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Comparison of the utility of barley retrotransposon families for genetic analysis by molecular marker techniques

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Abstract The Sequence-Specific Amplification Polymorphism (S-SAP) method, and the related molecular marker techniques IRAP (inter-retrotransposon amplified polymorphism) and REMAP (retrotransposon-microsatellite amplified polymorphism), are based on retrotransposon activity, and are increasingly widely used. However, there have been no systematic analyses of the parameters of these methods or of the utility of different retrotransposon families in producing polymorphic, scorable fingerprints. We have generated S-SAP, IRAP, and REMAP data for three barley (*Hordeum vulgare* L.) varieties using primers based on sequences from six retrotransposon families (*BARE* -1, BAGY-1, BAGY-2, Sabrina, Nikita and Sukkula). The effect of the number of selective bases on the S-SAP profiles has been examined and the profiles obtained with eight *Mse*I + 3 selective primers compared for all the elements. Polymorphisms detected in the insertion pattern of all the families show that each can be used for S-SAP. The uniqueness of each transposition event and differences in the historic activity of each family suggest that the use of multiple retrotransposon families for genetic analysis will find applications in mapping, fingerprinting, and marker-assisted selection and evolutionary studies, not only in barley and other *Hordeum* species and related taxa, but also more generally.

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Introduction

Retrotransposons are mobile genetic elements found throughout the plant kingdom (Kumar and Bennetzen 1999; Fedoroff 2000). They generally show widespread chromosomal dispersion, variable copy number and random distribution in the genome (Kumar et al. 1997; Kalendar et al. 1999). Retrotransposons move to new chromosomal locations via an RNA intermediate, and insert new cDNA copies back into the genome (Boeke et al. 1985; Boeke and Corces 1989). This mode of replication increases genome size, and contributes significantly to the total DNA of higher plants; at least 50% of the maize genome is composed of retrotransposons (Shirasu et al. 2000). Retrotransposons represent one of the most 'fluid' genomic components (Gribbon et al. 1999); large variations in retrotransposon copy number are observed over relatively short evolutionary time scales. Different retrotransposon families, each with its own lineage and structure, have the potential to have been active at distinct phases in the evolution of a species.

Retrotransposons consist of a conserved domain encoding products required for transposition, bounded by direct repeats (long terminal repeats, LTRs) that contain promoters required for replication, signals for RNA processing, and motifs necessary for the integration of the DNA daughter copies (Kumar and Bennetzen 1999; Suoniemi et al. 1997). The domain order in the polyprotein encoded between the LTRs defines retrotransposons as "*copia* -like" or "*gypsy* -like", after the type elements of *Drosophila melanogaster*, and defines

two ancient lineages shared by plants, animals and yeasts (Xiong and Eickbush 1990). The LTRs are unique to each retrotransposon family, and this specificity has been exploited in genetic analyses by using primers within the LTRs to amplify flanking regions. Because retrotransposons replicate via an RNA intermediate, they do not excise like DNA transposable elements as a part of their transposition: their insertion into a new site acts as an evolutionary stopwatch generating a marker in both location and time. Retrotransposons are subject to rearrangements; recombination between the LTRs can remove the element, leaving solo LTRs (Shirasu et al. 2000), and other rearrangements can disrupt the internal sequence (Lee et al. 1990), but the relationship between the LTR(s) and the integration point and flanking DNA is maintained. The nested integration of retrotransposons into each other provides data on the historical activity of the different families (Shirasu et al. 2000).

Where the retrotransposon density is high within a genome, LTR primers can be exploited for detection of their chance association with other retrotransposons (Inter-Retrotransposon Amplified Polymorphism, IRAP) or microsatellites (Retrotransposon-Microsatellite Amplified Polymorphisms, REMAP; Kalendar et al. 1999). When retrotransposon activity has led to new genome integrations, these can be used to distinguish reproductively isolated plant lines. In this case, amplified bands derived from the newly inserted elements will be polymorphic, appearing only in plant lines in which the insertions have taken place. The IRAP and REMAP methods, using the barley *BARE* -1 and other retrotransposons, have been demonstrated to provide suitably polymorphic markers for variety identification or breeding purposes (Kalendar et al. 1999; Manninen et al. 2000; Vicient et al. 2001; Boyko et al. 2002).

Alternatively, retrotransposons can be used in an AFLP-type reaction (Vos et al. 1995) termed Sequence-Specific Amplified Polymorphism (S-SAP; Waugh et al. 1997). In AFLP (Amplified Fragment Length Polymorphism), DNA is digested with restriction enzymes, adapters are ligated to the fragments thereby created, and primers complementary to the adapters are then used to amplify the fragments. Bases can be added to the 3′ ends of the primers to select for amplification of the subset of fragments in which the restriction site of the adapter is adjacent to those particular corresponding nucleotides. Electrophoresis of the amplicons visualizes polymorphisms in the presence or position of the selected fragments. In the S-SAP system, one likewise digests the genomic DNA and ligates adapters, but uses only one adapter primer for the final amplification. The second primer is designed to anneal to a conserved motif in a retrotransposon that is close to the joint with the flanking genomic DNA, generally within the LTR. Hence, the polymorphism that is visualized is for the presence of a retrotransposon at a given distance from a restriction site. Highly prevalent retrotransposons require the use of selective primers, either for the adapter primer or the retrotransposon primer or both, in the S-SAP reaction for efficient amplification and visualization.

In the S-SAP technique, the selective bases added to the primers reduce the complexity of the amplified DNA, depending on the copy number of the retrotransposon targets. The use of S-SAP has been described for barley (Waugh et al. 1997), wheat (*Triticum aestivum* L; Gribbon et al. 1999), pea (*Pisum sativa* L.; Ellis et al. 1998), and alfalfa (*Medicago sativa* L.; Porceddu et al. 2002). This paper describes the development, characterization and comparison of S-SAP fingerprints with six retrotransposon families: *BARE* -1, Sukkula, Sabrina, Nikita, BAGY-1 and BAGY-2 in barley, and examines the use of these elements for the IRAP and REMAP techniques as well.

