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PCR markers distinguish *Plantago major* subspecies

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Abstract *Plantago major* plants from several Scottish and Dutch locations were surveyed for their genetic variation using PCR markers, namely RAPD analysis, anchored inter-SSR PCR, and chloroplast PCR followed by RFLP analysis. The RAPD and inter-SSR markers showed a differentiation between the two subspecies of *P. major*. These results are discussed in relation to earlier results using allozyme electrophoresis, DNA fingerprinting, and chloroplast RFLP analysis.

Key words RAPD analysis · *Plantago major* · Subspecies · Inter-SSR PCR

Introduction

Populations of a species that are reproductively isolated, for instance by distance, may diverge from each other through drift and/or differential selection. If they have diverged sufficiently they may be called two ecotypes, forms or even subspecies. It is generally accepted that morphological characters and ecological niche are a good guideline to distinguish two forms or subspecies within a species (see for instance Mølgaard 1976). Recently molecular techniques have been used to study the extent of differentiation among populations, ecotypes, forms and subspecies. Different molecular markers show different levels of genetic divergence, depending amongst other things on the rate of evolution of the specific markers. The study of differentiation among closely related taxonomical units for a range of characters from morphological characters, allozymes to repetitive DNA not only sheds light on the classification of the taxonomical units under study but also teaches us about the evolution of the characters and molecules.

Plantago major is an almost cosmopolitan species. As the species is highly inbreeding with outcrossing rates between 0 and 0.08 (Wolff 1991 b), it means that every population can be regarded as an inbred line highly adapted to its specific habitat. This strong ecotypic adaptation of resource allocation and growth has been described by Van Dijk (1989). Within the species two subspecies are recognised, ssp. *major* and ssp. *pleiosperma*, and these two subspecies can easily be intercrossed (Van Dijk 1984). Although the general appearance is similar, several morphological characters discriminate the two subspecies, such as the number of seeds per capsule, the number of veins in the leaf and the shape of the scapes, while other characters that vary, such as number of inflorescences and leaf length, are less clearly related to one of the two subspecies (Mølgaard 1976; Van Dijk 1984; Wolff 1991 a).

Van Dijk and Van Delden (1981) performed allozyme studies on *P. major* collected from nine locations in the Netherlands. Both subspecies, recognised by the number of seeds per capsule, occurred at six of these locations. The two subspecies shared 27 invariable allozyme loci, and allele frequencies of the nine polymorphic loci where similar, except for three loci, *Pgm*-1, *Got*-1 and *Me*-1. This result was confirmed in a later study by Wolff (1991 b). Results from Van Dijk (1989) and Wolff (1991 a) suggest that the morphological differences between the two subspecies is maintained by selection since they occupy different ecological niches. Allozyme loci that differ between two

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subspecies may be linked to genes controlling these two ecotype-morphologies.

The high similarity for allozyme loci is in contrast with the results from a DNA fingerprinting study on plants from different locations, using the M13 DNA repeat as a probe (Wolff et al. 1994). The minisatellite variability uncovered with this technique showed low levels of polymorphism within each subspecies and an extreme difference between the two subspecies. Furthermore, chloroplast RFLP analysis revealed surprisingly high levels of variation between the two subspecies (Wolff and Schaal 1992). After studying 86% of the chloroplast genome with nine restriction enzymes, three length polymorphisms were detected between subspecies *pleiosperma* and subspecies *major*, originating from the Netherlands.

The absence of a breeding barrier, low allozyme differentiation and a resemblance in morphology (except for a few characters) is in contrast with the strong differentiation for minisatellite and cpDNA. Additional PCR markers, obtained with RAPD analysis and inter-SSR PCR, may shed some light on how far the two subspecies have diverged for other types of markers than those already studied.

