Genetic structure and post-pollination selection in biennal plants

Korbecka, G.

Chapter 4

Fine-scale genetic structure in *Echium vulgare* and *Cynoglossum officinale*

Korbecka G., Klinkhamer P. G. L, Vrieling K. and K. Wolff

ABSTRACT

The presence of a genetic structure in plant populations can lead to an increase of inbreeding. Pollinators tend to visit neighboring plants, causing crosses among related individuals (biparental inbreeding). We tested for fine-scale genetic structure in two species, pollinated by bumblebees, *Echium vulgare* and *Cynoglossum officinale* in order to estimate the amount of biparental inbreeding, using 7 polymorphic microsatellite loci per species. The slope of the regression line between pair-wise kinship coefficients and ln of physical distance was significantly negative for *E. vulgare* but not for *C. officinale.* Average kinship coefficients per distance class were significantly higher than zero for both species only in the first distance interval (including distances up to 1.48 meters for *E. vulgare* and up to 6.49 meters for *C. officinale*). This suggests a genetic structure at a very small scale, probably due to leptocurtosis of gene dispersal curves. The genetic structure of both species appeared to be very weak compared to data published for 17 herbaceous species with similar types of pollen and seed dispersal. The estimated amount of biparental inbreeding does not exceed 2 % for *E. vulgare* and *C. officinale*. We conclude, therefore, that the population genetic structure does not intensify inbreeding in the studied species.

INTRODUCTION

It is common among plant species that gene flow is restricted to dispersal of pollen and seeds. As these are often restricted to a limited area, more related plants tend to grow next to each other and a genetic structure is formed within populations (Loveless and Hamrick, 1984; Vekemans and Hardy, 2004 and references there in). The presence of such a structure may have many consequences. Firstly, the adaptive value of various traits may depend on it. If neighboring plants are close relatives, then a strategy, which is 'better for the neighbors' but worse for the focus individual may still be favored by selection if it increases inclusive fitness (Hamilton, 1964). For example, one can expect that the direction of selection on plant responses to intraspecific competition (e.g. allelopathy, root competition, overshadowing of the neighboring plants) depends on inclusive fitness. Similarly, resistance to herbivores can be considered. The production of high levels of chemical defenses may be profitable not only for the individual in focus, but also for the neighboring plants, if herbivores consider a group of plants rather than a single plant as one foraging patch. For some traits, it is relevant if a genetic structure is present in a particular life-stage. Klinkhamer et al. (2001) found that neighbors of flowering plants with high nectar production rate received more bumblebees' visits, irrespective of how much nectar they produced themselves.

Strategies of which the fitness consequences depend on inclusive fitness are not the only consequence of genetic structuring of the populations. Genetic structure among flowering plants may also result in intensified inbreeding. Pollinators like bumblebees often visit neighboring plants, as this optimizes their nectar uptake rate (Heinrich, 1979). In genetically structured populations, such a visitation pattern leads to crosses among related individuals. Even with low selfing due to autogamy and geitonogamy, the proportion of inbreeding that comes from crosses among related individuals (biparental inbreeding) may be substantial. Therefore, the association between

population structure and inbreeding becomes very relevant in species, which are predominantly outcrossing and suffering from inbreeding depression.

Many studies on genetic structure within populations included both juvenile and reproducing individuals, although there may be considerable differences across life stages (e. g. Parker et al., 2001). The genetic structure may decay as plants get older due to the thinning process or it may get stronger if a directional selection operates locally (Chung et al., 2003; Ueno et al., 2002). Therefore, if the genetic structure is studied in relation to biparental inbreeding only flowering plants should be included.

In this paper we test for the presence of a genetic structure in the flowering stage of two species of the Boraginaceae: *Echium vulgare* and *Cynoglossum officinale*, which are both self-compatible, monocarpic biennials pollinated by bumblebees. Inbreeding depression affects survival of rosette plants in both species (see chapter 6 in Melser, 2001). Moreover, in *E. vulgare* inbreeding depression has been detected during reproduction of the offspring. Plants derived from self-pollination have lower seed production and lower siring success compared to outcrossed plants (Melser et al., 1999).

In the studied dune area, both species disperse seeds mainly through gravity. As a consequence, groups of seedlings germinating are observed in a direct neighborhood of places where flowering plants stood the season before, suggesting that these are at least half sibs and a genetic structure is likely to exist in the field populations. The two species differ in flower structure and development. In *E. vulgare* autogamy is prevented by a spatial separation of anthers and stigma and protandry, which is not the case for *C. officinale*, where anthers and stigma are located closely together. Therefore, the latter species is expected to have more inbreeding. Preliminary measurements of selfing rates reported by Rademaker et al. (1999) and Vrieling et al. (1999) support this expectation. In *E. vulgare* the percentage of selfed offspring per mother varies between 0 and 33% (average: 12.5%), while in *C. officinale* it varied between 0 and 70% (average: 32.2%).

