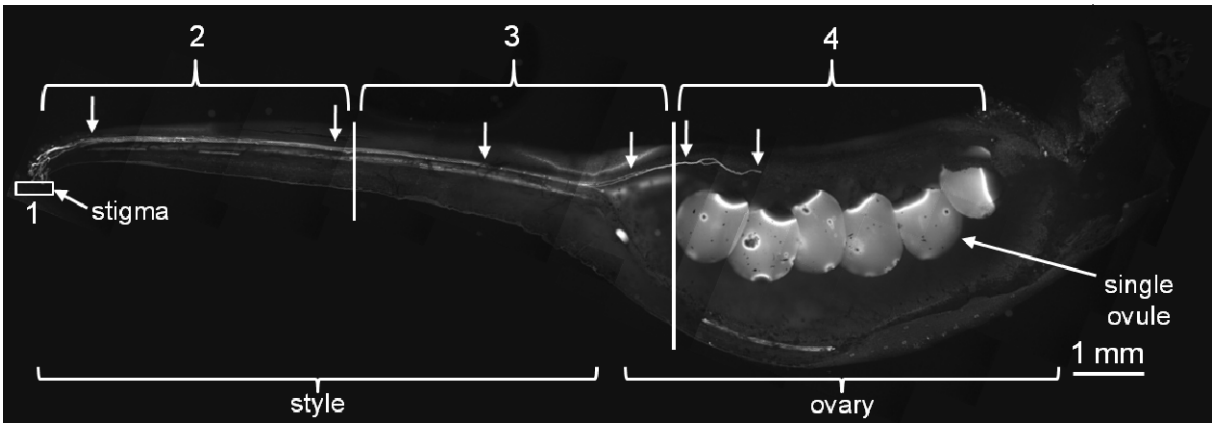


Figure 3.2: Different classes of pollen tube growth in one carpel of *H. niger* 48 hours after pollination with pollen of *H. foetidus*.
Black and white figure: (1) pollen visibly germinated but not growing into the stigmatic tissue, (2) pollen tube growing into the first half of the style, (3) pollen tube growing into the second half of the style and (4) pollen tube reaching the ovules. The pollen tube is indicated with arrows.

3.2.5 Embryo rescue experiments

Five embryo rescue experiments were carried out during the three flowering seasons, and crossing combinations were performed as shown in Table 3.1. Due to the availability and economic relevance of the various species, most of the crosses used *H. niger* or *H. x hybridus* as a maternal or paternal parent. The embryo rescue experiments focused mainly on the sucrose concentration in the *in vitro* culture medium and on the conditions during subsequent ovule culture.

Carpels were removed from the flower, surface disinfected in 70 % ethanol for 30 sec and in 2 % sodium hypochlorite with one drop Tween for 10 min and rinsed in sterilised water three times for one, two and five minutes (Figure 3.3). Single ovules were dissected from carpels

and cultured *in vitro* on a medium based on 1 x MS (Murashige and Skoog, 1962) prepared from macro- and micronutrient and vitamin stock solutions (Table 3.2). The medium was solidified with 0.4 % Gelrite (Duchefa, The Netherlands) at a pH of 5.8 (adjusted prior to autoclaving) and sterilised by autoclaving at 121 °C for 20 minutes (autoclave: Tuttnauer 3870 ELV, Biomedis, Gießen, Germany). Ovules from a single carpel were placed in each Petri dish. When the radicle emerged and hypocotyl elongation occurred, germinating ovules were transferred to light with a 16 h photoperiod. An ovule was scored as germinated, when the radicle emerged through the coat. Shoots were transferred onto MS medium supplemented with 0.2 mg/l BAP, 0.1 mg/l IAA and 0.1 mg/l GA_3. The number of crosses and carpels used for ovule culture differed each year.

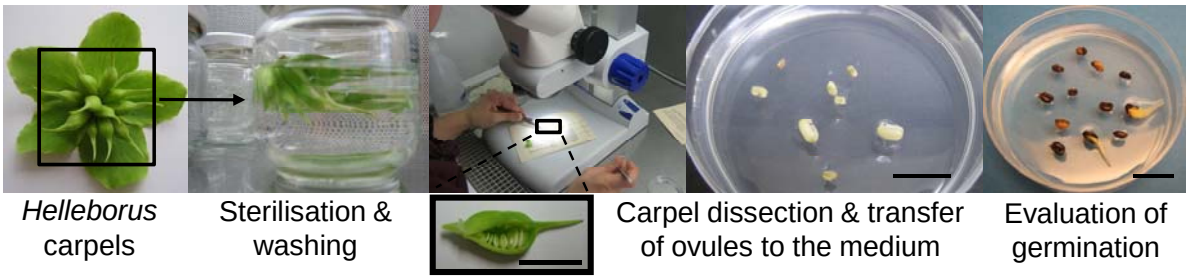


Figure 3.3: Schematic simplified overview from sterilisation of *Helleborus* carpels to ovule culture and evaluation of germination.
Helleborus carpels were separated from the flower, sterilised and washed. After washing, ovules were excised with the help of a stereomicroscope and transferred to medium. Evaluation of germination was carried out after different culture periods depending on the embryo rescue experiment. Bars represent 1 cm.

Table 3.2: Composition of macro-, micronutrients and vitamins in the *in vitro* culture media based on Murashige & Skoog (1962).

Components	Concentration	Molarity
Macroelements	**[mg/l]**	**[mM]**
NH_4NO_3	1650	20.61
KNO_3	1900	18.79
$CaCl_2$ x 2 H_2O	440	2.99
$MgSO_4$ x 7 H_2O	370	1.5
KH_2PO_4	170	1.25
Microelements		**[µM]**
H_3BO_3	6.20	100.27
$MnSO_4$ x H_2O	16.9	100
$ZnSO_4$ x 7 H_2O	8.6	29.91
KI	0.83	5
Na_2MoO_4 x 2 H_2O	0.25	1.03
$CuSO_4$ x 5 H_2O	0.025	0.10
$CoCl_2$ x 6 H_2O	0.025	0.11
FeNaEDTA	36.70	100
Vitamins		**[µM]**
Glycine	2.0	26.64
Myo-inositol	100	554.94
Nicotinic acid	0.5	4.06
Pyridoxine HCl	0.5	2.43
Thiamine HCl	0.1	0.30

Due to the low numbers of germinating ovules in relation to the numbers of cultured ovules, statistical analysis was not possible for all experiments; therefore, the data are presented as absolute numbers.

The five embryo rescue experiments are summarised in Table 3.3 according to the year of initiation, the variation in medium supplements and the culture conditions. Intraspecific crosses were performed only in experiment 1 and to a minor extent in experiment 2, which focused on interspecific crosses. The differences between these experiments are explained in detail in Chapters 3.2.5.1 and 3.2.5.2.

Table 3.3: Embryo rescue experiments that were carried out and their parameters during three years. Crossing combinations are marked in grew.

Year	Experiment no.	Crosses	Medium supplements	Temperature conditions
2007/ 2008	1	Intraspecific: *H. argutifolius* *H. foetidus* *H. x hybridus* *H. niger*	2.5 and 5 % sucrose	24 °C or 16 °C for 12 weeks → 6 °C for 11 weeks → back in 24 °C or 16 °C for 17 weeks
	2	Intra- and interspecific (Table 3.1)	2.5 and 5 % sucrose	24 °C
2008/ 2009	3	*H. x hybridus* x *H. niger*	2.5 % sucrose	20 °C
			2.5 % sucrose	20 °C for 12 weeks → 4 °C for 12 weeks → back in 20 °C
		H. niger x *H. x hybridus*	2.5 % sucrose,1 mg/l GA_3	20 °C
			2.5 % sucrose, 0.2 mg/l BAP	
			2.5 % sucrose, 1 g/l activated charcoal	
	4	Other interspecific crosses (Table 3.1)	2.5 % sucrose	20 °C for 12 weeks → 4 °C for 12 weeks → back in 20 °C
2009/ 2010	5	Interspecific crosses (Table 3.1)	2.5 % sucrose	20 °C for 12 weeks → 4 °C for 12 weeks → back in 20 °C

3.2.5.1 Embryo rescue with ovules from intraspecific crosses

To establish the embryo rescue procedure and the appropriate culture conditions for isolated ovules, carpels from intraspecific crosses within *H. argutifolius*, *H. foetidus*, *H. x hybridus* and *H. niger* were extracted weekly from three to six weeks after pollination in experiment 1 during the first year (2007/2008) (Table 3.1).

In this experiment, two culture media supplemented with 2.5 % or 5 % sucrose were compared and different temperature treatments were tested (Table 3.3, Figure 3.4). Ovules were cultured in darkness at 24 ± 1 °C or 16 ± 1 °C for 12 weeks, at which time the number of ovules in each temperature treatment was divided; one half was incubated at 6 ± 1 °C for 11 weeks, while the other half was incubated at the initial temperature (Figure 3.4). After this

incubation period the ovules were returned to their initial temperature and evaluated for germination after another 17 weeks.

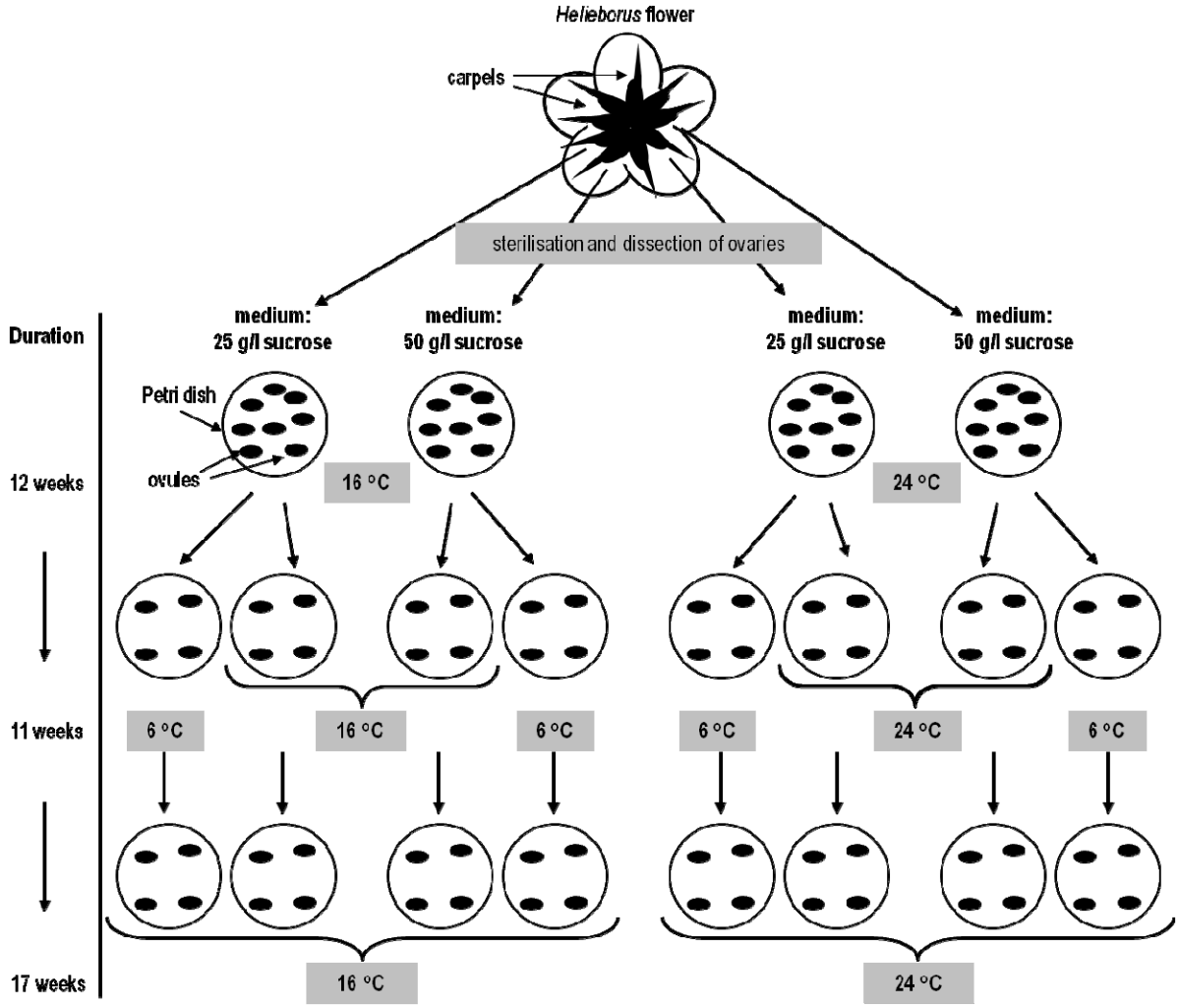


Figure 3.4: Schematic overview of medium and alternating temperature conditions during ovule culture in embryo rescue experiment 1 (2007/2008).

3.2.5.2 Embryo rescue with ovules from interspecific crosses

Embryo rescue experiment 2 was carried out in 2007/2008 (Table 3.3). In this experiment, ovules from intraspecific crosses were used as controls for comparison to ovules from interspecific crosses and all ovules remained at 24 ± 1 °C. Two culture media supplemented with 2.5 % or 5 % sucrose were compared.

Embryo rescue experiments 3 and 4 were carried out in 2008/2009 (Table 3.3). For experiment 3 crosses between *H. niger* and *H. x hybridus* were performed. Ovules were cultured under the conditions listed in Table 3.3. Either a constant temperature of 20 °C was combined with the application of different plant growth regulators, or a basal medium with 2.5 % sucrose was combined with a constant temperature of 20 °C or sequential temperature

phases from 20 °C to 4 °C to 20 °C (Table 3.3). In experiment 4, ovules from other crossing combinations (Table 3.1) were cultured on MS medium with 2.5 % sucrose at 20 ± 1 °C for 12 weeks, followed by 4 ± 1 °C and 20 ± 1 °C thereafter.

Ovules from interspecific crosses in embryo rescue experiments 3 and 4 (2008/2009) that did not germinate but that appeared to be viable were dissected under a stereomicroscope at 5 x magnification (Stemi 2000 C, Zeiss, Germany) after 60 to 67 weeks of culture and analysed for the presence and consistency of the endosperm and embryo. If embryos were found, then they were isolated, transferred to fresh medium and evaluated for further development.

For experiment 5, which was started in 2009/2010, only crossing combinations with genetically distant parental species were chosen. Again, ovules were cultured at 20 ± 1 °C for 12 weeks, followed by 4 ± 1 °C for 12 weeks and 20 ± 1 °C thereafter.

3.2.6 Hybrid identification

3.2.6.1 Hybrid identification by flow cytometry

Hybrids were identified by flow cytometry using propidium iodide staining and measurement of the nuclear DNA content in relation to their parental species. If the nuclear DNA contents of the parental species were sufficiently different (Chapter 2.3.2), then the hybrid would be expected to express an intermediate nuclear DNA content. To isolate nuclei, 0.6 cm^2 sections of leaf tissue from the putative hybrid and its parental plants were chopped together in a plastic Petri dish using a razor blade. No internal reference standards were used; the nuclear DNA contents were evaluated relative to each other. Further analysis was performed as described in Chapter 2.2.3.

3.2.6.2 Hybrid identification by RAPD analysis

Fresh young leaf tissue from *in vitro* shoots or roots of germinated potential hybrid seedlings was frozen in liquid nitrogen and ground to powder using a bead mill. Total plant DNA was extracted using the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The DNA concentration was determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA).

DNA was amplified by the RAPD technique (Williams et al., 1990). In addition to DNA from the putative hybrid, DNA samples from the parental plants were used to compare the amplification products of the hybrid with those of its parents. Amplification reactions were carried out in a volume of 20 µl containing 10 mM Tris (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$,

0.001 % gelatine, 100 µM of each deoxyribonucleoside triphosphate, 0.5 µM of each primer, 1 U Taq DNA Polymerase (FIREPol ® Solis Biodyne, Estonia) and 10 ng plant genomic DNA. Numerous random decamer primers were tested and 10 primers (Table 3.4; primer kits A, B and C, Carl Roth, Karlsruhe, Germany) were selected depending on the combination of hybrid and parental plants. Thermal cycling was conducted with a 5 min initial denaturation at 94 °C, followed by 35 cycles of 30 sec at 94 °C, 30 sec at 36 °C and 2 min at 72 °C and a final extension step for 10 min at 72 °C. Amplification products were separated by electrophoresis in 1.5 % agarose gels in 1 x TAE buffer (2 M Tris, 50 mM EDTA pH 8.0, 1 M glacial acetic acid; pH 8.44), detected by staining with ethidium bromide (10 µg/100 ml agarose gel) and visualised on a UV transilluminator. The banding patterns were evaluated by visual inspection. All amplification reactions were repeated at least twice.

Table 3.4: Informative RAPD primers used for identification of interspecific *Helleborus* hybrids.

Primer name (primer kits, Roth)	Sequence 5'→3'
A02	TGCCGAGCTG
A12	TCGGCGATAG
A14	TCTGTGCTGG
B3	CATCCCCCTG
B7	GGTGACGCAG
B10	CTGCTGGGAC
B13	TTCCCCCGCT
B16	TTTGCCCGGA
B19	ACCCCCGAAG
C14	TGCGTGCTTG

3.3 Results

3.3.1 Viability analysis of fresh and stored pollen

Pollen viability was determined by *in vitro* germination and/or staining with MTT. In the staining assay, the pollen colour varied from rose to dark pink for viable pollen. Yellow, grey, black or deformed pollen was regarded as dead (Figure 3.5 A-C).

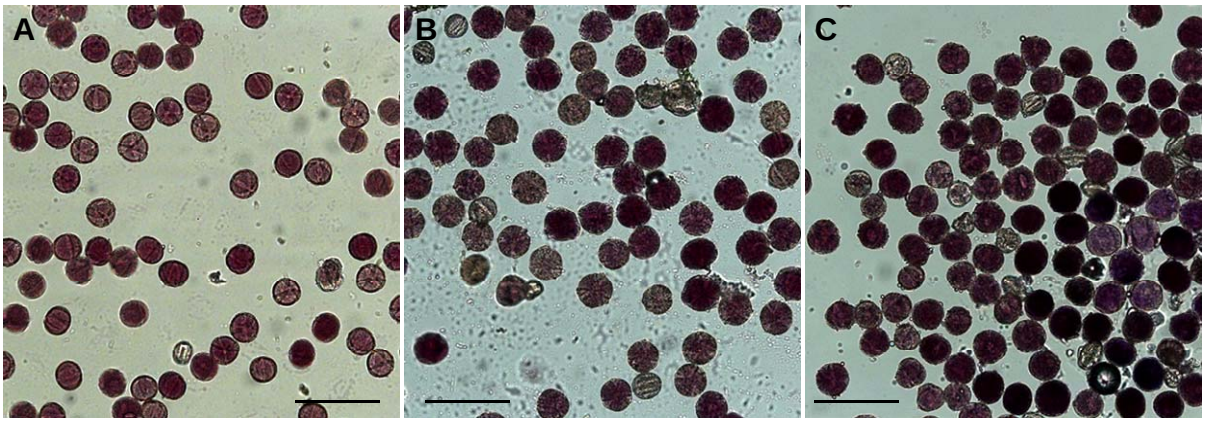


Figure 3.5: Viability staining of pollen of different *Helleborus* species with MTT.
Pollen of A: *H. argutifolius*, B: *H. x hybridus* and C: *H. niger* after nine months of storage at -20 °C. Bars represent 100 µm.

The viability of fresh and dried pollen was determined by *in vitro* germination and MTT staining for nine *Helleborus* species in 2007/2008 (Table 3.5). The stainability of fresh pollen was 67 % to 99 %, depending on the species. For eight species, however, pollen germination was low, between 0 % and 9.2 %. For *H. argutifolius* alone, a large percentage (54 %) of the pollen grains germinated *in vitro*. After drying, pollen stainability remained high (74 % to 99 %) and comparable to that of fresh pollen, whereas germinability decreased further, to 0 % to 5 %. Pollen drying was necessary for storage because fresh pollen moulded when stored for several weeks. No correlation between pollen stainability and *in vitro* germination was found.

Table 3.5: Comparison of stainability and *in vitro* germination of pollen of different *Helleborus* species directly after anther removal and after 24 hours of drying at room temperature.

	After anther removal		After 24 h drying	
	Pollen stainability [%]	Pollen germination [%]	Pollen stainability [%]	Pollen germination [%]
H. argutifolius	95	54	99	2.5
H. atrorubens	93	0	95	0
H. cyclophyllus	88	1.4	98	0.95
H. foetidus	98	0.8	98	0
H. x hybridus	81	6.8	96	0
H. lividus	96	6.4	99	5
H. niger	99	9.2	98	0
H. odorus	67	4.1	91	0
H. torquatus	81	0.3	74	0

As described in Chapter 3.2.2 pollen of six *Helleborus* species was stored at -20 °C for one year and subsequently used for intraspecific pollination. Pollen viability, which was determined by staining with MTT, varied from 17 % to 98 %, depending on the species (Table 3.6). Eight carpels per species were analysed 96 hours after pollination. For *H. argutifolius* and *H. foetidus* only, no pollen tubes were observed near the ovules, although pollen was found to be viable in the staining assay. In the case of *H. foetidus*, pollen was not found on the stigma. For *H. argutifolius*, pollen adhered to only four of the eight carpels. However, pollen stored for one year at -20 °C was found to be viable in the staining assay and was able to germinate on the stigmas of all carpels, to which it adhered (Table 3.6). Therefore, the staining assay was used for all subsequent pollen viability tests.

Table 3.6: Viability of pollen stored at -20 °C for one year and the respective pollen tube growth 96 hours after *in vivo* pollination of *Helleborus* flowers.

Species		Number of…			Pollen tube growth[a]			
	Pollen viability [%]	carpels analysed	stigmas with pollen	stigmas with germinated pollen	1	2	3	4
H. argutifolius	48	8	4	4	0	4	0	0
H. foetidus	86	8	0	0	0	0	0	0
H. x hybridus	76	8	8	8	0	2	0	6
H. niger	17	8	8	8	0	0	0	8
H. purpurascens	98	8	8	8	0	0	0	8
H. torquatus	89	8	8	8	0	0	0	8

[a](1) pollen visibly germinated but not growing into the stigmatic tissue, (2) pollen tube growing into the first half of the style, (3) pollen tube growing into the second half of the style and (4) pollen tube reaching the ovules.

Next, pollen viability was assessed via staining for eleven *Helleborus* species immediately after anther removal, after drying for 24 hours and after several durations of storage under different temperature conditions in 2008/2009 (Table 3.7).

Over all eleven species, the viability of fresh pollen varied from 22 % to 95 % (Table 3.7). After drying, pollen viability decreased in some cases and increased in others. During storage at 20 °C, pollen viability decreased rapidly. After two months of storage, viable pollen (2 %-50 %) was detected for only three of eleven species. Pollen viability did not decrease as rapidly at 4 °C as at 20 °C; viable pollen was detected for six species after two months at 4 °C (2 %-73 %). Thus, storage at either 20 °C or 4 °C would be adequate if pollen were to be stored for only a few days. When pollen must be stored for several weeks or months neither storage temperature was suitable. For long-term storage, a temperature of -20 °C was more suitable because after six months of storage at -20 °C, viable pollen was detected for nine of eleven species (7 %-86 %). Generally, the pollen viability of species belonging to section Chenopus and of their hybrid, *H. x sternii*, decreased faster than that of other species during storage at all temperatures.

Table 3.7: Pollen viability of different *Hellebours* species depending on temperature and duration of storage determined via staining with MTT.
Data are presented as mean ± standard deviation of n measurements.

Species	n	After removal	After drying	Pollen viability depending on temperature and duration of storage							
				20 °C		4 °C			-20 °C		
				4 weeks	2 months	4 weeks	2 months	6 months	4 weeks	2 months	6 months
H. argutifolius	3	95±8	88±13	0	0	0	0	0	76±13	24±28	0
H. atrorubens	3	82±13	57±42	17±11	0	52±38	47±37	0	83±3	86±9	86±13
H. croaticus	1	96	43	70	0	61	29	0	51	96	88
H. dumetorum	1	22	13	0	0	2	0	0	5	22	7
H. x hybridus	3	86±20	91±6	42±46	22±38	58±51	2±3	54±47	91±11	83±13	91±14
H. lividus	3	87±19	93±8	0	0	0	0	0	91±6	32±38	0
H. multifidus	2	58±59	91±5	8±11	2±3	9±1	26±37	7±10	73±36	71±28	97±1
H. niger	3	74±23	84±16	29±49	0	42±39	31±49	14±24	59±48	85±16	70±15
H. odorus	1	55	44	0	0	60	0	0	7	70	70
H. purpurascens	3	79±21	58±17	38±32	50±43	72±29	73±0	52±47	82±16	86±6	80±13
H. torquatus	1	68	52	0	0	12	0	0	77	71	80
H. x ballardiae	2	0	0	n.a.[a]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
H. x ericsmithii	2	0	0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
H. x sternii	3	85±16	83±8	0	0	0	0	0	56±21	0	0

[a] n.a. means not applicable

The pollen viability of three *Helleborus* hybrids was also tested. For *H. x ballardiae* and *H. x ericsmithii*, no stained pollen was observed, indicating that these hybrids are pollen sterile. Therefore, pollen of these hybrids was not stored. In contrast to *H. x ballardiae* and *H. x ericsmithii*, the hybrid *H. x sternii* exhibited a mean pollen viability of 85 % for fresh pollen. However, the pollen could not be stored for more than four weeks at any temperature

tested. Overall, the standard deviations between repeated measurements were high, especially for *H. x hybridus* and *H. multifidus*.

In a subsequent experiment, large numbers of anthers from *H. argutifolius*, *H. x hybridus* and *H. niger* were collected in 2009/2010 and stored in Petri dishes at -20 °C for nine months. Pollen viability based on MTT staining of fresh and dried pollen was between 80 % and 90 % on average over all three species (Figure 3.6). This range is comparable to the pollen viability determined for the same species in 2007/2008 (Table 3.5) and 2008/2009 (Table 3.7). After nine months of storage at -20 °C, pollen viability decreased to 71 % for *H. x hybridus* but remained between 90 and 100 % for *H. argutifolius* and *H. niger*.

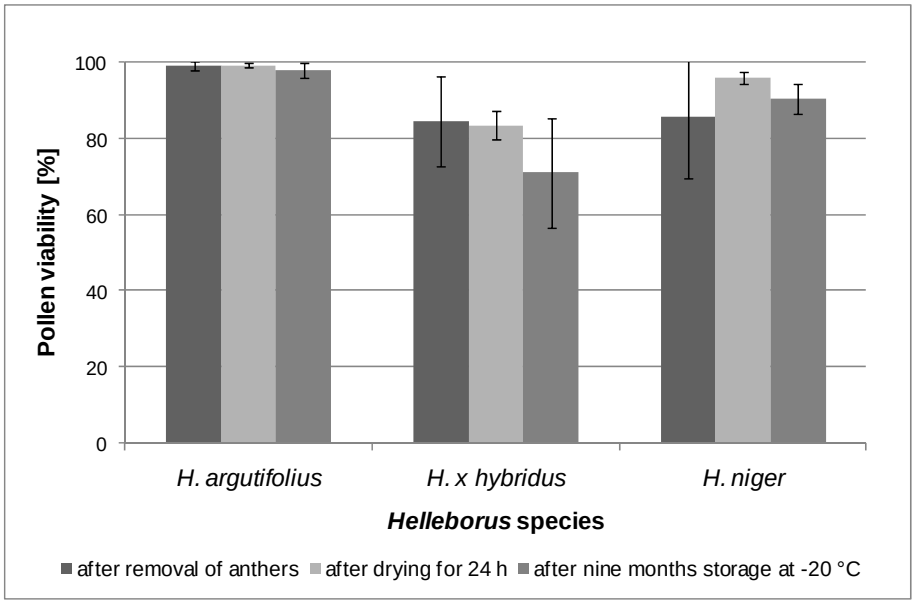


Figure 3.6: Viability of fresh, dried and stored pollen at -20 °C for nine months of three different *Helleborus* species determined with MTT staining.
Data are presented as mean ± standard deviation of four independent measurements.

In conclusion, all tested species produced viable pollen that in most cases could remain viable after up to six or nine months of storage at -20 °C. This storability is an important prerequisite for crosses between species with different flowering times.

3.3.2 Analysis of pollen tube growth *in situ*

To identify pre- and postzygotic crossing barriers between *Helleborus* species, the pollen tube growth was observed for the crossing combinations listed in Table 3.1.

Initially, pollen tube growth was compared for selected crosses 12, 24, 48 and 72 hours after pollination (Figure 3.7 A and B) to identify the time of fertilisation. After 12 hours, pollen tubes had grown into the first half of the style. After 48 hours, they reached the second half of

the style. After 72 hours, they reached the ovules in most of the observed carpels of *H. niger* in crosses using *H. niger*, *H. foetidus* or *H. x hybridus* as the pollen donor (Figure 3.7 A). The results were similar for crosses using *H. x hybridus* as the maternal parent and *H. x hybridus* or *H. niger* as pollen donors but were different for the crossing of *H. foetidus* with *H. x hybridus*, in which the pollen tubes grew more slowly and were not detected near the ovules (Figure 3.7 B).

