

INTERSPECIFIC HYBRIDISATION IN WOODY ORNAMENTALS

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VOORWOORD

Beste lezer,

Dat het leven bepaald wordt door toevalligheden, mocht ik ondervinden toen ik in mei 2003, heel onverwacht, een telefoontje kreeg van Prof. dr. ir. Erik Van Bockstaele met de vraag of ik wou werken aan een onderzoeksproject i.v.m. interspecifieke hybridisatie bij sierteeltgewassen. Van interspecifieke hybridisatie had ik toen nog amper gehoord, van sierteeltgewassen daarentegen des te meer. Hoe kan het ook anders, ik ben afkomstig uit Lochristi, de bloemengemeente van Vlaanderen. Ik moest dan ook geen twee keer nadenken over dit voorstel en begin juni 2003 was ik al aan de slag.

Tijdens de uitvoering van dit doctoraat, ondervond ik al snel dat de toevalligheden die het leven bepalen, zich ook continu uiten in het plantenveredelingswerk. Er bestaan dan wel veel technieken om de natuur een handje te helpen, uiteindelijk zijn vele nieuwe plantensoorten het gevolg van 'lucky findings'. Ook de planten die na kruisingen verkregen werden, vertoonden vaak 'toevallige' onverwachte fenomenen die dan weer aanleiding gaven tot diepgaander onderzoek.

Ik heb aan dit doctoraat meer dan vijf jaar met veel enthousiasme gewerkt. Werken met planten eist geduld, véél geduld, wat zeker niet mijn sterkste eigenschap is. Gelukkig kon ik het kruisingswerk (of beter gezegd monnikenwerk) combineren met diepgaander moleculair en cytogenetisch onderzoek. Scheen de zon, dan vond je mij terug tussen mijn planten op het veld, en bij regen zat ik knusjes in het labo. Tijdens de zomermaanden kwamen de kwekers van Best-Select geregeld de planten op het proefveld bekijken en selecteren. Het deed telkens deugd om te zien dat er in de markt interesse bestaat voor mijn kruisingsresultaten. En af en toe was er een internationaal congres of een studiedag, waarop ik mijn resultaten kon voorstellen aan collega-wetenschappers, hetgeen ook de nodige voldoening gaf. Waar ik zeker ook met plezier op terugkijk is mijn stage aan het PRI in Wageningen, waar Prof. dr. Ludmila Khrustaleva mij inwijdde in de wereld van de chromosomen.

Dit doctoraat kan op zichzelf ook een beetje beschouwd worden als een veredelingsproces. Ideeën en bestaande inzichten worden 'veredeld' tot een nieuwe heldere kijk. De uitwisseling van ideeën en informatie kunnen we opvatten als het maken van de kruisingen. Deze ideeën kwamen in de eerste plaats van mijn promotoren Prof. dr. ir. Erik Van Bockstaele en dr. ir. Johan Van Huylenbroeck. Ik wil hen graag bedanken voor de kansen die ze mij geboden hebben en de constructieve samenwerking. Ook dr. ir. Kristiaan Van Laecke en mijn collega-

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Wat de verdere generaties van mijn 'cultivar' betreft, hoop ik dat in de toekomst iemand dit doctoraat nog eens in de hand zal nemen en dat het op zijn beurt aanleiding zal geven tot weer nieuwe studies en inzichten. Met een beetje toeval... wie weet?

Naast toevalligheden zijn er ook zekerheden in het leven. Voor mij zijn dat mijn ouders, broer, zus, familie, vrienden en vooral mijn twee mannen in huis, Willem en Michiel. Zij zijn altijd de rots in de branding geweest tijdens dit soms wel stresserende schrijfproces. Bedankt!

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Lochristi, 3 december 2008

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SAMENVATTING

Binnen de sierboomteelt is productvernieuwing en –verbetering enorm belangrijk en dit vooral in het segment van de zogenaamde visueel attractieve planten. Gewenste karakteristieken hierbij zijn ziekteresistentie (duurzame teelten), winterhardheid, compacte groei, bloemkleur, bladvariaties,... Vaak zijn bepaalde kenmerken wel aanwezig binnen het geslacht, maar niet altijd binnen commercieel interessante soorten of cultivars. Interspecifieke kruisingen kunnen hier een oplossing bieden. Ook polyploidisatie kan aangewend worden om meer variatie te creëren in bestaande soorten. Moleculaire en cytogenetische technieken zijn beschikbaar om het hybride karakter van nakomelingen te analyseren. Tot op heden zijn deze moderne veredelings technieken bij houtachtige siergewassen vrijwel niet geëxploiteerd. Nochtans bieden ze een rijke bron voor nieuwe variatie, geven ze mogelijkheden tot inkruisen van gewenste kenmerken in commercieel aantrekkelijke planten en versnellen ze de selectie. In dit werk werd onderzocht wat de mogelijkheden zijn voor interspecifieke hybridisatie en ploëdiveredeling voor houtachtige siergewassen. Verschillende barrières komen hierbij voor en kunnen omzeild worden m.b.v. *in vitro* embryo rescue technieken, moleculaire en cytogenetische technieken. Nadien werden de beste strategieën bestudeerd voor de karakterisatie van bekomen hybride nakomelingen. Het hiervoor toepassen van morfologische, moleculaire (AFLP) en cytogenetische (GISH, Flowcytometrie) technieken werd geëvalueerd. In dit werk werden de geslachten *Hydrangea*, *Hibiscus* en *Buddleja* als case-studies gebruikt.

De ouderplanten die interessant waren om te gebruiken voor interspecifieke hybridisatie en ploëdiveredeling bij *Hydrangea*, *Hibiscus* en *Buddleja* bleken (cyto)genetisch zeer verschillend. Dit werd bevestigd door AFLP merkers, genoomgroottes en ploëdiegetallen. Voor verschillende *Hydrangea* soorten werd ook een karyotype opgesteld met aanduiding van de nucleolar organising regions (NOR). Hieruit kwamen veel verschillen in chromosoom profiel en NOR locaties naar voor. Daarnaast werden een aantal *Hydrangea* en *Buddleja* soorten teruggevonden die minder goed kiemend pollen produceerden. Al deze gegevens geven een indicatie over hoe kruisbaar de verschillende soorten binnen *Hydrangea*, *Hibiscus* en *Buddleja* zijn.

Succesvolle polyploidisatie, door toediening van oryzaline en trifluraline aan zaden en zaailingen, werd bekomen bij *B. globosa* ($2n = 2x = 38$). Hierdoor kan deze soort beter kruisbaar worden met *B. davidii* ($2n = 4x = 76$). Door gebruik te maken van een polyploidisatie protocol werd ook een nieuwe hexaploëde *H. syriacus* cultivar gecreëerd. *H. syriacus* ‘DVPazurri’ werd recent op de markt gebracht.

Bij het uitvoeren van interspecifieke kruisingen moeten heel wat pre- en postzygotische barrières omzeild worden. Het voorkomen van deze barrières werd in kaart gebracht bij interspecifieke hybridisatie bij *Hydrangea*, *Hibiscus* en *Buddleja*. Prezygotische barrières kwamen niet voor bij de 3 genera. Soortkruisingen werden wel verhinderd door allerlei postzygotische barrières. Abortie van de vruchten in een vroeg stadium van hun ontwikkeling kwam voor bij de 3 genera. Een *in vitro* embryo rescue protocol werd op punt gesteld voor

Hydrangea, *Hibiscus* en *Buddleja* (optimaal kiemingsmedium en maturatiestadium voor *in vitro* initiatie). Kieming van de embryo's verliep vlot bij *Hibiscus* en *Buddleja*. Bij *Hydrangea* werden er bijna geen gezonde embryo's aangetroffen in de vruchten. Bij *Hydrangea* en *Hibiscus* gingen vele F1 planten verloren door albinisme en groeiafwijkingen. Bij *Hydrangea* werden enkel echte hybriden gegenereerd na kruisingen tussen *H. macrophylla* en *H. serrata*. *Hibiscus* hybriden werden bekomen voor *H. syriacus* x *H. sinosyriacus* en *H. syriacus* x *H. paramutabilis*. Bij *Buddleja* werden verschillende F1 hybride generaties opgebouwd na interspecifieke kruisingen. Morfologische studies, AFLP analyses en/of genomgrootte bepalingen bevestigden het hybride karakter van de nakomelingen. Ondanks dat de F1 hybriden bij *Buddleja* en *Hibiscus* weinig fertiel waren, werden F2 en BC1 generaties bekomen voor *H. syriacus* x *H. paramutabilis*, *B. davidii* x *B. x weyeriana*, *B. x weyeriana* x *B. davidii* en *B. davidii* x *B. lindleyana*.

Belangrijk bij interspecifieke kruisingen is dat er bij de hybriden een introgressie plaatsvindt van het pollen DNA in het DNA van de nakomeling. Om dit te bestuderen kan gebruik gemaakt worden van de GISH (genomic *in situ* hybridisation) technologie. GISH is een genomkleuring techniek, die in staat is de parentale genomen/chromosomen in interspecifieke hybriden te onderscheiden. GISH is een recente techniek, die op dit moment al bij vele plantengenera aangewend wordt voor de identificatie van de oorsprong van chromosomen bij hybriden, gegenereerd in kruisingsprogramma's. GISH werd nog niet toegepast voor houtachtige siergewassen, die veelal gekarakteriseerd worden door kleine genomen en een groot aantal kleine chromosomen. In deze studie werd een standaard GISH protocol succesvol toegepast bij *B. x weyeriana* en F1 en F2 hybriden van *B. davidii* x *B. x weyeriana*. De oudergenomen konden duidelijk onderscheiden worden en recombinante chromosomen werden teruggevonden. Voor *Hibiscus* was meer optimalisatie nodig. Verschillende probe/block DNA verhoudingen en pre-annealing, met *H. syriacus* als probe gelabeld met biotine, waren niet succesvol. Wanneer de probe *H. syriacus* gelabeld werd met digoxigenine was het wel mogelijk de oudergenomen te onderscheiden in F2 hybriden van *H. syriacus* x *H. paramutabilis*. Ook hier werden recombinante chromosomen teruggevonden tussen *H. syriacus* en *H. paramutabilis*.

Naast het gebruik van geïnduceerde polyploidisatie en interspecifieke hybridisatie, levert het aanwenden van ongereduceerde gameten mogelijkheden om nieuwe genetische variatie in een veredelingsprogramma te realiseren. Zowel in F1 en F2 hybriden van *H. syriacus* x *H. paramutabilis* werden 2n gameten aangetroffen. De productie van ongereduceerde gameten werd ook gesuggereerd voor *B. globosa* en *B. lindleyana*.

De resultaten van dit doctoraatsonderzoek naar de mogelijkheden van interspecifieke hybridisatie bij houtachtigen geven een duidelijk antwoord op de vraag welke moderne technieken succesvol gebruikt kunnen worden bij de verdeling van houtachtige siergewassen. Een aantal openliggende vragen (bv. productie van 2n gameten bij hybriden, effect van hybridisatie op genomgroottes bij nakomelingen,...) zijn de moeite om verder uit te diepen. Deze studie leidde tenslotte ook tot de selectie van een aantal kandidaat cultivars. Verdere evaluatie ervan zal uitwijzen of deze candidvars uiteindelijk als nieuwe cultivar op de markt kunnen komen.

SUMMARY

In ornamentals, especially in the visually attractive plants segment, constant innovations and improvements of the existing cultivars are very important. Interesting characteristics are resistance, winter hardiness, compact growth, flower color, leaf shapes, ... Desired traits are often available in the genus, but not always within the commercial species and cultivars. Therefore, interspecific hybridisation can be a good solution. Also by polyploidisation more variation can be created within existing cultivars and species. Molecular and cytogenetic tools are available to determine the hybrid status of obtained seedlings. Up till now, these modern breeding techniques are rarely used for woody ornamentals. However, they offer also for woody plants a lot of opportunities to create new genetic variation, to introgress novel traits in commercially interesting plants and to speed up selection. In this dissertation the possibilities of interspecific hybridisation and ploidy breeding for woody ornamentals are investigated. Because of the frequent occurrence of multiple barriers an integrated approach is used with application of *in vitro* embryo rescue techniques, molecular and cytogenetic techniques. Also the best strategies to analyse the hybrid nature of seedlings are determined. Application of morphological, molecular (AFLP) and cytogenetic (GISH, flow cytometry) techniques is evaluated for this purpose. In this thesis *Hydrangea*, *Hibiscus* and *Buddleja* are used as case-study plants.

The parent plants, interesting to use in interspecific hybridisation and ploidy breeding programs within *Hydrangea*, *Hibiscus* and *Buddleja*, were (cyto)genetically very different. This was confirmed by AFLP markers, genome sizes and ploidy levels. For different *Hydrangea* species also a karyotype was constructed with indication of nucleolar organising regions (NOR). From this, many differences in chromosome profile and NOR locations came forward. In addition, some *Hydrangea* and *Buddleja* species produce pollen with a low germination capacity. All these information give an indication about the crossability of different species within *Hydrangea*, *Hibiscus* and *Buddleja*.

Successful polyploidisation by application of oryzalin and trifluralin on seeds and seedlings, was obtained for *B. globosa* ($2n = 2x = 38$). Because of this *B. globosa* could be better crossable to *B. davidii* ($2n = 4x = 76$). Also by using a polyploidisation protocol a new hexaploid *H. syriacus* cultivar 'DVPazurri' was created and recently commercialised.

When performing interspecific hybridisation a lot of pre- and postzygotic barriers have to be circumvented. The occurrence of crossing barriers for interspecific hybridisation within *Hydrangea*, *Hibiscus* and *Buddleja* was investigated. There were no prezygotic barriers observed for the 3 genera. However, interspecific crosses were hampered due to several postzygotic barriers. For the 3 genera abortion of the fruits in an early stage of their development was a major barrier. Therefore, an *in vitro* embryo rescue protocol was optimised for *Hydrangea*, *Hibiscus* and *Buddleja* (*in vitro* germination medium, stage for *in vitro* initiation). Germination was very good for *Hibiscus* and *Buddleja* embryos. For

Hydrangea, almost no healthy ovules could be found in the fruits. A lot of the obtained F1 seedlings of *Hydrangea* and *Hibiscus* were lost due to albinism and growth aberrations. For *Hydrangea* true hybrids were only generated after crosses between *H. macrophylla* and *H. serrata*. *Hibiscus* hybrids were obtained for *H. syriacus* x *H. sinosyriacus* and *H. syriacus* x *H. paramutabilis*. For *Buddleja* several F1 hybrid progenies were obtained after interspecific crosses. Morphological studies, AFLP analyses and/or genome size measurements confirmed the hybrid nature of the seedlings. Although the F1 hybrids of *Buddleja* and *Hibiscus* are almost sterile, F2 and BC1 progenies were obtained for *H. syriacus* x *H. paramutabilis*, *B. davidii* x *B. x weyeriana*, *B. x weyeriana* x *B. davidii* and *B. davidii* x *B. lindleyana*.

For introgressive breeding stable intergenomic recombination is necessary of pollen DNA in the DNA of the progeny plant. Genomic *in situ* hybridisation (GISH) can be used to monitor introgression. GISH is a genome painting technique which allows to distinguish parental genomes/chromosomes in interspecific hybrids. GISH is already applied in many plant genera for the identification of the origin of chromosomes in hybrids resulting from breeding programs, but is rarely used for woody ornamentals, often characterised by small genomes and a high amount of small chromosomes. In this study, a standard GISH protocol was successfully used for *B. x weyeriana* and F1 and F2 hybrids of *B. davidii* x *B. x weyeriana*. The parental genomes could be clearly distinguished and recombinant chromosomes were observed. For *Hibiscus*, more optimisation was needed. Using different ratios probe/block DNA and pre-annealing, using *H. syriacus* as probe labelled with biotin, was not successful. When *H. syriacus* was labelled as a probe with digoxigenin it was possible to distinguish between parental genomes in F2 hybrids of *H. syriacus* x *H. paramutabilis* and recombinant chromosomes between *H. syriacus* and *H. paramutabilis* were detected.

Beside use of induced polyploidy and interspecific hybridisation, also the application of unreduced gametes offers opportunities to create new genetic variation in a breeding program. In F1 and F2 hybrids of *H. syriacus* x *H. paramutabilis* unreduced gametes were observed. The production of unreduced gametes in *B. globosa* and *B. lindleyana* was also suggested.

The results of this PhD. investigation of the possibilities of interspecific hybridisation and ploidy breeding in woody plants give a clear answer to the question which modern techniques can be used in the breeding of woody ornamentals. Some unanswered questions (e.g. about production of unreduced gametes in hybrids, about effects of hybridisation on genome sizes of progenies,...) are interesting for further investigation. Finally, this study also has led to the selection of several candidate cultivars. After further evaluation it will be decided whether or not this candivars can be introduced into the market as new cultivars.

ABBREVIATIONS

AFLP	amplified fragment length polymorphism
BAP	6-benzylaminopurine
BC1-BC2	progeny of the first (BC1) and second (BC2) back cross generation
BEST-Select	Belgium shrub and tree selection
bp	base pairs
B5	Gamborg medium
CCD	charge-coupled device (camera)
cDNA	copy DNA
C-G-A-T	cytosin – guanin – adenin – thymin
CMA	chromomycin A
COL	colchicine
DAPI	4,6-diamino-2-phenylindole
DMSO	dimethylsulfoxide
DNA	deoxyribo nucleic acid
dUTP	2'-deoxyuridine 5'-triphosphate
FDR	first division restitution
FISH	fluorescence in situ hybridisation
FITC	fluorescein isothiocyanate
F1-F2	progeny of the first (F1) and second (F2) generation
GISH	genomic in situ hybridisation
ILVO	Institute for Agricultural and Fisheries Research
IMR	indeterminate meiotic restitution
INRA	Institut National de la Recherche Agronomique
ITS	internal transcribed spacer
2-Ip	isopentenyladenosin
kb	kilo base pairs
LDPE	low density polyethylene
LSD	least significant difference
McGISH	multicolor GISH
MS	Murashige and Skoog medium
M	metacentric (chromosome)
n, 2n	gametic (n) and somatic (2n) cell
NOR	nucleolar organising region
NTS	non-transcribed spacer
ORY	oryzalin
PAR	photosynthetic active radiation
PCR	polymerase chain reaction
PI	propidium iodide
PPM	plant preservative mixture TM
PRINS	primed <i>in situ</i> (DNA labelling)
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RHS	Royal Horticultural Society
RNA	ribo nucleic acid
rRNA	ribosomal RNA

SAPHO	association for the improvement of horticultural plants
SD	standard deviation
SDR	second division restitution
SDS	sodium dodecyl sulphate
SM	submetacentric (chromosome)
SSC	sodium chloride-sodium citrate
SSR	simple sequence repeat (microsatellites)
ST	subtelocentric (chromosome)
T-DNA	DNA of the tumor-inducing (Ti) plasmid
TRITC	tetramethyl rhodamine iso-thiocyanate
TRI	trifluralin
Tyr-FISH	tyramide-FISH
UPGMA	unweighted pair group method with arithmetic mean
WPM	woody plant medium
x, 2x, ...	number of chromosome sets
1C, 2C	DNA content of a genome with chromosome number n, 2n
1Cx, 2Cx,...	DNA content of a genome with x, 2x, ... chromosome sets

INTRODUCTION

OBJECTIVES AND OUTLINES OF THIS WORK

Both growers and consumers show an increasing interest in novel introductions in woody ornamental plants. Novelties in morphological characteristics (flower, growth habitus, leaf color, fruit, ...) and physiological features (winter hardiness, drought resistance, growth vigor, enhanced flowering period, multiplication rate, disease resistance,...) are very important. Nevertheless, breeding efforts of woody ornamental plants remain rather low. The main reasons are the long life cycles and the high cost involved in breeding woody plants. Moreover, for most species the market size is too small to justify the investment to operate large breeding programs. However, woody ornamentals have great potential for improvement also by using more advanced breeding techniques. Among the advanced breeding techniques, interspecific hybridisation and chromosome doubling technology are very powerful tools to produce superior plants. However, these techniques are not much exploited for woody ornamentals.

In this thesis the first main objective was to develop and evaluate different tools that might be used for breeding and selection of woody ornamentals. Interspecific hybridisation and chromosome doubling were attempted in 3 woody ornamental genera (*Hydrangea*, *Hibiscus* and *Buddleja*) in order to yield information on the occurrence and the overcoming of multiple pre- and postzygotic crossing barriers. These 3 genera are interesting for their horticultural value as well as for their genetic characteristics. They belong to the segment of visually attractive plants, which is an economically very important segment for the woody ornamentals. *Hydrangea*, *Hibiscus* and *Buddleja* species are also characterised by small genomes and/or chromosomes and a large (genetic) variation. Because of the frequent occurrence of multiple barriers an integrated approach is used with application of various techniques (*in vitro* embryo rescue technique, molecular and cytogenetic techniques) aiming for sexual fusion, regeneration and screening.

Another objective was to investigate the best strategies to characterise potential hybrids. Since the morphology of hybrids can be very unpredictable, molecular (AFLP) and cytogenetic techniques (flow cytometry, *in situ* hybridisation) are used (and evaluated) to unambiguously identify hybrids. Among the cytogenetic techniques, fluorescence and genomic *in situ* hybridisation (FISH and GISH) protocols were adapted for woody species, characterised by small genomes and chromosomes, to determine the hybrid nature and genomic rearrangements of obtained F1 seedlings.

A last aim of this thesis was to generate and select new valuable pre-breeding material within *Hydrangea*, *Hibiscus* and *Buddleja*.

In view of the practical work in this Ph.D., in chapter 1 a literature overview is presented about the breeding of woody ornamental plants and the cytogenetic tools that can be used in advanced breeding programs. Also a short overview about the genus and the breeding work of the woody ornamentals *Hydrangea*, *Hibiscus* and *Buddleja* is given.

In chapter 2 all general materials and methods are summarised. The more specific materials and methods are described in the particular chapters.

When starting a new breeding program, first the parent plants must be screened and completely characterised. In chapter 3 the parent species used within *Hydrangea*, *Hibiscus* and *Buddleja* are described morphologically together with their ploidy level, genome size, pollen germination capacity and AFLP fingerprint. In this chapter also a karyotype analysis and physical mapping of 45S rRNA genes of 3 *Hydrangea* parent species are made. All these (cyto)genetic and biological information should give indications on cross compatibility and help in decision making on specific cross combinations.

Polyploidisation is useful in a breeding program in order to obtain parent species with an equal ploidy level. Polyploidisation can also be applied to restore fertility of (almost) sterile hybrids or can be used as a breeding technique itself. In chapter 4 mitotic polyploidisation is performed on *Buddleja globosa* (diploid) in order to make it better crossable to *Buddleja davidii* (tetraploid). Further in this chapter mitotic polyploidisation is used as a breeding technique to create a new *Hibiscus* cultivar. Next to mitotic polyploidisation, also meiotic polyploidisation by use of unreduced gametes can generate a lot of variation within genera and species. Evidence for the occurrence of unreduced gametes in *Hibiscus* and *Buddleja* is gathered in chapter 5.

The possibilities of interspecific hybridisation of woody ornamentals in general and of *Hydrangea*, *Hibiscus* and *Buddleja* in particular are described in chapter 5. Interspecific crosses within the 3 genera are made. *In vitro* embryo rescue techniques are applied to overcome postzygotic barriers. The hybrid nature of the obtained F1 and F2 seedlings is characterised by means of AFLP, ploidy level, genome size measurements and morphological parameters.

Introgression is one of the main aims in interspecific hybridisation for introducing a restricted number of traits from the donor species into the recipient. Introgressive hybridisation leads to a transfer of neutral or adaptive traits from one species to another and is important for increasing genetic diversity in genera and species. Intergenomic recombination is essential for introgression. One of the most powerful and effective techniques to detect chromosomal recombination is the cytogenetic technique genomic *in situ* hybridisation (GISH). In chapter 6 GISH protocols are adapted and used to analyse introgression in *Hibiscus* and *Buddleja* hybrids.

Finally, in chapter 7 a general discussion and further perspectives are given.

CHAPTER 1

LITERATURE REVIEW*

1.1 Classical breeding of woody ornamentals

In woody ornamentals a lot of plant innovations were discovered by plant hunters and plant collectors. Especially in the 18th, 19th and early 20th century numerous novel plant species and genera were introduced from e.g. China, Japan and North-America. Several arboreta have been involved in these plant exploration activities. They played a major role in the initial characterisation and evaluation of landscape plant germplasm (Bean 1989a). Still today private nurserymen and some arboreta introduce new plants selected from ‘wild’ populations for their horticultural value (Pooler 2001). Another important source of novelties are ‘lucky findings’ in open pollinated seedling populations (Callaway and Callaway 2000). Seeds might be collected either from wild species or from existing cultivars. A last source of genetic variation are spontaneously occurring mutations (Callaway and Callaway 2000). Since most woody ornamental plants are vegetatively propagated, these mutations might often result in a stable new cultivar.

Reliable data of controlled breeding programs are only available from the last 50-100 years e.g. for outdoor roses (De Vries and Dubois 1996). Only in a limited number of woody plant genera a planned breeding and selection scheme with controlled pollinations is followed. The applications for European plant breeders’ rights show that apart from fruit crops as *Malus* and *Prunus*, the main breeding efforts in woody ornamental plants are in genera important for wood production (*Populus*, *Salix*) and cut flower or foliage production (*Hydrangea*, *Hypericum*, *Ilex*) or in pot plants (*Skimmia*, *Hebe*) and flowering ornamental shrubs (roses, *Rhododendron*, *Clematis*, *Hydrangea*, *Calluna*, *Weigela*,...) (Mac Cartheigh 2008).

Private breeding efforts in woody ornamental plants are in general very limited (with exception of roses and *Rhododendron*). They are focussed on breeding for aesthetic qualities and often do not involve multi-generation crossing programs to incorporate resistance to pests or tolerance to urban and other environmental stress. Only a few public institutions in Europe and United States have still active woody ornamental breeding programs. Reduced support levels have resulted in many public institutions reducing or eliminating their breeding

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programs. New private-public partnerships as in France (INRA-SAPHO; Arène *et al.* 2007) and Belgium (ILVO-BEST-Select cvba) may offer new chances.

Morphological screening and evaluation of seedlings is very common. But for practical breeding programs in woody ornamentals also the development and validation of robust, simple and reliable screening and selection protocols towards biotic and abiotic stress resistance became a priority. A first step is the complete screening of the available germplasm. Until now, selection was mainly focused on morphological criteria. Only the direct use of newly introduced landscape plant germplasm was evaluated in many field trials organised by arboreta or other institutions. Thus, plants that may have possessed some outstanding characteristics have often been discarded because of limitations in general adaptation or other characteristics. Plant germplasm should be characterised for all important traits, including disease (Paulin and Cadic 1999), insect or mite resistance (Gillman *et al.* 1999) and tolerance to various environmental stresses such as cold (Hakam *et al.* 2000) and drought (Cregg 2004). Secondly bio-assays and screening methods for selection towards both biotic (Leus *et al.* 2008) and abiotic stress (Cregg 2004) resistance are desired. Managing 'sustainable resistance' implies a good knowledge of the sources and mechanisms of resistance on the plant side and the evolutionary process and mechanisms of aggressivity and virulence on the pathogen side (Linde and Debener 2003; Leus *et al.* 2006; Dewitte *et al.* 2007).

1.2 Advanced breeding of woody ornamentals

During the last decades new breeding techniques were developed and used mainly in agricultural crops but some of these techniques have also a great potential in woody ornamentals. These modern techniques offer an extended potential to introduce new genetic variation, to screen for valuable germplasm or to enhance selection. One might expect that in the future these will allow a more efficient and straightforward breeding and selection.

1.2.1 Interspecific hybridisation

1.2.1.1 Introduction

Interspecific hybridisation is a very important source of evolution and domestication of ornamental plants. Controlled interspecific and even intergeneric hybridisations are by utmost interesting to combine desired traits from genetically distinct genotypes. Interspecific hybrids have the potential to capture hybrid vigor as well as to combine traits that do not occur within a single species (Van Tuyl and Lim 2003). They can alter not only the qualitative traits but

also the quantitative traits of the parent species. The transfer of alien genes by sexual means requires both the production of F1 hybrids and the subsequent fertility of these hybrids.

However, while natural hybrids can exist between species which flowering times overlap, pre- and postfertilisation barriers hinder the frequency of hybridisation. The difficulty of creating interspecific hybrids increases along with the phylogenetic distance between the parents (Sharma 1995). Often desirable sexual crosses are not successful due to genetic, environmental (Van Tuyl and De Jeu 1997) or geographical barriers. As suggested by Hogenboom (1973; 1975) breeding incompatibility between plants may be controlled by either 2 major mechanisms, incompatibility and incongruity.

1.2.1.2 Incompatibility

Incompatibility is typically involved in crosses between closely related genotypes (Franklin-Tong and Franklin 2000). (Self-)incompatibility has evolved to encourage outbreeding and heterozygosity in flowering plants (de Nettancourt 1977) and is defined as ‘the inability of a fertile hermaphrodite seed plant to produce zygotes after self-pollination’. Self-incompatibility is a relative simple and genetically (S-locus genes) defined example of cell-cell recognition in plants. Self-incompatible plants are able to distinguish between self-pollen and nonself-pollen within the pistil and arrest the further growth and germination of self-pollen (Newbegin *et al.* 1993). Self-incompatible pollen is rejected at some point in the pollination process specific to that species. This may happen at hydration, germination, during growth through the style, in the ovule, or even postfertilisation in some species (Wheeler *et al.* 2001). Most flowering plants are self-compatible, which is generally considered to be the primitive condition. However, self-incompatibility arose a number of times during the evolution of flowering plants (Bateman 1952; Franklin-Tong and Franklin 2000).

Nasrallah *et al.* (2007) demonstrated that hybrid self-fertility can result from epigenetic changes in expression of the S-locus genes that determine specificity in the self-incompatibility response. The loss of self-incompatibility in synthetic hybrids produced by self-fertile and self-incompatible species was analysed in two crucifer genera. Also aberrant processing of S-locus receptor kinase gene transcripts as detected in *Arabidopsis* hybrids and suppression of the S-locus cysteine-rich protein gene as observed in *Capsella* hybrids are two reversible mechanisms by which self-incompatibility might break down upon interspecific hybridisation to generate self-fertile hybrids (Nasrallah *et al.* 2007).

1.2.1.3 Incongruity

Interspecific incompatibility or incongruity, on the other hand, is the mechanism that prevents gene flow between species and establishes the limits to outbreeding (McCubbin and Kao 1996). Hogenboom (1973) suggests that interspecific incompatibility is the result of barriers determined by evolutionary divergence of physiology or morphology between species.

Incongruity occurs in interspecific crosses as a result of lack of genetic information in both parent plants, necessary to complete pre- and postfertilisation processes. Unlike self-incompatibility that is mainly a prefertilisation barrier, incongruity can cause both pre- and postfertilisation barriers (Raghavan 1997). In many interspecific crosses, inhibition of pollen tube growth operates in one direction, whereas the reciprocal cross is successful. This unilateral inhibition is termed ‘unilateral incongruity’ (Kuboyama *et al.* 1994).

Prefertilisation barriers

Some of the prefertilisation barriers after interspecific pollination are (i) inability of pollen to germinate on a foreign stigma, (ii) failure of the pollen tube to reach the ovule due to excessive length of the style or slow growth of the pollen tube which fails to reach the base of the style before the ovary abscises, (iii) bursting of the pollen tube in the style and (iv) failure of the pollen tubes to penetrate the micropyle and to realise fertilisation (DeVerna *et al.* 1987). Several *in vivo* and *in vitro* methods have been developed to overcome prefertilisation barriers. Cut-style pollination is often suitable for hybridisation between parent plants with different pistil lengths since pollen tube growth and development are often impeded in too short or too long styles (Williams and Rouse 1990). It was applied successfully in interspecific hybridisation within *Vriesea* (Vervaeke *et al.* 2001), *Tulipa* (Van Creij *et al.* 1997), *Fritillaria* (Wietsma *et al.* 1994) and *Lilium* (Van Tuyl *et al.* 1991; Fukai *et al.* 2005). Another method is style grafting where a compatible style is grafted on an incompatible ovary (Van Tuyl *et al.* 1991). In lilies, hormone treatment (auxines, cytokinins, gibberellins) of the pollinated style was efficient (Van Creij *et al.* 1997). In interspecific crosses of *Populus* treatment of the stigma with organic solvents such as hexane and ethyl acetate before pollination has been reported to be effective in overcoming prefertilisation barriers (Willing and Pryor 1976). Immunosuppressors such as salicylic acid have been used to produce hybrids in cereals (Mujeeb-Kazi 1981) and legumes (Chen *et al.* 1978). Pollination with mentor pollen (= pollen which is fully compatible with the intended seed parent but which has been treated by e.g. ionizing radiation) together with incongruent pollen was successful for obtaining the crossing *Diploaxis* x *Brassica* (Sarmah and Sarla 1995). Also *in vitro* pollination resulting in the production of viable seeds has been achieved following self-pollination as well as cross-pollination (Bhojwani and Raste 1996).

Postfertilisation barriers

The occurrence of postfertilisation barriers in interspecific crosses may result in deviations at different stages of embryo development. The embryo aborts in a premature stage, often due to the absence or retarded development of the endosperm (Sharma 1995). Both embryo and endosperm have to develop an equilibrium for sharing nutrients in an undisturbed developmental process. When the equilibrium in the development of the zygote and endosperm is disturbed an abortion of the young embryo or disintegration of the endosperm follows (Van Tuyl and De Jeu 1997). This abortion takes place in various stages of development of the young seed. Depending on the stage of embryo abortion *in vitro* media can replace the endosperm, allow maturation of the hybrid embryo and rescue the abortive embryo. This technique offers the possibility not only to rescue seedlings from more crosses,

but also to rescue more seedlings per cross (Zenkteler 1990). Embryo rescue is a promising tool for interspecific breeding and has been proven possible on *Capsella* (Monnier 1988), *Tulipa* (Van Creij *et al.* 1997), *Lilium* (Van Tuyl and Van Holsteyn 1996; Arzate-Fernandez *et al.* 1998), *Arachis* (Feng *et al.* 1996), *Alstroemeria* (De Jeu and Jacobsen 1995; Burchi *et al.* 1998) and *Camellia* (Hwang *et al.* 1992) among others. Reviews have been published by Sharma (1995), Sharma *et al.* (1996) and De Jeu (2000). Though embryo rescue is not always required to obtain hybrids, it might improve crossing efficiency significantly compared to natural crosses (Faure *et al.* 2002). In those crops in which the fruit is aborted before embryo culture can be applied, ovule/seed culture is an easy and fast method (Van Tuyl and De Jeu 1997). This technique is often called ‘in ovule culture’. The technique was applied to obtain interspecific hybrids in e.g. *Gossypium* (Stewart and Hsu 1977), *Lilium* (Van Tuyl *et al.* 1991), *Alstroemeria* (De Jeu and Jacobsen 1995) and *Delphinium* (Honda and Tsutsui 1997). Postfertilisation barriers occurring after successful embryo rescue are hybrid albinism (Yao and Cohen 2000), lack of hybrid growth vigor and hybrid breakdown (Shaked *et al.* 2001; Germana and Chiancone 2001). The main genetic causes of inviability of the hybrid include (i) genome disharmony and incompatible development cues, (ii) the deleterious, complementary action of one or a few gene(s) and (iii) cytoplasmic effects (Levin 1978). A possible solution towards the problem of hybrid albinism, hybrid breakdown and hybrid growth aberrations can be to perform the reciprocal cross or to use bridge plants.

Also F1 sterility in interspecific crosses is very common. Despite survival or even vigorous growth of F1 hybrids, sterility may result in a major barrier to gene flow between species, since it hampers further breeding. Errors in chromosome segregation at meiosis may cause F1 sterility (Heslop-Harrison 1999). Hybrids become sterile because homologous chromosomes are not conjugated, which prevents their correct distribution in two successive meiotic divisions. The resulting microspores contain aneuploid numbers of chromosomes and tend to be non-viable (Shamina *et al.* 1999). Among species or plants, the degree of F1 sterility is normally a fair measure of the closeness of the evolutionary relationship between the parents. F1 sterility can be overcome by polyploidisation (see 1.2.2).

1.2.1.4 Interspecific hybridisation within woody ornamentals

Also in breeding programs of woody ornamentals interspecific hybridisation and techniques to overcome hereby occurring barriers offer an extended potential to introduce new genetic variation. In many woody ornamental species natural occurring or man made interspecific hybrids are described and commercialised e.g. *Acer x conspicuum*, *Aesculus x carnea*, *Amelanchier x grandiflora*, *Berberis x media*, *Buddleja x weyeriana*, *Caryopteris x clandonensis*, *Cotoneaster x watereri*, *Cytisus x praecox*, *Forsythia x intermedia*, *Hypericum x inodorum*, *Ilex x meserveae*, *Mahonia x media*, *Photinia x fraseri*, *Populus x canadensis*, *Spirea x vanhouttei*, *Syringa x chinensis*, *Tilia x europaea*, *Viburnum x burkwoodii* (Hoffman 2005). In different species controlled interspecific hybridisation is attempted such as in *Rhododendron* (Eckhaut *et al.* 2003; 2007), *Populus* (Willing and Pryor 1976) and *Viburnum* (Hoch *et al.* 1995; 2004). Some well known intergeneric hybrids are *x Mahoberberis* and *x*

Cupressocyparis (Hoffman, 2005). In the *Genisteae* tribe research on intergeneric crosses is going on (Bellenot-Kapusta *et al.* 2006).

1.2.2 Ploidy breeding

1.2.2.1 Introduction

Polyploidy is the condition in which a normally diploid cell or organism acquires one or more additional sets of chromosomes. Allopolyploids are those polyploids that have arisen through the processes of interspecific hybridisation and chromosome doubling (not necessarily in this order). Autopolyploids are those polyploids that have arisen from conspecific parents (Soltis and Soltis 2000; Shaked *et al.* 2001). Allopolyploidy is more common than autopolyploidy.

Polyploidy has been considered as one of the most common phenomena associated with plant evolution (Soltis *et al.* 1992) and is an important mechanism of speciation and adaptation in plants (Ramanna and Jacobsen 2003). It has been estimated that 70% of the angiosperms (Stace 1993) and most of the economically useful plant species are polyploids. *Sedum suaveolens* (stonecrop) has the highest chromosome number of any angiosperm ($2n = 640 = 80x$) (Uhl 1978) and fern *Ophioglossum pycnostichum* the highest among all plants ($2n = 1260 = 84x$) (Löve *et al.* 1977). Many plant species, particularly the cultivated ones, such as potato, coffee, banana, wheat, cotton, alfalfa, sugarcane, maize, roses, chrysanthemum,... have gained polyploid genomes or undergone polyploidisation events during their evolution (Ayala *et al.* 2000). Some polyploids are of recent origin, while others are tens of million years old. The proportion of polyploid species has increased over time. Polyploidy is an active, continuous process contributing to an ever-increasing percentage of polyploids (Meyers and Levin 2006). Endoreduplication is the most common mode of somatic polyploidisation in plants and can be found in many cell types, especially in those undergoing differentiation and expansion (Joubès and Chevalier 2000). It appears to be most prevalent in plants with a small genome (Galbraith *et al.* 1991).

The development of (induced) polyploid forms is very important for the breeding of ornamentals and for the creation of more genetic variation. Polyploid plants may be found in agriculture and horticulture as they often possess superior characteristics over their diploid counterparts. For example, polyploids may have larger leaves and flowers, thicker stems and roots, darker green leaves, an increased width-to-length ratio of the leaves, a more compact growth habit and a higher tolerance to environmental stresses (Kehr 1996; Kermani *et al.* 2003). Polyploids are very common and successful because of their better adaptation on broader ecological amplitude, higher colonisation ability (higher outcrossing rate), higher selfing rate (Barringer 2007) and reduced inbreeding depression (deleterious alleles are masked by the extra genomes) (Soltis and Soltis 2000). Negative side effects of polyploidisation are sometimes infertility (however, this can also be positive), brittle wood, stunting and malformations. All these genotypic and phenotypic differences are caused

mainly by an increased cell size (Kondorosi *et al.* 2000), gene dosage (increasing heterozygosity) and allelic diversity (Ramsey and Schemske 1998).

Polyploidy has considerable effects on duplicate gene expression including silencing and up- or down regulation of one of the duplicated genes. The extra copies of essential genes can mutate and diverge, resulting in new traits (sometimes without compromising essential functions). Many expression alterations are organ-specific. Specific genes can be independently and repeatedly silenced during polyploidisation, whereas patterns for other genes appear to be more stochastic (Adams and Wendel 2005). Genomic change may occur very rapidly following polyploid formation at all levels of the genome (Leitch and Bennett 1997). Genomic *in situ* hybridisation (GISH, see 1.3.4.3) can be used to identify structural rearrangements upon polyploid formation.

1.2.2.2 Mitotic polyploidisation

Mitotic chromosome doubling can be achieved by treatment with colchicine, oryzaline, trifluralin or another mitotic inhibitor (Van Tuyl *et al.* 1992). Mitotic inhibitors hamper the formation of the spindle axis along which chromosomes are separated during anaphase. Although DNA is replicated, no new cell wall is formed, causing a doubled DNA content in the nucleus (Rao and Suprasana 1996). Most often colchicine is used but oryzalin and trifluralin are frequently suggested as more efficient alternatives and have been used successfully to produce (allo)polyploids in many crops (Verhoeven *et al.* 1990; Van Tuyl *et al.* 2000). Polyploidy can be induced starting from different types of explants such as shoot tips (Hamill *et al.* 1992; Van Duren *et al.* 1996; Adaniya and Shirai 2001), axillary node cuttings (Awolaye *et al.* 1994), buds (Notsuka *et al.* 2000), ovules (Gmitter and Ling 1991), callus (Roy *et al.* 2001) or complete plantlets (Geoffriau *et al.* 1997).

Induced polyploids might be a shortcut for crop improvement and mitotic polyploidisation has been used for several purposes (Ramanna and Jacobssen 2003). Firstly, altering the ploidy level of parent plants can overcome problems with interploidy crosses. For example, in roses mitotic polyploids were induced by use of colchicine and oryzalin (Kermani *et al.* 2003) or trifluralin (Zlesak *et al.* 2005). Secondly, as hybrids resulting from crosses between distantly related plant species often are sterile, mitotic polyploidisation is widely used to restore fertility in hybrids (Rose *et al.* 2000a; Lim *et al.* 2001a). And finally, in some cases creating sterile triploid cultivars might be a breeding goal itself. In *Weigela* triploid cultivars were obtained from crosses between diploid and a chromosome doubled seedling (Arène *et al.* 2007). Sterility caused by this so-called triploid block is not always complete as was demonstrated in roses (Van Huylbroeck *et al.* 2005).

Also in other woody ornamental crops such as *Rhododendron* (Vainola 2000), *Syringa* (Rose *et al.* 2000a), and *Platanus* (Liu *et al.* 2007) polyploidisation has been realised.

Mitotic allopolyploids produce homogenous gametes, due to the preferential pairing between homoeologous chromosomes (no recombination), which increases the predictability of the F₂,

but on the other hand limits the expression of recessive genes that are only present in the chromosomes of one of both parental species.

1.2.2.3 Meiotic polyploidisation

Meiosis includes 2 successive divisions of the nucleus with one round of DNA replication and leads to the formation of gametes with half of the chromosomes of the mother cell during sexual reproduction. Unreduced ($2n$) gametes originate due to deviating meiosis in plants. They possess the same chromosome number as somatic cells. In breeding, polyploids derived from unreduced gametes (= sexual polyploids) have frequently been shown to be more useful than the chemically induced polyploids for crop improvement (Ramanna and Jacobsen 2003), because only meiotic allopolyploidisation may result in direct introgression of desired genes. Moreover, $2n$ gametes are considered to be the driving force behind the formation of polyploids. As a genetic consequence, three types of $2n$ gametes can be obtained, corresponding to First Division Restitution (FDR, see Figure 1.1), Second Division Restitution (SDR, see Figure 1.1) and Indeterminate Meiotic Restitution (IMR).

Due to equatorial division of sister chromatides, FDR gametes contain an equal number of parental chromosomes. On the other hand, in the case of SDR, sister chromatides move to the same daughter cell. Therefore FDR gametes maintain maximum heterozygosity and SDR display maximum homozygosity in the absence of crossing over (for review see Veilleux 1985; Peloquin *et al.* 1999). Since IMR gametes display a mixture of FDR and SDR depending on the degree of bivalent formation, the genetic consequence of IMR is more complex than that of FDR or SDR (Lim *et al.* 2001a). Meiotic polyploidisation by FDR and IMR can result in high frequencies of homoeologous recombination and in stable introgression of desired traits (Lim *et al.* 2004).

The production of $2n$ gametes has been documented in a wide variety of angiosperm species (Harlan and de Wet 1975; Ramanna and Jacobsen 2003). But normally, the frequency of $2n$ gametes is rather low. In *Lilium*, the formation of $2n$ gametes is dramatically increased upon F1 hybrid formation (Van Tuyl *et al.* 1989), a phenomenon that is also observed in other crops (Van Tuyl and De Jeu 1997; Ramanna *et al.* 2003; Lim *et al.* 2003a; 2004; Barba-Gonzalez *et al.* 2005). In general, the mean frequency of $2n$ gametes found in studies of hybrids (27.52%) was nearly 50-fold greater than in nonhybrids (0.56%) (Ramsey and Schemske 1998). Because interspecific hybrids often experience severe meiotic irregularities involving poor chromosome pairing and non-disjunction, the normal (reduced) gametes of hybrids often possess unbalanced, aneuploid cytotypes and are thus inviable. This suggests that the effective frequency of $2n$ pollen may be even higher than estimated here (Ramsey and Schemske 1998). Therefore $2n$ gamete formation in F1 generation would provide a very convenient tool for efficient introgression of desired genes in F2 interspecific hybrids. Despite this recognition, there have been relatively little efforts made to use $2n$ gametes in crop breeding. Some progress, however, has been made in the case of autopolyploid crops such as potato and alfalfa among others (Ramanna and Jacobsen 2003). Unreduced gametes have

been used for increasing plant vigor, yield, disease resistance and other agronomic characters. Also in roses unreduced gametes gave rise to meiotic polyploids (Crespel and Gudin 2003; Crespel *et al.* 2006). The variation of $2n$ pollen production in roses could be related to environmental fluctuations. The mode of $2n$ pollen formation is genetically equivalent to a FDR mechanism, able to transmit a high percentage of the heterozygosity from the diploid parent ($2n$ pollen producer) to the tetraploid offspring. In case of allopolyploids, $2n$ gametes have been applied in the breeding programs of *Lilium*, *Alstroemeria* and *Vaccinium* (Lim *et al.* 2003b; Lyrene *et al.* 2003; Barba-Gonzalez *et al.* 2004). In *Lilium* and *Alstroemeria*, $2n$ gametes were useful for overcoming sterility in F1 interspecific hybrids, for inducing intergenomic recombination as well as introgression of alien chromosome segments as determined by GISH analysis (see 1.3.4.3).

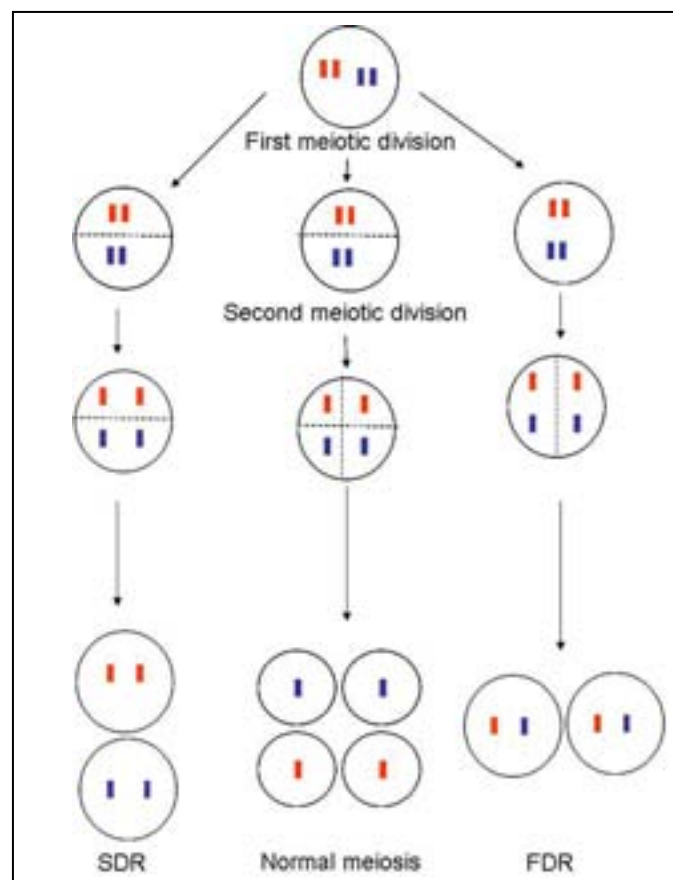


Figure 1.1: Schematic overview of FDR and SDR compared to normal meiosis.

In order to exploit the advantages of sexual polyploidisation, it is necessary to identify genotypes that produce $2n$ gametes in reasonably high frequencies to use them for the generation of progeny either through crossing or selfing. Unreduced gametes can be detected in 4 different ways (Bretagnolle and Thompson 1995). Firstly, unreduced pollen is generally associated with abnormally large pollen size. This association is due to the positive correlation between DNA content and cell volume which, in turn, influences pollen diameter. Secondly, $2n$ pollen can be screened flow cytometrically. With this method, direct

quantification of nuclear pollen DNA is possible. There are relatively few studies in which the nuclear DNA content of pollen was directly measured using flow cytometry (Bino *et al.* 1990; Pichot and Maâtaoui 2000; Sugiura *et al.* 2000; Pan *et al.* 2004). Thirdly, ploidy analysis of the progeny can reveal the presence of unreduced gametes in parent plants. And finally, techniques to stain fixed anthers/ovules can indicate abnormalities of the microsporogenesis which are related to unreduced gametes. Only the latter 2 methods can be used to detect egg cell formation (Lamote *et al.* 2002; Estrada-Luna 2004). Modes of origin of 2n pollen can be determined by analysis of microsporogenesis by GISH (see 1.3.4.3) and other staining methods (Barba-Gonzalez *et al.* 2005).

Several researchers also found that 2n pollen production is stimulated by environmental factors such as temperature (Lokker 2004), herbivory, wounding, water and nutrient stress (Ramsey and Schemske 1998), N₂O treatment (Akutsu *et al.* 2007) and caffeine treatments of immature flower buds (Olden 1954).

1.2.2.4 Haploid induction

In plant breeding also haploid induction can be important. Three main *in vitro* methods are frequently used to generate haploids (Bajaj 1990): (i) culture of excised ovaries and ovules, (ii) embryo culture after wide pollination and (iii) culture of excised anthers and microspores. Pierik (1999) also mentioned application of chemical treatments, temperature shocks, irradiation or environmental stress conditions on microspore or anther cultures to create haploid plants. From literature it is evident that the induction of haploids is largely restricted to a limited number of families: Solanaceae, Brassicaceae, Cucurbitaceae, Asteraceae, Poaceae, Liliaceae (Keller and Korzun 1996). In several ornamentals the production of haploids has been attempted in *Tulipa* (Van den Bulk *et al.* 1994), *Lilium* (Han *et al.* 1997), *Gerbera* (Miyoshi and Asakura 1996) and in roses (El Mokadem *et al.* 2002a; b). In pear successful haploidisation has been achieved by greenhouse selection of seedlings resulting from crosses involving pear varieties or hybrids and by *in situ* parthenogenesis induced by irradiated pollen (Bouvier *et al.* 1993). Haploidisation through anther and microspore culture is also achieved in *Malus* (Hofer 2005; Germana 2006).

1.2.3 Mutation breeding

Many plant species tend to mutate. Ornamental plants are ideal for the application of mutation induction techniques because many economically important traits e.g. flower characteristics or growth habit are easily monitored after mutagenic treatment. Furthermore, many ornamental species are heterozygous and vegetatively propagated. This allows detection, selection and conservation of mutants in the F1 generation. Mutation induction is especially advantageous for breeding woody species due to their long vegetative cycles. Leaf color mutants and dwarf forms are found in woody ornamentals and commercialised (Shum and

Preil 1998; Arène *et al.* 2007). *In vitro* mutagenesis was studied in roses (Ibrahim 1999). In *Forsythia* variation of yellow color induced by gamma rays has been described (Broertjes and Van Harten 1988). In *Rhododendron simsii*, 15 cultivars with new flower colors derived from mutation induction were released mainly during 1970s (Heursel 1980; Maluszynski *et al.* 1992). Most of these alterations also appeared as natural sports. In *Lantana depressa* 2 flower color mutants with yellow and white flowers have been marketed (Datta 1995). A new *Weigela* cultivar with brighter red flowers has been selected in France after mutagenic treatment (Broertjes and Van Harten 1988; Duron 1992). Also a *Caryopteris* mutant plant has been commercialised (Arène *et al.* 2007).

1.2.4 Genetic modification

Genetic transformation of plants in general requires profound molecular insight but allows a directed approach whenever a gene encoding for a desired trait is available (Schum and Preil 1998). In breeding programs for woody plants such biotechniques are today mainly focused on fruit trees (reviewed by Petri and Burgos 2005) and some forest trees from which poplar has become one of the most useful model systems for the study of woody plant biotechnology (Cseke *et al.* 2007). Once a transformant is isolated, vegetative propagation provides unlimited production of the desired transgenic line (Petri and Burgos 2005). In general woody plants are harder to transform and require a longer evaluation process compared to other crops. Transformations are focused on several traits such as herbicide, insect and virus resistance (Jong 2000), lignin biosynthesis, pigments (Davies *et al.* 2003), marker genes, life time after harvest (Serek *et al.* 2004), improved rooting and self-sterility suppression (Petri and Burgos 2005). Some of these traits are obtained by specific transgene expression into proteins. Others are achieved by silencing of transgenes or endogenous genes. In woody ornamentals genetic modification was reported mainly in roses (Van der Zalm *et al.* 1997; Dohm 2003; Debener *et al.* 2004; Katsumoto *et al.* 2007), *Forsythia* (Rosati *et al.* 1996; 2003), *Rhododendron* (Ueno *et al.* 1996; Duneman *et al.* 2002) and *Platanus acerifolia* (Li *et al.* 2007). Genes of interest were altered flower color, disease resistance and improved rooting. Recently also an increasing interest is seen to develop transgenic sterile cultivars of invasive ornamental woody plants (Li *et al.* 2004). The future of genetic transformation of woody plants however requires in general the development of genotype-independent protocols based on transformation of meristematic cells with high regeneration potential and/or use of regeneration promoting genes. Also public acceptance remains a bottle neck to come to a break-through.

1.2.5 *In vitro* breeding

In vitro clonal micropropagation is a widely used method and protocols are available for most species, although continued optimisation is still required for many crops, especially for woody

ornamentals (Brown and Thorpe 1995). However, for a lot of woody ornamental genera, *in vitro* micropropagation is commonly used. Besides for multiplication purposes, *in vitro* techniques are frequently used in polyploidisation experiments (see 1.2.2) and for embryo rescue after interspecific crosses (see 1.2.1). For the breeding of woody ornamentals, *in vitro* techniques are also used for somatic embryogenesis (Dunstan *et al.* 1995) and to create new genetic variation via somaclonal variation as is the case in e.g. *Rhododendron* (Samyn *et al.* 2002).

Another *in vitro* breeding technique is somatic hybridisation by the fusion of protoplasts allowing circumventing sexual crossing barriers between different species. Theoretically, fusion is possible between cells of completely different origin. However, the regeneration ability decreases as the phylogenetic difference between fusion parents increases. In symmetrical fusions the genetic contribution of both partners is equal, which often decreases the viability, regeneration capacity and fertility of the fusion products. This is the result of a 'gene conflict' caused by unbalanced genomes, as certain chromosomes repel one another, so cell division creates aneuploid cells that fail to develop any further. A solution is provided by asymmetric protoplast fusion. In this approach, the chromosomes of the donor protoplast are fragmented by γ -, X- or UV-irradiation (Hall *et al.* 1992) or ultracentrifugation. These fragments are to be integrated into the acceptor genome (Forsberg *et al.* 1998). Asymmetric hybrids tend to root better, to regenerate easier and to be more fertile owing to the smaller 'gene conflict'. Examples of crosses intermediated by protoplast fusion are found between *Rosa*, *Rubus* and *Prunus* (Squirrell *et al.* 2005), between different *Dianthus* species and between *Dianthus* and *Gysophila* (Nakano and Mii 1993a; 1993b; Nakano *et al.* 1996), between *Primula* species (Mizuhiro *et al.* 2001), between *Pelargonium* species (Nassour and Dorion 2003), between *Iris* species (Tsukatani *et al.* 2002), between *Lilium* species (asymmetric) (Horita *et al.* 2003), between *Dendranthema* and *Artemisia* (Furuta *et al.* 2004) and between *Citrus* and *Poncirus* (Cheng *et al.* 2007).

1.3 Characterisation of hybrids

Apomixis and the possibility of uncontrolled pollination make it necessary to confirm whether or not seedlings obtained from distant crosses are indeed desired hybrids. Different methods of hybrid verification based on morphological, molecular and cytological markers are commonly applied. The most useful are those which provide reproducible results and which may be used at the earliest possible stage of seedling development.

1.3.1 Morphological markers

Rieseberg and Ellstrand (1993) compiled a list of 46 studies that report morphological character expression in hybrids. This revealed several surprising tendencies. First, F1 hybrids

were shown to be a mosaic of both parental (45.2%) and intermediate (44.7%) morphological characters rather than just intermediate ones. An explanation for the high proportion of parental characters expressed in hybrids is that many morphological traits that differentiate closely related species display dominant inheritance patterns. Thus the expression of parental or intermediate traits in hybrids will depend on the nature of the genetic control of a particular character, as well as interactions with the environment. A second important finding was the high frequency of transgressive or novel characters observed in hybrids. Over 10% of morphological characters in F1 hybrids were transgressive and over 30% were transgressive in later-generation hybrids. The expression of transgressive characters was not restricted to a few interspecific combinations but rather seems to be a predictable feature of most first- (64%) and later-generation (89%) hybrids. Rieseberg and Ellstrand (1993) gave several explanations for this expression of novel or transgressive characters in hybrids including (i) an increased mutation rate in hybrids, (ii) the complementary action of new combinations of normal alleles, (iii) the placement of unexpressed (or expressed) alleles in a new genetic background, (iv) the fixation of recessive alleles present in the heterozygous form in the parents, (v) a reduced developmental stability and (vi) simple heterosis (overdominance). Thus the unpredictability of hybrid characters expression morphologically makes that the hybrid nature of F1 seedlings has also to be confirmed by molecular tools such as marker techniques or flow cytometry.

1.3.2 Molecular PCR-markers

A big challenge in breeding woody ornamentals is the long generation time. Molecular marker techniques provide an opportunity to screen plants at a young age for traits of interest. Some of the most widely used applications of PCR-based markers do not require the knowledge of the marker position on the genome of the plant studied. Therefore, RFLP, RAPD, AFLP and microsatellites are efficient for species and cultivar identification, for the control of effectiveness of a cross and for confirmation of hybrid status or inheritance of molecular markers linked with an interesting gene/trait in F1 progenies in an early stage after interspecific crosses (Arus 2000). For woody ornamentals isozyme analysis was used for the genus *Acer* (Tobolski and Kemery, 1992) and the genus *Tilia* (Maurer and Tabel, 1995). Also studies with RAPD markers in the genus *Syringa* (Marsolais *et al.* 1993), *Rhus* (Prakash and Van Staden, 2007) and AFLP-markers in roses (Zhang *et al.* 2001), *Syringa* (Pfosser *et al.* 2000) and *Rhododendron* (De Riek *et al.* 2008) are published. More recently ITS and SSR markers are used for roses (Yan *et al.*, 2005) and *Sorbus* (Oddou-Muratorio 2001). Molecular studies of the inheritance of specific traits and marker-assisted breeding are performed in roses (Debener *et al.* 2003; Linde *et al.* 2004; 2006).

Molecular markers are also used for other purposes such as phylogenetic, taxonomic and biodiversity studies. Molecular phylogeny and biodiversity studies reveal a lot of interesting information to breeders about intra- and interspecific relationships within a specific genus. These data can help for the selection of good parents to be used in breeding programs and for

the selection of interesting offspring plants. Phylogenetic results are published for roses (Wu *et al.* 2001; Koopman *et al.* 2008), *Ceanothus* (Jeong *et al.* 1997), *Hamamelis* (Li 2008), *Weigela* and *Diervilla* (Kim and Kim 1999), *Ilex* (Cuenoud *et al.* 2000) and *Rhododendron* (De Riek *et al.* 2008).

