Hybridization and introgression between *Brassica napus* and *Brassica rapa* in the Netherlands

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Cover photos by Sheila Luijten
Front: *Brassica napus*, *Brassica rapa* and F1-hybrids motorway A6 Almere-carpool
Back: *Brassica napus* Europoort

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Executive summary

In 2008 the COGEM (Committee for Genetic Modifications) started a project to investigate the risks of admixture of GM products with non-GM products. In the current report the results of sub-project IV are presented. Since this is the last sub-project of the main project "*Admixture at import*", the results from sub-projects I to III will be briefly summarized before presenting sub-project IV in more detail.

Sub-project I, *Admixture of GM and non-GM crops at import* (CGM 2009-03), recommended that for GM crop species that cannot establish in the Dutch environment, methods and equipment should be simplified and that admixture should be reduced to a minimum in the country of origin by packing the products at the production site and by training the involved persons working with GM products.

Sub-project II, *Transport chains and seed spillage of potential GM crops with relatives in the Netherlands* (CGM 2010-02) showed that information on seed transport of GM-variants into the Netherlands is poorly available, because no central registration exists. Therefore the transport chain of *Brassica napu*s was investigated. Seed import is mainly from European countries, while import from the GM producing countries is negligible. Seeds arrive by coasters and are either processed in a closed process by a hot pressing method (ADM and Cargill) in the port of arrival (90% of the seed import), or are transported by truck to smaller companies that use a cold pressing method. Seeds can also end up in bird and rodent food. Spillage in the open process is probably much higher than for the closed process. Estimated seed loss during transport ranges from 0.1% to 3.0%. Seed loss is not featured by any of the quality control systems or inspections regimes. Moreover, confusion arose while gathering information of the transport chain, because *B. napus* (rape or oilseed rape; in Dutch Koolzaad) is also referred to as "Raapzaad", the Dutch name for *Brassica rapa*, a closely related species. Apparently these two species are often treated as a single product.

Sub-project III, *A baseline study of the distribution and morphology of* Brassica napus *L. and* Brassica rapa *L. in the Netherland*s (CGM 2010-03) showed that in the Dutch environment *B. napus* is much less common than previously thought. Feral populations show a scattered distribution pattern across the Netherlands with exception of the North East of the province of Groningen where *B. napus* is frequently found in road verges. Populations are usually small and local, and found

in highly disturbed habitats. Larger populations can often be traced back to spillage during transport. *Brassica rapa*, however, is much more common than thought and is found mainly in the lowland western part of the country and in river valley areas. Populations are usually large and linearly shaped. Although *B. napus and B. rapa* are closely related, their morphology differs significantly. Both species can be best identified by combining several morphological traits and not by a single trait. Species determination based on morphological characters is not simple and previous publications referring to either of these species must be regarded with caution. With an updated identification card and the help of volunteers from FLORON a new baseline for the distribution of *B. napus* and *B. rapa* was established. Based on these findings we recommended that monitoring should involve a wider area than just the well-defined area where *B. napus* is cropped, processed or transshipped. Due to the scattered distribution pattern of *B. napus*, monitoring could become too complex for the "permitholder". A national "alarm system" would then be more suitable to monitor possible adverse effect of GM traits.

Sub-project IV, *Hybridization and accumulation of genes through outcrossing*, was executed by Sheila Luijten and Tom de Jong (Institute of Biology Leiden). The initial question of the sub-project was whether accumulation of (trans) genes occurs in feral *B. napus* populations through repeated outcrossing with GM *B. napu*s crops. Because feral populations of *B. napus* are found infrequently, the focus of project IV was shifted to populations of *B. rapa*, because they are much more common. We concentrated on the gene flow from *B. napus* to *B. rapa*. Cross-pollination between *B. napus* (AACC) and *B. rapa* (AA) will produce an F1-hybrid (AAC). The F1-hybrid will have 29 chromosomes, 20 AA chromosomes and 9 C chromosomes from *B. napus*. Due to this uneven number of chromosomes and the preference of pairing between A-chromosomes, gametes of the flowering F1-hybrid have 10 A-chromosomes supplemented with zero to nine unpaired C-chromosomes. Hybrids can be detected by counting the number of extra chromosomes. However, a small fraction of the progeny will have no extra chromosomes and is therefore indistinguishable from *B. rapa* at level of flow cytometry and chromosome counting. The first backcross generation (BC1) probably has a lower fitness than the F1, suggesting that the chance that a transgene from *B. napus* will be incorporated in a *B. rapa* population is small, but not zero. The probability of introgression depends on the position of the transgene. If a transgene is positioned on an A-chromosome in *B. napus* it can

potentially be transmitted to *B. rapa* in only two generations. If the transgene is located on one of the C-chromosomes introgression is less likely but still, the transgene can be incorporated into the A-genome after homeologous recombination between A- and C-chromosomes.

Results of this investigation

With the help of the exact location of fields cropped to *B. napus* between 2006 and 2008 and our own distribution data from 2009, we searched for *B. rapa* populations adjacent to or in the direct vicinity of a field cropped to *B. napus*. We also searched for populations in which both species grew intermixed. From the visited 89 fields cropped to *B. napus*, only 19 had a *B. rapa* population within a radius of 2.5 km. Only seven of these 19 locations were situated within 50 m from a *B. napus* field or transshipping site. In total we sampled 27 *B. rapa* populations of which five served as a control. These five locations were located at least 10 km away from a recent *B. napus* field and they grew in an area where cultivation of *B. napus* had been absent for decades. The remaining 22 *B. rapa* populations are therefore suspect for hybridization with *B. napus*. In only three out of these 22 sampled populations flowering hybrids were found, while non occurred in the five controls. The hybrids had a flow cytometry value and chromosome number exactly intermediate between *B. napus* and *B. rapa*. All hybrids belonged to the F1 generation and had an AAC-genome with 29 chromosomes. The percentage of F1-hybrid plants in the sample size varied from 11% to 23%. All three populations with hybrids were very disturbed ares with a mix of *B. rapa* with feral *B. napus*. The feral *B. napus* plants were probably either recruited by regular spillage of seeds or from a soil seed bank after disturbance. In the latter case this means that hybridization could occur anywhere where *B. rapa* occurs as a weed and *B. napus* was a crop in the past. All other *B. rapa* plants sampled had a flow cytometry value and chromosome number equal to *B. rapa*. No single individual was found with a measurement somewhere between the F1-hybrid and *B. rapa*, implying that no recent backcrosses were found, also not in the populations with F1-hybrids. It is not clear why backcrosses with some extra C-chromosomes were not found, because such plants are expected to occur in high frequencies, unless there is a strong selection against BC1 plants with extra C-chromosomes. In Canada and on an abandoned field in Denmark, plants with extra C-chromosomes were found frequently. At the same time in the UK, as in our research, only F1-hybrids were found. A possible explanation for the

absence of BC progeny could be the intensive management of the road verges, which could result in an unsuitable habitat for establishment for BC plants.

As mentioned earlier, cross-pollination between an F1-hybrid and *B. rapa* may also result in a few hybrids without extra C chromosomes. Through recombination in the F1 such hybrids may contain fragments of C-chromosomes. We used two alternative methods to detect if these introgressed plants exist. Firstly, a FISH method with a BAC containing a C-genome specific sequence (a C-genome specific retrotransposon) as fluorescent probe was used to detect the C-genome chromosomes. This method worked well for the F1-hybrids because it coloured all C-chromosomes. In one out of 22 *B. rapa* plants tested two of the 20 chromosomes showed a fluorescent signal, suggesting an incorporation of a large fragment from a C-chromosome into an A-chromosome as a result of homeologous pairing. The plant will be used for more elaborate investigation showing the size and nature of the introgressed chromatin. Secondly, with AFLP we selected markers that were 100% monomorphic in *B. napus, B. oleracea* and the F1-hybrid. If these markers are found in *B. rapa* populations with high introgression risk that are situated in the vicinity of a *B. napus* source (field or transshipping site), but not in the control *B. rapa* populations, this suggests gene flow from *B. napus* to *B. rapa*. We found no difference between the five control populations and the 22 populations with high introgression risk, suggesting that no recent gene flow has occurred. Our sample size and the number of markers are limited, and hence we may have missed introgressed plants. We cannot conclude that gene flow has never occurred between *B. napus* and *B. rapa*, this may have happened centuries ago and more sophisticated techniques might reveal more detail (see discussion below).

The original question of the COGEM was whether stacking of transgenes would occur in GM products found in the environment? And what would be the risk of introgession of these transgenes? For *B. napus* no GM plants are cultivated in the Netherlands. However, in Nature News (August 2010) researchers reported that *B. napus* was found growing everywhere along roads in North Dakota, USA (http://www.nature.com/news/2010/100806/full/news.2010.393.html). These genetically modified *B. napus* plants were found to contain either a herbicide resistant gene from Monsanto or from Bayer, and some *B. napus* plants even had both transgenes. This example is evidence of accumulation of transgenes in a feral

crop species. Although the scale of weediness mentioned in this news report seems to be very different from the Dutch situation documented in sub-project III, there are similarities between Canada and the Netherlands. Feral *B. napus* is mostly found in road verges, near petrol stations and grocery stores. *B. napus* thrives along roads in the US because it resists the herbicides and thus occupies a vacant niche. GM *B. napus* could well become common along Dutch roads when spraying with herbicides occurs frequently.

A transgene on a C-chromosome has a lower chance to be transmitted and can only be incorporated into *B. rapa* after recombination between A and C. It is therefore preferable to place the transgene on one of the C-chromosomes and not on the A-chromosomes. Spillage of seeds should be prevented. Since it takes about three months for *B. napus* because the seeds are fully ripe, mowing of the plants after flowering could already reduce establishment strongly.

Detailed information of the sequence of the C-genome is now available at the *Brassica* genome project (http://www.brassica.info/) and this information makes it possible to investigate in more detail how much gene flow has occurred between *B. napus* and *B. rapa* since the introduction of *B. napus* around the year 1500 (subproject III).

Nederlandse samenvatting

Begin 2008 heeft de COGEM (Commissie Genetische Modificatie) het project “Vermenging bij import” uitgeschreven. Dit rapport is het verslag van deelproject IV en omdat hiermee het gehele project wordt afgerond, worden eerst de resultaten van project I tot III samengevat, waarna project IV in meer detail wordt toegelicht.
Deelproject I, *Vermenging bij import* (CGM 2009-03), beveelt aan voor genetisch gemodificeerde (GM) soorten vermenging aan de basis van de transportketen te voorkomen door in het land van herkomst op de productieplaats de producten te verpakken en de werknemers te scholen omtrent het werken met GM producten.

Deelproject II *Transportketens* (CGM 2010-02) signaleert dat informatie over zaadtransport van GM-varianten slecht beschikbaar is, omdat deze gegevens niet centraal geregistreerd worden. Alleen voor Koolzaad (*Brassica napus*) zijn er voldoende gegevens beschikbaar over de transportketen. Het overgrote deel van de zaden wordt ingevoerd vanuit Europa en maar voor een klein deel uit landen met GM teelt. Zaden worden met zee- of binnenvaartschepen aangevoerd en dan of direct via een afgesloten proces met warme persing verwerkt bij de bedrijven ADM en Cargill (90% van de import), of zaden worden per vrachtauto getransporteerd naar kleinere perserijen (koude persing). De zaden zijn ook in vogel- en knaagdiervoer aanwezig. Zaadverlies bij het open proces is waarschijnlijk groter dan bij het gesloten proces. Exacte getallen van zaadverlies ontbreken, maar de schattingen lopen uiteen van 0.1% tot 3%. Het morsen van zaden in relatie tot transport en verwerking is voorspelbaar, maar waar vogelvoer in het milieu terecht komt is onvoorspelbaar. Bij kwaliteitsystemen van ketens wordt geen rekening gehouden met verliezen van zaden. Het rapport beveelt aan het systeem aan te passen om menging van non-GM en GM koolzaad te voorkomen. Bovendien wordt er gesignaleerd dat Koolzaad (Engels: rape of oilseed rape) ook onder de naam Raapzaad (*Brassica rapa*) ingevoerd wordt. Er wordt kennelijk in de handel geen duidelijk onderscheid gemaakt tussen deze twee soorten.