Species with higher inbreeding levels are more likely to form a genetic structure in a population (Loveless and Hamrick, 1984; Vekemans and Hardy, 2004). Therefore, we expect to find a stronger population structure in *C. officinale* compared to *E. vulgare*.

MATERIALS AND METHODS

Species description

E. vulgare is a tetraploid species: 2n = 4x = 32 (Gadella and Kliphuis, 1963; Litardiere, 1943). The inheritance is probably tetrasomic in this species (see appendix to chapter 5). Every plant produces 1-10 flowering stems each with up to 50 cymes and each cyme carries up to 20 flowers. Mean seed weight equals 2.7 mg with a mean length of 2.5 mm (van Breemen, 1984). Seeds disperse by gravity, although secondary dispersal by wind or transport with dried flowers in the fur and feathers of animals is possible. Seeds covered by sand remain viable many years and disturbance of the soil usually increases the number of germinating seedings of *E. vulgare* (van Breemen, 1984).

C. officinale is a diploid plant: 2n = 2x = 24 (Gadella and Kliphuis, 1963; Luque and Valdes, 1986). Every plant produces 1-3 flowering stems each with up to 25 cymes and each cyme carries up to 20 flowers. Seeds weigh on average 20 mg and are on

average 6 mm long (van Breemen, 1984). The seed is covered with hooked spines, which enable them to stick to the fur of animals resulting in dispersal over longer distances. In areas grazed by cattle such dispersal plays a significant role. However, in our study area the only largest herbivores are rabbits, which are believed to disperse only a small fraction of *C. officinale* seeds (Rademaker and de Jong, 1999). The majority of the seeds fall next to the mother plants and germinate within 1-2 years after maturation (Boorman and Fuller, 1984; van Breemen, 1984).

In our study areas, both species are predominantly visited by bumblebees (e.g. *Bombus pascuorum* S., *B. terrestris* L., *B. hypnorum* L., *B. pratorum* L.) (Rademaker, 1998)

Study sites

In spring 2001, we selected an *E. vulgare* population in the dune area of Meijendel (near The Hague, The Netherlands, 52˚8′N, 4˚20′E). This population was located within a rectangular area of 6 x 20 meters and was partly sheltered from the wind by shrubs of sea buckthorn (*Hippophae rhamnoides*). There were 115 flowering plants in the population and 50 of them were randomly chosen, numbered and mapped (fig. 1). We collected a sample of seeds and a leaf for DNA extraction at the peak of flowering.

A *C. officinale* population was sampled in the same dune area in 2003. The population grew in an understorey of a thicket. The predominant tree species in the thicket was *Crategus monogyna* with a small percentage of poplar trees (*Populus nigra, P. alba*) and *Sorbus aucuparia.* Smaller scrubs in the ticket consisted mainly of *Ligustrum vulgare*. The understorey was covered by mosses (~90% of a surface) with nettles (*Urtica dioica*) locally occurring at high density. In 2003, there were 288 flowering plants in the selected area of 40 x 45 meters. We numbered and mapped all the plants and sampled a leaf to dry in silica gel for DNA analysis. We randomly chose 103 plants for DNA extraction (fig. 2). After flowering, the plants were sampled together with their seeds. Twelve flowering plants did not set any full seeds.

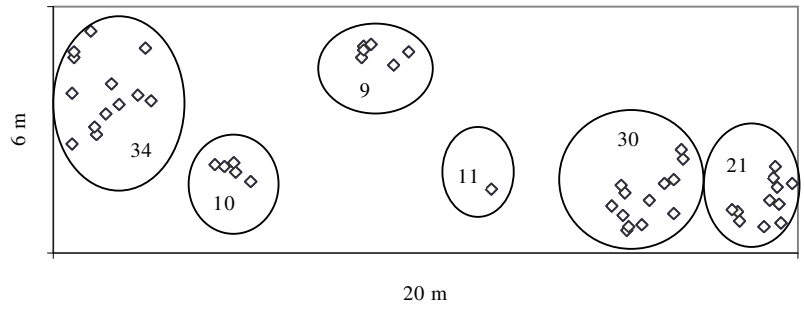

Fig. 1
Map of 49 flowering *E. vulgare* plants sampled for analysis of the population structure. A number next to each group of plants indicates how many flowering plants there were in total in each group. In total there were 115 flowering plants.

