Jour. Korean For. Soc. 89(4) : 536 - 542. 2000
韓國林學會誌 89(4) : 536 - 542. 2000

Linkage Analysis of both RAPD and I-SSR Markers using Haploid Genome from a Single Tree of *Pinus densiflora* S. et Z.[1]

Yong-Pyo Hong[2], Jae-Min Chung[2], Yong-Yul Kim[2] and Suk-Seong Jang[2]

소나무 單一 母樹의 半數體 게놈을 利用한 RAPD 및 I-SSR 標識子의 連關分析[1]

洪鎔杓[2] · 鄭載珉[2] · 金龍律[2] · 張錫成[2]

ABSTRACT

A linkage map for Japanese red pine (*Pinus densiflora*) was constructed on the basis of two DNA marker systems of random amplified polymorphic DNAs (RAPDs) and inter-simple sequence repeats (I-SSR). Haploid genomic DNAs were extracted from megagametophyte tissues of 96 individual seeds in a single tree. A total of 98 DNA markers including 52 RAPD markers amplified by 25 primers and 46 I-SSR markers amplified by 18 primers were verified as Mendelian loci showing 1 : 1 segregation in 96 megagametophytes which were χ^2-tested at 5% significance level. Of them, 63 segregating loci turned out to be linked into 20 linkage groups by the two-point analysis. However, 35 loci (17 RAPD and 18 I-SSR) of the 98 segregating loci did not coalesced into any linkage groups at a LOD of 3.0. The linked 63 loci were separated by an average distance of about 25.5 cM, which were spanned 1097.8 cM as a whole. The minimum and maximum map distances of the linkage groups were 4.3 cM and 54.9 cM, respectively. Incorporation of I-SSR loci into linkage map of RAPD loci resulted in extended and partially more saturated linkage blocks.

Key Words : Pinus densiflora S. et Z., *Genetic linkage map, Random Aamplified Polymorphic DNAs (RAPDs), Inter-Simple Sequence Rrepeats (I-SSRs), Haploid genome.*

要 約

소나무 단일개체에서 채취한 풍매종자 중 임의로 선택한 96개의 반수체 genome을 이용하여 RAPD 및 I-SSR PCR 증폭산물을 분석하였다. RAPD 분석용 primer 200개와 I-SSR 분석용 primer 90개를 screen하여 증폭산물의 분획양상이 선명한 RAPD primer 45개와 I-SSR primer 22개를 선택하여 PCR을 수행하였다. 45개의 RAPD primer중 25개와 22개의 I-SSR primer중 18개를 사용한 PCR 분석결과에서 멘델의 유전양식을 만족하는 52개 (2.08/primer)와 46개 (2.56/primer)의 다형성 유전자좌를 각각 확인하였다. 멘델의 유전양식을 만족하는 96개의 다형성 유전자좌를 대상으로 LOD 3.0에서 two-point 연관분석을 수행한 결과 총 63개(35개의 RAPD와 26개의 I-SSR)의 유전자좌가 20개의 연관군에 속하는 것이 확인되었다. 총 연관거리는 1097.8 cM이었으며, 유전자좌간 평균연관 거리는 25.5 cM, 최소 및 최대연관 거리는 각각 4.3 cM 및 54.9 cM이었다. 그리고 20개의 연관군 중 14개의 연

[1] Received on August 24, 2000.
[2] Division of Genetics & Physiology, Korea Forest Research Institute, Forestry Administration, Suwon 441-350, Korea
산림청 임업연구원 유전생리과.

관군이 RAPD와 I-SSR 유전자좌의 통합에 의해서 형성된 연관군이었다. 즉, 52개의 RAPD와 46개의 I-SSR 유전자좌를 각각 분석한 결과보다 길고 새로운 연관군이 형성되었다. 보다 정밀한 유전자 연관지도를 작성하기 위해서는 보다 많은 수의 DNA marker가 필요하다고 판단되며, 본 연구의 결과는 소나무의 유용 유전자의 확인 및 생장과 재질과 같은 유용형질에 대한 QTL의 위치를 결정하는데 기초자료가 될 것이다.

INTRODUCTION

Japanese red pine (*Pinus densiflora* S. et Z.), showing wide distribution throughout the northeastern Asia, is one of the major indigenous pines and of the important forest resources in Korea. Japanese red pine has been known to show high levels of genetic diversity and morphological variation such as height, bark color, main stem length, and crown shape among populations in south Korea (Kim and Lee, 1992; Kim et al., 1995).

