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Application of the RLGS Method to Large-Size Genomes Using a Restriction Trapper

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Abstract

We developed a method for producing restriction landmark genomic scanning (RLGS) profiles of large-size genomes, such as those of higher plants or amphibians using a restriction trapper. Use of the conventional RLGS method is limited to genomes smaller than 3×10^9 bp, because the larger genomic DNAs, especially those of more than 1×10^{10} bp, produce high background due to incorporation of radioactivity at non-specifically damaged sites. Our new method reduces the background levels by reducing genome complexity to 1/200–1/300 using a purification step to enrich DNA fragments carrying specific restriction landmarks at their ends using a restriction trapper. This step makes it possible to obtain RLGS patterns of larger genomes. Our paper describes the practical application for the RLGS method using a restriction trapper with the pine tree genome (3×10^{10} bp/haploid genome; *Pinus koraiensis* Sieb. et Zucc.) as an example.

Key words: restriction landmark genomic scanning (RLGS); restriction trapper; large-size genome; *Pinus koraiensis*

Restriction landmark genomic scanning (RLGS) was developed as a high-speed genome scanning method based on the concept that the cleavage sites of restriction enzymes can be used as landmarks.[1,2] This method employs the direct labeling of genomic DNA and high-resolution two-dimensional electrophoresis. One of the many advantages of the RLGS system is its applicability to genomes of all organisms because direct labeling, not hybridization or polymerase chain reaction (PCR), is employed as the detection system. Recently, a new genetic mapping method using RLGS was developed (RLGS spot mapping) which could theoretically be used for all types of genomes.[3] However, use of the conventional RLGS system is limited to genomes smaller than about 3×10^9 bp, because the larger ones, such as those of higher plants or amphibians, produce high background due to the incorporation of radioactivity at non-specifically damaged sites. Generally, in proportion to the genome size, the number of radioactive molecules from a single locus is reduced when a constant amount of genomic DNA is used as the starting material, although the background does not change. Therefore, a spot from a single copy locus would be masked by the background levels. To apply the RLGS system to the human or mouse genome which is about 3×10^9 bp, we developed a blocking technique and employed a DNA polymerase with no 3′ exonuclease activity, such as Sequenase Ver. 2.0 (USB, U.S.A.), for the labeling step.[1,2,4] However, these improvements were not enough for the larger genomes, especially those larger than 1×10^{10} bp. The maximal amount of DNA samples which can be subjected to RLGS is limited to 1 μg per one gel to prevent the "tailing" of spots and the signal/noise (S/N) ratio is low with these genomes. Therefore, even if the films are exposed for a long time and a sufficient amount of labeled sample is loaded onto the gel, the problem of background noise can not be solved.

Recently, we developed a new method for purifying DNA fragments with specific enzyme sites at their ends using a restriction trapper.[5,6] This purification step eliminates the unlabeled DNA fragments regardless of their fragment size, allowing the cloning of DNA fragments eluted from the punched-out gel. As the background of the RLGS pattern is generated by non-specific incorporation of radioactivity into DNA fragments which do not

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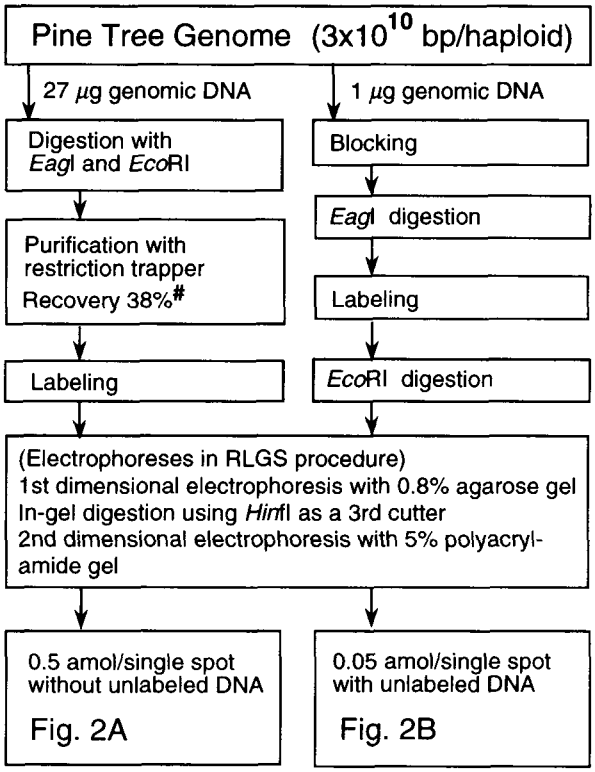