Materials and methods

Plant materials

Three barley varieties were studied: Wanubet, Ingrid, and Golden Promise. We chose these three varieties as well-established experimental materials that also have been used as the basis for breeding programs. They are important genotypes in the pedigrees of Canadian (Wanubet is a waxy, nude form of Betzes), German, and UK spring barleys, respectively. Golden Promise is also an easily-transformed barley variety with good combining ability in crosses. The question of sampling bias affecting marker polymorphism between these varieties was approached by computation of all pairwise distances from S-SAP data (not shown) on the varieties in the UK Recommended List and the Finnish National List, two distinct but internally representative sets. The distance between Ingrid and Golden Promise ranked 19th out of 903 possible distances (1st being the shortest distance), Wanubet vs. Golden Promise ranked 178th, and Wanubet vs. Ingrid ranked 239th. Hence, the varieties chosen represent a range of genetic diversity such as may be expected within the National List of spring barleys in Finland and the Recommended List of spring barleys in the UK. For each variety, DNA was extracted from freeze-dried leaf material collected as bulks of five plants by Keygene NV (Wageningen, Netherlands).

S-SAP analysis

The S-SAP method as described by Waugh et al (1997) was employed with the following modifications. *Eco*RI (5 U) was used in conjunction with *Mse*I (5 U) to digest 1 μg of genomic DNA. Enzyme-specific adapter and primer sequences for *Eco*RI and *Mse*I were as described by Vos et al. (1995). Pre-amplifications were carried out with *Eco*RI and *Mse*I primers having A and C, respectively, as selective nucleotides. Following pre-amplification, selective amplification was performed using an extended *Mse*I primer. This was used in combination with a retrotransposon-based primer labeled at the 5′ end with an IRD tag (MWG Biotech, Ebersberg, Germany) to allow fragment detection on the LICOR Dual Laser automated sequencer. Primers were chosen based on the following criteria: closeness to the LTR-genome joint; sequence conservation; functionality as PCR primers; performance in PCR. Primers were designed from the LTRs of six retrotransposon families and were used one at a time in conjunction with an *Mse*I primer. The primers used are listed in Table 1.

Amplified fragments were separated and analyzed on a LI-COR DNA analyzer Gene ReadIR 4200 (MWG-Biotech) following the method of Jackson and Matthews (2000) with minor modifications; PCR products were diluted 1:1 with loading buffer,

Table 1 Primers used in S-SAP, IRAP and REMAP analyses

Retrotransposon-based primers	Position in LTR[a]	Orientation[b]	Sequence[c]
BARE -1-5980	5′ terminus +A	Reverse	CTAGGGCATAATTCCAACAA
BARE-1-92460	5′ 146	Reverse	CTGGCTAGCCAACTAGAGGCTTGC
BARE-1-E1814	3′ 1	Forward	TTCCCATGCGACGTTCCCCAAC
BARE -1-C0700	5′ 371	Reverse	ACACACAAAGCATTCCTCCGG
Sabrina-C0945	3′ 420	Forward	GCAAGCTTCCGTTTCCGC
Sukkula-91673	5′ terminus	Forward	TGTGACAGCCCGATGCCGACGTTCC
Sukkula-E0228	5′ 6	Reverse	GGAACGTCGGCATCGGGCTG
Sukkula-9900	3′ 1	Forward	GATAGGGTCGCATCTTGGGCGTGAC
BAGY-1-C2043	3′ 1200	Forward	TCGTCGCCGGCGTCATCTCC
BAGY-1-C0651	5′ 8	Reverse	CTATACTTGATTAGGGTTCCAGGG
BAGY-1-4164	3′ 4164	Forward	CGATGTGTTACAGGCTGGATTCC
BAGY-2-C0589	5′ 35	Forward	TTCGACACTCTTACTTATCGAAAGG
BAGY-2-E0521	5′ 19	Forward	TCGAAAGGTCTATGATTGATCCC
BAGY-2-E0520	3′ 8	Reverse	CATGAAAGCATGATGCAAAATGG
Nikita-E2647	5′ 80	Forward	ACCCCTCTAGGCGACATCC
Nikita-57	3′ 57	Forward	CGCATTTGTTCAAGCCTAAACC
Mse I primers			
Basic *Mse* I primer	n.a.	–	GATGAGTCCTGAGTAA-
Selective 3′ bases	n.a.	–	-C; -CA, -CC, -CG, -CT; -CAA, -CAC, -CAG, -CAT, -CCA, -CCC, -CCG, -CCT, -CTA, -CTC, CTG, -CTT

[a]Position is given relative to the 5′ or 3′ end of the LTR as the number of nucleotides internal to the LTR terminus
[b]Forward primers match the sense or "upper" strand with respect to transcription; reverse primers match the antisense strand
[c]All sequences are given in the 5′ to 3′ direction

and Sequagel XR (National Diagnostics, Hull, UK) acrylamide was used without degassing. The labeled fragments are automatically converted to band images by the sequencer, then scored manually from a computer screen as present or absent. The intensity or contrast of the image was varied to ensure that all the band differences scored were true polymorphisms and not differences in band intensity. Only clearly polymorphic bands were recorded.

IRAP and REMAP Analyses

IRAP and REMAP analysis was performed as detailed previously (Kalendar et al. 1999) using the retrotransposon-derived primers listed in Table 1.

Results

Choice of retrotransposons for marker analysis

Several element families, *BARE* -1, Sukkula, Sabrina, Nikita, BAGY-1 and BAGY-2, were chosen for methodological development on the basis of their having been identified in barley. The *copia* -like (Kumar and Bennetzen 1999) *BARE* -1 family was the first full-length retrotransposon to be described for barley (Manninen and Schulman 1993) and it remains the best characterized. It is highly abundant, present in $16.6 \pm 0.6 \times 10^3$ copies (Vicient et al. 1999) and accounts for approximately 3% of the genome. It is actively transcribed (Suoniemi et al. 1996), translated and assembled into virus-like particles (Jääskeläinen et al. 1999), and is involved in genomic diversification within the genus and even over distances of hundreds of meters in cultivated barley (Vicient et al. 1999; Kalendar et al. 2000).

