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Contrasting patterns of genetic diversity in two tropical pines: *Pinus kesiya* (Royle ex Gordon) and *P. merkusii* (Jungh et De Vriese)

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Abstract We studied allozyme and chloroplast (cp) DNA variation in natural populations of *Pinus kesiya* and *P. merkusii* from Thailand and Vietnam. The results showed striking differences between the two species in the amount and distribution of allozyme variation. *P. kesiya* harboured considerable allozyme variation and showed weak interpopulational differentiation. In contrast, *P. merkusii* had very low intrapopulational variability but a high level of interpopulational differentiation. The average Nei's genetic distance separating the two species was exceptionally high (0.701) taking into account their close taxonomic placement in the same subsection *Sylvestres*. The constructed phylogenetic trees revealed very early divergence of *P. kesiya* and *P. merkusii*. The present analysis of cpDNA variation also confirmed the dissimilar character of these two species and was compatible with other evidence indicating the outstanding position of *P. merkusii* as compared to other Asian members of the subsection *Sylvestres*. Analysis of cpDNA variation in sympatric populations of *P. kesiya* and *P. merkusii* revealed that they are pure representatives of the species in question. This result indicates that despite an overlapping distribution *P. kesiya* and *P. merkusii* do not hybridise in nature. We suggest that the distinctive character of *P. merkusii* is a result of an early separation from other Eurasian pines. Despite spatial proximity, *P. kesiya* and *P. merkusii* are kept apart by strong reproductive barriers. The low genetic variability of *P. merkusii* may be explained by previous bottlenecks, reduced gene flow among populations, and an inbreeding due to small population size and asynchronous flowering.

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Introduction

Pinus kesiya (Royle ex Gordon) and *Pinus merkusii* (Jungh et De Vriese) are the southernmost of pines in Eurasia (Critchfield and Little 1966). *P. kesiya* extends from the mountains of the Indian Khasi states toward Yunnan (Mirov 1967; Armitage and Burley 1980). To the southeast it extends to Thailand, Burma, Laos and Vietnam. In Thailand, *P. kesiya* occurs in small patches. *P. merkusii* occurs in China, Laos, Cambodia, Thailand, Vietnam, Sumatra and the Philippines (Critchfield and Little 1966; Mirov 1967). The border of Yunnan is apparently the northernmost limit of *P. merkusii* (Mirov 1967). Similar to *P. kesiya*, the distribution of *P. merkusii* in Thailand is dispersed (Cooling 1968). Maps of the distribution of *P. kesiya* and *P. merkusii* have been published by several authors (Critchfield and Little 1966; Mirov 1967; Cooling 1968; Armitage and Burley 1980; Farjon 1984). In northern Thailand, the two species grow together. The relative distribution of the two pines appears to be controlled mainly by altitude (Cooling 1968; Armitage and Burley 1980). The usual pattern of altitudinal distribution is with *P. kesiya* at the higher (often above 1200 m) and *P. merkusii* at the lower elevations (50–1000 m) but edaphic conditions may locally cause inversion (Cooling 1968).

In the current taxonomy of the genus *Pinus* the two species are regarded as closely related and are placed in the subsection *Sylvestres* which also comprises all other Eurasian species from the subgenus *Pinus* (Critchfield and Little 1966; Mirov 1967; Farjon 1984). However, our chloroplast (cp) DNA-based analysis of phylogenetic relationships among Eurasian *Pinus* species has revealed a surprisingly high dissimilarity of *P. merkusii* from all other species of the subsection *Sylvestres* (Wang and Szmidt 1993). In the present study, we sought to determine whether the

Table 1 Geographic origin of the *P. kesiya* (Pk) and *P. merkusii* (Pm) populations analysed for allozyme (a) and cpDNA (cp) variation

