STAURT-ROGERS, C. and A. J. FLAVELL (2001): The evolution of Ty1-*copia* group retrotransposons in gymnosperms. Mol. Biol. Evol. **18**: 155–163.

STEBBINS, G. L. (1948): The chromosomes and relationship of *Metasequoia* and *Sequoia*. Science **108**: 95–98.

STEBBINS, G. L. (1950): Variation and Evolution in Plants. Columbia University Press, New York.

STEBBINS, G. L. (1971): Chromosome Evolution in Higher Plants. Edward Arnold, London.

SYRACH-LARSEN, C. and M. WESTERGAARD (1938): A triploid hybrid between *Larix deciduas* Mill. and *Larix occidentalis* Nutt. J. Genet. **36**: 523–530.

TAKASO, T. and P. B. TOMLINSON (1992): Seed cone and ovule ontogeny in *Metasequoia*, *Sequoia* and *Sequoiadendron* (Taxodiaceae, Coniferales). Bot. J. Linn. Soc. **100**: 15–37.

The Arabidopsis Genome Initiative. (2000): Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408**: 796–815.

THOMAS, G. and K. K. CHING (1968): A comparative karyotype analysis of *Pseudotsuga menziesii* (Mirb.) Franco, and *Pseudotsuga wisconiana* (Hayata). Silvae Genet. **17**: 138–143.

TODA, Y. (1996): Karyomorphological studies of the Taxodiaceae. Forest Genetics **3**: 141–146.

VAN DE PEER, Y., J. S. TAYLOR and A. MEYER (2003): Are all fishes ancient polyploids? J. Structural and Functional Genomics **2**: 65–73.

VEBLEN, T. T. and D. H. ASHTON (1982): The regeneration status of *Fitzroya cupressoides* in the Cordillera Pelada, Chile. Biol. Cons. **23**: 141–161.

WENDEL, J. F. (2000): Genome evolution in polyploids. Plant Mol. Biol. **42**: 225–249.

WOLFE, K. H. (2001): Yesterday's polyploids and the mystery of diploidization. Nature Rev. Genet. **2**: 323–341.

WRIGHT, J. W. (1976): Introduction to Forest Genetics. Academic Press, New York.

ZHANG, S., L. QI, C. CHEN, X. LI, W. SONG, B. CHEN and S. HANN (2004): A report of triploid *Populus* of the section Aigeiros. Silvae Genet. **53**: 69–75.

ZINNAI, L. (1952): Tetraploid plants in Japanese red pine (*Pinus densiflora* Sieb. Et Zucc.) discovered in transplant beds. J. Jap. For. Soc. **34**: 185–187.

ZINNAI, L. (1953): The morphological characters and the fertility of the pollen of a tetraploid Japanese red pine induced by the colchicines method. J. Jap. For. Soc. **35**: 245–248.

Reproductive Success of Pollen Derived From Selected and Non-Selected Sources and its Impact on the Performance of Crops in a Nematode-Resistant Japanese Black Pine Seed Orchard

By S. GOTO[1,*], A. WATANABE[2], F. MIYAHARA[3] and Y. MORI[3]

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Abstract

The reproductive success of pollen derived from selected and non-selected sources and its impact on the performance of orchard crops were evaluated, using five pairs of microsatellite markers, in a Japanese black pine (*Pinus thunbergii* Parl.) clonal seed orchard consisting of 16 nematode-resistant clones. The paternity of each open-pollinated seed was determined by comparing the genotypes of seeds from six clones (24 trees) with genotypes of the 16 orchard clones and two trees (N1, N2) representing other genotypes that had been inadvertently included in the orchard. Out of 384 seeds examined, the paternity of 316 seeds (82.3%) was assigned to the clones within the seed orchard. On average, the male reproductive success of orchard clones varied from 0.0% to 10.5%, and was significantly related to the male-flowering fecundity of each clone. It was not related to the synchrony of flowering phenology between mates. The expected proportions of seeds produced by clonal trees as a result of pollination by orchard clones, and by contaminating pollen originating from internal and external sources were estimated at 86.8%, 3.3% and 9.9%, respectively. Nematode-resistant seedlings of Japanese black pine were produced from surviving 2-yr seedlings that had previously been inoculated with pinewood nematode (*Bursaphelenchus xylophilus*). Without pollen contamination, the survival rate of seedlings produced by mating between resistant clones is expected to be 62.4%. However, in this orchard the figure was reduced to 57.5%, due to pollen contamination from both internal and external sources.

[1]) University Forest in Hokkaido, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yamabe, Furano, Hokkaido 079-1561, Japan.

[2]) Forest Tree Breeding Center, Independent Administrative Institution, Ishi, Juo, Hitachi, Ibaraki 319-1301, Japan.

[3]) Fukuoka Prefecture Forest Research and Extension Center, Toyoda 1438-2, Yamamoto-machi, Kurume, Fukuoka 839-0827, Japan.

[*]) Communicating author: SUSUMU GOTO, University Forest in Hokkaido, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yamabe, Furano, Hokkaido 079-1561, Japan. Phone +81-167-42-2111, Fax +81-167-42-2689, E-mail: gotos@uf.a.u-tokyo.ac.jp

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Key words: nematode-resistant, microsatellite, *Pinus thunbergii*, microsatellite, pollen contamination, reproductive success, seed orchard.

