Dissimilarity of contemporary and historical gene flow in a wild carrot (*Daucus carota*) metapopulation under contrasting levels of human disturbance: implications for risk assessment and management of transgene introgression

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- **Background and Aims** Transgene introgression from crops into wild relatives may increase the resistance of wild plants to herbicides, insects, etc. The chance of transgene introgression depends not only on the rate of hybridization and the establishment of hybrids in local wild populations, but also on the metapopulation dynamics of the wild relative. The aim of the study was to estimate gene flow in a metapopulation for assessing and managing the risks of transgene introgression.
- **Methods** Wild carrots (*Daucus carota*) were sampled from 12 patches in a metapopulation. Eleven microsatellites were used to genotype wild carrots. Genetic structure was estimated based on the F_{ST} statistic. Contemporary (over the last several generations) and historical (over many generations) gene flow was estimated with assignment and coalescent methods, respectively.
- **Key Results** The genetic structure in the wild carrot metapopulation was moderate ($F_{ST} = 0.082$) and most of the genetic variation resided within patches. A pattern of isolation by distance was detected, suggesting that most of the gene flow occurred between neighbouring patches (≤ 1 km). The mean contemporary gene flow was 5 times higher than the historical estimate, and the correlation between them was very low. Moreover, the contemporary gene flow in roadsides was twice that in a nature reserve, and the correlation between contemporary and historical estimates was much higher in the nature reserve. Mowing of roadsides may contribute to the increase in contemporary gene flow. Simulations demonstrated that the higher contemporary gene flow could accelerate the process of transgene introgression in the metapopulation.
- **Conclusions** Human disturbance such as mowing may alter gene flow patterns in wild populations, affecting the metapopulation dynamics of wild plants and the processes of transgene introgression in the metapopulation. The risk assessment and management of transgene introgression and the control of weeds need to take metapopulation dynamics into consideration.

Key words: *Daucus carota*, wild carrot, genetic structure, transgene introgression, gene flow, genetically modified crops, GM, metapopulation dynamics, migration rate, weed control.

INTRODUCTION

The rapid development of biotechnology offers new opportunities to ensure food supply. Novel traits can be introduced into crops by transgene technology more efficiently than by conventional crop breeding. Since the first commercialization of a genetically modified (GM) crop in 1996, the global area of GM crops has grown steadily and reached 170.3 million hectares in 2012 (James, 2012). Increasing numbers of GM crops with different traits are being produced and released into the environment. The introduction of new transgenes into crops has raised concerns about possible negative effects on the environment. Transgenes could move from crops into wild relatives via gene flow. Depending on the nature of the transgene and its product, such transgene flow may lead to unwanted ecological and evolutionary consequences in wild populations (Ellstrand, 2003a, b).

For instance, Snow *et al.* (2003) reported that an insect-resistant transgene that codes for the *Bacillus thuringiensis* (Bt) toxin could increase seed production in wild sunflower by up to 55 % owing to reduced herbivory. Such transgene introgression may increase the invasiveness of wild sunflower and have negative effects on local communities (Snow *et al.*, 2003). A study by Watrud *et al.* (2011) suggested that *Brassica* species containing herbicide resistance transgenes may become dominant in plant communities under herbicide drift. Schafer *et al.* (2011) found that *Brassica napus* with herbicide resistance transgenes had escaped from the cultivation fields and grew extensively on roadsides of North Dakota, USA. In addition, Londo *et al.* (2011) showed that, because of the stacking of herbicide and insect resistance transgenes in *B. napus*, the frequency of an unselected transgene can increase by hitchhiking with a selected transgene depending on the selective pressure. Therefore, the environmental

risks associated with transgene introgression need to be carefully assessed before the commercialization of GM crops.

The process of transgene introgression from crops into wild relatives involves several steps: first, the formation of crop–wild hybrids with a transgene through hybridization between crops and wild populations; second, the establishment of the transgene in local wild populations through backcrossing with wild plants; third, the spread of the transgene across the whole metapopulation of the wild species via pollen and seed dispersal. The majority of previous studies have focused only on evaluating the first two steps of transgene introgression (Meirmans *et al.*, 2009). By developing a metapopulation model for transgene introgression, Meirmans *et al.* (2009) showed that the frequency of the transgene in the metapopulation depends considerably on the population dynamics. High gene flow among populations and/or a high rate of population turnover (extinction/colonization) may lead to high transgene frequency. It is therefore recommended that studies on the environmental risk assessment of transgene introgression should incorporate the metapopulation dynamics of the wild relatives (Meirmans *et al.*, 2009).

Since the probability of transgene introgression depends on metapopulation dynamics, factors influencing metapopulation dynamics may also affect introgression. Wild populations close to crop fields are usually strongly affected by human disturbance. Habitat loss and fragmentation due to human disturbance may alter the level of gene flow among patches and the rate of patch turnover. If gene dispersal becomes limited under strong human disturbance, the distribution pattern of genetic diversity may change dramatically in the metapopulation. In this case, a newly emerged gene, such as a transgene in a local wild population, may not be able to spread through the metapopulation. Conversely, human-mediated dispersal may enhance connectivity among populations in areas where anthropogenic disturbance is high, which would lead to increased spread of an escaped transgene. However, it is difficult to study the effects of human disturbance and associated habitat changes on gene flow, because finding intact wild populations as controls is hard and the effects of other factors may interfere with those of human disturbance (Chiucchi and Gibbs, 2010). It would be ideal if we could compare gene flows from the same set of populations with and without human disturbance.

