

Genetic structure and differentiation of *Plantago major* reveals a pair of sympatric sister species

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Abstract

Seeds of the widespread weed *Plantago major* were collected from 10 European countries, as well as Trinidad and North America. The seed collections were from populations of two taxa which are ecologically rather than geographically separated and formally recognized as the subspecies *Plantago major* ssp. *major* and *P.m.* ssp. *intermedia* (also called *P.m.* ssp. *pleiosperma*). Eight polymorphic allozyme loci and 73 random-primed DNA fragments were scored, as well as 11 morphological characters. Complete concordance between morphological traits and genetic data provides evidence that these two taxa, although very similar, are distinct species. They are both widespread, they are broadly sympatric and capable of interbreeding. However, slight morphological and ecological differences coincide with genetic clustering of populations from widely separated locations. In addition, *P. major* and *P. intermedia* differ in their population structure: *P. intermedia* has greater genetic diversity among populations and less genetic variance within populations than *P. major*. We suggest that differences between the two species in their levels of selfing may explain the distinctive genetic structure of each species. We hypothesize a link between selfing rate and lifespan of the two taxa. *P. major* is characterized by lower genetic variation among populations, a higher rate of outcrossing, longer lifespan and production of fewer seeds per seed capsule. *P. intermedia* is more highly structured with much differentiation among populations, a higher rate of inbreeding and it often grows as an annual.

Keywords: evolution, genetic diversity, mating systems, *Plantago*, population structure, subspecies

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Introduction

In the study of plant population genetics advances have recently been made in understanding the theoretical effect of inbreeding on population structure (Charlesworth *et al.* 1993, 1997). However, satisfactory explanations of empirical data are not as advanced as single models fail to explain what plants are actually doing (Bonnin *et al.* 1996; Awadalla & Ritland 1997; Liu *et al.* 1998). Genetic structure of natural populations impacts on studies of differentiation, speciation, adaptation, the

evolution of mating systems and the tracing of population history.

In the past, studies have been constrained by the resolving power of the genetic techniques employed to study genetic variation. Studies using random amplification of polymorphic DNAs (RAPDs) frequently have found more genetic variation than studies of the same groups of organisms examined for allozyme variation (Hidayat *et al.* 1996; Haig *et al.* 1994; Macaranas *et al.* 1995; Burlando *et al.* 1996). In many investigations the increased ability to resolve genetic variation has revealed population structure previously unrecognized (although this is not always the case, i.e. Van Oppen *et al.* 1995).

The cosmopolitan and common weed, *Plantago major* L. has been extensively studied in the Netherlands and Denmark; thus much is known of its ecology, genetics, and reproductive biology (Mølgaard 1976; Van Dijk & Van

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Delden 1981; Van Dijk 1984; 1991b; Wolff 1991a; Wolff & Schaal 1992; Wolff *et al.* 1994). *P. major* is highly inbreeding but the genus to which it belongs contains species with a wide range of mating systems, from inbreeders to obligate outcrossers. *Plantago major* is wind-pollinated and its seeds, sticky when wet, are often dispersed by animals (Kuiper & Bos 1992). The worldwide distribution of *P. major* appears to have followed human movement from Europe (the genus name means footprint, a fitting term for the prostrate growth form of *P. major*, common on paths).

Although cosmopolitan in distribution, *P. major* is locally subdivided into ecotypes. Some authors have recognized these ecotypes as separate species, other authors recognized them as subspecies or varieties, in some cases using different names. Confusion arises indirectly from the fact that the concept of a 'species' has changed over the 200 years since Linnaeus described *P. major*, and

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Me-1, fast *Acph-2* and the null allele of *6Pgd-2*. However, we did observe three alleles not seen before: slow *6Pgd-1*, fast *6Pgd-2*, and slow *6Pgd-3*.

Population genetics

Cluster analysis using neighbour-joining (Saitou & Nei 1987) with PHYLIP (Felsenstein 1993) and printed using TreeView was performed with: (i) Euclidean distance (calculated with Arlequin (Schneider *et al.* 1997)) from the RAPD data; and (ii) arc distance (Cavalli-Sforza & Edwards 1967) calculated from the allozyme data using BIOSYS (Swofford & Selander 1981). The choice of distance measure had little effect on the structure of the resulting tree.

