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Genetic variability in *Plantago* species in relation to their ecology

3. Genetic structure of populations of *P. major, P. lanceolata* and *P. coronopus* *

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Summary. Populations of the three *Plantago* species *P. major, P. lanceolata* and *P. coronopus* were scored for allozyme variability. They showed normal variability levels compared with other, similar plant species. Differentiation among populations appeared to be rather low in comparison with other species, probably due to a considerable amount of long distance seed transport. In order to be able to make an analysis of small-scale gene flow, all (sub)populations were critically checked for the existence of population structure equilibrium. The allozyme variation was tested for neutrality by testing homogeneity of F values among loci: between populations (Lewontin-Krakauer test) and within populations. No systematic deviations from the prediction of the neutral theory could be established. From the population structure analyses, little gene flow in the species *P. major* (with high selfing levels) and *P. coronopus* could be concluded, whereas *P. lanceolata* showed relatively high levels of gene flow. The degree of homozygosity in the latter species was too high to explain with the available data. In *P. coronopus,* on the other hand, an unusually high number of heterozygotes were observed.

Key words: Allozyme variability – Population structure – Gene flow – Plantago

Introduction

The genetic variation which enables plant species to grow in a range of environmental conditions appears to concern variation in genes influencing morphology and development. Numerous studies of geographical and ecotypic variation within species have been attempted that deal with this kind of genetic variation, for instance the classic study of Clausen and Hiesey (1958) on *Potentilla glandulosa* (for more references see Bradshaw 1984). Following the discovery of a high degree of polymorphism at the enzyme level, intensive research began to investigate its functional meaning. A direct parallel with the previously mentioned type of genetic variation could not be established, however. On the contrary, it was even claimed by some authors that almost all allozyme variation was selectively neutral (Kimura 1983). In *Plantago major,* the two subspecies *major* and *pleiosperma* contain characteristic alleles at two allozyme loci (Van Dijk and Van Delden 1981). A detailed analysis of genetic variation in this species (Van Dijk 1984) indicated that subspecies-specific enzyme alleles occurred which were linked with genes influencing similar characters as studied by Clausen and Hiesey (1958). The high selfing rate appeared to be an important factor in the maintenance of gene complexes consisting of such fitness-affecting genes and the allozyme markers involved.

The apparent absence of an important role of allozyme variation in selectional processes should not be considered as negative. These neutral genes may be used as marker genes, both as chromosome markers, like in the *P. major* subspecies mentioned, or in determining population structure, using the method described by Van Dijk (1985 b, 1987). Van Dijk's method could lead to an estimation of small-scale gene flow, while population differentiation provides information on gene flow at a larger scale.

An important factor in determining the gene flow characteristics of a plant species is the breeding system.

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Selfing may strongly reduce gene flow in cases where seed dispersal is restricted, and gene flow would depend largely on pollen dispersal. The three *Plantago* species of this study have different breeding systems. One of them, *P. lanceolata*, is self-incompatible. This species also shows gynodioecy and protogyny, mechanisms which cannot be very important in a self-incompatible species in avoiding inbreeding. In the self-compatible species *P. coronopus* these mechanisms also exist, and here they may influence the degree of selfing considerably. *P. major* is protogynous only, and this mechanism seems to be rather ineffective in preventing self-fertilization because populations of this species are usually highly homozygous (Van Dijk and Van Delden 1981).

A major attempt of this study is the estimation of selfing rate, seed dispersal and pollen dispersal, and their respective consequences for the genetic structure of the populations of the three species. Equilibrium in the populations between gene flow and genetic drift is one of the major prerequisites for the use of the method described in Van Dijk (1987); the other prerequisite is the selective neutrality of allozyme variation.

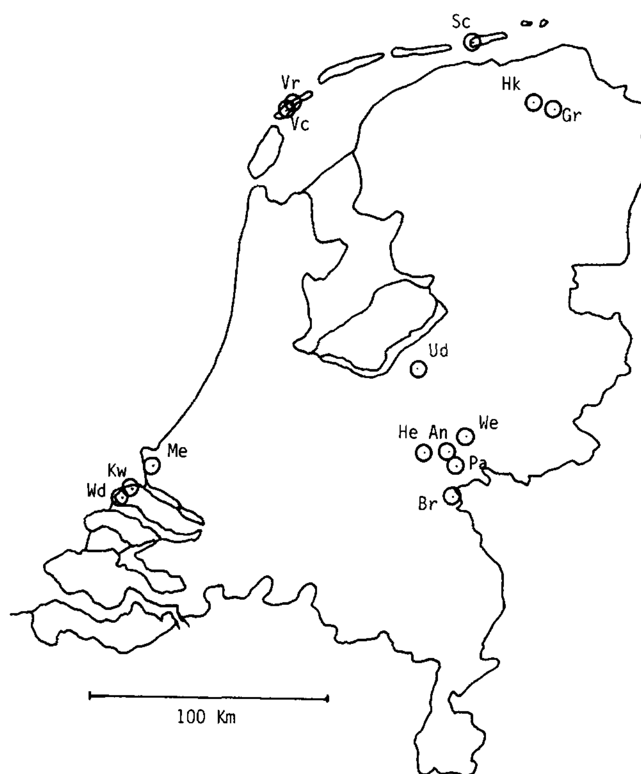

Fig. 1. The geographical positions of the various populations in The Netherlands. An (Angeren): Riverbank, flooded during the winter season. Grazed by cattle. Strongly variable densities of *P. major* ssp. *pleiosperma* among years; Br (Bruuk): Path across wet hay fields and wooded areas. *P. major* ssp. *major* in the middle of the path, *P. lanceolata* at both sides; Gr (Groningen): Lawn, partially trampled. *P. major* ssp. *major;* He (Heteren): Hayfield with *P. lanceolata.* Abondened cart track nearby with *P. major* ssp. *major;* Hk (Hoogkerk): Dikes around sedimentary field of a sugar factory, grazed by sheep. *P. major* is represented with both subspecies. Equivalent to population H in Van Dijk and Van Delden (1981); Kw (Kwade Hoek): Coastal meadow. *P. major* ssp. *pleiosperma* grows only at places exceptionally flooded by the sea. *P. coronopus* grows at lower places, with higher salt concentrations; Me (Merrevliet): Very wet, late mown hay field with *P. lanceolata;* Pa (Pannerden): Pasture near river, grazed by cattle, with *P. lanceolata;* Sc (Schiermonnikoog): Wet cart track near inner dune lake, grown with *P. major* ssp. *major;* Ud (Uddel): Sandy roadside with *P. lanceolata* and *P. coronopus.* The population is situated in an area with large heath fields; Vc (Vlieland-coast): Basalt dike along the Wadden Sea coast. *P. coronopus* grows between the stones; Vr (Vlieland-roadside): Very dense monoculture of *P. coronopus* along road from North Sea to Wadden Sea; Wd (Westduinen): Old inner dune pasture, extensively grazed by cattle. *P. lanceolata* and *P. coronopus;* We (Westervoort): Abondened railway yard with *P. lanceolata*