Figure 1. Protocols of the RLGS method for pine tree DNA with/without restriction trapper treatment.

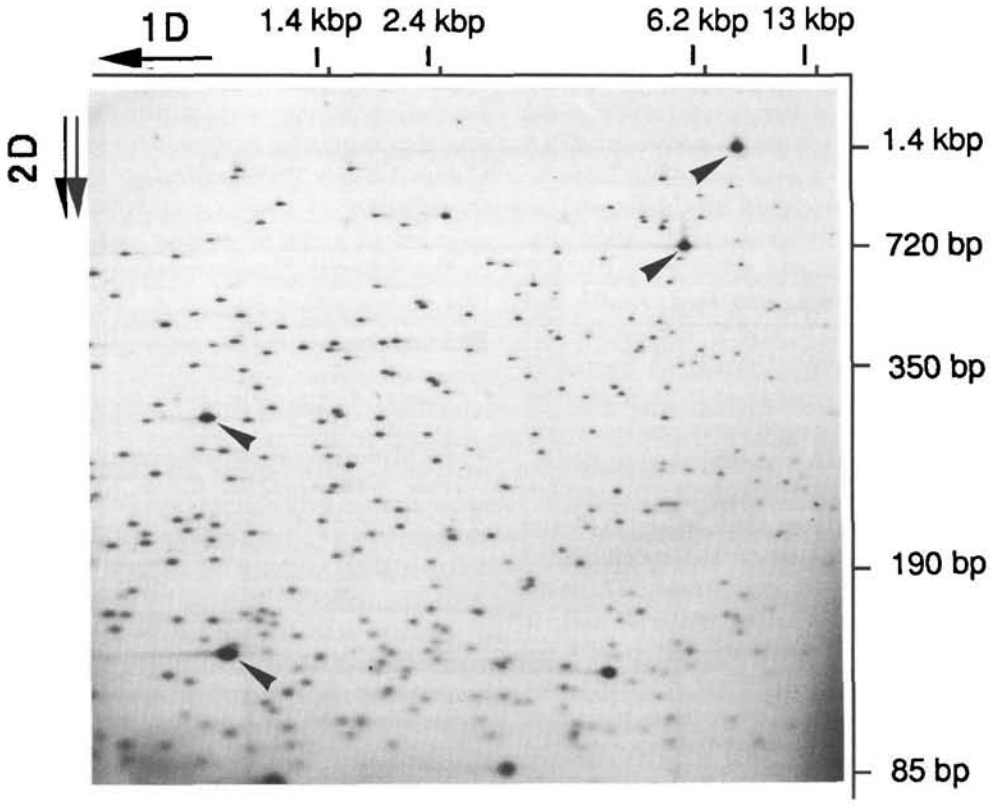
DNA preparation: Megagametophyte tissue was frozen and crushed to a powder at −196°C in liquid nitrogen. A 2-ml solution of 0.025% (w/v) Proteinase K (Merck, Germany), 10 mM Tris-HCl pH 8.0, 1% SDS, and 150 mM EDTA was added to the crushed powder and the sample was incubated at 55°C for 2 h. The sample was subsequently extracted with PCI solution (phenol/chloroform/isoamyl alcohol, 50:48:2) twice and was dialyzed against TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) buffer. After dialysis, the sample was treated with 1 μg/ml of RNase, and the DNA was recovered by ethanol precipitation. The DNA precipitant was resuspended in 50 μl of TE buffer. Twenty-seven μg of DNA prepared from one megagametophyte was subjected to pretreatment with restriction trapper and the subsequent RLGS procedure (Fig. 1 and 2A). One μg of the same DNA sample without restriction trapper treatment was subjected to the conventional RLGS to obtain a control pattern (Fig. 1 and 2B).