F-statistics were estimated using the program FSTAT (Weir 1990) with the allozyme data set, including bootstrap by loci to generate 95% confidence intervals. F_{IS} values for each population were compared using a Mann–Whitney *U*-test to determine if *P.m. ssp. intermedia* was significantly less outcrossing than *P.m. ssp. major* (one-tailed test). Measures of population heterozygosity (H_E) were calculated from the allozyme data using GelJock (Porter 1995), and genetic diversity (D_{ST}) (Nei 1973) calculated as $(H_S \cdot F_{ST}) / (1 - F_{ST})$. Total variability was expressed as $H_T = H_S + D_{ST}$. The RAPD and inter-SSR data were analysed with AMOVA (Excoffier *et al.* 1992) and Arlequin (Schneider *et al.* 1997). Randomised permutations we used to compare Φ_{ST} estimates for the two subspecies. Assignment of each population to one or other subspecies was randomised, keeping the number of populations constant and Φ_{ST} calculated for each taxon. Although RAPD data are dominant we used programs designed for haplotype frequencies because *P. major* is highly inbreeding; thus we assumed that the low level of hidden heterozygosity would have a negligible effect on the calculations. Stewart & Excoffier (1996) showed that Φ_{ST} estimates were little altered when 'allele' frequencies were estimated assuming a high selfing rate (0.9) and incorporated into AMOVA calculations.

Results

Morphological and genetic data were scored for each plant without knowledge of the morphological or ecological characteristics of the populations from which the seeds originated. During data analysis plants were assigned to one of two taxa using both genetic and morphological characters.

Morphology

Principal component analysis was used without prior classification of the plants, and using all 11 morphological

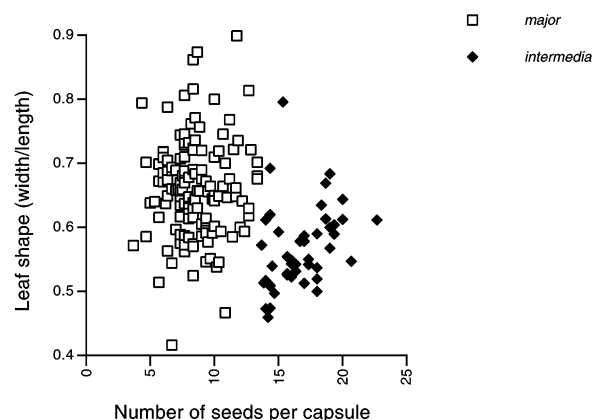


Fig. 1 The two taxa *Plantago major ssp. major* and *Plantago major ssp. intermedia* can be distinguished based on the relationship

characters this method did not produce clear and distinct clusters of plants. However, when the plants were coded into two groups based on the genetic results (see neighbour-joining tree below), then simple scatter plots of some morphological characters produced distributions that were almost nonoverlapping (Fig. 1). Discriminative analysis using the morphological characters assigned the plants to the two genetic groups with 100% accuracy. The mean number of seeds per capsule is the character that most successfully separates the plants into two groups, and used in combination with spike length or leaf width it can usually distinguish the two subspecies in our study.

The morphological and genetic data suggested that from the majority of locations a single subspecies had been collected. However, from the morphological analysis it appeared that two of the 112 seeds collected from Britain were within the *P.m. ssp. intermedia* group: one plant from Harwell and one from Aston Hill; the other plants from these populations are of the subspecies *major*. Two plants from Sibton with fewer than 12 seeds per capsule as expected for *P.m. ssp. major* had uncharacteristically narrow leaves and a small scape:spike ratio.

RAPD and inter-SSR DNA fragments

Between one and seven DNA fragments were scored as present or absent for each random primer. Only polymorphic markers were recorded; these are 22 anchored inter-SSR markers and 51 RAPD markers (data available from authors upon request). Twenty-five of these 73 markers (34.2%) were present in less than 10% or in more than 90% of the samples. Thirteen (17.8%) of the markers were specific (or nearly so) to one of the two subspecies (Table 2). The majority of these diagnostic fragments were amplified only in *P.m. ssp. major*.

Pairwise Euclidean distances were calculated from the 73 polymorphic markers and used to cluster plants that were genetically most similar. Two groups of plants were clearly distinguished by the neighbour-joining analysis of all RAPD and inter-SSR markers (Fig. 2). One group of 43 plants was identified as *P.m. ssp. intermedia*. Within this clade, plants cluster first with plants that were collected from the same location. The exception was one plant from LA airport (USA) which was more similar to plants from Trinidad than to plants from the same collection site. The

