Theor Appl Genet (1999) 98: 265–273

J. A. Dávila · Y. Loarce · E. Ferrer

Molecular characterization and genetic mapping of random amplified microsatellite polymorphism in barley

Received: 6 May 1998 / Accepted: 20 July 1998

Abstract This study has analyzed the molecular basis and genetic behaviour of the polymorphism generated by the amplification of barley genomic DNA with primers complementary to microsatellites. Primers anchored at the 5′ end, used alone or in combination with arbitrary sequence primers, generated random amplified microsatellite polymorphisms (RAMPs). Unanchored primers were also used as single primers in a microsatellite primed-PCR (MP-PCR). Twenty six randomly selected RAMP DNA fragments which showed polymorphism between the cultivars Steptoe and Morex were cloned and sequenced. All sequences showed the expected repeated motif at the end of the insert, with the number of repeats ranging from five to ten. Genomic sequences containing low numbers of microsatellite motifs were preferentially amplified; therefore, only a fraction of the polymorphism could be attributed to variation in the number of microsatellite motifs at the priming site. Some sequences contained either cryptic simple sequences or members of families of repeated DNA. Polymorphism at the internal cryptic simple sequences was detected by RAMP bands inherited as co-dominant markers. Four MP-PCR bands were cloned and sequenced. A number of repeats identical to the primer itself were found at each end of the insert. Two allelic bands were polymorphic for an internal microsatellite. The potential use of cloned bands as fingerprinting tools was investigated by employing them as hybridization probes in Southern blots containing digested barley DNA from a sample of cultivars. RAMP probes produced complex hybridization band patterns. MP-PCR probes produced either a highly variable single locus or low-copy number loci. Segregations for 31 RAMPs and three MP-PCR bands were studied in a population of 70 doubled-haploids from the Steptoe/Morex cross. One third of all markers were co-dominantly inherited. Markers were positioned on an RFLP map and found to be distributed in all barley chromosomes. The new markers enlarged the overall length of the map to 1408 cM.

Key works Microsatellites · Simple sequence repeats · RAMP · Mapping · Barley

Communicated by J. W. Snape

J. A. Dávila · Y. Loarce · E. Ferrer (✉)
Department of Cell Biology and Genetics, University of Alcalá,
Campus Universitario, E-28871 Alcalá de Henares,
Madrid, Spain
Fax: + 34-1-885 4799
E-mail: bcferrer@gene.alcala.es

Introduction

Microsatellites, also called simple sequence repeats (SSRs), are tandem repeated arrays of short core sequence. They are present in the vast majority of eukaryotic genomes. The total number of different dinucleotide blocks has been estimated for several plant species including some tropical trees (Condit and Hubbell 1991), *Brassica* (Lagencrantz et al., 1993), soybean (Morgante and Olivieri 1993), rice (Wu and Tanskley 1993) wheat (Roder et al. 1995; Ma et al. 1996), *Quercus* (Dow et al. 1995) and spruce (Pfeiffer et al. 1997). The number of sites ranged from 10^3 to 10^5 depending on the species and the repeat motif. Polymorphism produced by a variable number of tandem repeats has been demonstrated in a large number of plant species. This feature has made microsatellites a very attractive molecular marker for species with a narrow genetic base. Thus, in barley, highly polymorphic microsatellites have been developed (Saghai-Maroof et al. 1994; Becker and Heun 1995 a; Liu et al. 1996) and have been shown to discriminate between even closely related cultivars (Russell et al. 1997).

The labour-intensive procedures, which include the cloning and sequencing needed to develop this type of

marker, have promoted the use of alternative ways of detecting polymorphism associated with microsatellites. These methodologies are based on the use of primers complementary to SSRs. Multilocus profiles have been generated using different kinds of oligonucleotide containing simple sequence repeats as single primers (Gupta et al. 1994; Zietkiewicz et al. 1994; Weising et al. 1995 a; Charters et al. 1996; Nagaoka and Ogihara 1997) or in combination with arbitrary sequence oligonucleotides (Wu et al. 1994; Becker and Heun 1995 b; Matioli and Brito 1995; Sánchez de la Hoz et al. 1996; Dávila et al. 1998). These studies have shown the reproducibility of the patterns generated, the Mendelian inheritance of the polymorphic amplified bands and their usefulness in the investigation of the genetic relationships between accessions or cultivars of different plant species. Mapping of this kind of marker in maize (Gupta et al. 1994), *Arabidobsis* (Wu et al. 1995) and barley (Becker and Heun 1995 b) has been previously reported.

Less is known about the molecular nature of the polymorphism associated with these markers. The primer design allowed the evaluation of two alternatives. Firstly, when 5′-anchored oligonucleotides are used as primers, polymorphism will be produced in the variation of the number of repeats of the core sequence at each locus. Secondly, when 3′-anchored oligonucleotides are used, polymorphism was attributable not to variation at the priming site but to the variation of the inter-repeat sequence. Unanchored oligonucleotides will produce one or the other kind of product depending upon where they annealed with the SSR sequence. The first situation has already been tested since single-locus microsatellites have been isolated after cloning PCR products from the amplification of DNA with primers containing 5′-degenerated sequences (Fisher et al., 1996). In addition, all three types of oligonucleotides could detect dominant polymorphism (i.e. presence/absence) due to extensive variation at the priming site, as has been found using unanchored oligonucleotides (Weising et al. 1995 a).

In the present investigation, decamer 5′-anchored oligonucleotides used in combination with arbitrary sequence decamers were employed to produce barley DNA fingerprints. The polymorphism generated, termed random amplified microsatellite polymorphism (RAMP) (following Wu et al. 1994), was able to better reflect genetic relationships among barley cultivars than does random amplified polymorphic DNA (RAPD) (Sánchez de la Hoz et al. 1996; Dávila et al. 1998). The present work was undertaken to investigate the genetic and molecular nature of these polymorphic bands, and also those produced by using unanchored oligonucleotides as single primers [microsatellite primed-PCR (MP-PCR)] (Weissing et al. 1995 a). Mapping the polymorphic bands should indicate if microsatellite sequences are dispersed throughout the barley genome. Cloning and molecular characterization of RAMP and MP-PCR bands will show the sequences responsible for the polymorphism. Moreover, cloned polymorphic products would have the potential of either being converted into single-locus markers or into novel probes which can be used in barley fingerprinting.

