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Restriction patterns reveal origins of chloroplast genomes in *Brassica* amphiploids

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Summary. Chloroplast (ct) DNA from the three elementary *Brassica* species (*B. nigra* (L.) Koch, *B. oleracea* L. and *B. campestris* L.) and the three amphiploid *Brassica* species (*B. carinata* A. Br., *B. napus* L. and *B. juncea* (L.) Czern.) was digested with fifteen restriction endonucleases. In all species restriction sites for enzymes with GC-rich recognition sequences were less frequent and not as variable as for those with AT-rich sequences. Comparisons between species revealed two distinct groups of ct DNA fragment patterns: complex one, composed of *B. oleracea, B. napus, B. campestris* and *B. juncea* and complex two, composed of *B. nigra* and *B. carinata.* The patterns of *B. carinata* were virtually identical to those of *B. nigra* and those of *B. juncea* were virtually identical to those of *B. campestris* indicating not only where the ct genomes of *B. carinata* and *B. juncea* originated, but also how little these genomes have been altered since the origin of these amphiploids. Ct DNA in *B. napus* shows more homology with that of *B. oleracea* than with that of *B. campestris,* but the ct genome of this amphiploid has diverged more from that of its putative parent than have those of the other two amphiploids.

Key words: *Brassica* – Chloroplast DNA – Maternal inheritance – Amphiploids

Introduction

Electrophoretic patterns of organelle DNA digested with restriction endonucleases have become a valuable tool for comparing cytoplasms of higher plants. Studies incorporating this tool have, for example, established the strict maternal inheritance of chloroplast (ct) and mitochondrial (mt) DNA in certain species (Conde et al. 1979; Frankel et al. 1979; Vedel et al. 1981) and provided evidence for recombination of organelle DNA in parasexual hybrids (Belliard et al. 1979). Variations in restriction patterns of ct and mt DNA occur within species (Levings and Pring 1977; Rhodes et al. 1981) and are quite marked in some instances of maternally-inherited mutations (Pring et al. 1979). Diversity in organelle DNA has also been demonstrated between races of corn (Timothy et al. 1979), populations of tobacco (Scowcroft 1979) and species of wheat (Vedel et al.

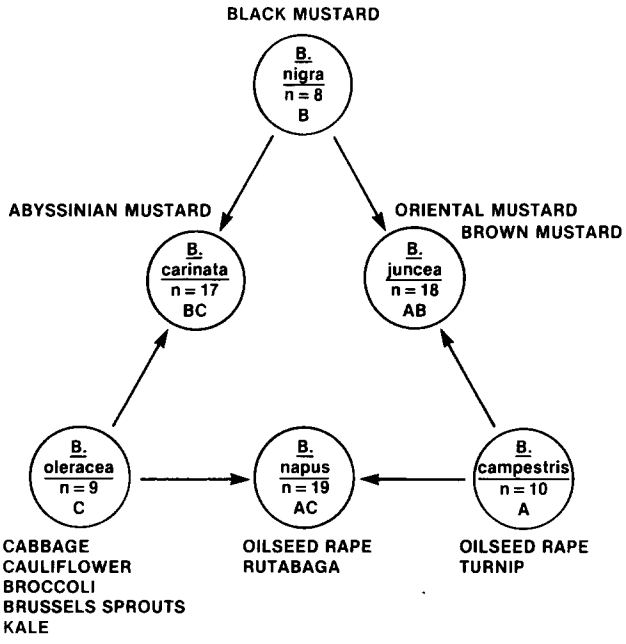

Fig. 1. Nuclear genomic relationships in *Brassica.* Arrows indicate origins of these genomes in amphiploids (adapted from U 1935). Included are some common names and crops associated with each species

1981). Recently Kung et al. (1982) used Sma I digests of ct DNA to construct a phylogeny of the ct genomes in *Nicotiana.*

To date such studies in *Brassica* have been very limited. Lebacq and Vedel (1981) reported that the Sal I restriction patterns of ct and mt DNA of *B. oleracea, B. napus, B. campestris, B. juncea, B. nigra* and *B. carinata* were simpler than those in other genera. Although the patterns generally supported the conventional taxonomic classification within *Brassica,* they could not indicate the cytoplasmic origin of the three amphiploid species. According to the triangle of U (1935) *B. nigra, B. oleracea* and *B. campestris* (n = 8, 9 and 10, respectively) are the elementary *Brassica* species and *B. carinata, B. juncea* and *B. napus* (n = 17, 18 and 19, respectively) are amphiploids derived from hybridization between these elementary species (Fig. 1). A review of taxonomic and cytogenetic studies of *Brassica* species (Prakash and Hinata 1980) reveals that the cytoplasm of this important genus is relatively unexplored.