No.	Marker	Locality	Region	Lat. (N)	Long. (E)	Altitude (m)
Pk-1	a	Mae Rid	Chiang Mai	18° 10′	98° 50′	1200
Pk-2	a, cp	Mae Sanaam	Chiang Mai	18° 04′	98° 10′	1100
Pk-3	a	Baw Luang	Chiang Mai	18° 09′	98° 20′	1100
Pk-4	a,cp	Huey Bong	Chiang Mai	18° 10′	98° 25′	800
Pk-5	a	Dai Suthep	Chiang Mai	18° 46′	98° 00′	1200
Pk-6	a	Dai Inthanon	Chiang Mai	18° 41′	98° 27′	1000
Pk-7	a	Samoeng	Chiang Mai	19° 00′	98° 45′	1100
Pk-8	a	Khun Kung	Chiang Mai	19° 19′	98° 49′	1000
Pk-9	a	Huey Tong	Chiang Mai	18° 30′	98° 35′	800
Pk-10	a	Ban Wat Chan	Chiang Mai	19° 05′	98° 18′	900
Pk-11	a	Nong Krating	Chiang Mai	17° 56′	98° 17′	1080
Pk-12	a	Pai	Mae Hong Son	19° 22′	98° 24′	700
Pk-13	cp	Bokaew	Chiang Mai	18° 00′	98° 12′	1000
Pk-14	cp	Omkoi	Chiang Mai	17° 52′	98° 20′	900
Pm-1	a	Ban Wat Chan	Chiang Mai	19° 05′	98° 18′	900
Pm-2	a	Non Krating	Chiang Mai	17° 56′	98° 17′	1080
Pm-3	a	Buntarik	Ubonratchatani	14° 46′	105° 25′	240
Pm-4	a, cp	Mae Sanaam	Chiang Mai	18° 10′	98° 10′	1100
Pm-5	cp	Otaran	Surin	14° 30′	103° 57′	180
Pm-6	cp	Nong Koo	Surin	14° 43′	103° 50′	180
Pm-7	cp	Omkoi	Chiang Mai	17° 56′	98° 20′	900
Pm-8	cp	Phumniyom	Chiang Mai	14° 44′	103° 51′	180
Pm-9	a	Rung-giong	Vietnam	17° 36′	106° 25′	40

previously observed dissimilarity of *P. merkusii* is also reflected at the allozyme level. For this purpose, we studied allozyme variation in natural populations of this species from Thailand and Vietnam and compared it to that found in *P. kesiya* populations occurring in Thailand. To test the possibility for hybridisation between the two species we have conducted a new analysis of cpDNA variation.

Materials and methods

Plant material

For allozyme analysis, seed samples were collected from 12 natural populations of *P. kesiya* and from five populations of *P. merkusii* from Thailand and Vietnam (Table 1). For cpDNA analysis, needle samples were collected from four populations of *P. kesiya* and five populations of *P. merkusii* (Table 1). The number of trees included in these collections ranged from 20 to 25. Populations of *P. kesiya* and *P. merkusii* from the Chiang Mai province in northern Thailand were sympatric. The remaining populations were allopatric. Seeds and needles were kept at –20°C until analysis. Random samples of 75–90 seeds were taken from seed collections and regarded as random samples of the zygote population. Allozyme variation was studied in macrogametophytes and their corresponding embryos. CpDNA variation was studied in 20–25 individuals from each of the nine sampled populations (Table 1).

Allozyme analysis

Enzyme extractions and electrophoresis were made as described previously (Szmidt 1984). Ten enzyme systems comprising 20 loci were scored (Szmidt 1984; Changtragoon and Finkeldey 1995). Four loci, *Adh*-1, *Got*-1, *Got*-2 and *Sdh*-2, showed poor reproducibility and were excluded from further analysis. The remaining 16 loci were assessed simultaneously in each macrogametophyte and corresponding embryo. In this report only the embryo data were utilised.

