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Mendelian Inheritance of Inter-Simple Sequence Repeats Markers in *Abies koreana* Wilson[1]

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구상나무에 있어서 Inter-Simple Sequence Repeats Marker의 遺傳樣式[1]

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ABSTRACT

Polymerase chain reaction(PCR)-based inter-simple sequence repeats(I-SSR) markers were analyzed in 48 megagametophytes of a single tree of *Abies koreana* Wils. Nineteen of the 35 primers, screened with 6 megagametophyte DNA and produced the clearest amplification products in the preliminary experiment, were used for PCR with 48 megagametophyte DNAs sampled from a single tree. On the basis of the chi-square test, a total of 51 amplicons, amplified by the 19 primers, were revealed to be segregated according to the Mendelian ratio(i.e., 1 : 1 segregation ratio) in the 48 megagametophytes at 5% significance level. Based on the linkage analysis, the observed 51 Mendelian loci turned out to be unlinked each other, which suggested that they are evenly distributed in the genome. However, majority of RAPD markers are known to belong to the independent linkage blocks, which frequently results in the amplification of RAPD markers from the restricted regions of the genome. Owing to the nature of even distribution of the 51 loci observed in this study, the I-SSR markers could give better resolution of estimating genetic diversity from the whole genome than RAPD markers. And I-SSR markers are also more suitable than RAPD markers for reconstructing phylogenetic relationship by a cladistic method which requires to fulfil the assumption of independent evolution of the different characters.

Key words : inter-simple sequence repeat markers, Abies koreana *WILS.*

要 約

구상나무 개체목으로부터 채취한 48개의 배유조직을 이용해서 PCR 방법에 의해 생성된 inter-simple sequence repeats(I-SSR) 표지자를 분석했다. 예비실험에서 6개의 배유조직을 이용해서 35개의 primer를 검색했으며, 그들 중에서 PCR 반응이 가장 잘되는 19개 primer를 선정해서 48개 배유조직을 이용한 본실험에 사용했다. 카이자승 검정 결과, 19개 primer에 의해 증폭된 51개의 증폭산물이 5% 유의 수준에서 멘델의 분리비(1 : 1)에 따라 차대에 유전됨을 확인할 수 있었다. 멘델 유전자좌로 확인된 51개 표지자들의 게놈내 분포양상을 확인하기 위해서 연관분석을 수행한 결과, 51개 유전자좌들이 상호간에 서로 연관되어있지 않은 것으로 확인되어 이들이 전체 게놈상에 고르게 분포하고 있음을 확인할 수 있었다. 본 연구에서 관찰된 51개 유전자좌들이 게놈상에 고르게 분포하고 있다는 특성 때문에 게놈상의 특정부위에 편중되지 않은 유전정보를 얻을 수 있다는 장점이 있다. 즉, 기존의 RAPD 표지자들 중 상당수가 독립적인 연관군을 형성하는 것으로 알려져 있기 때문에 이들 연관군이

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위치한 특정 부위의 DNA를 증폭하여 분석하는 RAPD 표지자에 비해서 I-SSR 표지자들이 유전 다양성을 추정하는데 더 유용한 표지자로 활용될 수 있을 것으로 생각되며, 이들 표지자들이 독립적인 진화의 과정을 겪을 것으로 기대되기 때문에 cladistic 방법에 의해 진화적 유연관계를 추정하는데 더 적합한 표지자로 생각된다.