We report here a comparison of *Brassica* cytoplasms based on restriction patterns of ct DNA with 15 enzymes. The patterns divide the genus into two broad groups and clearly indicate the origins of the ct genomes in the amphiploids.

Materials and methods

Two cultivars or accessions were analysed in each of *B. nigra, B. carinata* and *B. juncea* and three (one from each of cabbage, Brussels sprouts and kale) in *B. oleracea.* Twelve cultivars were analysed in *B. napus* (11 oilseed rape and one rutabaga) and six in *B. campestris* (five oilseed rape and one mustard cabbage). The oilseed rape cultivars originated from Canada, Europe, the Soviet Union, China and Japan.

Extraction of DNA

Chloroplast DNA was isolated according to the method of Tewari (1979) with modifications. Leaves (usually 300 g), harvested from 4–6 weeks-old plants following a dark period of 24–48 h, were homogenized in 100 g batches, each batch in 400 mls of buffer A (0.33 M sorbitol/50 mM Tris/3 mM EDTA/0.1% BSA, pH = 8.0) and filtered trough 4 layers of cheesecloth and 2 layers of Nitex® screen (20 μ). The extraction up to the point of lysis was carried out on ice. The filtrate was centrifuged at 2000 × g for 2 min at 4 °C and the crude chloroplast pellet was suspended in buffer A to a total volume of 60 mls (for 300 g of leaves), adjusted to 0.01 M $MgCl_2$ and digestd with DNAse I (Sigma), 100 μg/ml, for 1 h at 4 °C. DNAsing was terminated by adding 180 ml buffer B (0.3 M sucrose/50 mM Tris/50 mM EDTA, pH = 8.0) and centrifugation at 2,000 × g for 2 min in 2 × 250 ml bottles. The pellet was similarly washed two more times with buffer B at a total volume of 180 ml. The final pellet was suspended in 10–15 mls of 0.05 M Tris/0.02 M EDTA (pH = 8.0) and made 0.2 M NaCl and 2% SDS. The preparation was gently rocked at room temperature to disperse clumps and an equal volume of phenol saturated with 0.2 M Tris (pH = 8) was added. Further rocking was followed by the addition of 2–5 mls of chloroform : isoamyl alcohol (24 : 1), more rocking and centrifugation at 9000 × g. The clear supernatant was twice extracted in the same manner with equal volumes of chloroform : isoamyl alcohol and the DNA precipitated by adding 2–1/2 volumes of cold absolute ethanol and storing overnight at −20 °C. The precipitate was pelleted and dissolved in 200 μl of distilled water. Frequently DNA prepared in this manner could not be restricted without subsequent digestion with proteinase K (500 μg/ml, 10 mM Tris, pH = 7.5) at 40 °C for 1 h followed by extraction with phenol/chloroformisoamyl alcohol as above.

Occasionally the DNA co-precipitated with a viscous, yellow liquid which was removed with hydroxyapatite (HAP). Typically, 1 g of DNA-grade Biogel® (Bio-Rad) was suspended in PBI (0.01 M phosphate, pH = 6.8), loaded on a Pharmacia® PD-10 column and equilibrated with PUB2 (0.24 M phosphate/8.0 M urea/0.8% SDS, pH = 6.8). The DNA preparation was mixed with 8 volumes of PUBl (0.27 M phosphate/9.0 M urea/0.9% SDS, pH = 6.8) and stirred into the HAP. The column was washed with several volumes of PUB2 followed by 2–3 volumes of PB1. The DNA was eluted with 1 volume of PB2 (0.4 M phosphate, pH = 6.8), dialysed against 0.2 M NaCl and ethanol precipitated.

