

Full Length Research Paper

Genetic variation within and between three Vietnamese pine populations (*Pinus merkusii*) using random amplified polymorphic DNA (RAPD) markers

Ho Manh Tuong*, Nguyen Thu Giang, Chu Hoang Ha and Le Van Son

Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, Vietnam.

Received 26 February, 2016; Accepted 13 July, 2016.

Pinus merkusii is an important species in Vietnam with many economic and biological contributions. The information on diversity within and between populations of a species is necessary for plantation programs, breeding and conservation strategies. Genetic diversity of three Vietnamese populations (NA, QB and QN) was analyzed using the random amplified polymorphic DNA (RAPD) markers. Nine RAPD primers produced 82 markers, 77 of which were polymorphic with 93.9% of polymorphism. The results showed higher genetic variation within populations (72%) than between populations (28%) and low Nei's genetic differentiation index among populations (0.1867). The populations also clustered based on PCoA analysis where cluster I included NA and QB populations and Cluster II, the QN population. These results suggest that *P. merkusii* populations in Vietnam is necessary to develop the genetic resources.

Key words: DNA markers, genetic diversity, *Pinus merkusii*, random amplified polymorphic DNA (RAPD), Vietnam.

INTRODUCTION

Pinus merkusii is a tropical forest tree grown and planted naturally across the south Equator. This species is distributed naturally and artificially in Southeast Asia including Vietnam, Laos, Cambodia, Thailand, Malaysia, China and the Philippines (Cooling, 1968; Farjon, 2005; Razal et al., 2005; Theilade et al., 2000). It can grow from 30 to 2000 m above the sea level (Cooling, 1968; Santisuk, 1997). This species is used for the production of heavy wood, fuel, pulp, timber, resin and turpentine.

Resin which is used as material for medicine, paints, printing and perfume industry is one of its most important products (Hidayat and Hansen, 2002; Razal et al., 2005; Theilade et al., 2000). *P. merkusii* is one of the principal tree in reforestation, soil erosion control and rehabilitation in Vietnam and Indonesian Islands (Hidayat and Hansen, 2002; Razal et al., 2005). This species has been extremely exploited which reduced areas of its habitats, habitat quality which led to dramatic decrease in natural

*Corresponding author. Email: tuongcns@ibt.ac.vn. Tel: +84-4-37918003. Fax: +84-4-38363144.

Table 1. Population signal and location of three *Pinus merkusii* populations in Vietnam.

Population Sign	Sample size	Location	Latitude, longitude	Altitude (m)
NA	26	Nam Dam – Nghe An	18°40'45.0"N-105°29'19.7"E	100
QB	25	Quang Trach – Quang Binh	17°53'50.8"N- 106°23'03.9"E	50-70
QN	28	Uong Bi – Quang Ninh	21°04'08.9"N- 106°44'43.4"E	70-80

areas in the past decades (Bharali et al., 2012; Kumar et al., 2000). As a result, it is listed in the International Union for Conservation of Nature as the world's threatened forest tree species (UICN, 2011).

The degree of genetic diversity between populations is important for re-plantation breeding and conservation strategies. Genetic variation plays the crucial role for the long-term stability (Sharma et al., 2002). The exploitation of some plant species by human have been altered for a long time (Finkeldey and Hattermer, 2007). As a consequence, many plantations of forest species show low genetic diversity. Although, most studies of *P. merkusii* were provided by isozyme analysis (Changtragoon and Finkeldey, 1995; Suwarni et al., 1999; Siregar and Hattermer, 2004; Thao et al., 2013), DNA markers such as simple sequence repeat (ISS), inter simple sequence repeat (ISSR), RAPDs have been used successfully used for analysis of pines populations (Alrababah et al., 2011; Marquardt et al., 2007; Mariette et al., 2001; Nurtjahjningsih et al., 2007; Navascues and Emerson, 2007; Gauli et al., 2009; Thao et al., 2013; Thomas et al., 1999; Zhang et al., 2005). RAPD markers are useful tools to analyze the genetic diversity within and between plant populations (Fritsch and Rieseberg, 1996; Cruzan, 1998). Many species have been assessed using marker such as *Gentianella germanica* (Fischer and Matthies, 1998), rice (Qian et al., 2001) as well as pines (Alrababah et al., 2011; Lee et al., 2002; Xia et al., 2001; Zhang et al., 2005).