1.3.3 Ploidy level and genome size

One of the most astonishing features recognised about the nuclear genome of higher plants is the variability in the amount and type of nuclear DNA between different species. The variation in 1C DNA content in angiosperms ranges from about 0.01 pg in *Fragaria viridis* to 127.4 pg in *Fritillaria assyriaca* (Bennett 1985; Bennett and Leitch 2005). Part of such variation is due to numerical changes in chromosomes but in many other cases there is a widespread and substantial variation resulting from amplification and deletion of DNA sequences within the chromosomes (Sharma and Raina 2005). Although genome size differences among related species are widely accepted as a species evolutionary attribute, variability within a species is often queried. This scepticism is attributed to instrumental or methodical errors or differences in the content of cytosolic compounds (Greilhuber 2005). However, differentiation of genome sizes has been documented either on interspecific or intraspecific levels (reviewed by Knight *et al.* 2005). Inter- and intraspecific variation in nuclear DNA amount in angiosperm cells is related to cellular characteristics such as cell volume and mass, including pollen size, chromosome size, rate of mitosis and meiosis as well as to organism traits such as minimum generation time (Bennett 1972; 1998), breeding system (self-pollinated species have on average smaller genomes than primarily outcrossing species) and neighborhood size (Govindaraju and Cullis 1991), seed size (Thompson 1990) and rate of shoot growth (Grime and Mowforth 1982). It is also correlated to environmental conditions (Bennett 1976; Grime and Mowforth 1982). These observations have been taken as support for the notion that genome size may evolve in response to evolutionary forces (Cavalier-Smith 1985; 2005). Significant relationships have also been found in intra- and interspecific investigations between DNA amount and latitude (Bennett 1976), altitude (Rayburn and Auger 1990), temperature (Ceccarelli *et al.* 1993), fertiliser treatment (Cullis 1987) and seedling growth rate (Mowforth and Grime 1989).

The chromosomes of woody ornamentals mostly are small and can be difficult to view and to count. Light microscopy is therefore not always a practical method for determining ploidy levels. Compared to conventional chromosome counting, flow cytometry has become a very important tool in plant breeding. Flow cytometry provide a fast and accurate determination of nuclear DNA content that is related directly to ploidy level (Galbraith *et al.* 1983; Dolezel 1991; Dolezel and Bartos 2005). Flow cytometry can be used for mainly 3 purposes: (i) characterisation of available plant material, including screening of possible parent plants for breeding programs, (ii) ploidy level determination after polyploidisation experiments and (iii) offspring screening after interspecific crosses (Eeckhaut *et al.* 2005). Ploidy measurements are performed on DAPI (an AT base specific dye) stained nuclei. Estimates of the genome

sizes are obtained by flow cytometry using intercalating fluorochrome propidium iodide (PI) stained nuclei.

In interspecific and intergeneric crosses, resulting hybrids will normally exhibit an intermediate ploidy level and/or genome size compared with the parent plants. Genome size analysis by flow cytometry proved its use for the determination of interspecific hybrids in a lot of plant genera e.g. *Allium* (Van der Valk *et al.* 1991), *Coffea* (Barre *et al.* 1998), *Cucurbita* (Sisko *et al.* 2003), weedy *Hieracium* (Morgan-Richards *et al.* 2004; Suda *et al.* 2007) and weedy *Amaranthus* spp. (Rayburn *et al.* 2005). The flow cytometric confirmation of hybrid origin of regenerants seems to be superior to other available methods since it is relatively cheap and fast and enables evaluation of a large number of regenerants in a short period. However, differentiation of hybrids based on genome size is limited to sufficient differences among parental genomes within species used in hybridisation attempts (Van Tuyl and Boon 1997).

1.3.4 Cytogenetic markers: *in situ* hybridisation

1.3.4.1 Introduction

Next to incompatibility or incongruity between the parental species and the sterility of the F1 and BC1, also rare or negligible meiotic recombination between the alien chromosomes and one of its homoeologous counterparts, prevents the creation of a viable interspecific hybrid in which incorporation of alien chromatin into the recipient genome is accomplished (Chang and De Jong 2005). However, introgression is one of the main goals in interspecific hybridisation aiming to introduce a restricted number of traits from the donor species into the recipient. It is considered as widespread and very important in the evolution of flowering plants (Anderson 1949). Introgressive hybridisation can lead to a transfer of neutral or adaptive traits from one species to another, can lead to increased genetic polymorphism in one or both parental species or it can have a negative outcome such as an evolution of aggressive weeds or an extinction of rare species. Introgression breeding is important for increasing genetic diversity in genera and species. Introgression or gene flow between crops and wild species has been documented a lot, due to extensive activities in crop improvement (Ananthawat-Jonsson 2001).

Intergenomic recombination is essential for introgression (Lim 2000). During subsequent generations, the number of chromosomes from the donor species will diminish, ultimately leading to plants with single alien chromosomes or chromosome segments. To accept that introgressive hybridisation has occurred there must be evidence of the transfer of genetic material from one species into another. Selections can be made on the basis of specific traits, like disease resistance, aberrant plant phenotype and species-specific molecular markers. Also karyotype analysis to demonstrate the presence of an extra chromosome is often used, since sometimes alien chromosomes in the additions may be morphologically distinguishable (Chang and De Jong 2005). But the by far most important and powerful tool to visualise alien

chromosomes and to detect chromosomal recombination is the cytogenetic technique *in situ* hybridisation (Schwarzacher *et al.* 1989).

1.3.4.2 Karyotyping and fluorescence *in situ* hybridisation (FISH)

Eukaryotic chromosomes all follow similar mitotic and usually meiotic cycles of DNA replication, condensation, division and decondensation. The morphology of metaphase is relatively conserved and the size, centromere (primary constriction) position and the presence and location of the nucleolar organisers (secondary constriction) are characteristic for individual chromosome types.

One of the first applications of cytogenetic methods was the analysis of metaphase chromosomes, termed karyotyping. Until 1969 cytogeneticists were only focusing on the descriptive phase of chromosome research, mostly consisting of simple karyotype analysis and genetics and cytology of numerical and structural chromosome mutations (De Jong 2003). Karyotypes generally display chromosomes ordered in sequence of decreasing length. Microscopic identification of individual chromosomes has long been based on morphological characteristics like arm length, centromere position and secondary constrictions. Pardue and Gall (1970) observed that specific denaturing conditions like NaOH treatment and high temperature can alter the staining properties of chromosome regions. This discovery opened the possibilities of visualisation of banding profiles along chromosomes. Since then, Ag-NOR staining, C- (constitutive heterochromatin; Gill and Kimber 1974), N- (nucleolar heterochromatin; Gerlach 1977) and Q- (quinacrine)-banding techniques have been applied for plant karyotype analysis to identify chromosomes, to discriminate between the NOR-bearing satellite chromosomes and other chromosomes (De Jong 2003) and to recognise gene-rich and gene-poor regions on the chromosomes. In fluorescent chromosome banding, the fluorescent dyes bind directly to DNA either uniformly or basepair specifically and certain fractions of chromosomes or different types of heterochromatin can be differentiated and some information about the DNA sequence behind the chromosome elucidated (Schwarzacher 2003). When DNA differences between species are too large (very different karyotype, large sequence divergence), it can be an indication that interspecific crosses might not be successful.

For example the woody ornamental genera *Viburnum*, *Sambucus* and *Lonicera* have been investigated for chromosome number and karyomorphology including Giemsa-C-banding, fluorochrome banding (DAPI/CMA) and cold treatment (Benkouseppon and Morawetz 1993). Cold-induced chromosome regions are found in *Viburnum* and *Sambucus* and are identical with larger heterochromatin regions, shown by Giemsa-C-banding. Several karyological characters point to a closer relationship between *Viburnum* and *Sambucus*. These genera appear isolated from *Lonicera* which differ remarkably in karyomorphology (Benkouseppon and Morawetz 1993). Karyomorphology studies are published also for *Alstroemeria* (Buitendijk and Ramanna 1996), wheat (Endo and Gill 1984), *Rosa canina* (Lim *et al.* 2005),

Begonia (Oginuma and Peng 2002) among others and basic karyotypes of species up to 210 chromosomes are published (de Freitas *et al.* 2007).

Buongiorno-Nardelli and Amaldi (1969), John *et al.* (1969) and Pardue and Gall (1969) claimed the first successful *in situ* hybridisations of cell spreads and tissue preparations. Their insight came directly from the thought that chromosomal DNA could be made single stranded and allowed to hybridise *in situ* with labelled probe DNA molecules before the sites of hybridisation were detected. Their work was carried out with radioactively labelled satellite DNA and they showed that the sequences with particular base pair compositions were concentrated in particular regions of chromosomes, including the NOR. Later, non-radioactive and fluorescent (FISH) labelling were developed and opened the possibility to address chromatin regions (defined repetitive and single-copy sequences of nucleic acids) of individual chromosomes on the basis of DNA sequence information in addition to morphological features (Shubert *et al.* 2001). The method is based on the site specific hybridisation of single-stranded DNA molecules (probes labelled e.g. with biotin or digoxigenin) to denatured, complementary target sequences on cytological preparation like metaphase chromosomes or interphase nuclei (Figure 1.2). After fluorescence detection steps, the probe sequences become visible at the site of hybridisation.

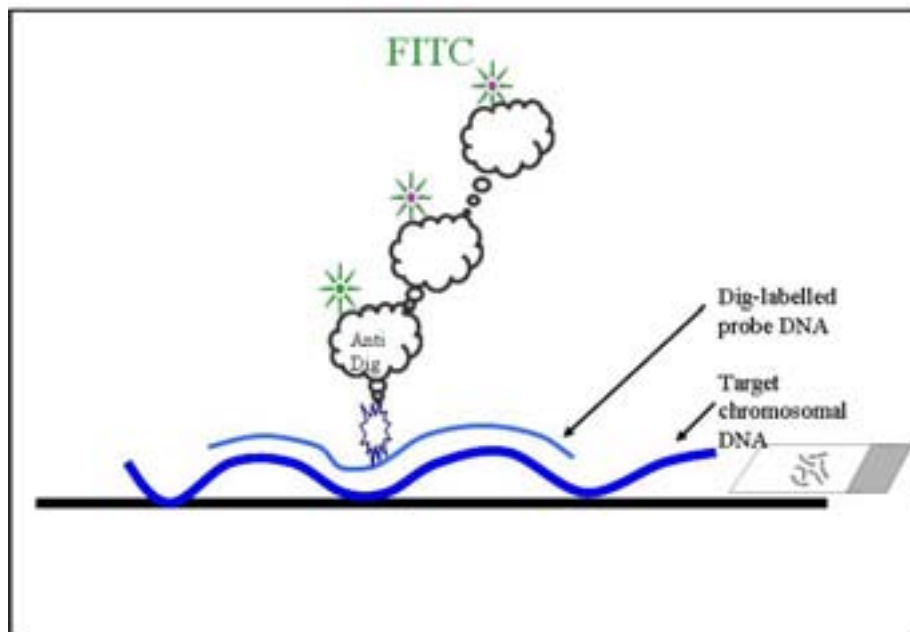


Figure 1.2: Principle of FISH. A single-stranded DNA probe labelled with e.g. digoxigenin hybridise to a denatured complementary target sequence on metaphase chromosomes. After detection with anti-digoxigenin antibody labelled with fluorescein isothiocyanate (FITC), the site of hybridisation can become visible under a fluorescent microscope equipped with proper filter set.

FISH of single-copy DNA sequences including disease-related and other economically important genes in plant species has become indispensable in map-based cloning and other physical mapping strategies. FISH is also valuable for identifying the sites of highly repetitive

genes e.g. ribosomal genes (rRNA genes) which are difficult to map by other methods (Leitch and Heslop-Harrison 1992; De Jong 2003). The localisation of this ribosomal DNA, using FISH play a major role in chromosome identification and karyotype analysis. rRNA genes have been isolated from many different plant species and used as probes for FISH (Schwarzacher 2003). In eukaryotes, rRNA genes are organised as families of tandemly repeated units located at one or a few chromosomal sites (Appels and Honeycutt 1986; Flavell 1986). Among them, 45S rDNA sequences were shown to be on the NOR of satellite chromosomes (Gerlach and Dyer 1980; Leitch and Heslop-Harrison 1992). On metaphase chromosomes, NORs can be identified as secondary constrictions and, due to the abundance of argyrophilic proteins, they can be visualised by silver staining (Goodpasture and Bloom 1975). However, during metaphase, only NORs that are transcriptionally active in the previous interphase, form secondary constrictions and can be silver stained (AgNORs) (Sumner 1982). The silent NORs are not excluded from nucleoli. Recent results from different laboratories indicated that nucleoli are also composed of silent NORs (Akhmanova *et al.* 2000; Sullivan *et al.* 2001; Strohner *et al.* 2001). By 45S FISH both the active and the silent NORs can be detected. The 45S rDNA repeat unit consists of highly conserved coding regions (18S, 5.8S and 25S rDNA), internal transcribed spacers and non-transcribed spacers. In flowering plants, the 45S rDNA repeat unit ranges from 7.5 kb in *Oryza sativa* to 18.5 kb in *Trillium tschonoskii* (Appels and Honeycutt 1986). Variation in non-transcribed spacer (NTS) length and sequence gives rise to the length heterogeneity of rDNA units within and between species. Since the 5S and 45S units are highly conserved, probes isolated originally from wheat can be used to localise the 45S and 5S genes in most eukaryotic species. rDNA, linked with the nucleolar bearing chromosomes, have been frequently detected in the karyotype of various crops (Jiang and Gill 1993; 1994), also for few woody ornamentals. Examples are *Aloe* (Adams *et al.* 2000), *Arabidopsis* (Maluszynska and Heslop-Harrison 1993), barley (Leitch and Heslop-Harrison 1992), *Brassica* (Fukui *et al.* 1998; Iwano *et al.* 1998; Kulak *et al.* 2002; Hasterok *et al.* 2006), *Crocus* (Frello *et al.* 2004), *Hyacinthella* (Puizina *et al.* 2003), *Lilium* (Lim *et al.* 2001b), *Medicago* (Cerbah *et al.* 1999), *Pinus* (Hizume *et al.* 2002), *Ribes* (Chiche *et al.* 2003), soybean (Shi *et al.* 1996), *Vicia* (Navratilova *et al.* 2003) and wheat (Mukai *et al.* 1991a; b) among others.

Also phylogenetic and evolutionary studies in different plant species have been based on the sequence analysis of 45S rDNA (Appels *et al.* 1980; Appels and Honeycutt 1986; Mukai *et al.* 1991a;b; Bachmann 1992).

Other repetitive sequences are retroelements, single sequence repeats or telomere associated sequences (Schwarzacher 2003). Despite their common components, retroelements are very heterogeneous and, because they evolve fast, are often found to be species specific and reflect species phylogeny and evolution (Heslop-Harrison 2000) and can be detected by using FISH.

A number of noticeable advances were made in reliably detecting tiny FISH signals in chromosome preparations and in improving FISH sensitivity for the detection of small hybridisation sites. Using a cooled charge-coupled device (CCD) camera, detection sensitivity can be increased 30-fold due to the integration of digital computer images and the intensity of the fluorescent signals (Wiegant *et al.* 1991). Another approach to increase FISH sensitivity is

primed *in situ* DNA labelling (PRINS; Koch *et al.* 1989). PRINS is based on PCR with target DNA in a chromosomal preparation in the presence of fluorescent-labelled nucleotides. A third method is based on peroxidase-mediated deposition of hapten- and fluorochrome-labelled tyramides (Tyr) and has been shown to considerably increase the signals of FISH (van Gijlswijk *et al.* 1997). Using this Tyr-FISH in transgenic shallots, Khrustaleva and Kik (2001) detected the position of T-DNA inserts on condensed metaphase chromosomes as small as 710 bp. Other FISH protocols with powerful signal enhancements are described by Lansdorp *et al.* (1996), Long and Komminoth (1997) and Zhong *et al.* (2001). DNA or cDNA probes as small as 500 bp can be visualised on metaphase chromosomes. The detection of very small chromosomal targets is no longer a technical limit. However, chromosome folding and the position of the DNA sequence inside the chromosome is the main factor limiting the accessibility of the probe to the chromosome target (De Jong 2003).

1.3.4.3 Genomic *in situ* hybridisation (GISH)

Among all specific adaptations of FISH, the use of total genomic DNA have become most useful in the discrimination of parental species in plant hybrids. This genome painting technique or GISH (Schwarzacher *et al.* 1989) is based on hybridisation with genomic DNA of one of the parental species as probe, labelled with e.g. biotin or digoxigenin (Figure 1.3). The addition of an excess amount of unlabeled DNA from the other parent (blocking DNA) substantially increases the specificity of probing and enables more closely related species to be distinguished.

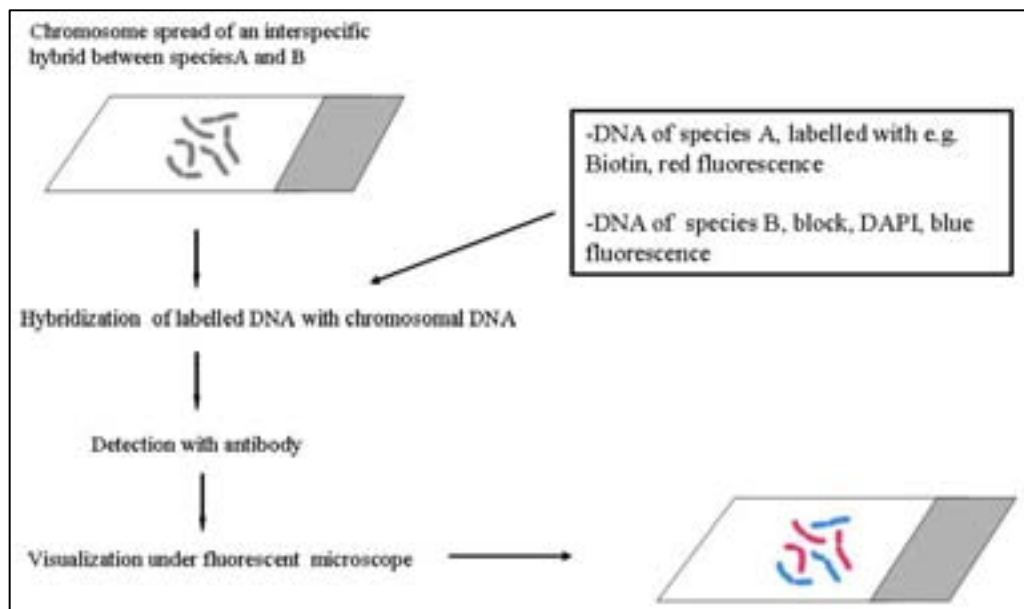


Figure 1.3: Principle of GISH.

GISH analysis of interspecific and intergeneric hybrids provides (i) discrimination of parental chromosomes, (ii) determination of the genome composition in BC progenies, (iii) identification of individual chromosome numbers, (iv) tracing of recombinant chromosomes or chromosome segments and (v) determination of the mechanism of genetic inheritance (Lim 2000). Monitoring of the introgressed alien chromosome segments in the subsequent generation is an important tool in assessing the processes of meiotic recombination, chromosome transmission and genetic analysis methods for confirming the best way of gene flow (Lim 2000).

The technique has been employed in many plant species and their interspecific and intergeneric hybrids such as in *Allium* (Hizume 1994; Keller *et al.* 1996; Khrustaleva and Kik 1998; 2000), *Gasteria x Aloe* (Takahashi *et al.* 1997), *Alstroemeria* (Kuipers *et al.* 1997; Kamstra *et al.* 1999a;b), *Arabidopsis* (Maluszynska and Heslop-Harrison 1993; Fransz *et al.* 1996;1998), banana (Osuji *et al.* 1997; D'Hont *et al.* 2000), *Brassica* (Nagpal *et al.* 1996), *Brassica napus x Raphanus sativus* (Snowdon *et al.* 1997), *Sinapsis alba x Brassica oleracea* (Wei *et al.* 2007), *Coffea* (Barre *et al.* 1998), grass (Bailey *et al.* 1993), *Lilium* (Karlov *et al.* 1999; Lim *et al.* 2000; Lim and Van Tuyl 2002; Van Tuyl *et al.* 2002; Lim *et al.* 2003a; b; Marasek *et al.* 2004; Barba-Gonzalez *et al.* 2006), *Lolium* (Thomas *et al.* 1994; Pasakinskiene *et al.* 1997), maize (Poggio *et al.* 1998), *Nicotiana* (Parokonny *et al.* 1992a), potato (Garriga-Caldere *et al.* 1997), rice (Fukui *et al.* 1997; Yan *et al.* 1999), soybean (Shi *et al.* 1996; Zhu *et al.* 1996), tomato (Parokonny *et al.* 1997; Zhong *et al.* 1998; Ji *et al.* 2004) among others. There exist many examples of successful gene transfer in wheat detected by using GISH (Schwarzcher *et al.* 1992; King *et al.* 1993; Miller *et al.* 1994; Chen *et al.* 1995). Disease-resistant genes from *Hordeum bulbosum* have been transferred into *Hordeum vulgare* background. Molecular cytogenetic analysis combining GISH and FISH has been undertaken to detect and to physically localise the introgressed chromatin on the chromosomes of the recombinant plants (Pickering *et al.* 1997; 1998; 2000). Humphreys and Pasakinskiene (1996) detected the introgression of genes conferring drought resistance from *Festuca arundinacea* to *Lolium multiflorum*. In *Quercus* the technique was used to study genome organisation and evolution in the genus (Zoldos 2008). So far, other GISH analyses on woody ornamentals have not been reported in literature.

If dispersed repeats of the parental sequences are too similar or extensive homogenisation of these parental sequences has occurred already within the hybrids, the chromatin of chromosomes belonging to the parental complements of synthetic interspecific hybrids or presumed allopolyploid species can no longer reliably be discriminated by GISH. However, GISH was useful to identify genomes in interspecific hybrids involving parent species sharing more than 80% sequence homology and having near identical karyotypes as in *Gibasis* (Parokonny *et al.* 1992b), *Solanum* (Wilkinson *et al.* 1995) and *Allium* (Irifune *et al.* 1995; Khrustaleva and Kik 1998). Increased stringency conditions, combined with an excess of unlabelled blocking DNA in the hybridisation mixture, can allow discrimination of genomes sharing up to 90-95% of sequence homology (Parokonny *et al.* 1997).

Multicolor GISH (McGISH) can be used to identify three or more genome origins simultaneously (Mukai *et al.* 1993; Khrustaleva and Kik 2000). Examples of McGISH can be

found in *Allium* (Khrustaleva and Kik 1998; 2000), *Beta* (Desel *et al.* 2002) and *Lilium* (Lim and Van Tuyl 2002).

Raising wide hybrids through somatic fusion is also a major tool for introgressing useful agronomic traits in cultivars where it is not possible to obtain desired hybrids by conventional hybridisation and/or embryo rescue cultural methods. GISH has been successfully utilised to discriminate different genomes involved in somatic hybrids between tomato and wild eggplant (Escalante *et al.* 1998), rice and its wild relatives (Shishido *et al.* 1998), tobacco and its wild relatives (Kenton *et al.* 1993; Kitamura *et al.* 1997), potato and tomato (Jacobsen *et al.* 1995).

The development of GISH also revolutionised the identification of component genomes in known or suspected allopolyploids (Bennett *et al.* 1992; Snowdon *et al.* 1997; Hoshi *et al.* 1994) and the unravelling of the structural rearrangements and genome re-organisation events that took place after polyploidisation (Kenton *et al.* 1993; Jellen *et al.* 1994; Yang *et al.* 1999). Examples are found in *Brassica* (Nagpal *et al.* 1996; Maluszynska and Hasterok 2005), *Aster* (Matoba *et al.* 2007), sugarcane and banana (D'Hont 2005) and wheat (Mukai *et al.* 1993).

1.4 The genus *Hydrangea*

1.4.1 Taxonomy and plant description (McClintock 1957)

Division: Magnoliophyta
Class: Dicotyledonae
Order: Cornales
Family: Hydrangeaceae

The genus *Hydrangea* includes at least 23 species naturally found in temperate regions of Eastern Asia, North America and in subtropical Central and South America (McClintock 1957; Morteau 2003). Several are cultivated as ornamentals (McClintock 1957; Dirr 1998). The *Hydrangea* genus is divided into the section *Hydrangea*, which contains the temperate climate species, and the section *Cornidia*, which contains the vining species from tropical and subtropical climates. As described by McClintock (1957) the cultivated species are all members of the section *Hydrangea* and have been placed into subsections *Americanae* (*H. arborescens* and *H. quercifolia*), *Asperae* (*H. involucrata* and *H. aspera*), *Calytranthae* (*H. anomale*), *Heteromallae* (*H. paniculata*) and *Macrophyllae* (*H. macrophylla*). The mountain *Hydrangea* (*H. macrophylla* subsp. *serrata*) is considered by some to be a separate species (*H. serrata*) closely related to *H. macrophylla*. Flowers of *Hydrangea* are hermaphrodite. *Hydrangea* is known as an allogamous genus (Morteau 2003). However, conflicting

information concerning self-incompatibility has been reported (Kudo and Niimi 1999; Reed 2000; 2004). In Figure 1.4 the most popular *Hydrangea* species are shown.

Hydrangea macrophylla Ser., which is grown both as a garden and florist's plant, is native to Asia. It is valued for its large corymbs that, depending on soil pH and cultivar, range in color from pink to blue and in intensity from pale to deeply colored. Flower buds are set on the previous year's growth. *Hydrangea macrophylla* has long been the most widely grown member of the genus. However, the popularity of several other *Hydrangea* species has greatly increased in recent years. In particular consumer interest in panicle (*H. paniculata* Sieb.) and oakleaf (*H. quercifolia* Bartr.) *Hydrangea* has grown during the last decades (Dirr 1998). *Hydrangea paniculata* which is native to China and Japan, grows 3 to 6 m in height and spread. Inflorescences are produced in mid-summer and consist of 15 to 20 cm long panicles of white to pale pink flowers. It is the most cold-hardy member of the genus. *Hydrangea quercifolia* is native to south eastern United States. Most plants grow 2 m or taller in height with an equal to wider spread. A few compact cultivars are available. Cream-colored conical inflorescences up to 30 cm length are produced in early summer and often turn in an attractive rose-color as they age. Mahogany red autumn foliage and exfoliating bark provide autumn and winter interest in the landscape (Dirr 1998). Inflorescence of *H. macrophylla*, *H. paniculata* and *H. quercifolia* consist of a combination of showy sterile and inconspicuous fertile flowers. Fertile flowers are small (< 10 mm in length) and have 2 to 4 styles (McClintock 1957).



Figure 1.4: The most popular ornamental *Hydrangea* species: *H. macrophylla* (A), *H. paniculata* (B) and *H. quercifolia* (C).

Phylogenetic analysis based on morphology (Hufford 1997; Mortreau 2003), plastid DNA restriction sites (Downie and Palmer 1992), sequences of the plastid genes *matK* and *rbcl* (Hufford *et al.* 2001) and combinations of plastid and nuclear DNA (Soltis *et al.* 2000) have provided valuable insights into the evolution of and the relationships in *Hydrangeaceae*.

Despite its high ornamental value, the genus *Hydrangea* has been poorly studied (cyto)genetically. However, (cyto)genetic knowledge is essential for the management of genetic resources and plant breeding. A study of *Hydrangea* species with molecular SSR markers (Rinehart *et al.* 2006, Jones and Reed 2006; Reed and Rinehart 2007) showed that there exists a large divergence between different *Hydrangea* species. Also the DNA content

varies among the different *Hydrangea* species. Cerbah *et al.* (2001) and Zonneveld (2004) reported the smallest genome size in *H. quercifolia* (1.95 pg.2C⁻¹). There is a 3-fold genome range between *H. quercifolia* and *H. paniculata* (7.00 pg.2C⁻¹). Genome size does not necessarily correlate with chromosome number or fertility. For *H. macrophylla* cultivars mostly 36 chromosomes were counted ($2n = 2x = 36$) but some triploid cultivars have been identified (Demilly *et al.* 2000; Cerbah *et al.* 2001; Zonneveld 2004). Both ploidy types appear fertile. *H. quercifolia*, which has a much smaller genome size, also has 36 chromosomes ($2n = 2x = 36$). *H. paniculata* is mostly tetraploid with 72 chromosomes. Aberrant chromosome numbers of $2n = 2x = 34$ and $2n = 2x = 30$ were found within *H. aspera* and *H. involucrata* respectively. Preliminary karyotypes of *H. macrophylla* and *H. aspera* are published (Cerbah *et al.* 2001; Mortreau 2003).

1.4.2 Breeding of *Hydrangea*

Since the introduction of *Hydrangea* in Europe, gardeners have improved the species by empirical crosses and selection. Cultivar development efforts have primarily relied on the selection of superior plants from open-pollinated seedling populations (Mallet *et al.* 1992; Mallet 1994; Dirr 1998). Compared to wild varieties, cultivars tend to have deeper colored flowers and more rigid and shorter stems (Cerbah *et al.* 2001). Recently a few *H. macrophylla* plants termed remontant or reflowering have been identified (Dirr 2004). Because they flower on the current year's growth, remontant *H. macrophylla* cultivars should flower reliably every year. Nowadays for *Hydrangea*, selections are also done for use as cut flower or pot plant.

Within *Hydrangea* also interspecific hybridisation could offer more opportunities for further improvements. According to McClintock (1957), natural hybrids in *Hydrangea* are rare. A hybrid between *H. hirta* and *H. scandens* has been reported from the Izu Peninsula of Japan (McClintock 1957). Another hybrid between *H. macrophylla* and *H. paniculata* was made in 1910, but was lost during World War I (Haworth-Booth 1959). Reed (2000) reported the development of an embryo rescue protocol for *Hydrangea*. *H. macrophylla* x *H. paniculata* hybrids could be obtained only by using this *in vitro* embryo rescue technique (Reed *et al.*, 2001). However, the obtained hybrids were lacking in vigor and were sterile (Reed, 2004). Production of *H. macrophylla* x *H. arborescens* hybrids required both embryo rescue and subsequent regeneration from callus culture. All plants obtained had aneuploid chromosome numbers (Kudo and Niimi 1999). Kudo *et al.* (2002) reported the production of putative *H. macrophylla* x *H. quercifolia* hybrids using embryo rescue, but no details are available about the vigor or fertility of this interspecific hybrid. The first report of a *Hydrangea* interspecific hybrid that was obtained without the use of embryo rescue was from Jones and Reed (2005). Reciprocal crosses were made between *H. involucrata* and *H. arborescens*. The crosses only were successful when *H. arborescens* was used as the maternal parent. Of more than 500 seeds, only 8 plants remained alive (Jones and Reed 2005; 2006).

Recently a hybrid *Hydrangea* x 'Innovalaur' SEMIOLA®, originating from a cross between *H. anomala* spp. *petiolaris* and *H. seemanii*, was released in France (<http://www.sapho.fr>). In

the same research group crosses were attempted between *H. macrophylla* and *H. paniculata* but were not successful (A. Cadic, personal communication).

Intergeneric crosses were made with *Dichroa febrifuga* and *Hydrangea macrophylla* (Reed *et al.* 2008). According to Rinehart *et al.* (2006) *Dichroa* is related to *H. macrophylla* but its relationship within *Macrophyllae* is not well defined.

In view of genetic modification of *Hydrangea*, a reproducible and efficient regeneration system of *H. macrophylla* plantlets, which is the first necessary step of genetic transformation depending on *Agrobacterium tumefaciens* DNA vector, was presented by Boccon-Gibon *et al.* (2000).

1.5 The genus *Hibiscus*

1.5.1 Taxonomy and plant description (Bean 1989b)

Division: Magnoliophyta

Class: Dicotyledonae

Order: Malvales

Family: Malvaceae

Hibiscus belongs to the *Malvaceae*. It is a polymorphic genus of some 250 species of trees, shrubs and herbs. *Hibiscus* is tropical and subtropical in distribution, with some species extending into the temperate regions of the world e.g. *Hibiscus syriacus* L., *Hibiscus sinosyracus* Bailey and *Hibiscus paramutabilis* Bailey (Bailey 1950; Bates 1965; Beers and Howie 1992). These three species are native to China (Bates 1965) and the similarity in their natural distribution pattern is an indication of a similar tolerance to environmental factors. According to the characteristics of an outcrossing plant summarised by Wyatt (1983), *Hibiscus* is an allogamous species, with hermaphrodite flowers. Han and Yeam (1993) reported seed set after self pollinations ranging from 0% to 100%, suggesting that the self-incompatibility in *Hibiscus syriacus* is modified by pseudo-self-compatibility. According to Allard (1960), *Hibiscus* species are largely self-compatible.

Hibiscus syriacus (Althea or Rose of Sharon) is the most popular species. About 40 different cultivars, with varying flower color and shape, are commonly in culture in Europe and a lot more genotypes are present in different collections (Yu and Yeam 1972; Van De Laar 1997). It is a deciduous shrub with more or less distinctly trilobate leaves (Figure 1.5) (Kim and Lee 1991). Flowering is from the end of July till the first part of October (Bailey 1950; Krüssmann 1962; Bean 1973). Most cultivars are tetraploid ($2n=4x=80$, Skovsted 1941). *Hibiscus sinosyracus* ($2n=4x=80$) has broader leaves compared to *Hibiscus syriacus*. The

leaves have short triangular lobes. The involucral bracts outside the calyx are as long as the calyx or even longer (Bates 1965; Bean 1973). *Hibiscus paramutabilis* has big round-shaped leaves and the flowers are larger (Figure 1.5) (Bates 1950). The chromosome number is $2n=4x=82$ (Niimoto 1966). Both *Hibiscus sinosyrriacus* and *Hibiscus paramutabilis*, are more vigorous in growth compared to *Hibiscus syriacus*. Observations of calyx, capsule and seeds suggest that *Hibiscus paramutabilis* can be classified close to *Hibiscus syriacus* and that *Hibiscus sinosyrriacus* is intermediate between both species. The genetic relationships among these three species and some morphological characteristics were published by Van Huylenbroeck *et al.* (2000). Also close relationships with a few other Chinese species, *Hibiscus mutabilis* and *Hibiscus indicus*, are suggested (Bates 1965).



Figure 1.5: Flower and leaf morphology of *H. syriacus* (A,B) and *H. paramutabilis* (C, D).

1.5.2 Breeding of *Hibiscus*

For winter hardy *Hibiscus*, breeding work is mainly done in *Hibiscus syriacus*. Recently some results of the Korean research on interspecific hybridisation between *H. syriacus*, *H. sinosyrriacus* and *H. paramutabilis* were published (Kyung and Kim 2001a; 2001b; Kyung *et al.* 2001a; 2001b). Also some breeding work involving more tropical species of *Hibiscus* is documented. Tachibana (1958) and Kuwada (1964) made successful crosses between *H. mutabilis* and *H. moscheutos*, but the F1 seedlings were sterile. Embryo rescue in *Hibiscus syriacus* has already successfully been performed following intraspecific tetraploid x octoploid pollination (Kim *et al.* 1996a; 1996b). Also ovule culture has been done in *Hibiscus* (Paek *et al.* 1989a; 1998b).

Attempts to create interspecific hybrids between *H. syriacus* and *H. rosa-sinensis* by somatic fusion, especially to introduce new flower colors and forms, were not successful so far (Yu *et al.* 1976; Paek *et al.* 1989c). Vazquez-Thello *et al.* (1996) tried to make frost tolerant *Hibiscus* by somatic hybridisation between *Hibiscus rosa-sinensis* and *Lavatera thuringiaca*. However, the fusion products did not regenerate into plants.

Next to interspecific breeding, also other techniques were used for breeding *Hibiscus*. For *Hibiscus syriacus* breeding work resulted in the development of polyploid cultivars, which in general have larger flowers and a longer flowering period (Egolf 1971; 1981; Shim *et al.* 1993). By mutagenesis, a new cultivar 'Shirasagi-no-yume' has been marketed in Japan (Maluszynski *et al.* 1992).

1.6 The genus *Buddleja*

1.6.1 Taxonomy and plant description (Bean 1989a)

Division: Magnoliophyta

Class: Dicotyledonae

Order: Gentianales

Family: Scrophulariaceae

The genus *Buddleja* L. has been placed in several families including Scrophulariaceae, Loganiaceae and Buddlejaceae (Rogers 1986; Griffiths 1994; Bhattacharyya and Johri 1998). Nowadays the genus is classified again in the family Scrophulariaceae (Olmstaed *et al.* 2001), with approximately 125 species naturally found in tropical and subtropical areas of Asia, Africa and North and South America (Leeuwenberg 1979; Norman 2000). The plants have a dense canopy of foliage and a generous flower display. The flowers are arranged in long panicles and the scent of them attracts many different butterflies and bees. The plant grows as a herbaceous perennial. According to the characteristics of an outcrossing plant summarised by Wyatt (1983), *Buddleja* is an allogamous species and the flowers have both female and male reproductive organs. Figure 1.6 presents some of the best known *Buddleja* species.

B. davidii Franch., the butterfly bush, is native to China and Japan and is the most commonly grown species for ornamental use in the gardens in Europe. It is a multi-stemmed and spineless shrub reaching a height of 2 to 4 m. The plant is deciduous to semi-deciduous when mature, but seedlings tend to retain their leaves. Hundreds of honey-scented white, pink, purple or blue flowers are borne in panicles (10 to 30 cm long) in early summer. The small flowers are hermaphrodite. *B. davidii* starts flowering and fruiting after 1 year, although some panicles may be present within the first year. There exist at least 45 cultivars, differing

principally in flower color or growth habit (Krüssman 1984; Philip 1992). Other well known species are *B. alternifolia* Maxim., *B. lindleyana* Fort. ex Lindl., *B. crispa* Benth. and *B. globosa* Hope. *B. globosa* has orange scented flowers in small spherical clusters.

Buddleja has come under scrutiny internationally and in various regions of the USA because of its potential for invasiveness.

Few molecular and cytogenetic data for *Buddleja* are published. In his cyto-taxonomic notes on *Buddleja*, Moore (1960) reported a basic chromosome number of $x = 19$. *Buddleja* species with 38 up to 228 chromosomes exist (Moore 1960). For example *B. davidii* is reported to have 76 chromosomes whereas *B. globosa* has 38 chromosomes. In a study of Hanson *et al.* (2001) and Zonneveld *et al.* (2005) a genome size of 0.86 pg.1C^{-1} for *B. globosa* and 1.45 pg.1C^{-1} for *B. davidii* was published.

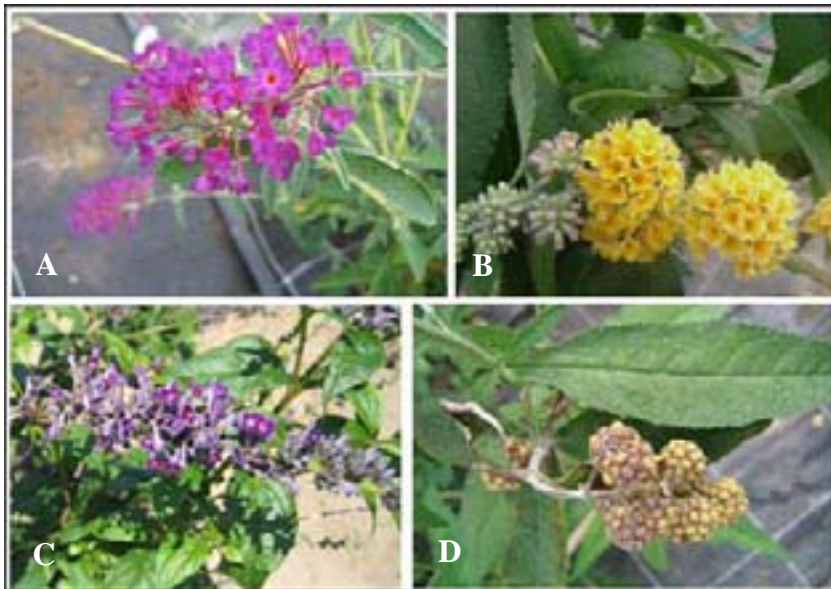


Figure 1.6: Some of the best known *Buddleja* species: *B. davidii* (A), *B. x weyeriana* 'Sungold' (B), *B. lindleyana* (C) and *B. globosa* (D).

1.6.2 Breeding of *Buddleja*

Initial breeding efforts focussed on dwarfism, silver-gray leaf color, flower color and branched-panicle (inflorescence) architecture (Tobutt 1992). Leeuwenberg (1979) and Maunder (1987) published extensive lists of known interspecific *Buddleja* hybrids. For example, the hybrid *B. x lewisiana* Everett (Maunder 1987) has been described between *B. madagascarensis* Lam. and *B. asiatica* Lour. Leeuwenberg (1979) also reported on a natural hybrid between *B. madagascarensis* and *B. indica* Lam.

The high genetic variability in *Buddleja* gives considerable opportunities to develop new and novel cultivars through interspecific crosses. Controlled interspecific hybridisation in *Buddleja* was first reported by Van De Weyer in the 1910s when he crossed *B. globosa* with

B. davidii var. *magnifica* to create *B. x weyeriana* Weyer. ex Rehd. (Van De Weyer 1920), having the yellow color of *B. globosa*. Cultivars of this hybrid ('Golden Glow', 'Moonlight', 'Sungold' and 'Honeycomb') are still popular (Dirr 1998). 'Lochinch' is an offspring of hybridisation between *B. davidii* and *B. fallowiana* Balf. and possesses the silver-grey foliage of *B. fallowiana* and the hardiness, habit and panicle size of *B. davidii* (Dirr 1998). Moore (1949) reported on a series of interspecific hybrids he produced in the 1940s. Species used in his breeding work included *B. alternifolia* Maxim., *B. asiatica*, *B. stenostachya* Reh. and Wils., *B. salviifolia* Fort. and *B. lindleyana*. Recent hybridisation in *Buddleja* has been restricted to *B. davidii* and *B. fallowiana* (Tobutt 1993), *B. davidii* and *B. lindleyana* (Elliott *et al.* 2004) and *B. indica* and *B. davidii* (Lindstrom *et al.* 2004). In none of the previous mentioned studies, *in vitro* techniques were used to enhance the efficiency of the interspecific hybridisation.

Because of differences in ploidy level and genome sizes within *Buddleja* species, polyploidisation might be useful to aid in interspecific breeding. Tetraploid *B. globosa* could already be obtained by application of colchicine to nodal sections (Rose *et al.* 2000b). The tetraploid plants showed remarkable morphological differences compared to the diploids. The tetraploids had shorter internodes resulting in more compact plants. The leaves were broader, thicker and more crinkled. Complete tetraploids could be visually distinguished from diploids. The tetraploids appeared to be more susceptible to frost. The size of the flowers did not differ, but the shape of the flowers was more elliptical, rather than spherical. Anthers developed poorly.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Plant material

The parent plants used in the interspecific crosses and ploidy experiments were chosen based on their morphological characteristics and on the breeding goals. In *Hydrangea*, the breeding goal was to enlarge the *H. paniculata* Sortiment with novel flower colors and leaf shapes. For *Hibiscus*, the main aim was to introgress more growth vigor into *H. syriacus*. For *Buddleja*, the breeding objective was to introgress yellow flower colors in *B. davidii*. Also compactness and sterility were very important features for *Buddleja*.

All parent plant material used in the interspecific hybridisation experiments is overviewed in Table 2.1. Within a species different cultivars were used to be able to evaluate cultivar dependency of crossing barriers. For the ploidy breeding experiments *Buddleja globosa* and *Hibiscus syriacus* cultivars were used.

The plant material was cultivated in greenhouses or outdoors in containers or in open ground. Normal nursery practices for irrigation, fertilisation and pest control were followed.

Sterile flowers of the *Hydrangea* species are single, except for *H. quercifolia* ‘Snow Flake’ which has double sterile flowers (Table 2.1). Inflorescences are lace-cap or panicle, with a lot of fertile flowers in it. All the *Hydrangea* parental species flower from the end of July.

Flowers of the *Hibiscus* species are single (Table 2.1) with a diameter between 8 cm (*H. syriacus* ‘Melwhite’) and 14 cm (*H. paramutabilis*). The *Hibiscus* species used in the experiments all flower in the same period (August-September).

Flowers of *Buddleja* species are single (Table 2.1) with a length of the panicle between 7.5 cm (*B. crispa*) and 25 cm (*B. alternifolia*). For *Buddleja*, there are some differences in flowering period. *B. alternifolia*, *B. crispa* and *B. globosa* flower earlier (June) compared to *B. davidii*, *B. x weyeriana* and *B. lindleyana* (July-August).

The plant material is described in more detail in Chapter 3.

Table 2.1: Overview of the plant material used in the experiments together with their flower morphology.

Genotype	Color (RHS color) ^w	Type ^y
<i>Hydrangea macrophylla</i> ‘Fasan’	red-pink to violet-blue ^x	S, lace-cap
<i>H. macrophylla</i> ‘Blaumeise’	purple-blue ^x	S, lace-cap
<i>H. macrophylla</i> ‘Bergfink’	red-pink to violet-blue ^x	S, lace-cap
<i>H. macrophylla</i> ‘Mariesii Perfecta’	blue-pink, light blue ^x	S, lace-cap
<i>H. paniculata</i> ‘Unique’	white	S, panicle
<i>H. paniculata</i> ‘White Moth’	white	S, panicle
<i>H. paniculata</i> ‘White Lace’	white	S, panicle
<i>H. paniculata</i> ‘Praecox’	white	S, panicle
<i>H. quercifolia</i> ‘Snow Flake’	white, autumn coloration	D, panicle
<i>H. quercifolia</i> ‘Sikes Dwarf’	white, autumn coloration	S, panicle
<i>H. quercifolia</i> ‘Tennessee Clone’	white, autumn coloration	S, panicle
<i>H. serrata</i> ‘Blue Bird’	purple-blue ^x	S, lace cap
<i>H. aspera</i> ‘Macrophylla’	purple (77A)	S, lace-cap
<i>Hibiscus syriacus</i> ‘Melwhite’	white	S
<i>H. syriacus</i> ‘Oiseau Bleu’	violet-blue (93B), purple-red centre (71A)	S
<i>H. syriacus</i> ‘Purple CV ₂ ’ ^v	purple (82B), red centre (53A)	S
<i>H. syriacus</i> ‘Red Heart CV’ ^v	pink (76B), red centre (53A)	S
<i>H. sinosyriacus</i> ‘Autumn Surprise’	white, red-purple centre (60B)	S
<i>H. sinosyriacus</i> ‘Lilac Queen’	light purple (76A), red centre (60A)	S
<i>H. paramutabilis</i>	white (56D), red centre (60B)	S
<i>Buddleja lindleyana</i>	purple (77A)	S, panicle
<i>B. globosa</i>	orange-yellow (23A)	S, spherical
<i>B. alternifolia</i>	purple (76A)	S, panicle
<i>B. davidii</i> ‘Royal Red’	purple (77A), orange centre (28B)	S, panicle
<i>B. davidii</i> ‘Nanho Purple’	purple (77A), orange centre (28A)	S, panicle
<i>B. davidii</i> ‘White Profusion’	white	S, panicle
<i>B. davidii</i> ‘White Ball’	white	S, panicle
<i>B. davidii</i> ‘Nanhoensis Alba’	white	S, panicle
<i>B. x weyeriana</i> ‘Sungold’ ^z	yellow (14A), orange centre (28A)	S, interrupted panicle
<i>B. crispa</i> ‘Moondance’	purple (76B)	S, panicle

^z *B. x weyeriana* is originating from a F2 selection after crossing *B. globosa* with *B. davidii*;

^y S = single; D = double ; ^x Depending on soil pH, the color may change; ^w see 2.4.2;

^v Shim *et al.* (1993)

2.2 Hand pollination

Hand pollination, preceded by emasculation (just before anthesis), was done from July till the end of September between 2003 and 2007, under controlled environmental conditions in an insect free plastic greenhouse. Intra- and interspecific pollinations were performed by rubbing anthers with releasing pollen over a susceptible stigma of a fully opened flower (Figure 2.1). To ensure good pollen adhesion on the stigma, pollinations were performed twice on 2 subsequent days. It was not possible to pollinate all flowers of inflorescences of *Buddleja* and *Hydrangea* at the same time, since individual flowers were not receptive at the same moment.

For *Buddleja*, some pollinations were performed with pollen that was stored for 2 months. For storage, pollen was harvested in the morning and dried under a lamp (Osram 40 W) during the day. Dried pollen was then stored at -20°C. Thawing and stimulating of the stored pollen was done overnight by illumination (Osram 40 W).

Whenever not impeded by various reasons, also reciprocal crosses were performed.

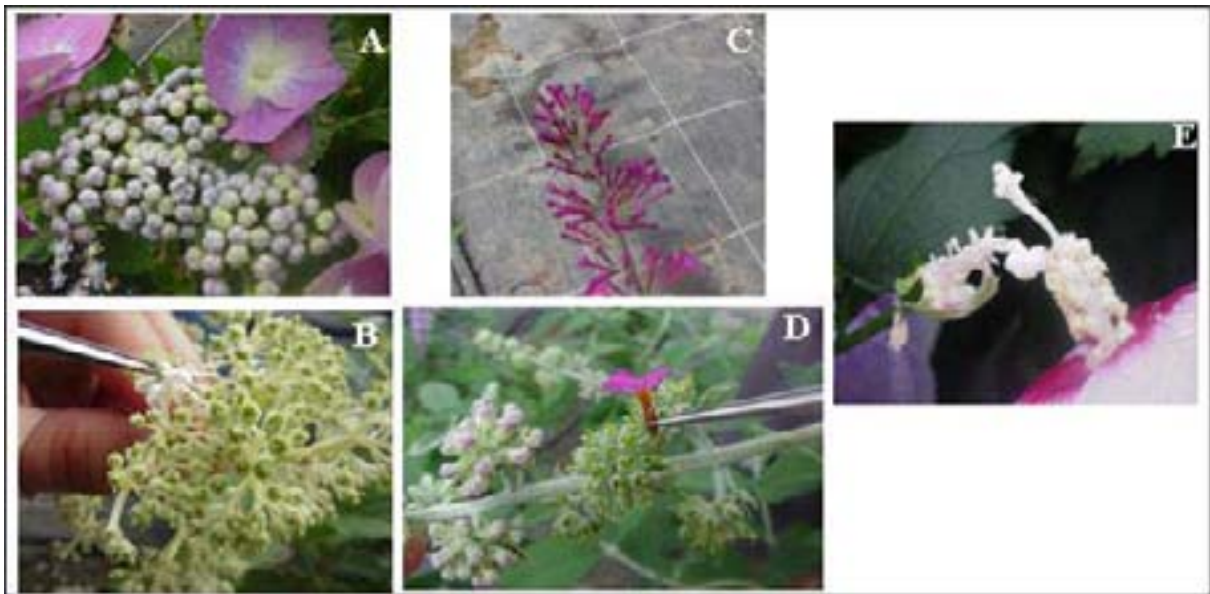


Figure 2.1: Emasculation stage (A, C) and hand pollination (B,D,E) of *Hydrangea* (A, B), *Buddleja* (C,D) and *Hibiscus* (E).

According to Reed (2004), stigmas of *Hydrangea* flowers are receptive to pollen from anthesis to 5 days after anthesis. In this study *Hydrangea* flowers were pollinated 1 to 5 days after emasculation. For *Buddleja*, flowers were pollinated 1 to 2 days after emasculation, as described by Lindstrom *et al.* (2004). No literature is available about the best time for pollination for *Hibiscus*. Pollinations within *Hibiscus* were performed 1 to 2 days after emasculation when the stigma became sticky.

2.3 *In vitro* manipulations

2.3.1 *In vitro* conditions and disinfection procedure

Preceding *in vitro* initiation, *in vivo* material was rinsed in 70% ethanol, sterilised for 20 min in a 10% (v/v) NaOCl solution with 0.005% (v/v) Teepol and finally rinsed 3 times in autoclaved water.

In vitro cultures were placed in a conditioned growth chamber at $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ under a 16 h photoperiod at $40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PAR, supplied by cool white fluorescent lamps (OSRAM L36W/31). Tissue culture plants were subcultured every 6 weeks.

2.3.2 *In vitro* media compositions

The media for germination of *Hibiscus* (Ghib), *Buddleja* (Gbud) and *Hydrangea* (Ghyd1) embryos or seeds are presented in Table 2.2. After the first 2 leaves appeared, *Hydrangea* seedlings were transferred to a new medium (Ghyd2; Table 2.2) enriched with BAP to obtain a better development. All media contained PPM ($1 \text{ mL}\cdot\text{L}^{-1}$ medium) (Plant Cell Technology, USA). For multiplication of *Buddleja* shoots medium Mbud (Table 2.2) was used.

Table 2.2: Different media compositions for *Hibiscus*, *Buddleja* and *Hydrangea*

Component	Ghib = Gbud	Ghyd1	Ghyd2	Mbud
Water	1000 mL	1000 mL	1000 mL	1000 mL
Macroelements	MS ^z	B5 ^y	B5	WPM ^x
Microelements	MS	B5	B5	WPM
Vitamins	MS	B5	B5	WPM
Fe-complex	MS	B5	B5	WPM
C-source	3% sucrose	3% sucrose	2% sucrose	3% sucrose
Hormones	-	-	2,5 μM BAP	25 μM 2-Ip
Agar	7 $\text{g}\cdot\text{L}^{-1}$ MC29	7 $\text{g}\cdot\text{L}^{-1}$ MC29	7 $\text{g}\cdot\text{L}^{-1}$ MC29	7 $\text{g}\cdot\text{L}^{-1}$ MC29
Active charcoal	-	-	-	2.5 $\text{g}\cdot\text{L}^{-1}$
pH	5.8	6	6	5.8

^z MS: Murashige and Skoog 1962; ^y B5: Gamborg *et al.* 1968; ^x WPM: Lloyd and McCown 1980

Media were autoclaved (121°C , 30 min, 500 hPa). Petri-dishes (5.5 cm diameter) were filled with 5 mL medium and sealed with LDPE (Low density polyethylene) foil. Meli-jars (De Proft *et al.* 1985) contained 100 ml medium/jar.

2.3.3 *In vitro* embryo rescue

Ten to eleven weeks after pollination most fruits started to abort. Therefore, 10 to 11 weeks after pollination, the resulting fruits were harvested and sterilised (see 2.3.1). The fruits were sliced longitudinally in the laminar flow under a binocular (1.6x to 5x magnification). Embryos (*Hibiscus*) or seeds (*Buddleja*, *Hydrangea*) were dissected out of the fruits and initiated *in vitro* on medium Ghib (*Hibiscus*), Gbud (*Buddleja*) or Hhyd1/Ghyd2 (*Hydrangea*) (see Table 2.2) in petri-dishes. When seedlings had developed the first true leaves, they were transferred to meli-jars (De Proft *et al.* 1985).

2.3.4 Acclimatisation

After careful removal of the agar, plantlets were acclimatised at least 4 weeks after rooting *in vitro* in plastic seedling trays, filled with peat mixture (Organic matter 20%, dry matter 25%, 1.5 kg.m⁻³ fertiliser: 12N:14P:24K + trace elements). They were kept in a fog unit (RH > 90%) during 3 weeks for acclimatisation.

2.4 Determination of morphology

2.4.1 Leaf morphology

To measure the different leaf parameters of *Hibiscus* leaves, fully developed leaves (between the 5th and 10th internode of newly formed shoots) were collected from three different plants of each genotype. The following indices were determined as described by Van Huylbroeck *et al.* (2000) (Figure 2.2): leaf length over width (L/B), angle of the leaf basis (α), relative width of the mid lobe (M/B), relative length of the side lobes (C/L) and relative depth of indentation of the lobes (A/L).

To measure leaf indices for *Buddleja* and *Hydrangea* leaves, 10 fully developed leaves of three different plants of each genotype were collected. Leaf morphology was described by measuring leaf length (L) and leaf width (W) and calculating the ratio L/W.

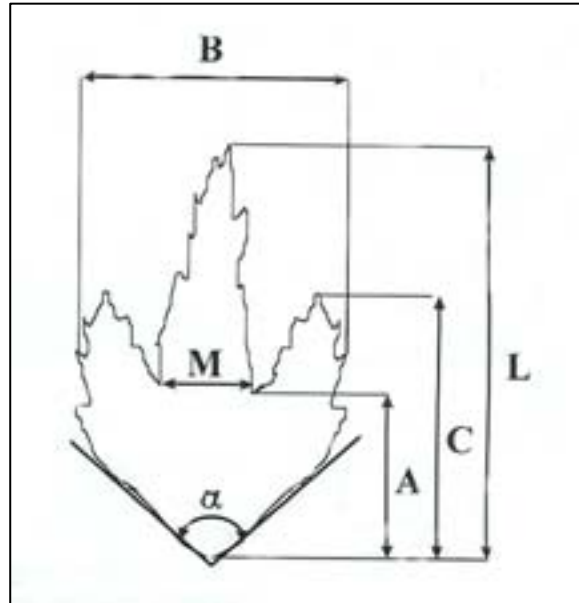


Figure 2.2: Schematic overview of the measured *Hibiscus* leaf parameters (Van Huylenbroeck *et al.* 2000).

2.4.2 Flower color

Flower colors of the *Hibiscus* and *Buddleja* plants were determined using the Royal Horticultural Society Color Chart (RHS colors). For *Hydrangea* plants flower colors were not determined since they are too much influenced by the environment (e.g. pH).

2.4.3 Statistical analysis

If possible, statistical analysis was performed in SPSS 11.5 for Windows. A one way ANOVA was conducted on the obtained data. If significant differences occurred, means were separated by the LSD ($p = 0.05$) method.

2.5 Flow cytometry

2.5.1 Ploidy measurements of leaf material

Flow cytometry involves the analysis of fluorescence and light-scattering properties of single particles during their passage within a narrow, precisely defined liquid stream (Galbraith *et al.* 1983; Dolezel *et al.* 1989; Dolezel 1991).

Flow cytometry was performed using a Partec PAS III. Young leaf samples (0.5 cm²) from one plant with a known ploidy level and from the plants with unknown ploidy level were taken. The samples were chopped with a sharp razor blade (Galbraith *et al.* 1983) in 400 µl buffer solution (0.1 M citric acid, 0.5% (v/v) Tween 20, pH 2.5; Otto 1990) for the isolation of the nuclei. The chopped sample was passed through a nylon filter of 50 µm mesh (CellTrics®, Partec). Afterwards, 800 µl of a second buffer (0.4 M Na₂HPO₄, 2 mg.L⁻¹ DAPI, pH 8.5; Otto 1990) was added. The mixture of the 2 buffers gave pH 7, which is the optimal pH for the staining of the nuclei with DAPI (Otto 1990).

After filtration the nuclear suspensions were passed through the flow chamber, filled with a sheath fluid (de-ionised water). The nuclei traversed the focus of an intense light beam, produced by a high-pressure mercury vapor lamp (Hg lamp 100 W). At a wavelength of 365 nm the nuclei, stained with DAPI, fluoresced. The excitation light was collected by a lens and converted to pulses of electrical current by a photomultiplier. The electronic signals were then digitised and the binary data were stored as one-dimensional histograms. The fluorescence intensity is linearly correlated with the amount of DNA that was stained with DAPI.

The first sample measured in the flow cytometer was the external standard (control plant), which gauges the apparatus. The voltage of the photomultiplier, which transfers the DAPI-fluorescence (depending on the DNA-content) into an electrical current, was adjusted in a way that the diploid peak was fixed at position 100 (channel number). Haploid peaks then occurred on position 50, tetraploid peaks at position 200 etc. The measurement of the external standard was repeated after every 10 samples to correct the voltage of the photomultiplier if necessary. At least 5000 nuclei are analysed per sample.

2.5.2 Ploidy measurements of pollen

Nuclear samples were prepared from mature pollen according to Van Tuyl *et al.* (1989) with modifications. Pollen were submersed in extraction buffer (0.1 M citric acid, 0.5% (v/v) Tween 20, pH 2.5; Otto 1990). After short centrifugation, the pollen pellet was transferred to a Petri dish (5 cm diameter). Pollen grains were chopped with a razor blade (Galbraith *et al.*, 1983) and 400 µl of the extraction buffer (Otto 1990) was added. The chopped material was

then passed through a 50 µm nylon filter (CellTrics®, Partec). The residue on the filter was washed with 800 µl staining buffer containing 0.4M Na₂HPO₄ and 2 mg.L⁻¹ DAPI (Otto 1990). The nuclear suspension was then analysed with a Partec PAS III flow cytometer (Hg lamp 100 W), according to the principle described in paragraph 2.5.1. At least 5000 nuclei are analysed per sample.

2.5.3 Genome size measurements

Genome sizes were measured with a Partec PAS III flow cytometer with an argon laser (488 nm) and with a Partec Cyflow Space with a green solid state laser (100 mW, 532 nm) according to the similar principle as described in paragraph 2.5.1.

Young leaf material (0.5 cm² of both the sample and the internal reference) was co-chopped in 500 µl extraction buffer (Partec, Cystain PI absolute P, according to the manufacturers protocol). The chopped material is then filtered through a nylon filter with 50 µm mesh (Celltrics®, Partec) and 2 ml staining solution is added. The staining solution contains (per sample) 2 ml staining buffer, 12 µl Propidium Iodide stock solution and 6 µl RNase stock solution (Partec, Cystain PI absolute P). The sample is incubated for at least 30 minutes in the dark before measuring with the flow cytometer. At least 5000 nuclei are analysed per sample. Obtained data were analysed using Flomax software.

For every *Buddleja*, *Hibiscus* and *Hydrangea* sample the reference plant *Pisum sativum* ‘Minerva Maple’ was used which has a genome size of 9.39 pg.2C⁻¹ (Johnston *et al.* 1999). At least 5 repetitions of each sample were measured. Genome sizes were calculated from the peak position ratios. According to Greilhuber *et al.* (2007) influence of plant cytosolic compounds on fluorochrome accessibility to nuclear DNA was tested. The peak position of the internal reference standard was located on (almost) identical fluorescence channels in all measurements, either with or without sample.

Definitions on genome size terminology are used as defined by Greilhuber *et al.* (2005).

