

# Molecular evidence for glacial expansion and interglacial retreat during Quaternary climatic changes in a montane temperate pine (*Pinus kwangtungensis* Chun ex Tsiang) in southern China

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**Abstract** It has been suggested that the eastern Asian temperate flora have responded to Quaternary climatic changes in a different way compared with temperate plants in Europe and Northern America. However, knowledge about their phylogeographic structure and evolutionary history is still limited. In this study we investigated mitochondrial DNA variation in 17 populations of *Pinus kwangtungensis*, a five-needled pine inhabiting in isolated mountains of southern China and northern Vietnam. A total of ten mitochondrial haplotypes (mitotypes) were characterized by the polymorphisms of two fragments (*cox-1-2* and *nad7* intron1), and total genetic diversity was high ( $h_T = 0.847$ ). The construction of phylogenetic relationships of the ten mitotypes detected three major, well-distinct clades, largely corresponding to four

population groups identified by SAMOVA analysis. SAMOVA also indicated that most genetic variance should be attributed to among-group differentiation ( $F_{CT} = 0.868$ ), consistent with the substantial genetic structure found within *P. kwangtungensis* ( $G_{ST} = 0.751$ ). The genetic distances of *P. kwangtungensis* weakly but significantly correlated with geographical distances ( $R = 0.228$ ,  $P = 0.03$ ), revealing a pattern of isolation-by-distance. Demographic analysis did not detect any bottleneck events in the recent history of *P. kwangtungensis*. These results clearly suggested that there were three major refugia for this montane temperate conifer during warm stages (along with other minor refugia), and that the species would have tracked Quaternary climatic changes by expanding to nearby lowlands in the glacial periods, and by retreating to mountaintops (the refugia) during interglacial warmer stages, experiencing fragmentation and isolation among refugia.

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*Pinus kwangtungensis* · Refugia · Quaternary

## Introduction

China has the most diverse temperate flora among all the regions in the northern temperate zone. This has been attributed to several reasons, most notably the presence of an unbroken continuity of vegetation types, the collision of the Indian subcontinent with the mainland of Asia, the occurrence in southern China of a larger area of subtropical climate with complex topography, and the reduced impact of late Tertiary and Quaternary climate cooling (Axelrod et al. 1996; Qian and Ricklefs 1999). In southern China, complex landforms sustain numerous temperate forests at

higher elevations (e.g., those mainly composed by conifers such as *Abies*, *Cathaya*, *Pinus*, or *Tsuga*), which are separated by extensive tropical and subtropical forests occupying lower altitudes. During cooler periods in the Quaternary, temperate forests would have shifted to lower elevations, allowing isolated patches to join together, whereas interglacial warm periods would have forced temperate forest back to “refugia” at higher elevations (Qian and Ricklefs 2000). Repeated cycles of fragmentation and expansion would have promoted population divergence and even resulted in rapid speciation in high-elevation flora in southern China, as would have occurred for the temperate flora of the whole of eastern Asia (Qian and Ricklefs 2000). However, this hypothesis is still awaiting verification, because of the general lack of fossil data in these latitudes (Liew et al. 1998; Liu et al. 2003). Other lines of research, for example genetic studies, may help to shed light on the dynamics of Chinese temperate species in relation to Neogene climatic changes.

Demographic history during glacial–interglacial cycles left strong genetic imprints in current plant populations. By tracing the geographic patterns of genetic polymorphisms (i.e., phylogeographic studies), much information can be acquired about whereabouts of small populations (refugia) and the spread routes from these refugia (Petit et al. 2008; Hu et al. 2009). It has been suggested that the phylogeographic structure of high-elevation species (particularly in subtropical and tropical areas; Hewitt 2000, 2004; Jaramillo-Correa et al. 2008; Petit et al. 2008) could be shaped by several cycles of interglacial allopatric fragmentation followed by glacial contiguous range expansion along an elevation gradient locally. This model, which can be regarded as a “phalanx” model of spread (Hewitt 1996), may result in multiple centers of genetic diversity, pronounced genetic structure, and sharp contact zones between colonizer fronts. In addition, the species could retain high levels of genetic diversity, because these spatially limited, slower expansions would limit the occurrence of population bottlenecks thus allowing more alleles to be conserved (Hewitt 1996, 1999; Ledig et al. 2000; Johansen and Latta 2003; Dechaine and Martin 2004, 2005; Jaramillo-Correa et al. 2006, 2008). This pattern contradicts the “pioneer” model (Hewitt 1996). Widely used to explain the Quaternary dynamics for a wide array of boreal and temperate taxa at high latitudes in the northern hemisphere (Hewitt 2000, 2004), this model assumes extensive latitudinal range shifts in response to climatic cycles (typically rapid expansions northwards in the interglacials and retreats southwards during the glacials). The interglacial long-distance latitudinal migrations from a few refugia are expected to cause substantial impoverishment of genetic diversity, because the northern, colonizing populations are subjected to repeated founder effects (Hewitt 1996, 1999). In contrast, the rear-

edge populations can maintain higher levels of polymorphism (but see Petit et al. 2003).