Analysis of DNA

Chloroplast DNA was digested as per suppliers' instructions with the following enzymes: Eco R1, Hind III, Pst I, Sal I, Sma I and Sph I (Boehringer Mannheim); Bam HI, Bcl I, Bgl II, Cla I, Kpn I, Pvu II, Sst I, Xba I and Xho I (Bethesda Research Laboratories). Digested samples were electrophoresed in submerged gels of 0.5 or 0.7% agarose in a Tris- acetate buffer (40 mM Tris and acetate/1 mM EDTA, pH = 7.8) Gels were stained for 1 h with ethidium bromide (0.5 μg/ml) and destained for 1/2 h. For each enzyme a pattern representative of a species or group of species was given a number starting from one at *B. oleracea* and moving counter- clockwise around the triangle of U.

Results and discussion

Two distinct and species-unrelated groups of restriction patterns were observed. One group had few fragments, showed very little variation between species and came from digests with enzymes containing G or C in at least four of the six nucleotides of the recognition sequence (Table 1; Fig. 2). Few fragments might be expected from such enzymes since the GC content of chloroplast DNA in higher plants ranges from 37 to 44% (Edelman 1981). The enzyme Bam Hl is an exception in this group in that its patterns have many fragments.

The simple pattern of these enzymes suggests that their sites occur in highly conserved, GC-rich regions of the genome. That such regions exist in chloroplast DNA of higher plants is demonstrated by sequence data (Dyer and Bowman 1979; Wildeman and Nazar 1980) and hybridization studies (Thomas and Tewari 1974) of rRNA.

The other group of restriction patterns had many fragments in all species and showed variation between and within species (Table 1; Figs. 3 and 4). Enzymes producing the more complex patterns have A or T in four of the six recognition sequence nucleotide pairs.

An examination of the differences in restriction patterns between species (Table 1; Figs. 2–4) reveals that there are two broad groups of ct DNAs in *Brassica; B.*

Table 1. Classification of electrophoretic patterns of chloroplast DNA extracted from six *Brassica* species and digested with 15 restriction endonucleases

Enzyme	Restriction patterns and species of *Brassica*					
	oleracea n=9	*napus* n=19	*campestris* n=10	*juncea* n=18	*nigra* n=8	*carinata* n=17
Sph I	1[a]	1	1	1	1	1
Sst I	1	1	1	1	1	1
Sal I	1	1	1	1	2	2
Xho I	1	1	1	1	2	2
Sma I	1	1	1	1	2	2
Kpn I	1	1	1	1	2	2
Pvu II	1	1	1	1	2	2
Xba I	1	1	2	2	3	3
Eco RI	1	1	2	2	3	4
Bam HI	1	1	2	2	3	3
Hind III	1	1	2	2	3	3
Pst I	1	2	2	2	3	3
Bgl II	1	2	2	2	3	3
Cla I	1	2	1	1	3	3
Bcl I	1	2	1	1	3	3

[a] For each enzyme a pattern is given a number (starting arbitrarily with one at *B. oleracea* and moving counter-clockwise around the triangle of U) and species which share the same pattern have the same number for this enzyme

oleracea, *B. napus*, *B. campestris* and *B. juncea* form one group, which we designate as complex one, and *B. nigra* and *B. carinata*, constitute complex two. Differences do occur within these two complexes but the differences within complexes are considerably less frequent than differences between them. This classification of ct DNA is supported by results from other research. Studies of chromosome pairing in hybrids between the three basic species (*B. nigra*, *B. campestris* and *B. oleracea*) by Mizushima (1950) caused him to conclude that *B. oleracea* and *B. campestris* are more closely related to each other than either is to *B. nigra*. The morphological classification of Schulz (1919) and chemotaxonomic studies of seed proteins by Vaughan et al. (1966) also lend support to a bipartite division of the ct genome in this genus. Thus, in *Brassica*, species relationships based on chromosome structure, morphology and seed proteins are reflected in the restriction patterns of ct DNA. Similarly a co-evolution of cytoplasmic and nuclear genomes in the races of maize has resulted in agreement between classifications based on conventional taxonomic criteria and those based on restriction patterns of ct and mt DNA (Timothy et al. 1979).