The information of genetic variation is essential for selecting, breeding and conservation strategies. This study tends to identify the level of genetic variation within and between three *P. merkusii* populations that help in breeding and conservation strategies to maintain the genetic diversity resources.

MATERIALS AND METHODS

Sample collection

Young clean leaves were collected from 79 *P. merkusii* lines from three populations at three provinces of Vietnam (Table 1 and Figure 1). All samples were stored in -80°C after collecting.

DNA isolation

Total DNA was isolated from young leaves following Doyle and

Doyle (1990) method. DNAs quality and quantity were accessed using NanoDrop Lite (Thermo scientific, USA), the DNA concentration was adjusted to 50 ng/μL for use in RAPD-PCRs.

PCR amplification for RAPD markers

Nine RAPD primers were selected for this study (Table 2). PCR reaction was performed in PCR vertiti@96well-Fast Thermal Cycler (Thermo Scientific, USA) with total volume of 25 μL containing: 1X polymerase chain reaction buffer, 0.25 mM dNTPs, 2 mM MgCl₂, 0.2 μM each primer F/R, 1 U Taq DNA polymerase, 100 ng DNA template and sterile ultrapure water. PCR thermal cycles consist of the following steps: 95°C for 4 min, followed by 35 cycles of denaturation at 92°C for 1 min, annealing at 35°C for 1 min and extension at 72°C for 1 min, final extension at 72°C for 10 min and 4°C for 30 min. Amplification products were analyzed by electrophoresis on 1.5% agarose gel with TAE buffer, stained with ethidium bromide, and photographed under ultraviolet light. The bands were scored by using PyElph software (Pavel and Vasile, 2012).

Genetic diversity analysis

DNA fragments were scored for presence (1) or absence (0), and analyzed using GenAlEx6. The data matrix was then subjected to analysis of molecular variance (AMOVA), principal coordinate analysis (PCoA) and the diagram for identifying the genetic diversity within and between three populations by GenAlEx software (Peakall and Smouse, 2006). Nei's gene diversity (*h*), Shannon' information index (*I*), mean observed number of alleles (*N_a*); mean effective number of alleles (*N_e*), percentage of polymorphic loci (*P*) and Nei's genetic distance between populations (*G_{st}*) were analyzed using Popgene software version 3.5 (Yeh et al., 2000).

RESULTS

Polymorphism of RAPD markers

Nine RAPD primers were able to amplify DNA fragments of 79 individuals of *P. merkusii* (Table 2). A total of 82 bands were obtained including 77 polymorphic bands with mean of 93.90%. The results also showed that the average number of markers were 9.1 for primer and the average number of polymorphic markers were 8.55 primer. The fragment sizes fluctuated between 200 and 1500 bp and the number of bands ranged from 4 (RA143) to 14 (OPE14). The PIC values of primers were high and ranged from 0.724 to 0.88 with an average of 0.82. Figure 2 showed the result of amplication for the portion



Figure 1. The locations of the three populations of *P. merkusii* (NA, QN and QB) in Vietnam.

Table 2. Primer sequences, length and number of amplified and polymorphic bands and PIC values of nine RAPD primers.

Primer	Sequence (5'-3')	Length (bp)	No. of amplified bands	No. of polymorphic bands	Polymorphism (%)	PIC
RA46	CCAGACCCTG	200-1200	11	9	81.81	0.88
RA143	TCGCGATAG	500-1000	4	4	100	0.724
RA159	G TTCACACGG	250-800	9	9	100	0.848
OPB10	CTGCTGGGAC	250-1400	10	10	100	0.855
OPD20	ACCCGGTCAC	300-1000	8	7	87.5	0.827
OPE14	TGCGGCTGAG	250-1200	14	14	100	0.829
OPF09	CCAAGCTTCC	400-1500	6	6	100	0.781
OPG13	CTCTCCGCCA	250-1500	10	9	90	0.875
OPR08	CCCGTTGCCT	250-1500	10	9	90	0.852
overall			82	77		
Average			9.1	8.55	93.90	0.82

PIC: Polymorphic information content.

of NA and QB populations with OPF09 and RA46 primers. These results indicated that all nine RAPD

primers are significant for assessing *P. merkusii* populations.

