Interspecific Hybridization in Poplars Using Recognition Pollen

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Introduction

In the development of plants suitable for intensive cultivation in new environments, interspecific hybridization has provided a means of combining together desirable characteristics in one individual. In poplars, SCHREINER, and STOUT in U.S.A., PICCAROLO in Italy (FAO, 1958) and PRYOR and WILLING (1965) in Australia have achieved some success using this approach. However interspecific incompatibility barriers prevent hybridization between white poplars (Section Leuce) and black poplars (Section Aigeiros) (FAO, 1958). Attempts to overcome this problem in poplars and other flowering plants have had only partial success. Pollen mixes of compatible (mentor) and incompatible types have been used for remote hybridization by MICHURIN (1950), TSITSIN (1962) and KARPOV (1966) with variable results. STETTLER (1968) however, developed a refinement of this method in which the compatible (mentor) pollen was first killed by gamma-radiation before use in the mix. This killed pollen was still able to produce pollen tubes, and it was believed, substances with a stimulatory function that would enable the incompatible viable pollen added at the same time to grow into the stigmas. However the percent of success with poplar hybrids was low (less than 1% of progeny). In this paper, we describe a simple method for overcoming incompatibility barriers using recognition *pollen*. This term is applied to the killed compatible pollen in the mix, rendered inviable or of reduced viability by repeated freezing and thawing, gamma-radiation or chemical treatment. Using branch cultures with scions grafted to rootstocks, we have obtained highly successful interspecific hybridizations in more than 12 experiments using the parents P. alba and P. deltoides.

The concept of recognition pollen is based on a considerable body of evidence now accumulating which shows that substantial amounts of protein are contained in the walls of pollen grains, especially in the inner layer, the intine (TSINGER and PETROVSKAYA-BARANOVA, 1961; KNOX and HESLOP-HARRISON, 1969, 1970), in all flowering plant pollens that have been tested. It is well-known that pollen grains release proteins when moistened (GREEN, 1894; STANLEY and LINSKENS, 1965; MÄKINEN and BREWBAKER, 1967). Immunofluorescence methods have recently been used to localise the diffusible antigenic proteins in these intine sites in pollen of Gladiolus gandavensis, Ambrosia spp. and Phalaris tuberosa (KNOX et al. 1970; KNOX 1971 a, b; KNOX and HESLOP-HARRISON, 1971 a, b). The heterogeneity of the antigens has been demonstrated in immunological studies e. g. WODEHOUSE, 1954; KNOX 1971 a, b). In Phalaris, the rapidly released antigens spread over the surface of the stigma adjacent to the pollen grains soon after alighting there (KNOX and HESLOP-HARRISON 1971 b) suggesting these antigens play a role in the recognition phenomena involved in compatibility reactions on the stigma. The experiments reported in this paper provide further evidence for such a role for the pollen wall proteins in interspecific matings of poplar.

Materials and Methods

Four female clones 601141, 60/156, 601160 and 60/166*) of the American Eastern Cottonwood, P. deltoides MARSH. (Section Aigeiros) and one female clone of the white poplar P. alba L. (Section Leuce) were selected and branches bearing flower buds taken into a glasshouse and grafted onto rooted cuttings of a semi-evergreen black poplar clone 65/27. The four deltoides clones are selections from seedling populations comprising several thousand seedlings. Seed from which clone 601141 originated was obtained from School of Forestry, State College, Mississippi, U.S.A. in 1960; 601156, 601160 and 601166 from the U.S. Forest Service, Delta Experiment Station, Stoneville, Mississippi in 1960. The grafts were made in winter 1970 (early August) about 3 - 4 weeks prior to natural flowering in the field. The grafts were "bottle-grafts", a safe and satisfactory technique for hybridization experiments. The understock was rooted about 4 months prior to grafting, consisting of cuttings 30-40 cm long, and 1.5—2.0 cm in diameter, transplanted after rooting into 20 cm plastic pots. The semi-evergreen clone 65/27*) is highly compatible with P. *deltoides* since it is a selection from the progeny of the cross P. deltoides X Chilean Semi-evergreen, a form of P. nigra var. italica, known as "Persistente" in Chile (PRYOR 1969). The use of grafted females ensured normal development of the embryos and complete ripening of the seeds, which is not always achieved by placing the branches of Aigeiros poplars in water-filled containers. Seeds were germinated in the glasshouse immediately on ripening in flats filled with perlite, and exposed to intermittent mist for 5 seconds every 10 minutes. Germination occurred within hours. Nutrient solutions were applied twice-weekly, and seedlings were transplanted when the first true leaf pair unfolded after about three weeks, into flats or pots containing 2 parts of sterilized soil, 1 part of peatmoss and 1 part of sand.