Deelproject III, *Monitoring verwilderde populaties* (CGM 2010-03) laat zien dat Koolzaad veel minder algemeen in Nederland is dan werd verondersteld. Vindplaatsen van 'wild' Koolzaad liggen verspreid over het hele land met een duidelijk zwaartepunt in Noordoost-Groningen. De populaties zijn meestal klein en liggen vrijwel altijd in de buurt van punten waar Koolzaad wordt gemorst of

verbouwd. Of deze planten zich blijvend vestigen is niet onderzocht, maar op enkele lokaties werden twee jaar achtereen planten waargenomen. Raapzaad blijkt veel algemener dan Koolzaad en wordt voornamelijk in het westen en de lagere delen van ons land waargenomen. De raapzaadpopulaties zijn meestal groot en langerekt. Beide soorten zijn nauwverwant en goed kruisbaar, omdat Koolzaad (2n=38, AACC) het genoom heeft van beide ouders: Raapzaad (2n=20, AA) en Kool (*Brassica oleracea,* 2n=18, CC). Op basis van hun genoom en hoeveelheid DNA per cel zijn Koolzaad en Raapzaad perfect te onderscheiden. Ook op basis van een combinatie van morfologische kenmerken zijn beide soorten te onderscheiden. Het morfologische onderscheid tussen Kool- en Raapzaad is niet altijd makkelijk of men maakt geen onderscheid. Enige argwaan naar de juistheid van het naamsgebruik van beide soorten in eerdere publicaties is daarom op zijn plaats. Op basis van de nieuwe verspreidingsgegevens van beide *Brassica* soorten, die mede verzameld werden door FLORON-vrijwilligers, stelden wij voor dat monitoring een groter gebied zou moeten beslaan dan waar Koolzaad geteeld, overslagen of verwerkt wordt. Een nationaal alarm systeem heeft een groter bereik dan het gebied van de vergunninghouder.

Voor deelproject IV "*Stapeling van genen door inkruising*" was de oorspronkelijke vraag of er via de "natuurlijke" koolzaadpopulaties in Nederland risico is op stapeling van genen doordat planten in het wild meerdere malen kruisen met GM Koolzaad op de akker. Uit deelonderzoek III bleek echter dat verwilderde koolzaadpopulaties niet algemeen voorkomen in Nederland en omdat Raapzaad wel veel voorkomt en makkelijk hybridiseert met Koolzaad, kan stapeling van genen ook optreden in het milieu via Raapzaad. Daarom is onderzocht hoe vaak hybriden voorkomen in raapzaadpopulaties en of er introgressie heeft plaatsgevonden door genenuitwisseling van Koolzaad (AACC) naar Raapzaad (AA). Wanneer Koolzaad met Raapzaad hybridiseert geeft dit een F1-hybride (AAC). Deze F1-hybride heeft 29 chromosomen, 20 A-chromosomen van Raapzaad en 9 ongepaarde C-chromosomen van Koolzaad. Bij terugkruising van de F1-hybride met de wilde soort Raapzaad, hebben de nakomelingen (BC1) allemaal het AA-genoom maar daarnaast ook een variabel extra aantal (van 1 tot 9) extra C-chromosomen of heel soms helemaal geen C-chromosomen. Hybride en BC1 nakomelingen met extra chromosomen kunnen gedetecteerd worden op basis van hun hoeveelheid DNA of chromosoomaantal per cel. Dit geldt niet voor hybriden met nul extra

chromosomen en deze planten zijn dus op dit niveau niet te onderscheiden van Raapzaad. Statistisch komt dit in 0.2% van de geslachtscellen voor, maar uit gepubliceerd experimenteel onderzoek blijkt dat in de BC1 zo'n 5-10% planten met nul C-chromosomen gevonden en dat is hoger dan statistisch verwacht wordt. Als een transgen op een A-chromosoom van Koolzaad ligt en het komt via kruising en terugkruising in een AA plant terecht dan is er dus sprake van transgenoverdracht van Koolzaad naar Raapzaad. Het is ook mogelijk dat een transgen op een C-chromosoom ligt en door genuitwisseling op het A-chromosoom terecht komt en op deze manier in een AA plant terecht komt. De BC1-hybriden zijn echter minder vitaal en dit verlaagt dit de kans op introgressie.

Resultaten uit het onderhavig onderzoek

Aan de hand van de ligging van de koolzaadvelden tussen 2006-2008 en onze eigen verspreidingsgegevens van Koolzaad en Raapzaad uit 2009 zijn we in 2010 op zoek gegaan naar (met Koolzaad gemengde) raapzaadpopulaties die in de directe nabijheid lagen van een koolzaadveld of overslaglokatie. De zoektocht naar mogelijke hybriden spitste zich hoofdzakelijk toe op het westelijke deel van Nederland, omdat Raapzaad vooral daar voorkomt, maar ook werd het grootste koolzaadproductiegebied in Noordoost-Groningen bezocht. In totaal zijn 23 raapzaadpopulaties, vier gemengde populaties van Koolzaad en Raapzaad, en één koolzaadpopulatie bemonsterd. Van de in het totaal 28 bemonsterde locaties zijn er vijf raapzaadpopulaties bemonsterd in een gebied waar op grond van de ligging en afstand tot koolzaadakkers het risico op uitkruising met Koolzaad in de laatste decennia vrijwel uitgesloten kon worden. Deze vijf lokaties worden als controlepopulaties beschouwd. Van de 27 onderzochte raapzaad-/gemengde populaties bevonden 19 populaties zich binnen een straal van 2.5 km van een koolzaadbron. Van die 19 populaties waren er 17 in de buurt van koolzaadvelden en bevonden er zich twee locaties in de buurt van een overslaglokatie. In slechts drie verstoorde populaties vonden we bloeiende planten met een flowcytometriegetal en chromosoomaantal dat precies intermediair was tussen dat van Raapzaad en Koolzaad. Dit zijn de F1-hybriden met genoom AAC. Het percentage F1-hybriden per steekproef varieerde van 11% tot 23%. Deze drie populaties met hybriden werden gekenmerkt door het samen voorkomen van Koolzaad en Raapzaad op lokaties waar de grond kortgeleden is bewerkt. Het Koolzaad in deze populaties met hybriden is vermoedelijk afkomstig van het morsen van zaden tijdens tranport of de

planten zijn gerecruteerd uit de zaadbank na verstoring van de grond. In het laatste geval is het lastig te voorspellen waar gemengde populaties te voorschijn zullen komen en dit bemoeilijkt de monitoring. Buiten de 3 hybride populaties hadden alle andere onderzochte planten de flowcytometriescore en het chromosoomaantal van Raapzaad en dus 2n=20 chromosomen. Er zijn geen planten gevonden met een flowcytometriescore of chromosoomaantal intermediair tussen de F1-hybride en Raapzaad. Er zijn dus geen recente terugkruisingen gevonden, ook niet in de populaties met F1-hybriden. Omdat terugkruisingen met extra C-chromosomen in hoge frequentie verwacht worden, concluderen wij dat deze planten óf niet gevormd worden, óf dat er sterke selectie tegen is zodat de zaden van de BC1 met extra C-chromosomen nooit een levenskrachtige, bloeiende plant opleveren. In Canada en op een verlaten akker in Denemarken werden wel frequent planten aangetroffen met extra C-chromosomen, terwijl men in Groot Britannië, net als in dit onderzoek, alleen F1-hybriden vond. In hetzelfde Canadese onderzoek werd bovendien een raapzaadplant gevonden die door introgressie herbicide-resistent geworden was, hetgeen laat zien dat er genenuitwisseling is van Koolzaad naar Raapzaad. Wij veronderstellen dat door het intensievere beheer van bermen en akkerranden in Nederland, populaties slechts kortlevend zijn en dat daarom terugkruising minder kans heeft.

Zoals boven al gemeld kan terugkruising van een F1-hybride met Raapzaad 5-10% nakomelingen opleveren met nul extra C chromosomen. Door recombinatie tussen het A- en C-genoom of uitwisseling van A- en C-chromosomen kan het toch voorkomen dat er in AA-planten DNA van het C-genoom voorkomt. We hebben twee alternatieve methoden gebruikt om na te gaan of zulke planten in Nederlandse raapzaadpopulaties voorkomen. Ten eerste, werd een BAC-FISH methode gebruikt om specifiek de C-chromosomen te kleuren. Van de 29 chromosomen die voorkomen in de F1-hybride lichtten er inderdaad negen helder op. Van de 22 raapzaadplanten (2n=20) onderzocht m.b.v. BAC-FISH, was er één plant waarbij twee van de 20 chromosomen kleurden. Dit wijst op chromosoomsubsitutie, waarbij 2 A-chromosomen zijn vervangen door twee C-chromosomen en het laat genenuitwisseling zien tussen Koolzaad en Raapzaad. Ten tweede, is met behulp van de AFLP-methode gezocht naar DNA markers die 100% monomorf (een allel homozygoot aanwezig in alle planten) zijn in Koolzaad, Kool en de F1-hybride. Als deze markers voorkomen in raapzaadpopulaties die in de buurt

groeien van een koolzaadbron (akker of overslagterrein), maar niet in de controlepopulaties, dan duidt dit op gene flow van Koolzaad naar Raapzaad. Er werd geen verschil gevonden tussen de verdachte en controle populaties. Dit suggereert dat er recent geen uitwisseling heeft plaats gevonden. We kunnen echter niet uitsluiten dat andere geavanceerde moleculaire methoden meer zichtbaar maken, bijvoorbeeld door gebruik te maken van specifieke Single Nucleotide Polymorphisms (SNPs) of specifieke sequenties van het C-genoom.

De vraag van de COGEM was oorspronkelijk of er stapeling van genen kan optreden in wilde koolzaadpopulaties. Tijdens het onderzoek verscheen op 6 augustus 2010 in Nature News een bericht over de “verwildering” van Koolzaad in de VS (http://www.nature.com/news/2010/100806/full/news.2010.393.html), waarbij op grote schaal in North Dakota GM Koolzaad langs wegen werd waargenomen. Vlak naast de weg zijn de GM koolzaadplanten de enige soort die het regelmatig herbicidengebruik overleeft. Er zijn zowel van Bayer als van Monsanto GM koolzaadlijnen in teelt die een (verschillend) herbicide-resistentiegen bevatten, maar in de berm werden ook planten waargenomen met beide type transgenen. Dit voorbeeld laat zien dat stapeling van genen in Koolzaad mogelijk is. Hoewel de schaalgrootte van de verwildering van Koolzaad in dit bericht tegenstrijdig lijkt met de resultaten uit ons deelproject III is dit bij nadere beschouwing niet zo. Net als in Nederland staan de koolzaadplanten in de VS vooral in de rand van de weg.

De strekking van ons rapport is dat in de Nederlandse situatie genenuitwisseling van Koolzaad naar Raapzaad lijkt mee te vallen, omdat 1) Koolzaad infrequent in het Nederlandse landschap aanwezig is en 2) kruisingen met Raapzaad meestal niet verder te gaan lijken dan de F1-hybride en 3) er tot nu toe maar één raapzaadplant is gevonden waarbij een groot stuk van het C-genoom aan twee A-chromosomen is toegevoegd. Dit zal nog verder onderzocht worden. Toch is hier wel het een en ander tegen in te brengen. Als bermen van akkers en wegen regelmatig worden bespoten met herbicide wordt dit habitat geschikt voor HR Koolzaad. Het nieuwe habitat zal des te sneller worden opgevuld naarmate er meer GM zaden worden gemorst. Uit de literatuur blijkt dat als men in het lab F1-hybriden kruist met Raapzaad, 5-10% van de nakomelingen nul extra C-chromosomen hebben. Deze planten zijn uiterlijk en cytogenetisch vergelijkbaar met het oorspronkelijk Raapzaad. Als het transgen in het A-genoom wordt ingebouwd is er dus een

behoorlijke kans dat het terecht komt in de andere soort. Voor een transgen op het C-genoom is deze kans veel kleiner omdat er minder C wordt doorgegeven en genen van het C-genoom alleen na recombinatie tussen A- en C-chromososmen in Raapzaad terecht kunnen komen. Het is dus aan te bevelen om transgenen op C-chromosomen te plaatsen en niet op A-chromosomen. Een andere aanbeveling, die in de verschillende deelrapporten terugkomt, is dat morsen van zaden beter voorkomen kan worden. Als het morsen van zaden niet voorkomen kan worden dan is maaien na de bloei wellicht afdoende om het Koolzaad te bestrijden.