Chiucchi and Gibbs (2010) used the approach of analysing a single data set with two analytical techniques that estimated gene flow in a snake species on different time scales: assignment tests that estimated gene flow over a few generations (Wilson and Rannala, 2003) and coalescent techniques that covered many generations (Beerli and Felsenstein, 2001). The coalescent-based technique provides a summary of gene flow over hundreds of generations and therefore goes back to the time before humans began to alter habitats significantly (Chiucchi and Gibbs, 2010). It therefore represents the gene flow pattern with less overall human disturbance than the contemporary estimate. Chiucchi and Gibbs (2010) found that contemporary and historical rates of gene flow were generally low and similar among populations. This result suggested that highly fragmented habitats resulting from strong human disturbance might not necessarily lead to considerable changes in gene flow among populations (Chiucchi and Gibbs, 2010). However, the observed similarity of contemporary and historical gene flow may have been a result of the extremely limited dispersal pattern of the snake species

studied (Chiucchi and Gibbs, 2010). For species with higher levels of gene flow, more studies are required to determine the roles that human disturbance play in metapopulation dynamics.

In Europe, only one GM crop is allowed to grow in fields for commercial production: *Bt* maize for insect resistance (James, 2012). One reason for the approval of *Bt* maize may be that the risks of transgene introgression into wild relatives are absent in Europe. For crops such as oilseed rape (*B. napus*) and carrot (*Daucus carota* ssp. *sativus*), however, the risks of transgene introgression are higher because their wild relatives are widely distributed in Europe and introgression can occur from crops into wild relatives (Devos *et al.*, 2004; Magnussen and Hauser, 2007). Wild carrot (*D. carota* ssp. *carota*) is the ancestor of the cultivated carrot and they can easily hybridize with each other in the field (Magnussen and Hauser, 2007). Wild carrots are distributed throughout Europe and the Mediterranean, North Africa, Central and Southwestern Asia, and Ethiopia (Dale, 1974; Heywood, 1983). They have been introduced into many parts of the world, e.g. America, New Zealand, Australia, China and Japan (Dale, 1974; Li and Xie, 2002; Hauser *et al.*, 2004; Umehara *et al.*, 2005). Wild carrots are noxious weeds in North America, China and Japan (Dale, 1974; Li and Xie, 2002; Umehara *et al.*, 2005; USDA, 2012). They can spread rapidly in areas disturbed by human activities, e.g. roadsides and wastelands (Umehara *et al.*, 2005). The cultivated carrot is attracting increasing attention for genetic modification owing to the great nutritional benefits of high carotenoid contents. Genetically modified carrots with different traits have been developed, e.g. resistance against biotic and abiotic stress (Chen and Punja, 2002; Kumar *et al.*, 2004) and increased nutritional content (Morris *et al.*, 2008), though none has been approved for commercialization yet. Given its already present propensities for weediness, the introgression of transgenes with fitness advantages into wild populations may turn the wild carrot into a more serious weed.

Previous studies have indicated that introgression could occur in the field from cultivated to wild carrots (Wijnheijmer *et al.*, 1989; Magnussen and Hauser, 2007). Cultivated and wild carrots can easily hybridize with each other and the hybrids can survive and reproduce in nature (Wijnheijmer *et al.*, 1989; Hauser and Shim, 2007; Magnussen and Hauser, 2007). In addition, the wild carrot is predominantly an outcrossing plant and its pollen can be dispersed by insects over a long distance (Rong *et al.*, 2010). Therefore, crop-specific alleles from cultivated carrots may spread easily within and between wild carrot populations. However, studies on gene flow among wild carrot populations and metapopulation dynamics are still lacking. According to the metapopulation model of introgression by Meirmans *et al.* (2009), such studies will provide further information about the overall chance of introgression from a crop species into a metapopulation of its wild relatives.

Here, in order to characterize the metapopulation dynamics, especially the effects of human disturbance on metapopulation dynamics, we compared historical and contemporary patterns of gene flow in a wild carrot metapopulation. We tested the null hypothesis that human disturbance did not change gene flow in the metapopulation and that contemporary gene flow was similar to historical gene flow in wild carrots. Then, we applied a metapopulation model of introgression to simulate the process of transgene introgression in the wild carrot

metapopulation. We aimed to answer the following questions: (1) What is the rate of gene flow in the wild carrot metapopulation? (2) Is contemporary gene flow equal to historical gene flow in the wild carrot metapopulation? (3) How does the rate of gene flow affect the chance of transgene introgression into the wild carrot metapopulation?

MATERIALS AND METHODS

Study site and sampling

Wild carrot (*Daucus carota*) is widely distributed in the Netherlands. It occurs predominantly on roadsides exposed to a high level of human disturbance. The study area, located in the province of South Holland, is one of the areas most disturbed by humans in the Netherlands (Fig. 1). It is next to the historic city of Leiden, at a distance of about 20 km north of The Hague and 40 km south of Amsterdam. In summer, flowering wild carrots are distributed almost continuously along roads; away from roadsides, e.g. in cultivation fields and old grasslands, they are rare. There are also some wild carrot populations in the dune nature reserves (areas close to the North Sea), where there is little human disturbance.