Pollen extraction: Branches bearing the male floral buds of P. alba L. var. pyramidalis BGE. (syn. P. bolleana LAUCHE) (nomenclature of REHDER, 1958), P. deltoides or P. nigra var. italica were brought into the glass house about 4-6 weeks prior to anthesis in the field, and placed in water-filled containers. Anthesis occurred 2 weeks later, and pollen release occurred over several days. Pollen was collected daily and dessicated over silica gel for 12—16 hours before storing dry at —18° C. Under these conditions poplar pollen remains viable for several years, and is the source of pollen used for the hybridization experiments. Recognition pollen was prepared in two ways; freezing and thawing or gamma-irradiation. Repeated freezing and thawing resulted in considerable reduction in viability as determined using the fluorochromatic reaction test of HESLOP-HARRISON and HESLOP-HARRISON (1970), with apparently little impairment

*) These clonal numbers refer to the code used in the Botany Department, Australian National University and are not registered with the International Poplar Commission.

of biological activity of the wall proteins. Dry pollen samples were frozen to —18° C., then thawed, the procedure being repeated 6—12 times. Gamma-irradiation was carried out by exposing dry pollen samples to varying doses of radiation up to 100,000 rads (the amount used in the experiments described here) from a ^{60}Co source. Viability checks using the method of HESLOP-HARRISON and HESLOP-HARRISON (1970) showed over 95% of the pollen lost its viability with exposures between 1,000 and 5,000 rads. Also preliminary trials have been carried out using chemical methods for the production of *recognition pollen*. In these, pollen samples were exposed to ether or anhydrous methanol for 2 minutes before the supernatant was decanted and the defatted killed pollen was rapidly air-dried. Viability tests showed the pollen was completely inviable after these treatments.

For use, the *recognition pollen* (of the compatible type) was thoroughly mixed dry with the fresh, viable incompatible pollen in 1 : 1 ratio. The pollen mix was then dusted on receptive stigmas using a small water colour brush. Whole inflorescences were treated at the same time.

Cytological observations of the growth of the pollen tubes after compatible and incompatible pollinations, were made using the combined squash technique and callose fluorescence method described by PREIL and REIMANN-PHILIPP (1969).

Observations

Using the *recognition pollen* method, we have produced more than 500 hybrid seedlings of the mating *P. deltoides* ♀ × *P. alba* ♂. These seedlings showed characteristics intermediate between the parents *(Fig. 1)* none showing exclusively maternal phenotype. The most striking difference between the two parents is perhaps the absence of white tomentum on the undersides of the leaves *(Figs. 2 and 4)* both in *P. deltoides*, and in the progeny where only slight tomentum appears on the very young leaves near the shoot tip *(Fig. 2)*. Leaf shape and position is also characteristically different in the parents *(Fig. 1)*, with *P. deltoides* having triangularshaped leaves, finely dentate at the margins; *P. alba* having deeply-5-lobed leaves, coarsely-dentate with prominent teeth at the margins; and the hybrid progeny having triangular leaves with coarsely-dentate, often very crinkled and folded margins. At the junction of lamina and petiole, the leaves of the hybrids are markedly cordate *(Figs. 2 and 4)* a feature not well developed in either parent. The leaf position varies from upright *(P. deltoides)* to hanging downwards *(P. alba)*, while the hybrids are intermediate *(Figs. 1 and 3)*. This is dependent on the angle between stem and petiole (leaf angle A), and petiole and lamina (leaf angle B). Angle A varies between the parents to some extent *(Fig. 1)* while angle B is markedly different. In *P. deltoides* the angle varies from 170—180°, in *P. alba* it lies between 70—120°, while in the hybrid, while mostly intermediate, paternal phenotypes for this character are also found *(Fig. 1)*. The shoots of the *deltoides* parent are strongly angled, while the *alba* parent has round shoots, and those of the hybrids are slightly-angled.

