

Contrasting coarse and fine scale genetic structure among isolated relic populations of *Kmeria septentrionalis*

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Abstract Trees of the Magnoliaceae family are of scientific, cultural and socio-economic importance. *Kmeria septentrionalis* Dandy (Magnoliaceae) is a dioecious tree, found in small, isolated, relic populations in Southern China, and is subject to extensive protection due to its rarity and high economic values. To improve conservation outcomes and in particular, germplasm collection guidelines, information on spatial genetic structure of the species is required. In this study, we investigated the spatial genetic structure and genetic diversity of 161 individuals of *K. septentrionalis* collected from five natural populations using AFLP molecular markers. Within-population genetic variation was measured, with percentage of polymorphic bands (PPB) ranged from 63% to 87%, while H_S (genetic diversity within population) varied from 0.185 to 0.244 with a mean of 0.215 ± 0.025 . Significant genetic differentiations were revealed between pairwise populations, indicating each population existing as an independent evolutionarily significant unit. Mantel test results showed no pattern of isolation-by-distance among populations separated by large distance. Fine scale spatial patterns of genetic variation suggested significant effects of

isolation-by-distance within population at distances of 22 m. The results of contrasting genetic structure at coarse and fine scale in *K. septentrionalis* may indicate restricted pollen flow and seed dispersal at fine scales, and separated evolution in isolated populations over long period of time at coarser scales. Finally, we make several suggestions for improved management practices that may assist in the conservation of this species.

Keywords AFLP · Conservation · Genetic structure · Isolation · *Kmeria septentrionalis* · Spatial autocorrelation analysis

Introduction

Species of the Magnoliaceae family are valued for their timber, medicinal and food products and for their ornamental value. They are also of exceptional scientific importance in studies of plant evolution and biogeography. This family display many characters that are considered evolutionarily primitive, and a disjunct geographical distribution today though the family occurred throughout Northern Hemisphere in the late Cretaceous and Tertiary periods (Cicuzza et al. 2007). The species of this important plant family act as indicators of conservation status of the Asian and American forests where they occur, demonstrating the impact of environmental change on an entire taxonomic group of plants. This is currently particularly important, during a time of unprecedented environmental change resulting from human activities, including widespread habitat loss and global warming (Matsuda et al. 2003; Cicuzza et al. 2007).

Over 40% of all Magnoliaceae species occur in Southern China (Cicuzza et al. 2007). A significant number of these

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are considered threatened because of habitat decline and, in some cases, overexploitation. Magnoliaceae species are increasingly attracting the interest of conservation biologists, and increasing efforts relevant to their conservation has been undertaken in recent years.

Kmeria septentrionalis Dandy (Magnoliaceae) is an endangered dioecious tree with a restricted distribution found in highly fragmented populations across Southern China (Pan 2007). *Kmeria septentrionalis* is a relic species of the Tertiary period (Law 1984) and its origin has been dated back to the middle Oligocene (ca. 30 Myr ago) (Nie et al. 2008). The species is listed one the most endangered species in China (<http://www.chinabiodiversity.com>), and a nature reserve (Guangxi Mulun National Nature Reserve) has been established for its protection since 1991. However, field surveys (Pan 2007) revealed that the populations are not sustainable in the long term, and the species is under a growing threat from illegal logging.

Various measures have been undertaken over recent years to improve the protection and conservation of the species (Pan 2007). Recent conservation efforts include re-introducing genetic material into populations of *K. septentrionalis*, and establishing *ex situ* conserved population and seed bank, at the South China Botanical Garden. However, the lack of genetic information for *K. septentrionalis* has posed a barrier for the design of effective sampling strategies, which will capture genetic variation within the *ex situ* population and seeds and minimize introduction of maladapted genotypes. In this study, we investigated population genetic diversity, genetic structure, and gene flow patterns among relic populations of *K. septentrionalis*. To make a practical contribution towards genetic guidelines for *ex situ* and *in situ* conservation, we generated Amplified Fragment Length Polymorphism (AFLP) data for *K. septentrionalis* populations to (1) estimate genetic diversity parameters, (2) examine the patterns of genetic differentiation in coarse and fine scale, with an attempt to identify possible evolutionarily significant units (ESUs) and their geographic ranges for future conservation, and (3) provide genetic guidelines for genetic materials collection for *ex situ* and *in situ* conservation.

Materials and methods

Sampling and AFLP procedure

Since *K. septentrionalis* is patchily distributed (Pan 2007), individuals of *K. septentrionalis* occurring within close geographical proximity, usually within a range of 500 m, were delimited as one population. At each site, except MLS (Fig. 1), samples were collected haphazardly from the entire population. A more detailed sampling regime was



Fig. 1 Location of of five populations of *Kmeria septentrionalis* included in this study. MG, MLS, ML, LB and LC are population codes

implemented for fine scale spatial autocorrelation analysis at the site MLS in which disturbance was not evident. At site MLS, 46 individuals were sampled within an area of 100 × 100 m. Positions of all sampled individuals were recorded and pairwise distances were measured using a tape measure. Fresh leaves of 161 individuals were collected from five relic populations in southern China (Table 1, Fig. 1). Leaves were dried with silica gel. Total genomic DNA was extracted following a modified CTAB protocol (Doyle and Doyle 1987) and purified with the Wizard[®] DNA Clean-Up System (Promega Corporation). The quantity and quality of DNA was assessed on a 1% agarose gel. DNA concentrations were adjusted to 50 ng/μl after purification.