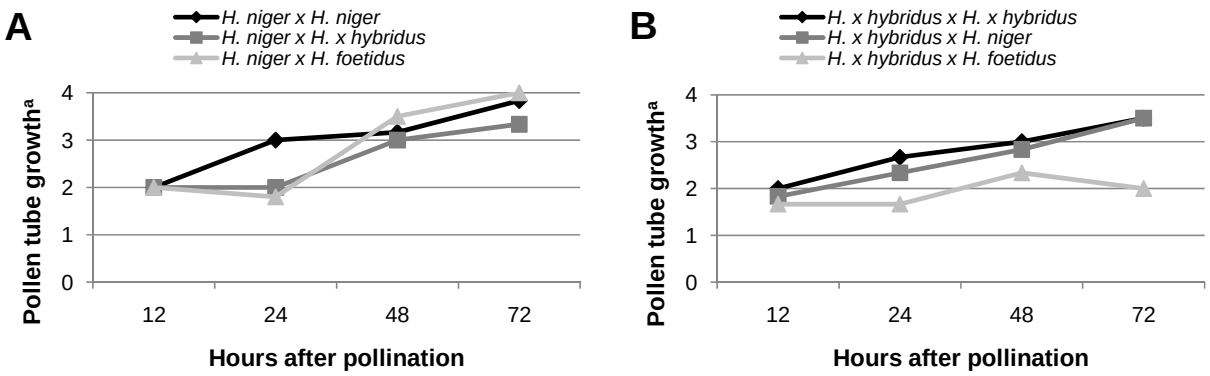


Figure 3.7: Mean pollen tube growth 12 to 72 hours after pollination of selected intra- and interspecific crossing combinations.
H. niger (A) or *H. x hybridus* (B) as maternal plant and *H. foetidus*, *H. x hybridus* and *H. niger* as pollen donors. Six carpels were observed for all crossings except *H. x hybridus* x *H. foetidus* were only three were examined.
[a](1) pollen visibly germinated but not growing into the stigmatic tissue, (2) pollen tube growing into the first half of the style, (3) pollen tube growing into the second half of the style and (4) pollen tube reaching the ovules.

From these and other observations of pollen tube growth during the first 72 hours after pollination (data not shown), the theoretical time of fertilisation was determined to be from 48 to 72 hours after pollination. Therefore, all further analyses presented in this chapter were carried out between 72 and 144 hours after pollination to determine whether the pollen tubes had reached the ovules. Examples of images showing pollen germination on the stigma, pollen tube growth up to the ovules and fertilisation are shown in Figure 3.8 A-C for intraspecific crosses of *H. niger* between 72 and 144 hours after pollination.

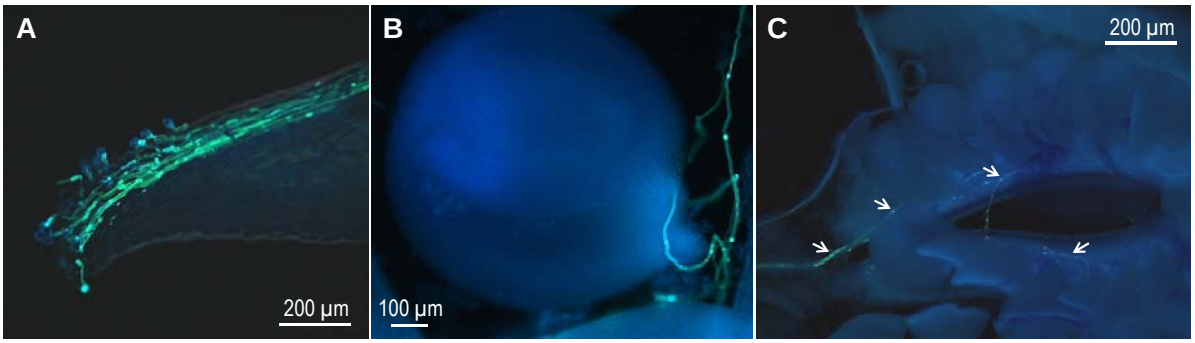


Figure 3.8: Pollen germination on the stigma and pollen tube growth observed in carpels of *H. niger* after intraspecific pollination.
A: stigma and style 72 hours, B: ovule 96 hours and C: ovary 144 hours after pollination, the pollen tube is indicated with arrows.

For most of the carpels observed, the pollen grains on the stigma and the pollen tubes in the style interfered with each other, making it impossible to count them (Figure 3.8 A). In some cases pollen germination was observed outside the stigma (Figure 3.9 (black and white figure)). Either the pollen germinated but did not grow into the style (Figure 3.9 A), or it germinated and grew into the style (Figure 3.9 B).

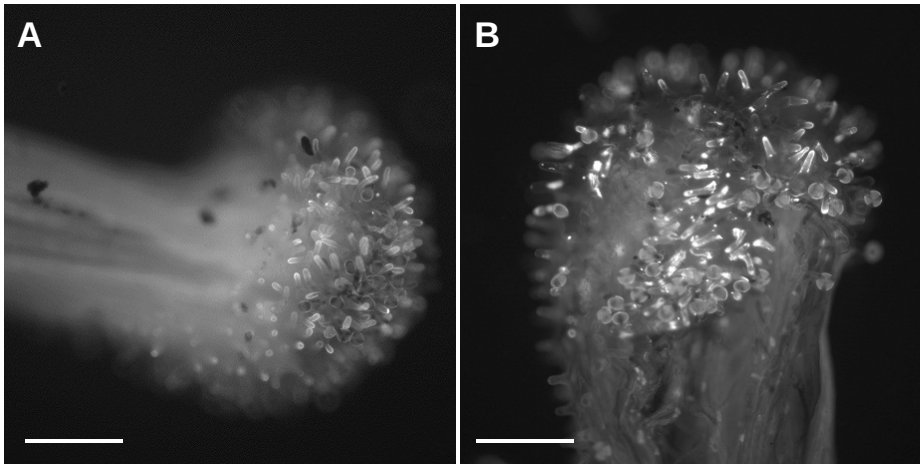


Figure 3.9: Pollen germination outside the stigma observed in carpels of two different crosses.
A: *H. x hybridus* x *H. lividus* 72 hours and B: *H. x hybridus* x *H. niger* 96 hours after pollination. Bars represent 200 µm.

Four *Helleborus* species, *H. foetidus*, *H. x hybridus*, *H. niger* and *H. odorus*, were selected based on their genetic distances to each other. The growth of pollen tubes in crosses between these species was analysed in greater detail. The genetic distances shown in the following tables represent averages of the genetic distances between several *H. x hybridus* or *H. niger* genotypes and the other species used, most of which were represented by only one plant.

The results for pollen tube growth from 72 to 144 hours after pollination from all three years of crosses between *H. foetidus*, *H. x hybridus*, *H. niger* and *H. odorus* were summarised for

each combination in Table 3.8. Pollen tube growth for each of the interspecific crosses was compared to that of intraspecific crosses of *H. x hybridus* and *H. niger*, for which pollen tubes reached the ovules after 72 hours in 67 % and 96 % of the analysed carpels, respectively. For crosses between *H. niger* and *H. x hybridus* (Table 3.8, Figure 3.10) pollen tubes were observed near the ovules in 87 % and 94 % of the analysed carpels, respectively, whereas pollen tubes were observed near the ovules in only 1 % and 4 % of the carpels for crosses between *H. foetidus* and *H. x hybridus.* Pollen tubes reaching the ovules were observed in 20 % to 42 % of the carpels for the crosses between *H. foetidus* and *H. odorus* (Table 3.8, Figure 3.11), between *H. niger* and *H. odorus* and between *H. foetidus* and *H. niger*. For *H. odorus* x *H. niger*, only five carpels were analysed; pollen tube growth was observed near the ovules in each one.

Table 3.8: Pollen tube growth for different intra- and interspecific crossing combinations summarised from 72 to 144 hours after pollination.
The table starts with intraspecific crosses followed by interspecific crosses, which are ordered by the genetic distance between the parental plants from the lowest to the highest.

	Genetic distance	♀	♂	Number of…			Pollen tube growth[a]			
				carpels analysed	stigmas with pollen	stigmas with germinated pollen	1	2	3	4
Intraspecific		*H. niger*	*H. niger*	28	28	28	0	0	1	27 (96 %)
		H. x hybridus	*H. x hybridus*	15	15	15	0	0	5	10 (67 %)
Interspecific	0.257	*H. niger*	*H. odorus*	53	53	53	0	30	1	22 (42 %)
		H. odorus	*H. niger*	5	5	5	0	0	0	5 (100 %)
	0.262	*H. x hybridus*	*H. foetidus*	91	80	71	0	56	14	1 (1 %)
		H. foetidus	*H. x hybridus*	49	37	24	0	21	1	2 (4 %)
	0.264	*H. niger*	*H. x hybridus*	194	193	193	0	0	10	183 (94 %)
		H. x hybridus	*H. niger*	208	208	208	1	1	26	180 (87 %)
	0.267	*H. foetidus*	*H. odorus*	49	49	49	0	38	1	10 (20 %)
	0.271	*H. niger*	*H. foetidus*	102	102	99	1	54	16	28 (28 %)
		H. foetidus	*H. niger*	45	36	34	5	8	3	18 (40 %)

[a](1) pollen visibly germinated but not growing into the stigmatic tissue, (2) pollen tube growing into the first half of the style, (3) pollen tube growing into the second half of the style and (4) pollen tube reaching the ovules.

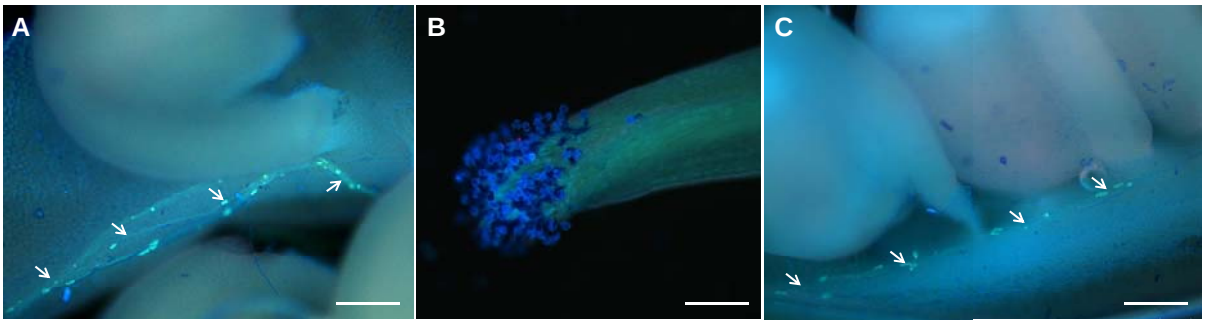


Figure 3.10: Pollen tube growth near the ovules and pollen on the stigma in hybridisations of *H. niger* x *H. x hybridus*.
A: pollen tubes near the ovules 72 hours, B: on the stigma and C: pollen tubes near the ovules 96 hours after pollination. The pollen tube is indicated with arrows. Bars represent 200 µm.

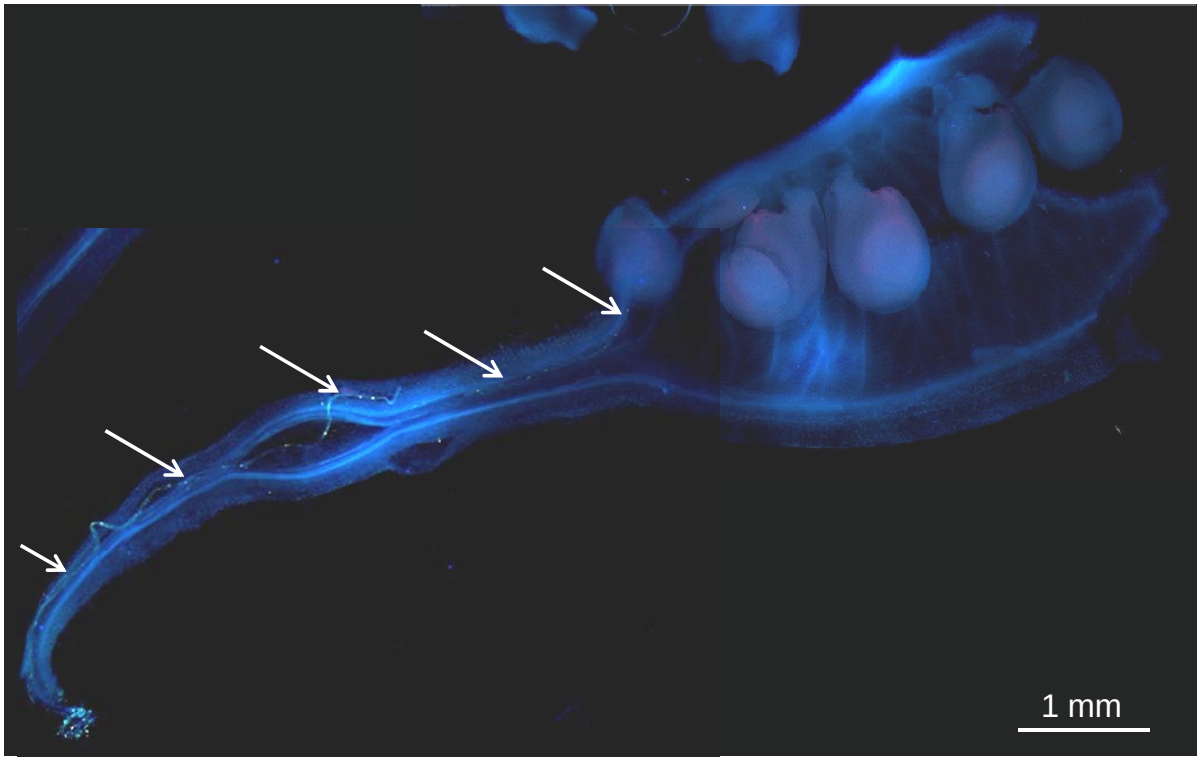


Figure 3.11: Pollen tube growth observed for hybridisation of *H. foetidus* x *H. odorus* 96 hours after pollination.
The pollen tube is indicated with arrows.

Combinations for which pollen tube growth was analysed in less depth are shown in Table 3.9. For 30 of the 35 observed crossing combinations, pollen tube growth was observed near the ovules in at least one of the analysed carpels, suggesting that fertilisation is possible. For the following crosses pollen tubes stopped growing before they reached the ovules: *H. argutifolius* x *H. niger*, *H. niger* x *H. torquatus*, *H. x hybridus* x *H. argutifolius*, *H. lividus* x *H. x hybridus* and *H. argutifolius* x *H. torquatus*.

Table 3.9: Pollen tube growth for different hybridisation combinations summarised from 72 to 96 hours after pollination.
The table starts with crosses, in which *H. niger* was involved, followed by crosses with *H. x hybridus* and *H. argutifolius*. Within these three groups crosses are ordered by the genetic distance between the parental plants from the lowest to the highest.

Genetic distance	♀	♂	Number of…			Pollen tube growth[a]			
			carpels analysed	stigmas with pollen	stigmas with germinated pollen	1	2	3	4
0.202	*H. niger*	*H. argutifolius*	6	6	4	0	2	0	2
	H. argutifolius	*H. niger*	9	7	6	2	2	2	0
0.218	*H. niger*	*H. lividus*	7	7	7	0	0	0	7
	H. lividus	*H. niger*	5	5	5	0	0	2	3
0.243	*H. niger*	*H. cyclophyllus*	6	6	6	0	0	0	6
0.247	*H. niger*	*H. torquatus*	8	8	8	0	8	0	0
	H. torquatus	*H. niger*	3	3	3	0	0	2	1
0.249	*H. niger*	*H. croaticus*	8	8	8	0	1	4	3
0.249	*H. niger*	*H. dumetorum*	5	5	5	0	0	0	5
0.253	*H. niger*	*H. atrorubens*	6	6	6	0	0	0	6
	H. atrorubens	*H. niger*	13	13	13	0	0	0	13
0.254	*H. niger*	*H. multifidus*	9	9	9	0	4	1	4
	H. multifidus	*H. niger*	5	5	5	0	0	1	4
0.277	*H. niger*	*H. purpurascens*	7	7	7	0	0	0	7
	H. purpurascens	*H. niger*	3	3	3	0	0	0	3
0.081	*H. x hybridus*	*H. torquatus*	8	8	8	0	0	4	4
0.082	*H. x hybridus*	*H. cyclophyllus*	5	5	5	0	0	0	5
0.093	*H. x hybridus*	*H. atrorubens*	6	6	6	0	0	0	6
	H. atrorubens	*H. x hybridus*	6	6	6	0	0	0	6
0.105	*H. x hybridus*	*H. odorus*	3	3	3	0	0	0	3
0.112	*H. x hybridus*	*H. croaticus*	5	5	5	0	0	4	1
0.114	*H. x hybridus*	*H. multifidus*	3	3	3	0	0	2	1
	H. multifidus	*H. x hybridus*	5	5	5	0	0	0	5
0.117	*H. x hybridus*	*H. dumetorum*	5	5	5	0	0	0	5
0.130	*H. x hybridus*	*H. purpurascens*	6	6	6	0	2	1	3
	H. purpurascens	*H. x hybridus*	3	3	3	0	0	0	3
0.255	*H. x hybridus*	*H. argutifolius*	8	7	7	0	4	3	0
	H. argutifolius	*H. x hybridus*	5	5	5	0	0	1	4
0.277	*H. x hybridus*	*H. lividus*	9	9	9	0	4	4	1
	H. lividus	*H. x hybridus*	5	5	5	0	5	0	0
0.069	*H. argutifolius*	*H. lividus*	5	5	5	0	0	1	4
	H. lividus	*H. argutifolius*	4	4	4	0	3	0	1
0.241	*H. foetidus*	*H. argutifolius*	3	3	3	0	1	1	1
0.244	*H. argutifolius*	*H. torquatus*	3	3	3	0	1	2	0
0.301	*H. argutifolius*	*H. multifidus*	3	3	3	0	0	1	2

[a](1) pollen visibly germinated but not growing into the stigmatic tissue, (2) pollen tube growing into the first half of the style, (3) pollen tube growing into the second half of the style and (4) pollen tube reaching the ovules.

In summary, 44 different crossing combinations (Table 3.8, Table 3.9) were evaluated for pollen tube growth near the ovules after 72 hours. For 39 analysed crosses, pollen tubes reached the ovules in at least one observed carpel, suggesting that fertilisation is possible.

3.3.3 Embryo rescue with ovules from intraspecific crosses

Ovules from intraspecific crosses of *H. argutifolius*, *H. foetidus*, *H. x hybridus* and *H. niger*, in which embryo development was assumed to occur, were isolated and cultured under different media and temperature conditions in embryo rescue experiment 1 (Table 3.3).

No differences in germination were observed between the two media, which differed in their sucrose concentrations. The results for both media are summarised below.

Over all species examined, 21 % of the ovules isolated from carpels sterilised three weeks after pollination remained in good condition. After four weeks, the corresponding percentage was 54 %; after five weeks, it was 58 %. For carpels isolated six weeks after pollination, 59 % of the ovules were not aborted. The numbers of germinated ovules for different temperature conditions during ovule culture are shown in Table 3.10. Overall, 37 germinated ovules were observed for *H. argutifolius*, one for *H. foetidus*, 16 for *H. x hybridus* and eight for *H. niger*. In total, 62 shoots were obtained, of which 86 % were derived from ovules isolated five and six weeks after pollination. Shoot cultures were obtained from all species (Figure 3.12). The temperature sequence of 16 °C, 6 °C and 16 °C was best for ovule culture. Therefore, alternating warm and cold temperatures were used for all subsequent embryo rescue experiments.

Table 3.10: Number of germinated ovules depending on the *Helleborus* species and the temperatures after 40 weeks of culture *in vitro*.

	Number of...		Number of germinated ovules				
			Temperature [°C][a]				
Species	flowers pollinated	ovules cultured	16	24	16→6→16	24→6→24	Sum
H. argutifolius	25	1015	3	1	28	5	37
H. foetidus	22	504	0	0	1	0	1
H. x hybridus	21	807	5	0	6	5	16
H. niger	16	1124	1	1	4	2	8

[a]Alternating temperatures: 1st temperature for 12 weeks, followed by 11 weeks at 6 °C and again 17 weeks at the 1st temperature

The results of this experiment have been published previously in greater detail by Meiners and Winkelmann (2010).

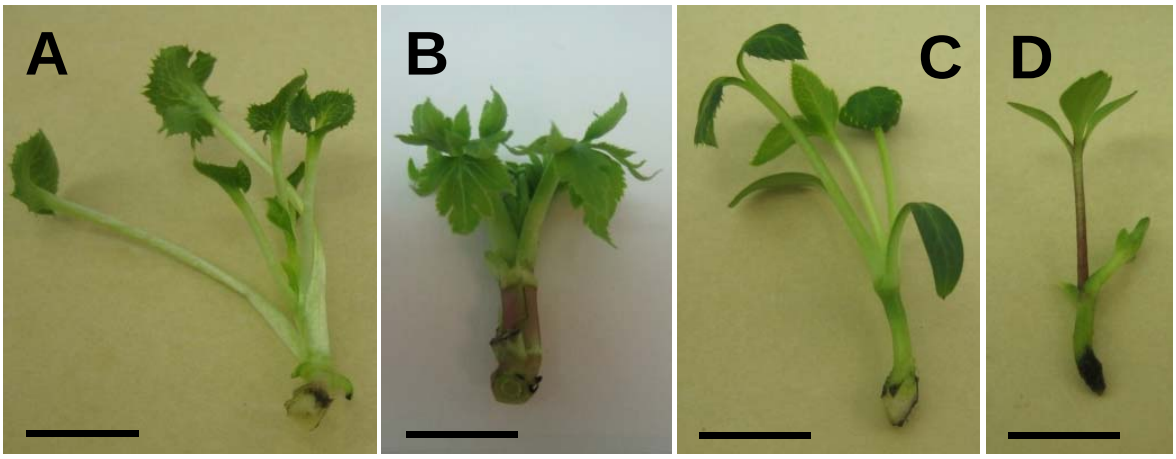


Figure 3.12: *In vitro* shoots obtained from intraspecific embryo rescue experiment 1.
A: *H. argutifolius*, B: *H. foetidus*, C: *H. x hybridus*, D: *H. niger*. Bars represent 1 cm.

3.3.4 Embryo rescue with ovules from interspecific crosses

During embryo rescue experiment 2 in 2007/2008 (Table 3.3), all ovules were cultured under the same temperature (24 °C). The results are shown in Table 3.11, in which they are ordered by crossing combinations and within each maternal species by the genetic distance between the maternal and the paternal species. Overall, 321 flowers were pollinated. Different numbers of flowers were pollinated for each combination, resulting in different numbers of cultured ovules (Table 3.11). The two tested sucrose concentrations (2.5 % and 5 %) showed no differences relating to ovule development, germination or embryo culture and were summed as in the experiment 1 with ovules from intraspecific crosses. The medium with 2.5 % sucrose was chosen for embryo rescue experiments 3-5 in 2008/2009 and 2009/2010.

Twelve weeks after ovule culture was initiated, all ovules turned brown to black and were divided into two groups. Dry and shrivelled ovules were either unfertilised or aborted and were classified as aborted, while turgescent and swollen ovules showing the shape of a seed were classified as not aborted (Figure 3.13 A-C). This classification of ovules was used in all subsequent embryo rescue experiments.

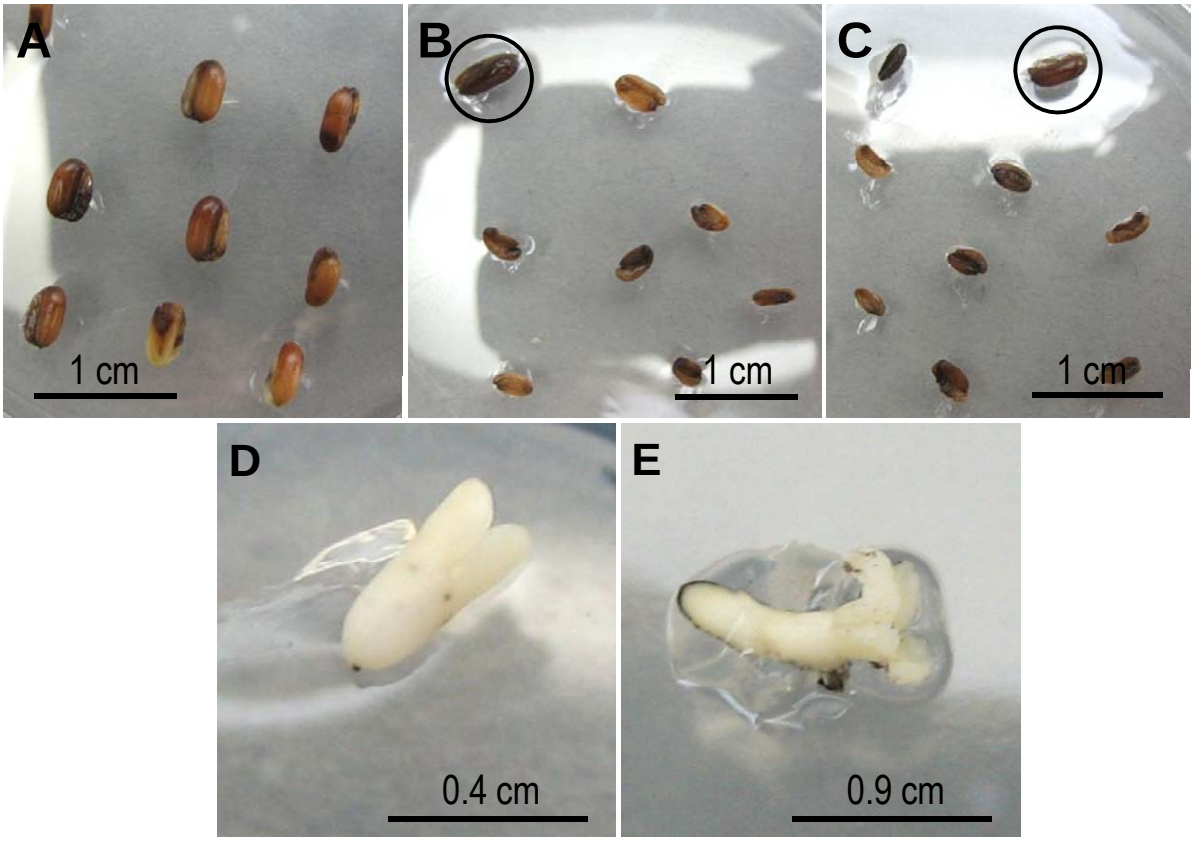


Figure 3.13: Classification of ovules and embryos during the embryo rescue experiment 2 (2007/2008). Ovules from the cross A: *H. niger* x *H. niger,* all not aborted, B: *H. niger* x *H. multifidus*, C: *H. niger* x *H. lividus* and embryos from the crosses D: *H. niger* x *H. argutifolius*, torpedo stage and E: *H. foetidus* x *H. argutifolius*, cotyledon stage. In B and C ovules classified as not aborted are encircled.

After 12 weeks of culture, 80 % of ovules obtained from carpels extracted three weeks after pollination from interspecific crosses were aborted. This proportion increased to 87 % for four, 83 % for five and 88 % for six weeks after pollination. Aborted ovules were discarded. Ovules that were not aborted were dissected after 14 to 18 weeks of culture to rescue embryos if they were present. Some of these embryos developed into shoots, while others stopped growing and turned brown. More embryos could be rescued from ovules that were isolated five or six weeks after pollination compared to ovules that were isolated three to four weeks after pollination.

Three interspecific hybrids developed from embryos obtained in experiment 2: one between *H. x hybridus* and *H. argutifolius* (Figure 3.14, Table 3.11) with a genetic distance of 0.255 between the parental species, and two between *H. foetidus* and *H. argutifolius* (Figure 3.15, Table 3.11) with a genetic distance of 0.241. Additionally, shoot cultures were obtained from ovules from intraspecific crosses of *H. niger* and *H. x hybridus* (Table 3.11).

Table 3.11: Overview of embryo rescue experiment 2 (2007/2008) pooled over all ovule isolation dates and media tested.

Within the table crossing combinations are ordered by their maternal parent and within three groups crosses are ordered by the genetic distance between the parental plants from the lowest to the highest.

Genetic distance	♀ x ♂	Number of ... flowers pollinated	carpels isolated	ovules cultured	ovules not aborted	ovules germinated after 14 weeks	embryos developed	embryos further developed into plants
	H. niger x H. niger	17	120	1517	504	3	2	0
0.202	*H. niger x H. argutifolius*	12	87	1336	188	0	3	0
0.218	*H. niger x H. lividus*	3	18	283	54	0	0	0
0.243	*H. niger x H. cyclophyllus*	3	18	259	11	0	0	0
0.247	*H. niger x H. torquatus*	6	36	591	44	0	0	0
0.253	*H. niger x H. atrorubens*	1	8	96	12	0	0	0
0.254	*H. niger x H. multifidus*	6	29	319	76	0	0	0
0.257	*H. niger x H. odorus*	4	18	278	19	0	0	0
0.264	*H. niger x H. x hybridus*	19	137	1987	232	0	0	0
0.271	*H. niger x H. foetidus*	14	86	1264	245	0	0	0
	H. x hybridus x H. x hybridus	20	79	763	250	1	6	2
0.255	*H. x hybridus x H. argutifolius*	10	41	366	30	0	1	1
0.262	*H. x hybridus x H. foetidus*	9	35	349	0	0	0	0
0.264	*H. x hybridus x H. niger*	44	181	1813	146	0	0	0
	H. foetidus x H. foetidus	22	37	431	237	0	5	0
0.241	*H. foetidus x H. argutifolius*	21	51	307	114	0	2	2
0.262	*H. foetidus x H. x hybridus*	23	53	433	58	0	0	0
0.271	*H. foetidus x H. niger*	43	102	868	242	0	0	0
	H. argutifolius x H. argutifolius	6	7	82	19	0	0	0
0.202	*H. argutifolius x H. niger*	21	62	670	143	0	0	0
0.241	*H. argutifolius x H. foetidus*	5	20	200	28	0	0	0
0.243	*H. cyclophyllus x H. niger*	2	2	21	1	0	0	0
0.247	*H. torquatus x H. niger*	1	2	23	10	0	0	0
0.254	*H. multifidus x H. niger*	6	12	117	25	0	0	0
0.277	*H. purpurascens x H. niger*	3	7	54	2	0	0	0
	Sum	**321**	**1248**	**14427**	**2689**	**4**	**19**	**5**

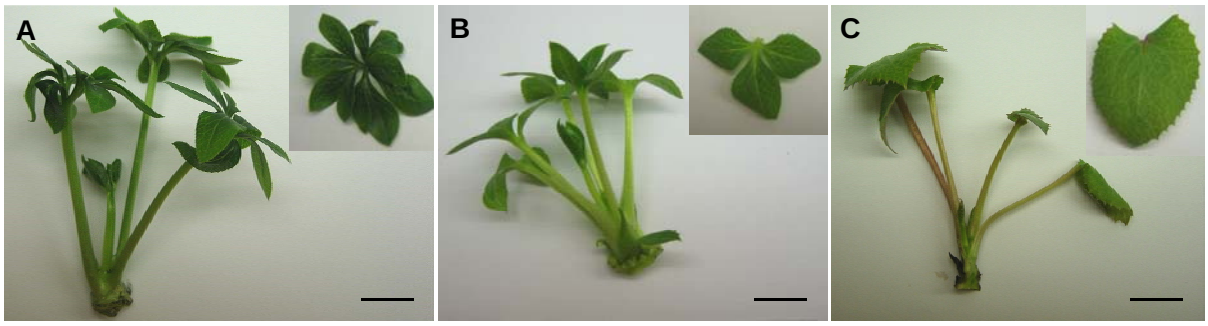


Figure 3.14: Morphology of *in vitro* shoots and leaves of the interspecific hybrid *H. x hybridus* x *H. argutifolius* in comparison to its parental species.
A: *H. x hybridus*, B: interspecific hybrid: *H. x hybridus* x *H. argutifolius*, C: *H. argutifolius*. Bars represent 1 cm.