Materials and methods

Adult plants from various locations in the Netherlands and Scotland were collected and grown in the greenhouse (see Table 1). At most locations the plants were clearly either subspecies *pleiosperma* or ssp. *major*; some locations, however, had both subspecies, using their habitat and growth form as an indication (Mølgaard 1976; Van Dijk 1989). Plants growing close to the river, in agricultural fields, or on a harbour landing had the characteristic growth habit of ssp. *pleiosperma*, namely double-bend inflorescences and ovate leaves, and more than 15 seeds per capsule. Plants growing in trodden areas and on road margins had the characteristic growth habit of ssp. *major*, namely straight inflorescences and round leaves, and less than 15 seeds per capsule. The subspecies designation of the plants (see Table 1) is based on field observations where the situation is either a clear ssp. *major* or a clear ssp. *pleiosperma* situation (number of seeds per capsule, growth habit and habitat).

DNA was isolated using a miniprep CTAB method according to Wolff (1996). RAPD analysis was performed on an MJ Research PTC-100 thermocycler, according to Wolff et al. (1995). Half a unit of Dynazyme (Flowgen) was used in each 25 µl reaction. The primers employed were OPA1 (CAG GCC CTT C), OPA2 (TGC CGA GCT G) and OPA10 (GTG ATC GCA G) and these were obtained through Operon (VH Bio). Anchored inter-SSR PCR was performed according to Zietkiewicz et al. (1994) with the modification of running the amplified DNA on a 1.4% agarose gel and visualisation of the DNA by ethidium bromide. The primers UBC887 (DVD TCT CTC TCT CTC TC), UBC889 (DBD ACA CAC ACA CAC AC) and UBC891 (HVH TGT GTG TGT GTG TG) were obtained through the University of British Columbia (UBC).

In an initial survey for variation of the chloroplast genome, chloroplast fragments of seven *P. major* samples (consisting of Dutch as well as Scottish representatives of both subspecies) were amplified with the nine primer sets as described by Demesure et al. (1995). The electrophoresis was done on 1.4% agarose gels. All primer sets resulted in a good amplification of a PCR fragment. These resulting fragments were digested with six restriction enzymes, namely *Alu*I, *Hae*III, *Hin*fI, *Hpa*I, *Rsa*I and *Taq*I. The total set of samples were only amplified with primer sets *trn*S and *trn*fM and digested with *Taq*I.

The presence and absence of polymorphic RAPD and inter-SSR PCR fragments were scored for all samples (see Table 1). For each primer the polymorphic fragments were numbered from the longer fragment downward.

Results

RAPD analysis and inter-SSR PCR generated reliable and reproducible polymorphic patterns. A total of 34 different bands were scored, of which ten were monomorphic and were therefore shared by the two subspecies. The occurrence of the polymorphic bands is shown in Table 1. Fragments mainly occurring in subspecies *major* are indicated with an m if present in an individual; fragments that are mainly found in subspecies *pleiosperma* are indicated with a p, whereas the presence of polymorphic fragments that occur in both subspecies is indicated with a $+$. Bands only found in Scottish *pleiosperma* populations are indicated with an s. The absence of a band is always indicated with a $-$.

Many of the polymorphic PCR fragments, 18 out of 24, are found predominantly and in high frequency in only one of the two subspecies (Table 1). Of the six remaining fragments that are shared between the subspecies, one (OPA1.3) is found in low frequencies in ssp. *major*, three (889.3 and 889.4, 891.3) are found in both subspecies and two (OPA10 fragments 3 and 5) are only present in all Scottish *pleiosperma* plants.

The NJ tree was made with the polymorphic RAPD and inter-SSR PCR fragments using the program RAPDistance (Armstrong et al. 1994), PHYLIP 3.5c (J. Felsenstein 1986–1995) and Treeview (Page 1996) and it is based on Nei's distances; samples with missing values were left out of this analysis. The resulting tree (Fig. 1) clearly shows that the two subspecies have diverged and that within the subspecies *pleiosperma* the Scottish populations are differentiated from the Dutch ones. Within the subspecies *major* the Scottish populations are indistinguishable from the Dutch populations with the primers employed.