Shirasu et al. (2000) identified the retrotransposons Nikita, Sukkula, Sabrina and BAGY-2, as well as *BARE* -1, in a 66-kb contiguous segment of the barley genome, where they were present mostly as solo LTRs and arranged predominantly as nested insertions. Based on the positions of the elements in the nests, one can reconstruct the order of insertion; elements that tend to interrupt other elements more than they themselves are interrupted may be considered as having been active more recently. Using this approach (A. Schulman, unpublished), we can characterize the relative activity of these retrotransposons as *BARE*-1 > Sukkula = BAGY-2 > Nikita > Sabrina. We were interested in the relative usefulness of these families as markers based on this measure of activity, and also in testing different classes of retrotransposons.

The Sukkula family is unusual in not possessing a protein-coding domain (Kalendar et al., submitted), although it is insertionally polymorphic and useful as a molecular marker (Manninen et al. 2000). The BAGY-2 family (Vicient et al. 2001) is a *gypsy* -like element in the plant *Athila* group, which is unusual in its possession of an *envelope* domain similar to those of retroviruses. Its insertion pattern is polymorphic in closely related European barleys. BAGY-1 is a *gypsy* -like element that does not possess an *env* domain (Panstruga et al. 1999). Nikita is not well characterized, although it has been described as an *Athila* -like, thus *env* -bearing, element (http://wheat.pw.usda.gov/ggpages/ITMI/Repeats/index.shtml). Although the copy numbers of these elements have not been well-defined (http://wheat.pw.usda.gov/

ggpages/ITMI/Repeats/nrTREP_list.html), preliminary data for Sukkula and BAGY-2 place their frequencies in the same range as that of *BARE* -1.

Variation in the fingerprinting efficiency of retrotransposon-derived primers in S-SAP

The 'quality' (band intensity versus background intensity, distribution and abundance of bands) of the profiles generated was highly dependent on the retrotransposon primer, and partly dependent on the selective bases added to the *Mse*I primer used in the S-SAP reaction following pre-amplification. A high quality profile displays abundant, intense bands against a low background, evenly distributed with at least 2–3 bp separating each band from its neighbors. A fingerprinting profile containing an even fragment distribution, easily scorable polymorphic bands and a small number of intense monomorphic bands that act as reference points allows a high multiplex ratio and reliable scoring.

Some S-SAP primer combinations resulted in extremely intense bands or many closely spaced bands in the lower molecular weight range. Other combinations produced very weak higher molecular weight patterns. As both of these situations mean that potentially informative bands may be missed, direct comparisons between primer combinations were most accurately made in the medium size range. To ensure consistency, only bands lying between the 146- and 358-bp molecular weight markers on the S-SAP gels were scored for comparative studies of the various retrotransposon-derived primers in S-SAP reactions. Table 2 summarizes the average number of bands observed between 146 and 358 bp when each of the sixteen retrotransposon primers was amplified in conjunction with each of the eight *Mse*I + 3 primers presented in Fig. 1. The number of

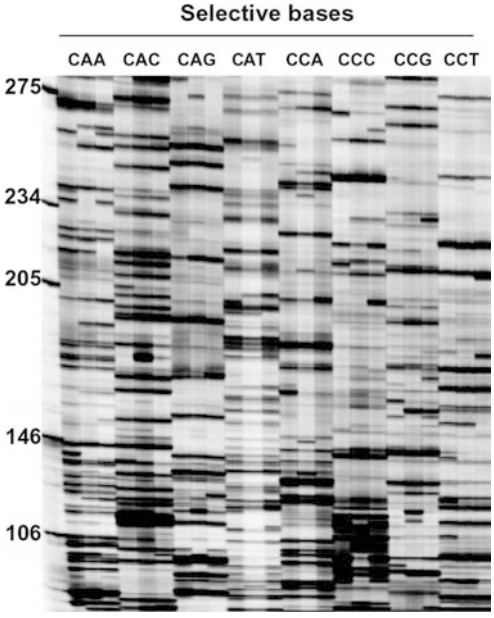

Fig. 1 Effect of altering the selective base composition of the *Mse*I primer on the S-SAP profile obtained. The retrotransposon primer was *BARE* -1-5908. The three barley varieties are shown in the same order (Wanubet, Ingrid, and Golden Promise) from left to right, in each case. The selective bases on the *Mse*I primer used are shown at the *top*. A 1-μl aliquot of the reaction carried out using the selective bases shown at the top was analyzed on a LICOR Gene ReadIR 4200. Reaction conditions were the same in all cases, with the exception of the *Mse*I primer used

Table 2 Characteristics of S-SAP profiles produced using each of the retrotransposon-derived primers

Retrotransposon primer	Mean number of bands (range) [a]	Mean percentage of polymorphic bands (range)	Distribution of bands through profile	Distribution of polymorphic bands	Background level	Clarity of profile[b]
BARE -1-5980 [c]	30.1 (23–37)	25.6 (8.8–35.5)	Even	Even	Low	1
BARE -1-92460	24.4 (1–39)	11.1 (0–23.1)	Uneven	Even	Quite high	4, 5
BARE -1-E1814	17.8 (15–26)	13.4 (5–23.1)	Even	Even	Quite high	4
BARE -1-C0700 [c]	9.25 (4-20)	9.9 (0-28.9)	Uneven	More large fragments	Low	4
Sabrina-C0945	12.4 (1–19)	24.0 (0–41.7)	Even	Even	Low	1
Sukkula-91673 [c]	19.4 (8-38)	9.7 (8-28.9)	Even	More large fragments	Low	2
Sukkula-E0228	23.6 (16–32)	20.95 (7.7–31.3)	Even	Even	Low	1
Sukkula-9900	20.75 (14–28)	18.7 (0–40)	Even	Even	Low	1, 5
BAGY-1-C2043	10.1 (3–15)	11.9 (0–33.3)	Even	Even	Low	3
BAGY-1-C0651	12.5 (7–25)	14.9 (0–37.5)	Even	Even	Low	1
BAGY-1-4164	17.8 (12–26)	13.4 (5–23.1)	Even	Even	Low	3
BAGY-2-C0589	26.88 (20–35)	24.5 (2.8–15.2)	Even	Even	Low	3
BAGY-2-E0521	9.5 (1–14)	10.8 (0–36.4)	Even	Even	Detectable	2
BAGY-2-0520	24.25 (15–33)	10.2 (0–20)	Even	Even	Low	2
Nikita-E2647	19 (11–28)	17.5 (7.7–35.3)	Even	Even	Low	3
Nikita-57	13 (11–16)	16.5 (0–30.8)	Uneven	Even	Detectable	3