Both interspecific hybrids showed intermediate leaf phenotypes during *in vitro* culture (Figure 3.14, Figure 3.15). The hybrid *H. x hybridus* x *H. argutifolius* had three-lobed leaves, whereas the leaves of the maternal plant were pedate with many segments (Figure 3.14). The hybrid *H. foetidus* x *H. argutifolius* had pedate leaves with approximately three to five segments, whereas the maternal plant had pedate leaves with more segments (Figure 3.15). Leaves from the paternal species of both hybrids, *H. argutifolius*, were rather obtuse to cordate.

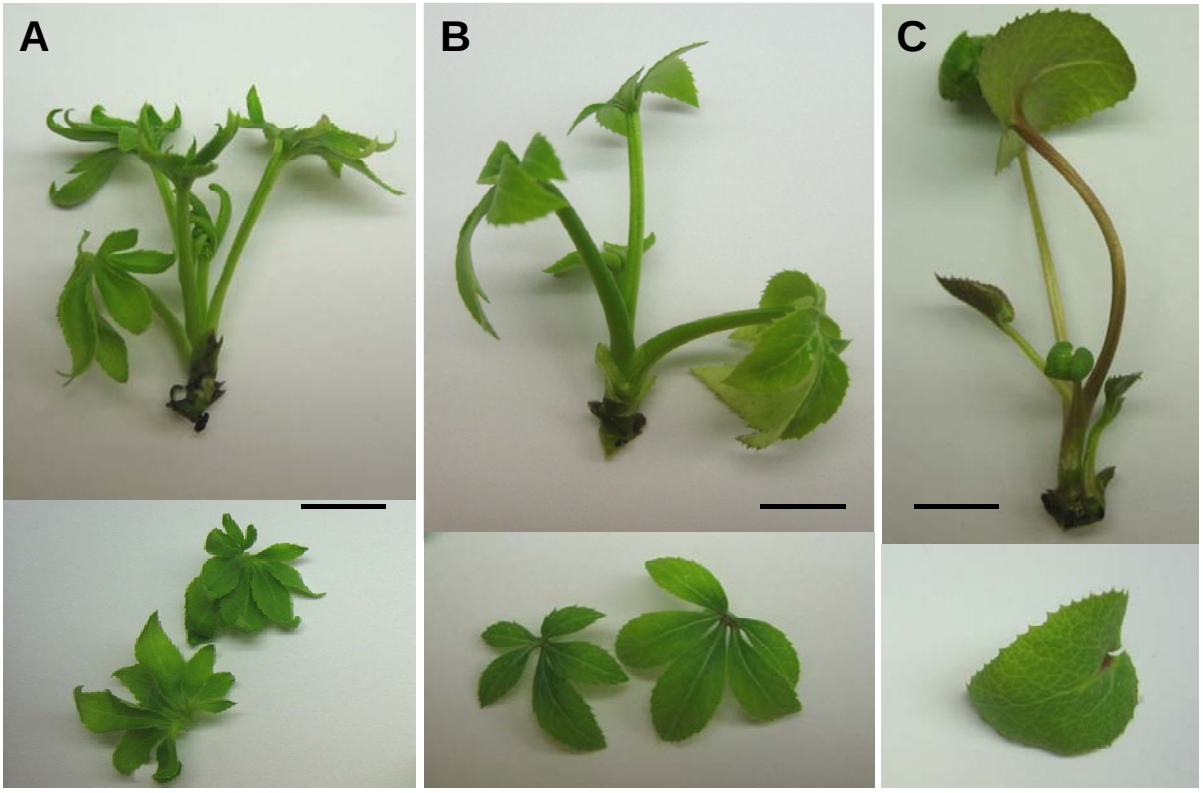


Figure 3.15: Morphology of *in vitro* shoots and leaves of the interspecific hybrid *H. foetidus* x *H. argutifolius* in comparison to its parental species.
A: *H. foetidus*, B: interspecific hybrid: *H. foetidus* x *H. argutifolius*, C: *H. argutifolius*. Bars represent 1 cm.

Embryo rescue experiments 3 and 4 (Table 3.3) were initiated in parallel in the winter of 2008/2009. In both experiments, ovules were isolated five to seven weeks after pollination.

In experiment 3 ovules from crosses between *H. niger* and *H. x hybridus* and between *H. x hybridus* and *H. niger* were cultured on different media (Table 3.3) and under different temperature conditions. No differences related to germination were observed; therefore, the data were pooled from across the different media and culture conditions.

All ovules from other crossing combinations in experiment 4 (Table 3.3) were cultured at 20 °C for 12 weeks, followed by 4 °C for 12 weeks and a return to 20 °C. Some ovules germinated during cold storage, but many ovules still showed no development. Therefore, after 34 to 41 weeks of culture, all ovules from both experiments (3 and 4) that were not aborted were again transferred to 4 °C for 12 weeks, some for the first and others for the second time. The results of both experiments are shown in Table 3.12 and Table 3.13.

Overall, 405 flowers were pollinated, 2299 carpels were isolated and 29251 ovules were cultured, among which an average of 7.4 % were not aborted (summary of Table 3.12 and Table 3.13).

At the final evaluation (after 60 to 67 weeks of culture), the total number of germinations was recorded, all remaining ovules were dissected and the following observations were made: ovules were either empty, filled with liquid endosperm or filled with white or brown coloured solid endosperm (Figure 3.16 A-D). Embryos were found in and rescued from only ovules with solid endosperm, but not all ovules with solid endosperm contained embryos (Figure 3.16 C-D). The predominant developmental stage of these embryos was heart-shaped (Figure 3.17, A-C), but torpedo (Figure 3.17, D) and cotyledon stages were also observed (Figure 3.17, E-F, Table 3.12, Table 3.13).

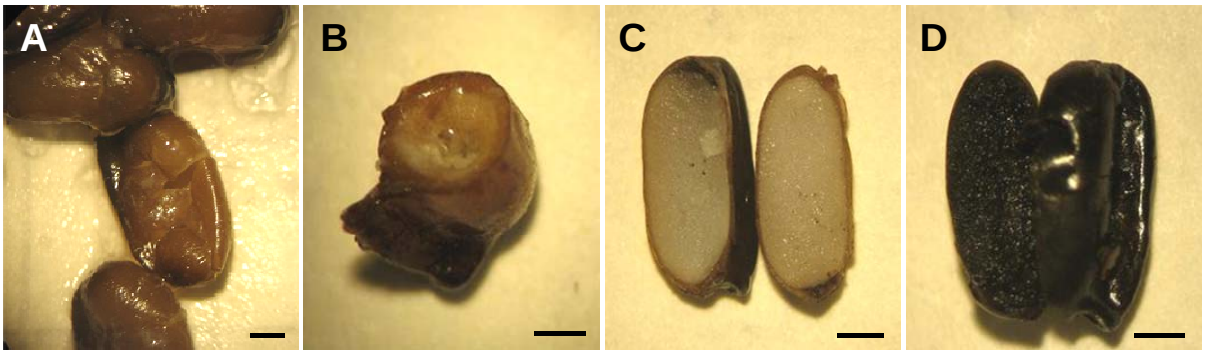


Figure 3.16: Ovules without embryos after 64 weeks of *in vitro* culture.
A: cut and empty, B: with liquid endosperm, C: with white solid endosperm and D: with brown solid endosperm. Bars represent 1 mm.

Figure 3.17: Ovules with embryos in different stages after 64 weeks of *in vitro* culture.
A-C: heart-shaped stages, indicated with arrows, D: torpedo stage, E-F: cotyledon stages. Bars represent 1 mm.

The results of experiments 3 and 4 are presented in two tables: Table 3.12, which lists the crosses that produced offspring, and Table 3.13, which lists the crosses that did not yield plantlets.

Most of the shoots that were obtained came from ovules that were isolated six or seven weeks after pollination. Only nine shoots developed from ovules isolated after five weeks of pollination: six shoots of *H. argutifolius* x *H. lividus* and one shoot each of *H. croaticus* x *H. multifidus*, *H. odorus* x *H. croaticus* and *H. purpurascens* x *H. croaticus*. All other shoots developed from ovules that were isolated six or seven weeks after pollination.

For crosses between the closely related sister species *H. argutifolius* and *H. lividus*, 26 % of the cultured ovules germinated and yielded in offspring (97 individuals) (Table 3.12). The shoots grown *in vitro* were morphologically similar to their parents, and no hybrid phenotype was identified at this stage.

Table 3.12: Overview of the crossing combinations used for embryo rescue experiments 3 and 4 (2008/2009), which led to embryo or plant development.
The results are pooled over all dissection dates and media used.

Genetic distance	♀ x ♂	Number of…										
		flowers pollinated	carpels isolated	ovules cultured	ovules not aborted	ovules germinated	ovules dissected	ovules with liquid ES[a]	ovules with solid ES	embryos rescued[b]	embryos developed into shoots	Sum of hybrid offspring
0.081	*H. x hybridus x H. torquatus*	14	68	700	84	6	78	45	11	7 (1G,5H,1T)	0	6
0.082	*H. x hybridus x H. cyclophyllus*	8	39	403	240	33	207	161	44	24 (22H,2C)	2	35
0.093	*H. x hybridus x H. atrorubens*	8	42	431	211	19	192	161	22	12 (11H,1C)	1	20
0.105	*H. x hybridus x H. odorus*	16	70	743	223	6	217	165	13	6 (1G,5H)	0	6
0.112	*H. x hybridus x H. croaticus*	11	44	486	136	14	122	103	6	5 (1G,4H)	0	14
0.114	*H. x hybridus x H. multifidus*	11	59	559	141	7	134	72	12	8H	1	8
0.117	*H. x hybridus x H. dumetorum*	10	52	509	19	1	18	10	3	2H	0	1
0.130	*H. x hybridus x H. purpurascens*	10	54	539	131	8	123	92	23	15H	0	8
0.264	*H. x hybridus x H. niger*	103	287	2827	105	11	94	36	34	23 (3G,17H,1T, 2C)	2	13
0.069	*H. argutifolius x H. lividus*	3	11	114	85	37	48	41	7	4 (3H,1C)	0	37
0.069	*H. lividus x H. argutifolius*	5	26	258	150	60	90	46	27	23 (8H,7T,8C)	0	60
0.097	*H. croaticus x H. multifidus*	2	10	112	3	1	2	0	0	0	0	1
0.106	*H. torquatus x H. croaticus*	1	5	44	19	2	17	15	1	0	0	2
0.115	*H. croaticus x H. odorus*	1	5	61	31	2	29	21	1	1H	0	2
0.115	*H. odorus x H. croaticus*	1	6	56	22	2	20	12	3	2 (1G,1H)	0	2
0.141	*H. purpurascens x H. croaticus*	1	1	8	4	1	3	0	0	0	0	1
	Sum	**205**	**779**	**7850**	**1604**	**210**	**1394**	**980**	**207**	**132**	**6**	**216**

[a]ES: endosperm; [b]developmental stages of embryos: G=globular, H=heart-shaped, T=torpedo, C=cotyledon

Table 3.13: Overview of the crossing combinations used for embryo rescue experiments 3 and 4 (2008/2009), which did not lead to embryo or plant development.
The results are pooled over all dissection dates and media used.

Genetic distance	♀ x ♂	Number of…							
		flowers pollinated	carpels isolated	ovules cultured	ovules not aborted	ovules dissected	ovules with liquid ES[a]	ovules with solid ES	embryos rescued[b]
0.202	*H. niger x H. argutifolius*	7	87	1298	0				
0.218	*H. niger x H. lividus*	5	64	1059	12	12	10	0	0
0.243	*H. niger x H. cyclophyllus*	6	74	1028	81	76	10	0	0
0.247	*H. niger x H. torquatus*	6	67	1104	10	10	10	0	0
0.249	*H. niger x H. dumetorum*	4	31	563	0				
0.249	*H. niger x H. croaticus*	13	79	1038	26	19	1	0	0
0.253	*H. niger x H. atrorubens*	7	89	1220	136	135	21	0	0
0.254	*H. niger x H. multifidus*	16	163	2116	33	32	7	0	0
0.257	*H. niger x H. odorus*	9	55	910	8	8	0	0	0
0.264	*H. niger x H. x hybridus*	37	315	4657	98	96	20	0	0
0.271	*H. niger x H. foetidus*[c]	13	165	2396	66	76	26	0	0
0.277	*H. niger x H. purpurascens*	15	106	1558	3	3	0	0	0
0.255	*H. x hybridus x H. argutifolius*	5	20	212	0	0	0	0	0
0.262	*H. x hybridus x H. foetidus*	3	9	96	0	0	0	0	0
0.277	*H. x hybridus x H. lividus*	4	18	185	18	12	6	2	0
0.241	*H. argutifolius x H. foetidus*	4	20	267	0				
0.244	*H. argutifolius x H. torquatus*	2	6	81	0				
0.251	*H. argutifolius x H. atrorubens*	1	3	43	0				
0.255	*H. argutifolius x H. x hybridus*	2	8	112	25	25	21	2	2 (1G,1H)
0.272	*H. argutifolius x H. purpurascens*	3	4	31	0				
0.301	*H. argutifolius x H. multifidus*	3	11	145	0				
0.093	*H. atrorubens x H. x hybridus*	2	10	105	0				
0.097	*H. atrorubens x H. croaticus*	2	8	82	0				
0.100	*H. atrorubens x odorus*	1	4	36	0				
0.124	*H. atrorubens x H. purpurascens*	3	9	85	0				
0.253	*H. atrorubens x H. niger*	2	5	43	0				
0.112	*H. multifidus x H. odorus*	2	8	94	20	20	15	1	1T
0.114	*H. multifidus x H. x hybridus*	2	5	46	12	19	3	0	0
0.132	*H. multifidus x H. purpurascens*	2	5	57	10	28	11	0	0
0.254	*H. multifidus x H. niger*	4	10	82	0				
0.301	*H. multifidus x H. argutifolius*	4	14	147	0				
0.095	*H. odorus xH. torquatus*	1	5	46	17	17	13	1	1H
0.112	*H. odorus x H. multifidus*	1	5	51	10	10	5	1	0
0.257	*H. odorus x H. niger*	2	10	91	1	1	0	0	0
0.105	*H. cyclophyllus x H. multifidus*	1	3	34	0				
0.249	*H. croaticus x H. niger*	3	16	176	1	1	0	0	0
0.277	*H. purpurascens x H. niger*	1	2	17	0				
0.286	*H. lividus x H. purpurascens*	2	7	90	0				
	Sum	**200**	**1520**	**21401**	**587**	**600**	**179**	**7**	**4**

[a]ES: endosperm
[b]developmental stages of embryos: G=globular, H=heart-shaped
[c]one offspring was identified as self-pollination

Crosses between species belonging to section Helleborastrum, especially those with *H. x hybridus* as the maternal parent (98 offspring), resulted in many interspecific hybrids (Table 3.12). Again, no hybrid phenotype was apparent at the *in vitro* shoots due to the morphological similarity of the parental species. For the crosses *H. multifidus* x *H. odorus* and *H. odorus* x *H. torquatus*, which did not produce offspring, embryos were found in the ovules (Table 3.13).

Intersectional crosses between *H. argutifolius* and *H. x hybridus* resulted in ovules containing embryos, but the embryos did not develop (Table 3.13). All ovules that were not aborted from interspecific crosses with *H. niger* as the maternal parent either were empty or contained liquid endosperm. No embryos were found (Table 3.13).

Hybrids between species belonging to different sections of *Helleborus* were obtained from the combination *H. x hybridus* x *H. niger*. Their regular development from germination to shoot is shown in Figure 3.18. Usually, the radicle emerges and elongates, and after the testa is pulled off, two cotyledons appear. The period from germination to the appearance of the primary leaves differed among most of the ovules. In summary, nine offspring that developed into shoots before the end of this project were obtained.

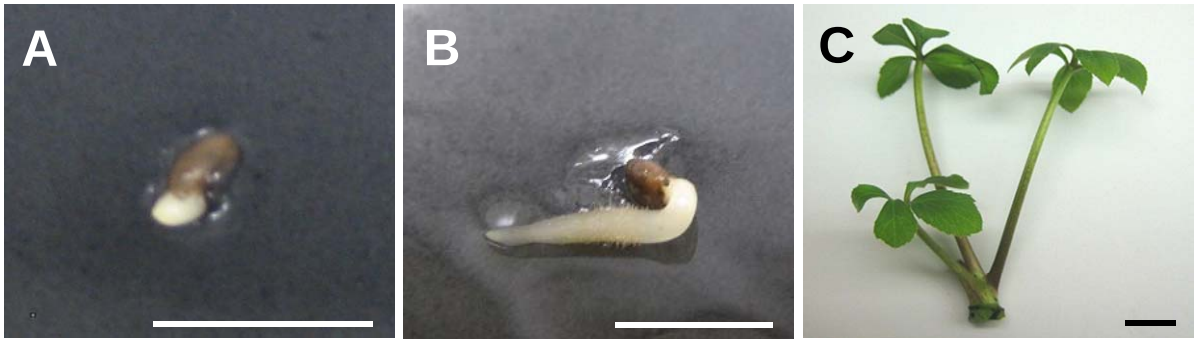


Figure 3.18: Development of one ovule of one *H. x hybridus* x *H. niger* combination.
A: radicle emergence at one ovule from genotype 2539.5 in November 2009, B: the same ovule from genotype 2539.5 in December 2009, C: shoot from genotype 2539.5 in October 2010. Bars represent 1 cm.

Similar to the intersectional hybrids *H. x hybridus* x *H. argutifolius* and *H. foetidus* x *H. argutifolius*, which were obtained from experiment 2 during the 2007/2008 flowering season, the *in vitro* shoots of *H. x hybridus* x *H. niger* showed leaves of an intermediate phenotype (Figure 3.19). The leaves of both parental species and of the hybrids were pedate (Figure 3.19 A and B). The leaves of *H. niger* were scarcely toothed at the margins and coarsely toothed at the apices of the leaflets (Figure 3.19 B). The segments were oblong or oblanceolate. Leaf segments from *H. x hybridus* were narrowly to broadly elliptic or

oblanceolate and coarsely serrate at the margins (Figure 3.19 A). The leaf margins of the hybrids were less serrate than those of *H. x hybridus*, and the shape of the leaf segments was intermediate between the parents (Figure 3.19 C-E).

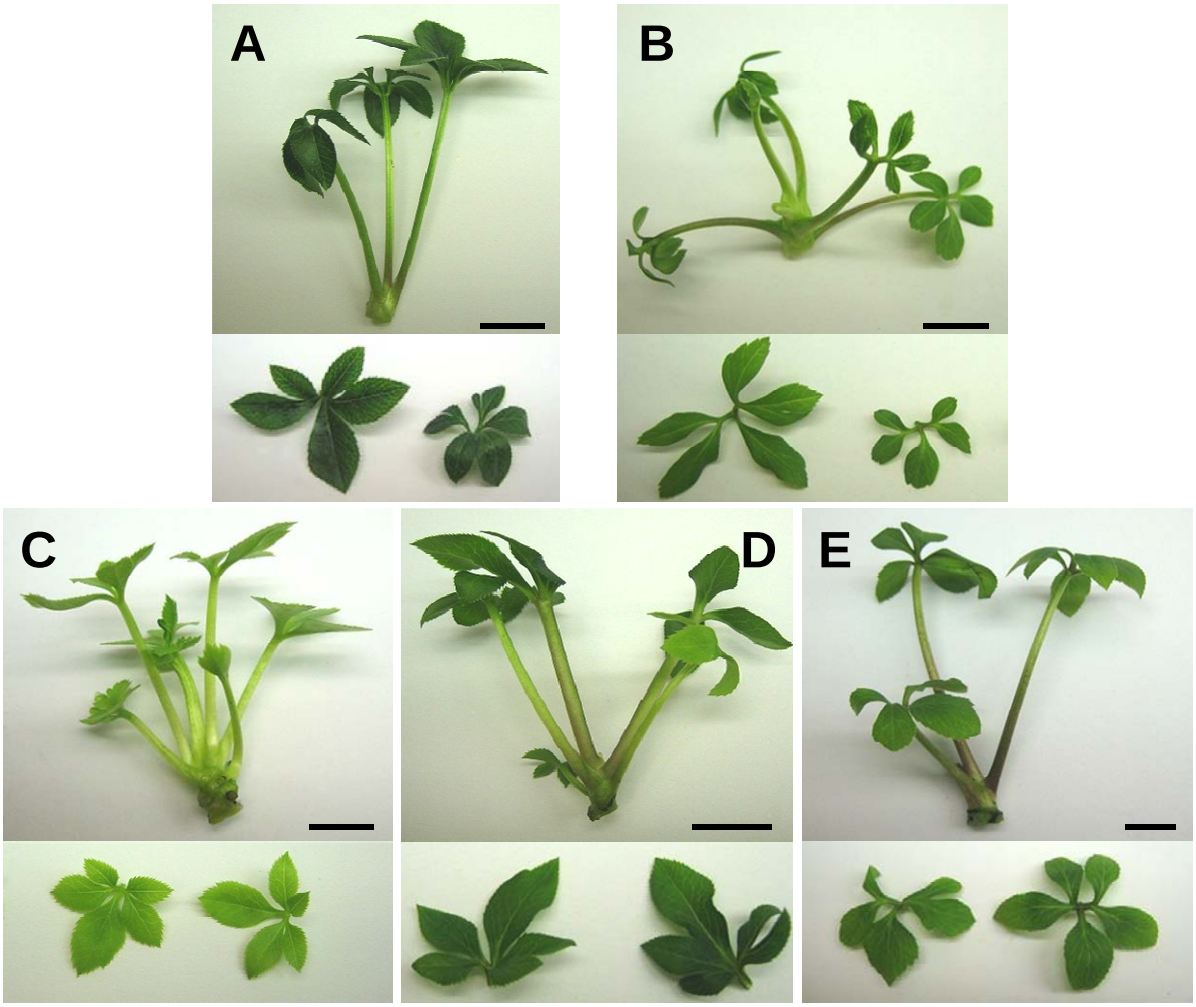


Figure 3.19: Comparison of *in vitro* shoots and leaves of *H. x hybridus* x *H. niger* hybrids with their parental species.
A: *H. x hybridus*, B: *H. niger*, C: *H. x hybridus* x *H. niger* 2321.6, D: *H. x hybridus* x *H. niger* 2520.5 and E: *H. x hybridus* x *H. niger* 2539.5. Bars represent 1 cm.

Due to the long time needed for ovule culture and the slow development of the seedlings, acclimatised greenhouse plants of all hybrids were not available for comparisons at the plant level at this time. It will be exciting to evaluate plant characteristics, especially flower morphology, at a later date.

In parallel to the regular development of *H. x hybridus* x *H. niger* offspring, some distinctive features were observed. Two germinated ovules and two embryos were stunted (Figure 3.20 A-D). Two seedlings of the same cross had only one cotyledon (Figure 3.20 E-F). Additionally, two of the 13 *H. x hybridus* x *H. niger* offspring died during development.

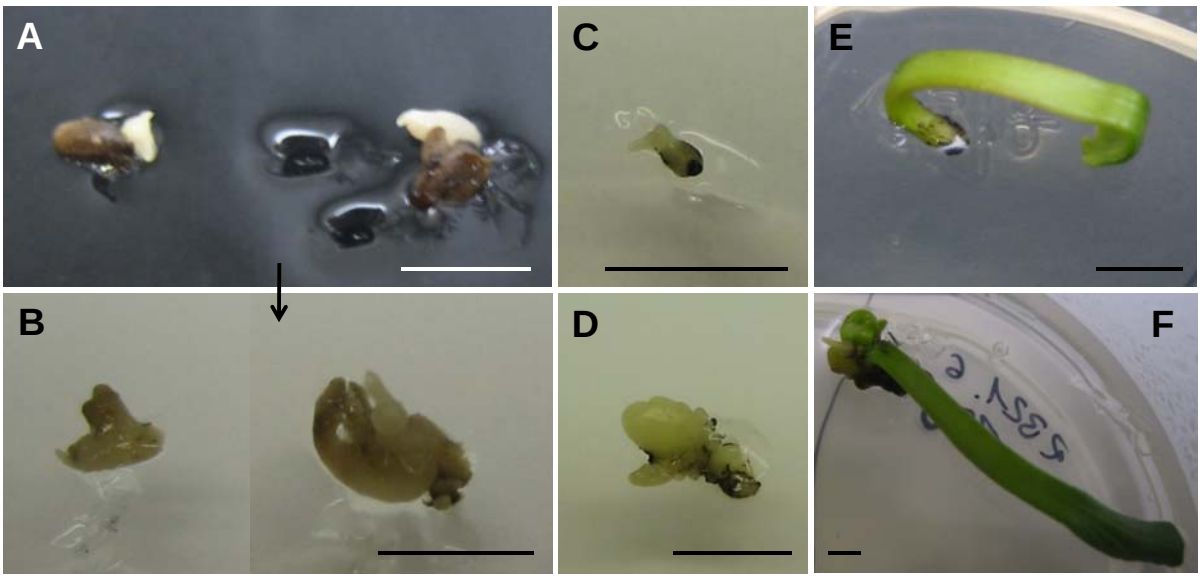


Figure 3.20: Developmental stages and characteristics of *H. x hybridus* x *H. niger* offspring.
A: germinated ovules from *H. x hybridus* x *H. niger* 2391.5 in December 2009 and B: in November 2010, developing embryos of C: *H. x hybridus* x *H. niger* 2391.5 and D: *H. x hybridus* x *H. niger* 2527.5, E and F: seedlings of *H. x hybridus* x *H. niger* 2321.6, which developed only one cotyledon. Bars represent 0.5 cm.

The final embryo rescue experiment (experiment 5) was carried out in 2009/2010. In this experiment, crosses were performed between *H. foetidus*, *H. niger*, *H. odorus* and *H. x hybridus* (Table 3.1, Table 3.3). In these crosses, the genetic distances between the parental plants were greater than 0.257. After 12 weeks at 20 °C followed by 12 weeks at 4 °C, all ovules were aborted and discarded.

Finally, 18 different interspecific hybrid offspring, representing 217 different genotypes (Table 3.14) were obtained during the three years of embryo rescue experiments 1-4 (Table 3.11, Table 3.12). From 16 of these hybrids, *in vitro* shoot cultures for propagation were successfully initiated, represented by 192 different genotypes (Table 3.14).

Table 3.14: Interspecifc hybrid offspring obtained from all five embryo rescue experiments.
The table is arranged according to genetic distances between the parental species from the lowest to the highest.