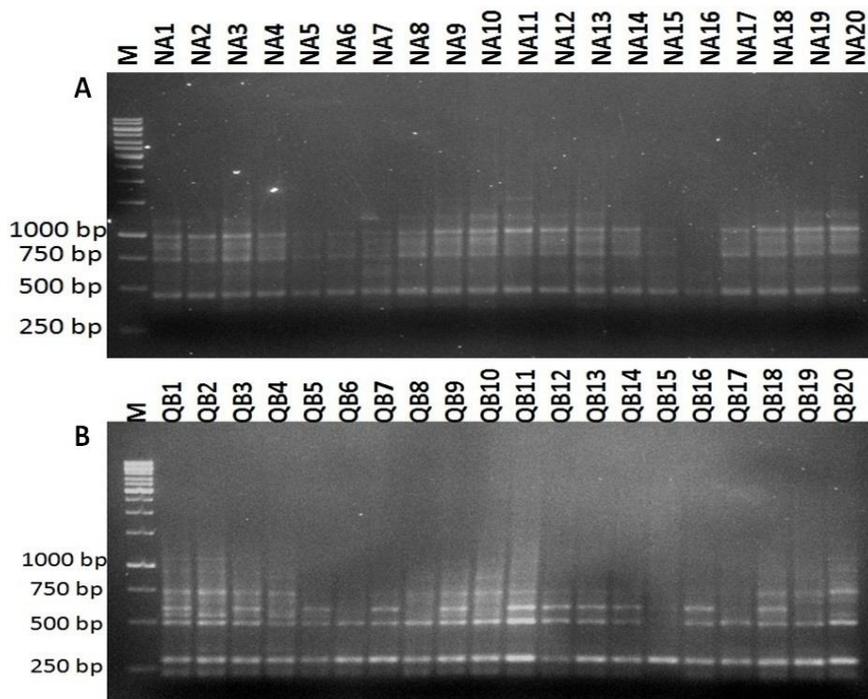


Figure 2. The RAPD profiles of 20 samples of NA population with OPF09 primer (A) and 20 samples of QB population with RA46 primer (B); M: DNA ladder 1kb (Thermo scientific).

Table 3. Genetic diversity within three *P. merkusii* populations.

Population	N	P (%)	Na	Ne	<i>I</i>	<i>h</i>
NA	26	73.33	1.627	1.401	0.360	0.238
QB	25	74.67	1.613	1.442	0.387	0.258
QN	28	69.33	1.507	1.430	0.359	0.242
overall		72.44	1.582	1.424	0.369	0.246

N: Number of samples; Na: number of alleles per locus; Ne: number of effective alleles per locus; *I*: Shannon' information index; *h*: Nei' gene diversity; *P*: percentage of polymorphic loci.

Genetic variation within populations

The genetic variation within population is shown in Table 3. The overall genetic diversity of Vietnam pine indicated that the percentage of polymorphic loci (*P*) was highest for QB (74.67%) and lowest for QN (69.33%) with mean of 72.44%. Furthermore, the NA population revealed the highest value in the number of alleles per locus with 1.627 and QN population allocated at the lowest point with 1.507. Meanwhile, the number of effective alleles per locus was the highest and lowest value at QB (1.442) and NA (1.401), respectively, with an average of 1.424.

Moreover, in QB population, the Shannon information

index (*I*) and Nei's gene diversity (*h*) reached the highest point of 0.387 and 0.258, respectively, and showed the lowest point at QN (0.359) and NA (0.238).

Genetic variation between populations

Genetic distance ranged from 0.042 (NA and QB) to 0.214 (NA and QN). The Nei's genetic differentiation (*G_{st}*) between populations which is 0.1867 was also calculated. The result indicated the low genetic variation among populations. Genetic diversity in total set of population (*H_t*) and average gene diversity within

Table 4. Nei' genetic distance of three *P. merkusii* populations.