Pines are important, and very often dominant, components of temperate forests over large parts of the northern hemisphere (Richardson and Rundel 1998). Mixed forests in the mountains of eastern Asia support about 25 pine species (with particular concentrations in China), representing one of the two greatest centers of pine species diversity (Price et al. 1998). *Pinus kwangtungensis* Chun ex Tsiang is a relatively widespread species among the five-needled pines in southern China and northern Vietnam. This cold-resistant pine grows on isolated mountaintops, cliffs, or slopes (700–1,900 m a.s.l.) in the Nanling Mountains, the mountains at the southeast edge of Yungui Plateau, the Dayaoshan Mountains, the Wuzhishan Mountains, and the mountainous areas of northern Vietnam. Pollen fossil records show that many upland conifers of southern China, including *Abies*, Cupressaceae, *Keteleeria*, *Pinus* (most likely including *P. kwangtungensis*), and *Tsuga*, were common in the subtropical coastal regions of southern China (that is, the lowland areas adjacent to the current, higher locations) during the last glacial maximum (Zheng 2000). The current distributional pattern and paleopalynological evidence suggest that *P. kwangtungensis* could have experienced glacial expansion (colonizing lower elevations, including coastal areas) and interglacial retreat (to the mountaintops) during the Quaternary climate oscillations. Thus, *P. kwangtungensis* may be useful for testing the suggested hypothesis of glacial expansion and interglacial retreat for the temperate flora of eastern Asia (Qian and Ricklefs 2000). Although this model could work for many conifers and other montane temperate species in southern China, studies in this direction are needed. To date, only a few phylogeographic surveys have been conducted on species of this region (Chiang et al. 2006; Wang and Ge 2006; Gong et al. 2008; Tian et al. 2008; Zhang et al. 2009), a situation very different from that in Europe and North America, where reports on this topic are now accumulating (Petit et al. 1997; Abbott et al. 2000; Abbott and Brochmann 2003; Petit et al. 2003; Hewitt 2004; Soltis et al. 2006).

A previous study based on a chloroplast minisatellite revealed clear population structure within *P. kwangtungensis* ( $F_{ST} = 0.540$ ) (Tian et al. 2008), indicating that genetic drift and local adaptation in isolated refugia promoted genetic divergence among populations. However, chloroplast genomes are inherited paternally and transmitted by pollen in pines (Petit et al. 2005). Gene flow mediated by the bisaccate pollen might be prevalent and distort the genuine historical signals (Liepelt et al. 2002; Chen et al. 2008). In contrast, mitochondrial DNA markers, which are maternally inherited, moved by seeds in pines, and unaffected by subsequent pollen movements, might be

more powerful for disentangling the imprints of past climate oscillations in *Pinus* species (Naydenov et al. 2007). In this study, we used the polymorphisms observed in the mitochondrial genome to examine the phylogeographic structure of *P. kwangtungensis*. Specifically, we are addressing the following questions:

- 1 Does *P. kwangtungensis* have an enhanced mitochondrial population structure compared with the chloroplast one?
- 2 How many refugia are there for *P. kwangtungensis* and
- 3 Is the hypothesis of glacial expansion and interglacial retreat under a phalanx model valid for *P. kwangtungensis*?

**Materials and methods**

**Population sampling**

Extensive field surveys were carried out throughout the entire range of *P. kwangtungensis* in China from 2004 to 2006. Fresh needles of *P. kwangtungensis* were collected from 17 populations across southern China (Table 1, Fig. 1). Except for Vietnam, the sampled populations covered most of the recorded sites of *P. kwangtungensis*. All samples were dried in silica gel and stored at -20°C

until they were processed. Populations were defined as groups of trees spaced about 50–100 m apart on a mountain.

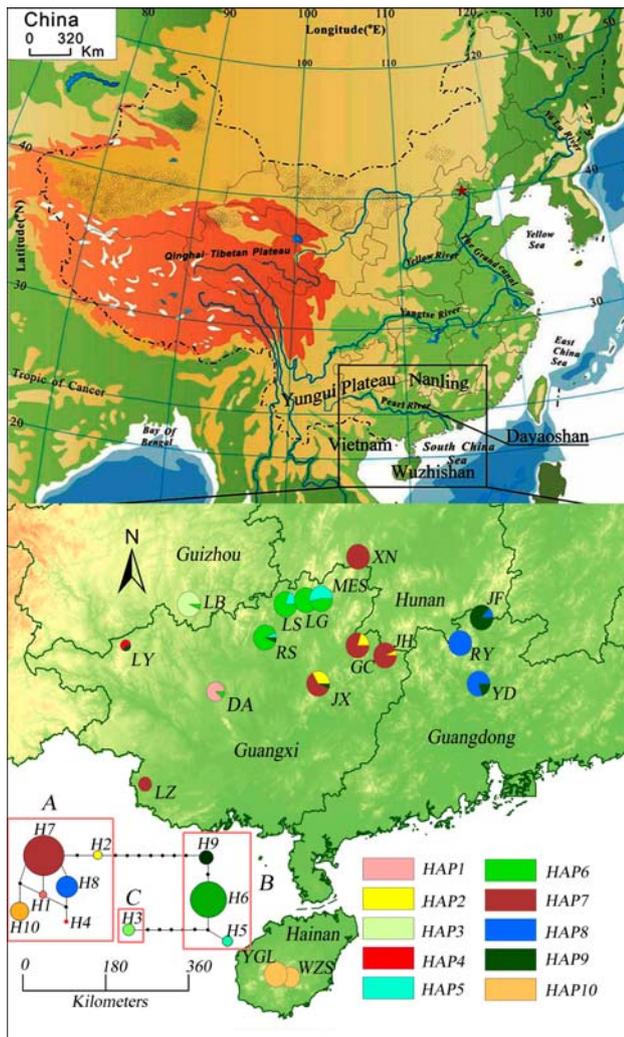
**DNA extraction, amplification, and sequencing**