Genetic distance	Crossing combination	Number of…		
		hybrid offspring	offspring died	shoot cultures established
0.069	*H. argutifolius x H. lividus*	37	2	35
0.069	*H. lividus x H. argutifolius*	60	4	56
0.081	*H. x hybridus x H. torquatus*	6	2	4
0.082	*H. x hybridus x H. cyclophyllus*	35	1	34
0.093	*H. x hybridus x H. atrorubens*	20	2	18
0.097	*H. croaticus x H. multifidus*	1	1	0
0.105	*H. x hybridus x H. odorus*	6	0	6
0.106	*H. torquatus x H. croaticus*	2	1	1
0.112	*H. x hybridus x H. croaticus*	14	6	8
0.114	*H. x hybridus x H. multifidus*	8	2	6
0.115	*H. croaticus x H. odorus*	2	0	2
0.115	*H. odorus x H. croaticus*	2	1	1
0.117	*H. x hybridus x H. dumetorum*	1	1	0
0.130	*H. x hybridus x H. purpurascens*	8	0	8
0.141	*H. purpurascens x H. croaticus*	1	0	1
0.241	*H. foetidus x H. argutifolius*	2	0	2
0.255	*H. x hybridus x H. argutifolius*	1	0	1
0.264	*H. x hybridus x H. niger*[a]	11	0	9
	Sum	217	23	192

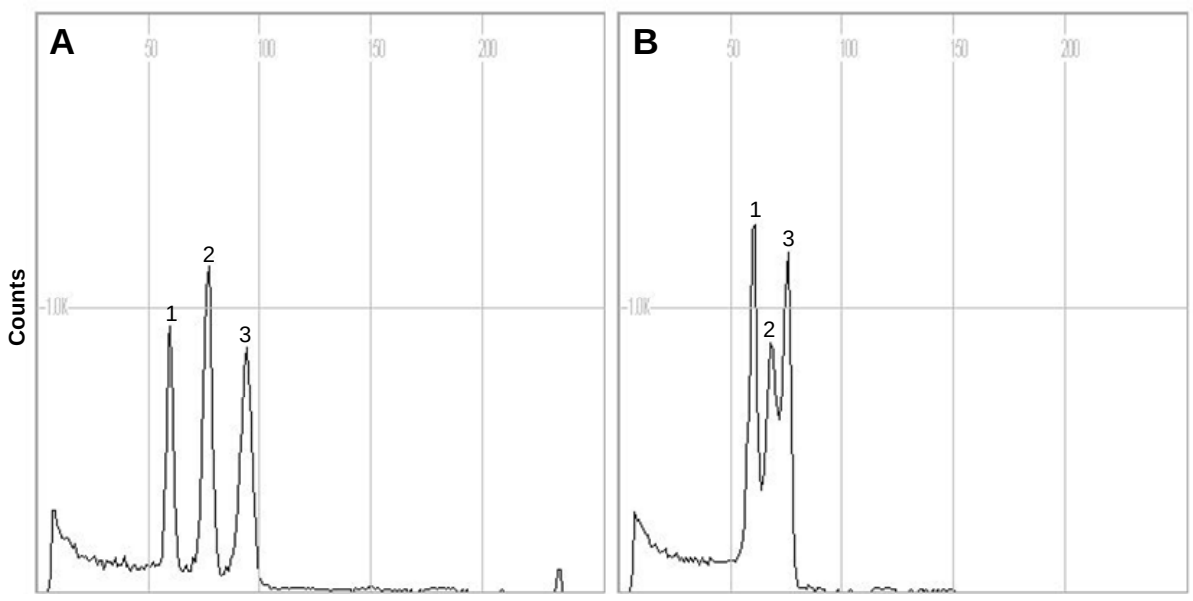
[a]two embryos were still developing at the end of the project

3.3.5 Hybrid identification

3.3.5.1 Hybrid identification by flow cytometry

As described in Chapter 2.4.2 flow cytometry is suitable for hybrid identification, only if the nuclear DNA contents of the parental species are sufficiently different. Two putative hybrids obtained by embryo rescue were analysed by flow cytometry.

The hybrid between *H. x hybridus* (28.7 pg/2C) and *H. argutifolius* (18.3 pg/2C) was definitively verified by having a genome size equal to the average of both parents (Figure 3.21 A). An interspecific hybrid between *H. foetidus* (22.0 pg/2C) and *H. argutifolius* was also analysed by flow cytometry (Figure 3.21 B). The difference in nuclear DNA content between the parental species was 3.7 pg, which is much smaller than that between *H. x hybridus* and *H. argutifolius* (Figure 3.21 A). Therefore, the peaks in the histogram were much closer together. However, the peaks were separate from each other, enabling the hybrid to be definitively identified. All hybrids whose parents differed in nuclear DNA contents by less than 3.7 pg were not verified by flow cytometry and, instead, were analysed using molecular markers.



Peak no.	Plants	Counts	Mean	CV
1	*H. argutifolius* (father)	3706	61.84	2.6
2	Hybrid	5254	79.01	2.2
3	*H. x hybridus* (mother)	4810	96.29	2.3

Peak no.	Plants	Counts	Mean	CV
1	*H . argutifolius* (father)	5329	62.86	2.8
2	Hybrid	4424	69.00	2.7
3	*H. foetidus* (mother)	5321	76.17	2.5

Figure 3.21: Hybrid identification by flow cytometry.
Histograms show the nuclear DNA contents of the parental species and the hybrid represented by the peak position 2 in A: *H. x hybridus* x *H. argutifolius* and B: *H. foetidus* x *H. argutifolius*. All nuclei were isolated from leaf tissue and stained simultaneously.

3.3.5.2 Hybrid identification by RAPD analysis

For all putative hybrids, especially those whose parental species had similar nuclear DNA contents, RAPD markers were used for hybrid verification. All parental species were screened with 25 different RAPD primers. For some crosses between species belonging to section Helleborastrum, 12 additional primers were tested. Banding patterns were evaluated, and primers were selected for hybrid identification if at least one specific DNA fragment for each parental species could be identified.

One example of hybrid verification based on banding patterns is shown in Figure 3.22 for *H. x hybridus* x *H. argutifolius* and *H. foetidus* x *H. argutifolius.* Three characteristic paternal bands were found in the hybrid *H. x hybridus* x *H. argutifolius*. The two independent *H. foetidus* x *H. argutifolius* hybrids had different banding patterns; one had four and the other had three fragments from the paternal plant.

Another example of RAPD analysis for hybrids between *H. x hybridus* and *H. niger* is shown in Figure 3.23. Although different maternal and paternal genotypes were used, one characteristic paternal *H. niger* DNA fragment was amplified in all hybrid offspring for both primers used (Figure 3.23 A-B).

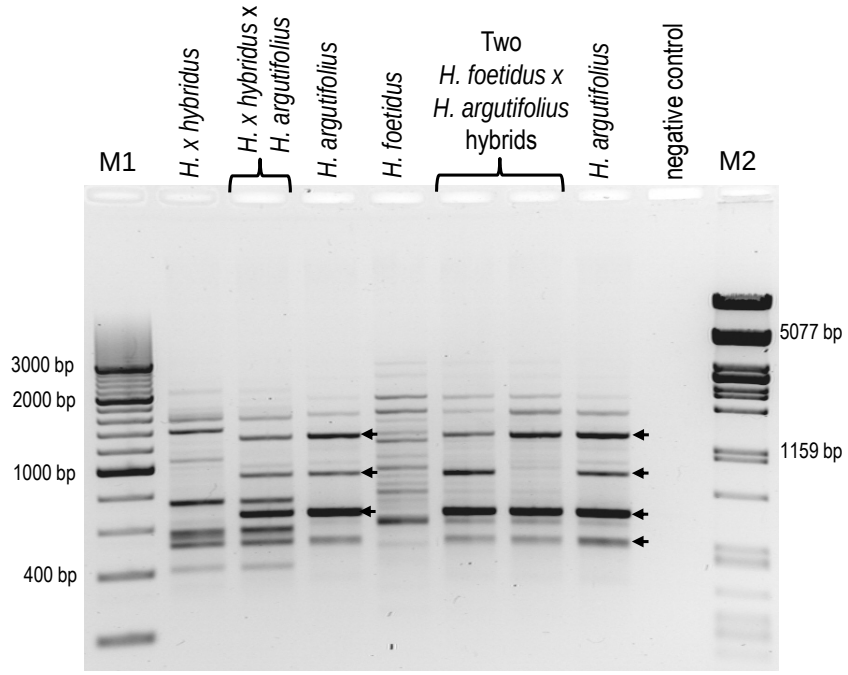


Figure 3.22: RAPD analysis banding pattern of one *H. x hybridus* x *H. argutifolius* and two *H. foetidus* x *H. argutifolius* hybrids and their parental species with primer B16.
M1: O'Range Ruler™ 200 bp DNA ladder (Fermentas), negative control = water, M2: λ/PstI DNA ladder. Characteristic pollen donor fragments are indicated with black arrows.

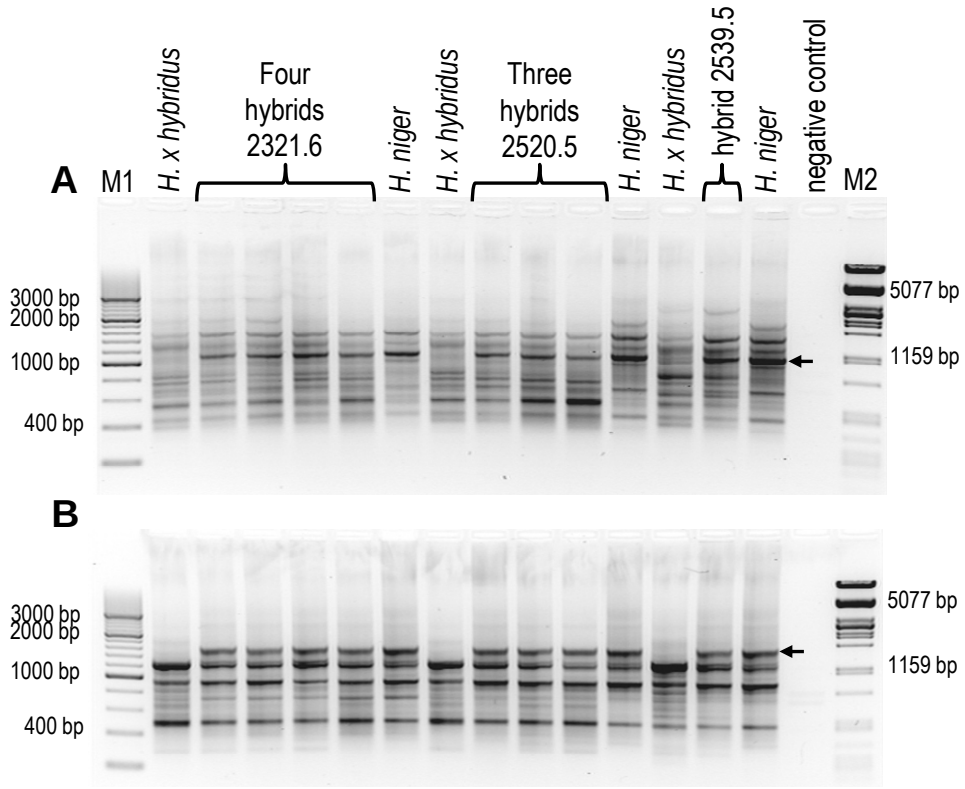


Figure 3.23: RAPD analysis banding pattern of eight *H. x hybridus* x *H. niger* hybrids and their parental genotypes.
A: primer A01, B: primer B10; M1: O'Range Ruler™ 200 bp DNA ladder (Fermentas); 2321.6, 2520.5 and 2539.5 are *H. x hybridus* x *H. niger* hybrids with different parental genotypes; negative control = water, M2: λ/PstI DNA ladder. Characteristic pollen donor fragments are indicated with black arrows.

Due to the higher genetic similarity of species belonging to section Helleborastrum compared to species from other sections, it was more difficult to identify primers that would amplify characteristic pollen donor fragments in putative hybrids between species in this section. The RAPD banding pattern of *H. x hybridus* x *H. odorus* offspring is shown in Figure 3.24. Two characteristic paternal bands that did not occur in the maternal DNA were amplified. Some offspring had both paternal bands, while others had only one. Thus, the hybrid banding patterns may look different even among hybrids between the same parental genotypes (Figure 3.24).

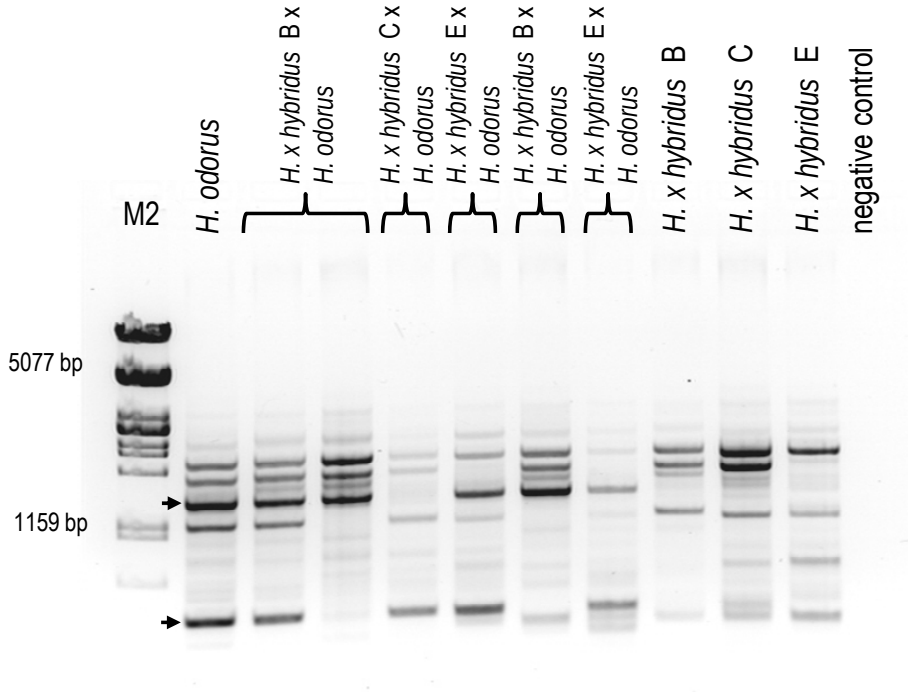


Figure 3.24: RAPD analysis banding pattern of six *H. x hybridus* x *H. odorus* hybrids and their parental genotypes with primer B 19.
M2: λ/PstI DNA ladder, negative control = water, *H. x hybridus* B, C and E represent different maternal genotypes. Characteristic pollen donor fragments are indicated with black arrows.

The DNA of the original maternal genotype was used for RAPD analysis, especially for offspring with *H. x hybridus* or *H. niger* as parental species, because the amplified banding patterns of different genotypes of these species differed slightly. The numbers of offspring tested, primers used, characteristics of the banding patterns and verified hybrids for each crossing combination are listed in Table 3.15.

Interspecific hybrids were successfully verified by RAPD analysis for 16 of the 18 different interspecific crossing combinations. Overall, 217 offspring were obtained, of which 157 were analysed using RAPD markers. All putative hybrids from crossing combinations for which shoot cultures were established were analysed using RAPD markers, except for the crossing

combinations *H. argutifolius* x *H. lividus*, *H. lividus* x *H. argutifolius*, *H. x hybridus* x *H. cyclophyllus* and *H. x hybridus* x *H. atrorubens*, of which fewer offspring than shoot cultures were tested for each combination due to the larger numbers of offspring. The offspring of *H. croaticus* x *H. multifidus* and *H. x hybridus* x *H. dumetorum* died before DNA could be isolated, so no hybrids were identified from these crosses. Additionally, some offspring of other combinations stopped growing and were discarded. DNA had already been isolated from some of these, but no DNA was available for others (Table 3.15).

Table 3.15: Hybrid verification of interspecific *Helleborus* hybrids by RAPD analysis.
The table is arranged according to genetic distances between the parental species from the lowest to the highest.

Genetic distance	Crossing combination	primers used	Number of…				
			total offspring[b]	offspring tested	possible paternal bands	Min/Max paternal bands in offspring[a]	Hybrids verified
0.069	*H. argutifolius x H. lividus*	B03	37 (2)	27	2	1/2	26
0.069	*H. lividus x H. argutifolius*	B03	60 (4)	38	2	1/2	37
0.081	*H. x hybridus x H. torquatus*	B13	6 (2)	4	1	1	4
0.082	*H. x hybridus x H. cyclophyllus*	B16	35 (1)	28	1-3[c]	1/3	28
0.093	*H. x hybridus x H. atrorubens*	B13	20 (2)	13	3	1/3	13
0.097	*H. croaticus x H. multifidus*	--	1 (1)	0			
0.105	*H. x hybridus x H. odorus*	B19	6 (0)	6	2	1/2	6
0.106	*H. torquatus x H. croaticus*	B13	2 (1)	1	1	1	1
0.112	*H. x hybridus x H. croaticus*	A12 B13	14 (6)	8	2 5	1/2 1/4	1 7
0.114	*H. x hybridus x H. multifidus*	B16	8 (2)	7	3	3	7
0.115	*H. croaticus x H. odorus*	C14	2	2	1	1	2
0.115	*H. odorus x H. croaticus*	B7	2 (1)	2	1	1	2
0.117	*H. x hybridus x H. dumetorum*	--	1 (1)	0			
0.130	*H. x hybridus x H. purpurascens*	A14	8	8	3	1/2	8
0.141	*H. purpurascens x H. croaticus*	B13	1	1	4	2	1
0.241	*H. foetidus x H. argutifolius*	B16	2	2	4	3/4	2
0.255	*H. x hybridus x H. argutifolius*	B16	1	1	3	3	1
0.264	*H. x hybridus x H. niger*	A02 B10	11	9	1 1	1 1	9

[a]minimum number of bands/maximum number of bands found in the offspring depending on each individual offspring genotype
[b]number of offspring, which died during development, is given in brackets
[c]depending on the maternal genotype

In summary, 155 offspring were successfully identified as hybrids. Identification failed for only one offspring each of the combinations *H. argutifolius* x *H. lividus* and *H. lividus* x *H. argutifolius*.

3.4 Discussion

3.4.1 Viability of fresh and stored pollen of different *Helleborus* species

The terms viability, stainability, vigour, fertility, germinability and fertilisation ability are all used to describe aspects of pollen functional ability, generally termed pollen viability (Dafni & Firmage, 2000). Therefore, it is important to know the context, in which the term pollen viability is used. Different methods can be used to assess pollen viability, but five essential approaches have been reviewed by Dafni & Firmage (2000): (1) measurement of respiration or chemical conductivity of pollen leachates; (2) staining techniques, either vital stains for the presence of cytoplasm or dyes that indicate enzyme activity; (3) *in vitro* or *in vivo* germination; (4) proline content; and (5) capacity to effect seed set. Germination tests and pollen staining techniques are often used in combination and have been assessed for their ability to measure pollen viability in different plant species (Rodriguez-Riano & Dafni, 2000; Trognitz, 1991).

Analysis of pollen viability by different methods

Two different viability tests were used to assess the viability of *Helleborus* pollen (Chapter 3.3.1). *In vitro* pollen germination was conducted for eight *Helleborus* species, but germination was extremely low when the tested pollen was dried. In parallel, MTT staining indicated pollen viability of 67 % to 99 % regardless of whether fresh or dried pollen was tested (Table 3.5). Due to the failure of *in vitro* germination to accurately assess pollen viability, MTT staining was used in subsequent analyses of the pollen viability of eleven *Helleborus* species and three hybrids (Table 3.7). All species examined had average pollen viabilities between 22 % and 95 % directly after anther removal and between 13 % and 93 % after 24 hours of drying at room temperature, depending on the species. Two hybrids, *H. x ballardiae* and *H. x ericsmithii*, were identified as pollen sterile, consistent with the observations of Mathew (1989), who has reported sterility in these hybrids.

Heslop-Harrison et al. (1984) have tested *in vitro* germinability and three different staining procedures with pollen from *H. niger* and other plant species: the fluorochromatic procedure (FCR) with fluorescein diacetate (FDA), lactophenol-acid fuchsin and tetrazolium chloride (TTC). FDA tests the integrity of the plasmalemma and esterase activity. All methods yielded viabilities between 90 % and 100 % for freshly released *H. niger* pollen. Pollen pre-treatment with DMSO reduced viabilities to between 30 % and 40 %, while pre-treatment with heat at

60 °C for 30 min resulted in 0 % viable pollen when estimated using FCR or *in vitro* germination, but 90 % to 100 % viable pollen when stained with lactophenol-acid fuchsin or TTC. Heslop-Harrison et al. (1984) concluded that FCR is excellent to predict potential germinability but is not useful to test pollen viability itself. Pollen may still be capable of functioning but may not be stained by FDA and may not be able to germinate without proper rehydration before testing (Heslop-Harrison et al., 1984). Furthermore, many environmental factors can affect pollen germination and pollen tube growth *in vitro*, including temperature, medium composition and especially the concentration of pollen grains on the medium, which is described as a population effect (Brewbaker & Kwak, 1963). Additionally, the pollen of some species is not amenable to *in vitro* germination at all (Stone et al., 1995). Heslop-Harrison et al. (1984) have demonstrated the proper *in vitro* germination of *H. niger* pollen. The failure of *in vitro* germination of *Helleborus* pollen in this study may be due to an inappropriate medium, incorrect pre-treatment procedures or an insufficient concentration of pollen grains. Anthers and pollen were limited in some species for which only one plant was available.

Several studies have reported conflicting results when using stainability and germinability in combination to determine the pollen viability of other plant species. On the one hand, pollen may exhibit reduced vigour (e.g., as estimated using stains) before it loses its ability to germinate, as has been demonstrated in *Nicotiana* (Shivanna et al., 1991). On the other hand, pollen viability assays based on enzyme activity may estimate high pollen viability because pollen may still contain active enzymes after having lost the ability to germinate (Dafni & Firmage, 2000). Pollen that has been classified dead may still be able to sire seed under natural conditions and vice versa (Dafni & Firmage, 2000). In conclusion, at least two different methods should be combined to evaluate pollen viability; if these methods produce conflicting results, then a third method is beneficial.

To explain the contrasting results of pollen viability assayed via *in vitro* germination and MTT staining in this study, a third method (via *in vivo* pollination) was tested. Pollen of the species *H. x hybridus*, *H. niger*, *H. purpurascens* and *H. torquatus* that had been stored for one year at -20 °C was used for intraspecific *in vivo* pollination (Chapter 3.3.1, Table 3.6). Pollen tubes reached the ovules, although the pollen viability determined by MTT was only 17 % for *H. niger*. In spite of their high viability, pollen tubes failed to reach the ovules in *H. foetidus* and *H. argutifolius*, possibly due to a lack of stigma receptivity or to a limited number of anthers resulting in insufficient pollen concentration on the stigma. MTT proved to

be applicable to determine pollen viability in *Helleborus*, as in other species (Rodriguez-Riano & Dafni, 2000; Trognitz, 1991), in contrast to *in vitro* germination, which failed to accurately assess pollen viability in this study. Following the *in vivo* germination experiment, MTT was chosen for all subsequent analyses of *Helleborus* pollen viability in this study.

Monitoring of pollen viability during storage

Pollen storage is a common and useful method for bridging temporal gaps between the flowering times of different species. Pollen viability during storage is mainly influenced by temperature and relative humidity. To identify suitable pollen storage conditions for the eleven *Helleborus* species, dried anthers were stored at 20 °C, 4 °C or -20 °C for four weeks, two months or six months (Chapter 3.3.1, Table 3.7). Pollen viability decreased rapidly when pollen was stored at 20 °C. After two months at 20 °C, viable pollen was found for only three of eleven species. The pollen of six species was viable after storage at 4 °C. When pollen was stored at -20 °C, pollen viability varied between 22 % and 86 % for all eleven species. Standard deviations between repeated measurements were quite large because only small numbers of anthers were stored. Smaller standard deviations were found in the second storage experiment, in which larger numbers of anthers from *H. argutifolius*, *H. x hybridus* and *H. niger* were dried and stored for nine months. The average viability observed in this second experiment was between 71 % and 98 % (Chapter 3.3.1, Figure 3.6).

In strawberries, genotypic differences in pollen viability have been determined during pollen storage at -18 °C over 12 months (Zebrowska, 1995). Depending on the viability assay and the genotype, pollen viability varied from 8 % to 81 %. For papaya, a pollen viability of about 30 % has been determined via *in vitro* germination after nine months of storage at -18 °C (Cohen et al., 1989).

To reduce humidity, pollen is often dried in a desiccator prior to storage. The negative effect of high relative humidity on pollen viability during storage has been demonstrated for papaya pollen (Cohen et al., 1989) and for *Papaver*, *Narcissus* and *Typha*, for which pollen viability and membrane integrity degraded more rapidly at 75 % than at 40 % relative humidity (Van Bilsen et al., 1994).

The results mentioned above are consistent with those obtained for *Helleborus* in this study, in which -20 °C was found to be best for long-term pollen storage. Desiccation of anthers was performed at room temperature and was necessary to prevent moulding during storage.

Using FCR, Vesprini et al. (2002) have found stable pollen viabilities between 80 % and 90 % for *H. bocconei* and *H. foetidus* pollen stored for 72 hours under temperatures fluctuating between 0 °C and 18 °C. Even at temperatures around -4 °C, pollen viability remained high for both species due to the conversion of cytoplasmic polysaccharides to mono- and disaccharides, a mechanism to sustain viability during pollen presentation at low temperatures (Vesprini et al., 2002). The results for *H. foetidus* and *H. bocconei* (Vesprini et al., 2002) are comparable to those obtained in this study, in which *Helleborus* pollen viabilities remained high during long-term storage. Additionally, the stable viability of *H. bocconei* and *H. foetidus* pollen during temperature fluctuations between 0 °C and 18 °C (Vesprini et al., 2002) indicates that anther drying at room temperature may not affect pollen viability negatively.

In summary, pollen grains obtained from eleven *Helleborus* species remained viable after long-term storage for six months (after 12 months in some species) when stored at -20 °C in a desiccated state. Pollen staining via MTT was an effective method to determine pollen viability for *Helleborus* species.

3.4.2 Pollen tube growth *in situ*

Even when pollen is viable and capable of functioning in the sense of effecting fertilisation, several additional factors influence successful pollination. Pollen lands on the stigmatic surface, adheres, hydrates, germinates and grows through the pistil to the ovules in the ovary. In the ovules, one sperm cell fuses with the egg cell to produce the zygote. Reproductive barriers may act at different stages from pollination to fertilisation. To obtain deeper insight into this process, important aspects of pollen adhesion, hydration and germination as well as pollen tube growth and guidance are summarised below.

Pollen adhesion and hydration

Initiation of pollination depends on the ability of the pollen grain to adhere to the stigmatic surface, which varies morphologically and in the presence or absence of exudates, which are important for adhesion in many species (Wheeler et al., 2001). Stigmas are classified as wet (e.g., *Solanaceae*) or dry (e.g., *Brassicaceae*) (Lord & Russell, 2002). The control of pollen acceptance by adhesion is more important for dry stigmatic surfaces than for wet stigmatic surfaces. Generally, the stigmas of the *Ranunculaceae* family are dry. In *H. foetidus* and *H. niger,* in particular, the stigmas are dry with unicellular papillae (Schill et al, 1985).

Therefore, only the mechanisms known from *Brassicaceae* and other plants with dry stigmas are considered further here.

In addition to the stigma, the pollen coat is essential for adhesion to the stigmatic surface. It contains many molecules that are involved in initial interactions with the stigma, preparing the way for adhesion, hydration and germination (Lord, 2003). Even isolated pollen coatings induce physiological changes on the stigmatic surface (Elleman & Dickinson, 1996). In *Arabidopsis thaliana*, stigmas bind conspecific pollen with much higher affinity than pollen from related species; this interaction occurs within seconds after pollination (Zinkl et al., 1999). Proteins involved in the recognition process on the stigmatic surface have been identified from self-incompatibility reactions, in which self pollen is actively rejected (Lord & Russell, 2002). In *Brassicaceae*, stigmatic molecules, e.g., SLR (S-locus related protein) and SLG (S-locus glycoprotein), as well as pollen coat molecules, e.g., the male determinant of self-incompatibility (a small cysteine-rich protein (SCR)) are involved in the adhesion of the pollen grain to the stigma (Lord, 2003). Studies in *Arabidopsis* suggest that adhesion is under polygenic control (Preuss et al., 1993).

Following adhesion, successful pollen tube growth depends upon the hydration of the pollen grain. Pollen hydration is regulated by controlling water flow from the stigma to the grain. Stigma exudates containing long-chain lipids act as signals to stimulate pollen hydration. In *Brassica*, a plasma membrane-localised aquaporin-like protein in the stigma may act as a water channel (Dixit et al., 2001). The diversity of stigmatic surfaces within plant families suggests that hydration mechanisms are likely to be divergent, although the results of hydration seem to be similar (Heslop-Harrison & Heslop-Harrison, 1992). In the pollen coat, glycine-rich proteins (GRPs), e.g., oleosin, may play a role in hydration (Lord & Russell, 2002; Lord, 2003). The complex interaction between the pollen and the stigmatic surface concerning adhesion or hydration may act as a prezygotic barrier, if it is not successful.

Pollen germination

After hydration, the pollen germinates. In this process, flavonols may function as signal molecules in some species (Taylor & Hepler, 1997). The pollen tube always enters a specialised extracellular matrix that is usually a combination of pollen coat secretions and stigma exudates (Lord & Russell, 2002). Pollen germination is known to be density dependent, and peptides responsible for promoting germination have been identified (Lord & Russell, 2002).

The initial penetration of the stigmatic surface varies considerably among species, stigmas and styles. The adhesion of the pollen grain to the stigmatic papillae determines the point of entry; enzymes probably facilitate the entrance of the pollen tube into the transmitting tract of the style (Lord, 2003). In solid styles with dry stigmas, pollen tubes must penetrate the cuticle, the outer lipidic cover of the stigmatic papillae. The initial penetration appears to vary among several species with dry stigmas. In the *Asteraceae*, for example, the pollen tubes appear to grow extracellularly until they reach the base of the papillae; in *Papaver rhoeas*, the pollen tube grows underneath the cuticle to the base of the papillar cell (Wheeler et al., 2001). When pollen tubes penetrate the stigmatic papillae, an increase in calcium secretion occurs at the site of pollen adhesion on the papillar surface in *Brassica* (Elleman & Dickinson, 1999). Calcium is assumed to play a key role in mediating pollen tube growth in the pistil (Reger et al., 1992); the calcium ion is necessary during pollen-stigma interactions, for pollen tube tip growth and for pollen tube directionality (Reger et al., 1992; Hepler et al., 2006). Additionally, calcium is involved in multiple roles during plant fertilisation (Hepler et al., 2006). For example, high concentrations of calcium released from the synergid vacuole during degeneration might induce gamete fusion (Punwani & Drews, 2008).