The initial survey of polymorphisms in the cp genome revealed that only the fragment amplified with primers *trn*S and *trn*fM showed a length polymorphism among the seven samples tested, which was easier visualised using *Taq*I as a restriction enzyme. The analysis of all samples showed that the majority of the ssp. *major* plants contain a longer *Taq*I fragment, except for EP6, whereas the majority of the ssp. *pleiosperma* plants have a smaller fragment, except for the Dutch PBA3 and the Scottish LW3. The presence of the longer fragment is indicated on the NJ tree with a $*$. One other length polymorphism (this time in the long *Taq*I fragment) was found in one Scottish *pleiosperma* plant, namely EP4.

Table 1 Genotypes of several *P. major* plants with their sampling sites for one cp RFLP, three inter-SSR PCR and three RAPD primers. The presence of bands is indicated with m, p, + or s (see text) and absence with a −. A dot means not determined

Sample	Site	State	ssp	cp	UBC887			UBC889						UBC891					OPA1			OPA2		OPA10				
					1	2	3	1	2	3	4	5	6	1	2	3	4	5	1	2	3	1	2	1	2	3	4	5
NPZ3	Path	NL	m	l	m	−	m	·	·	·	·	·	·	−	m	+	m	m	−	m	−	−	m	−	−	−	m	−
PBA3	Border	NL	Mix	l	−	−	−	p	−	−	−	p	−	p	−	−	−	−	p	−	−	p	−	p	p	−	−	−
PBA5	Field			l	m	−	m	−	m	−	+	−	−	−	m	+	m	m	−	m	−	−	m	−	−	−	m	−
PBA6				l	m	−	m	−	m	−	−	−	m	−	m	+	m	m	−	m	−	−	m	−	−	−	m	−
PBC2	Lawn	NL	m	l	m	−	m	p	−	−	+	p	−	−	m	+	m	m	−	m	−	−	−	−	−	−	m	−
HMB3	Lawn	SC	m	l	m	−	m	−	m	−	+	−	m	−	−	−	m	m	−	m	−	−	−	−	−	−	m	−
EP4	Coastal path	SC	m	l	m	−	m	−	m	+	+	−	m	−	−	−	m	m	−	m	−	−	m	p	−	−	m	−
EP6				s	m	−	m	−	m	+	+	−	m	−	−	−	m	m	−	−	+	−	m	−	−	−	m	−
EV1	Field	SC	m	l	m	−	m	−	m	+	+	−	m	−	−	−	m	m	−	m	−	−	m	−	−	−	m	−
EV2	entrance			l	m	−	m	−	m	+	+	−	m	−	m	−	m	m	−	m	−	−	m	−	−	−	m	−
NPZH3	Harbour landing	NL	p	s	−	p	−	p	−	+	+	p	−	p	−	−	−	−	p	−	−	p	−	p	p	−	−	−
NPZH6				s	−	p	−	p	−	+	+	p	−	·	·	·	·	·	p	−	−	p	m	p	p	−	−	−
GB4	Riverbank	NL	p	s	−	p	−	p	−	−	−	p	−	p	−	−	−	−	p	−	−	p	−	p	p	−	−	−
GB5				s	−	−	−	p	−	+	+	p	−	p	−	−	−	m	p	−	−	p	m	p	p	−	−	−
T5	Riverbank	NL	p	s	−	p	−	p	−	−	+	p	−	p	−	−	−	−	p	−	−	p	−	−	p	−	−	−
LW1	Agricultural	SC	p	l	−	p	−	·	·	·	·	p	−	−	−	+	−	−	p	−	−	p	−	p	p	s	−	s
LW2	field			l	−	−	−	−	−	−	+	−	−	−	−	+	−	−	p	−	−	p	−	p	p	s	−	s
LW3				l	−	p	−	p	−	+	+	p	−	p	−	+	−	−	p	−	−	p	−	p	p	s	−	s
LW4				s	−	p	−	p	−	·	+	−	−	p	−	+	−	−	p	−	−	p	−	p	p	s	−	s
Bord	Field	SC	p	l	−	−	−	p	−	−	+	−	−	p	−	+	−	−	p	−	−	p	−	−	p	s	−	s
BHV1	Path	NL	Mix	1	m	−	m	−	m	+	+	−	m	−	m	+	m	m	·	·	·	−	m	−	−	−	m	−
BHV3	along			s	−	−	−	p	−	+	+	p	−	p	−	−	−	−	p	−	−	p	−	p	p	−	−	−
BHV5	riverbank			s	−	p	−	p	−	−	+	p	−	p	−	−	−	−	p	−	−	p	−	p	p	−	−	−
BHV6				s	−	p	−	p	−	−	−	p	−	p	−	−	−	−	p	−	−	p	−	p	p	−	−	−
BHV7				s	−	−	−	p	−	+	−	p	−	p	−	−	−	m	p	−	−	p	−	p	p	−	−	−
BHV8				l	m	−	m	−	m	−	+	−	m	−	−	−	m	m	−	m	−	−	m	−	−	−	m	−
BHV9				l	m	p	m	−	m	−	+	−	m	−	m	−	m	m	−	−	+	−	m	−	−	−	m	−
BHV11				l	m	−	m	−	m	−	+	−	m	−	−	−	m	m	−	m	−	−	−	−	−	−	m	−
BHV12				l	m	−	m	−	m	−	+	−	m	−	−	−	m	m	−	−	+	−	m	−	−	−	m	−
BHV13				l	·	−	m	−	m	·	·	−	m	−	m	−	m	m	−	−	+	−	m	−	−	−	m	−