[a]The number of bands recorded in the size range between 146 and 358 bp (two of the molecular weight markers on the LICOR gel)
[b]Profiles were scored according to the folowing scheme: 1, clear profiles, bands easy to score; 2, clear profiles, but band intensities variable within tracks and more difficult to score; 3, clustered bands or background signal complicate profile, but informative bands may still be scored; 4, informative bands obscured by background signal or other bands and scoring informative bands is difficult; 5, informativeness and quality of profiles vary widely with each *Mse*I primer combination
[c]Amplified in conjunction with the four Mse-CAN and the four -CCN pimers. All others amplified in conjunction with the four Mse-CAN and the four -CTN primers

polymorphic bands is expressed as a percentage of the total number observed in the section of the profile between 146 and 358 bp. The characteristics of profiles generated with each retrotransposon-derived primer are also summarized in Table 2; they reflect the results with optimal *Mse*I primers. The number of selective bases on *Mse*I primers was also varied (Fig. 2). Figure 3 illustrates the results generated when Mse-CAC is used in conjunction with primers derived from each of the retrotransposon families from the three barley varieties.

As well as ensuring the consistency of the profiles by examining the same section of all gels, reproducibility of the S-SAP technique was confirmed by other experiments (not shown), in which six of the S-SAP primer combinations were used in an extensive study of the genetic uniformity of barley varieties. Cultivar Ingrid was used as a control on three gel runs for each of the six S-SAP primers, using independent DNA extractions derived from the same bulk leaf sample of Ingrid; the controls gave the same profile on all three gels with each of the six S-SAP markers. In addition, the uniformity of cultivar Ingrid was assessed with 23 individual plants of this variety, revealing only one variant among the six S-SAP combinations; a single plant lacked one band. This is more likely to be explained by variation within the variety than by a reproducibility problem with the S-SAP technique. The reproducibility of the S-SAP technique was demonstrated by the original authors (Waugh et al. 1997) and was shown to be comparable with that of the widely used AFLP technique (Vos et al. 1995).

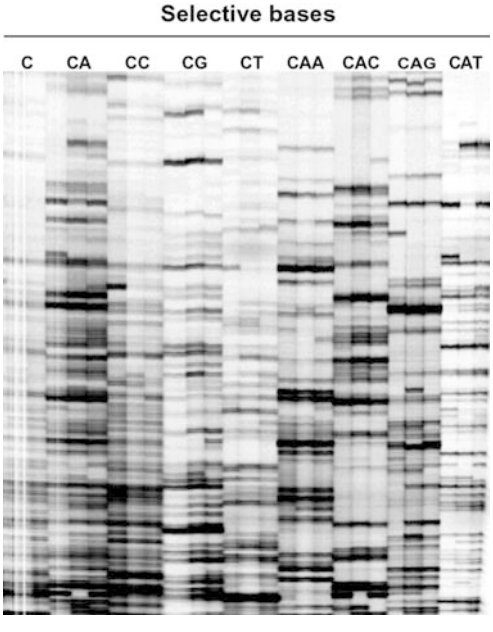

Fig. 2 Influence of the number of selective bases in the *Mse*I primer on the complexity of the S-SAP profile. The retrotransposon primer was Sukkula-9900. The reaction conditions for all the samples were the same; only the composition of the selective bases on the Mse primer was varied, as indicated at the *top* of each set of lanes. A 1-μl aliquot of each reaction was analyzed on a LICOR Gene ReadIR 4200. Sample order is the same as in Fig. 1

Profiles obtained with a *BARE*-1 5980 primer in combination with *Mse*I selective primers were consistently good. A large number of clear bands with a high percentage of polymorphisms were produced. The largest average number of scorable bands (30.1) was recorded, of which 25.6% were polymorphic. This is the highest proportion of polymorphic bands detected for any of the retrotransposon-derived primers used for S-SAP in this study. This is similar to the degree of polymorphism described by Waugh et al. (1997); in that case, in S-SAP profiles generated with the same primer, 26% of the approximately 36 bands obtained were polymorphic. Comparable results were thus obtained despite experimental differences in the choice of restriction enzyme, barley varieties and the molecular weight of the bands scored. S-SAP profiles using *BARE* -1 92460 and *BARE* -1 E1814 were of inferior quality to those generated with *BARE* -1 5980; a high background signal masked some of the large number of faint bands and made reproducible scoring of polymorphic bands difficult despite the high degree of polymorphism.

Sukkula-derived profiles were characterized by a large number of clear bands, of which a high percentage was polymorphic. The background signal detected was low and the distribution of bands through the profile was even, both highly desirable features if primers are to be used for extensive or routine screening. Though the profiles were not as informative as those obtained using the *BARE* -1 5980 primer, they contained averages of 19.4–23.6 bands, of which 9.7–20.95% (averages) were polymorphic. The banding patterns were of good quality and easy to score. The Sabrina-derived profile was of similarly high quality, though the numbers of bands were slightly lower than in Sukkula profiles. The proportion of bands displaying polymorphisms was more variable in Sabrina than in Sukkula profiles.

Fingerprints generated with BAGY-2 primers were characterized by a large number of clear bands evenly distributed up to over 600 bp in size with quite low background signal, though only fragments up to 358 bp were recorded for this study. The intensity of the bands was not even within each reaction. Some bands were strongly amplified while others remained very faint. The number of bands and the proportion of bands that displayed polymorphism were lower than in *BARE* -1 5980 S-SAPs, but similar to those in S-SAPs derived from the other retrotransposon primers.