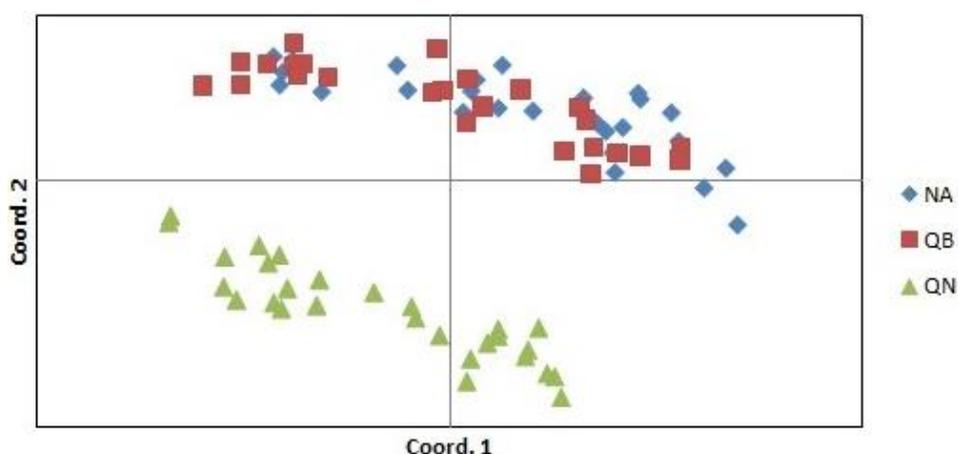
	NA	QB	QN	Ht	0.3131
NA	0000			Hs	0.2546
QB	0.042	0000		Gst	0.1867
QN	0.214	0.2	0000		

H_t Gene diversity in total set of populations; H_s average gene diversity within population; G_{st} Nei's genetic differentiation index among populations.

Table 5. Analysis of molecular variance (AMOVA) for three pines populations.

Source of variation	df	SS	MS	Est.Var.	Total variation (%)	P value
Among population	2	210.705	105.352	3.641	28	<0.001
Within populations	76	727.953	9.578	9.578	72	<0.001
Total	78	938.658		13.219	100	

df: Degree of freedom; p value: the probability of obtaining a more extreme component estimate by chance; SS: sums of square; Est.Var: estimate variation component

**Figure 3.** The clustering of three *P. merkusii* populations based on PCoA analysis. Coord.1 and coord.2 are the first and second components of PCoA analysis, respective.

population (H_s) accounted for 0.3131 and 0.2546, respectively (Table 4). Moreover, molecular variance (Table 5) obtained the low proportion between populations (28%) as well as high proportion within population (72%). These results demonstrated that the differentiation within and among populations was high (Table 5).

The genetic diversity of three populations was established based on PCoA analysis (Figure 3). The PCoA coordinate dimensions accounted for 22.85 and 19.74% of the total variation, respectively. The population divided into two groups where cluster I included NA and QB groups and cluster II contained QN group.

DISCUSSION

The RAPD markers were successful for analyzing genetic variation in Vietnam pine populations. All the nine RAPD primers expressed high number of bands and PIC (0.82). These results illustrated that RAPD method is a powerful technique for genetic variation analysis of Vietnamese populations. Nei' gene diversity (h) (0.246) and Shannon' information index (I) (0.369) of this study suggested a moderate genetic diversity within three Vietnamese pine populations. The moderate genetic diversity within population may be caused by the bottlenecks during the evolutionary processes and the inbreeding after

bottlenecked of small populations (Alrababah et al., 2011; Zhang et al., 2005).

The Nei's genetic differentiation index among populations (G_{st}) (0.1867) reflected narrow genetic variation among populations. The genetic distances among three populations were low, from 0.042 (NA and QB) to 0.214 (NA and QN). The low level of genetic distance among NA and QB populations was the characteristic of endangered species (Slatkin, 1985). Moreover, the results were caused by the genetic drift, migration, selection or isolation of gene resources (Fisher and Matthies, 1998; Sun and Wong, 2001; Gómez et al., 2010). Other causes were that the local communities selected and breeding of these plants from other locations (Gómez et al., 2010). Furthermore, the QN population was more different than NA and QB caused the geographic distance.

Interestingly, the molecular variation within population (72%) showed higher than between populations (28%) (Table 5). This result is crucial for outcrossing woody plants (Alvarrez et al., 2001; Hamrick and Godt, 1996; Heaton et al., 1999; Steiger et al., 2002). High level of genetic within populations is significant for breeding strategies. Alvarez et al. (2001) showed that cross pollinating species always have the genetic diversity higher than self-pollinating species (Alvarez et al., 2001). In the PCoA diagram (Figure 3), the first and second coordinate accounted for 22.85 and 19.74% of the total variation. The result showed low genetic variation among the *P. merkusii* populations.

In conclusion, this study suggests that three *P. merkusii* populations need effective conservation and breeding programs to develop the genetic diversity of resources of pines in Vietnam.

Conflict of Interests

The authors certify that there is no actual or potential conflict of interest in relation to this article.