Contrary to the findings of Lebacq and Vedel (1981), Sal I fragments six and seven (M.W. 4.5 and 2.2×10^6, respectively) were absent in our digests of *B. oleracea* DNA. In our examination of 23 cytoplasms in complex one with 15 enzymes we could not detect a difference in genome size as large as 6.7×10^6. This discrepancy may be due to cultivar differences or, since the combined weight of fragments six and seven equals exactly that reported for fragment four, the cultivar they examined may have included a mixture of plants containing two different cytoplasms, one of which contained a Sal site in that fragment of the ct genome.

Of particular interest are the restriction patterns from the three amphiploid species, *B. napus*, *B. juncea* and *B. carinata*. With two exceptions the patterns of *B. carinata* and *B. juncea* were identical to those of *B. nigra* and *B. campestris*, respectively (Table 1; Figs. 2–4). In one exceptional case the Eco RI digests of both accessions in *B. carinata* were slightly different from those of *B. nigra*, but still much more different from those of *B. oleracea*. In the other, the Bgl II pattern of a *B. juncea* cultivar is slightly different from that of *B. campestris*. This indicates that the ct genome of *B. carinata*

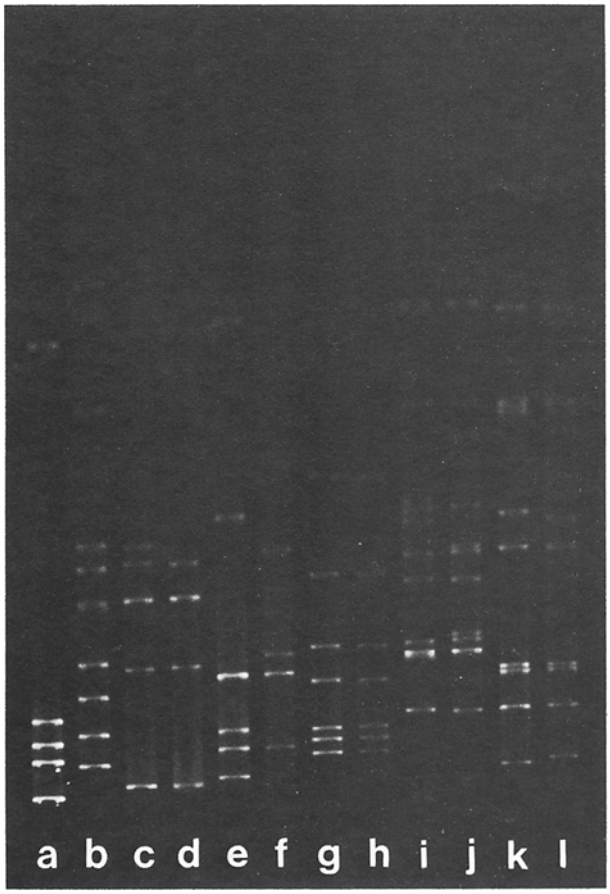

Fig. 2. Representative restriction patterns of chloroplast DNA digested with the first seven enzymes of Table 1. In lanes a and b *B. napus* DNA is digested with Sph I and Sst I respectively. The 10 remaining lanes consist of five pairs of digests of *B. napus* and *B. carinata* DNA in the following order: Sal I (*c, d*), Sma I (*e, f*), Kpn I (*g, h*), Xho I (*i, j*), and Pvu II (*k, l*) with the *B. napus* digest being the first of each pair. Sph I and Sst I show no differences betweeen species whereas the other five enzymes divide the species of the genus into complex 1 (represented by *B. napus*) and complex 2 (represented by *B. carinata*)