Amplifications using the BAGY-1 primers with *Mse*I selective primers produced a similar number of fragments to those obtained using the Sabrina primer. Signal intensity was highly variable, comprising very strong bands and very faint bands in the same track. With the exception of BAGY1-C0651, which generated good profiles, the bands appeared in clusters in which very strong bands masked weak ones. Although most bands could be scored, profiles of this nature are not ideal for

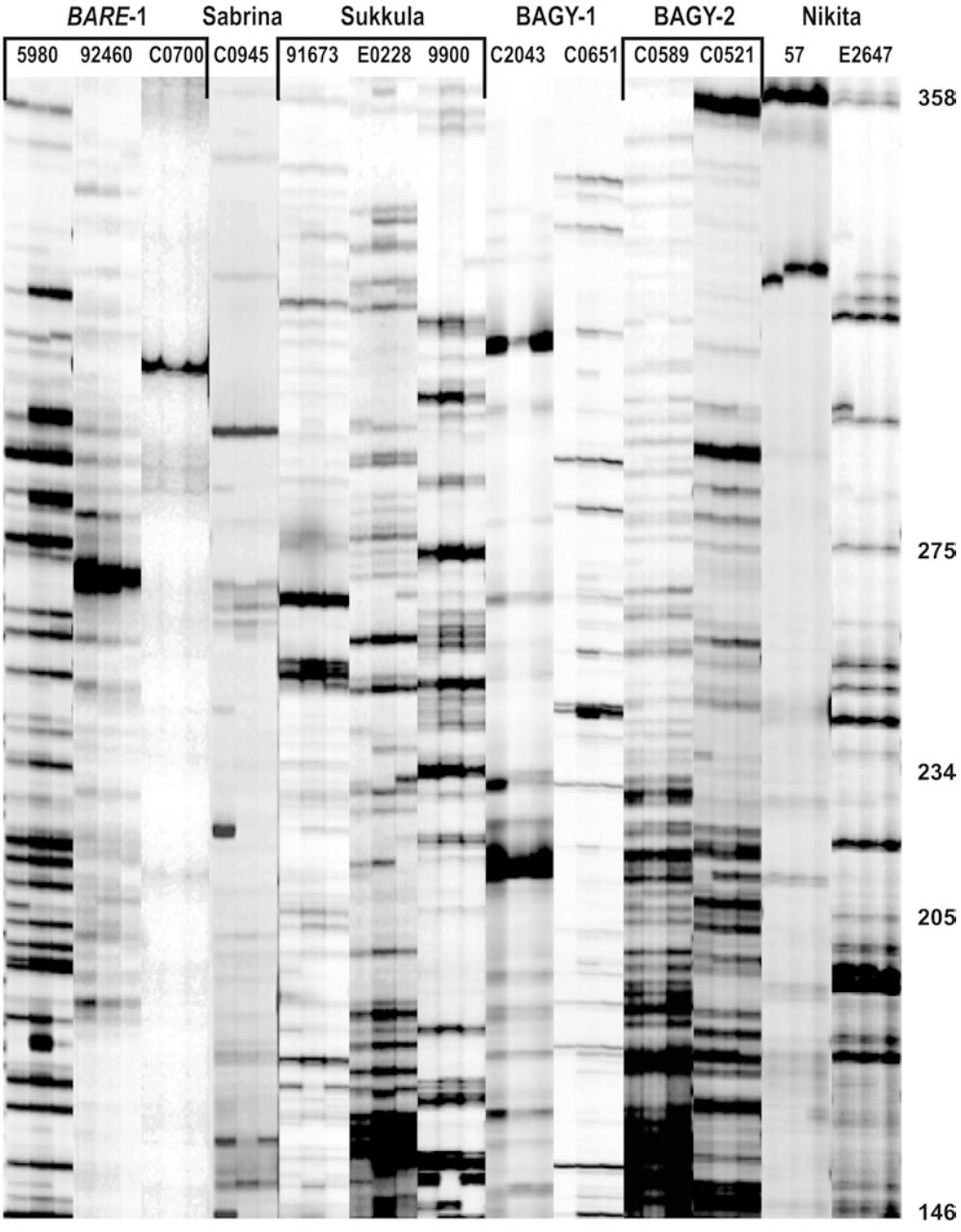

Fig. 3 Comparison of S-SAP profiles obtained with different retrotransposon families and primers. Each retrotransposon primer was used in combination with the Mse-CAC adapter primer. Each set of three lanes displays reactions from Wanubet, Ingrid, and Golden Promise (from left to right). A 1-μl aliquot of each reaction using the selective bases indicated at the *top* was analyzed on a LICOR Gene ReadIR 4200. With the exception of the DNA sample and the retrotransposon primer used in each combination, reaction conditions were the same in all cases

extensive use. S-SAPs using Nikita primers displayed similar unevenness in band intensity and high variation in the percentage of polymorphic bands in each primer combination.

Variation in the S-SAP fingerprint depending on the selective bases used in the *Mse*I + 3 primer

High copy-number retrotransposons require selective bases on the S-SAP primers in order to reduce the number of amplified and detectable bands to a manageable range, i.e., a number small enough to be resolved by electrophoresis. Each of the retrotransposon-derived primers was used in conjunction with one of eight selective *Mse*I primers, each with three selective nucleotides at the 3′ end (*Mse*I + 3); the last two selective bases differed in each primer (CNN). These are listed in Table 1. A section of the image displaying *BARE* -1-5980 plus eight *Mse*I primers is shown in Fig. 1. This illustrates the impact on the S-SAP profile of altering the composition of the selective bases at the 3′ end of the *Mse*I primer. All eight *Mse*I selective primers tested in combination with *BARE* -1 5980 produced highly polymorphic and reproducible profiles.

Widely differing profiles were obtained with different *Mse*I + 3 primers. Some selective primer combinations produced highly informative profiles with many clear differences between varieties, whilst another *Mse*I + 3 primer in combination with the same retrotransposon

primer might display few polymorphisms between varieties and ill-defined bands. This trend was reproducible between amplification reactions and gels (data not shown). However, the selective *Mse*I + 3 primers responsible for uninformative profiles with one retrotransposon primer were not universally poor. In conjunction with other retrotransposon primers they generated high-quality polymorphic fingerprints. For example, the *Mse*I + 3 primers that resulted in poor profiles in combination with the Sukkula-9900 retrotransposon primer produced clear and polymorphic fingerprints in combination with other retrotransposon primers. Hence, *Mse*I primer choice must be optimized for a particular element family.

The influence of the base composition of the selective nucleotides on the number of bands amplified is summarized in Table 2. The number of bands detected for each retrotransposon primer may vary from 8 to 38 (Sukkula-91673). from 20 to 35 (BAGY-2-C0589), or from 11 to 16 (Nikita 57). The percentage of bands that displayed polymorphisms between the three tested varieties was highly variable. This, however, was not linked to the 'quality' (background signal, band clarity and separation) of the profile. For example, although the quality of all the Sukkula-9900 profiles was excellent, the percentage of bands that were polymorphic varied from 0 to 40% and the number of bands between 146 and 358 bp ranged from 14 to 28. This variability demonstrates the need to screen a panel of primers when undertaking a genotyping program using S-SAP or similar techniques.