Introduction

Seed orchards are usually established by the selection, collection, and propagation of phenotypically or genotypically superior trees. Genetic gain calculations are based on complete panmixia in the orchard, taking no account of the possibility of pollination by non-selected trees. However, studies with genetic markers have demonstrated pollen contamination levels of over 30% in most seed orchards (WHEELER and JECH, 1992 for review; ADAMS *et al.,* 1997; STOEHR *et al.,* 1998; PAKKANEN *et al.,* 2000; MORIGUCHI *et al.,* 2004). Theoretically, if the superior trees in a seed orchard are all successfully pollinated by trees with non-selected characteristics, the genetic gain would decrease by up to 50%. Therefore, pollen contamination in open-pollinated seed orchards is potentially one of the most serious factors reducing the genetic efficiency of improved seeds. However, few studies have thoroughly examined the impact of pollen contamination on the performance of orchard crops through progeny testing (PLOMION *et al.,* 2001; GOTO *et al.,* 2002b; GRATTAPAGLIA *et al.,* 2004; STOEHR *et al.,* 2004).

For the successful management of seed orchards, and to prevent contamination, it is essential to identify the source of the pollen. Few reports, however, refer to the source of pollen contamination. ADAMS *et al.* (1997) suggested that 79% of the contamination within a block in a Douglas-fir seed orchard came from natural stands, while 21% was from other blocks in the orchard. In a Sugi (*Cryptomeria japonica*) seed orchard, about 10% of the contamination was derived from other blocks in the orchard (MORIGUCHI *et al.,* unpublished). Unfortunately, mis-planting and/or mis-labeling commonly occur within seed orchards (HARJU and MUONA, 1989; WHEELER and JECH, 1992; KAWAUCHI and GOTO, 1999; GOTO *et al.,* 2001). Occasionally, non-selected genotypes are included amongst the orchard trees as a result of mistakes during the collection or propagation of selected genotypes and as a result of rootstocks overtaking their grafts (VAN DE VEN and MCNICOL, 1995; KAWAUCHI and GOTO, 1999). Such non-selected trees within the orchard will be internal sources of both pollen and seed contamination. However, the level and influence of non-selected trees within the orchard are not yet fully understood.

The maximum gene diversity for a given number of clones occurs when mating among the seed orchard parents approaches panmixis, and all orchard genotypes contribute equally to the crop (KANG *et al.,* 2001). The contribution of clones as pollen donors has been evaluated, using allozyme or DNA molecular markers, for *Picea glauca* (SCHOEN and STEWART, 1986), *Pseudotsuga menziesii* (STOEHR *et al.,* 1998), *Pinus thunbergii* (GOTO *et al.,* 2002a), *Pinus contorta* (STOEHR and NEWTON, 2002), *Quercus robur* (BUITEVELD *et al.,* 2001), and *Cryptomeria japonica* (MORIGUCHI *et al.,* 2004). In general, the mating dynamics within orchard clones deviate significantly from panmixis. Not only inter-mate distances, but also several biological factors such as flowering fecundity and synchrony of flowering phenology between mates, affect the male reproductive success of orchard clones (EL-KASSABY *et al.,* 1984; SCHOEN and STEWART, 1986; ERICKSON and ADAMS, 1989; BURCZYK and PRAT, 1997; BURCZYK *et al.,* 2002). To improve the parental balance in a seed orchard, it is necessary to determine the strength of these influences on male reproductive success.

Japanese black pine (*Pinus thunbergii* Parl.) is a very common and important tree species that is often used in wind breaks to provide protection against sand and salt in coastal areas of Japan. However, during the last five decades the species has been severely affected by pine wilt disease, caused by the pinewood nematode (*Bursaphelenchus xylophilus*), especially in southwestern Japan. Because of the severity of damage caused by the pest and the importance of Japanese black pine, a research project was initiated in 1978 to examine the selection and production of pinewood nematode-resistant plant material. Within this project, sixteen resistant plus trees of Japanese black pine were selected from 14,620 candidates (FUJIMOTO *et al.,* 1989). Clonal seed orchards, consisting of these resistant clones, were established through grafting. These seed orchards have played an important role in the reforestation of the coastal area affected by pine wilt disease (TODA *et al.,* 1993). In the Kyushu district, Japan, Japanese black pine plants resistant to pine wilt disease have been produced by inoculating seedlings from the seed orchard with the pinewood nematode (TODA *et al.,* 1993).

In the previous study, we used random amplified polymorphic DNA (RAPD) markers (WILLIAMS *et al.,* 1990) for clonal checking of ramets in the Japanese black pine clonal seed orchard located in Fukuoka prefecture, Japan. As a result, we detected two non-selected trees (N1, N2) within the seed orchard (GOTO *et al.,* 2001). These trees will act as internal sources of pollen contamination of the seed orchard (internal pollen contamination, hereafter). In this seed orchard, male reproductive success of orchard clones and pollen contamination were assessed using 45 RAPD markers (GOTO *et al.,* 2002a). The results indicated that the male reproductive success of each orchard clone deviated significantly from the panmictic situation and that pollen contamination was around 2%. This value should be regarded as a minimum estimate of pollen contamination, because some contaminants are likely to have banding patterns indistinguishable from orchard genotypes due to the low polymorphism levels of RAPD markers. Moreover, the source of pollen contamination could not be determined. Recently, microsatellite markers that are co-dominant and characteristically exhibit high levels of polymorphism have been successfully applied to seed orchard studies (BUITEVELD *et al.,* 2001; CHARIX *et al.,* 2003; GRATTAPAGLIA *et al.,* 2004; MORIGUCHI *et al.,* 2004).

In this study, we used microsatellite markers to evaluate the reproductive success of pollen derived from selected and non-selected sources, including both internal and external pollen contamination. We discuss the influence of pollen contamination on the performance of orchard crops, especially on the resistance of seedlings to the pinewood nematode and on the cost of nematode-resistant Japanese black pine seedling production.