The roadsides in the study area are mowed once a year in autumn and the resulting hay is used for feeding livestock. Mowing used to be more frequent in this area but has been reduced in an attempt to provide food and habitats for insects and birds. The degree of human disturbance (e.g. mowing, traffic, urbanization) has increased dramatically during the last several hundred years.

We sampled 12 patches of wild carrots that were distributed throughout the wild carrot metapopulation, with a distance of at least 412 m between adjacent patches (Fig. 1). Three patches (WE, WP and WM; Fig. 1), are located in the dune nature reserve and the remaining nine patches are along roadsides. About 30 plants were randomly chosen from each patch (Table 1) and leaves were collected from each plant for DNA extraction. Leaf samples were placed in 2.0 mL tubes and stored at -80°C until DNA extraction.

DNA extraction and microsatellite genotyping

Genomic DNA of wild carrot was isolated using the DNeasy Plant Mini Kit (Qiagen, Venlo, the Netherlands). Eleven microsatellite markers were used for genotyping the samples; this was the same set of loci as used by Rong *et al.* (2010) excluding microsatellite locus 1–5E, because its number of alleles was too high for analyses by BAYESASS (Wilson and Rannala, 2003) and MIGRATE (Beerli, 2009). The detailed protocol was reported in Rong *et al.* (2010). The products amplified by PCRs with different fluorescent labels were multiplexed and run on a MegaBACE sequencer (GE Healthcare, Eindhoven, the Netherlands) with ROX 400 as an internal size standard. Alleles at each microsatellite locus were scored as fragment sizes using the MegaBACE Fragment Profiler software, version 1.2 (Amersham Biosciences, 2003). To reduce errors, samples with weak signals or unclear peaks were reamplified and rerun. Remaining samples with weak signals or unclear peaks were removed and regarded as missing data (less than 5 % per microsatellite locus).

FIG. 1. Study site and sampled patches of the wild carrot metapopulation. The study site is located between the North Sea and Leiden, the Netherlands. Wild carrots are widely distributed in this area, mainly along roads subject to strong human disturbance (e.g. regular mowing and heavy traffic). The open circles indicate the locations of the 12 wild carrot patches studied (WA – WP). There were a few wild carrot patches inside the dune nature reserve, subject to little human disturbance (e.g. WE, WM and WP).

TABLE 1. Sampling information of wild carrot patches

Patch	Location (latitude, longitude)	Density (m ⁻²) ¹	Average distance between adjacent samples (m)	Maximum distance between samples (m)
WA	52°11'16–17" N, 4°25'1–3" E	0.44	1.5	45
WB	52°10'53–54" N, 4°24'33–35" E	0.37	3.1	90
WC	52°9'47–48" N, 4°23'51–53" E	0.62	2.0	60
WD	52°9'33–34" N, 4°22'36–41" E	0.62	3.6	112
WE	52°11'22–23" N, 4°22'47–49" E	0.45	1.8	40
WF	52°9'29–30" N, 4°26'39–40" E	1.00	1.7	50
WH	52°10'58" –11" N, 4°25'18–22" E	0.56	3.7	108
WI	52°10'34–35" N, 4°25'53–56" E	1.29	2.1	58
WK	52°11'24–28" N, 4°24'35–39" E	1.20	4.2	125
WM	52°9'19–24" N, 4°22'44–52" E	0.40	6.7	210
WN	52°10'3–5" N, 4°26'49–52" E	1.95	2.2	77
WP	52°10'50–52" N, 4°22'54" –23'1" E	0.49	5.2	155

¹Average number of flowering individuals per m² in each patch.

Genetic structure

To summarize the diversity at the microsatellite loci, we used GENALEX 6.41 (Peakall and Smouse, 2006) to calculate the sample size (N), number of alleles (A) and effective alleles (A_e), Shannon's information index [$I = -\sum p_i \ln(p_i)$, where p_i is the frequency of the i th allele], observed and expected heterozygosity (H_o and H_e) and fixation index ($F_{IS} = 1 - H_o/H_e$). Exact tests for Hardy–Weinberg equilibrium were performed with GENEPOP 4.0 (Rousset, 2008). The Markov chain parameters for the tests were 10 000 dememorizations, 1000 batches and 10 000 iterations per batch. Analysis of molecular variance (AMOVA) was performed with Arlequin 3.5.1.3 (Excoffier and Lischer, 2010) and significance tests were based on 10 000 permutations. Under isolation by distance in a two-dimensional habitat, $F_{ST}/(1 - F_{ST})$ estimates for pairs of subpopulations (patches) are expected to be correlated approximately linearly with the logarithm of the pairwise geographical distance (Rousset, 1997; Fenster *et al.*, 2003). The regression slope b then equals $1/4\pi D\sigma^2$, where D may represent the global density of the metapopulation and σ may refer to gene dispersal among patches (Rousset, 1997; Fenster *et al.*, 2003). Pairwise $F_{ST}/(1 - F_{ST})$ ratios were calculated, tested for significance in different distance classes (0–1, 1–2, 2–3, 3–4 and 4–5.605 km) and regressed on the natural logarithm of spatial distance with SPAGeDi 1.3d (Hardy and Vekemans, 2002). Significance tests were based on 10 000 random permutations done for locations, individuals and genes, respectively.