ACKNOWLEDGEMENTS

This study was supported by Applied DNA Technology Department and National Key Laboratory of Gene Technology Institute of Biotechnology, Vietnam Academy of Science and Technology.

REFERENCES

- Alrababah MA, Al-Horani AS, Alhamad MN, Migdadi HM (2011). Genetic diversity of the eastern most fragmented Mediterranean *Pinus halepensis* Mill. populations. *Plant. Ecol.* 212(5):843-851.
- Alvarez AE, Van de wiel CCM, Smulders MJM, Vosman B (2001). Use of microsatellite to evaluate genetic diversity and species relationships in the genus *Lycopersicon*. *Theor. Appl. Genet.* 103(8): 1283-1292.
- Bharali S, Deka J, Saikia P, Khan ML, Paul A, Tripathi OP, Singha LB, Shanker U (2013). *Pinus merkusii* (Jungh et DeVriese) a vulnerable gymnosperm needs conservation. *NeBio.* 3(1):94-95.
- Changtragoon S, Finkelde R (1995). Patterns of genetic variation and characterization of the mating system of *Pinus merkusii* in Thailand. *For. Genet.* 2(2):87-97.
- Cooling EN (1968). Fast growing timber trees of the lowland tropics: *Pinus merkusii* No 4, 1 st ed. Oxford Forestry Institute, Oxford.
- Cruzan MB (1998). Genetic markers in plant evolutionary ecology. *Ecology* 79(2):400-412.
- Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. *Focus.* 12:13-15.
- Farjon A (2005). Pines: Drawings and Descriptions of the Genus *Pinus*, 2nd ed. Boston: Brill Leiden.
- Finkeldey R, Hattermer HH (2007). Tropical forest genetics. Springer, Berlin, Heidelberg, New York.
- Fischer M, Matthies D (1998). Experimental demography of the rare *gentianella germanica*: seed bank formation and microsite effects on seedling establishment. *Ecography* 21(3):269-278.
- Fritsch P, Rieseberg LH (1996). The use of random amplified polymorphic DNA (RAPD) in Conservation genetics. Smith TB, and Wayne RK, editors. In: Molecular Genetic Approaches In Conservation, pp. 54-73.
- Gauli A, Gailing O, Stefrmon VM, Finkeldey R (2009). Genetic similarity of natural populations and plantations of *Pinus roxburghii* Sarg. in Nepal. *Ann. For. Sci.* 66(7):703.
- Gómez A, Masselli S, Bueno MA (2010). Distribution of the genetic diversity of *pinus ayacahuite* (Ehrenberg) at the Communal Forest of Totonicapán, Guatemala. *Biorem. Biodiv. Bioavail.* 4(1):35-41.
- Hamrick JL, Godt MJW (1996). Effects of life history traits on genetic diversity in plant species, philosophical Transactions of the Royal Society(B) of London. *Biol. Sci.* 351(1345):1291-1298.
- Heaton HJ, Whitkus R, Gomez-pompa A (1999). Extreme ecological and phenotypic differences in the tropical tree chizopate (*Manilkara zapota* L) are not matched by genetic divergence: A random amplified polymorphic DNA (RAPD) analysis. *Mol. Ecol.* 8:627-632.
- Hidayat J, Hansen CP (2002). *Pinus merkusii* Jungh. et de Vriese. Seed leaflet 60 Daniada Forest Seed Center, Humlebaek, Denmark.
- IUCN. IUCN Red List of Threatened Species. Version 2011; 1. Available: <http://www.iucnredlist.org>.
- Kumar A, Walker S, Molur S (2000). Prioritisation of endangered species. Setting biodiversity priorities for India-World Wide Fund for Nature, India, New Delhi, pp341-386.
- Lee SW, Ledig FT, Johnson DR (2002). Genetic variation at allozyme and RAPD markers in *Pinus longaeva* of the White Mountains, California. *Am. J. Bot.* 89(4):566-577.
- Mariette S, Chagné D, Lézier C, Pastuszka P, Raffin A, Plomion C, Kremer A (2001). Genetic diversity within and among *Pinus pinaster* populations: comparison between AFLP and microsatellite markers. *Heredity.* 86(4):469-479.
- Marquardt PE, Echt CS, Epperson BK, Pubanz DM (2007). Genetic structure, diversity, and inbreeding of eastern white pine under different management conditions. *Can. J. For. Res.* 37:2652-2662.
- Navascues M, Emerson BC (2007). Natural recovery of genetic diversity by gene flow in reforested areas of the endemic Canary Island pine, *Pinus canariensis*. *For. Ecol. Manag.* 244(1-3):122-128.
- Nurtjahjaningsih ILG, Saito Y, Tsuda Y, Ide Y (2007). Genetic diversity of parental and offspring populations in a *Pinus merkusii* seeding orchard detected by microsatellite markers. *Bull. Tokyo. Univ.* 118:1-14.
- Pavel AB, Vasile CL (2012). PyElph a software tool for gel images analysis and phylogenetics. *BMC Bioinformatics* 13: 9.
- Peakall R, Smouse PE (2006). GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol.* 6(1):288-295.
- Qian W, Ge S, Hong DY (2001). Genetic variation within and among populations of a wild rice *Oryza granulata* from China detected by

