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No Trace of Introduced cpDNA of *Pinus thunbergii* in *Pinus densiflora* for. *erecta* Postulated as an Introgressive Hybrid between *Pinus densiflora* and *Pinus thunbergii**

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소나무와 곰솔간 移入交雜種으로 推定되어온 金剛松에 있어서 곰솔 cpDNA의 不在*

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ABSTRACT

Portions of chloroplast genes(*psb*D and *rbc*L) were amplified from *Pinus thunbergii*(Japanese black pine : black pine) and *Pinus densiflora*(Japanese red pine : red pine) by PCR and digested by a restriction enzyme, *Hae*Ⅲ, respectively. Two species specific cpDNA markers were identified. With the observed cpDNA markers, paternal inheritance of cpDNA in pine hybrids was verified in an artificial hybrid family between black pine(Chollanam 37) and red pine(Chungchongbuk 3). On the basis of paternal inheritance of chloroplast genome in a hybrid, 2 portions of cpDNA amplified from 115 individuals of *Pinus densiflora* for. *erecta* were screened to detect any traces of black pine specific cpDNA markers in *P. densiflora* for. *erecta* which has been postulated as an introgressive hybrid between red pine and black pine(Hyun *et al.*, 1967). All the analyzed individuals of *Pinus densiflora* for. *erecta* revealed the identical profiles of *Hae*Ⅲ digested *psb*D and *rbc*L genes to red pine. This result suggests that there is no introduced chloroplast genome of black pine in *Pinus densiflora* for. *erecta* and that there is no concrete evidence of treating *P. densiflora* for. *erecta* as an introgressive hybrid between red pine(♀) and black pine(♂).

Key words : Pinus densiflora *for.* erecta, Pinus thunbergii(*black pine*), Pinus densiflora(*red pine*), *chloroplast genes* - psb*D and* rbc*L*, *PCR*, *Introgressive hybrid*

要 約

소나무와 해송으로부터 엽록체상의 두 유전자 *psb*D와 *rbc*L를 PCR에 의해 증폭한 후 제한효소 *Hae*Ⅲ를 사용해서 절단했다. 두 개의 종 특이적 엽록체 DNA 단편이 확인되었고, 이 두 개의 표지자를 이용하여 소나무(충북3호)와 해송(전남37호)의 인공교잡 가계로부터 엽록체 DNA의 부계 유전양식이 확인되었다. 인공교잡 가계에 있어서 엽록체 DNA의 부계 유전양식을 근거로 해송으로부터 소나무로의 이입교잡에 의해 생겨났다는 가설(현신규 등, 1967)이 지배적인 금강송 115개체로부터 이입교잡에 의해 유입되어진 흔적을 구명하기 위하여 해송 특이 엽록체 DNA의 존재 여부를 검색하였다. 분석에 사용된 금강송 전 개체에서 소나무에서 관찰된 엽록체 DNA(*psb*D와 *rbc*L)의 절편 분획 양상과 동일한 절편 분획 양상이 확인되었다. 본 실험의 결과로부터는, 금강송에는 해송으로부터 유입된 엽록체 게놈의 흔적을 찾아 볼 수 없었으며, 따라서 금강송을 소나무(♀)와 해송(♂)의 이입교잡종이라고 간주할만한 확고한 증거를 제시할 수 없었다.

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INTRODUCTION

Chloroplast genome has been known to be paternally inherited in conifers unlike other plants (Neale *et al.*, 1986 ; Neale and Sederoff, 1989 ; Wagner *et al.*, 1989, 1992 ; Watanabe *et al.*, 1996). It is well established that the chloroplast genome evolves at a conservative rate(Palmer, 1987). The estimated evolutionary rate of chloroplast genes($1.0 - 3.0 \times 10^{-9}$ substitutions/site/year) is considerably below that observed for plant nuclear genes($5.0 - 30 \times 10^{-9}$ substitutions/site/year) (Wolfe *et al.*, 1987). In consideration of the relatively long generation time in conifers, the evolutionary rate of conifer chloroplast genes would be much lower than that estimated from flowering plants, which frequently provided the species specific chloroplast DNA markers with a few variants(Hong, 1991). The paternal inheritance of cpDNA was also verified in the artificial and the natural hybrids, *P. densi-thunbergii*, between black pine and red pine(Watanabe *et al.*, 1996).