Impact of the number of selective bases in the *Mse*I primer on the S-SAP profile

The retrotransposons from which primers were designed in this study are highly represented in barley. Although copy number has not been determined explicitly, our results (unpublished) indicate a similar order of magnitude for Sukkula and BAGY-2 as for *BARE*-1, which is present in 14,000 full-length copies in the barley genome (Vicient et al. 1999). In addition, the barley genome contains more than 1×10^5 solo LTRs of *BARE*-1; for the other elements this information is not available, although solo LTRs are known to be present (Shirasu et al. 2000). The larger the number of priming sites for S-SAP, the greater the number of selective bases needed to ensure that profiles remain 'readable'. For S-SAP profiles generated using any given retrotransposon primer, it may be expected that increasing the number of selective bases at the 3′ end of the *Mse*I primer will increase the degree of specificity in the reaction, as with AFLP (Vos et al. 1995) and as previously described for S-SAP in barley using the *BARE*-1 element (Waugh et al. 1997). Each selective base added to the enzyme-specific primer or to the retrotransposon derived primer theoretically reduces the complexity of the fingerprint by a factor of four, assuming that S-SAP does not assess the retrotransposon insertion site but the nearest *Mse*I site and that no mismatched priming ever occurs with the selective bases on the *Mse*I primer. The level of selection required for S-SAP using Nikita, Sukkula, Sabrina, BAGY-1, and BAGY-2 has not been determined previously.

The effect of selective base addition as reported for AFLP profiles in other species (Vos et al. 1995) was tested in S-SAP analyses of barley by varying the number of selective bases on the *Mse*I primer. Labeled, retrotransposon-derived primers were used in conjunction with *Mse*I + C, CA, CC, CG, CT, CAA, CAC, CAG or CAT to generate S-SAP profiles. A summary of the number of bands observed is given in Table 3. Figure 2 shows an example of the changing profile of Sukkula 9900 in response to 1, 2 or 3 selective nucleotides on the *Mse*I primer.

The predicted relationship between the number of selective bases on the *Mse*I primer and the number of amplified bands in the S-SAP profile was only observed with the Sabrina primer and Nikita-57 (Table 3). For these combinations, reducing the number of selective bases increased the number of bands. In BAGY-2 and BAGY 1-4164, *BARE*-1 and two Sukkula profiles (9900 and 91637), the opposite was true; increasing the number of selective bases on the *Mse*I primer increased the number of easily scored bands in the fingerprint. Another trend was observed in BAGY-1-C2043 and -C0651, Sukkula-E0228 and Nikita-E2647 based S-SAP profiles; increasing the number of selective bases from 1 to 2 increased the number of bands observed, but increasing the number of selective bases from 2 to 3 reduced the number of bands detected and made the remaining bands more difficult to score.

Efficacy of different retrotransposons for IRAP and REMAP fingerprinting

Table 4 summarizes the quality (as defined above for S-SAP), quantity and degree of polymorphism observed

Table 3 Impact of variation in the number of selective bases in the *Mse*I primer on the complexity of the S-SAP profile

Retrotransposon primer	Number of bands[a]		
	*Mse*I + 1	*Mse*I + 2	*Mse*I + 3
BARE-1-92460	4	25 ± 15.5	22.5 ± 7.2
BARE-1-E1814	10	11.5 ± 9.5	20.5 ± 7.8
BARE-1-C0700	9	7.3 ± 2.2	5 ± 2.6
Sabrina-C0945	19	13 ± 8	13.5 ± 3.9
Sukkula-91673	17	19.5 ± 6.5	32.75 ± 12.2
Sukkula-E0228	23	30.3 ± 4.3	24.5 ± 6.5
Sukkula-9900	7	21 ± 4.3	25 ± 4.2
BAGY-1-C2043	8	14.8 ± 2.2	10.75 ± 4.6
BAGY-1-C0651	6	13.5 ± 6.7	12.5 ± 8.4
BAGY-1-4164	10	15.3 ± 6.8	20.5 ± 7.8
BAGY-2-C0589	18	24.8 ± 0.96	27 ± 5.0
BAGY-2-E0521	3	5.5 ± 4.0	8.25 ± 5.0
BAGY-2-E0520	20	24.3 ± 10.6	26.5 ± 7.4
Nikita-E2647	23	27.3 ± 2.5	19.8 ± 7.0
Nikita-57	24	19.5 ± 7.6	12.8 ± 2.1

[a]Scored in the size range between 146 and 358 bp

Table 4 Efficacy of each retrotransposon primer for single-primer IRAP, IRAP with two primers, and REMAP

Primer	Single-primer IRAP				IRAP with a second LTR primer				REMAP			
	Band number (size, bp)	Percentage of bands polymorphic	Clarity of profile	Suitable for one-primer IRAP?	Band size (bp)	Percentage of bands polymorphic	Clarity of profile	Suitable for two-primer IRAP?	Band number (size in bp)	Percentage of bands polymorphic	Clarity of profile	Suitable for REMAP?
BARE-1-92460	10 (1000–4000)	10	Very clear	No	100–2500	30–60	Very clear	Yes	20–30 (100–2500)	30–50	Very clear	Yes
BARE-1-E1814	30+ (600–4000)	20	Poor	No	100–3000	20–40	Adequate with PAGE	Yes	40 (100–3000)	30–40	Adequate with PAGE	Yes
BARE-1-C0700	15 (1000–4000)	30	Clear bands	No	300–2000	20–30	Clear bands	Yes	15–20 (300–2000)	20–30	Clear	Yes
Sabrina-C0945	30 (1000–4000)	10	Clear	Yes	200–3000	20–30	Clear	Yes	20–30 (200–3000)	20	Clear	Yes
Sukkula-91673	30 (300–4000)	50	Very clear	Yes	100–3000	40–60	Very clear	Yes	20–25 (100–3000)	40–60	Very clear	Yes
Sukkula-E0228	40 (100–4000)	40	Very clear	Yes	100–2500	40–60	Very clear	Yes	25–30 (100–2500)	40–60	Very clear	Yes
Sukkula-9900	40 (500–4000)	60	Very clear	Yes	100–2500	40–80	Very Clear	Yes	20–30 (100–2500)	50–80	Very Clear	Yes
BAGY-1-C2043	20 (300–2000)	30	Clear	Yes	100–2500	40–60	Very Clear	Yes	10–15 (100–2500)	40–50	Very clear	Yes
BAGY-1-C0651	Not effective alone				300–2500	60	Very clear	Yes	Not effective			
BAGY-1-4164	20 (500–3000)	60	Very clear	Yes	100–3000	60	Very clear	Yes	30–40 (100–3000)	50	Clear	Yes
BAGY-2-C0589	Not effective alone				300–2500	40	Clear	Yes	15–25 (300–2500)	40	Clear	Yes
BAGY-2-E0521	Not effective alone				200–3000	10	Clear	Yes	Not effective			
BAGY-2-E0520	Not effective alone				200–3000	10	Clear	Yes	Not effective			
Nikita-E2647	15 (1000–4000)	10	Clear	No	200–2500	30–50	Very clear	Yes	Most bands derive from inter-SSR amplification			
Nikita-57	10 (1000–4000)	10	Very clear	No	100–3000	30–60	Very clear	Yes	(100–3000)	30–50	Very clear	Yes[a]