2.6 Microscopy

2.6.1 Pollen germination analysis

Open flowers were collected in the afternoon and pollen anthesis was stimulated by overnight illumination (Osram 40 W). Subsequently, the anthers of at least five flowers were submersed in a liquid germination medium according to Tian and Russell (1997) (10% (w/v) sucrose,

0.01% (w/v) H_3BO_3 , 0.01% (w/v) CaCl_2 , 0.02% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% (w/v) KH_2PO_4). The solution was vortexed in order to release the pollen and the anther debris was removed. After overnight (20 h, dark, room temperature) incubation of the pollen in the germination medium, a drop of the pollen solution was pipetted on a slide, covered with a cover slip and examined under a light microscope (LEICA DMIRB; 100x magnification). Germination of at least 300 pollen grains was analysed to determine the *in vitro* germination percentage of the pollen. Pollen grains were scored as germinated when the pollen tube length was at least twice the pollen grain length.

2.6.2 Pollen size and pollen viability measurements

To determine the pollen size, a pollen solution was prepared as described in paragraph 2.6.1, but no overnight incubation step was performed. The diameter of the pollen was measured digitally using the software Image Manager IM 500 (Leica). At least 300 pollen per genotype were measured to determine the typical pollen size range.

To determine the viability, the pollen was stained with acetocarmine, which only stains nuclear DNA of the viable pollen. Therefore, open flowers were collected and anthers of at least five flowers were submersed in a 0.5% (w/v) acetocarmine solution. The solution was vortexed in order to release the pollen, a drop of the pollen solution was pipetted on a slide, covered with a cover slip and examined under a light microscope (Leica DMIRB; 100x magnification). At least 300 pollen per genotype were examined. The diameter of the viable and unviable pollen was measured digitally using the software Image Manager IM 500 (Leica).

2.6.3 Identification of prefertilisation barriers

Characterisation of prefertilisation barriers was performed as described by Cuevas *et al.* (1994) and Ureshino *et al.* (2000). Pistils were harvested 72 h after pollination and fixed in FAA (formalin: 70% ethanol: acetic acid = 1:18:1) for 24 h. After fixing the pistils, they were rinsed in tap water and soaked in 4N NaOH for 24 h to soften the tissue. The softened pistils were washed 3 times in tap water and subsequently stained with a 0.1% solution of water-soluble aniline blue dye in 0.1 N K_3PO_4 for 3 h at room temperature.

For *Buddleja* and *Hibiscus*, the stained pistils were squashed on a glass slide under a cover glass. Because *Hydrangea* pistils were too thick, they were sliced before squashing on a glass slide under a cover slide. Pistil preparations were observed with a fluorescent microscope (Leica DM/IRB; 200x or 400x magnification). At least 4 (at random collected) pollinated pistils were analysed for the occurrence of prefertilisation barriers.

2.6.4 Analysis of microsporogenesis

Immature anthers were removed from young floral buds (9 mm) and excised on a microscopic slide in a drop of 1 μ M DAPI in a sodium citrate solution (0.3 M NaCl, 30 μ M sodium citrate, pH 7.0 in milliQ water). The anther debris was removed and a coverslip was added on the pollen preparation. The microspores were observed under light and fluorescence microscope (Leica DMIRB; 200x magnification). At least 150 microspores per genotype were examined in order to search for aberrations in tetrad formation.

2.6.5 Chromosome analysis and *in situ* hybridisation

Chromosome preparations

Chromosome preparation was performed using the cell spreading technique according to Pijnacker and Ferwerda (1984). Therefore, young root tips were collected in the early morning and pre-treated in a saturated α -bromonaphthalin solution overnight at 4°C. The material was then fixed in Carnoy solution [ethanol - acetic acid (3:1)] for at least 1 h and stored at -20°C until used. The material was rinsed in tap water before incubation in a pectolytic enzyme mixture (0.1% (w/v) pectolyase Y23, 0.1% (w/v) cellulase onozuka RS and 0.1% (w/v) cytohelicase in 10mM citrate buffer) for 2h 45min (*Hydrangea*), 30 min (*Buddleja*) or 40 min (*Hibiscus*) at 37°C. Squash preparations were made in a small drop of 10mM citrate buffer (10mM tri sodium citrate, 10mM citric acid, pH4.5) followed by a further squash in a drop of 60% acetic acid. Slides were then incubated on a heating plate (42°C) for 2 min. Carnoy solution was added to the slides and they were rinsed briefly in 98% ethanol. The slides were air-dried for at least 1 h. The best slides were selected under a phase contrast microscope (Leica DM IRB; 100x or 400x magnification) and stored at 4°C until use.

Construction and labelling of probe DNA

For 45S rDNA FISH, the clone pTa71 containing a 9 kb fragment of the 45S rDNA from wheat was used (Gerlach and Bedbrook 1979). Isolated DNA of 45S sequences from pTa71 was labelled with biotin-16-dUTP by nick translation (according to the manufacturers instructions, Roche). The labelling was checked afterwards by a dot blot.

For the probe construction for GISH, total genomic DNA was extracted from 2 g of young leaves as described by Rogers and Bendich (1988). The genomic DNA was sonicated to yield fragments ranging from 0.5 to 10 kb and labelled with biotin-16-dUTP or digoxigenin by nick translation. Blocking DNA was obtained by sonication of genomic DNA to fragments of 100-300 bp.

45S rDNA Fluorescence in situ hybridisation

The selected slides were pre-treated with 1 μ g.mL⁻¹ RNase A, 0.05 ng.mL⁻¹ pepsin and 4% (w/v) paraformaldehyde according to Leitch and Heslop-Harrison (1994). DNA denaturation and *in situ* hybridisation steps were performed according to Schwarzacher and Leitch (1993)

and Schwarzacher and Heslop-Harrison (1994). The hybridisation mixture contained 50% (v/v) deionised formamide, 10% (w/v) dextran sulphate, 2x SSC, 0.25% (w/v) SDS and probe DNA. The hybridisation mix was denatured for 10 min at 70°C and placed on ice for 5 min. After the hybridisation mixture was added to the slides a 5-min denaturation step at 80°C was carried out. Hybridisation was performed overnight in a humid chamber at 37°C. Subsequently, the slides were washed in 2x SSC at room temperature for 15 min, then transferred to 0.1x SSC at 48°C for 30 min and finally washed again in 2x SSC for 15 min at room temperature. Biotin-labelled DNA was detected with CY3-conjugated streptavidin (Jackson Immuno Research Laboratories, West Grove, Pa., USA) and amplified with biotinylated goat –antistreptavidin (Vector Laboratories, Burlingame, Calif., USA). The slides were (counter) stained with 1 µg.mL⁻¹ DAPI.

Chromosome analysis and karyotyping

After hybridisation or DAPI staining chromosome analysis was performed with a Nikon TE2000 Eclips epifluorescence microscope equipped with a custom Nikon DS-U1 color camera using a DAPI filter (excitation 355 nm), a TRITC filter (excitation 540-550 nm) and a FITC filter (excitation 495 nm). Images were captured by NIS software. Chromosome analysis was carried out on 10 well-spread metaphases of each genotype using the computer software MicroMeasure for Windows, version 3.3 (Reeves 2001). Chromosomes were characterised on the basis of chromosome length, arm length, centromeric index (Levan *et al.* 1964) and hybridisation signal.

For construction of a karyotype, chromosomes were arranged in order of decreasing length. Also the asymmetry of the karyotype [$(\sum \text{length short arm})/(\text{total chromosome complement}) * 100$] and the condensation index (length longest chromosome/length shortest chromosome in the same cell) was calculated.

2.7 AFLP analysis

DNA extraction of young leaf material was performed using the Qiagen DNeasy-kit (Hilden, Germany). AFLP reactions were run on an ABI 3130 DNA sequencer using the commercially available kit for fluorescent fragment detection (Perkin-Elmer 1995). EcoRI and MseI were used for DNA digestion. Selective amplification was done using 3 fluorescent labelled EcoRI-MseI primer combinations with 6 or 7 selective bases. For *Buddleja* primer combinations EcoRI-ACG_{hex} / MseI-CAA, EcoRI-ACA_{fam} / MseI-CTG and EcoRI-AGG_{hex} / MseI-CTT were used (Eeckhaut 2003). For *Hibiscus* selective amplification was done with primer combinations EcoRI-AAC_{fam} / MseI-CAT, EcoRI-ACA_{fam} / MseI-CTG, EcoRI-AAG_{hex} / MseI-CTA (Eeckhaut 2003). For *Hydrangea* different primer combinations with 6 or 7 selective nucleotides were tried: EcoRI-AGAG_{fam} / MseI-CAT, EcoRI-ACCC_{hex} / MseI-CAA, EcoRI-ACCG_{hex} / MseI-CTT, EcoRI-ACGT_{hex} / MseI-CAT, EcoRI-ACT_{fam} / MseI-CAT, EcoRI-AGG_{hex} / MseI-CTT, EcoRI-ACG_{hex} / MseI-CAA. The AFLP data were analysed with

the ABI Prism® GeneMapper™ software version 4.01. The number of markers uniquely present in each of the parent plants was checked for segregation in the offspring. The obtained AFLP data were analysed by multivariate analysis using Simple Matching Similarity Indices in SPSS 11.5 for Windows. Simple Matching Similarity Indices reflect the mutual similarity of 2 genomes and are calculated by $(C+D)/(A+B+C+D)$ with A = number of fragments uniquely present in genome 1, B = number of fragments uniquely present in genome 2, C = number of fragments present in both genomes and D = number of fragments absent in both genomes. These indices are commonly used for biodiversity evaluation (Karp *et al.* 1997). A phylogenetic tree (dendrogram) was constructed using clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The dendrogram is built stepwise by clustering the 2 most similar genotypes in a subsequent manner, each time reducing the number of groups by 1 unit, finally resulting into a 'tree' (Sneath and Sokal 1973).

In the dendrograms, F1 seedlings are named by a code containing the first 3 to 5 letters of each species involved in the cross.

CHAPTER 3

CHARACTERISATION OF PARENT PLANTS*

3.1 Morphology and (cyto)genetics of the parent plants

3.1.1 Introduction

In order to have indications on cross (in)compatibility between the species and to help in decision making on specific cross combinations, all (cyto)genetic and biological information of the species should be gathered. Little is known about the floral biology and (cyto)genetic characteristics of *Hydrangea*, *Hibiscus* and *Buddleja* species. Therefore, in this Chapter a brief morphological study was carried out focussed on flowering time and pollen fertility. The chosen parental species were also described for their ploidy level, genome size and AFLP fingerprint.

3.1.2 Specific materials and methods

All protocols used for the description of the parent plants are given in Chapter 2.

* Parts of this chapter are published in:

Van Laere K, Van Huylenbroeck J, Van Bockstaele E (2008) Karyotype analysis and physical mapping of 45S rRNA genes in *Hydrangea* species by fluorescence *in situ* hybridisation. *Plant Breeding* 127: 301-307

Van Laere K, Leus L, Van Huylenbroeck J, Van Bockstaele E (2008) Interspecific hybridisation and genome size analysis in *Buddleja*. Accepted for publication in *Euphytica*

3.1.3 Results

3.1.3.1 Pollen germination

For *Hydrangea* pollen germination percentage was cultivar dependent. Pollen of one cultivar of *H. quercifolia* ('Tennessee Clone') and one cultivar of *H. macrophylla* ('Bergfink') had a low germination, 2% and 5% respectively. The other cultivars of *H. quercifolia* and *H. macrophylla* had a pollen germination rate between 12% and 25%. *H. paniculata* cultivars had a pollen germination rate between 15% and 23%. *H. serrata* had pollen with the highest germination percentage (45%), while only 2% of *H. aspera* pollen germinated (Table 3.1).

Table 3.1: Overview of the pollen germination percentage (after 20 h incubation in germination medium) of the parental species used in the interspecific hybridisation and ploidy breeding experiments.

Genotype	Pollen germination percentage ^z (%)	Genotype	Pollen germination percentage ^z (%)
<i>Hydrangea macrophylla</i> 'Fasan'	12	<i>H. syriacus</i> 'Red Heart CV'	24
<i>H. macrophylla</i> 'Blaumeise'	16	<i>H. sinosyriacus</i> 'Autumn Surprise'	22
<i>H. macrophylla</i> 'Bergfink'	5	<i>H. sinosyriacus</i> 'Lilac Queen'	25
<i>H. macrophylla</i> 'Mariesii Perfecta'	24	<i>H. paramutabilis</i>	43
<i>H. paniculata</i> 'Unique'	15		
<i>H. paniculata</i> 'White Moth'	16	<i>Buddleja lindleyana</i>	1
<i>H. paniculata</i> 'White Lace'	20	<i>B. globosa</i>	2
<i>H. paniculata</i> 'Praecox'	23	<i>B. alternifolia</i>	30
<i>H. quercifolia</i> 'Snow Flake'	25	<i>B. davidii</i> 'Royal Red'	48
<i>H. quercifolia</i> 'Sikes Dwarf'	19	<i>B. davidii</i> 'Nanho Purple'	39
<i>H. quercifolia</i> 'Tennessee Clone'	2	<i>B. davidii</i> 'White Profusion'	55
<i>H. serrata</i> 'Blue Bird'	45	<i>B. davidii</i> 'White Ball'	<1
<i>H. aspera</i> 'Macrophylla'	2	<i>B. davidii</i> 'Nanhoensis Alba'	15
		<i>B. x weyeriana</i> 'Sungold' ^z	1
<i>Hibiscus syriacus</i> 'Melwhite'	37	<i>B. crispa</i> 'Moondance'	10
<i>H. syriacus</i> 'Oiseau Bleu'	56		
<i>H. syriacus</i> 'Purple CV ₂ '	19		

^z pollen was harvested early in the morning, in the first week of the flowering season

Pollen of the *Hibiscus* species had an *in vitro* germination rate between 19% and 47% (Table 3.1).

For some *Buddleja* species a low pollen germination (<5%) was observed as for *B. lindleyana*, *B. globosa*, *B. x weyeriana* and *B. davidii* 'White Ball' (Table 3.1). Because *B. alternifolia*, *B. crispa* and *B. globosa* flower earlier (flowering starts at the end of April) compared to the other species (flowering in June, July, August) used in the interspecific

crosses, it was tested whether or not the pollen could be stored for at least 2 months. Pollen storage was possible at -20°C for at least two months for *B. alternifolia* and *B. crispa* (Figure 3.1). For *B. globosa* no pollen germination was observed after 2 months of storage.

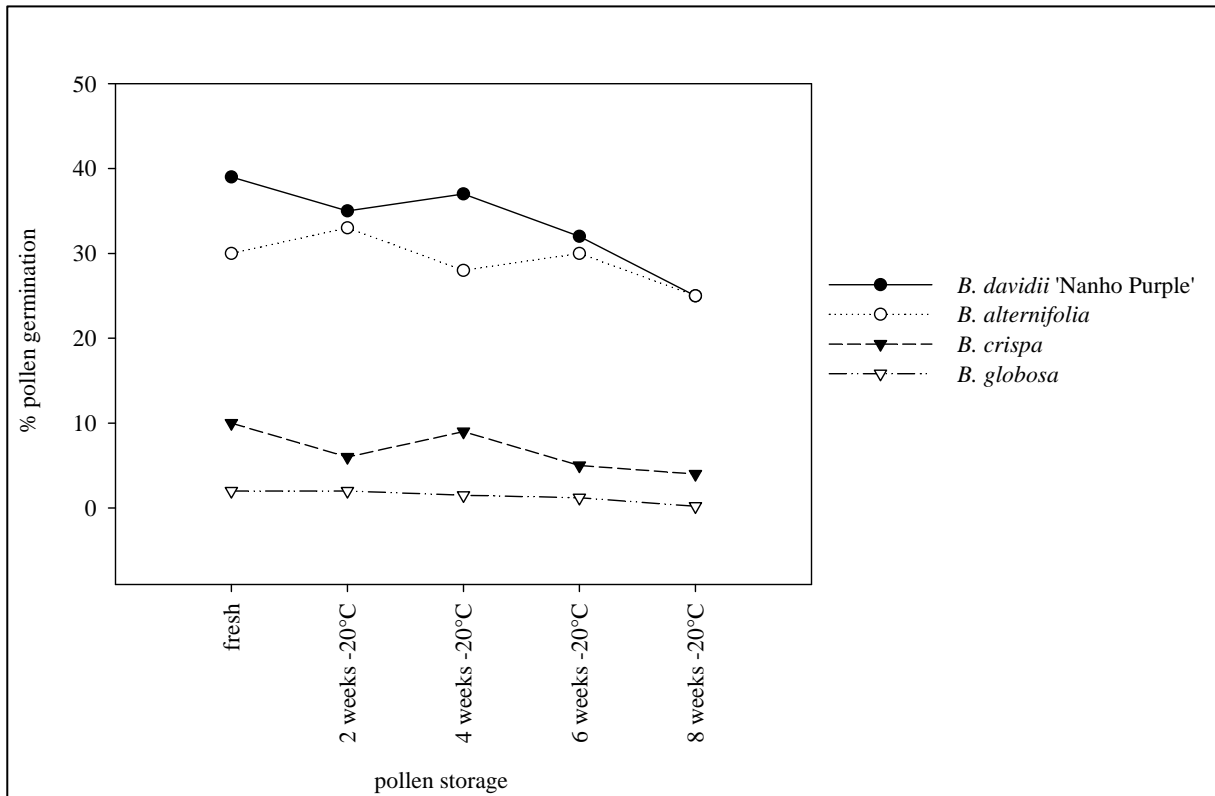


Figure 3.1: Pollen germination capacity of earlier flowering *Buddleja* species *B. globosa*, *B. crispa* and *B. alternifolia* after storage at -20°C (2, 4, 6 and 8 weeks), compared to *B. davidii* 'Nanho Purple', a species with a very good pollen germination.

3.1.3.2 Leaf morphology

General leaf morphology of *Hydrangea paniculata* is lanceolate. Leaves of *Hydrangea macrophylla*, *H. serrata* and *H. aspera* are also lanceolate but broader compared to *H. paniculata*. Leaves of *Hydrangea quercifolia* have the shape of oak leaves. *Hydrangea* is also a deciduous plant. As expressed by a higher L/W ratio (between 1.9 and 2.3), *Hydrangea paniculata*, *H. serrata* and *H. aspera* had more elliptical leaves compared to *H. macrophylla* (Table 3.2). Within *H. paniculata* there were also differences concerning L/W ratio, 'Unique' had the largest ratio (2.3), while 'Praecox' had a ratio of 1.9 (Table 3.2). Leaves of *H. macrophylla* were more rounded, but still lanceolate (L/W ratio between 1.3 and 1.5). Leaves of *H. quercifolia* were almost round and indented (Table 3.2).

Leaves of the *Buddleja* species are lanceolate. *B. crispa* has soft hairy leaves. Except for *B. davidii*, which is semi-deciduous, all other used *Buddleja* species are deciduous. *B. globosa* and *B. alternifolia* had the most oblonged leaves, with a L/W ratio of 4.1 and 5.1 respectively

(Table 3.2). The different *B. davidii* cultivars had different L/W ratios varying from 2.9 for ‘Royal Red’ to 4.0 for ‘Nanhoensis Alba’. *B. x weyeriana* had a lower L/W ratio (2.7). *B. crispa* and *B. lindleyana* showed the lowest L/W ratio, 1.7 and 2.2 respectively (Table 3.2).

Table 3.2: Leaf indices for *Buddleja* and *Hydrangea* parental species.

Genotype	L/W	Genotype	L/W
<i>Hydrangea macrophylla</i> ‘Fasan’	1.3	<i>Buddleja lindleyana</i>	2.2
<i>Hydrangea macrophylla</i> ‘Blaumeise’	1.4	<i>Buddleja globosa</i>	4.1
<i>Hydrangea macrophylla</i> ‘Bergfink’	1.4	<i>Buddleja alternifolia</i>	5.1
<i>Hydrangea macrophylla</i> ‘Mariesii Perfecta’	1.5	<i>Buddleja davidii</i> ‘Royal Red’	2.9
<i>Hydrangea paniculata</i> ‘unique’	2.3	<i>Buddleja davidii</i> ‘Nanho Purple’	3.2
<i>Hydrangea paniculata</i> ‘White Moth’	2.0	<i>Buddleja davidii</i> ‘White Profusion’	3.6
<i>Hydrangea paniculata</i> ‘White Lace’	2.0	<i>Buddleja davidii</i> ‘White Ball’	3.7
<i>Hydrangea paniculata</i> ‘Praecox’	1.9	<i>Buddleja davidii</i> ‘Nanhoensis Alba’	4.0
<i>Hydrangea quercifolia</i> ‘Snow Flake’	1.2	<i>Buddleja x weyeriana</i> ‘Sungold’	2.7
<i>Hydrangea quercifolia</i> ‘Sikes Dwarf’	1.0	<i>Buddleja crispa</i> ‘Moondance’	1.7
<i>Hydrangea quercifolia</i> ‘Tennessee Clone’	1.2		
<i>Hydrangea serrata</i> ‘Blue Bird’	2.1		
<i>Hydrangea aspera</i> ‘Macrophylla’	2.2		

Leaves of *Hibiscus* have triangular lobes. *H. paramutabilis* and *H. sinosyriacus* have broader leaves compared to *H. syriacus*. *Hibiscus* species are deciduous. Both *H. paramutabilis* and *H. sinosyriacus* had a smaller L/B ratio but a bigger angle of the leaf basis, compared to *H. syriacus* (Table 3.3). M/B ratio was the smallest for *H. syriacus* ‘Oiseau bleu’, all the other *Hibiscus* plants had a similar M/B. *H. syriacus* leaves were most indented as expressed by a lower A/L ratio (Table 3.3).

Table 3.3: Leaf indices for *Hibiscus* parental species.

Genotype	L/B	α	M/B	C/L	A/L
<i>H. syriacus</i> ‘Oiseau Bleu’	1.41	112.0	0.27	0.55	0.40
<i>H. syriacus</i> ‘Melwhite’	1.50	135.0	0.48	0.54	0.49
<i>H. syriacus</i> ‘Purple CV ₂ ’	1.57	100.2	0.44	0.54	0.48
<i>H. syriacus</i> ‘Red Heart CV’	1.98	98.4	0.48	0.56	0.52
<i>H. sinosyriacus</i> ‘Autumn Surprise’	1.01	180.4	0.46	0.69	0.59
<i>H. sinosyriacus</i> ‘Lilac Queen’	1.19	194.0	0.53	0.62	0.56
<i>H. paramutabilis</i>	0.88	242.0	0.43	0.70	0.64

3.1.3.3 (Cyto)genetic characteristics

Ploidy levels and genome sizes of the parental species used for ploidy breeding and interspecific hybridisation are summarised in Table 3.4. The results of the genome size measurements were similar on both flow cytometers used.

For *Hydrangea*, a lot of interspecific variation in genome sizes was observed. The smallest genome size was measured for *H. quercifolia* (on average 0.87 pg.1C^{-1}) while *H. macrophylla* and *H. paniculata* had the biggest genome sizes. For *Hydrangea macrophylla* and *H. paniculata* there was also a lot of intraspecific variation. Although *H. macrophylla* ‘Blaumeise’ and ‘Fasan’ were triploid cultivars (Figure 3.2), they had a similar genome size (3.24 pg.1C^{-1} and 3.28 pg.1C^{-1} respectively) as the diploid cultivars ‘Mariesii Perfecta’ and ‘Bergfink’ (3.21 pg.1C^{-1} and 3.40 pg.1C^{-1} respectively). *H. paniculata* which is tetraploid (Figure 3.2) also had an equal genome size (between 3.21 pg.1C^{-1} and 4.70 pg.1C^{-1}) compared to both the diploid and the triploid *H. macrophylla* cultivars. *Hydrangea aspera* had 34 chromosomes (Figure 3.2) instead of 36 for a diploid and had a lower genome size of 1.64 pg.1C^{-1} .

For *Hibiscus* there was almost no variation in genome size within different ploidy levels. The tetraploid species (Figure 3.2) and cultivars had a genome size between 2.13 pg.1C^{-1} for *H. syriacus* ‘Melwhite’ and 2.48 pg.1C^{-1} for *H. paramutabilis*. The octoploid *H. syriacus* cultivars ‘Purple CV₂’ and ‘Red Heart CV’ had a higher genome size of 3.71 pg.1C^{-1} and 4.88 pg.1C^{-1} respectively.

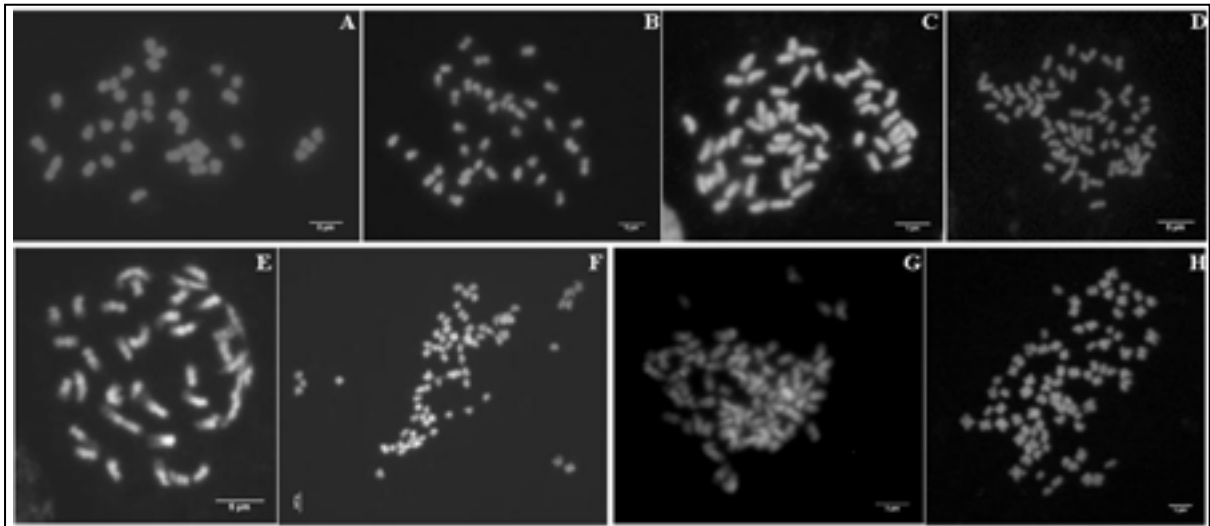


Figure 3.2: Metaphase spreads of chromosomes for (A) *Hydrangea aspera* ($2n = 2x = 34$), (B) *H. quercifolia* ($2n = 2x = 36$), (C) *H. macrophylla* ($2n = 3x = 54$), (D) *H. paniculata* ($2n = 4x = 72$), (E) *Buddleja lindleyana* ($2n = 2x = 38$), (F) *B. davidii* ($2n = 2x = 76$), (G) *B. x weyeriana* ($2n = 4x = 76$) and (H) *Hibiscus syriacus* ($2n = 4x = 80$). (bar = $5 \mu\text{m}$).

For *Buddleja* there was a lot of inter- and intraspecific variation in genome sizes. The genome sizes of the measured *B. davidii* cultivars varied from 1.36 pg.1C⁻¹ for ‘White Ball’ to 1.48 pg.1C⁻¹ for ‘Royal Red’ and ‘Nanho Purple’. All the *B. davidii* cultivars were tetraploid with 76 chromosomes (Figure 3.2). *B. lindleyana*, *B. alternifolia* and *B. crispa*, all diploid (Figure 3.2), had similar genome sizes of 0.94 pg.1C⁻¹, 0.92 pg.1C⁻¹ and 0.95 pg.1C⁻¹ respectively. *B. globosa*, also a diploid species had a higher genome size of 1.12 pg.1C⁻¹. The genome size of *B. x weyeriana* (1.78 pg.1C⁻¹) was much higher compared to *B. davidii*, although both species had the same chromosome number (Figure 3.2).

Table 3.4: Overview of the ploidy level, chromosome number and genome size.

Genotype	Ploidy level (2n)	Chromosome number	Genome Size (pg.1C ⁻¹)
<i>Hydrangea macrophylla</i> ‘Fasan’	3x	54	3.28 ± 0.06
<i>Hydrangea macrophylla</i> ‘Blaumeise’	3x	54	3.24 ± 0.01
<i>Hydrangea macrophylla</i> ‘Bergfink’	2x	36	3.40 ± 0.08
<i>Hydrangea macrophylla</i> ‘Mariesii Perfecta’	2x	36	3.21 ± 0.09
<i>Hydrangea paniculata</i> ‘Unique’	4x	72	3.43 ± 0.06
<i>Hydrangea paniculata</i> ‘White Moth’	4x	72	4.70 ± 0.00
<i>Hydrangea paniculata</i> ‘White Lace’	4x	72	3.69 ± 0.09
<i>Hydrangea paniculata</i> ‘Praecox’	4x	72	3.21 ± 0.08
<i>Hydrangea quercifolia</i> ‘Snow Flake’	2x	36	0.88 ± 0.05
<i>Hydrangea quercifolia</i> ‘Sikes Dwarf’	2x	36	0.86 ± 0.05
<i>Hydrangea quercifolia</i> ‘Tennessee Clone’	2x	36	0.87 ± 0.07
<i>Hydrangea serrata</i> ‘Blue Bird’	2x	36	2.04 ± 0.02
<i>Hydrangea aspera</i> ‘Macrophylla’	2x	34	1.64 ± 0.01
<i>Hibiscus syriacus</i> ‘Melwhite’	4x	80	2.13 ± 0.15
<i>Hibiscus syriacus</i> ‘Oiseau Bleu’	4x	80	2.34 ± 0.16
<i>Hibiscus syriacus</i> ‘Purple CV ₂ ’	8x	160	3.71 ± 0.03
<i>Hibiscus syriacus</i> ‘Red Heart CV’	8x	160	4.88 ± 0.08
<i>Hibiscus sinosyriacus</i> ‘Autumn Surprise’	4x	80	2.35 ± 0.05
<i>Hibiscus sinosyriacus</i> ‘Lilac Queen’	4x	80	2.33 ± 0.02
<i>Hibiscus paramutabilis</i>	4x	82	2.48 ± 0.08
<i>Buddleja lindleyana</i>	2x	38	0.94 ± 0.01
<i>Buddleja globosa</i>	2x	38	1.12 ± 0.04
<i>Buddleja alternifolia</i>	2x	38	0.93 ± 0.06
<i>Buddleja davidii</i> ‘Royal Red’	4x	76	1.48 ± 0.01
<i>Buddleja davidii</i> ‘Nanho Purple’	4x	76	1.48 ± 0.02
<i>Buddleja davidii</i> ‘White Profusion’	4x	76	1.41 ± 0.06
<i>Buddleja davidii</i> ‘White Ball’	4x	76	1.36 ± 0.03
<i>Buddleja davidii</i> ‘Nanhoensis Alba’	4x	76	1.46 ± 0.03
<i>Buddleja x weyeriana</i> ‘Sungold’	4x	76	1.78 ± 0.01
<i>Buddleja crispa</i> ‘Moondance’	2x	38	0.96 ± 0.03

An overall view of the genetic relationships between the different parental species of *Hydrangea*, *Hibiscus* and *Buddleja*, analysed by AFLP, is given in Figure 3.3.

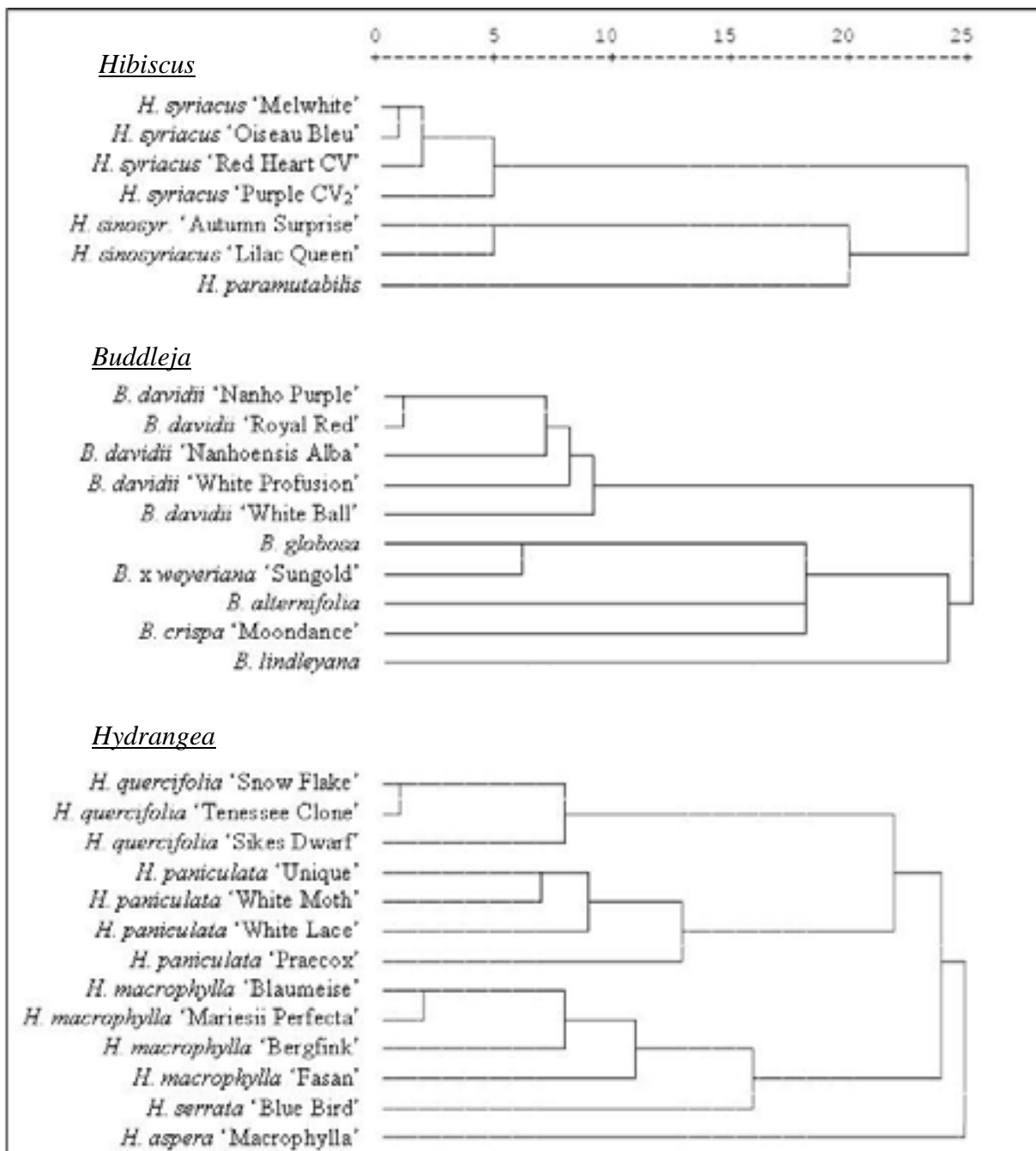


Figure 3.3: Dendrogram showing the genetic relatedness of the *Hibiscus*, *Buddleja* and *Hydrangea* parental species tested by AFLP. For *Hydrangea*, the dendrogram is shown which is constructed with the markers generated by the primer combinations with 6 selective nucleotides.

For *Hydrangea*, the 2 primer combinations with 6 selective nucleotides (E-ACG/M-CAA, E-AGG/M-CTT) yielded less markers (370) useful for scoring while the 2 primer combinations with 7 selective nucleotides (E-ACCG/M-CTT, E-ACGT/M-CAT) generated 397 markers. For distinguishing *H. macrophylla* and *H. serrata*, 2 species of the same taxonomic section,

all primer combinations were efficient. Although within the species there was genetic variation between cultivars, the different species were grouped in different clusters.

For *Hibiscus*, in total, the primer combinations E-AAC/M-CAT, E-ACA/M-CTG and E-AAG/M-CTA generated 362 markers that could be used for scoring. According to the AFLP data the 3 different *Hibiscus* species could be clearly differentiated. *H. sinosyriacus* showed a higher affinity to *H. paramutabilis* than to *H. syriacus*. Some degree of genetic variation was observed within the *H. syriacus* cultivars.

For *Buddleja* the crossing parents were very polymorphic. In total, the primer combinations E-ACA/M-CTG and E-AGG/M-CTT generated 303 markers that were used for scoring. The group of *B. davidii* cultivars came forward as a separate identity that was genetically different from the other *Buddleja* parent species. In this *B. davidii* cluster, a certain degree of genetic variation was observed. *B. x weyeriana*, originating from a cross between *B. globosa* x *B. davidii* (F2 selection) was closer related to *B. globosa* than to *B. davidii*.

3.1.4 Discussion

3.1.4.1 Morphological characteristics

In crossing schemes, it is necessary that parent plants have a similar pollen (and pistil) size in order to increase the success rate of pollinations. Also a similar flowering period is preferred, unless the pollen can be stored or flowering can be retarded or stimulated by temperature or light treatment. In our study, parental species for the different genera had the same type of flowers. Only *Hydrangea quercifolia* ‘Snow Flake’ had double flowers, while all other *Hydrangea* species had single flowers. The flowering period of the different parental species overlapped. Only for *Buddleja*, some earlier flowering species *B. crispa*, *B. alternifolia* and *B. globosa* were used in the crosses. So these species were not used as female parents in crosses with *B. davidii*, *B. lindleyana* or *B. x weyeriana*. Since the pollen of the earlier flowering species could be stored at -20°C for at least one month, they were only used as male parents in these crosses, which can be a problem due to unilateral incongruity.

Also pollen fertility and viability is of high importance for breeding success. Tests for germination capacity on *in vitro* media provide direct and reliable assessment of viability of the pollen (Heslop-Harrison *et al.* 1984; Heslop-Harrison 1999). In this study, some parental species of *Hydrangea* and *Buddleja*, *H. macrophylla* ‘Bergfink’, *H. quercifolia* ‘Tennessee Clone’, *H. aspera*, *B. lindleyana*, *B. globosa*, *B. davidii* ‘White Ball’ and *B. x weyeriana*, had a very low pollen germination capacity ($\leq 5\%$). Differences in pollen viability and germination capacity were noticed among individuals of the same species as well as among samples from the same individual (Shivanna and Rangaswamy 1992). Fertility of pollen varied also according to the flowering season (early, middle or late periods) and at different

day times (Shivanna and Rangaswamy 1992). Probably the way of collecting the pollen might explain mostly the low germination capacity of the pollen. In our study, pollen was collected and anthesis was stimulated by overnight illumination. It could be that the pollen was collected too early. However, by collecting and analysing the pollen in a standardised way, a classification could be made for species and cultivars with higher or lower pollen germination capacity.

3.1.4.2 (Cyto)genetic characteristics

Different ploidy levels are present among species of *Buddleja* and *Hydrangea*. *B. globosa*, *B. lindleyana*, *B. crispa* and *B. alternifolia* are diploid species, while *B. davidii* is tetraploid. *Hydrangea quercifolia*, *H. aspera* and *H. serrata* are diploid, while for *H. macrophylla* both diploid and triploid cultivars are used. *H. paniculata* is tetraploid. Differences in ploidy level are often an important incongruity barrier in interspecific hybridisation resulting in malformation of endosperm and the inhibition of germination (Badger 1988). Endosperm absence or retarded development often causes spontaneous abortion of the fruits (Sharma 1995). Polyploidisation offers sometimes a solution towards the problem of parent species having a different ploidy level (Stebbins 1971). Rose *et al.* (2000b) already succeeded in doubling the chromosome number of *B. globosa* in the view of breeding experiments with *B. davidii*. Eeckhaut (2003) and Duron and Morand (1978) obtained chromosome doubled plants of *B. davidii*. For other *Buddleja* species or for *Hydrangea* species polyploidisation was not reported so far.

For *Hibiscus* there was not much variation on genome sizes between the different species. On the contrary, for *Buddleja* and *Hydrangea* a lot of inter- and intraspecific variation on genome sizes was observed, which indicate difficulties when performing interspecific crosses. The genome sizes measured in our study were comparable with the genome sizes found in literature for *H. macrophylla* (3.33 pg.1C⁻¹ for triploid cultivars and 2.15 or 2.28 pg.1C⁻¹ for diploid cultivars), *H. paniculata* (1.89 pg.1C⁻¹ for diploid cultivars), *H. quercifolia* (0.98 pg.1C⁻¹), *H. serrata* (2.15 pg.1C⁻¹) and *H. aspera* (1.51 pg.1C⁻¹) (Zonneveld *et al.* 2005; Cerbah *et al.* 2001) and for *B. davidii* (1.45 pg.1C⁻¹) and *B. globosa* (0.86 pg.1C⁻¹) (Hanson *et al.* 2001; Zonneveld *et al.* 2005). Differences in genome size within a complex of species are also often correlated with ecological factors such as altitude (Caceras *et al.* 1998) or can be a response to physical, chemical or genetic stresses as a strategy for adaptation to a changing environment, as was suggested for light effects on *Helianthus annuus* by Price and Johnston (1996). Ecological and geographical differentiation of genome sizes has been documented either on interspecific or intraspecific levels for a lot of other genera and species (Ohri 1998; Knight *et al.* 2005). Most of the *Hydrangea* species originate from Asia, which may be considered as the center of origin of the genus. Only a few species exist in North and South America (Cerbah *et al.* 2001), e.g. *H. quercifolia*. In our study, for *Hydrangea* the smallest genome size was of *H. quercifolia* (0.87 pg.1C⁻¹), originating from North or South America. The different ecological and geographical selective pressures could have led to a decrease of the DNA content in American species (Cerbah *et al.* 2001). Also for *Buddleja*, different

geographic origin could have led to a decrease or increase of the genome size, like *B. davidii* originating from China and *B. globosa* from Peru and Chile. Significant differences in genome size were likewise found for example in *Musa acuminata* (Lysak *et al.* 1999). These authors proposed a relation with the geographical origin of accessions. Nevertheless, the occurrence of important internal chromosome restructurings involving the amounts of tandemly or interspersed DNA repeated sequences also influences the genome size of plants. This genome fraction can evolve rapidly and is responsible for a major change of DNA content (Flavell 1986; Uozu *et al.* 1997).

The chromosome numbers of the *Hibiscus*, *Hydrangea* and *Buddleja* species are very high. This corresponds to the general trend that, in flora of the temperate zone, trees and shrubs have higher basic numbers than the perennial herbs (Cerbah *et al.* 2001). One hypothesis is that a high primary basic chromosome number ($x = 18$ for *Hydrangea*, $x = 19$ for *Buddleja*, $x = 20$ for *Hibiscus*) is ancestral for the genus. Another hypothesis is that the genera are ancient polyploids. For example for *Hydrangea*, the primary basic chromosome number would be $x = 6$ or $x = 9$ and $x' = 18$ would then be of secondary polyploid origin. Schoennagel (1931) studied the chromosomal phylogeny of the *Saxifragaceae*, which included the genus *Hydrangea*, and considered that the basic chromosome number of the *Saxifragaceae* was $x = 7$, from which a basic chromosome number of $x = 9$ would be derived. Soltis *et al.* (1993) considered an original basic chromosome number of $x = 7$ or $x = 11$ for the family on the basis of *rbcL* sequences. The odd chromosome number observed for *H. aspera* can be the result of a decreasing dysploidy (Cerbah *et al.* 2001). Although chromosome numbers are high, genome sizes of the genera are considered as low. This implicates that chromosomes of the *Hydrangea*, *Hibiscus* and *Buddleja* species are small.

Van De Weyer (1920) made crosses between *B. globosa* \times *B. davidii* var. *magnifica*. From a greyish-white, violet-tinted F1 seedling seeds were harvested and sown. Among the resulting F2 seedlings, one yellowish-flowered plant was selected and named *B. x weyeriana* 'Golden Glow' (Van De Weyer 1920). *B. x weyeriana* 'Sungold', which was used in our experiments, is a sport from *B. x weyeriana* 'Golden Glow' (De Vogel 1967). Based on our morphological characteristics and AFLP analysis, the hybrid nature of *B. x weyeriana* 'Sungold' could be confirmed. It was expected that *B. x weyeriana* is a triploid plant with 57 chromosomes. However chromosome counting revealed 76 chromosomes for *B. x weyeriana*. The genome size of *B. x weyeriana* (1.78 pg.1C^{-1}) was even higher than the genome size of the tetraploid *B. davidii* cultivars (on average 1.44 pg.1C^{-1}). Based on these findings *B. x weyeriana* can be assumed to be a tetraploid ($2n=4x=76$). Different hypotheses might explain these observations: (i) *B. x weyeriana* is originating from an interspecific cross between a $2n$ -gametes producing *B. globosa* and a normal gamete producing *B. davidii*. *B. globosa* would then produce egg cells (GG) with a gametophytic genome size of 2.24 pg and *B. davidii* pollen (DD) would have a genome size of 1.44 pg , resulting in an F1 ancestor (GGDD) with a genome size of 1.84 pg.1C^{-1} (more or less similar to the value measured in this study: 1.78 pg.1C^{-1}), which is then selfed. (ii) *B. x weyeriana* is originating from a triploid F1 ancestor (GDD) producing diploid gametes (GD with a genome size of 1.84 pg) which is then selfed, resulting in an F2 plant (GGDD) with a genome size of 1.84 pg.1C^{-1} . (iii) *B. x weyeriana*

resulted from a backcross of the F1 ancestor (GDD) producing triploid gametes (GDD; 2.56 pg) with *B. globosa* producing normal gametes (G), also resulting in an F2 plant (GGDD) with a genome size of 1.84 pg.1C⁻¹ (again more or less similar to 1.78 pg.1C⁻¹, measured in this study). Although they were not observed in *Buddleja* so far, unreduced gametes occur in most plant species (Lim *et al.* 2003). This makes hypothesis (i) to be considered as the most probable one. But also the formation of diploid [hypothesis (ii)] and triploid [hypothesis (iii)] gametes by triploids, is far from unusual (Otto and Whitton 2000) e.g. in roses (Van Huylenbroeck *et al.* 2005). In all three cases of origin, *B. x weyeriana* is a tetraploid with a GGDD genome composition.

Finally, for the parental species of each genera the genetic relatedness was analysed using AFLP. For *Hibiscus* the results were in agreement with Van Huylenbroeck *et al.* (2000). They also concluded that *H. paramutabilis* and *H. sinosyriacus* were closer related to each other than to *H. syriacus*. Within *Hydrangea*, *H. macrophylla* and *H. serrata* are the most closely related, which was expected since they belong to the same subsection (McClintock 1957). In taxonomic notes, *H. macrophylla* and *H. serrata* are sometimes described as 2 different species (Wilson 1923; Haworth-Booth 1984) and in other studies as 1 species (McClintock 1957). According to McClintock (1957) some cultivars of *H. macrophylla* and *H. serrata* are not distinguishable in morphology, which can indicate that these cultivars are hybrids resulting from crosses between *H. macrophylla* and *H. serrata* (Zonneveld 2004). It is a well known phenomenon of hybrids that they produce unreduced gametes (Ramanna and Jacobsen 2003). Crosses between unreduced gametes producing *H. macrophylla* or *H. serrata* cultivars and normal gametes producing *H. macrophylla* could explain the existence of triploid *H. macrophylla* cultivars like 'Fasan' and 'Blaumeise' (Zonneveld 2004). Genetic distance with the other species, *H. quercifolia*, *H. paniculata* and *H. aspera*, is much greater. This is in agreement with the study of Rinehart *et al.* (2006) where SSR markers were used to estimate genetic distance between different *Hydrangea* species. Based on our AFLP data, *Buddleja x weyeriana* is more closely related to *B. globosa* than to *B. davidii*. All other species are genetically very different. So it can be concluded that the species within the genera *Hibiscus*, *Hydrangea* and *Buddleja* are genetically very different, which is an indication for difficult interspecific hybridisation.

3.2 Karyotyping of *Hydrangea* species

3.2.1 Introduction

Cytogenetic incompatibility among the species is a very important barrier to obtain viable interspecific hybrids (Reed *et al.* 2001). Hence, a better cytogenetic understanding of the genus can help in breeding programs. Therefore, in the second part of this chapter, a complete karyotype is constructed of *Hydrangea macrophylla*, *H. paniculata* and *H. quercifolia*. On these karyotypes the sites of the 45S rRNA genes are localised using FISH. The physical and genetical localisation of the rRNA genes on the *Hydrangea* karyotype can provide a first link between physical and genetic maps, which can lead to a better understanding of the genetic structure of *Hydrangea*. The chromosome portraits made from *Hydrangea* can be used to trace chromosome behaviour in the interspecific hybrids resulting from breeding work between *H. macrophylla*, *H. paniculata* and *H. quercifolia*.

3.2.2 Specific materials and methods

Hydrangea macrophylla ‘Fasan’ ($2n = 3x = 54$), *H. paniculata* ‘Unique’ ($2n = 4x = 72$) and *H. quercifolia* ‘Sikes Dwarf’ ($2n = 2x = 36$) were used for karyotype analysis. In the 45S rDNA FISH experiment $1.25 \text{ ng} \cdot \mu\text{l}^{-1}$ probe DNA was used in the hybridisation mix.

3.2.3 Results

3.2.3.1 Karyotype analysis

The average of the total length of the (monoploid) metaphase complement was $47.027 \mu\text{m}$ for *H. macrophylla* ‘Fasan’, $37.263 \mu\text{m}$ for *H. paniculata* ‘Unique’ and $46.038 \mu\text{m}$ for *H. quercifolia* ‘Sikes Dwarf’ (Table 3.5). The karyotypes of the 3 species were very different. The karyotype of *H. macrophylla* ‘Fasan’ consisted of 6 metacentric (M), 8 submetacentric (SM) and 4 subtelocentric (ST) chromosomes. The *H. paniculata* ‘Unique’ karyotype included 7 M, 10 SM and 1 ST chromosomes. And the karyotype of *H. quercifolia* ‘Sikes Dwarf’ consisted of 6 M, 10 SM and 2 ST chromosomes. The difference between the longest and the shortest chromosome in the karyotype was the biggest for *H. quercifolia* ‘Sikes Dwarf’ ($1.935 \mu\text{m}$) and the smallest for *H. paniculata* ‘Unique’ ($1.331 \mu\text{m}$). The 3 karyotypes expressed an asymmetric index between 64.29% for *H. paniculata* ‘Unique’ and 66.45% for

H. macrophylla ‘Fasan’ and a condensation index between 1.782 for *H. macrophylla* ‘Fasan’ and 2.078 for *H. quercifolia* ‘Sikes Dwarf’.

Table 3.5: Summary of the morphometric and karyotypic data (monoploid metaphases) for *H. macrophylla* ‘Fasan’, *H. paniculata* ‘Unique’ and *H. quercifolia* ‘Sikes Dwarf’. Data are averages \pm SD (n = 10).

	<i>H. quercifolia</i> ‘Sikes Dwarf’	<i>H. macrophylla</i> ‘Fasan’	<i>H. paniculata</i> ‘Unique’
Chromosome number	2n = 2x = 36	2n = 3x = 54	2n = 4x = 72
Total chromosome complement (μm) ^z	46.038 \pm 0.345	47.027 \pm 0.242	37.263 \pm 0.377
Length of longest chromosome (μm)	3.730 \pm 0.521	3.436 \pm 0.294	2.742 \pm 0.517
Length of shortest chromosome (μm)	1.795 \pm 0.225	1.928 \pm 0.282	1.411 \pm 0.306
Asymmetry of the karyotype (%)	65.04	66.45	64.29
Condensation index	2.078 \pm 0.373	1.782 \pm 0.258	1.943 \pm 0.489
Chromosome formula	2 ST+10 SM+6 M	4 ST+8 SM+6 M ^y	1 ST+10 SM+7 M

^z total chromosome complement on haploid level; ^y ST = subtelocentric chromosome, SM = submetacentric chromosome, M = metacentric chromosome

3.2.3.2 Chromosome localisation of 45S rDNA

For all analysed species, it was very difficult to determine secondary constrictions on the DAPI stained metaphases. But after hybridisation with pTa71 probe (45S rDNA), the NOR were clearly visible (Figure 3.4). FISH of 45S rDNA with *H. macrophylla* ‘Fasan’ revealed a signal (3 spots on homologous chromosomes) on the secondary constriction (NOR) of chromosome 2, one of the SM chromosomes of the karyotype (Table 3.6).

Table 3.6: Summary of position and abundance of 45S rDNA repeats on chromosomes of *H. macrophylla* ‘Fasan’, *H. paniculata* ‘Unique’ and *H. quercifolia* ‘Sikes Dwarf’. Data are averages \pm SD (n = 10).

Genotype	Chromosome number	Length of short arm (μm)	Length of long arm (μm)	Centromeric index
<i>H. macrophylla</i> ‘Fasan’	2	1.131 \pm 0.149	2.082 \pm 0.199	0.348 \pm 2.792
<i>H. paniculata</i> ‘Unique’	2	1.090 \pm 0.199	1.531 \pm 0.211	0.415 \pm 0.027
<i>H. paniculata</i> ‘Unique’	5 ^z	0.997 \pm 0.203	1.357 \pm 0.204	0.425 \pm 0.035
<i>H. paniculata</i> ‘Unique’	11	0.682 \pm 0.132	1.322 \pm 0.251	0.340 \pm 0.023
<i>H. quercifolia</i> ‘Sikes Dwarf’	3	1.327 \pm 0.214	1.829 \pm 0.278	0.419 \pm 0.039
<i>H. quercifolia</i> ‘Sikes Dwarf’	8	1.123 \pm 0.141	1.511 \pm 0.201	0.427 \pm 0.036

^z No NOR was detected here

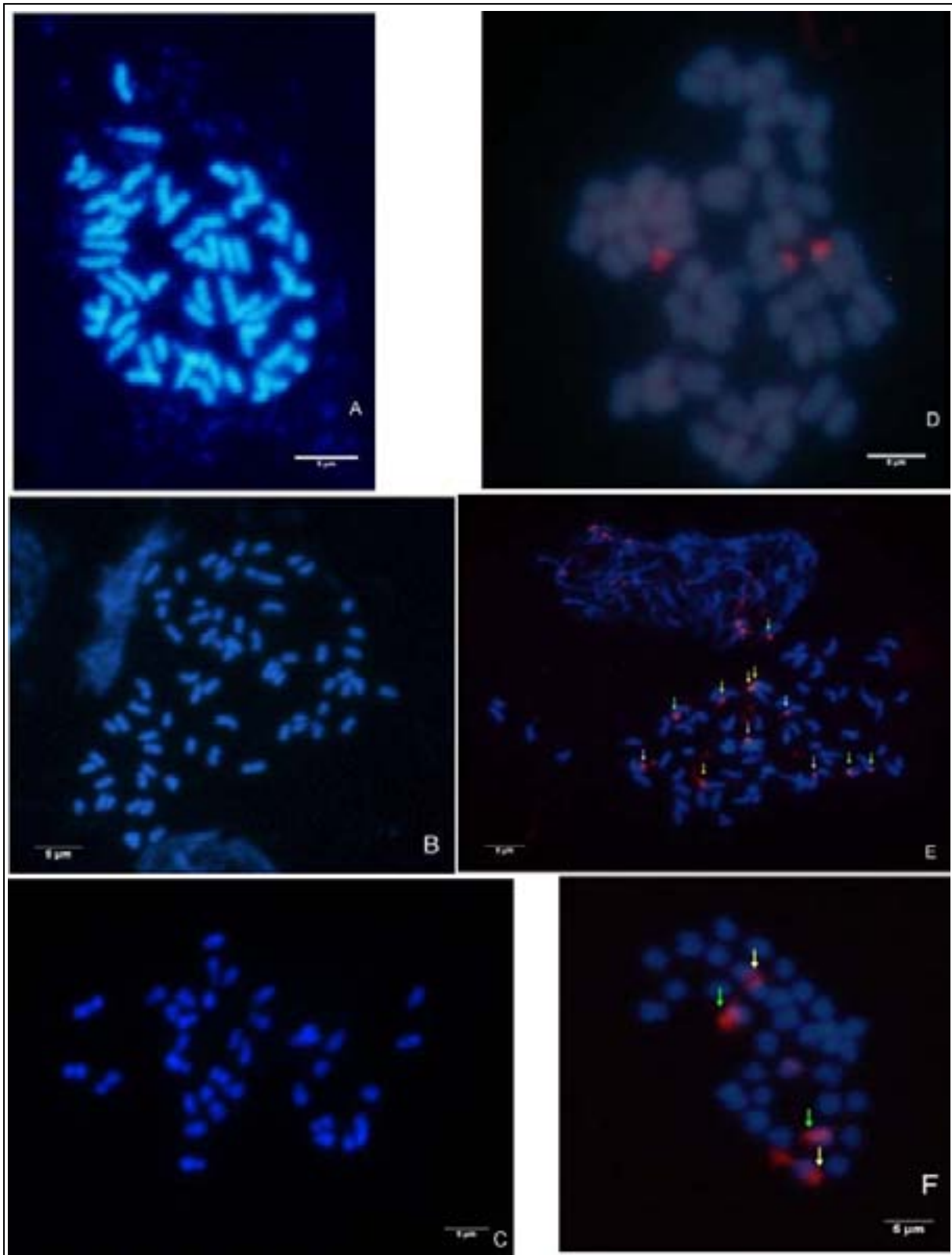


Figure 3.4: DAPI-stained metaphase of *H. macrophylla* 'Fasan' (A), *H. paniculata* 'Unique' (B) and *H. quercifolia* 'Sikes Dwarf' (C). Hybridisation with pTa71 probe (containing 45S rDNA) on a metaphase spread of *H. macrophylla* 'Fasan' (D), *H. paniculata* 'Unique' (E) and *H. quercifolia* 'Sikes dwarf' (F). For *H. macrophylla* 45S rRNA genes are located on one locus of chromosome 2 of the karyotype, for *H. paniculata*, 45S rRNA genes are detected on one locus of chromosome 2 (yellow arrows), chromosome 5 (green arrow) and chromosome 11 (blue arrow) of the karyotype. For *H. quercifolia*, 45S rRNA genes are visualised on one locus chromosome 3 (yellow arrows) and chromosome 8 (green arrows) of the karyotype. Images were taken with 1000x magnification (bar = 5 µm).

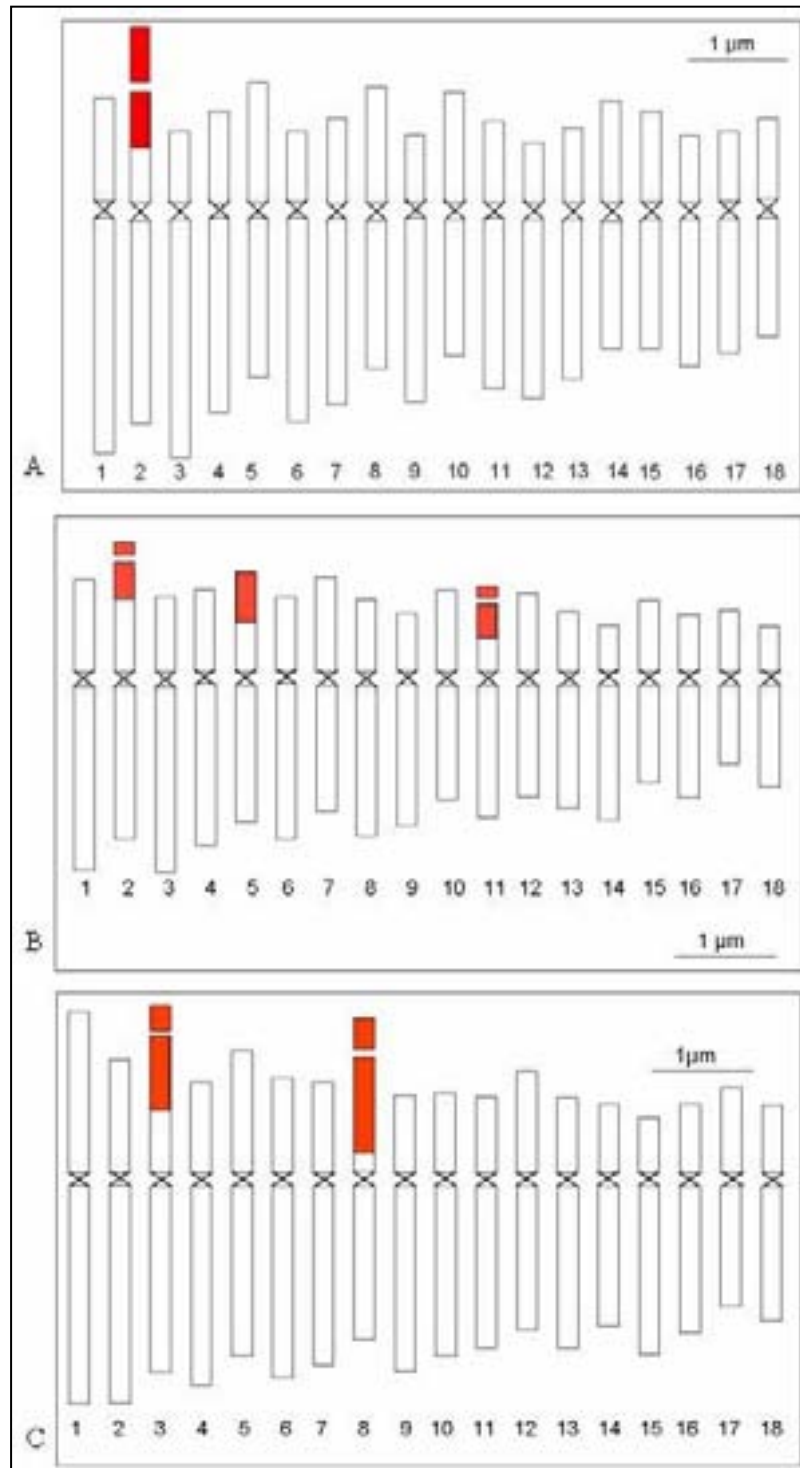


Figure 3.5: Idiogram of *H. macrophylla* 'Fasan' (A), *H. paniculata* 'Unique' (B) and *H. quercifolia* 'Sikes Dwarf' (C), with 45S rDNA signals and NORs indicated. For *macrophylla*, chromosomes 3, 6, 9 and 12 are subtelocentric (ST), chromosomes 1, 2, 4, 7, 11, 13, 16 and 17 submetacentric (SM), chromosomes 6, 8, 10, 14, 15 and 18 metacentric (M). For *paniculata*, chromosome 14 is ST, chromosomes 1, 3, 4, 6, 8, 9, 11, 13, 16 and 18 SM, chromosomes 2, 5, 7, 10, 12, 15 and 17 M. For *quercifolia*, chromosomes 4 and 15 are ST, chromosomes 2, 6, 7, 9, 10, 11, 13, 14, 16 and 18 SM, chromosomes 1, 3, 5, 8, 12 and 17 M.