Since 1959, various breeding programs have been applied to the improvement of this species, which has been mainly relied on the selection of plus trees showing desirable phenotypes such as superior wood quality and superior height and volume growth. However, most of the phenotypic traits are known to be controlled by more than one gene, namely polygenic and quantitative traits, whose expression is also affected by different environments so called, genetic by environmental interaction. Therefore, when a selection is performed mainly based on the phenotypic data, the well-defined and complicated statistical designs are required to estimate the genetic parameters for the practice of breeding such as breeding value. Furthermore, to estimate such genetic parameters for forest trees which are long-lived plants with long generation time, it requires long time and large running expenses.

A solution of saving time and costs for tree breeding is marker assisted selection in which individuals, containing genetic markers tightly linked to the genes controlling quantitative traits, are selected directly in the early stage of tree breeding programs. To perform the effective marker assisted selection, identification of the genetic markers linked to the target traits for breeding is prerequisite, which could be accomplished by the construction of saturated linkage map with both genetic markers and quantitative traits linked loci. Recent development of various molecular genetic marker systems allowed such approach to be proceeded. For forest tree species, major efforts on preparing genetic linkage map of DNA markers have been devoted to conifers (Binelli and Bucci, 1994; Devey et al., 1994, 1996; Gocmen et al., 1996; Jermstad et al., 1998; Kaya and Neale, 1995; Kubisiak et al., 1995, 1996; Lee, 1998; Mukai et al., 1995; Neal et al., 1994; Nelson et al., 1993; Tsumura et al., 1993; Yazdani et al., 1995) on account of the advantage of having segregating haploid genomes of megagatophytes which has the same maternal genetic complement as the embryo contained in the same seed. By analyzing a large number of magagemetophytes from a single tree, segregation of the DNA variants and recombination between verified Mendelian loci can be tested directly without the need for controlled pollinations (Guries et al., 1978; Tulsieram et al., 1992; Kubisiak et al., 1995).

In this study, we intended to construct a single tree linkage map of a plus tree of Japanese red pine (Kyungbuk No. 5) with two DNA marker systems of random amplified polymorphic DNAs (RAPDs) and inter-simple sequence repeats (I-SSRs), which could be served as a template for the future mapping of quantitative traits linked loci.

MATERIALS AND METHODS

1. DNA extraction

Seeds were collected from a single tree of Kyungbuk No. 5 which was ranked as the highest one on the basis of evaluation for both growth and specific gravity of the selected 58 families of

Japanese red pines in Korea. Haploid genomic DNAs were extracted from megagametophyte tissues of 96 individual seeds by a modified CTAB procedure (Hong et al., 1998). The amount of DNA was directly quantified with DynaQuant™ 200 (HOEFER Phamacia Biotech Inc.).

2. RAPD and I-SSR marker assay

For RAPD analysis, 20$\mu\ell$ of PCR mixtures contained 5$\mu\ell$ of the template DNA(5ng/$\mu\ell$) was composed of 20mM Tris-HCl, pH 7.4 at 25℃, 1.875mM of $MgCl_2$, 0,1mM EDTA, 1mM DTT, 100mM KCl, 0.1% Triton X-100, 2mM of each dNTPs, 40ng of 10-mer primers (Operon, USA), 0.0025%(v/v) Bovine Serum Albumin (BSA), and 0.6 unit of *Taq* DNA polymerase (Advanced Biotechnologies). PCR profile with the lid-heated DNA thermal cycler PTC-200 (MJ Research, USA) was designed as follows; initial pre-denature at 94 ℃ for 5 min, followed by 45 cycles of 94℃ for 5 sec (denaturation), 36℃ for 30 sec (annealing), and 72℃ for 1 min (extension). After 45 cycles of thermocycling, a final extension at 72℃ for 10 min was performed to insure full synthesis of the target templates.

For I-SSR analysis, the total volume of reaction mixtures for PCR amplification was 20$\mu\ell$ which contained 25ng of template DNA, 0.2mM each of dNTPs, 0.0025% of BSA (Boeringer Mannheim, Germany), 5$\mu\ell$ of 1.5*u*M primers (UBC Biotec. Lab., Canada), 1.6$\mu\ell$ of 25mM $MgCl_2$, 0.6 unit of *Taq* DNA polymerase (Advanced Biotechnologies). The lid-heated DNA thermal cycler PTC-200 (MJ Research, USA) was used for PCR with the follow -ing temperature profile : 5 min at 94℃ for 1 cycle for initial denaturation; 45 cycles of 30 sec at 94℃ (denaturation), 30 sec at 52℃ (annealing). and 1 min at 72℃ (extension). A final extension was performed at 72℃ for 10 min.

