A baseline study of the distribution and morphology of *Brassica napus* L. and *Brassica rapa* L. in the Netherlands

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Cover photos by Sheila Luijten
Front: Volunteer *Brassica napus* in northeast of Groningen
Back: Massive flowering of *Brassica rapa*

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Executive summary

In Europe, the cultivation of genetically modified (GM) *Brassica napus* (Dutch: “Koolzaad”) is prohibited, but the import of GM *B. napus* seeds is permitted. Until March 2009 no GM *B. napus* was imported as reported by the consent holders. Since March 2009 several GM *B. napus* events are authorised for import. However, given this short period, no official records for seed imports into the Netherlands are available. and thus whether import of GM *B. napus* has occurred during the last 12 months is unclear. However, the bulk of the seed imports of *B. napus* are from within Europe. It is known that *Brassica napus* can become established outside cropped fields in the ruderal landscape and is able to cross with a number of other crucifers belonging to the Dutch flora, in particular *Brassica rapa* (Dutch: “Raapzaad”). These characteristics are taken into account in the environmental risk analysis for genetically modified *B. napus*. Data on the occurrence of *B. napus* in the Netherlands might aid in the refinement of the risk analyis for GM *B. napus*.

The existing impression that *B. napus* is widespread in this country is presently under discussion, because of the confusion surrounding identification of *B. napus* and its closest relative, *B. rapa.* As a result, the current distribution of the species in the Netherlands is poorly understood. In this study we examine the morphological differences between *B. rapa* and *B. napus* and the presence of *B. napus* in the ruderal landscape to establish a baseline for its distribution. Against this background, recommendations are given how these results may be incorporated into the monitoring plans of (un-)expected adverse environmental effects as the results of the commercial releases of GM *B. napus* as required by EU directive 2001/18/EC.

Brassica napus (2n=38) is an allotetraploid derived from a hybridisation of *B. rapa* (2n=20) and *B. oleracea* (2n=18). This close relationship makes it hard to distinguish the first two (*B. oleracea* is easier to distinguish), but the difference in chromosome number permits identification with complete certainty. For the purpose of morphological re-examination, we collected a total of 78 accessions. The collection included old and new *B. napus* cultivars and seeds of *B. napus* and *B. rapa* from roadsides all over the Netherlands. We grew plants until flowering and seed set in an experimental garden in Leiden and, as a backup, in a greenhouse with natural light. On each plant we then measured 45 morphological traits to determine how much both species varied. Although the majority of traits differed significantly between the two species, they all exhibited an overlap, making it problematical to decide species identity on the basis of any one particular trait. Nevertheless, using statistical methods (discriminant analysis) nearly 100% of the plants could be assigned to the correct species. This method is too complicated for general use, however, and so a simplified “Libelle Method” was developed. With this method, which sums yes-no answers to a series of morphological questions, we hope to simplify species identification for a wider group than just the experienced florist. Even with this method, though, analysis does not yield 100% correct identifications. This shows that species identification based on morphological traits is by no means straightforward, so that data on *B. napus* distribution in the Netherlands as well as elsewhere in Europe needs to be treated with caution.

In the spring and early summer of 2008 and 2009 we searched for *B. napus* in areas where plants are likely to arise from seed spillage, i.e. around cultivation areas, harbours and processing facilities. For each observation we noted the location using GPS and counted the

number of *B. napus* plants and, if present, those of *B. rapa* and other relevant crucifers. In case of doubt, a leaf sample was collected to analyse the amount of DNA in the cells using flow cytometry. In 2009, the search was facilitated by the knowledge of the exact location of *B. napus* production fields in the years 2005-2008. Additionally, in 2009 numerous FLORON volunteers undertook cycling tours of approximately 15-20 km throughout the Netherlands in search of *B. napus* and *B. rapa*, quantifying the presence of each according to the FLORON abundance scale. To properly identify *B. napus* and *B. rap*a, we had previously developed an identification brochure, which was distributed to all the volunteers. Eventually, 85 of the 133 volunteers returned their data. The results of both investigations were very similar and showed that *B. rapa* is much more common and often present in large numbers along linear landscape elements, mainly in the western part of the country and in river valley areas. Observations of *B. napus*, on the other hand, were scattered fairly widely across the Netherlands, but in contrast to *B. rapa* observations were very local and usually concerned only a small number of plants (< 25). *Brassica napus* typically occurs in highly disturbed habitats and can often be traced back directly to seed spillage through human activity. Such activities include i) cultivation of *B. napus* as a crop, which occurs throughout the Netherlands with an emphasis on Groningen (outside of this region *B. napus* was rarely seen in road verges), ii) transport losses of seeds from trucks or freight trains, iii) seed losses near transshipment locations, iv) sowing of bird feed. Why the weediness of these two rather similar species differs so strongly is not clear and is the subject of an ERGO project currently being carried out at Leiden University in co-operation with the University of Amsterdam.

The possibility of hybridisation between *B. napus* and *B. rapa* is an important aspect of the risk analysis, because these crosses provide a route by which a transgene can escape from the crop into the natural vegetation. In this respect, the phenomenon of potential hybridisation is particularly relevant when *B. napus* is grown as a crop and *B. rapa* occurs as a nearby weed. While *B. napus* is mainly self-pollinating, *B. rapa* is strictly self-incompatible. As a result, the *B. napus* crop sires a high fraction of the seeds of any *B. rapa* individuals along the field edge. Although this relevant topic is beyond the scope of this project high numbers of hybrid seeds were found in fruits of *B. rapa* and a limited number of hybrid plants were found in the vicinity of a *B. napus* field. How well subsequent backcrosses to *B. rapa* fare and whether introgression occurs in the field is currently being investigated.

With respect to monitoring of (un)expected adverse environmental effects of GM plants, based on our findings of non-genetically modified *B. napus* we make the following recommendations. Because *B. napus* is found in the ruderal landscape near transshipment locations, along transportation routes and in road verges in the agricultural matrix with *B. napus* cultivation, it is advisable to focus on the areas with highest environmental exposure where substantial seed spillage could occur. This wider area will probably extend beyond the capabilities of the permit holder to conduct these monitoring activities themselves, thus the necessity for the involvement of other existing monitoring networks becomes apparent. It is important to harmonise the work of these different networks in order to have robust and statistically amendable data sets that can properly identify any adverse ecological impacts if they occur. Moreover, any changes to the natural environment will only be detectable if there is baseline information available on its natural state prior to introduction of the GMO at the location in question and in its surroundings. Monitoring the wildlife at GMO locations may not only be relevant in the case of cultivation, but since *B. napus* can spontaneously cross with *B. rapa*, and to a far lesser extent with other crucifers, it may be important as well that these feral plants would also be registered for all *B.*

napus-related activities as they may provide an escape path into the wild flora. The extensiveness of monitoring will need to depend on the scale on which seeds are potentially lost. In the case of import of *B. napus* seeds for processing, seed losses occur on the road from the harbour to the nearest pressing plant, which is a small and relatively well-defined area. As these considerations make clear, monitoring activities in the case of GM *B. napus* could be extensive. It could be argued that such monitoring becomes too complex for the permit holder and is probably best done by an independent organisation. Away from cropping areas, it is far less straightforward to monitor *B. napus* populations, as these are scattered and typically short-lived and may fall outside the responsibility of the permit holder. For the latter situation, a national “alarm system” based on a network of volunteers might provide a solution. With our new identification key it would be fairly easy to distinguish the two *Brassica* species, and with some training and expertise from a monitoring organisation this kind of alarm network could constitute a good alternative. In this way more focus can be directed. Thus the improved knowledge on the baseline distribution and morphology of *B. napus* can be incorporated into existing and future monitoring plans for commercial releases of GM *B. napus* in order to apply focus to those areas where adverse environmental effects are most likely.

Nederlandse samenvatting

De teelt van genetisch gemodificeerd (GM) koolzaad (*Brassica napus*) in Europa is op dit moment verboden, maar import van zaad van een aantal GM koolzaadlijnen is wel toegestaan, en wel sinds maart 2009. Voor die tijd vond geen import plaats van GM koolzaad. Tot op heden zijn nog geen rapportages (jaarlijkse monitoringverslagen) van de vergunninghouders beschikbaar die cijfers bevatten van de hoeveelheid GM zaad dat voor koolzaad geïmporteerd wordt naar Nederland. Wel is duidelijk geworden dat het overgrote deel van de zaden dat naar Nederland wordt geïmporteerd afkomstig is uit Europa. Koolzaad is geen inheemse plantensoort en het is bekend dat het zich kan vestigen in het Nederlandse landschap. Daarnaast kan de soort kruisen met een aantal andere kruisbloemigen (*Brassicaceae*) en wel in het bijzonder met het inheemse raapzaad (*Brassica rapa*). Deze kenmerken worden meegenomen in de milieurisicoanalyse van genetisch gemodificeerd koolzaad. Data over de verspreiding van koolzaad in Nederland kan helpen om de risicoanalyse voor GM koolzaad te verfijnen.

Het algemene beeld dat Nederland vol staat met koolzaad wordt de laatste tijd in twijfel getrokken vanwege mogelijke verwarring met raapzaad. De beschrijving in de flora is niet duidelijk in het onderscheid tussen beide soorten en uiteindelijk wordt er meestal een keuze gemaakt ten gunste van koolzaad, omdat dit het algemene beeld bevestigt. Door deze verwarring is het onduidelijk hoeveel koolzaad er voorkomt in ons landschap. Om hier duidelijkheid in te krijgen zijn in dit project de morfologische verschillen tussen koolzaad en raapzaad en de verspreiding van deze soorten in Nederland onderzocht om een nieuwe baseline vast te stellen van het voorkomen van koolzaad in Nederland. Aan de hand van de uitkomst geven wij aanbevelingen hoe onze resultaten kunnen worden ingepast in monitoringsplannen voor ongewenste ecologische effecten zoals die zijn vereist door de EU Directive 2001/18/EC bij het commercieel op de markt brengen van GM koolzaad.

Koolzaad (2n=38) is een allotetraploïd die ontstaan is door (kunstmatige) hybridisatie van raapzaad (*Brassica rapa*; 2n=20) en kool (*Brassica oleracea*; 2n=18). Door sterke verwantschap tussen koolzaad en raapzaad is het lastig om beide soorten op morfologische kenmerken van elkaar te onderscheiden, maar het verschil is duidelijk aan te tonen door meting van de hoeveelheid DNA met behulp van flowcytometrie. Om duidelijkheid te krijgen in de morfologie van beide soorten zijn in totaal 45 uiterlijke kenmerken onderzocht van 78 accessies. Deze collectie bestond uit een reeks oude en recente koolzaadlijnen en uit planten opgekweekt uit zaad van koolzaad en raapzaad uit bermen van diverse locaties in Nederland. De planten zijn vanuit zaad opgekweekt en buiten in een proeftuin van de Universiteit Leiden opgegroeid tot het stadium van vruchtzetting. Een controle-experiment werd uitgevoerd in een kas met natuurlijk licht. Alhoewel het merendeel van de 45 onderzochte kenmerken significant verschillend was, vertoonden alle kenmerken overlap en dat maakt de determinatie op basis van een enkel kenmerk onmogelijk. Met behulp van statistische methoden (discriminant analyse) was het mogelijk om bijna 100% van de planten toe te wijzen aan de juiste soort. Omdat deze statistische methode te gecompliceerd is voor algemeen gebruik, hebben we een andere eenvoudige en objective methode ontwikkeld: de Libelle Methode. Met behulp van de deze methode, die het aantal ja- en nee-antwoorden op een serie morfologische vragen optelt, is de determinatie van beide soorten toegankelijk voor een grotere groep dan alleen ervaren floristen. Ook de Libelle methode geeft niet altijd een correcte determinatie. Onze resultaten laten zien dat een 100% goede determinatie van beide soorten niet altijd eenvoudig is, hetgeen betekent dat oudere verspreidingsgegevens van koolzaad in Nederland, en wellicht ook elders

in Europa, fouten zullen bevatten.

In het voorjaar en begin van de zomer van 2008 en 2009 hebben we gezocht naar koolzaadplanten in gebieden met een hoge waarschijnlijkheid van het morsen van zaden, bijv. teelt- en overslagterreinen en in de buurt van olieperserijen. Voor iedere observatie werd de locatie vastgelegd met een GPS en het aantal koolzaadplanten geteld. Ook telden we het aantal planten van raapzaad en eventuele andere relevante kruisbloemigen. Bij twijfel aan de juiste soort, koolzaad of raapzaad, werd een stukje blad verzameld om met behulp van flow-cytometrie de hoeveelheid DNA vast te stellen. In 2009 werd de zoektocht naar koolzaad vergemakkelijkt doordat we de beschikking hadden over de precieze ligging van de koolzaadvelden in de jaren 2005-2008. Daarnaast hebben we een inventarisatieproject opgezet om met behulp van active vrijwilligers van de Stichting FLORON, op landelijke schaal kool- en raapzaad te inventariseren langs fietsroutes. Speciaal voor dit doel is een determinatiekaart ontwikkeld die verstuurd is aan al deze vrijwilligers. De fietstochten waren 15-20 km lang en de hoeveelheid kool-en/of raapzaad langs de route werd vastgelegd m.b.v. de FLORON abundantieschaal. Van de 133 deelnemende vrijwilligers gaven er 85 hun resultaten door. De resultaten van beide studies waren zeer vergelijkbaar en lieten zien dat raapzaad veel algemener is dan koolzaad. De populaties van raapzaad zijn meestal veel groter en lijnvormig, met name in de laagveengebieden in het westen van het land en in het rivierengebied. In het oosten en zuiden van ons land, op de hogere zandgronden, is raapzaad niet echt algemeen. Koolzaad wordt daarentegen wel overal in Nederland waargenomen (zowel landelijk als stedelijk). Koolzaadpopulaties zijn meestal klein in aantal (< 25 planten). Deze soort wordt meestal gevonden op plaatsen waar de bodem verstoord is geweest en de aanwezigheid van de planten is dikwijls te herleiden tot het morsen van zaad bij teelt, overslag en transport en het strooien van vogelvoer. In de directe nabijheid van koolzaadvelden in Noordoost-Groningen werden planten waargenomen, maar dit was nauwelijks het geval in de rest van het land. Waarom het onkruidkarakter van koolzaad en raapzaad zo duidelijk verschilt is niet duidelijk. Dit is het onderwerp van een ERGO-project dat momenteel uitgevoerd wordt bij de Universiteit Leiden in samenwerking met de Universiteit van Amsterdam.

De mogelijke hybridisatie tussen koolzaad en raapzaad is een belangrijk aspect van de risico-analyse, omdat via kruisingen een transgen kan ontsnappen van het cultuurgewas naar zijn nauwe verwanten in de natuurlijke vegetatie. Daarom is het belangrijk om de omvang te verruimen van het te monitoren gebied. Dit aspect is met name relevant in situaties waar raapzaad in de randen of bermen voorkomt en koolzaad geteeld wordt of daar waar regelmatig koolzaad gemorst wordt. De kans dat raapzaad bestoven wordt door koolzaad is groot omdat raapzaad een obligate kruisbestuiver is en koolzaad een zelfstuiver. De kans is met name hoog voor kleine populaties raapzaad, door een gebrek aan raapzaad- en een overdaad van koolzaadstuifmeel. Tijdens dit onderzoek hebben we gevonden dat voor alleenstaande raapzaad planten vlak naast het gewas, soms de helft van het aantal zaden in vruchten van raapzaad was bestoven door koolzaad uit de aangrenzende akker. Ook zijn we hybride planten tegengekomen in bermen. In hoeverre verder terugkruisingen in het wild leiden tot hybriden en introgressie van koolzaadgenen in raapzaad is onduidelijk. Hier wordt momenteel onderzoek naar gedaan.

Wij geven de volgende aanbevelingen. Koolzaad wordt gevonden in bermen nabij overslag, langs transportroutes en in bermen waar veel koolzaad geteeld wordt. Daarom raden wij aan om de aandacht te richten op die gebieden waar het meeste zaad wordt gemorst. Dit is een

groter gebied dan waar nu in eerste instantie de aandacht ligt en dit overschrijdt zeer waarschijnlijk het vermogen van de verantwoordelijke vergunninghouder van GM koolzaad om de monitoring zelf uit te voeren. Dit betekent dat de betrokkenheid van andere bestaande monitoringnetwerken vanzelfsprekend wordt. Het is belangrijk om de werkwijze van deze netwerken zo af te stemmen dat mogelijk ongewenste ecologische effecten op een statistisch betrouwbare wijze herkend worden. Een effect op het milieu kan echter alleen maar vastgesteld worden indien de natuurlijk staat van het milieu in de baseline is vastgesteld voor de introductie van de GMO. Monitoring van alleen "wildlife" op GMO locaties is misschien niet voldoende bij teelt, omdat koolzaad kan uitkruisen. Het is belangrijk om deze ferale planten op te merken in gebieden waar koolzaad gerelateerde activiteiten plaatsvinden, omdat dit een ontsnappingsroute kan zijn voor transgenen. Monitoring hangt af van de schaal waarop zaden gemorst worden. In het geval van import van koolzaad voor de verwerking zullen zaadverliezen voorkomen langs de weg van de haven naar de olieperserijen. Deze route is meestal kort en het betreft een goed afgebakend gebied. Zoals deze overwegingen duidelijk maken, kunnen monitoringsactivteiten dus intensiever blijken dan in eerste instantie gedacht wordt. Men kan zich dus afvragen of monitoring niet te complex wordt voor de vergunninghouder en dan beter door een onafhankelijk organisatie uitgevoerd kan worden. Buiten de teeltgebieden is het veel minder gemakkelijk om de effecten van GMO koolzaad op de omgeving te monitoren, omdat koolzaad verspreid over Nederland wordt waargenomen in kleine populaties die waarschijnlijk een korte levensduur hebben. Bovendien bevinden die locaties zich ver de plek waar zaden in grote aantallen worden ingevoerd. Voor deze situatie is het wellicht goed om een landelijk alarmsysteem te realiseren dat bijvoorbeeld bestaat uit vrijwillige waarnemers. Met onze nieuwe determinatiekaart zou het relatief makkelijk moeten zijn om beide Brassica soorten te kunnen onderscheiden. Een dergelijk alarmsysteem zou met training en de expertise van een monitoringsorganisatie een goed alternatief kunnen zijn. De hier geleverde kennis van de baseline wat betreft de verspreiding en morfologie van koolzaad kan worden ingepast in bestaande en toekomstige monitoringsplannen die nodig zijn bij het toelaten van GM koolzaad op de markt.

1. General introduction

Since the introduction of genetically modified (GM) crops, the area devoted to their cultivation worldwide has steadily grown. To date, GM crops have been grown mainly in countries outside Europe (United States, Argentine, Canada, Brazil, China and South Africa). There is no cultivation of GM crops in the Netherlands, although it is permitted to import e.g. GM corn and GM *B. napus* products. With the introduction of GM organisms, weediness and crossing with wild relatives has become a subject of widespread discussion. For crops like corn or cotton that do not establish feral populations and do not cross with plants in the wild flora of Europe, this is of no serious concern. For *Brassica napus* the situation differs, though, for it can hybridise with other (native) species, and especially with its close European ally *Brassica rapa*. In a addition, it is found in a variety of ruderal habitats in the agricultural and urban landscape. The ferality of *B. napus* is still unclear, although the species is included in several European floras, suggesting it is naturalised in many countries. In the light of the ongoing debate on GM crops, it is pointed out in several floras that distribution maps may be unreliable, as *B. napus* could have been confused with *B. rapa* (Preston *et al*. 2002, Van der Meijden 2005). In this report we focus on the distribution of *B. napus* in the Netherlands, in order to provide a new baseline for its distribution, and on the identification of *B. napus* and *B. rapa* [1].

1.1. Origin of *Brassica napus* L.

In 1935 the Korean botanist U, working in Japan, created crosses between different Brassicas, thus resynthesising species that were already known. *Brassica napus* L. turned out to be an allotetraploid (2n=38) derived from a hybridisation event between *Brassica rapa* L. (2n=20, AA genome) and *Brassica oleracea* L. (2n=18, CC genome) (Fig.1).