Materials and methods

The species

Plantago major is a wind-pollinated self-compatible perennial. Two subspecies are distinguished: ssp. *major,* $\leq$ 13 seeds per capsule, and ssp. *pleiosperma,* > 13 seeds per capsule. The latter subspecies is often annual in practice due to high winter mortality. Two important ecotypes within the ssp. *major* are the roadside (trampling resistant) type and the lawn type (adapted to mowing and grazing). Genetic differences between the subspecies and ecotypes have been described earlier (Van Dijk 1984).

Plantago lanceolata is a wind-pollinated, self-incompatible, gynodioecious perennial. Insect visits are also reported (Stelleman 1982). Ecotypic differentiation is less clear than in *P. major* (Wolff and Van Delden 1987); the plants from the populations Wd, Br and Ud are smaller than the plants from the other populations, and the Wd plants are late-flowering and form many secondary rosettes. The species occurs mainly in pastures, hay fields and roadsides.

Plantago coronopus is a wind-pollinated, self-compatible, gynodioecious perennial. Because mortality is high after having flowered, this species is very often biennial or annual. No ecotypic differentiation could be noticed (K. Wolff, in preparation). In The Netherlands, this species is almost completely restricted to coastal areas.

The populations

The populations sampled are situated in The Netherlands as shown in Fig. 1.

Electrophoresis

Electrophoresis methods were described by Van Dijk and Van Delden (1981) for *P. major*, and by Van Dijk (1985a) for *P. lanceolata.* The methods used for *P. major* were also used for *P. coronopus.* Contrary to these descriptions, the electrophoresis system of Poulik (1957) was used for *Acph,* with subsequent shaking the gel with sodium acetate staining buffer instead of boric acid. This electrophoresis system gave a better result than the previously used sodium borate buffer pH 9.

Table 1. Survey of the loci variable in at least one species with linkage relationships and description of alleles. L = linkage group, ? = not yet determined, S = number of subunits

Locus	*P. major*		*P. lanceolata*		*P. coronopus*[a]		
	L	Alleles	L	Alleles	L	S	Alleles
Acph-1	–	–	–	–	1	1	S (30), N (35), F (40)
-2	5	N, F	–	–	1	2	S (46), N (50)
Adh	–	–	–	–	1	1	S (21½), N (23), F (25)
Est-1	–	–	–	–	2	?	N (9), O
-2	–	–	–	–	3	?	N (18), O
-3	–	–	–	–	?	1	S (29), I (33), N (38), O
-4	4	N, F	3	A, B, C, D, E, F, G, O	+1, +4	1	N (43), F (47), O
-5			2	A, B, C, D, E, F, O			
Got-1	2	S, I, F	2	S, I, F	–		–
-2	4	S, N, F	4	S, F	–		–
Gpi-1	?	N, F	1	S_1, S_2, I, F	?	2	N (22), F (26)
Idh	–	–	2	S, N, F	4	2	S (33), N (35½)
Lap-1	–	–	1	S, I, F	–	–	
-2	–	–	4	S_1, S_2, N, F	2	1	S (36), N (40)
Me-1	3	S, N	–	–	–		–
6Pgd-1	–	–	5	S, N, F	–		–
-2	4	N, O	5	S, N, F	5	2	S (26), F (29½)
-3	–	–	4	S, N, F	–		–
Pgm-1	1	S_1, S_2, N	5	S, N, F	?	1	N (30), F (33)
-2	–	–	3	S_1, S_2, S_3, N, F_1, F_2	+1, +2	1	S (38), I (41), N (44)
Shdh	3	S_1, S_2, N, F	–	–	–		–
To-2	–	–	3	N, F	–		–

[a] The electrophoretic mobilities of the *P. coronopus* alleles are given in *parentheses* (bromophenol blue being 100). Invariable loci are *indicated by* – Est-5 has not been detected in *P. major* and *P. coronopus*

Statistical test of homogeneity of F values between loci within a population

(1) The mean F value was computed as the weighted average over loci, weighted by $N_j(1-\Sigma p_i^2)_j$, N_j being the number of individuals scored for the j-th locus and $(1-\Sigma p_i^2)_j$ being the gene diversity of the j-th locus. (2) The expected numbers of heterozygotes and homozygotes for each locus were calculated from the mean F value and N_j. (3) The observed and expected numbers of heterozygotes and homozygotes for all loci were used to calculate a χ^2 having $N-1$ degrees of freedom if N is the number of loci.

Results

Survey of the electrophoretic variation in the three species

The variable enzyme loci, their alleles and their linkage relationships, have been described for *P. major* and *P. lanceolata* in Van Dijk and Van Delden (1981) and Van Dijk (1985 a), respectively. These data are summarized in Table 1, together with a description of the electrophoretic mobilities of the alleles of the variable loci of *P. coronopus*. The linkage relationships between the variable loci of the latter species are partially presented, as the result of a preliminary investigation. The genetic basis of the allozyme variation in *P. coronopus* has been checked for 10 loci by crosses for Mendelian segregation. The remaining 3 variable loci (*Est*-3, *Gpi*-1 and *Pgm*-1) show staining patterns which are clearly interpreted, making non-Mendelian variation unlikely.