The hybrid seedlings were obtained from 12 experiments, four of which utilised gamma-irradiated *recognition pollen*, and eight with freeze-thawed *recognition pollen*. The progeny were quite variable in vigour, some probably showing increased vigour over parental seedlings of similar age, and some being dwarf and of much reduced vigour. The appearance of a sample of hybrids showing the range of phenotypes, is illustrated in *Fig. 3*. Abnormalities were shown by about 50% of progeny, including chlorophyll defects, loss of apical dominance and depressed growth whether they were produced using freeze-thawed or gamma-irradiated *recognition pollen*. Presumably these forms

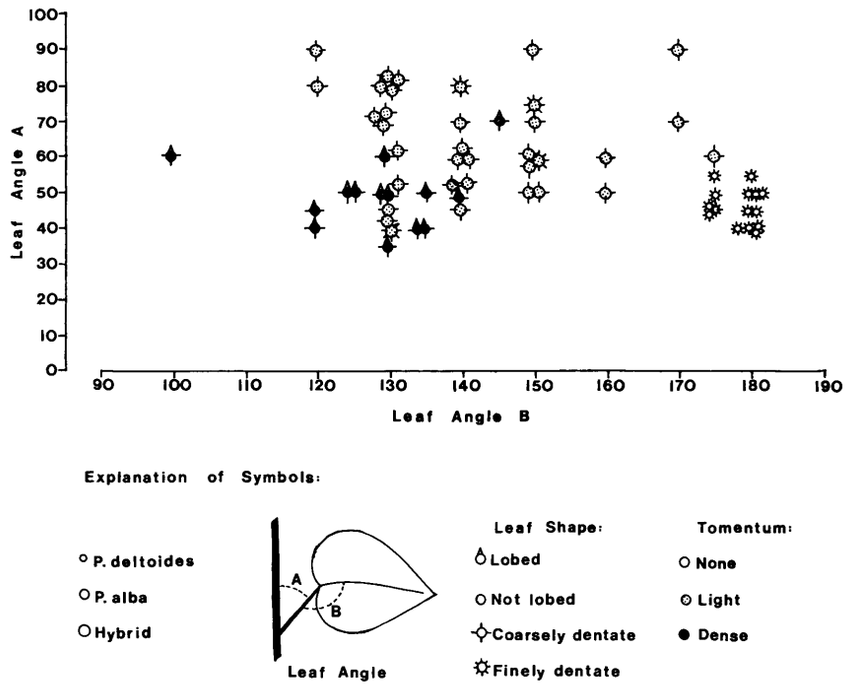


Fig. 1. — Scatter diagram showing principal morphological features of seedlings of *P. deltoides*, *P. alba* and a random sample from the hybrid progeny. The phenotypes of the female parent, *P. deltoides* are clustered together at right of diagram, while those of the pollen parent, *P. alba* are scattered and represented by dark circles because of their dense white tomentum.

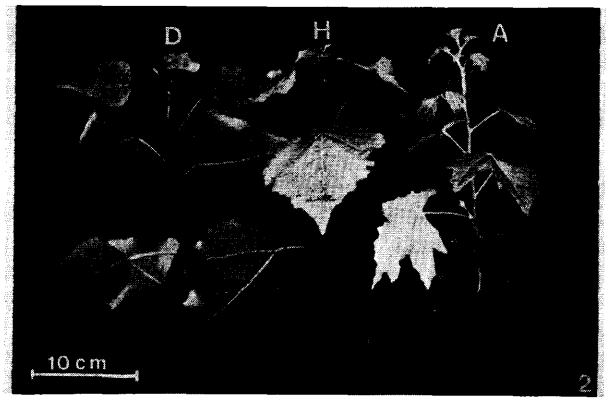


Fig. 2. — Characteristic appearance of seedlings of *P. deltoides* (D), *P. alba* (A) and hybrid (H). Note the white tomentum around the shoot apex and on underside of leaves which are pendulous in *P. alba*, and glabrous and erect in *P. deltoides*.