The simplest way to create such an AACC allotetraploid is to cross an AA with a CC plant to produce an AC hybrid, then apply colchine, a toxin that interferes with cell division, and then induce self-pollination. Although this somatic mutation is the simplest way to generate

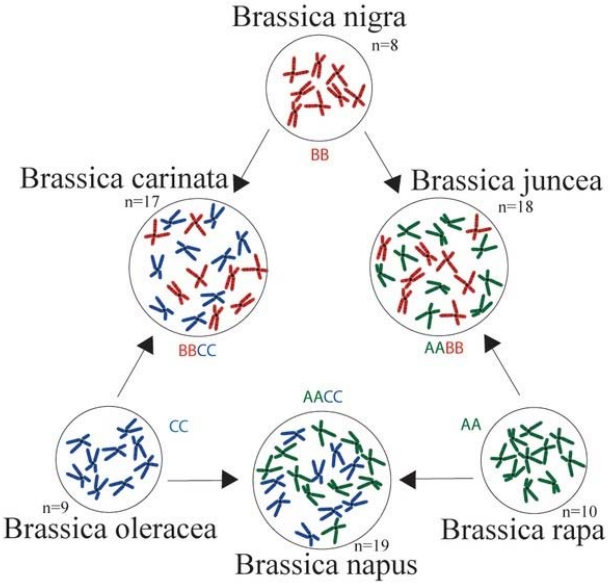

Figure 1. The triangle of U (1935). *Brassica napus* is an allotetraploid, containing both the AA genome of *Brassica rapa* and the CC genome of *Brassica oleracea*.

[1] Because of the profusion of non-scientific names for *Brassica napus* and *Brassica rapa* in various documents in the literature and within organisations, to avoid confusion we shall use the scientific names only.

tetraploid hybrids, it is not certain that this is the route most frequently followed in nature. Gametes are frequently unreduced and a fusion of AA pollen and CC eggs could also produce an allotetraploid, either directly or through a triploid bridge species (Levin 2000). It is now thought that the AA and CC genomes diverged about 3.75 million years ago (Inaba & Nishio 2002) and that they are still quite similar. On 9 November, 2009 Bayer Crop science announced the sequencing of the entire *B. napus* genome, and thus also of the A and C genomes, (http://www.baynews.bayer.de). Within the *B. napus* genome the AA and CC genomes have remained distinct (Howell *et al*. 2008), suggesting that homeologous recombination (between a chromosome of the A and C set) and subsequent mixing on one chromosome of chunks of DNA from A and C chromosomes is a rare phenomenon.

In 1753 *Brassica napus* was first described by Carl Linnaeus (http://www.linnean-online.org/7723/). In addition, he described *B. campestris* (http://www.linnean-online.org/7716/), which he collected in the wild in Sweden, and a cultivated type that he named *B. rapa*. His *B. rapa* included cultivars that later became denoted as subspecies, such as ssp. *rapa* (turnip, with edible swollen roots, in Dutch "knolraap" or "stoppelknol"), spp. *oleifera* (turnip rape or Chinese colza, used for seed-extracted oil; in Dutch "raap" or "raapzaad"), spp. *pekinensis* or spp. *chinensis* (Chinese cabbage, a vegetable, in Dutch "Chinese kool" or "pak choi kool"), and spp. *perviridis* (spinach mustard, again a vegetable, in Dutch "raapsteeltjes"). Metzger (1833, cited in Oost *et al*. 1987) made a detailed study of the *Brassica*s and concluded that *B. rapa* and *B. campestris* should be regarded as a single species. He grouped the two species under the name *B. rapa*. Later studies confirmed Metzger's conclusions by showing similarity in chromosome numbers and demonstrating fertility of the hybrid offspring. Based on molecular analysis, Takuno *et al*. (2007) suggested that the primitive cultivated type of *B. rapa* originated from the wild type in Europe or Central Asia and was then transferred to Eastern Asia, where it was further modified by breeding. These authors note that the wild *B. rapa* ancestor is common in Europe, while such wild populations are absent in Asia.

Finds of seeds near Neolithic and Bronze Age sites suggest that *B. rapa* may already have been an oil-bearing "tolerated weed" as far back as 9500 BC (Zohary & Hopf 2000). Seeds of *B. rapa* have frequently been found in archaeological excavations (Relational Archaeobotanical Database Advanced Research (RADAR), van Haaster & Brinkkemper 1995); the oldest find in the Netherlands is from Wateringen (south of The Hague) and dates to between 3700 and 3600 BC. A careful description of the seeds of different *Brassica* species is provided by Brinkkemper (1993), who found *B. rapa* seeds in many samples taken near Iron Age farms in Spijkenisse.

The exact origin of *B. napus* is unknown, but several sources mention that the species was already present in the early Middle Ages (OECD consensus document, Anonymous 1997). For instance, Westhuis (2008) cites historical sources that claim that as early as 1421 AD *B. napus* (i.e. "koolzaad") was a popular crop in Holland. In view of the confusion about the common names of *B. napus* and *B. rapa* such historical sources should probably be treated with caution. However, the 15th century dating is consistent with the few archaeological reports of *B. napus* seeds that are included in the RADAR database.

It seems likely that the first *B. napus* hybrid was formed when *B. rapa* and *B. oleracea* were cultivated alongside one another, as suggested by Zohary & Hopf (2000). Other authors point to Southern Europe as the region where the natural distributions of *B. rapa* and *B. oleracea* meet and where hybridisation may have occurred. Since no natural populations of *B. napus* are known, this question about species origin will probably remain unanswered. *B. napus* can be resynthesised by crossing *B. oleracea* and *B. rapa* (Song *et al*. 1993). Molecular evidence has shown that this hybridisation event has occurred several times in the past; Song and Osborn

(1992) found that cytoplasms of two *B. rapa* types, from *B. oleracea* and of *B. montana*, were present in different *B. napus* cultivars, suggesting at least four hybridisation events in the past.

1.2. Hybridisation

Fitzjohn *et al*. (2007) have reviewed the available data on reproductive compatibility and found that 23 species were able to successfully hybridise with *B. napus*. However, the rate of hybrid production varied among species and studies. Crosses in both directions between *B. napus* and *B. rapa* were very successful, especially when *B. napus* was the female parent in the cross. In contrast, crosses between *B. napus* and *B. oleracea* were difficult in both directions. Besides successful experimental hybridisation with other *Brassica* species like *B. juncea* and *B. nigra*, 15 successful intergeneric crosses have been reported with *B. napus*. Spontaneous and natural hybridisation experiments involving *B. napus* are far fewer in number (Fitzjohn *et al*. 2007), but may occur with species that are also common and native to the Dutch flora (*B. rapa*, *B. nigra*, *B. oleracea*, *Sinapis arvensis*). Introgression, incorporation of transgenes in populations of wild relatives, is only possible after repeated backcrosses. Successful backcrosses in the lab have been reported between *B. napus* and its progenitors, *B. rapa* and *B. oleracea*, with other Brassica species like *B. juncea* and *B. nigra* and with certain other species (e.g. *Sinapis arvensis*). Although many studies (Fitzjohn *et al*. 2007) demonstrate that hybridisation and backcrosses between *B. napus* and other species are possible, whether introgression actually occurs in the field probably also depends on the fitness of the crosses and on local ecological and environmental conditions, including pollinator availability and chances of establishment. In the case of a cross between *B. rapa* (AA) and *B. napus* (AACC), the hybrid has the AAC genotype.

Leflon *et al*. (2006) made crosses between *B. napus* (AACC) and *B. rapa* (AA) to produce an AAC hybrid, which they subsequently backcrossed to AA. In these backcrosses the C chromosomes are unpaired, but these unpaired chromosomes of the C genome can still be passed on to future generation as singles, by homeologous pairing or adhering to sets of A chromosomes. All these phenomena were observed in the backcrosses made by Leflon *et al*. (2006). If C chromosomes were transmitted as univalent to gametes, one would expect that in a cross between AAC and AA on average 4.5, i.e. half of all nine C chromosomes, would be transmitted to the offspring. The frequency of C chromosomes in the offspring deviated significantly from the expected binomial distribution around this mean of 4.5, however (Leflon *et al*. 2006). Apparently, some chromosomes are more readily lost than others. Nevertheless, the simple idea that C chromosomes are lost at a rate of around 50% in each subsequent cross to an AA plant is close to reality.

If *B. napus* can cross with *B. rapa* in the field and the hybrids are fertile, we should find plants with an AA genome but with different numbers of extra C chromosomes. How often these backcrosses occur and under what ecological conditions introgression arises is not well understood.

Table 1. Extra chromosomes (on top of the expected 2n=20) in crosses between AAC and AA plants, data from 3 crosses pooled (284 plants in total). From Table 3 in Leflon *et al*. (2006).

	0	1	2	3	4	5	6	7	8	9
Obs. freq. %	3.5	5.1	11.8	9.9	12.2	14.7	14.8	10.8	11.2	5.9
Exp. freq.%	0.2	1.8	7.0	16.4	24.6	24.6	16.4	7.0	1.8	0.2

1.3. Identification and distribution of *Brassica napus* and *Brassica rapa*

Of the three native parental species of *B. napus*, *Brassica rapa* has the widest European geographical distribution, occurring throughout Europe from the lowlands to the mountains (OECD consensus document, Anonymous 1997). *Brassica oleracea* only occurs along the Mediterranean coast from Greece to Spain and along the Atlantic coastlines of Spain, France, England and Helgoland. *Brassica montana* has the narrowest distribution: the Mediterranean coastal area between Italy and Spain. Whether *B. napus* should now be considered a native species in Europe is not clear. With respect to this species the OECD consensus document states: "In Europe, it is predominantly the winter form which has become a common yellow crucifer found along roadsides, on waste sites and cultivated ground, on docks, in cities and towns, on tips, and on arable fields and along riverbanks. In the British Isles, it has been naturalised wherever oilseed rape is grown." Whether *B. napus* is indeed common in the Netherlands and forms more or less stable "naturalised" populations without further anthropogenic seed input is one of the central questions of the present research project.

According to the last, 23rd edition of the Heukels' Flora (Van der Meijden 2005), *B. napus* and *B. rapa* are both common in the Netherlands, while the wild form of cabbage *B. oleracea* subsp. *oleracea* is only known from a few coastal sites. This Flora notes that *B. napus* has often been confused with *B. rapa* and that previous references to *B. napus* were often in error. This error is also apparent in FLORON[2], observations since 1975, which at first sight suggest that *B. rapa* has greatly increased in number from 1990 onwards (Figure 2). The 21st edition of the Heukels' Flora, dating from 1990, included an unclear drawing of the two species. The 22nd edition of the same Flora, dating from 1996 and edited by Ruud van der Meijden, explicitly warned that *B. rapa* was often mistaken for *B. napus*. It also included a clear picture of the position of the buds of the two species in relation to the flowers. This apparently led to an increase in the number of observations of *B. rapa*. Since 1998 the ratio at which observations of the two species are reported to FLORON has remained more or less constant. The FLORON species checklists of 1974 mentioned neither *B. napus* nor *B. rapa* (Odé 2009, personal communication) and several Dutch botanists have confirmed to us that in the period before 1990 the two species were generally both referred to as "koolzaad", the name now reserved for *B. napus*.

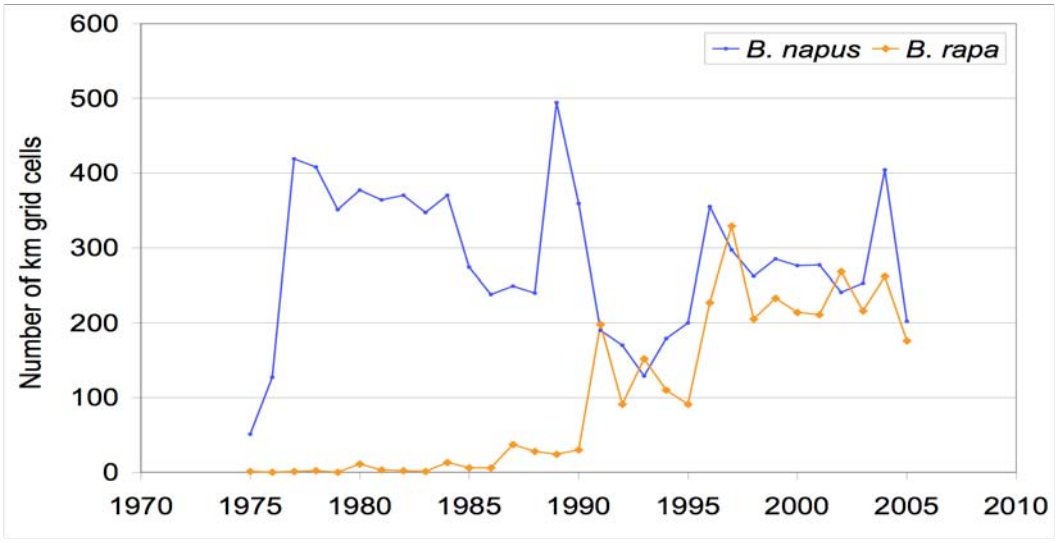

Figure 2. Yearly observations of *Brassica napus* and *Brassica rapa* in the Netherlands from 1975 to 2005, as recorded in the FLORON database. The number of observations (number of kilometre grid cells) fluctuates from year to year.

[2] An organisation that collects plant distribution data with the help of volunteers

It is highly unlikely that *B. rapa* was initially a rare species in the Netherlands that only occasionally escaped from cropland, as Figure 2 seems to suggest. Finds of *B. rapa* seeds are reported in the RADAR archaeological database, for example, and several old European floras (e.g. “Withering’s British plants”, MacGillivray 1856) pointed out that *B. napus* escapes from fields and is not indigenous, whereas the wild form of *B. rapa* is. The remark in the OECD consensus document that *B. rapa* is native throughout Europe is, in our opinion, entirely correct, also for the Netherlands.

The confusion in species identification cited above means the present distribution and relative abundance of *B. napus* and *B. rapa* in the Netherlands are essentially unknown. In short, it is not known whether *B. napus* is to be deemed part of the Dutch flora or whether it is only temporary populations that are established from spilt seeds, with such populations readily going extinct in the absence of further human interference.

1.4. Project aims

Identification problems may have led to overestimation of the presence of *B. napus* in the Netherlands. In addition, no information is available on the number of plants per observation or the persistence of any feral populations. To improve understanding of the presence of feral *B. napus* in the Netherlands these issues are addressed in the present study. The envisaged steps of the research were formulated as follows:

1. We will re-examine the identification of *B. napus* and *B. rapa* by measuring morphological characteristics in a field experiment, focusing on traits that are easy to measure and combine, so that anyone engaged in monitoring will be able to tell the two *Brassica*s apart.

2. We will visit areas where *B. napus* could potentially have established from seed spillage and record the number of plants and, if possible, the persistence of populations.

3. With the help of volunteers from FLORON we will update the presence of *B. napus* and *B. rapa* country-wide. We will provide volunteers with an identification brochure based on our preliminary measured morphological characteristics and ask them to give an estimate of the number of plants according to the FLORON abundance scale.

4. Depending on the outcome of the distribution data, we will suggest how monitoring plans for *B. napus* can be developed that measure whether this species is increasing in abundance.

5. An attempt will be made to find hybrids between *B. napus* and *B. rapa* at sites where both species occur, i.e. along land cropped to *B. napus* with a wild population of *B. rapa* available. Hybrids can be recognised at the genetic level by analysing the amount of DNA in the cell using flow cytometry; hybrids will have levels intermediate between the parental species.

2. Identification of *Brassica napus* and *Brassica rapa*

To properly investigate the distribution of *B. napus* one needs to be able to distinguish *B. napus* from *B. rapa* in the field on the basis of morphological characteristics. Since not all the morphological traits described in the Heukels' Flora were useful in discriminating between the two species, a new identification brochure was created. This brochure was distributed to FLORON volunteers in the spring of 2009.

2.1. First impressions of morphological traits and identification brochure

By way of initial re-examination of identification traits, we sampled and measured 2-3 plants per site of both *Brassica* species, collected during our first inventory of feral populations of *B. napus* at the start of the project in the summer of 2008. We sampled *B. napus* plants from wild populations and from cropland. *Brassica rapa* was only collected from wild populations. Later in the season, mature fruits were collected from a subset of the sample sites.

For this initial analysis the selected traits were based on the Heukels' Flora (23^{e} edition). The following traits were measured: ***a***) colour of the basal leaves and upper leaves (glaucous or grassy-green), ***b***) presence of hairs on the leaves (yes or no), ***c***) clasping of the stem by the upper leaves (approx. 50 % or approx. 100%), ***d***) position of the buds relative to the open flowers (above, in between, under), ***e***) various measures of flower size (length and width), ***f)*** length and width of the petal limb, ***g***) overlap of the sepals (yes or no), ***h***) position of the sepals (adjacent, 45^{o} angle, 90^{o} angle), ***i***) length and width of the siliqua, and ***j***) length of the beak. From the total length and width of the flower we calculated the diameter (***k***) and from the total length of the siliqua and beak the ratio of beak to siliqua (***l*)**. Flower characters (***e,f,g,h***) and the beak-siliqua ratio (***l***) are extra measures that are not given in the most recent Heukels' Flora. For the purpose of illustration we prepared photographs of all these traits.

From the initial investigation, in which species identity was confirmed by flow cytometry data (for method, see 2.2.2), it became clear that certain characters were less discriminating than the Flora suggested, in particular the position of the buds relative to the open flowers and the position of the sepals. Several other traits proved rather impractical. For instance, the colour of the basal leaves is a good discriminating trait, but these leaves soon start to wither as flowering proceeds. Clasping of the stem by the upper leaves appeared to be a good trait that can also be observed during flowering. This seems to be the case for the overlap of the petals and the beak-siliqua ratio, too.

The results were used to prepare an identification brochure (Figure 3). On one side of the brochure are detailed descriptions of the traits, illustrated by photographs and drawings, while the other side provides a summarised description of the two *Brassica* species and four other common yellow-flowering look-alike Brassicaceae. We did not anticipate any confusion with these species or other look-alikes, because *B. rapa* and *B. napus* are the only two yellow crucifers in the Dutch flora with sessile, (deeply) broadened cordate leaves on the stem.

Figure 3. Detailed identification brochure for *Brassica napus* and *Brassica rapa* and four other common yellow-flowering crucifers.

2.2. Materials and methods: Differences between *Brassica napus* and *Brassica rapa*

2.2.1. Quantification of identification traits of cultivated plants

To quantify the morphological traits we collected seeds in the summer of 2008 from wild populations (*B. napus* and *B. rapa*) and two cropped fields (*B. napus*). From the CGN (Wageningen) we ordered 32 old Dutch cultivars dating back to the period 1899-1985, two cultivars from the USA, three from Canada, three from the Ukraine, and three from Russia. Recent cultivars of *B. napus* were ordered from three breeding companies: Pioneer (lines PR-45D03 and PR-46W31), Limagrain (Ladoga) and Deutsche Saat Veredelung (DVS) (lines Oase, Billy, Hornet and Lioness). In addition, we cultivated tetraploid *B. rapa*. In total we grew 78 lines. The full list of cultivars and wild populations is given in Appendix 1.

For each cultivar or sampled population 15 seeds were germinated in petri-dishes at 20^0 C in a climate chamber. After germination, ten seedlings per cultivar/population were planted out in small pots in another climate chamber and kept at 20^0 C under an 8hr/16hr night/day regime until the plants were large enough to be planted outdoors. In November 2008 the plants were planted out in an experimental garden, being planted at random in 10 blocks. The area was fenced off to keep out rabbits. Later a bird net was installed to exclude pigeons and prevent further damage to the young plants. In December 2008 a backup of the experiment was set up in a climate chamber. This backup allowed us to examine whether a trait is consistently different between the two species or whether this depends on environmental conditions. Because of the limited space in the climate chamber, only seven plants per cultivar/population were planted in pots. The plants were initially kept at 20^0 C under an 8hr/16hr night/day regime. After one month the night temperature was set to 10^0 C and the day temperature to 15^0 C, but the night/day regime was kept the same. In March 2009 the backup experiment was placed outside in a temporary greenhouse to give the plants more growing space and “more realistic” growing conditions than in the climate chamber. Traits were measured in May and June in the garden and in June, July and August in the greenhouse.

A total of 45 traits were assessed and scored. These involved colour, structure and hairs on basal, middle and upper leaves, depth of the broadened cordate leaf and the amount of leaf

clasping around the stem, and various measurements of flower and fruit characteristics. The full list of measurements is given in Table 2.

2.2.2. Species identification via chromosome levels

Because *Brassica napus* (2n=38) is a hybrid between *Brassica rapa* (2n=20) and *Brassica oleracea* (2n=18) the two species can be accurately distinguished from the amount of DNA they contain per cell, which is higher in *B. napus* than in *B. rapa* (Figure 4).

The amount of DNA in a cell can be estimated using flow cytometry. With this technique we verified the species identity of all the plants in the field experiment, along with a subset of the plants sampled in the first flowering season in 2008. From each measured plant a young leaf was sampled. Leaves were analysed by an independent laboratory (www.iribov.nl) specialised in flow cytometry. These samples were analysed in conjunction with a reference sample that served as a standard (*Brassica oleracea*). With the aid of Hans de Jong and Xianwen Ji of the Wageningen University Laboratory of Genetics, we verified that the chromosome numbers corresponded well with the flow cytometry data (Appendix 2). Leflon *et al*. (2006) used flow cytometry on the backcrosses of AAC hybrids to the parents; they also found that cytometric values corresponded closely (r^2=0.99) to chromosome numbers.