Materials and methods

Plant material

Two barley cultivars, Steptoe and Morex, were used for screening polymorphism. Seventy doubled-haploid plants from the cross between these two cultivars were employed for mapping the amplified polymorphic bands. Thirteen barley cultivars (Alexis, Apex, Aramir, Blenheim, Hauters, Igri, Ingrid, Malta, Natasha, Plaisant, Triumph, Vogelsanger Gold, and Volla) were also used to assess the level of polymorphism detected by these markers. DNA was extracted from young leaves according to Sharp et al. (1988).

RAMP and MP-PCR assays

5′-Anchored oligonucleotides containing a dinucleotide repeat, either $GC(CA)_4$ and $GT(CA)_4$ or $CCGG(AC)_8$, were used in combination with an arbitrary sequence oligonucleotide from Operon (kits R, S and T). The PCR reaction mixture consisted of 25 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM of each primer, 100 μM of each dNTP and 0.5 U of *Taq* polymerase, in a 25-μl vol. PCR reactions were performed in a Perkin Elmer Cetus Model 480 DNA Thermal Cycler. The amplification protocol was: 1 min at 94°C, 2 min at 36°C and 2 min at 72°C for 40 cycles, followed by a final extension of 5 min at 72°C, for $GT(CA)_4$ and $GC(CA)_4$; and 1 min at 94°C, 1 min at 60°C and 2 min at 72°C for 30 cycles, followed by a final extension for 5 min at 72°C, for $CCGG(AC)_8$. Amplification products were fractionated by electrophoresis on non-denaturing 7% polyacrylamide gels and silver-stained following Bassam et al. (1991).

Oligonucleotides containing $(GGAT)_4$, $(GATA)_4$ and $(GTG)_5$ were used as single primers in reactions conducted as above, except that the annealing temperatures were 52°C, 42°C and 52°C respectively. Amplification products were fractionated by electrophoresis on 1.8% agarose gels and stained with ethidium bromide.

Cloning and sequencing amplified bands

Specific polymorphic bands were excised directly from either the polyacrylamide or agarose gels. Excised bands from polyacrylamide gels were added to 100 μl of elution buffer (50 mM KCl, 100 mM Tris-HCl, pH 9.0 and 0.1% Triton X-100). After 30 min at 95°C, 1 μl was taken as a template for re-amplification of the band. DNA was eluted from low-melting-point agarose gels using Geneclean II (BIO 101, Inc). RAMP and ISSR bands were ligated into the pCR-Script cloning vector (Stratagene) according to the manufacturer's instructions. DNA sequencing was carried out by an ABI Prism-377 automatic sequencer. Sequences were analyzed using the Dnasis program. The search for homologous sequences at the GenBank and EMBL databases was carried out using the GCG programs (University of Wisconsin).

Southern analysis

Several cloned RAMP and MP-PCR bands were used as hybridization probes. Fifteen micrograms of DNA from each of the barley

cultivars were digested with *Dra*I, *Eco*RI or *Hin*dIII under the conditions recommended by the manufacturer. Fragments were fractionated by electrophoresis on 0.8% agarose gels and transferred to charged nylon membranes (Boehringer Mannheim) following the manufacturer's instructions. Probes were labelled with digoxigenin-11-dUTP (Boehringer Mannheim) using PCR reactions (Hoisington et al. 1994). Pre-hybridization and hybridization was carried out at 65°C in 5 × SSC, 0.02% SDS, 0.01% sodium lauryl sarcosine and 0.3% blocking reagent (Boehringer Mannheim). The hybridization solution also contained 100 ng/ml of PCR-labelled probe. Membranes were washed twice with 0.15 × SSC, 1% SDS at room temperature for 5 min, and three times at 65°C for 15 min. Detection of hybridization was performed by an enzymatic reaction using anti-digoxigenin-AP and CDP-Star (Boehringer Mannheim) following the manufacturer's instructions. For mapping fragments generated by probe 4F4, DNA from the 70 doubled-haploid lines was digested with *Dra*I, blotted and hybridized as above.

Single-locus PCR-amplifications

From the sequence of two polymorphic bands (T14GT0 and 2F4), forward and reverse primers were designed using the PRIMER software. Optimization of each reaction was carried out by trying a range of annealing temperatures and $MgCl_2$ concentrations. PCR amplifications were performed in a total volume of 25 µl containing 25 ng of DNA, 0.2 µM of each primer, 100 µM of each dNTP,10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 to 2.5 mM $MgCl_2$ and 0.5 U of *Taq* polymerase. Samples were processed through 35 cycles consisting of 94°C for 1 min, with an annealing temperature for 45 s, and at 72°C for 45 s. Annealing temperatures were 58°C for T14GT0 and 60°C for 2F4. Amplification products were fractionated on a 3.5% Metaphor agarose gel (FMC) and stained with ethidium bromide. Alternatively, 0.48 µM of fluorescent-dUTP was added to the reaction and the products visualized using GeneScan software and an ABI Prism automatic sequencer.

Mapping analysis

The segregation data for the polymorphic bands detected in this work were combined with RFLP and isozyme mapping data kindly provided by P. Hayes (University of Oregon, USA) using the Mapmaker program (Lander et al. 1987) at LOD scores of 3.

Results

Molecular characterization of cloned RAMP and MP-PCR bands

Thirteen primer combinations consisting of a 5′-anchored oligonucleotide, $GC(CA)_4$ or $GT(CA)_4$, plus an arbitrary sequence oligonucleotide were used to amplify DNA from Steptoe and Morex. Figure 1a shows RAMPs obtained with different primer combinations. $CCGG(AC)_8$ was used as single primer in PCR amplification. Thirty of the fifty one polymorphic bands detected were excised from the polyacrylamide gels and cloned into the pCR-Script vector. Similarly, oligonucleotides $(GGAT)_4$, $(GATA)_4$ and $(GTG)_5$ were used as single primers to amplify DNA from both cultivars. The four products from the amplification with primer $(GGAT)_4$ – three polymorphic, (1F4, 2F4 and 3F4) and one monomorphic (4F4), (Fig. 1 b) – were excised from the agarose gels and cloned. All the cloned inserts were identical in size to the corresponding PCR products (data not shown).

