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Use of 5′-anchored primers for the enhanced recovery of specific microsatellite markers in *Brassica napus* L.

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Abstract Microsatellite markers have assumed great significance in biological research. The isolation and characterisation of microsatellites involves DNA library construction and screening, DNA sequencing, primer design and PCR optimisation. When a microsatellite is situated close to the beginning or end of a cloned fragment, specific primers cannot be designed for one of the flanking sequences, thus hindering the utilisation of such microsatellites as markers. The present approach was to use one 5′-anchored primer complementary to the microsatellite sequence in combination with one specific Cy5-labelled primer with a view to retrieving useful microsatellites, which would otherwise be lost. Six pairs of a 5′ anchored primer and a specific primer were used across a set of 31 *Brassica napus* winter cultivars and one accession each of five additional *Brassica* species. Using laser fluorometry a single labelled product was observed after amplification with each of four primer pairs, and one primer pair gave two labelled products. Three products corresponded in size with the products expected if 5′ anchoring was effective, indicating the amplification of locus-specific full-length products including all of the microsatellite repeats. All six primer pairs showed polymorphisms across the *Brassica* species examined, but only one primer pair showed polymorphisms within *B. napus*, making it useful for genetic analysis in rapeseed cultivars. The other primer pairs could be useful in studying gene introgression into *B. napus* or for investigating interspecific crosses involving different *Brassica* species.

Key words Anchored primers · Microsatellites · Simple sequence repeats · Polymorphism · PCR

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Introduction

In the recent past microsatellites or simple sequence repeats (SSRs) have assumed an increased significance as molecular markers in biological research. They have been shown to be useful for DNA fingerprinting (Charters et al. 1996), analysis of genetic diversity (Powell et al. 1996), genome mapping (Liu et al. 1996), paternity analysis (Amos et al. 1993), population studies (Saghai Maroof et al. 1994) and a variety of other purposes. Microsatellite markers have also been linked to economically important traits and could be of great importance in plant breeding (Koh et al. 1996). These markers are abundant and highly polymorphic in most species. They are usually inherited in a co-dominant manner and have a high information content. Above all, the analysis of SSRs is PCR based, requiring only nanogram quantities of DNA, and is amenable to automation.

Several different approaches have been followed for the utilisation of microsatellites as molecular markers. One of these is based on using single primers complementary to microsatellite sequences with two to four additional, non-repeat bases at the 5′ or 3′ end (Zietkiewicz et al. 1994). This approach results in complex fingerprints of so called inter-SSR markers comprised of a large number of amplification products with each product flanked by two microsatellite loci with opposite orientations. The additional non-repeat bases serve to anchor the annealing of the primer to the 5′ or 3′ end of the microsatellite sequences and to reduce the complexity of the fingerprint, making the results more amenable to analysis. 5′ anchoring should have the additional advantage of retaining any polymorphisms within the microsatellites in the amplification products, but the

observation that 5´-anchored primers produce more complex amplification products than comparable 3´-anchored primers with a high background of minor bands (Zietkiewicz et al. 1994) strongly indicates that 5´ anchoring is not always effective, allowing the primer to slip to the 3´ end of the microsatellite during PCR.

A second, more specific approach, is based on designing primer pairs complementary to unique DNA sequences flanking an individual microsatellite locus, allowing the specific amplification and analysis of this locus (Litt and Luty 1989; Weber and May 1989). The initial development-costs of specific microsatellite markers are high, since this approach necessitates the isolation and characterisation of microsatellite loci. This typically involves DNA library construction and screening with microsatellite-specific probes, isolating and sequencing positive clones, primer design and the selection of polymorphic microsatellites. The recovery rate of useful microsatellites is generally low due to non-polymorphic loci, primer pairs failing to give a good amplification, and clones positive in the screening which nevertheless do not contain microsatellites. Additional sources of loss are clones in which the microsatellite is located near the cloning site. For these microsatellites it is often not possible to design specific primers for both flanking regions.