[a]Effective combinations require SSR primer screening

for various retrotransposon families in IRAP and REMAP analyses with barley templates. Profiles resulting from *BARE* -1 primer combinations in REMAP were consistently good. Due to the high copy number of *BARE* -1 LTRs in the genome, IRAP generates too many bands to be easily scored by agarose gel, particularly with a 3′ LTR primer; in these cases polyacrylamide gel electrophoresis (PAGE) can be used to improve resolution of bands (Table 4). The *BARE* -1 5′ LTR primer produces predominantly large products (2.5–4.0 kb long). One solution to this problem (data not shown), applicable for highly prevalent retrotransposons, is to add selective nucleotides to the 3′ end of the *BARE* -1 primers used for IRAP. The single selective base of primer *BARE* -1-5980, however, is insufficient for this purpose.

In REMAP reactions generally, some PCR products could in principle be derived from the SSR primer alone. In control experiments for REMAP where the retrotransposon primer was left out of the reaction, we found an effect of the type of SSR primer used. Primers based on 2-nt repeats generated large inter-SSR (ISSR) products (from 1500 to 3000 bp in size), whereas primers for 3-nt repeats generated a range of fragments from 300 to 3000 bp. Addition of the retrotransposon primer to the reaction suppressed the inter-SSR bands. In REMAP analyses, inter-SSR products were not seen with any SSR primer in combination with a *BARE* -1 primer.

Sukkula-derived profiles were typified by a large number of clear bands, many of which were polymorphic in IRAP. Carrying out IRAP with two different (5′ LTR and 3′ LTR) Sukkula primers yields a very high degree of polymorphism and bands of high quality. The REMAP method with Sukkula is effective, but the products are less polymorphic than those obtained with *BARE* -1.

Amplifications with BAGY-1 primers yield products similar in quantity and quality to those obtained from Sukkula. Furthermore, when IRAP is carried out with BAGY-1 and Sukkula primers in combination, highly polymorphic patterns are obtained. As for Sukkula, mixed 5′ LTR and 3′ LTR primers for BAGY-1 yielded clear and highly polymorphic patterns. By comparison, the primers used for BAGY-2 in IRAP were not effective alone (Table 4). Combinations of 5′ LTR and 3′ LTR BAGY-2 primers, or BAGY-2 with other retrotransposon primers, are, however, effective in IRAP. The REMAP results were highly dependent on the particular BAGY-2 primer used. Our interpretation of the BAGY-2 data is that the primers are not yet optimized, due to a lack of sufficient sequence data for this retrotransposon family. Fingerprints from Nikita and Sabrina were of similar quality. Nikita was not effective for single-primer IRAP, although Sabrina was. Both worked well in two-primer or mixed-primer reactions. In REMAP, only the Nikita primer constructed for the 3′ end of the LTR was effective. The Sabrina 5′ LTR primer gave good results in REMAP.

Discussion

We found that the retrotransposons used for S-SAP analyses differed in their utility, and that primers derived from each of the elements yielded fingerprints of varying degrees of informativeness. The composition of the selective bases on the MseI primer has a strong impact on the number of bands detected and the level of polymorphism shown by the profile. For example, although the quality of all the Sukkula 9900 profiles was excellent, the percentage of bands that were polymorphic varied from 0 to 40%, and the number of bands observed in the size range between 146 and 358 bp ranged from 14 to 28. This variability demonstrates the need to screen a panel of primers when undertaking a fingerprinting program using S-SAP or similar techniques. The S-SAP system is a highly informative marker system that may be applied to genome analysis for many purposes. It is robust and reproducible, but it requires prior knowledge of retrotransposon LTR sequences for primer design. In species where retrotransposon LTRs have been identified, the use of a multi-retrotransposon approach in the molecular analysis is particularly informative, because each retrotransposon may have a unique tranpositional history (Kumar et al. 1997), and wider genome coverage is achieved.

The features that determine the quality of IRAP and REMAP fingerprinting are equivalent to those for S-SAP described above. Sharp bands of equal intensity against a low background are important. The REMAP method employs anchored simple sequence repeat (ISSR or microsatellite) primers. Hence, some bands in REMAP may be amplification products derived solely from the SSR primers. Generally, these products are minimized when the retrotransposon primer is identical or closely complementary to the templates, and has a high GC content at its 3′ end. This suppression of inter-SSR amplification may be due to the high sequence conservation in retrotransposons compared to SSR regions, or to the relative distribution of SSR regions and particular retrotransposons in the genome. The IRAP method is influenced by the degree of activity of a particular retrotransposon family and by the interspersion or nesting of the various retrotransposon families.