The number of loci concluded from the enzyme staining patterns was the same in *P. coronopus* and *P. major:* 36 in both cases. In *P. lanceolata,* 3 additional loci were postulated. The use of the same designation for the loci in the three species does not necessarily mean that they are evolutionary homologous, e.g., *Est-4* in one species may be homologous to *Est*-5 in the other. The three species are probably too remote in an evolutionary sense to expect similar linkage relationships between homologous loci. The fact that *P. coronopus* has a haploid set of five chromosomes, whereas in *P. major* and *P. lanceolata* this figure is six, forms a justification for this assumption.

A set of populations has been sampled for each species in order to estimate allele frequencies. *P. major* (Table 2) is represented by four large populations which are supposed to exist for longer periods: at least one decade (Hk) to several decades (Br, He and An). The populations Br and He belong to the subspecies *major*, An to the subspecies *pleiosperma*. The Hk population is a nearly random mixture of members of both subspecies; the allele frequencies for each subspecies are shown separately. In addition to these large

Table 2. Allele frequencies in the *P. major* populations[a]

Locus	Allele	Br, m (180)	He, m (42)	Hk, m+p (47)	Hk, m (15)	Hk, p (32)	An, p (118)	Gr, m (20)	Sc, m (35)	Kw, p (18)
		Population, subspecies (*m* = ssp. *major; p* = ssp. *pleiosperma*) and sample size								
Acph-2	N	1.000	1.000	0.894	0.933	0.875	1.000	0.950	1.000	1.000
	F	–	–	0.106	0.067	0.125	–	0.050	–	–
Est-4	N	0.747	0.429	0.798	0.867	0.766	0.934	1.000	1.000	–
	F	0.253	0.571	0.202	0.133	0.234	0.066	–	–	1.000
Got-1	S	–	–	0.394	0.067	0.547	0.893	–	–	0.778
	I	–	–	0.362	0.167	0.453	0.091	–	–	0.222
	F	1.000	1.000	0.244	0.766	–	0.016	1.000	1.000	–
Got-2	S	0.003	–	0.011	0.033	–	–	–	–	–
	N	0.994	1.000	0.861	0.867	0.859	0.579	1.000	1.000	1.000
	F	0.003	–	0.128	0.100	0.141	0.421	–	–	–
Me-1	S	0.031	0.048	0.394	0.333	0.422	0.296	0.350	1.000	–
	N	0.969	0.952	0.606	0.667	0.578	0.704	0.650	–	1.000
Pgm-1	S_1	0.139	0.107	–	–	–	0.008	–	–	–
	S_2	0.358	0.429	0.085	0.267	–	0.008	0.250	–	–
	N	0.503	0.464	0.915	0.733	1.000	0.984	0.750	1.000	1.000
Shdh	S_1	0.003	–	0.053	0.033	0.063	0.012	–	–	–
	S_2	0.208	0.048	0.106	0.067	0.125	0.313	–	–	–
	N	0.700	0.952	0.820	0.833	0.812	0.667	1.000	1.000	1.000
	F	0.089	–	0.021	0.067	–	0.008	–	–	–

[a] The loci *Gpi*-1 and *6Pgd*-2 were not variable in these population samples

and stable populations, three small populations have been included in Table 2 which are known to be relatively recently founded (Gr, Sc and Kw). Their allozyme variation is very low compared with the large populations. They are usually fixed for the most frequent allele, but sometimes for a less frequent one, as is to be expected when the number of colonizers which start a population is low. The differences between ssp. *major* and ssp. *pleiosperma* will be discussed later.

The seven *P. lanceolata* populations sampled (Table 3) represent six relatively large and old populations (He, Pa, Br, Wd, Me and We) of which Wd is more patchily distributed than the others. The seventh one, Ud, is isolated from other *P. lanceolata* populations by large heath fields, and was founded rather recently. In comparison with the three small and recently founded *P. major* populations, the genetic variability in Ud is not drastically reduced. This may be caused by the impossibility for a self-incompatible plant species like *P. lanceolata* to found a population by a single individual. The possibility of long distance seed transport, combined with the selective advantage of new *SI* alleles, will restore genetic variation after colonization by a few individuals more quickly than in the self-compatible species *P. major.*

P. coronopus is a species restricted in The Netherlands to coastal areas and is more separately distributed than the other two species, which belong to very abundant plant communities. Three populations (Kw, Vc and Vr) are large and stable. The Wd population occurs in an environment which has been stable during centuries, but the *P. coronopus* populations in this area are more or less isolated from each other and are fluctuating in size. The Ud population is an inland population, situated far away from the nearest coastal population (Fig. 1). This population appears to be completely invariable, even for leaf dentation, whereas *P. coronopus* is usually highly variable for this character. This population, therefore, may have been initiated by a single individual. The allele frequencies of the *P. coronopus* populations are given in Table 4.

The genetic variability of the populations is described by the degree of polymorphism (P), the mean number of alleles per locus (A), gene diversity (H_e; the level of heterozygosity expected under Hardy-Weinberg conditions) and the effective number of alleles per locus (A_e). Both latter measures are based on the sum of squares of the allele frequencies of a locus, Σp_i^2. The effective number of alleles equals $1/\Sigma p_i^2$; the gene diversity $= 1-\Sigma p_i^2$. Table 5 summarizes the various measures of genetic variability in the populations of the three species. Only data from the large populations have been used.

There are no strong differences between the population variabilities within the same species. In *P. major,* the two subspecies have about the same values. The higher values of P and A in the ssp. *pleiosperma* An population, compared with Br and He, are biased by