Amplification products of both RAPD and I-SSR PCRs were fractionated on 2% agarose gels in 1X TBE buffer, stained with ethidium bromide, and photographed over UV transiluminator. DNA size was calculated by comparing with 100 base-pair DNA ladder (GIBCO BRL, USA) which was loaded in two separate lanes on the same gel for electro - phoresis. In order to identify primers revealing polymorphisms among megagametophyte DNAs, 150 RAPD and 100 I-SSR primers were screened with randomly selected 5 megagametophyte DNAs. Primers, revealed clear amplification products and polymorphisms in 5 megagametophyte DNAs, were selected for PCR with 96 megagametophyte DNAs. For both RAPD and I-SSR markers, variants of amplification products were observed as presence (+) versus absence(-) of the amplicon of the same size. In case of unclear resolution of the amplocons, they were treated as missing data. After computer scanning of photographs, the presence of amplicons of the same size was verified via computer image analysis (Diversity One™, *pdi*, USA).

3. Linkage analysis

An 1 : 1 segregation of the observed amplicon variants was tested via chi-square test at 5% significance level. For RAPD and I-SSR markers, verified as Mendelian loci, linkage analysis was performed using a computer program of Mapmaker /EXP 3.0 (Lincoln et al., 1992). Linked loci were assigned to groups with a LOD (logarithm of the odds ratio) score of 3.0 and maximum map distance of 50 cM (centiMorgan). Once loci were assigned to a given linkage group, the most likely marker orders within the group were determined. Orders of the loci within each linkage group were ascertained by rippling of the linked loci. Map distances were computed using the Kosambi's mapping function.

RESULTS AND DISCUSSION

Of the 200 primers for RAPD and 90 primers for I-SSR screened in the preliminary experiment, 45 RAPD primers and 22 I-SSR primers were used for PCR with 96 megagametophytes of 'Kyungbuk No. 5', a single plus tree of Japanese red pine. Of them, PCR with 25 RAPD and 18 I-SSR primers

showed informative and reproducible polymorphic amplicons. The number of polymorphic fragments per primer were ranged from 1 to 5 for RAPD primers and from 1 to 6 for I-SSR primers. Fragment size of the scored amplicons was ranged from about 375 to 2,050bp (Fig. 1 and 2). From the chi-square tests of 1 : 1 segregation the observed polymorphic amplicons at $P<0.05$, 52 RAPD (2.08 amplicons per primer) and 46 I-SSR (2.56 amplicons per primer) amplicons were turned out to be Mendalian loci, whereas 35 markers were deviated 1 : 1 segregation and excluded in this analysis.

Linkage analysis with the 52 RAPD loci alone revealed 13 linkage groups of total map distances of 453.3 cM. Forty six I-SSR loci were distributed into 9 linkage groups covering total map distance of 254.6 cM. When linkage analysis was performed with both 52 RAPD and 46 I-SSR loci, 63 of the 98 loci were coalesced into 20 linkage groups by the two-point analysis. The 35 (17 RAPD and 18 I-SSR) loci that were not associated with any linkage groups at a LOD of 3.0 might be assigned to linkage groups if more number of loci are verified and included in the linkage analysis in future. The 20 linkage groups consisted of 6 groups with 2 loci, 9 groups with 3 loci, 3 groups with 4 loci, 1 group with 5 loci and 1 group with 7 loci (Fig. 3). Twenty linkage groups spanned 1097.8 cM with 63 loci separated by an average distance of about 25.5 cM in Kosambi's map units. The minimum and maximum map distances between linked loci were 4.3 cM and 54.9 cM, respectively. Although direct comparison of total map distances between different pine species might not have much meaning, total map distance observed in this study was much shorter than that (1469.8 cM of 17 linkage groups including 98 RAPD loci) observed in Japanese black pine (Kondo et al., 2000) and that (1689.2 cM of 15 linkage groups including 124 RAPD loci) observed in *Pinus densiflora* for. *multicaullis,* a forma characterized by 'multi-stem' of *Pinus densiflora* (Lee 1998). The short total map distance observed in this study might be resulted from the unlinked 35 Mendelian loci and the map could be much improved by incorporating more number of loci which might help those unlinked loci be associated with present linkage groups. Fourteen of the 20 linkage groups were tightly combined by incorporation of I-SSR loci into RAPD loci, which resulted in construction of a new and longer linkage groups than a unique method applied. The markers within linkage groups was well ordered by three and multipoint analysis.