In the previously published applications of S-SAP, IRAP, and REMAP, primers have generally been designed to anneal at or near the terminus of the LTR, except for the pea *PDR1*element where the LTRs are short (Ellis et al. 1998). For S-SAP, this is done primarily for two reasons. Firstly, *Mse*I is a frequent-cutter that could lead to the adapter primer amplifying monomorphic internal bands from within the retrotransposon. Secondly, at the end of the retrotransposon where the primer faces the flanking genomic DNA, setting its annealing site too far back from the terminus would generate bands that are too long to be effectively resolved on sequencing gels. Likewise, for IRAP, primers located at the termini of LTRs will produce shorter

products from elements near each other in the genome; the internal priming direction would in any case synthesize through the element and generate very long products, if any.

Nevertheless, Shirasu et al. (2000) showed that retroelements can be heavily nested in the barley genome, as they are in maize (SanMiguel et al. 1996). Retrotransposon primers could therefore detect polymorphisms resulting from nested insertions within the element. The efficacy of a primer in this application would depend on favored insertional positions for a particular family of elements, as well as sequence conservation at the annealing site. Here, we have included four primers (*BARE* -1-C0700, Sukkula-91673, BAGY-1-C2043, BAGY-1-4164) that anneal more than 1000 bp internal to the LTR termini in order to test their efficacy with nested insertions. All four of these primers work well for both IRAP and REMAP, but are more variably effective in S-SAP. This may be due to the dependence of S-SAP in this application not only upon the position of the nested insertions but also on the distribution of restriction sites within the nesting element. Furthermore, old, degenerate elements or regions of poor conservation could display restriction site and annealing site polymorphisms that would affect the results. In the case of the BAGY-1 primers, degeneracy is unlikely, because of the effectiveness of the element in IRAP and REMAP. The Sabrina-C0945 primer is located 420 bp from the LTR terminus, yet produces good polymorphism in fragments that are shorter than that. Given the ancient and recently inactive status of Sabrina as deduced from the structures of the nested sets, these polymorphisms are most likely to result from nesting or restriction site variation.

In S-SAP, the abundance of the retrotransposons in the barley genome may be estimated from the fingerprints by varying the number of selective bases on the *Mse*I primers. The number of primer binding sites in the genome is fixed for each retrotransposon; thus, changing the retrotransposon primer used in S-SAP will alter the fingerprint complexity depending on the abundance of the chosen element and its genomic distribution. As with AFLP (Vos et al. 1995), an abundance of target sites for the primers results in a lack of specificity in the PCR due to high competition in the reaction. Such profiles are characterized by a high level of background and the visualization of a small number of clear bands. These patterns are difficult to score and usually harbor few useful bands. Because the frequency of retrotransposon LTRs is fixed, increasing specificity can only be achieved by the addition of selective bases to the 3′ end of the *Mse*I primer, or to the retrotransposon-derived primer, or to both. This probably diminishes the competition in the PCR by lowering the number of amplicons, thus reducing the background signal and improving the resolution of the amplified bands.

The number of bands detected (as distinct from the total number of products amplified) with *BARE* -1, Sukkula and BAGY-2 primers increased with the addition of selective bases to the *Mse*I primer. Increasing the specificity of the *Mse*I primers in these cases probably helped to reduce the competition within the PCR and increase the concentration of the amplified products, so that clearer banding patterns were obtained. This is consonant with the great abundance of the retrotransposons in the genome, and the consequently high number of potential primer binding sites and products. Conversely, increasing the selective bases on the *Mse*I primers in conjunction with the Sabrina primers or the Nikita 57 primer may have created PCR conditions that were too specific for the amplification of a large number of bands. This implies that the priming sites for the Sabrina, Nikita 57, and BAGY-1 primers are less prevalent in the genome than are *BARE* -1, BAGY-2 and Sukkula. The efficacy of any particular selective *Mse*I primer is highly dependent on the retrotransposon family it is used with. This suggests that differences may exist in preferences for insertion sites or regions between retrotransposons in barley.

Although the novel retrotransposon markers described here do not offer any significant advantage over *BARE* -1 in terms of band frequency or polymorphism levels detected within barley cultivars, they may be of use in certain applications. Phylogenetic resolution between closely related germplasm accessions benefits from increasing the number of polymorphic markers scored, and several retrotransposons can be used together in S-SAP, IRAP, or REMAP to achieve this purpose. Similarly, the density of markers on a recombinational map can be increased by pooling data from several retrotransposons. However, polymorphism *per se* is only one of the factors that determines the utility of a retrotransposon family for marker applications; another is transpositional activity during the epoch when two lines or populations diverged from a common ancestor. Using different element families offers the possibility to increase phylogenetic resolution on the basis of such differences.

The retrotransposons chosen for analysis here may be distinct in their pattern of activity over evolutionary time. Shirasu et al. (2000) described *BARE* -1 as being both an ancient and an active retroelement; in the 66-kb sequence that was examined, all the flanking 5-bp repeat units for *BARE* -1 are perfectly conserved. This implies that these retrotransposon copies have not been in position long enough for mutational events to alter the flanking sequences. In contrast, Sabrina elements are described as 'ancient but recently inactive elements' because, in the same 66-kb barley sequence, *BARE* -1 and Sukkula elements interrupt the Sabrina elements (Shirasu et al. 2000). A recent analysis (Wei et al. 2002) of retrotransposon insertions in a 261-kb segment of the barley genome is consistent with this view. The study estimated the ages of the retrotransposon insertions from the incidence of base-pair substitutions in the LTRs. The five full-length *BARE* -1 elements inserted respectively 0.64, 0.89, 0.93, 1.69, and 2.05 million years ago, whereas the sole Sabrina element inserted 2.42 million years ago. A retrotransposon that has been ac-

tive recently in evolutionary terms will be polymorphic between individuals within a species. Retrotransposons that have been inactive for a long time will be less polymorphic at the intra-specific level, but may be more informative at the inter-specific or higher taxonomic levels. A combination of ancient and active retrotransposons can therefore provide a comprehensive evaluation of the evolutionary history of a genome and the relationships between taxa.

We intend to study the map distribution of these markers, in order to determine whether some elements occur in specific regions of the barley genome. This may be of scientific interest and of potentially practical value if some elements can be assigned to chromosome regions that are poorly covered by other markers. There is also a possibility that the different elements will be differently distributed in the wild relatives of barley, such as *H. spontaneum*. Therefore these would be useful markers for marker-assisted selection, for example in those cases where alien introgressed chromosome segments need to be selectively reduced in size, or eliminated.

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