Table 3. Allele frequencies in the *P. lanceolata* populations

Locus	Allele	He (90)	Pa (80)	Ud (97)	Br (88)	Wd (93)	Me (72)	We (100)
Est-4[a]	A	0.004	–	–	0.005	–	0.005	0.028
	B	0.033	0.048	0.036	0.045	0.051	0.068	0.075
	C	0.271	0.289	0.090	0.501	0.288	0.303	0.197
	D	0.150	0.100	0.149	0.141	0.135	0.162	0.170
	E	0.201	0.214	0.574	0.156	0.315	0.182	0.252
	F	0.044	0.096	0.036	0.040	0.056	0.036	0.083
	G	–	–	–	–	0.009	0.005	–
	O	0.297	0.253	0.115	0.112	0.146	0.239	0.195
Est-5[a]	A	0.040	0.027	0.009	0.034	0.019	0.072	0.034
	B	0.121	0.373	0.239	0.218	0.116	0.180	0.190
	C	0.085	0.119	0.327	0.124	0.233	0.126	0.114
	D	0.133	0.141	0.107	0.068	0.194	0.186	0.139
	E	0.330	0.260	0.098	0.107	0.273	0.287	0.309
	F	–	–	0.004	0.013	–	–	–
	O	0.291	0.080	0.216	0.436	0.165	0.149	0.214
Got-1	S	0.512	0.226	0.313	0.242	0.255	0.282	0.317
	I	0.418	0.695	0.687	0.747	0.707	0.683	0.648
	F	0.070	0.079	–	0.011	0.038	0.035	0.035
Got-2	S	0.466	0.598	0.943	0.618	0.788	0.582	0.554
	F	0.534	0.402	0.057	0.382	0.212	0.418	0.446
Gpi-1	S_1	0.296	0.207	0.211	0.359	0.188	0.304	0.208
	S_2	0.005	0.030	–	0.051	0.005	0.014	0.005
	I	0.479	0.293	0.660	0.264	0.468	0.344	0.406
	F	0.220	0.470	0.129	0.326	0.339	0.338	0.381
Idh	S	–	–	–	0.011	–	0.041	0.015
	N	1.000	0.994	1.000	0.978	0.973	0.939	0.970
	F	–	0.006	–	0.011	0.027	0.020	0.015
Lap-1	S	0.171	0.184	0.248	0.218	0.207	0.154	0.137
	I	0.570	0.551	0.639	0.437	0.440	0.530	0.595
	F	0.259	0.265	0.113	0.345	0.353	0.316	0.268
Lap-2	S_1	–	0.012	–	0.017	–	–	–
	S_2	–	–	–	–	–	–	0.005
	N	0.837	0.768	0.887	0.776	0.855	0.815	0.827
	F	0.163	0.220	0.113	0.207	0.145	0.185	0.168
6Pgd-1	S	–	–	–	0.056	0.005	–	0.010
	N	1.000	0.993	1.000	0.938	0.995	0.986	0.990
	F	–	0.007	–	0.006	–	0.014	–
6Pgd-2	S	0.077	0.164	0.067	0.171	0.117	0.052	0.027
	N	0.923	0.829	0.933	0.817	0.883	0.933	0.973
	F	–	0.007	–	0.012	–	0.015	–
6Pgd-3	S	–	–	–	–	–	–	0.005
	N	0.953	0.983	0.979	1.000	0.995	1.000	0.923
	F	0.047	0.017	0.021	–	0.005	–	0.072
Pgm-1	S	0.051	0.075	0.020	0.051	0.027	0.042	0.010
	N	0.927	0.919	0.980	0.949	0.973	0.958	0.980
	F	0.022	0.006	–	–	–	–	0.010
Pgm-2	S_1	0.028	–	0.005	0.006	0.011	0.015	0.064
	S_2	0.143	0.141	0.113	0.174	0.140	0.094	0.074
	S_3	0.016	0.026	0.119	0.034	0.005	–	–
	N	0.648	0.730	0.407	0.522	0.527	0.471	0.659
	F_1	0.039	0.013	0.299	0.028	0.027	0.072	0.025
	F_2	0.126	0.090	0.057	0.236	0.290	0.348	0.178
To-2	N	0.948	0.878	1.000	0.972	0.989	0.959	0.946
	F	0.052	0.122	–	0.028	0.011	0.041	0.054

Population and mean sample size given in column headers.

[a] The frequencies of the O-alleles are calculated from the increase in fixation index compared with the mean *F* value of the loci without O-alleles in each population

Table 4. Allele frequencies in the *P. coronopus* populations

Locus	Allele	Kw (149)	Wd (135)	Vc (90)	Vr (132)	Ud (70)
Acph-1	S	0.029	0.063	0.033	–	–
	N	0.955	0.791	0.967	1.000	1.000
	F	0.016	0.146	–	–	–
Acph-2	S	–	–	0.028	0.210	–
	N	1.000	1.000	0.972	0.790	1.000
Adh	S	0.163	0.145	0.048	0.127	–
	N	0.749	0.816	0.753	0.817	1.000
	F	0.088	0.039	0.199	0.056	–
Est-1[a]	N	0.697	0.180	0.805	0.908	1.000
	O	0.303	0.820	0.195	0.092	–
Est-2[a]	N	0.565	0.602	0.167	0.354	–
	O	0.435	0.398	0.833	0.646	1.000
Est-3[a]	S	0.011	0.011	–	0.022	–
	I	0.195	0.387	0.264	0.388	–
	N	0.667	0.514	0.736	0.590	1.000
	O	0.127	0.088	–	–	–
Est-4[a]	N	0.734	0.606	0.928	0.780	1.000
	F	0.209	0.260	0.072	0.220	–
	O	0.057	0.134	–	–	–
Gpi-1	N	0.980	0.890	0.853	0.739	1.000
	F	0.020	0.110	0.147	0.261	–
Idh	S	0.076	0.125	–	–	–
	N	0.924	0.875	1.000	1.000	1.000
Lap-2	S	0.010	0.023	0.076	0.135	–
	N	0.990	0.977	0.924	0.865	1.000
6Pgd-2	S	0.272	0.121	0.582	0.543	–
	F	0.728	0.879	0.418	0.457	1.000
Pgm-1	N	1.000	1.000	1.000	0.961	1.000
	F	–	–	–	0.039	–
Pgm-2	S	0.142	–	0.065	0.018	–
	I	–	0.066	–	–	–
	N	0.858	0.934	0.935	0.982	1.000

Population and mean sample size given in column headers.

[a] The frequencies of the O-alleles are calculated from the increase in fixation index compared with the mean *F* value of the loci without O-alleles in each population

the occurrence of two ssp. *major* individuals in the sample, containing the subspecies-specific alleles *Pgm*-1 S_1 and S_2 and *Got*-1 *F*. The mixed population Hk is considerably more variable, which is not surprising because both subspecies possess specific alleles (Van Dijk and Van Delden 1981). The three species appear to have almost no overlap in the variability ranges of their populations, *P. coronopus* being intermediate between *P. major* and *P. lanceolata*.