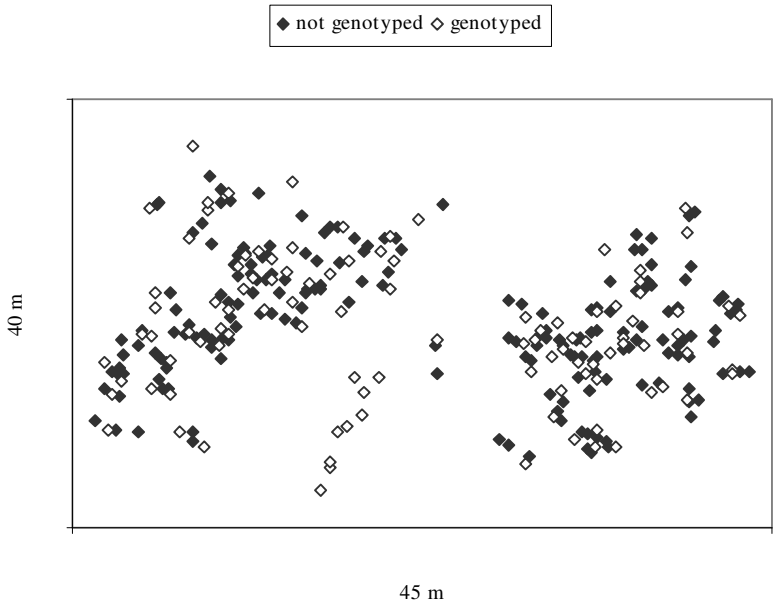

Fig. 2
Map of 288 flowering plants in *C. officinale* population. Open diamonds indicate plants genotyped and included into the analysis of a genetic structure.

Seed germination

We germinated the seeds from the selected flowering plants in order to have enough material for DNA extraction. In *E. vulgare* this germination was a part of a larger experiment, where 20 seeds were germinated from every flowering plant. The seeds were randomized and put on a thin layer of wet sand in replica plates. Then the plates were sealed with parafilm and placed in a climate room (day: 16h, 20^{o}C; night: 8h, 15^{o}C; 70% humidity). The germination percentage per mother equaled on average 89.3% (SE=1.53). We did not include non-germinating seeds into the paternity analysis. However, differential survival can not strongly bias the results. In chapter 5, we have shown that selfed seeds have only 16% lower germination compared to outcrossed seeds.

In *C. officinale*, we germinated only 1 seed per plant (91 seeds in total). The seeds were placed on wet filter paper in replica plates for 24 hours in the same climate room conditions as seeds of *E. vulgare*. The seed coat was removed from the seeds in order to monitor germination. Plates were monitored every day. After about a week one green cotyledone was taken for extraction. Seven seeds/seedlings that were infected by bacteria or fungi or/and did not show a proper germination were frozen at –20^{o}C and successfully genotyped later.

Microsatellite analysis in E. vulgare

The fifty selected plants and two seedlings per flowering plant were genotyped with 7 microsatellite loci. DNA extraction, PCR conditions and characterization of six microsatellite loci followed Korbecka et al.(2003). The 7th locus *E2-83* contained a dinucleotide repeat (GA) and was amplified using forward primer AACCCGACACA-TCCAGCTAC and reverse primer TGGGCCTTATGTAAGTAGTGCT yielding fragments between 180 and 212 base pairs. The forward primer was labeled with a TAMRA label. Locus specific annealing temperature for *E2-83* was 60°C in all 30 cycles.

In the majority of the cases, we were not able to determine the exact genotypes of these tetraploid plants because of a poor correlation between strength of signal and the number of copies of alleles. Therefore, we scored the microsatellites in a dominant fashion noting only the presence of alleles in individuals, without a number of copies per allele.

PCR for 6 loci, apart from locus *E2-83,* were done twice for flowering plants to test repeatability. Out of 300 PCRs, 7 failed in the first round, 5 of them failed again in the 2nd round. The five failed PCRs were from the same flowering plant, which was excluded from analysis together with its two seedlings. All PCRs that were successful twice gave the identical microsatellite pattern.

In order to get a reliable estimate of selfing rate, twenty seedlings were excluded from analysis because their PCRs failed for 4 or more loci. For the final analysis 49 flowering plants and 78 seedlings were used.