After penetration of the stigma, the pollen tube grows through the style towards the ovule. The pollen tube carries the sperm cells, which are endocytosed into the larger tube cell. The pollen tube grows by tip growth through the extracellular matrix of the transmitting tract of the pistil. The total volume of the pollen tube increases, but the cytoplasmic volume stays almost constant by periodic deposition of callose plugs (Taylor & Hepler, 1997). A variety of ions and proteins are involved in pollen tube growth (Hepler et al., 2006; Malhó et al., 2006). For example, ROP (Rho-related GTPase from plants) proteins are involved in pollen tube growth in *Arabidopsis* (Kumar & McClure, 2010). The stigma and style are organs specialised for mate selection and have complementary roles in secreting material to support the growth of compatible pollen tubes and discourage the growth of undesired pollen. After pollen germination, in particular, interactions between pollen tubes and the style tissue may act as reproductive barriers even for interspecific crosses.

Pollen tube guidance

The mechanism whereby the pistil supports the growth of the pollen tube from the stigma to the target embryo sac is called pollen tube guidance. Sporophytic pollen tube guidance describes growth from the stigma to the base of the style supported by the sporophytic cells of the pistil. Some candidate substances for sporophytic pollen tube guidance have been

identified. In the styles of *Nicotiana tabacum*, TTS (transmitting-tissue specific) proteins, which are arabinogalactan proteins, have been suggested to be involved in pollen tube guidance (Higashiyama & Hamamura, 2008). A stigma-stylar cysteine-rich adhesin (SCA) is expressed in the styles and pistils of lilies and is assumed to be involved in pollen tube adhesion in the style (Lord & Russell, 2002). In *Arabidopsis*, plantacyanin, which is expressed in the style, and an appropriate water gradient on the stigma are assumed to guide the pollen tubes, perhaps in combination with other chemoattractants (Higashiyama & Hamamura, 2008). GABA (γ-aminobutyric acid), which forms a concentration gradient in the pistil and is most concentrated at the inner integument of the ovule, may also be a sporophytic guidance candidate (Palanivelu et al., 2003).

During gametophytic guidance, the pollen tube is conveyed by the gametophyte. The pollen tube is the male gametophyte, and pollen specific glycoproteins in the pollen tube wall and receptor kinases in the pollen tube plasma membrane have been described as candidates for pollen/pistil interactions during pollen tube growth in the style (Lord & Russell, 2002). In summary, the growth of pollen tubes in the pistil is guided by both the female sporophyte and the male gametophyte.

Other than the male gametophyte, the female gametophyte plays the most important role in pollen tube guidance because it conveys the pollen tube to the ovule. A specific signal from the synergid cells navigates pollen tubes in close proximity to the embryo sac. The participation of the synergid cell in pollen tube attraction has been confirmed in *Torenia fournieri* (Higashiyama et al., 2001) and *Arabidopsis* (Kasahara et al., 2005). In *Arabidopsis in vitro* systems, pollen tubes that had grown within 100 μm of an unfertilised ovule turned sharply towards the ovule (Palanivelu & Preuss, 2006). Before pollen tubes gain the competence to respond to the synergid chemical signal, they require contact with the pistil during pollen tube growth in the style (Lord & Russell, 2002). Additionally, the attractant from the synergid cells is species-preferential, meaning that each species uses a different molecule or combination of substances and/or different concentrations (Higashiyama et al., 2006). In *Torenia*, most embryo sacs had received pollen tubes from interspecific crosses at a considerable time after pollination (e.g., a few days). In intergenic crosses, some embryo sacs of *Lindernia* had received pollen tubes from *T. fournieri*, suggesting that fertilisation was delayed in these crosses (Higashiyama et al., 2006). Generally, the species preferentiality of the attraction signal from the synergid cell acts as a reproductive barrier in interspecific hybridisation.

Recently, two cysteine-rich polypeptides (CRPs) in a subgroup of defensin-like proteins called LUREs, which are derived from the synergid cell, have been identified as competent to attract pollen tubes in *Torenia* (Okuda et al., 2009). Guided by the synergid cell attractant, the pollen tube enters the embryo sac through the micropyle. In the ovule, the pollen tube growth must be arrested, a process in which the FER protein (FERONIA receptor-like kinase), which is expressed by the synergids, is involved (Escobar-Restrepo et al., 2007). This process may also act as reproductive barrier. The pollen tube ruptures and releases the sperm cells, one of which fuses with the egg cell to produce the zygote while the other fuses with the central cell to generate the endosperm, which supplies nutrition to the embryo. Seed development is initiated by this double fertilisation and requires the development of the seed coat, embryo and endosperm. All reproductive barriers that may occur up to the point of fertilisation are referred to as prezygotic.

Pollen tube growth in *Helleborus* species

Interspecific incompatibility is not as well understood as self-incompatibility, but it is thought that related mechanisms are involved. The results of both are similar: pollen adhesion, hydration, germination and/or pollen tube growth is inhibited.

Helleborus flowers are protogynous and therefore prevent self-pollination, but they are self-compatible. In intra- and interspecific crosses, pollen tube growth was observed from 12 to 72 hours after pollination, and pollen tubes reached the ovules after 72 hours for pollinations with con- and heterospecific pollen in this study (Chapter 3.3.2). It was not possible to quantify pollen tubes, but relatively speaking, higher frequencies of pollen tubes were observed in crosses with conspecific pollen compared to crosses with heterospecific pollen, as has been demonstrated in *Trifolium* (Chen & Gibson, 1972). However, no abnormal pollen tube growth occurred in *Helleborus*.

Pollen adhesion, germination and penetration into the style as well as pollen tube growth were evaluated by staining with aniline blue, focusing on nine different crossing combinations between the species *H. foetidus*, *H. x hybridus*, *H. niger* and *H. odorus*, with parental genetic distances ranging from 0.257 to 0.264 (Chapter 3.3.2, Table 3.8). In all crosses, pollen tubes were observed near the ovules in at least one carpel. In crosses between *H. niger* and *H. x hybridus* pollen tubes reached the ovules in 87 % and 94 % of the analysed carpels, suggesting that fertilisation can occur. In crosses with *H. niger* as the maternal or paternal parent, pollen tubes reached the ovules in 28 % to 100 % of the analysed carpels.

Interestingly, pollen adhered to only on 36 of 45 stigmas in *H. foetidus*. Germination was observed on 34 of these stigmas, and pollen tubes reached the ovules in 18 of them. Some kind of barrier may be assumed to exist in this crossing combination. In general, crosses with *H. foetidus* as the maternal parent or pollen donor seemed to be problematic. In crosses between *H. x hybridus* and *H. foetidus*, pollen tubes were observed near the ovules in only 1 % and 4 % of the analysed carpels. For *H. x hybridus* x *H. foetidus*, pollen adhered to 80 of 91 stigmas, and pollen germinated and grew inside the style in 56 of these. For the reciprocal cross, pollen adhered to only 37 of 49 stigmas, and pollen germinated and grew inside the style in 21.

In summary, some problems with pollen adhesion occur in combinations of *H. foetidus* and *H. x hybridus* and in the majority of carpels of with germinated pollen, pollen tubes stopped growing in the style suggesting that an interaction between the pollen tube and the style tissue inhibits further growth. The failure of pollen to adhere to the stigma in crosses involving *H. foetidus* may also be a result of differences in pollen size because a possible correlation between pollen size or shape and stigma morphology has been suggested for *Helleborus* species (Nowicke & Svarla, 1983). The pollen of *H. foetidus* is smaller than that of *H. x hybridus* and *H. niger*. Thus, *H. x hybridus* and *H. niger* pollen may have difficulty adhering to the stigmatic surface of *H. foetidus* if it does not fit between the papillae and vice versa. In conclusion, prezygotic barriers are likely to exist between *H. foetidus* and *H. x hybridus* and some type of mate selection takes place.

Nevertheless, pollen tubes reaching the ovules were observed, albeit to a lesser extent in certain combinations. In some cases, ovule fertilisation was observed within *Helleborus* ovaries, but such observations were strongly dependent on the preparation of the carpel for microscopic analysis. Therefore, fertilisation could not be evaluated by this method.

It is possible that pollen tube guidance by the female gametophyte may have failed due to species preferentiality of the signal, as reported for *Torenia* (Higashiyama et al., 2006). In that case, the pollen tubes might reach the ovules, but fertilisation would fail due to the lack of guiding signals from the synergid cells. However, an argument against this possibility is the fact that several *Helleborus* hybrids already exist or were obtained in this study.

In conclusion, the results described above and observations of 35 additional crosses (Chapter 3.3.2, Table 3.9) show that in the majority of analysed carpels, pollen tubes reached the ovules and were usually observed in close proximity to the ovule, suggesting that fertilisation

can occur. It may be concluded that the crossing barriers between these *Helleborus* species are mainly postzygotic. Further details concerning gametophytic pollen tube guidance and fertilisation are discussed together with the embryo rescue results in the next chapter.

3.4.3 Embryo rescue to overcome postzygotic hybridisation barriers

Based on the evaluation of *in situ* pollen tube growth, fertilisation and embryo development were assumed to occur. On the basis of the information concerning the failure of hybridisation attempts between certain species combinations (Mathew, 1989; Oenings, personal communication), postfertilisation barriers were inferred to prevent zygote development, leading to embryo abortion. Therefore, the embryo rescue technique was chosen as a technique to overcome postzygotic crossing barriers. Embryo rescue means the isolation of complete ovaries, ovules or embryos before embryo abortion and their subsequent culturing *in vitro* on a medium (Winkelmann et al., 2010). An embryo rescue technique involving ovule culture was used for interspecific crosses within the genus *Helleborus*.

3.4.3.1 Selection of date after pollination for ovule isolation

Overall, five embryo rescue experiments were performed within three years (Chapters 3.3.3, 3.3.4, Table 3.3). Two experiments were carried out during the first flowering season in 2007/2008. In these experiments, the dates after pollination that were most suitable for ovule isolation and the optimal culture conditions were identified.

In experiment 1, ovules from intraspecific crosses of four species were used to compare different culture conditions and to identify suitable dates for ovule isolation after pollination (Chapters 3.3.3). The advantage of the use of intraspecific crosses was that normal embryo development could be assumed. Even in intraspecific crosses, 79 % of ovules on average across all species were aborted when ovules were isolated three weeks after pollination. After four weeks, the percentage of aborted ovules was only 46 %. This percentage was 42 % after five weeks and 41 % after six weeks. Isolation of the ovules after three or four weeks was too early, assuming that most of them were fertilised and able to develop.

Although experiment 2 showed that ovule abortion was 87 % on average (80 % to 91 % depending on the ovule isolation date) across all crossing combinations for ovules from interspecific crosses (Chapter 3.3.4), ovule isolation dates of five to seven weeks after pollination were chosen for the experiments 3 and 4 in 2008/2009. After five to seven weeks, embryo development was assumed to be sufficient for survival and embryos were not yet aborted.

Ovule isolation dates of four, six or eight weeks after pollination have been tested in intraspecific crosses of *Tulipa gesneriana* (Van Creij et al., 2000). The germination percentages increased with increasing ovule ages. *Tulipa* usually requires 13 weeks for seed maturation, which is similar to the seed maturation time in the plant in *Helleborus*. Immature seeds from selfed lentils have been isolated 15 to 25 days after pollination (Fratini & Ruiz, 2006), and the percentages of shoot formation increased with ovule age, a result similar to that observed in *Tulipa*. For *T. gesneriana* x *T. kaufmanniana* crosses, ovules were isolated from three to 13 weeks after pollination. Seedlings were produced only from five weeks onward, and seven to nine weeks after pollination was best (Custers et al., 1995). The results obtained in the experiments with *Tulipa* are similar to those obtained with *Helleborus*, in which most of the shoots obtained developed from ovules that were isolated five or more weeks after pollination. Comparable results have also been obtained for crosses between *Lilium* spp. (Ikeda et al., 2003). In these crosses, more plants developed when ovules were isolated five or ten days after pollination than when they were isolated three days after pollination.

In conclusion, the ovule isolation date is various for each plant species due to different durations that are needed for seed maturation in the plant. Therefore, it has to be determined for the regarded plant species, in particular.

3.4.3.2 Influence of sucrose concentration in the medium

In addition to the influence of ovule age, the effect of sucrose concentration in the culture medium was tested in both experiments in 2007/2008 (Chapters 3.3.3, 3.3.4, Table 3.3). Media supplemented with 2.5 % or 5 % sucrose were compared, and no differences in ovule development or germination percentage were observed.

In *Lilium* spp., differences in basal media and sucrose concentrations affected the development of ovules isolated three or five days after pollination but did not affect the development of ovules isolated ten days after pollination (Ikeda et al., 2003). The effects of sucrose concentration also depended on ovule age in *Alstroemeria* (Buitendijk et al., 1995), for which 6 % to 12 % sucrose were suitable for seven and 14-day-old ovules, while 4 % to 8 % sucrose was best for 21-day-old ovules. For interspecific *Tulipa* crosses, three different sucrose concentrations (3 %, 6 % or 9 %) have been tested; 6 % resulted in slightly higher germination percentages than 3 % or 9 %, but this effect is independent of ovule age (Van Creij et al., 2000). For immature selfed lentil seeds, lower concentrations were optimal; 1 % sucrose was more suitable than 2 % or 3 % sucrose (Fratini & Ruiz, 2006).

According to Sharma et al. (1996), sucrose is the most commonly used carbon source for embryo culture. Immature embryos require higher osmotic strength of the medium compared to mature ones. In addition, a high osmotic concentration of the medium is assumed to prevent precocious germination of young embryos and to support normal embryonic growth. Therefore, sucrose concentrations show a wide range from 0.5 % to 18 % in embryo rescue culture media, depending on the species. For *Helleborus*, however, no differences were found between media containing 2.5 % and 5 %, even for ovules of different ages, suggesting that either the sucrose concentration has no effect or that other concentrations should be tested.

3.4.3.3 Influence of temperature during ovule culture

In addition to ovule age and sucrose concentration, different temperatures during embryo culture were tested for intraspecific *Helleborus* crosses (Chapter 3.3.3). The temperatures used for *Helleborus* ovules in this study were chosen according to Niimi et al. (2006), who have determined the stages of embryo development in *H. niger* seeds under different dormancy breaking temperature treatments. At constant temperatures of 15 °C or 25 °C for 24 weeks, embryos developed into the torpedo or cotyledon stage but failed to germinate. Alternating temperatures of 15 °C or 25 °C for eight weeks followed by 4 °C for 16 weeks, or 15 °C for 16 weeks and 4 °C for eight weeks, resulted in the highest germination percentage for *H. niger* seeds (Niimi et al., 2006). Warm temperatures are assumed to encourage embryo development, while cold temperatures are needed to break morphophysiological dormancy (Chapter 1.2). Alternating temperatures were also more successful than constant temperatures promoting the germination of *Helleborus* ovules from intraspecific crosses. The temperature sequence from 20 °C to 4 °C and back to 20 °C was chosen for subsequent embryo rescue experiments in combination with ovule isolation dates of five to seven weeks after pollination and ovule culture on a medium supplemented with 2.5 % sucrose.

Embryo rescue experiments 3 and 4 (2008/2009) used ovules from several interspecific crosses (Chapter 3.2.3, Table 3.1). The majority of ovules went through two cold treatments at 4 °C for twelve weeks with temperatures of around 20 °C in between. Over all interspecific crossing combinations, 214 ovules that had received at least one out of two cold treatments germinated after around 60 weeks of culture.

3.4.3.4 Stages of embryo abortion

After 12 weeks of ovule culture, ovules were classified in aborted and not aborted (Chapter 3.3.4). Aborted ovules were discarded. Even after 60 weeks, many ovules were not aborted and remained in good condition. Therefore, all remaining ovules were dissected and evaluated for the presence or absence and consistency of the endosperm and for the presence or absence and developmental stage of embryos. Of all *Helleborus* ovules dissected, no embryos were found in 93 % of all dissected ovules. Only 11 % of the dissected ovules had solid endosperm and 7 % had embryos (Chapter 3.3.4). Embryo abortion must have occurred at a later stage in these ovules.

Embryo abortion in *Ipomoea* has been classified into two types (Mont et al., 1993): (i) early abortion, which occurred at the pre-globular stage or earlier, where the embryo was not visible; and (ii) late abortion, which occurred after the pre-globular stage, where the aborted embryo was visible and the detachment of the suspensor from the nucellus and/or degeneration of the embryo can be seen. If the embryo was aborted early, then the integuments did not develop further. If the embryo reached the globular stage, then fruit maturation began in parallel with seed coat development. When the embryo was aborted late, the integuments were almost completely developed, like a normal seed coat. Nutritional barriers due to deficient endosperm tissue development by unbalanced chromosomal sets and deficient suspensor development have been discussed as the main causes for embryo abortion in interspecific hybrids of *Ipomoea* (Mont et al., 1993).

Two possible abortion levels may also occur in *Helleborus*. Ovules that were shrivelled and dry 12 weeks after culture initiation were aborted early, whereas those that remained in good condition after 60 to 67 weeks of culture were aborted late. This explanation would be feasible for the presence of seeds or ovules that were classified as in good condition but that did not germinate and turned out to be empty or to contain liquid endosperm. The development of the integument and later the seed coat also indicated that egg fertilisation must have occurred; otherwise, the ovule would not need to grow during ovule culture, as it did in *Helleborus*. The pollen tube guidance signal produced by the female gametophyte may not be suitable due to species preferentiality when different species are crossed, but this barrier does not completely inhibit fertilisation in *Helleborus*. It remains difficult to determine the causes of embryo abortion in *Helleborus*.

Empty seeds, seeds containing liquid, viscous or solid endosperm and seeds containing endosperm and embryo have also been found in interspecific crosses of *Actinidia* species

(Hirsch et al., 2001). The percentages of empty seeds and seeds without embryos were higher in crosses between species from different sections than in crosses within sections at the same ploidy level (Hirsch et al., 2001). The seed classification for *Actinidia* was consistent with that of *Helleborus* ovules, which were classified as empty or containing liquid or solid endosperm. For both *Helleborus* and *Actinidia*, no embryos were found in ovules containing liquid endosperm. The endosperm must be solid in *Helleborus* and either viscous or solid in *Actinidia* to produce embryos. For *Actinidia*, the seed dissection date was selected to correspond to seeds in which the embryos had reached the torpedo developmental stage, because it was not possible to recover plantlets from earlier stages. In *Helleborus*, the predominant embryo developmental stage after 60 to 67 weeks of culture was heart-shaped, suggesting that dormancy was involved or that the embryos were somehow inhibited.

In persimmon, the predominant embryo developmental stage was globular regardless of the embryo isolation time (from 40 to 150 days after pollination), but embryo viability was best, if embryos were isolated from 60 to 80 days after pollination (Leng & Yamamura, 2006). Although persimmon embryos did not differ in developmental stage, the isolation date affected subsequent development. This observation is comparable to the results for *Helleborus* ovule dissection, but no conclusions can be drawn relating to the embryological stage at ovule isolation.

In *Phaseolus*, embryo abortion occurred at different developmental stages, from globular to early cotyledon, depending on the maternal parent (Ndoutoumou et al., 2007). The use of *P. coccineus* as the maternal species resulted in higher rates of embryo abortion than those observed in the reciprocal crosses. In *Phaseolus,* embryo development was slower in interspecific crosses than in self-pollinations. Deficient endosperm development and in some cases hypertrophy of the suspensor have been discussed as the causes of embryo abortion in *Phaseolus*.

3.4.3.5 Embryo abortion due to disturbance of endosperm development

Seed abortion in interspecific crossings has been studied in *Arabidopsis* (Bushell et al., 2003). Three possible explanations have been given for seed abortion: (i) allelic incongruity (negative interactions among the products of divergent gene sequences); (ii) genome shock (widespread preprogrammed changes to genomic structure or gene expression); and (iii) parental imprinting (due to ploidy imbalance or divergent expression patterns of imprinted genes or both). According to Bushell et al. (2003), allelic incongruity and genome shock can explain the failure of hybridisation at any stage of development from zygote formation

onward, while parental imprinting can account only for failure at the stage at which imprinting occurs, which is mainly endosperm development in flowering plants. Parental imprinting can be briefly defined as the process by which a gene comes to be expressed differently in an individual depending on whether the gene is derived from the individual's mother or father (Haig & Westoby, 1991).

How is imprinting involved in endosperm development? During the fertilisation process, one sperm cell fuses with the egg cell to form a zygote, while the other sperm cell unites with the two polar nuclei to form the triploid endosperm. The nuclear genomes of the embryo and endosperm are identical except that the endosperm has two doses of maternal genes for every dose of paternal genes (Haig & Westoby, 1991). As a consequence, the female parent may have a more important role in determining the characteristics of this nutrient source (Roach & Wulff, 1987). Maternal and paternal genomes have different gene expression patterns in the endosperm, and both are required for normal development. The parental genomic ratio that is usually required for normal endosperm development is 2m:1p (a nuclear ratio of two maternal (m) genomes to one paternal (p) genome). The endosperm functions as an intermediary in the transfer of nutrients from maternal tissues to the embryo. The success or failure of crosses between species at the same ploidy level results in a regular parental genomic ratio of 2m:1p, but the balance between maternal and paternal genomes may be disturbed in crosses between species. During endosperm development, paternally derived genes might act to increase the final number of endosperm cells, and maternally derived genes might counteract this tendency (Haig & Westoby, 1991). The terms 'maternal excess' or 'paternal excess' are usually used when parental genome ratios are 4m:2p or 2m:2p, for example. When these terms are applied to crosses between species at the same ploidy level, the endosperm may show 'maternal excess' in the relations between the expression of genes at some loci and 'paternal excess' in the interactions between other loci (Haig & Westoby, 1991). The complementary pattern would be predicted in the reciprocal cross.

Endosperm breakdown is the major cause of seed failure in interspecific crosses after successful fertilisation has occurred, while embryo death is only a secondary effect (Haig & Westoby, 1991). The failure of endosperm development in ovules from interspecific crosses has been observed in several genera (Buitendijk et al., 1995; Ikeda et al., 2003; Liu et al., 2006). In ovules from crosses between *Lilium* spp., ovule enlargement has been observed 14 weeks after culture initiation (Ikeda et al., 2003). Enlarged ovules contained milky-white endosperm and embryos, but not all expanded ovules contained visible endosperm and

embryos. Ovules containing liquid endosperm have also been observed in *Leucadendron* (Liu et al., 2006). Furthermore, mechanisms leading to endosperm breakdown and disturbed embryo development have been described in *Alstroemeria* (Buitendijk et al., 1995). In ovules from interspecific *Alstroemeria* crosses, embryo and endosperm development that was comparable to ovule development in self-pollinated flowers took place from four to 14 days after pollination (Buitendijk et al., 1995). *Alstroemeria* seeds usually require 56 days to mature (Buitendijk et al., 1995). Between 14 and 18 days after pollination, endosperm development began to stagnate due to failure of cellularisation. From 18 days after pollination onward, ovule development was retarded (based on ovule size), and embryo degeneration occurred due to the failure of endosperm development (Buitendijk et al., 1995).

Compared to *Lilium*, *Leucadendron* and *Alstroemeria,* the majority of *Helleborus* ovules dissected in this study after 60 to 67 weeks of culture were either empty (31 %) or contained liquid endosperm (58 %) (Chapter 3.3.4). For ovules that appeared to be in good condition but that were empty or contained liquid endosperm, endosperm breakdown may be an explanation for embryo abortion. The *Helleborus* crosses involved species at the same ploidy level. Therefore, the parental genome ratio was assumed to be 2m:1p and normal endosperm development was expected. Nevertheless, parental imprinting, which might result in divergent expression patterns of imprinted genes, is the most plausible explanation for the failure of endosperm development in *Helleborus*. Whether or to what extent maternal effects are involved in parental imprinting in ovules from interspecific *Helleborus* crosses is not known. As a consequence of endosperm degeneration, embryos were aborted due to lack of nutrients.

In addition to parental imprinting, allelic incongruity or genome shock are possible explanations for ovule abortion in *Helleborus* crosses, especially at later developmental stages.

3.4.3.6 Culture of rescued embryos

The isolated *Helleborus* embryos were transferred to *in vitro* culture medium. Six of 136 embryos developed into plantlets within the next three months (Chapter 3.3.4, Table 3.12). The remaining embryos, most of which were at the heart-shaped stage, did not develop at all. In *Actinidia* crosses, normal embryo development has been described for embryos that had reached at least the torpedo stage at the time of embryo isolation (Hirsch et al., 2001). Similarly, the isolated *Helleborus* embryos also may have been too young or the culture medium may not have been suitable. Sharma et al. (1996) have stated that embryo requirements related to medium composition depend on the developmental stage. In addition,

the failure to develop further may have been due to dormancy. Interestingly, the three intersectional hybrids between *H. x hybridus* and *H. argutifolius* and between *H. foetidus* and *H. argutifolius* from embryo rescue experiment 2 developed from isolated embryos. The main difference between the experiments performed in different years was that in experiment 2 (2007/2008), embryos were isolated after 14 to 18 weeks of culture at warm temperatures, while in experiments 3 and 4 (2008/2009), embryos were excised after two cold storage periods and an overall culture period of 60 to 67 weeks. Inhibition of embryo development may be less intense if the embryo is separated from the ovule at an earlier stage. To draw further conclusions, it would be helpful to know the timing of ovule development in interspecific crosses after isolation from the carpels to determine the time, at which the embryo and endosperm become visible. Detailed observations of embryo and endosperm development under different temperature treatments are needed.

3.4.3.7 Selective postfertilisation ovule abortion

In addition to ovule abortion at a later stage during ovule culture, ovule abortion may be initiated by the maternal plant. The plant itself influences fertilisation and postfertilisation behaviour and may affect embryo rescue.

Without doubt, the parental plants must be healthy and well nourished. After fertilisation, *Helleborus* sepals turn green and become photosynthetically active, a process in which gibberellins (Ayele at al., 2010) and cytokinins (Salopek-Sondi et al., 2002), which may also affect seed development, are involved. Hellebore flowers differ in carpel number and ovule number within carpels and also in flower number at a given point in time or over the whole flowering season. Recently, within-carpel and among-carpel competition during seed development has been investigated in *H. foetidus* (Parra & Sánchez-Lafuente, 2010). After fertilisation, ovules within carpels, among carpels and among flowers on the same plant compete for maternal resources. According to Parra & Sánchez-Lafuente (2010), ovules in the upper part of the carpel are less likely to mature than basal ones, and if they do mature, they are lighter in mass. The abortion of upper ovules increases with carpel number. Selective postfertilisation ovule abortion may be due to sibling rivalry or maternal control of resource investment in the offspring with the highest potential fitness (Parra & Sánchez-Lafuente, 2010).

In summary, in the context of *Helleborus* embryo rescue, postfertilisation ovule selection might result in a reduced number of viable ovules due to competition for resources or might be a mechanism that aims to promote the production of more successful offspring. It would be

interesting to determine whether the number of pollinated flowers per plant is positively correlated with seed abortion. In any case, the importance of the conditions during plant culture and ovule maturation should not be underestimated.

3.4.4 Interspecific *Helleborus* hybrids from embryo rescue

The embryo rescue technique was successfully used to overcome postzygotic crossing barriers between *Helleborus* species in this study. A total of 217 hybrids were obtained, of which 203 were from interspecific crosses within sections (Chapter 3.2.5.2). Crosses between the two species from section Chenopus, *H. argutifolius* and *H. lividus*, resulted in the largest number of offspring (97). Within section Helleborastrum (the largest *Helleborus* section, comprising 16 species), 106 hybrids were generated, 98 of which had *H. x hybridus* as the maternal parent. In addition to the intrasectional hybrids, 14 hybrids of parental species belonging to different *Helleborus* sections were obtained. These offspring include one *H. x hybridus* x *H. argutifolius* hybrid, two *H. foetidus* x *H. argutifolius* hybrids and 11 *H. x hybridus* and *H. niger* hybrids (Chapter 3.2.5.2). The new hybrids obtained and confirmed during this study are listed in combination with the known and the unconfirmed hybrids in Table 3.16.

Various fertile hybrids are already known to exist within section Helleborastrum (Mathew, 1989). Species such as *H. atrorubens*, *H. torquatus*, *H. cyclophyllus* and *H. odorus* may already have been used to introduce characteristics into *H. orientalis*, resulting in contemporary 'garden hybrids' that are known as *H. x hybridus*. It has been assumed that no crossing barriers exist within this section (Mathew, 1989), but no information is available regarding the crossing efficiency (e.g., the number of offspring from one flower compared to the number of ovules). The advantage of the Helleborastrum hybrids obtained during this study is that the parental genotypes are known and their characteristics can be directly compared to those of their hybrid offspring. For the so-called 'garden hybrids', only suggestions referring to trait origins have been made by Mathew (1989).

Hybridisation between *H. argutifolius* and *H. lividus* seems to be as easy hybridisation as within Helleborastrum, but does not occur naturally due to habitat isolation.

According to Mathew (1989), *H. foetidus* hybridises with members of section Chenopus, as observed in this study (Table 3.16), producing sterile offspring. For the intersectional hybrids between *H. niger* and the stemmed species, *H. x ballardiae* and *H. x nigercors*, hybrid sterility has been observed in this study and in earlier studies (Mathew, 1989). Additionally, hybrid weakness has been reported for other intersectional combinations (Mathew, 1989). Hybrid

sterility and weakness indicate that postzygotic barriers act between *Helleborus* species, at least between those belonging to different sections, justifying the use of embryo rescue techniques.

Table 3.16: *Helleborus* hybrids, reports of putative hybrids (Mathew, 1989) and confirmed interspecific hybrids obtained via embryo rescue within this thesis.
Confirmed hybrids obtained via embryo rescue within this thesis are coloured grey.