For *H. paniculata* ‘Unique’ 45S rDNA signals were detected on the secondary constriction (NOR) of chromosomes 2 (M) and 11 (SM) (each on 4 homologous chromosomes). Another 45S rDNA signal was detected on chromosome 5 (4 homologous chromosomes), but no NOR was visualised here (Table 3.6). Chromosome 5 is another M chromosome of the karyotype. FISH of 45S rDNA with *H. quercifolia* ‘Sikes Dwarf’ revealed a hybridisation signal on the secondary constriction (NOR) of chromosomes 3 and 8 (each on 2 homologous), 2 of the M chromosomes of the karyotype (Table 3.6).

For all three species, the hybridisation signal always was located on 1 locus and distally on the short arm of the chromosomes. The idiograms of the 3 *Hydrangea* species, with indication of the NOR are presented in Figure 3.5.

3.2.4 Discussion

Although the chromosomes of *Hydrangea* were in general very small, they were very well distinguishable and the haploid karyotype could be constructed. Submetacentric chromosomes were mostly present in all 3 karyotypes. However, clear differences in the karyotypes of the 3 species could be observed. The chromosomes in the karyotype of *H. paniculata* ‘Unique’ were smaller compared to the other 2 analysed species. The karyotype of *H. quercifolia* ‘Sikes Dwarf’ had the biggest difference between the largest and the shortest chromosome. The total haploid metaphase complement of *H. paniculata* ‘Unique’ was the smallest. For *H. macrophylla* ‘Fasan’ and *H. paniculata* ‘Unique’ a weak negative correlation could be observed between the total chromosome complement and the total DNA content (see Table 3.4). *H. macrophylla* ‘Fasan’ had a smaller total DNA content of $3.28 \text{ pg} \cdot 1\text{C}^{-1}$ compared to $3.43 \text{ pg} \cdot 1\text{C}^{-1}$ of *H. paniculata* ‘Unique’. However *H. macrophylla* ‘Fasan’ had a bigger total chromosome complement compared to *H. paniculata* ‘Unique’. For *H. quercifolia* ‘Sikes Dwarf’ a strong negative correlation between total chromosome complement and total DNA content was revealed. *H. quercifolia* ‘Sikes Dwarf’ had the smallest DNA content ($0.86 \text{ pg} \cdot 1\text{C}^{-1}$) but the total chromosome complement was almost the same as for *H. macrophylla* ‘Fasan’. Assuming that DNA condensation is equal along all chromosomes of the karyotype, $1 \mu\text{m}$ of a chromosome contains 0.046 pg DNA in *H. macrophylla* ‘Fasan’ and in *H. paniculata* ‘Unique’ and 0.019 pg DNA in *H. quercifolia* ‘Sikes Dwarf’. This means that DNA condensation is 2.5 times higher for *H. macrophylla* ‘Fasan’ and *H. paniculata* ‘Unique’ chromosomes compared to *H. quercifolia* ‘Sikes Dwarf’ chromosomes. It is known that chromosome condensation differs among plants. For instance, Khrustaleva and Kik (2001) revealed that the chromosome condensation in onion is 6 times higher than in tomato. Difference in geographic origin or divergent evolution of species encouraged by a reproductive isolation and different selection pressures might be reasons for these differences in DNA condensation. The *Hydrangea* species used in our study all belong to the same taxonomic section *Hydrangea*, but they have a different geographic origin. *H. quercifolia*

originates from North or South America while most other *Hydrangea* species originate from Asia.

Cerbah *et al.* (2001) also suggested a karyotype of *H. macrophylla*. Their karyotype contained 6 metacentric chromosomes, but had 4 submetacentric (versus 8 in our karyotype) and 8 subtelo-centric (versus 4 in our karyotype) chromosomes. However, the chromosomes in their study were not always well-distinguishable. The different staining method used for the chromosome spreads preparation, Feulgen staining in the study of Cerbah *et al.* (2001) versus DAPI staining in our study, might explain the difference in karyotype for *H. macrophylla*. Feulgen staining is effective in all species but in plants with small chromosome size, like *Hydrangea*, it can give weak staining results (Maluszynska 2003). For species with small chromosomes fluorescent staining with DAPI is specially recommended (Maluszynska 2003). Also in the karyotype of *H. aspera* (Mortreau 2003), submetacentric chromosomes are mostly present. Again, Feulgen staining was used to stain the chromosome slides.

The variability among *H. macrophylla* 'Fasan', *H. paniculata* 'Unique' and *H. quercifolia* 'Sikes Dwarf' is also expressed by use of the 45S rDNA FISH probe. In plants and animals, 45S rDNA usually is associated with a NOR. This region has a rich GC content (Schweizer 1976; Sumner 1990; Ebert *et al.* 1996). A NOR region is also associated with a secondary constriction (satellite) in the chromosome. For chromosome 2 of *H. macrophylla* 'Fasan', chromosome 2 and 11 of *H. paniculata* 'Unique' and chromosome 3 and 11 of *H. quercifolia* 'Sikes Dwarf' a NOR region was detected, associated with a secondary constriction. However, on chromosome 5, only a 45S rDNA signal was detected but no NOR (no secondary constriction). This is not an exception. For instance in *Allium cepa*, rRNA genes were located on the NOR-bearing chromosome 6 but also on the smallest chromosome 8, which does not possess a NOR (Ricroch *et al.* 1992). Secondary constrictions are naturally fragile and they are sometimes lost during preparation. However, it may also reflect differences in the activity of the rRNA genes. *In situ* hybridisation permits the detection of both active and inactive rRNA genes (Zurita 1998).

The difference in strength of the hybridisation signal observed among *H. macrophylla* 'Fasan', *H. paniculata* 'Unique' and *H. quercifolia* 'Sikes Dwarf', can be explained as a difference in the copy number of the rDNA (Maluszynska and Schweizer 1989). Satellite repeats also are known to undergo rapid changes in copy numbers, resulting in their accumulation in a single species or a genus (Macas *et al.* 2002). In plants, the copy number of the rDNA is rather high, between 600 and 8500 in a haploid genome (Lapitan 1991). Although FISH is not a completely quantitative technique, the intensity of the signal can be related to the copy number of similar structures (Leitch and Heslop-Harisson 1992).

The satellites (NOR) were always attached distally to the short arm of a chromosome. This is in agreement with Lima-de-Faria (1979) who observed that satellites are generally attached to the short arm of a NOR chromosome. Lima-de-Faria (1979) analysed the NORs in over 700 species and reported that in 87% of the cases, the NOR was located on the short arm of the chromosome.

rDNA probes corresponding to the site of ribosomal RNA genes were detected through FISH in many crops (Mukai *et al.* 1991). Species specific sequence together with FISH are useful markers to identify the rest of chromosomes, which can not be distinguished through banding techniques (Kamstra *et al.* 1997). The number and position of rDNA sites in *Hydrangea* species constituted a cytological marker to characterise the species. The chromosome portraits of *Hydrangea* species can be used to trace chromosome behaviour in the interspecific hybrids resulting from breeding work. Also the occurrence of postfertilisation barriers to interspecific hybridisation could result from a differentiation of species genomes (Buitendijk and Ramanna 1996). For *Hydrangea* species there are a lot of differences in the karyotypes, which can indicate difficult interspecific hybridisation.

3.3 Conclusion

A brief morphological study was carried out for the parental species. Most species had the same type of flower. Also flowering time of the species overlapped for most species. Pollen of some *Buddleja* and *Hydrangea* species had a low germination capacity *in vitro*. So possibly these species will not be successful as crossing parent in interspecific crosses.

It was showed that genome size measurements, ploidy determination, AFLP and karyotype analysis were efficient techniques to give a lot of (cyto)genetic information for parental woody ornamental species in general and for *Hydrangea*, *Hibiscus* and *Buddleja* in particular. Large variation in ploidy level and genome sizes were observed especially for *Hydrangea* and *Buddleja* species. For the 3 genera the species were genetically very divergent as was revealed by AFLP analysis. By karyotype analysis for *Hydrangea* dissimilarities were observed between species for chromosome profile and 45S rDNA sites. The great (cyto)genetic differences between the parental species might indicate difficult interspecific hybridisation within *Hydrangea*, *Hibiscus* and *Buddleja*.

The choice of crossing parents for experimental interspecific hybridisation is critical. So far, in most breeding programs choices are mainly based on morphological parameters. A better insight in the (cyto)genetic structure of different species will give new possibilities to predict the success rate of interspecific crosses. Interspecific hybridisation is therefore a matter of making compromising choices based on the morphological and (cyto)genetic knowledge that is available about candidate parental species. It should be ideal to have a genetic distance that gives a prediction on how crossable 2 species are. This can help in a more direct and specific interspecific breeding program. When the differences between the parental species are too large, interspecific crosses will not be successful. Besides these, also basic information on the floral characteristics (floral biology, way of reproduction, biology of the pollen, best time for pollination, ...) of the possible crossing parents remains necessary.

CHAPTER 4

PLOIDY BREEDING*

4.1 Introduction

Ploidy manipulation can be applied in a breeding program for 3 main reasons: (i) to obtain parent plants with an equal ploidy level (more efficient interspecific crosses), (ii) to restore fertility of often (almost) sterile F1 hybrids and (iii) as a breeding goal itself. Polyploidy can be induced artificially in a cell by chemicals such as colchicine, oryzalin and trifluralin or obtained from crosses between plants which produce unreduced gametes.

To obtain a yellow flowered *Buddleja* hybrid with a *B. davidii*-like morphology, a cross was attempted between *B. globosa* (yellow-orange flowers; $2n = 2x = 38$) and *B. davidii* ($2n = 4x = 76$). By doubling the chromosome amount of *B. globosa* it is expected that the cross with *B. davidii* will be more efficient. Therefore, in the first part of this chapter chromosome doubling in *B. globosa* is performed by applying oryzalin and trifluralin on *in vitro* seedlings and shoots.

Basic chromosome number of *Hibiscus syriacus* is $x = 20$ and most cultivars are tetraploid, $2n = 4x = 80$ (Skovsted 1941). However, in literature frequently the term diploid is used instead of tetraploid. Breeding work resulted also in hexaploid (so called triploid) and octoploid (so called tetraploid) cultivars, which in general grow very vigorously, have larger flowers and a longer flowering period, like 'Diana', 'Hélène', 'Pink Giant' and 'Melrose' (Egolf 1971, 1981, Van Huylenbroeck *et al.* 2000). These cultivars also produce few seeds. Because of this reduced seed production, flowering is never inhibited during the season. The hexaploid cultivars were positively evaluated in different judging reports of the *H. syriacus* assortment (Van De Laar 1997; Van Huylenbroeck *et al.* 1998). However, today all commercial hexaploid cultivars have white to pink flower colors. In the second part of this chapter the goal is to generate a hexaploid *H. syriacus* cultivar with a deep blue flower color and vigorous growth.

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Van Huylenbroeck J, Van Laere K, Leus L. Breeding strategies for woody ornamentals: selection towards disease resistance (2006) Accepted for publication in the proceedings of the international plant propagators society conference: 'Sustainable Plant Production', Grobbendonk, Belgium (29/08/06 – 01/09/06)

4.2 Specific materials and methods

4.2.1 Plant material

Seed(ing)s and shoots of *Buddleja globosa* ($2n = 2x = 38$) and seedlings of 2 tetraploid cultivars of *Hibiscus syriacus* ('Oiseau Bleu' and 'Melwhite') and 2 octoploid cultivars of *H. syriacus* ('Red Heart CV' and 'Purple CV₂') were used. The morphological and (cyto)genetic characteristics of these species and cultivars are described in Chapter 3.

4.2.2 Application of mitotic inhibitors

4.2.2.1 Mitotic inhibitors

The mitosis inhibitors colchicine (COL), oryzalin (ORY) and trifluralin (TRI) were used. Appropriate ORY and TRI solutions were prepared based respectively on the commercial herbicides Surflan and Treflan (DowElancoTM), containing 40.4% active ORY and 45.9% active TRI respectively. COL was available in crystallised form. For treating plant material, 0.5% (v/v) DMSO and 0.05% (v/v) Teepol were added to all solutions. The surfactant 'wets' the epidermal cell surface while DMSO increases cell permeability, so jointly they increase the effectiveness of the solution. For *in vitro* applications anti-mitotic solutions were filter sterilised (0.22 µm).

4.2.2.2 *In vitro* polyploidisation of *B. globosa*

For the treatment of *B. globosa* 4 different *in vitro* experiments were performed. In a first experiment seeds were sterilised and sown on medium Gbud (Table 2.2). Resulting seedlings were treated when 2 cotyledons had emerged with 5 µl of 0.3 mM TRI, 3 mM TRI, 0.3 mM ORY or 3 mM ORY during 3 subsequent days (= droplet). After treatment, the seedlings were transferred to fresh medium. In a second experiment seeds were sterilised and sown on medium Gbud (Table 2.2) enriched with 3 µM, 10 µM, 100 µM or 1mM ORY or with 3 µM, 10 µM, 100 µM or 1 mM TRI (= seed sowing). Six weeks after treatment, the surviving seedlings were transferred to fresh medium without mitosis inhibitors. In a third experiment shoots of *B. globosa* were sterilised and initiated *in vitro* on medium Mbud (Table 2.2). After 1 multiplication cycle, shoots were transferred to fresh Mbud medium enriched with 10 µM, 100 µM or 1 mM ORY or with 10 µM, 100 µM or 1 mM TRI (= solid shoots). After 6 weeks the surviving shoots were transferred to fresh Mbud medium without mitosis inhibitors. The 4th experiment was the same as the 3rd experiment but liquid media was used instead of solid

media. *In vivo* shoots of *B. globosa* were initiated *in vitro* on solid Mbud medium (Table 2.2) (= liquid shoots). After 1 multiplication cycle, the shoots were transferred to liquid medium Mbud (= Mbud medium without agar and charcoal) for 3 days (on a rotor at 250 rpm). After 3 days 10 μ M, 100 μ M or 1 mM ORY or 10 μ M, 100 μ M or 1 mM TRI was added to the medium. The shoots were incubated for 3 days on a rotor (250 rpm). After treatment, the shoots were transferred to fresh solid Mbud medium.

For each treatment, 100 seeds or shoots were used. Also 100 seeds or shoots were not treated as a control. The ploidy level of the surviving plantlets was measured by flow cytometry (see 2.5.1). Of each plantlet 4 leaves were sampled (at random). Plants that had an altered ploidy level were acclimatised in the greenhouse (see 2.3.4). Acclimatised plants were planted on the field and leaf morphology was described (see 2.4.1).

4.2.2.3 *In vivo* polyploidisation and ploidy breeding of *H. syriacus*

Hibiscus seedlings were treated *in vivo* in 3 different ways with a 0.2% (w/v) COL solution. A first treatment consisted of putting a drop of the COL solution on top of young meristems of the seedlings during 10 subsequent days. In total 315 seedlings were treated. In a second treatment, young seedlings were immersed in the COL solution for 2 (53 seedlings) or 4 (59 seedlings) hours. The last treatment was an incubation of the seedlings on a filter paper moistened with the COL solution for 4 or 24 hours. The 4 hours treatment was done for 87 seedlings, the 24 hours treatment for 70 seedlings. In total 40 seedlings were not treated as a control.

After treatment the seedlings were transferred back to the greenhouse. The ploidy of the surviving treated seedlings was determined using flow cytometry (see 2.5.1). From the obtained octoploid ‘Oiseau Bleu’ seedlings, 6 blue flowered genotypes were selected and used in crosses (see 2.2) with tetraploid *H. syriacus* ‘Oiseau Bleu’ and ‘Melwhite’ and with ovtoploid *H. syriacus* ‘Red Heart CV’ and ‘Purple CV₂’. Resulting seeds were harvested when fully matured and sown in the greenhouse. The ploidy level of the obtained seedlings was determined using flow cytometry (see 2.5.2). Leaf parameters of candivars (= candidate cultivar) were described (see 2.4.1) and the length of the different branches was measured of 10 branches per genotype.

4.3 Results

4.3.1 *In vitro* polyploidisation of *B. globosa*

4.3.1.1 Treatment of seed(ling)s

From seeds sown on germination medium without mitotic inhibitors about 65% germinated, resulting in young seedlings that could be treated with mitotic inhibitors. At first, after treatment with TRI and ORY, seedlings seemed to be burned (Figure 4.1). However, after about 2 weeks healthy shoots were produced by the treated seedlings.

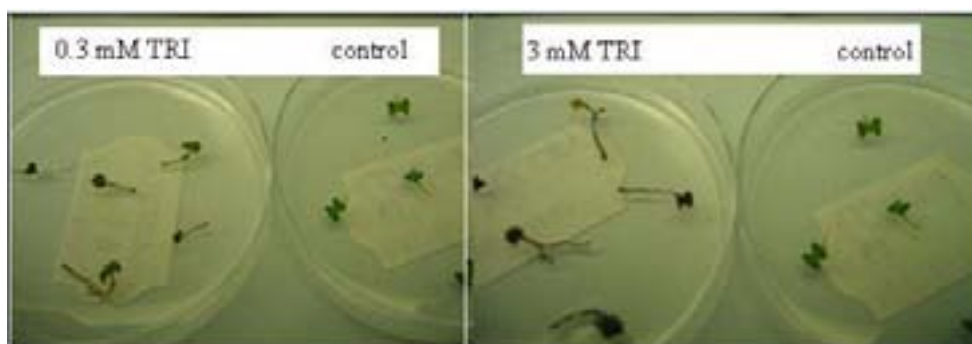


Figure 4.1: Seedlings of *B. globosa* after treatment with 0.3 mM and 3 mM TRI, compared to control seedlings.

In general, after treatment with 0.3 mM TRI, most seedlings survived. A treatment with 3 mM ORY was most harmful. Depending on the product and used concentration, after 1 month treated seedlings produced between 1 and 3.7 shoots, compared to 6 shoots in the control seedlings (Table 4.1). All produced shoots were analysed with the flow cytometer to determine the ploidy level. About 22% of the tested shoots were tetraploid, mostly obtained after treatment with 0.3 mM TRI (Table 4.1). About 15% of the shoots were mixoploid, but when measuring the ploidy level again after 1 month, these mixoploid plants were determined as diploids. Finally, of the 84 tetraploid *in vitro* seedlings, only 18 plants could be acclimatised and planted on the field for further evaluation (Table 4.1).

After sowing the seeds on germination medium enriched with mitotic inhibitors in total about 44.5% of the seeds germinated, compared to 80% of the control seeds (Figure 4.2). Only for treatments with 100 μ M and 1 mM ORY, tetraploid seedlings were observed after measurement of the surviving seedlings with the flow cytometer (Table 4.1). After treatment with 10 μ M ORY or TRI also 28 triploids and 68 mixoploids (in total) were found. A second

flow cytometric screening 1 month later revealed that more than 50% of them reverted to the original diploid ploidy level. Treatment with 100 μ M ORY or TRI resulted in 2 triploids and 10 mixoploids (in total). Finally, 23 tetraploid *B. globosa* seedlings could be acclimatised and planted on the field for evaluation (Table 4.1).

Table 4.1: Effect of the different TRI and ORY *in vitro* treatments on the doubling of the chromosome number of *B. globosa*.

Treatment	% Surviving seedlings or shoots	# Produced shoots per plant	# Tested Shoots (3 months after treatment	2x Plantlets (3 months after treatment)	4x Plantlets (3 months after treatment	4x plants on the field (1 year after treatment)
Droplet 0.3 mM ORY	25	3.7	93	46	4	-
Droplet 3 mM ORY	2	1	2	1	1	-
Droplet 0.3 mM TRI	58	3.1	183	94	76	18
Droplet 3 mM TRI	29	3.5	102	96	3	-
Droplet control	98	6	588	588	-	-
Seed sowing 3 μ M ORY	56	nr ^z	nr	56	-	-
Seed sowing 10 μ M ORY	39	nr	nr	7	-	-
Seed sowing 100 μ M ORY	40	nr	nr	23	10	5
Seed sowing 1 mM ORY	30	nr	nr	5	25	18
Seed sowing 3 μ M TRI	47	nr	nr	47	-	-
Seed sowing 10 μ M TRI	79	nr	nr	15	-	-
Seed sowing 100 μ M TRI	66	nr	nr	61	-	-
Seed sowing 1 mM TRI	0	nr	nr	-	-	-
Seed sowing control	80	nr	nr	80	-	-
Solid shoots 10 μ M ORY	78	5.1	398	398	-	-
Solid shoots 100 μ M ORY	52	4.8	250	250	-	-
Solid shoots 1 mM ORY	26	3.6	94	94	-	-
Solid shoots 10 μ M TRI	85	4.7	400	400	-	-
Solid shoots 100 μ M TRI	74	2.3	170	170	-	-
Solid shoots 1 mM TRI	36	3.2	115	115	-	-
Control solid shoots	82	6	492	492	-	-
Liquid shoots 10 μ M ORY	65	4.8	312	312	-	-
Liquid shoots 100 μ M ORY	63	4.5	284	284	-	-
Liquid shoots 1 mM ORY	45	3.9	176	176	-	-
Liquid shoots 10 μ M TRI	68	3.9	265	265	-	-
Liquid shoots 100 μ M TRI	62	3.8	236	236	-	-
Liquid shoots 1 mM TRI	43	4.0	172	172	-	-
Control liquid shoots	71	6.2	440	440	-	-

^z not relevant

In an early stage, the obtained tetraploid seedlings of *B. globosa* grew faster than the control seedlings (Figure 4.3). But after 6 to 8 months the growth stagnated and a lot of the tetraploid *B. globosa* plants died in the first year. In general, leaves of the tetraploid plants were broader and larger compared to diploid control plants (Figure 4.3). More detailed analysis of leaf morphology of the surviving tetraploids (12 plants) revealed a L/W ratio of 2.35, while L/W

was 3.00 for control plants. Tetraploid *B. globosa* plants did not flower yet, so far no crosses with tetraploid *B. davidii* cultivars could be made.

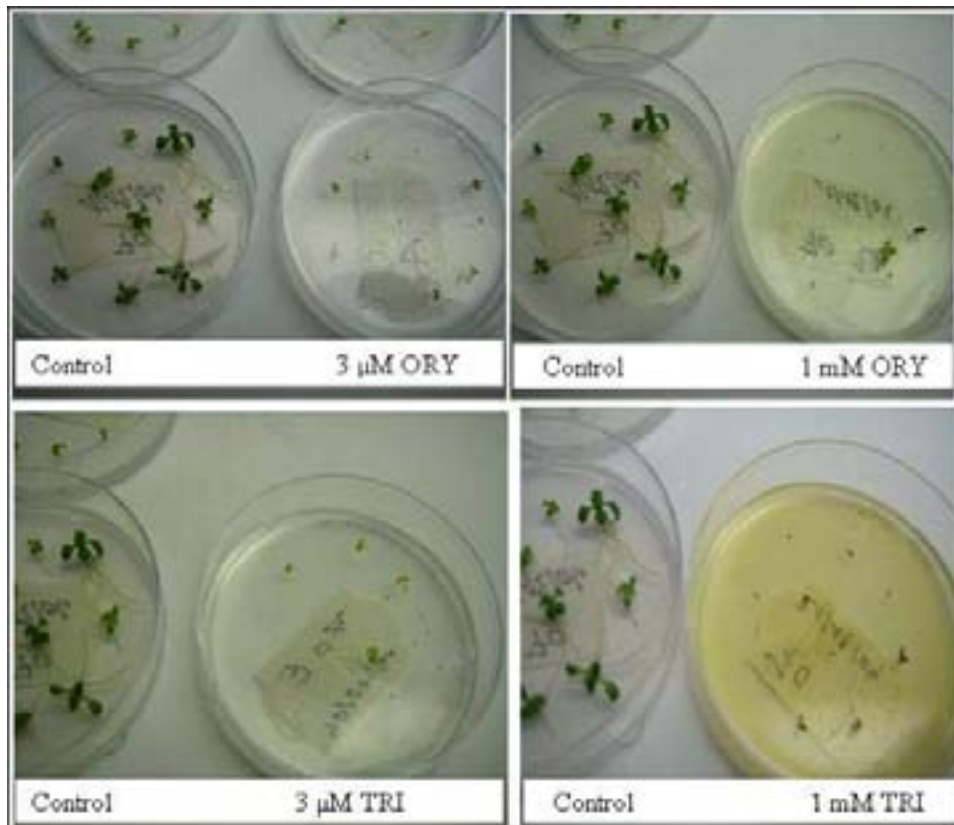


Figure 4.2: Germination of *B. globosa* seeds on medium enriched with different concentrations of different mitosis inhibitors (TRI, ORY), compared to control seeds.



Figure 4.3: Morphology of tetraploid *B. globosa* seedlings compared to diploid control seedlings. In the beginning tetraploid seedlings grew more vigorous. Leaves of tetraploids are larger and broader.

4.3.1.2 Treatment of shoots

After treatment of shoots on solid medium the amount of surviving shoots was similar as for the control shoots. After 1 month, treated shoots produced between 2.3 and 5.1 secondary

shoots depending on the treatment (Table 4.1), while control shoots formed on average 6 secondary shoots. However, none of the tested shoots had an altered ploidy level. When using liquid medium surviving and multiplication rate of the treated shoots was a little lower compared to the control shoots. But also here, none of the tested shoots had an altered ploidy level.

4.3.2 *In vivo* polyploidisation and ploidy breeding of *H. syriacus*

The results of the polyploidisation of *H. syriacus* ‘Oiseau Bleu’ are summarised in Table 4.2. The droplet method was most efficient to double the chromosome numbers of *H. syriacus*. Almost 25% of the treated seedlings were octoploid. After 2 h immersion in the COL solution 5.6% of the treated seedlings were octoploid, while 4 h immersion resulted in 5% octoploid seedlings. Incubation on filter paper moistened with the COL solution was not effective. The droplet and the immersion methods also yielded few hexaploid, decaploid and dodecaploid seedlings (data not shown).

Table 4.2: Effect of the different COL *in vivo* treatments on the doubling of the chromosome number of *H. syriacus* ‘Oiseau Bleu’.

Treatment	# Treated seedlings	Surviving seedlings	# 4x Seedlings	# 8x Seedlings
Droplet	315	267	189	78
2 h immersion	53	53	50	3
4 h immersion	59	58	55	3
4 h filter paper	87	87	87	-
24 h filter paper	70	70	70	-
Control	40	40	40	-

From the obtained octoploid ‘Oiseau Bleu’ seedlings, 6 blue flowered genotypes were selected and used in crosses with tetraploid *H. syriacus* cultivars in order to create hexaploids. An overview of the performed crosses is given in Table 4.3. Pollination of the tetraploid cultivars ‘Melwhite’ and ‘Oiseau Bleu’ with the octoploid seedlings resulted in 82 fruits and 1351 seeds. The reciprocal crosses yielded 17 fruits and 154 seeds. Control crosses between octoploid *H. syriacus* cultivars ‘Purple CV₂’ and ‘Red Heart CV’ x octoploid seedlings resulted in 35 seeds and 464 seeds, while the reciprocal control crosses gave only 10 fruits and 112 seeds. Crosses with *H. syriacus* ‘Melwhite’ generated most fruits and seeds. Overall germination capacity was high, between 70% and 100% for crosses with tetraploid cultivars and octoploid seedlings and between 48% and 78% for control crosses. Only in 1 control cross (octoploid seedling x ‘Red Heart CV’) there was no germination of the seeds. Determination of the ploidy level showed that all converted seedlings from crosses between tetraploid cultivars and octoploid selections were hexaploid, indicating that the converted seedlings were true hybrids.

After 2 subsequent selection cycles, finally one blue flowering candivar was selected. Morphological characteristics of this selection were compared to *H. syriacus* ‘Oiseau Bleu’ (Table 4.4). Growth vigor of the selected candivar was significantly higher than the tetraploid cultivar ‘Oiseau Bleu’. After 1 year, the clones had formed branches of ± 59 cm, while the branches of *H. syriacus* ‘Oiseau Bleu’ were only 25.8 cm at that time. Also compared to other commercial tetraploid *H. syriacus* cultivars, like ‘Marina’, the candivar was more vigorous in growth. Leaf (Table 4.4) and flower morphology of the hexaploid candivar and the *H. syriacus* ‘Oiseau Bleu’ were more or less the same.

Table 4.3: Overview of the performed crosses between different *H. syriacus* cultivars and chromosome doubled *H. syriacus* ‘Oiseau Bleu’ seedlings.

♀	Cross	♂	# Fruits	# Seeds	Seeds per fruit	# resulting seedlings	# First selected seedlings	# Second selected seedlings
<i>H. syr.</i> ‘Melwhite’		8x	72	1300	18.0	1167	18	-
<i>H. syr.</i> ‘Oiseau Bleu’		8x	10	51	5.1	36	23	1
<i>H. syr.</i> ‘Purple CV ₂ ’		8x	12	127	10.6	99	-	-
<i>H. syr.</i> ‘Red Heart CV’		8x	23	340	14.7	253	-	-
8x ^z	<i>H. syr.</i> ‘Melwhite’		16	152	9.5	144	-	-
8x	<i>H. syr.</i> ‘Oiseau Bleu’		1	2	2.0	2	-	-
8x	<i>H. syr.</i> ‘Purple CV ₂ ’		1	10	10.0	0	-	-
8x	<i>H. syr.</i> ‘Red Heart CV’		9	102	11.3	49	1	-

^z different (blue flowering) 8x genotypes were used in the crosses

Table 4.4: Average length of the branches and leaf indices for the blue flowering *H. syriacus* ‘Oiseau Bleu’ and the hexaploid selection ‘DVPazurri’ (n = 10).

Characteristic	<i>H. syriacus</i> ‘Oiseau Bleu’	Hexaploid selection ‘DVPazurri’
Length branches (cm) ^z	25.8 a	59.2 b
(leaf) α (°)	118.4 a	108.7 a
(leaf) M/B	0.37 a	0.31 a
(leaf) L/B	1.50 a	1.35 a
(leaf) C/L	0.60 a	0.56 a
(leaf) A/L	0.46 a	0.40 a

Another benefit of the hexaploid selection is its sterility. No fruit formation is observed. As a consequence flowering is not inhibited during the season.

The selected candivar was introduced on the market in 2008, under the name *Hibiscus syriacus* ‘DVPazurri’. In Figure 4.4 the morphology of ‘DVPazurri’ is presented.



Figure 4.4: Morphology of the selected hexaploid candivar ('DVPazurri') compared to 2 commercial tetraploid *H. syriacus* cultivars 'Oiseau Bleu' and 'Marina'.

4.4 Discussion

The presented results prove that it is possible to alter the ploidy level of *Buddleja* and *Hibiscus*. For *B. globosa* chromosome doubling was reported before by Rose *et al.* (2000b). Eeckhaut (2003) and Duron and Morand (1978) got polyploid forms of *B. davidii*. Detailed information on polyploidisation on *Hibiscus* was not reported so far. Polyploidisation was performed in a number of other ornamental species like *Lilium* (Van Tuyl and Lim 2003) or woody species including rose (Kermani *et al.* 2003), mulberry (Chakraborti *et al.* 1998), *Rhododendron* (Vainola 2000), pear (Kadota and Niimi 2002), pomegranate (Shao *et al.* 2003) and *Platanus* (Liu *et al.* 2007).

The favoured method for polyploidisation is to apply the mitotic inhibitor on *in vitro* cultured explants. In our study polyploidisation only was successful when applying ORY, TRI or COL on seeds and seedlings both *in vitro* and *in vivo*. For *Buddleja*, both the type of mitotic inhibitor as well as the concentration in which the mitotic inhibitor was applied had an influence on the results. Eeckhaut (2003) only had success when treating *B. davidii* seeds with ORY and TRI to yield chromosome doubled plants. Also for example in *Platanus*, treatment of (ungerminated) diploid seeds with COL was the most efficient method to obtain tetraploid seedlings (Liu *et al.* 2007). Nevertheless, other plant material can be treated with mitotic inhibitors. Rose *et al.* (2000b) treated nodal explants of *B. globosa* with COL. Duron and

Morand (1978) got a polyploid mutant of *B. davidii* 'Opera', named Courtabud' OPERETTE®, by applying COL on meristem cultures. Treatment of meristem cultures should also be tried on *B. globosa* in order to enhance the efficiency of chromosome doubling. In our study, treatment of *B. globosa* seeds with ORY or TRI had an efficiency of approximately 8%. In the study of Rose *et al.* (2000b) nodal sections of *B. globosa* were treated with 0.01%, 0.05% or 0.1% COL during 1, 2 or 3 days with an efficiency up to 60%. So it would also be useful to repeat our experiments with COL. In our experiments for *Hibiscus* the droplet method on young seedlings was most effective. The other methods, immersion of seedlings in COL solution or incubation on filter paper moistened with COL solution, were less (or not) effective. In *Lilium* (Takamura *et al.* 2002), *Nerine* (Van Tuyl *et al.* 1992), *Rosa* (Khosravi *et al.* 2008) and *Buddleja* (Rose *et al.* 2000b) scales or nodal sections were also immersed/soaked in mitotic inhibitor solution, resulting in chromosome doubled plants. Probably seeds/seedlings are not able to incorporate the mitotic inhibitor very efficiently when immersed in the solution. Therefore application of the mitotic inhibitor directly to the apical meristem might be more efficient.

Although Rose *et al.* (2000b) obtained successful polyploid *B. globosa* after treatment of nodal explants, in our study on *Buddleja globosa* applying mitotic inhibitors on nodal explants was not effective at all. Also in other ornamental plants polyploidisation was obtained by treatment of nodal sections e.g. roses (Kermani *et al.* 2003; Khosravi *et al.* 2008) and *Syringa* (Rose *et al.* 2000a). Treatment of nodal sections has some advantage compared to treatment of seed(lings), since with nodal sections 1 known genotype is used. For our breeding goal however, introgression of the yellow color of *B. globosa* and/or a chromosome doubled crossing partner were requested. In this case, it is not a problem that a polyploid form was obtained from seeds. If more complex specific qualities were wanted, polyploid plants should be obtained from nodal sections because only then the genotype is exactly known.

In our experiments, also mixoploid *Buddleja* and *Hibiscus* were obtained after polyploidisation. Mixoploids can be sectoral or periclinal (1 or 2 of the 3 histogenic layers are fully polyploid) cytochimeras. If the LII layer is tetraploid, a chimera will develop diploid gametes (Tilney-Basset 1986). Although the purpose of this experiment was the induction of plants with a fully doubled chromosome set, the number of mixoploids obtained should also be considered. They as well are an indication for the efficiency of the mitotic inhibitors. None of the chimeras, when growing into flowering plants had shifted into solid tetraploids.

The obtained tetraploid *B. globosa* seedlings were morphologically different from their diploid counterparts. In the beginning all tetraploids grew faster than the diploid controls. However, a lot of them died in the first year (probably because the tetraploids were more susceptible to frost). Also the leaf morphology was different. Considerable changes in morphology and fertility were also observed by Rose *et al.* (2000b) after their polyploidisation experiment on *B. globosa*. Their tetraploids had shorter internodes, broader, thicker and more crinkled leaves, greater frost susceptibility, elliptical inflorescences and poor anther development. Also in other genera morphological differences were observed after

treatment of plantlets with mitosis inhibitors. For example, treatment of diploid *Platanus* resulted in tetraploids with more compact growth habit and broader and thicker leaves (Liu *et al.* 2007).

In this study no crosses could be performed between the chromosome doubled *B. globosa* and *B. davidii*, because the tetraploid *B. globosa* did not flower yet. Since Van De Weyer (1920) reported that the yellow color is a recessive trait, occurring only in the second generation of a *B. globosa* x *B. davidii* cross, the creation of an F2 might be required to obtain yellow flowering hybrids.

The presented results also proved that it is possible to create more variation in *Hibiscus* by a ploidy breeding strategy. One blue flowering hexaploid selection *H. syriacus* ‘DVPazurri’ is brought in the market in 2008. This new cultivar is more vigorous in growth compared to other *H. syriacus* cultivars and is sterile, this in agreement with earlier observations in other hexaploid *H. syriacus* cultivars (Egolf 1979; 1981). Because the plants are sterile, they have a longer flowering period. Also other hexaploid *H. syriacus* cultivars such as ‘Diana’, ‘Helene’ and ‘Melrose’ are known to have an extended flowering period till half of Oktober (Van Huylenbroeck *et al.* 1998; Egolf 1979; 1981), while most tetraploid *H. syriacus* cultivars have a flowering season of 6 to 8 weeks in August and September (Van Huylenbroeck *et al.* 1998). Creating sterile triploid cultivars is also described in other species and resulted in new cultivars. For example for *Weigela* triploid selection have been get such as ‘Courtalor’ CARNAVAL®, ‘Courtamon’ FELINE® and ‘Courtared’ LUCIFER® (www.sapho.fr).

4.5 Conclusion

In this chapter polyploidisation protocols were performed for *Buddleja* and *Hibiscus*. These protocols can also be used for chromosome doubling of other woody ornamental species in order to enhance breeding efficiency.

For *Buddleja globosa*, treatment of young seedling with 0.3 mM TRI (droplet) and treatment of seeds with 100 µM and 1mM ORY was most successful to obtain chromosome doubled (tetraploid) *B. globosa* plants. For *Hibiscus syriacus*, chromosome doubled seedlings were obtained after treatment of young seedlings (droplet) with COL (0.2%). Treatment of shoots with mitotic inhibitors yielded no results. By following a polyploidisation protocol and performing interploidy crosses, a new hexaploid cultivar *H. syriacus* ‘DVPazurri’ could be selected and commercialised

It should be checked regularly if the generated tetraploid *B. globosa* is stable and if the gametes are chromosome doubled before using them in further breeding with *B. davidii*.

CHAPTER 5

INTERSPECIFIC HYBRIDISATION*

5.1 Introduction

In the breeding of ornamentals, the role of interspecific hybridisation in creating genetic variation is very important. However, interspecific incompatibility (both pre- and postzygotic) can occur for a wide variety of reasons (Van Tuyl en De Jeu 1997). Incompatibility and incongruity are determined by both genetic and environmental conditions and are therefore different for each plant genus or species. Understanding the nature of fertilisation barriers is a necessary key before manipulations can be designed to achieve hybridisation. Embryo abortion is a common cause of incongruity. Embryo rescue refers to a number of *in vitro* techniques which purpose is to promote the development of an immature or weak embryo into a viable plant. Embryo rescue procedures have been very successful in a wide range of plant materials (Sharma *et al.* 1996). Depending on the organ cultured, embryo rescue may be referred to as embryo, seed or ovary culture. Type of medium, conditions of temperature and light and time of *in vitro* initiation are the most important factors for successful embryo rescue (Sharma *et al.* 1996).

Considerable genetic variation can be generated if viable 2n gametes can be used for further crossing. The production of 2n gametes has been documented in a wide variety of angiosperm species (Ramanna and Jacobsen 2003). It is also known that interspecific hybrids in a lot of

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Van Huylenbroeck J, Van Laere K, Eeckhaut T, Van Bockstaele E (2004) Interspecific hybridisation in flowering shrubs. *Acta Hort.* 651: 55-62

Van Laere K, Van Huylenbroeck J, Van Bockstaele E (2006) Breeding strategies to increase genetic variability within *Hibiscus syriacus*. *Acta Hort.* 714: 75-81

Van Laere K, Van Huylenbroeck J, Van Bockstaele E (2007) Interspecific hybridisation between *Hibiscus syriacus*, *Hibiscus sinosyriacus* and *Hibiscus paramutabilis*. *Euphytica* 155: 271-283

Van Huylenbroeck J, Van Laere K, Eeckhaut T (2008) An integrated approach to overcome crossing incongruity in woody ornamentals. *Acta Hort.* 766: 355-359

Van Huylenbroeck J, Van Laere K, Leus L (2006) Breeding strategies for woody ornamentals: selection towards disease resistance. Accepted for publication in the proceedings of the international plant propagators society conference: 'Sustainable Plant Production', Grobbendonk, Belgium (29/08/06 – 01/09/06)

Van Laere K, Van Huylenbroeck J, Van Bockstaele E (2007) Modern breeding techniques: the *Hydrangea* case. Accepted for publication in the proceedings of the International *Hydrangea* conference 'Hydrangea 2007', Gent, Belgium (16 – 19/08/07)

Van Laere K, Leus L, Van Huylenbroeck J, Van Bockstaele E (2008) Interspecific hybridisation and genome size analysis in *Buddleja*. Accepted for publication in *Euphytica*

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genera frequently produce viable unreduced pollen, derived from abnormal meiotic division (Ramanna and Jacobsen 2003).

In this chapter the objectives were (i) to determine barriers occurring after interspecific crosses within the woody ornamentals *Hydrangea*, *Hibiscus* and *Buddleja*, (ii) to apply embryo rescue techniques for woody plants in order to overcome postzygotic barriers, (iii) to analyse the hybrid nature of obtained F1 and F2 seedlings by use of AFLP marker analysis, flow cytometry and morphological parameters and (iv) to investigate the occurrence of unreduced gametes in *Hibiscus* hybrids by different approaches (analysis of the pollen size, flow cytometry on the pollen, analysis of microsporogenesis and measurements of the ploidy level of the F2 progeny).

5.2 Specific materials and methods

All species and cultivars described in Chapter 3 were used in interspecific crosses within *Hydrangea*, *Hibiscus* and *Buddleja*. The protocols used for hand pollination, identification of prefertilisation barriers (germination of the pollen and pollen tube growth through the style), *in vitro* embryo rescue and characterisation of F1 and F2 hybrids are given in Chapter 2.

From the obtained F1 and F2 *Hibiscus* progenies, 6 F1 hybrids *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis*, 1 F1 hybrid *H. syriacus* ‘Red Heart CV’ x *H. paramutabilis* and 5 F2 hybrids, obtained by self pollinations of the F1 seedlings *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis*, were analysed for the occurrence of unreduced gametes. Plants used in the experiments were grown in the greenhouse under normal conditions. Protocols for pollen size and pollen viability measurements, analysis of DNA content of pollen and investigation of microsporogenesis are described in Chapter 2.

5.3 Results

5.3.1 Interspecific hybridisation within *Hydrangea*

5.3.1.1 Pre- and postzygotic barriers

In total over the different years, 21052 interspecific and 1116 intraspecific pollinations were made. A detailed overview of the different crosses is given in Table 5.1.

Table 5.1: Overview of the different interspecific crosses in *Hydrangea* spp. during different years, number of obtained fruits, seeds and F1 seedlings.

Female parent	Cross Male parent	# Polli- nations	styles in which pollentube growth was observed / analysed styles	Obtained fruits	Success- rate polli- nations (%)	Seeds <i>in</i> <i>vitro</i>	Seedlings <i>in vitro</i>	Acclimatised seedlings
<i>H. ser.</i> 'Blue Bird'	<i>H. macr.</i> 'Mariesii Perfecta'	178	na ^z	9	5.06	912	118	107
<i>H. ser.</i> 'Blue Bird'	<i>H. macr.</i> 'Blaumeise'	184	na	14	7.61	1920	- ^w	
<i>H. ser.</i> 'Blue Bird'	<i>H. macr.</i> 'Fasan'	187	na	- ^y	0.00			
<i>H. macr.</i> 'Blaumeise'	<i>H. ser.</i> 'Blue Bird'	280	na	14	5.00	633	43	38
<i>H. macr.</i> 'Fasan'	<i>H. ser.</i> 'Blue Bird'	125	na	8	6.40	64	-	
<i>H. ser.</i> 'Blue Bird'	<i>H. pan.</i> 'White Moth'	104	na	18	17.31	3581	-	
<i>H. ser.</i> 'Blue Bird'	<i>H. pan.</i> 'Unique'	142	na	-	0.00			
<i>H. pan.</i> 'White Moth'	<i>H. ser.</i> 'Blue Bird'	450	na	27	6.00	426	-	
<i>H. pan.</i> 'Unique'	<i>H. ser.</i> 'Blue Bird'	991	na	-	0.00			
<i>H. ser.</i> 'Blue Bird'	<i>H. quer.</i> 'Sikes Dwarf'	135	na	-	0.00			
<i>H. macr.</i> 'Blaumeise'	<i>H. quer.</i> 'Sikes Dwarf'	398	na	52	13.07	2873	-	
<i>H. macr.</i> 'Fasan'	<i>H. quer.</i> 'Sikes Dwarf'	329	na	49	14.89	1988	-	
<i>H. macr.</i> 'Bergfink'	<i>H. quer.</i> 'Sikes Dwarf'	265	15/15	154	58.11	- ^x		
<i>H. macr.</i> 'Bergfink'	<i>H. quer.</i> 'Tennessee Clone'	193	5/5	110	56.99	-		
<i>H. macr.</i> 'Mariesii Perfecta'	<i>H. quer.</i> 'Sikes Dwarf'	750	11/15	258	34.40	-		
<i>H. macr.</i> 'Mariesii Perfecta'	<i>H. quer.</i> 'Tennessee Clone'	384	4/5	301	78.39	-		
<i>H. quer.</i> 'Sikes Dwarf'	<i>H. macr.</i> 'Fasan'	386	na	17	4.40	-		
<i>H. quer.</i> 'Sikes Dwarf'	<i>H. macr.</i> 'Blaumeise'	123	na	-	0.00			
<i>H. quer.</i> 'Sikes Dwarf'	<i>H. macr.</i> 'Mariesii Perfecta'	333	10/13	134	40.24	-		
<i>H. quer.</i> 'Tennessee Clone'	<i>H. macr.</i> 'Mariesii Perfecta'	133	10/10	71	53.38	-		
<i>H. macr.</i> 'Fasan'	<i>H. pan.</i> 'Praecox'	195	na	19	9.74	4269	-	
<i>H. macr.</i> 'Fasan'	<i>H. pan.</i> 'White Lace'	361	na	41	11.36	3767	-	
<i>H. macr.</i> 'Fasan'	<i>H. pan.</i> 'Unique'	337	na	33	9.79	168	-	
<i>H. macr.</i> 'Fasan'	<i>H. pan.</i> 'White Moth'	109	na	31	28.44	3	1	- ^y
<i>H. macr.</i> 'Blaumeise'	<i>H. pan.</i> 'Unique'	409	na	60	14.67	109	1	-
<i>H. macr.</i> 'Blaumeise'	<i>H. pan.</i> 'Praecox'	200	na	11	5.50	57	-	
<i>H. macr.</i> 'Blaumeise'	<i>H. pan.</i> 'White Lace'	349	na	39	11.17	53	-	
<i>H. macr.</i> 'Blaumeise'	<i>H. pan.</i> 'White Moth'	120	na	41	34.17	-		
<i>H. macr.</i> 'Mariesii Perfecta'	<i>H. pan.</i> 'White Moth'	350	na	136	38.86	884	-	
<i>H. macr.</i> 'Mariesii Perfecta'	<i>H. pan.</i> 'Unique'	204	na	203	99.51	-		
<i>H. macr.</i> 'Bergfink'	<i>H. pan.</i> 'White Moth'	104	5/5	80	76.92	-		
<i>H. pan.</i> 'White Lace'	<i>H. macr.</i> 'Fasan'	437	na	63	14.41	-		
<i>H. pan.</i> 'White Lace'	<i>H. macr.</i> 'Blaumeise'	345	na	13	3.77	280	-	
<i>H. pan.</i> 'Unique'	<i>H. macr.</i> 'Fasan'	1266	7/11	356	28.12	-		
<i>H. pan.</i> 'Unique'	<i>H. macr.</i> 'Blaumeise'	574	4/5	75	13.07	416	2	-
<i>H. pan.</i> 'Unique'	<i>H. macr.</i> 'Mariesii Perfecta'	884	na	22	2.49	780	-	

Table 5.1: continued

Female parent	Cross	Male parent	# Pollinations ^z	Pre-zygotic barriers	Obtained fruits	Success-rate pollinations (%)	Seeds <i>in vitro</i>	Seedlings <i>in vitro</i>	Acclimatised seedlings
<i>H. pan.</i> 'Unique'		<i>H. macr.</i> 'Bergfink'	635	na	-	0.00			
<i>H. pan.</i> 'White Moth'		<i>H. macr.</i> 'Fasan'	613	4/5	215	35.07	1	-	
<i>H. pan.</i> 'White Moth'		<i>H. macr.</i> 'Blaumeise'	336	na	45	13.39	-		
<i>H. pan.</i> 'White Moth'		<i>H. macr.</i> 'Mariesii Perfecta'	495	na	35	7.07	774	14	11
<i>H. pan.</i> 'White Moth'		<i>H. macr.</i> 'Bergfink'	650	na	-	0.00			
<i>H. pan.</i> 'Unique'		<i>H. quer.</i> 'Sikes Dwarf'	65	11/11	-	0.00			
<i>H. pan.</i> 'Unique'		<i>H. quer.</i> 'Sikes Dwarf'	1153	na	231	20.03	344	1	-
<i>H. pan.</i> 'Unique'		<i>H. quer.</i> 'Tennessee Clone'	700	na	414	59.14	-		
<i>H. pan.</i> 'White Moth'		<i>H. quer.</i> 'Sikes Dwarf'	1705	3/6	542	31.78	120	-	
<i>H. pan.</i> 'White Moth'		<i>H. quer.</i> 'Tennessee Clone'	610	na	30	4.92	926	-	
<i>H. pan.</i> 'White Lace'		<i>H. quer.</i> 'Sikes Dwarf'	435	na	50	11.49	-		
<i>H. quer.</i> 'Sikes Dwarf'		<i>H. pan.</i> 'White Moth'	238	na	50	21.01	8	2	-
<i>H. quer.</i> 'Sikes Dwarf'		<i>H. pan.</i> 'White Lace'	95	na	-	0.00			
<i>H. quer.</i> 'Sikes Dwarf'		<i>H. pan.</i> 'Unique'	135	na	40	29.63	-		
<i>H. pan.</i> 'Unique'		<i>H. asp.</i> 'Macrophylla'	610	na	410	67.21	-		
<i>H. pan.</i> 'White Moth'		<i>H. asp.</i> 'Macrophylla'	370	na	39	10.54	1666	6	4
<i>H. ser.</i> 'Blue Bird'		<i>H. ser.</i> 'Blue Bird'	158	na	-	0.00			
<i>H. macr.</i> 'Fasan'		<i>H. macr.</i> 'Blaumeise'	178	na	20	11.24	3089	-	
<i>H. macr.</i> 'Fasan'		<i>H. macr.</i> 'Fasan'	107	5/30	5	4.67	32	-	
<i>H. macr.</i> 'Blaumeise'		<i>H. macr.</i> 'Fasan'	75	na	18	24.00	-		
<i>H. pan.</i> 'Unique'		<i>H. pan.</i> 'Unique'	260	na	250	96.15	1250	210	192
<i>H. pan.</i> 'Unique'		<i>H. pan.</i> 'White Lace'	338	na	-	0.00			

^z na: not analysed; ^y no fruits were observed; ^x no developed seeds could be found in the fruits; ^w no seeds germinated *in vitro*; ^v no seedlings could be acclimatised

Several cross combinations yielded no seeds and were therefore analysed for the occurrence of prezygotic barriers. However, in most cases, the growth of the pollen tube through the style to the ovaria could be observed (Table 5.1).

The interspecific crosses between *H. serrata* and *H. macrophylla* yielded 45 fruits (4.7% successful pollinations), while pollinations between *H. serrata* and *H. paniculata* and between *H. serrata* and *H. quercifolia* yielded 45 (2.7% successful pollinations) and 0 fruits respectively. Interspecific crosses between *H. macrophylla* and *H. quercifolia* resulted in 38.3% successful pollinations. Crossing *H. paniculata* and *H. macrophylla* yielded 17.2% fruits. Crosses between *H. paniculata* and *H. quercifolia* were successful in 26.4% and between *H. paniculata* and *H. aspera* in 45.8%.

The intraspecific crosses and self pollinations yielded no fruits for *H. serrata*, 43 fruits (11.9% successful pollinations) for *H. macrophylla* and 250 fruits (41.8% successful pollinations) for *H. paniculata* (Table 5.1).

Although a lot of fruits were obtained, almost no well developed seeds were found in the fruits. By use of *in vitro* embryo rescue the germination percentage of the initiated seeds could be increased from 0% (*in vivo* sowing) to 0.7% and 3.5% (*in vitro* embryo rescue) of interspecific and intraspecific seeds respectively. A lot of the obtained *in vitro* seedlings were lost due to albinism and growth aberrations. Acclimatisation rate of the healthy *in vitro* seedlings was 85.1% for interspecific seedlings versus 91.4% for intraspecific seedlings (Table 5.1). So far, only the seedlings resulting from the crosses between *H. macrophylla* and *H. serrata* could be planted in the field for further evaluation.

5.3.1.2 Characterisation of the obtained hybrids

The progeny resulting from the crosses between *H. macrophylla* and *H. serrata* showed a lot of variation in morphology. Some seedlings were more compact, the leaf shape and flower morphology varies for the different plants,... Therefore, the seedlings were not morphologically characterised in detail.

For AFLP analysis of *H. serrata* ‘Blue Bird’ x *H. macrophylla* ‘Mariesii Perfecta’, leaf material of 36 randomly chosen F1 seedlings was harvested. The primers generated in total 388 marker bands. *H. serrata* showed only 18 unique markers, while *H. macrophylla* could be characterised by 22 unique markers. All F1 seedlings showed an equal share of markers that could be traced back to each of the crossing parents, indicating the hybrid nature of the offspring. Figure 5.1 gives an overall view of the genetic relationships between the plants analysed by AFLP markers. The group of F1 hybrids was clearly genetically different from both *H. serrata* and *H. macrophylla*.

AFLP analysis of 8 *H. paniculata* ‘White Moth’ x *H. macrophylla* ‘Mariesii Perfecta’ seedlings revealed in total 126 unique markers for *H. paniculata* ‘White Moth’ and 117 unique markers for *H. macrophylla* ‘Mariesii Perfecta’. The seedlings inherited only between 10 to 15 of the 117 unique markers of the male parent *H. macrophylla*, while between 77 and 87 of the unique *H. paniculata* markers were inherited (Table 5.2). The hybrid nature of the seedlings could thus not clearly be proven by AFLP marker analysis. There were also between 13% and 30% markers in the seedlings, which were not found in the parent plants and between 17% and 31% markers that were present in both parent plants but not in the offspring. A dendrogram of the F1 seedlings is presented in Figure 5.2.

Also 2 F1 seedlings resulting from a cross between *H. paniculata* ‘White Moth’ x *H. aspera* ‘Macrophylla’ were analysed by AFLP. The primers generated 115 unique markers for *H. paniculata* ‘White Moth’ and 158 for *H. aspera* ‘Macrophylla’. Also here only few markers of the male parent were inherited by the seedlings (Table 5.2 and Figure 5.2). Between 14% and 26% markers were seen in the seedlings which were not found in the parent plants and between

23% and 62% of the markers that were present in both parent plants were not observed in the seedlings.

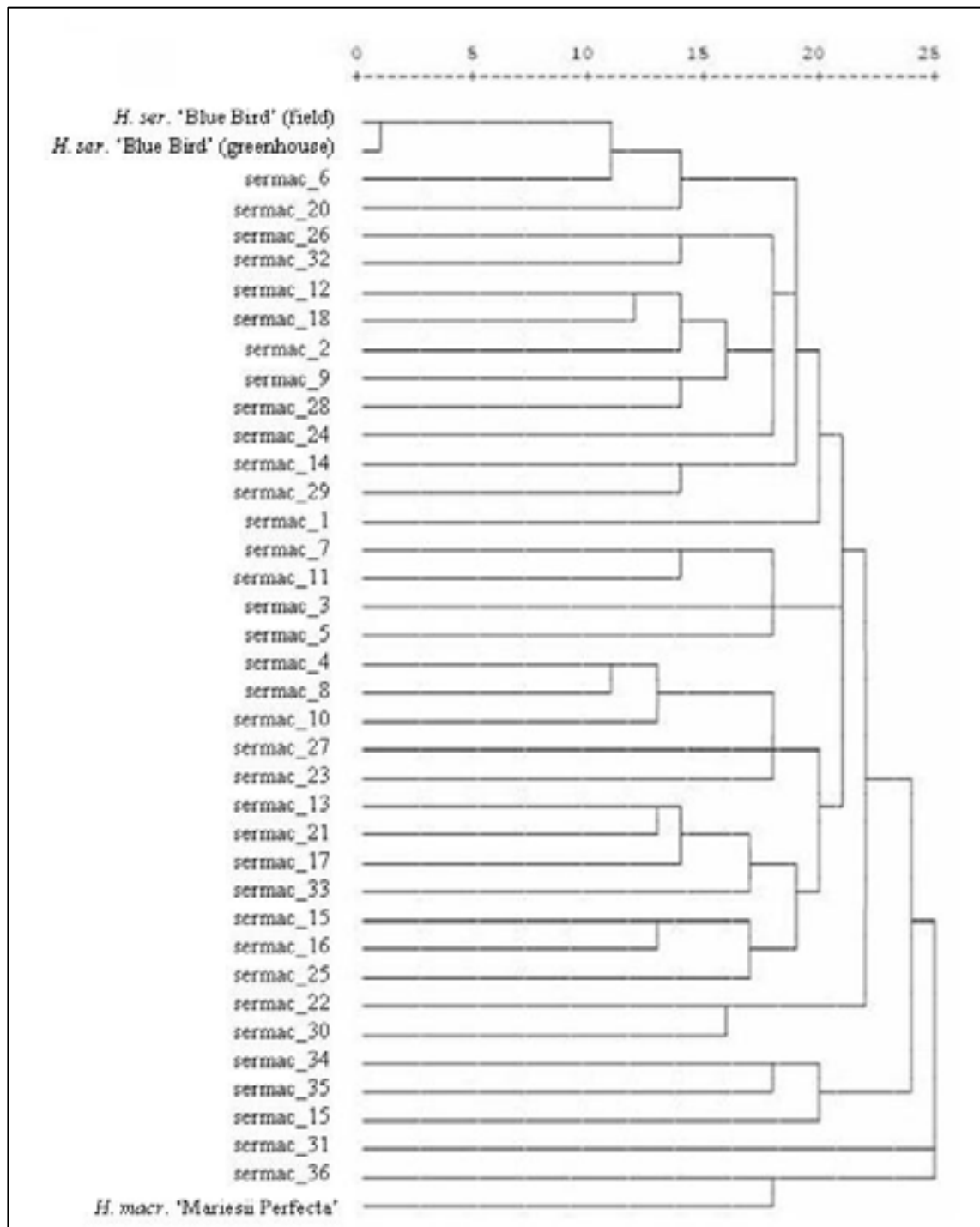


Figure 5.1: Dendrogram showing the genetic relatedness of 36 *H. serrata* 'Blue Bird' x *H. macrophylla* 'Mariesii Perfecta' F1 seedlings (sermac) and the parent plants tested by AFLP.

Table 5.2: Distribution of AFLP markers that are unique to one of the crossing parents after interspecific *H. paniculata* ‘White Moth’ x *H. macrophylla* ‘Mariesii Perfecta’ and *H. paniculata* ‘White Moth’ x *H. aspera* ‘Macrophylla’ crosses.