Gedetailleerde informatie over de opbouw van het C genoom komt nu beschikbaar via het *Brassica* genome project (http://www.brassica.info/) en zulke informatie maakt het mogelijk om in de naaste toekomst in meer detail te kijken hoeveel 'gene flow' er tussen de soorten heeft plaatsgevonden sinds de introductie van koolzaad, zo rond het jaar 1500 (zie deelproject III).

1 General introduction

Hybridization between cultivated crops and their wild relatives can lead to introgression of genes from one species to another, providing a potential for preserving and recombining (modified) traits through time (Ellstrand et al. 1999). For crops like corn, potato or cotton, that do not establish feral populations and do not cross with related European plant species the introgression risk is of no serious concern. For *Brassica napus* the situation is different though. In various countries all over the world non-transgenic and transgenic *B. napus* is found in road verges in the agricultural landscape, along transportation routes, at harbours or as a volunteer within other crops (Pessel et al. 2001, Lutman et al. 2003, Crawley & Brown 2004, Menzel 2006, Yoshimura et al. 2006, Von der Lippe and Kowarik 2007, Kawata et al. 2008, Knispel et al. 2008, 2010, Pivard et al. 2008, Warwick et al 2008, Nishizawa et al. 2009). In the Netherlands Luijten & De Jong (2010) found that *B. napus* had a scattered distribution pattern and local population sizes were generally small, although several larger populations were also found. Although exact measures of spillage of viable seeds are not available for the Netherlands, overall loss is estimated to range from 0.1% to 3.0 % (Tamis & de Jong 2010). When viable seeds of GM organisms are lost repeatedly, populations have a higher chance to become established in the environment. Depending on the lifespan of the feral population, stacking of different (trans)genes might be a possibility. So far, this scenario is only scarcely investigated, probably because the number of different modified traits in cropped *B. napus* is still limited. However, in North Dakota feral *B. napus* plants were found carrying two herbicide resistant traits from two different suppliers (http://www.nature.com/news/2010/100806/full/news.2010.393.html). This event is an example of the stacking of two traits within a crop species. The same may occur in wild relatives, because it is well known that *B. napus* can hybridise with related species, especially with its congener *Brassica rapa* (Warwick et al. 2003, Andersen & De Vicente 2010).

The extent of interspecific hybridization and exchange of (modified) traits depends on the similarity of the genome of the species in question. Within the genus

Brassica several diploid and tetraploid cultivated species share closely related genomes (U 1935). A close genomic similarity occurs between *Brassica rapa* (AA; *n*=10) and *Brassica oleracea* (CC; *n*=9) and hybridization between those species resulted, after duplication of the AC-genome, in the allotetraploid *Brassica napus* (AACC; 2*n*=38). *Brassica napus* is now a worldwide important crop species. Cross-pollination between *B. rapa* (AA) and *B. napus* (AACC) gives rise to a triploid F1-hybrid (AAC, 2*n*=29). This F1-hybrid will have 20 A-chromosomes and 9 C-chromosomes. Because all C-chromosomes are unpaired, problems may arise at gamete production during meiosis. During meiosis of the flowering F1-hybrid plant, gametes have either no extra C-chromosomes (only 10 A-chromosomes), or one to nine C-chromosomes on top of the 10 A-chromosomes. In a theoretical situation without selection every C-chromosome has 50% chance for transmission to a particular gamete. The average number of C-chromosomes in a gamete of an AAC plant will then be 4.5 and follows a binomial distribution. In that theoretical case only 0.2 % of the gametes will have, by chance, zero extra chromosomes, and also 0.2% will have nine extra chromosomes. The majority of gametes will have three to six extra chromosomes. Due to a higher abortion rate of embryos with an intermediate number of C-chromosomes, relatively more seeds with zero, one or two extra chromosomes will survive as compared to the theoretical distribution (Lu et al. 2001). In crosses between AAC hybrids and *B. rapa* (AA) Lu and Kato (2001) found 5-10% offspring with zero C-chromosomes, the other 90-95% of the plants had one or more C-chromosomes. The few progeny with only 20 AA-chromosomes that derive from a hybrid AACxAA cross will be indistinguishable from *B. rapa*. When the transgene is located on one of the A-chromosomes in *B. napus*, and this particular A-chromosome is transmitted from a hybrid to an AA offspring, then introgression has become a fact. If the transgene is located on the C -chromosome, recombination must occur between one of the C- and one of the A-chromosomes for a transgene to be transmitted to the AA-genome. Due to a high similarity, pairing between homologous A-chromosomes is largely favoured over pairing with C-chromosomes. C chromosomes remain mostly univalent and are transmitted to the backcrossed progeny with variable frequencies (Leflon et al. 2006). An analysis of the metaphase I in pollen mother cells of an AAC hybrid by Leflon et al. (2010) showed that bivalents were indeed mostly formed by A-chromosomes, but they also observed recombination between A- and C-chromosomes and even among C-

chromosomes, the latter must be non-homologous recombination and likely does not contribute to the gametes formed. Backcrossed progeny with a few extra chromosomes as observed in crosses under controlled conditions in the greenhouse, have been reported from the field also. Backcrossed plants with one to three extra chromosomes have been found in a mixed population of *B. rapa* and feral *B. napus* in Canada (Warwick et al. 2008) and in Denmark (Hansen et al. 2001). In Canada Warwick et al. (2008) detected in that same population introgression of an herbicide resistant transgene from *B. napus* into the gene pool of a *B. rapa* plant.

How frequently introgression of (trans)genes into weedy relatives occurs depends on several factors, *i.e.* the sympatry and flowering time (Wilkinson et al. 2003; Simard et al. 2006), the genetic background, the performance of the F1 and backcrossed hybrids, the number of backcrosses, habitat suitability, life span of the weedy population, and the time-scale of sympatric occurrence (Jørgensen et al. 2009; Warwick et al. 2009). Due to an increasing use of GM *B. napus* as a crop, hybrid progeny between *B. napus* (male line) and *B. rapa* (female line) has been studied extensively. The F1 progeny has a higher seed production but lower pollen viability than the parents. Survival of the hybrids was similar to that of the conspecific offspring (Hauser et al. 1998a). Similar results were found by Jenkins et al. (2001) and Vacher et al. (2004). The BC1 hybrids were less fit than the F1 and had reduced seed production, high early embryo abortion and low pollen viability. Hauser et al. (1998b) concluded that the fitness of the BC1 hybrids is expected to be highly variable because of segregation and recombination. Therefore some BC1 hybrids may be as fit as their parents.

Research goals

We searched for hybrids between *B. napus* and its wild relative *B. rapa*. Hybridization is most likely to occur in situations where *B. rapa* grows in small populations in close vicinity of extensive populations of *B. napus*, because *B. rapa* is self-incompatible and thus only able to outcross with other plants. Due to pollen scarcity in small populations of *B. rapa,* outcrossing with *B. napus* is likely to occur when *B. napus* pollen is abundant. How much hybridization has occurred in the Netherlands is still unknown, but in a previous investigation (Luijten & De Jong 2010) F1-hybrids were found as flowering plants at one site and among the seed progeny within fruits harvested on several *B. rapa* plants growing adjacent to a field

cropped to *B. napus*. For the current investigation we only focused on established plants. We have not focused on seeds within fruits collected from *B. rapa* plants growing adjacent fields cropped with *B. napus*.

To estimate hybridization from *B. napus* into *B. rapa* the following questions were addressed:

1) How common are hybrids?

2) Is there introgression from *B. napus* into *B. rapa*?

3) Which (environmental) factors favour hybridization?

Several techniques were used to search for hybrids: flow cytometry, Amplified Fragment Length Polymorphism (AFLP) fingerprinting, chromosome counting and chromosome painting with C-genome specific Bacterial Artificial Chromosomes (BACs). With the first three methods it is possible to find F1-hybrids and possibly also backcrosses, but demonstrating introgression of (parts of) the C-chromosome of into *B. rapa* chromosomes is most directly shown by Fluorescent In Situ Hybridization (FISH) with BACs. The use of genome painting with total genomic DNA or with species specific DNA sequences is now common place in introgressive hybridization programs and especially powerful if the donor and recipient species have diverse repetitive sequences (Chang and De Jong 2005). In this manner chromosome additions and chromosome substitutions can be demonstrated unequivocally (Kantama et al., 2007; Lim et al., 2006).

The occurrence of natural hybrid plants depends on the sympatric distribution of both *Brassica* species, which will be related to the distance to the nearest *B. napus* plants and the cultivation or processing of *B. napus* within radius (classes) of 1, 2 and 5 km. Establishment of hybrids was also investigated in relation to environmental factors, such as the amount of bare soil and grass cover, vegetation height, soil characteristics as moisture, acidity, nitrogen (Ellenberg indicator values) and the life history of the accompanying species at the sample site.

2 Results

Number of sample locations

To find as many sites as possible with putative hybrids between *B. napus* and *B. rapa* we searched for *B. rapa* or mixed populations of both *Brassica* species in road verges, in field margins adjacent to *B. napus* crops and near transshipping locations. During the survey we examined 89 fields cropped to *B. napus* and two transshipping locations. The presence of *B. rapa* populations in proximity of these cropping fields was not very high. Only 17 putative *B. rapa* populations could be sampled in the vicinity of the 89 fields cropped to *B. napus* and two in proximity of a transshipment location. Moreover, most of the sample sites did not occur directly adjacent to a cropping field or transshipment location. The closest distance to *B. napus* activities ranged from 5m to 2.5km. Of the 19 putative hybrid *B. rapa* populations only seven occurred within a distance of 50m to a *B. napus* pollen source. From these 19 sample sites 17 consisted only of plants that could be identified as *B. rapa*. The other two populations consisted of *B. rapa*, *B. napus* and plants that could be hybrid because of a deviating morphology. All these sites have in common that *B. napus* had grown there or was cultivated at least once between 2005 and 2008.

In a previous investigation Luijten & De Jong (2010) already found a mixed population of *B. napus*, *B. rapa* and F1-hybrids near Almere in 2009 (site Almere Carpool). In the area around Almere *B. napus* was cropped extensively in the 80-ties, but cultivation of *B. napus* between 2005 and 2008 was more or less absent. Besides the sample site Almere carpool, another three sites were sampled to investigate the presence of hybrids. A total of four sites were sampled in this region, two consisted of only *B. rapa*, one of *B. napus* and one contained both *Brassica* species (Almere carpool)

Table 1. Overview of the sampled locations, the population sizes of *B. rapa* and *B. napus*, the number of plants sampled for various analyses (flow cytometry, AFLP, chromosome countings/BAC-FISH) and sampling of ecological field data and various parameters indicating the distance and presence of *B. napus* in relation to the sampled location. (suspect vs control group *B. rapa*: 1= suspect group, 2= control group)

