

## Introgression between *Plantago major* L. subspecies *major* and subspecies *intermedia* (Gilib.) Lange. in a British population

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### ABSTRACT

We measured morphological characters of *Plantago major* from a British population in which subsp. *major* and subsp. *intermedia* both occur, and from 'pure' populations from different parts of Europe as standards, grown in uniform conditions. In the British population, most individuals fall within the variation of subsp. *major*, but individuals resembling subsp. *intermedia* occur as a minority. We attempted to quantify interspecific gene flow between *major* and *intermedia*, and tested the hypotheses that the amount of variation can be explained by minority effects, as functions of the relative frequency or density of the two taxa. The first hypothesis is upheld, but evidence for the effect of density is equivocal. A higher rate of gene flow occurs between these two taxa than expected if they are mostly self-fertilised. We suggest that the taxa do not merit specific rank as no breeding barrier seems to separate the species when they become sympatric.

**KEYWORDS:** genetic variation, hybridization, introgression, *Plantago*, Greater Plantain, plant speciation, population structure.

### INTRODUCTION

Introgressive hybridization has been defined as the repeated backcrossing of a natural hybrid to one or both parental populations and usually occurs in the direction of one parent only (Anderson 1949). It results in the transfer of genes from one parent to another across a partial breeding barrier (Anderson & Hubricht 1938; Anderson 1949, 1953; Harrison 1993). This can result in one of the parents becoming more variable, having incorporated some genes from the other parent, which may allow the introgressed parent to colonise a new environment.

There are some good examples which clearly describe introgression in the literature, where molecular techniques were used to confirm

previously doubtful examples. Abbott *et al.* (1992) used isozymes to confirm that ray-florets in groundsel *Senecio vulgaris* had arisen as a result of introgression from the ray-floreted *Senecio squalidus*. Introgression essentially occurs at the chromosome level. Eshed *et al.* (1992) revealed that when chromosomal segments were recombined into the genome of *Lycopersicon esculentum* (tomato) after hybridization with *L. pennellii*, the new chromosomal inserts survived through repeated recombination and backcrossing although the size of inserts reduced between the first and the sixth backcross generation.

In *Plantago*, maternally inherited chloroplast DNA (cpDNA) was analyzed by comparing DNA fragment patterns produced by seven restriction endonucleases in four species of *Plantago* (Hooglander *et al.* 1993). In this study it was shown that the larger 70bp DNA fragment present in Netherlands *Plantago major* L. subsp. *major* (hereafter referred to as '*major*') but absent in subsp. *intermedia* (Gilib.) Lange (hereafter referred to as '*intermedia*'), was not observed in a few individuals identified morphologically as *major* from sites where two taxa grow together. Outside the UK, *intermedia* and *major* tend to be separated ecologically, the former taxon being more typical of open saline sites, and has been said to differ biologically, being more often annual or short-lived and self-fertilising than *major* (van Dijk 1989; van Dijk *et al.* 1988). Thus, it is likely that these taxa are usually isolated spatially in countries such as Denmark and the Netherlands, and more often maintain their integrity there.

Nevertheless, Hooglander *et al.* (1993) suggested that natural successive hybridization and backcrossing onto *intermedia* mothers had occurred, with the more outcrossing *major* acting as pollen donors, resulting in introgression from *intermedia* to *major*.

In a population from north-east England (Metrocentre, Gateshead), morphological clustering in the two taxa *major* and *intermedia* is also not clear cut. The aim of the experiments reported in this paper was to investigate the possible occurrence of hybridization and introgression in these mixed populations. We reasoned that the offspring of mothers, some of the seeds of which had been fertilized by pollen from the other taxon or hybrids, would produce offspring that were more variable than those mothers which had not hybridized. By comparing the variability of offspring grown in a standard environment between mothers from different taxa, from areas with differing frequencies of the taxa, and from areas with different densities of adult plants, we were able to investigate parameters which affected rates of hybridisation and introgression.