DNA sequences from all cloned PCR products were determined. A total number of 26 RAMP sequences and four MP-PCR sequences were obtained. All the 26 cloned sequences from bands amplified with primers $GT(CA)_4$ or $GC(CA)_4$ contained a $(CA)_n$ microsatellite at one end of the insert and the nucleotide sequence of the arbitrary primer at the other. The length of the microsatellites ranged from five to eight repeats for 13 sequences. The remaining sequences contained a microsatellite with a number of repeats equal to that of the primer. The four sequences analyzed from the amplification with primer $CCGG(AC)_8$ contained an $(AC)_n$ microsatellite at one end of the insert and a $(TG)_n$ at the other. The number of repeats ranged from eight to ten. None of the four cloned bands amplified with

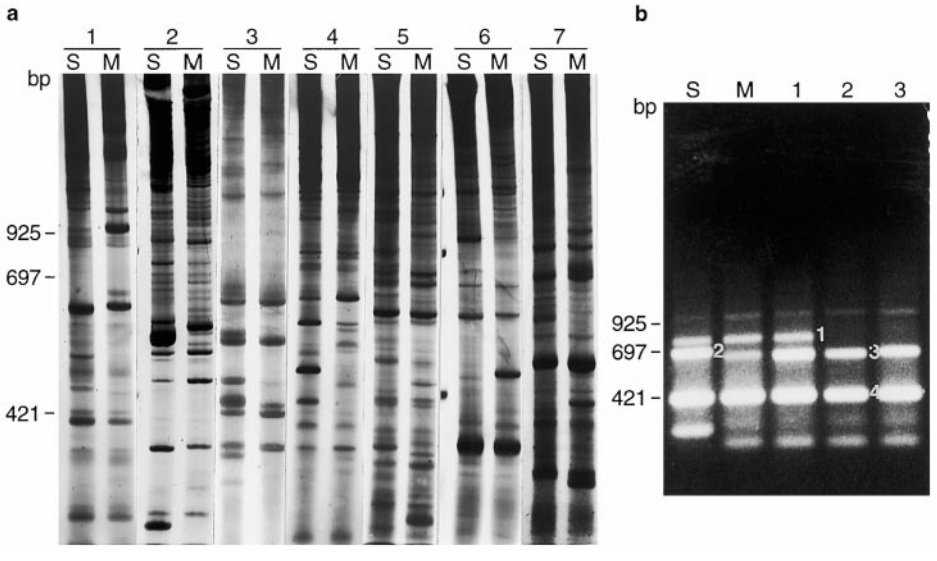

Fig. 1a, b Amplification patterns using different primers. **a** RAMP patterns of Steptoe (*S*) and Morex (*M*) with different primer combinations. *1* $GT(CA)_4$ and Operon S1; *2* $GC(CA)_4$ and Operon R10; *3* $GT(CA)_4$ and Operon S9; *4* $GT(CA)_4$ and Operon T6; *5* $GT(CA)_4$ and Operon T14; *6* $GT(CA)_4$ and Operon S20; *7* $GT(CA)_4$ and Operon T1. **b** ISSR patterns using primer $(GGAT)_4$. *Numbers* indicate the four bands, named F4, which were subsequently cloned. *S*: Steptoe; *M*: Morex. *Lane 1* Alexis; *2* Apex; *3* Aramir

primer $(GGAT)_4$ contained more than four repeats at each end, indicating that these bands were inter-repeat sequences.

Sequences were examined for additional microsatellites in the internal sequence. A perfect $(TC)_n$ microsatellite was found in sequences 2F4 and 3F4. These sequences were otherwise identical except for the number of repeats of these microsatellites, $(TC)_{13}$ and $(TC)_7$ for 2F4 and 3F4 respectively. Sequences considered microsatellite-like with short tracks of imperfect or irregular repeats were also found. For instance, A-rich regions were present in S9GT2A [$AGC(A)_7T(A)_5$]; and R4GT2 [$(A)_4C(A)_6T(A)_4$] whilst T14GT0 contained the sequence $(CA)_6TA(CA)_4$. Long repeat units were found in two cloned sequences. R4GT3 showed four tandem repeats of a unit of 27 nucleotides, and clone T6GT1 presented two tandem repeats of a unit of 113 nucleotides.

Sequences contained within the cloned RAMP were analyzed for similarity to previously reported sequences by a database search. For most of the clones no significant homology to any known sequence was detected. Clone S10GT4 with a size of 227 bp showed 69% homology in 160 bp with a *Secale cereale* R173 retrotransposon-like sequence (Rogowsky et al. 1992), S15GT3 A with a size of 292 bp showed 85% homology in 190 bp to the *Hordeum vulgare* BARE-1 copia-like retrotransposon (Manninen and Schulman 1993), and S20GT with a size of 459 bp showed 70% homology in 118 bp to the barley gene for thiol protease aleurin (Whitier et al. 1987).

Southern hybridization of cloned RAMP and MP-PCR bands

Five cloned RAMP and four cloned MP-PCR bands were used in Southern-blot experiments to screen for restriction fragment length polymorphism (RFLP) among barley cultivars. Clones R10GC1, S9GT2 and S9GT5 gave typical patterns of dispersed repeat sequences with a variation not easily scorable among the cultivars (Fig. 2 a). Clones T6GT1 and R4GT3 generated complex patterns of highly variable bands (Fig. 2 b). The four cloned MP-PCR bands used as probes resulted from the amplification of DNA with $(GGAT)_4$. 1F4 gave a single-locus pattern of hybridization and only two alleles were scored among the cultivars. Steptoe and Morex presented the same allele (data not shown). 2F4 and 3F4 detected the same hybridization patterns. Each cultivar was represented by a single band though differences in their lengths were difficult to score (data not shown). Polymorphism was detected for Steptoe and Morex. 4F4 identified a pair of bands for most of the cultivars. Both were polymorphic between Steptoe and Morex, and the study of their segregation in the doubled-haploid population indicated that each band corresponded to one locus. For one RFLP, 4F4-A, six different alleles were determined in a sample of 13 barley cultivars. The second locus, 4F4-B, was more difficult to score but at least five alleles were detected in the cultivar sample (Fig. 3).