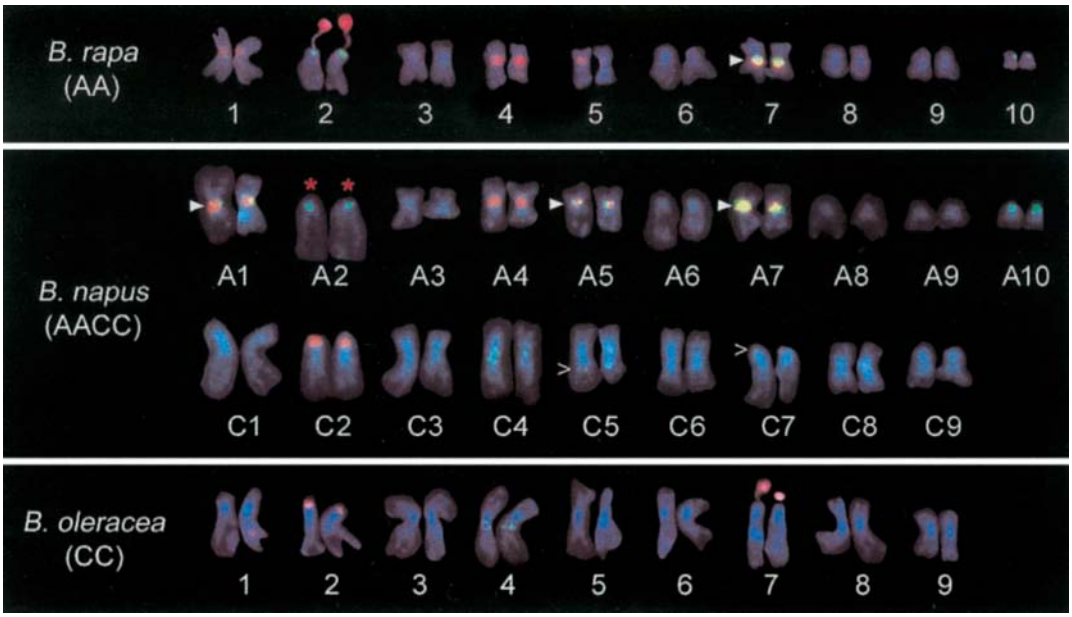

Figure 4. Karyotype of *Brassica rapa* (n=10), *Brassica napus* (n=19) and *Brassica oleracea* (n=9). Note that the CC chromosomes are, on average, larger than the AA chromosomes. Reproduced with permission from Snowdon *et al.* (2002).

2.2.3. Statistical analysis

In both experiments plants were grouped by the variable 'species'. Hybrid plants were excluded from the analyses. Only plants reaching the flowering/fruiting stage were added to the analysis. Some plants or lines in the greenhouse remained at the rosette stage and were thus eliminated from the dataset.

Interspecies differences were tested either with a Chi^2 test (nominal values) or with an ANOVA (continuous data). For the former, we used the program SPSS 17.0 and a program available on the web (http://www.quantitativeskills.com/sisa/).

To investigate differences between Dutch cultivars and between the species *B. napus* and *B. rapa* we used a discriminant analysis (SPSS 17.0), testing cultivars from CGN and nine recent

cultivars. The analysis was performed separately for the garden and the greenhouse experiment. In all cases we used a variable selection method for stepwise discriminant analysis that chooses variables for entry into the equation on the basis of how much they lower Wilks' lambda. At each step, the variable that minimises the overall Wilks' lambda is entered. Variables were entered into the model if their F value exceeded the entry value (F=3.84) and removed if the F value was less than the removal value (2.71). Missing values were replaced by the mean group value. Traits that were not significantly different between *B. napus* and *B. rapa* were not included in the analysis.

2.3. Results: Differences between *Brassica napus* and *Brassica rapa*

In both experimental setups, most of the variables (cf. Table 2) were significantly different for the two species. In the garden, though, no differences in leaf colour were found for the rosette and middle leaves of the plants, nor for the length of the beak. In the greenhouse, the hairiness of the middle leaf on the abaxial side was not significant. At neither growing condition was adaxial-side hairiness or upper-leaf surface texture significantly different. Overall, though, we can conclude that the two species exhibit many differences that are statistically significant. An explanation of the measured traits underlying this conclusion, with graphs, is provided in Appendix 3.

Taking the full range of variation into account, however, all the traits measured do exhibit a degree of overlap. Although the means are different, certain plants will have characters falling within the range of the other species. It is therefore not possible to distinguish the two species based on any single morphological character. Although seed size was not included in the overall analysis, the average seed size (diameter) of *B. napus* (2.063 mm ± 0.25 mm) is greater than that of *B. rapa* (1.59 ± 0.15 mm).

To test how well a combination of morphological traits can classify individuals as *B. napus* or *B. rapa*, a discriminant analysis was performed with the traits that were always significantly different. This analysis showed that more than at least 95% of the plants were correctly classified to the proper species in both experimental settings. In the experimental garden virtually all the *B. napus* plants were correctly classified (99.5%), while the percentage for *B. rapa* was slightly lower (95.5%). In the greenhouse 98.4% of all *B. rapa* plants and 97% of the *B. napus* plants were correctly classified.

The number of selected variables in the stepwise discriminant analysis differed between the garden (9) and the greenhouse (8). Three variables were the same in both sets: the presence of at least one leaf entirely clasping the stem, the presence of hairs on the midrib on the abaxial side of the leaf, and the presence of scent. Of the remaining variables, in the garden three were related to the flowers: width of the petal limb, shape of the inflorescence, length of the sepal, and two to the leaf: length of the part of the upper leaf extending beyond the stem and hairs on the abaxial side of the middle leaf.

Of the remaining selected traits in the greenhouse, two were flower-related: the presence of overlapping petals and the length of the flower; three were leaf-related: hairs on the midrib and on the abaxial side of the upper leaf, and colour of the rosette; and one was fruit-related: the ratio of beak to total fruit length.

We can conclude that both species can be correctly identified with high certainty based on a subset of the morphological characters measured. With the statistical method employed, however, this subset of traits is partly similar and partly different between plants grown in the experimental garden or in the greenhouse. A descriptive key for identifying the two *Brassica* species is provided in Appendix 4.

Table 2. Total number of morphological traits measured for *B. napus* and *B. rapa* in the experimental garden and greenhouse. Traits that are significantly different between the two species are given in black. Further legend: not significantly different in (1) the garden (blue), (2) the greenhouse (orange), (3) both locations (pink) and * not measured in the greenhouse.

1.	**Basal leaves**
1a.	Colour: glaucous or grassy-green (1)
1b.	Number of paired lobes (end lobe not included)
1c.	Hairs on the adaxial surface of the leaf
1d.	Hairs on the midrib on the abaxial surface of the leaf
1e.	Other hairs on the abaxial surface of the leaf
1f.	Leaf texture: smooth or rough
1g.	Lumps on the adaxial surface
2.	**Middle leaf (midway between the bottom leaf and the last leaf before the flowering stalk)**
2a.	Colour: glaucous or grassy-green (1)
2b.	Hairs on the adaxial surface of the leaf
2c.	Hairs on the midrib on the abaxial surface of the leaf
2d.	Other hairs on the abaxial surface of the leaf
2e.	Leaf texture: smooth or rough
2f.	Amount of clasping of the broadened cordate base (%)
2g.	Depth of the broadened cordate base (mm)
2h.	Part of the broadened cordate base extending beyond the stem (mm)
3.	**Top leaves (leaves in the lower part of the inflorescence, but not a bract of a flowering stalk)**
3a.	Colour: glaucous or grassy-green
3b.	Hairs on the adaxial surface of the leaf (3)
3c.	Hairs on the midrib on the abaxial surface of the leaf
3d.	Other hairs on the abaxial surface of the leaf (2)
3e.	Leaf texture: smooth or rough (3)
3f.	Amount of clasping of the broadened cordate base (%)
3g.	Depth of the broadened cordate base (mm)
3h.	Part of the broadened cordate base extending beyond the stem (mm)
4.	**Presence of at least one other leaf completely clasping the stem**
5.	**Hairs on the stem (yes or no)**
6.	**Shape of the inflorescence at the level of the open flowers: elongated or compact**
7.	**Position of the buds (above, in between, below)**
8.	**Position of the sepals (adjacent to the petal base, at a 45^{o} angle, or a 90 ^{o} angle)**
9a.	**Overlap of the petals (yes or no)**
9b.	**Percentage of petal overlap**
10.	**Scent (yes or no)**
11.	**Flower measurements**
11a.	Flower size (top open flower) in mm
11b.	Diameter of the flower
11c.	Length of the flower
11d.	Width of the flower
11e.	Length of the yellow petal limb (in Dutch: ”plaat”)
11f.	Width of the yellow petal limb
11g.	Length of the whitish petal base, or claw (in Dutch: “nagel”)
11h.	Length of the sepal
12.	**Fruit measurements**
12a.	Total length of the siliqua
12b.	Length of the beak (1)
12c.	Width of the siliqua
12d.	Ratio of beak to total fruit length
12e.	Angle between the siliqua and the pedunculus *
12f.	Angle between the siliqua and the pedicellus *
12g.	Angle between the pedunculus and the pedicellus *

2.4. The simplified "Libelle Method" for distinguishing *Brassica napus* from *Brassica rapa*

This method involves giving a simple score of either +1 or -1 for each trait that is measured. We refer to it as the "Libelle Method" because this simple scoring procedure is similar to that used in personality tests in popular magazines like Libelle. *Brassica napus* traits are scored as +1 and *B. rapa* traits as -1. The total sum per plant yields a final score. One important assumption is that for all traits a value must be given, for otherwise the total sum may be in error owing to a deficient number of values. A total of 18 traits were included in this analysis, giving a maximum score of +18 for *B. napus* and -18 for *B. rapa*. The total score per plant was calculated for a subset of plants for which all traits could be measured. This total score is based on the following traits: 1a, c, d, e, f, g, 2a, b, c, d, e, 3f, 4, 5, 6, 9a, 10 and 11g. These traits were chosen because they consisted primarily of nominal values, except for the percentage of leaf clasping of the upper leaf and the length of the whitish petal base, or claw. The results are reported in Figure 5 and the associated questions are given in Appendix 5.

In Figure 5 the plants are classified on the basis of cultivar/population number (x-axis). An interesting result is that the score for *B. rapa* is more negative for the greenhouse data than for the garden data. Also the range of the score for *B. rapa* is larger for the field (-12 to +9 than for the greenhouse (-18 to -7). The main score for *B. napus* lies between +10 and +18 and seems clearly separated from that for *B. rapa*. Even with this method, though, we were unable to fully discriminate between the two species. Nevertheless, Figure 5 shows that if we classify all plants with a score over +10 as *B. napus* and those with a score below zero as *B. rapa* our classification is 100% correct. Also with this method not all plants can be correctly classified, as there is overlap between *B. napus* plants and *B. rapa* grown in the experimental garden. This suggests that identification can indeed be problematical and that this is due to the greater variability in the traits of *B. rapa*. This method is still a try-out version and could be modified to weigh characters and include more characters. As it is easy to use and yields fairly consistent results, however, it is probably preferable to the discriminant analysis.

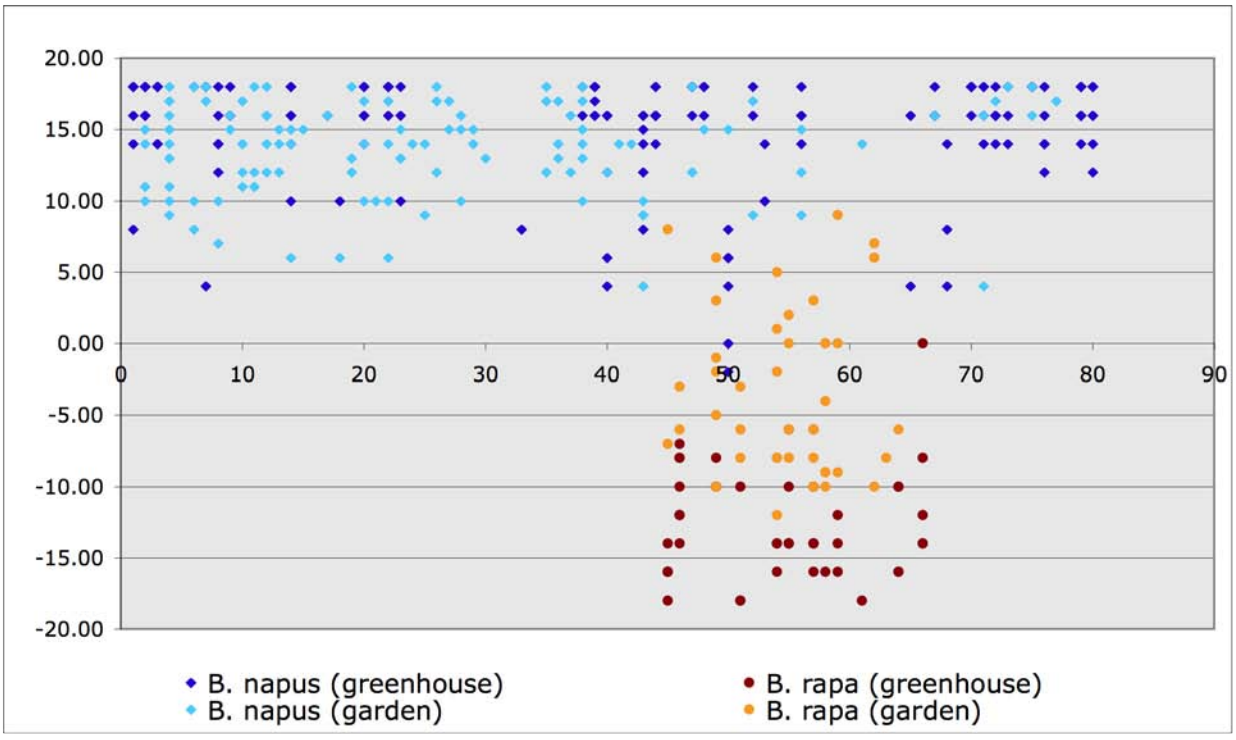

Figure 5. Total score per plant for all cultivars/populations of *Brassica napus* and *Brassica rapa.* On the x-axis the cultivar/population number: 1-38, 41 and 42 are the CGN cultivars, 40, 44-67, 79 and 80 the wild populations, and 43, 53, 68-76 the recent cultivars (see also Appendix 1).

2.5. Differences between *Brassica napus* cultivars

The question we had addressed was whether morphological traits could be successfully used to classify plants as the right cultivar. To this end we included all the cultivars obtained from CGN as well as the recent cultivars and excluded the field collection of *B. napus*. The discriminant analysis classified only 10.2 % of the plants in the correct group. The variables that were included in the stepwise analysis were: 1. length of the beak, 2. length of the part of the leaf lobe exceeding the stem, 3. width of the fruit, 4. clasping of the middle leaf and 5. depth of the broadened cordate base of the middle leaf. Because 89.8% of the plants could not be classified as the correct cultivar, it is not possible to recognise different cultivars in the field. These results should be viewed with caution, however, because the sample size per cultivar varied between only 3 and 10.

Of all the cultivars grown in the greenhouse as well as the CGN and recent cultivars obtained, only 15.8% could be correctly classified to cultivar level. Only three variables were included in the stepwise analysis: 1. the part of the cordate leaf base of the middle leaf extending beyond the stem, 2. the length of the whitish petal base (claw) and 3. the width of the yellow petal limb. Again, the small percentage of the plants classified to their proper cultivar shows that identification between cultivars in the greenhouse is difficult.

The only discriminative variable that both experimental settings have in common is the length of the cordate middle leaf base extending beyond the stem. It must be concluded that identification of the different cultivars is difficult, although this finding is to be viewed with due caution, because of the low sample size per cultivar. From the morphological traits measured, it was not possible to distinguish between old and recent cultivars, nor between plants collected in the field.

3. Dutch distribution of *Brassica napus* and *Brassica rapa*

While this chapter is concerned with the Dutch distribution of *B. napus*, the results presented do not provide a complete picture for the Netherlands as a whole, as this was not feasible within a period of two years. We therefore focused on the presence of feral plants or populations in regions with *B. napus*-related activities (i.e. import/export and cultivation areas). To extend the distribution data to the national scale would require the help of numerous FLORON volunteers.

3.1. How and where to investigate the presence of feral *Brassica napus*

In 2008 several regions with a potential for feral *Brassica napus* were visited (Figure 6). We concentrated on areas with import/export activities (harbours of Rotterdam and Amsterdam) and areas of past and present cultivation of *B. napus* (Groningen, Flevoland, Haarlemmermeer, Beemster and Wieringermeer). At the start of the project in spring 2008 no information was available on the exact location of field recently cropped with *Brassica napus*.

At the end of 2008, however, we obtained precise information about the location of fields where *B. napus* or *B. rapa* had been grown in the years 2005 – 2008 from the organisation 'Gegevensmanagement Dienst Regelingen Assen (LNV)' via dr. W.L.M. Tamis (CML). In most cases *B. napus* had been grown, with only a few instances of *B. rapa* cultivation, but in the LNV data these had been pooled. With this information we could focus more accurately on the

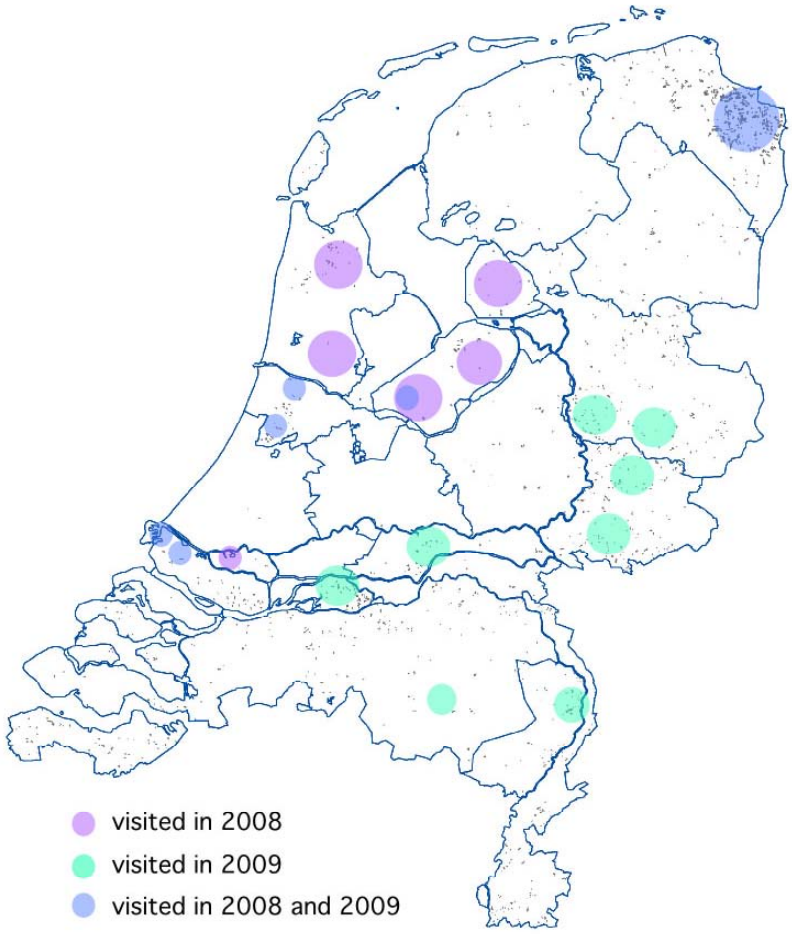

Figure 6. The *Brassica napus* areas investigated in 2008 (purple), 2009 (green) and both years (blue). The small grey dots represent fields of *Brassica napus*/*Brassica rapa* in the years 2005-2008.