Genotype	Unique markers inherited from female parent	Unique markers inherited from male parent
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_1	87/126	14/117
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_2	86/126	13/117
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_3	83/126	15/117
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_4	78/126	10/117
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_5	79/126	11/117
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_6	77/126	13/117
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_7	84/126	13/117
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_8	86/126	12/117
<i>H. pan.</i> ‘White Moth’ x <i>H. aspera</i> ‘Macrophylla’_1	74/115	15/158
<i>H. pan.</i> ‘White Moth’ x <i>H. aspera</i> ‘Macrophylla’_2	40/115	16/158

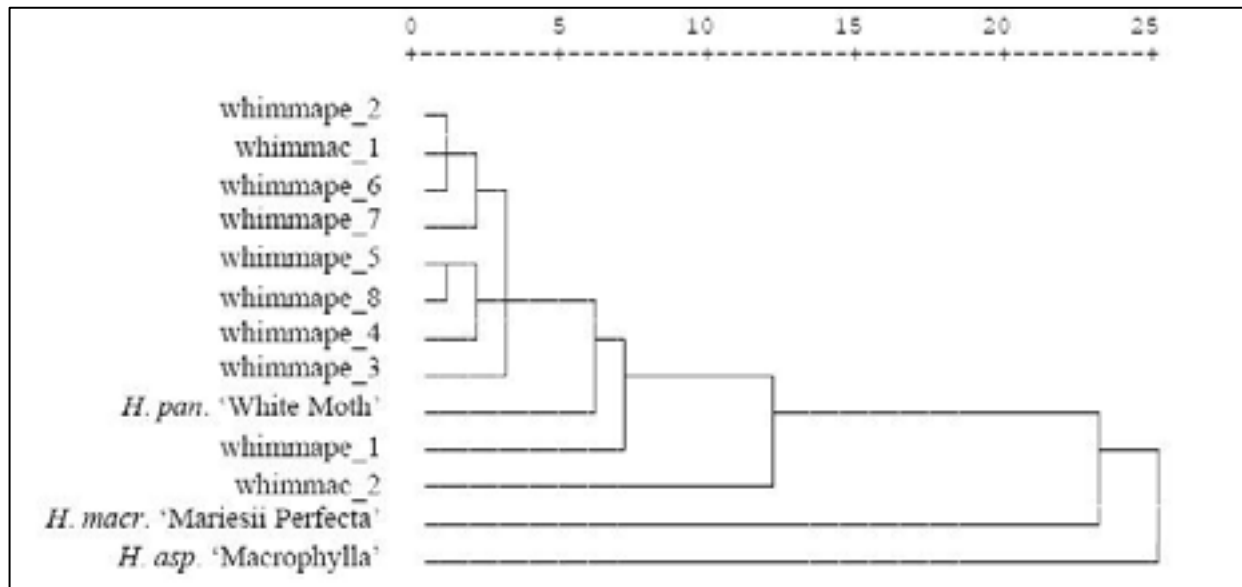


Figure 5.2: Dendrogram showing the genetic relatedness of F1 seedlings *H. paniculata* ‘White Moth’ x *H. macrophylla* ‘Mariesii Perfecta’ (whimmape), F1 seedlings *H. paniculata* ‘White Moth’ x *H. aspera* ‘Macrophylla’ (whimmac) and the parent plants tested by AFLP.

Results of the genome size measurements were similar on both flow cytometers that were used (Partec Pas III and Partec Cyflow Space). In Table 5.3 the genome sizes and chromosome numbers of the F1 seedlings and the parent plants are summarised.

The parent plants of the cross *H. serrata* ‘Blue Bird’ x *H. macrophylla* ‘Mariesii Perfecta’ (both diploid) had a genome size of 2.04 pg.1C⁻¹ and 3.21 pg.1C⁻¹. Nine of the tested F1 seedlings had a genome size between 2.08 pg.1C⁻¹ and 2.16 pg.1C⁻¹ (Table 5.3), while a more intermediate genome size of 2.63 pg.1C⁻¹ was expected. One seedling had a genome size (3.15 pg.1C⁻¹) similar to *H. macrophylla*.

For the F1 seedlings of *H. paniculata* ‘White Moth’ (4.70 pg.1C⁻¹) x *H. macrophylla* ‘Mariesii Perfecta’ (3.21 pg.1C⁻¹) a genome size of 3.96 pg.1C⁻¹ was expected based on the parent plants. Measurements of the seedlings revealed an intermediate (but closer to the genome size of *H. macrophylla*) genome size between 3.31 pg.1C⁻¹ and 3.46 pg.1C⁻¹. For 3 F1 seedlings 72 chromosomes could be counted, for 2 F1 seedlings only 66 chromosomes were observed. For the other 3 seedlings the chromosomes could not be counted because no good root material was available.

The 2 F1 seedlings *H. paniculata* ‘White Moth’ x *H. aspera* ‘Macrophylla’ had an intermediate genome size of 3.31 pg.1C⁻¹ and 3.26 pg.1C⁻¹, compared to the parent plants *H. paniculata* ‘White Moth’ (4.70 pg.1C⁻¹) and *H. aspera* ‘Macrophylla’ (1.64 pg.1C⁻¹), as was expected. The F1 seedlings had 72 chromosomes.

Table 5.3: Genome size of F1 seedlings resulting from crosses between *H. serrata* ‘Blue Bird’, *H. macrophylla* ‘Mariesii Perfecta’, *H. paniculata* ‘White Moth’ and *H. aspera* ‘Macrophylla’ and of the parent plants (results of genome sizes are averages \pm SD, n = 5 to 15).

Genotype	Ploidy level (2n)	Chromosome number	Genome size (pg.1C ⁻¹)
<i>H. serrata</i> ‘Blue Bird’	2x	36	2.04 \pm 0.02
<i>H. macrophylla</i> ‘Mariesii Perfecta’	2x	36	3.21 \pm 0.09
<i>H. paniculata</i> ‘White Moth’	4x	72	4.70 \pm 0.00
<i>H. aspera</i> ‘Macrophylla’	2x	34	1.64 \pm 0.01
<i>H. ser.</i> ‘Bleu Bird’ x <i>H. macr.</i> ‘Mariesii Perfecta’_1 (sermac_1)	2x	36	2.08 \pm 0.05
<i>H. ser.</i> ‘Bleu Bird’ x <i>H. macr.</i> ‘Mariesii Perfecta’_3 (sermac_3)	2x	36	2.11 \pm 0.03
<i>H. ser.</i> ‘Bleu Bird’ x <i>H. macr.</i> ‘Mariesii Perfecta’_5 (sermac_5)	2x	36	2.11 \pm 0.06
<i>H. ser.</i> ‘Bleu Bird’ x <i>H. macr.</i> ‘Mariesii Perfecta’_7 (sermac_7)	2x	36	3.15 \pm 0.01
<i>H. ser.</i> ‘Bleu Bird’ x <i>H. macr.</i> ‘Mariesii Perfecta’_9 (sermac_9)	2x	36	2.02 \pm 0.05
<i>H. ser.</i> ‘Bleu Bird’ x <i>H. macr.</i> ‘Mariesii Perfecta’_11 (sermac_11)	2x	36	2.16 \pm 0.05
<i>H. ser.</i> ‘Bleu Bird’ x <i>H. macr.</i> ‘Mariesii Perfecta’_13 (sermac_13)	2x	36	2.11 \pm 0.02
<i>H. ser.</i> ‘Bleu Bird’ x <i>H. macr.</i> ‘Mariesii Perfecta’_15 (sermac_15)	2x	36	2.09 \pm 0.07
<i>H. ser.</i> ‘Bleu Bird’ x <i>H. macr.</i> ‘Mariesii Perfecta’_17 (sermac_17)	2x	36	2.08 \pm 0.05
<i>H. ser.</i> ‘Bleu Bird’ x <i>H. macr.</i> ‘Mariesii Perfecta’_19 (sermac_19)	2x	36	2.11 \pm 0.03
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_1	^z	-	3.46 \pm 0.11
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_2	aneuploid	66	
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_3	4x	72	3.31 \pm 0.01
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_4	-	-	na
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_5	-	-	3.34 \pm 0.04
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_6	aneuploid	66	3.36 \pm 0.04
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_7	4x	72	3.39 \pm 0.09
<i>H. pan.</i> ‘White Moth’ x <i>H. macr.</i> ‘Mariesii Perfecta’_8	4x	72	3.31 \pm 0.11
<i>H. pan.</i> ‘White Moth’ x <i>H. aspera</i> ‘Macrophylla’_1	4x	72	3.31 \pm 0.02
<i>H. pan.</i> ‘White Moth’ x <i>H. aspera</i> ‘Macrophylla’_2	4x	72	3.26 \pm 0.04

^z plants could not be analysed because no good leaf or root material was available.

5.3.2 Interspecific hybridisation within *Hibiscus*

5.3.2.1 Pre- and postzygotic barriers

In total over the different years, 803 interspecific pollinations were made between *H. syriacus* and *H. sinosyriacus* and 1624 between *H. syriacus* and *H. paramutabilis*. Also 944 intraspecific *H. syriacus* crosses were performed. In Table 5.4 detailed information about the obtained fruits in each cross combination can be found.

The crosses *H. syriacus* x *H. sinosyriacus* resulted in 15 fruits (2.19% successful pollinations), while the reciprocal cross with *H. sinosyriacus* as female parent yielded no fruits. Also *H. paramutabilis* could only be used successfully as male parent in a cross with *H. syriacus*, resulting in 239 fruits (14.71% successful pollinations). Crosses between *H. sinosyriacus* and *H. paramutabilis* were completely unsuccessful. In the control crosses of *H. syriacus* x *H. syriacus* 84 fruits were obtained (8.90% successful pollinations).

One *H. syriacus* x *H. sinosyriacus*, 1 *H. sinosyriacus* x *H. syriacus*, 1 *H. syriacus* x *H. paramutabilis* and 1 *H. paramutabilis* x *H. syriacus* cross were analysed for prezygotic barriers. Only in the last cross no growth of the pollen tube through the style could be observed (Table 5.4).

Fruits of *H. syriacus* x *H. paramutabilis* contained on average 13.6 seeds and fruits of *H. syriacus* x *H. paramutabilis* 13.8. The fruits of the *H. syriacus* control crosses yielded on average 14.6 seeds (Table 5.4).

A comparison in efficiency between *in vitro* embryo rescue and *in vivo* sowing was made for several cross combinations (Table 5.4). For these interspecific crosses in general more plantlets were obtained by *in vivo* sowing. For the tested *H. syriacus* x *H. sinosyriacus* cross germination rate *in vitro* was 50% versus 52% *in vivo*, while for *H. syriacus* x *H. paramutabilis* this was 66% and 83% respectively. For the tested intraspecific control cross germination rate was 7.8% *in vitro* and 73% *in vivo*. A lot of the generated seedlings were lost during acclimatisation due to total and variegated albinism and growth aberrations. Of the obtained *in vitro* *H. syriacus* x *H. paramutabilis* seedlings 30% could be finally planted in the field versus 75% of the *in vivo* seedlings. For the *H. syriacus* x *H. sinosyriacus* seedlings this was 75% and 90% respectively. In the control cross 100% of the *in vitro* seedlings and 88% of the *in vivo* seedlings could finally be transferred to the field.

For some *H. syriacus* x *H. sinosyriacus* crosses seeds were only sown *in vitro*. For this cross combination germination rate was on average 86%. Due to losses of *in vitro* seedlings (albinism, growth aberrations; Figure 5.3), acclimatisation rate was only 37%. Also seeds of 1 *H. syriacus* x *H. paramutabilis* cross were only sown *in vitro*. Germination rate was 4% and acclimatisation rate was 100%. Seeds from control crosses which were only sown *in vitro* germinated for 53% of the cases and the acclimatisation rate was 46% (Table 5.4).

In general, no genotype effect was present for germination and acclimatisation rates.

Table 5.4: Overview of the different interspecific crosses in *Hibiscus* spp. during different years, number of obtained fruits, seeds and F1 seedlings.

Female parent	Cross Male parent	# Pollinations	styles in which pollentube growth was observed/analysed styles	Obtained fruits	Success-rate pollinations (%)	Embryos <i>in vitro</i> (<i>in vivo</i>)	Seedlings <i>in vitro</i> (<i>in vivo</i>)	Acclimatised Seedlings on the field
<i>H. syr.</i> 'Oiseau Bleu'	<i>H. sino.</i> 'Lilac Queen'	177	3/3	2	1.13	24	22	6
<i>H. syr.</i> 'Melwhite'	<i>H. sino.</i> 'Lilac Queen'	210	na ^z	4	1.90	12 (70)	10 (40)	3 (31)
<i>H. syr.</i> 'Melwhite'	<i>H. sino.</i> 'AS'	11	na	- ^y	0.00			
<i>H. syr.</i> 'Purple CV ₂ '	<i>H. sino.</i> 'Lilac Queen'	157	na	7	4.46	94	79	31
<i>H. syr.</i> 'Purple CV ₂ '	<i>H. sino.</i> 'AS'	14	na	2	14.28	8 (8)	- ^x (1)	- ^w
<i>H. syr.</i> 'Red Heart CV'	<i>H. sino.</i> 'Lilac Queen'	116	na	-	0.00			
<i>H. sino.</i> 'AS' ^v	<i>H. syr.</i> 'Oiseau Bleu'	50	3/3	-	0.00			
<i>H. sino.</i> 'AS'	<i>H. syr.</i> 'Red Heart CV'	12	na	-	0.00			
<i>H. sino.</i> 'AS'	<i>H. syr.</i> 'Purple CV ₂ '	23	na	-	0.00			
<i>H. sino.</i> 'Lilac Queen'	<i>H. syr.</i> 'Melwhite'	21	na	-	0.00			
<i>H. sino.</i> 'Lilac Queen'	<i>H. syr.</i> 'Red Heart CV'	12	na	-	0.00			
<i>H. syr.</i> 'Oiseau Bleu'	<i>H. paramutabilis</i>	554	5/8	103	18.59	890 (766)	592 (660)	471 (641)
<i>H. syr.</i> 'Melwhite'	<i>H. paramutabilis</i>	400	na	88	22.00	485 (485)	226 (334)	200 (226)
<i>H. syr.</i> 'Purple CV ₂ '	<i>H. paramutabilis</i>	370	na	43	11.62	300 (300)	298 (300)	170 (298)
<i>H. syr.</i> 'Red Heart CV'	<i>H. paramutabilis</i>	300	na	5	1.67	26	1	1
<i>H. paramutabilis</i>	<i>H. syr.</i> 'Oiseau Bleu'	21	0/1	-	0.00			
<i>H. sino.</i> 'Lilac Queen'	<i>H. paramutabilis</i>	20	na	-	0.00			
<i>H. syr.</i> 'Oiseau Bleu'	<i>H. syr.</i> 'Melwhite'	207	5/5	8	3.86	51 (201)	4 (147)	4 (130)
<i>H. syr.</i> 'Oiseau Bleu'	<i>H. syr.</i> 'Red Heart CV'	71	na	7	9.86	222	165	5
<i>H. syr.</i> 'Melwhite'	<i>H. syr.</i> 'Oiseau Bleu'	291	na	40	13.75	447	121	115
<i>H. syr.</i> 'Melwhite'	<i>H. syr.</i> 'Purple CV ₂ '	60	na	-	0.00			
<i>H. syr.</i> 'Melwhite'	<i>H. syr.</i> 'Red Heart CV'	67	na	-	0.00			
<i>H. syr.</i> 'Purple CV ₂ '	<i>H. syr.</i> 'Melwhite'	88	na	6	6.82	115	90	66
<i>H. syr.</i> 'Purple CV ₂ '	<i>H. syr.</i> 'Red Heart CV'	61	na	7	11.48	107	91	48
<i>H. syr.</i> 'Red Heart CV'	<i>H. syr.</i> 'Melwhite'	46	4/5	6	13.04	44	36	2
<i>H. syr.</i> 'Red Heart CV'	<i>H. syr.</i> 'Oiseau Bleu'	53	6/8	10	18.87	38	13	3

^z na: not analysed; ^y no fruits were observed; ^x no seeds germinated *in vitro*; ^w no seedlings could be acclimatised; ^v 'AS' = 'Autumn Surprise'

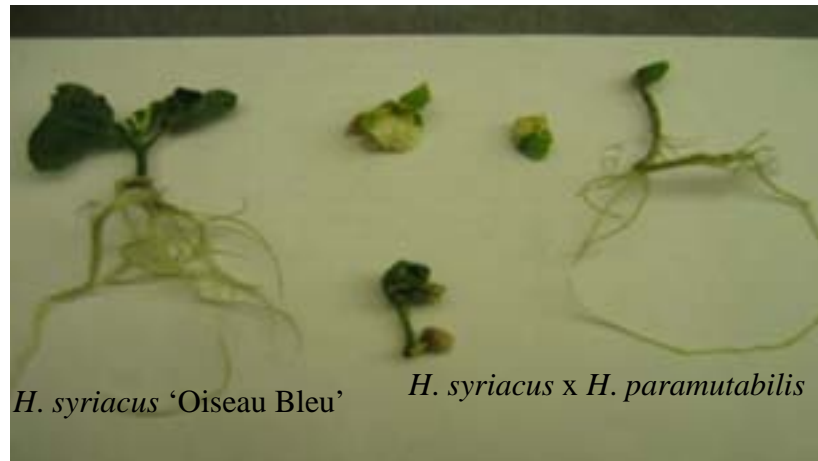


Figure 5.3: In vitro seedlings of *H. syriacus* 'Oiseau Bleu' and F1 hybrids *H. syriacus* x *H. paramutabilis*. A lot of the F1 hybrids could not be acclimatised due to albinism and growth aberrations.

5.3.2.2 Morphological characterisation of the obtained hybrids

Flowering seedlings with intermediate characteristics were found in F1 progenies of *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis*, *H. syriacus* 'Red Heart CV' x *H. paramutabilis*, *H. syriacus* 'Purple CV₂' x *H. paramutabilis*, *H. syriacus* 'Melwhite' x *H. paramutabilis* and *H. syriacus* 'Melwhite' x *H. sinosyriacus* 'Lilac Queen' (Table 5.5). All *H. syriacus* x *H. paramutabilis* F1 hybrids looked similar. Morphology and genetics of the F1 progeny of *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* and *H. syriacus* 'Melwhite' x *H. sinosyriacus* 'Lilac Queen' were described in detail.

Table 5.5: General morphological analysis of the F1 seedlings obtained after interspecific crosses within *Hibiscus*.

Female parent	Cross Male parent	# Plants on the field	# Plants with intermediate characteristics (based on leaf morphology)	# Plants with <i>H. syriacus</i> characteristics (based on leaf morphology)
<i>H. syr.</i> 'Oiseau Bleu'	<i>H. sino</i> 'Lilac Queen'	6	0	6
<i>H. syr.</i> 'Melwhite'	<i>H. sino</i> 'Lilac Queen'	34	18	16
<i>H. syr.</i> 'Purple CV ₂ '	<i>H. sino</i> 'Lilac Queen'	31	0	31
<i>H. syr.</i> 'Oiseau Bleu'	<i>H. paramutabilis</i>	1112	1060	52
<i>H. syr.</i> 'Melwhite'	<i>H. paramutabilis</i>	446	432	14
<i>H. syr.</i> 'Purple CV ₂ '	<i>H. paramutabilis</i>	468	430	38
<i>H. syr.</i> 'Red Heart CV'	<i>H. paramutabilis</i>	1	0	1

Leaf morphology

Leaf morphology of the *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* seedlings (Table 5.6) changed since F1 hybrids became older. In total 4 different leaf types could be distinguished

(Figure 5.4). The one year old hybrids all showed a uniform intermediate leaf type 1 with L/B comparable to *H. paramutabilis* and C/L the same as *H. syriacus* (Table 5.6 and Figure 5.4). The leaf angle and the relative width of the mid lob (M/B ratio) were higher than in both parent plants. The indentation of the lobes (A/L) was intermediate. When the hybrid seedlings were two years old, two types of leaves could be distinguished on the same plant. The first type had a same M/B, A/L and C/L ratio compared with the leaves of a one year old seedling. They had a larger L/B ratio and a smaller leaf angle. The second leaf type was more indented than the first one as was expressed by a lower A/L and M/B ratio. Hybrids that were three years old showed four different types of leaves (Table 5.6 and Figure 5.4). Type 1 and type 2 leaves were statistically the same as type 1 and type 2 leaves of a two year old seedling. Type 3 leaves were again more indented than type 2 leaves as shown in the A/L, C/L and M/B ratios. The 4th leaf type was asymmetric, as it was indented only at one side (see Figure 5.4). The side that was not indented expressed a similar A/L and C/L ratio as type 1 leaves, while the A/L ratio of the indented side was lower than for *H. syriacus*.

Also on the three year old hybrid seedlings of *H. syriacus* ‘Melwhite’ x *H. sinosyriacus* ‘Lilac Queen’ the same four different types of leaves could be distinguished on the same plant (Figure 5.4). Type 1 leaves had an L/B ratio, leaf angle and indentation comparable with *H. sinosyriacus*, the M/B ratio was intermediate. Indentation increased in type 2 and type 3 leaves, while the 4th leaf type was indented only at one side (Table 5.7).

Table 5.6: Leaf indices for *H. syriacus* ‘Oiseau Bleu’, *H. paramutabilis* and *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis* F1 hybrids (oispar) for different plant ages and leaf types (n=10). (C1, A1: measured at most indented site; C2, A2: measured at less indented site).

Genotype	Leaf-type	L/B	α	M/B	C ₁ /L	A ₁ /L	C ₂ /L	A ₂ /L
<i>H. syriacus</i> ‘Oiseau Bleu’		1.41 e ^z	112 a	0.27 b	0.55 ab	0.40 c	0.56 bc	0.41 b
<i>H. paramutabilis</i>		0.88 ab	242 d	0.43 d	0.70 d	0.64 f	0.71 d	0.65 g
oispar (1 year old)	type 1	0.92 bc	274 e	0.50 ef	0.51 a	0.49 de	0.52 ab	0.49 de
oispar (2 year old)	type 1	1.04 d	190 b	0.52 f	0.56 b	0.53 e	0.55 bc	0.53 f
	type 2	0.92 bc	196 bc	0.35 c	0.62 c	0.47 d	0.59 c	0.47 cd
oispar (3 year old)	type 1	0.98 cd	201 bc	0.47 de	0.55 ab	0.53 e	0.56 bc	0.53 ef
	type 2	0.91 b	212 c	0.34 c	0.56 b	0.41 c	0.57 c	0.43 bc
	type 3	0.82 a	236 d	0.18 a	0.48 a	0.25 a	0.49 a	0.24 a
	type 4	0.92 bc	192 b	0.38 c	0.61 c	0.32 b	0.58 c	0.53 ef

^z means indicated by the same symbol are not statistically different (LSD 0.05).

Table 5.7: Leaf indices for *H. syriacus* ‘Melwhite’, *H. sinosyriacus* ‘Lilac Queen’ and *H. syriacus* ‘Melwhite’ x *H. sinosyriacus* ‘Lilac Queen’ F1 hybrids (mellilac) for different plant ages and leaf types (n=10). (C1, A1: measured at most indented site; C2, A2: measured at less indented site).

Genotype	Leaf-type	L/B	α	M/B	C ₁ /L	A ₁ /L	C ₂ /L	A ₂ /L
<i>H. syriacus</i> ‘Melwhite’		1.50 d ^z	135 b	0.48 b	0.54 a	0.49 c	0.54 a	0.49 ab
<i>H. sinosyriacus</i> ‘Lilac Queen’		1.19 c	194 cd	0.53 c	0.62 bc	0.56 d	0.62 bc	0.56 cd
mellilac (3 year old)	type 1	1.03 b	180 c	0.48 b	0.62 bc	0.59 d	0.62 bc	0.59 d
	type 2	0.98 ab	206 d	0.36 a	0.63 c	0.50 c	0.63 c	0.50 b
	type 3	0.97 a	201 d	0.33 a	0.60 bc	0.45 b	0.60 b	0.45 a
	type 4	0.94 a	208 d	0.33 a	0.56 a	0.33 a	0.77 d	0.52 bc

^z means indicated by the same symbol are not statistically different (LSD 0.05).

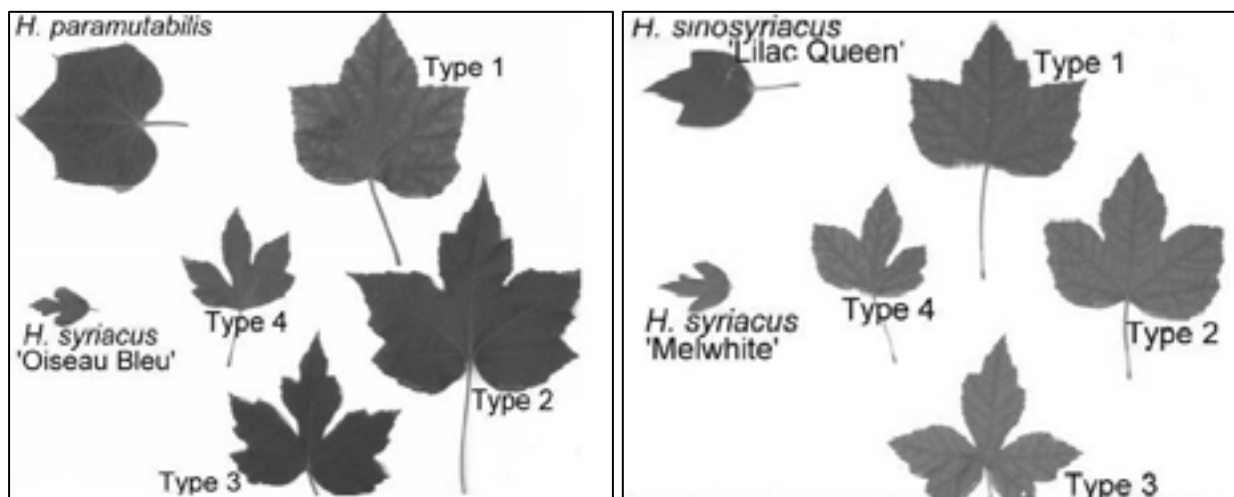


Figure 5.4: Leaf morphology of four different leaf types of the 3-year old *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* and *H. syriacus* 'Melwhite' x *H. sinosyrriacus* 'Lilac Queen' hybrids compared to the leaf morphology of the different parents.

Flower morphology

Compared to both parent plants, the F1 hybrid seedlings of *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* had bigger flowers, while the flower color was intermediate (Table 5.8 and Figure 5.5). Flower colors of all the F1 hybrids belonged, according to the RHS color chart, to the purple group. Within this group, three subgroups could be observed: 48.5% of the seedlings belonged to purple group 76A, 24.5% to purple group 76B and 27.0% to purple group 76D (Table 5.8). The same 3 subgroups were observed in the F1 hybrid seedlings of *H. syriacus* 'Melwhite' x *H. sinosyrriacus* 'Lilac Queen': 57.1% of the seedlings had a flower color according to the purple group 76A, 28.6% to the purple group 76B and 14.2% to the purple group 76D (Table 5.9). Flowers of these hybrids were also bigger compared to their parents (Table 5.9 and Figure 5.5).

Table 5.8: Flower characteristics of *H. syriacus* 'Oiseau Bleu', *H. paramutabilis* and the *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* F1 hybrids (oispar) obtained after interspecific crosses made in 2003.

Genotype	Petals	Basal spot on the petals	% of seedlings	Diameter flowers (cm) ^z	Type of flower
<i>H. syriacus</i> 'Oiseau Bleu'	Violet-purple group, 93B	Red-purple group, 71A		10.5 a	Single
<i>H. paramutabilis</i>	56D (white)	Red group, 45A		14.0 b	Single
oispar	Purple group, 76A	Red-purple group, 60A	48.5	16.0 c	Single
	Purple group, 76B	Red-purple group, 60B	24.5	16.0 c	Single
	Purple group, 76D	Red-purple group, 60B	27.0	16.0 c	single

^z means indicated by the same symbol are not statistically different (LSD 0.05)



Figure 5.5: Flower colors of F1 progeny of *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* and *H. syriacus* 'Melwhite' x *H. sino.* 'Lilac Queen' compared to the parent plants (F1-A: flower color 76A, F1-B: flower color 76B, F1-C: flower color 76D).

Table 5.9: Flower characteristics of *H. syriacus* 'Melwhite', *H. sino.* 'Lilac Queen' and the *H. syriacus* 'Melwhite' x *H. sino.* 'Lilac Queen' F1 hybrids (mellilac) obtained after interspecific crosses made in 2003.

Genotype	Petals	Basal spot on the petals	% of seedlings	Diameter flowers (cm) ^z	Type of flower
<i>H. syriacus</i> 'Melwhite'	Purple group 76A – 76D	Red-purple group 60A		8 a	Single
<i>H. sino.</i> 'Lilac Queen'	56D (White)	/		11 b	Single
mellilac	Purple group, 76A	Red-purple group, 60A	57.1	15.2 c	Single
	Purple group, 76B	Red-purple group, 60B	28.6	15.2 c	Single
	Purple group, 76D	Red-purple group, 60B	14.2	15.2 c	single

^z means indicated by the same symbol are not statistically different (LSD 0.05)

Growth vigor

Of the F1 progeny from *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* 95.3% grew very vigorous. The other 4.7% of the progeny looked the same as *H. syriacus*, indicating they were probably the result of a self pollination of *H. syriacus*. Of the F1 progeny from *H. syriacus* 'Melwhite' x *H. sino.* 'Lilac Queen' 52.9% of the seedlings grew very vigorous. The other 47.1% of the seedlings looked morphologically like the mother parent *H. syriacus* 'Melwhite', a strong indication that they were the result of a self pollination of *H. syriacus*.

The growth vigor of the F1 progeny of *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* and *H. syriacus* 'Melwhite' x *H. sino.* 'Lilac Queen' was higher compared to the *H. syriacus* cultivars and *H. paramutabilis*. After 1 year the F1 progeny *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* had formed on average branches of 57 cm and *H. syriacus* 'Melwhite' x *H. sino.* 'Lilac Queen' of 59 cm. At that time branches of *H. syriacus* 'Oiseau Bleu' and

‘Melwhite’ were on average 25.8 cm and 28.6 cm respectively and *H. paramutabilis* had branches of 41 cm.

Fertility

Analysis of the pollen revealed for the *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis* progeny a germination capacity of 1.9%, although the viability of the pollen was 73.98% (Table 5.10). The parent plants *H. syriacus* ‘Oiseau Bleu’ and *H. paramutabilis* had a germination capacity of the pollen of 56% and 43% respectively. About 75% of the pollen of *H. syriacus* ‘Melwhite’ x *H. sinosyriacus* ‘Lilac Queen’ was viable but the pollen germination capacity was only 5.3%, while the parent plants *H. syriacus* ‘Melwhite’ and *H. sinosyriacus* ‘Lilac Queen’ had pollen germination capacities of 37% and 25% respectively (Table 5.10).

Table 5.10: Viability and germination capacity of the pollen of *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis* and *H. syriacus* ‘Melwhite’ x *H. sinosyriacus* ‘Lilac Queen’ compared to the parent plants (results of pollen viability and pollen germination are averages, n = 300).

Genotype	Viability of the pollen (%)	Germination capacity of the pollen (%)
<i>H. syriacus</i> ‘Oiseau Bleu’	97.7	56.0
<i>H. syriacus</i> ‘Melwhite’	98.2	37.0
<i>H. sinosyriacus</i> ‘Lilac Queen’	85.0	25.0
<i>H. paramutabilis</i>	89.6	43.0
<i>H. syriacus</i> ‘Oiseau Bleu’ x <i>H. paramutabilis</i>	74.0 ^z	1.9 ^z
<i>H. syriacus</i> ‘Melwhite’ x <i>H. sinosyriacus</i> ‘Lilac Queen’	75.2 ^y	5.3 ^y

^z averages of different genotypes resulting from crosses between *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis*; ^y averages of different genotypes resulting from crosses between *H. syriacus* ‘Melwhite’ x *H. sinosyriacus* ‘Lilac Queen’

5.3.2.3 Molecular analysis of the obtained hybrids

For the AFLP analysis of *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis*, leaf material of 19 randomly chosen F1 seedlings was harvested. They all had intermediate characteristics as described above. The 3 primers generated in total 551 marker bands that could be used for scoring. The 2 crossing parents *H. syriacus* and *H. paramutabilis* appeared to be very polymorphic. The tested seedlings showed an equal share of markers that could be traced back to each of the crossing parents indicating the true hybrid nature of the offspring. Figure 5.6 gives an overall view of the genetic relationships between the analysed plants. The group of F1 hybrids (indicated in blue in Figure 5.6) clearly was genetically different from both *H. syriacus* and *H. paramutabilis* (parent plants are indicated in red in Figure 5.6). The hybrids were more related to each other than to one of the crossing parents.

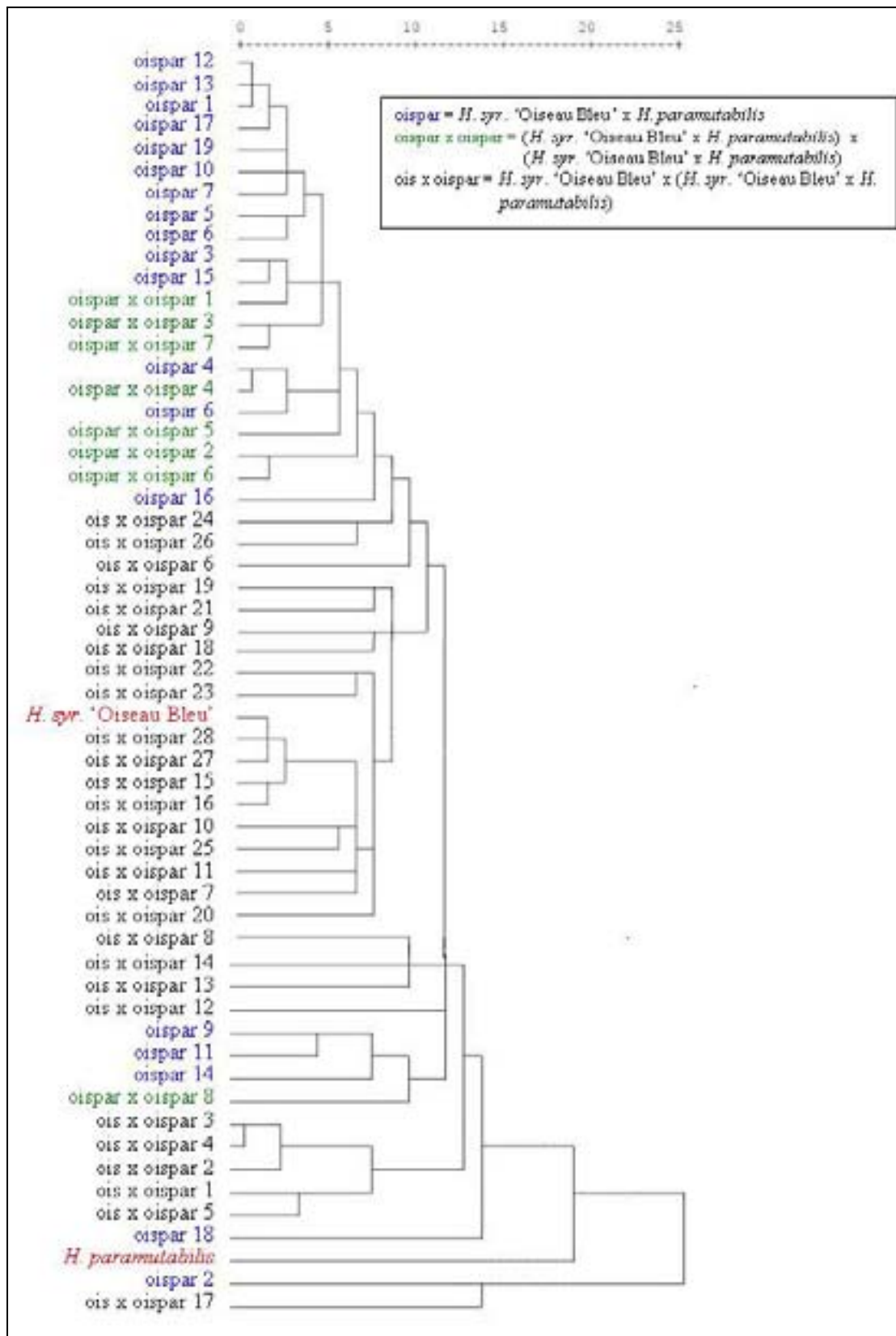


Figure 5.6: Dendrogram showing the genetic relatedness of *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* F1 (oispar), BC1 (ois x oispar) and F2 (oispar x oispar) seedlings (resulting from selfings of different F1 oispar plants) and the parent plants tested by AFLP.

For the AFLP analysis of *H. syriacus* 'Melwhite' x *H. sinosyriacus* 'Lilac Queen', leaf material of 10 at random chosen F1 seedlings was harvested, all having intermediate characteristics as described above. Also here, enough unique markers could be found from both parent plants, indicating the true hybrid nature of the seedlings. Clustering of the seedlings as presented in a dendrogram (Figure 5.7) also demonstrated the hybrid origin of the seedlings.

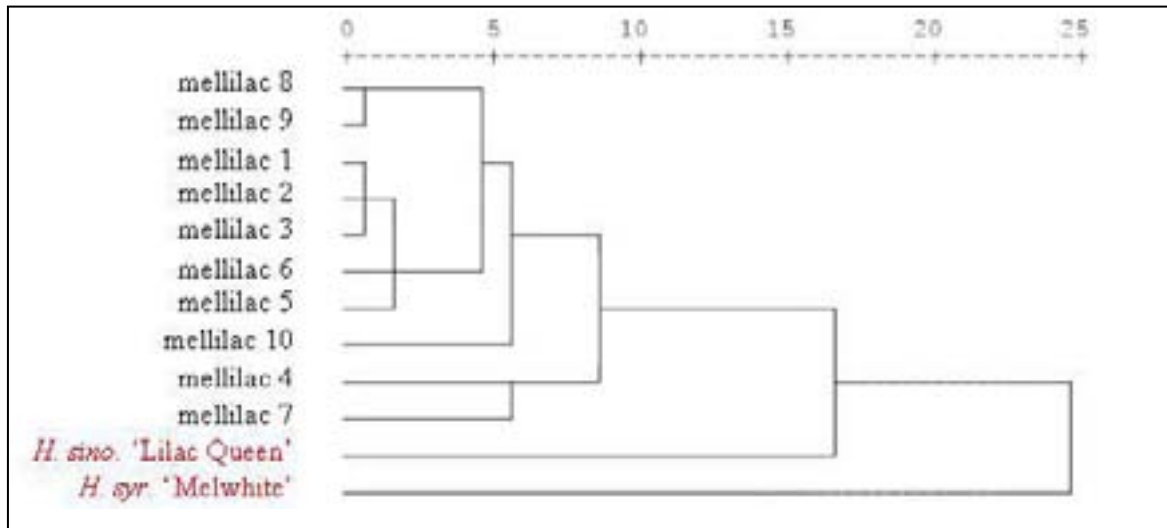


Figure 5.7: Dendrogram showing the genetic relatedness of *H. syriacus* 'Melwhite' x *H. sinosyriacus* 'Lilac Queen' F1 seedlings (mellilac) tested by AFLP.

Results of the genome size measurements were similar on both flow cytometers that were used (Partec Pas III and Partec Cyflow Space). In Table 5.11 the genome sizes and chromosome numbers of the F1 seedlings and the parent plants are summarised.

The parent plants *H. syriacus* 'Oiseau Bleu' ($2n = 4x = 80$) and *H. paramutabilis* ($2n = 4x = 82$) had a genome size of $2.34 \text{ pg} \cdot 1\text{C}^{-1}$ and $2.48 \text{ pg} \cdot 1\text{C}^{-1}$ respectively. The 19 analysed F1 seedlings, all having 81 chromosomes (Figure 5.8), had a genome size between $2.18 \text{ pg} \cdot 1\text{C}^{-1}$ and $2.45 \text{ pg} \cdot 1\text{C}^{-1}$.

The parents *H. syriacus* 'Melwhite' and *H. sinosyriacus* 'Lilac Queen' both had 80 chromosomes and a genome size of $2.13 \text{ pg} \cdot 1\text{C}^{-1}$ and $2.33 \text{ pg} \cdot 1\text{C}^{-1}$ respectively. The 10 tested F1 seedlings had 80 chromosomes and a genome size between $2.12 \text{ pg} \cdot 1\text{C}^{-1}$ and $2.44 \text{ pg} \cdot 1\text{C}^{-1}$ (Table 5.11).

Table 5.11: Genome size and ploidy measurements of parent plants and F1 hybrids resulting from crosses between *H. syriacus* 'Oiseau Bleu' and *H. paramutabilis* (oispar) and between *H. syriacus* 'Melwhite' and *H. sinosyriacus* 'Lilac Queen' (mellilac) (results of genome sizes are averages \pm SD, n = 5 to 15).

Genotype	Ploidy level (2n)	Chromosome number	Genome size (pg.1C ⁻¹)
<i>H. syriacus</i> 'Oiseau Bleu'	4x	80	2.34 \pm 0.08
<i>H. syriacus</i> 'Melwhite'	4x	80	2.13 \pm 0.02
<i>H. sinosyriacus</i> 'Lilac Queen'	4x	80	2.33 \pm 0.02
<i>H. paramutabilis</i>	4x	82	2.48 \pm 0.08
oispar 1	4x	81	2.45 \pm 0.04
oispar 2	4x	81	2.37 \pm 0.01
oispar 3	4x	81	2.31 \pm 0.01
oispar 4	4x	81	2.34 \pm 0.02
oispar 5	4x	81	2.37 \pm 0.09
oispar 6	4x	81	2.18 \pm 0.09
oispar 7	4x	81	2.40 \pm 0.01
oispar 8	4x	81	2.32 \pm 0.07
oispar 9	4x	81	2.44 \pm 0.06
oispar 10	4x	81	2.29 \pm 0.10
oispar 11	4x	81	2.43 \pm 0.03
oispar 12	4x	81	2.38 \pm 0.03
oispar 13	4x	81	2.32 \pm 0.01
oispar 14	4x	81	2.32 \pm 0.05
oispar 15	4x	81	2.35 \pm 0.01
oispar 16	4x	81	2.37 \pm 0.09
oispar 17	4x	81	2.35 \pm 0.05
oispar 18	4x	81	2.43 \pm 0.04
oispar 19	4x	81	2.39 \pm 0.01
mellilac 1	4x	80	2.44 \pm 0.14
mellilac 2	4x	80	2.32 \pm 0.05
mellilac 3	4x	80	2.30 \pm 0.02
mellilac 4	4x	80	2.29 \pm 0.09
mellilac 5	4x	80	2.37 \pm 0.07
mellilac 6	4x	80	2.22 \pm 0.09
mellilac 7	4x	80	2.25 \pm 0.09
mellilac 8	4x	80	2.28 \pm 0.01
mellilac 9	4x	80	2.12 \pm 0.02
mellilac 10	4x	80	2.30 \pm 0.01

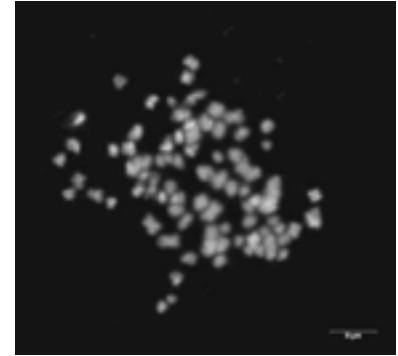


Figure 5.8: Metaphase spread of chromosomes for a *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* F1 seedling (bar = 5 μ m). Eighty-one chromosomes could be counted.

5.3.2.4 F2 generation of *H. syriacus* x *H. paramutabilis*

Despite the low germination vigor of the *H. syriacus* x *H. paramutabilis* pollen (Table 5.10), a small F2 generation was obtained from both *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis* hybrids and *H. syriacus* ‘Red Heart CV’ x *H. paramutabilis* hybrids. After *in vitro* embryo rescue, 3 F2 seedlings resulting from selfing of *H. syriacus* ‘Red Heart CV’ x *H. paramutabilis* and 11 F2 seedlings of *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis* could be acclimatised (Table 5.12). Also two F2 plants from backcrosses of *H. syriacus* ‘Red Heart CV’ x (*H. syriacus* ‘Red Heart CV’ x *H. paramutabilis*) (Table 5.12) and 156 F2 plants from backcrosses of *H. syriacus* ‘Oiseau Bleu’ x (*H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis*) were obtained. The dendrogram showing the genetic relationships within the F1, F2 and BC1 progeny is given in Figure 5.6.

Table 5.12: Overview of the different interspecific crosses in *Hibiscus* spp., number of obtained fruits, embryos and F2 seedlings. Numbers are the accumulation of the results over different crossing years.

Female parent ^z	Cross	Male parent ^z	# Pollinations	Obtained fruits	Embryos <i>in vitro</i>	Germinated embryos <i>in vitro</i>	Acclimatised seedlings
<i>H. syr.</i> ‘RH’ x <i>H. par.</i>		<i>H. syr.</i> ‘RH’ x <i>H. par.</i>	588	13	21	4	3
<i>H. syr.</i> ‘OB’ x <i>H. par.</i> ^x		<i>H. syr.</i> ‘OB’ x <i>H. par.</i>	1842	89	47	13	11
<i>H. syr.</i> ‘OB’ x <i>H. par.</i>		<i>H. syr.</i> ‘Oiseau Bleu’	1	. ^y			
<i>H. syr.</i> ‘Oiseau Bleu’		<i>H. syr.</i> ‘OB’ x <i>H. par.</i>	1653	69	57	173	156
<i>H. syr.</i> ‘OB’ x <i>H. par.</i>		<i>H. syr.</i> ‘Oiseau Bleu’	1015	-			
<i>H. syr.</i> ‘Red Heart CV’		<i>H. syr.</i> ‘RH’ x <i>H. par.</i>	1579	143	172	2	2

^z ‘OB’ = ‘Oiseau Bleu’, ‘RH’ = ‘Red Heart CV’, *par.* = *paramutabilis*; ^y no fruits were observed; ^x different genotypes of *H. syr.* ‘Oiseau Bleu’ x *H. paramutabilis* were used in the pollinations

5.3.2.5 Evidence for the occurrence of unreduced gametes in F1 and F2 *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis* hybrids**Ploidy determination and genome size estimation of F2 and BC1 hybrids**

The F1 hybrids *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis* were all tetraploid with a genome size between 2.32 pg.1C⁻¹ and 2.48 pg.1C⁻¹ (Table 5.11). For the 1 obtained F1 hybrid *H. syriacus* ‘Red heart CV’ x *H. paramutabilis* (2n = 6x = 120) a genome size of 3.58 pg.1C⁻¹ was measured. However, all the F2 hybrids *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis*, resulting from self pollinations of the F1 progeny, were hexaploid (Table 5.13 and Figure 5.9) with a genome size between 3.49 pg.1C⁻¹ and 3.59 pg.1C⁻¹. The 10 analysed BC1 seedlings *H. syriacus* ‘Oiseau Bleu’ x (*H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis*) had a genome size between 2.18 pg.1C⁻¹ and 2.38 pg.1C⁻¹ and for the 2 BC1 hybrids *H. syriacus* ‘Red Heart CV’ x (*H. syriacus* ‘Red Heart CV’ x *H. paramutabilis*) an average genome size of 3.86 pg.1C⁻¹ was measured (Table 5.13).

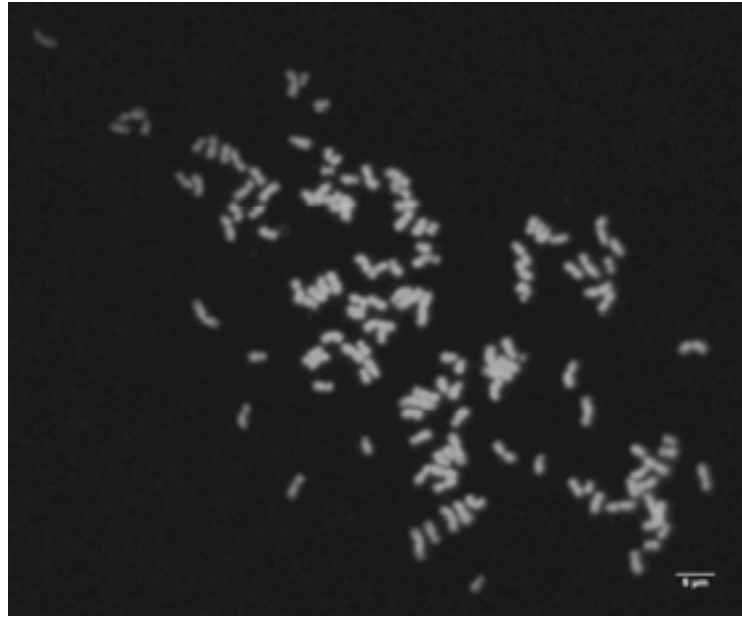


Figure 5.9: Metaphase spread of chromosomes for a *H. syr.* 'Oiseau Bleu' x *H. paramutabilis* F2 seedling (bar = 5 μ m) in which 120 chromosomes could be counted.

Because of the unexpected higher ploidy level and genome sizes of the F2 hybrids, 7 F1 and 5 F2 hybrids *H. syriacus* x *H. paramutabilis* were randomly chosen for further analysis for the occurrence of unreduced pollen.

Pollen size and pollen viability measurements

Hibiscus pollen grains were spherical. *H. syriacus* 'Oiseau Bleu' ($2n = 4x$) had a pollen diameter between 110 μ m and 180 μ m. The octoploid *H. syriacus* 'Red Heart CV' had bigger pollen with a diameter between 140 μ m and 210 μ m. For *H. paramutabilis*, the range of pollen size was broader with 150 μ m difference between the smallest (80 μ m) and the largest (230 μ m) pollen (Table 5.13). In *H. paramutabilis* 2 pollen populations were detected: a first population of smaller pollen with a distribution peaking at 130-140 μ m and a second one with a distribution peaking at 180-190 μ m (Figure 5.10).

Pollen of all F1 and F2 hybrids were also spherical. The F1 hybrids *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* and *H. syriacus* 'Red Heart CV' x *H. paramutabilis* had a comparable pollen diameter distribution with a difference of 80 μ m to 120 μ m between the smallest and the largest pollen. For the F2 hybrids *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* the range of the pollen diameter was broader (Table 5.13) (80 μ m to 230 μ m). For some F1 and F2 hybrids 2 pollen populations could clearly be distinguished (Figure 5.10). In 2 of the analysed F2 hybrids a small amount of pollen (< 1%) had a very large diameter of 250 μ m (Figure 5.10).

Acetocarmine staining showed that pollen with a diameter below 110 μ m was not viable. In general the larger pollen fraction (as from 140 μ m) was more viable compared to the smaller one. In total, more than 97% of the pollen of the *H. syriacus* parents was viable, compared to 90% for *H. paramutabilis* (Table 5.13). Pollen viability of the F1 hybrids *H. syriacus* 'Oiseau

Bleu' x *H. paramutabilis* ranged between 65% and 88%, while for the F2 hybrids this was higher (between 80% and 89%). In the F1 hybrid *H. syriacus* 'Red Heart CV' x *H. paramutabilis* about 95% of the pollen was viable (Table 5.13).

Table 5.13: Overview of the ploidy level, genome size and pollen characteristics of the *H. syriacus* and *H. paramutabilis* parents and their analysed F1 and F2 hybrids (results of genome sizes are averages \pm SD, n = 5 to 15; results of pollen viability and pollen diameter are averages, n = 300).

Genotype	Ploidy level (2n)	Chromosome number	Genome Size (pg.1C ⁻¹ \pm SD)	Viability of Pollen (%)	Pollen diameter (μ m)
<i>H. syriacus</i> 'Oiseau Bleu'	4x	80	2.34 \pm 0.08	97.7	110-180
<i>H. syriacus</i> 'Red Heart CV'	8x	160	4.88 \pm 0.08	98.6	140-210
<i>H. paramutabilis</i>	4x	82	2.48 \pm 0.05	89.6	80-230
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F1) -1 (oispar-1)	4x	81	2.45 \pm 0.04	65.2	100-220
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F1) -2 (oispar-2)	4x	81	2.37 \pm 0.01	88.2	100-180
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F1) -3 (oispar-3)	4x	81	2.32 \pm 0.01	66.1	90-210
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F1) -4 (oispar-4)	4x	81	2.32 \pm 0.01	65.7	80-200
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F1) -5 (oispar-5)	4x	81	2.35 \pm 0.08	84.8	90-210
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F1) -6 (oispar-6)	4x	81	2.37 \pm 0.09	^z	-
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F2) -1 (oispar-oispar-1)	6x	120	3.49 \pm 0.01	80.7	100-230
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F2) -2 (oispar-oispar-2)	6x	120	3.53 \pm 0.02	-	-
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F2) -3 (oispar-oispar-3)	6x	120	3.59 \pm 0.02	86.1	100-250
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F2) -4 (oispar-oispar-4)	6x	120	3.58 \pm 0.01	88.7	80-250
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F2) -5 (oispar-oispar-5)	6x	120	3.53 \pm 0.05	-	-
<i>H. syr.</i> 'Red Heart CV' x <i>H. par.</i> (F1)	6x	120	3.58 \pm 0.08	94.3	100-210
<i>H. syr.</i> 'Oiseau Bleu' x oispar -1 (ois-oispar-1)	nc ^y	nc	2.18 \pm 0.04	-	-
<i>H. syr.</i> 'Oiseau Bleu' x oispar -2 (ois-oispar-2)	nc	nc	2.38 \pm 0.05	-	-
<i>H. syr.</i> 'Oiseau Bleu' x oispar -3 (ois-oispar-3)	nc	nc	2.38 \pm 0.01	-	-
<i>H. syr.</i> 'Oiseau Bleu' x oispar -4 (ois-oispar-4)	nc	nc	2.24 \pm 0.05	-	-
<i>H. syr.</i> 'Oiseau Bleu' x oispar -7 (ois-oispar-5)	nc	nc	2.20 \pm 0.08	-	-
<i>H. syr.</i> 'Oiseau Bleu' x oispar -8 (ois-oispar-6)	nc	nc	2.32 \pm 0.04	-	-
<i>H. syr.</i> 'Oiseau Bleu' x oispar -9 (ois-oispar-7)	nc	nc	2.28 \pm 0.03	-	-
<i>H. syr.</i> 'Oiseau Bleu' x oispar -10 (ois-oispar-8)	nc	nc	2.25 \pm 0.04	-	-
<i>H. syr.</i> 'Oiseau Bleu' x oispar -11 (ois-oispar-9)	nc	nc	2.26 \pm 0.07	-	-
<i>H. syr.</i> 'Oiseau Bleu' x oispar -12 (ois-oispar-10)	nc	nc	2.34 \pm 0.03	-	-
<i>H. syr.</i> 'Red Heart CV' x (<i>H. syr.</i> 'Red Heart CV' x <i>H. par.</i>) -1	nc	nc	3.88 \pm 0.07	-	-
<i>H. syr.</i> 'Red Heart CV' x (<i>H. syr.</i> 'Red Heart CV' x <i>H. par.</i>) -2	nc	nc	3.84 \pm 0.03	-	-

^z no flowering observed so far; ^y not counted because no good root material was available

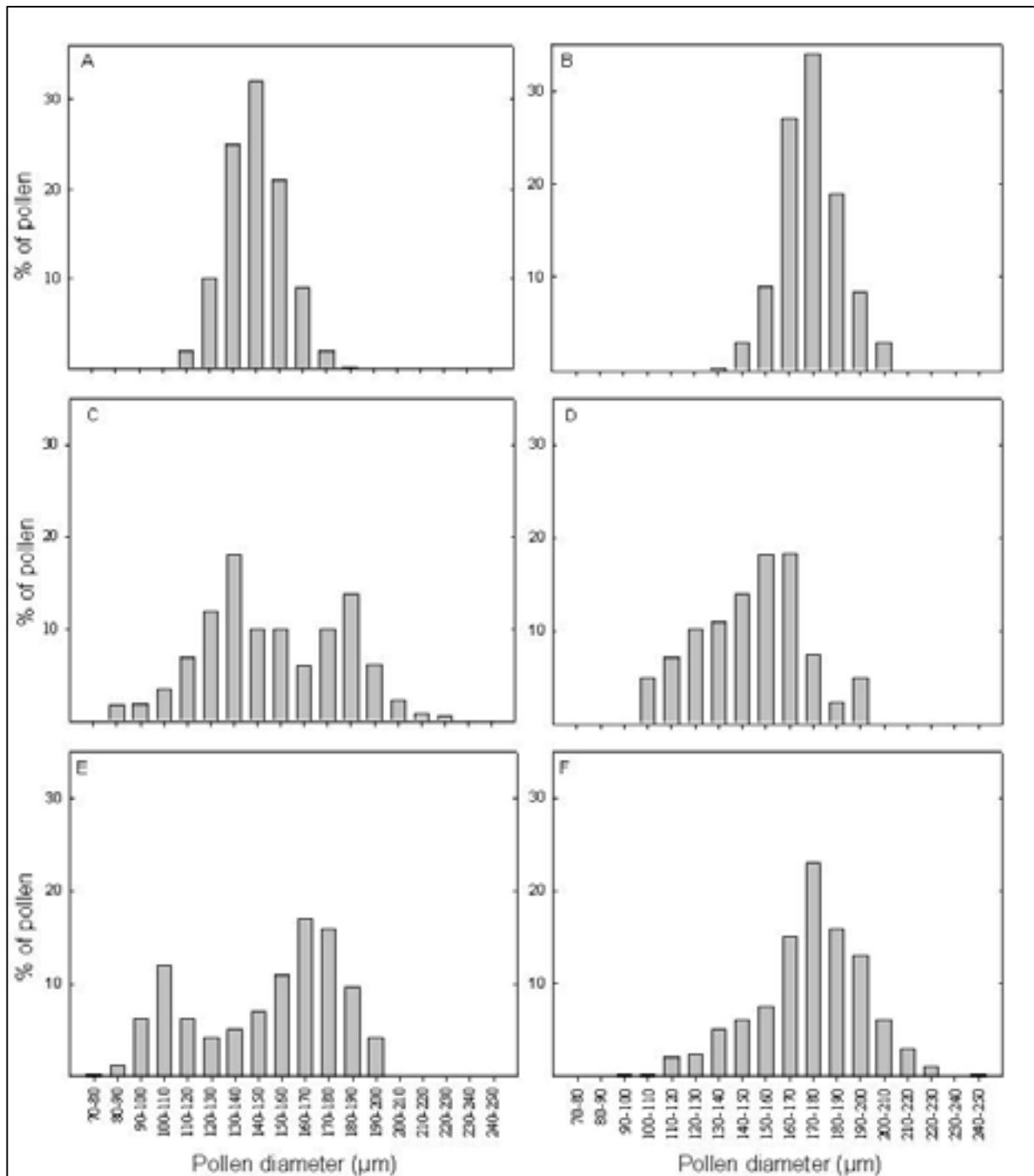


Figure 5.10: Pollen size distribution of the *H. syriacus* 'Oiseau Bleu' (A), *H. syriacus* 'Red Heart CV' (B), *H. paramutabilis* (C) parents and 2 different F1 hybrid seedlings (D, E) and 1 F2 hybrid seedling (F) hybrids *H. syriacus* x *H. paramutabilis* (n = 300).

Flow cytometric analysis of the pollen

In all investigated F1 genotypes, flow cytometric analysis of the pollen resulted in 1 single peak (2C) obtained from nuclei with equal fluorescence intensity compared to the leaf reference (Figure 5.11). However, in 2 of the analysed F2 hybrids *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis*, a small peak at the 4C level (4C/2C between 8% and 10%) was detected, indicating that unreduced pollen might be present (Figure 5.11).

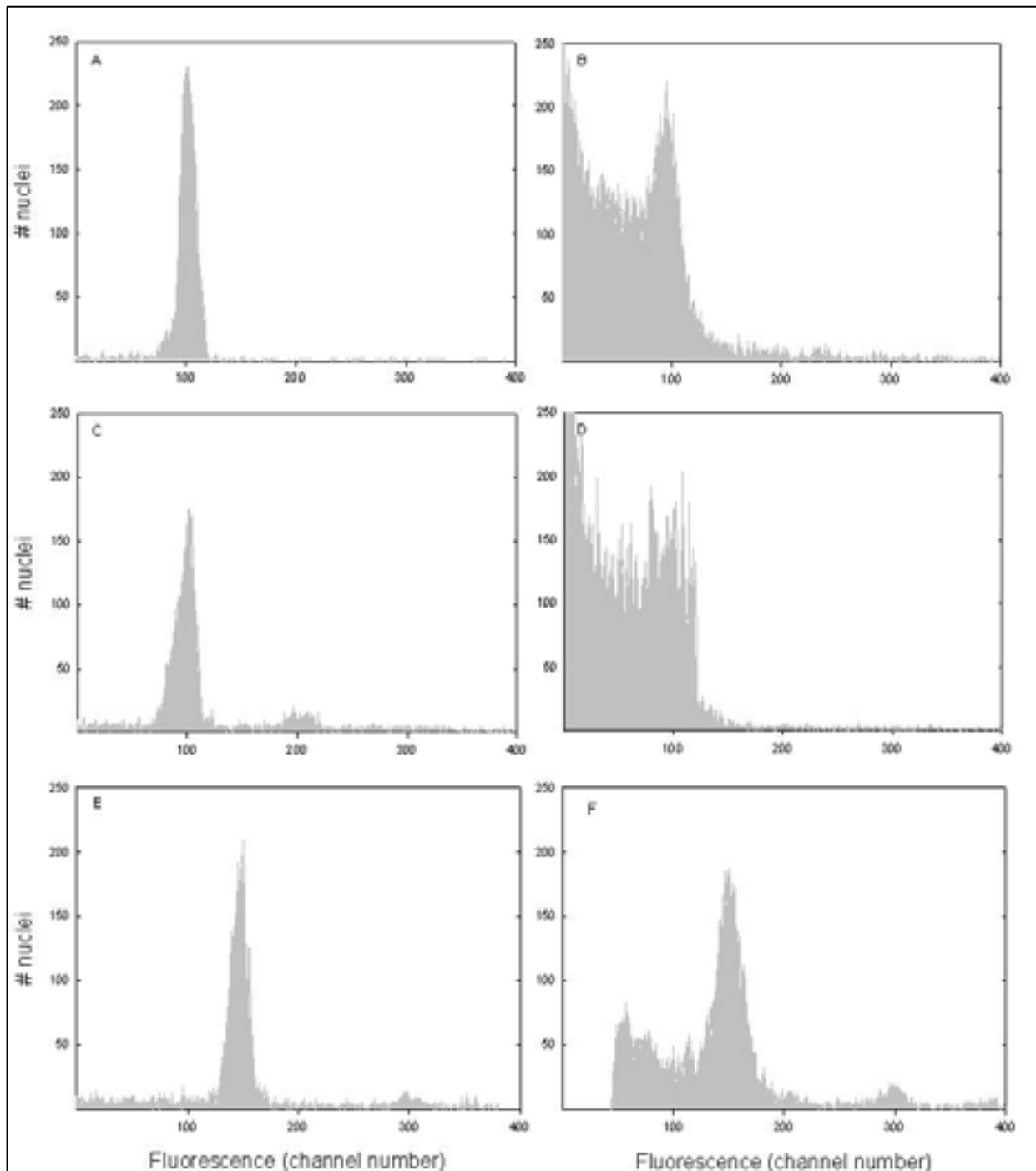


Figure 5.11: Flow cytometric analysis of leaf and pollen nuclei. A: somatic leaf tissue of *H. syriacus* 'Oiseau Bleu' (tetraploid), B: Normal (reduced) pollen nuclei of *H. syriacus* 'Oiseau Bleu' showing the peak according with the generative (2C) nuclei populations. C: somatic leaf tissue of a F1 hybrid *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* (tetraploid), D: Normal (reduced) pollen nuclei of a F1 hybrid *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis*, E: somatic leaf tissue of a F2 hybrid *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* (hexaploid), F: Reduced (2C) and unreduced generative pollen nuclei (4C) of a F2 hybrid *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis*.