Location	XCO (Rijks Driehoek coordinates)	YCO (Rijks Driehoek coordinates)	# *B. rapa* plants	# *B. napus* plants	# samples flowcytometry	# samples AFLP	# samples chromosome counting	Suspect vs control group *B. rapa*	closest distance to *B. napus* field or transshipping location	Years to which *B. napus* was cropped at least one time	# recent *B. napus* fields within a radius of 1 km, 2 km and 5 km (cumulative)
Wieringen	122614	537450	20	0	5	5	5	1	5 m	2006-2010	3, 10, 13
Elshout	137059	413132	1	0	1	1	1	1	10 m	2006-2008	3, 3, 14
*Meeuwen	128750	415800	50	35	9	9	-	1	10 m	2006-2010	4, 5, 13
Wijk en Aalburg 1	139928	418333	500	0	5	4	5	1	10 m	2006-2010	0, 1, 12
Wijk en Aalburg 2	132706	416882	30	1	8	7	2	1	10 m	2006-2010	5, 10, 36
*Europoort	67982	440945	20	2	7	7	7	1	50 m	-	0, 0, 0
Waalwijk	134670	412346	30	0	7	7	-	1	50 m	2006-2008	2, 3, 11
Haarlemmermeer	107393	484311	100	0	8	8	2	1	200 m	2006-2008	4, 8, 13
Schiphol	108700	484050	10	0	1	1	1	1	200 m	2006-2008	2, 5, 10
Wijk en Aalburg 3	133388	416317	50	0	4	4	3	1	450 m	2006-2010	5, 9, 39
Nieuw Vennep	102713	474382	20	0	1	1	1	-	800 m	2006-2010	3, 7, 16
Farmsum	256936	593301	40	0	2	2	2	1	900 m	2006-2010	0, 7, 59
Lelystad, Vliegveld	163900	497900	15	0	3	3	3	1	1 km	2008-2010	1, 1, 1
Numansdorp	91248	416049	10	0	4	4	2	1	1 km	2006-2008	0, 5, 16
Sleeuwijk	124000	424200	180	0	10	10	1	1	1 km	2006-2010	0, 4, 17
Zuidbroek	253650	576850	2	0	1	1	1	1	1 km	-	0, 0, 0
Eemshaven	252963	606381	13	0	5	2	2	1	1.5 km	2006-2008	0, 2, 4
Noordzeekanaal	108383	495306	300	0	11	9	2	1	2.4 km	2006-2008	0, 0, 2
Hoeksewaard	80568	421983	200	0	10	10	-	1	2.5 km	2006-2008	0, 0, 5
Almere Haven (A6)	143106	485002	150	0	4	3	3	1	8.6 km	1982/1987	0, 0, 0
*Almere, Carpool	148500	487500	100	250	12	12	10	1	11 km	1982/1987	0, 0, 0
Vogelweg (Flevoland)	150200	483750	1000	0	10	10	1	1	10 km	1982/1987	0, 0, 0
Almere Poort	138519	484012	0	75	5	4	3	-	10 km	1982/1987	0, 0, 0
Abcoude	125815	475142	400	0	10	10	2	2	15 km	-	0, 0, 0
De Meern, Carpool	130303	453684	300	0	8	8	3	2	14 km	-	0, 0, 0
Durgerdam	125801	488288	100	0	8	8	2	2	12 km	-	0, 0, 0
Hilversumse Meent	137400	474650	100	0	11	11	1	2	7 km	-	0, 0, 0
Tienhoven	131016	463981	300	0	12	12	4	2	17 km	-	0, 0, 0
total # of plants					**182**	**173**	**69**				
total # of locations					**28**	**28**	**25**				

- Location with F1-hybrids

All sites mentioned until now have a high probability for hybridization between *B. rapa* and *B. napus*. For comparison, another five "control" sites of *B. rapa* were added (Abcoude, De Meern, Durgerdam, Hilversumse Meent and Tienhoven). These sites were sampled in areas where no cropping or transshipping of *B. napus* was observed for more than a decade and we judged beforehand that recent hybridization was unlikely on these sites.

In total we sampled 28 populations (Table 1): one site with only *B. napus*, 23 sites with only *B. rapa* and four sites with both *B. rapa* and *B. napus*. Sample size per location varied from 1 to 12 plants. Flower buds for chromosome counting and BAC-FISH analysis could not be sampled in all populations because sometimes plants had already finished flowering at the time of sampling. Exact location of sampling, population size, distance to a *B. napus* activity and sample size per analysis technique to detect hybridization is listed in Table 1.

Sampling was not random. To detect putative hybrids sampling focused on plants with a deviating morphology when present at the sampling site. Besides these aberrant plants also plants with *B. rapa* and *B. napus* appearance were sampled at these sites. Also from sites without plants with deviating morphology specimens were sampled.

Flow cytometry measures and chromosome counts

For flow cytometry a total of 180 plants (28 populations) were analysed to measure the relative DNA amount per cell. For *B. rapa* the relative DNA amount varied from 1.02-1.09, for *B. napus* from 2.41-2.46 and for the hybrids from 1.71-1.73. The chromosome numbers were in close agreement with the relative DNA amount. The number of chromosomes per cell was counted on a total of 65 plants (24 populations) We found plants with 20 chromosomes (*B. rapa*), 38 chromosomes *(B. napus*) and plants with 28 or 29 chromosomes (hybrids). The flow cytometry data of hybrids were exactly intermediate between the DNA amount of the parents. The same holds for the chromosome numbers. This suggests strongly that all hybrid plants we found belonged to the F1 generation. During this survey we did not find plants that were intermediate between *B. rapa* (AA) and the F1-hybrid (AAC) in DNA content and chromosome numbers. This was equal for all tested locations. Therefore recently backcrossed (BC1) hybrids to *B. rapa* were not detected.

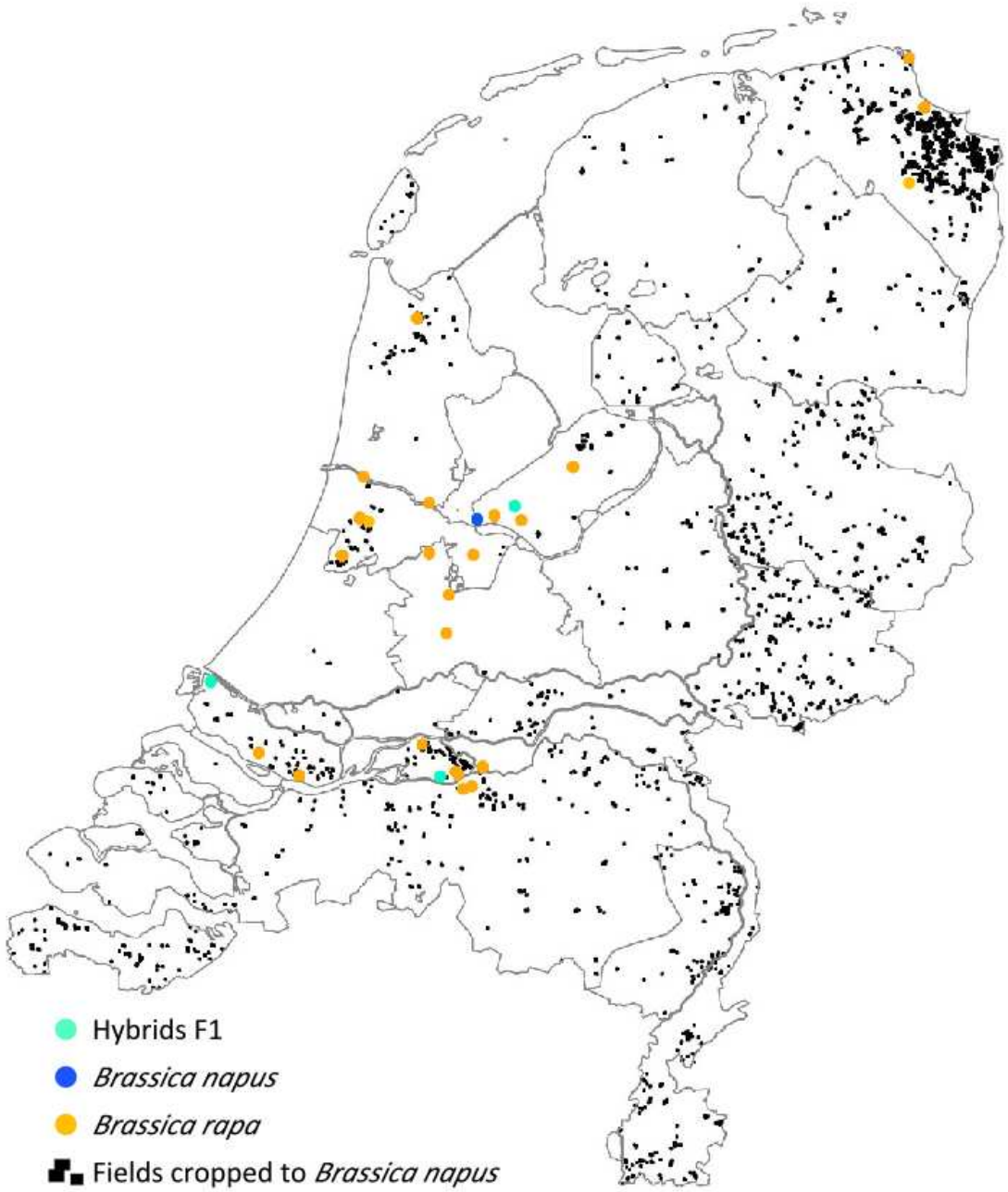

Figure 2. Sample sites with only *B. napus* and only *B. rapa* and those where F1-hybrids were found (together with the parental taxa).

Locations with hybrids

According to the flow cytometry measures and chromosome counts only three locations out of the 28 tested sites contained F1-hybrids (Figure 2). The three sites with F1-hybrids were (i) a road verge near a carpool area along the highway A6 near Almere (referred to as Almere Carpool), (ii) a road verge in between two roads in the village Meeuwen and (iii) a road verge opposite a cargo train transshipment location in Europoort (Rotterdam). The percentage of hybrid plants in the sample per location was 22%, 11% and 23%, and the corresponding number of *B. rapa* plants per site was 100, 50 and 20, respectively.

Not all sites with F1-hybrids were in the close vicinity of a *B. napus* field or transshipment location (Figure 3). Here we show the sample locations with *B. rapa* in relation to the nearest distance of a *B. napus* activity. Locations are based on the number of *B. rapa* plants (N=27). The location with only *B. napus* (Almere Poort) is not included.

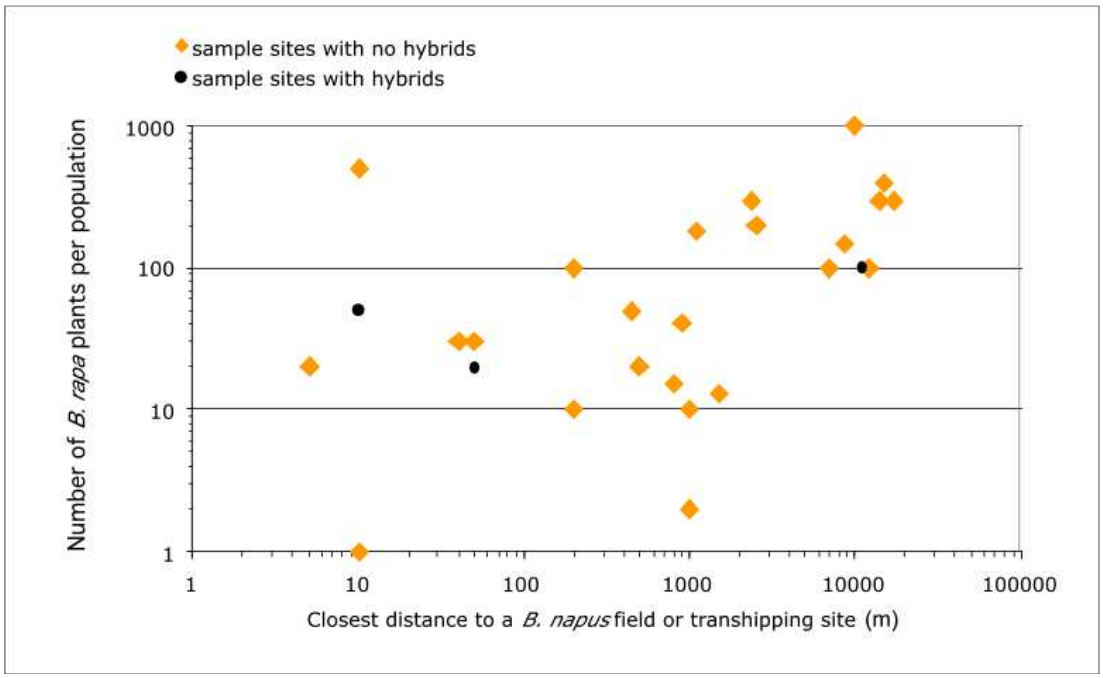

Figure 3. Overview of the sample sites, presented as population sizes of *B. rapa* in relation to the closest distance to a *B. napus* field or transshipping site.