Microsatellite analysis in C. officinale

We genotyped the 103 flowering plants and 91 seedlings with 7 microsatellite loci*: C2-19, C2-42, C2-43, C2-62, C2-72, C3-41* and *C3-79.* DNA extraction and PCR conditions followed Korbecka and Wolff (2004). Multiplexing allowed performing only 3 PCRs per individual to amplify all 7 loci. All 309 PCR for flowering plants and 273 PCRs for seedlings were successfully amplified.

We did not repeat PCRs because the microsatellites appeared to be very reliable and easy to score (Korbecka – unpublished data). Heterozygotes gave equally strong signals from both alleles, apart from locus *C2-42* where the signal intensity appeared to be negatively correlated with allel size.

Test for Hardy-Weinberg (HW) equilibrium

We tested for HW equilibrium in order to support our results on genetic structure. If there is a genetic structure, both nearest-neighbor pollination and selfing will lead to a departure from HW equilibrium. In *C. officinale*, we tested for HW equilibrium using a program ARLEQUIN (Schneider et al., 2000). For *E. vulgare* this analysis could not be done because we did not know the exact genotypes.

Selfing rate

Direct estimate in *E. vulgare* and *C. officinale*

We screened the seedlings for the presence of alleles that were not detected in the mother. If we found such alleles in at least one locus, the seedling was classified as outcrossed. This method overestimates the selfing rate because we are not able to detect outcrossing if the pollen has only alleles that are present also in the maternal plant.

However, we assume that this overestimation is minimal as we use 7 microsatellite loci for each species and most of these loci were very polymorphic (Tab. 1 and 2). Population selfing rate in *E. vulgare* was estimated based on offspring from 32 mothers with 2 seeds genotyped and 14 mothers with 1 seed. Three mothers had all seeds excluded from analysis due to too many failed PCR's. In *C. officinale* we used all the 91 seedlings.

Indirect estimate in *C. officinale*

In *C. officinale* we calculated the selfing rate indirectly based on the inbreeding coefficient $s = 2F/(1+F)$ (Hartl and Clark, 1989), where s is selfing rate (indirect estimate). The inbreeding coefficients for each locus based on observed (H_{obs}) and expected (H_{exp}) heterozygosities was calculated according to the following formula: $F = 1\text{-}H_{obs}/H_{exp}$ (Hartl and Clark, 1989). Then, we used averaged value of F to calculate the indirect estimate of selfing rate. Both self-pollination and biparental inbreeding will influence this estimate. By comparing the direct and indirect estimates of selfing rates we can get an indication of biparental inbreeding.

Genetic structure analysis

We tested genetic structure in both species using the program SPAGEDI (Hardy and Vekemans, 2002). In *E. vulgare*, data for individuals with two or three alleles in a certain locus were encoded as 'incomplete genotypes' with 2 or 1 unknown alleles respectively. The percentage of 'incomplete genotypes' for the parents varied between 67 and 90% depending on the locus. The frequencies of both alleles in an individual with two known alleles are assumed by SPAGEDI to be equal 0.5. A consequence of this way of encoding data is an inaccurate calculation of allele frequencies. The frequencies of common alleles will be underestimated and the frequencies of rare alleles - overestimated. However, on average, it does not bias the estimation of kinship coefficients.

We ran an analysis of genetic structure defining the number of distance classes, in such a way that each class had the same sample size (the same number of pair wise distances). We performed analysis with 6-10 distance classes, but we present correlograms based on analysis with 7 distance classes as a compromise between sample size per class and the physical distance covered per each class. We calculated pairwise kinship coefficients according to Loiselle et al. (1995). The significance of average kinship coefficients ($\hat{F}$) in every distance class was tested using permutation tests (one-sided test: H_0: $\hat{F} = 0$; H_1: $\hat{F} > 0$: 1000 permutations). We regressed the kinship coefficients against the natural logarithm of physical distance (Vekemans and Hardy, 2004). Permutation tests were used to test if the slope of these regression lines ($\hat{b}_F$) were significantly negative, as expected if isolation by distance occurs. These tests were also one-sided (H_0: $\hat{b}_F = 0$; H_1: $\hat{b}_F < 0$; 1000 permutations)

Vekemans and Hardy (2004) suggested that neither the values of kinship coefficients nor the slope of regression line should be used to compare genetic structure in different species because these values are arbitrary and depend on the sampling scheme. They proposed *Sp* statistics as an objective measure of a genetic structure for interspecific comparisons. *Sp* can be interpreted as a reciprocal of neighborhood size. Therefore, a low *Sp* value means that the neighborhood size is large and genetic

structure is weak. We calculated this statistics using a formula including the ploidy level (k = 2 for diploids, k = 4 for tetraploids) (pers.comm. – Hardy): $Sp = k/2 * (-\hat{b}_{F}/(1-\hat{F}_1))$, with $\hat{F}_1$ is the average kinship coefficient in the first distance interval. The calculated *Sp* values for *C. officinale* and *E. vulgare* were compared with data presented by Vekemans and Hardy (2004). We chose the 17 herbaceous species that were both animal pollinated and dispersing seeds by gravity for this comparison.