Differentiation among populations and subpopulations

As a measure of population differentiation, the ratio $D_{ST}/\bar{H}_e$ will be used (Brown 1979); its scale ranges from zero to infinity. This measure is biased by a limited number of subpopulations and lower sample sizes. When corrected for both k (the number of

Table 5. Levels of allozyme variation

Species	Population	% Polymorphic loci (P)	Mean number of alleles per locus (A)	Mean effective number of alleles per locus (A_e)	Mean gene diversity per locus (H_e)
P. major	Br	13.9	1.25	1.08	0.042
	He	11.1	1.14	1.07	0.035
	Hk	19.4	1.31	1.12	0.065
	An	16.7	1.28	1.08	0.048
	mean	15.3	1.24	1.09	0.047
P. lanceolata	He	30.8	1.77	1.36	0.131
	Pa	35.9	1.82	1.36	0.134
	Ud	28.2	1.67	1.28	0.104
	Br	33.3	1.90	1.35	0.134
	Wd	35.9	1.79	1.37	0.124
	Me	33.3	1.82	1.41	0.133
	We	35.9	1.87	1.39	0.128
	mean	33.3	1.81	1.36	0.127
P. coronopus	Kw	30.6	1.44	1.15	0.088
	Wd	30.6	1.44	1.16	0.093
	Vc	30.6	1.33	1.13	0.078
	Vr	30.6	1.36	1.15	0.093
	mean	30.6	1.40	1.15	0.088

Table 6. Differentiation between populations and subpopulations

Species or population		Number of (sub-)populations (k)[a]	Harmonic mean of sample sizes ($\bar{N}_h$)	$\frac{D_{ST}}{\bar{H}_e}$	Unbiased estimate of population differentiation[b]
P. major		4	67.7	0.379	0.491
P. major ssp. *m*		3	31.2	0.107	0.128
P. major ssp. *p*		2	50.3	0.077	0.134
P. lanceolata		7	87.6	0.045	0.041
P. coronopus		4	121.9	0.127	0.161
P. lanc.	Wd	2 (90 m)	46.5	0.016	0.010
	We	2 (25 m)	44.9	0.029	0.036
P. cor.	Kw	2 (200 m)	67.1	0.015	0.015
	Wd	2 (5 m)	65.9	0.100	0.185
	Vr	2 (1,300 m)	54.1	0.062	0.106

[a] The distances between the pairs of subpopulations are given in parentheses

[b] $\frac{D_{ST}}{\bar{H}_e} \cdot \frac{k}{k-1} - \frac{1}{\bar{N}_h}$

subpopulations) and $\bar{N}_h$ (the harmonic mean of the populations sample sizes), the estimate of population differentiation becomes unbiased, and is thus made comparable for different situations. This unbiased measure is:

$$\frac{D_{ST}}{\bar{H}_e} \cdot \frac{k}{k-1} - \frac{1}{\bar{N}_h}$$

Table 6 shows that the population differentiation in *P. major* is the highest of the three species. The genetic difference of the two subspecies with respect to loci *Got*-1 and *Pgm*-1 will partially cause this differentiation, because populations of both subspecies are included. Between populations of the same subspecies (using Br, He and Hk-m for ssp. *major* and An and Hk-p for ssp. *pleiosperma*), the degree of differentiation is distinctly lower, and is about equal in both subspecies. Both *P. coronopus* and the *P. major* subspecies are considerably more differentiated than *P. lanceolata.* Brown (1979) published a list of $D_{ST}/\bar{H}_e$ values for a number of species, subdivided into inbreeders and outbreeders, having mean values of 1.06 and 0.20, respectively. The values found here are much lower, but do not fall outside the range of the species listed.

Population diversity appears to be present at distances as low as a kilometer or less, to a degree which is already the same as the diversity over the whole country (a few hundreds of kilometers, see Tables 6 and 7). In some cases, deviating (sub)populations enhance population differentiation values: *P. lanceolata* population Ud and a part of *P. coronopus* population Wd, Wd-1, which have distinctly lower gene diversities than the other (sub)populations. Especially in *P. lanceolata,* genetic variation is, for the greatest part, already present within each population, and any large-scale geographic pattern in allele frequencies appears to be absent. This is also true for the other two species, to a lesser extent.

The occurrence of a thoroughly mixed population (Hk) of the two subspecies of *P. major* enables the estimation of the differentiation between these subspecies, undisturbed by a component of geographical distance. The $D_{ST}/\bar{H}_e$ value for the couple Hk-m/Hk-p, corrected for $\bar{N}_h$ only, is 0.105.

Tests for the neutrality of the allozyme variation

Data pointing to a low level of population differentiation do not discriminate between the possible causes: much gene flow and little genetic drift on the one hand, or similar selective forces on the other hand. A test for neutrality will provide more insight. Neutrality is also a necessary condition in the estimation of small-scale gene flow.

Neutrality will be tested at the interpopulation level and at the intrapopulation level. The test of Lewontin and Krakauer (1973) checks whether loci show comparable variances in allele frequencies among populations, as measured by their F_{ST} values. The expected variance in F_{ST} under neutral conditions is a function of the mean F_{ST} value and the number of populations (k): $V_e = 2\bar{F}_{ST}^2/(k-1)$. The populations have to be unrelated (Robertson 1975), and the allele frequencies

have to be distributed whether uniformly between 0 and 1 or normally. Both conditions were probably fulfilled in the set of populations under study. Alleles with mean frequencies below 0.01 have been omitted in the test. The test results (Table 8) indicate deviations in all three species, although not significant in *P. major* and *P. coronopus.* All ratios of observed to expected variance are higher than unity. In *P. major,* the loci *Pgm*-1 and *Got*-1 are known to be linked with loci causing fitness differences between the subspecies (Van Dijk 1984). Their exclusion lowers the ratio drastically, from 1.63 to 0.68. In *P. lanceolata,* the large number of alleles allows for a more powerful test of neutrality. The ratio between observed and expected variance is significantly different from unity, taking all seven populations. A closer look at the building-up of the variance by various alleles points to a few alleles with deviating frequencies in the Ud population, which has already been mentioned as probably being disturbed by genetic drift. Excluding this population, the ratio decreases to a non-significant level, from 1.76 to 1.16. Clearly, not selection but genetic drift in one of the populations is disturbing the similar behaviour of the various loci. Because *P. coronopus* also contains a subpopulation (Wd-1) which is thought to be disturbed by genetic drift, the test has been conducted for the four populations, using Wd-2 as representative of Wd; this lowers the ratio from 1.46 to 0.89.