Purification of EagI fragments using restriction trapper: *EagI* restriction trapper was prepared by Japan Synthetic Rubber Co., Ltd. and Nippon Roche K. K. as described elsewhere.[5] The final stored mixture of restriction trapper was adjusted to 10% (w/v) in a buffer containing 10 mM Tris-HCl pH 7.5, 1 mM EDTA, and 0.1% SDS (suspension buffer) and was stored at 4°C. The precise protocol for the purification method of DNA fragments using restriction trapper is also described elsewhere.[5] Briefly, 5 μl of 10% (w/v) restriction trapper (with 40 pmoles of hairpin linker fixed on the surface of beads) was washed twice with TS buffer (10 mM Tris-HCl pH 7.5, 250 mM NaCl) as pretreatment to exclude SDS. Twenty-seven μg of *EagI* - *EcoRI* digested genomic DNA was ligated to the *EagI* restriction trapper with 350 units of T4 ligase at 18°C for 2 h in 160 μl of ligation buffer. After inactivation of the ligase by heating at 65°C, the latex beads were washed twice with TS buffer. These beads were then treated with 25 units of *EcoRI* in 200 μl of high buffer (100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT) to cleave and remove the *EcoRI*-*EcoRI* ligated fragments. After washing twice with TS buffer, the target fragments (*EagI*-*EcoRI* fragments) were eluted by digestion with 20 units of *EagI*. After centrifugation, the supernatant was subjected to phenol extraction and ethanol precipitation. The precipitant was dissolved in 20 μl of distilled water, and the purified DNA was end-labeled at the *EagI* site by filling reaction with Sequenase Ver. 2.0 in the presence of [α-^{32}P]dGTP and [α-^{32}P]dCTP, according to a method described elsewhere.[2] *HinfI* was used for in-gel digestion before the second dimensional polyacrylamide gel electrophoresis. #, see reference 5.

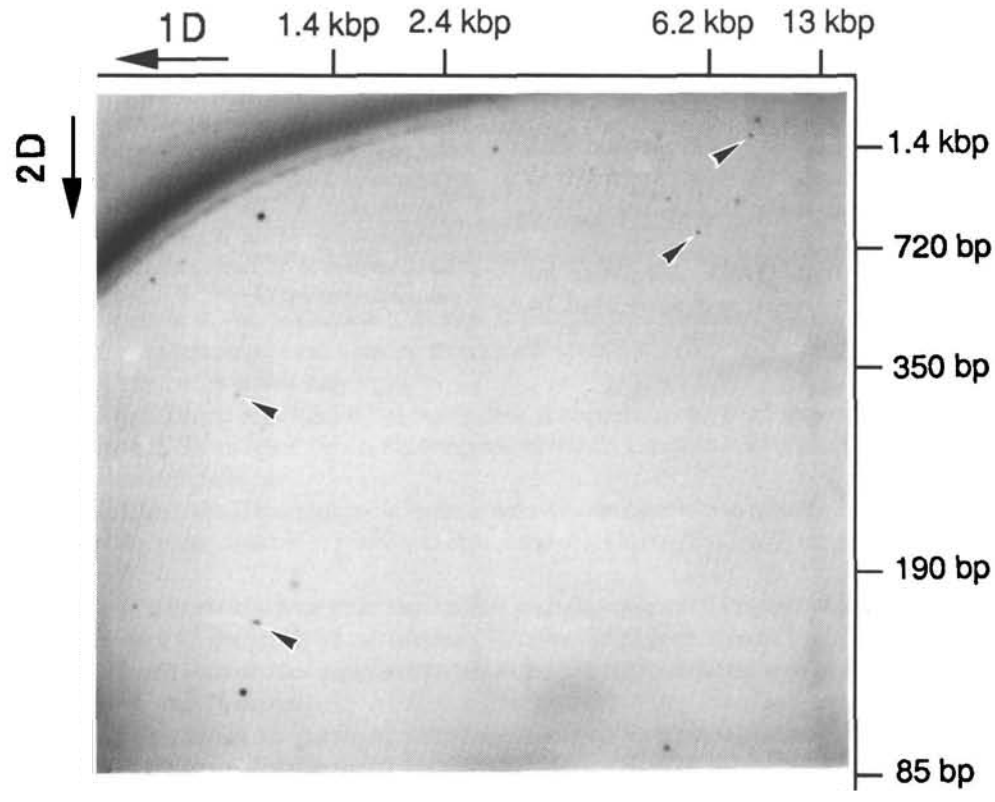
carry restriction landmark sites, background noise should be reduced. Thus, our purification procedure using a restriction trapper should be useful for producing clear RLGS patterns of genomes larger than 1×10^{10} bp.