Genomic DNA was extracted using a modified CTAB procedure (Doyle and Doyle 1987). Preliminary screening for polymorphisms was conducted using 15 pairs of universal mtDNA primers (the primer sequences are available upon request) over a panel of six individuals from different populations. Sequence polymorphisms were observed only in two fragments, cytochrome oxidase subunit 1 gene (*cox-1-2*) and NADH dehydrogenase subunit 1 gene (*nad7* intron1). The forward and reverse primers of the screened fragments were 5'-GATGCAGCGGAACCATGGCA-3' and 5'-TCCGATACCATTGATGTCC-3' (*cox-1-2*), and 5'-GGAACCGCATATTGGATCAC-3' and 5'-GTTGTACCGTAAACCTGCTC-3' (*nad7* intron1). Amplification reactions were carried out in a volume of 10 µl containing of 10× PCR buffer (10 mM Tris-HCl, pH 8.3 at 25°C), 1.0 mM MgCl<sub>2</sub>, 1.25 µM each primer, 0.25 µmol/L each dNTP, 0.5 U ex-Tag DNA polymerase, and 10–50 ng template. Amplification was carried out in a Tgradient 96 U thermocycler (Biometra, Göttingen, Germany) as follows: 4 min at 94°C followed by 11 cycles at a 0.5°C descending series of annealing temperatures each cycle

**Table 1** Genetic diversity and mitotype frequency in 17 populations of *P. kwangtungensis*

Population	N	N <sub>h</sub>	Genetic diversity	Mitotypes											
				H1	H2	H3	H4	H5	H6	H7	H8	H9	H10		
Longsheng (LS)	15	2	0.3429 ± 0.1278					3	12						
Jinxiu (JX)	15	3	0.5619 ± 0.0954		5						9			1	
Gongcheng (GC)	15	2	0.3429 ± 0.1278		3						12				
Rongshui (RS)	15	3	0.2571 ± 0.1416					1	13					1	
Lingui (LG)	15	1	0						15						
Maoershan (MES)	15	2	0.5333 ± 0.0515					8	7						
Xinning (XN)	15	1	0								15				
Jianghua (JH)	15	2	0.1333 ± 0.1123		1						14				
Ruyang (RY)	15	1	0									15			
Jiufeng (JF)	15	2	0.2476 ± 0.1307									2	13		
Yingde (YD)	15	2	0.3429 ± 0.1278									12	3		
Wuzhishan (WZS)	10	1	0												10
Yinggeling (YGL)	15	1	0												15
Libo (LB)	15	2	0.1333 ± 0.1123			14				1					
Leye (LY)	3	2	0.6667 ± 0.3143				2							1	
Duan (DA)	9	2	0.2222 ± 0.1662	8						1					
Longzhou (LZ)	5	1	0									5			
Total	222		0.8467 ± 0.0107	8	9	14	2	12	49	55	29	19	25		

N<sub>h</sub>, number of haplotypes; H, mitochondrial haplotype



**Fig. 1** Distribution of the ten mitotypes within and among populations of *P. kwangtungensis*. Pie sizes are proportional to the corresponding sample sizes. *Bottom left* are gene genealogies of the ten mitotypes, splitting into three major clades (A, B, and C). The size of the pie is proportional to the haplotype frequency found in the species

(58–53°C for *cox-1-2*; 68–63°C for *nad7* intron1), and 26 cycles with an annealing temperature of 54°C in *cox-1-2* and 56°C in *nad7* intron1. Denaturation was at 94°C (1 min) and extension was at 72°C (2 min) in all cycles, with a final 10 min extension at 72°C to end the reaction.

PCR products of *cox-1-2* and *nad7* intron1 were used for sequencing directly. Sequencing reactions were conducted with the forward and reverse primers of *cox-1-2* and designed sequencing primers of *nad7* intron1 (52 bp-U 5'-GGCCTCGTTATCCCACTGT-3'; 540 bp-U 5'-CTTG GGGTCGAGCAATAAAT-3') using the DYEnamic ET terminator kit (Amersham Pharmacia Biotech), following the manufacturer's procedure. Sequencing was done on a Megabase 1,000 automatic DNA sequencer (Amersham

Pharmacia Biotech) after the sequencing reaction products were purified by precipitation with 95% ethanol and 3 M sodium acetate (pH 5.2).