In order to estimate the amount of biparental inbreeding we have to know the frequency distribution of pollen dispersal distances within the population. Such data were not available, we used therefore the approach proposed by Vekemans and Hardy (2004): we will assume that pollen dispersal is restricted to the first distance class. Then the maximum estimate of biparental inbreeding is equal to the kinship coefficient in the first distance class.

RESULTS

Microsatellite analysis

The microsatellite loci used in this study were more variable in *E. vulgare* than in *C. officinale* (Tab. 1 and 2). The average number of alleles per locus equalled 5.7 (40/7) and 3.4 (24/7) in the studied species, respectively.

HW equilibrium

In *C. officinale*, the observed heterozygosities in all seven loci were lower than expected on the basis of non-random mating among the flowering plants. A significant deviation from HW equilibrium was found in 5 loci (Tab. 2). The average inbreeding coefficient (F) equals 0.226.

Selfing rate

In *E. vulgare*, the population selfing rate equals 5.43 % (N = 46 flowering plants). In *C. officinale* the population selfing rate equaled: 35.16% (N = 91 flowering plants). The indirect estimate of selfing rate in *C. officinale* based on inbreeding coefficient gave a very similar estimate 36.87%. The indirect estimate of selfing rate is not much higher that the direct one suggesting that biparental inbreeding is rare.

Tab. 1
Alleles and their approximated frequencies detected in 7 microsatellite loci in a population of 49 flowering *E. vulgare* plants. The allele frequencies were calculated by SPAGEDI.

Locus	Number of alleles	Above: allele lengths (bp) Below: approx. allele frequencies									
E3-46	7	220	222	224	226	228	230	234			
		0.23	0.27	0.12	0.03	0.16	0.06	0.12			
E3-40	6	178	181	187	190	193	196				
		0.15	0.11	0.31	0.10	0.30	0.03				
E2-11	2	242	249								
		0.55	0.45								
E3-84	5	294	297	300	303	306					
		0.02	0.01	0.43	0.45	0.09					
E2-83	10	180	190	198	200	202	204	206	208	210	212
		0.14	0.05	0.13	0.07	0.01	0.19	0.05	0.18	0.13	0.04
E3-91	6	169	181	184	187	193	196				
		0.26	0.13	0.06	0.08	0.35	0.12				
E3-56	4	268	269	271	286						
		0.39	0.26	0.23	0.13						

Tab. 2
Alleles, their frequencies and hererozygosities of 7 microsatellite loci in a population of 103 flowering *C. officinale* plants.

Locus	H_{obs}	H_{exp}	F	Number of alleles	Above: allele lengths (bp) Below: allele frequencies				
C2-72	0.40	0.56**	0.29	4	91	97	99	101	
					0.38	0.07	0.54	0.01	
C3-79	0.41	0.59**	0.31	5	188	191	208	214	217
					0.06	0.05	0.53	0.35	0.005
C2-43	0.41	0.50*	0.19	3	128	130	136		
					0.18	0.15	0.67		
C2-19	0.39	0.54*	0.27	3	115	117	131		
					0.03	0.49	0.48		
C2-62	0.32	0.39	0.17	3	167	169	171		
					0.24	0.01	0.75		
C3-41	0.17	0.19	0.14	2	133	136			
					0.90	0.10			
C2-42	0.41	0.51*	0.20	4	110	112	116	124	
					0.65	0.04	0.04	0.27	

H_{obs}, observed heterozygosity; $H_{exp,}$ expected heterozygosity; F inbreeding coefficient
* statistically significant deviation from Hardy-Weinberg equilibrium (P<0.05)
** statistically significant deviation from Hardy-Weinberg equilibrium after Bonferroni correction (P<0.0071).

Genetic structure

1. Regression analysis

In *E. vulgare*, the slope of the regression between kinship coefficients and the natural logarithm of physical distance was significantly lower than zero, indicating the presence of a weak genetic structure ($y = -0.0039 x + 0.0091$; $r^2 = 0.0049$; N = 1176; permutation test: P = 0.023; Fig.3). Such a significant genetic structure was not detected in *C. officinale* ($y = -0.0053 x + 0.0131$; $r^2 = 0.0003$; N = 5253; permutation test: P = 0.101; Fig. 3).