A geographic type of red pines, *P. densiflora* for. *erecta*, which grows in Kangwon-Kyungbuk region of Korea, was first classified by Uyeki (1928). Although this pine could be distinguishable from red pine on the basis of some morphological characteristics, overall phenotype is very similar to that of red pine. There have been extensive studies on *P. densiflora* for. *erecta* since Hyun *et al.*(1967) proposed the hypothesis of introgressive hybridization between black pine and red pine for inferring the origin of this pine. However, this hypothesis was mainly derived from the observation of the variation in resin duct position(i.e., medial position for black pine vs. external position for red pine : reviewed in Hyun *et al.*, 1967). In that study, they observed the typical resin duct position of black pine in some individuals of *P. densiflora* for. *erecta*. However, there have been several studies on variation of the resin duct position, which showed continuous variation of resin duct position among individuals of the plus trees of red pine from both Korea(34 trees) and Japan(22 trees)(Ahn, 1972), and among needles within individuals(Ryu *et al.*, 1985). This observation suggested that the observed variation in resin duct position of *P. densiflora* for. *erecta* could not be a critical evidence for verifying the hypothesis of the introgression of black pine to red pine. Furthermore, there have been several studies on allozyme variation in the populations of red pine, black pine, and *P. densiflora* for. *erecta*(Son *et al.*, 1989, 1990a, 1990b ; Kim and Lee, 1992 ; Kim *et al.*, 1993), which also failed to provide any qualitative evidence of introgressive hybridization. However, the paternal inheritance and conservative nature of cpDNA makes it possible to test the introgressive hybridity of *P. densiflora* for. *erecta* by providing qualitative evidence of being presence or absence of species unique genetic markers. If the hypothesis were true, the present populations of *P. densiflora* for. *erecta* should contain chloroplast genome of black pine which had been inherited from black pine, the hypothetical paternal parent. Moreover, if the introgression happened via recurrent backcrossing, the chloroplast genome of red pine(i.e., pollen recipient) should be replaced by that of black pine.

In the present study, 2 species specific cpDNA markers were analyzed in an artificial hybrid family, of which some progenies(73%, 15/19) showed potential resistance against pine gall midge in a field test, to verify paternal inheritance of cpDNA and to confirm hybridity by verifying the paternal parent of red pine. And 115 individuals of *Pinus densiflora* for. *erecta* were also screened to detect any traces of black pine specific cpDNA markers in *P. densiflora* for. *erecta* which has been postulated as an introgressive hybrid between red pine and black pine(Hyun *et al.*, 1967).

MATERIALS AND METHODS

A total of 115 individuals of *P. densiflora* for. *erecta* - 103 individuals from Kyungsangbukdo(Uljin and Bongwha) and 12 from Kangwondo(Inje and Myungju) - and 9 red pines from Kyungsangbukdo(Kyungju) were sampled. Genomic DNAs were extracted by modified CTAB DNA prepa-

Table 1. Primers used for amplification of *psb*D and *rbc*L genes

Gene	Primers (forward and reverse)	Source
*psb*D	5' - TGTCACCAAAAACAGAGACT - 3' 5' - TTCCATACTTCACAAGCAGC - 3'	*Pseudotsuga menziesii* (Hipkins *et al.* 1990)
*rbc*L	5' - TATGACTATAGCCCTTGGTA - 3' 5' - TAGAACCTCCTCAGGGAATA - 3'	*Nicotiana tabacum* (Shinozaki *et al.* 1986)

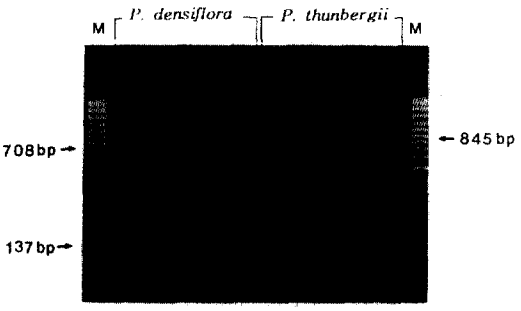

Fig. 1. PCR-RFLP profiles showing 2 species specific cpDNA markers between red pine and black pine - *Hae*III digested *psb*D amplicons. 'M' denotes DNA size marker of 100bp ladder.