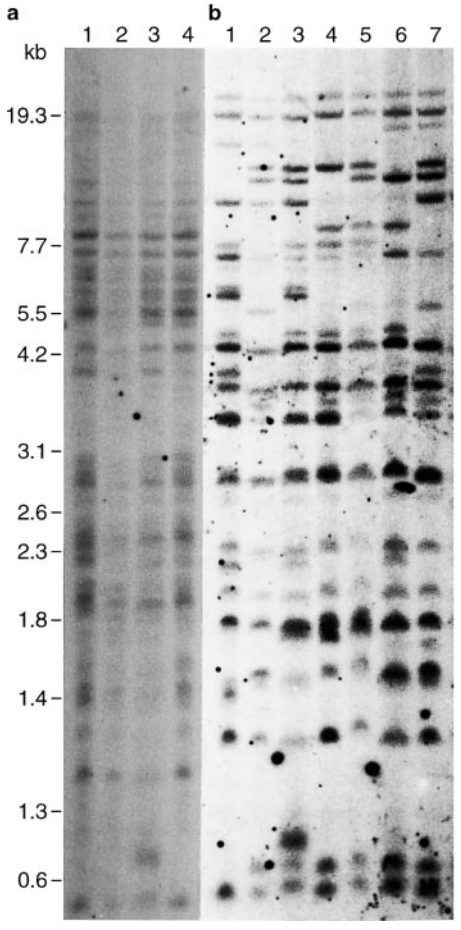

Fig. 2 Hybridization patterns detected by **a** probe S9GT2 and **b** Probe T6GT1 in a *Dra*I digest of genomic DNA from barley cultivars. *Lane 1* Blenheim; *2* Volla; *3* Natasha; *4* Hauters; *5* Plaisant; *6* Igri and *7* Apex

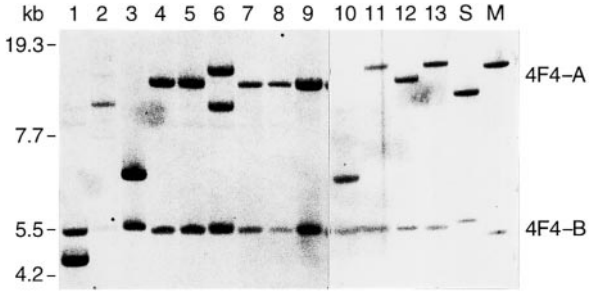

Fig. 3 Hybridization pattern detected by probe 4F4 in a *Dra*I digest of genomic DNA from 13 barley cultivars plus Steptoe (*S*) and Morex (*M*). *4F4-A* and *4F4-B* indicate the two loci analyzed in the cross Steptoe and Morex. *Lane 1* Aramir; *2* Apex; *3* Blenheim; *4* Alexis; *5* Natasha; *6* Vogelsanger Gold; *7* Ingrid; *8* Triumph; *9* Volla; *10* Malta; *11* Plaisant; *12* Hauters; *13* Igri

Table 1 Microsatellite primer sequences, repeat motif, number of alleles per locus, product sizes and diversity index in a sample of 15 barley cultivars

Locus	Forward primer	Backward primer	Repeated motif	Number of alleles	Range of PCR product sizes	Diversity index
2F4	TAGAAGGAGGAGGAGCACAC	TACTCACACACTCACCACCG	$(TC)_n$	5	149–165	0.73
T14GTO	ATGGTGTAGTGGTGAGATGC	TAGATCCATGAGGTTGTSCC	$(CA)_n(A)_n$	5	298–302	0.67

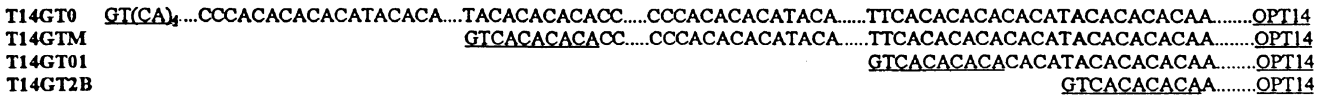

Fig. 4 Sequences of co-segregating bands produced by amplification with primers $GT(CA)_4$ and Operon T14. The internal annealing sites in the largest sequence, T14GT0, are *underlined*

PCR amplification of specific RAMP and MP-PCR bands

For two of the sequenced clones, T14GT0 and 2F4, pairs of oligonucleotides (20-mers) were synthesized and used as PCR primers (Table 1). Clone 2F4 contained a $(TC)_{13}$ microsatellite and clone T14GT0 contained an imperfect microsatellite-like sequence (Fig. 4). Both pairs of primers produced a single band in each cultivar. Polymorphism among the 13 barley cultivars, plus Steptoe and Morex, was scored by fluorescent-labelling of the amplified fragments which were detected with an automated sequencer. High levels of variability were observed for both microsatellites whose expected gene diversities (Nei, 1973) were 0.73 and 0.67 for 2F4 and T14GT0 respectively. Steptoe and Morex were characterized by different alleles for the 2F4 microsatellite.

Segregation and mapping

The 13 primer combinations used to amplify DNA from Steptoe and Morex produced 247 bands. The frequency of polymorphic bands was 31.1%. Segregations for 51 polymorphic bands were analyzed in 70 doubled-haploids; 96.8% of the bands segregated in the expected 1:1 ratio. Within an amplification profile, co-segregation of bands was observed on several occasions. Sequencing of these co-segregating bands showed that an amplified fragment of DNA could produce shorter fragments if internal sites for any of the primers were present. Figure 4 shows an example of these sequences. However, for other co-segregating bands, sequence homology was not found. Only one of the segregations was further analysed. A total number of 35 RAMPs were mapped onto an RFLP and isozyme barley map (Kleinhofs et al. 1993) (see Fig. 5.). Ten out of thirty five markers were scored as co-dominant. The sequences of four allele pairs of these co-dominant markers were obtained. The alleles showed variations in the number of repeats at the CA di-nucleotide motif (S9GT3), in the number of repeats at internal imperfect repeats (S9GT2, S15GT3), or in the addition/deletion of three nucleotides (R10GC2). Moreover, segregations were analyzed for the microsatellite 2F4 and the RFLPs 4F4-A and 4F4-B. These loci also showed Mendelian inheritance. However, 4F4-A showed no linkage to any other marker so that only 4F4-B could be mapped.