- RAPD and ISSR. *Theor. Appl. Genet.* 102(2-3):440-449.
- Razal RA, Tolentino ELT Jr, Carandang WM, Nghia NH, Hao PS, Luoma-Aho T (2005). Status of genetic resources of *Pinus merkusii* (Jung et De Vriese) and *Pinus kesiya* (Royle ex Gordon) in Southeast Asia. Los Baños, the Philippines & Serdang, Malaysia, University of the Philippines Los Baños College of Forestry and Natural Resources & International Plant Genetic Resources Institute (IPGRI) Regional Office for Asia, the Pacific and Oceania.
- Santisuk T (1997). Geographical and ecological distributions of the two tropical pines, *Pinus kesiya* and *Pinus merkusii*, in Southeast Asia. *Thai For. Bull. (Bot)*. 25:102-121.
- Sharma K, Degan B, Wuehlisch GV, Singh NB (2002). Allozyme variation in eight natural population of *Pinus roxburghii* Sarg. in India. *Silvae. Genet.* 51(5-6):246-253.
- Siregar IZ, Hattermer HH (2004). Patterns of genetic structure and variation of Merkus pine (*Pinus merkusii*) in Indonesia. *J. Trop. For. Sci.* 16(2):160-172.
- Slatkin M (1985). Rare alleles as indicators of gene flow. *Evolution.* 39(1):53-65.
- Steiger D, Nagal C, Moore P, Morden C, Osgood R, Ming R (2002). ALFP analysis of genetic diversity within and among *Coffea arabica* cultivars. *Theor. Appl. Genet.* 105(2-3):209-215.
- Sun M, Wong KC (2001). Genetic structure of three orchid species with contrasting breeding systems using RAPD and allozyme markers. *Am. J. Bot.* 88(12):2180-2189.
- Suwarni E, Dancik BP, Khasa D (1993). Inheritance of isozyme variants in seed tissues of *Pinus merkusii* Jung et De Vriese. *Biol. Chem. Genet.* 37(11-12):369-375.
- Thao DV, Widyatmoko AYPBC, Guan L, Gotoh E, Watanabe A, Shiraiishi S (2013). Isolation and characterization of tetranucleotide microsatellite markers for *Pinus merkusii*. *Conserv. Genet. Resour.* 5(2):433-436.
- Theilade I, Graudal L, Kjaer E (2000). Conservation of genetic resources of *Pinus merkusii* in Thailand. Technical Note No. 58. Danida Forest Seed Centre, Humlebaek, Denmark.
- Thomas BR, Macdonald SE, Hicks M, Adams DL, Hodgetts RB (1999). Effects of reforestation methods on genetic diversity of lodgepole pine: an assessment using microsatellite and randomly amplified polymorphic DNA markers. *Theor. Appl. Genet.* 98(5):793-801.
- Xia M, Zhou XF, Zhao SD (2001). RAPD analysis on genetic diversity of natural populations of *Pinus koraiensis*. *Acta. Ecol. Sin.* 21(5):730-737.
- Yeh FC, Yang R, Boyle TJ, Ye Z, Xiyun JM (2000). POPGENE 32, Microsoft Window-based Freeware for Population Genetic Analysis. Molecular Biology and Biotechnology Centre, University of Alberta; Edmonton, Canada.
- Zhang ZY, Chen YY, Li DZ (2005). Detection of low genetic variation in a critically endangered Chinese Pine, *Pinus squamata*, using RAPD and ISSR markers. *Biochem. Genet.* 43(5-6):239-249.