Though RAPD marker analysis is relatively simple, quick, and inexpensive molecular method, its lack of reproducibility has been still mentioned by a number of authors (Kaya and Neale, 1995; Binelli and Bucci, 1994; Gocmen, 1996). In present

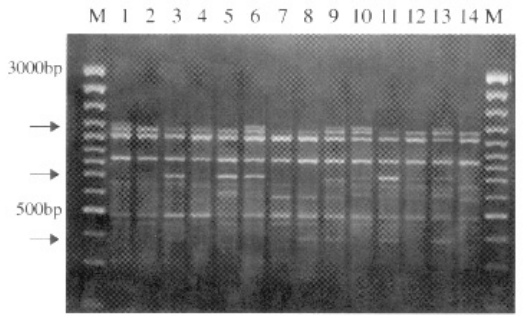

Fig. 1. Example of RAPD profiles of 14 megagametophyte DNA amplified with primer OPY-02. Arrows indicate 1 : 1 segregating polymorphic markers. M denotes DNA size marker of DNA 100bp ladder.

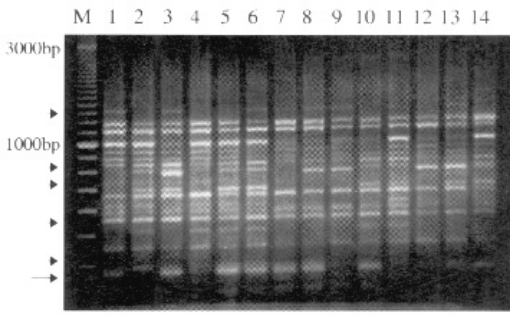

Fig. 2. Example of I-SSR profiles of 14 megagametophyte DNA amplified with primer UBC primer #810. Arrows indicate 1:1 segregating polymorphic markers. M denotes DNA size marker of 100bp ladder.