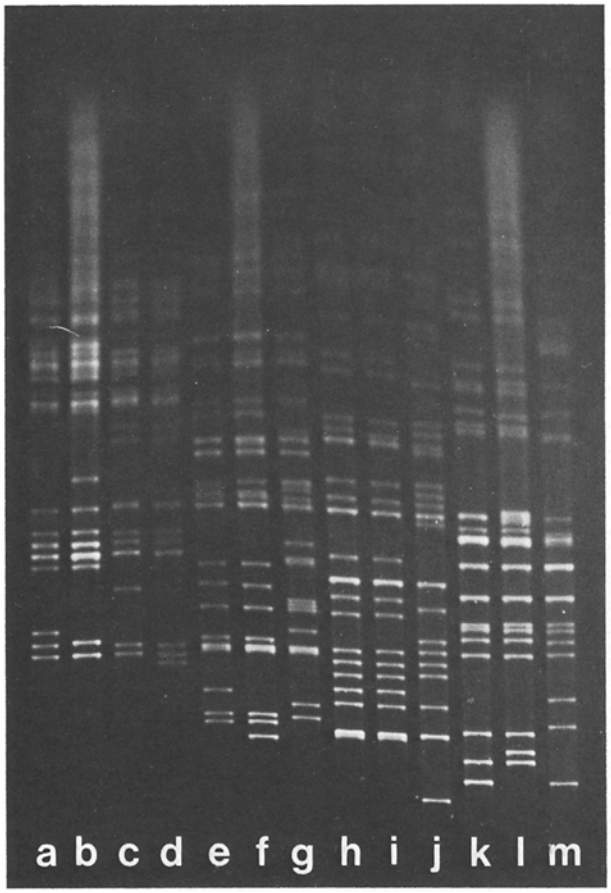

Fig. 3. Patterns for Eco Rl (*a–d*), Bam Hl (*e–g*), Hind III (*h–j*) and Xba I (*k–m*) show not only the major differences between the two complexes, but also differences within these complexes, in particular those between *B. napus* and *B. campestris*. Lanes *a, e, h* and *k* contain digests of *B. napus* DNA, lanes *b, f, i* and *l, B. campestris* DNA and lanes *d, g, j* and *m, B. carinata* DNA. The only variation found within complex 2 is illustrated in lanes *c* (*B. nigra*) and *d* (*B. carinata*)

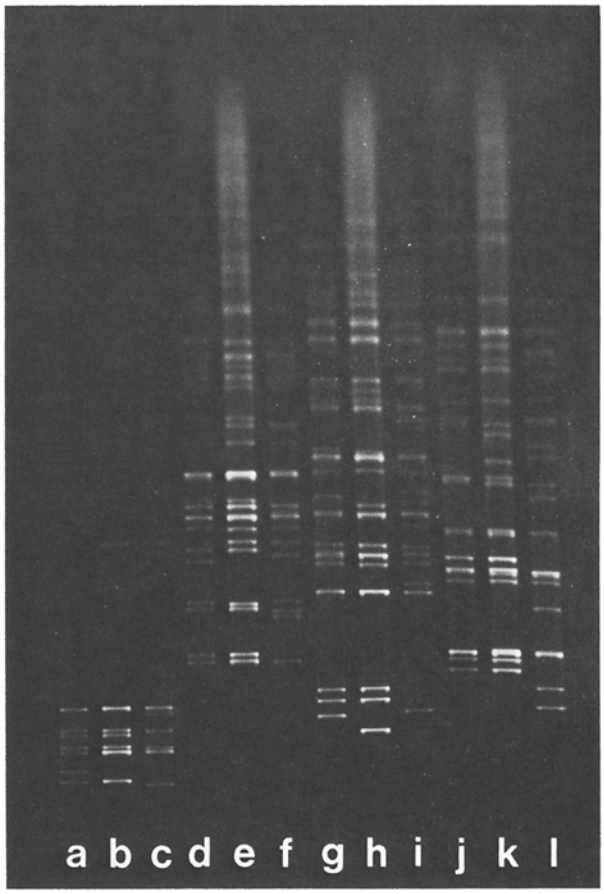

Fig. 4. Alterations in the chloroplast DNA of *B. oleracea* (lanes *a, d*) have been detected with Pst I (*a–c*) and Bgl II (*d–f*) and alterations which have occurred in *B. napus* but not in *B. oleracea* or *B. campestris* have been detected by Bcl I (*g–i*) and Cla I (*j–l*). Digests are as follows: *B. oleracea* (*a, d*), *B. napus* (*b, g, j*), *B. campestris* (*e, h, k*) and *B. carinata* (*c, f, i, l*)

is derived from *B. nigra* and that of *B. juncea* from *B. campestris* and that the ct genomes of these two amphiploids may have evolved little since their time of origin.