	Name	Crossing combination	Combination of characteristics
Confirmed hybrids	Orientalis hybrids	intraspecific *H. orientalis* crosses	variation of sepal shape and colours
	Orientalis hybrids, garden hybrids	interspecific crosses between species within section Helleborastrum, in which *H. orientalis* is involved	variation of sepal shape and colours, especially dark colours, double flowers
	H. x sternii	*H. argutifolius* x *H. lividus,* also possible reciprocal: *H. lividus* x *H. argutifolius*	fertile intermediates referring to growth type and flowers between the parental species
	H. x ballardiae *H. x nigercors* *H. x ericsmithii*	*H. niger* x *H. lividus* *H. niger* x *H. argutifolius* *H. niger* x *H. x sternii*	sterile intermediates referring to growth type and flowers between the parental species
Unconfirmed putative hybrids	*H. x jourdanii*	*H. foetidus* x *H. viridis*	differences in growth type and flower morphology
	--	*H. foetidus* x *H. argutifolius*	sterile offspring, differences in growth type and flower morphology
	-- 'Walberton's Rosemary'	*H. niger* x *H. orientalis* *H. niger* x *H. x hybridus*	differences in growth type and leaf and flower morphology; especially sepal colour and shape
	--	*H. niger* x *H. viridis*	differences in growth type and leaf and flower morphology
	--	*H. niger* x *H. purpurascens*	differences in growth type and leaf and flower morphology
Confirmed hybrids obtained via embryo rescue in this study	*H. x sternii*	*H. argutifolius x H. lividus* *H. lividus x H. argutifolius*	fertile intermediates referring to growth type and flowers between the parental species
	Intrasectional hybrids within Helleborastrum, in which *H. x hybridus* is involved	*H. x hybridus x H. atrorubens* *H. x hybridus x H. croaticus* *H. x hybridus x H. cyclophyllus* *H. x hybridus x H. dumetorum* *H. x hybridus x H. multifidus* *H. x hybridus x H. odorus* *H. x hybridus x H. purpurascens* *H. x hybridus x H. torquatus*	increase in variation of flower morphology: flower size and number, sepal colour (*H. atrorubens*, *H. torquatus*) and shape, nectaries; scent of *H. odorus*
	Intrasectional hybrids within Helleborastrum	*H. croaticus x H. multifidus* *H. croaticus x H. odorus* *H. odorus x H. croaticus* *H. purpurascens x H. croaticus* *H. torquatus x H. croaticus*	sepal colour (*H. purpurascens*, *H. torquatus*) and shape, nectaries, foliage, scent of *H. odorus*
	Intersectional hybrids	*H. foetidus x H. argutifolius*	differences in growth type and leaf and flower morphology
		H. x hybridus x H. argutifolius	differences in growth type and leaf and flower morphology; especially sepal colour and shape
		H. x hybridus x H. niger	differences in growth type and leaf and flower morphology; especially sepal colour and shape

For the hybrids between *H. x hybridus* and *H. niger* obtained in this study, some malformation during development was observed (Figure 3.20). Two germinated ovules developed little after germination and two isolated embryos developed very slowly. Additionally, two seedlings from the same cross had only one cotyledon. These weaknesses could be interpreted as a postzygotic barrier. Hybrid fertility has not yet been evaluated.

More reciprocal crosses were performed between *H. x hybridus* and *H. niger* than between other combinations of species due to the higher economic importance and availability of plant material of these species. Interestingly, 11 hybrids were obtained for *H. x hybridus* x *H. niger*, while ovules from *H. niger* x *H. x hybridus* crosses were aborted early or contained liquid endosperm due to endosperm breakdown. Fertilisation was assumed to occur because pollen tubes were observed near the ovules in all crossing combinations using *H. niger* as the maternal parent, and the ovules were observed to enlarge. For all crosses with *H. niger* as maternal the parent, even the crosses between *H. niger* and *H. argutifolius* or *H. lividus* exhibited aborted ovules and endosperm degeneration due to unknown reasons. Similarly, for intergeneric hybridisations between *Anemone coronaria* and *Ranunculus asiaticus*, hybrids have been obtained only from crosses with *Anemone* as the maternal parent via rescue of immature achenes (Dhooghe et al., 2010).

One possible explanation for these observations may be unilateral cross compatibility, meaning that a given cross is successful while the reciprocal cross fails, probably due to maternal effects. Unilateral compatibility has been described for certain crosses in *Hibiscus* (van Laere et al., 2007), *Curcurbita* (Sisko et al., 2003) and *Dianthus* (Nimura et al., 2003). For *Dianthus*, stunted endosperm development occurred when *D. japonicus* was used as the maternal parent. The cause of unilateral cross compatibility may be parental imprinting that results in endosperm breakdown, as discussed above. Additional maternal effects will be regarded in Chapter 5.1.

It is not definitively known whether unilateral cross compatibility exists between *H. niger* and *H. x hybridus*. From other sources, one may infer that *H. niger* x *H. x hybridus* is more successful than the reciprocal cross (Peter Oenings, personal communication). Additionally, in December 2009, the cultivar `Walberton's Rosemary´ was reported to be the first *H. niger* x *H. x hybridus* hybrid sharing morphological characteristics of both parents (Rice, 2009). Therefore, it seems more likely that the conditions used during plant growth or ovule culture were not suitable. Medium composition and temperature requirements may be species specific due to differences in geographical distribution. Lower temperatures (5 °C) during plant

growth in the 2008/2009 flowering season seemed to be beneficial for embryo rescue compared to the temperatures used during other years (12 °C in 2007/2008 and 2009/2010).

Genetic distance between parental species

The genetic distances between *Helleborus* species and their impact on hybridisation are discussed below.

The genus *Helleborus* is divided into six sections based on morphological characters. Four sections are monospecific, section Chenopus comprises two species, and section Helleborastrum comprises 16 species (Chapter 1.1.1). The molecular genetic analysis of the genus using AFLP markers supports the division of *Helleborus* into six sections (Chapter 2.3.3). The genetic distances between the species were estimated and the nuclear DNA content of each species was determined. Both factors are thought to be useful to predict hybridisation success.

Helleborus hybrids were obtained via embryo rescue, mostly within sections but also between sections (three combinations) (Chapter 3.3.4). The genetic distances between the parents of these three hybrids were quite large (0.241–0.264) compared to those between other combinations of species within sections (0.069-0.141). Therefore, the genetic distance between genotypes is not a clear predictor of hybridisation success in *Helleborus*; it only represents a trend. Genome size is also not a valuable predictor; hybrids between species with large differences in genome size already exist (e.g., between sections Helleborus and Chenopus) and were obtained in this project (e.g., *H. x hybridus* x *H. argutifolius*). Zonneveld (2001) has stated that if species of section Helleborastrum were regarded as one highly variable species due to their near lack of differences in nuclear DNA content, the division into sections would become meaningless because almost all sections would be monospecific.

However, although the genetic distances and genome sizes failed to directly predict hybridisation success, both factors indicated crossing combinations that were problematic in some way. Therefore, these factors are valuable indicators of the possibility of recovering hybrid offspring from a particular combination; crosses between parental plants with large differences in genome size or large genetic distances must be carried out more carefully and large numbers of crosses must be performed. Furthermore, the genetic distances make it possible for the first time to directly compare the relationships of different combinations of parental species. For example, *H. x hybridus* is equally genetically distant from *H. foetidus* and *H. niger*.

Intergeneric hybridisations between other members of the family *Ranunculaceae* (*Anemone coronaria* x *Ranunculus asiaticus)*, as members of the *Ranunculaceae*, have already been successful (Dhooghe et al., 2010), indicating that large genetic distances can be overcome within this plant family.

3.4.5 Hybrid verification

In addition to morphological characters, chemical and molecular characters and differences in genome size can be used for hybrid identification. Flow cytometry was tested for the identification of *Helleborus* hybrids. The method was effective only if the genome sizes of the parental species were sufficiently different. Among the hybrids obtained in this study, only those between sections Chenopus and/or Griphopus and Helleborus or Helleborastrum met this criterion. Hybrids between *H. x hybridus* and *H. argutifolius* as well as between *H. foetidus* x *H. argutifolius* were successfully identified by flow cytometry (Chapter 3.3.5.1). For hybrids within section Chenopus and within or between sections Helleborus and Helleborastrum, flow cytometry was not suitable. Therefore, these hybrids were analysed using RAPD markers.

Hybrids were obtained from 18 different interspecific crossing combinations. For 16 of these, 157 genotypes, of which 155 were verified as hybrids, were tested by RAPD analysis (Chapter 3.3.5.2). Hybrid identification failed only for *H. argutifolius* x *H. lividus* and one reciprocal cross. RAPD primers have already been proven to be effective for the identification of interspecific hybrids from embryo rescue experiments in *Lens* (Fratini & Ruiz, 2006) and *Dianthus* (Nimura et al., 2003). The larger the genetic distance between the parental plants, the easier it was to identify primers that amplified polymorphic bands. For closely related species, several RAPD primers had to be tested to amplify characteristic pollen donor fragments that were not present in the maternal genotypes. In *Dianthus*, one out of 40 primers tested amplified two characteristic pollen parent fragments and was therefore chosen for hybrid verification. Similarly to *Dianthus*, 25 primers were initially used for parental screening in *Helleborus*. For some closely related species (*H. x hybridus*, *H. purpurascens* and *H. atrorubens*), 12 additional primers were tested to obtain suitable primers for certain combinations. Interspecific *Helleborus* hybrids were successfully identified using RAPD markers.

RAPD markers are a suitable molecular marker system for hybrid identification due to their biparental inheritance and the almost unlimited number of independent markers (depending on the number of primers). In contrast to cytoplasmic or RNA markers, RAPDs are

distributed throughout the genome, which is beneficial in the analysis of hybrids. Additionally, molecular character expression appears to be much more predictable than morphological character expression, which is influenced by the environment, because molecular characters follow the rules of inheritance (Rieseberg & Ellstrand, 1993). First generation hybrids have been shown to be mosaics of both parental and intermediate morphological characters rather than solely intermediate ones (Rieseberg & Carney, 1998). The expression of parental or intermediate characters in hybrids depends on how these characters are genetically controlled and on interactions with the environment (Rieseberg & Carney, 1998). The expression of morphological characters is less predictable in later generation hybrids than in first generation hybrids due to the accumulation of extreme characters. Transgressive morphological characters have been found in first generation hybrids and to a greater degree, in later generation hybrids (Rieseberg & Ellstrand, 1993).

In *Helleborus* hybrids, morphological character expression was observed only by the appearance of *in vitro* leaves that resembled both parent plants to some extent. It will be interesting to examine and analyse the adult plants with regard to their horticultural value if they display transgressive characteristics related to flower colour, sepal shape, fertility or sterility, foliage and resistance against *C. hellebori.*

4 Morphological and molecular genetic analysis of hellebore leaf spot disease (*Coniothyrium hellebori*) isolates from different geographic origins

4.1 Introduction

Helleborus species are widely distributed in botanical, public and private ornamental gardens. They are quite variable with respect to growth type, scent, leaf morphology and especially flower colour and structure. The plants' appearance can be strongly affected by symptoms of the hellebore leaf spot disease, caused by the fungus *Coniothyrium hellebori*. The first description of *C. hellebori* was made by Cooke & Massee (1887), who observed symptoms on leaves of *H. niger*.

Coniothyrium-like fungi are widespread and are classified depending on the host, conidiomatal structure, mode of conidiogenesis and conidium morphology. *Coniothyrium* species have drawn attention as 'biocontrol agents' (Gerlagh et al., 1999), but they are also known to be common colonisers of different hosts. Examples include *C. fuckelii,* which colonises stems of *Rubus* spp., *C. wernsdorffiae,* the causal agent of rose brand canker (Ellis & Ellis, 1997) and *C. leucospermi,* which causes leaf spots of *Proteaceae* (Taylor & Crous, 2001).

In hellebores, small, black fruiting bodies, the pycnidia, which bear the spore masses, are formed in the necrotic leaf spots either on the upper or lower leaf surface (Figure 4.1). The spores are spread mainly by water, wind and wind-blown rain. Whereas some diseases are mainly important during propagation, hellebore leaf spot is more problematic at the cultivation and final growth site, where the fungus has optimal conditions to develop and remains for many years. The most effective method to prevent infection is to remove and destroy infected leaves immediately. Otherwise, the spores remain in the leaf material over the winter, and the plants are infected again in the following spring.

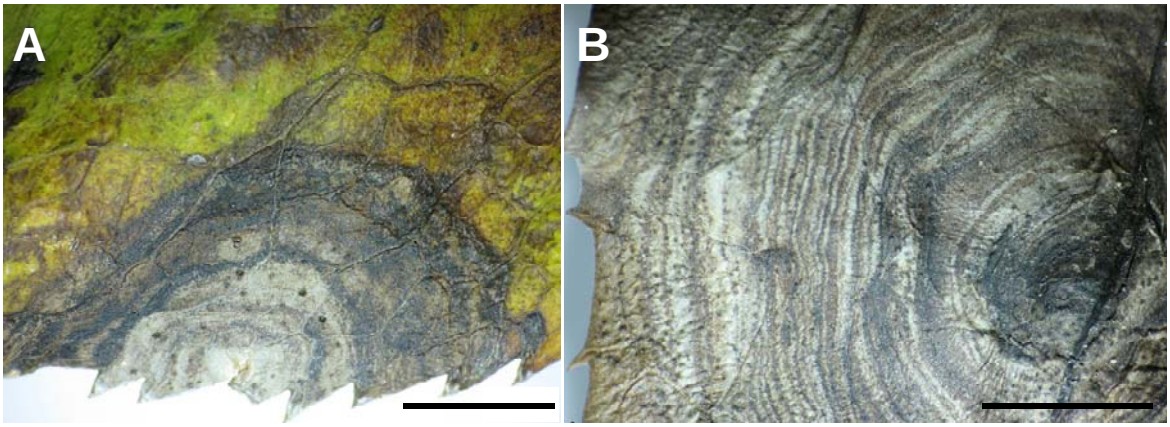


Figure 4.1: Necroses at the leaf margins of *H. x hybridus* caused by *C. hellebori* (A and B).
Bars represent 5 mm.

Although *C. hellebori* is the most common disease of hellebores, reports on its biology and genetics are rare. According to Pape (1928), hellebore leaf spot is distributed in many European countries and in North America. The disease was observed on different *Helleborus* species like *H. niger*, *H. argutifolius*, *H. foetidus*, and *H. viridis,* and Pape (1928) described plants growing under inappropriate soil conditions to be preferentially infected. In addition to the species mentioned above, other species are probably affected as well. A short description of the fungus's *in vitro* characteristics can be found in Van der Aa & Vanev (2002) and in Young & Alcorn (1981), who compared *C. hellebori* to other *Coniothyrium* species mainly from jojoba.

Currently, no information concerning the morphological, pathogenic or molecular genetic characteristics of the pathogen is available. Knowledge regarding pathogenicity and fungal genetic diversity is very important for *Helleborus* breeding to evaluate the susceptibility of *Helleborus* species and to develop reliable inoculation methods for disease resistance tests. The objectives of this study were (i) to determine variability in morphology and cultural characteristics of different isolates of this pathogen, (ii) to identify a suitable method for spore production, (iii) to perform pathogenicity tests and (iv) to determine molecular genetic relationships based on RAPD (Random Amplified Polymorphic DNA) markers of geographically diverse *C. hellebori* isolates in comparison to the mycoparasite *Coniothyrium minitans*.

4.2 Materials and Methods

4.2.1 Isolates and cultures

Symptomatic leaf samples from *Helleborus* hosts were collected during this study; these samples came primarily from Germany, but also from Austria, Canada, Great Britain, Switzerland and the United States (Table 4.1). The fungus was isolated from leaf sections bearing pycnidia on a mixture of potato dextrose (PDA) and carrot juice agar (1.95 % PDA (Oxoid), 2.5 % carrot juice, 0.9 % agar). Two isolates, no. 1 and no. 2 (Table 4.1), were obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig. Additionally, for comparisons, the *C. minitans* strain CON-m-91-08 was used, which was kindly provided by Prophyta GmbH, Malchow, Germany. *C. hellebori* mycelium cultures were transferred to tomato mash agar (20 % tomato mash, 0.3 % $CaCO_3$, 1.5 % agar, pH 7.2).

Table 4.1: Collection of *C. hellebori* isolates and *C. minitans* according to their geographic locations and plant hosts.
Conidial sizes of four single-spore isolates of the isolates no. 20 and 24 were compared.

Isolate No.	Geographic location/name	Country[a]	Plant host
1	DSMZ 1205	Unknown	Unknown
2	DSMZ 62472	Germany	Unknown
3	Kiel	Germany	*H. niger*
4	Ellerhoop	Germany	*H. niger*
5	Bad Zwischenahn	Germany	*H. x hybridus*
6	Berlin	Germany	*H. niger*
7	Osnabrueck	Germany	*H. x hybridus*
8	Muenster	Germany	*H. niger*
9	Muenster	Germany	*H. niger*
10	Muenster	Germany	*H. niger*
11	Thale	Germany	*H. niger*
12	Huenxe-Drevenack	Germany	*H. niger*
13	Radevormwald	Germany	*H. x hybridus*
14	Cologne	Germany	*H. niger*
15	Rottenburg a. d. Laaber	Germany	*H. x hybridus*
16	Weihenstephan	Germany	*H. x hybridus*
17	Weihenstephan	Germany	*H. x hybridus*
18	Weihenstephan	Germany	*H. x hybridus*
19	Munich	Germany	*H. x hybridus*
20.1	Pendling/Thiersee	Austria	*H. niger*
20.2	Pendling/Thiersee	Austria	*H. niger*
20.3	Pendling/Thiersee	Austria	*H. niger*
20.4	Pendling/Thiersee	Austria	*H. niger*
21	Kesswil	Switzerland	*H. x hybridus*
22	Oberrieden	Switzerland	*H. x sternii*
23	Wisley	Great Britain	*H. x hybridus*
24.1	Philadelphia	USA	*H. niger*
24.2	Philadelphia	USA	*H. niger*
24.3	Philadelphia	USA	*H. niger*
24.4	Philadelphia	USA	*H. niger*
25	Toronto	Canada	*H. niger*
26	*Coniothyrium minitans* CON-m-91-08	Unknown	

[a]German samples are arranged from North to South

For the establishment of single-spore isolations, spore suspensions from pycnidia induced on oat flake agar (3 % oat flakes, 1.5 % agar) were plated on water agar. After three days, single germinating conidia were isolated under a microscope and transferred to tomato mash agar. Single conidial isolates were established from each origin and were at least duplicate in number, except for the isolate from Wisley, Great Britain (No. 23, Table 4.1) from which 15 single conidial isolates were initiated. The cultures were stored at approximately 20 °C in the dark and subcultured by transferring young mycelium of actively growing colonies onto fresh agar plates every two months. Permanent cultures were applied from the single spore isolates to preserve the cultures in a young stage. Therefore, sterile filter paper stripes were put onto solid medium. The plate was inoculated by mycelia discs, and when the mycel had grown over the filter paper, the paper was removed, dried in an exsiccator and stored in small tubes at -20 °C.

4.2.2 Morphological comparisons of *C. hellebori* isolates

4.2.2.1 Mycelial growth rate and colony morphology

Growth rates and temperature requirements for the 25 *C. hellebori* isolates and *C. minitans* were determined on tomato mash agar. The tomato mash agar plates were inoculated with discs (5 mm in diameter) from young mycelia of actively growing isolates and placed upside down at the centre of the plates. The plates were maintained at temperatures ranging from 5 °C to 30 °C, at 5 °C intervals, for 10 days in the dark. Four plates were incubated for each isolate at each temperature. Diameter measurements were obtained every other day twice from each colony, at right angles to each other. Final evaluation of radial mycelial growth, colony colour and morphology was carried out after ten days when the first isolates reached the margin of the plate.

4.2.2.2 Pycnidial production and spore germination

Pycnidial production was examined for isolates no. 2, 3, 16, 20 and 24 on tomato mash and oat flake agar at 20 °C in the dark or with 12 and 24 h illumination at approximately 20 µmol/m²s. There were four replicates for each isolate. The plates were observed every three days, and the final examination was carried out after 24 days. Pycnidial production was classified into qualitative groups according to the abundance of pycnidia: (0) no pycnidia, (1) rare pycnidia, (2) sparse pycnidia, (3) many pycnidia, (4) the dish was covered with pycnidia.

To identify adequate temperature conditions for spore germination, conidia from isolates no. 18, 20 and 24 were suspended in sterile water and plated on water agar. The plates were

incubated at 10 °C, 15 °C, 20 °C, 25 °C and 30 °C in the dark and at 20 °C under light for 24 h. The dishes were monitored for conidial germination after 4 h, 8 h and 24 h.

4.2.2.3 Conidial size

Conidia from each isolate were harvested from two to three week old colonies grown on oat flake agar at 20 °C in the dark. The length and width of 100 individual conidia of each of the 25 *C. hellebori* isolates and *C. minitans* were measured (Axio Scope.A1, Zeiss, Oberkochen, Germany) at 400 x magnification. To evaluate the variability of conidial size within the same origin of an isolate, four independent single spore isolates were used for the isolates from Pendling/Thiersee (no. 20) and Philadelphia (no. 24) (Table 4.1).

4.2.3 Pathogenicity trials

All 25 *C. hellebori* isolates were used in inoculation experiments of *Helleborus* plants and small leaf discs. For each isolate, pycnidia were induced on oat flake agar. Three 18 months old *H. niger* plants derived from seeds of the cultivar 'COSEH 2040' were inoculated with each isolate. Additionally, three leaf discs (13 mm in diameter from young plant parts and 14 mm in diameter from old leaf parts) of the same plants used for inoculation were put with the adaxial side down on sterile filter paper in a Petri dish in three replicates, and the whole leaf disc experiment was repeated once. The plants and the leaf discs were inoculated by spraying with spore suspensions with a concentration of 10^6 conidia ml^{-1} until they were dripping wet, except for isolate no. 1, which was not able to sporulate. Thus, for this isolate, mycelial plugs were transferred onto the leaves and the leaf discs. All plants were covered with plastic bags, and the dishes with the leaf discs were closed to prevent desiccation of the inoculum and promote infection. Control inoculations were carried out with sterile water and also with *C. minitans*. The inoculated plants and leaf discs were cultured in a climate chamber at 20 °C in 95 % humidity for 36 h in the dark. Subsequently, a photoperiod of 12/12 h with an illumination of 1000 lux was used. After one week, the plastic covers were removed. The leaf discs were evaluated for symptoms after seven and fourteen days and classified according to their degree of browning. The plants were observed for five weeks on a weekly basis and a final evaluation was carried out after five weeks.

4.2.4 DNA extraction and RAPD PCR analysis

Freshly grown mycelium (0.01 to 0.1 g fresh mass) was harvested from one plate, transferred to a reaction tube and thoroughly homogenised in buffer at room temperature with a micropestle. The extraction procedure followed a protocol developed for plant leaf material

by Dorokhov and Klocke (1997). The protocol was modified in the following ways: (i) 0.2 mg RNase A was added before incubation at 65 °C and (ii) in the second DNA pellet washing step, isopropanol was replaced by 70 % ethanol. After drying the pellets, they were dissolved in 25 µl of sterile bi-distilled water. DNA was extracted in two independent biological replicates.

DNA was amplified by the RAPD technique (Williams et al., 1990). In addition to all 25 *C. hellebori* isolates and *C. minitans* as the outgroup, nine other single spore cultures of isolate no. 23 were used to evaluate the genetic variability within one origin. Amplification reactions were carried out in a volume of 20 µl containing 10 mM Tris (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001 % gelatine, 100 µM of each deoxyribonucleoside triphosphate, 0.5 µM of each primer, 1 U Taq DNA Polymerase (FIREPol ® Solis Biodyne, Estonia) and 5 ng fungal genomic DNA. In total, 40 different random decamer primers (primer kits B and C, Carl Roth, Karlsruhe, Germany) were used. Thermal cycling was conducted with 5 min initial denaturation at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C and a final extension step for 10 min at 72 °C. Amplification products were separated by electrophoresis in 1.5 % agarose gels in 1 x TAE buffer (2 M Tris, 50 mM EDTA pH 8.0, 1 M glacial acetic acid; pH 8.44), detected by staining with ethidium bromide (10 µg/100 ml agarose gel) and visualised on a UV transilluminator. Reproducibility of the results was ensured by repeating the PCR amplification reactions of all isolates twice based on the two different DNA extractions.

4.2.5 RAPD data analysis

The banding patterns of each primer were evaluated by visual inspection. Reproducible bands were included and transformed into a 0/1 matrix. Genetic distances and phenograms were computed using the PHYLIP 3.69 software package (http://evolution.genetics.washington.edu/phylip.html). The pairwise distances between the analysed isolates were calculated using the similarity index of Nei and Li (1979). On the basis of the distance matrix of the genetic distances, a cluster analysis was carried out using the unweighted pair group method with arithmetic mean (UPGMA). Relative branch support was assessed by bootstrap analysis (Felsenstein, 1985) with 1000 replicates. The phenograms were displayed with the TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

4.2.6 Statistical analyses

Radial mycelial growth was presented separately for each isolate and as the average of all isolates depending on the temperature in one graph. The influence of temperature on the average mycelial growth of all *C. hellebori* isolates was analysed using the Wilcoxon rank sum test modified for heterogeneous variances. Additionally, statistical analyses by ANOVA and Tukey's test were carried out to detect significant differences in mycelial growth between the *C. hellebori* isolates cultured at the same temperature. The conidial length and width of all single conidial isolates were examined separately for statistical differences with analysis of variance (ANOVA) and Tukey's test. The pycnidial production on different media and the disease severity were presented as boxplots. To detect significant differences, the Kruskal-Wallis rank sum test and Wilcoxon rank sum test modified for heterogeneous variances were used. P values were adjusted according to Holm (1979). For all statistical analyses, the R software package version 2.11.0 was used.

4.3 Results

4.3.1 Morphological comparisons of *C. hellebori* isolates

4.3.1.1 Mycelial growth characteristics

C. hellebori hyphae grew at temperatures from 5 °C to 25 °C in the dark (Table 4.2, Figure 4.2). Optimum mycelial growth occurred at 20 °C; the maximal radial mycelial growth rate occurred for isolate no. 16 and was 3.4 mm/day, and the minimal rate was 2.4 mm/day for isolate no. 12. *C. minitans* grew slightly slower (Table 4.2). The largest difference in radial mycelial growth among the isolates was observed at 25 °C (Figure 4.2): some isolates grew slower at this temperature than at 5 °C, such as no. 12, 14, 17, 21 and 25, whereas five isolates grew faster at 25 °C than at 15 °C (Table 4.2). Almost no growth occurred at a temperature of 30 °C. The highest radial mycelial growth of all isolates was recorded at 20 °C, for which the average mycelial growth differed significantly from growth at all other temperatures. Significant differences between the mycelial growth of the *C. hellebori* isolates were detected at all temperature levels (Table 4.2).

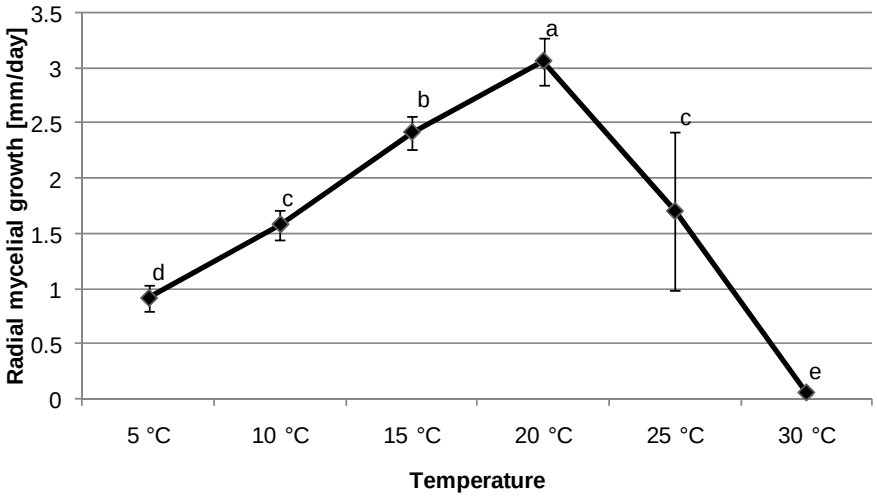


Figure 4.2: Average radial mycelial growth of 25 *C. hellebori* isolates after incubation at different temperatures for 10 days.
Vertical bars represent standard deviations. Different letters above the mean indicate statistical differences (P≤0.05) between the temperatures by Wilcoxon rank sum tests modified for heterogeneous variances.

The colony colour differed only slightly, appearing as different shades of grey in the colony centre with almost pure white margins at all temperatures (Figure 4.3). The mycelium was floccose, and the abundance of aerial mycelium varied depending on the temperature and the isolate. Pycnidia production was observed at a temperature of 15 °C for four isolates and

occurred up to 25 °C. At 25 °C, pycnidia were found either in concentric rings, scattered within the mycelium or both for 25 isolates. Only isolate no. 1 was not able to sporulate.

Table 4.2: Average radial mycelial growth of each *C. hellebori* isolate and *C. minitans* on tomato mash agar at different temperatures.
The number of plate repetitions was n=4.