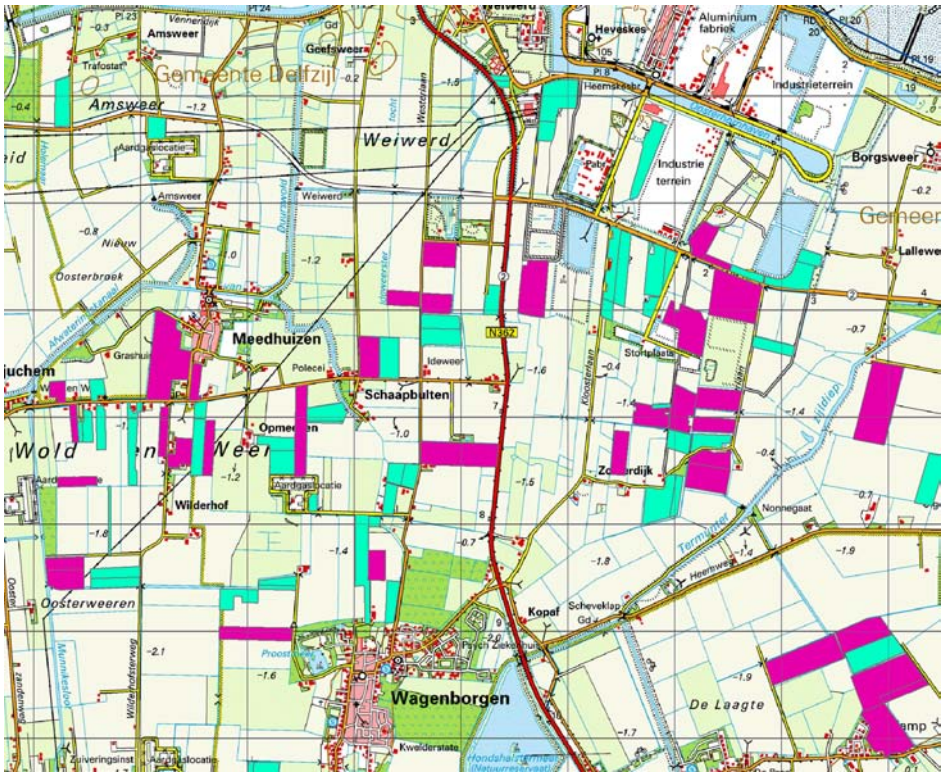

Figure 7. Cropped fields of two different years projected onto a topographical map.

presence of *B. napus* in 2009 than was possible in 2008. The data were presented by means of shape files showing the distribution of fields on a white background. To investigate the possible establishment of feral plants near recent *B. napus* fields (2005 – 2008) these shape files were projected onto a topographical map; see for example Figure 7. To combine different layers of information on a landscape scale we used the program ArcGIS. This software was made available to us at the GIS studio of the Institute of Biodiversity and Ecosystem Dynamics (IBED) at the University of Amsterdam.

It was striking to discover that cultivation of *B. napus* was spread out rather evenly across the Netherlands, although the highest density of cropland was in the north-east of the northern province of Groningen (Figure 7). In the west of the country cultivation of *B. napus* is more local (i.e. Haarlemmermeer and Wieringermeer), while in the east and south it is less local. In Flevoland and the Noordoost Polder, too, *B. napus* fields were rather scattered.

With this information on the precise position of fields cropped to *B. napus* in 2009 we reinvestigated several areas, viz. Groningen and the Haarlemmermeer, to assess the presence of feral populations in the Netherlands (Figure 6). We did not revisit the areas in the provinces of Noord-Holland, Flevoland or the Noordoost Polder, but instead examined several new areas in the east and south of the country: the Twente and Achterhoek regions and the vicinity of Deventer, Tiel (Betuwe area), Wijk en Aalburg, Venlo, and Eindhoven. We also revisited the Rotterdam and Amsterdam harbour areas (see Figure 6. Observations of *B. napus* en route to and from the target areas were also recorded.

The location of the plants was recorded with GPS and in 2008 and 2009 the number of plants was counted. In 2008 traits were also recorded and 2-3 reference plants were collected. Traits were noted according to the morphological traits listed in the species descriptions for *B. napus* and *B. rapa* in the standard Dutch Flora (Heukels' Flora 23^{e} ed.). If identification appeared to be problematic, leaf material of that plant was collected and the amount of DNA

analysed by the company Iribov using flow cytometry. As observations proceeded, it became clear that other traits could be added to improve the distinguishability of *B. napus* and *B. rapa*.

3.2. Presence of *Brassica napus* in relation to transshipment and cultivation

- Transshipment areas

In the Rotterdam harbour area are several companies, both old and new, that transship bulk loads of seeds of various cereals and other crops. At the time of our visit it was not known which of them handled *B. napus* seeds, but at three locations we found *B. napus* plants (Table 3). The plants were growing in the verge of the road, along the railway or on stony slopes along the water outside the property (Maashaven and Beneluxhaven) or only on the property (Botlek). On our second visit in 2009 the road verges had already been mown and plants were found only along the railway near the transshipment company in the Beneluxhaven. The latter location appeared to be very close to ADM Europoort, a large company that imports *B. napus* for warm crushing. At all the sites other yellow-flowering crucifers were found: *B. rapa* and *B. nigra* (Maashaven and Beneluxhaven), *Sinapis arvensis* (all three localities) and *Diplotaxis tenuifolia* (Beneluxhaven). The number of *B. napus* plants varied considerably from site to site, ranging from 50 to 500 specimens.

In the harbour area in Westpoort (Amsterdam) the location of companies processing *B. napus* was unknown at the time of our visit. During the flowering season of 2008 and 2009 various road verges in the area were explored, but with very little result. We found *B. napus* at only two sites, in close proximity to one another: a derelict plot on the edge of a building site, and the verge of the main road connecting Amsterdam Westpoort to IJmuiden. In 2009 only five plants were found, all on the building site. This location has no direct relationship with any processing of *B. napus*, but the area is well-known to birdwatchers and perhaps *B. napus* seeds were introduced by way of birdseed mixtures. The number of plants per site in this region ranged from 5 to 15. Here, *B. napus* grew together with *B. rapa*, *B. nigra* and *Sinapis arvensis*.

In 2008 and 2009 in the Farmsum industrial and harbour area near Delfzijl, in the north-east of Groningen province, we found *B. napus* plants in very small numbers scattered throughout the area: in flowerbeds, on road verges and along a quay, for example. This quay was very close to a cold-crushing oil processing company. In 2008 the plants were mown during fruiting and in 2009 all the vegetation at this site was treated with a herbicide. The number of plants per site In N.E. Groningen ranged from 1 to 150. No *B. rapa* was found in this area, although we did observe *B. nigra* and *Sinapis arvensis* at *B. napus* sites. In a verge of a road leaving the Farmsum area several hundred plants were found in 2009.

Table 3. Presence or absence of *Brassica napus* at three locations with silos in the Rotterdam harbour area in the years 2008 and 2009.

Location	Maashaven		Botlek		Beneluxhaven	
Years of visits	2008	2009	2008	2009	2008	2009
On the property			yes			
Road verge			no	mown	yes	mown
Railway verge			no	mown	yes	yes
Quay	yes	mown				

- Cultivation areas

Observations of *B. napus* in cultivation areas are reported in Table 4, showing the number of sites with *B. napus* in each of the regions investigated in the year 2008 and 2009. In most of these regions (7 out of 11) we encountered no *B. napus* plants. In regions where the species was observed the number of sites was fairly small, except in the Oldambt Polder in N.E.

Groningen. In this area *B. napus* is occasionally found in road verges and ruderal habitats around the area of cultivation, as well as in more urban areas (Figure 8). Since *B. napus* is cultivated here at a higher density than elsewhere in the Netherlands, there seems to be a relationship with seed spillage during harvesting.

Three regions were visited twice: Haarlemmermeer and the sites in Flevoland and northeast Groningen. Only in two of these was *B. napus* observed in both flowering seasons. In Groningen all the revisited sites except two were still occupied one year later in 2009. The presence of *B. napus* in two consecutive flowering seasons may be an indication of possible establishment of a population or ferality of *B. napus*. However, this should be investigated or monitored in more detail for several years to assess whether plants derive from new seed input or from seed production by established plants.

In three regions north of the Noordzeekanaal in the province of Noord-Holland (Beemster, Alkmaar and Wieringermeer) we found no *B. napus* in road verges while driving through the agricultural area, but we did find *B. rapa* frequently and sometimes this species is locally very abundant. In the provinces of Flevoland and Noordoost Polder the number of *B. napus* observations was very small. Along the route three sites were observed, two in Flevoland (a motorway exit near Lelystad, and a sloping bank at a carpooling car park near an exit to Almere) and one in the Noordoost Polder (an exit near Kampen). The *B. napus* plants we did find in 2008 were all on ruderal sites in road verges (Lelystad exit and carpooling car park). The distribution map of *B. napus/B. rapa* plots showed that the density of plots in these areas was rather low. Only the carpooling site near Almere was reinvestigated in 2009. At this site we found a mixed population of *B. napus*, *B. rapa* and hybrids, making identification difficult. To be sure of correct species identification, plants were therefore checked for their DNA amount using flow cytometry. In 2009 this site was visited again, because of the possible presence of hybrids of the two *Brassica* species. This is a ruderal site and the origin of *B. napus* here is unclear.

Table 4. Number of *Brassica napus* observations in cultivation regions in the years 2008 and 2009 and presence/absence of *Brassica rapa* in the direct vicinity. Legend: x = presence of *B. rapa*; 0 = absence of *B. rapa*; - = no information of presence *B. rapa.*

Locations	2008	2009	*Brassica rapa*	Site description
Beemster/Alkmaar	0	-	x	
Wieringermeer	0	-	x	
Haarlemmermeer	0	0	x	
Flevoland	2	2	x	road verge, ruderal
Noordoost Polder	1	-	x	road verge, ruderal
Groningen (north-east)	17	35	0	road verge, ruderal, urban
Achterhoek	-	0	0	
Twente	-	0	0	
Deventer e.o.	-	2	-	urbanisation
Betuwe (west of Tiel)	-	0	x	
Wijk en Aalburg (west of)	-	0	x	
North of Venlo	-	4	-	road verge, ruderal, urban
Eindhoven	-	3	x	motorway road verge, urban

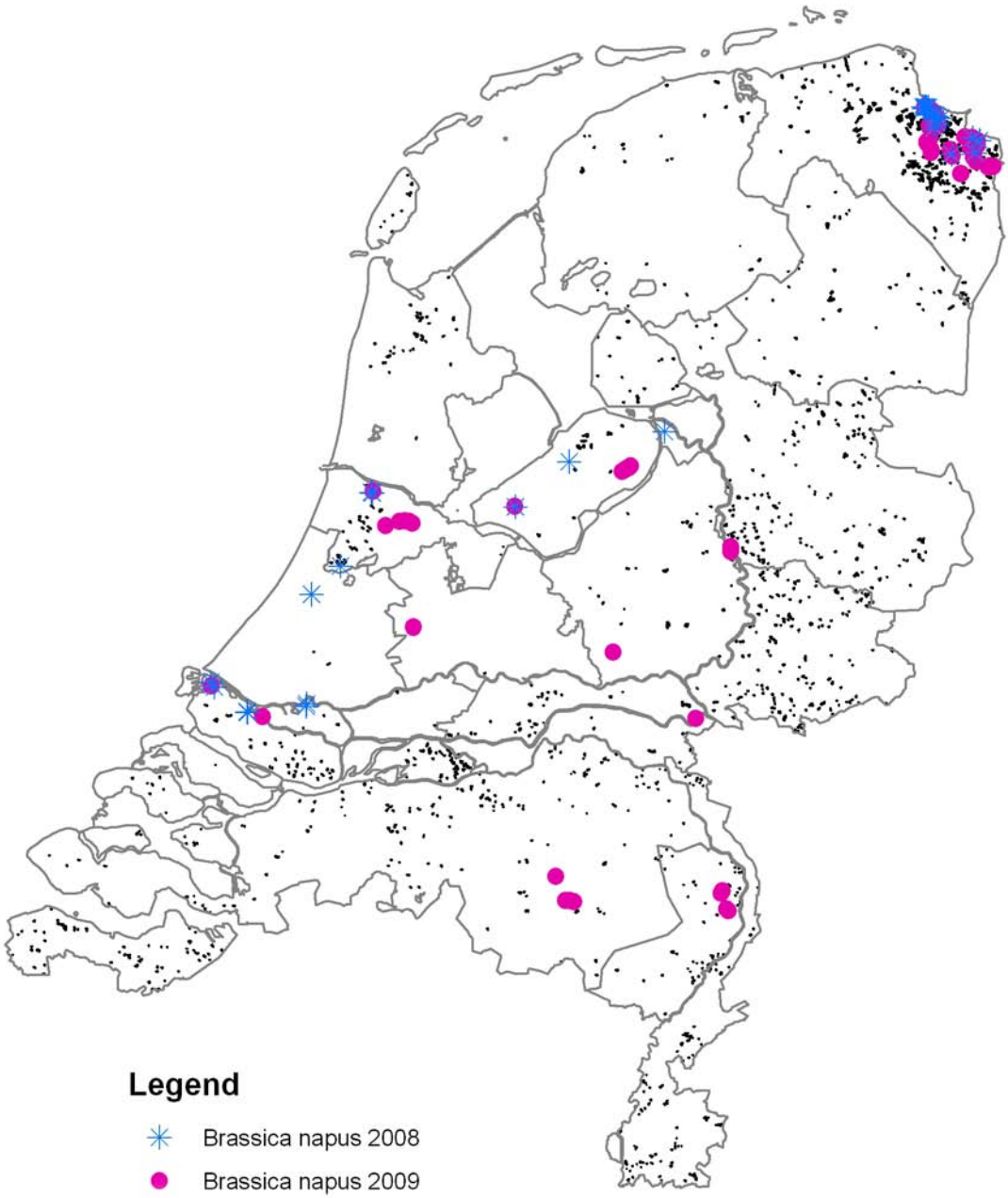

Figure 9. Observations of *Brassica napus* in the Netherlands in the years 2008 and 2009. The distribution of cropped fields in the years 2005-2008 is indicated by the small black dots.

Although, except for Groningen, our search without the knowledge of the exact location of the cultivated *B. napus* fields yielded very little result in 2008, the knowledge of the cropped fields with *B. napus* had little impact on the results in 2009 (Table 4). The only region in 2009 where we did find *B. napus* in the direct vicinity of *B. napus* cultivation was in an agricultural area north of Venlo. All the other sites were situated in an urban or ruderal setting. The presence of *B. rapa* was also extremely low in these regions, except for the Betuwe area and the area west of Wijk en Aalburg. Intensive mowing of the verges along roads in these agricultural regions combined with the lower density of *B. napus* fields (less chance of seed spillage) may explain the low number or even absence of feral *B. napus* there. In this respect these areas differ from N.E. Groningen, where seed spillage might be greater because of the higher density of *B. napus* fields, although in most cases the number of plants per site is relatively low. Of the ruderal sites in N.E. Groningen, very few populations seem to be self-sustaining. Here we found both young plants and flowering ones. This is in itself surprising for a supposedly annual species, but might be related to the time of germination. A mere two observations are of course too few to draw any conclusion about the persistence of these small populations.

Apart from harbours and agricultural regions, scattered individuals were also found along the Amsterdam-Eindhoven motorway. In both 2008 and 2009 up to 500 plants were spotted between the rails at the train stations of Woerden and Ede/Wageningen, probably the result of seed spillage from a freight train. Figure 9 shows our observations of *B. napus* in the Netherlands in 2008 and 2009.

So far, observation of *B. napus* in the ruderal landscape has been attributed solely to the spillage of seeds from cultivation, transportation and transhipment. 'Birdseed' mixtures also contain *B. napus* seeds, however, including seeds from countries with sometimes wide cultivation of GM varieties. When a small sample of plants raised from *B. napus* seeds in birdseed were screened for glyphosate resistance, all the plants died thus indicating that no GM varieties were present (Appendix 6).

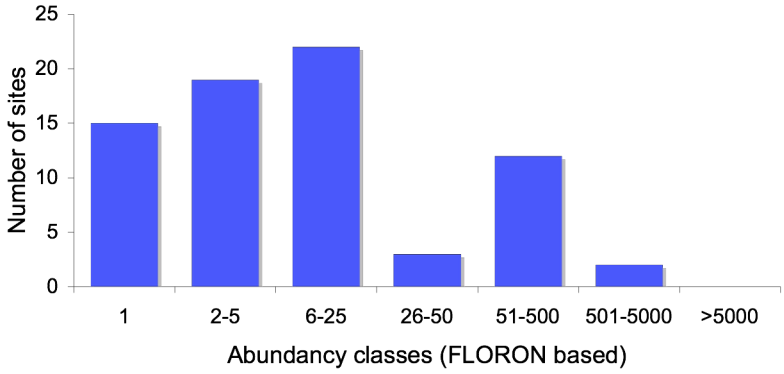

Figure 10. Number of sites per abundancy class for our own observations of *Brassica napus* pooled for the years 2008 and 2009.

3.3. Number of *Brassica napus* plants per site

At most sites the number of plants per site is low, varying from a single specimen up to 25 plants (Figure 10). Sites with several hundred plants seem to be the exception rather than the rule. Sites with a high number of plants, i.e. around 500 plants or more, were found along roads (e.g. Biddinghuizen and Farmsum) and railways (Woerden, near a transshipment company in Europoort). These sites are most likely associated with large-scale seed spillage. Owing to the

short timescale of the project, it is not possible to conclude whether the plants are derived solely from yearly input or from a combination of seed input through spillage and seed production. The timescale of this project is too short to yield any information on the longevity of the presence of the plants (ferality).

3.4. Country-wide volunteer cycling project

In cooperation with FLORON, the Dutch national florists' organisation, a project was developed to enlist the aid of enthusiastic volunteers. This project was entitled "Fietsen voor Koolzaad", i.e. "Cycling for Oilseed Rape". As identification of both species of interest is problematical and the species traits described in the standard Dutch Flora do not suffice, the brochure we had developed in 2008 on the basis of measured morphological traits was sent to all FLORON volunteers as well as to other plant working groups (e.g. KNNV, IVN). To involve as many people as possible, we set out cycle tours near their home town, asking volunteers to cycle a distance of about 15 km (Figure 11), although some were longer. If fields cropped to *B. napus* were present in the vicinity, the tour was guided along them. These were not marked on the final map handed out to each participant, though. This printed version of the tour on a topographical map with an overlay of a km^2 grid with Amersfoort coordinates was prepared by Sheila Luijten (Plant Ecology, IBL) and Ruud Beringen (FLORON). A form was also distributed on which to fill in observation data, viz. the location of the plants (GPS or read from the map), the number of plants (FLORON abundancy classes) and the presence of any *B. napus* plots in the direct vicinity. Participants were explicitly asked to note the abundance not only of *B. napus* and *B. rapa*, but also of *Sinapis arvensis*, *Brassica nigra*, *Diplotaxis tenuifolia* and *Raphanus raphanistrum*.

Figure 11. Example of a cycle tour near Almere.

To inform participants, online information about the project "Fietsen voor Koolzaad" was posted on a special section of the FLORON website, with downloads of the identification brochure, additional Brassicaceae identification tables, instructions on filling in the data form, an empty data form, etc.

3.5. Volunteers' observations on distribution of *Brassica napus* and *Brassica rapa*

After the University of Leiden had published a newsflash about the cycling project on their website, it received a lot of media attention on (mainly local) radio stations and in newspapers. After 133 volunteers had enrolled we stopped the appeal for help, because it became unfeasible to produce any more cycle tours at such short notice and the goal of 70 participants had already been nearly doubled. Fortunately, the tours were distributed fairly evenly across the Netherlands (Figure 12).

Of the 133 participants, 90 returned their form to us. Not all participants were sure about their identification of *B. napus* and *B. rapa*. Some sent

Figure 12. The distribution of the cycle tours and cultivation fields of *Brassica*.

in photographs or plant material if identification appeared to be problematic and in those cases we were able to correctly identify the species. We were not able to verify each and every result, though, because not all volunteers sent in materials (photos or plants). The total number of sites reported was lower for *B. napus* (n=232) than *B. rapa* (n=806). In only 27 observations both speccies were recorded. These observations are per site and not pooled per km^2 and may consequently comprise one or more observations per square kilometre. On 10 tours no *B. napus* or *B. rapa* was observed. From 43 cycle tours we did not receive any information, or participants informed us they were unable to cycle for personal reasons. Besides the data from the cycle tours we received another 75 individual observations from project participants. The results are presented in Figure 13. Of the total number of observations 22% were of *B. napus* and 78% of *B. rapa*. This is a very different picture from that in the records in FLORON's national database, which in recent years shows an average ratio of fifty-fifty. To compare our data with those of FLORON we adjusted our data to the km^2 level. Even then, though, our results still show more *B. rapa* observations than *B. napus* (Figure 13).