RAMP markers were scattered on the seven barley chromosomes. Only five RAMPs were mapped at the same site as other previously mapped RFLPs markers. Therefore, 30 RAMP loci have been added to the RFLP map. Chromosomes 3, 5 and 6 presented the largest number of RAMPs (seven), whereas chromosome 4 was characterized by only two markers. RAMP markers were distributed over the chromosome maps with no obvious clustering. Several markers mapped to chromosome areas not covered by RFLPs. For example, T1GT on chromosome 1, R10GC2 on chromosome 3, R8GC3 on chromosome 4 and R10GC3 on chromosome 6. One marker, S18GT2, mapped at the tip of chromosome 1. The two 4F4 mapped loci were closely linked on the short arm of chromosome 2.

Discussion

It has been pointed out (Wu et al. 1994; Zietkiewicz et al. 1994; Becker and Heun 1995b; Weising et al. 1995a; Sánchez de la Hoz et al. 1996) that the use of primers containing microsatellite motifs would be an efficient tool to simultaneously show inter-individual variation at different microsatellite loci. Moreover, to better reveal the variation in the number of repeats at each microsatellite locus, anchored primers with SSR motifs at the 3′ end and non-repetitive bases at the 5′ end should be preferentially employed. In the present study, this kind of primer was used singly or in combination with arbitrary sequence oligonucleotides to

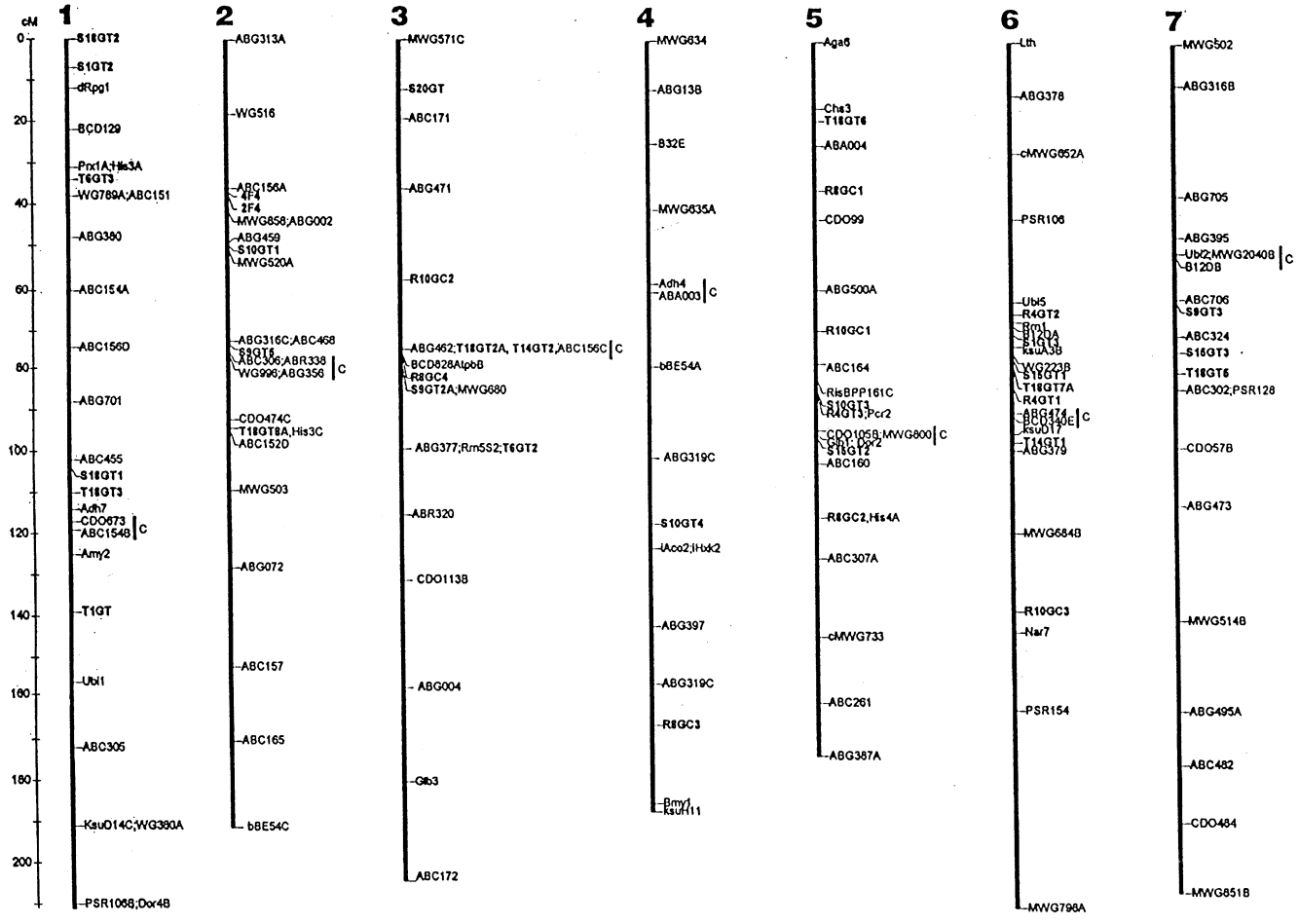

Fig. 5 Linkage map of barley based on the linkage data from the Steptoe/Morex cross (Kleinhofs et al. 1993). Only a reduced number of RFLP loci have been included. The RAMP and MP-PCR loci mapped in this study are in *bold*. The scale to the left of the chromosomes shows map distances in centiMorgans (cM). The approximate centromere positions are shown by *a vertical bar with a C*

amplify DNA from two barley cultivars, Steptoe and Morex. The cloning and sequencing of polymorphic fragments revealed several features with respect to the molecular nature of these fragments and the efficiency of this approach.