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Materials and Methods

Seed orchard

The orchard used in this study was the clonal Japanese black pine seed orchard located near Ogoori, in Fukuoka prefecture, Japan. The orchard (0.5 ha in size) contains 16 nematode-resistant clones selected during the project on nematode-resistance of Japanese black pines (FUJIMOTE *et al.,* 1989). Ramets were multiplied by grafting, then planted 5 x 5 m apart and distributed throughout the orchard according to GIERTYCH (1965). The seed orchard was established in 1988. When established, the seed orchard contained 200 trees and in 2002, at the time of seed collection for this study, 159 mature trees were present within it. The male-flowering fecundity of each tree was recorded during May 1999, the flowering season. We assigned a male-flowering fecundity score (from 1 to 5) by visually inspecting the pollen shedding, where 1 and 5 indicated the lowest and highest pollen production, respectively. Flowering phenology was also observed during the flowering season, from 11th April to 7th May 2001, at intervals of two or three days. Thus, the synchrony of phenology between pairs of clones was investigated.

Plant material and DNA analysis

In October 2002, we collected 384 open-pollinated seeds from 24 maternal trees (*Fig. 1*), including six clones (16 seeds x 4 ramets x 6 clones). Needles of 16 orchard clones and non-selected trees (N1, N2) were also collected for the paternity analysis. For each sample, DNA was extracted using a DNeasy Plant Mini Kit (Qiagen Co LTD) or a Plant Genomic DNA Mini kit (Viogene-Biotek Corp.), from the hypocotyls of each germinated seed or from approximately 150 mg of needles from the adult trees. Microsatellite loci were amplified using five primer pairs. Two primer pairs (pde5 and pde14) were adapted from LIAN *et al.* (2000), and additional three primers were developed (bcpd006, bcpd015 and bcpd309) by Watanabe et al. (unpublished). The clone-sequences of bcpd006, bcpd015 and bcpd309 contained repeat $(AT)^5(GT)^{21}$, $(TA)^4(TG)^{20}$, and $(AT)^4(GT)^{19}$, respectively. The primer sequences were as follows: bcpd006, forward 5'TATAGTATTGTATGTCTTGAATG, bcpd006; reverse 5'CATCATTGTTATTGCTATCC; bcpd015, forward 5'CAATAACAAATGGTTCCATG; bcpd015, reverse 5'CTAAGGTATTTTTTTCCTCCG; bcpd309, forward 5' GATGTGTCATCTATCCATCCC; bcpd309, reverse 5' ATCTGTGTGGCTCATATTCG. Genotypes were scored according to PCR product length at each locus using a Prism 3100 Genetic Analyzer (ABI) and GeneScan analysis software (ABI).

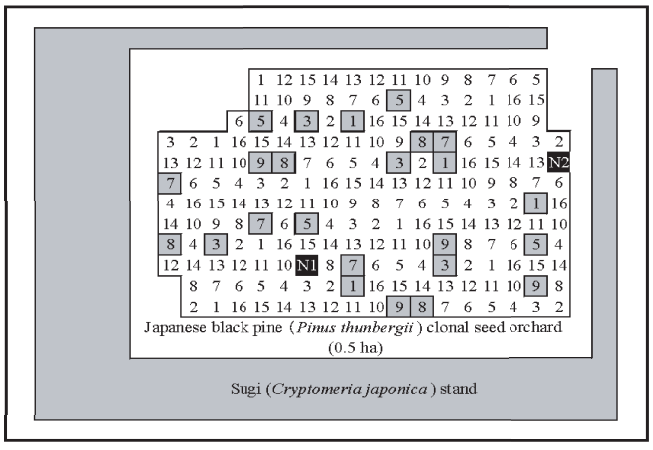

Figure 1. – Layout of the orchard clones and location of maternal and non-selected trees (N1, N2). Nos. 1–16 indicate the designated number of the orchard clones; 1: Namikata(t)-73, 2: Obama(t)-30, 3: Tosashimizu(t)-63, 4: Ooita(t)-8, 5: Ei(t)-425, 6: Tsuyazaki(t)-50, 7: Misaki(t)-90, 8: Ooseto(t)-12, 9: Namikata(t)-37, 10: Tanabe(t)-54, 11: Yasu(t)-37, 12: Shima(t)-64, 13: Yoshida(t)-2, 14: Sendai(t)-290, 15: Mitoyo(t)-103, 16: Bizen(t)-143. The squares represent maternal trees used for seed collection in this study.

Paternity assignment

Genotypes of 384 seeds and 18 candidate paternal parents were determined at five microsatellite loci and paternity of each seed was assigned based on simple exclusion method (DOW and ASHLEY, 1996). Possible paternal alleles at every locus, inferred by subtracting the maternal alleles from the offspring alleles, were compared with the 18 candidates. Genotypes that did not share possible paternal alleles at the locus were considered not to be progeny of the candidate paternal parent. If all 18 genotypes were excluded (no match), we considered that their paternal parent was outside the orchard, thus indicating external pollen contamination. When all but one candidate genotype was excluded (an exact match), we designated that genotype as the paternal parent. Null alleles can cause serious problems when using microsatellite markers, because they can lead to exclusion of the "true" paternal parent (MORIGUCHI *et al.,* 2004). Therefore, if a mismatch was detected in the genotypes of maternal clones and their seeds, we assumed the existence of a null allele for the paternity analysis. In such cases, when both the maternal and offspring genotypes were homozygous, they were treated as heterozygotes possessing one null allele. In such cases, the true parent must not be excluded from the possible parents (DOW and ASHLEY, 1996).

Estimating pollen dispersal from a non-selected tree

The observed genetic contribution (P_O) of a non-selected tree was defined as the proportion of the seeds sired by a non-orchard tree as determined by the paternity analysis. The density of pollen dispersed from individual trees decreases rapidly with distance from the source (WANG *et al.,* 1960; SILEN, 1962). We assumed that the expected genetic contribution (P_E) of a non-selected tree is according to normal distribution, with variance τ^2, and can be described by the equation:

$$P_E = \alpha EXP(-(d^2/2\tau^2))$$

where α is a constant and d is the distance from the non-selected tree to mother tree. We calculated the sum of squares error of the difference between the observed (P_O) and expected genetic contributions (P_E). We then used the Solver function in Microsoft Excel to optimize the parameters α and τ to minimize this error. We applied the model with the best fit to the 159 mature trees in the seed orchard, and thus estimated the internal pollen contamination for the total crop.