#### Data analysis

Because the mitochondrial genome was regarded as a single locus, the two fragments were pooled to define mtDNA haplotypes (mitotypes). A haplotype frequency map was constructed using ArcGIS 8.3. Observed numbers of mitotypes and mitochondrial diversity estimates ( $H$ , equivalent to the expected heterozygosity,  $H_E$ , for diploid data; Weir 1996) were calculated for each population. Total genetic diversity ( $h_T$ ), within-population diversity ( $h_S$ ) were calculated with Arlequin 2.000 (Schneider et al. 2000).

To determine phylogenetic relationships among mitotypes, a statistical parsimony network was constructed with the software TCS version 1.13 (Clement et al. 2000). Substitutions and indels were assumed to evolve with equal possibility although they may exhibit different mutation rates. Rates of mtDNA evolution are very slow (Wolfe et al. 1987), and, therefore, differences between substitution and indel mutation rates are unlikely to affect the resolution of intraspecific phylogenetic relationships over short timescales (Gonzales et al. 2008; Ronikier et al. 2008). Each indel was regarded as having originated independently of other indels in network construction. However, in order to evaluate the effects of complex indels on phylogeographic inference, several analyses in this study were also conducted based only on substitutions within the two mitochondrial fragments.

The geographical structure of genetic variation at mitochondrial DNA in *P. kwangtungensis* was investigated using three approaches. First, we evaluated whether there is a phylogeographic signal in the haplotype distribution of *P. kwangtungensis* by comparing  $G_{ST}$  with  $N_{ST}$  using the software HAPLONST (Pons and Petit 1996). A higher  $N_{ST}$  than  $G_{ST}$  usually indicates the presence of phylogeographic structure (Pons and Petit 1996), with closely related haplotypes being found more often than less closely related haplotypes in the same area. Second, we tested for a pattern of isolation by distance (IBD) according to Rousset (1997) method: a Mantel test with 10,000 random permutations was performed between the matrix of pairwise population differentiation statistics ( $F_{ST}$ ) and that of the natural logarithm of the geographical distances (Rousset 1997). Pairwise  $F_{ST}$  values between populations were estimated using Arlequin 2.000, and geographical distances between populations were calculated with the software Geodis 2.4 (Posada et al. 2000). Third, a simulated annealing procedure implemented in the SAMOVA algorithm (Dupanloup et al. 2002) was used to define groups of populations that

are geographically homogeneous and maximally differentiated from each other. The software iteratively seeks the composition of a user-defined number  $K$  of groups of geographically adjacent populations that maximizes  $F_{CT}$ , the proportion of total genetic variance due to differences among groups of populations. This software also enables identification of genetic barriers between groups of populations. We ran the software for  $K = 2-9$  starting from 10,000 random initial conditions.

To evaluate the influence of recent historical events on the demography of *P. kwangtungensis*, the software Bottleneck version 1.2 (Piry et al. 1999) was used to determine whether populations have experienced a recent reduction in their effective population sizes. Three tests, namely a “sign test”, a “standardized differences test” (Cornuet and Luikart 1996), and a “Wilcoxon sign-rank test” (Luikart et al. 1998), were performed under the assumption of the infinite allele model (IAM), stepwise mutation model (SMM), and two-phase model (TPM).

## Results

### Sequence characteristics, haplotype network and haplotype distribution

The aligned sequences of *cox-1-2* for 222 individuals sampled from 17 populations of *P. kwangtungensis* were 1,415 bp in length, with only two variable sites characterizing two haplotypes. The aligned sequence of *nad7* intron1 was 1,428 bp in length, comprising of 14 complex indels and two substitutions. Incorporating these complex indels, nine haplotypes were recognized in *nad7* intron1 among all individuals of *P. kwangtungensis*. The variable sites within the two fragments are shown in supplementary material Table 1. Mitotype sequences defined by the two mitochondrial fragments were deposited in the GenBank under the accession numbers EF114116–EF114117 (*cox-1-2*) and EF114118–EF114126 (*nad7* intron1).

Because mitochondrial genomes of plants inherit as a single copy (Schaal et al. 1998), the two fragments were combined directly and ten different mitotypes were determined. A haplotype network and a haplotype distribution map were constructed and are shown in Fig. 1. Three distinct clades can be clearly identified in the haplotype network (A, B, and C) (Fig. 1). Within the haplotype cladogram, clade A consisted of six mitotypes (H1, H2, H4, H7, H8, and H10) and was widely distributed in northern Guangdong Province (populations JF, RY and YD), southern Hunan Province (XN and JH), east to west Guangxi Zhuang Autonomous Region (GC, JX, LY, DA, and LZ), and Hainan Island (WZS and YGL) (Fig. 1). The haplotypes of this clade were mostly interconnected by one

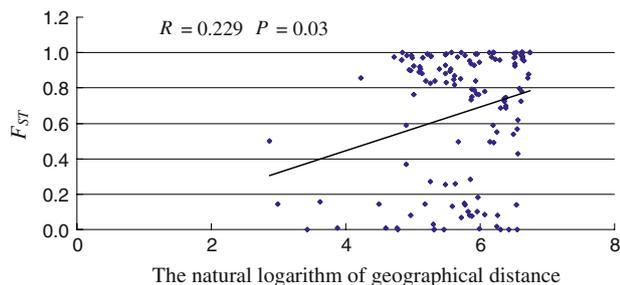
or two mutations. The H7 haplotype was possibly an ancestral one, because it was located in the central position of the network and occurred at high frequency in five populations (XN, JH, GC, JX, and LZ). Because clade A was widespread, subdivision was detected within this clade: H8 only occurred in northern Guangdong (JF, RY, and YD); H2 and H7 were distributed in southern Hunan (XN and JH) and Guangxi (GC, JX, and LZ); H10 remained restricted to Hainan Island (WZS and YGL); and, finally, H1 and H4 were only found in DA and LY populations, respectively.