For about half of the fragments sequenced from DNA amplifications using 5′-anchored primers, the number of di-nucleotide motifs was the same as for the primer itself. This could be attributed to inefficient anchorage of the primers which, favored by the low-stringency conditions, slipped toward the 3′ end of the microsatellite during PCR (Fisher et al. 1996). Although such an effect might be operating, the following suggest that there are alternative explanations. The template of the largest fragment, T14GT0, contained six $(GT)_n$ sites [DNA sequences of four fragments amplified with the same pair of primers were examined (Fig. 4)]. Four of them annealed with the primer $GT(CA)_4$ and produced four fragments which were sequenced. From the study of these sequences it was clear that A/G and AT/GT mismatches were allowed, but not GG/GT. Moreover, fragment T14GT01, which has a $(GT)_6$ site at one end, was amplified by the anchorage of the $GT(CA)_4$ primer at the 5′ end of the first di-nucleotide. These results indicate that although a certain primer-template mismatching at the 5′ end is tolerated, the anchor sequence seems to be able to site the primer at the 5′ end of the di-nucleotide tract. Other observations corroborated this conclusion. For example, bands on the polyacrylamide gels were discrete (Fig. 1) with no associated bands to indicate an annealing of the primer to different internal di-nucleotides of the microsatellite. Further, primers $GC(CA)_4$ and $GT(CA)_4$ in combination with the same Operon primer produced different profiles with few fragments of the same length (data not shown but see Sánchez de la Hoz et al. 1996). Also, the sequenced fragments from high-stringency amplifications with primer $CCGG(AC)_8$ presented the same features regarding the number of di-nucleotides at the microsatellite end as that amplified with the short primers $GC(CA)_4$ or $GT(CA)_4$. Moreover, none of the products sequenced from amplifications with the unanchored primer $(GGTA)_4$ produced an increment in the number of

repeated units over that contained by the primer, as has also been reported in different species using this type of primer (Weising et al. 1995 a). Overall, these observations suggest that genome sequences with a low number of di-nucleotide motifs were the preferential annealing sites for the 5′-anchored primers employed in these experiments. The greater abundance of shorter microsatellites over the larger could be the best explanation for these results. Fisher et al. (1996), used 5′-anchored primers with seven non-repeated nucleotides at the 5′ end to amplify DNA from *Pinus radiata*. They produced a library of amplified fragments which was screened with a labelled oligonucleotide containing the repeated di-nucleotide motif to select clones with large microsatellites. They found 1.5% positive clones. The present results are similar since from 30 sequenced fragments only one with a larger microsatellite (containing eight repeat units) was found.

All the RAMP fragments sequenced were the result of two priming events. This can be explained taking into account that, during PCR, single-stranded DNA products, with complementary sequences at each end, could establish a secondary structure that would complicate their amplification, in comparison with the amplification of products with a different sequence at each end.

Several of the RAMP sequences examined revealed the presence of irregular direct repeats containing combinations of different repeat types. Most of them could not be classified as compound microsatellites due to the short number of repeat units for each motif. These sequences have been called "cryptically simple repeats" (Tautz et al. 1986). The association of $(CA)_n$ repeats with other SSRs, with repeat units of between two and seven nucleotides or $(A)_n$ mononucleotide repeats, has also been reported for the porcine genome (Wilke et al. 1994). Similarly, very long regions that appear to be composed of mixed simple-sequence structures have frequently been found in eukaryotic genomes (Zischler et al. 1992). Cryptically simple sequences could be exploited for DNA fingerprinting, as pointed out by Tautz (1993). In *Drosophila* populations it has been shown that they can be polymorphic and are probably produced by DNA slippage during replication and recombination (Tautz 1989). In the present study one-third were found to be co-dominant RAMP markers. Sequence differences between four Steptoe and Morex pairs of alleles were based on either different numbers of perfect repeats at one end of the fragment or the addition/deletion of bases which took part in the cryptically simple sequence. The irregular direct repeats of one of these sequences (T14GT0) (Fig. 4) was specifically amplified in a sample of barley (Table 1) to confirm the utility of cryptically simple repeats for disclosing polymorphism in closely related cultivars. Association between different SSRs was also detected for MP-PCR bands amplified with primer $(GGAC)_4$. Two allelic sequences, 4F2 and 4F3, presented an internal perfect microsatellite $(TC)_n$, highly variable among the barley cultivars analyzed (Table 1). In contrast, most of the sequences obtained from dominant RAMP markers, apart from the short $(CA)_n$ stretch at one end of the fragment, showed no association with irregular direct repeats. These RAMP polymorphisms could have been caused by sequence divergence at the priming sites, rearrangements that either altered the orientation of the primer, or because they were present between the two priming sites, as suggested for the origin of RAPD polymorphism (Paran and Michelmore 1993). A similar origin could be attributed to the polymorphisms scored as dominant markers amplified by unanchored primers in MP-PCR.

Seven RAMP bands examined were associated with repeats other than SSRs in the barley genome. This observation was not unexpected given the high proportion of repeated DNA in eukaryotic genomes, including barley. Similar associations have been reported for fragments amplified using minisatellite core sequences in wheat (Bebeli et al. 1997) and rice (Zhou et al. 1997), and for inter-*Alu* sequences in the human genome (Kass and Batzer 1995). By analyzing these RAMP sequences it was possible to distinguish between dispersed and tandem repeats. Dispersed repeats homologous to the known *copia*-like retrotransposon BARE and to R173 were present in the RAMP fragments S15GT3A and S10GT4 respectively. However, for most of the cloned RAMP bands, no homology with previously reported sequences was found. The nature of these sequences was determined by using the cloned bands as hybridization probes in Southern blots containing digested DNA from barley cultivars. Together with the typical patterns of conserved dispersed families (Fig. 2 a), two bands containing tandem repeats (S9GT2 and T6GT1) showed highly polymorphic profiles for the barley cultivars (Fig. 2 b). Variation was attributed to the different number of tandem repeats (manuscript in preparation). The possibility of converting these multilocus profiles into single-locus markers, either by designing specific primers or by increasing the hybridization stringency, is currently being explored. The above results show that cloned RAMP bands could be an additional source of markers useful in DNA fingerprinting for barley germplasm identification.