Analysis of microsporogenesis

During microsporogenesis, cytokinesis and cell wall formation started only after telophase II, after the division of the 4 nuclei ('simultaneous type' of meiosis). This resulted in 4 microspores (tetrads) (Figure 5.12). In all investigated genotypes, a very high number of tetrads were observed (Table 5.14). In *H. syriacus* 'Red Heart CV' and 1 F1 hybrid *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis*, about 6% triads were observed, although this was not reflected in a bigger pollen size or the flow cytometric detection of 2n pollen. In contrast, for the 2 F2 hybrids *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis*, in which 2n pollen was detected flow cytometrically, more than 6% diads were observed during microsporogenesis (Table 5.14 and Figure 5.12). In F1 and F2 also polyads were observed sporadically, but were not scored.

Table 5.14: Overview of the % tetrads, triads and diads observed during the meiosis of *H. syriacus* and *H. paramutabilis* plants and their analysed F1 and F2 hybrids (n = 150).

Genotype	% tetrads	% triads	% diads
<i>H. syriacus</i> 'Oiseau Bleu'	100.0	0.0	0.0
<i>H. syriacus</i> 'Red Heart CV'	93.3	6.7	0.0
<i>H. paramutabilis</i>	99.4	0.1	0.2
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F1) -1 (oispar-1)	98.2	1.6	0.0
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F1) -2 (oispar-2)	91.1	6.4	1.0
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F1) -3 (oispar-3)	98.6	1.2	0.1
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F1) -4 (oispar-4)	96.9	1.4	1.3
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F1) -5 (oispar-5)	99.1	0.0	0.5
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F1) -6 (oispar-6)	- ^z	-	-
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F2) -1 (oispar-oispar-1)	91.5	0.5	6.7
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F2) -2 (oispar-oispar-2)	-	-	-
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F2) -3 (oispar-oispar-3)	96.1	2.9	0.5
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F2) -4 (oispar-oispar-4)	87.8	1.5	6.9
<i>H. syr.</i> 'Oiseau Bleu' x <i>H. par.</i> (F2) -5 (oispar-oispar-5)	-	-	-
<i>H. syr.</i> 'Red Heart CV' x <i>H. par.</i> (F1)	98.1	1.7	0.1

^z no flowering was observed so far

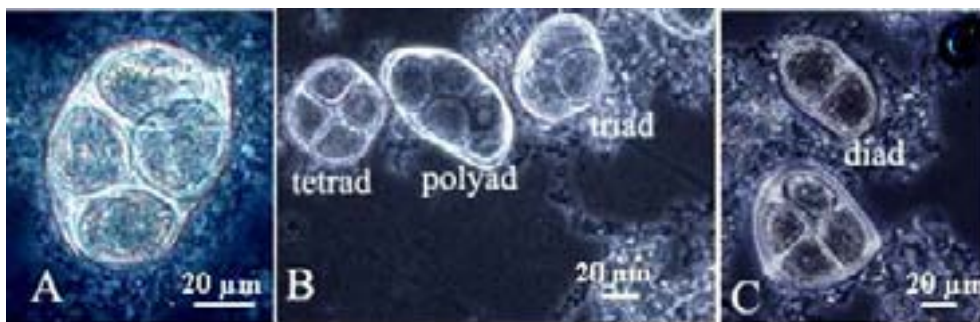


Figure 5.12: Microsporogenesis in *Hibiscus*. (A) normal tetrad of *H. syriacus* 'Oiseau Bleu'. (B) tetrads, triads and polyads observed in the F1 hybrids (C) diads in the F2 hybrids (bar = 20 μm).

5.3.3 Interspecific hybridisation within *Buddleja*

5.3.3.1 Pre- and postzygotic barriers

In total over the different years, 21258 interspecific and 2974 intraspecific pollinations were made (Table 5.15). In general, a lot of abortion of the fruits in an early stage of their development was observed. In total 1939 interspecific (overall success rate of crosses: 9.1%) and 893 intraspecific (overall success rate of crosses: 30.0%) fruits were obtained (Table 5.15). For the crosses that yielded fruits, *B. davidii* x *B. globosa* had the lowest success rate (0.22%) and *B. lindleyana* x *B. davidii* the highest (33.8%). For the intraspecific crosses, the success rate for *B. lindleyana* (9.2%) and *B. x weyeriana* (0.4%) crosses was much lower compared to *B. davidii* (34.1%).

Several interspecific and intraspecific crosses were analysed for the occurrence of prezygotic barriers (Table 5.15 and Figure 5.13). In most tested styles the growth of the pollen tube could be observed, indicating that there were no prezygotic barriers for the performed crosses.

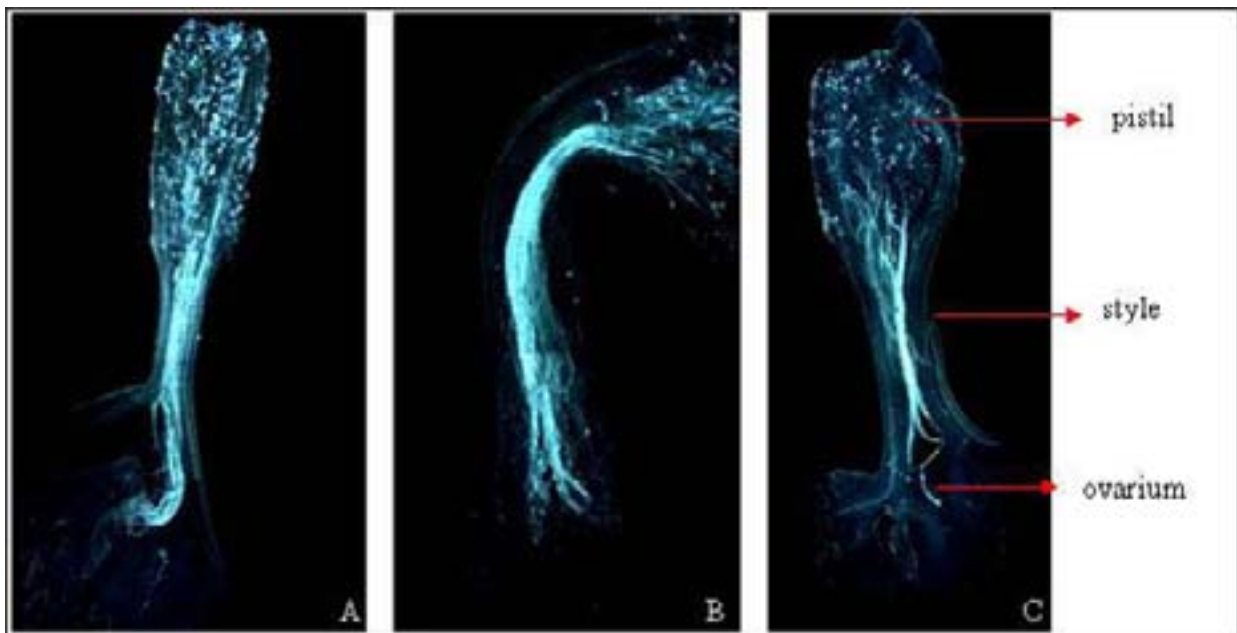


Figure 5.13: Anilineblue staining of pollen tubes (male parent) growing through the style of the female parent (72 h after pollination) for the crosses *B. davidii* 'White Profusion' x *B. lindleyana* (A, B) and *B. davidii* 'White Ball' x *B. lindleyana* (C).

When using *B. davidii* as female parent in interspecific crosses, between 1.00 and 14.33 seeds per fruit could be harvested. Fruits of *B. x weyeriana*, *B. lindleyana*, *B. crispa* and *B. alternifolia* used as female parents in interspecific crosses contained between 1.00 and 10.18 seeds. When using them in intraspecific crosses, *B. davidii* fruits contained on average 42.02 seeds, while *B. x weyeriana* and *B. lindleyana* contained 2.00 and 26.25 seeds respectively.

Germination of the *in vitro* sown seeds occurred normally within 2 weeks. Despite the high number of seeds that were harvested, for *B. lindleyana* x *B. davidii*, *B. davidii* x *B. alternifolia*, *B. alternifolia* x *B. lindleyana*, *B. weyeriana* x *B. alternifolia*, *B. davidii* x *B. crispa*, *B. crispa* x *B. davidii* and *B. crispa* x *B. weyeriana* no F1 seedlings were generated. For the other interspecific crosses germination rate was between 6.6% for *B. weyeriana* x *B. lindleyana* and 95.9% for *B. alternifolia* x *B. crispa* (Table 5.15). Intraspecific *B. davidii* and *B. lindleyana* seeds germinated for 78.9% and 58.3% respectively, while intraspecific crosses of *B. x weyeriana* did not result in F1 plants (Table 5.15).

Only for intraspecific *B. davidii* crosses *in vitro* embryo rescue was compared to *in vivo* sowing (Table 5.15). By use of *in vitro* embryo rescue germination capacity of the seeds could be increased from 1.6% *in vivo* to 78.1% *in vitro*.

Overall, 86.3% of the generated seedlings could be acclimatised (Table 5.15).

5. Interspecific hybridisation

Table 5.15: Overview of the different interspecific crosses in *Buddleja* spp. during different years, number of obtained fruits, seeds and F1 seedlings.

Female parent	Cross Male parent	# Pollinations	styles in which pollentube growth was observed/analysed styles	Obtained fruits	Success-rate pollinations (%)	Seeds <i>in vitro</i> (<i>in vivo</i>)	Seedlings <i>in vitro</i> (<i>in vivo</i>)	Acclimatised seedlings
<i>B. weyer.</i> 'Sungold'	<i>B. dav.</i> 'Royal Red'	256	na ^z	6	2.34	8	6	6
<i>B. weyer.</i> 'Sungold'	<i>B. dav.</i> 'NP'	300	4/4	27	9.00	174	121	91
<i>B. weyer.</i> 'Sungold'	<i>B. dav.</i> 'WP'	258	8/8	23	8.91	53	27	24
<i>B. weyer.</i> 'Sungold'	<i>B. dav.</i> 'White Ball'	758	na	7	0.92	48	9	9
<i>B. weyer.</i> 'Sungold'	<i>B. dav.</i> 'NA'	767	na	121	15.78	633	220	220
<i>B. dav.</i> 'Royal Red'	<i>B. weyer.</i> 'Sungold'	150	11/11	15	10.00	10	9	9
<i>B. dav.</i> 'NP' ^v	<i>B. weyer.</i> 'Sungold'	255	na	7	2.75	9	8	7
<i>B. dav.</i> 'WP' ^v	<i>B. weyer.</i> 'Sungold'	125	na	1	0.80	8	7	4
<i>B. dav.</i> 'White Ball'	<i>B. weyer.</i> 'Sungold'	766	na	10	1.31	4	- ^w	
<i>B. dav.</i> 'NA' ^v	<i>B. weyer.</i> 'Sungold'	905	na	14	1.55	13	-	
<i>B. lindleyana</i>	<i>B. dav.</i> 'Royal Red'	134	na	13	9.70	515	-	
<i>B. lindleyana</i>	<i>B. dav.</i> 'NP'	287	na	22	7.67	220	-	
<i>B. lindleyana</i>	<i>B. dav.</i> 'WP'	756	10/10	405	53.57	984	-	
<i>B. lindleyana</i>	<i>B. dav.</i> 'White Ball'	450	9/9	65	14.44	177	-	
<i>B. lindleyana</i>	<i>B. dav.</i> 'NA'	611	na	251	41.08	84	-	
<i>B. dav.</i> 'Royal Red'	<i>B. lindleyana</i>	260	10/10	15	5.77	15	5	1
<i>B. dav.</i> 'NP'	<i>B. lindleyana</i>	425	15/15	28	6.59	42	29	20
<i>B. dav.</i> 'WP'	<i>B. lindleyana</i>	1570	19/19	186	11.85	96	27	15
<i>B. dav.</i> 'White Ball'	<i>B. lindleyana</i>	1119	9/13	19	1.70	49	-	
<i>B. dav.</i> 'NA'	<i>B. lindleyana</i>	951	na	96	10.09	303	-	
<i>B. lindleyana</i>	<i>B. weyer.</i> 'Sungold'	212	na	- ^y	0.00			
<i>B. weyer.</i> 'Sungold'	<i>B. lindleyana</i>	357	na	98	27.45	895	59	54
<i>B. dav.</i> 'Royal Red'	<i>B. globosa</i>	350	na	-	0.00			
<i>B. dav.</i> 'NP'	<i>B. globosa</i>	290	na	-	0.00			
<i>B. dav.</i> 'WP'	<i>B. globosa</i>	281	na	-	0.00			
<i>B. dav.</i> 'NA'	<i>B. globosa</i>	145	na	3	2.07	42	20	19
<i>B. alternifolia</i>	<i>B. globosa</i>	268	na	-	0.00			
<i>B. globosa</i>	<i>B. alternifolia</i>	76	na	-	0.00			
<i>B. weyer.</i> 'Sungold'	<i>B. globosa</i>	187	na	-	0.00			
<i>B. lindleyana</i>	<i>B. globosa</i>	86	na	-	0.00			
<i>B. dav.</i> 'WP'	<i>B. alternifolia</i>	307	na	7	2.28	68	-	
<i>B. dav.</i> 'White Ball'	<i>B. alternifolia</i>	727	na	23	3.16	44	-	
<i>B. dav.</i> 'NA'	<i>B. alternifolia</i>	580	na	1	0.17	8	-	

Table 5.15: continued

Female parent	Cross Male parent	# Polli- nations	styles in which pollentube growth was observed/ analysed styles	Obtained fruits	Success- rate polli- nations (%)	Seeds <i>in vitro</i> (<i>in vivo</i>)	Seedlings <i>in vitro</i> (<i>in vivo</i>)	Acclimatised seedlings
<i>B. lindleyana</i>	<i>B. alternifolia</i>	118	na	-	0.00			
<i>B. alternifolia</i>	<i>B. lindleyana</i>	91	na	15	16.48	150	-	
<i>B. weyer.</i> 'Sungold'	<i>B. alternifolia</i>	408	na	82	20.10	835	-	
<i>B. alternifolia</i>	<i>B. weyer.</i> 'Sungold'	31	na	-	0.00			
<i>B. dav.</i> 'White Ball'	<i>B. crispa</i> 'Moondance'	1674	na	15	0.90	65	-	
<i>B. dav.</i> 'NA'	<i>B. crispa</i> 'Moondance'	1406	na	122	8.68	421	-	
<i>B. dav.</i> 'WP'	<i>B. crispa</i> 'Moondance'	269	na	68	25.28	- ^x		
<i>B. crispa</i> 'Moondance'	<i>B. dav.</i> 'White Ball'	721	na	297	41.19	47	-	
<i>B. crispa</i> 'Moondance'	<i>B. dav.</i> 'NA'	753	na	179	23.77	-		
<i>B. crispa</i> 'Moondance'	<i>B. dav.</i> 'WP'	244	na	226	92.62	-		
<i>B. crispa</i> 'Moondance'	<i>B. weyer.</i> 'Sungold'	120	na	5	4.17	6	-	
<i>B. alternifolia</i>	<i>B. crispa</i> 'Moondance'	454	na	125	27.53	365	350	350
<i>B. dav.</i> 'Royal Red'	<i>B. dav.</i> 'Royal Red'	200	na	6	3.00	8	-	
<i>B. dav.</i> 'Royal Red'	<i>B. dav.</i> 'NP'	355	12/15	167	47.04	198 (7500)	106 (214)	312
<i>B. dav.</i> 'Royal Red'	<i>B. dav.</i> 'WP'	258	na	130	50.39	(6003)	(120)	110
<i>B. dav.</i> 'NP'	<i>B. dav.</i> 'NP'	350	10/15	16	4.57	38	19	16
<i>B. dav.</i> 'NP'	<i>B. dav.</i> 'Royal Red'	380	na	235	61.84	248 (21000)	184 (170)	331
<i>B. dav.</i> 'NP'	<i>B. dav.</i> 'WP'	80	na	-	0.00			
<i>B. dav.</i> 'WP'	<i>B. dav.</i> 'Royal Red'	663	na	246	37.10	377 (240)	370 (-)	277
<i>B. dav.</i> 'WP'	<i>B. dav.</i> 'NP'	240	na	50	20.83	(1200)	(75)	65
<i>B. dav.</i> 'WP'	<i>B. dav.</i> 'White Ball'	40	na	26	65.00	-		
<i>B. lindleyana</i>	<i>B. lindleyana</i>	173	na	16	9.25	420	245	132
<i>B. weyer.</i> 'Sungold'	<i>B. weyer.</i> 'Sungold'	235	na	1	0.43	2	-	

^z na: not analysed; ^y no fruits were observed; ^x no healthy seeds could be found in the fruits; ^w no seeds germinated *in vitro*; ^v 'NP' = 'Nanho Purple', 'WP' = 'White Profusion', 'NA' = 'Nanhoensis Alba'

5.3.3.2 Morphological characterisation of the obtained hybrids

Flowering F1 seedlings of *B. davidii* 'Nanho Purple' x *B. x weyeriana*, *B. x weyeriana* x *B. davidii* 'Royal Red', *B. davidii* 'Nanho Purple' x *B. lindleyana* and *B. davidii* 'White Profusion' x *B. lindleyana* were morphologically characterised in detail. Generated seedlings resulting from other cross combinations were still smaller and/or did not flower yet. They were therefore so far not morphologically characterised.

Leaf morphology

The *B. davidii* x *B. x weyeriana* seedlings all had a uniform morphology with an average L/W of the leaves comparable with *B. davidii* (Table 5.16). The *B. x weyeriana* x *B. davidii* seedlings had an average L/W ratio higher than both parent plants.

The F1 seedlings resulting from the cross *B. davidii* x *B. lindleyana* had an intermediate leaf size (Table 5.16 and Figure 5.14).

Table 5.16: Leaf morphology, pollen germination and fruit formation of the F1 hybrids *B. davidii* x *B. x weyeriana*, *B. x weyeriana* x *B. davidii* and *B. davidii* x *B. lindleyana* compared to the parent plants (results for pollen germination are averages, n = 300, results for fruit formation are averages \pm SD, n = 20; results for L/W are averages \pm SD, n = 10).

Genotype	Average leaf length / leaf width (L/W) ^z	<i>In vitro</i> germination rate of pollen (%)	Average fruit formation (%) ^z
<i>B. x weyeriana</i> ‘Sungold’	2.9 \pm 0.3	1.2	57.3 \pm 3.9
<i>B. davidii</i> ‘Nanho Purple’	3.2 \pm 0.4	39.0	73.0 \pm 3.9
<i>B. davidii</i> ‘Royal red’	3.4 \pm 0.6	15.4	73.3 \pm 2.2
<i>B. davidii</i> ‘White Profusion’	3.5 \pm 0.5	44.3	84.3 \pm 3.3
<i>B. lindleyana</i>	2.2 \pm 0.2	0.5	49.7 \pm 3.3
<i>B. x weyeriana</i> x <i>B. davidii</i>	3.7 \pm 0.3	0.8	31.1 \pm 4.3
<i>B. davidii</i> x <i>B. x weyeriana</i>	3.4 \pm 0.5	40.0	72.1 \pm 2.9
<i>B. davidii</i> x <i>B. lindleyana</i>	3.1 \pm 0.2	1.7	4.5 \pm 2.3

^z no statistical analysis could be performed because of to large SD

Flower morphology

The length of the flower panicle of the *B. davidii* x *B. x weyeriana* seedlings was similar to both parent plants (Table 5.17). The flower color differed compared to the parent plants (Table 5.17). The germination capacity of the pollen was on average 40% and the fruit formation about 72%, which was comparable to *B. davidii* (Table 5.16).

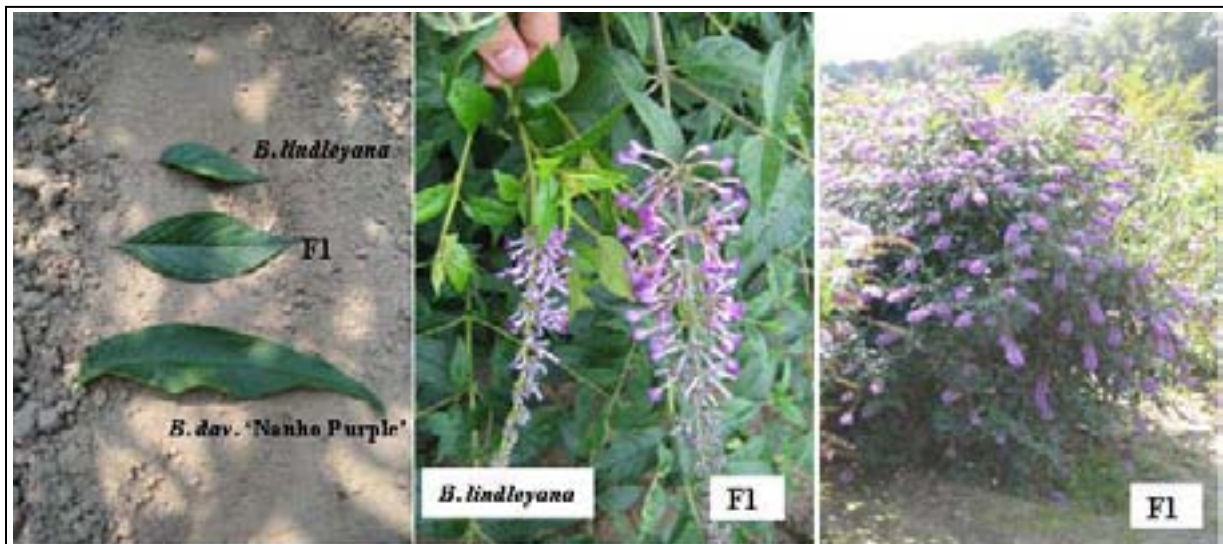
Seedlings resulting from the reciprocal cross *B. x weyeriana* x *B. davidii* had larger flower panicles compared to both the parent plants (Table 5.17). The flower color depended on the *B. davidii* cultivar used in the cross (Table 5.17) and the panicle shape was intermediate compared to the parent plants (Figure 5.15). These hybrids were less fertile since on average, only 0.8% of the pollen of the *B. x weyeriana* x *B. davidii* hybrids germinated *in vitro* and the fruit formation was about 31% (Table 5.16).

The flower color of the *B. davidii* x *B. lindleyana* F1 seedlings depended on the *B. davidii* cultivar that was used in the cross. The inflorescence of the F1 hybrids was similar to *B. lindleyana*, but the flower panicle of the F1 seedlings was longer (Table 5.17, Figure 5.14). The *B. davidii* x *B. lindleyana* seedlings were rather compact and almost sterile, with an *in vitro* germination capacity of the pollen of 1.7% and a fruit formation of 4.5% (Table 5.16).

Table 5.17: Flower morphology of the F1 hybrids *B. davidii* x *B. x weyeriana*, *B. x weyeriana* x *B. davidii* and *B. davidii* x *B. lindleyana* compared with the parent plants (results of panicle length are averages \pm SD, n = 20).

Genotype	Flower color	Average flower panicle length (cm) ^z
<i>B. x weyeriana</i> 'Sungold'	yellow	11.5 \pm 1.4
<i>B. davidii</i> 'Nanho Purple'	purple RHS 80 - 82	7.2 \pm 0.9
<i>B. davidii</i> 'Royal Red'	red-Purple RHS 74	9.2 \pm 1.3
<i>B. davidii</i> 'White Profusion'	white	11.6 \pm 2.4
<i>B. lindleyana</i>	purple RHS 80 - 82	12.1 \pm 2.3
<i>B. x weyeriana</i> x <i>B. davidii</i> 'White Profusion'	25% white, 75% purple RHS 80 - 82	15.6 \pm 2.5
<i>B. x weyeriana</i> x <i>B. davidii</i> 'Nanho Purple'	purple RHS 80 - 82	15.5 \pm 2.7
<i>B. davidii</i> 'Royal Red' x <i>B. x weyeriana</i>	purple RHS 87 - 88	11.6 \pm 2.1
<i>B. davidii</i> 'White Profusion' x <i>B. lindleyana</i>	8% white, 92% purple 80-82	15.6 \pm 1.9
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> *	purple (RHS 80 - 82)	15.8 \pm 2.2

^z no statistical analysis could be performed because of to large SD

Figure 5.14: Leaf and flower morphology of *B. davidii* 'Nanho Purple' x *B. lindleyana* F1 seedlings.

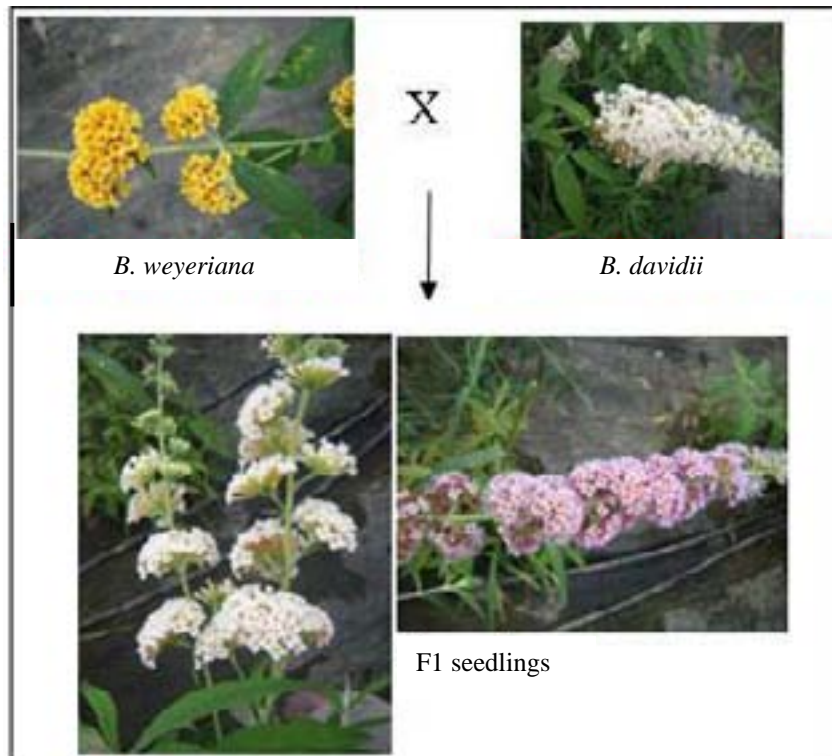


Figure 5.15: Flower morphology of F1 seedlings resulting from *B. x weyeriana* x *B. davidii* crosses.

5.3.3.3 Molecular analysis of the obtained hybrids

AFLP analysis was performed to confirm the hybrid status of *B. x weyeriana* x *B. davidii*, *B. davidii* x *B. x weyeriana*, *B. davidii* x *B. lindleyana* and *B. alternifolia* x *B. crispa*.

Figure 5.16 gives an overview of the genetic relationships between hybrids resulting from *B. x weyeriana* and *B. davidii* crosses. *B. x weyeriana* and *B. davidii* ('Nanho Purple' and 'Royal Red') appeared to be very polymorphic. The two tested *B. davidii* 'Nanho Purple' x *B. x weyeriana* F1 seedlings inherited 65% and 70% of the unique markers of *B. davidii* and 40% and 44% of the unique markers of *B. x weyeriana*. The two tested *B. x weyeriana* x *B. davidii* 'Royal Red' F1 seedlings inherited 26% and 32% of the unique markers of *B. x weyeriana* and 34% and 44% of the unique markers of *B. davidii*.

For AFLP analysis of 15 randomly chosen F1 seedlings resulting from a cross between *B. x weyeriana* 'Sungold' x *B. davidii* 'Nanhoensis Alba', the primer combinations generated in total 636 marker bands, with 64 markers unique for *B. x weyeriana* 'Sungold' and 92 markers unique for *B. davidii* 'Nanhoensis Alba'. The F1 seedlings inherited between 27 and 44 unique markers from the female parent and between 24 and 46 unique markers from the pollen donor. Figure 5.16 shows the dendrogram that indicates the relatedness of the F1 progeny and the parent plants. The group of hybrids was genetically more related to each other than to the parent plants, proving the hybrid nature of the seedlings.

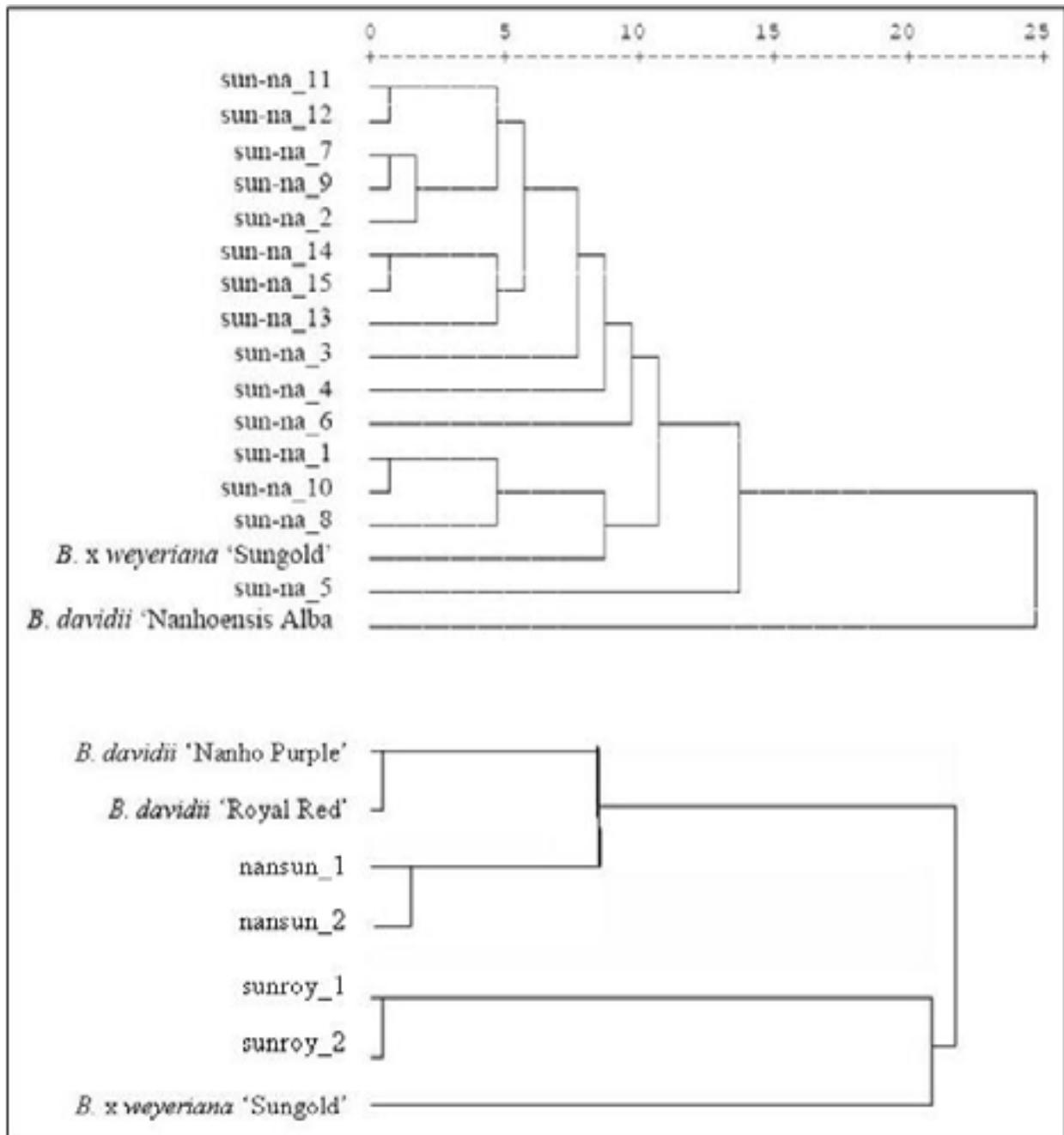


Figure 5.16: Dendrogram showing the genetic relatedness of the *B. davidii* 'Nanho Purple' x *B. x weyeriana* 'Sungold' (nansun), the *B. x weyeriana* 'Sungold' x *B. davidii* 'Royal Red' (sunroy) and the *B. x weyeriana* 'Sungold' x *B. davidii* 'Nanhoensis Alba' (sun-na) F1 seedlings and the parent plants tested by AFLP.

Also for *B. davidii* x *B. lindleyana* crosses the parent species were very polymorphic. Both parent plants had 40 unique markers. Between 79% and 95% of the unique markers of the male parent *B. lindleyana* and between 56% and 71% of the unique markers of the female parent *B. davidii* were inherited by the F1 seedlings. Figure 5.17 gives an overview of the genetic relationships between hybrids and parent plants.

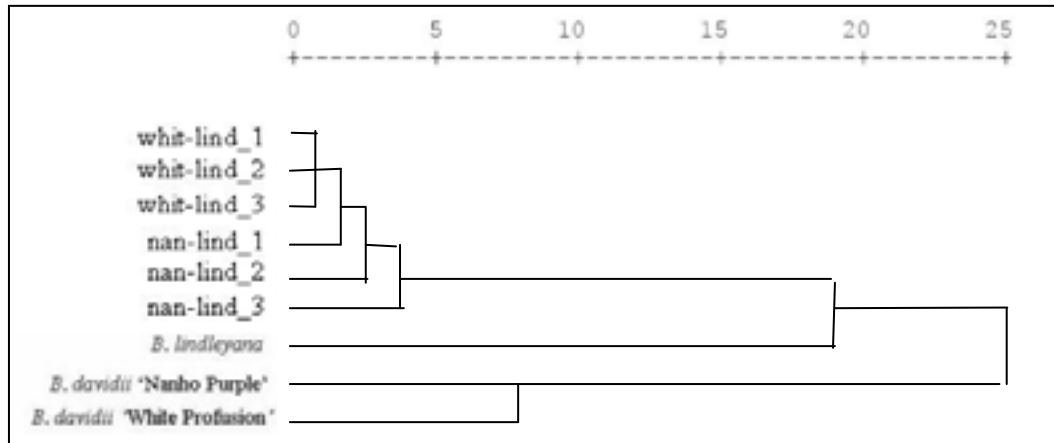


Figure 5.17: Dendrogram showing the genetic relatedness of the *B. davidii* x *B. lindleyana* (whit-lind and nan-lind) F1 seedlings and the parent plants tested by AFLP.

Ten randomly chosen F1 seedlings resulting from a cross *B. alternifolia* x *B. crispa* were analysed by AFLP. *B. alternifolia* was characterised by 79 unique marker bands, while for *B. crispa* 60 unique markers could be identified. The F1 seedlings inherited between 23% and 76% of the unique markers of the female parent and between 23% and 80% of the unique markers of the male parent. In Figure 5.18 the dendrogram is shown of the F1 seedlings and the parent plants. The hybrids are clearly genetically different from both the parent plants.

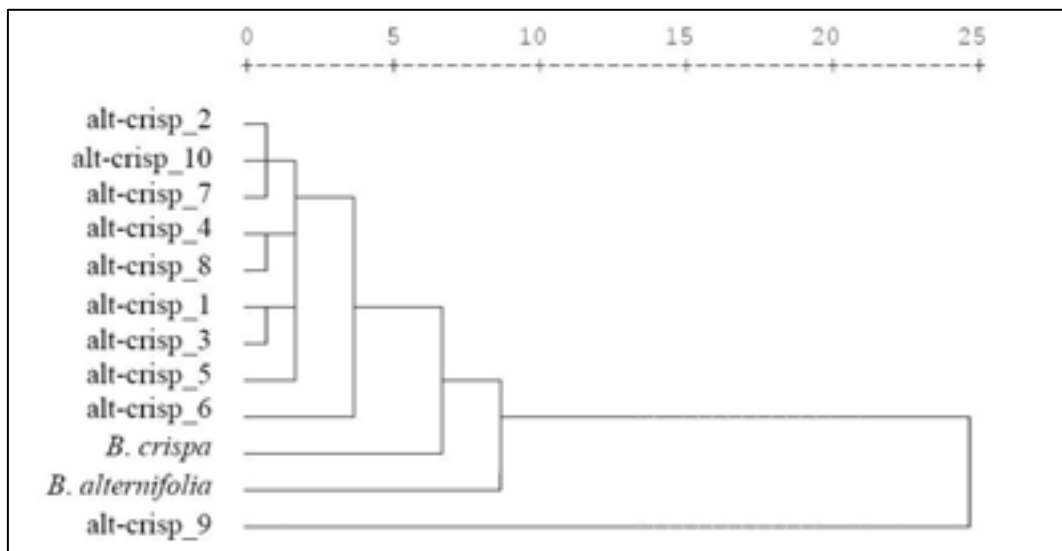


Figure 5.18: Dendrogram showing the genetic relatedness of the *B. alternifolia* x *B. crispa* (alt-crisp) F1 seedlings and the parent plants tested by AFLP.

For the plants analysed by AFLP also genome sizes and chromosome numbers were measured. In Table 5.18 the genome sizes and chromosome numbers of the F1 seedlings and the parent

plants are summarized. The results of the genome size measurements were similar on both flow cytometers that were used (Partec Pas III and Partec Cyflow Space).

For the F1 seedlings resulting from *B. x weyeriana* x *B. davidii* ‘Royal Red’ and *B. x weyeriana* x *B. davidii* ‘Nanhoensis Alba’, a genome size of 1.62 pg.1C^{-1} was expected, based on the genome sizes of *B. x weyeriana* (1.78 pg.1C^{-1}), *B. davidii* ‘Royal Red’ and ‘Nanhoensis Alba’ (1.46 pg.1C^{-1}). Genome size measurements revealed for the F1 seedlings *B. x weyeriana* x *B. davidii* ‘Royal Red’ a genome size of 1.55 pg.1C^{-1} and for the F1 seedlings *B. x weyeriana* x *B. davidii* ‘Nanhoensis Alba’ a genome size between 1.57 pg.1C^{-1} and 1.72 pg.1C^{-1} (Table 5.18). All these F1 hybrids also had 76 chromosomes (Figure 5.19). However, for the F1 hybrids of the reciprocal cross *B. davidii* ‘Nanho Purple’ x *B. x weyeriana* a genome size of 1.30 pg.1C^{-1} (average) was measured. This is lower than might be expected from the genome sizes of the parent plants. These plants were also considered to be tetraploid as 76 chromosomes were counted (Figure 5.19).

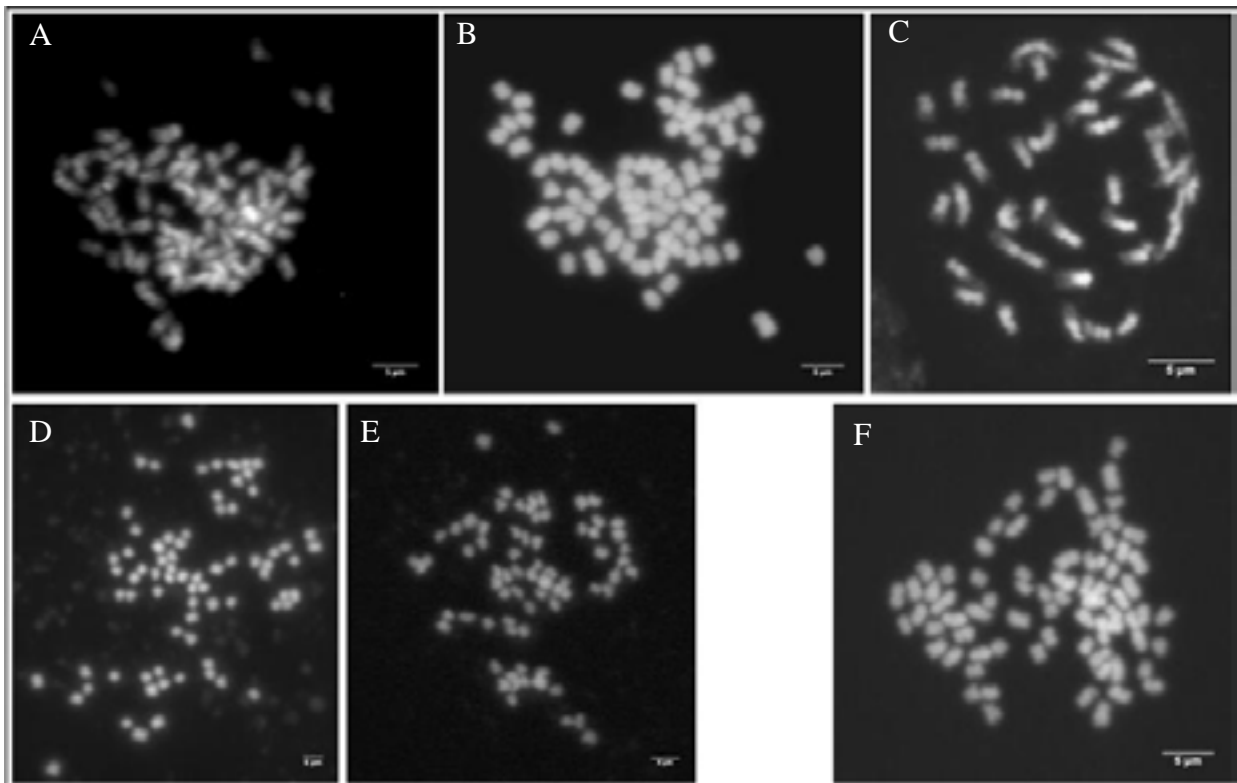


Figure 5.19: Metaphase spreads of chromosomes for (A) *B. x weyeriana* (76 chromosomes), (B) *B. davidii* (76 chromosomes), (C) *B. lindleyana* (38 chromosomes), (D) an F1 seedling resulting from a cross *B. davidii* x *B. x weyeriana* (76 chromosomes), (E) an F2 seedling of *B. davidii* x *B. x weyeriana* (76 chromosomes) and (F) an F1 seedling of *B. davidii* x *B. lindleyana* (76 chromosomes) (bar = 5 µm).

For the F1 hybrids *B. davidii* ‘Nanho Purple’ x *B. lindleyana* and *B. davidii* ‘White Profusion’ x *B. lindleyana*, a genome size of 1.21 pg.1C^{-1} and 1.18 pg.1C^{-1} respectively was expected, based on the sizes measured in the parent plants (0.94 pg.1C^{-1} for *B. lindleyana*, 1.41 pg.1C^{-1} for *B. davidii* ‘White Profusion’ and 1.48 pg.1C^{-1} for *B. davidii* ‘Nanho Purple’). However, after

measuring the genome sizes of 28 F1 seedlings, higher genome sizes were determined, varying between 1.50 pg.1C⁻¹ and 1.69 pg.1C⁻¹. All the *B. davidii* x *B. lindleyana* hybrids had 76 chromosomes (Figure 5.19, Table 5.18). F2 seedlings (see 5.3.3.4) resulting from self pollinations of different F1 hybrids of *B. davidii* ‘Nanho Purple’ x *B. lindleyana* had a higher genome size between 1.71 pg.1C⁻¹ and 1.85 pg.1C⁻¹. Also in the F2 progeny 76 chromosomes were counted.

Table 5.18: Genome size measurements and ploidy level of parent plants and F1 and F2 hybrids resulting from interspecific crosses between *B. davidii*, *B. x weyeriana*, *B. lindleyana*, *B. alternifolia*, *B. crispa* and *B. globosa* (results of genome sizes are averages \pm SD, n = 5 to 15).

Genotype	Ploidy level (2n)	Chromosome number	Genome size (pg.1C ⁻¹)
<i>B. davidii</i> ‘White Profusion’	4x	76	1.41 \pm 0.06
<i>B. davidii</i> ‘Royal Red’	4x	76	1.46 \pm 0.01
<i>B. davidii</i> ‘Nanho Purple’	4x	76	1.48 \pm 0.01
<i>B. davidii</i> ‘Nanhoensis Alba’	4x	76	1.46 \pm 0.03
<i>B. davidii</i> ‘White Ball’	4x	76	1.36 \pm 0.03
<i>B. lindleyana</i>	2x	38	0.94 \pm 0.01
<i>B. globosa</i>	2x	38	1.12 \pm 0.04
<i>B. x weyeriana</i> ‘Sungold’	4x	76	1.78 \pm 0.01
<i>B. alternifolia</i>	4x	38	0.93 \pm 0.06
<i>B. crispa</i> ‘Moondance’	4x	38	0.96 \pm 0.03
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. x weyeriana</i> (F1) - 1 (nansun_1)	4x	76	1.31 \pm 0.01
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. x weyeriana</i> (F1) - 2 (nansun_2)	4x	76	1.28 \pm 0.01
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. x weyeriana</i> (F2) - 1 (nansun-nansun-1)	4x	76	1.26 \pm 0.01
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. x weyeriana</i> (F2) - 2 (nansun-nansun-2)	4x	76	1.37 \pm 0.01
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. x weyeriana</i> (F2) - 3 (nansun-nansun-3)	4x	76	1.37 \pm 0.01
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. x weyeriana</i> (F2) - 4 (nansun-nansun-4)	4x	76	1.26 \pm 0.01
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. x weyeriana</i> (F2) - 5 (nansun-nansun-5)	4x	76	1.32 \pm 0.01
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. x weyeriana</i> (F2) - 6 (nansun-nansun-6)	4x	76	1.22 \pm 0.03
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. x weyeriana</i> (F2) - 7 (nansun-nansun-7)	nc ^z	nc	1.26 \pm 0.02
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. x weyeriana</i> (F2) - 8 (nansun-nansun-8)	4x	76	1.41 \pm 0.02
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. x weyeriana</i> (F2) - 9 (nansun-nansun-9)	nc	nc	1.32 \pm 0.02
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. x weyeriana</i> (F2) - 10 (nansun-nansun-10)	nc	nc	1.26 \pm 0.02
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. x weyeriana</i> (F2) - 11 (nansun-nansun-11)	nc	nc	1.32 \pm 0.01
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Royal Red’ (F1) -1 (sun-roy_1)	4x	76	1.55 \pm 0.01
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Royal Red’ (F1) -2 (sun-roy_2)	4x	76	1.55 \pm 0.02
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-1 (sun-na_1)	4x	76	1.60 \pm 0.05
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-2 (sun-na_2)	nc	nc	1.60 \pm 0.05
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-3 (sun-na_3)	4x	76	1.66 \pm 0.09
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-4 (sun-na_4)	nc	nc	1.62 \pm 0.06
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-5 (sun-na_5)	4x	76	1.61 \pm 0.08
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-6 (sun-na_6)	nc	nc	1.58 \pm 0.06
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-7 (sun-na_7)	4x	76	1.61 \pm 0.05
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-8 (sun-na_8)	nc	nc	1.63 \pm 0.03
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-9 (sun-na_9)	nc	nc	1.62 \pm 0.06
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-10 (sun-na_10)	4x	76	1.57 \pm 0.05
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-11 (sun-na_11)	nc	nc	1.60 \pm 0.08
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-12 (sun-na_12)	nc	nc	1.72 \pm 0.10
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-13 (sun-na_13)	nc	nc	1.65 \pm 0.10
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-14 (sun-na_14)	nc	nc	1.72 \pm 0.04
<i>B. x weyeriana</i> x <i>B. davidii</i> ‘Nanhoensis Alba’ (F1)-15 (sun-na_15)	4x	76	1.64 \pm 0.01
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. lindleyana</i> (F1) - 1 (nan-lind_1)	4x	76	1.69 \pm 0.01
<i>B. davidii</i> ‘Nanho Purple’ x <i>B. lindleyana</i> (F1) - 2 (nan-lind_2)	4x	76	1.69 \pm 0.01

Table 5.18: continued

Genotype	Ploidy level (2n)	Chromosome number	Genome size (pg.1C ⁻¹)
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F1) - 3 (nan-lind_3)	nc	nc	1.60 ± 0.02
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F1) - 4	nc	nc	1.60 ± 0.02
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F1) - 5	nc	nc	1.50 ± 0.02
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F1) - 6	nc	nc	1.50 ± 0.05
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F1) - 7	nc	nc	1.69 ± 0.01
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F1) - 8	4x	76	1.60 ± 0.03
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F1) - 9	4x	76	1.50 ± 0.05
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F1) - 10	nc	nc	1.69 ± 0.03
<i>B. davidii</i> 'White Profusion' x <i>B. lindleyana</i> (F1) - 1 (whit-lind_1)	nc	nc	1.69 ± 0.01
<i>B. davidii</i> 'White Profusion' x <i>B. lindleyana</i> (F1) - 2 (whit-lind_2)	nc	nc	1.60 ± 0.02
<i>B. davidii</i> 'White Profusion' x <i>B. lindleyana</i> (F1) - 3 (whit-lind_3)	4x	76	1.50 ± 0.01
<i>B. davidii</i> 'White Profusion' x <i>B. lindleyana</i> (F1) - 4	nc	nc	1.60 ± 0.03
<i>B. davidii</i> 'White Profusion' x <i>B. lindleyana</i> (F1) - 5	nc	nc	1.50 ± 0.05
<i>B. davidii</i> 'White Profusion' x <i>B. lindleyana</i> (F1) - 6	4x	76	1.64 ± 0.02
<i>B. davidii</i> 'White Profusion' x <i>B. lindleyana</i> (F1) - 7	4x	76	1.60 ± 0.02
<i>B. davidii</i> 'White Profusion' x <i>B. lindleyana</i> (F1) - 8	4x	76	1.60 ± 0.02
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F2) - 1	4x	76	1.78 ± 0.02
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F2) - 2	nc	nc	1.81 ± 0.05
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F2) - 3	4x	76	1.85 ± 0.11
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F2) - 4	nc	nc	1.73 ± 0.10
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F2) - 5	nc	nc	1.71 ± 0.00
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F2) - 6	nc	nc	1.75 ± 0.06
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F2) - 7	nc	nc	1.79 ± 0.07
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F2) - 8	nc	nc	1.72 ± 0.02
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F2) - 9	nc	nc	1.72 ± 0.06
<i>B. davidii</i> 'Nanho Purple' x <i>B. lindleyana</i> (F2) - 10	4x	76	1.79 ± 0.03
<i>B. alternifolia</i> x <i>B. crispa</i> 'Moondance' (F1) - 1 (alt-crisp_1)	2n	38	0.89 ± 0.08
<i>B. alternifolia</i> x <i>B. crispa</i> 'Moondance' (F1) - 2 (alt-crisp_2)	nc	nc	0.86 ± 0.04
<i>B. alternifolia</i> x <i>B. crispa</i> 'Moondance' (F1) - 3 (alt-crisp_3)	nc	nc	0.87 ± 0.09
<i>B. alternifolia</i> x <i>B. crispa</i> 'Moondance' (F1) - 4 (alt-crisp_4)	2n	38	0.89 ± 0.08
<i>B. alternifolia</i> x <i>B. crispa</i> 'Moondance' (F1) - 5 (alt-crisp_5)	nc	nc	0.91 ± 0.07
<i>B. alternifolia</i> x <i>B. crispa</i> 'Moondance' (F1) - 6 (alt-crisp_6)	nc	nc	0.92 ± 0.08
<i>B. alternifolia</i> x <i>B. crispa</i> 'Moondance' (F1) - 7 (alt-crisp_7)	nc	nc	0.92 ± 0.06
<i>B. alternifolia</i> x <i>B. crispa</i> 'Moondance' (F1) - 8 (alt-crisp_8)	2n	38	0.91 ± 0.06
<i>B. alternifolia</i> x <i>B. crispa</i> 'Moondance' (F1) - 9 (alt-crisp_9)	nc	nc	0.87 ± 0.04
<i>B. alternifolia</i> x <i>B. crispa</i> 'Moondance' (F1) - 10 (alt-crisp_10)	2n	38	0.95 ± 0.05
<i>B. davidii</i> 'Nanhoensis Alba' x <i>B. globosa</i> (F1) - 1 ^z	nc	nc	1.59 ± 0.08
<i>B. davidii</i> 'Nanhoensis Alba' x <i>B. globosa</i> (F1) - 2	nc	nc	1.58 ± 0.10
<i>B. davidii</i> 'Nanhoensis Alba' x <i>B. globosa</i> (F1) - 3	nc	nc	1.58 ± 0.09
<i>B. davidii</i> 'Nanhoensis Alba' x <i>B. globosa</i> (F1) - 4	nc	nc	1.53 ± 0.07
<i>B. davidii</i> 'Nanhoensis Alba' x <i>B. globosa</i> (F1) - 5	nc	nc	1.57 ± 0.08
<i>B. davidii</i> 'Nanhoensis Alba' x <i>B. globosa</i> (F1) - 6	nc	nc	1.53 ± 0.06
<i>B. davidii</i> 'Nanhoensis Alba' x <i>B. globosa</i> (F1) - 7	nc	nc	1.57 ± 0.04
<i>B. davidii</i> 'Nanhoensis Alba' x <i>B. globosa</i> (F1) - 8	nc	nc	1.54 ± 0.03
<i>B. davidii</i> 'Nanhoensis Alba' x <i>B. globosa</i> (F1) - 9	nc	nc	1.56 ± 0.07
<i>B. davidii</i> 'Nanhoensis Alba' x <i>B. globosa</i> (F1) - 10	nc	nc	1.58 ± 0.05

^z nc: chromosomes are not counted for these plants because no good root material was available; ^y No AFLP is performed on this progeny

The hybrids *B. alternifolia* x *B. crispa* were expected to have a genome size of 0.95 pg.1C⁻¹. Measurements by flow cytometry indicated a genome size between 0.86 pg.1C⁻¹ and 0.95 pg.1C⁻¹ for the F1 progeny (Table 5.18). F1 seedlings all had 38 chromosomes.

Finally the genome size of 10 F1 seedlings of *B. davidii* ‘Nanhoensis Alba’ x *B. globosa* was determined. A genome size of 1.29 pg.1C⁻¹ was expected, based on the parent plants (1.46 pg.1C⁻¹ for *B. davidii* and 1.12 pg.1C⁻¹ for *B. globosa*). Nine of the tested seedlings had a genome size between 1.53 pg.1C⁻¹ and 1.58 pg.1C⁻¹. One seedling had a genome size of 1.86 pg.1C⁻¹ (Table 5.18).

5.3.3.4 F2 generation of crosses between *B. davidii*, *B. x weyeriana* and *B. lindleyana*

From the F1 *B. davidii* ‘Nanho Purple’ x *B. x weyeriana* and *B. x weyeriana* x *B. davidii* ‘Royal Red’ seedlings, an F2 generation was obtained by both self pollination and back crossing with each parent (Table 5.19). All together 4472 *B. x weyeriana* x *B. davidii* and *B. davidii* x *B. x weyeriana* flowers were self pollinated with a success rate of 0.85% and 2.78% respectively (Table 5.19). In total 126 seeds could be initiated *in vitro* but only the *B. davidii* x *B. x weyeriana* seeds germinated and resulted in 61 acclimatised F2 plants.

Table 5.19: Overview of the different interspecific crosses in *Buddleja* spp., number of obtained fruits, seeds and F2, BC1 seedlings. Numbers are the accumulation of the results over different crossing years.

Cross			# Pollinations	Obtained fruits	seeds <i>in vitro</i>	Seedlings <i>in vitro</i>	Acclimatised seedlings
Female parent ^z	Male parent ^z						
<i>B. weyer</i> ‘SG’ x <i>B. dav.</i> ‘RR’	<i>B. weyer</i> ‘SG’ x <i>B. dav.</i> ‘RR’	(F2)	1993	17	45	-	
<i>B. dav.</i> ‘NP’ x <i>B. weyer</i> ‘SG’	<i>B. dav.</i> ‘NP’ x <i>B. weyer</i> ‘SG’	(F2)	2479	69	81	70	61
<i>B. dav.</i> ‘Nanho Purple’	<i>B. weyer</i> ‘SG’ x <i>B. dav.</i> ‘RR’	(BC1)	205	11	10	4	4
<i>B. dav.</i> ‘White Profusion’	<i>B. weyer</i> ‘SG’ x <i>B. dav.</i> ‘RR’	(BC1)	260	36	369	285	266
<i>B. dav.</i> ‘Royal Red’	<i>B. dav.</i> ‘NP’ x <i>B. weyer</i> ‘SG’	(BC1)	244	110	110	32	32
<i>B. dav.</i> ‘White Profusion’	<i>B. dav.</i> ‘NP’ x <i>B. weyer</i> ‘SG’	(BC1)	362	30	876	557	473
<i>B. weyer</i> ‘Sungold’	<i>B. weyer</i> ‘SG’ x <i>B. dav.</i> ‘RR’	(BC1)	258	16	27	1	1
<i>B. weyer</i> ‘Sungold’	<i>B. dav.</i> ‘NP’ x <i>B. weyer</i> ‘SG’	(BC1)	306	35	266	190	155
<i>B. weyer</i> ‘SG’ x <i>B. dav.</i> ‘RR’	<i>B. dav.</i> ‘Nanho Purple’	(BC1)	250	9	89	- ^x	
<i>B. weyer</i> ‘SG’ x <i>B. dav.</i> ‘RR’	<i>B. weyer</i> ‘Sungold’	(BC1)	202	8	53	-	
<i>B. weyer</i> ‘SG’ x <i>B. dav.</i> ‘RR’	<i>B. dav.</i> ‘White Profusion’	(BC1)	120	- ^y			
<i>B. weyer</i> ‘SG’ x <i>B. dav.</i> ‘RR’	<i>B. globosa</i>	(BC1)	140	-			
<i>B. dav.</i> ‘NP’ x <i>B. weyer</i> ‘SG’	<i>B. dav.</i> ‘Nanho Purple’	(BC1)	201	29	292	269	267
<i>B. dav.</i> ‘NP’ x <i>B. weyer</i> ‘SG’	<i>B. dav.</i> ‘Royal Red’	(BC1)	203	2	2	2	- ^w
<i>B. dav.</i> ‘NP’ x <i>B. weyer</i> ‘SG’	<i>B. dav.</i> ‘White Profusion’	(BC1)	164	35	340	336	336
<i>B. dav.</i> ‘NP’ x <i>B. weyer</i> ‘SG’	<i>B. globosa</i>	(BC1)	109	2	20	-	
<i>B. dav.</i> ‘NP’ x <i>B. lindleyana</i>	<i>B. dav.</i> ‘NP’ x <i>B. lindleyana</i>	(F2)	179	19	200	184	160
<i>B. dav.</i> ‘WP’ x <i>B. lindleyana</i>	<i>B. dav.</i> ‘WP’ x <i>B. lindleyana</i>	(F2)	737	-			

^z NP = ‘Nanho Purple’, WP = ‘White Profusion’, ‘RR’ = ‘Royal Red’, ‘SG’ = ‘Sungold’; ^y no fruits were observed; ^x no seeds germinated *in vitro*; ^w no seedlings could be acclimatised;

The backcrosses were more successful. Of the 3024 performed crosses, 323 fruits could be harvested and 2454 seeds were initiated *in vitro* (Table 5.19). A BC1 population was made for several (*B. davidii* ‘Nanho Purple’ x *B. x weyeriana*) x *B. davidii*, *B. davidii* x (*B. x weyeriana* x *B. davidii* ‘Royal Red’), *B. davidii* x (*B. davidii* ‘Nanho Purple’ x *B. x weyeriana*) and *B. x weyeriana* x (*B. x weyeriana* x *B. davidii* ‘Royal Red’) cross combinations.

An F2 generation was also made from *B. davidii* x *B. lindleyana* by controlled self pollinations. In total 916 self pollinations were performed which yielded 19 fruits, containing on average 10.5 well developed seeds. About 92% of the *in vitro* initiated seeds germinated and resulted in 160 acclimatised F2 *B. davidii* ‘Nanho Purple’ x *B. lindleyana* seedlings.

For the *B. davidii* ‘Nanho Purple’ x *B. x weyeriana* F2 hybrids different genome sizes were measured, between 1.22 and 1.41 pg.1C⁻¹. Chromosome counting revealed for all F2 plants 76 chromosomes despite genome size differences (Table 5.18, Figure 5.19).

So far, none of the F2 seedlings flowered.

5.4 Discussion

5.4.1 Occurrence of interspecific pre- and postzygotic barriers

The possible existence of prezygotic barriers can be verified by microscopic evaluation of pollen tube growth through the style of the female parent (Cuevas *et al.* 1994). For most tested crosses in this study, pollen tube growth through the style towards the ovaria could be observed, followed by the formation of fruits. Therefore, it could be concluded that in none of the case study species prezygotic barriers were the reason for failure of interspecific crosses.