Isolate No.	Radial mycelial growth [mm/day] Mean					
	5 °C	10 °C	15 °C	20 °C	25 °C	30 °C
1	1.1 b[a]	1.7 bc	2.4 c	3.1 cd	2.4 c	0.08 ab
2	0.9 d	1.6 cd	2.5 bc	3.3 b	1.5 g	0.1 a
3	0.8 de	1.7 bc	2.6 ab	3.2 bc	1.5 g	0.08 ab
4	0.8 de	1.7 bc	2.5 bc	3.0 ef	1.0 i	0.08 ab
5	1.0 c	1.6 cd	2.4 ce	2.9 ef	2.7 a	0.05 b
6	0.8 de	1.4 e	2.3 e	3.0 de	1.8 f	0.05 b
7	0.8 de	1.5 de	2.4 c	3.2 cd	2.1 de	0.05 b
8	0.8 de	1.7 bc	2.6 ab	3.2 bc	2.0 e	0.08 ab
9	0.8 de	1.4 e	2.4 ce	3.1 de	1.0 i	0.05 b
10	0.9 de	1.6 cd	2.5 bc	3.2 cd	1.2 h	0.05 b
11	1.0 bc	1.6 cd	2.4 c	3.1 cd	2.7 a	0.05 b
12	0.8 de	1.4 e	2.1 f	2.4 h	0.7 j	0.05 b
13	1.0 bc	1.6 d	2.4 cd	2.8 g	1.6 g	0.05 b
14	1.1 ab	1.8 a	2.4 ce	3.2 bc	0.7 j	0.05 b
15	0.8 de	1.4 e	2.2 f	2.9 ef	2.6 ab	0.05 b
16	1.2 a	1.8 ab	2.7 a	3.4 a	2.5 b	0.05 b
17	0.9 de	1.5 de	2.4 cde	2.9 ef	0.7 j	0.08 ab
18	0.8 e	1.5 de	2.3 de	3.0 e	1.2 h	0.05 b
19	1.1 ab	1.8 abc	2.5 bc	2.9 f	2.4 c	0.1 a
20	1.0 c	1.7 bc	2.6 b	3.3 b	2.2 d	0.07 b
21	1.0 bc	1.4 e	2.3 e	3.2 cd	0.8 j	0.05 b
22	0.8 de	1.6 d	2.5 c	3.1 d	1.9 e	0.1 a
23	0.9 de	1.5 de	2.4 c	3.1 cd	2.5 bc	0.05 b
24	1.0 c	1.7 c	2.6 ab	3.2 bc	2.2 d	0.05 b
25	0.8 de	1.4 e	2.1 f	2.7 g	0.6 k	0.1 a
26	0.4	0.8	1.4	2.2	0.9	0.05

[a]within columns, values followed by the same letter are not significantly different (P≤0.05) within the temperature treatment by Tukey's test.

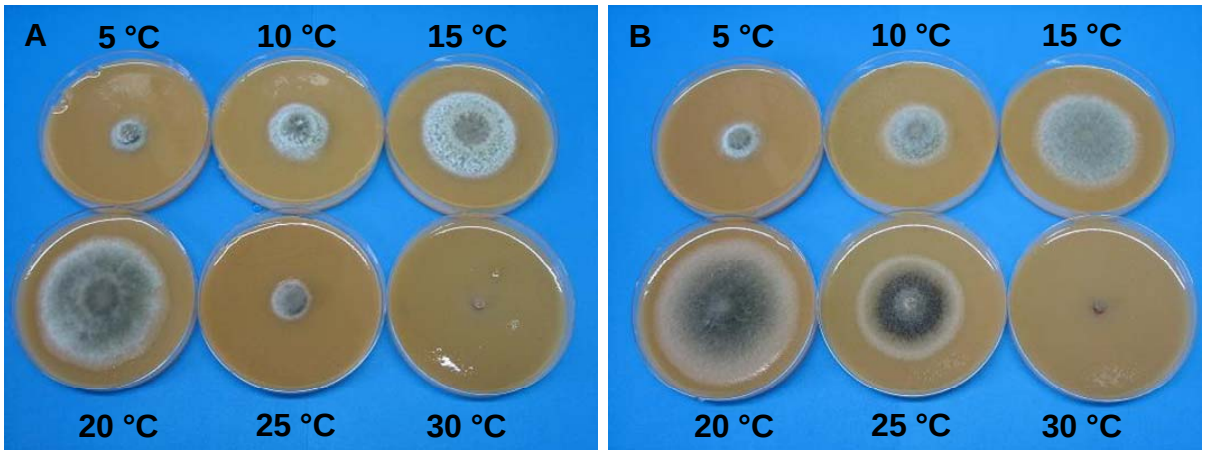


Figure 4.3: Colony morphology of two *C. hellebori* isolates cultured at different temperatures.
A: isolate no. 17 (Weihenstephan) and B: isolate no. 24 (Philadelphia) after 10 days of growth on tomato mash agar at different temperatures in the dark. Plate diameter was 9 cm.

4.3.1.2 Pycnidial production and spore germination

To determine the conditions suitable for pycnidia production, five isolates were selected and cultured either on tomato mash or oat flake agar under different illumination conditions. The radial mycelial growth rate during the first ten days was between 2.2 mm/day and 2.9 mm/day on average over all isolates and did not differ between the two media or among different illumination conditions (data not shown). The abundance of aerial mycelium was similar on tomato mash and oat flake agar with 24 h illumination, but it was reduced on oat flake agar with 12 h illumination (Figure 4.4); there was almost no aerial mycelium on oat flake agar when plates were incubated in the dark (Figure 4.4).

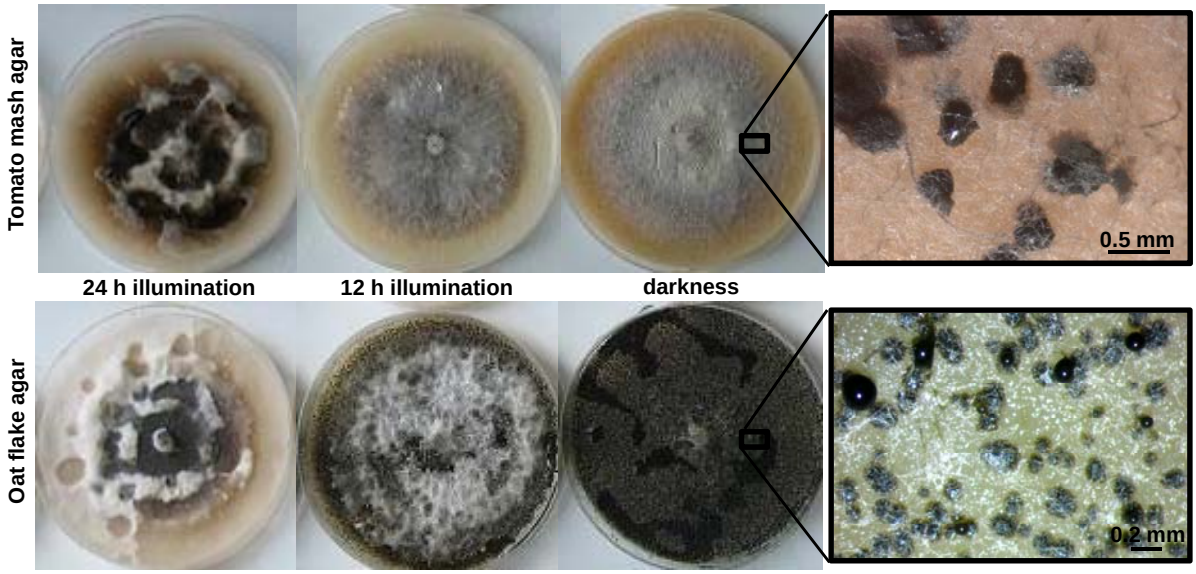


Figure 4.4: Comparison of pycnidial production of isolate no. 16 (Weihenstephan) on different media and under different illumination conditions.
The magnified pictures were obtained from other plates and represent examples. Plate diameter was 9 cm.

Pycnidia were produced in all treatments, but their amounts differed strongly (Figure 4.5). Overall, more pycnidia were recorded on oat flake agar in comparison to tomato mash agar and plates cultured in the dark (Figure 4.5). The highest number of pycnidia was produced on oat flake agar in the dark. The differences in pycnidial production between the two media and between the culture in the dark and the culture at 24 h illumination were significant. Pycnidia were either immersed in the agar or embedded in the mycel. Their shapes ranged from globose to ellipsoidal (Figure 4.6 A). On plates covered with pycnidia, an exudation of blackish-brown conidial slime was observed (Figure 4.4).

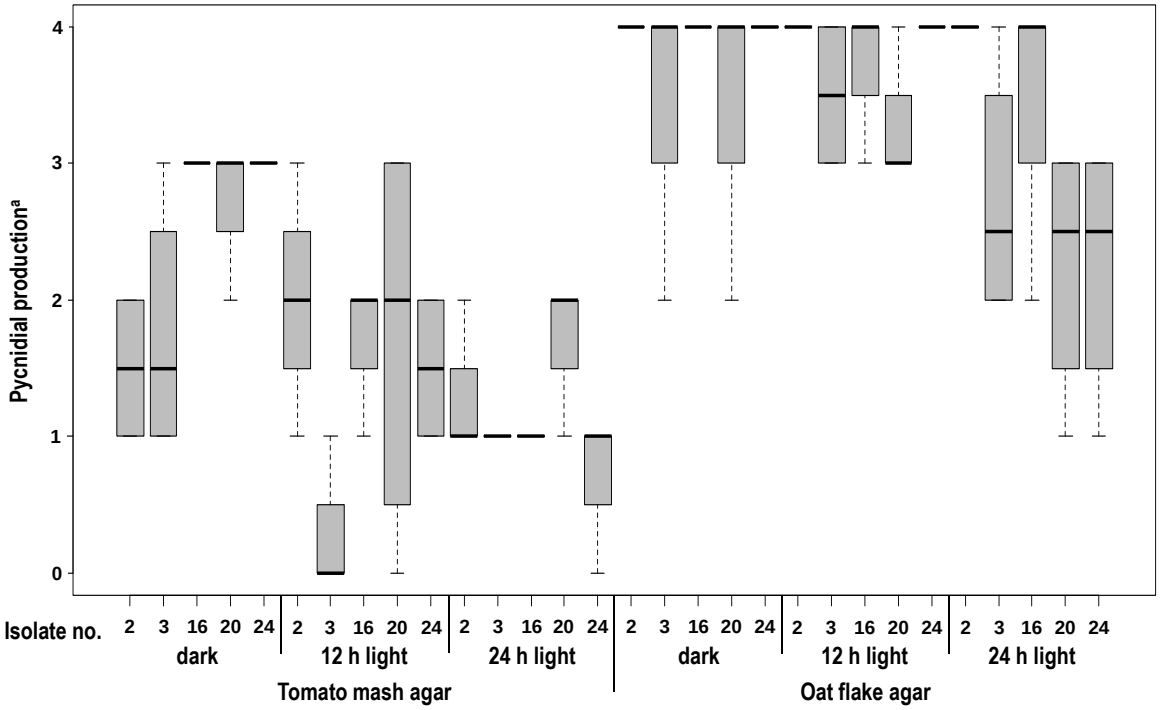


Figure 4.5: Pycnidial production on different media and unter different illumination conditions at 20 °C after 24 days.
The lines within the boxes show the median, and the upper and lower hinges represent the first and the third quartiles, respectively. Whiskers below and above the box mark the locations of the minimum and maximum, respectively. The number of plate repetitions was n=4.
[a]pycnidial production: (0) no pycnidia, (1) rare pycnidia, (2) sparse pycnidia, (3) many pycnidia, (4) the Petri dish is covered with pycnidia

Spore germination was analysed under different temperature conditions. After 8 h, the first germinating conidia were observed for all three isolates on water agar plates incubated at 15 °C to 25 °C. Germination percentage increased up to 80-100 % during the first 24 h. No differences regarding spore germination were observed for incubation at 20 °C under light compared to darkness. Below 15 °C and above 25 °C, germination was reduced (data not shown).

4.3.1.3 Conidial size

The average conidial size was determined for 24 *C. hellebori* isolates and *C. minitans* using 100 spores derived from several different pycnidia (Table 4.3). Conidia were unicellular, smooth-walled, ellipsoidal and pale brown to dark brown with partially obtuse apices and rounded bases (Figure 4.6 B). Conidial length varied from 3.6 µm to 4.2 µm between the four single-spore isolations of isolate no. 20 and single-spore isolate no. 24.3. For all the other isolates, length ranged from 4.5 µm to 5.1 µm and width ranged from 2.6 µm to 2.9 µm. Only

two single-spore isolations of isolate no. 20 differed significantly in length and width from the other isolates examined. The other two single-spore isolations of isolate no. 20 were in fact shorter compared to other isolates, but the width was similar corresponding to a lower length/width ratio. In comparison to *C. hellebori,* spores of *C. minitans* differed significantly with spore sizes of 5.6 µm and 3.7 µm in length and width, respectively.

Table 4.3: Length and width of conidia of *C. hellebori* isolates and *C. minitans*.

Isolate No.	Geographic location/name	Conidial size [µm]	
		Length[a]	Width[a]
1	DSMZ 1205	ND[b]	ND
2	DSMZ 62472	4.7 de	2.6 cde
3	Kiel	4.5 e	2.7 cde
4	Ellerhoop	4.8 cd	2.8 bc
5	Bad Zwischenahn	5.1 bc	2.8 bc
6	Berlin	4.6 e	2.7 cd
7	Osnabrueck	5.0 bcd	2.8 cd
8	Muenster	4.8 cd	2.6 cd
9	Muenster	4.8 cd	2.8 bc
10	Muenster	4.7 de	2.6 de
11	Thale	4.7 de	2.7 cde
12	Huenxe-Drevenack	4.7 de	2.6 de
13	Radevormwald	5.0 bc	2.8 bc
14	Cologne	4.8 cd	2.6 e
15	Rottenburg a. d. Laaber	5.0 bc	2.8 c
16	Weihenstephan	5.1 b	2.6 cde
17	Weihenstephan	5.0 bc	2.7 cd
18	Weihenstephan	5.1 b	2.6 cde
19	Munich	5.1 b	2.9 b
20.1	Pendling/Thiersee	4.2 f	2.6 de
20.2	Pendling/Thiersee	3.8 g	2.3 g
20.3	Pendling/Thiersee	3.6 g	2.2 g
20.4	Pendling/Thiersee	4.1 f	2.6 de
21	Kesswil	4.9 bcd	2.7 cde
22	Oberrieden	4.9 cd	2.6 cde
23	Wisley	5.0 bc	2.6 de
24.1	Philadelphia	4.9 cd	2.7 cd
24.2	Philadelphia	4.9 bcd	3.0 b
24.3	Philadelphia	4.2 f	2.4 f
24.4	Philadelphia	4.6 de	2.7 cde
25	Toronto	4.7 cd	2.7 cd
26	*Coniothyrium minitans* CON-m-91-08	5.6 a	3.7 a

[a]Within columns, values followed by the same letter are not significantly different (P≤0.05) by Tukey's test
[b]ND means not detected, as the isolate was not able to sporulate

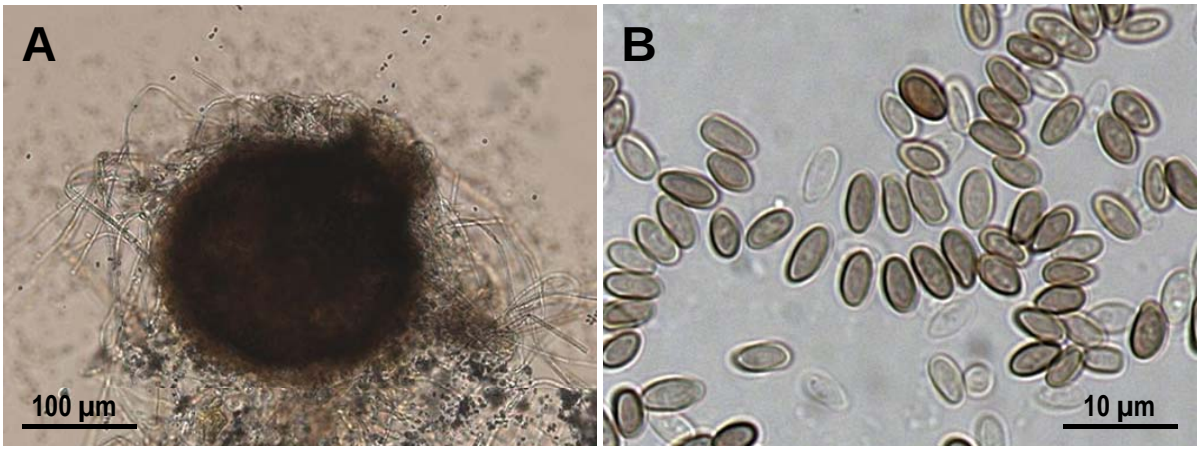


Figure 4.6: Pycnidium and spores of one *C. hellebori* isolate.
A: pycnidium and B: spores of *C. hellebori* isolate no. 24 (Philadelphia).

4.3.2 Pathogenicity of *C. hellebori* isolates

Infections were induced by all *C. hellebori* isolates on 18 month old plants of *H. niger* as well as on leaf discs from young and old leaves. Different symptoms were observed at the whole plant level. Seven days after inoculation, small black spots, lesions and the die-back of the youngest freshly developed leaf were recorded as symptoms (Figure 4.7). Later on, the die-back of the inoculated leaves was also observed until the 35th day of the experiment as symptoms accumulated. The disease severity differed slightly depending on the isolate, but these differences were not significant due to large variances (Figure 4.8). At each plant, one leaf lobe of one young and one old leaf was cut off and used for the leaf disc assay. Therefore, these leaves were wounded at their petioles. The cutting site at the petiole browned, and these leaves faded already 14 days after inoculation.

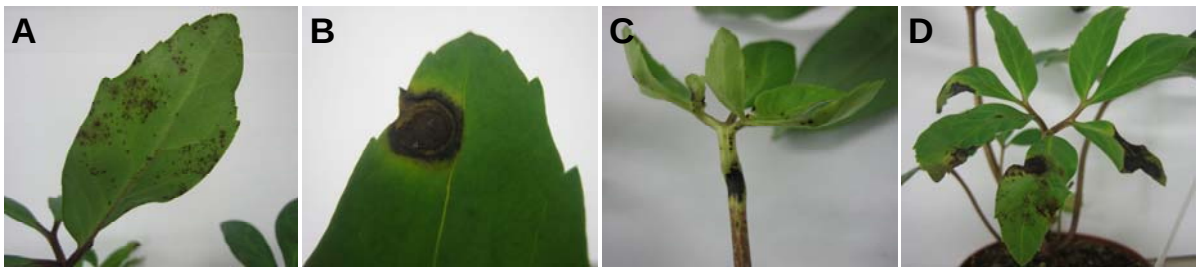


Figure 4.7: Symptoms occurring on infected hellebores during the pathogenicity trial.
A: small black spots, B: lesion, C: symptoms on the youngest leaf that developed after inoculation and D: small black spots and lesions.

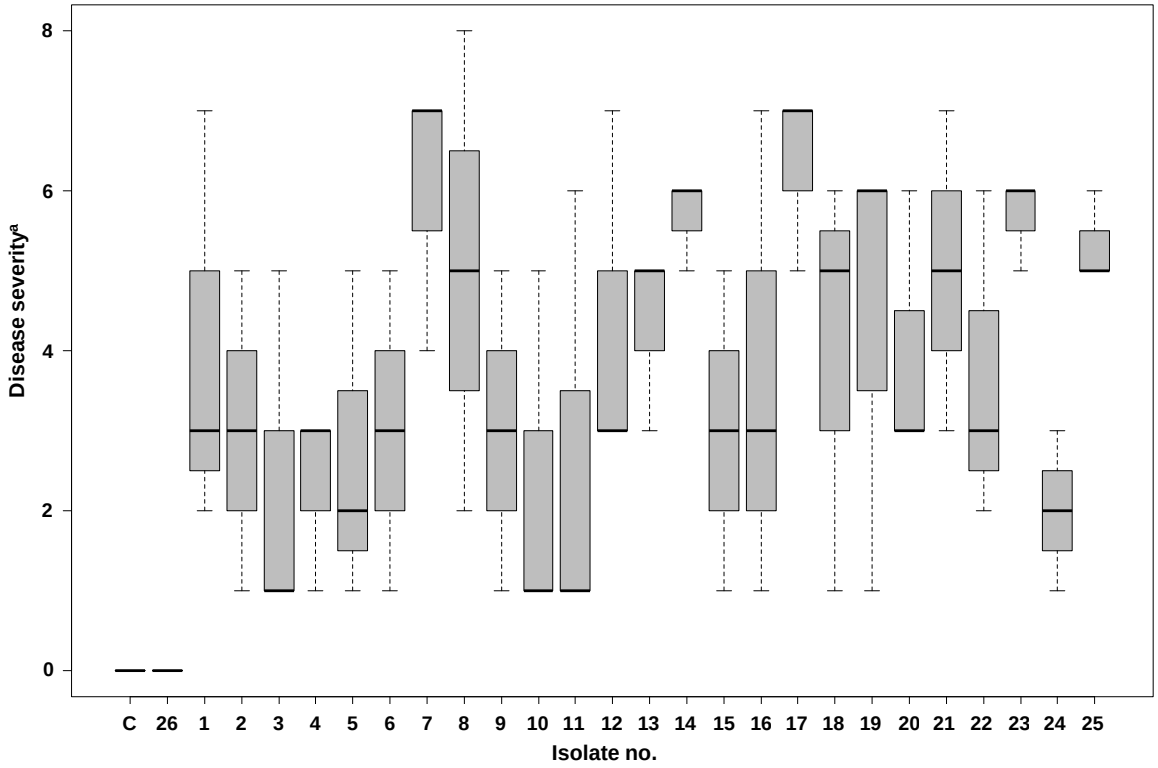


Figure 4.8: Disease severity classes depending on the *C. hellebori* isolate 35 days after inoculation of *H. niger* plants.
The lines within the boxes show the median, and the upper and lower hinges represent the first and the third quartiles, respectively. Whiskers below and above the box mark the locations of the minimum and maximum, respectively. The number of plant repetitions was n=3. Isolate C indicates control with sterile water.
[a]Nine different classes of disease severity were distinguished: (0) no symptoms, (1) small black spots, (2) small black spots and die-back of the youngest leaf, (3) small black spots and lesions (>0.5 cm²), (4) small black spots, die-back of the youngest leaf and lesions (>0.5 cm²), (5) small black spots and die-back of the inoculated leaves, (6) small black spots and die-back of the youngest and the inoculated leaves, (7) small black spots, lesions (>0.5 cm²) and die-back of the inoculated leaves, (8) small black spots, lesions (>0.5 cm²), die-back of the youngest and the inoculated leaves.

On the leaf discs, different stages of browning were noted seven days after inoculation (Figure 4.9 A) in both repetitions. The symptoms progressed much faster than they did on the plants. The leaf discs obtained from young leaves were browning faster than those from old leaves. After 14 days, the leaf discs were totally brown, and all inoculated leaf discs bore pycnidia (Figure 4.9 B) except for isolate no.1. Therefore, no statistical analysis was carried out for the leaf disc assay. Control inoculations did not develop symptoms.

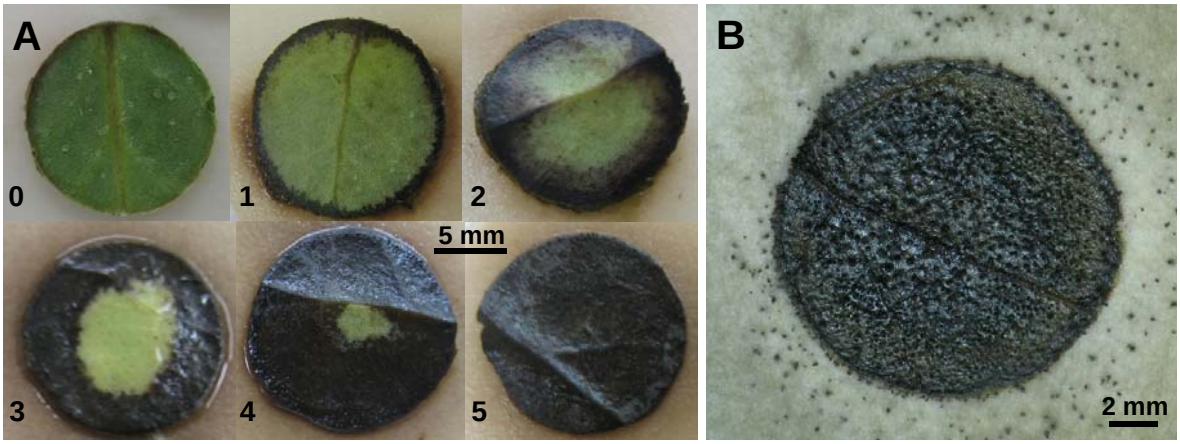


Figure 4.9: Pathogenicity trial by leaf disc assay.
A: different stages of browning after seven days, numbers next to the discs represent classes of browning; B: brown leaf disc covered with pycnidia 14 days after inoculation.

4.3.3 Genetic relationships based on RAPD analysis

Of the 40 RAPD primers employed, 37 were able to amplify distinct fragments in *C. hellebori* and *C. minitans* DNA (Table 4.4). In total, 394 DNA fragments were produced, of which 159 (40 %) were polymorphic between the *C. hellebori* isolates. Depending on the primer, between two and 19 DNA fragments of various intensities were amplified (Table 4.4), ranging in size from approximately 0.2 kb to 2 kb. Bands were reproducible in repeated amplifications.

The banding patterns of the 10 different single spore cultures of isolate no. 23 did not differ. Therefore, only one single-spore isolate of no. 23 was integrated in genetic distance determination.

Pairwise genetic distances between 25 *C. hellebori* isolates and *C. minitans* were calculated based on Nei and Li's coefficients using all 394 fragments. The average genetic distance among all *C. hellebori* isolates was 0.022. The genetic distance between the *C. hellebori* isolates and *C. minitans* ranged from 0.238 to 0.261. Within the *C. hellebori* isolates, the distance varied from 0.002 between isolates no. 21 and 9 or 14 to 0.034 between no. 1 and 9. Based on the Nei and Li coefficients matrix, a phenogram was calculated by the UPGMA cluster method (Figure 4.10).

Table 4.4: Number of amplified and polymorphic bands obtained from RAPD analysis of different *C. hellebori* isolates dependent on the primer.
These data were used for genetic distance estimation and cluster analysis.

Primer name (primer kits, Roth)	Sequence 5'→3'	No. of amplified bands	No. of polymorphic bands between *C. hellebori* isolates
C1	TTCGAGCCAG	13	2
C2	GTGAGGCGTC	10	1
C3	GGGGGTCTTT	9	4
C4	CCGCATCTAC	11	7
C5	GATGACCGCC	14	5
C6	GAACGGACTC	12	5
C7	GTCCCGACGA	12	6
C8	TGGACCGGTG	13	2
C9	CTCACCGTCC	15	7
C10	TGTCTGGGTG	9	1
C11	AAAGCTGCGG	13	5
C12	TGTCATCCCC	19	15
C13	AAGCCTCGTA	15	4
C14	TGCGTGCTTG	16	7
C15	GACGGATCAG	13	4
C16	CACACTCCAG	8	2
C17	TTCCCCCCAG	4	3
C18	TGAGTGGGTG	6	2
C19	GTTGCCAGCC	11	4
C20	ACTTCGCCAC	16	2
B1	GTTTCGCTCC	13	5
B2	TGATCCCTGG	7	4
B3	CATCCCCCTG	5	4
B4	GGACTGGAGT	6	3
B5	TGCGCCCTTC	11	6
B6	TGCTCTGCCC	12	9
B7	GGTGACGCAG	12	6
B8	GTCCACACGG	10	3
B10	CTGCTGGGAC	8	2
B11	GTAGACCCGT	11	5
B12	CCTTGACGCA	9	3
B13	TTCCCCCGCT	2	0
B15	GGAGGGTGTT	10	3
B17	AGGGAACGAG	12	4
B18	CCACAGCAGT	13	6
B19	ACCCCCGAAG	6	3
B20	GGACCCTTAC	8	5
		∑= 394	∑= 159 (40%)

In the dendrogram, two major clades emerged. One clade represented the outgroup *C. minitans*, which was clearly distant from all *C. hellebori* isolates. The second clade comprised the *C. hellebori* isolates and could be divided into two subgroups (100 % bootstrap), (I) one comprising 12 isolates (85 % bootstrap) and (II) the other one 13 isolates (86 % bootstrap). The isolates no. 15-19 from Southern Germany were all grouped in the first subgroup of the *C. hellebori* clade, and those from Muenster, together with most isolates from Northern Germany, were in the second subgroup. The second subgroup was again subdivided into two groups: (i) isolate no. 20 (Pendling/Thiersee) and isolate no. 11 (Thale) (genetic

distance 0.017), in addition to (ii) other isolates from Germany and other countries. The two isolates obtained from the German Collection of Microorganisms and Cell Cultures were also found in the different subgroups I and II with a genetic distance of 0.023. Isolates from the American continent were grouped in the different subgroups; the same was true for the isolates from Switzerland.