DNA analysis

Purified cpDNA from single trees of *P. kesiya* and *P. merkusii* was prepared using the protocol described by Szmidt et al. (1986). In our previous study, we have identified numerous restriction enzyme-probe combinations that detected haplotypes characteristic of the two species (Wang and Szmidt 1993). Nine of these combinations were employed in the present study. CpDNA samples were digested separately with *Bcl*-I, *Bgl*-II, and *Dra*-I (Boehringer, Mannheim) and hybridized to three cpDNA probes from *P. contorta*: PcK32 (10.5 kb), PcH132 (11.0 kb) and PcH157 (4.3 kb) (Lidholm and Gustafsson 1991). Methods for digestion, separation, DNA transfer and hybridization were as described previously (Wang and Szmidt 1990, 1993). The DNA size marker used was the BRL 1-kilobase (kb) ladder.

Statistical methods

Allozyme frequencies, expected and observed heterozygosities, gene-diversity statistics and genetic-distance measures (Cavalli-Sforza and Edwards 1967; Nei 1987) were calculated using release 1.7 of the BIOSYS-1 program (Swofford and Selander 1981). A locus was considered polymorphic if the frequency of the most common allele did not exceed 0.95. Two methods of phylogenetic inference were used to analyze allozyme data. In the first of these, we employed a Wagner distance procedure (Farris 1972) for tree reconstruction. The second method employed the neighbour-joining procedure described by Saitou and Nei (1987). A chord distance coefficient (Cavalli-Sforza and Edwards 1967) was used in both methods.

Results

Allozyme variation

Mean allozyme frequencies for *P. kesiya* and *P. merkusii* are given in Table 2. Allozyme frequencies and other statistics for the individual populations can be obtained upon

Table 2 Average allozyme frequencies in *P. kesiya* and *P. merkusii*. N = sample size

Locus	*P. kesiya*	*P. merkusii*	Locus	*P. kesiya*	*P. merkusii*
Aco-1			*Mnr*		
N	1079	359	N	1080	382
1	0.032	0.000	1	1.000	1.000
2	0.967	1.000			
3	0.001	0.000	*Pgd*-1		
			N	1077	426
Adh-2			1	0.120	0.000
N	1080	442	2	0.877	0.982
1	1.000	0.002	3	0.000	0.001
2	0.000	0.998	4	0.001	0.000
			5	0.002	0.016
Aph					
N	1080	442	*Pgd*-2		
1	1.000	0.000	N	1077	443
2	0.000	1.000	1	0.655	0.000
			2	0.344	0.033
Fes			3	0.000	0.997
N	1055	432			
1	0.498	0.049	*Pgi*-1		
2	0.009	0.951	N	1080	442
3	0.494	0.000	1	1.000	1.000
Gdh			*Pgi*-2		
N	1079	354	N	1079	434
1	0.027	0.931	1	0.002	0.559
2	0.973	0.003	2	0.004	0.391
3	0.000	0.021	3	0.989	0.051
4	0.000	0.045	4	0.005	0.000
Lap-1			*Pgm*-1		
N	1078	420	N	1076	442
1	0.920	0.993	1	0.090	0.000
2	0.080	0.004	2	0.906	1.000
3	0.000	0.004	3	0.001	0.000
			4	0.002	0.000
Lap-2					
N	1061	408	*Pgm*-2		
1	0.726	0.102	N	1079	442
2	0.274	0.887	1	0.001	0.006
3	0.000	0.011	2	0.999	0.994
Mdh-1			*Sdh*-1		
N	1080	407	N	1077	381
1	0.000	0.026	1	0.789	0.001
2	0.000	0.974	2	0.199	0.001
3	1.000	0.000	3	0.003	0.953
			4	0.000	0.045