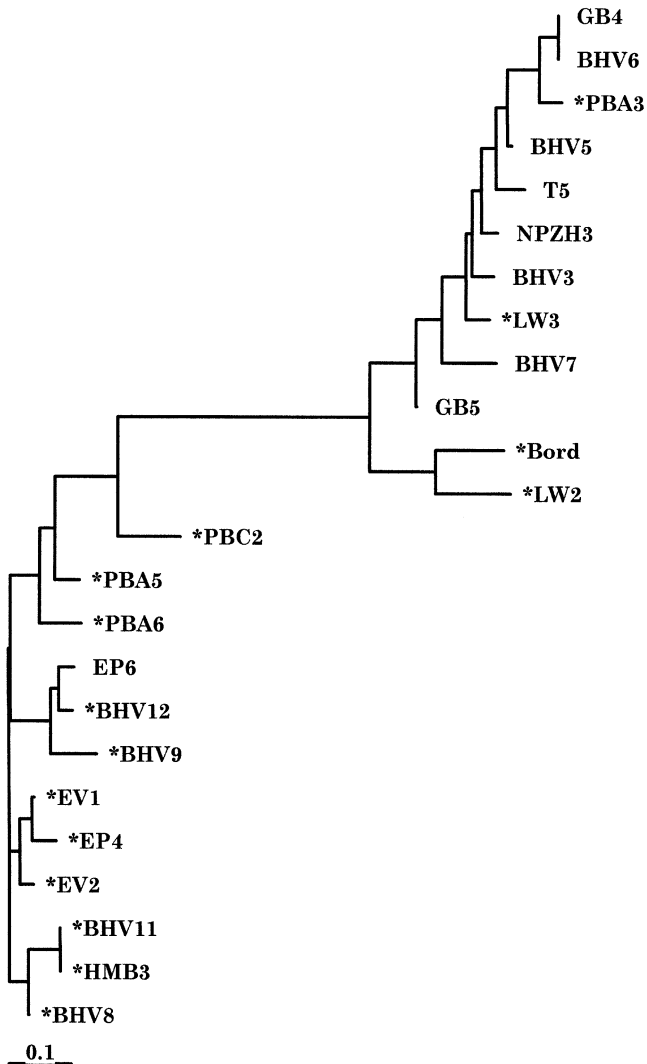

Fig. 1 NJ tree of the Dutch and Scottish *P. major* plants, based on Nei's distance using polymorphic RAPD and inter-SSR PCR fragments. A * means that a longer chloroplast PCR fragment was present