AFLP analysis

For the AFLP analysis the initial dataset of the greenhouse plants was merged with the plants sampled in the field, because the initial AFLP dataset consisted of predominantly *B. napus* samples and the field dataset of predominantly *B. rapa* plants. The final AFLP dataset for analysis consisted of 235 plants: 64 *B. napus* plants from the greenhouse cultivated in 2009, 6 *B. napus* from the field, 156 *B. rapa* and 5 F1-hybrids. 4 *B. oleracea* variants were added (Brussels sprouts (n=2), Cauiflower (n=1) and Broccoli (n=1)). The *B. rapa* plants grown in the greenhouse were not added to the final AFLP dataset, because no information of the locations is present, but were used to define *B. rapa* specific markers.

A total of 122 markers were scored. From these 122 markers 99 markers were either A or C specific, because they were present in *B. rapa*, *B. napus* and the F1-hybrid but not in *B. oleracea* (A-specific). Or the marker was present in *B. oleracea*, *B. napus*, the F1-hybrid but not in *B. rapa* (C-specific). These 99 markers were either monomorphic (one allele) or polymorphic (more alleles). Unfortunately, the C-genome was based on four *B. oleracea* plants only. To define a marker A or C we assume that the bp-sequence of the scored markers is the same among all four groups. From these 99 markers Nei genetic distances were calculated among all 235 individuals to determine the genetic relationship among *B. rapa*, *B. napus, B. oleracea* and the F1-hybrids. A PCOA (principal co-ordinate analysis) was performed to visualise this relationship (Figure 4). This method can detect wether within the *B. rapa* group all plants are similar or that some subgroup resembles *B. napus*, which would suggest a history of introgression. The percentage of variation explained by the first three axis was respectively 80.68%, 4.67% and 4.16% (sum = 89.51%). *Brassica rapa* and *B. napus* are two distinct clusters. The F1-hybrids and *B. oleracea* are more genetically related to *B. napus*. This is logical because the F1-hybrids are AAC and share many C markers with *B. napus*. The number of markers scored per species varies as follows: *B. rapa* (38-54), *B. oleracea* (56-58), *B. napus* (71-86) and the F1-hybrid (80-87). The PCOA does not only shows that *B. rapa* is genetically separated from *B. napus*, *B.oleracea* and the F1-hybrid, it also suggests that none of the *B. rapa* plants examined is derived from a recent backcross. Such plants would be intermediate between *B. rapa* and the F1-hybrid. Figure 4 shows that no *B. rapa* plants are visualized between the *B. rapa* cluster and the other three taxa. The figure also shows that the control *B. rapa* populations are well-mixed with the *B.*

rapa populations growing in vicinity of *B. napus* activity (field/transshipment site). The position of *B. oleracea* seems rather puzzling, because one would expect that *B. oleracea* is less related to *B. rapa* than is suggested by figure 4. This position could be explained by the fact that not all markers used in this analyis are A- or C-specific.

Annex 1 gives the frequency for each of the 99 scored markers. From these 99 markers 31 were not found in *B. oleracea* and thus could represent the A-genome. These markers were also present in *B. napus* and the F1-hybrid. None of these markers were 100% monomorphic in *B. rapa*, nor in *B. napus* but some of those were monomorphic in the F1-hybrids. It is therefore difficult to define which markers are A-specific. This was different for markers representing the C-genome. From the 40 monomorphic markers in *B. napus*, 23 were also monomorphic in *B. oleracea* and 20 in the F1-hybrid. We therefore assume that these 23 markers are C-specific. From these 23 markers, 13 were indeed not found in *B. rapa.* The frequency of the remaining 10 markers varied from 0.01-1.00 in *B. rapa*. One of these markers is not very informative, because it is found in all groups.

For each *B. rapa* plant the sum of these 10 markers was taken and the result is given in figure 5. Here we compare the *B. rapa* plants from high-risk populations (purple and orange) with the plants from the control populations (blue). The number of C-markers among *B. rapa* plants varied from 2 to 7.

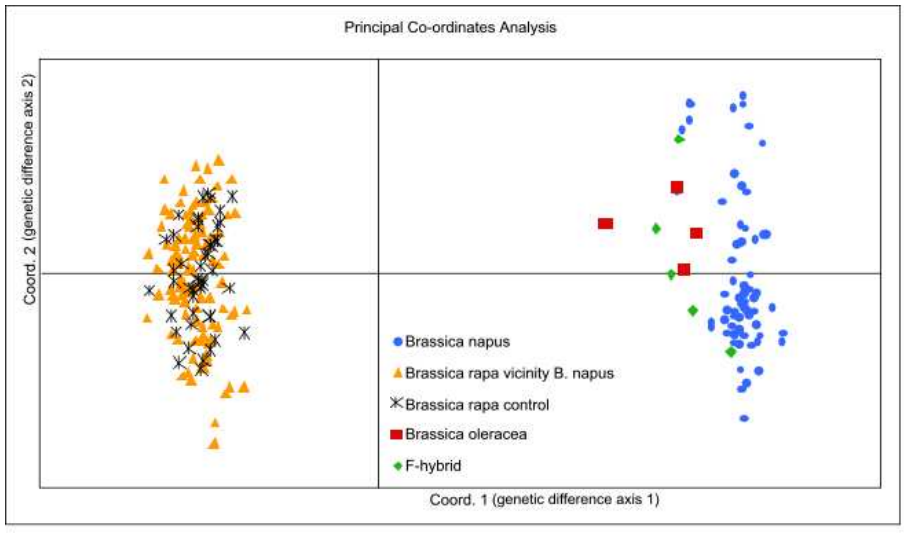

Figure 4. PCOA based on Nei genetic distances showing the genetic relationship of *B. napus*, *B. oleracea*, *B. rapa* and the F1-hybrid. For *B. rapa* the two groups are given, control versus sampled in the vicinity of a *B. napus* activity (field/transshipment site).

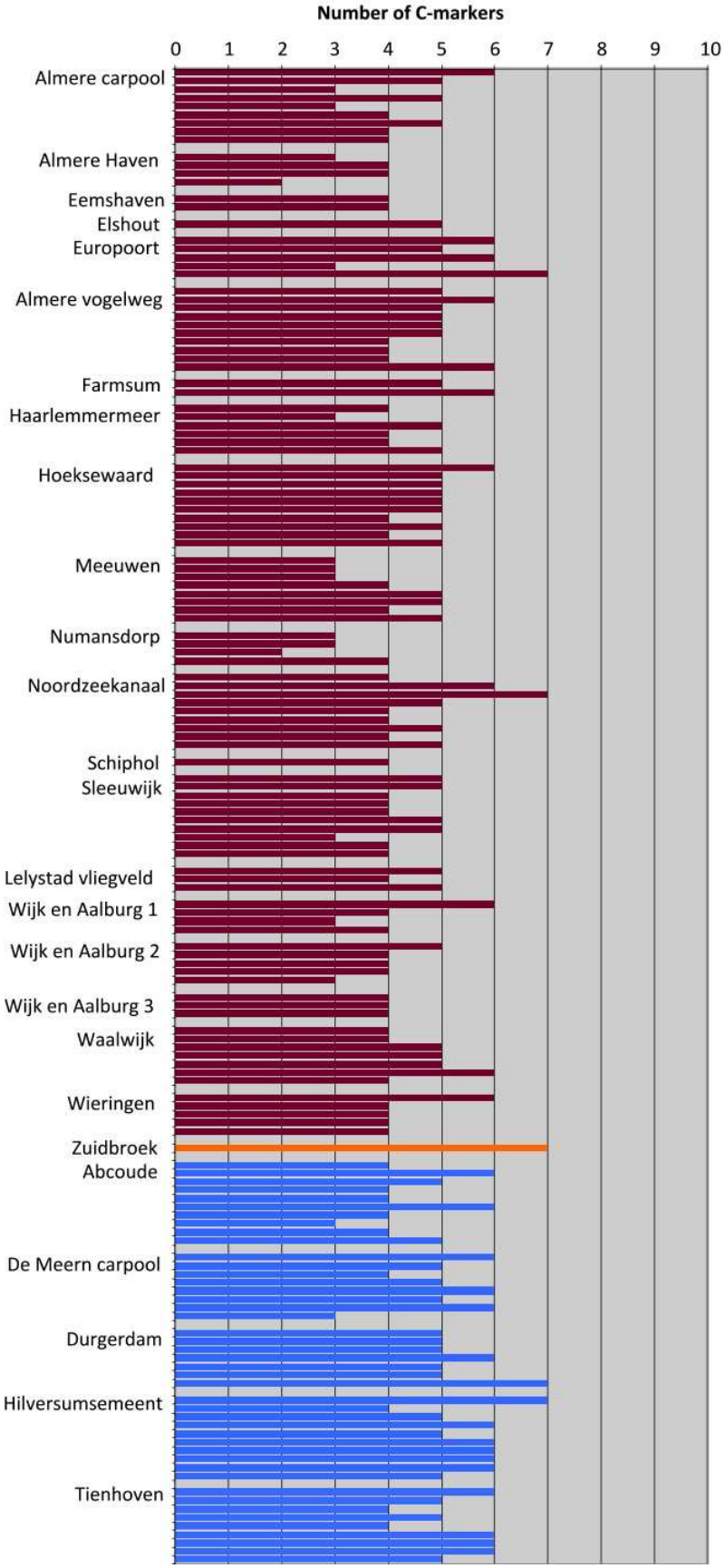

Figure 5. The total number of C-markers per *B. rapa* plant in different populations based on the 23 C-markers.

The results are in agreement with the PCOA analysis. In all *B. rapa* populations, those with high introgression risk and the control populations, there seems to be the same probability of encountering some markers from the C-genome. Based on the population means no significant difference was found between the high-introgression-risk group and the control group (Anova P=0.135). Although not significant, the number of markers tended to be higher in the control group, in contrast to our expectation. For the high-introgression-risk sites only population Zuidbroek (N=1, marker sum = 7) deviated from the group mean. It may also be the case that the 10 bands present in the AFLP gels of *B. rapa* reflect DNA fragments that are equal in length to the C-bands but different in DNA sequence. Because we have not sequenced the 23 C-genome bands we cannot rule out this possibility. If this is the case the occasional occurrence of C-genome bands in *B. rapa* is a matter of chance and does not represent any introgression event.

Painting of the C-genome with BAC-FISH

The painting protocol with the repeat probe for the C-genome was first tested in a FISH experiment on mitotic chromosome spreads of *Brassica rapa* (AA) and *B. oleracea* (CC) as controls. Under the conditions described we found no signal or only weak background signals on the AA genome and strong signals on all C-chromosomes. We then used the FISH on root tip material of four F1-hybrids that we kept in the greenhouse under optimal growing conditions. Figure 6 shows two examples of the controls and two AA plants. Chromosomes completely painted by red color are from the C-genome (*B. oleracea*); uncoloured chromosomes represent the A-genome, chromosomes painted with few red dots also belong to the A-genome, since some C-genome specific sequences may contain repetitive sequences that could hybridize with chromosome-segments of the A-genome. The figure shows that *B. rapa* plant WR01 with 2n=20 chromosomes has very little or no signals. The few fluorescent foci represent background and a very low level of cross hybridization of the probe on the one set of A-chromosomes. The Zuidbroek, Zuid04 AA-plant, collected in the northeast of the province of Groningen, showed clear signals on two chromosomes, which were clear in the metaphase complements as well as in a part of the interphase nuclei, as two bright fluorescing domains. Further experiments are now done to explore in more depth the nature and precise position of the C-genome signal on the chromosomes. Based on Nei genetic distances this Zuidbroek AA-plant falls within the main *B. rapa* cluster (Figure 4), but

taking only the 10 monomorphic putative C-markers into account, this plant has seven markers (Figure 5), just like four other plants (Noordzeekanaal03, Europoort06, Durgerdam07 and Hilversumsemeent01), which is the highest number found. Only Europoort06 was painted for the C-genome, but the result was negative.