Table 7. Geographic distance and genetic differentiation for some combinations of populations

Species	Populations	(Mean) distance in km	Unbiased estimate of population differentiation
P. major	Br-He	27	0.096
	Br-Hk-m	165	0.089
	He-Hk-m	146	0.193
	An-Hk-p	147	0.134
P. lanceolata	He-Pa-Br-We	18	0.026
	Wd-Me	15	0.008
	(He, Pa, Br, We)-(Wd, Me)	130	0.022
P. coronopus	Kw-Wd	10	0.112
	Vc-Vr	2½	0.043
	(Kw, Wd)-(Vc, Vr)	175	0.161

The neutral behaviour of the allozyme loci within populations will be checked by the test which is described in "Materials and methods". This test has been carried out for all populations, and for those subpopulations that were different from each other. The loci with null alleles, and the loci which were suspected to have null alleles, were omitted. In the 19 subpopulations tested, two appear to be significantly deviating from the expectations under neutrality (Table 9). In *P. lanceolata,* Br ($P=0.017$) *Lap*-2 and *Pgm*-2 have too many homozygotes and *Got*-2 has too many heterozygotes. It is, in this respect, interesting to note that *Lap*-2 and *Got*-2 belong to the same linkage group, and *Pgm*-2 to a different one. In *P. coronopus,* Vr-2 ($P=0.004$), *Pgm*-1 and *Gpi*-1 showed too many homozygotes, and *Est*-3 too many heterozygotes. A closer look at the underlying causes points out that the contribution of *Pgm*-1 is brought about by the occurrence of one small part of the Vr-2 population area dominated by the F-allele (genotypes: 4×FF, 2×NF, 1×NN), whereas the F allele is completely absent in the rest of the population sample (and even in all other population samples). The deviation in the genotype ratios in *Gpi*-1 and *Est*-1 exists over the entire population area.

Population structure and gene flow estimates

An estimation of the gene flow parameters σ_p (standard devation of the axial normal distribution of pollen dispersal), σ_s (the same for seed dispersal), and t (outcrossing rate) from population structure data has been described by Van Dijk (1987). The two necessary conditions for obtaining reliable estimates of these gene flow parameters – selective neutrality of the genetic variation used, and equilibrium for gene flow and genetic drift – are fulfilled for most populations.

In all *P. lanceolata* and *P. coronopus* populations, and in *P. major* Br, the position coordinates of the

Table 8. The Lewontin-Krakauer test for neutrality of the allozyme variation

Species	Number of populations	F_{ST}	Expected var. in F_{ST}	Observed var. in F_{ST}	Ratio obs./exp.	df	Level of significance
P. major	4	0.2156	0.0310	0.0504	1.63	11	ns
P. lanceolata	7	0.0369	0.000453	0.000800	1.76	29	*
P. coronopus	4	0.0699	0.003255	0.004796	1.47	18	ns
P. major minus *Pgm*-1 and *Got*-1	4	0.109	0.0079	0.0054	0.68	7	ns
P. lanceolata minus Ud	6	0.0234	0.000220	0.000255	1.16	29	ns
P. coronopus minus Wd-1	4	0.0900	0.005405	0.004826	0.89	17	ns

* $P<0.01$

Table 9. Test for homogeneity of homozygosity levels of loci within populations

Species	Population	Mean F	df	χ^2
P. major	Br	0.753	4	4.80
	He	0.415	3	3.85
	Hk	0.855	6	6.78
	An	0.897	5	7.07
P. lanceolata	He	−0.014	8	2.40
	Pa	0.099	10	17.43
	Ud	0.114	7	5.59
	Br	0.085	9	20.44[a]
	Wd	0.083	10	4.43
	Me	0.083	9	7.52
	We-1	0.039	10	4.89
	We-2	0.059	9	5.07
P. coronopus	Kw-1	0.113	6	1.30
	Kw-2	0.143	6	5.26
	Wd-1	0.365	5	3.54
	Wd-2	0.045	6	8.00
	Vc	−0.037	8	1.00
	Vr-1	0.056	5	2.35
	Vr-2	0.061	8	23.30[b]

[a] $P < 0.05$; [b] $P < 0.01$

Table 10. Homozygosity and local differentiation within populations, and mean values for the loci with the highest gene diversities

Species	Population	Number of loci used	F_{IT}	$F_r(0)$
P. major	Br	3	0.749	0.541
	He	3	0.369	0.261
	An[a]	5	0.892	0.255
P. lanceolata	He	6	−0.011	0.001
	Pa	6	0.092	−0.071
	Ud	6	0.118	0.031
	Br	6	0.090	0.002
	Wd	6	0.084	0.025
	Me	6	0.081	0.014
	We-1	6	0.049	0.096
	We-2	6	0.045	0.292
	We-1+2	6	0.047	0.179
P. coronopus	Kw-1	6	0.122	0.275
	Kw-2	6	0.149	0.209
	Kw-1+2	6	0.140	0.254
	Wd-1	6	0.327	0.801
	Wd-2	6	0.055	0.247
	Vc	6	−0.050	0.072
	Vr-1	6	0.113	−0.059
	Vr-2	6	0.047	0.177
	Vr-1+2	6	0.064	0.182

[a] Mainly juveniles used instead of only adults

sampled individuals are known. In the *P. major* populations, He and An data were only available about whether or not plants were growing at (almost) zero distance from each other. Only the most variable loci in each population were used for the gene flow estimates, because less variable loci do not appreciably contribute. All average values of F (F_{IT}'s and F_r's) over loci have to be weighed by the gene diversities of the loci, and, if there are differences in sample sizes among loci, by the products of sample size and gene diversity. In Table 10 a survey is given of the mean F_{IT} and $F_r(0)$ values in the various (sub)-populations. $F_r(0)$ is a measure of the degree of local differentiation (Van Dijk 1987). In all populations except for *P. major* An, only adults were sampled. In the An population, the *P. major* ssp. *pleiosperma* individuals behave like annuals in practice, due to the high winter mortality. Therefore, sampling juveniles may be acceptable in this situation. The finding of a high degree of local differentiation in the An population is surprising, because the river, flooding the banks during the winter season, was supposed to mix the surface layer, including the seeds, thoroughly.