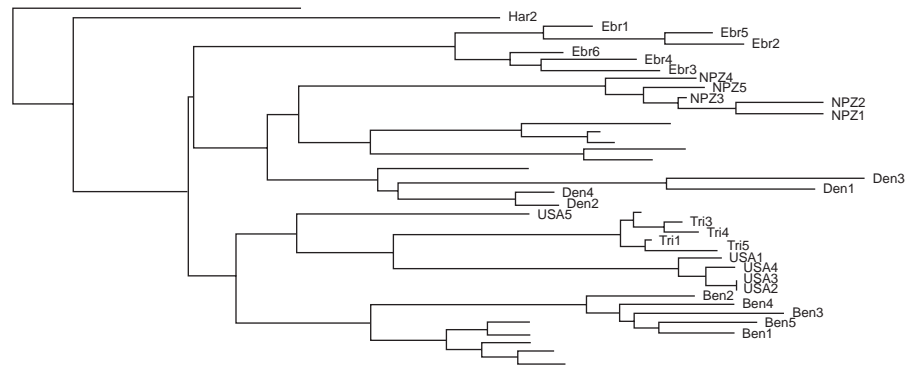


Fig. 2 Neighbour-joining tree (Saitou & Nei 1987) showing the genetic relationship between *Plantago major* populations using pairwise Euclidean distances calculated from 73 RAPD and inter-SSR markers (Schneider *et al.* 1997).

P.m. ssp. intermedia), these frequency differences cannot be viewed as significant.

The allozyme data for the *Plantago* samples were divided into two groups, corresponding to the two subspecies, and by excluding some of the *P.m. ssp. major* populations from Britain these groups were made similar in both their geographical coverage and sample size. The nine populations of *P.m. ssp. intermedia* were from Britain, Denmark, Spain, USA, Trinidad and two sites in Greece and the Netherlands. The nine populations of *P.m. ssp. major* were from Austria, Belgium, Greece, Germany, Switzerland, Spain, and three sites in Britain. Thus *F*-statistics can be directly compared for the similarly geographically widespread groups (Table 4). Significantly more genetic variation was described by among-population differences in *P.m. ssp. intermedia* ($F_{ST} = 0.777$) than by among-population differences in *P.m. ssp. major* ($F_{ST} = 0.232$). In contrast, *P.m. ssp. intermedia* had a lower mean expected heterozygosity (H_S) than *P.m. ssp. major*. The distribution of within-population allozyme variability (H_E) for these two taxa revealed that *P.m. ssp. major* had a greater range of mean expected heterozygosity per population (Fig. 3). Total genetic diversity (H_T) of *P.m. ssp. intermedia* was slightly higher than total genetic diversity in *P.m. ssp. major* (Table 4). The level of inbreeding within each population (F_{IS}) revealed *P.m. ssp. intermedia* to have a significantly greater rate of selfing than *P.m. ssp. major* ($P < 0.10$). The F_{ST} value for *P.m. ssp. intermedia* was skewed down by a single locus (*6Pgd-1*) in a single population (Ebro, Spain); removal of this population reduces

our estimation of outcrossing in *P.m. ssp. intermedia* to zero, significantly lower than the 10.3% rate of outcrossing in *P.m. ssp. major*.

Discussion

In contrast to some earlier genetic studies of *Plantago major* (Van Dijk & Van Delden 1981), this study has resolved enough genetic variation to unequivocally distinguish the two subspecies and to resolve population structure. Our analysis clearly separates the plants into two taxa, showing complete concordance of the morphological and genetic data sets. In addition, within *P.m. ssp. intermedia* there is concordance of genetic similarity and physical proximity. Both the allozyme and DNA data provide estimates of F_{ST} that indicate that these two taxa have quite distinct population structures with much more diversity among populations of *P.m. ssp. intermedia* than seen among populations of *P.m. ssp. major*.

Species or ecotypes?

There is no consistency in the way botanists use subspecies and varieties to classify organisms. Frequently, subspecies are used to define regionally separated races that are morphologically differentiated (McDade 1995) rather than to classify ecotypes which are separated by habitat (Stace 1989; Hamilton & Reichard 1992; Stuessy 1994), as is the case here. We consider that *P. major* and *P. intermedia* should be regarded as separate species in agreement with Gilbert 1809 and shall refer to them thus for the remainder of this study. Our data suggest that the two taxa are genetically differentiated even in sympatry, and have distinct patterns of genetic population structure. There is no absolute measure of genetic difference that determines what are separate species. However, comparison with similar studies and examination of genetic evidence in tandem with other information such as the ecology, morphology and mating system allows for an educated view of the independence of the taxa, their cohesion and their potential for separate evolutionary fates. This study can, for instance, be compared with a study of Howard *et al.* (1997) in which they compared two oak species using RAPDs. The two oaks are considered to be good species that can be discriminated by

700 different primers and only found 14 markers with six primers that were species specific. The two *Plantago* taxa studied here have as many markers that discriminate them in widespread sympatry, in addition to the morphological and ecological characters.