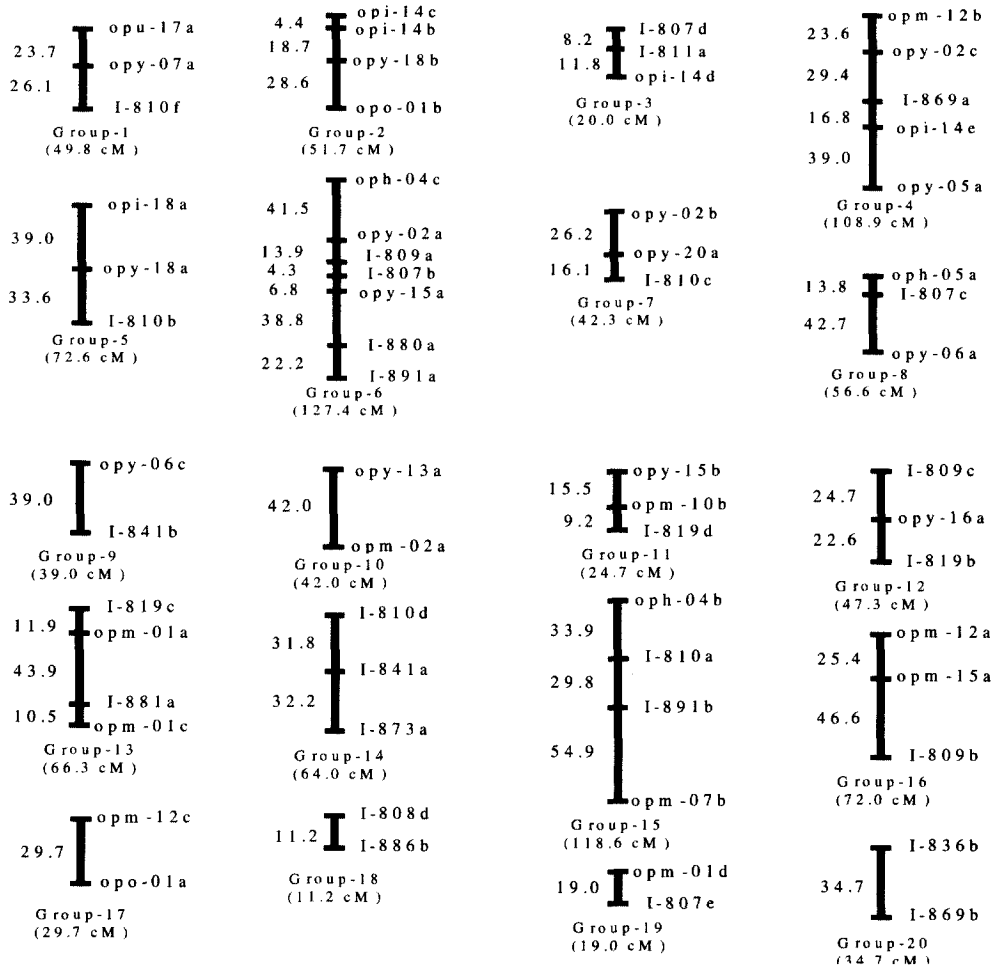

Fig. 3. Genetic linkage map of *Pinus densiflora*. ID of DNA markers are listed on the right and map distance in centiMorgan on the left. Sixty-three of the 98 incorporated loci of RAPD and I-SSR were mapped to 20 linkage groups, while the remaining 35 loci were unlinked, using a LOD (logarithm of the odds ratio) score of 3.0 and maximum recombination distance of 50cM. The 20 linkage groups covered a total of 1097.8 cM.

study, the low number of loci investigated might be resulted in not only the unsaturated linkage map but also more number of linkage groups than the number of typical haploid chromosome number (n=12) in the *Pinus*. For the construction of an efficiently saturated linkage map of Japanese red pine, it should be considered that the linkage analysis should be performed with a large number of Mendelian loci of various DNA markers. Lee (1998) presented that 'multi-stem' character which was the major mophological trait in *P. densiflora* for. *multicaullis* is controlled by one major gene. Knowledge of the positions of marker loci within or among species can permit the comparative study and manipulation of important genes or of quantitative trait loci (QTLs). Moreover, Williams and Neale (1992) reported that DNA marker-assisted selection for improving wood specific gravity could shorten the generation interval and improve selection response in loblolly pine (*Pinus taeda*). Also, Kaya et al.(1999) suggested that QTLs of annual height- and diameter-increment growth in loblolly pine were controlled at least in part, by a few genes of large effects. To identify the QTLs

for the annual growth of diameter and height, and wood quality in Japanese red pine, saturated linkage map should be prepared using mapping population of a full-sib family.

REFERENCES

1. Aravanopoulos, F.A. 1998. Analysis of genetic linkage in *Salix exigua* Nutt. Silvae Genetica 47 : 127-131.
2. Barreneche, T., C. Bodenes, C. Lexer, J.F. Trontin, S. Fluch, R. Streiff, C. Plomion, G. Roussel, H. Steinkellner, K. Berg, J.M. Favre, J. Glossl and A. Kremer. 1998. A genetic linkage map of *Quercus robur* L. (pendunculate oak) based on RAPD, SCAR, microsatellite, minisatellite, isozyme and 5S rDNA. Theor. Appl. Genet. 97 : 1090-1103.
3. Binelli, G. and G. Bucci. 1994. A genetic linkage map of *Picea abies* Karst., based on RAPD markers, as a tool in population genetics. Theor. Appl. Genet. 88 : 283-288.
4. Devey, M.E., T.A. Fiddler, B.H. Liu and S.J. Knapp. 1994. An RFLP linkage map for loblolly pine based on a three-generation outbred pedigree. Theor. Appl. Genet. 88 : 273-278.
5. Devey, M.E., J.C. Bell, D.N. Smith, D.B. Neale and G.F. Moran. 1996. A genetic linkage map for *Pinus radiata* based on RFLP, RAPD and microsatellite markers. Theor. Appl. Genet. 92 : 673-679.
6. Gocmen, B., K.D. Jermstad, B.D. Neale and Z. Kaya. 1996. Development of random amplified polymorphic DNA markers for genetic mapping in Pacific yew (*Taxus brevifolia*). Can. J. For. Res. 26 : 497-503.
7. Grattapaglia, D. and R. Sederoff. 1994. Genetic linkage maps of *Eucalyptus grandis* and *E. europhyulla* using a pseudo-testcross mapping strategy and RAPD markers. Genetics 137 : 1121-1137.
8. Guries, R.P., S.T. Friedman and F.T. Ledig. 1978. A megagametophyte analysis of genetic linkage in pitch pine (*Pinus rigida* Mill.). Heredity 40 : 309-314.
9. Hong, Y.P., K.S. Kim, E.R. Noh, E.M. Shin and Z.S. Kim. 1998. No trace of introduced cpDNA of *Pinus thunbergii* in *Pinus densiflora* for *erecta* postulated as an introgressive hybrid between *Pinus densiflora* and *Pinus thunbergii*. Jour. Korean. For. Soc. 87 : 543-548.
10. Jermstad, K.D., D.L. Bassoni, N.C. Wheeler and D.B. Neale. 1998. A sex-averaged genetic linkage map in coastal Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco var. '*menziesii*' based on RFLP and RAPD markerds. Theor. Appl. Genet. 97 : 762-770.
11. Kaya, Z. and D.B. Neale. 1995. Utility of Random Amplified Polymorphic DNA (RAPD) markers for linkage mapping in Turkish red pine (*Pinus brutia* Ten.). Silvae Genetica 44 : 110-116.
12. Kaya, Z., M.M. Sewell and D.B. Neale. 1999. Identification of quantitative trait loci influencing annual height- and diameter- increment growth in loblolly pine (*Pinus taeda* L.). Theor. Appl. Genet. 98 : 586-592.
13. Kim, Y.Y., J.O. Hyun, K.N. Hong, T.B. Choi and K.S. Kim. 1995. Genetic variation of natural population of *Pinus densiflora* in Korea based on RAPD marker analysis. Korean J. Breed. 27 : 23-48.
14. Kim, Z.S and S.W. Lee. 1992. Genetic structure of natural populations of *Pinus densiflora* in Kangwon-Kyungbuk region. Korean J. Breed. 24 : 48-60.
15. Kosambi, D. 1994. The estimation of map distances from recombination values. Ann. Eugen. 12 : 63-67.
16. Kubisiak, T.L., C.D. Nelson, W.L. Nance and M. Stine. 1995. RAPD linkage mappping in a longleaf pine x slash pine F1 family. Theor. Appl. Genet. 90 : 1119-1127.
17. Kubisiak, T.L., C.D. Nelson, W.L. Nance and M. Stine. 1996. Comparison of RAPD linkage maps constructed for a single longleaf pine from