INTRODUCTION

Abies koreana WILS., a unique alpine forest tree species in Korea, is distributed over only limited areas(Mts. Chiri, Dukyu, and Halla) of the high mountains in Korea. Conservation of this unique Korean *Abies* species is one of the urgent problems in terms of maintaining the genetic diversity. Assessing genetic diversity is a prerequisite for establishing the strategies for the gene conservation to provide buffering effects over the unpredictable environmental changes in future. Several genetic markers have been adopted to monitor the genetic variation in the natural populations of forest trees(Adams and Demeke, 1993 ; Ali *et al.*, 1990 ; Hong *et al.*, 1993a ; Neale and Adams, 1982 ; Strauss *et al.*, 1992 ; Tsumura *et al.*, 1992). Molecular genetic markers, which have been known to provide more number of markers than biochemical markers(Adams and Joly, 1980a, 1980b ; Copes *et al.*, 1981 ; El-Kassaby *et al.*, 1982 ; Forde and Blight, 1964 ; Kim *et al.*, 1992 ; Kim *et al.*, 1994 ; Millar *et al.*, 1983 ; Mitton *et al.*, 1979 ; Weber and Stettler, 1981), have been extensively applied to assessing genetic diversity in forest trees(Adams and Demeke, 1993 ; Ahuja *et al.*, 1993 ; Carlson *et al.*, 1991 ; Devey *et al.*, 1993 ; Hong *et al.*, 1993b ; Hong *et al.*, 1995 ; Mukai *et al.*, 1995 ; Ponoy *et al.*, 1994 ; Strauss *et al.*, 1992 ; Tsumura *et al.*, 1996). Based on the recent studies with random amplified polymorphic DNA(RAPD) markers, the genetic relationships of tree species have been reconstructed with better resolution. And the linkage maps of tree species also have been more saturated(Adams and Demeke, 1993 ; Carlson *et al.*, 1991 ; Castiglione *et al.*, 1993 ; Dieter *et al.*, 1992 ; Grattapaglia *et al.*, 1994 ; Isabel *et al.*, 1992 ; Plomion *et al.*, 1994 ; Roy *et al.*, 1992 ; Tsumura *et al.*, 1992 ; Tulsieram *et al.*, 1992 ; Vicario *et al.*, 1995).

As the sequence information on the genome has been accumulated, it has been realized that there are large numbers of simple sequence repeats(SSRs) distributed over the whole genome [e.g., over 50,000 copies of $(AG)_n$ and $(CT)_n$ simple sequences in pines ; reviewed in Tsumura *et al.*, 1996]. SSRs are composed of tandem repeats of 2 to 4 bp in the genome. A PCR-based molecular genetic marker system of SSR markers has been developed on the basis of the variation in the number of such simple repeats. However, in order to analyze SSR markers, flanking sequences of the SSRs should be known to prepare target specific SSR primers, which should be accomplished by a series of biotechnical procedures of cloning, Southern hybridization, and DNA sequencing etc. To overcome the limitation of preparing target specific primers for SSR markers, inter-simple sequence repeats(I-SSR) has been developed. For the generation of I-SSR markers, intervening sequences between identical SSRs could be amplified by using a portion of SSRs. These primers are composed of a few copies(e.g., 8 copies in general) of simple repeats with the specific arbitrary anchors 1 to 3 bases at the 3'-end(I-SSR primers). I-SSR could be amplified by using a single I-SSR primer that anneals both to the simple sequence repeats in sense strand and to the identical se-quences in antisense strand within the range of amplifiable distance(i.e., <2 kb) by PCR in the genome. Though I-SSR markers are known to be dominant markers(i.e., presence or absence of amplicons) in general, they revealed the Mendelian segregation ratio in the megagametophytes of Dougals-fir and sugi families(Tsumura *et al.*, 1996). I-SSR marker is known to be advantageous over another PCR-based genetic markers of RAPD in terms of reproducibility, which is ensured by relatively higher annealing temperature. The problem of dominant phenotypes(i.e.,

undistinguishable phenotype of heterozygous state from homozygous state with presence of amplicon in diploid genome) of the I-SSR markers could be overcome by the haploid nature of conifer megagametophytes. In the present study, the inheritance mode of I-SSR markers in 48 megagametophytes of a single tree was verified in order to assess genetic diversity in the populations of *A. koreana* in Korea. The distribution pattern of I-SSR marker loci in the genome was also surveyed via linkage analysis.