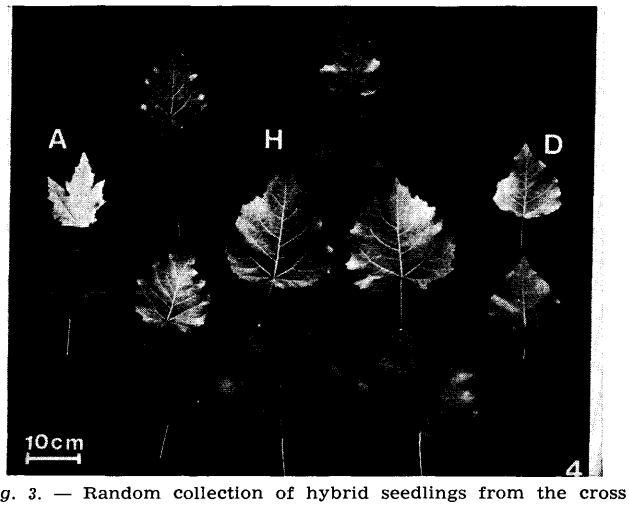


Fig. 3. — Random collection of hybrid seedlings from the cross *P. deltoides* × *P. alba*, 16 weeks after germination. — *Fig. 4.* — Leaf patterns of parental seedlings of *P. deltoides* (D), *P. alba* (A) and from five of the hybrids (H). Underside of each pair of leaves is uppermost, each pair is from the same individual.

arose through genetic imbalance of the genome in the hybrid.

However, the nature of the *recognition pollen* influenced seed-setting *(Table 1)*. The number of seeds per capsule was reduced by 60% in the hybrids produced with freeze-thawed *recognition pollen*. Gamma-irradiated *recognition pollen* further depressed seed set, reduced by almost 90% with corresponding reduction in viability of the seedlings.

Table 1. — Effects of various pollen mixes on seed setting and seed viability in the mating *P. deltoides* ♀ × *P. alba* ♂.

Pollen mix	Mean no. of seeds per capsule	% viable seedlings
Viable *deltoides*	35	92
Freeze-thawed *deltoides* + viable *alba*	11	74
Freeze-thawed *deltoides* only	0	0
Gamma-irradiated *deltoides* + viable *alba*	5	52
Gamma-irradiated *deltoides* only	0	0

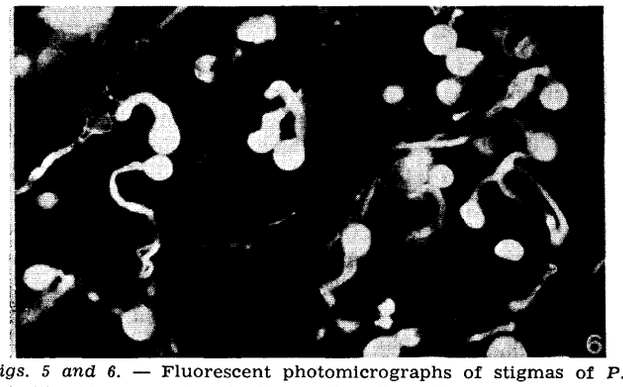
Further experiments have been carried out using the chemically-prepared *recognition pollen*. Pollen coat materials (tryphine or pollenkitt) being largely lipid, are removed but the pollen wall proteins remain unaffected in exposure periods of about 2 minutes. These matings have produced excellent seed set, but have not yet been harvested. Matings in the reciprocal direction have also been carried out with similar results.

Further evidence on the nature of the incompatibility mechanism is provided by observations of pollen tube growth in compatible and incompatible pollinations. In compatible matings of *P. deltoides* ♀ × *P. yunnanensis* ♂ (Section *Tacamahaca)* 24 hours after pollination, pollen tubes were abundant, penetrating between the stigmatic cells *(Fig. 5)* in squash preparations using the technique of Preil and Reimann-Philipp (1969). In contrast, after 24 hours in incompatible pollinations using pollen of *P. alba*, most pollen grains had germinated but the tubes had grown only over the surface of the stigma, and had failed to penetrate, *(Fig. 6)*.

Discussion

(a) Recognition substances and incompatibility: —

A number of hypotheses have been developed to explain the well-established genetic phenomena of incompatibility in physiological terms (see reviews by Linskens and Kroh, 1967, and Townsend, 1971). Most of these depend on the assumption that pollen grains release substances interacting with analogous materials in the stigma to bring about the various phenomena recorded. In support of a determinative role for the pollen grain in these processes, Mäkinen and Lewis (1962) showed that the S alleles of *Oenothera organensis* produced specific S protein forming a major part of the mobile protein of the pollen grain. Later, Lewis, Burrage and Walls (1967) used immunological methods to detect antigens associated with the expression of particular S alleles diffusing out of pollen placed on agar containing specific antiserum. A considerable load of extracellular diffusible proteins was detected using cytochemical methods in the pollen grain walls of over sixty flowering plants tested (Knox and Heslop-Harrison, 1970) including those with antigenic activity (Knox, Heslop-Harrison and Reed, 1970) occurring in short-term leachates of whole pollen. These proteins show rapid diffusion to the external surface from their intine sites when moistened (Knox, 1971 a, Knox and Heslop-Harrison, 1971 a) and pollen of *Phalaris* has been shown to release antigens which spread over the stigmatic surface adjacent to the grains within 5—10 minutes of arrival (Knox and Heslop-Harrison, 1971 b). Within this period, pollen tubes penetrated the stigma in