The lower local differentiation in the *P. major* He population compared with the Br population is due to the unusually high density of large plants in the former population, which reduces the level of selfing. The highly locally differentiated Br population is probably the more normal situation in path and roadside populations.

In *P. lanceolata,* the degree of local differentiation is very low in all populations, except We. *P. coronopus* lies roughly between the two other species. The Wd-1 subpopulation, already mentioned as probably having been influenced by genetic drift, shows an extraordinary high level of local differentiation. This may be the result of a recent increase in numbers, starting with a few individuals, each of which has surrounded itself with a number of (genetically related) descendants.

To be able to estimate gene flow levels from the data, knowledge about population density of reproducing individuals, population dimensionality and yearly turnover is necessary. Populations are usually two-dimensional at a small scale, but in the case of roadsides or coastal zones, a changeover to one dimension will happen at distances larger than the width of the zone. If the population is situated within a small area, both dimensions are limited. Table 11 gives the necessary information.

In *P. major,* the only population which could be analysed completely is Br. This population has a very high F_{IT} (Table 10), whereas $F_r(0)$ is lower. Because the contribution of local differentiation to F_{IT} cannot exceed $F_r(0)$, the selfing rate must be considerable. The estimation of σ_p in such situations is not very reliable using a sample size like this (Van Dijk 1987). For this reason, a range of σ_p values has been taken within reasonable limits (Table 12). The values of t and σ_s can then be estimated, and appear to be rather insensitive for the σ_p value chosen. Thus, t will be approximately

Table 11. Demographic and dimensional information

Species	Population	Approximate density N/m²	Max.[a] width (m)	Max.[a] length (m)	Approximate yearly turnover (%)[b]
P. major	Br	34	2	–	25
P. lanceolata	He	20	–	–	5
	Pa	10	–	–	23
	Ud	30	1	–	47
	Br	20	2	–	41
	Wd	50	2	4	50
	Me	50	–	–	20
	We	20	–	–	28
P. coronopus	Kw	50	–	–	80
	Wd	20	1.5	3	80
	Vc	20	5	–	80
	Vr	500	1	–	80

[a] –: Means that no effective limitations in the appropriate dimension are present

[b] The turnover values are for the greater part based on demographic work of Van der Toorn, Haeck and Mook (unpublished)

Table 12. Estimates of gene flow and neighbourhood parameters in *P. major*

Population	σ_p (cm)	t	σ_s (cm)	N_e	M (cm)
Br	20	0.175	6.5	2.7	11.0
	50	0.165	6.3	2.5	19.7
	100	0.155	6.2	2.4	35.7
He		0.5 –0.6			
An		0.06–0.07			

0.165 and σ_s about 6.3 cm. The local effective population size N_e, analogous to Wright's (1943) neighbourhood size with respect to genetic drift, is about 2.5. The mean gene transport per generation (M) is dependent on σ_p, and lies between about 10 and 40 cm if σ_p is between 20 and 100 cm. In the populations He and An, estimates of t can also be made; the ranges shown in Table 12 are from almost no pollen flow to a very high pollen flow.

In *P. lanceolata,* almost no local differentiation is observed in six populations. Nevertheless, the F_{IT} values are distinctly positive in all populations except He. This is an unexpected result, because this species is self-incompatible, so that selfing is not a realistic cause of the positve F_{IT}'s. Although the self-incompatibility system can be broken down at high temperatures (JMM Van Damme, personal communication), the occurrence of selfing in natural situations seems to be rather unlikely. If t values are estimated, however, they are found between 0.84 and 0.9 in the populations Pa, Ud,

Table 13. Estimates of gene flow and neighbourhood parameters in *P. lanceolata*

Population	σ_p if t=1 (cm)	σ_s if t=1 and σ_p is large (cm)	N_e	M (cm)
He	200–∞	70	365	>200
Pa	[a]	–	–	–
Ud	[a]	70	70	–
Br	[a]	400	600	–
Wd	[a]	40	120	–
Me	[a]	75	500	–
We	70	8.4	7.9	63

[a] Even a very small value of σ_p ($\rightarrow 0$) cannot explain the homozygosity observed if t=1 (see text)

Br, Wd and Me. In the He population, the combination of $F_r(0)$ and F_{IT} values are compatible with t=1 and a high level of gene flow (Table 13). as is the We population, but with a considerably lower level of gene flow. The reason for higher local differentiation and low seed transport in the We population may be that this population is neither mown nor grazed (both actions promote seed transport). Though the Ud population is also not situated at a place which is used agriculturally, it still has a low level of local differentiation.

In *P. coronopus,* the reverse situation is met: F_{IT} is lower than $F_r(0)$ in all populations. Only in Kw does the outcome of the gene flow analysis lead to acceptable parameter values, but with t close to one. In Wd-2 and Vr, local differentiation cannot be explained by the σ_p levels estimated, but needs lower levels which are, however, incompatible with t values lower than one (Table 14). In Vc, F_{IT} is negative, also leading to t>1. Because t cannot be larger than one, a different mechanism must be responsible for the low homozygosity levels. Because *P. coronopus* is self-compatible, values of t lower than one are expected, although the occurrence of completely and partially male sterile plants (varying in frequency from 8.7% to 18.4%), the stronger protogyny and the smaller number of flowers per spike favours cross-fertilization more than in *P. major.*

Discussion

Allozyme variation appears to be present in all three species of this study, in amounts compatible with other similar plant species (Hamrick et al. 1979). The levels of variability are sufficiently high to use the differences in allele frequencies between and within populations for the estimation of large-scale and small-scale gene flow. In both cases, selective neutrality of the genetic variation involved and the existence of equilibrium

Table 14. Estimates of gene flow and neighbourhood parameters in *P. coronopus* (all distances in cm)

Population	Estimated parameters					Examples using reasonable value of σ_p and σ_s				
	σ_p	t	σ_s	N_e	M	σ_p	t	σ_s	N_e	M
Kw	15	0.975	3.3	3.8	13.8					
Wd-2	35	0.985	[a]	–	30.8	15	1.22[b]	3	3.0	15.2
Vc	∞	1.08[b]	5.2	5.7	∞	50	1.10[b]	8.2	8.7	47.6
Vr	20	0.994	[a]	–	17.7	5	1.19[b]	2	10.0	5.4

[a] Even a very small value of σ_s ($\rightarrow 0$) cannot explain the degree of local differentiation observed if the values of σ_p and t are like estimated (see text)

[b] Because t cannot exceed 1, there must be an unknown factor which promotes heterozygosity if the σ_p values are like estimated or proposed (see text)

between gene flow and genetic drift are necessary conditions. Deviations from equilibrium may be recognised by a smaller genetic variability, unusual allele frequencies and/or a high local differentiation. *P. lanceolata* Ud and *P. coronopus* Wd-1 do not appear to be in equilibrium.