MATERIAL AND METHODS

1. DNA extraction and PCR amplification

Total genomic DNAs were extracted from 48 megagametophytes of a single tree of *A. koreana* by a modified CTAB method(Hong *et al.*, 1991). The total volume of reaction mixture for PCR amplification was 20$\mu\ell$ which contained 5ng of template DNA, 0.2mM each of dNTPs, 0.0025% of BSA(Boeringer Mannheim, Germany), 5$\mu\ell$ of 1.5μM primers, 1.2$\mu\ell$ of 25mM $MgCl_2$, 1 unit of *Taq* DNA polymerase(Promega). The lid-heated DNA thermal cycler PTC-200(MJ Research) was used for PCR with the following temperature profile : 5 min. at 94℃ for 1 cycle for initial denaturation ; 45 cycles of 30 sec. at 94℃ for denaturation, 30 sec. at 52℃ for annealing, and 1 min. at 72℃ for extension. A final extension was performed at 72℃ for 10 min. PCR products were fractionated on 2% agarose gels in 1X TBE buffer, stained with EtBr, and photographed over UV transiluminator. DNA size was calculated by comparing with 100 base-pair DNA ladder(GIBCO BRL) which was loaded in two separate lanes on the same gel for electrophoresis.

2. Data analysis

Amplification fragment variants were observed as presence versus absence of the amplicon of the same size. After computer scanning of the photographs, the presence of the amplicons of the same size was verified via computer image analysis(Diversity One, pdi). An 1 : 1 segregation of the observed amplicon variants was tested via chi-square test at 5% significance level. Linkage analysis was performed using a computer program of MapMaker/QTL(V.1.1., Lincoln *et al.*, 1992).

RESULTS AND DISCUSSION

Nineteen of the 35 primers, screened with 6 megagametophyte DNA and showed clear amplification products in the preliminary experiment, were used for PCR with 48 megagametophyte DNAs of a single tree(Fig. 1). A total of 351 amplicons were successfully generated by 19 primers and 12 of them were monomorphic. On the basis of chi-square tests, 51 of the 339 amplicon variants(i.e., presence or absence of the amplification product of the same size) were revealed to be segregated according to the Mendelian ratio(i.e., 1 : 1 segregation ratio) at 5% significance level(Table 1). The 1 : 1 segregation of the I-SSR amplicon variants in the megagametophytes suggested that each amplicon variant could be verified as an allele in a single locus. An averaged number of the segregating loci/primer was 2.8, which was relatively larger than(1.6 loci/primer) that in the previous study with Douglas-fir and sugi families(Tsumura *et*

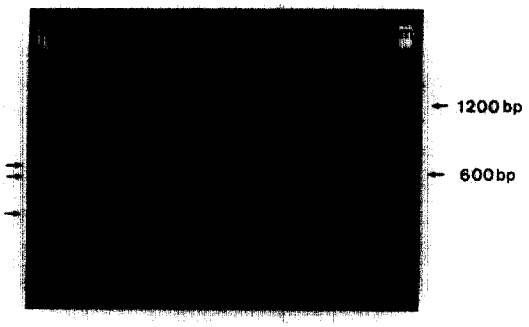

Fig. 1. Example of I-SSR profiles amplified from 16 megagametophyte DNAs using the UBC primer # 845. Arrows with dot denote the amplicons verified as the Mendelian markers, which did not show the deviation from the expectation of 1 : 1 segregation ratio(i.e., presence vs. absence of the amplification product of the same size) in the 48 megagametophytes. "m" denotes the DNA size marker 100bp ladder.

Table 1. Number of Mendelian loci identified by 19 SSR primers

Primers	No. of loci	Primers	No. of loci
#812 : (GA)8-A	3	#835 : (AG)8-YC	6
#813 : (CT)8-T	2	#841 : (GA)8-YC	4
#816 : (CA)8-T	2	#842 : (GA)8-YG	1
#817 : (CA)8-A	5	#844 : (CT)8-RC	3
#818 : (CA)8-G	3	#845 : (CT)8-RG	4
#822 : (TC)8-A	3	#847 : (CA)8-RC	2
#823 : (TC)8-C	3	#855 : (AC)8-YT	3
#824 : (TC)8-G	2	#887 : DVD-(TC)7	3
#825 : (AC)8-T	1	#890 : VHV-(GT1)7	1
#826 : (AC)8-C	3		

Numbers written in subscript denoted the copy number of the simple sequences in the parenthesis.
Y=(C,T), R=(A,G), D=(A,G,T),
V=(A,C,G), H=(A,C,T)

al., 1996).