Contemporary and historical gene flow

Contemporary rates of gene flow among patches were estimated with BAYESASS 1.3 (Wilson and Rannala, 2003). BAYESASS uses assignment tests in a Bayesian framework and a Monte Carlo Markov chain (MCMC) to estimate the migration rates among populations over the last several generations (Wilson and Rannala, 2003). Each MCMC run was performed with 2×10^6 iterations after a burn-in of 1×10^6 and with a sampling frequency of 2000. To maximize the log likelihood values, delta values were adjusted so that the accepted numbers of changes were 40–60 % of the total number of iterations. In order to minimize convergence problems, we carried out 36

MCMC runs with different initial seeds and selected the one with the lowest deviance for further analysis (Faubet *et al.*, 2007).

The historical rates of gene flow among patches were estimated with MIGRATE 3.2.7 (Beerli, 2009). MIGRATE uses coalescent theory to estimate effective population size θ (equals $4N_e\mu$ for nuclear data) and gene flow $M = m/\mu$ over a long period of time (within the past $4N_e$ generations), where N_e is the effective population size, μ is the mutation rate and m is the migration rate (Beerli and Felsenstein, 2001). Wild carrot is usually annual or biennial in the Netherlands; the historical estimate may therefore summarize gene flow over hundreds of years in the past, which is much longer than the duration of strong human disturbance in the current urbanized landscape (e.g. mowing). MIGRATE uses either a maximum likelihood (ML) or a Bayesian analysis with an MCMC to estimate parameters (Beerli and Felsenstein, 2001; Beerli, 2006, 2009). Here we used the Bayesian method because it has been shown that this method works better than the ML approach in accuracy and coverage (Beerli, 2006). We used the Brownian motion mutation model for the microsatellite loci and uniform prior distributions (minimum = 0, maximum = 100, delta = 10) for parameter estimates. For the Markov chain settings, samples were taken every 100 cycles, and 10 000 genealogies were recorded after a burn-in of 10 000. For the parameter estimates, the results of three replicated runs were combined. In order to increase the efficiency of the MCMC, heating was turned on by running four parallel chains at the temperatures of 1.0, 1.5, 3.0 and 1 000 000.0 and swapping between them. To perform the analysis within a reasonable time, we parallelized the MIGRATE run at the Chinese Academy of Sciences and Max Planck Society Partner Institute for Computational Biology. Assuming a mutation rate μ of 5×10^{-4} for microsatellite loci (Chiucchi and Gibbs, 2010), the migration rate m was calculated as $M\mu$ with M values estimated from the MIGRATE run.

The pairwise mean migration rates between patches estimated with BAYESASS were compared with those estimated with MIGRATE. To test the correlation between contemporary and historical migration rate matrices, a Mantel test was performed using mantel.rtest ('ade4' library) with 10 000 permutations in R (R Development Core Team, 2010). Mantel tests were also performed for the correlation between migration rate and the natural logarithm of spatial distance. To further evaluate the effects of human disturbance, gene flow estimates among patches in the

roadsides under high human disturbance were compared with those in the dune nature reserve under low human disturbance.

Model simulation

To assess the influence of migration rate on the processes of transgene introgression into a metapopulation, we performed model simulations using the program INTROGRESSION (Meirmans *et al.*, 2009; <http://www.patrickmeirmans.com/software/>). Mirroring the sampling setup, we modelled a metapopulation consisting of 12 wild carrot populations all with the mean effective population size calculated by MIGRATE. We assumed that one of the simulated wild carrot populations was adjacent to a GM carrot root production field and received pollen from this field. As an estimate of the rate of hybridization between the crop and the wild population we used the data from Magnussen and Hauser (2007). They found four hybrids out of 71 wild carrot plants growing close to carrot root production fields. These hybrids were most likely F_2 or backcross individuals sired by pollen from flowering bolters in carrot fields. Therefore, we set the rate of pollen flow from the GM carrot field to the adjacent wild carrot population to $4/71 = 0.056$. To compare the effects of different migration rates in the metapopulation on the risk of transgene introgression, the migration rates among wild carrot populations were set to the mean of the historical and contemporary gene flow estimates respectively. According to Meirmans *et al.* (2009), in the model simulation the fitness of the homozygous wild-type allele was set to 1, the fitness of the homozygous transgene was $(1 + s)$ and the fitness of the heterozygote was $(1 + 0.5 \times s)$. To simulate the effects of selection on the risk of transgene introgression, the selection coefficient of the transgene (s) was set to 0 or 0.2 in the program. The extinction rate of populations was set to 0. The processes of transgene introgression were simulated stochastically with 20 replicates for each parameter combination.