Remarkably, however, the data for *B. napus*, the third amphiploid, is different and more complex. Of the 12 cytoplasms examined, those of three oilseed rape varieties ('ATR-5Tw', 'Bronowski' and 'Isuzu-natane') gave restriction patterns identical to those of *B. campestris* with all 15 enzymes. 'ATR-5Tw' was derived by backcrossing the *B. napus* cultivar 'Tower' onto a wild triazine resistant biotype of *B. campestris* as female (Beversdorf et al. 1980). The consistent *B. campestris* pattern of ct DNA in ATR-5Tw demonstrates for the first time, to our knowledge, the strict maternal inheritance of ct DNA in this genus. Certain seed quality characteristics of 'Bronowski' suggest that it also originated from the same interspecific cross, a process in which it could have acquired a *B. campestris* cytoplasm (K. Downey, pers. commun.). The *B. campestris* patterns of 'Isuzu-natane' are not surprising given the history of breeding oilseed rape in Japan. The introduction of *B. napus* to that country at the end of the last century, followed by extensive hybridization of that species with the already well-established cultivars of *B. campestris*, explain why more than 60% of the registered Japanese oilseed rape cultivars are from interspecific crosses (Namai et al. 1980). As well, the *B. campestris* patterns of 'Bronowski' and 'Isuzu-natane' ct DNA are consistent with the roles these varieties play in cytoplasmic male sterility in *B. napus* (Erickson et al., in preparation).

The patterns of the other nine *B. napus* cultivars (eight oilseed rape and one rutabaga) and both of the two possible parental genomes (*B. oleracea* and *B. campestris*) were identical with seven enzymes (Fig. 2). However, with the remaining eight enzymes three sets

of patterns arose: in one set (Fig. 3) with Bam Hl, Eco Rl, Hind III and Xba I all nine *B. napus* cultivars were the same as those of *B. oleracea* and both differed from *B. campestris*; in another set (Fig. 4) with Bgl II and Pst I the *B. napus* cultivars resembled those of *B. campestris* and both species differed from *B. oleracea*; in the third set (Fig. 4) with Bcl I and Cla I *B. oleracea* and *B. campestris* appeared to be identical and both differed from *B. napus*. It is important to note that these nine cultivars behaved as a group with each enzyme and in no case did one cultivar resemble *B. campestris* and another, *B. oleracea* with a given enzyme. Depending on the enzyme the ct DNA of these nine cultivars resembled that of either *B. oleracea* or *B. campestris* or was different from both. This is evidence of a *B. napus* ct genome distinct from that of *B. oleracea* and *B. campestris* but sharing homologies with both.

In the remainder of this report we refer to the ct genome of these nine cultivars as that belonging to *B. napus*. The uniformly *B. campestris* patterns of the other three *B. napus* cultivars are probably due to their origins in *B. campestris* × *B. napus* crosses which occurred long after *B. napus* had evolved as a separate species. Because this cross is much easier than *B. oleracea* × *B. napus*, it is likely that other *B. napus* cultivars, aside from those derived by somatic cell fusion, will be found to have *B. campestris* ct genomes, but that very few will be found to have *B. oleracea* ct genomes.

The four sets of *B. napus* restriction patterns based on 15 enzymes, can be presented symbolically as follows, where O, C and N represent the ct DNA patterns of *B. oleracea*, *B. campestris* and *B. napus* respectively:

Set 1 : O = N = C (seven enzymes, Fig. 2)
Set 2 : O = N + C (four enzymes, Fig. 3)
Set 3 : O + N = C (two enzymes, Fig. 4)
Set 4 : O = C + N (two enzymes, Fig. 4).

The large degree of homology between the ct genomes of the three species is apparent not only from the identical patterns of set one, but also from those of the other sets where the differences between species involve only one or two bands (Figs. 3 and 4). However, the *B. napus* banding patterns conform more often to those of *B. oleracea* (set two) than to those of *B. campestris* (set three). This suggests that *B. napus* ct DNA derived from *B. oleracea*. The differences between *B. napus* and *B. campestris* (set two) would thus be due to divergence between *B. oleracea* and *B. campestris* in the pre-*B. napus* period and to alterations in *B. campestris* during the *B. napus* period. If this hypothesis is correct, the differences in patterns of set three could be most simply explained as resulting from alterations in the *B. oleracea* genome in the *B. napus* period and those of set four, from alterations in the *B. napus* genome in the same period.

In summary, by means of restriction patterns we have been able to characterize and determine relationships among the ct genomes of *Brassica* species. Differences in these patterns are useful not only for determining cytoplasmic diversity and evolution but also for detecting and locating recombinations within the ct genome following transformation or fusion experiments.

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