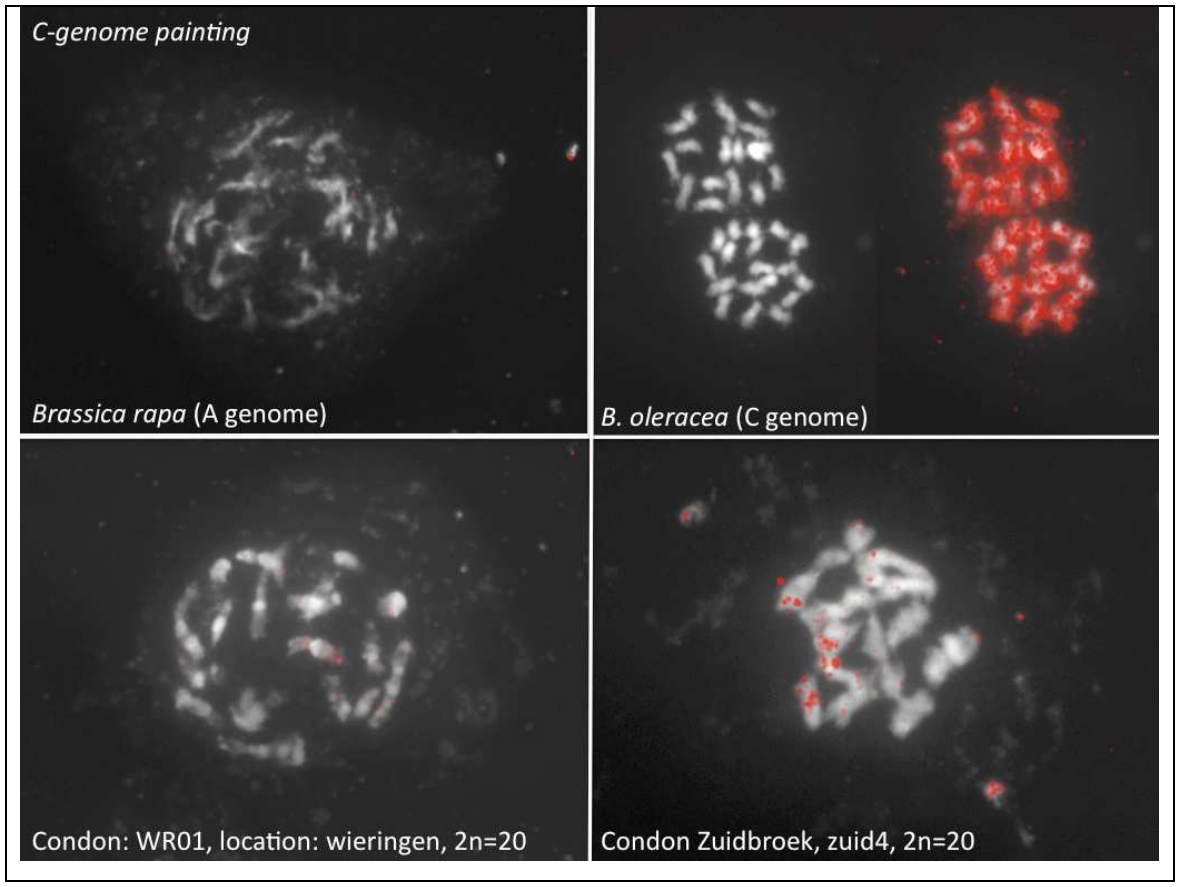

Figure 6. Painting of the C-genome of four different plants: *B. rapa*, *B. oleracea*, and two *B. rapa* plants sampled near a *B. napus* activity. The red colour highlights the C-genome.

Presence of hybrids and external parameters

Among the 28 populations sampled, only three populations contained F1-hybrids. Due to this low number of sites with hybrid plants it is not possible to predict on the basis of ecological parameters statistically why at some sites hybrids are found and at other sites not. Annex 2 gives the measurements at sites with or without hybrids for ecological parameters measured in the field (vegetation height, bare soil and cover of grasses) and the derived abiotic measures (nitrogen, moist and acidity) and the life history of the species composition at the sample location.

3 Discussion

Sympatry is a key factor for hybridization to occur between species. In the Netherlands the distributions of *B. napus* and *B. rapa* do not overlap completely (Luijten and De Jong 2010). Cropping of *B. napus* is more extensive in the east, while *B. rapa* is found more in the western lowland areas of our country. For our search for hybrids we concentrated mainly on the lowland part of the Netherlands and then on those areas where cropping of *B. napus* had occurred in the years 2005-2008. Occurrence of a *B. rapa* population in the vicinity of a field cropped to *B. napus* (distance ≤ 2.5 km) was only 19% (17 *B. rapa* populations out of 89 fields cropped to *B. napus*). From the 27 investigated sites only seven sites were located within 50 meter distance from a cropping field or transshipment site. This suggests that gene flow from *B. napus* fields to weedy populations of *B. rapa* is hindered to a large extent due to lack of sympatry between both *Brassica* species. A similar result was found in the UK, where sympatry between natural *B. rapa* populations and fields cropped to *B. napus* was also rather infrequent (Wilkinson et al. 2001, Allainguillaume et al. 2006).

Hybridization of *B. rapa* with *B. napus*

Only three out of the 27 investigated *B. rapa* populations contained hybrids (locations Meeuwen, Europoort and Almere carpool) and these plants were all F1-hybrids (AAC), having 20 A-chromosomes from *B. rapa* and 9 C-chromosomes from *B. napus* (2n=29 chromosomes). All other tested plants had either 20 AA-chromosomes, representing *B. rapa* (or AA-plants) or had 38 chromosomes representing *B. napus* (20 A-chromosomes plus 18 C-chromosomes; AACC). The percentage of hybrids in the sample size per location was 22% (Carpool Almere), 11% (Meeuwen) and 23% (Europoort). The number of chromosomes counted per cell was in full agreement with the amount of DNA in a cell. No plants were found with an intermediate number of chromosomes or DNA amount between the F1-hybrid and *B. rapa*. The lack of plants with extra C-chromosomes suggests that recently backcrossed plants were not found. AFLP data show a similar pattern in

which all *B. rapa* plants group together in one cluster that is genetically well separated from *B. napus*, *B. oleracea* and the F1-hybrids.

Our result is similar to Wilkinson et al. (2000) and Allainguillaume et al. (2006). In the UK only F1-hybrids were found and no plants with extra C-chromosomes (backcrossed progeny). However, these authors only investigated *B. rapa* populations in natural riverbanks growing less than five meters from a field cropped to *B. napus* and excluded mixed populations from the analysis. The percentage of F1-hybrids in *B. rapa* populations in the UK was not very high and varied from 0 to 1.5%. In Canada Simard et al. (2006) found higher percentages of F1-hybrids in field margins (1.1 - 17.5%) and fewer F1-hybrids (0 - 1.1%) in populations located less than 10 m from a *B. napus* field. They also found that hybridization decreased as the density of *B. rapa* increased. In contrast to the result of the UK and Canada, F1-hybrids in the Netherlands were not found in adjacent populations with only *B. rapa*. Most of the Dutch investigated *B. rapa* populations were located further away than 10 meters from a *B. napus* field in the year of investigation.

That no F1-hybrid plants were found in most *B. rapa* populations is nevertheless surprising because F1-hybrid seeds are easily formed in fruits in adjacent *B. rapa* populations through pollination by *B. napus* (i.e. Landbo et al. 1996, Warwick et al. 2003, Allainguillaume et al. 2006, Simard et al. 2006, Luijten & De Jong, 2010) and several *B. rapa* populations grew within flight distances of pollinators. Besides decreasing 'gene flow' with increasing distance between crop and wild relatives in adjacent populations, the reduction in plant fitness might be another reason that hybrids are not found in large numbers. While F1-hybrids typically grow and survive well, the quality of their pollen and seeds per fruit is often reduced. Backcrossed progeny do generally worse than parents (Hauser et al. 1998a, 1998b, Jenkins et al. 2001). In addition, competition in dense vegetation with perennials could hinder hybrid establishment in *B. rapa* populations adjacent to *B. napus* fields. The dense vegetation cover in most adjacent verges of *B. napus* fields is probably unsuitable for *B. napus* to establish also.

In the Netherlands F1-hybrids were only found in mixed populations of feral *B. napus* and *B. rapa*. Here the soil is open due to recent disturbance, which gives the opportunity for both *Brassica* species to create temporary populations. This seems to be an important condition for the formation of F1-hybrid plants. For locations

Almere carpool and Europoort we observed the presence of a mixed stand in 2008, 2009 and 2010 (population Meeuwen was only visited in 2010). For these mixed populations co-occurrence of both *Brassica* species is more stable than for *B. rapa* populations adjacent a *B. napus* field, because cropping of *B. napus* is not fixed to the same field year after year. Mixed populations of *B. rapa* and feral *B. napus* in road verges can establish through recent seed loss of *B. napus* during harvest of neighbouring *B. napus* fields (Meeuwen), or during transport from or to transshipment sites (Europoort). The mixed population at the location Almere carpool is probably related to a seed bank formed through 'historic' cultivation of *B. napus*, because cropping of *B. napus* was much more extensive around Almere and Lelystad in the 80-ties than it is nowadays. In 2010 new *B. napus* populations in disturbed soil were encountered in Flevoland: three sites near a building construction site south of Almere, one along the highway A6 near Lelystad and one large population (± 500 plants) in the verge of a diverted road near the new railway track Lelystad - Zwolle. The soil seed bank of *B. napus* could therefore be extensive in Flevoland and create temporary populations of various sizes after the soil is disturbed.

It is known that seeds from *B. napus* can survive in the seed bank up to ten years inside as well as outside arable fields (Schlink 1998, Pessel et al. 2001, Lutman et al. 2003, Pivard et al. 2008). These data and those from a previous study (Luijten & De Jong 2010) show that the presence of mixed stands of both *Brassica* species is more the exception than the rule. Also the persistence of these mixed populations with hybrids remains unclear because very few studies are available. The fact that very few mixed populations were found seems to be positive finding, but it is difficult to predict where these populations will appear. The distance between the field cropped to a GM species and its wild relative becomes unimportant as both species are preserved in the same local seed bank. This will have consequences for monitoring strategies.

Backcrossing and introgression

The impression may arise that hybrid plants with extra C-chromosomes are only found under controlled conditions and not in the Dutch environment. Although no plants with extra C-chromosomes were found in the Netherlands and neither in the mixed populations, backcrossed hybrids were found in a mixed population in

Denmark (Hansen et al. 2001, 2003) and in Canada (Warwick et al. 2008). In Denmark, backcrossed plants in the mixed population grew in an organically grown field as weeds for 11 years. The mixed population consisted for nearly 50% of backcrossed plants while only one F1-hybrid was found among 102 plants tested. Hybrids from the field were initially identified with AFLP analysis (Hansen et al. 2001), but the offspring of the backcrossed progeny was later identified with chromosome counting and AFLP analysis (Hansen et al. 2003). Among the offspring, plants were found with 0, 1 or 2 extra C-chromosomes. Most *B. rapa* plants carried also a *B. rapa*-chloroplast, but two *B. rapa*-like plants carried a *B. napus*-chloroplast. Another example of introgression is found in Canada where cropping of herbicide resistent (HR) *B. napus* is quite extensive (Warwick et al. 2008). *Brassica rapa* grows here as a weed in cropping fields or in adjacent verges. In a mixed stand of *B. rapa* and feral *B. napus* the number of hybrids declined over a period of five years, but the HT transgene persisted in the mixed population. Backcrossing to *B. rapa* had resulted eventually in an introgression event of the HT trait into *B. rapa*. How many generations were needed to incorporate the HT transgene in *B. rapa* is not known exactly.

According to the flow cytometry data and chromosome numbers we did not find plants that have recently been backcrossed in the sense that they carry some extra chromosomes. Only plants with 20 chromosomes were found, suggesting that all these plants are AA i.e. *B. rapa*. However, we did find 23 AFLP markers that were monomorphic for *B. napus* and *B. oleracea* that occurred in *B. rapa* with variable frequencies in all sample sites. These markers may show introgression from the C-genome into the A-genome of *B. rapa* if we assume that the AFLP fragments found in *B. rapa* have the same DNA sequence as those found in the C-genome of *B. oleracea*. Since plants sampled from sites in the vicinity of a *B. napus* activity and the control sites have similar frequency of C-markers, recent introgression is unlikely. Instead the markers may reflect historic introgression events. *Brassica napus* and *B. rapa* have already been traded and grown for 500 years in Europe (Van Haaster and Brinkkemper 1995, Zohary and Hopf 2000).