During the development of specific microsatellite markers for rapeseed (*Brassica napus* L.) a number of clones of this type were found. In order to still utilise the microsatellites in these clones for marker development, combinations of 5´-anchored primers complementary to the microsatellite sequences with Cy5-labelled specific primers were applied for the amplification of these loci. The present study was then conducted to: (1) see if a combination of an anchored primer with a specific primer gives a locus specific amplification product, (2) test if the 5´ anchoring works in normal PCR conditions on genomic DNA, and (3) see if these microsatellite loci show polymorphisms in rapeseed and an extended panel of *Brassica* species.

Materials and Methods

Plant material and DNA isolation

Thirty one winter cultivars of rapeseed (*B. napus* L.), representing the full range of genetic diversity found within the winter cultivars, along with one accession each of *Brassica oleracea*, *Brassica rapa*, *Brassica nigra*, *Brassica juncea* and *Brassica tournefortii* were used in the present study. Total DNA was extracted from 5 to 10 g of young leaves from plants grown in the greenhouse according to the procedure described in Uzunova et al. (1995).

Isolation and sequencing of microsatellites

Two small insert DNA libraries were developed by cloning *Sau*3AI fragments of 400–800 bp, derived from total DNA of the winter rapeseed varieties ‘Mansholt’s Hamburger Raps’ and ‘Samourai’, respectively, into the *Bam*HI site of lambda phage vector ZAP express (Stratagene, La Jolla, Calif., USA). To identify clones containing dinucleotide repeats the library was screened with synthetic GA/CT and CA/GT polynucleotides (Amersham Pharmacia Biotech, Freiburg, Germany). Random-primed labelling of the probes and hybridisation to filters from the plated phage library were performed according to Uzunova et al. (1995). After two screening steps, purified plaques were converted into pBK-CMV plasmids via the in vivo excision system of ZAP express. These clones were screened a third time by colony hybridisation. From positive clones, the DNA was isolated with a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) and sequenced by cycle sequencing using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech). The products obtained after sequencing were separated on an automatic DNA sequencer (ALF Express, Amersham Pharmacia Biotech).

Primer design and PCR analysis

Specific primers were designed for the available flanking regions of the microsatellites with the Oligo Primer analysis software Version 6 using the default parameters of this program: primer length 16–22 nucleotides, 3´ dimer delta G –3.5 kcal/mol, 3-bp maximum dimer, 3´ stability –5.5 to –9.4 kcal/mol, oligo T_m 49.2 to 65.5°C, % GC 30–60. 5´-anchored primers were designed in such a way that in addition to the microsatellite sequence per se 4–6 bp complementary to the sequence immediately 5´ to the microsatellite were included. For each microsatellite locus an anchored primer was used in combination with a Cy5 fluorescent labelled specific primer to facilitate detection of the amplification product by fluorometry in the automated DNA sequencer. The amplification was done in a PTC-100 programmable thermocycler (MJ Research, Mass., USA) using a touch down PCR programme (Kresovich et al. 1995). This programme included a denaturing step of 60 s at 94 °C and an extension step of 45 s at 72°C. The initial annealing was done for 30 s at 65°C and the annealing temperature was subsequently reduced by 1°C every two cycles until a final temperature of 55°C was reached which was maintained for the remaining 20 cycles of amplification. The PCR was performed in a volume of 10 µl containing 25 ng of genomic DNA, 0.5 µM each of the two primers, 0.2 mM of the dNTPs, 1.5 mM $MgCl_2$, 1×PCR buffer, and one unit of *Taq* polymerase (Amersham Pharmacia Biotech). The mixture was covered at the top by one drop of mineral oil. The PCR products were separated on a 6% polyacrylamide gel in the automatic DNA sequencer.