Figs. 5 and 6. — Fluorescent photomicrographs of stigmas of *P. deltoides* 24 hours after dusting with pollen of *P. yunnanensis*, a compatible mating *(Fig. 5)*, and *P. alba*, an incompatible mating *(Fig. 6)* showing callose localization in the pollen tubes which appears white in these photographs. In *Fig. 5* the plane of focus is below surface of stigma and the pollen tubes here penetrated directly through the stigmatic tissue. In *Fig. 6* the plane of focus is on the stigma surface and the tubes have grown over the surface for varying distances and have not penetrated. (×133).

compatible matings but failed to do so in incompatible matings. This evidence supports the involvement of these antigenic proteins in recognition phenomena at the surface of the stigma, and the term *recognition substances* has been applied (KNOX and HESLOP-HARRISON, 1971 b) to pollen-borne materials concerned with regulating the breeding system.

The observations presented in this paper show that pollen grains play a determinative role in compatibility reactions on the stigma. The genetic evidence supporting gametophytic incompatibility systems indicates that the pollen grains must play a role, but direct evidence has not previously been obtained. In these poplar experiments (and in those of STETTLER, 1968) it is clearly demonstrated that killed compatible pollen is capable of providing recognition substances essential for stigma penetration and growth of the incompatible pollen tubes. One interpretation of these experiments, in the light of the evidence presented in support of the role of recognition substances, is that these substances in the killed pollen diffuse out of their extracellular sites as in a compatible mating, spreading over the stigma surface. Where incompatible but viable pollen lies adjacent, its pollen tube can then successfully penetrate into the stigma, growing through regions of the stigma now invested by the compatible recognition substances. The incompatible pollen thus effectively "borrows" recognition material from the killed pollen. Evidence from immunological studies of the poplar pollen wall proteins to be presented elsewhere (KNOX, in preparation) shows that the intine-borne antigens spread over the surface of the stigmas in both compatible and incompatible matings in a manner very similar to that described for *Phalaris* (KNOX and HESLOP-HARRISON, 1971 b). Further studies of the antigenic relationships of the pollen wall proteins from various poplar species are in progress.

(b) Implications of the recognition pollen method for hybridization of poplars and other plants: —

In their extensive programmes of remote hybridization, MICHURIN (1950) and later TSITSIN (1962) and KARPOV (1966) used mixes of viable compatible and incompatible pollen in attempts to increase the success of their "mentor" methods. STETTLER (1968) experimented with mixes of poplar pollen in which the compatible type had been killed by gamma-radiation, in the belief that even inviable pollen grains released stimulatory substances essential for pollen tube growth. However his percentage success in terms of hybrid progeny was low. In the experiments reported in this paper, similar techniques have been used except that new methods have been found to produce *recognition pollen* in the belief that it is the pollen wall proteins that are released and control recognition phenomena on the stigma surface. Freezing and thawing, while it did not always kill all the pollen, nevertheless so reduced its viability and pollen tube growth, that it could not compete with the incompatible viable pollen. We believe that considerably less damage is done to the wall proteins by these procedures, as shown by the increase in seed-setting *(Table 1)*. The more recent chemical methods for the production of *recognition pollen* show considerable promise. These methods have the advantage of simplicity and rapidity with more certainty that the recognition pollen is killed.

A feature of the present results is the high success in production of hybrids, with no full parental types in the progeny and apparently no haploid types as reported by STETTLER (1968) in matings with *P. trichocarpa* (Section *Tacamahaca)*. This was achieved both with freeze-thawed *recognition pollen* and with gamma-irradiated pollen similar to that described by STETTLER (1968), who did not achieve a high level of hybrid seed production. The difference in these results is presumably attributable to the use of grafted female branch cultures which readily produced fertile seed. The water culture method of VON WETTSTEIN (1944) used by STETTLER (1968) is not so reliable under our conditions especially with *Aigeiros* poplars.