#### MATERIALS AND METHODS

##### MORPHOLOGY

Four separate field populations (45 plants) were collected near the Metrocentre, Gateshead, UK (NZ220630) and taken into cultivation. The four populations were separated by short distances. Population (A) was collected near the River Tyne which is tidal here. The distance between population (A) and population (B) is 35 m; there is 15 m between population (B) and (C), whilst population (C) and (D) are nearly 40 m apart. Habitats in this area are highly ruderal and are dominated by human activities. Each of the four populations appeared to contain some individuals showing characteristics of both *major* and *intermedia*. Although these taxa are reported to differ in up to 12 characters (Hegi 1914, Van Dijk 1984, 1989), average seed number per capsule is the most reliable discriminant, being non-overlapping between the taxa and easily quantifiable (Van Dijk 1984, 1989, Morgan-Richards & Wolff 1999). Individuals were classified primarily on the basis of average seed number per capsule, using a discontinuity in the data distribution as the discriminant (El-Bakatoushi 2004) (*major*  $\leq 10.4$ , *intermedia*  $\geq 10.5$ ). This character, which has also been considered an important discriminant in all earlier studies of these taxa, is strongly correlated with both major principal components of the variation, and with 7 of the 12 other characters employed (Table 2). Pure

populations used for comparison were collected as seed by K. Wolff from districts where only one taxon occurs. These comprised two *intermedia* populations from Denmark (NYA) and the Netherlands (NPZH) and two *major* populations from Italy (Salt.2) and the Netherlands (NPZ) (details in El-Bakatoushi, 2004).

Using the same cultivated material, El-Bakatoushi (2004) compared characters known to vary between the taxa before and after cultivation, and also examined cross-correlation between these characters. As a result of this study, 13 morphological characters were selected which did not change markedly after cultivation and all of which showed some significant character state correlation between individuals (Table 2). These characters were scored after two years growth in standard conditions in pots under glass at Moorbank Gardens (University of Newcastle) (Table 1).

Principal Component Analysis (PCA) was used for the multivariate analysis of the morphological characters, using Minitab V.12.1 (1998). PCA is the most mathematically natural ordination technique (Gauch 1982; Digby & Kempton 1987). The use of PCA enabled us to examine multivariate taxonomic relationships in a manner unbiased by choice of character. It was also possible to combine both continuous and multistate characters in the analysis. By means of cross-correlation between character states and principal components, we were able to examine the relative contribution of each of the 13 characters to these components (Table 2).

##### EXPERIMENTAL HYBRIDIZATION AND HYBRID FERTILITY

*Plantago major* is protogynous, and flowering acropetal so that stigmas are exerted from flowers 1–3 days before pollen is shed (El-Bakatoushi 2004). Experimental cross-pollination was achieved by applying dehiscing anthers to stigmas exerted from the lowest flowers on a solitary spike, and then excising the remaining flowers and enclosing the cross-pollinated flowers in a polythene bag. Reciprocal crosses were made between the two taxa using both individuals from the Metrocentre, and the 'pure' control populations. Pollen stainability of samples of 300 grains was examined at  $\times 400$  magnification using 45% acetocarmine in experimental hybrids and in mother-daughter lines.

TABLE 1. MORPHOLOGICAL CHARACTERS AND CHARACTER STATES RECORDED FOR *PLANTAGO MAJOR* AND SUBSP. *INTERMEDIA* AFTER TWO YEARS CULTIVATION

1. Leaf blade length / length of leaf blade to base from point of maximum width)
2. Degree of angle at blade / petiole interface (0–180°)
3. Number of main veins in longest leaf (lower surface of leaf)
4. Leaf margin
  0. Teeth well marked (4+)
  1. Teeth slightly apparent (1–4)
  2. Undulate toothed
  3. Entire
5. Leaf indumentum (upper surface, between middle and next vein)
  0. Densely covered with hairs
  1. Somewhat covered with hairs
  2. Slightly covered with hairs
  3. Glabrous
6. Leaf indumentum (Lower surface, between middle and next vein)
  0. Densely covered with hairs
  1. Somewhat covered with hairs
  2. Slightly covered with hairs
  3. Glabrous
7. Width/length of longest leaf.
8. Ratio of flowering spike diameter, 3 mm from bottom of spike/ 3 mm from top.
9. Ratio calyx length/length fruiting pedicle
10. Capsule apex
  0. Obtuse
  1. Intermediate
  2. Acute
11. Capsule length (mm) (lowest flower)
12. Seed number (average from five capsules)
13. Average length of five seeds (mm)

TABLE 2. REGRESSION  $R^2$  BETWEEN FREQUENCY OF *INTERMEDIA*, DENSITY, INDEX, COEFFICIENT VARIATION OF *MAJOR* AND COEFFICIENT VARIATIONS (CV)

	Frequency <i>intermedia</i>	Density both spp.	cv <i>major</i>	cv <i>intermedia</i>
Frequency <i>intermedia</i>	1			
Density both spp.	ns	1		
cv <i>major</i>	0.701***	ns	1	
cv <i>intermedia</i>	0.346*	0.318*	0.341	1

Unstained grains were considered unviable. The proportion of ovules setting apparently viable seed was examined by dissecting the contents of three capsules in a drop of water on a slide under a dissecting microscope. Infertile seeds were dark in colour, shrunken, and lacked mucilage.