ration(Hong, 1991). The amount of DNA was indirectly quantified by agarose gel electrophoresis with the known quantity standard of uncut λ-DNA. Primers for *psbD* and *rbcL* genes were synthesized(Bioneer, Korea ; Table 1). Twenty-five $\mu\ell$ of PCR reaction mixture was composed of 20mM Tris-HCl, pH7.4 at 25℃, 1.875mM of $MgCl_2$, 0.1mM EDTA, 1mM DTT, 100mM KCl, 0.1% Triton X-100, 200uM of each dNTPs, 1μM of each primer, 0.25%(v/v) BSA, 1 unit of *Taq* DNA polymerase(Biometra, Germany), and 25ng of template DNA. PCR thermocycling was performed with DNA Engine PTC-200(MJ Research) as follows : 1 cycle of the initial denaturation at 94℃ for 5 minutes, followed by 45 cycles of 3 temperature cycling - denaturation at 94℃ for 45 seconds, annealing at 55℃ for 45 seconds, and extension at 72℃ for 1 minute, and 1 cycle of final extension at 72℃ for 10 minutes. PCR products were purified by EtOH precipitation and digested with *Hae*III(Promega, USA) at 37℃ overnight. Restriction enzyme digested PCR products were fractionated in 2.0% agarose gel, prepared with 1X TBE buffer, at 6V/cm for 3hrs. After electrophoresis, fractionated DNAs were stained with EtBr and photographed over the UV transilluminator. DNA size marker of 100bp ladder was fractionated in the same gels.

Table 2. Restriction site changes observed in 2 cpDNA genes of red pine and black pine.

Gene	Enzyme	Site changes*	Korean red pine	black pine
*psb*D	*Hae*III	845→708+137	O	X
*rbc*L	*Hae*III	478→300+178	O	X

* DNA sizes were referred to Tsumura *et al.* (1995).

RESULTS AND DISCUSSION

Species specific cpDNA profiles for red pine and black pine were generated by *Hae*III digestion of the amplified cpDNAs, a portion of *psb*D (Fig. 1) and *rbc*L genes(figure not shown), respectively. Red pine could be distinguished from black pine by one additional restriction site in both genes(Table 2). All the members of a hybrid family showed the same profile as that of red pine which was served as a paternal parent in an artificial hybridization(Fig. 2). This observation suggests that chloroplast genome is paternally inherited in the artificial hybrids and that all the hybrid progenies were reproduced by interspecies hybridization between black pine(♀) and red pine(♂).

The red pine specific pattern of the restriction profiles was observed in all the individuals of *P. densiflora* for. *erecta* and red pine analyzed in this study(Fig. 3). Any traces of cpDNA of black pine was not observed in any individuals of *P. densiflora* for. *erecta* analyzed in this study, which suggests that the previous postulation of the introgressive hybridization between red pine(♀) and black pine(♂) for the explana-

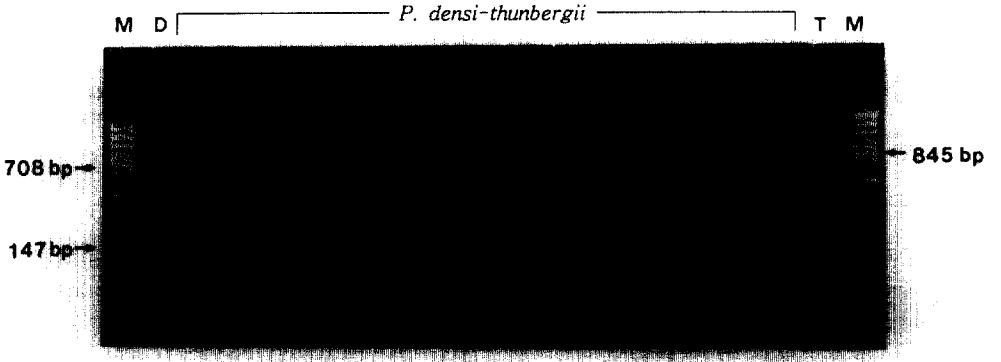

Fig. 2. An example of PCR-RFLP profiles showing paternal inheritance mode of chloroplast genome in artificial hybrids - *Hae*III digested *psb*D amplicon.
'D' denotes red pine of paternal parent, 'T' denotes black pine of maternal parent, and 'M' denotes DNA size marker of 100bp ladder.