Results

Six 5´-anchored primers along with their corresponding Cy5 fluorescent-labelled specific primers (Table 1) were tested against 31 winter cultivars of rapeseed as well as five additional *Brassica* spp. In the case of four of the six primer pairs, MD38, MD46, MR157 and MR174, only one amplification product per genotype was observed in the rapeseed varieties (Table 2). One primer pair, MR146, produced two products, a major and a minor product, clearly separated by size, and the remaining primer pair, MD40, produced one major product of 114 bp and a continuum of minor products, ranging from 110 bp to 128 bp.

In Table 2 the observed sizes of the amplification products are compared to the sizes expected respectively when 5´ anchoring is successful or not successful. In two cases, primer pairs MD38 and MD46, the single amplification products correspond in size to the full-length products expected when anchoring works. In ad-

Table 1 Sequenced clones, SSR motifs, repeat sequences, primer pairs designed and number of anchor bases

Clone	SSR motif	Repeat	Primer pairs	No. of anchor bases
pMD38	GA/TC	$(AG)_{12}$	AAATGTGAGAGAGAGAGAGAG[a] TAATAGAACACGTTGGAGCTTA[b]	6
pMD40	GA/TC	$(GA)_{19}A(GA)_3$	ATCTGAGAGAGAGAGAGAGAG AGGGCGTAGGTGAACAAT	4
pMD46	GA/TC	$(TC)_{15}$	TTGGATCTCTCTCTCTCTCTC TCTCTCCCTCGCTCTCATAATAA	5
pMR146	GA/TC	$(AG)_2G(AG)_{13}$	ACTCAGAGGAGAGAGAGAGAGA ATGCGATGAACAGAGTTACAG	4
pMR157	GA/TC	$(CT)_{15}TT(CT)_4$	AAAACTCTCTCTCTCTCTCTC ATTACGAAAATGCCCTGACGA	4
pMR174	GA/TC	$(AG)_{13}$	GATCAGAGAGAGAGAGAGAGA TAACGATGGTGCTCTAAGTGT	4

[a] 5′-anchored microsatellite sequence complementary primer
[b] Cy5-labelled specific primer

Table 2 The expected and observed sizes of the amplification products in relation to anchoring of the 5′-anchored primers

Primer pair	Expected PCR product size [bp][a]		Observed PCR product sizes [bp][b]	Polymorphisms in rapeseed
	With anchoring	Without anchoring		
MD38	83	76	83	+
MD40	128	110	**114**[c] 110–128	–
MD46	80	68	80	–
MR146	97	84	**97** 82	+
MR157	141	119	119	–
MR174	188	181	185	–

[a] On the basis of the sequenced clone
[b] In 'Mansholt' or 'Samourai', depending on the origin of the sequenced clone
[c] Bold figures: major amplification product when two are present

dition, the major amplification product of primer pair MR146 is also identical in size to the expected full-length product. On the other hand, the minor product of this primer pair and the single product of primer pair MR157 correspond in size to the products expected when anchoring fails. The product of primer pair MR174 and the major product of primer pair MD40 are intermediate in size and do not correspond to one of the expected products. On the other hand, the minor products of primer pair MD40 cover the full range of products between the sizes of the products expected with and without anchoring.

All six primer pairs tested showed polymorphisms across the *Brassica* species (Fig. 1). Among the rapeseed cultivars significant polymorphism was only observed with primer pair MD38 which showed two alleles producing clearly distinguishable bands at 83 bp and 80 bp (Fig. 2) with allele frequencies of 0.52 and 0.48. In addition, with the major product of primer pair MR146 a null allele was observed in one of the rapeseed varieties, indicating a limited degree of polymorphism at the corresponding locus. The other four primer pairs did not show any polymorphisms among the rapeseed cultivars tested.