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Impact of pollen contamination on the orchard crop

Nematode-resistant Japanese black pine is produced by inoculating seedlings from the orchard with the pinewood nematode and selecting those that show the resistance. The survival rate of inoculated seedlings is about 62.4% when the only parents are orchard clones and there has been no pollen contamination (GOTO et al., 2002b). Seedlings sired as a result of pollen contamination have a decreased survival rate: only 25.0% (GOTO *et al.,* 2002b). Based on the survival rates determined by GOTO *et al.* (2002b), we calculated the actual survival rate (As) in this seed orchard as follows:

$$As = MO \times 0.624 + \{IntPC + ExtPC\} \times 0.250$$

where MO is the proportion of seeds produced by mating between orchard clones, and IntPC and ExtPC represent the proportions of internal and external pollen contamination, respectively. Recently, about 50,000 seedlings in Fukuoka prefecture, Japan, have been inoculated with the pinewood nematode. Of these, 31,200 (50,000 x 0.624) seedlings would be expected to survive in the absence of pollen contamination. The economic loss was calculated by comparing the real and ideal situations (i.e. with and without pollen contamination), assuming the cost of each nematode-resistant plant to be 500 Japanese yen.

Table 1. – Allelic diversity of microsatellite loci used in this study.

Locus	k	$H_{(O)}$	$H_{(E)}$
bcpd006	9	0,833	0,765
bcpd015	11	0,944	0,900
bcpd309	12	0,500	0,844
pde5	5	0,333	0,659
pde14	11	0,722	0,862
Mean	9,6	0,666	0,806

Allelic diversity is caluculated using 18 parental clones in *Pinus thunbergii*. k: the number of alleles, $H_{(E)}$: expected heterozygosity, $H_{(O)}$: observed heterozygosity. Total exclusionary power for the second parent is caluculated as 0.993 using computer program CERVUS 2.0 (MARSHALL et al., 1998).

Results

Allelic diversity of microsatellite loci

The number of alleles for the 18 candidate genotypes ranged from 5 to 12, with an average of 9.6 (*Table 1*). The average expected heterozygosity over the five loci was 0.806. The total exclusion probability for the second parent was 0.993 (MARSHALL *et al.,* 1998). The high polymorphism of the microsatellite loci allows precise determination of paternity. When comparing the genotype of each seed with its maternal clone, a mismatch was detected in seeds at the bcpd006 and bcpd309 loci. This may be caused by the presence of a null allele. Therefore, we assumed the presence of a null allele in these loci in the paternity determinations.

Male reproductive success of orchard clones and influencing factors

Out of 384 seeds examined, the paternity of 316 seeds (82.3%) was assigned to the clones within the seed orchard. Overall, internal pollen contamination by N1 and N2 amounted to 3.6% and 0.0%, respectively (*Table 2*). External pollen contamination affected 9.9% of the samples overall. The paternity of 16 seeds (4.2%) was assigned to multiple clones, therefore these 16 seeds were excluded from the subsequent analysis. The mean male reproductive success of the six parental clones varied widely, from 0% for Ei(t)-495 and Bizen(d)-143 to 10.5% for Tsuyazaki(t)-50. The selfing rate of the six parental clones ranged from 0.0% to 20.3%, with an

Table 2. – Male reproductive success of orchard clones and internal pollen contamination by non-selected trees (N1, N2) and external pollen contamination.

		Parental clones						
ID	Orchard clone	Namikata(t)-73	Tosashimizu(t)-63	Ei(t)-495	Misaki(t)-90	Ooseto(t)-12	Namikata(t)-37	mean
1	Namikata(t)-73	**0,203**	0,016	0,143	0,016	0,067	0	0,074
2	Obama(t)-30	0,109	0,066	0,032	0,048	0,067	0,017	0,056
3	Tosashimizu(t)-63	0,031	**0,066**	0,016	0,032	0,017	0,086	0,041
4	Ooita(t)-8	0,016	0,082	0,111	0,113	0,100	0,138	0,093
5	Ei(t)-495	0	0	**0**	0	0	0	0
6	Tsuyazaki(t)-50	0,109	0,066	0,175	0,145	0,083	0,052	0,105
7	Misaki(t)-90	0,156	0,033	0,048	**0,065**	0,117	0,052	0,078
8	Ooseto(t)-12	0,156	0,098	0,111	0,032	**0,133**	0,086	0,103
9	Namikata(t)-37	0,094	0,115	0,063	0,065	0,117	**0,121**	0,096
10	Tanabe(t)-54	0	0	0,016	0,065	0,067	0,034	0,030
11	Yasu(t)-37	0,016	0,033	0,095	0,065	0,017	0,034	0,043
12	Shima(t)-64	0,016	0,115	0,032	0,048	0,033	0,034	0,046
13	Yoshida(t)-2	0	0,016	0	0,032	0,017	0	0,011
14	Sendai(t)-290	0,031	0,131	0,032	0,065	0,100	0,103	0,077
15	Mitoyo(t)-103	0,016	0	0	0	0	0	0,003
16	Bizen(t)-143	0	0	0	0	0	0	0
	Internal pollen contamination (N1)	0	0,016	0,016	0,081	0,050	0,069	0,036 *
	Internal pollen contamination (N2)	0	0	0	0	0	0	0 *
	External pollen contamination	0,047	0,148	0,111	0,129	0,017	0,172	0,099 *

Bold font: Selfing, * Internal and external pollen contamination overall samples.