However, from the AFLP data alone it is not possible to be certain that a marker found in *B. rapa* is indeed representing a particular base pair sequence of a similar marker only found in on the C-genome and not on the A-genome. After a mutation

some DNA fragments isolated from *B. rapa* may have, by chance, the same length and would thus appear at almost the same position on the gel as C-specific bands of *B. oleracea*, even though the base pair sequence might be different. It would therefore have been better to have these bands sequenced. Such services are provided by Keygene but were not feasible within the current project. Instead of using AFLPs it would nowadays also be possible to develop molecular markers highly specific to the C-genome by using the huge amount of upcoming sequencing data from the *B. napus* and *B. rapa* consortia. Focusing on specific genetic regions that are conserved in both species, one can reveal many unique SNPs or one could focus on larger DNA sequences that are truly unique for A and C genomes. Detailed information of the sequence of the C genome is nowadays available at the website of the *Brassica* genome project (http://www.brassica.info/).

Homeologous substitution

The chromosome painting experiments were more conclusive on possible introgression of C-genome chromosomes into the AA *B. rapa* germ plasm. The technology is strong in demonstrating the occurrence of whole or large parts of alien chromosomes in monosomic additions and other hybrids containing a varying number of donor chromosomes in the background of a related species. The method is based on the discrimination of transposable elements that are unique or sufficiently different for one of the species in the hybridization, in this case to all C-chromosomes. Genome painting can identify the transfer of large parts of chromosomes, but cannot demonstrate the presence of small homeologous segments in hybrids. However, genome painting experiments on metaphase I complements of these hybrids can show whether A-C bivalents are formed, and thus if homeologous recombination between A- and C-genome chromosomes can take place. The existence of the hybrid with fragments from C on two of its chromosomes is still difficult to explain. Further painting studies under more stringent hybridization studies as well as analyses of meiosis of these hybrids are expected to explain more about the mechanisms behind these chromosome substitutions. That chromosome substitution in the *Brassica* family is not rare by itself has been shown a few years ago in painting studies of different *Boechera* apomicts (Kantama et al., 2007). Our study is, to our knowledge, the first one that reports homeologous substitution in nature in *B. rapa*.

The BACs used in this study to paint specific C-genome chromosomes contain a retrotransposon (most likely a Cy3/gypsy type) that is unique for the *B. oleracea* genome. Other teams, for instance Heslop-Harrison and co-workers from Leicester University, have comparable C-genome specific PCR products. So, more additional tools can now be created to reveal molecular markers for monitoring C-genome chromatin in *B. rapa* recipients.

Introgression risk

Finding *Brassica* hybrids in the Netherlands is still like finding a needle in a haystack, because sympatry between wild *B. rapa* and fields cropped to *B. napus* or feral populations seems rather low and no stages beyond the F1-hybrid were found. The fact that such hybrids are rare suggests that gene flow from *B. napus* to *B. rapa* may not occur in the Netherlands. However, *B. napus* could become more common along roadsides, especially when roadsides that are now bare because of spraying with herbicides are colonised by seeds of GM *B. napus* that are resistent against the herbicide, like the current situation in the US and Canada. Also even with a low chance of hybridization new allels can be introduced into the *B. napus* populations and, when they pose a selective advantage these genes will increase in frequency. From a laboratory experiment it became clear that if the F1-hybrid is backcrossed with *B. rapa,* 5% to 10 % of the backcross progeny has no extra C-chromosomes and hence resemble *B. rapa* cytogenetically (Lu and Kato 2001, Leflon et al. 2006). This percentage of 5 to 10% is too high to neglect. If a transgene is incorporated on an A-chromosome and without further selection against hybrids, the chance is 5-10% that the trangene is present in *B. rapa* in the BC1. After this initial hurdle a new allele with a selective advantage could rapidly increase in frequency. In practice this chance may be lower due to selection against hybrids but it is still substantial. Recent studies also show that recombination occurs more frequently in tetraploid and triploid *B. napus* than in diploid plants, especially between homologous chromosomes (Leflon et al. 2006, 2010, Nicolas et al. 2008). A transgene on a C-chromosome has a lower chance to be transmitted and can only be incorporated after recombination between chromosomes of the A and C set. Introgression risks are therefore much lower when transgenes are placed on C-chromosomes and not on A-chromosomes of *B. napus*. This recommendation has already been made several times by the COGEM, but because under the current legislation the applicant does not need to report in the Environmental Risk Assesment where in

the genome the transgene is placed, this recommendation is still without any consequences. Another recommendation that was mentioned in all other sub-projects, is that spillage of seeds should be prevented. Since it takes about three months after flowering for *B. napus* before the seeds are fully ripe, mowing of the plants before fruiting could already reduce the establishment strongly. We observed that a large (c. 50 flowering plants) *B. napus* population along a newly constructed roadside in Utrecht in 2009 had disappeared and was replaced by grass already in 2010 after mowing.

Concluding remarks

1. Co-occurrence of native *B. rapa* and cropped *B. napus* does not occur very frequently. Not many *B. rapa* populations were found in close vicinity to a *B. napus* site (cropping fields/transshipment site) and the number of *B. rapa* populations mixed with feral *B. napus* This reduces the possibility of gene flow between these two closely related plant species.
2. In three out of 27 sampled *B. rapa* locations F1-hybrids (AAC) were found (11% of the locations sampled). On these sites *B. rapa* grew together with feral *B. napus* while on the remaining sites, except for one, no feral *B. napus* was found.
3. The three sites with F1-hybrids were all characterised by an open soil due to recent disturbance. All sites were located in verges of a public road. These sites were either in close vicinity of the cultivation of *B. napus* (Meeuwen), in close vicinity of a transshipping location (Europoort) or in an area of historic extensive cultivation of *B. napus* (Almere carpool). In the latter situation, monitoring becomes more problematic, because it is rather unpredicatible where *B. napus* populatuion will emerge from the seed bank.
4. No plants with extra C-chromosomes are found, suggesting that no recent backcrossed plants have established. All sampled *B. rapa* plant had 20 chromosomes and a relative DNA amount typical for *B. rapa*. This suggests that these plants have the AA-genome. There could be several reasons why plants with more than 20 chromosomes are not found: i.e. reduced fitness of hybrids and backcrossed plants, low seed production or reduced establishment in unsuitable habitat
5. On two chromosomes of one out of the 22 AA-plants analysed genome painting highlighted large parts of the C-genome. This is most likely the result of

homeologous pairing between A- and C-chromosomes. It is not clear when this exchange has taken place, but it is an example of introgression.

6. It was not possible to predict which ecological parameters may enhance the establishment of hybrid plants, because too few sites with hybrid plants were found to perform a reliable statistical analysis.

Although introgression or accumulation of GM-traits could not be demonstrated in this survey, examples of introgression or gene stacking are found in other countries with a history of cropping *B. napus*.

Recommendations

1. It is preferable to insert the transgene on one of the C-chromosomes only and not on A-chromosomes, because a transgene on a C-chromosome has a lower chance to be transmitted, because it can only be incorporated after recombination between A and C.
2. One should prevent spillage of seeds wherever feasible.
3. Mowing of *B. napus* plants in road verges before the fruits are ripe is a possible method to prevent seed production.

4 Material and methods

Locations

In spring 2010 a search was performed to find hybrid or introgressed plants between *B. napus* and *B. rapa*. From a previous investigation (Luijten & De Jong 2010) it became clear that *B. rapa* is predominantly found in the western lowland part of the Netherlands and in the river valleys. The cultivation of *B. napus* is predominantly located in the east and south of our country, and especially in the northeast of Groningen (Gegevensmanagement Dienst Regelingen Assen, LNV). From a previous investigation in 2009 (Luijten & De Jong 2010) several sites were suspect of having hybrids (Wijk en Aalburg and Europoort) or F1-hybrids were already detected (Almere Carpool). These sites were revisited in 2010. Besides these locations new sites were searched with the help of the location of *B. napus* cultivation fields in the years 2005-2008 (Dienst Regelingen, LNV). We especially focused on areas where *B. napus* was cropped for several years and where *B. rapa* could potentially grow in not too large populations (Figure 1).

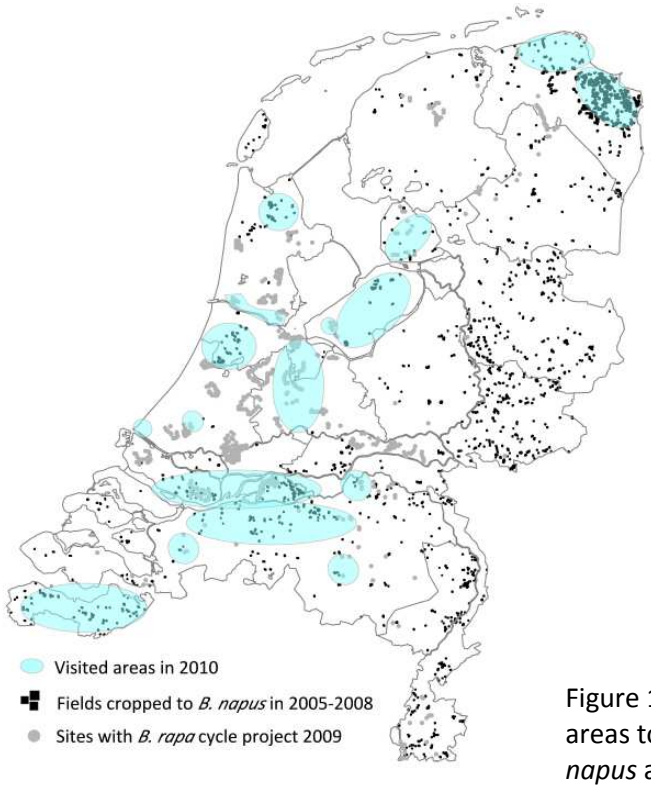

Figure 1. Overview of the investigated areas to find putative hybrids between *B. napus* and *B. rapa*.

Sampling

At each site we collected per sampled plant leaf material for flow cytometry and AFLP analysis, and 10 to 20 inflorescences with very small green flowerbuds to count the number of chromosomes and for painting of the C-genome chromosomes. We sampled especially those plants with a deviating morphology. A leaf was collected in a ziplock bag for flow cytometry analyses of DNA amounts. An additional small sample of leaf was sampled in 2 ml tubes to which a small glass ball was added for DNA extraction in the molecular lab. During transport leaves were kept cold in a cooling box. Leaves sampled for flow cytometry analysis were sent within 1-5 days after sampling to IRIBOV BV (http://www.iribov.nl). Leaf samples for AFLP analysis were kept at -80^{o}C until DNA extraction. For cytogenetic analyses we fixed the young flowerbuds in Carnoy's fixation solution (freshly prepared absolute ethanol (3 parts) : 1 glacial acetic acid (1 part)). After 24 hrs of fixation, when all plant material had turned white, the fixation fluid was replaced with 70% of ethanol to preserve the material until analysis. These tubes with flowerbuds were sent to dr. Hans de Jong (Laboratory of Genetics, Wageningen University).

At each sampling site a form was filled in recording the GPS coordinates, the number of *B. napus* and *B. rapa* plants, the percentage of bare soil, the average height of the vegetation, distance to the closest *B. napus* field or other *B. napus* activity (processing or transshipment location), species list with abundances (%). Usual plot size was approximately 10 m^2.