The principle and precise protocol of the RLGS method have been described elsewhere.[1,2] Figure 1 shows the standard procedure for RLGS with purification by a restriction trapper and for conventional RLGS. The RLGS profile of a large-size genome (pine tree, 3×10^{10} bp; *Pinus koraiensis* Sieb. et Zucc.) with restriction trapper treatment and its comparative profile produced by the conventional method are shown in Fig. 2A and 2B,

Figure 2. RLGS patterns of "Ohtaki 121", plus tree of *Pinus koraiensis* Sieb. et Zucc. with/without restriction trapper treatment. The arrowheads indicate the spots which correspond to multicopy DNA fragments. Twenty-seven μg and 1 μg of pine tree DNA were used as starting materials in A and B, respectively. A: RLGS pattern of pine tree genomic DNA with restriction trapper treatment. B: RLGS pattern of the same genomic DNA as A produced by the conventional RLGS method.



A: Restriction Trapper (+), 3 days



B: Restriction Trapper (−), 42 days

respectively. In the RLGS pattern without restriction trapper treatment (Fig. 2B), clear spots can not be seen because the spots from the single-copy loci were hidden by the background and only a few spots (arrowheads) could be detected which correspond to multicopy DNA fragments by autoradiography for 42 days (Fig. 2B).

On the other hand, the spots could be clearly and reproducibly seen in the pattern with the purification step (Fig. 2A). About 350 spots on one gel can be detected by autoradiography for 3 days by using *Eag*I, *Eco*RI, and *Hin*fI, as restriction enzyme A, B, and C, respectively.[1,2] Thus, almost all non-specifically labeled DNA fragments were eliminated and the copy number of DNA fragments on each spot from the specific single locus was increased when a constant amount of DNA was subjected to RLGS. This dramatic reduction of background is due to the high specificity of the purification (more than 10^6-fold) by the restriction trapper, which is based on the specificity of the restriction enzyme.[5] In this experiment, 27 μg of genomic DNA was used as a starting material and, it has been reported that in the purification step with restriction trapper, 38% of the total *Eag*I fragment can be recovered.[5] As the pine tree genome size is ten times larger than those of mammals, we tried loading onto the gel the *Eag*I fragments from pine tree DNA whose copy number is equivalent to that produced from 1 μg of human genome.

Our data demonstrate that RLGS with a restriction trapper can be applied to genomes of all organisms without size limitations. A high-speed genome mapping method, RLGS spot mapping, has recently been developed,[3] and a newly imprinted gene in mice has been discovered using RLGS with methylation-sensitive restriction enzyme (RLGS-M).[7] The RLGS method with restriction trapper treatment offers a novel approach to the study of large-size genomes using RLGS-based DNA analyses.

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