RESULTS

Genetic structure

The genetic diversity estimates were similar among wild carrot patches (Table 2). The global Hardy–Weinberg tests with GENEPOP indicated that all wild carrot patches studied showed significant deficits in heterozygotes, suggesting genetic structure or self-fertilization within patches. Genetic differentiation among wild carrot patches was moderate but highly significant (overall $F_{ST} = 0.082$, $P < 0.00001$), with most of the genetic variation occurring within patches (91.8 %). Linear regression of pairwise $F_{ST}/(1 - F_{ST})$ on the natural logarithm of spatial distance between patches was significant ($P < 0.05$), showing a pattern of isolation by distance (Fig. 2). In addition, random permutations in different distance classes showed that only within 1 km were values of $F_{ST}/(1 - F_{ST})$ significantly different ($P < 0.01$). These results suggest that most of the gene flow might occur between adjacent wild carrot patches, leading to lower genetic differentiation between them than between wild carrot patches further apart. The regression slope b was 0.022, with a jack-knifed standard error (SE) over all loci of 0.011. Therefore, $4\pi D\sigma^2$ was 45 with the 95 % confidence interval (CI, estimated with 2SE) from 23 to infinity. This estimate also suggested that most gene flow might occur among neighbouring individuals when they were distributed continuously.

Contemporary and historical gene flow

The mean contemporary gene flow among pairs of patches ranged from 0.0008 to 0.0898 (overall mean = 0.0032; Fig. 3). In this study, the numerical estimates of gene flow shown refer to migration rate (m). The highest gene flow 0.0898 (95 % CI 0.0082–0.1828) was from WM to WD with the closest distance of 412 m apart. The mean gene flow was lower than 0.01 when the distance between patches was greater than 3.3 km (Fig. 3). The

FIG. 2. Isolation by distance in a wild carrot metapopulation. The open circles indicate the pairwise observed values of $F_{ST}/(1 - F_{ST})$ of wild carrot patches. The broken line represents the regression line of $F_{ST}/(1 - F_{ST})$ on the natural logarithm of spatial distance (km) between patches. The filled squares represent mean values of pairwise $F_{ST}/(1 - F_{ST})$ of wild carrot patches in different distance classes (0–1, 1–2, 2–3, 3–4 and 4–5.605 km) between patches, and bars indicate standard errors.

FIG. 3. Pairwise contemporary and historical gene flow among wild carrot patches. Open circles represent contemporary gene flow estimates calculated with BAYESASS. Filled circles indicate historical gene flow estimates calculated with MIGRATE. It is assumed that gene flow is asymmetrical, e.g. gene flow from WM to WD is not the same as that from WD to WM. Therefore, gene flow estimates from both directions are shown.

mean historical gene flow ranged from 0.0003 to 0.0012 (overall mean = 0.0006) among patches. The mean effective population size was 804. The highest historical gene flow estimate 0.0012 (95 % CI 0.0001–0.0023) was from WE to WD, with a distance of about 3.4 km apart. Mantel tests between migration rate and the natural logarithm of geographical distance were insignificant for both contemporary and historical estimates.

The contemporary gene flow estimates were always higher than the historical estimates for the same pair of patches (Fig. 3). Moreover, the Mantel test indicated that there was no correlation between the contemporary and historical gene flow estimates ($r = 0.029$, $P = 0.36$). The three patches in the dune nature reserve, WM, WE and WP, had the lowest contemporary immigration rates (Table 2).

When the dataset was separated into two subsets, one containing only patches in the roadsides and the other with patches in the dune nature reserve, the mean contemporary gene flow among patches in the roadsides (0.0030) was twice that among patches in the dune nature reserve (0.0015), while historical estimates were similar (0.0006 versus 0.0007). Additionally, the Mantel tests indicated low correlation between contemporary and historical gene flow among patches in the roadsides ($r = -0.106$, $P = 0.72$)

and high correlation among patches in the dunes ($r = 0.999$, $P = 0.17$). The contemporary gene flow estimates between patches in the roadsides and those in the dune nature reserve were asymmetrical. On average, the contemporary gene flow from the patches in the nature reserve to those in the roadsides (0.0062) was five times higher than the estimate in the opposite direction (0.0012). However, the historical estimates were symmetrical (0.0007 from the dunes to the roadsides and 0.0006 in the opposite direction).

Model simulation

In general, a transgene would ultimately become fixed in a wild metapopulation when every generation transgene flow occurred from a GM field to an adjacent wild population and such processes kept taking place for tens or hundreds of generations. When the transgene conveyed a strong selective advantage, it would quickly become introgressed and reach fixation in the population adjacent to the crop field (Fig. 4, top row). However, the spread of the transgene to the other populations was not only determined by the selection coefficient, but also strongly determined by the migration rate among patches (Fig. 4). With the average historical migration rate (0.0006),

TABLE 2. Sample size, genetic diversity and migration rate estimates of wild carrot patches over 11 microsatellite loci. Mean $\pm$ standard error of sample size (N), numbers of alleles (A) and effective alleles (A_e), Shannon's information index (I), observed heterozygosity (H_o), expected heterozygosity (H_e) and fixation index (F_{IS}) were calculated using GENALEX 6.41. Contemporary and historical immigration rates (m) of each patch were calculated with BAYESASS and MIGRATE, respectively. Patches in bold face were located in nature reserves.