Segregation of the rest of the observed amplicon variants(288 amplicon variants) showed the deviations from the Mendelian ratio at 5% significance level, which might be resulted from non-specific binding of SSR primers on account of their simple composition of tandem repeats. This result does not coincide with the study of Tsumura *et al.*,(1996) where almost all the observed amplicon variants were under the simple Mendelian inheritance in Douglas-fir and sugi families. This discrepancy might be due to the selective scoring of the amplicons by Tsumura *et al.*, (1996) in which unstable or weak bands were not scored although there appeared to be many of them(discussed but data were not shown). Based on this observation, precaution should be taken while applying I-SSR markers to estimating population genetic parameters. Especially, when researchers are planning to analyze diploid genomes, simple scoring of the observed amplicons as the Mendelian genetic markers without verifying 1 : 1 segregation in the progeny should be avoided, because it could lead to incorrect estimation of population genetic parameters. Furthermore, the proportion of the Mendelian loci among the polymorphic I-SSR amplicons observed in this study(15%) was much lower than RAPD markers observed in other tree species, ranging from 33 to 86%(Bucci and Menozzi, 1990 ; Tulsieram *et al.*, 1992 ; Bradshaw *et al.*, 1994). This suggests that the simple interpretation of I-SSR amplicons as genetic markers without verifying an 1 : 1 segregation in the progeny might be more erroneous than that of RAPD markers. However, the Mendelian inheritance of I-SSR amplicons was verified from only 48 megagametophytes in this study. This suggested that some of the remaining polymorphic I-SSR amplicons, deviated from 1 : 1 segregation at 5% level, might be verified as Mendelian loci if more number of megagametophytes were analyzed for the segregation test.

Based on the linkage analysis(LOD=3.0), the observed 51 Mendelian loci turned out to be unlinked each other, which suggested that they are evenly distributed on the chromosomes(Kashi *et al.*, 1997). However, majority of RAPD markers are known to belong to the independent linkage blocks, which may frequently result in the amplification of RAPD markers from the restricted regions of the genome. For example, there are 33 RAPD linkage blocks which are composed of 147 loci in *Pinus rigitaeda*(In preparation, Kim, 1998). Owing to the nature of even distribution of the 51 loci observed in this study, the I-SSR markers generated from the whole genome could give better resolution of estimating genetic diversity than RAPD markers generated from the linkage blocks of the restricted portion of the genome. The evenly distributed nature of the I-SSR markers in the genome has an important meaning in studying population genetics because the evolutionary forces may have acted on the whole genome through evolution. Therefore, estimation of population genetic parameters on the basis of the genetic markers observed from the whole genome is expected to be more reliable than that estimated on the basis of those observed from the restricted portion of the genome such as linkage blocks.

Another benefit of the even distribution of the I-SSR markers is that those markers could connect the scattered linkage blocks constructed with RAPD markers in conifers whose genomes are relatively larger than those of other plants.

For example, there are 33 RAPD linkage blocks constructed with 147 of 266 Mendelian loci, generated by 83 primers, in *Pinus rigitaeda*(In preparation, Kim 1998). Of the 33 linkage blocks, only 6 blocks were composed of more than 7 loci. In considering of the 12 chromosomes of haploid genome in pines, there are too many linkage blocks which may be untied on account of the several factors such as the limited number of analyzed RAPD loci, large genome size of pines, and the restricted portion of the amplifiable sequences by RAPD PCR. Some of the untied linkage blocks could be connected by the inclusion of I-SSR markers, which may be more evenly distributed on the whole genome than RAPD markers, in the linkage analysis. I-SSR markers are also more suitable than RAPD markers for reconstructing phylogenetic relationship by cladistic method(e.g., parsimony method) which requires to fulfil the assumption of inde-pendent evolution of the different characters.

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