AFLP amplification

For the AFLP analysis *B. napus* and both parental species, *B. rapa* and *B. oleracea*, were screened to represent the three genomes AACC, AA and CC. For the AFLP analysis two kinds of sample sets were used. One sample set consisted of plants grown in the greenhouse in 2009. This sample set consisted of 13 different accessions of *B. napus* (N= 64 plants), four natural populations of *B. rapa* (N=20 plants) and ten hybrid plants grown from seed sampled in fruits on *B. rapa* plants growing adjacent a field cropped to *B. napus*. This group of plants consists predominantly of *B. napus*. The second group consisted of plants sampled in the field in 2010. Since we focus in the cross-pollination from *B. napus* tot *B. rapa*, this field data set consists nearly only of plants belonging to the species *B. rapa*. For *B.*

oleracea three varieties of *B. oleracea* (broccoli (N=1), cauliflower (N=1) and Brussels sprouts (N=2)) were screened. We sampled eight plants from a supposedly wild *B. oleracea* population at the Afsluitdijk near Breezanddijk, but since AFLP and flow cytometry clearly showed that the samples were *B. napus* instead of *B. oleracea*, these samples were excluded from the analysis.

AFLP analysis was performed according to the protocol described by Vos et al. (1995) with minor modifications. DNA was extracted using a modified method of the CTAB procedure of Doyle and Doyle (1987). Genomic DNA was double digested using *Mse*I and *Eco*RI restriction enzymes, carried out overnight (16 hrs) at 37°C. The digestion mixture contained genomic DNA, 5U EcoRI, 5U *Mse*I, 0.01 mg BSA, and 4 µl 10X restriction buffer (New England Biolabs react 4) in a final volume of 40 µL adjusted with distilled H_20. After the digestion step 10μL ligation mix was added to each sample. The ligation mix contained 5 pmol *Eco*RI adaptor, 45 pmol *Mse*I adaptor, 2.5 pmol ATP, 2U T4 DNA ligase (New England Biolabs) and 1 µL 10x restriction buffer (New England Biolabs react 4) in a final volume of 10 µL adjusted with distilled H_2O. The total end volume per sample after adding the ligation mixture to the restricted samples was 50 µl. Ligation/digestion took place at 37°C for 16 hours. The restriction/ligation mixture was diluted (1:2.5) and pre-amplified with AFLP pre-selective primers with one selective nucleotide at the 3' end (*Eco*+A and *Mse*+C). All PCR reactions were carried out using AFLP core mix (Applied Biosystems). The PCR conditions used for pre-selective amplification were based on a touchdown program: one step at 72°C for 2 minutes, followed by 12 cycli of 30 sec at 94°C., 30 seconds of annealing, starting at 65°C and an extension step for one minute at 72°C. The annealing temperature was subsequently reduced by 0.7°C for the next 12 cycles and was continued at 56°C for 22 cycles, followed by a final step at 60°C for 30 minutes. The pre-amplification product was diluted 20 times and then used as template for the selective amplification. The PCR conditions for selective amplification were the same as the pre-selective PCR except that the first step at 72°C for 2 minutes was replaced by a step at 94°C for 1 minute. Three primer combinations were used (*Mse*+CAA/*Eco*+ACA, *Mse*+CTT/*Eco*+AAG and *Mse*+CAA/*Eco*+AAG). The selective E-ANN primers were labeled with a fluorescent FAM label at the 5' end of the primer. All reactions were performed in a Tgradient PCR machine (Biometra). Reproducibility was checked by repeating the complete

AFLP protocol for one sample per population for each primer combination. Samples were run on a MegaBace 1000 capillary sequencer (Amersham Biosciences).

Chromosome counting and genome painting (BAC-FISH)

Chromosome slides and FISH protocol followed the methods described in Szinay et al. (2008) and Xiong and Pires (2011) with the following details. Flower buds were washed in (2 × 3 minutes) purified (MQ) water, then 10 mM Sodium Citrate buffer, pH 4.5 (1 × 3 minutes, then treated with a pectolytic enzyme mixture (1% Cytohelicase, 1% Pectolyase and 1% Cellulase RS) in a 10 mM Sodium citrate buffer for 2 hours at 37 °C. After digestion, flower buds were washed in MQ and kept the Sodium Citrate buffer on ice or in 4 °C fridge until further treatment. Using fine needles we placed the soft material onto a dry clean slide, covered it with a drop of acetic acid 45% and dissected the material until cells were completely separated. We then placed the slide on a hot plate (55 °C) for 2 minutes, while stirring the drop gently with a needle. The slides were fixed with ice-cold Carnoy's solution, air dried, and the quality of the preparation was checked under the phase contrast microscope equipped with no-cover glass objectives. Slides with a few cell divisions or poor spreading of the nuclei were discarded.

We used the BAC BNIH 123L05 from the *B. napus* library (Isobel Parkin, pers. comm.) that was donated by Professor Chris Pires (Division of Biological Sciences, University of Missouri, Columbia MO, 65211, USA). The repeats in the BAC allowed use to identify the C-genome chromosomes (Xiong and Pires 2011). The BAC DNA was labeled with biotin-dUTP by standard Nick-translation.

Slides were treated with 1% formaldehyde for extra fixation, followed by a short rinse in 2 × saline citrate solution (SSC). Then we incubated the slides with RNAse (100 µg/mL in 2×SSC) on the slide at 37 °C for 1 hour to degrade RNA, and washed the slides with 2×SSC and treated them with pepsin to remove part of the proteins that cover the chromosomes and nuclei. The slides were fixed with 1% formaldehyde. After the fixation, the slides were washed with 2×SSC and dehydrated with an ethanol series (70%, 90%, 100%).

A probe labelled with Biotin-dUTP was mixed with the hybridization mixture (50% formamide, 20% dextran sulfate), the mixture was denatured for 10 minutes at 100^{o}C and stored on ice before being added to the slides. Each slide that was covered with 20 µL probe mixture was denatured on a hot plate at 80 °C for 3 minutes. Hybridization was carried out overnight at 37 °C. After hybridization, slides were washed three times at 42^{o}C in a solution of 50% formamide/2×SSC for 5 minutes, followed by two washes of 5 minutes in 2×SSC.

The Biotin-dUTP labelled probe was detected by Texas Red-conjugated Avidin. The signal was amplified with Biotinylated anti-Avidin and Texas Red-conjugated Avidin. After the detection and amplification steps the slides were dehydrated with ethanol series (70%, 90%, 100%). Slides were air-dried, then stained with 12 µL DAPI inVectashield (50 µg/mL), and finally studied under a Zeiss Axoplan fluorescence microscopy, equipped with epifluorescence illumination and plan apochromatic optics. Images were captured with a Photometrics camera and combined in a multicolour layer mode using Genius Software of Applied Imaging.

Statistics

AFLP data were analysed with the genetic analysis package GenAlex 6 (Peakall & Smouse, 2006). The genetic relationship among all individuals was calculated based on Nei genetic distances. The result was visualized by performing a principal coordinates analysis (PCOA).

A oneway analysis of variance based on population means was used to test if the number of C-markers differed between the high-risk introgession group and the control group. The high-risk introgression group are *B. rapa* populations in the vicinity a *B. napus* activity (cropping and transhipment site). The control group are *B. rapa* populations for which we believe that a *B. napus* activity has been absent for several decades

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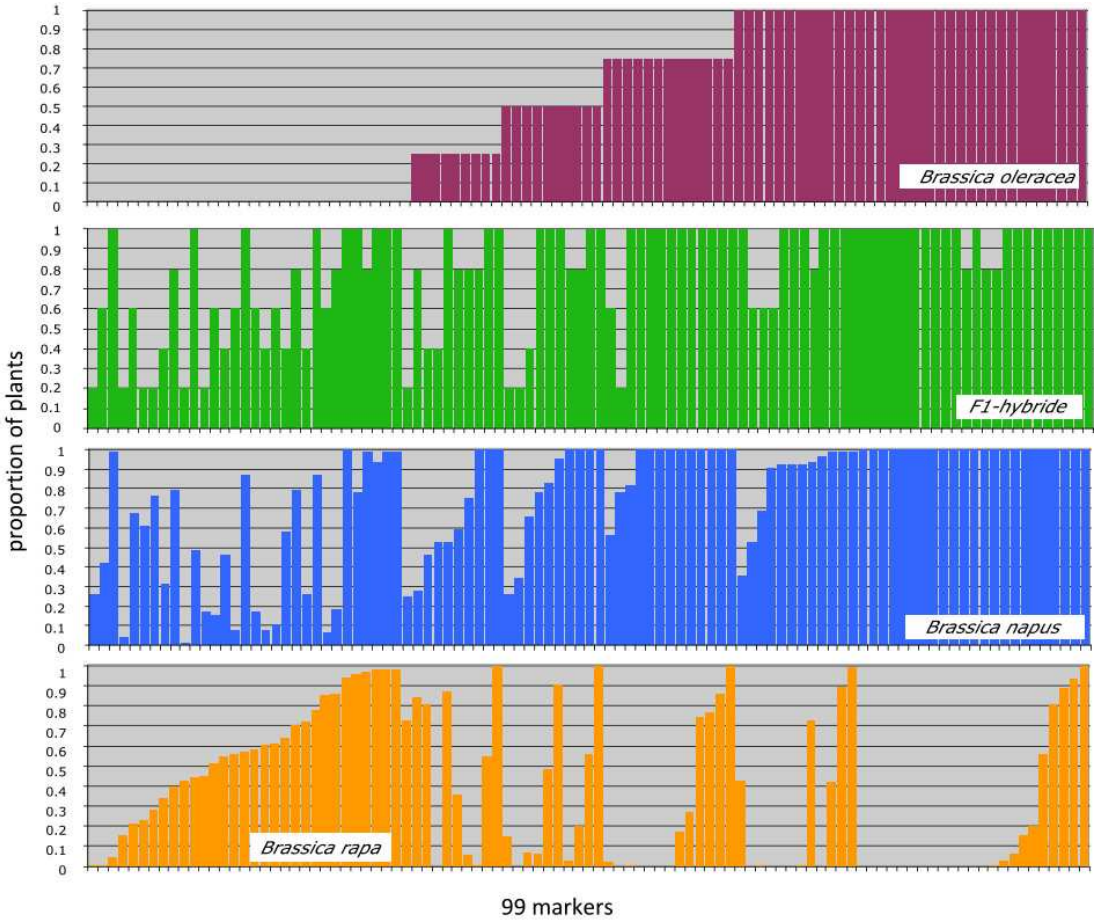
Annexes

Annex 1.

The proportion of plants for *B. rapa*, *B. napus*, *B. oleracea* and *F1-hybrid* per locus (marker) for the 99 markers representing either the A- and C-genome.



Annex 2.

Mean values and range for the measured ecological parameters and distances between *B. napus* and *B. rapa* for sites with and sites without hybrids.

	***B. rapa* site without hybrids (n=22)**		***B. rapa* with hybrids (n=3)**	
Ecological variables	mean ± se	range	mean ± se	range
Vegetation height (cm)	52.5 ± 5.28	20 - 100	45.0 ± 17.6	25 - 80
Grass cover (%)	51.1 ± 4.74	15 - 98	55.0 ± 21.8	15 - 90
Bare soil (%)	9.23 ± 2.58	0 - 35	23.3 ± 18.3	5 - 60
Relative nitrogen (Ellenberg)	0.51 ± 0.07	0.19 - 1.72	0.21 ± 0.04	0.15 - 0.30
Relative acidity (Ellenberg)	0.57 ± 0.12	0.10 - 2.77	0.20 ± 0.05	0.11 - 0.27
Relative moisture (Ellenberg)	0.44 ± 0.08	0.17 - 1.92	0.18 ± 0.04	0.11 - 0.24
Relative life history (Ellenberg)	0.38 ± 0.06	0.12 - 1.53	0.15 ± 0.02	0.12 - 0.20
***Brassica napus* related variables**				
Closest *B. napus* plants (m)	3889 ± 1175	5 - 17000	4.0 ± 3.0	1 - 10
Closest B. napus activity (m)	4298 ± 1219	5 - 17000	3687 ± 3657	1 - 11000
# *B. napus* fields within 1 km	1.24 ± 0.38	0 - 5	2.0 ± 1.0	1 - 4
# *B. napus* fields within 2 km	3.04 ± 0.79	0 - 10	2.33 ± 1.33	1 - 5
# *B. napus* fields within 5 km	10.9 ± 3.26	0 - 59	5.0 ± 4.0	1 - 13