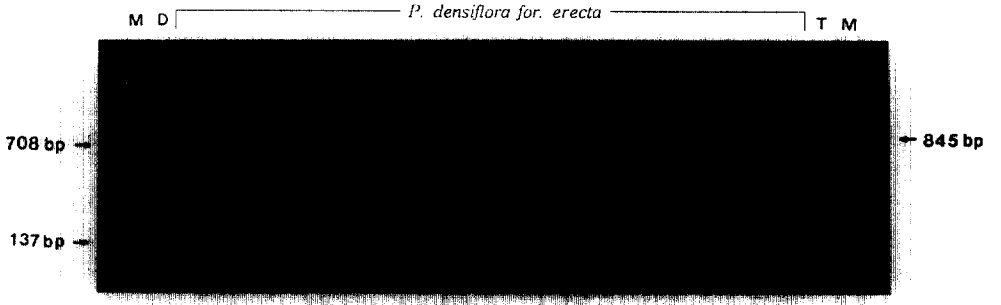

Fig. 3. An example of PCR-RFLP profiles of *P. densiflora* for. *erecta* showing identical pattern to red pine - *Hae*III digested *psb*D amplicon.
'D' denotes red pine, 'T' denotes black pine, and 'M' denotes DNA size marker of 100bp ladder.

tion of the origination of *P. densiflora* for. *erecta* may not be true. As a hypothetical explanation for the origination of *P. densiflora* for. *erecta* (Hyun *et al.*, 1967), Hyun *et al.* proposed that natural hybrids(i.e., *P. densi-thunbergii*) had been reproduced in the populations of red pines by fertilization with the migrated pollens of black pine. And the hybrid progenies were recurrently backcrossed with neighboring red pines on account of the selective disadvantage in fertility among hybrid progenies(Hyun *et al.*, 1967), which resulted in introgression of black pine to red pine.

Though red pine and black pine may be the most closely related species, they are not genetically homologous enough to hybridize freely in fact(Ahn, 1972 ; Ryu *et al.*, 1985 ; Shiraishi and Watanabe, 1995 ; Watanabe *et al.*, 1996). For example, two pines differ by 6 of 385 nucleotides of a portion of chloroplast *rbc*L gene(Shiraishi and Watanabe, 1995). In spite of comparing sequence variation in the small portion of chloroplast genome, theoretically, they might have diverged at least 6×10^9 years ago on the basis of the observed sequence difference(Wolfe *et al.*, 1987), which suggests that they are not so closely related phylogenetically as expected. Some investigations on fertility in the artificial hybridization also revealed that there is certain level of reproductive isolation, though not complete(Ahn, 1972 ; Ryu *et al.*, 1985). From the observation in natural hybrids, there is also restricted reproductive combination in hybridization, in which red pine is served as maternal parent and black pine as paternal parent(Watanabe *et al.*, 1996). Those investigations suggest that hybridization between red pine(♀) and black pine(♂) - proposed hybrid

in the hypothesis of introgression(Hyun *et al.*, 1967) could not happen frequently in nature.

If the hypothesis of the introgression were true, both nuclear and chloroplast genomes of black pine should be introduced to red pine. As backcross is repeated, nuclear genome of black pine is continuously diluted as a half per generation in successive introgressive hybrids. If hybrids of the first generation were reproduced a couple of hundred or thousand years ago and the generation time of the hybrids were assumed to be roughly 10 years, it might not easy to find any traces of black pine nuclear genes in the present populations of introgressive hybrids. This may be the reason why several extensive investigations on allozyme variants failed to identify any black pine specific alleles in the populations of *P. densiflora* for. *erecta*(Son *et al.*, 1989, 1990a, 1990b ; Kim and Lee, 1992 ; Kim *et al.*, 1993). However, chloroplast genome of red pine is replaced by a single hybridization with that of black pine on account of the paternal inheritance of chloroplast genome in pine hybrids. Even though backcross had been repeated to result in introgression, chloroplast genome of black pine should have remained intact in introgressive hybrids. As a result, chloroplast genome of black pine should be fixed in the resulting introgressive hybrids, which should provide qualitatively different cpDNA markers between red pine and *P. densiflora* for. *erecta* of the postulated introgressive hybrid. In contrast to the hypothetical expectation, any cpDNA markers of the black pine were not observed in the analyzed individuals of *P. densiflora* for. *erecta* in this study.

In conclusion, on the basis of cpDNA marker analysis, there is no concrete evidence of treating *P. densiflora* for. *erecta* as an introgressive hybrid between red pine(♀) and black pine(♂).