On the contrary, a lot of postzygotic barriers were determined after interspecific crosses within *Hydrangea*, *Hibiscus* and *Buddleja*. Spontaneous abortion of the fruits in an early stage of their development was a problem for the 3 genera. The introduction of an *in vitro* step to rescue hybrid embryos from abortion and to allow further maturation was therefore urged. In this study, for *Buddleja* and *Hibiscus* this *in vitro* embryo rescue step was very efficient to increase germination vigor of the hybrid embryos. However, for *Hydrangea* the major problem was the lack of germination of the immature seeds, probably due to endosperm malformation, although this was not quantified. Almost no developed seeds were found in the resulting fruits after interspecific *Hydrangea* crosses. Embryo culture is already successfully applied in many other ornamental species where pollinated flowers could remain on the plant for a notable time as in *Alstroemeria* (Buitendijk *et al.* 1992) and *Lilium* (Van Tuyl *et al.* 1991). It was also already successfully performed before in *Hibiscus* (Kim *et al.* 1996a; 1996b) and *Hydrangea* (Reed *et*

al. 2001; Reed 2004, Kudo and Niimi 1999; Kudo *et al.* 2002). To our knowledge seed culture was not reported in *Buddleja* so far.

Total, variegated or virescent albinism (and growth aberrations) are the major barriers to obtain viable hybrids. This phenomenon of chlorosis is frequently observed in interspecific crosses as in *Rhododendron* (Michishita *et al.* 2002) and *Zantedeschia* (Yao and Cohen 2000). Although it was not an obstacle after interspecific crosses within *Buddleja*, it obviously was for interspecific hybridisation within *Hydrangea* and *Hibiscus*.

The *Hibiscus* and *Buddleja* F1 hybrids obtained in our study had a very low pollen germination percentage. This is a well known phenomenon after interspecific hybridisation. According to Heslop-Harrison (1999) errors in chromosome segregation at meiosis may cause F1 sterility. Failed chromosome segregation leads to an unbalanced chromosome number in the gametes which results in sterility. One of the most striking consequences of a disturbed synapsis is the abortion of the malformed embryo sac in many hybrids, causing female sterility. Microspores resulting from a disturbed meiosis usually are non-viable (Van Tuyl and De Jeu 1997; Sharmina *et al.* 1999). Allopolyploidisation can be used to restore the fertility of the hybrids (Chen *et al.* 2002).

5.4.1.1 Interspecific hybridisation within *Hydrangea*

Although the genome sizes for *H. macrophylla* and *H. serrata* were different (Table 3.4) and for *H. macrophylla* both diploid and triploid cultivars were used, for *Hydrangea* unambiguous identified hybrids were obtained after crosses between *H. serrata* and *H. macrophylla*. The dendrogram of the *Hydrangea* parent plants (Figure 3.3) shows a close genetic relationship between *H. macrophylla* cultivars and *H. serrata*, which make them more compatible and result in more successful interspecific crosses. Also in literature *H. serrata* and *H. macrophylla* are described to be closely related (Wilson 1923; McClintock 1957; Haworth-Booth 1984; Zonneveld 2004). F1 seedlings resulting from crosses between *H. paniculata* ‘White Moth’ x *H. macrophylla* ‘Mariesii Perfecta’ and between *H. paniculata* ‘White Moth’ x *H. aspera* (although *H. aspera* had a low germination capacity of the pollen) were also obtained. Two possible reasons for failure of other cross combinations are differences in the genome size of the *Hydrangea* species ranging from 0.86 pg.1C⁻¹ in *Hydrangea quercifolia* to 4.70 pg.1C⁻¹ for *Hydrangea paniculata* (Table 3.4) and ploidy differences within the species used for the interspecific crosses.

In agreement with Mortreau (2003) postzygotic barriers observed after our interspecific crosses manifested in non-germination of the seeds. An F1 progeny could only be obtained using *in vitro* embryo rescue. Fruits were harvested 10 to 11 weeks after pollination and seeds were initiated *in vitro*. However, in the fruits only few healthy seeds could be observed, most seeds were aborted. Harvesting the fruits earlier was tried, but was not successful. Also Reed (2004) and Reed *et al.* (2001) reported about *Hydrangea macrophylla* x *Hydrangea paniculata* hybrids that only could be obtained by using *in vitro* embryo rescue. However, their hybrids were lacking in vigor and were sterile. Production of *H. macrophylla* x *H. arborescens* hybrids even

required both embryo rescue and subsequent regeneration from callus culture. Kudo *et al.* (2002) reported producing putative *H. macrophylla* x *H. quercifolia* hybrids using embryo rescue, but no details are available about the vigor or fertility of this interspecific hybrid. The first report of a *Hydrangea* interspecific hybrid that was obtained without the use of embryo rescue was from Jones and Reed (2005). Reciprocal crosses were made between *H. involucrata* and *H. arborescens*. The crosses only were successful when *H. arborescens* was used as the female parent. Of more than 500 seeds, only 8 plants remained alive (Jones and Reed 2005; 2006). Recently a hybrid *Hydrangea* x 'Innovalaur', originating from a cross between *H. anomala* spp. *petiolaris* and *H. seemanii*, was released in France (<http://www.sapho.fr>). In the same research group crosses were attempted between *H. macrophylla* and *H. paniculata* but were not successful (A. Cadic, personal communication). Some intergeneric crosses were made recently with *Dichroa febrifuga* and *Hydrangea macrophylla* (Reed *et al.* 2008). According to Rinehart *et al.* (2006) *Dichroa* is closely related to *H. macrophylla* but its relationship within *Macrophyllae* is not well defined.

In our study also self pollinations and intraspecific crosses (between different cultivars of the same species) were performed for *H. serrata*, *H. macrophylla* and *H. paniculata*. The intraspecific crosses yielded no results. For the self pollinations fruits were obtained for *H. macrophylla* and *H. paniculata*, but only for *H. paniculata* healthy seeds could be found in the fruits, resulting in F1 seedlings after *in vitro* embryo rescue. *Hydrangea* is known as an allogamous genus (Mortreau 2003). However, conflicting information concerning self-incompatibility in *Hydrangea* has been reported. Reed (2000) obtained few viable seeds from self pollinations of *H. macrophylla*, *H. paniculata* and *H. quercifolia*, while Kudo and Niimi (1999) obtained large numbers of viable seeds from self pollinations of *H. macrophylla*. According to Reed (2004) *H. paniculata* and *H. quercifolia* have a gametophytic self-incompatibility system. Pollen germinated freely after self pollination in both species but self-pollen tubes grew much slower than cross-pollen tubes. By the time pollen tubes had reached the bottom of the style in cross pollinations, self-pollen tubes had grown only one-fourth of the way down the style. Also no or low seed set following self pollinations has been observed. However, self-incompatible species sometimes produce self-seed (Reed 2004). Pseudo-self-incompatibility is defined as the limited or occasionally full seed set following incompatible pollination of a plant known to possess self-incompatibility (Ascher 1976). Studying the effects of high temperature and end-of-season pollinations may help to explain differences in self-incompatibility assessments in *Hydrangea*. The possibility that there may be self-compatible *Hydrangea* cultivars should be considered (Reed 2004).

5.4.1.2 Interspecific hybridisation within *Hibiscus*

For *Hibiscus*, interspecific hybrids between *H. syriacus*, *H. paramutabilis* and *H. sinosyriacus* were obtained. However, crossing compatibility was unilateral and only successful when *H. syriacus* was used as the mother parent. The success rate of a cross was also cultivar dependent. In our study, crosses with *H. syriacus* 'Red Heart CV' were less successful compared to crosses with other *H. syriacus* cultivars. Differences in compatibilities in crosses among different

Hibiscus species were also reported by Kyung and Kim (2001a; 2001b). Unilateral incongruity has been described in the *H. mutabilis* x *H. moscheutos* crosses of Kuwada (1964) and Tachibana (1958) and in many other species, like *Lilium* (Van Tuyl *et al.* 1991) and *Bromeliaceae* (Vervaeke *et al.* 2001). Self pollinations and intraspecific crosses for *H. syriacus* in this study were indeed successful. Similar percentage of fruits and amount of seeds in the fruits were obtained compared to interspecific crosses. Although more BC1 seedlings were obtained by back crosses, both BC1 and F2 (self pollinations of F1) could be generated. So based on our results *Hibiscus* can be considered having a reproductive system which is autogamous but mixed with allogamous. These findings are in agreement with Allard (1960) who reported that *Hibiscus* species are largely self-compatible and with Han and Yeam (1993) who observed from 0% to 100% seed set after self pollinations in *Hibiscus syriacus*.

In vitro embryo rescue was compared with direct *in vivo* sowing of the seeds in the greenhouse, since it was possible for the fruits to stay on the plant until they were fully matured. For the embryo rescue, the embryos were isolated 10 to 11 weeks after pollination. They had developed by then to the torpedo stage or in some cases even to the cotyledonary stage and could easily be isolated from the seeds. So it was possible to perform embryo culture instead of seed culture. In our study both *in vitro* embryo rescue and *in vivo* sowing were successful, since for the two methods F1 seedlings were obtained. Although higher amounts of seedlings from *H. syriacus* x *H. sinosyriacus* crosses could be generated *in vitro*, a lot of them were lost during acclimatisation due to albinism and growth aberrations. Performing the reciprocal cross or use of bridge plants can be a solution as shown in *Zantedeschia* (Yao *et al.* 1994; 1995). However, in our study the reciprocal crosses were hampered by unilateral incongruity.

5.4.1.3 Interspecific hybridisation within *Buddleja*

Interspecific hybrids between *Buddleja davidii* x *B. x weyeriana*, *B. x weyeriana* x *B. davidii*, *B. davidii* x *B. lindleyana*, *B. davidii* x *B. globosa* and *B. alternifolia* x *B. crispa* were generated after *in vitro* seed culture of manually pollinated flowers. When using *B. davidii* as female and *B. lindleyana* or *B. x weyeriana* as male parent, crosses were less successful and less seeds per fruit could be initiated *in vitro* compared to the reciprocal cross. The pollen of *B. lindleyana* and *B. x weyeriana* has proven to have less germination percentage compared to *B. davidii* (Chapter 3), probably explaining the lower fruit formation and less amount of obtained seeds. Although the cross *B. lindleyana* x *B. davidii* yielded a lot of fruits, the *in vitro* initiated seeds did not germinate. This is in agreement with Elliott *et al.* (2004) who also only had success when using *B. lindleyana* as male parent in crosses with *B. davidii*. For all our crosses involving *B. globosa* (only used as male parent) there was a lot of abortion of the fruits in an early stage of their development. Only few fruits, seeds and seedlings were obtained. *B. globosa* pollen seemed to have a low germination capacity (Chapter 3) and because it flowered earlier than *B. davidii*, stored pollen had to be used. However, Van De Weyer (1920) successfully crossed *B. globosa* (female) with *B. davidii* (male), resulting in *B. x weyeriana*.

Within the species used in the interspecific *Buddleja* crosses, different ploidy levels occur. *B. globosa*, *B. lindleyana*, *B. alternifolia* and *B. crispa* are diploid species ($2n = 2x = 38$), while *B. davidii* and *B. x weyeriana* are tetraploid ($2n = 4x = 76$). Differences in ploidy level can be the incongruity barrier in interspecific *Buddleja* hybridisation resulting in malformation of endosperm, the inhibition of germination (Badger 1988) or spontaneous abortion of the fruits (Van Tuyl *et al.* 1991; Pickersgill *et al.* 1993; Sharma 1995). Polyploidisation offers sometimes a solution towards the problem of parent species having a different ploidy level (Stebbins 1971). Doubling the chromosome number of *B. globosa* in the view of breeding experiments with the tetraploid *B. davidii* was already successful (Rose *et al.* 2000b; Chapter 4). Successful crosses between doubled *B. globosa* and *B. davidii* were reported by Rose *et al.* (2001). Their first seedling to flower had yellow flowers tinted with mauve.

In agreement with Elliott *et al.* (2004) and Tobutt (1993), in this study F1 seedlings were obtained after self pollinations and intraspecific crosses with *B. davidii*. In contrast to Elliott *et al.* (2004), in this work also seedlings were obtained after self pollinations and intraspecific crosses with *B. lindleyana*. In addition, both self pollinations and back crosses of F1 progenies were successful. So based on these results it can be concluded that *Buddleja* has a reproductive system which is a mix between self and cross pollination.

5.4.2 Characterisation of F1 hybrids

Combination of AFLP analysis and morphological screening have proven to be useful to determine the hybrid character of obtained F1 seedlings. When parental species differ enough in genome size and ploidy level, also genome size measurements and chromosome counting was efficient to characterise F1 and F2 hybrids.

By AFLP analysis the hybrid nature of the *Hydrangea macrophylla* x *H. serrata* progeny was proven. The hybrids were not tightly clustered, but they were all genetically different from both the parent plants. On the contrary, genome sizes of *H. macrophylla* x *H. serrata* seedlings were not intermediate compared to the parent plants, they were similar either to *H. macrophylla* or to *H. serrata*. AFLP analysis on *H. paniculata* ‘White Moth’ x *H. macrophylla* ‘Mariesii Perfecta’ and *H. paniculata* ‘White Moth’ x *H. aspera* ‘Macrophylla’ did not yield convincing results to determine the hybrid nature of the seedlings. Almost no unique markers of the pollen donor (between 8.5% and 12.8% for *H. paniculata* x *H. macrophylla* and between 9.5% and 10.1% for *H. paniculata* x *H. aspera*) was inherited in the F1 seedlings. However, up to 30% fragments, which were not present in the parental species, were seen in the F1 seedlings and up to 62% of the markers found in both parents were not observed in the F1 seedlings. These findings indicate genome rearrangements in the seedlings due to interspecific hybridisation. Some recent studies indicate that the process of wide hybridisation may induce rapid and extensive genetic and epigenetic changes in some plant species and genomic stasis in others (Han *et al.* 2003). Changes included disappearance of parental hybridisation fragments, simultaneous appearance of novel fragments and loss of parental fragments (Han *et al.* 2003). Some of these changes

occurred as early as in the F1 hybrid, whereas others occurred after allopolyploid formation. It is suggested that DNA methylation and localised recombination at the DNA level are probably the main causes for the genomic changes (Kashkush *et al.* 2002; Madlung *et al.* 2002; Han *et al.* 2003; Keyte *et al.* 2006; Marfil *et al.* 2006). Genomic changes after hybridisation, resulting in unexpected AFLP results, are also observed in allopolyploids of Triticeae (Han *et al.* 2003), hybrids between *Solanum tuberosum* x *Solanum kurtzianum* (Marfil *et al.* 2006) and between *Helianthus annuus* x *Helianthus tuberosus* (Natali *et al.* 1998). After chromosome counting it could be concluded that most of the analysed F1 seedlings resulting from *H. paniculata* 'White Moth' x *H. macrophylla* 'Mariesii Perfecta' and *H. paniculata* 'White Moth' x *H. aspera* 'Macrophylla' had a similar chromosome amount as the female parent (72 chromosomes). In 2 of the tested F1 *H. paniculata* x *H. macrophylla* seedlings only 66 chromosomes could be determined. One hypothesis can be that the F1 seedlings are substitution lines with only few chromosomes of *H. macrophylla* and *H. aspera* in a *H. paniculata* genome. Substitution lines are also often found in hybrids between *Lolium* and *Festuca* (Kopecky *et al.* 2008). In Festulolium cultivars differences in the proportion of parental chromatin are present (Kopecky *et al.* 2008). Also chromosome elimination and aneuploidy are frequently observed in hybrids between *Lolium* and *Festuca* (Kopecky *et al.* 2008). Depending on the proportion of parental chromosomes present in the hybrids, genome sizes will vary. Another hypothesis might be that the F1 seedlings with the same chromosome amount of the female parent are not hybrids but resulting seedlings of uncontrolled self pollination of *H. paniculata*. The 2 seedlings with 66 chromosomes are than the only aneuploid hybrids. Kudo and Niimi (1999) also obtained aneuploid hybrids after crosses between *H. macrophylla* and *H. arborescens*. Their hybrids had $2n = 42$ chromosomes, whereas the chromosome numbers of *H. macrophylla* and *H. arborescens* were $2n = 52$ and $2n = 38$ respectively. Aneuploid hybrids were also observed among others in *Lycopersicon* (DeVerna *et al.* 1990) and sugarcane (Heinz and Mee 1971). However the AFLP results are in contradiction with this hypothesis. And also the genome sizes of the F1 seedlings from both cross combinations were in between the genome size of the parental species. For seedlings resulting from a self pollination of the female parent, a same genome size of this female parent would be expected. GISH should be applied on the F1 seedlings *H. paniculata* 'White Moth' x *H. macrophylla* 'Mariesii Perfecta' and *H. paniculata* 'White Moth' x *H. aspera* 'Macrophylla' to determine the hybrid nature and to unravel the genome composition of the F1 seedlings.

Hibiscus hybrids are known to be easy to detect at the seedling stage because they tend to blend the genotypic and phenotypic characteristics of both parents thereby becoming distinguishable (Akpan and Hossain 1996). Morphological screening on the field of the *Hibiscus* F1 seedlings obtained in this study together with AFLP analysis revealed that most of the *in vitro* and *in vivo* *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* seedlings were true interspecific hybrids. But of the progeny obtained from *H. syriacus* 'Melwhite' x *H. sinosyriacus* 'Lilac Queen' crosses only about 50% of the obtained seedlings were true hybrids as proven by morphological and genetic analysis. The other 50% of the F1 seedlings *H. syriacus* 'Melwhite' x *H. sinosyriacus* 'Lilac Queen' as well as all seedlings from other *H. syriacus* x *H. sinosyriacus* cross combinations were unwanted self pollinations of *H. syriacus*. Probably these self pollinations were the green vigorous growing seedlings *in vitro*, while the aberrant (albino) plantlets which could not be

acclimatised, might have been hybrids. In both *H. syriacus* x *H. paramutabilis* and *H. syriacus* x *H. sinosyriacus*, true hybrids had larger flowers compared to their parent plants and flower color was intermediate. On adult plants of both hybrid progenies four different leaf types could be observed completely at random. Leaf dimorphisms were described before for some other plant genera. Juvenile plants or parts of plants of *Hedera helix* bear alternately arranged, palmately lobed leaves while the mature leaf is entirely ovate or rhombic (Wareing and Frydman 1976; Metcalfe 2005). Similar observations of different foliage types are reported in many species of *Populus* (Critchfield 1960) and also in *Morus Alba* (Everett 1960). It is not clear whether or not this phenomenon on the *Hibiscus* F1 seedlings can be explained by the change from juvenile to adult phase. Changes in leaf shape have never been seen in seedlings of the parental species *H. syriacus*, *H. sinosyriacus* or *H. paramutabilis*. So most probably the leaf polymorphisms in the hybrids are caused by genetic modifications after interspecific hybridisation. Despite the low fertility of the *H. syriacus* x *H. paramutabilis* hybrids, a small F2 progeny could be obtained so far. However, a larger flowering F2 progeny is needed to study segregation of leaf types and flower colors.

Morphological screening of the obtained *Buddleja* F1 seedlings on the field revealed that *B. davidii* x *B. x weyeriana*, *B. x weyeriana* x *B. davidii* and *B. davidii* x *B. lindleyana* seedlings were true interspecific hybrids. Especially the *B. davidii* x *B. lindleyana* seedlings have very interesting features as they turned out to be almost sterile. Sterility is often wanted to counteract the invasive character of some cultured plants. *B. davidii* is the most commonly cultivated species in the genus *Buddleja*, yet it possesses many of the characteristics associated with invasive plants: the seeds are small, winged and wind-dispersed. Seeds germinate quickly and the resulting plants reach maturity rapidly. Development of sterile *Buddleja* hybrids will address the problem of invasiveness found for most *B. davidii* cultivars (Lindstrom *et al.* 2002; 2005). Also AFLP and genome size measurements on the *Buddleja* F1 seedlings proved their hybrid character. Genome size measurements on *B. alternifolia* x *B. crispa* F1 seedlings were not useful to analyse the hybrid character, since the parent plants had a similar genome size. The F1 hybrids *B. x weyeriana* x *B. davidii* had an intermediate genome size, compared to the crossing parents, proving again the hybrid nature of the F1 seedlings. On the contrary, the reciprocal F1 and F2 hybrids *B. davidii* x *B. x weyeriana* had a genome size that was lower than expected. *B. x weyeriana* itself contains genomic DNA of *B. davidii* and *B. globosa* with *B. globosa* having a lower gametophytic genome size than *B. davidii*. It is possible that the F1 seedlings inherited relatively more *B. globosa* DNA than *B. davidii* DNA of the *B. x weyeriana* parent, resulting in lower genome sizes for the F1 and F2 hybrids. The variation in genome size among the tested F1 and F2 hybrids might be explained by chromosome rearrangements occurring after interspecific hybridisation between *B. davidii* and *B. x weyeriana*. Genome sizes and ploidy level of *B. davidii* x *B. lindleyana* and *B. davidii* x *B. globosa* F1 seedlings was higher than expected based on the parent plants, indicating the occurrence of unreduced gametes in *B. lindleyana* and *B. globosa* (see 5.4.3).

5.4.3 Occurrence of unreduced gametes

With *B. davidii* having 76 chromosomes and *B. lindleyana* having 38 chromosomes, it was presumed that the F1 hybrids of *B. davidii* x *B. lindleyana* would be triploid with 57 chromosomes. But chromosome counting revealed 76 chromosomes for all seedlings. Moreover, the genome size of these F1 seedlings was higher than the expected genome size based on the parent plants. Also ploidy level and the genome sizes of *B. davidii* x *B. globosa* were higher than was foreseen. By this it can be suggested that *B. lindleyana* and *B. globosa* both produce unreduced gametes.

When *B. lindleyana*, producing unreduced gametes with a $1C = 2Cx$ genome size of 1.88 pg, is crossed with *B. davidii*, producing normal gametes with a $1C = 2Cx$ nuclear DNA content of 1.46 pg, the F1 hybrids are expected to have a $1C = 2Cx$ nuclear DNA content of 1.67 pg, which is similar to the genome size measured in this study for most F1 seedlings. The suggestion of *B. lindleyana* producing unreduced gametes was also made by Elliott *et al.* (2004) who made crosses with *B. davidii* and *B. lindleyana*. In their experiments the hybrid was unexpectedly fertile. They assumed that the hybrids were tetraploid (instead of triploid) and thus the result of a cross with $2n$ -gametes producing *B. lindleyana*. Also with our AFLP analysis it was very remarkable that the hybrids *B. davidii* x *B. lindleyana* inherited a lot (between 79% and 95%) of the *B. lindleyana* markers (see 5.3.3.3). This amplifies the possibility for *B. lindleyana* of producing unreduced gametes.

Normally, seedlings of *B. davidii* x *B. globosa* are expected to have a genome size of approximately $1.25 \text{ pg} \cdot 1C^{-1}$. If *B. globosa* would produce unreduced gametes ($1C = 2Cx$ DNA content of 2.24 pg), F1 seedlings *B. davidii* x *B. globosa* would have a genome size of $1.85 \text{ pg} \cdot 1C^{-1}$. However, a genome size of $1.56 \text{ pg} \cdot 1C^{-1}$ (average) was measured for the F1 seedlings. So possibly, *B. globosa* produces unreduced gametes, but also other genome reorganisations after interspecific hybridisation could have occurred. The fact that *B. globosa* produces unreduced gametes was already suggested before (Chapter 3). *B. x weyeriana* is originating from a cross *B. globosa* x *B. davidii* and has also a higher genome size than expected. However in this case *B. globosa* was used as female parent while in our cross *B. globosa* was used as male parent. So probably *B. globosa* produces both $2n$ pollen and $2n$ egg cells. There are examples in which one and the same genotype produces both $2n$ eggs and $2n$ pollen simultaneously, but such cases are rare (Ramanna and Jacobsen 2003). For example both $2n$ eggs and $2n$ pollen have been reported to occur in hybrids of emmer wheat x *Aegilops squarrosa* (Fukuda and Sakamoto 1992), *Triticum turgidum* x *Secale cereale* and *Triticum turgidum* x *Aegilops squarrosa* (Xu and Joppa 1995) and *Alstroemeria* interspecific hybrids (Ramanna and Jacobsen 2003) among others. But commonly plants produce either only $2n$ eggs or $2n$ pollen. In Chapter 3 it was also observed that pollen of *B. globosa* had a very low germination capacity *in vitro*. It is not clear whether it concerned unreduced pollen or normal pollen. If *B. globosa* produces viable unreduced gametes (even in a small frequency), then it is possible to perform crosses with *B. davidii* on tetraploid level without polyploidisation of *B. globosa*, which is more advantageous for the creation of genetic variation.

Another hypothesis can be that *B. x weyeriana* and the F1 hybrids *B. davidii* x *B. globosa* are not true hybrids but (as a consequence of incompatibility and ploidy differences between the parent plants) are substitution lines with only one or a few chromosomes of *B. globosa* in a *B. davidii* genome, resulting in a slightly higher genome size than *B. davidii*. However, a high amount of *B. globosa* DNA has been detected in *B. x weyeriana* both by AFLP marker analysis (Chapter 3) and GISH (Chapter 6). The F1 hybrids *B. davidii* x *B. globosa* has not been tested yet by AFLP or GISH, because they are still small *in vitro* seedlings.

The fact that there is variation in the genome sizes of the F1 seedlings can indicate the occurrence of chromosome rearrangements after interspecific hybridisation between *B. davidii* and *B. lindleyana* and between *B. davidii* and *B. globosa*.

Also the production of unreduced gametes in F1 and F2 hybrids of *Hibiscus syriacus* x *H. paramutabilis* was shown. The traditional approach to estimate unreduced pollen production is based on pollen morphology. For the *Hibiscus syriacus* parent plants, pollen size and ploidy level were correlated. *Hibiscus syriacus* 'Oiseau Bleu', tetraploid, had smaller pollen than *Hibiscus syriacus* 'Red Heart CV', an octoploid. However, for *Hibiscus paramutabilis* and the F1 hybrids *H. syriacus* x *H. paramutabilis*, pollen size and ploidy level of the pollen did not show any correlation. The observed reduced viability of the smaller pollen might indicate that the smaller sized pollen observed in *H. paramutabilis* and the F1 hybrids *H. syriacus* x *H. paramutabilis* was degenerated pollen, while the larger pollen was the normal reduced pollen. Two F2 hybrids produced a small fraction (< 1%) of pollen with an outranging diameter. This large pollen fraction possibly concurred with the 2n gametes detected by flow cytometry. It is known that variation in pollen size is not necessarily ploidy-based (Dajoz *et al.*, 1995). Also Pichot and Maâtaoui (2000) and Sugiura *et al.* (2000) showed that ploidy-morphology associations tend to be imperfect, with discrepancies between frequency estimates based on flow cytometry and morphology.

Flow cytometry proved to be a powerful and fast tool to examine the presence of viable unreduced pollen in *Hibiscus*. No unreduced pollen could be detected in samples of the *H. syriacus* and *H. paramutabilis* parent plants and of the F1 hybrids. Flow cytometric analysis resulted in only 1 single peak in the histogram, derived from pollen nuclei with equal DNA content as somatic leaf tissue. This signal is derived from the generative nucleus, which is in the post replication stage (G2) of cell division and gives rise to a 2C peak. The generative cell finally divides into 2 sperm nuclei, but the time of division is dependent on the plant family (Russel, 1991). Although in several other genera, 2 peaks from both generative and vegetative nuclei are observed, the occurrence of a single 2C peak was also observed in roses by Roberts (2007). It remains unclear what happens with the signal of the vegetative nuclei, but it is suggested that these nuclei are more easily damaged during rigorous chopping because of their larger size (Roberts 2007; Jacob *et al.* 2001) or that they break down as soon as they are released from the pollen since they are programmed to degenerate (Raven *et al.* 1992). However, in 2 of the tested F2 hybrids the production of unreduced gametes (between 8% and 10%) could be detected. In these plants a second peak derived from the generative nuclei of unreduced gametes was observed. Also for some other genera there are several ploidy-based studies on pollen development and unreduced pollen production with flow cytometry (Van Tuyl

et al. 1989; Bino *et al.* 1990; Pichot and Maâtaoui 2000; Sugiura *et al.* 2000; Jacob *et al.* 2001; Pan *et al.* 2004; Roberts 2007).

Presence of unreduced pollen can also be demonstrated by abnormalities during meiosis, mainly diads (two 2n pollen) or triads (one 2n pollen and two n pollen). For the F1 hybrids and the parent plants, (almost) no diads could be observed. A low fraction of triads (about 6%) was observed in *H. syriacus* 'Red Heart CV' and in 1 F1 hybrid *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis*, but the formation of unreduced gametes could not be proven by the other techniques. This might indicate that the 2n microspore of the triads degenerated before the final pollen was formed. The occurrence of a very small fraction of polyads in the F1 hybrids could be explained by aberrant chromosome pairing at meiosis which in turn resulted in unbalanced chromosome segregation and the formation of micronuclei, typically seen in interspecific hybrids. As unbalanced chromosome segregation leads to unviable pollen production (Bretagnolle and Thompson 1995), these polyads contribute to the production of small unviable pollen in hybrids. For the F2 hybrids on the contrary, more than 6% of diads could be observed in 2 of the analysed plants, which is a clear proof of the presence of unreduced pollen in these genotypes.

Both the parent plants *H. syriacus* 'Oiseau Bleu' and *H. paramutabilis* and their F1 hybrids were tetraploid with a genome size varying between 3.32 pg.1C⁻¹ and 2.48 pg.1C⁻¹, while all the F2 hybrids were hexaploid with a genome size between 3.49 pg.1C⁻¹ and 3.59 pg.1C⁻¹. Because none of the aforementioned approaches (pollen size measurements, flow cytometry on the pollen and study of meiosis of the pollen), revealed unreduced pollen production of the F1 hybrids, it should be concluded that the F1 hybrids produce unreduced egg cells.

5.5 Conclusion

The possible occurrence of pre- and postzygotic barriers was analysed for interspecific hybridisation within woody ornamentals using 3 different 'case-study-plants', *Hydrangea*, *Hibiscus* and *Buddleja*. Many interspecific crosses were performed within the 3 genera. It was sometimes difficult to draw conclusions of the huge amount of data that was thereby obtained since the optimal fertilisation period and conditions of the plants were not always well evaluated and not all possible cross combinations could be made. However, some remarkable trends could be figured out.

No prezygotic barriers were observed after crosses within the 3 genera. However, interspecific crosses were hampered by several postzygotic barriers. For all 3 genera a major barrier was the abortion of the fruits in an early stage of their development. An *in vitro* embryo rescue step enhanced the efficiency of the interspecific hybridisation within the genera. Germination of the *in vitro* initiated seeds/embryos was very good for *Hibiscus* and *Buddleja*. For *Hibiscus* also a lot of F1 seedlings were obtained without an *in vitro* step. For *Hydrangea* there was a lack of germination of the immature seeds. Almost no well developed seeds were found in the resulting

fruits after interspecific *Hydrangea* crosses. Albinism was observed for F1 seedlings of *Hydrangea* and *Hibiscus*.

For *Hydrangea* only true hybrids were obtained from crosses between *H. macrophylla* and *H. serrata*, 2 species within the same subsection. For *Hibiscus* hybrids were generated from crosses between *H. syriacus*, *H. sinosyriacus* and *H. paramutabilis*, while for *Buddleja* different F1 progenies were obtained after interspecific crosses.

Morphology of F1 seedlings can be very unpredictable e.g. the leaf polymorphisms in the *Hibiscus* hybrids, which were caused by genetic modifications after interspecific hybridisation. Therefore, for the characterisation of the F1 seedlings, a combination of different techniques is needed to clearly determine the hybrid nature. AFLP analysis has proven to be useful to determine the hybrid character of most obtained F1 seedlings. When parental species differ enough in genome size and ploidy level, also genome size measurements and chromosome counting was very efficient to characterise F1 hybrids. Only obtained F1 seedlings between *H. paniculata* x *H. macrophylla* and *H. paniculata* x *H. aspera* would not unambiguously be determined as true hybrids. GISH should be applied to clarify whether or not they are hybrids.

Although the F1 seedlings obtained after interspecific crosses within *Buddleja* and *Hibiscus* were not very fertile, an F2 and BC1 progeny could be generated for *H. syriacus* x *H. paramutabilis*, *B. davidii* x *B. x weyeriana*, *B. x weyeriana* x *B. davidii* and *B. davidii* x *B. lindleyana*. Some F1 and F2 hybrids are selected and multiplied for further evaluation in the field.

By using 2n gametes considerable genetic variation can be generated which opens new possibilities in breeding work. To use 2n gametes in commercial breeding programs, an efficient selection of valuable genotypes producing unreduced gametes should be used. In this study evidence for the occurrence of unreduced gametes was found for *B. globosa*, *B. lindleyana* and the F1 hybrids *H. syriacus* x *H. paramutabilis*. More investigation should be done to efficiently use these 2n gametes in the further breeding program of *Buddleja* and *Hibiscus*.

CHAPTER 6

GISH AS A TOOL TO CHARACTERISE HYBRIDS WITH SMALL GENOMES AND CHROMOSOMES*

6.1 Introduction

Interspecific hybridisation is an important strategy in the breeding of ornamentals. Effective identification of parental genomes in hybrids is essential for determination of genomic origin of a chromosome or chromosomal segments and thus for a better understanding of the selected breeding lines. Nowadays molecular markers are widely used for monitoring alien genetic material in a plant genome (Garcia *et al.* 1995; Fedak 1999; Yamagishi *et al.* 2002). However, a large number of markers that represent different chromosome regions should be used to analyse a complete chromosome. Moreover, a molecular marker approach often does not provide an answer whether one or multiple copies of a particular gene/chromosome are present in a plant. In this respect, GISH is a powerful tool to differentiate chromosomes of different parental genomes in allopolyploid species, interspecific hybrids and their backcross progenies as well as to trace intergenomic chromosome rearrangements (Schwarzacher *et al.* 1989; Parokony *et al.* 1997; Takashi *et al.* 1997). GISH is mostly used for plant species with large-sized chromosomes (Karlov *et al.* 1999; Khrustaleva and Kik 2000; Marasek *et al.* 2006). GISH often failed to decorate entire chromosomes of plants with very small genomes (Lim *et al.* 1998; D'Hont *et al.* 2000; Brutovska *et al.* 2000). It was suggested that the critical genome size, below which a uniform labelling of chromosomes by GISH is difficult to achieve, might be $\sim 0.6 \text{ pg.1C}^{-1}$ (Raina and Rani 2001). Ali *et al.* (2004) reported on an adaptation of the GISH technique in *Arabidopsis*, characterised by an extremely small genome. By using a high probe concentration, they obtained detectable hybridisation signals on entire chromosomes.

Woody ornamentals in general and *Buddleja* and *Hibiscus* in particular are commonly characterised by small genomes and a high number of small-sized chromosomes (Chapter 3, Chapter 5).

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Van Laere K, Khrustaleva L, Van Huylenbroeck J, Van Bockstaele E (2008) GISH as a tool to characterise hybrids with small genomes and chromosomes. In preparation

In this chapter the GISH technique is optimised for application in woody ornamentals in order to be able to monitor introgression of alien DNA in hybrids resulting from interspecific breeding work. A detailed study was performed using GISH (i) to prove the hybrid origin of *B. x weyeriana*, (ii) to analyse parental genome composition in F1 and F2 hybrids of *B. davidii* x *B. x weyeriana* and in F1 and F2 hybrids of *H. syriacus* x *H. paramutabilis*, (iii) to determine whether homoeologous recombination has occurred in the F2 hybrid plants, (iv) to get evidence of 2n gamete formation in *B. globosa*.

6.2 Specific materials and methods

6.2.1 Plant Material

For *Buddleja* the hybrid cultivar *B. x weyeriana* ‘Sungold’, 1 F1 (nansun-1, Chapter 5) and 1 F2 (nansun-nansun-1, Chapter 5) hybrids *B. davidii* ‘Nanho Purple’ x *B. x weyeriana* ‘Sungold’ were used. For *Hibiscus* 1 F1 (oispar-1, Chapter 5) and 1 F2 (oispar-oispar-1, Chapter 5) hybrids between *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis* were analysed.

6.2.2 Genomic *in situ* hybridisation and detection

Biotinylated genomic DNA of *B. globosa* and *H. syriacus* and digoxigenated genomic DNA of *H. syriacus* was used as a probe (see 2.6.5). Genomic DNA of *B. davidii* and *H. paramutabilis* was used as blocking DNA (see 2.6.5).

DNA denaturation and *in situ* hybridisation steps were performed according to Schwarzacher and Leitch (1993), Leitch and Heslop-Harrison (1994) and Schwarzacher and Heslop-Harrison (1994). The hybridisation mix contained 50% (v/v) deionised formamide, 10% (w/v) dextran sulphate, 2 x SSC, 0.25% (w/v) SDS, probe DNA and block DNA. For *B. x weyeriana* 25 ng probe DNA and 3 µg block DNA was used. For the F1 hybrid *B. davidii* x *B. x weyeriana* 25 ng probe DNA and 2 µg block DNA was added, while for the F2 hybrid this was 25 ng probe DNA and 4.5 µg block DNA. For the F1 hybrid of *Hibiscus* 25 ng probe DNA and 4.5 µg block DNA was used. For the F2 hybrid *H. syriacus* x *H. paramutabilis* different experiments were tried: 75 ng probe DNA (biotin) - 3.85 µg block DNA, 50 ng probe DNA (biotin) - 3.85 µg block DNA, 35 ng probe DNA (biotin) - 4.40 µg block DNA, 35 ng probe DNA (biotin) - 1.5 µg block DNA and 25 ng probe DNA (digoxigenin) - 4.5 µg block DNA. The hybridisation mix was denatured for 10 min at 70°C and placed on ice for 5 min. After the hybridisation mixture was added to the slides a 5-min denaturation step at 80°C was carried out.

For the F2 hybrid *H. syriacus* x *H. paramutabilis* also pre-annealing was tried with 75 ng or 40 ng probe DNA (biotin) and 3.85 µg block DNA. The hybridisation mix was boiled for 5 minutes and incubated for 1 h at 58°C. The slides were denatured in pre-heated formamide solution (50% formamide in 2x SSC) at 80°C for 2 min. The hybridisation mix was added to pre-denatured slides.

Hybridisation was done overnight at 37°C in a humid chamber. Subsequently, the slides were washed in 2 x SSC for 15 min at room temperature, followed by 0.1 x SSC for 30 min at 48°C (80% stringency) and 2 x SSC for 15 min at room temperature. Biotin-labelled DNA was detected with CY3-conjugated streptavidin and amplified with biotinylated goat-antistreptavidin. Digoxigenin-labelled DNA was detected by anti-Dig FITC (sheep) and anti-sheep-FITC (rabbit). Finally, the chromosomes were counterstained with 1 µg.mL⁻¹ DAPI and analysed by fluorescence microscope (see 2.6.5).

Chromosome analysis and analysis of the hybridisation signals was carried out on 5 to 10 well-spread metaphases of each genotype. Chromosomes were characterised on the basis of chromosome length, arm length, centromeric index (Levan *et al.* 1964) and hybridisation signal (see 2.6.5).

6.3 Results

6.3.1 GISH on *Buddleja* hybrids

6.3.1.1 Parental genome composition of *B. x weyeriana* 'Sungold'

According to the literature *B. x weyeriana* 'Sungold' is a mutant from an F2 selection of *B. globosa* x *B. davidii* (Van De Weyer 1920; De Vogel 1967). Therefore karyotype analysis and GISH study on this crossing parent was carried out first.

Analysis of the chromosome profile (only DAPI stained metaphases) of *B. x weyeriana* 'Sungold' revealed a total chromosome complement of 41.41 ± 0.33 µm, with a length of the largest chromosome of 3.16 ± 0.44 µm and the shortest chromosome 1.42 ± 0.29 µm (Figure 6.1). *B. x weyeriana* had 12 metacentric and 7 submetacentric chromosomes (Figure 6.1), with an overall condensation index of 0.021 pg.µm⁻¹.

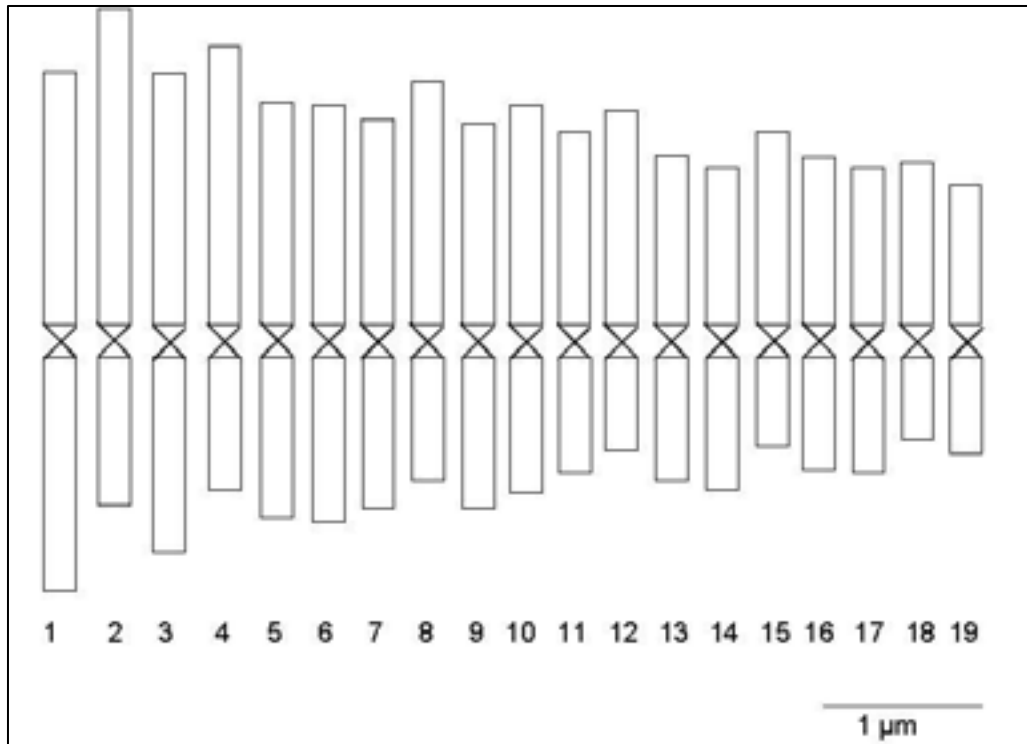


Figure 6.1: Idiogram of *B. x weyeriana* 'Sungold'. Chromosomes 1, 3, 5, 6, 7, 9, 10, 13, 14, 16, 17 and 19 are metacentric. The other chromosomes are submetacentric (bar = 1 μ m).

Figure 6.2 shows the result of GISH using total genomic DNA of *B. globosa* as a probe hybridised on *B. x weyeriana* 'Sungold'. The 2 parental genomes could be clearly identified. GISH revealed 36 chromosomes inherited from *B. davidii* and 28 chromosomes from *B. globosa*. The other 12 chromosomes were recombinant chromosomes between *B. globosa* and *B. davidii*, with the position of 5 recombination points located distally on the long arm, 2 recombination points distally on the short arm, 2 on the distal and proximal positions on the long arm and the last 3 recombination points interstitial on the long arm. Hence, crossing-over events occurred randomly in distal, interstitial and proximal regions of chromosomal arms.

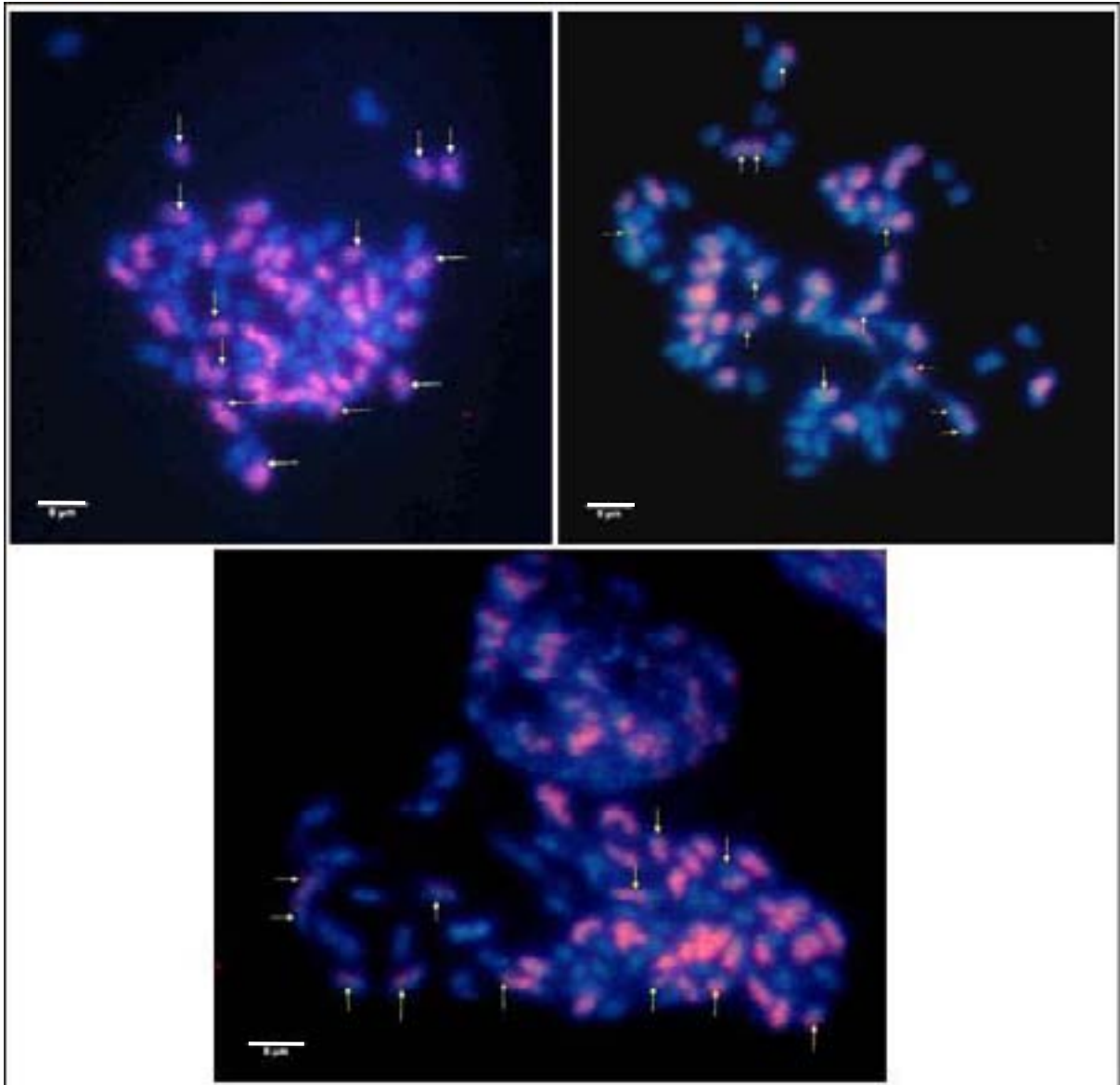


Figure 6.2: Discrimination of chromosomes originating from *B. globosa* (red fluorescence) and *B. davidii* 'Nanho Purple' (blue fluorescence) in the genome of *B. x weyeriana* 'Sungold' using GISH. The recombinant chromosomes are indicated by an arrow (bar = 5 µm).

6.3.1.2 Parental genome composition of F1 and F2 hybrids of *B. davidii* x *B. x weyeriana*

For the F1 hybrid (nansun-1) also an idiogram was constructed (Figure 6.3). The F1 hybrid had a total chromosome complement of $50.03 \pm 0.16 \mu\text{m}$. The largest chromosome had a length of $3.49 \pm 0.34 \mu\text{m}$ and the shortest chromosome was $2.00 \pm 0.27 \mu\text{m}$. The chromosome profile contained 10 metacentric and 9 submetacentric chromosomes, with a condensation of $0.013 \text{ pg} \cdot \mu\text{m}^{-1}$.

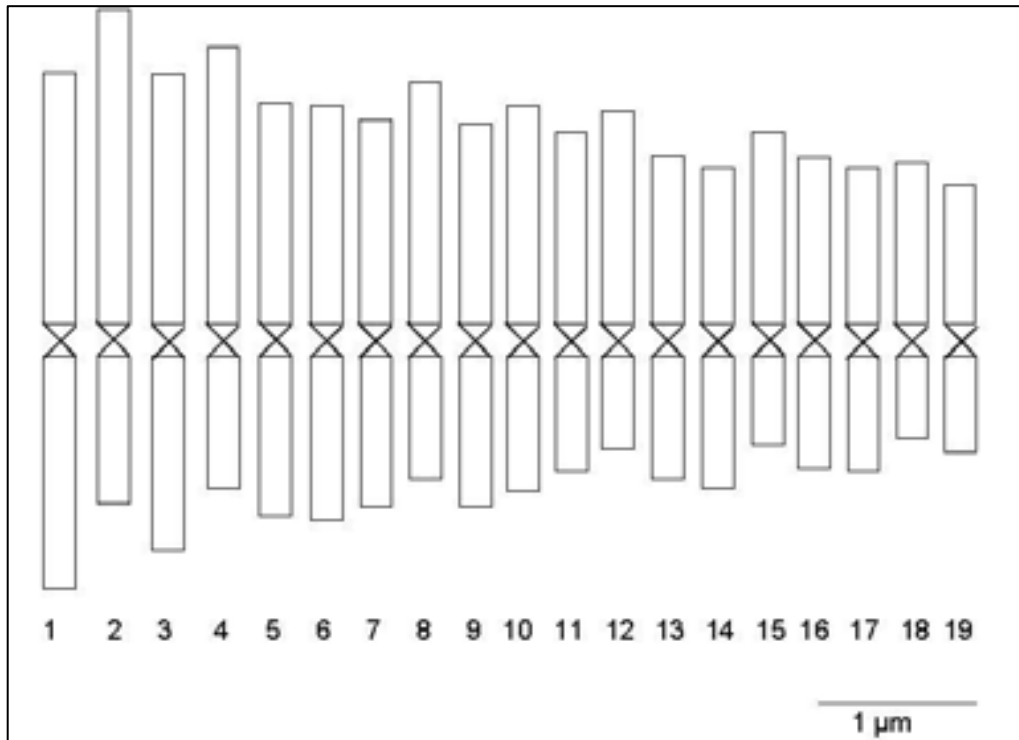


Figure 6.3: Idiogram of *B. davidii* 'Nanho Purple' x *B. x weyeriana*. Chromosomes 1, 3, 5, 6, 7, 9, 13, 14, 17 and 19 are metacentric. The other chromosomes are submetacentric (bar = 1 µm).

For GISH analysis on the F1 hybrid nansun-1 *B. globosa* was used as a probe because *B. x weyeriana* is a hybrid between *B. globosa* and *B. davidii* as was proved by GISH analysis of *B. x weyeriana* (see 6.3.1.1). Therefore, when using genomic DNA of *B. x weyeriana* as a probe too much cross hybridisation was expected.

The 2 different genomes could be clearly distinguished. GISH on the F1 hybrid *B. davidii* x *B. x weyeriana* 'Sungold' revealed 16 chromosomes completely belonging to *B. davidii*, all the other chromosomes contained recombination points with *B. globosa* DNA. On 4 recombinant chromosomes a large hybridisation signal (= *B. globosa* DNA) could be distinguished, on the other 56 recombinant chromosomes smaller places of introgression of *B. globosa* DNA was observed. Introgression was at random on the chromosomes (Figure 6.4).

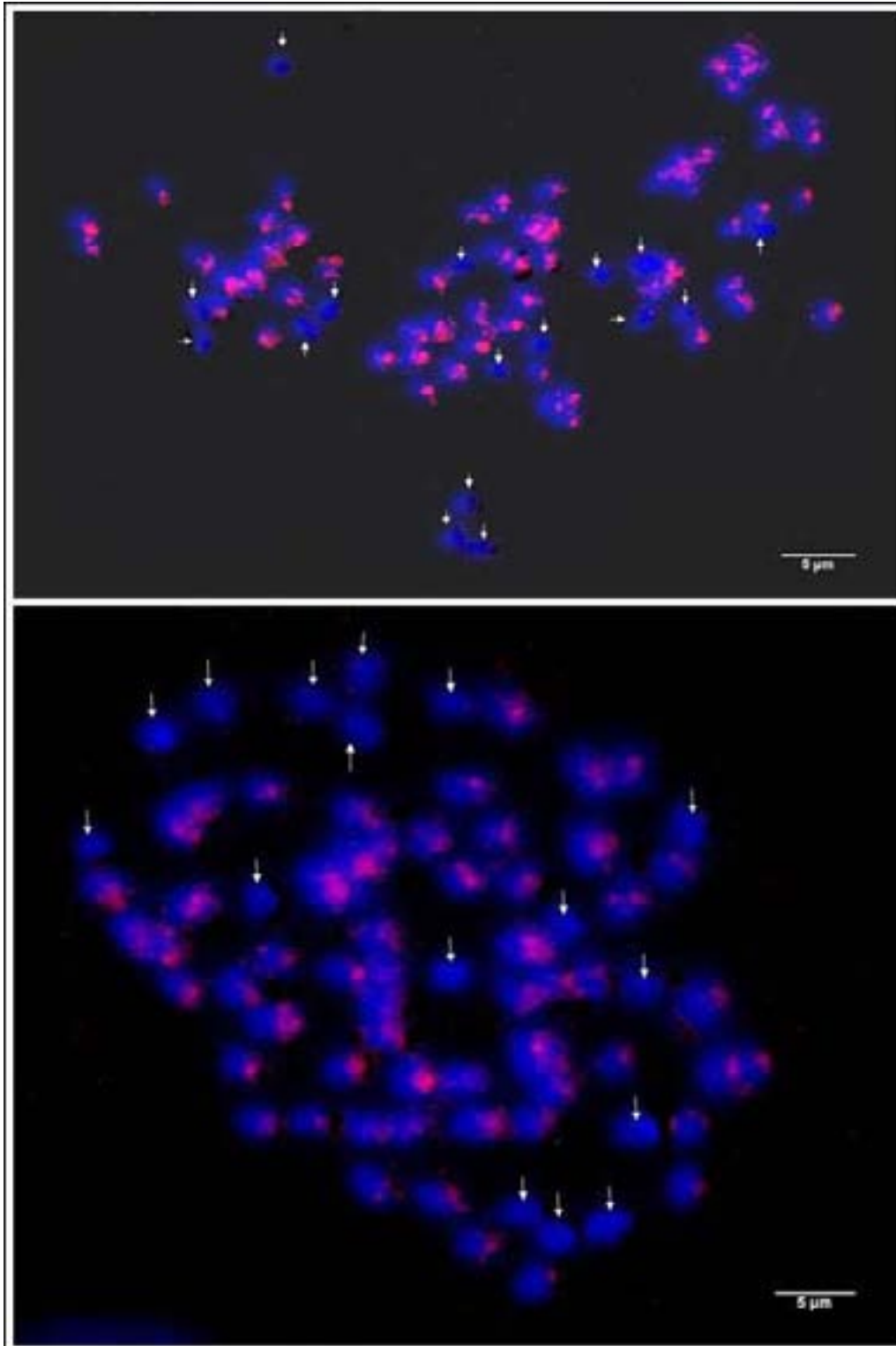


Figure 6.4: Discrimination of chromosomes originating from *B. globosa* (red fluorescence) and *B. davidii* 'Nanho Purple' (blue fluorescence) in the genome of the F1 hybrid *B. davidii* 'Nanho Purple' x *B. x weyeriana* 'Sungold' (nansun-1) using GISH. The chromosomes completely belonging to *B. davidii* are indicated by an arrow (bar = 5 µm).

In the analysed F2 hybrid *B. davidii* 'Nanho Purple' x *B. x weyeriana* (nansun-nansun-1), only 10 chromosomes could be distinguished completely belonging to *B. davidii*. All the other chromosomes have at random introgression of *B. globosa* DNA (Figure 6.5).

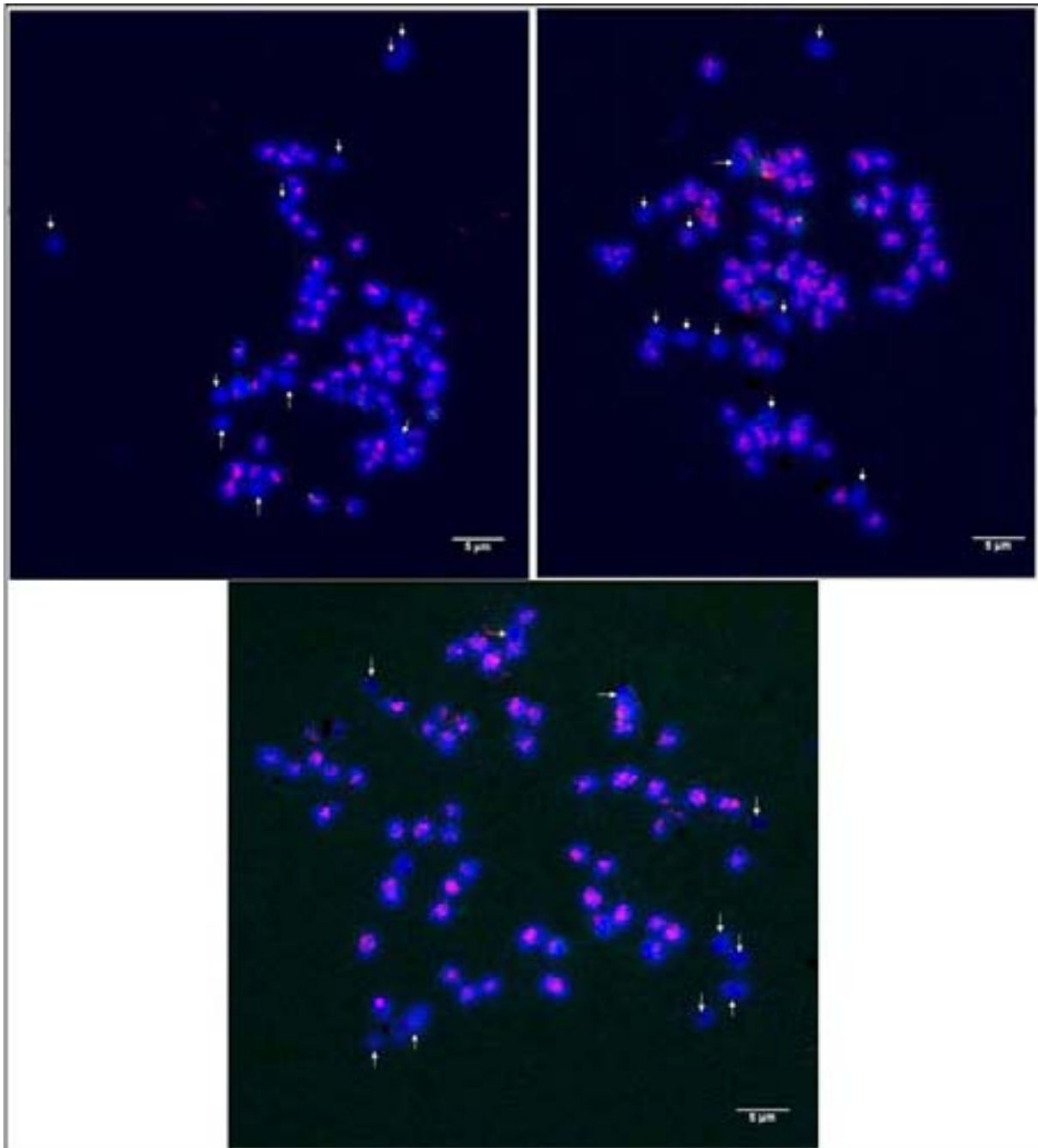


Figure 6.5: Discrimination of chromosomes originating from *B. globosa* (red fluorescence) and *B. davidii* 'Nanho Purple' (blue fluorescence) in the genome of the F2 hybrid *B. davidii* 'Nanho Purple' x *B. x weyeriana* 'Sungold' (nansun-nansun-1) using GISH. The chromosomes completely belonging to *B. davidii* are indicated by an arrow (bar = 5 μm).

6.3.2 GISH on *Hibiscus* hybrids

6.3.2.1 Parental genome composition of F1 hybrid of *H. syriacus* x *H. paramutabilis*

As described in Table 5.11, the F1 hybrid *H. syriacus* x *H. paramutabilis* had 81 chromosomes. In Figure 6.6 the idiogram of the F1 seedlings is shown. The total chromosome complement is $69.79 \pm 0.31 \mu\text{m}$, with a length of the longest chromosome of $4.89 \pm 0.59 \mu\text{m}$ and the shortest chromosome $2.28 \pm 0.33 \mu\text{m}$. The idiogram contains 14 metacentric chromosomes, 6 submetacentric chromosomes and 1 unpaired (*H. paramutabilis*) chromosome.