#### VARIATION IN SEED NUMBER AS A MEANS OF QUANTIFYING INTROGRESSION

Thirteen 5 × 5 m areas were selected by eye from near the Metrocentre, and a single fruiting spike from every fruiting plant within each area

was collected. The number of fruiting spikes per 25 m<sup>2</sup> patch were counted, and the average seed number per capsule calculated for five capsules of each spike. Spikes were described as *intermedia* or *major* on the basis of average seed number per capsule. The discriminants used (*major* ≤10.4, *intermedia* ≥10.5) were based on previous findings in the literature, and a slight discontinuity observed in the distribution of the Metrocentre population data for this attribute. On the basis of this, the frequency of each taxon within the area was calculated.

For each area, two mothers were selected, one *major* and one *intermedia* (but in areas containing only *major*, two *major* mothers were selected) and 20 seedlings of each were grown to maturity (see seed germination) so that the coefficient of variation (cv) of seedling seed number could be calculated for each. This involved the raising of  $2 \times 13 \times 20 = 520$  seedlings.

#### RESULTS

##### MORPHOLOGY

The first two components of the PCA represented 97.8 % and 1.9% of the total variation respectively. Of the 13 characters used (Table 1), six showed a significant correlation with both major principal components, and 10 showed a significant correlation with at least one. Three characters, all relating to fruits, did not significantly influence principal components, namely character 9 (relative length of fruiting pedicel), 10 (capsule apex shape) and 11 (capsule length), although each of these showed some internal coherence with other characters used. The characters which mostly strongly influenced the two most important components were characters 2 (angle of blade to petiole) and 12 (seed number per capsule), two characters which traditionally have been important in the separation of the two taxa.

Figure 1 represents the first two axes of PCA. The limits of variation in the standard populations are shown. The Metrocentre population encompassed the variation of the four 'pure' populations used as standards, and no less than 40% of individuals (18/45) fell outside the morphological limits of these standards, and can be considered to be morphologically intermediate between the taxa. Only three Metrocentre individuals corresponded clearly to *intermedia*, so that 24 individuals (53%) corresponded to *major*. No clear or distinct morphological clusters could be detected amongst or between Metrocentre populations, although the pure populations show distinct clustering. Most individuals of the intermediate phenotype falling outside the limits of the standard populations tended to resemble *major* more than *intermedia*, perhaps suggesting that most backcrossing occurs to *major*.

##### HYBRID FERTILITY

No significant difference was found in either seed set or pollen fertility between either parent and the offspring of crosses, or between the offspring of crosses and of selfs. Both the percentage of stainable pollen, and the proportion of ovules set as seed were invariably in excess of 80%. However, there was some evidence for significant matrilineal heritability in pollen stainability for selfed offspring from the Metrocentre population ( $r^2 = 11.6^{***}$ ), and for seed set after crosses in both Metrocentre and 'pure' populations ( $r^2 = 15.7^*$  and  $r^2 = 46.5^{***}$  respectively).

##### THE COEFFICIENT OF VARIATION OF MAJOR AND INTERMEDIA SEED NUMBER IN QUADRATS

The regression between coefficient of variation (cv) of *major* seed number and frequency of *intermedia* per 25 m<sup>2</sup> quadrat showed that no less than 70.1 % of the variability in seed number in *major* offspring could be attributed to the frequency of *intermedia* in that quadrat (Fig. 2 & Table 2). This suggested that considerable ongoing hybridisation occurred between *major* and *intermedia*. There was no significant effect between the density of fruiting plants per quadrat and offspring seed number cv (Table 2). However, the correlation between cv of *intermedia* offspring seed number and density of plants/quadrat was just significant (Table 2). There was also a positive relationship between the frequency of *intermedia* and cv of seed number in *intermedia* offspring ( $r^2 = 0.346^*$ ) (Table 2). The correlation between coefficient of variation of seed number of *intermedia* and cv of seed number of *major* was also slightly significant ( $r^2 = 0.341^*$ ) (Table 2).