Patch	N	A	A_e	I	H_o	H_e	F_{IS}	BAYESASS	MIGRATE
WA	29.8 $\pm$ 0.6	7.5 $\pm$ 1.0	3.4 $\pm$ 0.5	1.38 $\pm$ 0.16	0.54 $\pm$ 0.06	0.63 $\pm$ 0.05	0.13 $\pm$ 0.09	0.016	0.0078
WB	29.5 $\pm$ 0.2	6.8 $\pm$ 0.9	3.4 $\pm$ 0.5	1.35 $\pm$ 0.15	0.56 $\pm$ 0.07	0.64 $\pm$ 0.05	0.15 $\pm$ 0.09	0.064	0.0069
WC	29.8 $\pm$ 0.3	5.0 $\pm$ 0.7	2.4 $\pm$ 0.3	1.02 $\pm$ 0.14	0.46 $\pm$ 0.08	0.53 $\pm$ 0.06	0.15 $\pm$ 0.09	0.017	0.0062
WD	30.5 $\pm$ 0.5	5.2 $\pm$ 0.8	2.7 $\pm$ 0.4	1.09 $\pm$ 0.16	0.42 $\pm$ 0.08	0.55 $\pm$ 0.06	0.26 $\pm$ 0.10	0.126	0.0070
WE	33.9 $\pm$ 0.4	5.9 $\pm$ 1.0	2.6 $\pm$ 0.5	1.08 $\pm$ 0.16	0.43 $\pm$ 0.07	0.51 $\pm$ 0.06	0.17 $\pm$ 0.09	0.014	0.0071
WF	27.9 $\pm$ 0.5	6.8 $\pm$ 1.1	3.9 $\pm$ 0.7	1.40 $\pm$ 0.18	0.54 $\pm$ 0.08	0.65 $\pm$ 0.05	0.18 $\pm$ 0.09	0.029	0.0068
WH	28.5 $\pm$ 0.5	6.3 $\pm$ 0.7	3.7 $\pm$ 0.6	1.35 $\pm$ 0.17	0.52 $\pm$ 0.07	0.63 $\pm$ 0.07	0.15 $\pm$ 0.09	0.046	0.0068
WI	28.3 $\pm$ 0.4	6.5 $\pm$ 0.7	3.5 $\pm$ 0.5	1.35 $\pm$ 0.13	0.50 $\pm$ 0.06	0.66 $\pm$ 0.05	0.23 $\pm$ 0.08	0.017	0.0064
WK	30.5 $\pm$ 0.2	6.0 $\pm$ 0.7	2.8 $\pm$ 0.5	1.18 $\pm$ 0.14	0.47 $\pm$ 0.06	0.56 $\pm$ 0.05	0.17 $\pm$ 0.05	0.023	0.0065
WM	42.9 $\pm$ 0.5	6.4 $\pm$ 1.0	3.1 $\pm$ 0.6	1.18 $\pm$ 0.18	0.44 $\pm$ 0.08	0.57 $\pm$ 0.07	0.23 $\pm$ 0.08	0.012	0.0059
WN	35.5 $\pm$ 0.5	7.0 $\pm$ 1.1	3.2 $\pm$ 0.5	1.31 $\pm$ 0.17	0.50 $\pm$ 0.07	0.61 $\pm$ 0.06	0.16 $\pm$ 0.08	0.041	0.0062
WP	29.3 $\pm$ 0.3	5.1 $\pm$ 0.8	3.1 $\pm$ 0.5	1.13 $\pm$ 0.17	0.50 $\pm$ 0.08	0.57 $\pm$ 0.08	0.13 $\pm$ 0.06	0.016	0.0072

FIG. 4. Transgene frequency changes over generations simulated with a metapopulation model. Broken lines indicate transgene frequencies in a wild population adjacent to a GM field. Solid lines represent mean transgene frequencies in other wild populations which are not adjacent to the GM field in the metapopulation. Dotted lines indicate the mean transgene frequencies in the whole metapopulation. (Left) Migration rate among populations is 0.0006 (mean historical estimates of MIGRATE); (right) migration rate among populations is 0.0032 (mean contemporary estimates of BAYESASS); (top) the transgene has a selection advantage of 0.2 in wild populations; (bottom) the transgene is neutral.

the spread of the transgene to the other patches was much slower than with the average higher contemporary migration rate (0.0032). However, this difference was most clear at relatively long time scales (40–100 generations). Within the first 20 generations, a

typical length of employment of a cultivar, the difference was present but less pronounced. When the transgene conveyed a selective advantage in wild populations ($s = 0.2$), after the first 20 generations the transgene frequency in the other wild

patches reached 0.002 with the historical rate and 0.011 with the contemporary rate (Fig. 4). When the transgene was neutral, the processes of transgene introgression into the whole metapopulation became extremely slow.

DISCUSSION

Genetic structure and gene dispersal

We observed a moderate but highly significant genetic structure in the wild carrot metapopulation. Most of the gene flow occurred among geographically close patches (Fig. 2), indicating a pattern of isolation by distance in the wild carrot metapopulation. The value of $4\pi D\sigma^2$ may be referred to as the neighbourhood size (N_b) in the context of a continuous population (Fenster *et al.*, 2003). In a previous study, we measured gene dispersal and neighbourhood size within a single wild carrot patch (Rong *et al.*, 2010), one of the patches also investigated in this study (WM). The gene dispersal estimate σ in WM ranged from 4 to 12 m (95 % CI 3–25 m) and the neighbourhood size N_b from 42 to 73 (95 % CI 28–322) (Rong *et al.*, 2010). The neighbourhood size estimated from WM is quite similar to the value of $4\pi D\sigma^2$ calculated based on the whole wild carrot metapopulation in this study, i.e. 45 (95 % CI 23 to infinity). This similarity suggests that genes disperse over the wild carrot metapopulation in the way that would be expected in a continuous population. This may be due to the dominance of wild carrots along the roads and the high contemporary gene flow among patches. In the study site, however, some wild carrot patches (e.g. WC, WD and WF) were separated. Moreover, the patches in the dune nature reserve were isolated. The contemporary gene flow was higher among patches in the roadsides than in the dunes and between them the contemporary gene flow was asymmetrical. Therefore, compared with a continuous population model, a metapopulation model may be more appropriate means of accounting for the uneven distribution and gene flow patterns. In WM, weak but significant genetic structure was detected within 14 m and the chance of biparental inbreeding may be high owing to the limited seed dispersal around mother plants and the predominantly outcrossing nature (about 96 % outcrossing) of wild carrot (Rong *et al.*, 2010). In this study, all wild carrot patches generally showed significant deficits in heterozygotes, implying inbreeding within patches, which is in line with the previous study.