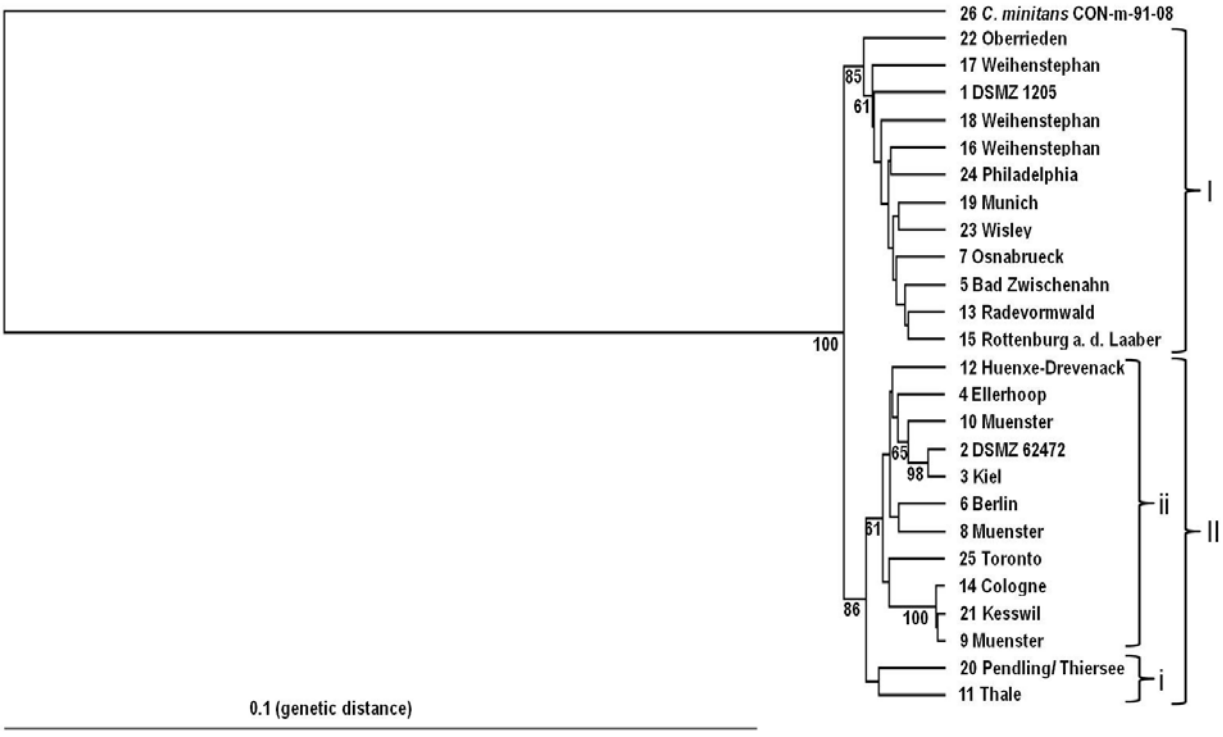


Figure 4.10: UPGMA phenogram based on Nei and Li similarity indices from 394 RAPD markers for 25 *C. hellebori* isolates and *C. minitans* as the outgroup.
The bootstrap percentages >50 % calculated from 1000 bootstraps are shown below the branches.

4.4 Discussion

For the first time, morphological comparisons, pathogenicity tests and molecular genetic studies on the intraspecific diversity of *C. hellebori* were carried out in this study. The evaluation of morphological characteristics of this pathogen revealed only very few differences between the different *C. hellebori* isolates. All isolates were similar in their cultural characteristics, with the best mycelial growth occurring at a temperature of 20 °C and growth stopping at 30 °C. Considerable growth differences between the isolates were only found at 25 °C.

Conidial measurements carried out in this study were consistent with those from Cooke and Massee (1887), but conidia were reported to be larger in other examinations (Pape, 1928; Young & Alcorn, 1981). Variability in conidial morphology has already been reported in the case of *C. leucospermi,* in which conidia varied in size, colour and wall ornamentation depending on whether the culture was *in vitro* or *in vivo* and on age (Taylor & Crous, 2001). *C. minitans* was clearly distinguishable in conidial and cultural characters.

Spore germination and pycnidial production of all isolates were observed in a broad temperature spectrum (15 °C to 25 °C), which is in agreement with observations in nature, where the first disease symptoms can be observed from May and increase during the summer. This result indicates that temperatures that range from 15 °C to 25 °C promote infection, whereas higher temperatures reduce infection. Culturing on oat flake agar at 20 °C in the dark was optimal for the mass production of conidia *in vitro*, an observation that will be important for the establishment of resistance tests on *Helleborus*. In these tests, temperatures between 15 °C and 20 °C should be guaranteed for conidia mass production and the infection of plants.

During this study, only the anamorphic stage of the fungus was observable, and no sexual state was found, suggesting that *C. hellebori* predominantly exists in its asexual form, as was also reported for *C. zuluense* (van Zyl et al., 2002a).

Young and Alcorn (1981) found that pre-wounding greatly enhanced plants' susceptibility. To gain further insight into the infection of *Helleborus* plants with leaf spot disease, the inoculation of plants with injured leaves and leaf discs representing strongly wounded tissue was performed in this study. In agreement with Young and Alcorn (1981), leaf discs and wounded leaf parts browned and faded 14 days after inoculation, while intact leaves were not that much affected in this study. One symptom observed on plants in this study, but not in naturally occurring infections, were the small black spots, probably representing small

necroses where the fungus was not able to overcome a barrier. All 25 *C. hellebori* isolates were verified as pathogens of *Helleborus* by means of Koch's postulates, but no differentiation among different levels of virulence was possible.

Phylogenetic analyses of RAPD marker data, which turned out to reveal highly reproducible band patterns, confirmed the similarity of the collected *C. hellebori* isolates (Figure 4.10). Genetic relationships using RAPD markers were successfully determined for several other fungal species, such as *Aspergillus* species and their teleomorphs (Abu Seadah & El Shikh, 2008), *Coniothyrium minitans* (Grendene et al., 2002) as well as plant pathogens like *Fusarium culmorum* (Gargouri et al., 2003), *Ophiostoma ulmii* (Solla et al., 2008) and different *Colletotrichum* species (Martín & García-Figueres, 1999; Denoyes-Rothan et al., 2003). In comparison to genetic similarities of 62-86 % estimated between *C. zuluense* isolates (van Zyl et al., 2002b), a plant pathogen causing stem canker of *Eucalyptus*, the genetic distances calculated for *C. hellebori* were lower, corresponding to higher similarity, and were more comparable to distances determined within and between *Colletotrichum* species (Martín & García-Figueres, 1999).

The RAPD data analysis in this study showed that all *C. hellebori* isolates formed a single clade that was separate from *C. minitans*. In this clade, two subgroups were identified, but no correlation with geographic location could be identified. The genetic distances of isolates outside Europe were similar to those collected in Europe. Originally, those isolates must have been imported from Europe, because hellebores are not native to America.

Interestingly, 10 of 12 isolates in the first subgroup of the *C. hellebori* clade were derived from *H. x hybridus* or *H. x sternii* plants, whereas 11 of 13 isolates in the second subgroup were obtained from *H. niger*. Therefore, a correlation between the isolates' position in the phenogram and its *Helleborus* host species cannot be excluded. In addition, the two groups correlate more or less with conidial length. Conidia of the first subgroup were longer with 4.9 µm minimal length, 5.0 µm on average and 5.1 µm maximum compared to a minimal 4.2 µm, average 4.7 µm and maximal length of 4.9 µm in the second subgroup. The isolates from the first subgroup grew on average more rapidly at 25 °C [(0.72-)2.07(-2.71) mm/day] than those from the second subgroup [(0.58-)1.36(-2.68) mm/day]. The range of radial mycelial growth at 25 °C overlapped almost entirely for both groups, but was differing on average between these groups. However, the isolates of the two clades did not differ in pathogenicity on *H. niger* as shown and discussed before.

In summary, two *C. hellebori* groups were clearly identified in the phenogram: isolates in the first subgroup were predominantly obtained from *H. x hybridus* and *H. x sternii*, had longer conidia and their mycelia tended to grow on average more rapidly at 25 °C compared to isolates in the second subgroup. These observations may lead to the assumption that hellebore leaf spot disease may be caused by two subspecies. The reports on co-occurrence of more than one species or at least varieties associated with the same disease have increased since phylogenetic approaches have been used to study species isolates (Douhan et al., 2008). Especially in species, in which sexual reproduction was not known to occur, cryptic species were identified by phylogenetic studies (Cortinas et al., 2006; Le Gac et al., 2007).

Two species associated with stem canker of *Eucalyptus,* which was formerly described as *Coniothyrium zuluense* (Wingfield et al., 1996), were revealed on the basis of phylogenetic analyses. The two species identified (Cortinas et al., 2006), of which one was described as cryptic, differed slightly in certain morphological characters as it was found also for *C. hellebori* in this study. However, to resolve whether two species or at least species varieties causing hellebore leaf spot disease were identified, supplementary analyses would be necessary (Chapter 5.3).

For now, the small genetic distances, the knowledge about the two distinct groups and the lack of differences in virulence, represent important information, especially for the establishment of resistance tests. Future tests on the response of *Helleborus* species and hybrids to *C. hellebori* will be facilitated by inoculations with a mixture of only a few isolates from the two different subgroups. Thus, the results of this study regarding the establishment of appropriate conditions for *C. hellebori* culture, sporulation and plant inoculation on the one hand and the determination of molecular genetic relationships of diverse isolates on the other hand are fundamental for future breeding endeavours to achieve resistant or more tolerant *Helleborus* species (Chapter 5.4).

5 Conclusions and Outlook

Three different studies with various main focuses were conducted within this thesis (Chapters 2, 3 and 4). The results of these studies have already been discussed directly in the corresponding chapter. Here, some conclusions are drawn relating to different issues in this thesis. In addition, an outlook for future research topics based on the results from this thesis and the conclusions is given.

5.1 Postzygotic isolation mechanisms and maternal effects within the *Helleborus* genus

Postzygotic barriers during embryo development leading to embryo abortion have successfully been overcome by embryo rescue, but additional postzygotic isolation mechanisms like hybrid sterility or hybrid breakdown may occur. It is known that hybrid sterility is the most common form of postzygotic reproductive isolation in plants (Ouyang et al., 2010). Additionally, extensive variability in viability and fertility has been observed within and between hybrid generations from the same interspecific cross (Rieseberg & Carney, 1998).

Hybrids between species belonging to the same *Helleborus* section were described as fertile (Mathew, 1989); therefore, fertility is assumed for the intrasectional hybrids within the sections Chenopus and Helleborastrum that were obtained during the experiments described in this thesis. In comparison, for the intersectional hybrids *H. x ballardiae*, *H. x nigercors* and *H. x ericsmithii*, hybrid sterility has been observed (Mathew, 1989), suggesting that the intersectional hybrids *H. x hybridus* x *H. argutifolius*, *H. foetidus* x *H. argutifolius* and *H. x hybridus* x *H. niger* may also be sterile. However, evaluation of the *Helleborus* hybrids obtained during this thesis for additional postzygotic isolation mechanisms cannot be carried out until the plants are flowering. Nevertheless, various causes of hybrid sterility are described below.

Hybrid sterility may be caused by genetic factors and/or chromosomal rearrangements during meiotic pairing, which might occur in crosses between chromosomally divergent species (Rieseberg & Carney, 1998). In addition, chromosomal instabilities have been observed in crosses between genetically distant species, even though the parental species had the same chromosome numbers (Dhooghe et al., 2010). Intergeneric hybrids between *Anemone* and *Ranunculus* turned out to be mixoploid, suggesting that genome instability led to the elimination of parental chromosomes (Dhooghe et al., 2010). With regard to *Helleborus*

hybrids, the same chromosome numbers were determined for *Helleborus* species. Further analyses of the hybrids will be required to assess genome stability.

According to the Dobzhansky-Muller model, postzygotic reproductive barriers result from a deleterious interaction between functionally divergent genes from the hybridising species (Dobzhansky, 1937). Studies of the genetic basis of hybrid sterility and hybrid breakdown in rice have revealed that several mechanisms are involved. In rice, sterility involves a cytoplasmic gene that causes both male and female sterility (Li et al., 1997). Interactions between a pair of complementary genes that lead to greatly reduced fertility also have been described (Li et al., 1997). Approximately 50 loci have been identified as controlling hybrid fertility in rice, including loci that cause female gamete abortion, those that induce pollen sterility or those that cause both (Ouyang et al., 2010).

In *Helleborus*, the basis of sterility, male or female or both, has not been described. In this study, pollen viability was assessed for *H. x ballardiae* and *H. x ericsmithii*. The pollen was deformed and not stainable, thus at least male sterility was supposed, perhaps due to the above-mentioned mechanisms or cytoplasmic male sterility, which also presents some type of gene interaction. Cytoplasmic male sterility (CMS) is a maternally inherited condition in which a plant is unable to produce functional pollen (Schnable & Wise, 1998). The restoration of fertility relies on nuclear genes that suppress cytoplasmic dysfunction. In conclusion, the combination of the maternally-inherited cytoplasm and the nuclear restoring genes results in fertility or sterility. If sterility of *Helleborus* hybrids is caused by CMS, compatible nuclear restorer genes would be either missing or in a homozygous recessive condition.

In addition to CMS, the genome-plastome combination may also influence other traits. In the case of interspecific hybrids between *Senecio jacobaea* x *S. aquaticus*, the hybrids display higher fitness than the parental species and the reciprocal cross (Kirk et al., 2005). The higher fitness was attributed to maternal benefits, which could be conferred by the non-nuclear inheritance of genetic material and organelles from the maternal plants (Kirk et al., 2005).

In addition to cytoplasmic maternal effects, there may also be phenotypic maternal effects as a result from the environment or the genotype of the maternal parent (Roach & Wulff, 1987). These influences can either be structural or physiological and could come from the tissues surrounding the embryo and endosperm, which are all maternal in origin (Roach & Wulff, 1987). These tissues, the integuments of the ovule and the wall of the ovary eventually form the seed coat, fruit, and other seed structures. These are known to be important determinants

of seed dormancy, dispersal and germination traits. Variation in these structures could determine the mature phenotype of an individual (Roach & Wulff, 1987).

Maternal effects could affect endosperm development (Chapter 3.4.3), could be the result of the non-nuclear inheritance of genes or structures in the cytoplasm (Chapter 5.1) or could be phenotypic (Chapter 5.1) and greatly impact the success of certain crossing combinations. These effects are relevant, if the reciprocal cross fails to produce hybrid offspring or if the offspring exhibit lower fitness. Maternal effects may have occurred in the work described in this thesis in the cross between the two *Helleborus* species *H. x hybridus* and *H. niger*. Crosses using *H. x hybridus* as a maternal parent were successful in hybrid offspring production, though the reciprocal cross failed. In addition, all interspecific crosses with *H. niger* as the seed parent failed. Therefore, it is not known whether there is a broad maternal effect or whether environmental conditions played a role. Hybrids with *H. niger* as the maternal parent already exist (Table 3.16). Further experiments will be necessary to examine this issue.

5.2 Influence of seed dormancy on embryo rescue

Seed maturation in hellebores has to be regarded in combination with seed dormancy. In *Helleborus*, as a member of the *Ranunculaceae*, morphological dormancy (MD) and morphophysiological dormancy (MPD) are apparent (Chapter 1.2). For *Helleborus* it has been observed that warm temperatures are needed during embryo growth (Nimii et al., 2006); this treatment is supposed to break MD. In seeds with MPD the underdeveloped embryos have physiological dormancy that prevents germination (Baskin & Baskin, 2004). Eight types of MPD exist, which are distinguished based on the level of physiological dormancy: non-deep, intermediate and deep (Table 1.3). These classes are divided into two categories, simple and complex. In seeds with simple MPD, warm temperatures are necessary for embryo growth, while cold temperatures are needed for seeds with complex MPD.

Studies on seed dormancy-breaking treatments have already been carried out to identify suitable conditions for the germination of *Helleborus* seeds (Lockhart & Albrecht, 1987; Nimii et al., 2006; McElhannon et al., 2008). It is known that the embryo is still underdeveloped in a mature seed released by the plant and that the embryo needs an appropriate amount of time and warm temperatures to develop further (Nimii et al., 2006). Gibberellin treatments have not been more successful to break the dormancy of *Helleborus* seeds than warm-cold stratifications (Oenings, personal communication). Therefore, MPD is

supposed to be simple, and if cold stratification is needed to break the physiological component, it is probably deep (Table 1.3). In conclusion, for *Helleborus* a deep simple MPD can be assumed. The process of seed maturation in the plant is still totally unknown.

In the embryo rescue experiments of this thesis, most of the embryos that were rescued from ovules failed to develop further *in vitro*. This result can be explained by the fact that the physiological dormancy was not broken, although the ovules received a warm-cold stratification treatment before embryo excision. *Helleborus* seed dormancy and embryo development depend on different temperatures. It will be interesting to know whether physiological dormancy is only located in the embryo or if it is also imposed by the tissues surrounding the embryo. A better understanding of this issue would be helpful for the development of dormancy breaking strategies, which is of major interest in *Helleborus* propagation and breeding.

Observations of immature seeds from intraspecific crosses at different stages during this study revealed that an embryo is only visible if the endosperm was solidified, as it was also found in the embryo rescue experiments; but solid endosperm with embryos was not found until approximately the 10th week after pollination (data not shown). Therefore, it will be interesting to gain a deeper understanding of seed maturation in the plant; e.g., which embryological stages and what endosperm texture are found at what time. With this knowledge, it will be easier to optimise the periods of different temperature treatments during *in vitro* culture and maybe shorten the process of embryo rescue.

5.3 The cause of hellebore leaf spot disease

The hellebore leaf spotting pathogen *C. hellebori* was analysed in detail within this project for the first time. Isolates from different geographical origins revealed to have low variability with repect to morphological characteristics and genetic distances. Only asexual stages were observed during the morphological examinations of the *C. hellebori* isolates. The 25 analysed isolates were distinguished into two groups by RAPD analysis (Chapter 4.3.3). These two groups correlated with the *Helleborus* host species and morphological features such as conidial length and mycelial growth at 25 °C. Therefore, it is proposed that two species, one possibly cryptic, or at least subspecies are associated with hellebore leaf spot.

Two species were identified by phylogenetic analyses to be associated with *Coniotyhrium* canker of *Eucalyptus*, formerly *Coniothyrium zuluense*. These species are *Teratosphaeria gauchensis* and *T. zuluensis* (Crous et al., 2009). These two species correlated geographically:

T. gauchensis was found to be in South America, Hawaii and two isolates were found in Uganda, whereas *T. zuluensis* was found in African countries and South-East Asia (Cortinas et al., 2006). Both species share similar morphological characteristics, but small differences in spore size and growth at different temperatures according to their geographic origin were found.

Similarly, two groups were identified by RAPD analysis within isolates obtained from hellebore leaf spot disease. Therefore, it is assumed that two subspecies or varieties are associated with the disease. DNA sequence comparisons of different genes will be necessary for confirmation. The most common genes used for phylogenetic analyses of fungal species reside in the ribosomal RNA (rRNA) gene cluster including the internal transcribed spacer (ITS) regions ITS1 and ITS2, the inter-genic spacer IGS, 5.8S rRNA, 18S rRNA, and 26S rRNA genes (Xu, 2006). These multi-copy genes are supposed to be highly conserved within a species, though they can be quite variable between species (Xu, 2006). Other commonly used genes are the mitochondrial ATPase subunits, beta-tubulin (BT) and elongation factor (EF-1) (Cortinas et al., 2006). To determine whether cryptic species are involved in hellebore leaf spot disease, in which sexual reproduction is supposed to be absent, experiments to induce sexual stages could be carried out. Sequences of the mating type-loci (*MTL*) could also be analysed because mating type loci have already been analysed from many fungi that were supposed to exist predominantly in its asexual form (Kueck & Poeggeler, 2009).

Fundamental knowledge of hellebore leaf spot disease has been obtained in this study. A collection of 25 isolates from different geographic origins was established and characterised with molecular genetics. To gain a deeper insight of this pathogen, supplementary analyses could be performed as mentioned above.

5.4 Resistance of interspecific *Helleborus* hybrids to hellebore leaf spot disease

The relationship between *Helleborus* hybrids and hellebore leaf spot disease will now be addressed. There remains a lack of knowledge of symptom development in the host and in the differences in susceptibility and resistance between *Helleborus* species. The mechanisms influencing hellebore susceptibility remain unknown. The general aspects of hybrid vigour, especially with regard to diseases and pests, are explained in the following.

The fitness of hybrids is often compared to that of the parental plants. Hybrid genotypes are highly heterogeneous with regard to fitness, within as well as between generations (Rieseberg

& Carney, 1998). In addition, ecological factors can influence fitness making it even more difficult to obtain reliable data on hybrid fitness. In particular for species with strong post-fertilisation reproductive barriers, the average viability and fertility of early hybrid generations is predicted to be lower than that of the parental species due to the break-up of adaptive gene combinations (Dobzhansky, 1937 in Rieseberg & Carney, 1998). In contrast, the fitness of certain hybrid genotypes appears to be equivalent to or exceed that of the parents for some fitness parameters due to heterosis effects. Lifetime fitness (long term evaluation) was not determined in most studies (Rieseberg & Carney, 1998). Enhanced hybrid fitness should be reasonable, if post-fertilisation barriers are weak, a fact being not conflictive with Dobzhansky's (1937) model.

The responses to diseases and pests are also expected to vary. The response of hybrids to herbivores and pathogens can vary considerably, depending partially on the hybrid generation or the hybrid genotype, as was described for fitness in general. Increased susceptibility, as well as enhanced resistance compared to the parent plants, was observed. Fritz et al. (1994) advanced different hypotheses based on the inheritance of resistance in F_1 hybrids: (i) the additive hypothesis: hybrid resistance does not differ from the midparent value, (ii) dominance hypothesis: hybrid resistance is similar to that of one parent, (iii) the hybrid-susceptibility hypothesis: hybrids are less resistant than either parent and (iv) the hybrid-resistance hypothesis: increased resistance compared to both parents due to heterosis.

Hybrids may have a transgressive or unique morphology and chemical traits. They may exhibit novel resistance traits that are not found in either parental species. Some chemical defences indicate heterosis in hybrids (Fritz et al., 1999). Secondary compounds are often inherited additively or complementarily (Rieseberg & Carney, 1998) and could be one possible general source of resistance in hellebores. Hellebores are famous for their secondary metabolites, e.g., glycosides, saponin and steroids (Colombo et al., 1990). All plant parts of hellebores are poisonous, and allergic reaction may be triggered in people that touch the plants. Some of these aspects should in future analyses be connected to the plant-pathogen system *Helleborus–C. hellebori*.

In the raspberry, it has been demonstrated that cultivars with hairy canes are less infected by *Coniothyrium fuckelii,* which causes stem canker in *Rubus* spp., and other diseases affecting *Rubus* spp. (Jennings, 1982). Gene *H,* whose recessive alleles produce non-hairy canes, was identified as being responsible for cane hairiness. The gene may be closely linked with several gene complexes that individually confer resistance, or it may itself confer resistance through

pleiotropic effects (Jennings, 1982). To improve resistance characteristics, interspecific *Rubus* hybrids were obtained by crossing species of *R. pileatus, R. mesogaeus, R. coreanus* and *R. lasiocarpus*. These showed resistance during inoculation experiments, but the F_1 hybrids were largely susceptible (Jennings, 1979), exemplifying the hybrid-susceptibility hypothesis.

In *Helleborus*, differences between species and interspecific hybrids in symptom occurrence and development have been described (Peter Oenings, personal communication). Wounded leaves were found to be more affected than intact ones, and less susceptible genotypes were identified within interspecific hybrids of *H. x ericsmithii* (*H. niger* x *H. sternii*) (Peter Oenings, personal communication). The variability in *H. x ericsmithii* susceptibility to *C. hellebori* was also found to depend on the genotype (Peter Oenings, personal communication) as reviewed in general by Rieseberg & Carney (1998).

There have not yet been any *Helleborus* species or genotypes identified that act either susceptible or resistant on *C. hellebori*. A substancial analysis of the *Helleborus* species would be valuable and necessary to identify sources of resistance against *C. hellebori*. The collection of 25 *C. hellebori* isolates and the system of *in vitro* mass spore production that are described in this thesis (Chapter 4) fulfil fundamental prerequisites for *Helleborus* resistance screenings. Further, the identification of phenotypic classes of resistant and susceptible plants, which could be used for controlled crossing experiments resulting in a segregating population for the resistance trait, could clarify the inheritance of hellebore leaf spot resistance.

5.5 Conformance of the three thesis objectives

The three main objectives of this thesis and the methods that were used to achieve them were described in Chapter 1.6. Here, the objectives are listed again and their achievement is briefly explained.

1. To obtain interspecific hybrids, fundamental knowledge of the relatedness of *Helleborus* species was acquired.

   Cytological analyses determined the same chromosome number of 2n=32 for all analysed *Helleborus* species (Chapter 2.3.1). Nuclear DNA contents were estimated for 21 *Helleborus* species (Chapter 2.3.2). Species of the two sections Chenopus and Griphopus differed strongly in nuclear DNA content from the other species; therefore, interspecific hybrids with one parent belonging to one of these sections can be easily identified with flow cytormetry. Genetic relationships within the genus were determined for 19 *Helleborus* species (Chapter 2.3.3). The genus' division into subgenera and sections was reflected in the phenogram based on 1109 AFLP marker fragments. Genetic distances between species provide novel information to compare relationships between two species directly.

2. Interspecific hybridisations often suffer from pre- or postzygotic crossing barriers. Therefore, crossing barriers needed to be localised and methods to overcome these barriers were established.

   The viability of fresh pollen and pollen stored for at least nine months was determined via staining. High viability was observed, sufficient for pollination of flowers, even if the pollen was stored for one year at -20 °C (Chapter 3.3.1). Hybridisation barriers were identified as mainly postzygotic; for a few crossing combinations prezygotic barriers may be present as well but do not prevent pollen tube growth to the ovules (Chapter 3.3.2). Postzygotic barriers in crosses between different *Helleborus* species were successfully overcome with *in vitro* culturing of isolated ovules stored at different temperatures (Chapter 3.3.4). Intra- and intersectional hybrids were obtained and confirmed by flow cytometry and RAPD analysis (Chapter 3.3.5).

3. Because disease tolerance against *C. hellebori* is a major concern, biological insights into this pathogen are essential and were obtained.

   A collection of 25 *C. hellebori* isolates from different geographical locations was established *in vitro* and morphological characteristics were compared (Chapter 4.3.1). Spore production could be induced by culturing the isolates on oat flake agar (Chapter 4.3.1). Spores were used for a first pathogenicity test on *H. niger* (Chapter 4.3.2). Molecular genetic analyses of the *C. hellebori* isolates were performed using RAPD analysis (Chapter 4.3.3). This analysis revealed low genetic variability and identified two different groups of *C. hellebori* isolates.

In conclusion, the thesis' objectives were achieved and novel information was obtained that can serve as the basis for future projects.

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11/2007-02/2011	Promotion an der Staatlichen Forschungsanstalt für Gartenbau Weihenstephan in Freising im Bereich Zierpflanzenbau und Pflanzenzüchtung (11/2007-05/2009) und an der Leibniz Universität Hannover an der Naturwissenschaftlichen Fakultät im Institut für Zierpflanzen- und Gehölzwissenschaften, Abteilung Baumschule (06/2009-02/2011)
10/2005-10/2007	Studium Gartenbauwissenschaften (Master of Science), Leibniz Universität Hannover Masterarbeit: Entwicklung eines Real-time PCR basierten Verfahrens zur absoluten Quantifizierung von Geminivirus Genomkomponenten
10/2002-09/2005	Studium Gartenbauwissenschaften (Bachelor of Science), Leibniz Universität Hannover Bachelorarbeit: Versuche zur Regeneration und Transformation verschiedener *Vaccinium*-Spezies Ausgezeichnet mit dem Förderpreis des Arbeitskreises deutscher In-vitro-Kulturen im Jahr 2006

Erweiternd zum Studium

08/2006-09/2006	Deutsche Sammlung von Mikroorganismen und Zellkulturen, Pflanzenvirologie, Braunschweig Virusdiagnose, Klonierungsarbeiten, Methoden der Virusübertragung
09/2005 & 03/2006	Pflanzenschutzamt für Gartenbau, Oldenburg amtliche Mittelprüfungen, Diagnose von Pflanzenpathogenen
08/2004-09/2004	Klemm und Sohn GmbH & Co. KG, Stuttgart Kreuzungsarbeiten an *Osteospermum*, Tätigkeiten im Labor
02/2004-04/2004	Institut für Zierpflanzenzüchtung, Bundesanstalt für Züchtungsforschung, Ahrensburg Techniken der In-vitro-Kultur sowie Protoplastenfusion
08/2003	Arche Noah, Gesellschaft zur Erhaltung und Verbreitung der Kulturpflanzenvielfalt, Österreich, Langenlois Sortenerhaltung, Saatguternte und -behandlung

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04/2005-10/2007	Konrad-Adenauer-Stiftung, Stipendiatin

Studienbegleitende Tätigkeiten

05/2006-10/2007	Bildungswerk der Konrad-Adenauer-Stiftung, Hannover Tätigkeit als Tagungsleiterin
10/2004-03/2006	Leibniz Universität Hannover, Wirtschaftswissenschaftliche Fakultät, Lehrstuhl Marktanalyse und Agrarpolitik, studentische Hilfskraft

Berufsausbildung und Berufserfahrung

09/2003	Bruns-Pflanzen-Export GmbH, Bad Zwischenahn, Gärtnerin Koordination des Gartencenterversandes
06/2002-09/2002	Hochschule Wädenswil, Schweiz, Praktikum Projekt zur Entwicklung integrierter Pflanzsysteme
02/2002-05/2002	Bruns-Pflanzen-Export GmbH, Bad Zwischenahn, Gärtnerin Koordination des Gartencenterversandes und der Pflanzenverladung
08/2001-12/2001	Atlantic Nurseries, New York, USA, Gärtnerin Stauden-, Gehölz, Zierpflanzen- und Topfpflanzenproduktion
07/2001	Johann Bruns Baumschulen, Bad Zwischenahn, Gärtnerin Produktion und Pflege verschiedener Containerkulturen
08/1999-06/2001	Johann Bruns Baumschulen, Bad Zwischenahn Ausbildung zur Gärtnerin

Engagement

04/2005-08/2007	BHGL, Studentensprecherin Hannover
04/2003-03/2007	Universitäres Engagement Studentische Vertretung in Studienkommission, Prüfungsausschuss und Studentischem Rat der Leibniz Universität Hannover

Publikationen

02/2011	Meiners, J., Debener, T., Schweizer, G., Winkelmann, T. (2011). Analysis of the taxonomic subdivision within the genus *Helleborus* by nuclear DNA content and genome-wide DNA markers. *Scientia Horticulturae* 128: 38–47
06/2007	Meiners, J., Schwab, M., Szankowski, I. (2007). Efficient in vitro regeneration systems for *Vaccinium* species. *Plant Cell, Tissue and Organ Culture* 89: 169–176

Sprachen

Englisch	Fließend in Wort und Schrift
Französisch	Grundkenntnisse