#### DISCUSSION

Using evidence from a variety of molecular markers, Morgan-Richards & Wolff (1999) suggested that *major* and *intermedia* are sufficiently distinct to warrant specific rank, despite evidence of widespread hybridisation outside the British Isles (Hooglander *et al.* 1993, Wolff & Morgan-Richards 1999). However, the taxa are not so distinct in the British Isles (Kay 2002) and we doubt if morphologically 'pure' populations of *intermedia* often occur here. The scattered and largely ruderal distribution of *intermedia* in the

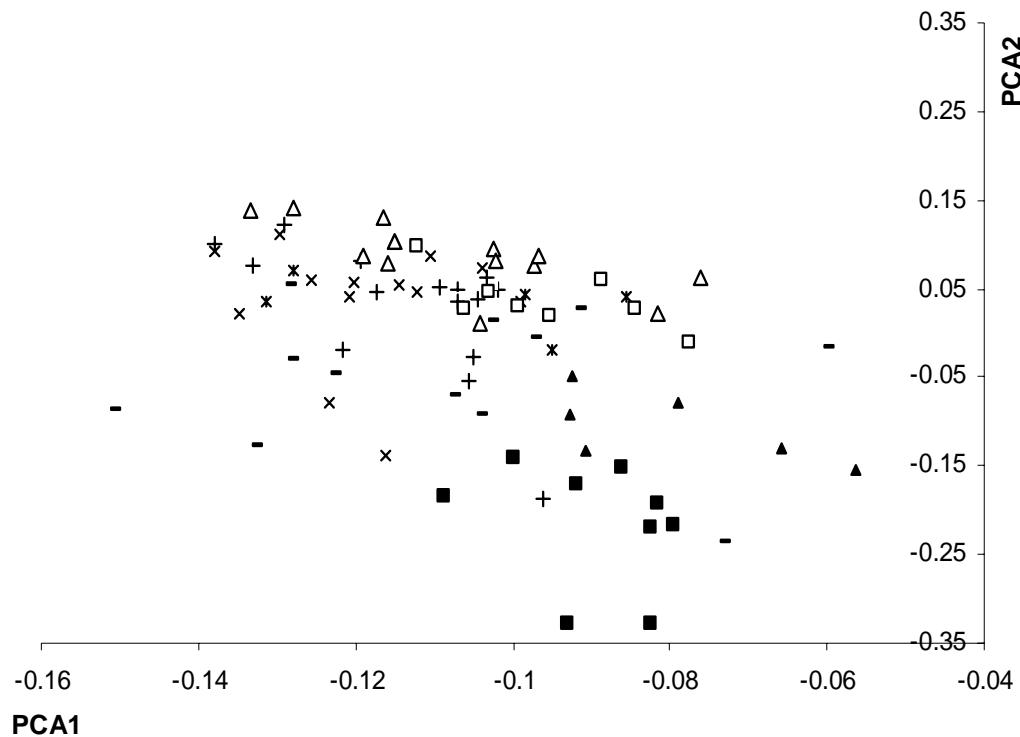


FIGURE 1 The first two axis of PCA for natural populations with four pure populations.  
 + population A, \* population B, ■ population C, x population D  
 ■ standard *intermedia* (NYA), ▲ standard *intermedia* (NPZH)  
 △ standard *major* (Salt2), □ standard *major* (NPZ)

British Isles clusters around centres of population such as London, Bristol, Belfast, the west Midlands and the East Midland cities (Kay 2002). We suspect that *intermedia* may be largely of recent and adventive origin in these islands.

There is no evidence of a genetic barrier between the taxa. Both taxa are diploid with  $2n = 12$  (Clapham *et al.* 1987) and this number was also obtained from many individuals of both taxa in the Metrocentre population (El-Bakatoushi 2004). All the individuals from the Metrocentre population we investigated had a high level of pollen stainability (>80%), seed set and fertility was high amongst manually made crosses, and the fertility of offspring of these crosses did not vary from that of their parents. Nevertheless, morphological differences between the taxa in British populations persisted after cultivation, suggesting that many characteristics which separate the two

taxa *major* and *intermedia* are genetically controlled (El-Bakatoushi 2004).

In the Metrocentre population, intermediate, putatively hybrid individuals tended to resemble *major* from standard populations more than *intermedia* and this may be an indication that the hybrids have backcrossed to *major* individuals, which dominate in the community. (Another less likely hypothesis, i.e. that *major* characters are overwhelmingly dominant to *intermedia* characteristics was disproved after crosses between the taxa were reported by El-Bakatoushi (2004)).