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Table 3. – Regression analysis of the relationship between male reproductive success and synchrony of flowering phenology, male flowering fecundity of each orchrad clone. The independent variables in the regression is male reproductive success of orchard clone.

Variables	R^2	Adjusted R^2	Standardized coefficient	t	P
Constant	0,426	0,413	-	0,389	0,698
Male flowering fecundity			0,652	8,286	<0.001
Synchrony of flowering phenology			-0,016	-0,199	0,843

average of 9.8%. The mean flowering fecundity score ranged from 0.09 for Mitoyo(t)-103 to 2.15 for Tsuyazaki(t)-50, excluding Bizen(t)-143 (data not shown). The clone Bizen(t)-143 was introduced to this orchard in 2000, later than the other clones, therefore male flowers of this clone were very rare during the year of seed collection (2001). The average overlap of the flowering periods of parental clones and paternal candidates was about four days. There was no distinct difference in synchrony between pairs: except that the non-selected tree N2 flowered after most of the orchard's female flowers were finished. Regression analysis indicated that flowering fecundity of orchard clones was strongly associated with male reproductive success, whereas synchrony of flowering phenology was not correlated with it (*Table 3*).

Estimates of pollen contamination from internal and external pollen sources

The observed genetic contribution of N2 in this study was 0.0%. This tree produced very few male flowers and its flowering did not coincide with most of the female flowers of the orchard clones. Therefore, we assumed that the influence of N2 as a male parent in this seed orchard could be ignored. We, therefore, calculated the expected genetic contribution (P_E) based on N1 as the sole internal source of pollen contamination. Parameters α and τ were assigned values of 12.5% and 17.5 m, respectively, to minimize the sum of squares error of the difference between P_O and P_E (*Fig. 2*). When we applied this model to all of the 159 mature trees within the seed orchard, the proportion of seeds sired by N1 was estimated as 3.3% of the total orchard crop. In contrast, outside pollen contamination reaching each female parent tree was not related to their distance from the edge of the seed orchard. Therefore, we applied the amount of external pollen contamination determined in this study (9.9%) to the whole orchard crop.

Impact of pollen contamination on the orchard crop

In this study, the proportion of seedlings produced by mating between orchard clones was estimated at 86.8% for the total crop. Internal and external pollen contamination was estimated at 3.3% and 9.9%, respectively. Therefore, the actual survival rate (A_S) was predicted to be 0.575 (0.868 x 0.624 + (0.033 + 0.099) x 0.250). When 50,000 seedlings are inoculated, 31,200 (50,000 x 0.624) seedlings will survive without pollen contamination. However, only 28,730 (50,000 x 0.575) seedlings will survive in the real situation. Assuming the price of nematode-resistant seedlings is 500 Japanese yen, the gross cost of the difference is 15,600,000 minus 14,365,227 Japanese yen. Therefore, the economic loss was calculated as 1,234,733 Japanese yen (corresponding to over 9,000 Euro$).

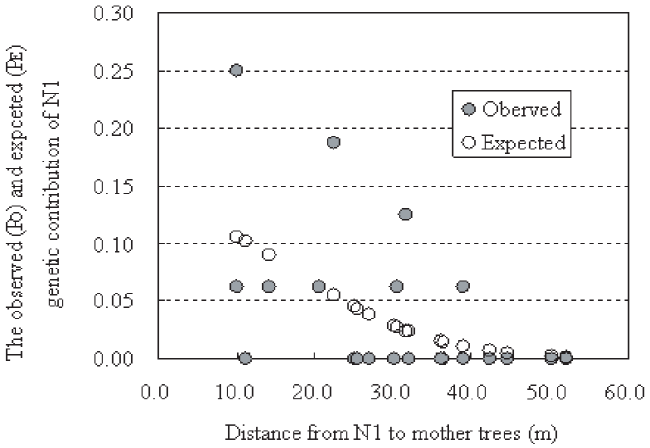

Figure 2. – The observed (P_O) and expected (P_E) genetic contributions of N1 to the seeds for each of the maternal trees and the distance from N1 to each maternal tree. The observed genetic contribution (P_O) of the non-selected tree N1 was defined as the proportion of the seeds sired by N1, determined by paternity analysis. The expected genetic contribution (P_E) was based on a normal distribution with variance τ^2: where a is a constant and d indicates the distance from N1 to each mother tree. The optimized parameters α and τ were 12.5% and 17.5 m, respectively.

Discussion

The exclusion probability over the five loci was high (0.993), indicating that high levels of polymorphism guarantee precise paternity assignment for orchard crops. In this study, out of 384 seeds surveyed, the paternity of 368 seeds (95.8%) could be assigned using only five pairs of microsatellite markers. GOTO *et al.* (2002a) identified the male parent of 559 out of 648 seedlings (86.3%) using 45 RAPD markers. Therefore the use of microsatellite markers greatly improved the precision and efficiency of the paternity analysis.