request from the authors. Of the 16 loci examined, nine were polymorphic (0.95 criterion) in at least one population of *P. kesiya* and *P. merkusii* (data not shown). In *P. kesiya*, the most polymorphic loci were *Fes*, *Pgd*-2 and *Sdh*-1. In *P. merkusii*, most loci (except *Pgi*-2) showed low polymorphism. The *Aph* locus was fixed for alternative alleles in the two taxa (Table 2). Differences among the investigated species were further accentuated by the occurrence of unique alleles at the *Fes*, *Pgd*-1, *Pgd*-2, *Mdh*-1 and *Adh*-2 loci (Table 2). A summary of the genetic-diversity measures at the 16 loci in the investigated populations of *P. kesiya* and *P. merkusii* is given in Table 3. In *P. kesiya* populations, mean expected (H_e) and observed (H_o) heterozygosities were relatively high and ranged between 0.117 and 0.160 and 0.130 and 0.166 respectively. In contrast, *P. merkusii* populations had much lower genetic variation (H_O=0.016–0.075; H_E=0.019–0.083).

Table 3 Genetic variability measures in the investigated populations of *P. kesiya* (Pk) and *P. merkusii* (Pm). H_O = observed heterozygosity, H_E = unbiased expected heterozygosity, G_{ST} = coefficient of genetic differentiation. Standard errors in parentheses

Population species	Mean sample size	H_O	H_E	G_{ST}
Pk-1	89.9 (0.1)	0.160 (0.050)	0.166 (0.049)	
Pk-2	89.7 (0.3)	0.127 (0.041)	0.133 (0.044)	
Pk-3	90.0 (0.0)	0.130 (0.044)	0.137 (0.044)	
Pk-4	89.5 (0.4)	0.141 (0.040)	0.153 (0.044)	
Pk-5	89.8 (0.2)	0.144 (0.048)	0.144 (0.044)	
Pk-6	89.0 (0.8)	0.128 (0.042)	0.145 (0.047)	
Pk-7	89.9 (0.1)	0.117 (0.041)	0.132 (0.045)	
Pk-8	89.9 (0.1)	0.149 (0.045)	0.152 (0.047)	
Pk-9	89.5 (0.1)	0.148 (0.047)	0.151 (0.046)	
Pk-10	89.6 (0.3)	0.145 (0.043)	0.151 (0.045)	
Pk-11	89.5 (0.4)	0.142 (0.044)	0.145 (0.046)	
Pk-12	89.8 (0.1)	0.133 (0.041)	0.130 (0.040)	
P. kesiya	1067 (1.8)	0.130 (0.042)	0.148 (0.045)	0.023
Pm-1	87.6 (1.4)	0.046 (0.027)	0.055 (0.029)	
Pm-2	72.2 (6.6)	0.016 (0.016)	0.019 (0.019)	
Pm-3	88.4 (1.1)	0.036 (0.021)	0.047 (0.030)	
Pm-4	82.6 (0.3)	0.075 (0.027)	0.083 (0.025)	
Pm-9	85.3 (3.8)	0.060 (0.028)	0.055 (0.025)	
P. merkusii	415.9 (7.7)	0.043 (0.015)	0.073 (0.034)	0.337

Distinct differences between the two taxa were found with respect to the apportionment of gene diversity within and among their populations. The gene diversity due to differences among populations (G_{ST}) was much lower in *P. kesiya* than in *P. merkusii*. Average unbiased and chord genetic distances (Cavalli-Sforza and Edwards 1967; Nei 1978) among populations of the investigated taxa are presented in Table 4. Conspecific distances among *P. kesiya* populations were lower than similar distances among *P. merkusii* populations. The unbiased and chord distances between *P. kesiya* and *P. merkusii* were extremely high (Table 4).

Phylogenetic relationships

The shortest Wagner tree (1.261) obtained in this study is shown in Fig. 1. Two highly diverged clusters corresponding strictly to taxonomic status were revealed. The first cluster comprised all sampled populations of *P. kesiya* and the other cluster comprised all sampled populations of *P. merkusii*. The *P. kesiya* cluster showed much less divergence than the cluster comprising populations of *P. merkusii*. Similar to the Wagner tree, populations of *P. kesiya* clustered separately from populations of *P. merkusii* on the neighbour-joining tree (data not shown).