Clade B differed from clade A by eight mutations, and included three distinct mitotypes (H5, H6, and H9). H6 was connected with H9 and H5 each by two mutations. The H5 and H6 haplotypes were mostly restricted to northern Guangxi (MES, LG, LS, and RS) (Fig. 1), except for occasional occurrence in a population of southern Guizhou (LB). However, within clade B, H9 was exceptionally widespread along the distribution area of *P. kwangtungensis*, occurring in west, central and east populations (LY, RS, JX, JF, and YD). In the light of its central position within the network and widespread distribution, H9 possibly represented an ancestral haplotype within the whole cladogram. Clade C consisted of only one mitotype (H3), which was endemic to population LB. This clade was differentiated from clade B by seven mutations.

When only substitutions within *cox-1-2* and *nad7* intron1 were considered, only five mitotypes were characterized. The haplotype network was quite simple, because of the limited sequence variation (supplementary material Fig. 1). The distribution pattern of the five mitotypes (supplementary material Fig. 1) was basically consistent with that of the ten mitotypes (that incorporating the indels), except for the lack of genetic distinctiveness of the LB population. This discrepancy is obviously due to the additive resolution of the indels within *nad7* intron1.

### Genetic diversity and genetic structure

The total mitochondrial diversity in *P. kwangtungensis* detected in this study was high ( $h_T = 0.847$ ) (Table 1). Nevertheless, the genetic diversity within populations had a relatively low value ( $h_S = 0.223$ ). Six populations (RY, XN, LG, LZ, WZS, and YGL) were fixed for one mitotype ( $H = 0$ ). Nine populations (JF, YD, JH, GC, MES, LS, LB, DA, and LY) possessed only two mitotypes ( $H = 0.133-0.667$ ). The most variable populations were RS and JX, where three distinct mitotypes were detected ( $H = 0.257$  and  $0.562$ , respectively). The level of population subdivision of *P. kwangtungensis* was high with  $G_{ST} = 0.751$  and  $N_{ST} = 0.798$ . The  $U$  test showed that  $N_{ST}$  was not significantly larger than  $G_{ST}$  ( $U = 0.88$ ,  $P > 0.05$ ). A test for isolation-by-distance indicated the existence of a weak but

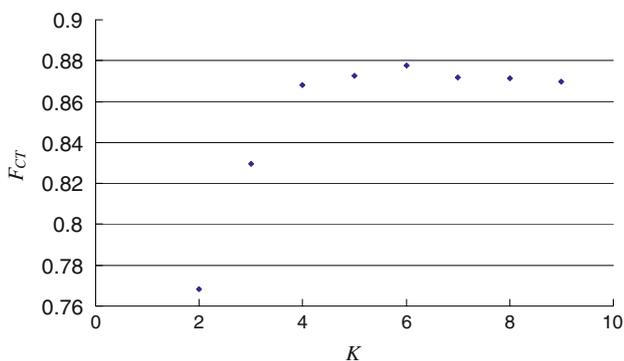


**Fig. 2** Correlations between pairwise  $F_{ST}$  value and the natural logarithm of geographical distance between populations

significant correlation between genetic and geographical distances ( $R = 0.229$ ,  $P = 0.03$ ) (Fig. 2).

Although only five mitotypes were characterized by the substitutions, the total mitochondrial diversity represented by these few mitotypes was also relatively high ( $h_T = 0.647$ ). The genetic structure revealed by the five mitotypes was also high ( $G_{ST} = 0.673$ ), but  $N_{ST}$  (0.792) was significantly larger than  $G_{ST}$  ( $U = 4.93$ ,  $P < 0.05$ ), suggesting that the complex indels within *nad7* intron1 had significant effects on the phylogeographic structure of *P. kwangtungensis*.

To determine groups of populations that are geographically homogeneous and maximally differentiated from each other, we defined the number of groups  $K$  from 2 to 9. The results showed that the  $F_{CT}$  values increased very quickly with  $K$ , reaching almost plateau values at  $K = 4$  and with at least one of the groups containing a single population (LB) ( $F_{CT} = 0.868$ ; Fig. 3). Thus, the configuration of  $K = 4$  was retained as the most possible number of groups (G1–G4 in Table 2). The four groups determined by SAMOVA analysis can be clearly associated with the structure of the haplotype network and the distribution of mitotypes across southern China (Table 2, Fig. 1). G1 and G2 included all populations in which mitotypes belonging to clade A are the most abundant. If only three groups were delimited, then groups 1 and 2 merged into one group



**Fig. 3** The correlations between the number of groups ( $K$ ) and genetic variance ( $F_{CT}$ ) in SAMOVA analysis

(Table 2). The splitting of those populations characterized by mitotypes of clade A into two groups (G1 and G2) in SAMOVA analysis may represent the differentiation of Hainan populations from mainland populations, both genetically and geographically. G3 comprised the populations exhibiting exclusively the mitotypes of clade B (MES, LG, LS, and RS) plus population JF (which showed a mitotype of clade A in low proportion). G4 consisted only of LB, in which the unique mitotype belonging to clade C (H3) is dominant. Notably, the distributions of the three haplotype clades showed quite sharp boundaries: no overlapping of mitotypes was observed among the four groups of populations, with the exceptions of H6 and H9 haplotypes only (Fig. 1; Table 1).