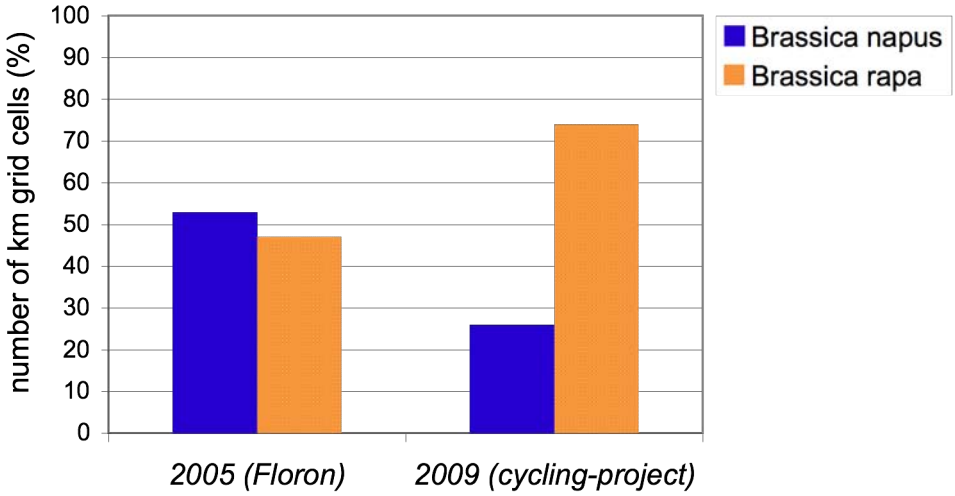

Figure 13. Proportion of *Brassica napus* and *Brassica rapa* observations for the 2009 cycling project compared with FLORON records (year 2005 only).

The resultant distribution map (Figure 14) shows that most reports of *B. napus* are observations of single populations and are scattered throughout the Netherlands, while *B. rapa* observations derive mainly from the western part of the country. Besides these single observations of *B. rapa*, many participants observed no other target species. This result was expected, especially in the provinces of Utrecht, Noord- and Zuid-Holland, where we ourselves had often encountered very large *B. rapa* populations on roadsides. Surprisingly, in the province of Noord-Holland, around Alkmaar and Hoorn, some participants noted only *B. napus* in large numbers, with no observations of *B. rapa* at all. From our investigation in 2008 in these regions and the absence of *B. napus* cultivation, it seems likely that the volunteers wrongly identified the Brassica species as *B. napus*. As we were unable to subsequently check these data, though, we have included these observations in the results. It shows that even with the aid of our identification brochure correct species identification remains difficult. This might also be the case for the observations in the north-east of Flevoland, although we did find several hundred *B. napus* plants along a main road there. These observations should clearly be checked in the coming years.

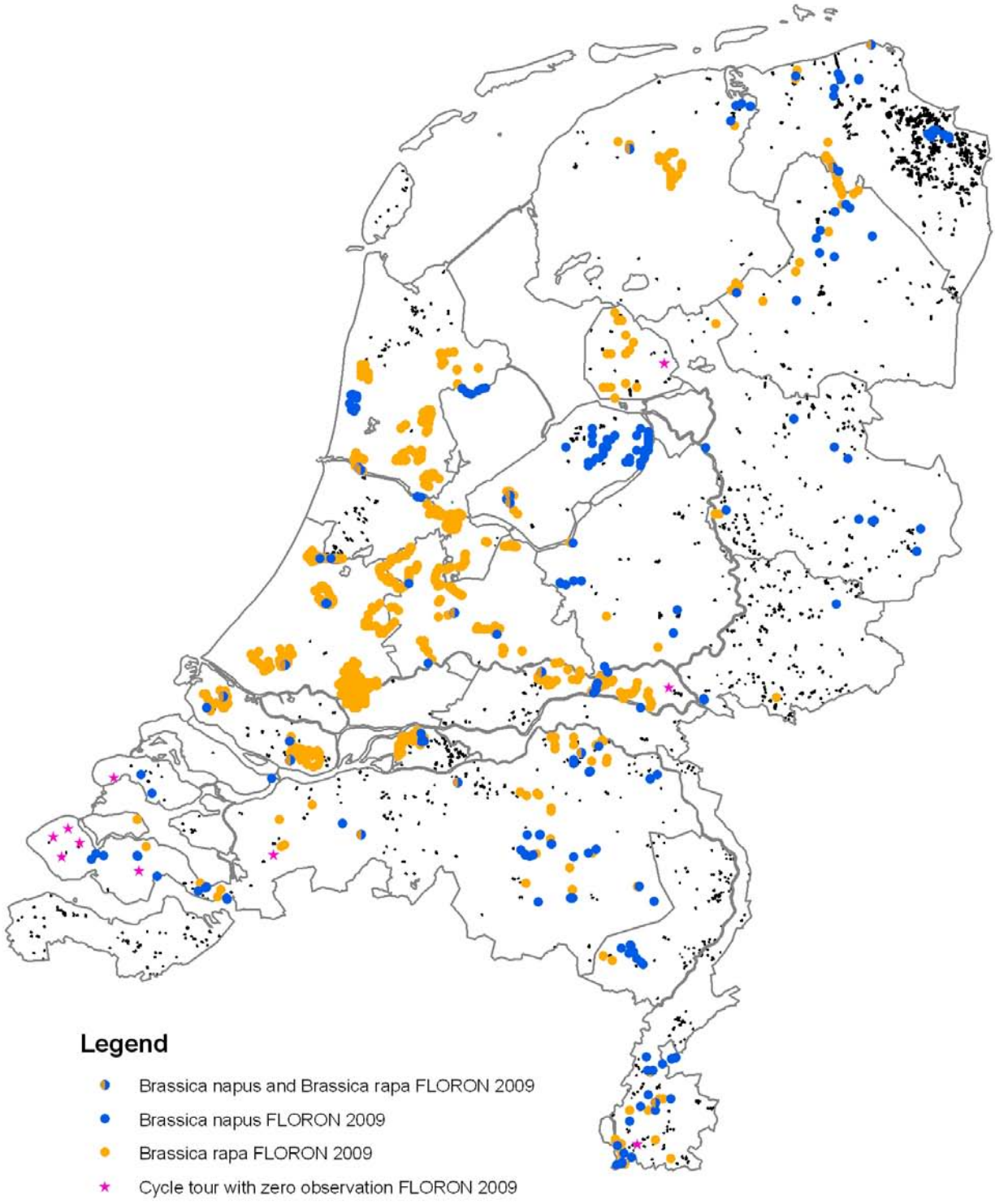

Figure 14. Distribution of total number of *Brassica napus* and *Brassica rapa* populations obtained. from observations from the cycling project "Fietsen voor Koolzaad" including individual observations and cycling tours with zero observations, all in 2009. The distribution of cropped fields in the years 2005-2008 is indicated by the small black dots.

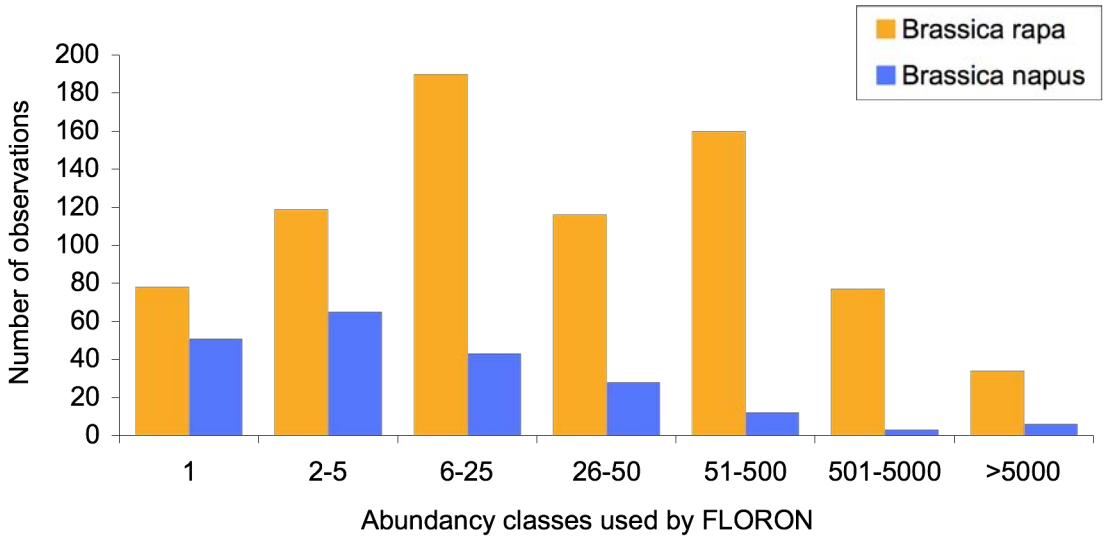

Figure 15. Number of sites per abundancy class for *Brassica napus* for the year 2009 ("Fietsen voor Koolzaad" project).

The cycling project data show not only that *B. rapa* is more common than *B. napus*, but also that a higher number of plants is generally observed per *B. rapa* observation than in the case of *B. napus* (Figure 15). This latter result is in agreement with our own findings. A number of observations suggested the presence of several thousands plants at a single location, however. These sites were described as former *B. napus* fields that had been converted to an industrial site, or a soil depot. These data were not checked. Another observation concerned several *B. napus* sites along a road near Biddinghuizen. This is close to one of our own observations and is most likely accurate.

Although many participants gave no description of the sites in question, some did. These descriptions showed that *B. napus* was found in road verges, in ruderal areas, on abandoned arable land, around main road or motorway exits, and in urban areas. Some participants found *B. napus* in verges that had been sown with "wildflower mixtures". The number of *B. napus* observations was no greater on cycling tours in the direct vicinity of fields cropped to *B. napus*, except for the tours in N.E. Groningen. This result is very similar to our own, although it is not always clear where the plants come from.

The distribution data also show that on some cycling tours both *Brassica* species were found. At these sites gene flow may occur form *B. napus* to *B. rapa*.

4. Occurrence of hybrids

In situations where *Brassica rapa* is growing at the edge of the fields cropped to *B. napus*, especially if population size is small, cross-pollination may occur, because *B. rapa* is self-incompatible while *B. napus* is mainly selfing. At sites like these there is the greatest chance of hybrids being found. There is no morphological information available on the identification of hybrids, however, so the only effective method was to examine the amount of DNA using flow cytometry.

Among the seeds (first generation) derived from *B. rapa* plants growing in a verge along one *B. napus* plot we did find large numbers of hybrids (around 50%). In the climate chamber all the plants at the rosette stage looked very similar and showed traits of *B. rapa*: grassy-green leaves with hairs on both sides of the leaf. Rosette plants of *B. napus* are glaucous in colour, hairless and have a smooth (waxy) surface.

In the Westpoort area of Amsterdam only 2 hybrids out of a total of 91 plants were found among offspring. Hybrids were assessed as being intermediate between *B. rapa* and *B. napus* and are therefore F_1 hybrids.

We carried out a pilot study searching for hybrids within *B. rapa* stands in verges along former *B. napus* fields, a prime habitat where hybrids are likely to be found. Few were found, however. At one site we found one hybrid among 66 plants, and at another site 3 hybrids among 50 plants. A third site, a mixed stand of both *Brassica* species near the aforementioned carpooling site near Almere, contained 15 hybrids out of 36 plants. At this site we experienced identification problems. Levels of DNA varied and, since DNA content was higher than in the F1, these plants might be backcrosses to *B. napus*. These results require further investigation, though.

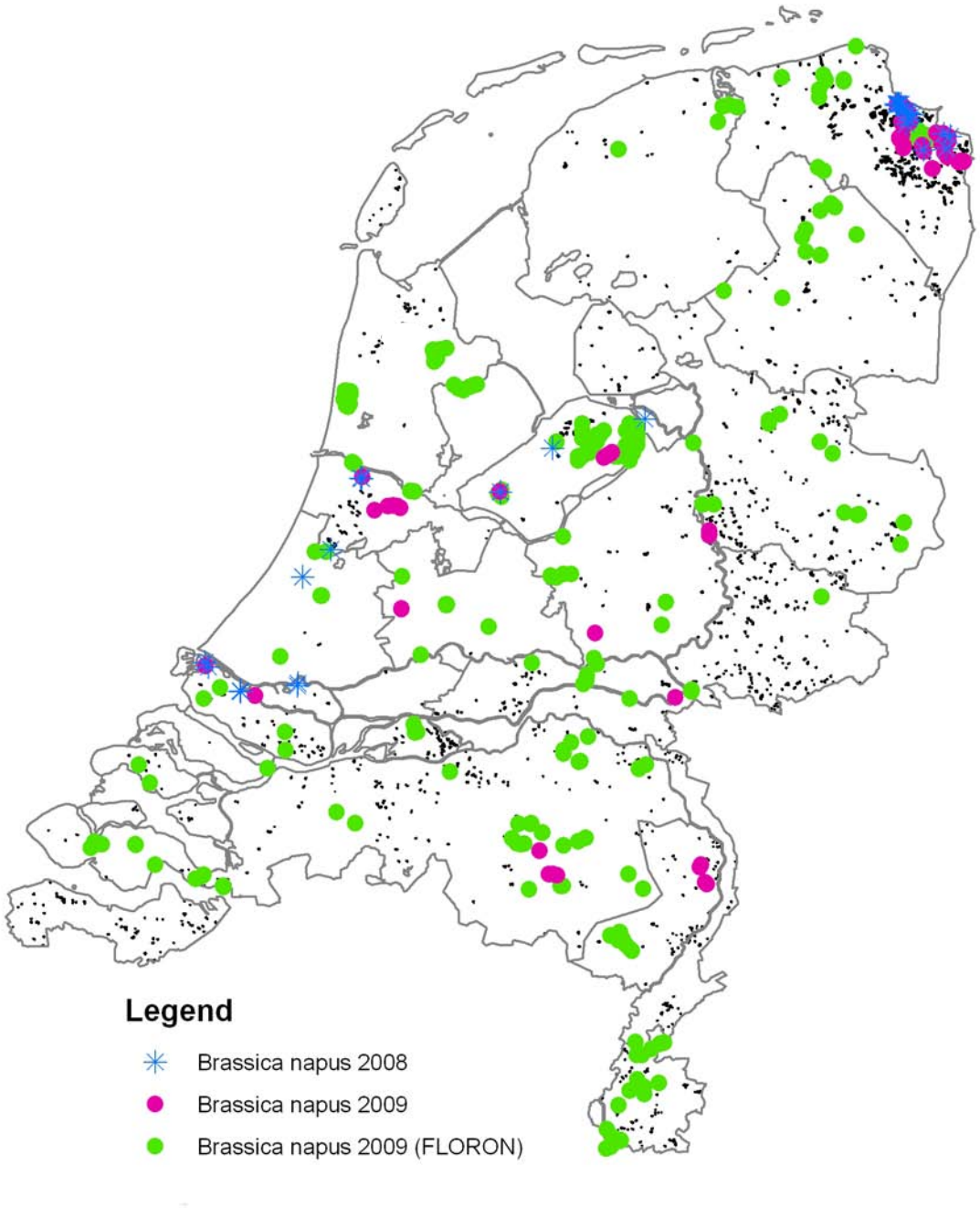

Figure 16. Distribution of *Brassica napus* based on our own observations (years 2008 and 2009) and those of FLORON volunteers (year 2009). The distribution of fields cropped to *B. napus* in the years 2005-2008 is indicated by the small black dots.

5. Discussion and conclusions

5.1. Species identification

In the Netherlands identification of *B. napus* and *B. rapa* has been problematic and plagued by the prejudice that the yellow-flowering *Brassica* generally encountered is or was *B. napus*. In a new identification brochure we clarified the morphological differences between the two species and drawn attention to the fact that *B. rapa* has in the past often been mistaken for *B. napus*. This brochure was sent to numerous FLORON volunteers as well as a number of professionals. The new insights are important for those collecting species distribution data for input to the national species distribution database (NDFF).

Although identification capability has been greatly improved, it is still sometimes problematic for volunteers and species inventories are best carried out by experienced florists. Observations of *B. napus* uploaded to open websites collecting species distribution data for the Netherlands (for instance, waarneming.nl) should be treated with caution, because the floristic skills of the observer are usually unknown and this may lead to erroneous data, with *B. rapa* or even *Sinapis arvensis* sometimes being mistaken for *B. napus*. In the absence of clear photos showing relevant traits, such observations are therefore still unreliable. That misidentifications are still made is not entirely surprising. These mistakes might also be made in other European countries, because in books and on websites the species in question may be illustrated with incorrect photos (e.g. for the Bundesamt für Naturschutz (BfN) website (www.floraweb.de)).

As our garden and greenhouse trials demonstrated, *Brassica napus* and *Brassica rapa* can be distinguished statistically, as almost all the traits measured showed significant differences. For all the traits there was an overlap between the two species, however, making identification based on any one trait problematic. Using a subset of traits, though, successful identification of the appropriate species should be possible. With the traits measured in this study, over 95% of the plants could be classified as the right species. Three traits identifying *B. rapa* emerged as common features of the two datasets: at least one leaf entirely clasping the stem, hairs on the midrib on the abaxial side of the middle leaf, and scent, suggesting that the other traits in question are influenced by the environment.

5.2. Species distribution

Our results show that *B. rapa* is much more common than *B. napus* in the Netherlands. This finding is in contrast to the species distribution data available in the National Flora and Fauna Database/FLORON and contradicts the general notion that the yellow-flowering crucifer commonly seen in Dutch road verges is *B. napus*. It seems very likely that *B. napus* was confused with *B. rapa*, rendering previous distribution data unreliable. The latest edition of the Heukels' Flora of The Netherlands (Van der Meijden 2005) also questioned the naturalised status of *B. napus*, suggesting that *B. napus* has probably not been as feral as always believed.

In that respect, our findings differ from those in other countries. In Germany, in the agricultural area around Bremen, *B. napus* is also reported to be more common than *B. rapa* (Menzel 2006). The distribution data in the New Atlas of the British Flora (Preston *et al*. 2002) shows that *B. napus* is held to be more common than *B. rapa* in the UK; nonetheless, accurate identification of this species is thought to be unreliable, especially prior to 1965. Wilkinson *et al*. (2000) even mention that *B. napus* has often been mistaken for *B. rapa* and that the latter is probably less common than initially thought, being found mainly along riverbanks (Wilkinson *et al*. 2000), which is in complete opposition to our findings. While in the Netherlands, too, *B. rapa* is mainly found in the verges of roads and small canals in the lowland semi-natural (grazed)

grassland areas and the river valley region, it is also observed on disturbed soils along roads and motorways and on similar open soils on undeveloped land. On the higher, sandy soils in the eastern part of the Netherlands, *B. rapa* appears to be very rare or even absent. Population sizes vary widely, ranging from several plants to a few hundred or even more. On the sides of roads and canals in the lowland grasslands, and on disturbed soils around roadworks, for example, populations of *B. rapa* can extend for several hundred metres or even kilometres, sometimes at high densities.

Brassica napus observations are scattered fairly widely across the country (cf. Figure 16). Plants are generally found on disturbed, ruderal, roadside sites on bare, open soils, in flower beds and in pavements. Unlike *B. rapa* sites, most *B. napus* sites harbour fewer than 25 plants and are quite local and small-scale. The number of plants per site in the Netherlands shows a similar pattern to that in Germany around Bremen, where nearly 80% of the sites fall in the category 1-25 plants per site (Menzel 2006). Within a 30 km radius of Osnabrück, the number of plants generally ranged from a few individuals to over a thousand (Elling *et al*. 2009).

The relatively few sites with numerous *B. napus* plants are probably related to the spillage of seeds during transport, import/export transhipment or seed processing. At such sites one can find up to several hundreds of plants along roads, railways or harbour quays. Along the outgoing lane from the city, or in our case the transhipment company, we found many more plants than along the incoming lane (cf. Crawley & Brown 2004, Von der Lippe & Kowarik 2007), suggesting spillage of seeds during transport and adherence of seeds somewhere on the outside of lorries or cargo trains. Spillage of seeds during transfer from dockside import/export facilities (i.e. terminals) to seed-processing companies proves to be a pathway for environmental escape of GM seeds near the former (Yoshimura *et al*. 2006) and along transportation routes to the latter (Nishizawa *et al.* 2009), or simply in ruderal (non-crop-distributed) areas (Yoshimura *et al.* 2006).

Investigation of road verges in areas where *B. napus* had been cultivated at least once from 2005 to 2008 revealed very few observations of *B. napus*, except in the north-east of Groningen. In this part of the Netherlands *B. napus* cultivation occurs at higher densities than in the rest of the country, and more sites with *B. napus* were found. Although most sites had an average of 25 plants or less, several road verges harboured 100 plants or more. In these cases, spillage during sowing or harvesting is probably the direct cause of the presence of *B. napus* outside the cropped fields. In other parts of the country, very few *B. napus* plants were found in road verges along (former) fields. From the present study it is unclear whether this result is due to the lower density of such fields or the lack of suitable growing conditions in well-maintained road verges or a combination of the two, or some other explanation.