The *recognition pollen* method has therefore great potential as a tool for the production of hybrids in situations where the two parents are sufficiently closely related for their genomes to be compatible, yet cannot be hybridized because of incompatibility barriers. For example, the white poplars of Section *Leuce* have good timber quality, and are all interfertile, but incompatibility barriers prevent hybridization with the black poplars of Section *Aigeiros*, useful for their rapid growth rates in lower latitudes (PRYOR and WILLING, 1965). The *recognition pollen* method has made easily possible hybrid production which opens the way for the production of clones involving the genetic material of *P. alba* in combination with species of the *Aigeiros* group. Work is in progress to determine whether similar methods can be used to involve the genetic material of aspen *P. tremula*, also in the Section *Leuce*, in similar combination.

Pollen-wall proteins have been detected in all angiosperm pollens so far tested, and in the pollen of a species of *Pinus* (KNOX and HESLOP-HARRISON, 1970). It seems likely therefore that the *recognition pollen* method can be used to overcome incompatibility barriers in other groups of plants, both where the barrier is interspecific and intraspecific.

Recently, we have begun experiments with *Cosmos bipinnatus* which is known to have a sporophytic incompatibility system (Crowe 1954) with most individuals being self incompatible, and have achieved considerable success in using *recognition pollen* from compatible plants to carry out self-pollinations. Since *Pinus* species are known to have extracellular pollen-wall proteins (Knox and Heslop-Harrison, 1970) the use of this technique needs investigation in *Pinus* where incompatibility systems exist, and large pollen samples can be readily obtained.

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Summary

The *recognition pollen* method is described, and has enabled successful hybridization of the black poplar, *P. deltoides* and the white poplar, *P. alba*. In these species, hybridization is normally prevented by incompatibility barriers. For use, the *recognition pollen* (killed pollen of the compatible type produced by freeze-thawing, gamma-irradiation or by treatment with methanol or ether) is mixed with fresh incompatible pollen and the mix dusted on the stigmas. All the progeny were hybrid. The determinative role played by pollen grain recognition substances in the control of interspecific incompatibility reactions on the stigma is demonstrated by these experiments. The *recognition pollen* method appears to have widespread applications in plant improvement.

Zusammenfassung

Es wird eine Hilfspollen-Methode beschrieben, die eine erfolgreiche Kreuzung von *Populus deltoides* und *P. alba* ermöglichte. Beide Arten sind normalerweise unverträglich. Hilfspollen war auf verschiedene Weise getöteter Pollen der Art, die als Mutter benutzt werden sollte. Dieser wurde zur Bestäubung mit dem sonst unverträglichen Pollen der anderen Art 1 : 1 gemischt. Alle danach entstandenen Nachkommen waren Bastarde. Von dem toten Hilfspollen abgegebene Stoffe an die Narben müssen diese Kreuzungen zum Erfolg geführt haben.

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Multivariate analysis of variation in needles among provenances of Pinus kesiya Royle ex Gordon (syn. P. khasya Royle; P. insularis Endlicher)

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Introduction

Pinus kesiya Royle ex Gordon (syn. *P. khasya* Royle; *P. insularis* Endlicher) is currently the major exotic species used for afforestation in Zambia (Cooling and Endean, 1966; Jones, 1968), and it is important or promising for many other tropical countries. It is high on the list of priorities for action by the FAO Panel of Experts on Forest Gene Resources and an international provenance trial is in progress with 19 seed lots collected in the Philippines by Turnbull[3]) during 1969 (Burley and Turnbull, 1970). It is a wide-ranging species in southeast Asia occurring naturally from Assam, where it is commonly called *P. khasya*, to the Philippines, where it is known as *P. insularis (Figure 1)*. Limited evidence from studies in the natural habitat and in exotic plantations suggests that these are geographic races of the same species.

The nomenclatural situation is confused because various recognised authorities have adopted different names, *e. g. P. khasya* (Dallimore and Jackson, 1948); *P. kesiya* (Dallimore, Jackson and Harrison, 1966). Until an authoritative ruling has been given on the historical priority, these two names must be accepted as equivalent (see Burley, 1971).

The present study was designed to examine further the relationships among several provenances of this species, grown as exotics under relatively uniform conditions, using anatomical and morphological characteristics of needles

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