2. Permutation tests for average kinship coefficients per distance class.

In an analysis dividing data into 7 distance intervals for both species, the average kinship coefficients in the first distance class ($\hat{F}_1$) equaled 0.0169 and 0.0145 for *C. officinale* and *E. vulgare* respectively and they were significantly higher than zero (permutation tests, $P<0.05$). The first distance class in this analysis with 7 classes included pairwise distances between plants up to 1.48 m and 6.49 meters for *E. vulgare* and *C. officinale*, respectively. The average kinship coefficient was consistently higher than zero in the first distance classes if analysis was done with 6-9 distance classes for *E. vulgare*, and with 6-8 classes for *C. officinale*.

3. Biparental inbreeding

Assuming that pollen dispersal is limited to the first distance class, we conclude that biparental inbreeding equals 1.69% and 1.45% for *C. officinale* and *E. vulgare*, respectively.

4. Sp statistics

Sp values equal 0.0054 and 0.0079 for *C. officinale* and *E. vulgare,* respectively.

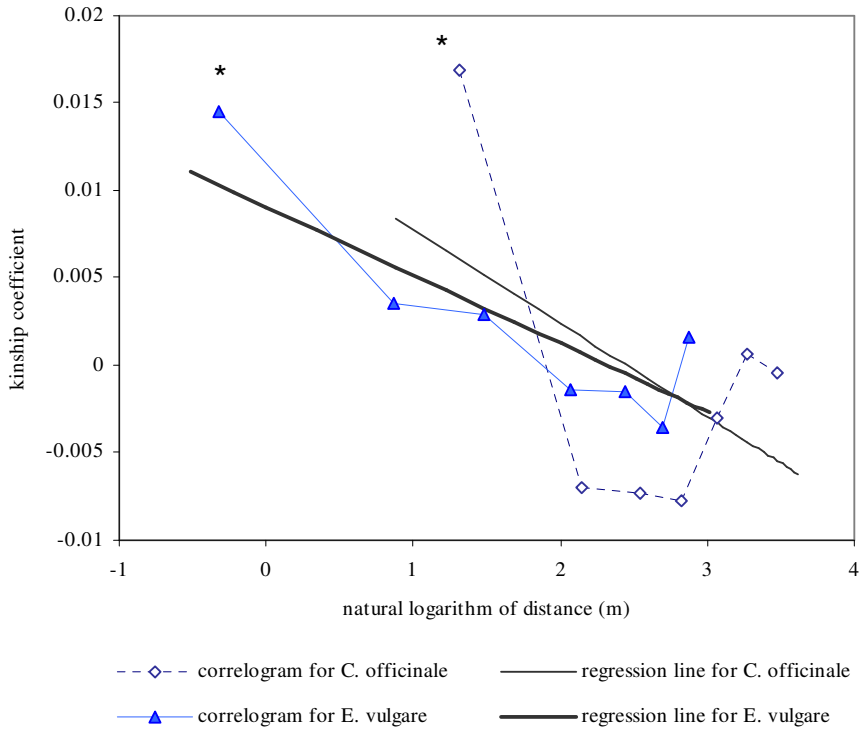

Fig. 3
Correlograms (average kinship coefficients per distance class plotted against a mean natural logarithm of distance in a class) and regression lines (between pairwise kinship coefficients and natural logarythm of distance) for *E. vulgare* and *C. officinale*

* -average kinship coefficient significantly higher than zero (permutation test, P<0.05)

DISCUSSION

Biparental inbreeding

We detected a low level of biparental inbreeding (<2%) which may be an over estimate because we assumed that the pollen dispersal is restricted to the first distance class. This means that crosses among related individuals are rare and do not contribute to the inbreeding in *E. vulgare* and *C. officinale* in the field.

High levels of biparental inbreeding are more likely to be detected in species with higher selfing rates. The reason for it is that these species are more likely to form a strong genetic structure (Loveless and Hamrick, 1984; Vekemans and Hardy, 2004). One of the few studies presenting experimental measurement of biparental inbreeding has reported a level of biparental inbreeding as high as 30% in *Aquilegia canadensis* (a perennial with, on average, 78 % selfing in the field, (Griffin and Eckert, 2003). In another study, Kelly and Willis(2002) found little or no biparental inbreeding in two populations of *Mimulus guttatus.* However, previous report on a genetic structure in this species have shown that the neighboring plants are not related (Sweigart et al., 1999). The experimental design used by Kelly and Willis (2002) and Griffin and Eckert (2003) is based on comparing the levels of apparent selfing in two groups of plants. The first group includes plants randomly transplanted within the population and the second (control) group includes plants only dug out and planted back in the places where they grew originally. This design allows for a more accurate estimation of the amount of biparental inbreeding and certainly more studies using this method are desirable.