### Demographic history

To infer whether *P. kwangtungensis* had suffered recent reductions of its effective population size (i.e., population bottlenecks), three tests (sign test, standardized differences test, and Wilcoxon sign-rank test) were carried out. The results showed that *P. kwangtungensis* populations did not deviate significantly from mutation-drift equilibrium under SMM, IAM, and TPM, suggesting that the occurrence of recent bottleneck events is unlikely for this Asian endemic pine.

## Discussion

### Genetic diversity

Mitochondrial DNA diversity ( $h_T = 0.847$ ) revealed in *P. kwangtungensis* is high relative to that reported for other species of Pinaceae (e.g., Burban and Petit 2003; Jaramillo-Correa et al. 2006; Aizawa et al. 2007; Chen et al. 2008). This high diversity might be attributed to the high frequency of indels within *nad7* intron1. Within mitochondrial genome of plants, nucleotide substitutions are very rare, but indels are frequent and responsible for most of their sequence variation (Day et al. 2004). If the indels of *nad7* intron 1 are excluded from the analysis, the two detected substitutions within this fragment allow the characterization of only three distinct mitotypes. However, when substitutions within *cox-1-2* are also considered, up to five haplotypes could be inferred from the two fragments, and the mitochondrial DNA diversity would reach 0.647. Thus, the high genetic variability may be intrinsic to *P. kwangtungensis*, because the same pattern is also detected in chloroplast ( $h_T = 0.629$ , Tian et al. 2008) and nuclear sequence data (Z.-Y. Zhang et al. unpublished).

High polymorphism may reflect the wide distribution of *P. kwangtungensis* in southern China, with large and stable

**Table 2** The composite populations under different user-defined number *K* (2–9) of groups of populations in SAMOVA analysis

GN \ PN	2	3	4	5	6	7	8	9
XN			G 1					
GC								
JH								
JX								
LZ								
DA								
LY								
YD								
RY								
WZS				G 2				
YGL								
LB			G 4					
JF			G 3					
LG								
MES								
LS								
RS								

PN, population name; GN, the number of group

population sizes. In contrast with many pine species distributed in Europe and North America (e.g., *P. sylvestris* and *P. resinosa*, Echt et al. 1998; Dvornyk et al. 2002), which experienced drastic reductions in population sizes during the Quaternary glacial periods, *P. kwangtungensis* could have expanded its range in southern China during these colder episodes (see discussion below). During the Quaternary warming stages (the interglacials), populations of *P. kwangtungensis* would be effectively sheltered in the mountainous areas of southern China (such as occurs in the present day). This would have allowed the maintenance of relatively large population sizes, despite retreat to mountaintops, thus retaining high genetic diversity. Further support for this inference comes from the evidence that demographic bottlenecks were not detected at the species level in this study. Tian et al. (2008) also suggested that most populations of *P. kwangtungensis* were stable demographically in recent history.

The retention of ancestral polymorphisms is an alternative explanation for the high genetic variation found in *P. kwangtungensis*. Pines have a long generation time and

are outbreeding species. These features maximize the possibility of allele coalescence preceding ancient lineage divergence, which may result in increased intraspecific genetic diversity, especially in recently radiated clades (Bouillé and Bousquet 2005; Syring et al. 2007). *P. kwangtungensis* belongs to a closely related species group, which recently radiated in eastern Asia (Eckert and Hall 2006). Within this species group, comprehensive sharing of genealogies has been documented (Syring et al. 2007; Z.-Y. Zhang et al. unpublished data). Thus, it is very likely that ancestral polymorphisms of progenitors are still retained in *P. kwangtungensis*. Furthermore, given the ability of many *Pinus* species to form fertile hybrids, the probability of introgression with closely related species cannot be ruled out.

#### Phylogeographic structure and potential refugia

Despite the high total mitotype variation, within-population diversity in *P. kwangtungensis* is relatively low ( $h_s = 0.223$ ). Consequently, most of the species' diversity should

be attributed to population genetic differentiation ( $G_{ST} = 0.751$ ). When genetic distances among haplotypes (ordered haplotypes) are considered, genetic differentiation is similarly high ( $N_{ST} = 0.798$ ) but not significantly higher than unordered haplotypes ( $U = 0.88$ ,  $P > 0.05$ ), implying lack of phylogeographic structure (Pons and Petit 1996). This result seems to be inconsistent with the haplotype network, the geographic distribution of haplotypes, and the results of SAMOVA which revealed clear phylogeographic structure (Fig. 1; Table 2). This incongruence could be caused by the incorporation of the complex indels with *nad7* intron1, which blurs the difference between  $N_{ST}$  and  $G_{ST}$ . By comparing the haplotype networks in Fig. 1 and supplementary material Fig. 1 it can be stated that the complex indels with *nad7* intron1 increase the genetic distances among haplotypes. Pons and Petit (1996) pointed out that when differences between haplotypes separated by more than three nucleotide sites are considered,  $N_{ST}$  is no longer larger than  $G_{ST}$ . In *P. kwangtungensis*, when merely substitutions within the two mitochondrial fragments are considered,  $N_{ST}$  is significantly larger than  $G_{ST}$  ( $U = 4.93$ ,  $P < 0.05$ ).