Having said that we consider these two species to be genetically distinct, we recognize that clustering of these plants into two groups could be a genetic artefact rather than evidence of common ancestry. It is possible that the markers that distinguish the two species in this study are all linked to selected traits (such as width of leaves and the number of seeds per capsule). The maintenance of ecotypes via selection against hybrids was suggested by Van Dijk & Van Delden (1981) who used allozymes to study Dutch populations of these species. Selection against ecotype hybrids has been documented in other plant species (e.g. Nagy 1997). With 73 random markers analysed, most of which probably originate in the nuclear DNA (Lorenz *et al.* 1994), it seems unlikely that the structure observed could be dominated by markers linked to traits under strong selection because recombination would break up linkage groups. However, because these species have high selfing rates, it may only require 13 or 14 markers that are loosely linked to alleles under strong selection to produce quite different frequencies of these markers, and create the divergence observed (Charlesworth *et al.* 1997). If 13 markers in our study were linked to the number of seeds per capsule then one might see genetic clustering of plants that concurred with taxonomy. Thus, background selection and selective sweeps could reduce genetic variability through hitch hiking or linkage of markers to alleles under negative or positive selection and thus produce random clustering of populations. However, we found specific markers in each species,

not on a local scale but on a European scale, which could well be specific on a world scale. Linkage of all diagnostic DNA markers would have to be maintained in populations from Greece to Trinidad (over many generations) for the clustering of the DNA phenotypes to concur with taxonomic groups. The selection for ecotypes as an explanation for our two genetic clusters is being tested with crossing experiments to follow the transmission of morphological traits and RAPD and inter-SSR markers in hybrid plants.

Compelling evidence that there are two taxa represented in our study does not only rely on 13 diagnostic markers, but also on the dramatic difference in the genetic structure of the two groups (Fig. 2). The difference in the Φ_{ST} estimates for the two species is greater than expected from a random division of this data into two taxa. We therefore feel that the consistency over a wide geographical range indicates that each taxon is distinct with the genetic cohesion of a full species. Hybrids that are observed in the field and produced in the greenhouse (Mølgaard 1976), and gene flow seen at Sibton, confirm that occasional hybridization does occur. Potentially lower fitness of hybrids might restrict gene flow

Table 5 Estimates of outcrossing rates calculated using the fixation index (t_F) and multilocus estimation from a progeny analysis (t_m) for two Dutch populations of *Plantago intermedia* and *P. major* (1 & 2) (Wolff 1991b) and results from this study (3)

| | F_{IS} | t_F | t_m |
|--------------------------|----------|-------|-------|
| <i>P. intermedia</i> (1) | 0.889 | 0.059 | 0.013 |
| <i>P. intermedia</i> (2) | 0.948 | 0.027 | 0 |
| <i>P. intermedia</i> (3) | 0.927 | 0.037 | |
| <i>P. major</i> (1) | 0.747 | 0.145 | 0.076 |
| <i>P. major</i> (2) | 0.750 | 0.143 | 0.016 |
| <i>P. major</i> (3) | 0.818 | 0.103 | |

difference in degree of variation and the different structure of the variation may well require more than one causal factor.

1. *P. major* may have been derived from *P. intermedia* and thus represent a subset of the genetic variation within *P. intermedia*.

2. *P. major* may have lost variation due to a more recent or more severe bottleneck and/or due to background selection and selective sweeps.

3. *P. major* may have a greater rate of outcrossing than *P. intermedia*.

4. The rate of molecular evolution in *P. major* might be slower than within *P. intermedia*.

The distinct population genetic structure seen here could be explained by just a small difference in rate of outcrossing because the frequency of outcrossing is the most important determinant of population genetic structure, affecting both genetic diversity within populations and genetic diversity among them (Barrett & Harder 1996). Previous studies of both natural populations and offspring experiments of *P. major* and *P. pleiosperma* (= *P. intermedia*) have found significant variation in their levels of selfing (Table 5), in agreement with this study. As seen in other inbred plant species (Wolff & Schaal 1992; Charlesworth *et al.* 1993; Hamrick & Godt 1996; Liu *et al.* 1998), a higher rate of outcrossing in *P. major* would result in homogenizing of the genetic variation compared to *P. intermedia* where a higher rate of inbreeding would lead to more marked differentiation among populations. Local selection would tend to increase diversity between populations more in the inbreeding taxon than the outbreeder (Charlesworth *et al.* 1997).

The change in level of outcrossing in *Plantago*, in tandem with slight ecological shifts suggests that these changes, and perhaps others, as yet unrecorded, have changed the selection pressure in favour of greater outcrossing in *P. major* (or alternatively in favour of greater inbreeding in *P. intermedia*). Such changes provide the opportunity to identify factors that promote and maintain sex (Hurst & Peak 1996). In this example lifespan may be

a key component in determining the rate of outcrossing. *P. intermedia* is able to complete its life cycle in one year (summer) and is less hardy in the winter than *P. major* which often occurs as a perennial (Mølgaard 1976; Kuiper & Bos 1992). We believe that viewing *P. major* and *P. intermedia* as independent taxa is essential for the development of ideas towards understanding what we have observed.

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