Mating between orchard clones deviated significantly from panmixis ($\chi^2 = 60.6$, $p < 0.001$). The male reproductive success of each clone varied from 0.0 to 10.5% in this seed orchard (*Table 2*). Studying the same seed orchard, GOTO *et al.* (2002a) found a greater deviation from panmixis ($\chi^2 = 218.0$, $p < 0.001$) than reported here. There is greater parental balance in a mast year than in a year of poor seed production (SEIDO, 2001). However, the seeds were collected in 1997 (GOTO *et al.,* 2002a) and 2002 (this study), both of which were mast years, so this

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cannot explain the difference in deviation from panmixis. Parental balance may be improved as the seed orchard matures. Flowering phenology often affects male reproductive success (EL-KASSABY *et al.,* 1984; ERICKSON and ADAMS, 1989; BURCZYK and PRAT, 1997; BURCZYK *et al.,* 2002). The orchard clones used in this seed orchard have been selected from a relatively restricted area (southwestern Japan), and the flowering of the clones in the orchard is almost synchronous (TODA *et al.,* 1994). This explains the absence of a relationship between synchrony of flowering phenology and male reproductive success. In contrast, the fecundity of male flowers differs between clones and is closely related to the male reproductive success of each clone (*Table 3*). Such relationships have been recorded in a white spruce seed orchard (SCHOEN and STEWART, 1986) and a Douglas-fir seed orchard (BURCZYK and PRAT, 1997). The same clones contributed most as male parents to the orchard crop in both this study and the previous RAPD-based study by GOTO *et al.* (2002a). The male-flowering characteristics of Japanese black pine appear to be genetically stable (TODA *et al.,* 1993). Therefore, clones that display lower fecundity and lower male reproductive success should be replaced by newly selected clones in order to improve the parental balance.

The level of self-fertilization detected in this seed orchard (9.8% on average) is similar to that the levels recorded in other seed orchards (BURCZYK and PRAT, 1997; STOEHR *et al.,* 1998; SEIDO, 2001; STOEHR and NEWTON, 2002; MORIGUCHI *et al.,* 2004). In conifers, self-fertilization adversely affects embryo development, seed germination, and survival of young seedlings, due to inbreeding depression (LIAN *et al.,* 2001). TANG and IDE (1998) investigated the genetic variation in both seeds and 1-, 2- and 3-year-old seedlings of *Chamaecyparis obtusa*. They found that both observed and expected heterozygosities were higher in older seedlings than in young ones. They suggested that natural thinning of inbred offspring, including those produced through self-fertilization, occurred prior to their transplantation in the nursery. Amongst 2-yr seedlings in the nursery, GOTO *et al.* (2002a) detected only 1.5% that was the result of self-fertilization. Therefore, selfing should not be a serious problem in this seed orchard.

We assessed the influence of non-selected trees within the seed orchard on pollen contamination. The internal pollen contamination, from N1, affected 3.3% of the total crop. Pollen dispersion from N1 was estimated based on a normal distribution with variance $\tau = 17.5$ m (*Fig. 2*). The curve of pollen dispersion of single tree seems reasonable when compared with data from other wind-pollinated tree species, such Japanese red pine (*Pinus densiflora*) (LIAN *et al.,* 2001), *Quercus petraea* and *Q. robur* (STREIFF *et al.,* 1999). If all 159 mature trees within the seed orchard contribute equally to the orchard crop, the genetic contribution of each tree would be 1/159 (0.6%). Therefore, the expected genetic contribution (P_E) of N1 is remarkably high. This tree was a heavy producer of pollen (Y. Mori and F. Miyahara, personal observation), which may account for its high genetic contribution. In contrast, N2 did not contribute to reproduction within the seed orchard. N1 was located in the center of the orchard, whereas N2 was at the edge (*Fig. 1*). Moreover, the flowering fecundity of N2 was very low, and its flowering phenology was not synchronized with most of the orchard clones. Thus, the influence of a non-orchard tree within the seed orchard may depend on its flowering characteristics and its location within the orchard.

The estimate of external pollen contamination in this study was lower than values reported previously in other seed orchards, where it can exceed 30% (ADAMS *et al.,* 1997; STOEHR *et al.,* 1998; PAKKANEN *et al.,* 2000; CHAIX *et al.,* 2003; MORIGUCHI *et al.,* 2004). External pollen contamination usually depends on the density of conspecific natural stands around the seed orchard. Since no natural pine forests exist within at least 1 km of the seed orchard, we suggest that the lack of adjacent natural populations of Japanese black pines may prevent high levels of external pollen contamination.

Pollen contamination effects on progeny performance are likely orchard specific, and therefore, each orchard should be tested for these effects (STOEHR *et al.,* 2004). GRATTAPAGLIA *et al.* (2004) identified the paternity of offspring produced by one female tree in a progeny testing field of superior growth in *Eucalyptus*. They showed that the proportion of offspring sired by external pollen donors in the selected population was significantly higher than in a non-selected tree population. On the other hand, STOEHR *et al.* (2004) made the controlled pollination with outside orchard pollen and inside orchard pollen on trees of a Douglas-fir costal-interior transition zone seed orchard, and they reported that the trees height difference due to pollen source were statistically non-significant. In case of the Japanese black pine seed orchard consisting of nematode-resistant clones, pollen contamination will strongly influence the performance of orchard crops, because the resistance against the nematode was remarkably lower in seedlings derived from non-selected populations than in those from selected populations (FUJIMOTO *et al.,* 1989; TODA *et al.,* 1993; GOTO *et al.,* 2002b). We estimated the internal and external pollen contamination to the total of orchard crops was 3.3% and 9.9%, respectively. In spite of relatively low level of pollen contamination, the survival rates following inoculation will decrease from 62.4% to 57.5% and the financial loss was calculated as 1,234,733 Japanese yen (corresponding to over 9,000 Euro$). Thus, the impact of pollen contamination from internal and external sources is serious. Clonal checking of ramets within the seed orchard is essential for determining the source of internal pollen contamination (GOTO *et al.,* 2001). Several orchard management techniques, such as supplemental mass pollination (SMP) (STOEHR *et al.,* 1998) and gibberellin $A_{4/7}$ treatment (TODA *et al.,* 1993), can enhance orchard clones' pollen production. Rigorous clonal checking and application of these techniques should be encouraged to prevent internal and external pollen contamination in Japanese black pine seed orchards.

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Literature cited

ADAMS, W. T., V. D. HIPKINS, J. BURCZYK and W. K. RANDALL (1997): Pollen contamination trends in a maturing Douglas-fir seed orchard. Can J For Res **27**: 131–134.