The highly significant relationship between variability in seed number in *major* offspring and frequency of *intermedia* in the same quadrat suggested that this introgression is ongoing, reflecting a high rate of gene flow between two taxa and helped to confirm that some *major* individuals were introgressed towards *intermedia*. However, we also found a

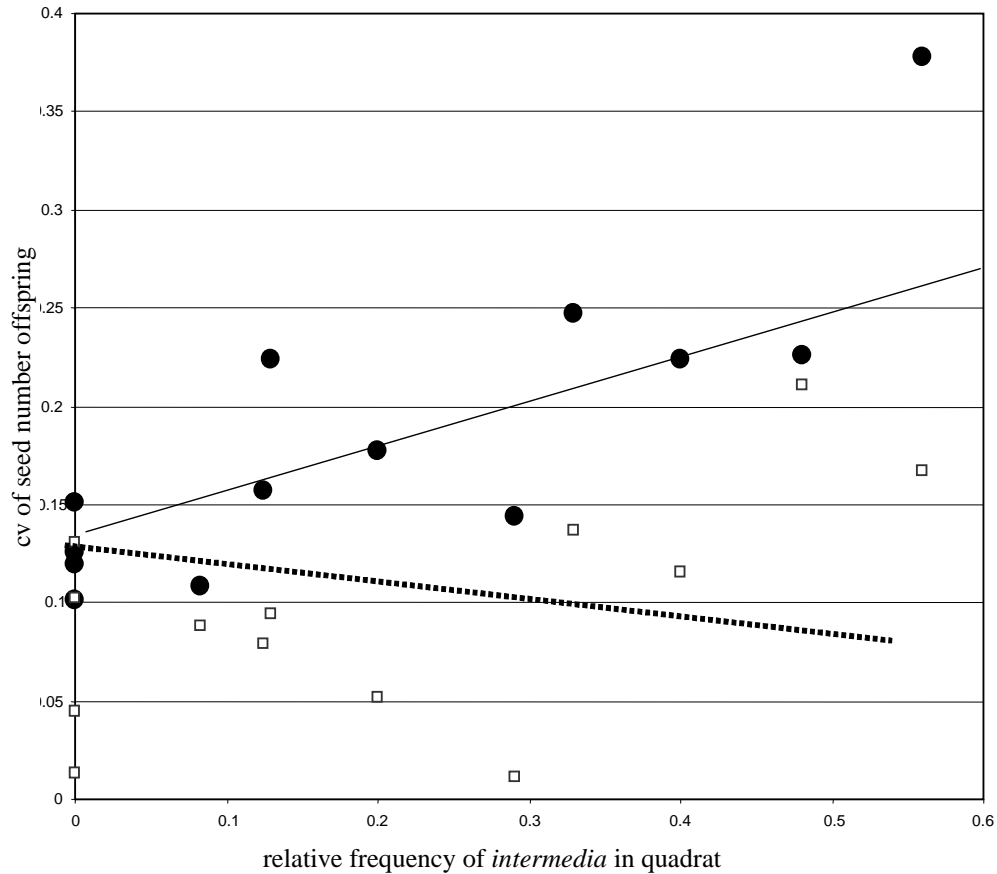


FIGURE 2 Scattergram between relative frequency of *intermedia* in quadrat and coefficient of variation of seed number of *major* ( $r^2 = 0.701$ ), and *intermedia* offspring, showing lines of best fit. ● cv of seed number in *major* offspring, □ cv of seed number in *intermedia* offspring

positive relationship between coefficient of variation of seed number in *intermedia* and frequency of *intermedia* per quadrat. This could have been an artefact caused by a greater variability in seed number in *intermedia* than in *major*.

The variability in *intermedia* seed number in general may have resulted from a greater tendency for *intermedia* individuals to receive outcrossed pollen from the numerically dominant plants of *major*. This suggestion was supported by the slightly positive relationship which occurred between density of plants per quadrat and the coefficient of variation for *intermedia* seed number. Minority effects occur when two potentially interfertile taxa or demes co-occur at very different frequencies, so that pollen arriving on stigmas of the minority taxon will originate overwhelmingly from the dominant taxon (Fowler & Levin 1984).

Individuals of *intermedia* apparently suffered from minority effects at the Metrocentre which were maximised at high density, causing any outcrossed pollen to tend to originate from *major*. Consequently, one would expect immigrant *intermedia* to lose their identity over successive generations, when it occurs, as here, in a minority

*Major* and *intermedia* cannot be seen as separate species in the localities from which the mixed UK populations were collected, as it seems that genes of the infrequent and possibly adventive *intermedia* are readily diluted by the native taxon *major*. A high rate of gene flow occurred between these taxa, and both taxa could readily occupy the same open habitat. On this evidence the rank of subspecies is ideal for both taxa, even if the taxa sometimes behave more as one would expect of species in some non-British parts of their range.

## ACKNOWLEDGMENTS

We gratefully thank the University of Newcastle Fund for support to Ranya El-Bakatoushi.

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(Accepted December 2005)