The clear structuring of *P. kwangtungensis* into three mitotype clades and four genetically differentiated geographical groups may help to trace the demographic history of the species. Phylogeographic data suggest the existence of several isolated refugia (“interglacial refugia”; Bennett and Provan 2008), in which mtDNA divergence gradually accumulated at each warm period of the Quaternary. Up to three major (interglacial) refugia can be hypothesized for *P. kwangtungensis* in southern China. The long genetic distances occurring among the three mitotype clades (7 or 8 mutations) may imply that the fragmentation of the ancestral genetic pool is not recent, and thus the suggested refugia could have been in place since the early interglacial periods of the Quaternary. The first major refugium might have been located in southern Hunan and east Guangxi, where populations JH, GC, and JX are distributed. The area includes the Mengzhuling and Dupangling Mountains (in the central range of Nanling Mountains) and the Dayaoshan Mountains, and is represented by haplotypes H2 and H7 (which are the most ancestral in clade A) (Fig. 1). The second major refugium might include populations MES, LG, LS, and RS, an area delimited by the Yuechengling Mountains (in the west range of the Nanling Mountains) and the Jiuwandashan Mountains (a range located at the southeast edge of Yungui Plateau). The H5 and H6 mitotypes are concentrated in this putative refugium. The third major refugium was probably situated in the southeast of Yungui Plateau, which corresponds to the location of LB population, the only place where the H3 haplotype can be found.

According to the genetic data, populations within each refugia could have experienced different demographic

histories along the Quaternary climatic cycles. Populations of the second (which are genetically quite homogeneous; Fig. 1; Table 2) but particularly the third refugium probably experienced very little range expansion during the glacial periods (the climatic episodes favorable for such a montane, cold-tolerant species). In contrast, populations of the first refugium might have expanded their range more extensively (but still on a limited scale, of just a few hundreds of kilometers), colonizing the lowlands of Guangxi and Guangdong, and even crossing the Qiongzhou Strait and reaching Hainan Island (this shallow strait was passable during the glacial periods because of the marine regressions; Ferguson 1993). This relatively large spread probably took place at one of the last glacial stages of the Quaternary, which were by far the most intense throughout the Quaternary (Raymo 1994), that is, the most favorable periods for demographic expansion of *P. kwangtungensis*. After this expansion, the advent of the next immediate interglacial period caused the spreading populations of the first refugium to retreat to the nearby mountains looking for more favorable conditions at higher elevations, thus disrupting the newly gained distribution area into several smaller parts. This produced several minor, secondary refugia, where further differentiation occurred during the last few warm stages of the Quaternary. The fact that different mitotypes within clade A characterize specific groups of populations is indicative of the existence of several minor refugia. For example, LY is characterized by H4, DA by H1, the populations of east Guangxi, southwest Guangxi, and southern Hunan (GC, JX, LZ, JH and XN) by H2 and H7, the easternmost populations (JF, RY and YD) by H8, and the Hainan populations (WZS and YGL) by H10. The recentness of this expansion from the first refugium and the further genetic subdivision is supported by the small genetic distances among the mitotypes of clade A (one or two mutations), which are much shorter than those among the three major clades.

Remarkably, the clear among-group differentiation of *P. kwangtungensis* is also supported by chloroplast DNA markers ( $F_{ST} = 0.540$ ) (Tian et al. 2008). The 17 populations of *P. kwangtungensis* were similarly clustered into three groups according to chloroplast genetic distance, although the components of each group were different, because of the paternal inheritance of chloroplast markers in pines. This pattern is in sharp contrast with the chloroplast population structure of many other conifers (Jaramillo-Correa et al. 2006; Chen et al. 2008), further suggesting that the isolation events among the three major refugia are significant (repeated at each warmer stage of the Quaternary) and the phylogeographic signals are still evident, despite the strong dispersal ability of pines' pollen. However, albeit a similar genetic pattern was revealed by both chloroplast and mitochondrial markers, the latter

offers a much clearer phylogeographic structure (e.g., clearer boundaries between groups and restricted distribution range of mitotypes which is important for identifying the localities of refugia), highlighting the need for maternally inherited markers in deciphering the evolutionary histories of pines during the Quaternary.