Contemporary and historical gene flow

The contemporary gene flow we found was on average 5 times higher than the historical gene flow and there was no correlation between them (Fig. 3). The historical estimates summarize gene flow over many generations, which represents a period much longer than the time over which contemporary gene flow occurred. If environmental factors only lead to small random fluctuations in gene flow frequency through time, it is expected that the levels of contemporary and historical gene flow are similar and significantly correlated with each other, as shown by Chiocchi and Gibbs (2010). Moreover, Epps *et al.* (2013) indicated that genetic structure based on F_{ST} may not be affected by recent human disturbance over a few generations and may therefore reflect the historical gene flow pattern. They found

that the genetic structure of African buffalo herds was weak, suggesting strong historical gene flow, while recent gene flow was restricted, probably due to recent human disturbance. Therefore, F_{ST} could represent the ‘historical’ gene flow pattern, although it should be much more ‘recent’ than the estimate with MIGRATE. The isolation-by-distance pattern was detected based on F_{ST} but not on the contemporary gene flow, suggesting that gene flow in the past was more restricted. The long-term gene flow estimates with MIGRATE were also not correlated with geographical distance. This finding may be an effect of the metapopulation dynamics of wild carrots over a longer time period; for instance, patches could become extinct and be recolonized. The observed inconsistency between contemporary and historical gene flows in this study suggests that environmental factors may have changed in the near past, leading to the increase in the contemporary gene flow.

The most important changes in environmental factors in this case may be due to the increased human disturbance over time, especially the regular mowing along roads. Wild carrot can produce a large number of small seeds, which are covered with bristles that aid dispersal by wind or animals (Umehara *et al.*, 2005; Manzano and Malo, 2006). Seeds were mostly scattered within several metres around mother plants (Umehara *et al.*, 2005; Rong *et al.*, 2010). During mowing, humans may help to transport carrot seeds over long distances along roads, resulting in high gene flow among patches. On the other hand, mowing may lead to more bare areas and shift the vegetation towards plants that have a higher tolerance of the frequent loss of aboveground plant parts (Berendse and Elberse, 1989; Kleijn, 2003). Wild carrot is regarded as an early successional invader of bare areas (Eckardt, 1987; Kleijn, 2003; Umehara *et al.*, 2005) and it grows faster and larger than many other species (Eckardt, 1987). It can be an annual, biennial or perennial monocarpic plant (Lacey, 1986). It may regenerate the aboveground parts quickly after mowing in the period of vegetative growth (Harrison and Dale, 1966) and its seeds can lie dormant for several years in the soil (Dale, 1974; Lacey, 1982). Hauser and Shim (2007) showed that wild carrots survived and flowered at the highest frequency at a site subjected to recent human disturbance with very sparse vegetation, and much less frequently at older sites with low or dense vegetation. In our study area, wild carrots flower and disperse seeds in summer. Therefore, mowing in autumn after seed ripening may promote seed dispersal between patches and reduce the overall density of vegetation along roads, which may facilitate the germination and establishment of wild carrots in autumn. No obvious dormancy was detected in wild carrot seeds sampled at the study site, whereas many young seedlings of wild carrots were found along roads in autumn (J. Rong, pers. obs.). Similarly, mowing may be the main reason that wild carrots have become invasive in wastelands and roadsides under human disturbance in different parts of the world (Umehara *et al.*, 2005). The three wild carrot patches in the dune nature reserve where there was no mowing (WM, WE and WP) had lower contemporary immigration rates than all other patches in the roadsides (Table 2), also supporting the hypothesis that mowing increases gene flow among patches. Wild carrot patches in the dune nature reserve may be more stable and isolated from those along the roads, resulting in less gene flow from outside the patches. The mean contemporary gene flow in the roadsides under high human disturbance was twice that in the dunes under low human disturbance, and the

correlation between contemporary and historical estimates was much higher in the dunes. Such results also suggested that recent human disturbance in the roadsides may account for the increase in contemporary gene flow among patches. In addition, the mean contemporary gene flow from the wild carrot patches in the dunes to those in the roadsides was five times higher than that in the opposite direction, while the historical estimates were symmetrical. Suppose the numbers of migrants in the two directions were the same at the beginning. Because the survival and reproduction of wild carrots were much better in the roadsides with regular mowing than in the dunes without mowing, as discussed above, the number of 'effective' migrants could be much higher in the roadsides than in the dune, leading to asymmetrical gene flow between the two types of wild carrot patches. On the other hand, historically, the effects of human disturbances such as mowing were negligible in both subsets and therefore the gene flow between them was symmetrical.