More simple polymorphic patterns were observed using MP-PCR bands as probes (Fig. 3). Only one or two loci per probe were detected. Apart from the microsatellite sequence presented in bands 4F2 and 4F3, no other repeated motif was enclosed in the MP-PCR bands sequenced. It is unlikely that length polymorphism detected in Southern blots with probes 4F2 and 4F3 (in the range of 5.5 kb) is due to variation in the number of repeats of the short microsatellite. This polymorphism, and also that detected by 4F4, is better attributed to variation in the number of GGCA repeats flanking genomic sequences homologous to the probe.

When used as probes, synthetic oligonucleotides containing a di-, tri- or tetra-nucleotide motif have revealed highly polymorphic DNA-fingerprints in a large number of plant genomes (for a review see Weising et al. 1995 b). Cloning of fragments hybridizing with $(GATA)_4$ in tomato has shown the existence of long stretches of DNA with the GATA motif in tandem (Vosman and Arens 1997). The genomic sequences targeted by the F4 probes could, therefore, correspond to a single-locus or to low-copy number loci flanked by the GGCA motif.

An average of 11 bands per reaction were scored in amplifications from Steptoe and Morex DNA when using a 5′-anchored primer in combination with a RAPD primer. Of these, an average of 5.86 were polymorphic between the two cultivars and an average of two markers per reaction were scored as co-dominant. These figures are slightly higher than those obtained using similar kinds of primers with the cultivars Proctor and Nudinka (Becker and Heun 1995 b). The level of polymorphism revealed by the 13 primer combinations employed in the present work is similar to that detected by RFLP markers using 85 random genomic probes, and half of the value obtained with 45 single-locus SSRs in the same barley cross (Liu et al., 1996). Taking into account that RAMP assays did not require initial investment in terms of probe or sequence information, this approach could be an alternative to the generation of large numbers of polymorphic markers in a short time. Moreover, the fact that a subset of RAMP markers segregated co-dominantly is an advantage in the construction of linkage maps using F_2 populations over other multilocus methods such as RAPD or AFLP.

Co-segregation was found for some RAMPs within the same gel. Sequence information of the co-segregating bands has revealed the existence of specific DNA fragments with more than two annealing sites for primers (Fig. 4). Alternatively, co-segregating fragments lacking sequence homology would be located tightly linked in their genomic locations with a reduced possibility of recombination.

The 35 RAMP loci mapped by linkage analysis were dispersed over all seven barley chromosomes, with a range of 1–7 loci per chromosome (Fig. 5). The two MP-PCR loci were closely linked. Some of the markers located on chromosomes 3, 5 and 6 were observed in small clusters around the centromere. Similar results were observed in an inter-simple-sequence-repeat map of maize (Gupta et al. 1994), in the location of GATA- and GACA- containing loci in tomato (Arens et al. 1995), and in a SSR map of barley (Liu et al. 1996). It is possible that the pericentromeric repeated DNA sequences were rich in different simple sequence repeats. Alternatively, the clustering could be the consequence of reduced recombination in pericentromeric areas of barley chromosomes, increasing the density on the linkage map for markers in these regions. The 32 new loci added to the Steptoe/Morex RFLP map has enlarged the overall length of the map by 164 cM to a total of 1408 cM. Several RAMPs mapped to large gaps in the RFLP linkage map improve the coverage of certain chromosome regions. These effects have been previously observed when different types of molecular markers were combined in barley. For instance, RAPD and RFLP (Giese et al. 1994); RAMP and RFLP (Becker and Heun 1995 a, b); RFLP and SSR (Liu et al. 1996); and AFLP and RFLP (Waugh et al. 1996).

Acknowledgments We thank P. Hayes for supplying the doubled-haploid lines and the RFLP data for the Steptoe/Morex population. This work was supported by a grant from the European Union (BIO2-CT92–0486). J.A.D. was funded by fellowships from the *Ministerio de Educación y Cultura* and *Fundación Caja de Madrid* (Spain).

References

Arens P, Odinot P, van Heusden AW, Lindhout P, Vosman B (1995) GATA- and GACA- repeats are not evenly distributed throughout the tomato genome. Genome 38:84–90

Bassam BJ, Caetano-Anollés G, Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem 196:81–88

Bebeli PJ, Zhou Z, Somers DJ, Gustafson JP (1997) PCR primers with minisatellite core sequences yields DNA fingerprinting probes in wheat. Theor Appl Genet 95:276–283

Becker J, Heun M (1995 a) Barley microsatellites: allele variation and mapping. Plant Mol Biol 27:835–845

Becker J, Heun M (1995 b) Mapping of digested and undigested random amplified microsatellite polymorphism in barley. Genome 38:991–998

Charters YM, Robertson A, Wilkinson MJ, Ramsay G (1996) PCR analysis of oilseed rape cultivars (*Brassica napus* L. ssp. *oleifera*) using 5′-anchored simple sequence repeat (SSR) primers. Theor Appl Genet 92:442–447

Condit R, Hubbell SP (1991) Abundance and DNA sequence of two-base repeat regions in tropical tree genomes. Genome 34:66–71

Davila JA, Sánchez de la Hoz MP, Loarce Y, Ferrer E (1998) The use of random amplified microsatellite polymorphic DNA and coefficients of parentage to determine genetic relationships in barley. Genome (in press)

Dow BD, Ashley MV, Howe HF (1995) Characterization of highly variable (GA/CT)*n* microsatellites in the bur oak, *Quercus macrocarpa*. Theor Appl Genet 91:137–141

Fisher PJ, Gardner RC, Richardson TE (1996) Single-locus microsatellites isolated using 5′-anchored PCR. Nucleic Acids Res 24:4369–4371

Giese H, Holm-Jensen G, Mathiassen H, Kjaer B, Rasmussen SK, Bay H, Jensen J (1994) Distribution of RAPD markers on a linkage map of barley. Hereditas 120:267–273