#### Molecular evidence for glacial expansion and interglacial retreat of *P. kwangtungensis*

The “Refuge” theory proposed by Haffer (1969) suggests that populations were isolated when their habitats were disjoined because of cyclic expansions and contractions during alternating warm and cold episodes of the Quaternary. For cold-adapted, high-elevation species, particularly in subtropical and tropical areas, the Quaternary glaciations could have provided opportunities for range expansion at lower elevations, whereas during the interglacials their ranges were strongly restricted to reduced and fragmented refugia in high-altitude regions (Dechaine and Martin 2004; Yuan et al. 2006; Jaramillo-Correa et al. 2006, 2008). Indeed, in the current interglacial period (Holocene), many cold-adapted species exist at high elevations of southern China surviving as relicts. For instance, several relict *Abies* species (e.g., *A. beshanzuensis*, *A. yuanbaoshanensis*, and *A. ziyuanensis*), which are adapted to humid and cold environments, are disjunctly distributed along several fragmented mountaintops of southern China (Xiang 2001). Fossil pollen records suggest, however, that *Abies* species were common in lowland forests of central and southern China during the last glacial maximum (Xiang 2001).

Because there are several pines (e.g., *P. massoniana*, *P. fenzeliana*) with different ecological requirements co-distributing with *P. kwangtungensis* in southern China and it is difficult to assign pine pollen to specific taxa, the paleopalynological evidence for the glacial expansion of montane pines such as *P. kwangtungensis* is necessarily vague (Yu et al. 1998; Zheng 2000). However, demographic history during glacial–interglacial cycles may leave strong genetic imprints on plant populations, which provide much information about their evolutionary histories during the Quaternary (Hewitt 1996, 2004; Petit et al. 2008). Most aspects of the herein characterized phylogeographic structure of *P. kwangtungensis* are in agreement with the hypothesis of Qian and Ricklefs (2000) of glacial expansion and interglacial retreat for the temperate flora of eastern Asia, and also with the phalanx colonization model of Hewitt (1996, 1999).

First, as expected, multiple centers of genetic diversity (namely refugia), strong phylogeographic structure, and clear boundaries between groups have been observed for *P. kwangtungensis*. Although glacial refugia could also

lead to multiple centers of genetic diversity, strong phylogeographic structure and, especially, clear boundaries between groups can seldom be observed (Hewitt 1996). It is, therefore, more likely these genetic features observed in *P. kwangtungensis* can be attributed to the repeated events of population fragmentation and isolation during the interglacials, which led to genetic isolation and further among-refugia divergence (Hewitt 1996; Jaramillo-Correa et al. 2006, 2008). During the glaciations, range expansions from interglacial refugia over short distances involved only limited gene flow among populations, resulting in clear boundaries between groups and weak (but still detectable) isolation-by-distance (Johansen and Latta 2003).

Second, this study did not identify any southern populations harboring high genetic diversity, an expected feature of the pioneer model with southern glacial refugia. On the contrary, several southern populations, such as LZ, WZS, and YGL, are represented by only one haplotype. In addition, the mitotypes within these populations belong to clade A, suggesting they might be derived from northern populations by downward (i.e., descending mountains) and southward expansion during perhaps one of the last glacial stages (the most intense ones) of the Quaternary. These southern populations, nevertheless, may have behaved as minor, secondary refugia, during further warming stages, as they are doing in the current interglacial period (Holocene).

Third, Hewitt (1996) pointed out that if the mountains acting as interglacial refugia are large, many alleles and genomes may survive. Furthermore, because phalanx colonizations occur over short distances only, one would expect much less dispersal bottlenecks and loss of allelic diversity (Hewitt 1996; Johansen and Latta 2003; Dechaine and Martin 2004, 2005). In our study, the demographic stability and high genetic diversity suggested for *P. kwangtungensis* probably reflects the slow, spatially limited range shifts tracking the Quaternary climatic changes, thus minimizing dispersal bottlenecks and loss of allelic diversity (Hewitt 1996, 1999; Johansen and Latta 2003; Dechaine and Martin 2004, 2005). The rugged topography of southern China would have provided many suitable habitats for the survival of this pine species both during the glacials (lowlands and valleys) and the interglacials (mountaintops). Taken together, a scenario of repeated interglacial fragmentation (isolation among interglacial refugia) followed by glacial expansion locally is the most likely for *P. kwangtungensis* during the Quaternary climatic changes.

The suggested strategy for *P. kwangtungensis* of surviving in mountaintop isolated interglacial refugia during warming stages (similar to current days) and locally expanding to lowland areas during glacial stages might be common for montane temperate plants in subtropical and

tropical latitudes. Several taxa (mainly conifers) with a common pattern (with some variations) of multiple refugia, strong genetic differentiation, and evidence of limited demographical expansion have been reported in recent years in subtropical China (Wang and Ge 2006; Gong et al. 2008; Tian et al. 2008; Zhang et al. 2009). Because the Quaternary glaciations were much less prevalent in Asia than in Europe and North America, and the topography of China is extremely heterogeneous (Qian and Ricklefs 2000), the montane temperate flora in southern China could have responded to the Quaternary glacial-interglacial cycles in a different way from in Europe and North America. This study provides empirical evidence in support of the Qian and Ricklefs (2000) hypothesis, pointing out that the phalanx model may be more plausible for interpreting the phylogeographic structure of montane temperate plants in southern China. However, validation of this hypothesis requires more phylogeographic studies to be conducted on different taxa, and integration of phylogeographic and fossil data is demanded to elucidate the evolutionary history of the montane temperate flora in southern China (Petit et al. 2008).

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