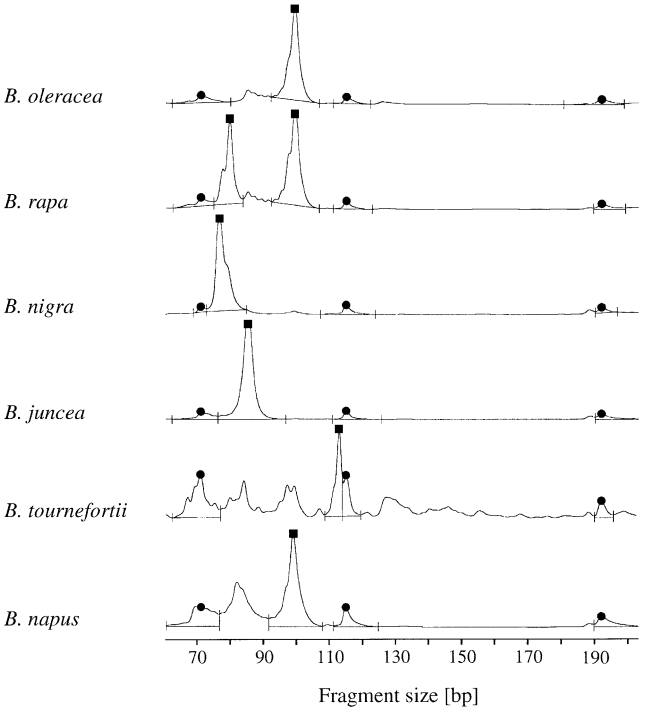

Fig. 1 Fragment analysis of marker MR146 in six *Brassica* species. Peaks labelled by *filled circles* represent size standards of 71, 115 and 192 bp. Peaks labelled by *filled squares* represent amplification products of the microsatellite locus MR146

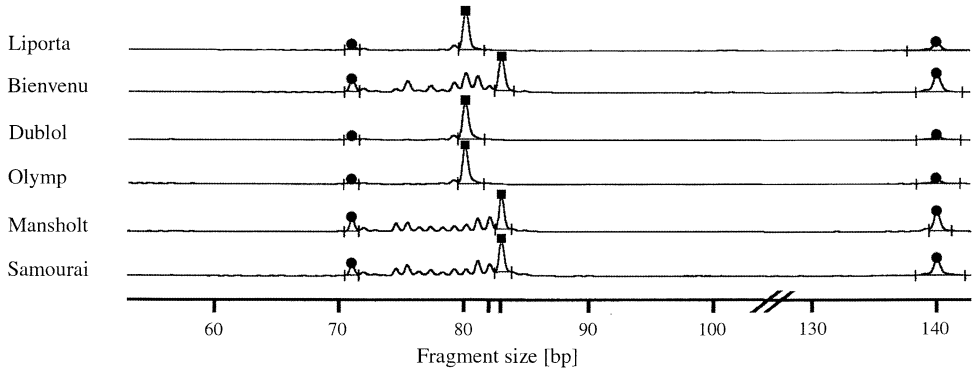

Fig. 2 Allelic diversity of marker MD38 in rapeseed. Peaks labelled by *filled circles* represent size standards of 71 and 140 bp. Peaks labelled by *filled squares* represent amplification products of the microsatellite locus MD38. The six rapeseed varieties showing the two alleles of MD38 at 80 and 83 bp are a subset of the 31 rapeseed varieties used to characterise the microsatellite markers

Discussion

The isolation and characterisation of microsatellites is a time- and resource-consuming process and the recovery rate of useful, locus-specific microsatellite markers is generally low. During the development of microsatellite markers some clones are regularly encountered in which the microsatellite is close to one end of the cloned fragment, leaving only a short stretch of unique sequence between the repeats and the cloning site. Due to constraints on primer design it is often not possible to find useful primers in these short regions. On the other hand, inter-SSR fingerprinting approaches like the one described by Zietkiewicz et al. (1994), using 5′ or 3′-anchored microsatellite complementary primers, have shown that microsatellite sequences like oligo-GA or oligo-GT make good primers. In the present study, 5′-anchored microsatellite complementary primers were therefore used in combination with Cy5-labelled specific primers as an approach to recover useful microsatellite markers from clones where it was not possible to design two specific flanking primers.