For most sites we have presence data of *B. napus* for one flowering season only. From this single-observation event it is not possible to give any information about the persistence of these plants as self-sustaining populations or the status of ferality in the Netherlands. To properly analyse whether our sites are indeed feral populations, they should be monitored for more than two flowering seasons. From the literature it is known that plants can persist for several years at the same location and are described as feral populations in Germany, France and the UK (i.e. Pessel *et al*. 2001, Crawly & Brown 2004, Dietz-Pfeilstetter *et al*. 2006, Menzel 2006, Pivard *et al*. 2008, Elling *et al*. 2009). However, the persistence of these populations seems to depend on seed inputs other than those from the population itself. A variety of seed inputs are cited in the aforementioned studies, such as the cropped field, losses during transport, the local seed bank, or forage seed mixtures. In the Netherlands 68% (13 out of 19) of the *B. napus* sites identified in 2008 were also occupied in 2009. These were several localities in N.E. Groningen, a road verge near the dockside transhipment company at Europoort, a building site in Amsterdam Westpoort,

the A10 motorway south of Amsterdam, the train stations of Ede-Wageningen and Woerden and one ruderal site near Almere. At all these sites a mixture of seed spillage and local seed production may well be involved.

Brassica napus is not a common weed in the Netherlands and observations are scattered across the country. The number of plants per observation is generally less than 25 and it is apparently hard for *B. napus* to establish large populations. There are many possible explanations for this, including verge mowing time (reduction of local seed production), lack of open spots (limited opportunity for germination and establishment) and vegetation density (competition). The large numbers of plants found near transhipment facilities and along various roads show that seeds are spilled into the environment in the course of transportation. The results obtained in the present study can serve as new baseline for the distribution of *B. napus* in the Netherlands.

From the distribution data obtained it is clear that *B. napus* and *B. rapa* can occur in sympatry. A small pilot study to find hybrids showed a high percentage of hybrids in fruits sampled on *B. rapa* plants flowering along a *B. napus* field. In two verges along land formerly cropped to *B. napus*, sampling of growing *B. rapa* showed a limited number of hybrids. The highest number of hybrids was found in mixed stands of *B. napus* and *B. rapa* on a ruderal site near Almere. With some plants, identification on the basis of morphological traits proved problematic. That hybridisation occurs between the two *Brassica* species is not a new finding. As yet, however, there has been only limited study of the effects of ecologically advantageous or disadvantageous modified traits introgressing into wild relatives in the natural environment for genetically modified crops (Warwick *et al*. 2009) and for *B. napus* in particular (Jørgensen *et al*. 2009).

The improved knowledge on the baseline distribution of *B. napus* can incorporated into existing and future monitoring plans for commercial releases GM. *B. napus* in order to apply focus to those areas where potential adverse environmental effects may occur most likely.

6. Monitoring of *Brassica napus* in the Netherlands

From this study focusing on the presence of *B. napus* in the natural environment in the Netherlands we conclude that this species is far less common in the ruderal landscape than previously thought and suggested by national species distribution data and the OECD consensus report. The number of plants found per site is fairly limited, although sites with several hundred plants can be found. In our judgement the suggestion made in the latest edition of Heukels' Flora (Van der Meijden 2005) that plants are not naturalised but establish, sometimes repeatedly from spilled seeds reflects the situation fairly accurately. Plants can sometimes establish a second generation from seed, but all sites and single plants we encountered were in highly disturbed areas like harbours, the verges of roads and railways and near places where birds were fed, strongly suggesting that human activities were essential for initiating the population. In some cases the origin of the plants still remains unclear, though. There is a partial overlap in the distribution of *B. napus* and the native *B. rapa* populations was found in contact areas.

The timescale of this project (a maximum of two flowering seasons) is too short to draw any conclusions as to whether *B. napus* sites are indeed feral, self-sustaining populations or merely temporarily present and (very) short-lived. The distribution data reported here can be taken as a new baseline for the distribution of *B. napus* in the Netherlands and reflect the fact that both GM and non-GM *B. napus* can escape to the natural environment.

In the European Union, an 'environmental risk assessment' (ERA) is mandatory for imports of food or feed derived from or containing GM plants and for outdoor cultivation of such plants. An ERA needs to indicate whether the GM plants have the potential to become more weedy or invasive than their comparator (often the parent). When a GM plant has obtained an authorisation for cultivation or import in Europe, 'post market environmental monitoring' (PMEM) is mandatory to assess any adverse effects the GM plant might have on the environment during import or cultivation. The implementation of monitoring is the responsibility of the authorisation holder. Monitoring of environmental impacts has two elements (Sanvido *et al*. 2005): case-specific monitoring (CSM) and general surveillance (GS). CSM, which is not obligatory unless the ERA identifies a particular risk, must address particular hypotheses vis-à-vis impacts contingent upon specific events identified in the environmental risk assessment. In GS, of which the implementation is also the responsibility of the permit holder, there is no specific hypothesis regarding any impacts of the GM plant, and this form of surveillance is always required in the EU. According to EU legislation, the objective of CSM and GS is to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO or its use in the ERA are correct and to identify the occurrence of adverse effects of the GMO or its use on human health or the environment which were not anticipated in the ERA, respectively. However, such potential effects may be wide-ranging in character, and either visible or largely hidden (Rotteveel & Den Nijs 2008), and plants may well emerge outside the agricultural environment. Together, this makes detection of possible adverse effects difficult for permit holders solely.

Brassica napus seeds and pollen become dispersed into the environment and plants can easily move beyond the monitoring area of the permit holder, i.e. the sites where the GMO is being cultivated, transshipped or processed. From this investigation it has become clear that non-GM *B. napus* is also found in the adjacent ruderal landscape, because seeds are lost along the verges of connecting roads in farming regions (especially in north-east Groningen), as well as along transport routes and near transhipment sites. If the observed distribution of non-GM *B. napus* reflects the potential distribution of GM *B. napus,* there is a need to extend the monitoring area beyond the agricultural environment. The present General Surveillance plan will then not

suffice for detecting possible establishment of GM *B. napus* as an invasive plant on roadsides and in disturbed habitats outside the agricultural system.

Besides adverse effects potentially affecting fauna, impacts on flora can also be expected, viz. hybridisation with close relatives and *B. rapa* in particular (Andersson & de Vicente 2010). Although the modest presence of *B. rapa* in *B. napus* cultivation areas might suggest little risk of introgression. However, the combination of low population size and self-incompatibility makes the former species highly susceptible to *B. napus* pollen, because of *B. rapa* pollen locally forming a minority compared with the *B. napus* pollen. Recording the presence of *B. rapa* in the direct vicinity of GM *B. napus* is therefore important for ascertaining the potential for introgression into the wild flora. Such monitoring will certainly involve extra effort and cost.

The good news is that we did not find many large populations of *B. napus* in the Netherlands, as had been previously imagined and suggested in the OECD consensus report. Reports supporting the latter (such as reported finds on waarneming.nl) could be traced back to confusion with *B. rapa* or could not be confirmed. Although large stands of plants were sometimes observed, the short timescale of this study makes it impossible to draw any bold conclusions about the establishment and persistence of the species at such sites.

6.1. Seed imports to the Netherlands

Seeds of *B. napus* are imported to the Netherlands in a variety of contexts. The vast majority, over 90%, is imported for warm crushing for oil production, which takes place only at ADM Europoort (Rozenburg) and Cargill (Amsterdam) (Tamis & de Jong 2010). These seeds are unloaded from large ocean-going vessels and factory sites are fenced off. There is probably little escape of seeds to the surrounding area and any establishing populations can easily be spotted and destroyed as necessary. The bulk of the remaining 10% of the seeds are imported for cold crushing, again for oil production, carried out at several locations in the Netherlands (Figure 17).

All these seed-crushing industries are near open water (sea, canal), where the seeds are brought in by ship and then transported by road to the pressing plant. Loss of seeds is localised and could be monitored by keeping an eye on the few roads connecting the harbour or docking berth of the ship with the crushing plant

The third route by which seeds of *B. napus* cultivars enter the Netherlands and subsequently the natural environment is as a component of animal feed, including seed mixtures for bird-feeding.

Finally, seeds of *B. napus* may potentially be imported as a contaminant of seed mixtures of other species imported from North America. However, our preliminary impression as to whether putative GM seeds of *B. napus* occur in seed mixtures was negative (Appendix 3).

In addition, seeds can escape from fields cropped to *B*. *napus*. As Figure 16 shows, these are to be found not only in Groningen and the Flevopolder, the traditional areas of *B. napus* cultivation, but right across the Netherlands. This finding may be of relevance in case of cultivation of GM *B. napus* in the Netherlands as also these areas should be considered in the monitoring plans.

6.2. Monitoring recommendations

In general, we suggest that monitoring should not be restricted to localised sites where genetically modified *B. napus* is cultivated, transhipped or processed, but be extended to the immediately vicinity. The cross-compatibility of *B. napus* with other related other crucifers and *B. rapa* in particular provides an opportunity for modified traits in *B. napus* to introgress through

pollination into volunteers. The inclination towards monitoring seems rather low, because imports of GM *B. napus* currently appear to be zero (Tamis & De Jong, 2010) and its cultivation is still prohibited. However, it is important to discuss the feasibility of incorporating the above monitoring suggestions in future plans and which parties bear responsibility, whether full or in part: the permit holder and/or the other monitoring networks.

The objective of monitoring plan is to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO or its use in the ERA are correct and to identity the occurrence of adverse effects of the GMO or its use on human health or the environment which were not anticipated in the ERA (Dir. 2001/18/EC). However such adverse effects in the natural environment, but such effects can only be detected if they embody a difference from the situation prior to introduction of the GMO (the baseline). Since the environment is not static baseline data should preferably know the natural changes of the environment before hand, This might not be feasible, however, because it is unknown where GM plant volunteers will arise.

The most likely place for GM plants to appear in the Netherlands due to imports is near the import harbours and along the connecting roads to crushing industries. At these locations it would be useful to monitor for the occurrence of GM *B. napus* and especially whether such plants form substantial, expanding populations and whether they exhibit traits intermediate between *B. napus* and *B. rapa* if the latter species has populations in the direct vicinity.

Regardless of the monitoring strategy adopted, it is recommended that it be conducted by botanical experts using the identification key we developed and that, when doubts arise, species determination should be verified using flow cytometry. Even with the FLORON folder issued in 2009, some FLORON volunteers mistook *B. rapa* for *B. napus* and on the website waarneming.nl observations of *B. napus* often prove to be incorrect.

Because *B. napus* populations are predominantly small and local, and input of seeds occurs at a small number of well-defined places, it will be fairly easy to detect new populations if and when these populations begin to expand and become invasive. This will depend, though, on the efficiency and extensiveness of the monitoring network.

Figure 17. Location of warm (large dots) and cold crushing plants in the Netherlands (Tamis & de Jong 2010).

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Appendices

Appendix1:
List of used cultivars and wild populations of *Brassica napus* and *Brassica rapa*

Appendix 2:
Cytometry analysis

Appendix 3:
Quantitative and qualitative analysis of morphological differences between *Brassica napus* and *Brassica rapa*

Appendix 4:
A descriptive key for identification of *Brassica napus* and *Brassica rapa*

Appendix 5:
The simplified "Libelle Method" for species identification

Appendix 6:
Testing *Brassica* seedlings for the presence of glyphosate resistance

Appendix 1

List of used cultivars and wild populations of *Brassica napus* and *B. rapa*.

Acc. nr	Species	Origin	abbr.	Name or sample site	Country	Year
1	*B. napus*	1	6879-1	6879-1	UKR	1980
2	*B. napus*	1	aa	CGN13917	NL	1976
3	*B. napus*	1	ab	6880	UKR	1980
4	*B. napus*	1	ake	Akela	NL	1966
5	*B. napus*	1	altex	Altex	CAN	-
6	*B. napus*	1	balt	Baltia	USR	1974
7	*B. napus*	1	bar	Barenza	NL	1974
8	*B. napus*	1	bb	6884	UKR	1980
9	*B. napus*	1	beli	Belinda	NL	1984
10	*B. napus*	1	bgs	Blauwe Groninger Snijmoes	NL	1980
11	*B. napus*	1	blako	Blako	NL	1954
12	*B. napus*	1	blue	Blue Siberean	NL	-
13	*B. napus*	1	bpt	Bpt(7) J derks	NL	1982
14	*B. napus*	1	brid	Bridger	USA	1985
15	*B. napus*	1	cas	Cascade	USA	1985
16	*B. napus*	1	gele	Friese Gele	NL	1954
17	*B. napus*	1	ggs	Groene Groninger Snijmoes	NL	1979
18	*B. napus*	1	hanna	Hanna	NL	-
19	*B. napus*	1	hgr	Hollandse Gele Roodkop	NL	1960
20	*B. napus*	1	lb	Limburgse Bladkool	NL	1974
21	*B. napus*	1	lon	Lonto	NL	1974
22	*B. napus*	1	mae	Maessen Bladkool	NL	1974
23	*B. napus*	1	manholt	Manholts Hamburger	NL	1899
24	*B. napus*	1	mara	Mara	NL	1979
25	*B. napus*	1	ow1	Ow1	NL	1982
26	*B. napus*	1	r731	R731	NL	1975
27	*B. napus*	1	ramon	Ramon	NL	1983
28	*B. napus*	1	ramp	Rampal	NL	1979
29	*B. napus*	1	sjg	Sjg	NL	1982
30	*B. napus*	1	sn	7230	NL	1977
31	*B. napus*	1	ss	6888	USR	1981
32	*B. napus*	1	tow	Tower	CAN	-
33	*B. napus*	1	trit	Triton	CAN	-
34	*B. napus*	1	velox	Velox	NL	1967
35	*B. napus*	1	vert	Vertis	NL	1977
36	*B. napus*	1	viva	Viva	NL	1974
37	*B. napus*	1	vrl	Vrl	NL	1982
38	*B. napus*	1	win	Windal	NL	1959
39	*B. napus*	1	xx	6886	UKR	1980
40	*B. napus*	1	zbg	Zandbult Groningen	Wild	2008
41	*B. napus*	1	zls	Zls	NL	1982
42	*B. napus*	1	zs	Zs	NL	1982
43	*B. napus*	5	ak44	Koolzaadakker A44	Field	2008
44	*B. napus*	2	awg	Afslag Woldendorp (paaltjes)	Wild	2008
45	*B. rapa*	2	bak44	Wegberm koolzaadakker A44	Wild	2008
46	*B. rapa*	2	be	Beemster	Wild	2008
47	*B. napus*	2	botlek	Botlek		2008
48	*B. napus*	2	fg	Farmsum bedrijventerrein	Wild	2008
49	*B. rapa*	2	fiets	Fietspas halfweg	Wild	2008
50	*B. napus*	2	fln	Flevoland napus	Wild	2008
51	*B. rapa*	2	flr	Felvoland rapa	Wild	2008
52	*B. napus*	2	fnig	Farmsum nigraplanten	Wild	2008
53	*B. napus*	5	gak	Koolzaadakker Groningen	Field	2008
54	*B. rapa*	2	hk	Heemskerk	Wild	2008
55	*B. rapa*	2	hull	Berm afslag boot naar Hull	Wild	2008
56	*B. napus*	2	kdg	Kade farmsun groningen	Wild	2008
57	*B. rapa*	2	kr	Kruislaan (Science Park, A'am)	Wild	2088
58	*B. rapa*	2	ma	Spoor Maarsen-Breukelen	Wild	2008

59	*B. rapa*	2	rak44	Rand koolzaadakker A44	Wild	2008
60	*B. rapa*	2	tom	Tom (viaduct A2 Maarsen)	Wild	2008
61	*B. napus*	2	vog1	Vogelhuis 1 (Maarsenbroek)	Garden	2008
62	*B. rapa*	2	vog2	Vogelhuis 2 (Maarsenbroek)	Garden	2008
63	*B. napus*	2	wa1n	Westpoort Adam 1 (kaapstadweg)	Wild	2008
64	*B. rapa*	2	wa1r	Westpoort Adam 1 (kaapstadweg)	Wild	2008
65	*B. napus*	2	wa2n	Westpoort Adam 2 (westpoortweg	Wild	2008
66	*B. rapa*	2	wa2r	Westpoort Adam 2	Wild	2008
67	*B. napus*	2	wmg	Windmolen Groningen	Wild	2008
68	*B. napus*	3	bil	Billy DSV	Breeder	2005
69	*B. rapa (4n)*	3	buko	Buko (KWS) Winterrübsen	Breeder	2007
70	*B. napus*	3	hor	Hornet DSV (Eurograss)	Breeder	2005
71	*B. napus*	3	ladoga	Ladoga (Limagrain)	Breeder	2005
72	*B. napus*	3	lion	Lioness DSV (Eurograss)	Breeder	2003
73	*B. napus*	3	oase	Oase DSV (Eurograss)	Breeder	
74	*B. rapa (4n)*	3	perko	Perko PVH (KWS) Winterrübsen	Breeder	2007
75	*B. napus*	3	pr45	PR45D03 (Pioneer Hi-Bred N-EU)	Breeder	2006
76	*B. napus*	3	pr46	PR46W31 (Pioneer Hi-Bred N-EU)	Breeder	2003
77	*B. napus*	4	vrn	*Brassica napus* var. *liforum*	Breeder	
78	*B. napus*	4	vrr	*Brassica rapa*, Nootzoet	Breeder	

Legend:

1. Cultivars obtained from CGN Wageningen
2. Wild populations
3. Recent cultivars (Pioneer, DSV, Limagrain)
4. Producer of (garden) seeds
5. *Brassica napus* field

Appendix 2
Cytometry analysis

As Table A2-1 shows, flow cytometry permits identification of the two species *Brassica napus* and *B. rapa,* but also can detect AAC hybrids. The cytometric value of the observed hybrid (1.8) is approximately intermediate between the parentals AA (1.1) and AACC (2.5). A surprising result is to find plants with 28 chromosomes in the field where 20A+9C= 29 chromosomes are expected and to find one *B. napus* cultivar with 2n=36 instead of 2n=38. The 38 chromosomes found for tetraploid *B. rapa* (cultivar Perko) instead of 40 chromosomes might be an error (missing of a small chromosome?). The relative amount of DNA is approximately duplication of diploid *B. rapa*. The cultivar Perko has a different morphology from *B. napus* and resembled *B. rapa* more closely, while tetraploid *B. rapa* is sturdier.

Table A2-1. Comparison of the relative amount of DNA (IRIBOV) and counts of chromosome numbers (De Jong & Ji, University of Wageningen) for different field collections of *Brassica rapa*, *B. napus* and possible hybrids.

code	seed plant, origin	DNA amount	chromosomes
A44-11-3	*B. rapa*, near field	1.1	20
HZ-2-2	*B. rapa*, roadside cycle track	1.1	20
WA2-18	*B. rapa*, Westpoort Amsterdam	1.09	20
WA2-18	*B. rapa,* Westpoort Amsterdam	1.11	20
WA2-23	*B. rapa*, Westpoort Amsterdam	1.08	20
WA2-23	*B. rapa*, Westpoort Amsterdam	1.09	20
BAK44-3	*B. rapa*, roadside opposite *B. napus* crop	1.09	20
BAK44-3	*B. rapa*, roadside opposite *B. napus* crop	1.1	20
HK-30	*B. rapa*, Heemskerk	1.07	20
HK-30	*B. rapa*, Heemskerk	1.09	20
RAK44-1	Hybrid on edge of *B. napus* crop	1.8	28
RAK44-1	Hybrid on edge of *B. napus* crop	1.8	28
GRakker 5-5	*B. napus*, Groningen field	2.48	36
GRakker 5-5	*B. napus*, Groningen field	2.49	36
AK44 2-1	*B. napus*, highway A44 field	2.48	38
AK44 2-1	*B. napus*, highway A44 field	2.53	38
Perko	*B. rapa* (4n), cultivar	2.23	38
Perko	*B. rapa* (4n), cultivar	2.14	38
Vreeken bv.	*B. napus* var. *liforum*, cultivar	2.52	38
Vreeken bv	*B. napus* var. "Nootzoet", cultivar *	2.45	38
F-13-3	*B. napus*, Talud Almere	2.48	38
F-13-3	*B. napus*, Talud Almere	2.53	38

* Sold as Raapzaad, Nootzoet (*Brassica rapa*), number 394300.

Appendix 3

Quantitative and qualitative analysis of morphological differences between *Brassica napus* and *Brassica rapa*.

The following 45 traits were measured to determine how both species varied morphologically.