Neutrality of the allozyme variation has been tested on different levels. Although its validity has been criticized (Ewens and Feldman 1976), the Lewontin-Krakauer test may be the most suitable test for selective neutrality among populations. The results indicate that this test is not very powerful with the numbers of alleles, and especially with the numbers of populations used. Even in the most ideal species for this test, in view of the greater number of populations and loci studied, *P. lanceolata*, the occurrence of one isolated and relatively small population (Ud) severely disturbed the outcome. Using only similar populations with respect to levels of variability, thus avoiding populations like *P. lanceolata* Ud and *P. coronopus* Wd-1, the test did not reject neutrality in any of the species. In *P. major*, the special character of the loci *Pgm*-1 and *Got*-1 has to be kept in mind in all cases where both subspecies are involved. The test for homogeneity in deviation from Hardy-Weinberg equilibrium among loci also did not suggest selective differences, with the exception of one subpopulation being significant at the 0.01 level.

Assuming that selection has no disturbing influence, the degree of population differentiation can give information about the gene flow between populations. Long distance gene flow is undoubtedly possible in the three species. *P. major* and *P. lanceolata* have sticky seeds, allowing transport by animals or men. Their ability to colonize new areas quickly has been proven by history: both *P. lanceolata* and *P. major* ssp. *major* have been introduced in Northwest Europe, together with agriculture, about 6,000 years ago, and both species became cosmopolitan after the migration of Europeans world wide. The recently established *P. major* populations Gr, Sc and Kw also illustrate the ability to colonize new sites. Direct evidence is present in the Br population containing one Got-2^{SF} individual, while all other individuals are Got-2^{NN}, and in the An population containing two ssp. *major* individuals. In *P. coronopus* a seed polymorphism exists: one of the usually five seeds remains in the capsule lid and is thus able to be transported across water surfaces (Schat 1981).

If each population receives in each generation mN individuals from other unrelated populations (m being the fraction of migrating individuals and N the population size), the resulting F_{ST} would be about $1/(4mN+1)$ as in Wright's (1943) island model. *P. lanceolata* would have an mN value of about 10, *P. major* and *P. coronopus* of about 2 to 3. The real number of immigrants to obtain a particular F_{ST} may be larger if neighbouring populations are related, or when the immigrants belong to a different ecotype not able to contribute as many genes to the next generation as the native ecotype. Especially between populations of *P. major*, a species in which many ecotypes are distinguishable (Van Dijk 1984), this latter mechanism may restrict the effective gene flow when compared with the real gene flow. However, the two subspecies, which can be considered to represent the most extreme ecotypes, have similar allele frequencies on other loci than *Pgm*-1 and *Got*-1 (see population Hk, Table 2). This suggests that gene flow is possible between ecotypes and subspecies, with subsequent selection against parts of the genome, while other, non-selected parts are introduced into the other ecotype.

The possibility of long-distance seed transport does not necessarily mean that gene flow within populations is markedly influenced. Quantitatively, these actively dispersed seeds may be almost negligible when compared with the large amounts of passively dispersed seeds staying near the female parent plant. In *P. major*, the high degree of local differentiation points to such a situation. Nevertheless, long-distance seed transport may play an important part, in addition to the gene

exchange levelling allele frequencies among populations, in keeping populations homogeneous over larger distances, breaking down clines and preventing the complete local loss of genetic variation. In *P. lanceolata,* the majority of populations shows almost no internal differentiation, suggesting that in this situation active seed transport through hay-making or by cattle may be much more important. This is consistent with the finding of a distinct local differentiation within the unmanaged We population.

The values of σ_p and σ_s as estimated in the various populations of the three species by the method of Van Dijk (1987) are in agreement with expectations. *P. lanceolata,* having the tallest spikes, appears to disperse its pollen and seeds over larger distances than the more prostrate *P. major* or *P. coronopus.* In *P. major,* the gene flow is additionally reduced by the high level of selfing, diminishing the role of pollen flow. The degrees of heterozygosity, however, are not in agreement with the combinations of pollen flow and the level of local differentiation in nearly all populations of *P. lanceolata* and *P. coronopus. P. lanceolata,* being self-incompatible and showing a high level of gene flow, was expected to be almost in Hardy-Weinberg equilibrium, with F_{IT} values close to zero. *P. coronopus,* self-compatible and markedly restricted in gene flow, was expected to have fairly positive F_{IT} values. The mean F_{IT} value of the *P. coronopus* populations (minus Wd-1) appeared, however, to be even lower than the mean F_{IT} value of the *P. lanceolata* populations (Table 9).

Brown (1979), comparing data of many plant species with respect to their F_{IT} values, noted that inbreeding species often showed too many heterozygotes, whereas outbreeding species tended to have too many homozygotes. He called this the "heterozygosity paradox". The situation in this study is similar with respect to *P. coronopus* and *P. lanceolata.* The self-compatible species *P. major* does not behave abnormally.

In *P. coronopus,* heterozygosity is compatible with t = 1, or higher (Table 14). If t is lower than one, which should be checked by direct measurements. the excess of heterozygotes will be even more pronounced. Selection in favour of heterozygotes (in general, because various linkage groups are involved) is the only available explanation, and should be validated in a further experiment.

A shortage of heterozygotes in an obligatory outcrossing species like *P. lanceolata* can be brought about in several ways (see Brown 1979). The most common one, restricted neighbourhoods, has already been taken into account by the method used. Selection against heterozygotes in all linkage groups does not appear likely. An alternative explanation is a Wahlund effect as a result of two groups of plants with different allele frequencies being mixed. This case also requires further investigation.

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