Discussion

The PCR-generated polymorphic markers are useful tools to study populations of the two subspecies of *P. major* and to group the plants into the two subspecies. Within the species *P. major* substantial polymorphism was revealed using three RAPD and three inter-SSR primers, as 24 of the 34 bands were polymorphic. The results of other primers were not included in the study but showed a similar outcome. Most of the variation found, 19 out of 24 polymorphic bands, was variation between subspecies and between countries of origin, as can be expected from a highly inbred plant species (Wolff 1991 b). Of these 19 bands there were seven that were occasionally seen in the "wrong" subspecies and this may indicate gene flow. Some locations have individuals from both subspecies (PBA and BHV). Seed counts of plants from the mixed locations showed they were correctly identified as being one or the other subspecies. As the two groups are clearly clustered in different branches of the NJ tree it appears that the variation within each group is smaller than the variation between the two groups.

The fact that most polymorphic RAPD and inter-SSR PCR fragments are almost subspecies-specific does not coincide with the results found using allozyme electrophoresis. The allozymes showed no subspecies-specific alleles and only two allele-frequency differences between Dutch populations of the two subspecies. The RAPD and inter-SSR results are also not totally in concordance with the DNA fingerprinting results where it was shown that the two subspecies had very dissimilar DNA fingerprint patterns. The RAPD and inter-SSR results take an intermediate position between the allozyme and fingerprint results. It is not known to what extent the RAPD and inter-SSR fragments generated consist of repetitive DNA. If a substantial number of them amplify repetitive DNA this may explain the discordance with the allozymes (encoded by functional non-repetitive DNA) and the DNA fingerprint data, (solely based on tandemly repeated DNA motifs). Williams et al. (1993) suggested that fragments from all copy number classes would be amplified.

The polymorphic cp PCR/RFLP fragment showed a distinction between the Dutch populations of the two subspecies, except for the mixed PBA population, which is at the border of an agricultural field and a roadside. Apparently gene flow has taken place between the ssp. *major* and ssp. *pleiosperma* plants in this population, although the nuclear DNA seems to be totally *pleiosperma* DNA, based on the RAPD and inter-SSR markers. This phenomenon is referred to as chloroplast capture and has been reported in many other plant species (Kron et al. 1993; Rieseberg 1995). However, in the Scottish populations the haplotypes are not at all restricted to one subspecies. One *major* population (EV) has both haplotypes and the *pleiosperma* populations either only have the "Dutch *major*" haplotype (Bord) or both haplotypes (LW).

The differentiation of the two subspecies for many RAPD and inter-SSR bands, but not for others, can be explained in three ways. Firstly, the two subspecies may have diverged relatively recently and differentiation for the different markers has occurred at different rates, leaving some markers that are still shared between the two subspecies. Secondly, functional DNA, like allozyme loci and undoubtedly some RAPD and inter-SSR amplicons, do not allow high mutation rates as there is strong selection against non-synonymous substitutions due to the lowered functionality of the resulting mutant alleles. On the other hand it is known that repetitive DNA can diverge relatively fast between species, subspecies and even populations (King 1993).

Thirdly, in nature some hybrids are formed (Van Dijk and Van Delden 1981) and in these hybrids recombination takes place between some parts of the genomes of the two subspecies. It can be hypothesised that some parts of the genome have linkage blocks containing QTLs for ecotypic differentiation. Selection for these QTLs is strong as populations and subspecies are highly adapted. Markers in these blocks may therefore be different between the two subspecies due to hitchhiking with the selected QTLs (Van Dijk and Van Delden 1981). The latter two explanations are favoured as it has been shown in a study by Wolff and Schaal (1992) that the three cp restriction fragment length polymorphisms between the two subspecies point to a long divergence time.

Information on the repetitiveness of bands discriminating the two subspecies, as well as bands occurring in both subspecies, needs to be gained to confirm or contradict the hypotheses mentioned. Furthermore, the occurrence and fitness of hybrids in the field and in artificial populations needs to be studied to ascertain the strength of natural selection.

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