1. Basal leaves
- 1a. Colour: glaucous or grassy-green (1)
- 1b. Number of paired lobes (end lobe not included)
- 1c. Hairs on the adaxial surface of the leaf
- 1d. Hairs on the midrib on the abaxial surface of the leaf
- 1e. Other hairs on the abaxial surface of the leaf
- 1f. Leaf texture: smooth or rough
- 1g. Lumps on the adaxial surface

2. Middle leaf (midway between the bottom leaf and the last leaf before the flowering stalk)
- 2a. Colour: glaucous or grassy-green (1)
- 2b. Hairs on the adaxial surface of the leaf
- 2c. Hairs on the midrib on the abaxial surface of the leaf
- 2d. Other hairs on the abaxial surface of the leaf
- 2e. Leaf texture: smooth or rough
- 2f. Amount of clasping of the broadened cordate base (%)
- 2g. Depth of the broadened cordate base (mm)
- 2h. Part of the broadened cordate base extending beyond the stem (mm)

3. Top leaves (leaves in the lower part of the inflorescence, but not a bract of a flowering stalk)
- 3a. Colour: glaucous or grassy-green
- 3b. Hairs on the adaxial surface of the leaf (3)
- 3c. Hairs on the midrib on the abaxial surface of the leaf
- 3d. Other hairs on the abaxial surface of the leaf (2)
- 3e. Leaf texture: smooth or rough (3)
- 3f. Amount of clasping of the broadened cordate base (%)
- 3g. Depth of the broadened cordate base (mm)
- 3h. Part of the broadened cordate base extending beyond the stem (mm)

4. Presence of at least one other leaf completely clasping the stem
5. Hairs on the stem (yes or no)
6. Shape of the inflorescence at the level of the open flowers: elongated or compact
7. Position of the buds (above, in between, below)
8. Position of the sepals (adjacent to the petal base, at a 45^{o} angle, or a 90 ^{o} angle)
9a. Overlap of the petals (yes or no)
9b. Percentage of petal overlap
10. Scent (yes or no)

11. Flower measurements
- 11a. Flower size (top open flower) in mm
- 11b. Diameter of the flower
- 11c. Length of the flower
- 11d. Width of the flower
- 11e. Length of the yellow petal limb (in Dutch: ”plaat”)
- 11f. Width of the yellow petal limb
- 11g. Length of the whitish petal base, or claw (in Dutch: “nagel”)
- 11h. Length of the sepal

12. Fruit measurements
- 12a. Total length of the siliqua
- 12b. Length of the beak (1)
- 12c. Width of the siliqua
- 12d. Ratio of beak to total fruit length
- 12e. Angle between the siliqua and the pedunculus *
- 12f. Angle between the siliqua and the pedicellus *
- 12g. Angle between the pedunculus and the pedicellus *

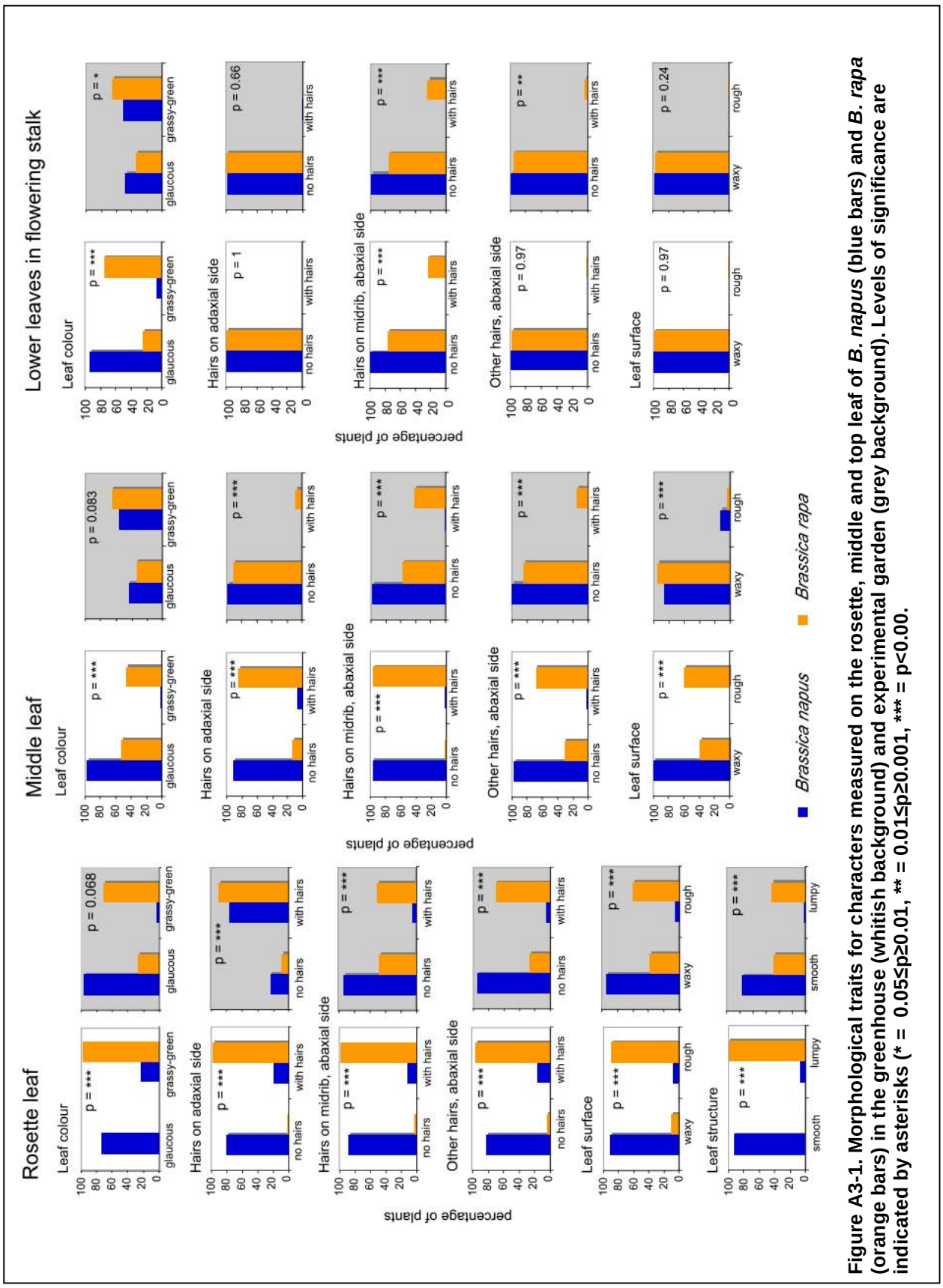

Figure A3-1. Morphological traits for characters measured on the rosette, middle and top leaf of *B. napus* (blue bars) and *B. rapa* (orange bars) in the greenhouse (whitish background) and experimental garden (grey background). Levels of significance are indicated by asterisks (* = 0.05≤p≥0.01, ** = 0.01≤p≥0.001, *** = p<0.00.

Leaf traits

There were significant differences in most of the traits of the leaves of *B. napus* and *B. rapa* (Figure A3-1). In the greenhouse the rosette leaves of the two species differed significantly with respect to all traits, but outside in the experimental garden the colour of these leaves was not significantly different (Chi-square, p=0.068). The trait of colour (blue-green (=glaucous) versus grassy-green) proved difficult to score, especially outside in the experimental garden.

The rosette leaves of *B. rapa* have hairs on both sides of the leaf. On the adaxial side small lumps are visible from which the hairs protrude. When the leaf is rubbed between the fingers the surface feels rough. The rosette leaves of *B. napus* are smooth and hairless.

The middle leaf has similar traits to the rosette leaves. Here, too, leaf colour proved to be an ambiguous trait. On plants outside in the experimental garden the middle leaf showed no significant differences, while in the greenhouse the leaf colour of the middle leaf did. The middle leaf of *B. rapa* is generally hairier than in the case of *B. napus*.

There were more similarities in the traits of the top leaf between the two *Brassica*s in the greenhouse than in the experimental garden. The leaf colour of the middle leaf showed a significant difference, though, being glaucous for *B. napus* and grassy-green for *B. rapa*. The leaves of both species feel smooth to the touch and are generally hairless, although a significant number of *B. rapa* plants was observed with hairs on the midrib on the abaxial side of the leaf.

The presence of hairs seems to differ between the two experimental locations. In the greenhouse more *B. rapa* plants were observed with hairs. On plants growing outside, hairs are easily lost.

The following traits were not significantly different between *B. napus* and *B. rapa*: number of paired side lobes on the leaf, total length of the middle leaf and total length of the top leaf.

Traits associated with the clasping of the stem by leaves, the depth of the broadened cordate leaf base and the distance this base extends beyond the stem (Figure A3-2) do show highly significant differences: p = *** (Anova). The percentage of stem-clasping was higher for *B. rapa* (80-90%) than for *B. napus* (approx. 50%).

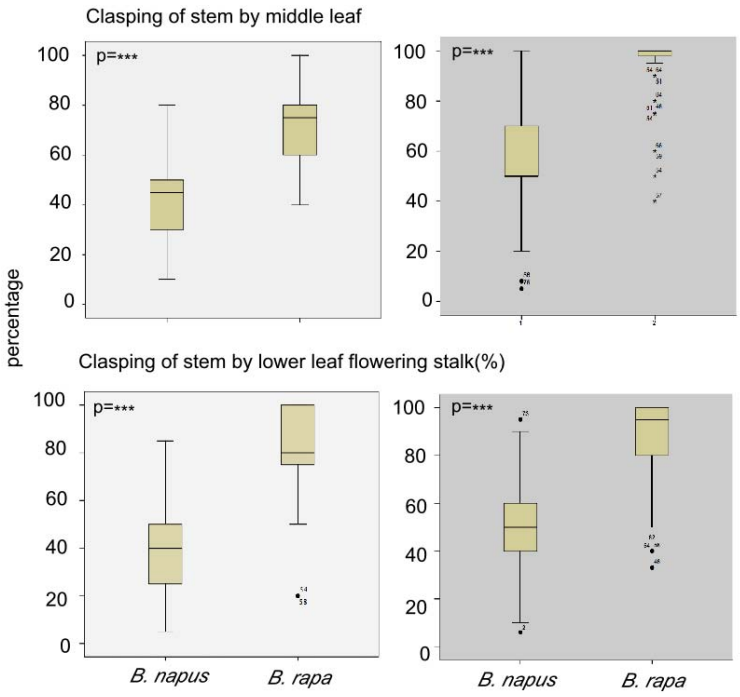

Figure A3-2. Stem-clasping of the middle and top leaves of *B. napus* and *B. rapa* in the greenhouse (whitish background) and experimental garden (grey background). Levels of significance are indicated by asterisks (* = p<0.001).**

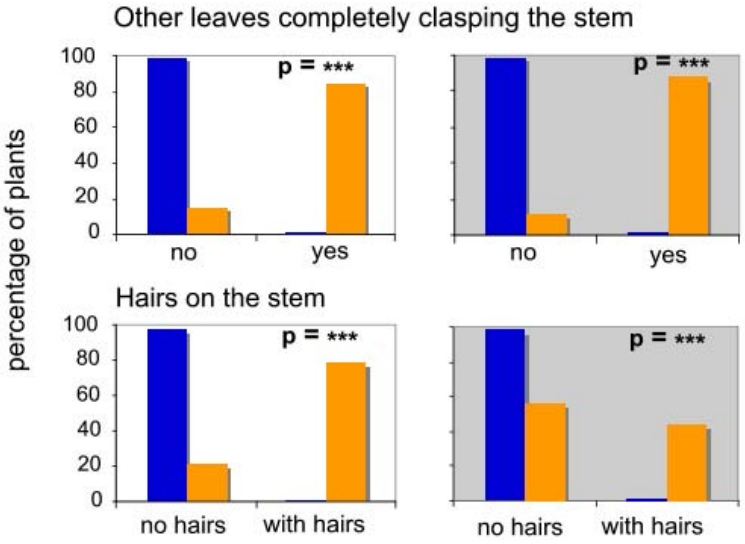

Figure A3-3. Presence of clasping leaves other than the top leaf and presence of hairs on the stem of *B. napus* (blue) and *B. rapa* (orange) in the greenhouse (whitish background) and experimental garden (grey background). Levels of significance are indicated by asterisks (* = p<0.001).**

Because the top leaf did not always completely clasp the stem, the presence of other completely clasping leaves was recorded. The results are reported in Figure A3-3 and indicate that this trait is significantly associated with *B. rapa* but not with *B. napus*, for which there is on average only 50% stem-clasping.

The stem of *B. napus* is predominantly hairless (Figure A3-3), while some *B. rapa* plants have hairs on their stem. These hairs are usually found on the lower half of the plant along the extension of the midrib of the leaves.

Floral traits

All the traits associated with the inflorescence show significant differences between *B. napus* and *B. rapa* (Figure A3-4). At both experimental sites, garden and greenhouse, the inflorescence of *B. napus* has an extended shape, which means the open flowers are widely separated (Figure A3-5, Photo 2). In *B. rapa* the open flowers are close together without any open space in between them (Figure A3-5, Photo 1).

Figure A3-5. Typical shape of the inflorescence.

Photo 1. *B. rapa*

Photo 2: *B. napus*

The position of the flower buds with respect to the open top flowers is significantly different between the two *Brassica*s, but the pattern is less clear. As Figure A3-4 shows, in *B. napus* the position of the buds ranges from above the open top flowers to in between them, in *B. rapa* from below the top flowers to in between them. The arrows indicate the trait as cited in the standard Heukels' Flora.

The position (or angle) of the sepal with respect to the base of the petal is not as straightforward as the Flora states, especially not in *B. rapa*. According to the Heukels' Flora the angle of the sepal is 45^{o} in *B. napus* and 90^{o} in *B. rapa*. In the experimental garden, most *B. rapa* plants had similarly positioned sepals to *B. napus*, viz. at an angle of 45^{o}. In the greenhouse, however, the angle of the sepal varied from 45^{o} to 90^{o}, with a tendency towards the latter.

The position of the buds and sepals is thus not particularly constant and appears to vary considerably with growing conditions.

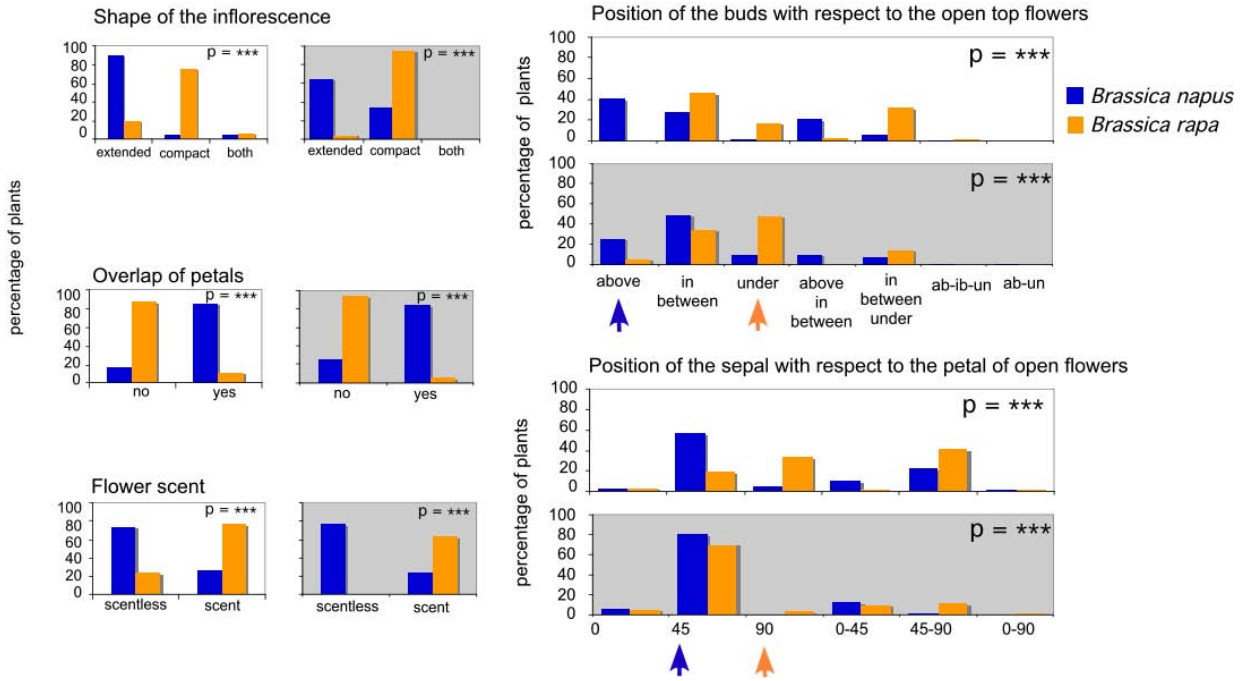

Figure A3-4. Floral traits of *B. napus* (blue) and *B. rapa* (orange) in the greenhouse (whitish background) and experimental garden (grey background). The arrows indicate the trait as cited in the Heukels' Flora. Levels of significance are indicated by asterisks (* = p≤0.001).**

The fourth floral trait, overlap of the petals, shows a clearly significant difference between *B. napus* and *B. rapa*. Most *B. napus* plants have flowers with overlapping petals, which is not generally the case with *B. rapa*. At the same time, though, some flowers of *B. rapa* do have overlapping petals within the range of *B. napus* (Figure A3-5). Most flowers of *B. napus* have an overlap of 5 to 10 (15) percent.

The flowers of *B. rapa* have significantly more scent than *B. napus*. However, not all flowers of *B. napus* are scentless. We found that while the scent of *B. rapa* was strong and (sickly) sweet, the flowers of *B. napus* had a weak, sweet scent. This differed among observers (male<>female), however, so this trait is rather subjective.

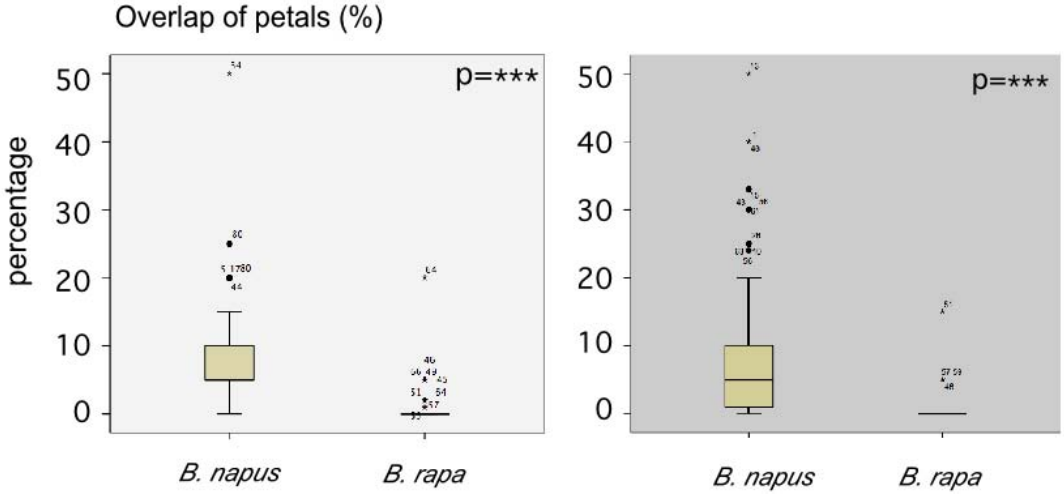

Figure A3-5. Percentage overlap of petals in *B. napus* and *B. rapa* in the greenhouse (whitish background) and experimental garden (grey background). Levels of significance are indicated by asterisks (* = p<0.001).**

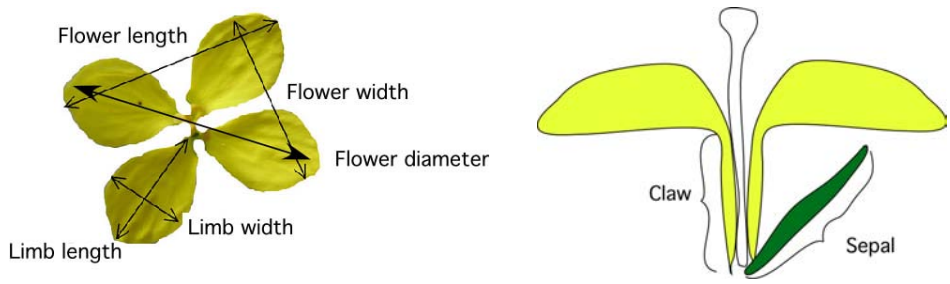

Figure A3-6. Flower traits measured: diameter, length and width of the flower; length and width of the limb; length of the sepal; length of the claw.

Flower measurements

Brassica napus has significantly larger flowers than *B. rapa*. The traits measured are shown in Figure A3-6. Although the diameter, length and width of the flower, the length and width of the petal limb and the length of the sepal are significantly greater in *B. napus*, we did observe an overlap in size between the two species (Figure A3-7). The length of the petal claw seemed to be fairly constant under both growing conditions (greenhouse versus garden).