BUITEVELD, J., E. G. BAKKER, J. BOVENSCHEN and S. M. G. DE VRIES (2001): Paternity analysis in a seed orchard of *Quercus robur* L. and estimation of the amount of background pollination using microsatellite markers. For Genet **8**: 331–337.

BURCZYK, J., D. PRAT (1997): Male reproductive success in *Pseudotsuga menziesii* (Mirb.) Franco: the effects of spatial structure and flowering characteristics. Heredity **79**: 638–647.

BURCZYK, J., W. T. ADAMS, G. F. MORAN and A. R. GRIFFIN (2002): Complex patterns of mating revealed in a *Eucalyptus regnans* seed orchard using allozyme markers and the neighbourhood model. Mol Ecol **11**: 2379–2391.

CHAIX, G., S. GERBER, V. RAZAFIMAHARO, P. VIGNERON, D. VERHAEGEN and S. HAMON (2003): Gene flow estimation with microsatellites in a Malagasy seed orchard of *Eucalyptus grandis*. Theor Appl Genet **107**: 705–712.

DOW, B. D. and M. V. ASHLEY (1996): Microsatellite analysis of seed dispersal and parentage of saplings in bur oak, *Quercus macrocarpa*. Mol Ecol **5**: 615–627.

EL-KASSABY, Y. A., A. M. K. FASHLER and O. SZIKLAI (1984): The role of reproductive phenology and its impact on genetically improved seed production in a Douglas-fir seed orchard. Silvae Genetica **33**: 120–125.

ERICKSON, V. J. and W. T. ADAMS (1989): Mating success in a coastal Douglas-fir seed orchard as affected by distance and floral phenology. Can J For Res **19**: 1248–1255.

FUJIMOTO, Y., T. TODA, K. NISHIMURA, H. YAMATE and S. FUYUNO (1989): Breeding project on resistance to pine-wood nematode – An outline of the research and the achievement of project for ten years. Bull For Tree Inst **7**: 1–84 (in Japanese with English summary).

GIERTYCH, M. M. (1965): Systematic lay-outs for seed orchards. Silvae Genetica **14**: 91–94.

GOTO, S., F. MIYAHARA and Y. IDE (2001): A fast method for checking the genetic identity of ramets in a clonal seed orchard by RAPD analysis with a bulking procedure. Silvae Genetica **50**: 271–275.

GOTO, S., F. MIYAHARA and Y. IDE (2002a): Monitoring male reproductive success in a Japanese black pine clonal seed orchard with RAPD markers. Can J For Res **32**: 983–988.

GOTO, S., F. MIYAHARA and Y. IDE (2002b): Contribution of pollen parents to the nematode-resistance in seedlings of Japanese black pine. J Jpn For Soc **84**: 45–49 (Japanese with English summary).

GRATTAPAGLIA, D., V. J. RIBEIRO and G. D. S. P. REZENDE (2004): Retrospective selection of elite parent tree using paternity testing with microsatellite markers: an alternative short term breeding tactics for *Eucalyptus.* Theor Appl Genet **109**: 192–199.

HARJU, A. and O. MUONA (1989): Background pollination in *Pinus sylvestris* seed orchards. Scand J For Res **4**: 513–520.

KANG, K. S., D. LINDGREN and T. J. MULLIN (2001): Prediction of genetic gain and gene diversity in seed orchard crops under alternative management strategies. Theor Appl Genet **103**: 1099–1107.

KAWAUCHI, H. and S. GOTO (1999): Monitoring clonal management in nematode-resistant Japanese black pine seed orchard in Kagoshima prefecture. J Jpn For Soc **81**: 338–340 (Japanese with English summary).

LIAN, C., M. MIWA and T. HOGETSU (2000): Isolation and characterization of microsatellite loci from the Japanese red pine. Mol Ecol **9**: 1186–1188.

LIAN, C., M. MIWA and T. HOGETSU (2001): Outcrossing and paternity analysis of *Pinus densiflora* (Japanese red pine) by microsatellite polymorphism. Heredity **87**: 88–98.

MARSHALL, T. C., J. SLATE, L. E. B. KRUUK and J. M. PEMBERTON (1998): Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol **7**: 639–655.

MORIGUCHI, Y., H. TAIRA, N. TANI and Y. TSUMURA (2004): Variation of parental contribution in a seed orchard of *Cryptomeria japonica* determined using microsatellite markers. Can J For Res **34**: 1683–1690.

PAKKANEN, A., T. NIKKANEN and P. PULKKINEN (2000): Annual variation in pollen contamination and outcrossing in a *Picea abies* seed orchard. Scand J For Res **15**: 399–404.

PLOMION, C., G. LEPROVOST, D. POT, G. VENDRAMIN, S. GERBER, S. DECROOCQ, J. BRACH, A. RAFFIN and P. PASTUSZKA (2001): Pollen contamination in a maritime pine polycross seed orchard and certification of improved seeds using chloroplast microsatellites. Can J For Res **31**: 1816–1825.

SCHOEN, D. J. and S. C. STEWART (1986): Variation in male reproductive investment and male reproductive success in white spruce. Evolution **40**: 1109–1120.

SEIDO, K. (2001): Genetic and breeding studies on pollen management in Hinoki (*Chamaecyparis obtusa*) seed orchard. Bull Yamanashi For Res Inst **22**: 13–64 (in Japanese with English summary).

SILEN, R. R. (1962): Pollen dispersal considerations for Douglas-fir. J For **60**: 790–795.

STOEHR, M. U. and C. H. NEWTON (2002): Evaluation of mating dynamics in a lodgepole pine seed orchard using chloroplast DNA markers. Can J For Res **32**: 469–476.