LITERATURE CITED

1. Ahn, K.Y. 1972. Studies on the species crossabilities in the genus *Pinus* and principal characteristics of F_1 hybrids. J. Kor. For. Soc. 16 : 1-32.
2. Hipkins, V.D., C.H. Tsai and S.H. Strauss. 1990. Sequence of the gene for the large subunit of ribulose 1, 5-bisphosphate carboxylase from a gymnosperm, Douglas-fir. Plant Mol. Biol. 15 : 505-507.
3. Hong, Y.P. 1991. Chloroplast DNA variability and phylogeny in the California closed cone pines. Ph.D. Thesis. Oregon State University.
4. Hyun, S.K., K.H. Koo and K.Y. Ahn. 1967. Introgressive hybridization in red pine in the eastern part of South Korea I. Res. Rep. Inst. For. Gen. Korea. 5 : 43-52.
5. Kim, Z.S., S.W. Lee, J.W. Hwang and K.W. Kwon. 1993. *Pinus densiflora* for. *erecta* Can it be treated genetically as a distant group? Reconsideration based on allozyem data. J. Kor. For. Soc. 82(2) : 166-175.
6. Kim, Z.S. and S.W. Lee. 1992. Genetic structure of natural populations of *Pinus densiflora* in Kangwon-Kyungbuk region. Korean J. Breed. 24(1) : 48-60.
7. Neale, D.B., N.C. Wheeler and R.W. Allard. 1986. Paternal inheritance of chloroplast DNA in Douglas-fir. Can. J. For. Res. 16 : 1152-1154.
8. Neale, D.B. and R.R. Sederoff. 1989. Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. Theor. Appl. Genet. 77 : 212-216.
9. Palmer, J.D. 1987. Chloroplast DNA evolution and biosystematic used of chloroplast DNA variation. Am. Nat. 130 : S6-S29.
10. Ryu J.B., S.H. Hong and H.G. Chung. 1985. Introgressive hybridization of *Pinus densiflora* in Korea by the position of resin duct in needle. J. Kor. For. Soc. 69 : 19-27.
11. Shiraishi, S. and A. Watanabe. 1995. Identification of chloroplast genome between *Pinus densiflora* Sieb et Zucc. and *P. thunbergii* Parl. based on the polymorphism in *rbc*L gene. J. Jpn. For. Soc. 77 : 429-436.
12. Son, D.S., C.Y. Kwon and S.J. Park. 1990a. The characteristics of selected trees of putative hybrid pines between *P. thun-*

bergii and *Pinus densiflora*. J. Kor. For. Soc. 79(2) : 127-137.

13. Son, D.S., S.J. Park and J.W. Hwang. 1990b. The variation of isozymes and molphological characteristics of needle, cone and seed according to resin duct index in *Pinus densiflora* and *P. thunbergii*. J. Kor. For. Soc. 79(4) : 424-430.
14. Son, D.S., S.C. Hong, J.K. Yoe and J.B. Ryu. 1989. Genetic variation of isozymes in populations of *Pinus densiflora* and *P. thunbergii* naturally distributed in Kyungpook province. J. Kor. For. Soc. 78(4) : 345-359.
15. Uyeki H. 1928. On the physiognomy of *Pinus densiflora* growing in Korea and silvicultural treatment for its improvement. Bull. Agr. and Forestry Coll. Suwon. Korea. No. 3. 263p.(in Japanese)
16. Tsumura Y., K. Yoshimura, N. Tomaru, and K. Ohba. 1995. Molecular phylogeny of conifers using RFLP analysis of PCR-amplified specific chloroplast genes. Theor Appl Genet. 91 : 1222-1236.
17. Wagner, D.B., D.R. Govindaraju, C.W. Yeatman and J.A. Pitel. 1989. Paternal chloroplast DNA inheritance in a diallel cross of jack pine(*Pinus banksiana* Lamb.). J. Hered. 80 : 483-485.
18. Wagner, D.B., W.L. Nance, C.D. Nelson, T. Li, R.N. Patel and D.R. Govindaraju. 1992. Taxonomic patterns and inheritance of chloroplast DNA variation in a survey of *Pinus echinata, Pinus elliottii, Pinus palustris* and *Pinus taeda*. Can. J. For. Res. 22 : 683-689.
19. Watanabe, A., S. Shiraishi, E. Kawase, T. Toda and T. Nasu. 1996. Genome analysis of *Pinus* X *densi-thunbergii* Uyeki using DNA molecular markers-verification of its hybridity. J. Jpn. For. Soc. 78 : 293-300.
20. Wolfe, K.H., W.H. Li and P.M. Sharp. 1987. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proc. Natl. Acad. Sci. USA. 84 : 9054-9058.