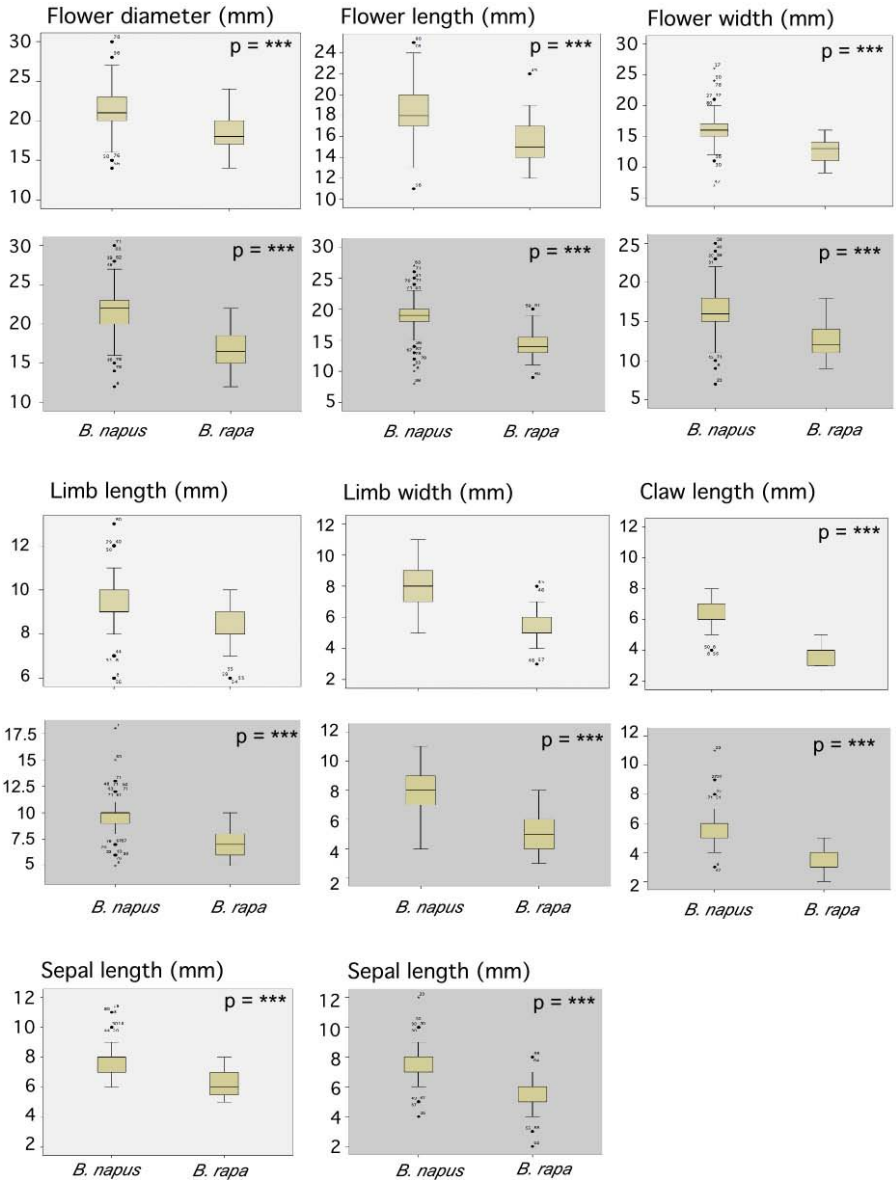

Figure A3-7. Various flower measurements of *B. napus* and *B. rapa* in the greenhouse (whitish background) and experimental garden (grey background). Levels of significance are indicated by asterisks (* = p<0.001).**

Fruit measurements

The fruits of *B. napus* are significantly longer and thicker than those of *B. rapa* (Figure A3-8). The beak of *B. rapa* fruits was found to be significantly longer for plants grown in the greenhouse. In plants grown outside in the experimental garden the length of the beak was similar for both species. Relative to total fruit length, the beak of *B. rapa* fruits was proportionally larger (approx. 25-30%) than for *B. napus* (20-25%) under both growing conditions.

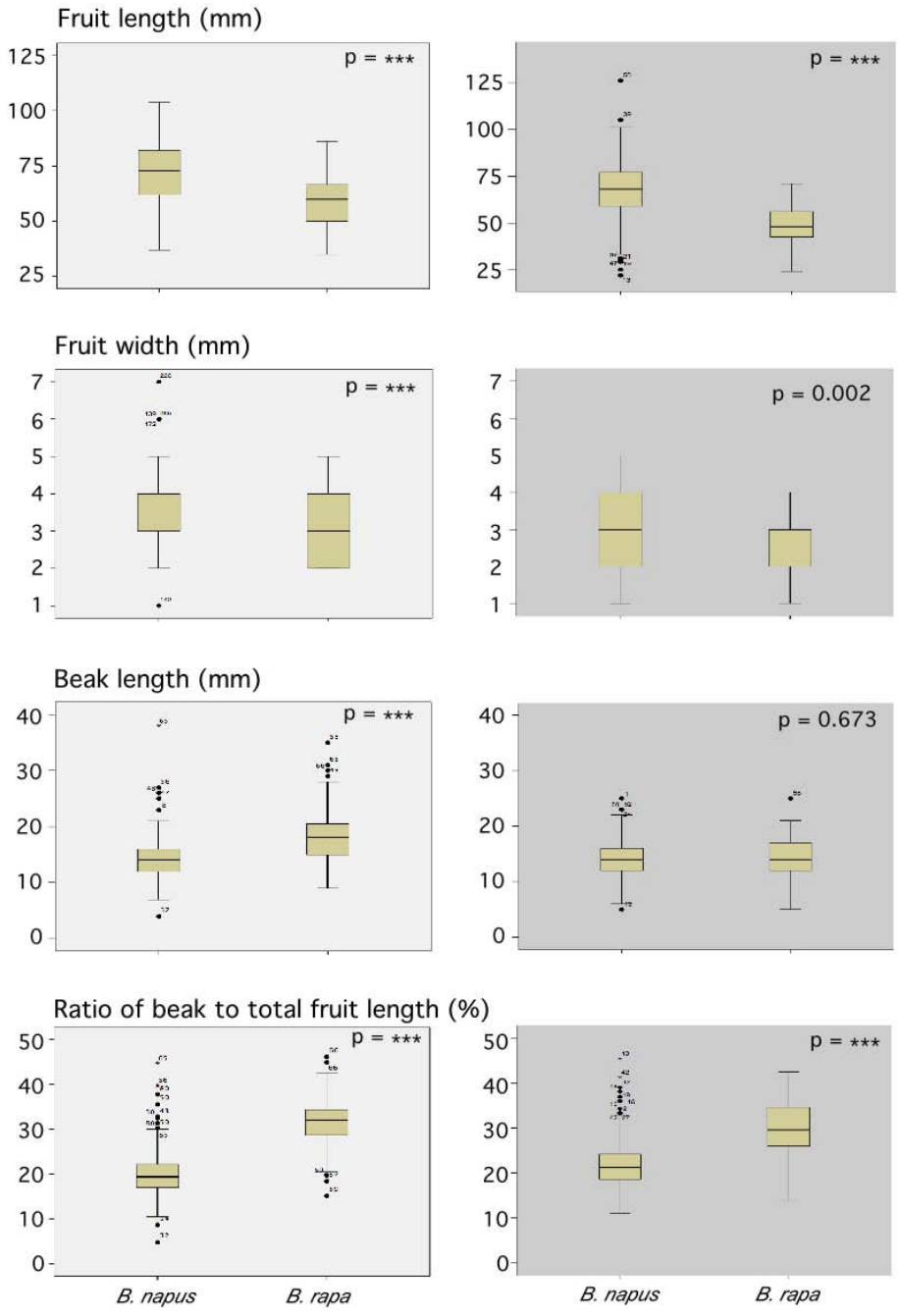

Figure A3-8. Various fruit measurements of *B. napus* and *B. rapa* in the greenhouse (whitish background) and experimental garden (grey background). Levels of significance are indicated by asterisks (* = p<0.001).**

The angle of the fruits with respect to the flowering stalk (pedunculus) is significantly different for the two species, although here too there is a degree of overlap (Figure A3-9). Nonetheless, the fruits of *B. rapa* generally tend to be more inclined towards the flowering stalk than those of

B. napus. The angle between the pedicellus of the fruit and the fruit itself is on average 25° for *B. rapa* and 15° for *B. napus* 15 degrees.

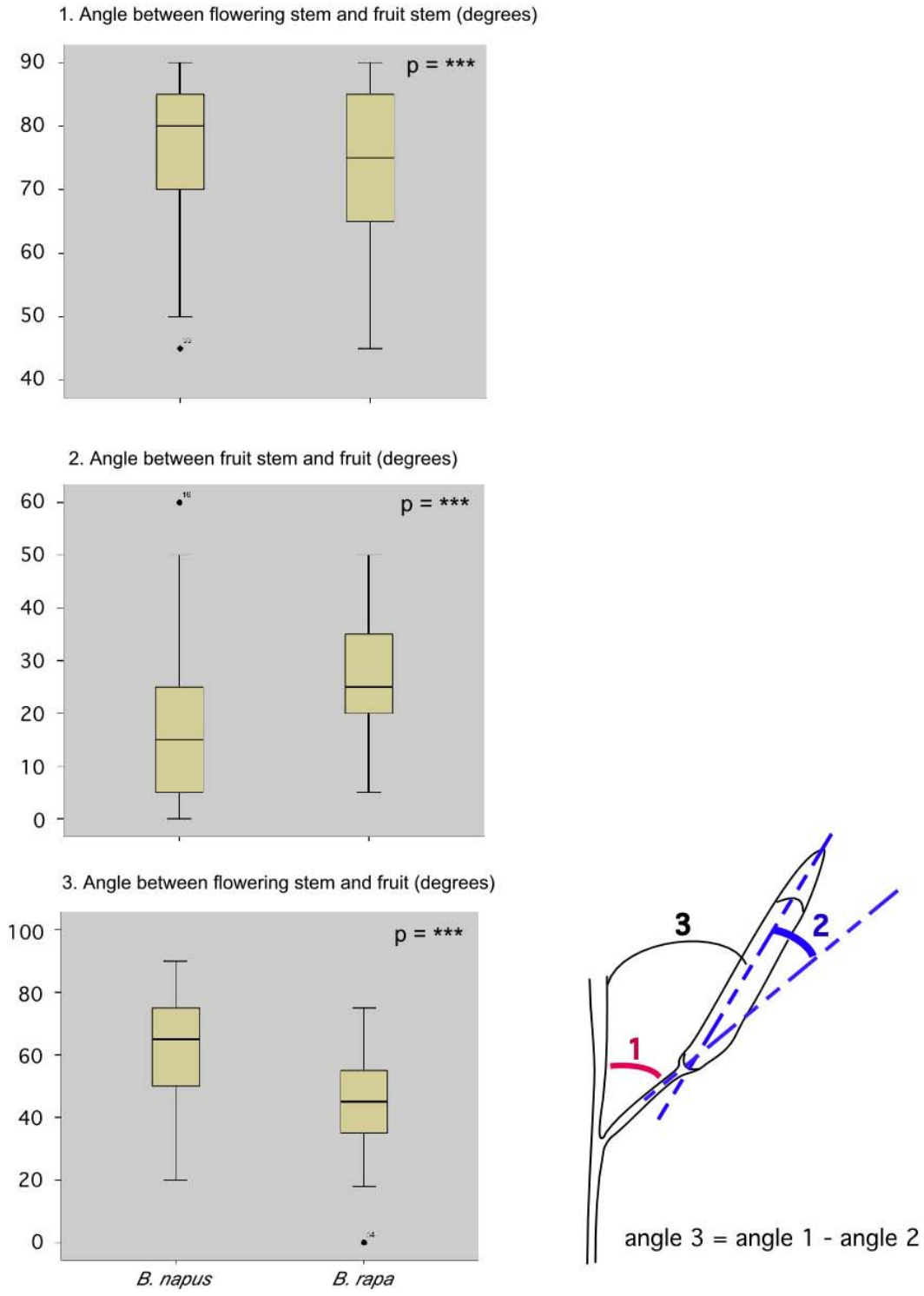

Figure A3-9. Various measurements of the angle between the fruit stem (pedicellus) and flowering stalk (pedunculus) of *B. napus* and *B. rapa* in the greenhouse (whitish background) and experimental garden (grey background). Levels of significance are indicated by asterisks (* = p<0.001).**

Appendix 4

A descriptive key for identification of *Brassica napus* and *Brassica rapa*

Brassica napus	*Brassica rapa*
Basal rosette leaves glaucous (blue-green) and generally glabrous, with a waxy surface. Leaves smooth and thick (cabbage-like).	Basal rosette leaves grassy-green with numerous hairs with a slightly bulbous base, giving impression of whitish dots when viewed from above. Leaves rough and thin and flexible (*Sinapis arvensis*-like).
Stem leaves glabrous.	Stem leaves with some coarse hairs on ribs (usually midrib on abaxial side).
Stem glabrous.	Stem sometimes with a few coarse hairs along extension of midrib.
No leaves clasping stem entirely; generally, all leaves less than 60% stem-clasping.	At least one leaf (generally upper) clasping stem entirely.
Inflorescence elongated, with open flowers loosely distributed on stalk of inflorescence below buds.	Inflorescence compact, with open flowers grouped together at top of inflorescence, overtopping buds or at same level.
Petals mostly in an imperfect cross because of petal overlap.	Petals usually in a perfect cross and not overlapping.
Width of petal limb: 7-9 (4-11) mm.	Width of petal limb: 4-6 (3-8) mm.
Length of petal claw: 5-6 (4-7) mm.	Length of petal claw: 3-4 (2-5) mm.
Ratio of beak to total fruit length: 18-25% (10-30%)	Ratio of beak to total fruit length: 25-35% (20-40%).
Mature fruits relatively thick: approx. 5 mm.	Mature fruits slender: approx. 3 mm.

Appendix 5

The simplified "Libelle Method" for species identification

1. Basal leaves
   - 1a. Colour of the leaves: glaucous = +1 or grassy-green = -1
   - 1c. Hairs on the adaxial surface of the leaf: yes = -1 or no = +1
   - 1d. Hairs on the midrib on the abaxial surface of the leaf: yes = -1 or no = +1
   - 1e. Other hairs on the abaxial surface of the leaf: yes = -1 or no = +1
   - 1f. Leaf texture: rough = +1 or smooth = -1
   - 1g. Lumps on the adaxial surface: yes = -1 or no = +1

2. Middle leaf (halfway between the bottom leaf and the last leaf before the flowering stalk)
   - 2a. Colour of the leaf: glaucous = +1 or grassy-green = -1
   - 2b. Hairs on adaxial surface of the leaf: yes = -1 or no = +1
   - 2c. Hairs on the midrib on the abaxial surface of the leaf: yes = -1 or no = +1
   - 2d. Other hairs on the abaxial surface of the leaf: yes = -1 or no = +1
   - 2e. Leaf texture: rough = +1 or smooth = -1

3. Top leaves (leaves in the lower part in the inflorescence, but not the bract of a flowering stalk)
   - 3f. Amount of clasping of the lower leaves in the inflorescence: less than 75% = +1; more than 75% = -1

4. Presence of at least one leaf completely clasping the stem: yes = -1 or no = +1
5. Hairs on the stem: yes = -1 or no = +1
6. Shape of the inflorescence at the level of the open flowers: elongated = +1 or compact = -1

9a. Overlap of the petals: yes = -1 or no = +1

10. Scent: yes = -1 or no = +1

11. Flower measurements
   - 11g. Length of the whitish petal base, or claw (Dutch: *nagel*): 5-6 (4-7) mm =+1 or 3-4 (2-5) mm = -1

Brassica napus is characterised by +1 values and *B. rapa* by -1 values. A value of +18 is *B. napus* and a value of -18 is *B. rapa.* However, lower values than +18 or higher values than -18 are observed. According to our results presented in the main text *B. napus* values fall in the range of +18 to +10 while *B. rapa* falls in the range of -18 tot -5.

Appendix 6.

Testing *Brassica napus* seedlings for the presence of glyphosate resistance

To gain an preliminary impression of whether GM seeds of *B. napus* occur in seed mixtures, we treated seedlings from a variety of seed sources with Roundup. We opted for glyphosate resistance because herbicides with this active ingredient are available to the public and are routinely sold at garden centres as Round-up® (Monsanto) or Clear-up® 360 N (Bayer). We did not test gufosinate resistance, but this GM *B. napus* is cultivated in Northern America.

Materials and methods

A total of 24 sets of *Brassica* seeds were examined for the presence of GM seeds (Table A6-1). Seeds were obtained via commercial suppliers or extracted from other seed crops (flax, millet, canary grass (*Phalaris canariensis*, in Dutch: *kanariezaad*) or from bird-feed seed mixtures obtained from pet shops or collected in the field. Seedlings were grown in 10 x 10 x 10 cm pots in a climate-controlled chamber (LD: 16/8 hours, 25.0 ± 0.2 °C, 70 % RH, light intensity 80 umol/m2/s) and provided with sufficient water. It was not possible to follow plants until the flowering stage to be certain of the species, and the species name given below is based on seed and seedling characteristics and is tentative.

After a period of approximately 3-4 weeks, when plants had grown sufficient leaf area, they were tested. Prior to each test, leaves were gently rubbed to remove water-repellent cuticula. The above-ground part of each plant was then briefly immersed in a standard solution (10 ml/l) of glyfosate concentrate. A series of preliminary experiments had previously demonstrated that, using this method, non-resistant plants showed clear signs of deterioration within 7 to 10 days, with plants dying in approximately 14 days or shortly thereafter. Due to availability, plants were tested in different series. In addition to each series of test plants, two sets of 10 control plants of *B. napus* (Vreekens zaden and liforum 392600) were tested. From each supplier, 5 plants were treated with the same herbicide solution as the test plants and 5 were treated with water. Between 2 and 3 weeks after treatment the numbers of surviving plants were scored.

Surviving plants underwent a second treatment with more concentrated glyfosate (20ml/l) to test resistance more rigorously and were then scored again.

Results

A total of 841 plants were tested. Only one batch, D15 Canada *Brassia sp.*, contained plants that were resistant to the glyfosate treatment (Table A6-1). Two plants survived the first glyfosate treatment (10ml/l), but not the second (20ml/l). Statistical analysis showed that the number of GM plants in the seed mixture in question is significantly ($p = 0.042$, asympt.) higher then the 0.9% limit set by EU regulations.

Discussion

Only two plants (from a batch of 36) survived the first glyphosate treatment. However, neither plant survived the second, more rigorous treatment. If plants are resistant, a double dose is not expected to kill them (Nicholas de Schrijver, personal communication). What is lacking is confirmation whether GM plants do survive our glyphosate treatment of submerging the whole plant. With that reservation, our results suggest that there were no plants in our sample that wereentirely resistant to glyphosate, whether naturally or by way of genetic modification.

Table A6-1. Number and percentage of plants surviving glyfosate treatment

Seed	n tested	n survivors	% survivors
Control 1, *B. napus* (supplier= Vreekens)	94	0	**0.0**
Control 2, B. *napus* var. liforum 392600	93	0	**0.0**
Rapeseed imported from Uruguay	27	0	**0.0**
D15 Flax from Canada, *Brassica juncea*	4	0	**0.0**
D15 Flax from Canada, *Brassica nigra*	9	0	**0.0**
D10 Canary grass Canada, *Brassica sp.*	8	0	**0.0**
Rapeseed imported from Uruguay	26	0	**0.0**
D 15 Flax from Canada, *Brassica sp.*	36	2	**5.6**
D 16 Millet from USA, *Brassica sp.*	4	0	**0.0**
D 14 supplier 2122, article 200133 Frankrijk *B. napus* bulk	30	0	**0.0**
D13 supplier 4717, article 200133, France, *B. napus* bulk	27	0	**0.0**
D 21 supplier 6135, article 200233 Canada, *B. rapa* bulk	33	0	**0.0**
Aviary bulk seed mixture from G. Dolderman store, Montfoort	20	0	**0.0**
Aviary bulk seed mixture from G. Dolderman store, Montfoort	59	0	**0.0**
Canary seed mixture from Deli Nature premium Beduco NV (Belgium), containing *B. rapa, B. napus, B. nigra* guaranteed 'natural'	58	0	**0.0**
Pigeon seed mix from Discus	24	0	**0.0**
B. napus collected 2009 at Woerden railway station	28	0	**0.0**
Seed mixture with *B. rapa/B. napus* distributed by Stichting Natuur en Milieu	62	0	**0.0**
Brassica napus, Natuur en Milieu	98	0	**0.0**
Brassica rapa, Natuur en Milieu	48	0	**0.0**