CpDNA variation

All nine endonuclease-probe combinations employed in this study detected restriction-fragment patterns that dis-

Table 4 Average unbiased and chord distances for *P. kesiya* and *P. merkusii*

Species	No. of populations	Unbiased distance		Chord distance	
		P. kesiya	*P. merkusii*	*P. kesiya*	*P. merkusii*
P. kesiya	12	0.003		0.064	
Range		(0.000–0.017)		(0.035–0.114)	
P. merkusii	5	0.711	0.035	0.632	0.182
Range		(0.663–0.790)	(0.001–0.071)	(0.598–0.659)	(0.099–0.254)

Table 5 CpDNA fragments detected by the nine enzyme-probe combinations in *P. kesiya* and *P. merkusii* (fragment size in kb)

Enzyme	Probe					
	PcH157		PcH132		PcK32	
	P. merkusii	*P. kesiya*	*P. merkusii*	*P. kesiya*	*P. merkusii*	*P. kesiya*
Bcl-I	–	0.40	–	0.39	0.75	0.75
	0.50	–	1.10	1.10	1.85	1.85
	0.70	–	2.25	–	3.30	3.30
	1.60	1.60	–	4.80	3.90	–
	2.00	–	5.00	–	–	4.00
	2.20	2.20	7.00	7.00		
	–	2.70				
	–	4.10				
	7.00	–				
Bgl-II	0.75	–	0.55	0.55	0.40	0.40
	–	0.80	0.75	0.75	0.35	0.35
	0.85	–	–	0.90	0.52	0.52
	1.20	–	1.00	–	0.75	0.75
	1.50	1.50	–	1.20	1.25	1.25
	–	1.80	1.65	–	1.35	1.35
			2.40	2.40	–	2.20
			–	2.65	3.05	–
			–	2.80	–	3.10
			4.00	–	3.60	–
Dra-I	–	0.85	0.55	0.55	–	0.60
	–	2.35	2.20	–	–	0.70
	–	3.70	3.60	–	1.20	1.20
	–	–	–	5.80	1.35	1.35
	6.90				1.55	–
					–	2.45
					–	2.80
					2.90	–
					4.15	–
					–	4.50

tinguished *P. kesiya* from *P. merkusii*. The restriction fragments identified by these combinations are listed in Table 5. Fragments hybridising to the *Bcl*-I/PcH157 and *Bgl*-II/PcH132 combinations are presented in Fig. 2 a and b. The fragment patterns observed in the present study were identical with those detected in our previous study (Wang and Szmidt 1993). Analysis of cpDNA variation in sympatric populations of *P. kesiya* and *P. merkusii* did not detect hybridisation between the two species. All analysed individuals of *P. kesiya* showed identical fragment patterns diagnostic for that species. Similarly, patterns diagnostic for *P. merkusii* were found in all analysed individuals of that taxon.

Discussion

All Eurasian *Pinus* species from the subgenus *Pinus* are placed in a single subsection *Sylvestres* (section *Pinus*) which suggests relatively recent and common ancestry. However, our cpDNA-based analysis of phylogenetic relationships among Eurasian *Pinus* species has placed the southernmost member of this subsection *P. merkusii* in a separate, monotypic clade (Wang and Szmidt 1993). Similarly, on the cladogram presented by Farjon (1984), *P. merkusii* appears as the most distinct Asian species within the subsection *Sylvestres*. Striking differences between the two