In PCRs with combinations of 5′-anchored microsatellite primers and specific primers two types of products can be expected: (1) locus-specific products due to amplification with the specific primer and the 5′-anchored microsatellite primer, and (2) single primer products as a result of amplification with the anchored primer alone. The latter will be similar to the products in inter-SSR fingerprints and are likely to be built at the expense of the former, especially since inter-SSR fingerprints usually comprise a large number of different products. The results presented clearly show that all six primer combinations analysed produced amounts of locus-specific products easily detectable in laser fluorometry. It is quite likely that inter-SSR fingerprinting products from amplification with the anchored primer alone were also built in the PCRs but these products went undetected since only the specific primers were labelled for fluorometry.

Microsatellite complementary primers with additional non-repeat bases at the 5′ end are designed to include the full length of the microsatellite repeats, with all possible polymorphisms in the final amplification products, by anchoring the primers to the 5′ end of the repeat sequences. In practise, Zietkiewicz et al. (1994) observed significantly more complex amplification patterns with a high background of minor products with 5′-anchored primers compared to 3′-anchored primers, indicating that 5′ anchoring may not always work. As a consequence the primer should slip to the 3′ end of the microsatellite repeats during consecutive rounds of PCR.

Of the six primer pairs tested, three, MD38, MD46 and MR146, produced products corresponding in size to the products expected by 5′-anchored primers, indicating the amplification of full-length products including all of the repeat sequences. One of these primer pairs, MR146, also produced an additional product of a size identical to the size expected with the microsatellite primer aligned to the 3′ end of the repeat sequence, indicating that in this case 5′ anchoring was only partially successful. The same may have been the case with primer pair MD40, where a continuum of DNA fragments ranging in size from the full-length product to the product expected without anchoring was produced, and primer pair MR174, where a product of intermediary size was built. In the case of primer pair MR157 anchoring obviously failed completely since only a product corresponding in size to the product expected without anchoring was observed. The success of 5′ anchoring was correlated with the number of anchor bases in the microsatellite complementary primers. While the anchored primers of primer pairs MD38 and MD46, which produced only a single full-length product each, comprised six and five anchor bases, respectively, the anchored primers of the four remaining primer pairs, in which cases anchoring was only partially or not at all successful, comprised only four anchor bases each. This result indicates that there might be a lower limit of five non-repeat bases required for fully successful 5′ anchoring.

Of the three full-length products only one proved to be polymorphic in the 31 rapeseed varieties tested, revealing two distinct length alleles. A single additional polymorphism was observed for another of the three products in the form of a null allele in one of the rapeseed varieties. This limited degree of polymorphism is well within the range observed in rapeseed. In an earlier study (Uzunova and Ecke 1999) using 11 sequence-specific primer pairs only seven gave reproducible amplification products representing eight independent loci. Three of these loci proved to be monomorphic in the

same set of rapeseed varieties as has been used in the present study. The remaining five loci showed between two and four alleles, including null alleles at four loci. While only one primer pair tested in the present study will be useful in the genetic analysis of conventional rapeseed varieties due to the limited degree of polymorphism in *B. napus*, all six primer pairs revealed polymorphisms between the different *Brassica* species. These could be used for analysing interspecific crosses, characterising resynthesised rapeseed genotypes or for introgressions of chromosomal segments from other *Brassica* species into *B. napus*.

The results presented show that combinations of 5´-anchored microsatellite complementary primers with specific Cy5-labelled primers provide an useful approach to utilise microsatellites as markers, which would otherwise be lost by virtue of being near the beginning or end of a cloned fragment. This approach may still be further improved by recognising that there may be a lower limit of five or six non-repeat bases for a successful 5´ anchoring of the microsatellite complementary primer. In the present study this approach has been used to recover useful microsatellite markers in rapeseed and related *Brassica* species but there is no reason why the approach should not work equally well with other plant species or with animals.

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