Gupta M, Chyi Y-S, Romero-Severson J, Owe JL (1994) Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. Theor Appl Genet 89:998–1006

Hoisington D, Khairallal M, González-de-León D (1994) Laboratory protocols:CIMMYT applied molecular genetics laboratory, 2nd edn. CIMMYT, Mexico DF

Kass DH, Batzer MA (1995) Inter-*Alu* polymerase chain reaction: advancements and applications. Anal Biochem 228:185–193

Kleinhofs A, Killian A, Saghai-Maroof MA, Biyashev RM, Hayes P, Chen FQ, Lapitan N, Fenwick A, Blake TK, Kanazin V, Ananiev

E, Dahlee L, Kudrna D, Bollinger J, Knapp SJ, Liu B, Sorrells M, Heun M, Franckowiak JD, Hoffman D, Skadsen R, Steffenson BJ (1993) A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. Theor Appl Genet 86: 705–712

Lagercrantz U, Ellegre H, Andersson L (1993) The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates. Nucleic Acid Res 21: 1111–1115

Lander ES, Green P, Abrahamsom J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174–181

Liu Z-W, Biyashev RM, Saghai-Maroof MA (1996) Development of simple sequence repeat DNA markers and their integration into a barley linkage map. Theor Appl Genet 93: 869–876

Ma ZQ, Röder M, Sorrells ME (1996) Frequencies and sequence characteristics of di-, tri, and tetra-nucleotide microsatellites in wheat. Genome 39: 123–130

Manninen I, Schulman AH (1993) BARE-1, a *copia*-like retroelement in barley (*Hordeum vulgare* L.). Plant Mol Biol 16: 1089–1091

Matioli SR, Brito RA de (1995) Obtaining genetic markers by using double-stringency PCR with microsatellites and arbitrary primers. BioTechniques 19: 752–755

Morgante M, Olivieri AM (1993) PCR-amplified microsatellites as markers in plant genetics. Plant J 3: 175–182

Nagaoka T, Ogihara Y (1997) Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor Appl Genet 94: 597–602

Nei M (1973) Analysis of gene diversity in subdivided populations. Am Nat 106: 238–292

Paran I, Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theor Appl Genet 85: 985–993

Pfeiffer A, Olivieri AM, Morgante M (1997) Identification and characterization of simple-sequence repeats in Norway spruce (*Picea abies* K.). Genome 40: 411–419

Röder M, Plaschke J, König SU, Börner A, Sorrells ME, Tanskley SD, Ganal MW (1995) Abundance, variability and chromosomal location of microsatellites in wheat. Mol Gen Genet 246: 327–333

Rogowsky PM, Lin JY, Manning S, Taylor C, Landgridge P (1992) Structural heterogeneity in the R173 family of rye-specific repetitive DNA sequences. Plant Mol Biol 20: 95–102

Russell J, Fuller J, Young G, Thomas B, Taramino G, Macaulay M, Waugh R, Powell W (1997) Discriminating between barley genotypes using microsatellite markers. Genome 40: 442–450

Saghai-Maroof MA, Biyashev RM, Yang GP, Zhang Q, Allard RW (1994) Extraordinarily polymorphic microsatellite DNA in barley: spcies diversity, chromosomal locations and population dynamics. Proc Natl Acad Sci USA 91: 5466–5470

Sánchez de la Hoz MP, Dávila JA, Loarce Y, Ferrer E (1996) Simple sequence repeat primers used in polymerase chain reaction amplifications to study genetic diversity in barley. Genome 39: 112–117

Sharp, PJ, Kreiss M, Shewry P, Gale MD (1988) Location of β-amylase sequences in wheat and its relatives. Theor Appl Genet 75: 286–290

Tautz D (1989) Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acids Res 17: 6463–647

Tautz D (1993) Notes on the definition and nomenclature of tandemly repetitive DNA sequences. In: Pena SDJ, Chakraborty R, Epplen JT, Jeffreys AJ (eds) DNA fingerprinting: state of the science. Birkhäuser, Basel, Boston Berlin

Tautz D, Trick M, Dover G (1986) Cryptic simplicity in DNA is a major source of genetic variation. Nature 322: 652–656

Vosman B, Arens P (1997) Molecular characterization of GATA/GACA microsatellite repeats in tomato. Genome 40: 25–33

Waugh R, Bonar E, Baird P, Lawrence P, Harrower B, Booth A, Khalil W, Hayes P, Graner A, Thomas WTB, Ellis RP, Powell W (1996) Conservation of AFLP marker order in different barley populations. In: Slinkard AI, Scoles G, Rossnagel B (eds), Proc Vth Int Oat Conf and VIIth Int Barley Genet Symp, vol 2. University Extension Press, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, pp 397–399

Weising K, Atkinson RG, Gardner RC (1995 a) Genomic fingerprinting by microsatellite-primed PCR: a critical evaluation. PCR Methods Appl 4: 249–255

Weising K, Nybom H, Wolff K, Meyer W (1995 b) DNA fingerprinting in plants and fungi. CRC Press, Boca Raton, Florida

Whittier RF, Dean DA, Rogers JC (1987) Unpublished. Data from EMBL, accession number X05167

Wilke K, Jung M, Che Y, Geldermann H (1994) Porcine $(GT)_n$ sequences: structure and association with dispersed and tandem repeats. Genomics 21: 63–70

Wu K-S, Tanskley SD (1993) Abundance, polymorphism and genetic mapping of microsatellites in rice. Mol Gen Genet 241: 225–235

Wu K-S, Jones R, Danneberger L, Scolnik PA (1994) Detection of microsatellite polymorphism without cloning. Nucleic Acids Res 22: 3257–3258

Zhou Z, Bebeli PJ, Somers DJ, Gustafson JP (1997) Direct amplification of minisatellite region DNA with VNTR core sequences in the genus *Oryza*. Theor Appl Genet 95: 942–949

Zischler H, Kammerbauer C, Studer R, Grzeschik K-H, Epplen JT (1992) Dissecting $(CAC)_5/(GTG)_5$ fingerprints into individual locus-specific, hypervariable components. Genomics 13: 983–990

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple-sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20: 176–183