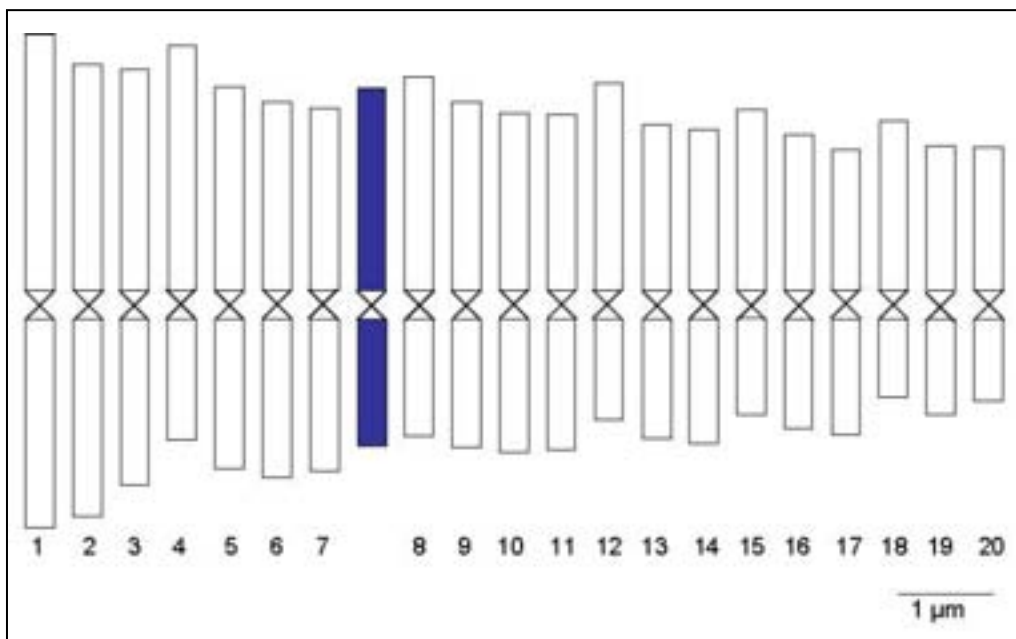


Figure 6.6: Idiogram of the F1 hybrid *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis*. Chromosomes 1, 2, 3, 5, 6, 7, 9, 10, 11, 13, 14, 16, 17 and 19 are metacentric, while chromosomes 4, 8, 12, 15, 18 and 20 are submetacentric. The unpaired (*H. paramutabilis*) chromosome is indicated in blue (bar = $1 \mu\text{m}$).

GISH analysis was performed on 1 F1 seedling of *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* (oispar-1) using genomic DNA of *H. syriacus* as probe DNA and genomic DNA of *H. paramutabilis* as block DNA. The two parental genomes could be clearly identified. GISH analysis revealed, as expected, 40 chromosomes of *H. syriacus* and 41 chromosomes of *H. paramutabilis* (see Figure 6.7).

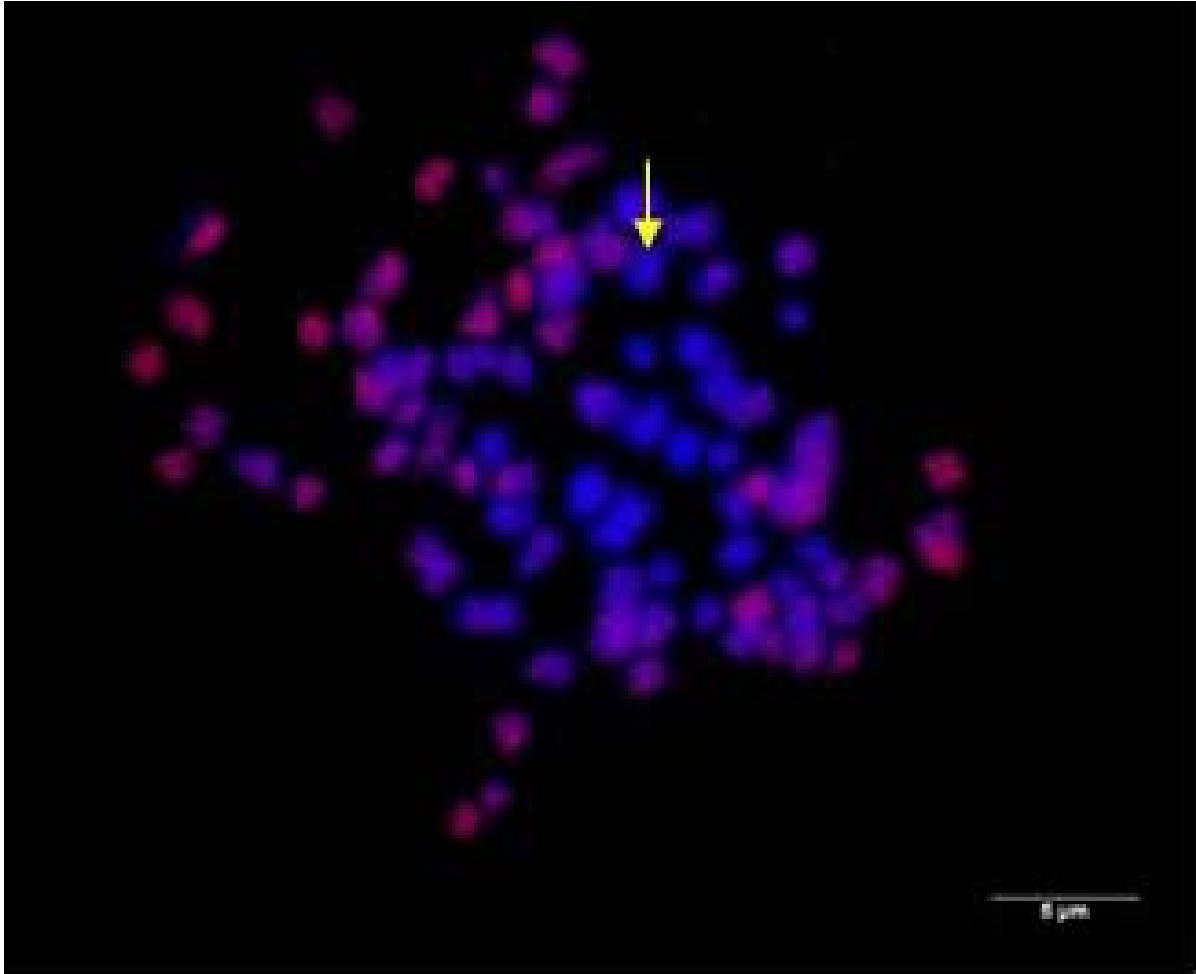


Figure 6.7: Discrimination of chromosomes originating from *H. syriacus* ‘Oiseau Bleu’ (red fluorescence) and *H. paramutabilis* (blue fluorescence) in the genome of the F1 hybrid *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis* (oispar-1) by use of GISH. The unpaired chromosome of *H. paramutabilis* is indicated by an arrow (bar = 5 μ m).

6.3.2.2 Parental genome composition of F2 hybrid of *H. syriacus* x *H. paramutabilis*

Finally, GISH was performed on 1 F2 seedling *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis* (oispar-oispar-1). It was very difficult to distinguish the parental genomes in the F2 hybrid and several experimental set-ups were carried out.

In a first experiment different ratios probe/block DNA were tried. Figure 6.8 shows the results of GISH using *H. syriacus* as a probe, labelled with biotin hybridised on F2 *H. syriacus* ‘Oiseau Bleu’ x *H. paramutabilis* hybrid. When using 75 ng probe and 3.85 μ g block DNA, some chromosomes were distinguished completely belonging to *H. syriacus* and some completely belonging to *H. paramutabilis*. But on several chromosomes cross hybridisation of probe DNA to homologous DNA sequences of both parents frequently took place. This made it difficult to distinguish between recombination and cross hybridisation.

By trying to use different combinations of amounts of probe DNA and block DNA, the results were still not convincing enough to analyse (Figure 6.8). It was difficult to distinguish

between signal arising from the *H. syriacus* chromosome segment and cross hybridisation signal.

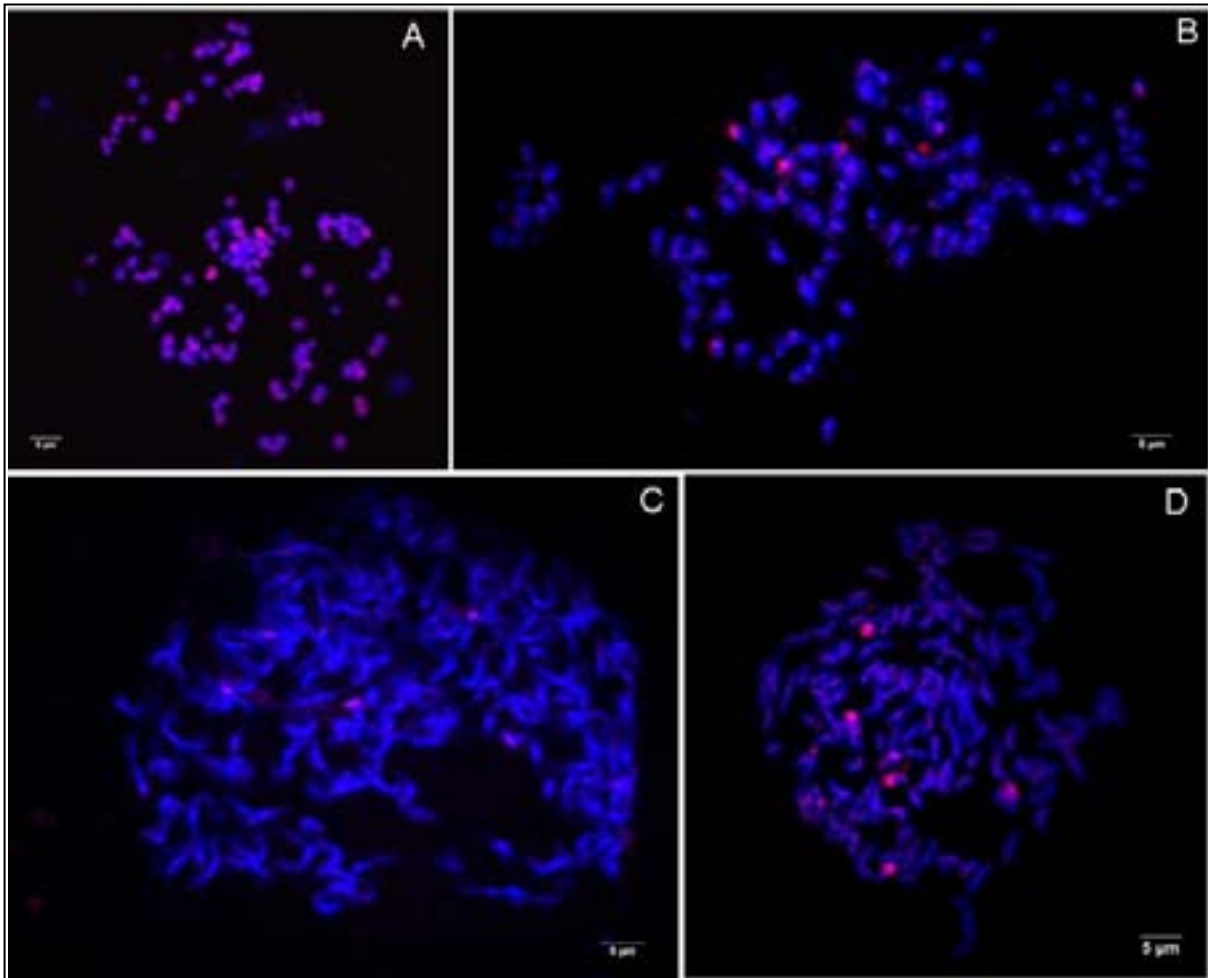


Figure 6.8: Discrimination of chromosomes originating from *H. syriacus* 'Oiseau Bleu' (red fluorescence) and *H. paramutabilis* (blue fluorescence) in the genome of the F2 hybrid *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* (oispar-oispar-1) (bar = 5 µm) by use of GISH. (A) use of 75 ng probe DNA (*H. syriacus*) and 3.85 µg block DNA, (B) use of 50 ng probe DNA (*H. syriacus*) and 3.85 µg block DNA, (C) use of 35 ng probe DNA (*H. syriacus*) and 4.40 µg block DNA, (D) use of 35 ng probe DNA (*H. syriacus*) and 1.50 µg block DNA.

In a second experiment pre-annealing of the probe was performed before hybridisation. The use of pre-annealing reduced the cross hybridisation. However, a lot of the signal was lost and almost no *H. syriacus* DNA was detectable. When using 75 ng probe, 7 recombinant chromosomes could be distinguished, with recombination points distal or interstitial on the long arm of the chromosomes. When using 40 ng probe, 10 recombinant chromosomes were determined, with recombination points also distal or interstitial on the long arm of the chromosomes. The results of the GISH experiments using pre-annealing of probe and block DNA are shown in Figure 6.9.

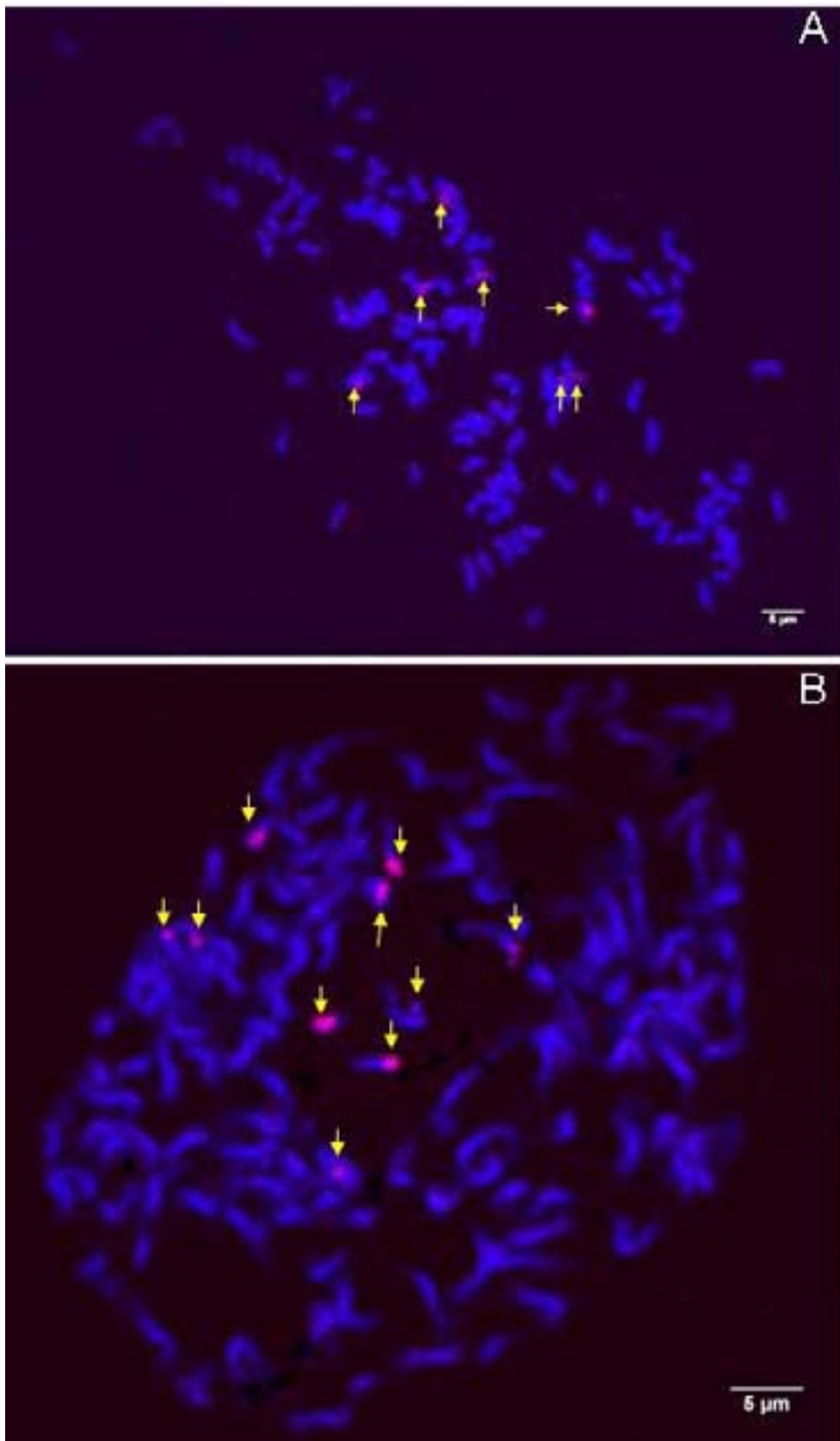


Figure 6.9: Discrimination of chromosomes originating from *H. syriacus* 'Oiseau Bleu' (red fluorescence) and *H. paramutabilis* (blue fluorescence) in the genome of the F2 hybrid *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* (oispar-oispar-1) using GISH (bar = 5 μm). (A) use of pre-annealed 75 ng probe DNA (*H. syriacus*) and 3.85 μg block DNA, (B) use of pre-annealed 40 ng probe DNA (*H. syriacus*) and 3.85 μg block DNA. Recombinant chromosomes are indicated by an arrow.

A third experiment, labelling the *H. syriacus* 'Oiseau Bleu' with digoxigenin (instead of biotin), yielded more reliable results. It was possible to distinguish 31 chromosomes belonging to *H. syriacus* and 64 chromosomes belonging to *H. paramutabilis*. The other 25 chromosomes were recombinant chromosomes with recombination points located at random on the chromosomes (Figure 6.10).

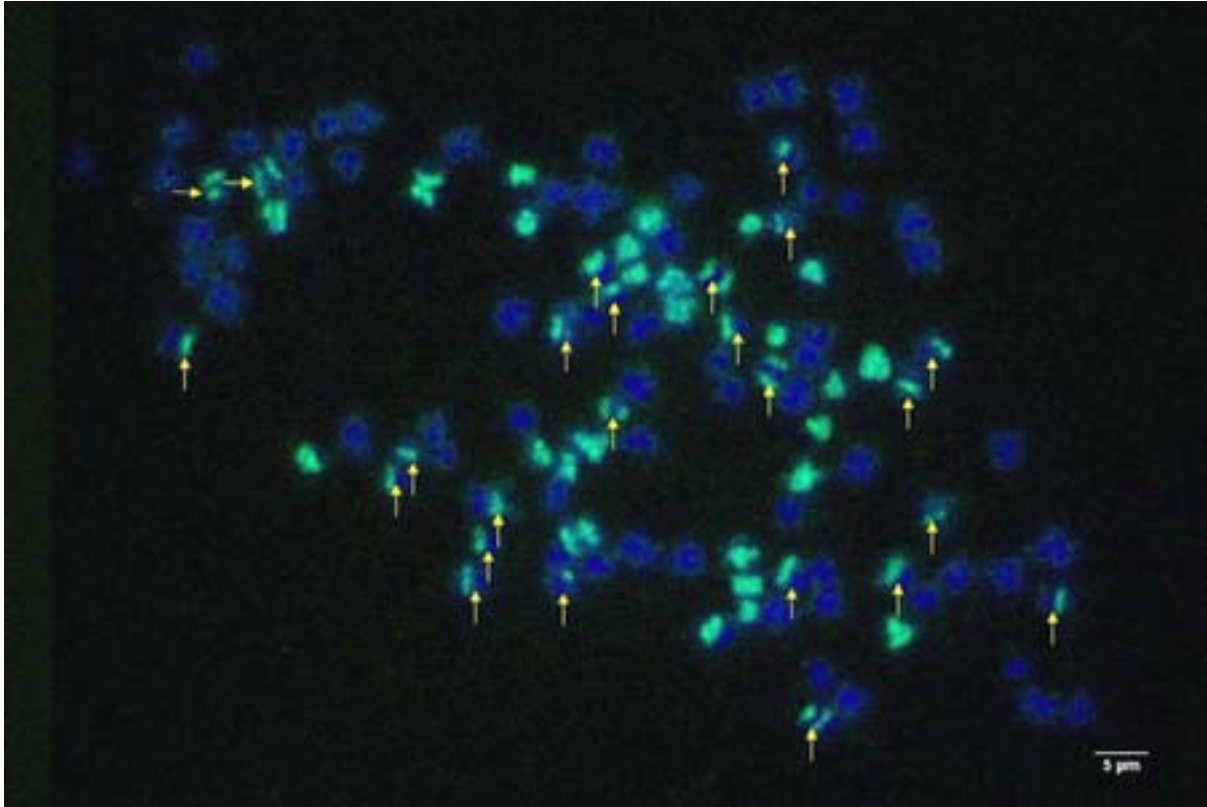


Figure 6.10: Discrimination of chromosomes originated from *H. syriacus* 'Oiseau Bleu' (green fluorescence) and *H. paramutabilis* (blue fluorescence) in the genome of the F2 hybrid *H. syriacus* 'Oiseau Bleu' x *H. paramutabilis* (oispar-oispar-1) using GISH. Recombinant chromosomes are indicated by an arrow (bar = 5 μm).

6.4 Discussion

6.4.1 GISH technique adaptation to woody ornamentals

In this study the standard GISH protocol was adapted to the woody ornamentals *Buddleja* and *Hibiscus*, characterised by small genomes and/or a high number of small chromosomes. Diploid and tetraploid *Buddleja* species ($x = 19$) had a genome size between 0.93 pg.1C^{-1} and 1.48 pg.1C^{-1} (Table 3.4) and chromosome lengths between $1.42 \mu\text{m}$ and $3.49 \mu\text{m}$ were measured. Tetraploid *Hibiscus* ($x = 20$) had genome sizes between 2.13 pg.1C^{-1} and 2.48

pg. $1C^{-1}$ (Table 3.4) and chromosomes were between 2.28 μm and 4.89 μm . For woody ornamentals, successful GISH experiments were only reported before in *Quercus* (Zoldo 2008). Moreover, in the past, GISH often failed to decorate entire chromosomes of plants with very small genomes. The level of differentiation is dependent on the signal strength from labelled probe DNA. Sometimes cross hybridisation between the two genomes makes it very critical to distinguish between the chromosomes of these two genomes. Therefore, detection of recombination and pairing can be extremely difficult if not impossible because of small genomes and small chromosomes (Haider *et al.* 2002). It was suggested that the critical genome size, below which a uniform labelling of chromosomes by GISH is difficult to achieve, might be ~ 0.6 pg. $1C^{-1}$ (Raina and Rani 2001). Within the genera *Brassica* (Fahleson *et al.* 1997), *Coffea* (Raina *et al.* 1998), *Musa* (D'Hont *et al.* 2000), *Hypericum* (Brutovska *et al.* 2000) and in the woody ornamental *Rubus* (Lim *et al.* 1998) detectable GISH signals were mainly restricted to pericentromeric heterochromatin blocks and sometimes to nucleolus organisers (NORs) where repetitive DNA sequences are clustered (Ali *et al.* 2004). Genome sizes of these genera are between 0.15 pg. $1C^{-1}$ and 1.58 pg. $1C^{-1}$ and basic chromosome numbers are $x = 8$ to $x = 11$ (Dickson *et al.* 1992; Cros *et al.* 1994; Nagpal *et al.* 1996; Asif *et al.* 2001; Hanson *et al.* 2002). Also in *Lycopersicon*, another genus with a small genome between 0.85 pg. $1C^{-1}$ and 1.23 pg. $1C^{-1}$ and basic chromosome number of $x = 12$ (Arumuganathan and Earle 1991), there was a lack of resolution of recombination segments and a lack of clear differentiation between different genomes (Haider *et al.* 2002).

The sensitivity and efficiency of GISH depend on several variables, for which optimal conditions must be determined: (i) the probe construction and hybridisation conditions, (ii) the type and efficiency of probe labelling, (iii) the tissue preparation and (iv) the method used for signal detection (Chevalier *et al.* 1997).

An important hybridisation condition is the ratio probe/block DNA. In our experiments ratios probe/block were not lower than 1/80. Usually, blocking DNA is used in excess ratios, ranging in final concentrations from 25-fold up to 100-fold the amount of that of the probe. In interspecific and intergeneric cereal hybrids and in *Crocus* cultivars, parental genomes could be distinguished by lower amounts of blocking DNA (Schwarzacher *et al.* 1989; Ørgaard *et al.* 1995). The genome size of cereals and *Crocus* is rather large (Bennett and Smith 1971; 1976; Olszewska and Osiecka 1982; Brandizzi *et al.* 1996; Vogel *et al.* 1999) and have high number of species specific repetitive DNA (Schwarzacher *et al.* 1989). It is known that GISH relies largely on the hybridisation of genome specific repetitive DNA sequences (Heslop-Harrison 2000). Genotypes with small genomes have a relatively small amount of repetitive DNA (Haider *et al.* 2002), which is not favourable for discriminating parental chromosomes by GISH. Therefore, as a consequence of the small genome size of *Buddleja* and *Hibiscus* and lack of repetitive DNA, higher probe/block ratios should be used. In our study on *Buddleja* hybrids, standard ratios probe (*B. globosa*)/block (*B. davidii*) DNA between 1/80 and 1/180 were used successfully (Figure 6.2 – 6.5).

For *Hibiscus* different ratios probe (*H. syriacus*)/block (*H. paramutabilis*) DNA between 1/40 and 1/125 were tested. Increasing the ratio between probe and block DNA did not result in

acceptable results. It was not possible to distinguish between real signal and cross hybridisation. Therefore pre-annealing was performed before hybridisation. The pre-annealing blocks out common DNA sequences between the different genomes, hence increasing species specificity of the probes (Ananthawat-Jonsson and Reader 1995). When using pre-annealing GISH on the *Hibiscus* F2 hybrid, a lot of *H. syriacus* DNA was not visible anymore. Only a few recombination spots were detected. GISH relies on the hybridisation of genome specific repetitive DNA sequences (Heslop-Harrison 2000). While genes are often similar over large taxonomic distances, repetitive DNA motifs vary in both sequence and abundance even between closely related species. It has been suggested that these evolutionary changes in repetitive DNA, its genome organisations and chromosomal structure are possibly correlated with speciation (Dean and Schmidt 1995; Heslop-Harrison 2000). So most probably there is a high level of homology between the 2 parental genomes, *H. syriacus* and *H. paramutabilis*, which results in a lot of loss of *H. syriacus* DNA in the GISH experiments with pre-annealing for the hybrids of *H. syriacus* x *H. paramutabilis*.

A different approach was used by Ali *et al.* (2004) to perform GISH in *Arabidopsis*, having an extremely small genome, between 0.16 pg.1C⁻¹ and 0.25 pg.1C⁻¹, and a basic chromosome number of x = 5. By using a high probe concentration (up to 15 µg per slide) and a long hybridisation time, they obtained detectable hybridisation signals on entire chromosomes. To prevent cross hybridisation the authors used BAC clones bearing species specific repetitive DNA sequences for blocking. For woody ornamentals no BAC clones are available. Therefore, for species with lack of genetic information, the best approach is to search for an optimal ratio probe/block DNA.

Another important condition that needs to be optimal for GISH analysis is the probe labelling and detection system. Our GISH experiment on the *Hibiscus* F2 hybrid demonstrated an advantage of the digoxigenin detection system compared to the biotin system. Emphasis has been placed on the false-positive results that are obtained when biotin is used to label a probe, because endogenous biotin may sometimes interfere with specific signals (Chevalier *et al.* 1997). Digoxigenin is an interesting alternative because it is present exclusively in *Digitalis* plants as a secondary metabolite (Chevalier *et al.* 1997).

6.4.2 GISH on *Buddleja* and *Hibiscus* hybrids

GISH analysis, using genomic DNA of *B. globosa* as a probe, proved the hybrid origin of *B. x weyeriana* 'Sungold', originating from a cross between *B. globosa* and *B. davidii*, since number of chromosomes belonging to *B. globosa* could be distinguished in *B. x weyeriana*. Furthermore, based on the GISH results for *B. x weyeriana* 'Sungold' it was clear that *B. globosa* produces unreduced gametes. In *B. x weyeriana* 36 chromosomes were distinguished belonging to *B. davidii*, 28 chromosomes to *B. globosa* and 12 recombinant chromosomes. It was expected to observe maximum 19 chromosomes of *B. globosa* in *B. x weyeriana*, since *B.*

globosa is diploid ($2n = 2x = 38$). In F1 and F2 hybrids of *B. davidii* x *B. x weyeriana* 'Sungold' it was shown that all *B. globosa* DNA was introgressed into the *B. davidii* chromosomes after 2 crossing generations. Also the amount of *B. globosa* DNA diminished along further generations.

For *Hibiscus*, it was more difficult to distinguish between the parental genomes and to observe recombination. In the F1 hybrids of *H. syriacus* x *H. paramutabilis* the parental genomes could clearly be distinguished by GISH using biotinylated *H. syriacus* as probe DNA. However, for the F2 hybrids, the level of differentiation achieved in our study was dependent on the modifications made in the hybridisation mixture. GISH using different ratios probe/block DNA with biotinylated *H. syriacus* as probe DNA yielded no clear results due to cross hybridisation. When using pre-annealing before hybridisation, only few spots of *H. syriacus* DNA was observed. However, it was expected that also in the F2 hybrid more than a few spots of *H. syriacus* DNA would be present, since both the F1 and F2 hybrids look morphologically more or less the same and in the F1 hybrid an equal amount of *H. syriacus* and *H. paramutabilis* DNA was seen. On the contrary, GISH analysis on the *Hibiscus* F2 hybrid with *H. syriacus* used as probe labelled with digoxigenin (probe/block DNA ratio 1/180) revealed several chromosomes completely belonging to *H. syriacus* and recombinant chromosomes between *H. syriacus* and *H. paramutabilis*.

The differences in genomes (size, amount of polymorphic markers) of *Buddleja* and *Hibiscus* species did not prevent chromosome pairing and crossing over in meiosis as was shown by GISH in *B. x weyeriana*, the F1 and F2 hybrids *B. davidii* x *B. x weyeriana* and the F2 hybrid *H. syriacus* x *H. paramutabilis*. The most widely accepted model for recombination is the homology-dependent double-strand break repair model and it is thought that recombination occurs predominantly between DNA stretches which have sufficient homology (Schnable *et al.* 1998). This means that there is still a considerable amount of homology between the different *Hibiscus* and *Buddleja* genomes. On the other hand, the demonstration of the possibility of distinguishing between the genomes of *B. globosa* and *B. davidii* and between the genomes of *H. syriacus* and *H. paramutabilis* indicates a considerable divergence of the middle and highly repetitive DNA sequences of the species. This variation in the repetitive DNA is generally considered as source of differentiation among species and genera (Dean and Schmidt 1995) and allows the discrimination between the chromosomes of the parental genomes in interspecific hybrids by means of GISH (Schwarzacher *et al.* 1992). The recombination points in *Buddleja* and *Hibiscus* hybrids were mostly located distally on the long arm but also a lot of the recombination points were randomly distributed along the chromosomes.

6.5 Conclusion

In this chapter it was proved that GISH can be used successfully on the woody ornamentals *Buddleja* and *Hibiscus* hybrids. *Hibiscus* and *Buddleja* are characterised by a small genome (between 0.93 pg.1C⁻¹ and 1.48 pg.1C⁻¹ for *Buddleja* and between 2.13 pg.1C⁻¹ and 2.48 pg.1C⁻¹ for *Hibiscus*) and/or a high amount of small chromosomes (between 1.42 µm and 3.49 µm for *Buddleja* and between 2.28 µm and 4.89 µm for *Hibiscus*). By GISH it was possible (i) to prove the hybrid nature of *B. x weyeriana*, (ii) to distinguish between the parental genomes in *B. davidii* x *B. x weyeriana* (F1 and F2) and *H. syriacus* x *H. paramutabilis* (F1 and F2), (iii) to observe recombinant chromosomes between the parental genomes and (iv) to confirm the production of unreduced gametes in *B. globosa*.

Our adaptation on the GISH protocol for woody ornamentals, with small genomes/chromosomes and with no sequence information, consisted of searching (i) the optimal probe/block ratio and (ii) the best labelling and detection system for the probe. In our protocol probe/block ratios were not lower than 1/80. Digoxigenin seemed to be better for labelling the probe compared to biotin, since for *Hibiscus* hybrids, parental genomes could only be clearly distinguished using a digoxigenylated probe. For *Buddleja* GISH was successful with a biotinylated probe. In our GISH experiments on *Buddleja* and *Hibiscus* stringency conditions of 80% - 85% were successfully used.

In future, GISH technology can provide also for woody ornamentals a useful strategy for characterising breeding lines and speeding up the breeding process. Application of GISH for interspecific breeding will allow (i) to determine the hybrid origin of genotypes, (ii) to visualise recombination events on particular chromosomes, (iii) to perform quantitative analysis of introgressed chromosomal segments and (iv) to distinguish the copy number of particular chromosomes in a plant.

CHAPTER 7

GENERAL DISCUSSION, CONCLUSION AND PERSPECTIVES

The main objective of this PhD. study was the development and evaluation of different tools that might be used for breeding and selection of woody ornamentals. Nowadays, advanced breeding techniques like interspecific hybridisation, polyploidisation, marker-assisted breeding, ... are mainly used in seed propagated agricultural crops. However, some of these techniques might also have a great value for improvement of woody ornamentals, since these modern techniques offer an extended potential to introduce new genetic variation, to screen for valuable germplasm or to speed up selection (Uhlinger 1982; Van Tuyl and De Jeu 1997).

An interspecific breeding program consists of different steps: (i) description of the available germplasm and choosing proper parent plants, (ii) making interspecific crosses and (iii) determination of the hybrid nature of obtained seedlings and further selection.

So far, in most breeding programs choices of parental species are mainly based on morphological parameters. However, a better insight in the (cyto)genetic structure of different species is very important to better predict the success rate of interspecific crosses. In this study it was shown that genome size and ploidy measurements, AFLP studies and karyotype analysis are important tools to gather (cyto)genetic information about the available germplasm for a breeding program within woody ornamentals in general and within *Hydrangea*, *Hibiscus* and *Buddleja* in particular. Within these 3 genera significant differences in ploidy levels and/or genome sizes were present. Information on the genetic variation measured by AFLP markers was generated and for *Hydrangea* species a karyotype with indication of 45S rDNA sites (NOR-sites) was constructed. Great differences were observed of chromosome profiles and NOR sites between the species. Besides (cyto)genetic analysis, also pollen germination was investigated of candidate parent species. These measurements determined some *Buddleja* and *Hydrangea* species producing pollen with low germination capacity.

All these (cyto)genetic and biological differences give indications on cross compatibility and help in decision making on specific cross combinations. To optimise future breeding efforts it should be interesting to be able to appreciate a (genetic) distance that would give a fair prediction of what taxons are crossable or not before making crosses. A profound preparatory study of possible crossing parents can contribute to a more direct and specific breeding program. For this reason a better insight in the floral characteristics (floral biology, breeding system, biology of the pollen, best time for pollination, ...) can generate additional valuable information.

In interspecific breeding, prezygotic incongruity can be sometimes observed. Microscopic evaluation of pollen tubes can verify their existence. Several techniques, such as cut-style and

grafted-style techniques, use of mentor pollen,... exist to overcome prezygotic barriers. In this research project, no prezygotic barriers were detected based on analysis of pollen tube growth. However, interspecific crosses were hampered by several postzygotic barriers. Observed (cyto)genetic and biological differences between parental species explained cross-(in)compatibility in some cases. The appearance of postzygotic incongruity was variable and differed for the 3 genera. The major barrier was the abortion of the fruits in an early stage of their development. This can be due to endosperm malformation (Johnston *et al.* 1980). In this PhD. study an *in vitro* embryo rescue protocol was successfully applied on *Hydrangea*, *Hibiscus* and *Buddleja*. This embryo rescue enhanced the efficiency of interspecific crosses. Immature ovules/embryos were initiated on *in vitro* germination media 10 or 11 weeks after pollination. Germination of the *in vitro* initiated embryos was very good for *Hibiscus* and *Buddleja*. For *Hibiscus* also good germination rates were obtained even when no *in vitro* step was performed. For *Hydrangea* almost no well developed ovules were found in the resulting fruits after interspecific *Hydrangea* crosses.

The fact that no complex media were requested offers perspectives to routinely apply embryo rescue in breeding programs of many woody ornamental genera. It also might be valuable to consider environmental effects to enhance fertilisation possibilities. Sullivan (1989) reported on the beneficial effects of high temperatures and humidity levels (although he did not provide quantitative data). In *Lilium* high temperatures proved to be advantageous for pollination and fruit development (Van Tuyl and De Jeu 1997). Treatment of ovaries with auxines, cytokinins or gibberellins also is known to improve seed and fruit formation (Pittarelli and Stavely 1975; Van Tuyl and De Jeu 1997).

After seedlings are obtained, albinism is frequently a major barrier for generating viable hybrids (Yao and Cohen 2000). It was not a problem for *Buddleja*, but it was observed for F1 seedlings of *Hydrangea* and *Hibiscus*. Also decreased hybrid vigor was observed in some cross combinations of *Hydrangea* and *Hibiscus*. These diverse growth aberrations are not due to inhibited chloroplast development but rather to unbalanced new gene combinations (Shaked *et al.* 2001; Germana and Chiancone 2001). However, it was sometimes difficult to draw conclusions on these barriers since not all possible cross combinations could be made (i.e. reciprocal crosses). In future hybridisation programs, it might be recommended to test different cultivars of parent species in cross combinations and to make all reciprocal crosses.

Next to interspecific hybridisation, also polyploidisation can be a shortcut for crop improvement since it allows (i) to overcome problems with interploidy crosses and (ii) to restore fertility of often sterile hybrids and (iii) can be a breeding goal itself. In this study successful chromosome doubling was performed in *Buddleja* and *Hibiscus*. The chromosome amount of *B. globosa* could be doubled, which makes it (theoretically) better crossable to *B. davidii*. However, it should be checked if the generated tetraploid *B. globosa* is stable and if the gametes are chromosome doubled before using them in further breeding with *B. davidii*. In *Hibiscus*, chromosome doubling followed by a backcross with a non-doubled cultivar finally resulted in a new hexaploid *H. syriacus* cultivar 'DVPazurri' which was introduced into the market. This new cultivar is very vigorous in growth and, as a consequence of being

sterile, has a longer flowering period. This result shows the possibilities of such ploidy breeding techniques for woody plants.

Creating fertile allopolyploids by chromosome doubling of sterile hybrids does not always serve the purpose of introgression of alien genes. First, the backcrossing of an allotetraploid with a diploid parent produces a triploid which is not always suitable for further crossing. Secondly, due to autosyndetic pairing in an allopolyploid no intergenomic recombination occurs. Therefore, the interest of breeders in using somatically doubled allopolyploids diminishes (Ramanna and Jacobsen 2003). However, more genetic variation can be generated if unreduced gametes from genotypes and F1 hybrids could be used in crosses (Ramanna and Jacobsen 2003). In this work strong indications were found for the fact that *B. globosa* might produce unreduced gametes. This was concluded based on (i) a higher genome size and ploidy level than expected for *B. x weyeriana*, an F2 selection resulting from a cross *B. globosa* x *B. davidii*, (ii) crosses between *B. davidii* x *B. globosa*, which resulted in F1 seedlings with a higher genome size than presumed and (iii) GISH analysis of *B. x weyeriana* revealing 28 chromosomes (instead of maximum 19) completely belonging to *B. globosa* ($2n = 2x = 38$) plus 12 recombinant chromosomes between *B. globosa* and *B. davidii*. If *B. globosa* produces viable unreduced gametes (even in a small frequency), then it should be possible to perform crosses with *B. davidii* on tetraploid level without polyploidisation of *B. globosa*. This is more advantageous for the creation of genetic variation. However, it is suggested to confirm the production of unreduced pollen in *B. globosa* by analysis of the pollen and egg cells. Unreduced gametes were also found in *B. lindleyana*. Crosses between *B. davidii* ($2n = 4x = 76$) and *B. lindleyana* ($2n = 2x = 38$) resulted in tetraploid F1 hybrids instead of triploids. Moreover, AFLP analysis showed that the hybrids *B. davidii* x *B. lindleyana* inherited a lot of the *B. lindleyana* markers, which amplifies the possibility for *B. lindleyana* of producing unreduced gametes. Also in this case the production of unreduced gametes should be proved by pollen and egg cells analysis. Finally, unreduced gametes were observed in F1 and F2 hybrids of *H. syriacus* x *H. paramutabilis*. By using different detection methods it could be demonstrated that the F1 hybrids produce unreduced egg cells while in the F2 hybrids unreduced pollen was detected.

So far, in woody ornamentals, little efforts have been made to use $2n$ gametes in crop breeding. However, in our study it was concluded that unreduced gametes also occur in woody ornamentals, even in higher frequencies than was expected. These results open new perspectives to use $2n$ gametes in further breeding programs in *Hibiscus* and *Buddleja* and in woody ornamentals in general. To exploit these opportunities completely a fast detection method should be optimised. It might also be very interesting if unreduced gametes could be induced in selected woody ornamental genotypes and hybrids. It is found that $2n$ pollen production is stimulated by environmental factors such as temperature (Lokker 2004), herbivory, wounding, water and nutrient stress (Ramsey and Schemske 1998), N_2O treatment (Akutsu *et al.* 2007) and caffeine treatments of immature flower buds (Olden 1954). Techniques should be tried out and further developed for woody ornamental species.

In this dissertation different methods for hybrid verification based on morphological, molecular and cytological markers were applied and evaluated. Only for *Hibiscus* and

Buddleja it was possible to determine the hybrid nature of the obtained F1 seedlings by use of morphological parameters. For *Hydrangea* morphological markers were not able to unambiguously characterise hybrids. It is known that interspecific hybrids often look more alike one of their parents and are sometimes rather difficult to recognise when young (Rieseberg 1995). Rieseberg (1995) argues that hybrids are not always morphologically intermediate to their parents because the expression of parental versus intermediate character states in hybrids depends on the nature of the genetic control of a particular character, as well as on interactions with the environment. Moreover, morphology of F1 seedlings can be very unpredictable. Therefore, for the characterisation of F1 seedlings a combination with molecular techniques is needed to clearly determine the hybrid nature.

When parental species differ enough in genome size and ploidy level, genome size measurements and ploidy determination by flow cytometry was very efficient to characterise F1 and F2 hybrids. Normally in interspecific crosses the resulting hybrids will exhibit an intermediate ploidy level and/or genome size compared with the parent plants, as was showed for *Buddleja* progenies. Only for *Hibiscus*, genome sizes between the parental species did not differ enough to be used for detection of the hybrid character of the F1 seedlings. Flow cytometry has the advantage to be a fast, easy and reliable method to screen large hybrid populations. However, for *Buddleja* and *Hydrangea* sometimes conflicting results were obtained. In future, more research should be done on the effects of interspecific hybridisation on the genome of hybrids.

Also AFLP analysis was useful to determine the hybrid character of most obtained F1 seedlings resulting from interspecific crosses within *Hydrangea*, *Hibiscus* and *Buddleja*. AFLP is a reliable technique and no prior sequence information is required. Molecular markers are widely used for monitoring alien genetic material in a plant genome (Garcia *et al.* 1995; Fedak 1999; Yamagishi *et al.* 2002) and offer many opportunities to characterise hybrids in a lot of woody ornamental breeding programs. However, by AFLP dominant markers are generated which do not provide an answer whether one or multiple copies of a particular gene/chromosome are present in a plant. SSR markers could be an alternative for AFLP since they generate co-dominant markers. However, SSR markers are not available for *Hibiscus* and *Buddleja*. For *Hydrangea*, SSR markers recently were developed (Rinehart *et al.* 2006).

If the parental species possesses different $2n$ values, chromosome counting and karyotyping by conventional staining methods are very reliable. A more recent cytogenetic technique, GISH, is more reliable and is able (i) to distinguish whether one or multiple copies of a specific gene/chromosome are present in the plant, (ii) to differentiate chromosomes of different parental genomes in allopolyploid species, interspecific hybrids and their backcross progenies and (iii) to trace intergenomic chromosome rearrangements (Schwarzacher *et al.* 1989; Parokonny *et al.* 1997; Takashi *et al.* 1997). In this study the GISH technology was adapted for the woody ornamentals *Buddleja* and *Hibiscus*. GISH was successfully used in *B. x weyeriana*, *B. davidii* x *B. x weyeriana* (F1 and F2) and *H. syriacus* x *H. paramutabilis* (F1 and F2). Parental genomes could be distinguished and recombinant chromosomes were observed. Our results clearly showed that GISH might be valuable also for species with small genomes and/or small chromosomes, although in literature it was described that GISH often

failed in such species (D'Hont *et al.* 2000; Brutovska *et al.* 2000; Lim *et al.* 1998). This opens new perspectives to use GISH in woody ornamental plant breeding to monitor intergenomic recombination, which is essential for introgression of desired genes in interspecific hybrids. Besides, GISH can also be interesting to unravel the mode of origin of unreduced gametes.

Another important objective of this PhD. study was to generate and select new valuable pre-breeding material in *Hydrangea*, *Hibiscus* and *Buddleja*. In *Hydrangea* true hybrids were only obtained from crosses between *H. macrophylla* and *H. serrata*. From a genetic point of view these 2 species are very related. Fertilisation barriers between more distinct genera could not be overcome. Only few F1 seedlings were obtained from crosses between *H. paniculata* and *H. macrophylla* and *H. paniculata* and *H. aspera*. But these seedlings contained only few genetic material of the male parent. The main objective of the breeding program of *Hydrangea*, to enlarge the *H. paniculata* assortment with novel flower colors and leaf shapes, was not achieved. However, in the progeny of *H. serrata* x *H. macrophylla* several plants with other interesting characteristics were selected for further evaluation. For *Hibiscus*, interspecific hybrids were generated from crosses between *H. syriacus*, *H. sinosyriacus* and *H. paramutabilis*. The hybrids were characterised by increased growth vigor, new leaf types and larger flowers compared to the existing *H. syriacus* cultivars. Valuable pre-breeding material from F1 and F2 populations was selected and multiplied for further observation. For *Buddleja* F1 progenies were obtained after interspecific crosses between *B. davidii* and *B. x weyeriana*, between *B. davidii* and *B. lindleyana*, between *B. davidii* and *B. globosa* and between *B. alternifolia* and *B. crispa*. For other cross combinations fertilisation barriers could not yet be overcome. Some interesting pre-breeding material from F1 and F2 progenies of *B. davidii* x *B. lindleyana* and *B. alternifolia* x *B. crispa* was selected for further evaluation. Novel characteristics of these selections are mainly compactness, sterility and new flower morphology. In the F1 progenies no yellow flowering seedlings could be selected yet. In the future breeding program of *Buddleja*, more crosses will be made between *B. davidii* and yellow flowering species (*B. globosa*, *B. x weyeriana*) to introgress a yellow flower color in *B. davidii*.

F1 seedlings obtained after interspecific crosses within *Buddleja* and *Hibiscus* had low fertility. For breeding, sterility has advantages since sterile plants are not invasive (which is a big problem for some woody ornamental species i.e. *Buddleja*), are often more growth vigorous and have an increased flowering period (i.e. *H. syriacus* 'DVPazurri'). So in future breeding, sterility can be an important goal for breeding programs. On the other hand, sterility may result in a barrier to gene flow between species, since it hampers further crossing (Heslop-Harrison 1999).

This study demonstrated that the creation of interspecific hybrids, along with the chromosome doubling technology offers opportunities for woody ornamental breeding programs. It was shown that (i) genome size and ploidy measurements together with AFLP analysis are useful to gather (cyto)genetic information about candidate woody parent plants to allow a more specific and direct breeding program, (ii) embryo rescue enhances the efficiency of interspecific crosses within several woody genera, (iii) mitotic and meiotic (2n gametes)

polyploidisation can generate a lot of genetic variation and (iv) flow cytometric measurements, AFLP analysis and GISH can be applied to characterise interspecific hybrids within woody ornamentals and to speed up selection.

A better understanding of the phenomena associated with allopolyploids and interspecific hybrids would lead to important innovations in the commercial assortment of many ornamental genera. One might expect that in the future these will allow a more efficient and straightforward breeding and selection.

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- European white oaks *Quercus petraea* (Matt.) Liebl. And *Q. robur* L. In Sharma AK, Sharma A (eds) Plant genome: biodiversity and evolution, Volume 1, Part E: Phanerogams –Angiosperm. Science Publishers. pp. 43-78
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CURRICULUM VITAE

Opleiding

- 2003 – 2006 Doctoraatsopleiding in de toegepaste biologische wetenschappen
Universiteit Gent - Faculteit Bio-ingenieurswetenschappen
- 2002 - 2005 Geaggregeerde voor het secundair onderwijs in de toegepaste biologische wetenschappen
Universiteit Gent - Faculteit Bio-ingenieurswetenschappen
- 1997-2002 Bio-ingenieur in de cel- en genbiotechnologie
Universiteit Gent - Faculteit Bio-ingenieurswetenschappen
- 1991-1997 ASO Grieks - Wiskunde
Sint - Bavohumaniora, Gent

Loopbaan

- 2003 – heden: Wetenschappelijk attaché - ILVO - Eenheid Plant - Toegepaste Genetica en Veredeling - Melle
- Organisatie en opvolging van de veredeling van boomkwekerijgewassen
 - Onderzoeksproject: 'Interspecifieke hybridisatie bij sierboomgewassen' (IWT-VIS-CO project nr. 020802, samenwerking met BEST-Select cvba).
- Januari – juni 2003: Wetenschappelijk medewerker - Tibotec-Virco - Mechelen
- Tibotec-Virco is een farmaceutisch bedrijf dat onderzoek verricht naar remmende stoffen tegen HIV en tuberculose.
 - 'Integrase-screening project': Ontwikkelen van een secundaire radioactieve assay voor het testen van stoffen op hun werking tegen HIV (screenen naar Integrase remmende stoffen).

Publicaties

Artikels in boeken:

- Eeckhaut T, Van Laere K, De Riek J, Van Huylbroeck J (2006) Overcoming interspecific barriers in plant breeding. In: Teixeira Da Silva JA (ed.) Floriculture, ornamental & plant biotechnology: advances and topical issues (1st edition), Global Science Books, London, UK, pp. 540 – 551

 Artikels met peer review

- Van Laere K, Van Huylenbroeck J, Van Bockstaele E (2007) Interspecific hybridization between *Hibiscus syriacus*, *Hibiscus sinosyriacus* and *Hibiscus paramutabilis*. *Euphytica* 155: 271-283
- Van Laere K, Van Huylenbroeck J, Van Bockstaele E (2008) Karyotype analysis and physical mapping of 45S rRNA genes in *Hydrangea* species by fluorescence *in situ* hybridization. *Plant Breeding* 127: 301-308
- Van Laere K, Leus L, Van Huylenbroeck J, Van Bockstaele E (2008) Interspecific hybridisation and genome size analysis in *Buddleja*. Aanvaard voor publicatie in *Euphytica*
- Van Laere K, Dewitte A, Van Huylenbroeck J, Van Bockstaele E (2008) Evidence for the occurrence of unreduced gametes in interspecific *Hibiscus* hybrids. Aanvaard voor publicatie in *Journal of Horticultural Science and Biotechnology*
- Van Laere K, Khrustaleva L, Van Huylenbroeck J, Van Bockstaele E (2008) GISH as a tool to characterise hybrids with small genomes and chromosomes. In voorbereiding

Artikels in proceedings van wetenschappelijke congressen:

- Van Huylenbroeck J, Van Laere K, Eeckhaut T, Van Bockstaele E (2004) Interspecific hybridization in flowering shrubs. *Acta Hort.* 651: 55-62
- Van Laere K, Van Huylenbroeck J, Van Bockstaele E (2006) Breeding strategies to increase genetic variability in *Hibiscus syriacus*. *Acta Hort.* 714: 75-81
- Eeckhaut T, Van Huylenbroeck J, Van Laere K, Van Bockstaele E (2006) Interspecific hybridization in woody ornamentals: how to deal with barriers? *Acta Hort.* 725: 117-126
- Van Huylenbroeck J, Van Laere K, Eeckhaut T (2008) An integrated approach to overcome crossing incongruity in woody ornamentals. *Acta Hort.* 766, 355-359
- Van Huylenbroeck J, Van Laere K, Leus L (2006) Breeding strategies for woody ornamentals: selection towards disease resistance. Aanvaard voor publicatie in proceedings of the international plant propagators society conference: 'Sustainable plant production', Grobbendonk, België (29/08/06 - 01/09/06)
- Van Laere K, Van Huylenbroeck J, Van Bockstaele E (2007) Modern breeding techniques: the *Hydrangea* case. Aanvaard voor publicatie in proceedings of the international *Hydrangea* conference 'Hydrangea 2007', Gent, België (16 - 19/08/07)
- Van Laere K, Khrustaleva L, Van Huylenbroeck J, Van Bockstaele E (2008) Molecular cytogenetic tools for interspecific breeding within woody ornamentals. Aanvaard voor publicatie in *Acta Hort.*
- Van Huylenbroeck J, Van Laere K (2008) Breeding strategies for woody ornamentals. Aanvaard voor publicatie in *Acta Hort.*

Vulgariserende artikels

- Van Laere K, Van Huylenbroeck J (2004) Interspecifieke hybridisatie van sierboomkwekerijgewassen. *Sierteeltonderzoek in Vlaanderen*, p. 14
- Van Laere K, Van Huylenbroeck J (2005) Interspecifieke hybridisatie bij sierboomkwekerijgewassen. *Sierteeltonderzoek in Vlaanderen*, p. 44
- Van Laere K, Van Huylenbroeck J (2006) Interspecifieke hybridisatie bij sierboomkwekerijgewassen. *Sierteeltonderzoek in Vlaanderen*, p. 51
- Van Laere K, Van Huylenbroeck J (2008). Veredeling van sierbomen: zoektocht naar gezonde en attractieve nieuwigheden. *Groencontact* 34(1): 10-13

Andere:

- 6-maandelijkse IWT-voortgangsverslagen i.v.m. de resultaten van het onderzoeksproject ‘interspecifieke hybridisatie bij sierboomteeltgewassen’ (IWT-VIS-CO 020802)
- De Riek J, Lamote V, Eeckhaut T, De Keyser E, Van Laere K, Van Huylenbroeck J (2006) Erfelijkheidslcer in de praktijk. Cursus, pp. 185

Congressen, symposia en studiedagen

Studieverblijven in het buitenland:

- Mei 2004: Plant Research International, Wageningen, Nederland: GISH-technologie (Dr. Ludmila Khrustaleva)

Internationale congressen met actieve bijdrage:

- Van Huylenbroeck J, Van Laere K, Eeckhaut T, Van Bockstaele E. Interspecific hybridisation in flowering shrubs (poster) Eucarpia 21st international symposium, section ornamentals: ‘Classical versus molecular breeding of ornamentals’, Freising-Weihenstephan, Duitsland (25-29 augustus 2003)
- Van Laere K, Van Huylenbroeck J, Van Bockstaele E. Embryo rescue and *in vitro* polyploidisation as tools for overcoming postfertilisation barriers in interspecific crosses (voordracht) COST 843 WG1 meeting: ‘Competence, gene transfer and expression: special aspects of micropropagation’, Heraklion, Griekenland (18-21 november 2004)
- Van Laere K, Van Huylenbroeck J, Van Bockstaele E. *In vitro* and microscopic tools for interspecific hybridization in woody ornamentals (voordracht) COST 843 final meeting: ‘Plant quality enhancement through tissue culture’, Stara Lesna, Slovakije (28 juni - 3 juli 2005)
- Van Laere K, Van Huylenbroeck J, Van Bockstaele E. Karyotyping of *Hydrangea* species by 45S rDNA FISH and computer-aided chromosome analysis (poster) XV FESPB (Federation of European Societies of Plant Biology) congress, Lyon, Frankrijk (17 -21 juli 2006)
- Van Laere K, Van Huylenbroeck J, Van Bockstaele E. Breeding strategies to increase genetic variability in *Hibiscus syriacus* (voordracht) Eucarpia 22th international symposium, section ornamentals: ‘Breeding for beauty’, San Remo, Italië (15 - 19 september 2006)
- Van Laere K, Van Huylenbroeck J, Van Bockstaele E. Modern breeding techniques: the *Hydrangea* case (voordracht) International *Hydrangea* conference ‘Hydrangea 2007’, Gent, België (16 - 19 augustus 2007)
- Van Laere K, Khrustaleva L, Van Huylenbroeck J, Van Bockstaele E. Molecular cytogenetic tools for interspecific breeding within woody ornamentals (voordracht) 1st international symposium on woody ornamentals of the temperate zone, Pruhonice, Tsjechië (26 - 30 mei 2008)
- Van Huylenbroeck J, Van Laere K. Breeding woody ornamentals at ILVO, Belgium (poster) 1st international symposium on woody ornamentals of the temperate zone, Pruhonice, Tsjechië (26 - 30 mei 2008)

Internationale congressen met passieve deelname:

- Eeckhaut T, Van Huylenbroeck J, Van Laere K, Van Bockstaele E. Interspecific hybridisation in woody ornamentals: How to deal with barriers? 5th international symposium on *in vitro* culture and horticultural breeding, Debrecen, Hungary (13-17 september 2004)
- Van Huylenbroeck J, Van Laere K, Eeckhaut T. An integrated approach to overcome crossing incongruity in woody ornamentals. 27th international horticultural congress of ISHS: 'Global horticulture diversity and harmony', Seoul, Korea (13 - 19 augustus 2006)
- Van Huylenbroeck J, Van Laere K, Leus L. Breeding strategies for woody ornamentals: selection towards disease resistance. International plant propagators society conference: 'Sustainable plant production', Grobbendonk, België (29 augustus - 1 september 2006)

Deelname aan andere studiedagen en symposia

- 28 november 2003: BSHS Symposium: pot plants: Production, protection and selection. PCS, Destelbergen, België
- 10 december 2004: BSHS Symposium: Chains in Horticulture_Theorie and Practice. WICC, Wageningen, Nederland
- 13 mei 2005: BPTCG symposium: Novel approaches in crop improvement. CLO-DvP, Melle, België
- 17 maart 2006: NVPW Symposium. WICC, Wageningen, Nederland
- 26 oktober 2006: 20th BPTCg meeting: Tissue Culture - Facing the future. Gembloux Agricultural University, Gembloux, België
- 9 november 2006: Workshop fluorescence microscopy. Olympus, Aartselaar, België
- 22 november 2006: BSHS Symposium: the future of Horticultural science and education in a European perspective. EESC, Brussel, België
- 9 maart 2007: Bijeenkomst van de studiekering voor plantenveredeling: Celbiologie: nieuwe ontwikkelingen en toepassingen binnen de veredeling. Wageningen, Nederland.
- 23 november 2007: BPBA meeting: Epigenetics and somaclonal variation in plants. Espace Vinçotte, Parc Scientifique Créalys, Gembloux, België
- 17-18 april 2008: Sierteeltcongres 'Uitdagingen in de sierteeltsector en wat de wetenschap daartoe kan bijdragen', Gent, België

Begeleiden van eindwerken

- Interspecifieke hybridisatie bij de sierteeltgewassen *Buddleja*, *Hibiscus* en *Hydrangea*. Tim Sekeet, HoGent, CTL-BME, Ind. Ing. Biotechnologie (2004-2005)
- Stage algemene plantenveredeling. Gilles Allaert, HoGent, tuinbouw hogeschool, campus Melle, graduaat landbouw en biotechnologie (februari-mei 2005)
- Stage algemene plantenveredeling. Harm Hebing, HAS Ten Bosch, Nederland (september – december 2005)
- Interspecifieke hybridisatie bij sierteeltgewassen. Nancy De Winne, HoGent, CTL-BME, Ind. Ing. Biotechnologie (2005-2006)
- Veredelings technieken en interspecifieke hybridisatie bij sierteeltgewassen. Koen Eyskens, Katholieke Hogeschool Kempen, Departement IIBT, Master Bio (2006-2007)

- Ondersteunende technieken bij de veredeling van *Hibiscus*, *Hydrangea*, *Ligustrum* en *Buddleja*. Yff Van Den Driessche, HoGent, CTL-BME, Ind. Ing. Tuinbouw (2006-2007)
- Interspecifieke hybridisatie bij sierteeltgewassen. Wouter Boone, HoGent, CTL-BME, Ind. Ing. Biotechnologie (2006-2007)
- Interspecifieke hybridisatie bij sierboomgewassen. Ellen Ydens, HoGent, CTL-BME, Ind. Ing. Tuinbouw (2007-2008)
- Chromosoomverdubbeling bij houtachtige sierteeltgewassen. Anneleen Verhaeghe, HoGent, CTL-BME, Ind. Ing. Tuinbouw (2007-2008)

Begeleiden van buitenlandse bezoekers

- Peter Oenings, Heuger Blumen, Münsterstrasse 46, 49219 Glandorf, Duitsland (september 2005): algemene veredelingstechnieken (embryo rescue, prefertilisatie onderzoek, ploëdieveredeling, protoplastveredeling, ...)
- Prof. Dr. Ludmila Khrustaleva, Russian State Agrarian University – Moscow Timiryazev Agricultural Academy (juni-augustus 2007): Cytogenetische technieken (GISH / FISH /chromosoomkleuringen / karyotypering) bij sierteeltgewassen (*Hibiscus*, *Buddleja*, *Hydrangea*)

Andere activiteiten

- Juli – december 2002: Schrijven IWT-project: *Moleculair-genetisch onderzoek naar de bloei- inductie bij Spathiphyllum floribundum* (IWT-beurs werd niet toegekend)
Promotoren: Prof. Dr. ir. Erik Van Bockstaele en Prof. Em. Dr. ir. Pierre Debergh
- Oktober 2004: Wetenschapsweek ‘Klonen van planten’
- 20 april 2005: CLO-DvP, Melle: Geven van workshop ivm. doorbreken van barrières bij soortkruisingen: Chromosoomverdubbeling *in vitro*
- Januari – februari 2006: ILVO – eenheid Plant, Melle: NCBL cursus ‘Erfelijkheidsleer in de praktijk’: doceren speciale veredelingstechnieken.