STOEHR, M. U., B. L. ORVAR, T. M. VO, J. R. GAWLEY, J. E. WEBBER and C. H. NEWTON (1998): Application of a chloroplast DNA marker in seed orchard management evaluations of Douglas-fir. Can J For Res **28**: 187–195.

STOEHR, M. U., J. E. WEBBER, C. C. A. HOLLEFREUND and R. A. PAINTER (2004): Potential pollen contamination effects on progeny from an off-site Douglas-fir seed orchard: 9-year field results. Can J For Res **34**: 981–984.

STREIFF, R., A. DUCOUSSO, C. LEXER, H. STEINKELLNER, J. GLOESSL and A. KREMER (1999): Pollen dispersal inferred from paternity analysis in a mixed oak stand of *Quercus robur* L. and *Q. petraea* (Matt.) Liebl. Mol Ecol **8**: 831–841.

TANG, D.-Q. and Y. IDE (1998): Detection of genetic variation among seed and seedlings of *Chamaecyparis obtusa* using allozyme markers. J For Res **3**: 35–38.

TODA, T., K. NSHIMURA, M. TAJIMA (1994): Some characteristics of the resistant pine clones to the pine-wood nematode (IX) – Flowering period of male and female flowers of resistant pine clone –. Kyushu Br Jpn For Soc **47**: 53–4 (In Japanese).

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edited by Thünen Institute of Forest Genetics

TODA, T., M. TAJIMA, K. NISHIMURA and H. TAKEUCHI (1993): Resistance breeding to the pine wood nematode in Kyushu district – Progress of study after selection of the resistant clones. Bull For Tree Inst **11**: 37–88 (in Japanese with English summary).

VAN DE VEN, W. T. G. and R. J. MCNICOL (1995): The use of RAPD markers for the identification of Sitka spruce (*Picea sitchensis*) clones. Heredity **75**: 126–132.

WANG, C.-W., T. O. PERRY and A. G. JOHONSON (1960): Pollen dispersion of slash pine (*Pinus elliotii* Engelm.) with special reference to seed orchard management. Silvae Genetica **9**: 78–86.

WHEELER, N. C. and K. S. JECH (1992): The use of electrophoretic markers in seed orchard research. New Forests **6**: 311–328.

WILLIAMS, J. G. K., A. R. KUBELIK, K. J. LIVAK, J. A. RAFALSKI and S. V. TINGEY (1990): DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res **18**: 6531–6535.

Seed Source Variation in Morphology, Germination and Seedling Growth of *Jatropha curcas* Linn. in Central India

By H. S. GINWAL[1,*], S. S. PHARTYAL[1], P. S. RAWAT[2] and R. L. SRIVASTAVA[3]

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Abstract

The aim of the study was to determine source variation in *Jatropha curcas* seeds collected from ten locations in Central India. A significant seed source variation was observed in seed morphology (colour, size and weight), seed germination (viability, germination percent, germination energy, germination value) and seedling growth parameters (survival percentage, seedling height, collar diameter, leave/plant, and seedling biomass). The seed source of Chhindwara (M.P.) was found as the best source in comparison to others. The phenotypic and genotypic variance, their coefficient of variability and broad sense heritability also showed a sizeable variability. This offers a breeder ample scope to undertake screening and selection of seed sources for the desired traits. Further, high percentage of heritability coupled with moderate intensity of genetic gain, was observed for seed germination traits, which signifies that germination is under strong genetic control and good amount of heritable additive genetic component can be exploited for improvement of this species.

Key words: Jatropha curcas, variation, seed source, variability, heritability, genetic gain, seed germination, germination energy.

Introduction

Jatropha curcas Linn. (physic nut or Ratanjot), a genus of family *Euphorbiaceae*, is believed to be a native of Mexico and Central America. It has been introduced in Africa and Asia and is now cultivated worldwide. Portuguese introduced physic nut as an oil yielding plant in India. It is a multipurpose, deciduous, small tree (or large shrub), reported to be cultivated in drier sites of central and western parts of India. Recently, it has also been introduced in the northern and southern states under massive plantation work to enhance livelihood of rural people and simultaneously to develop wasteland. *Jatropha curcas* is a prominent species with vide variety of uses. Seeds, leaves and bark are used in traditional medicine and for veterinary purposes. The oil has a strong purgative action and is also widely used for skin diseases and to soothe rheumatic pain. A decoction of leaves is used against cough and as an antiseptic after birth (HELLER, 1996).

In recent years energy conservation and its alternative production has acquired significant importance in the wake of the world energy crisis. Since the oil crisis of the 1970s and recognition of the limitations of world oil resources, most of the oil importing countries including India has been highly motivated to develop alternative sources of energy to meet their domestic needs from natural resources. *J. curcas* has been found highly promising species which can yield oilseed as a source of energy in the form of bio-diesel owing to its short gestation period, hardy nature, high quality oil content, etc. The oil can also be used in soap and candle industries and its by-product glycerine can be used in the pharmaceutical industry.

Considering vast semi-wild distribution of *J. curcas* in different parts of India, it would be expected to have considerable genetic variation. Sufficient information on such aspect is lacking in this species in spite of its many uses. Environmental factors in combination with genetic and physiological factors play important role in determination of plant potential for seed quality. These characters appear to be under strong genetic control (ROY *et al.,* 2004). Depending on the species, germination

[1]) Division of Genetics and Tree Propagation, Forest Research Institute, PO- I.P.E., Kaulagarh Road, Dehradun-248195, UA, INDIA.

[2]) Research and Coordination Section, Forest Research Institute, PO- New Forest, Dehradun-248006, UA, INDIA.

[3]) Arid Forest Research Institute, PO-Krishi Mandi, New Pali Road, Jodhpur-342005, Rajashthan, INDIA.

*) Authors for corresponding: ginwalhs@rediffmail.com

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