Comparison of the genetic structure among the species

According to data reported by Vekemans and Hardy (2004), *Sp* values for the17 herbaceous species, that were both animal pollinated and dispersing seeds by gravity, varies between 0.00471 for self-incompatible *Arabidopsis halleri* and 0.26316 for *Phaseolus lunatus* (a predominantly selfing plant), with a mean at 0.04328. A comparison of these *Sp* values to the values calculated for our two study species (0.0054 for *C. officinale* and 0.0079 for *E. vulgare*) confirms that the detected genetic structure in populations of flowering plants of these species is very weak. Interestingly, the genetic structure in *C. officinale* is weaker than in *E. vulgare*, which is contrary to our expectation. We can explain this only by more effective seed dispersal in *C. officinale*.

Why is the genetic structure in E. vulgare and C. officinale so weak?

In the analysis of the genetic structure in *E. vulgare* and *C. officinale*, the permutation tests show that the kinship coefficients are significantly higher than zero only for plants in the smallest distance class. This may indicate that there is a genetic structure at a very small scale (up to 1.48 meters for *E. vulgare* and up to 6.49 meters for *C. officinale*). This kind of structure may arise when the gene dispersal curve is leptocurtic, which means that there are essentially two kinds of dispersal: short and long distance dispersal. This explanation is likely for our study species. For example, seeds of *C. officinale* disperse by gravity within a distance up to 1.4 meters from a maternal plant (Boorman and Fuller, 1984). A part of the seeds may disperse secondarily to large distances by means of animals or run-off water. A similar line of reasoning may be used

for pollen dispersal. For example, Richards (1997) described that species with flowers pollinated by animals like bees or butterflies often have leptocurtic pollen dispersal curves due to clumped distribution of the flowering plants, presence of plant patches with various amount of reward and pollinator preferences for more rewarding patches. Pollinator movements within a patch would lead to short distance pollen dispersal and movements among patches – long distance dispersal.

The weak genetic structure in both species may also be a result of a thinning process. High mortality of seedlings and young rosettes has been recorded in *E. vulgare* and *C. officinale* (Jong and Klinkhamer, 1988; Klemow and Raynal, 1985). Therefore, we can not exclude that a genetic structure is more prominent in younger life stages.

ACKNOWLEDGEMENTS

The authors thank Olivier Hardy for advice on genetic structure analysis and Henk Nell and Hans de Heiden for field assistance. The study of *E. vulgare* population was supported by project no. 805-36-044 of the Life Sciences Foundation (SLW), which is subsidised by the Netherlands Organisation for Scientific Research (NWO). The study of *C. officinale* population was supported by Marie Curie Fellowship of the European community programme Human Potential (contract number: HPMT-CT-2001-00272).

REFERENCES

Boorman, L. A., and R. M. Fuller. 1984. The comparative ecology of two sand dune biennials: *Lactuca virosa* L. and *Cynoglossum officinale* L. *New Phytologist*, 96:609-629.

Chung, M. Y., B. K. Epperson, and M. G. Chung. 2003. Genetic structure of age classes in *Camellia japonica* (Theaceae). *Evolution*, 57:62-73.

Gadella, T. W. J., and E. Kliphuis. 1963. Chromosome numbers of flowering plants in the Netherlands II. *Acta Bot. Neerl.*, 12:195-230.

Griffin, C. A. M., and C. G. Eckert. 2003. Experimental analysis of biparental inbreeding in a self-fertilizing plant. *Evolution*, 57:1513-1519.

Hamilton, W. D. 1964. Genetical Evolution of Social Behaviour I. *Journal of Theoretical Biology*, 7:1-&.

Hardy, O. J., and X. Vekemans. 2002. SPAGEDI: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes*, 2:618-620.

Hartl, D. L., and A. G. Clark. 1989. *Principles of population genetics*. Sinauer Associates, Sunderland, Massachusetts.

Heinrich, B. 1979. Resource heterogeneity and patterns of movement in foraging bumblebees. *Oecologia*, 40:235-245.

Jong, T. J. d., and P. G. L. Klinkhamer. 1988. Population ecology of the biennials *Cirsium vulgare* and *Cynoglossum officinale* in a coastal sand dune area. *Journal of Ecology*, 76:366-382.