both haploid and diploid mapping populations. Forest Genetics 3 : 203-211.

18. Lee, J.H. 1998. A genetic linkage map of *Pinus densiflora* Sieb. et Zucc. for *multicaulis* Uyeki based on Random Amplified Polymorphic DNAs (RAPD). M.S. Thesis, Seoul National University (in Korean).
19. Lincoln, S., M. Daly and E. Lander. 1992. Constructing genetic maps with MAPMAKER/EXP 3.0 Whitehead Institute Technical Report. 3rd edition.
20. Mukai, Y., Y. Suyama, Y. Tsumura, T. Kawahara, H. Yoshimaru, T. Kondo, N. Tomaru, N. Kuramoto and M. Murai. 1995. A linkage map for sugi (*Cryptomeria japonica*) based on RFLP, RAPD and isozyme loci. Theor. Appl. Genet. 90 : 835-840.
21. Neal, D.B., C.S. Kinlaw and M.M. Sewell. 1994. Genetic mapping and DNA sequencing of the loblolly pine genome. Forest Genetics 1 : 197-206.
22. Nelson, C.D., W.L. Nance and R.L. Doudrick. 1993. A partial genetic linkage map of slash pine(*Pinus elliottii* Engelm. var. *elliottii*) based on random amplified polymorphic DNAs. Theor. Appl. Genet. 87 : 145-151.
23. Szmidt, A.E. and X.R. Wang. 1993. Molecular systematics and genetic differentiation of *Pinus sylvestris* (L.) and *P. densiflora* (Sieb. et Zucc.). Theor. Appl. Genet. 86 : 159-165.
24. Tsumura, Y., Y. Ogihara, T. Sasakuma and K. Ohba. 1993. Physical map of chloroplast DNA in sugi, *Cryptomeria japonica*. Theor. Appl. Genet. 86 : 166-172.
25. Tulsieram, L.K., J.C. Glaubitz, G. Kiss and J.E. Carlson. 1992. Single tree genetic linkage mapping in conifers using haploid DNA from megagametophytes. Biotechnology 10 : 686-690.
26. Williams, C.G. and D.B. Neale. 1992. Conifer wood quality and marker-aided selection : a case study. Can. J. For. Res. 22 : 1009-1017.
27. Yazdani, R., F.C. Yeh and J. Rimsha. 1995. Genomic mapping of *Pinus sylvestris* (L.) using random amplified polymorphic DNA markers. Forest Genetics 2 : 109-116.