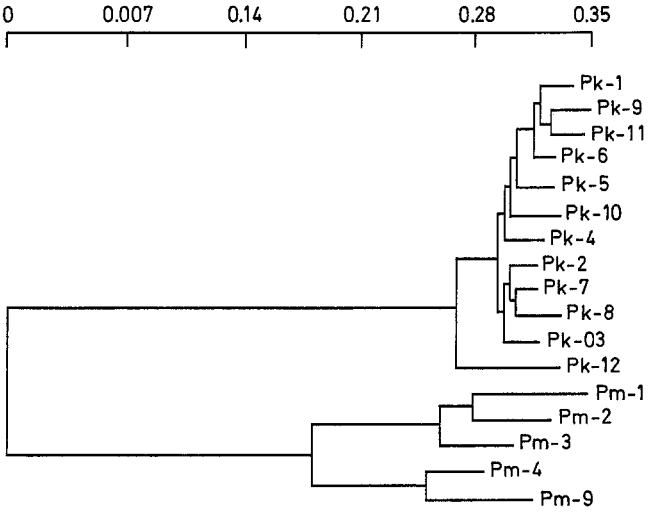

Fig. 1 Wagner tree based on chord distances among the investigated populations of *P. kesiya* (*Pk*) and *P. merkusii* (*Pm*)

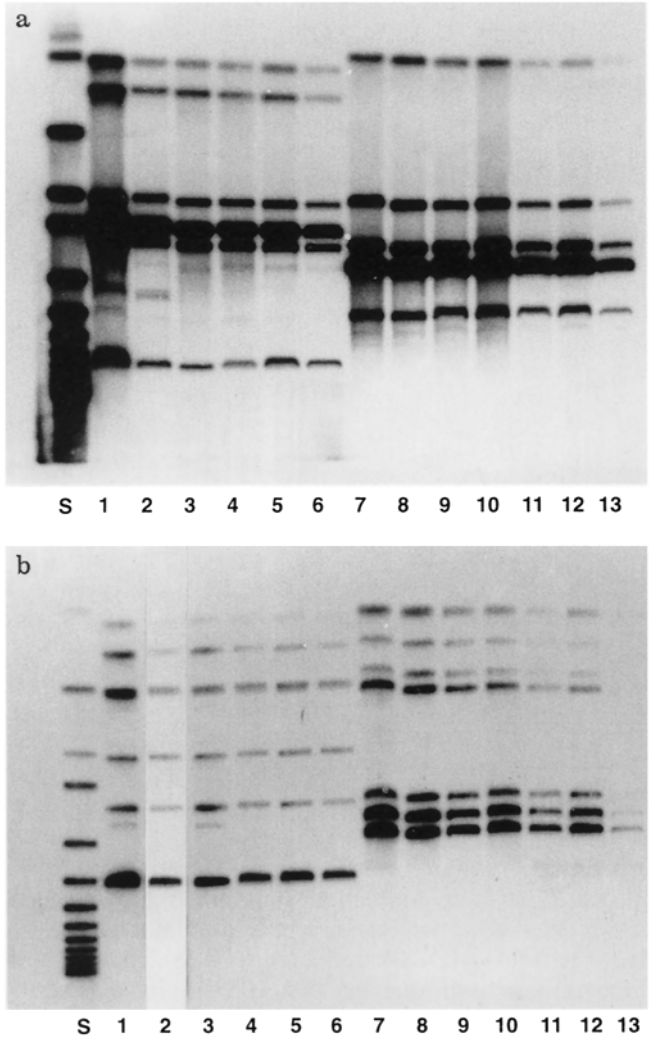

Fig. 2a, b Hybridisation patterns detected by *Bcl*-I/PcH157 (**a**) and *Bgl*-II/PcH132 (**b**) enzyme-probe combinations in *P. merkusii* and *P. kesiya* individuals from regions of sympatry. *S:* DNA standard; *lanes 1–6*: *P. merkusii*; *lanes 7–13*: *P. kesiya*