Kelly, J. K., and J. H. Willis. 2002. A manipulative experiment to estimate biparental inbreeding in monkeyflowers. *International Journal of Plant Sciences*, 163:575-579.

Klemow, K. M., and D. J. Raynal. 1985. Demography of two facultative biennial plant species in an unproductive habitat. *Journal of Ecology*, 73:147-167.

Klinkhamer, P. G. L., T. J. de Jong, and L. A. Linnebank. 2001. Small-scale spatial patterns determine ecological relationships: an experimental example using nectar production rates. *Ecology Letters*, 4:559-567.

Korbecka, G., K. Vrieling, J. Squirrell, M. L. Hale, and K. Wolff. 2003. Characterization of six microsatellite loci in *Echium vulgare* (Boraginaceae). *Molecular Ecology Notes*, 3:274-276.

Korbecka, G., and K. Wolff. 2004. Characterization of nine microsatellite loci in *Cynoglossum officinale* (Boraginaceae). *Molecular Ecology Notes*, 4:229-230.

Litardiere, R. d. 1943. Recherches caryologiques et caryo-taxonomiques sur les Boraginacees. II Nombres chromosomiques dans le genre Echium. *Boissiera*, VII:155-165.

Loiselle, B. A., V. L. Sork, J. Nason, and C. Graham. 1995. Spatial genetic structure of a tropical understory shrub, *Psychotria officinalis* (Rubiaceae). *American Journal of Botany*, 82:1420-1425.

Loveless, M. D., and J. L. Hamrick. 1984. Ecological determinants of genetic structure in plant populations. *Annual Review of Ecology and Systematics*, 15:65-95.

Luque, T., and B. Valdes. 1986. Caryological study of Spanish *Boraginaceae* III. *Cynoglossum* L. s. str. *Willdenowia*, 15:485-496.

Melser. 2001. *Selective seed abortion and offspring quality. PhD thesis*. Leiden University, Leiden.

Melser, C., A. Bijleveld, and P. G. L. Klinkhamer. 1999. Late-acting inbreeding depression in both male and female functions of *Echium vulgare* (Boraginaceae). *Heredity*, 83:162-170.

Parker, K. C., J. L. Hamrick, A. J. Parker, and J. D. Nason. 2001. Fine-scale genetic structure in *Pinus clausa* (Pinaceae) populations: effects of disturbance history. *Heredity*, 87:99-113.

Rademaker, M. C. J. 1998. *The optimal sex-allocation strategy in relation to plant size for the hermaphroditic species Echium vulgare and Cynoglossum officinale. PhD thesis.* Leiden University, Leiden.

Rademaker, M. C. J., and T. J. de Jong. 1999. The shape of the female fitness curve for *Cynoglossum officinale*: Quantifying seed dispersal and seedling survival in the field. *Plant Biology*, 1:351-356.

Rademaker, M. C. J., T. J. de Jong, and E. van der Meijden. 1999. Selfing rates in natural populations of *Echium vulgare*: a combined empirical and model approach. *Functional Ecology*, 13:828-837.

Richards, A. J. 1997. *Plant breeding systems*. 2nd ed. Chapman & Hall, London.

Schneider, S., D. Roessli, and L. Excoffier. 2000. *ARLEQUIN ver. 2.000: A software for population genetic data analysis.* University of Geneva, Switzerland.

Sweigart, A., K. Karoly, A. Jones, and J. H. Willis. 1999. The distribution of individual inbreeding coefficients and pairwise relatedness in a population of Mimulus guttatus. *Heredity*, 83:625-632.

Ueno, S., N. Tomaru, H. Yoshimaru, T. Manabe, and S. Yamamoto. 2002. Size-class differences in genetic structure and individual distribution of *Camellia japonica* L. in a Japanese old-growth evergreen forest. *Heredity*, 89:120-126.

van Breemen, A. M. M. 1984. Comparative germination ecology of three short-lived monocarpic boraginaceae. *Acta Botanica Neerlandica*, 33:283-305.

Vekemans, X., and O. J. Hardy. 2004. New insights from fine-scale spatial genetic structure analyses in plant populations. *Molecular Ecology*, 13:921-935.

Vrieling, K., P. Saumitou-Laprade, J. Cuguen, H. van Dijk, T. J. de Jong, and P. G. L. Klinkhamer. 1999. Direct and indirect estimates of the selfing rate in small and large individuals of the bumblebee pollinated *Cynoglossum officinale* L (Boraginaceae). *Ecology Letters*, 2:331-337.