Implications for transgene introgression and weed control

Genetically modified carrots have been produced and some transgenes may have selective advantages in wild populations, such as herbicide and disease tolerance (Chen and Punja, 2002). If transgene flow occurs from GM into wild carrot populations, transgenes with selective advantages may become introgressed in the wild metapopulation, leading to unwanted ecological consequences such as increased resistance to herbicides and diseases. Carrots are bred to be biennial: to grow vegetatively in the first year before winter for root production and to produce seeds in the second year after cold treatment. It is known that in carrot seed production fields pollen dispersal from adjacent wild carrots may lead to the contamination of carrot seeds (Dale, 1974; Wijnheijmer *et al.*, 1989; Magnussen and Hauser, 2007). Similarly, Wijnheijmer *et al.* (1989) showed that wild carrots adjacent to a carrot seed production field were morphologically intermediate between typical wild and cultivated carrots, probably due to hybridization between the two types. Though carrots normally do not flower in root production fields, a low frequency of early flowering plants has been found in many root production fields in Denmark (Hauser *et al.*, 2004) and the Netherlands (Wijnheijmer *et al.*, 1989; J. Rong, pers. obs. in the field and communications with farmers and breeders). These flowering carrots in root production fields, so-called bolters or weedy carrots, may originate from cultivated carrots or hybrids between cultivated and wild carrots (Wijnheijmer *et al.*, 1989; Hauser *et al.*, 2004). The bolting problem in carrots is very similar to the case of bolting beets, in which flowering weed beets in root production fields mostly resulted from hybridization between cultivated and wild beets in seed production fields (Ellstrand, 2003b). In both cases, bolting can cause severe reduction in production because the roots of the flowering plants become woody and useless. In addition, gene flow may occur from flowering bolters in cultivation fields to flowering wild plants close by. Using amplified fragment length polymorphism analysis, Magnussen and Hauser (2007) identified putative F_2 or backcross offspring between cultivated and wild carrots in some wild carrot populations close to carrot root production fields in Denmark. Their study suggested not only that hybridization occurred between cultivated and wild carrots but also that the hybrids produced were able to survive and reproduce in wild populations (Magnussen and Hauser,

2007). These results were supported by a 3-year field experiment showing that F_1 hybrids between cultivated and wild carrots can survive and reproduce in wild habitats in Denmark (Hauser and Shim, 2007).

If transgenes move from cultivated carrot fields into nearby wild carrot patches via pollen or seed dispersal, they are likely to become established in local patches (Magnussen and Hauser, 2007). Because wild carrots are distributed widely, they form a dynamic metapopulation, as shown in our study, with human disturbance being one major determinant of the gene flow among patches. Our model simulation showed that a transgene may quickly become fixed in the wild patch next to the cultivation field. Higher contemporary gene flow among patches could accelerate the process of transgene introgression into the whole metapopulation (Fig. 4). However, such processes are slow even when the transgene has a strong selective advantage. Model simulations by Meirmans *et al.* (2009) indicated that if GM crops were cultivated widely, a higher proportion of patches in the wild metapopulation would be in direct contact with the GM crops, which could also accelerate the process of introgression. In addition, they showed that even when cultivation of the GM crop stopped after 15 generations (being replaced by a new variety, for instance), the transgene may continue to spread and go to fixation in the wild metapopulation. Therefore, although the initial processes are slow, after a certain number of generations the frequency of the transgene may quickly increase in the wild metapopulation and the associated negative effects, if any, may become increasingly obvious. Again, it is recommended that, when assessing the risk of transgene introgression, the metapopulation dynamics of the transgene should be taken into consideration over a long time scale. The most effective way of minimizing the chance of transgene introgression may be to reduce the frequency of the transgene in wild populations next to cultivation fields. In the case of the carrot, spatial isolation between cultivation fields and wild populations alone may not be sufficient to reduce hybridization because genes can be dispersed over a long distance (Fig. 3), as also indicated by Rong *et al.* (2010). It may be more effective to reduce the number of flowering carrots (weedy carrots) in root production fields or to grow spring carrot varieties (e.g. 'Bospeen' in the Netherlands) that are harvested before the peak flowering period of wild carrots. For carrot seed production, it would be ideal if it could be conducted in regions with no wild carrots, which may dramatically reduce the chance of transgene introgression from cultivated to wild carrots. At the same time, seed contamination by pollen dispersal from wild carrots will be reduced, as also will the bolting problem in carrot root production fields, as suggested by the control of weed beets (Ellstrand, 2003b).

For the control of the wild carrot as a roadside weed, it may be more effective to pull or mow wild carrots while flowering or mow close to the ground before flowering than to mow after flowering (Dale, 1974; Eckardt, 1987), because the latter may facilitate seed dispersal and germination, promoting the invasiveness of wild carrot. However, mowing in summer may be in conflict with other aims of habitat management, such as the provision of food and habitats for insects and birds. As discussed above, survival and reproduction in wild carrots may depend on vegetation density, with the highest rates at very sparse densities and much reduced rates at high densities. Thus, it may help to maintain the wild vegetation at a certain density or to introduce diverse plant species into bare lands to provide competition for wild carrots.

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