species in the amount and distribution of allozyme variation found in the present study provide additional evidence for the distinct character of *P. merkusii*. *P. kesiya* harboured relatively high allozyme variation and showed weak interpopulational differentiation. This result is concordant with a previous study on allozyme variation in this species (Boyle et al. 1991). Similar levels and patterns of allozyme variation have been found for other Asian *Pinus* species (Szmidt 1982; Son et al. 1989; Wang et al. 1990; Szmidt and Wang 1993). In contrast, *P. merkusii* had very low intrapopulational variability but a high level of interpopulational differentiation. The average Nei's genetic distance separating the two species was exceptionally high (0.711) and was similar to distances found among *Pinus* species from different subsections occurring in North America (Karalamangala and Nickrent 1989). Much lower genetic distances were reported by Millar et al. (1988) for *Pinus* species from the subsection *Oocarpae*. The constructed phylogenetic trees revealed very early divergence of *P. kesiya* and *P. merkusii*. The present analysis of cpDNA variation confirmed the dissimilar character of these two species observed previously (Wang and Szmidt 1993). Our present findings are also compatible with other morphological, physiological, and biochemical evidence indicating the outstanding position of *P. merkusii* as compared to other Asian members of the subsection *Sylvestres* (Cooling 1968; Farjon 1984; Weissmann and Lange 1987).

We believe that the distinct character of *P. merkusii* observed in this and other studies is a result of an early separation and prolonged isolation of this species from other Asian pines. During the Jurassic and Cretaceous periods, tropical pines were present in southeastern Asia (Mirov 1967). It is therefore possible that *P. merkusii* has developed in southeastern Asia in the Tertiary. During the Quaternary glaciation the climate in Asia has been benign enough to permit continuation of pines in this region (Mirov 1967). Thus, while the Quaternary glaciation destroyed all pine species in the northern part of the Hemisphere, *P. merkusii* could have survived the general deterioration of climate characteristic of the second half of the period. In fact, *P. merkusii* appears better adapted to a warm climate than other pines from southeastern Asia and at present is the only pine that occurs south of the Equator. By contrast, *P. kesiya* could have colonised southeastern Asia from the north, probably very recently, and has not yet spread very far west (Frankis, personal communication).

Our present analysis of cpDNA variation in sympatric populations of *P. kesiya* and *P. merkusii* revealed that they are pure representatives of the species in question. This result suggests that, despite overlapping distributions, *P. kesiya* and *P. merkusii* do not hybridise in nature. To our knowledge, *P. merkusii* has never been successfully crossed with any other *Pinus* species. Genetic barriers separating *P. merkusii* from other *Pinus* species could arise as a result of its prolonged isolation during the Quaternary glaciation.

The high inter-population variability found in *P. merkusii* is to be expected for a species that now has a fragmented range and was probably even more fragmented in

the recent past (Mirov 1967; Frankis, personal communication). There is also some evidence for similar differentiation among populations of *P. merkusii* with respect to morphological and chemical characteristics (Cooling 1968 and references therein; Coppen et al. 1993). Pronounced morphological differences exist between the Sumatran populations and Asian mainland populations of *P. merkusii* (Frankis, personal communication). The low intrapopulational variability of *P. merkusii* found in the present study may be explained by previous bottlenecks, reduced gene exchange between sparsely distributed populations, and an increased opportunity for inbreeding due to small population size and asynchronous flowering. Forest fires play an important role in the regeneration of *P. merkusii*. This suggests that individual populations could have been established by only a few founders. Unusually high selfing rates in *P. merkusii* populations from Thailand have been found (Changtragoon and Finkeldey 1995). We studied only populations from Thailand. It is thus important that future work includes additional populations from other parts of its distribution. As demonstrated by this and other studies, the evolution of *Pinus* species in Asia has been particularly complex and involved various mechanisms of population divergence such as isolation and interspecific gene exchange (Wang and Szmidt 1990; Wang et al. 1990; Szmidt and Wang 1993; Wang and Szmidt 1994). Continued research on this group of taxa could provide much novel information relevant to a better understanding of plant evolution.

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