AFLP profiles were generated according to an established protocol (Vos et al. 1995). Negative controls and five replicates were included. Preselective amplification (20 μl reactions) was performed using primer pairs with a single selective nucleotide (*MseI*-C and *EcoRI*-A). The *EcoRI* selective primers were fluorescently (6-FAM) labeled. Individuals from different populations were used for selective primer screening. Five primer pairs (*MseI*-CAG/*EcoRI*-ACT, *MseI*-CTG/*EcoRI*-ACT, *MseI*-CTA/*EcoRI*-AGC, *MseI*-CAG/*EcoRI*-ACC, *MseI*-CAC/*EcoRI*-ACC, from 48 primer combinations) produced clear and reliable profiles, and were used for selective amplification. The amplified products were visualized on an ABI PRISM 377 DNA sequencer using 5% denaturing polyacrylamide gel with an internal size standard (ABI ROX50, PE Applied Biosystems). DNA fragments were sized by GENESCAN V2.1 (PE Applied Biosystems). Fragments from 50 to 500 bp were scored as present or absent, ambiguous bands identified from negative controls and replicate comparisons were excluded. A presence (1)/absence (0) matrix was constructed using MG V1.0 (Zhou and Peter 2003).

Table 1 Location, population details and sample size for five *Kmeria septentrionalis* populations included in this study

Code	Position	Altitude (m)	Sample size	Population size	H_S	PPB (%)	Site descriptions
LB	25°28'41"N, 108°04'02"E	679	29	40	0.185	63.01	Logged, small saplings or seedlings
ML	25°02'02"N, 107°58'24"E	550	15	30	0.195	67.22	Near village, few paths
MLS	25°03'03"N, 107°58'01"E	557	46	300	0.220	79.85	Well-protected with mature trees, saplings and seedlings)
LC	24°52'04"N, 108°49'21"E	400	37	100	0.232	79.1	Logged, mature trees, saplings and seedlings
MG	22°43'07"N, 103°54'32"E	870	34	40	0.244	86.92	Logged, saplings and seedlings
Means	–	–	–	–	0.215 (0.025)	75.22 (9.83)	–

Standard deviations were indicated in brackets for mean values

H_S genetic diversity within populations, PPB percentage of polymorphic bands

Data analysis

To investigate population genetic diversity and structure, the following parameters were calculated using POPGENE 1.32 (<http://www.ualberta.ca/~fyeh/>): percentage of polymorphic bands (PPB); total genetic diversity (H_T); genetic diversity within population (H_S); Nei's unbiased genetic distance (Nei 1978).

To test genetic differentiation, the overall population differentiation coefficients of F_{ST} and pairwise F_{ST} were calculated with AFLP-SURV 1.0 (Vekemans et al. 2002), using the non-uniform prior Bayesian method (assuming Hardy–Weinberg equilibrium). This method generates the most accurate allele frequencies for small to moderate populations from dominant data (Zhivotovsky 1999). One thousand permutations of the data were performed to test the significance of F_{ST} at 0.05 levels against the null hypothesis that there is no genetic differentiation among the populations (i.e. $F_{ST} = 0$) using AFLP-SURV 1.0, Vekemans et al. 2002). Following Rousset (1997), Patterns of isolation-by-distance were assessed by a correlation of $F_{ST}/(1 - F_{ST})$ with \log_{10} distance for all pairs of populations. Since population pairs are not independent, a Mantel test (Mantel 1967) was used to evaluate significance in GenAlEx 6.1 (Peakall and Smouse 2006) with 999 permutations.

The fine scale spatial genetic structure within population MLS was assessed by spatial autocorrelation analysis using GenAlEx 6.1 (Peakall and Smouse 2006), following the procedures of Smouse and Peakall (1999) that allow the multivariate analysis of individual spatial genetic structure for multilocus data sets. Evenly distance interval was set at 9 m which resulted in at least 30 pairs of population at each distance class (Escudero et al. 2003). For all pairs of individuals within a distance class, a correlation coefficient r was calculated. Upper and lower confidence limits, generated by 999 random permutations of the data, represent the 95% confidence interval (CI) about the null hypothesis

of no spatial structure. In addition, 95% CIs of r were estimated by bootstrapping.

Results

Level of genetic diversity

Five primer combinations amplified a total of 665 fragments in 161 individuals (Table 1). H_S ranged from 0.185 to 0.244, with an average of 0.215 ± 0.025 . Total genetic diversity across all populations (H_T) was 0.249. PPB varied from 63% to 87% with an average of $75\% \pm 9.83\%$. Population LC was the most genetically diverse in terms of H_S and PPB (Table 1). The H_S and PPB of population MLS was higher than that of the neighbouring ML population.

Genetic differentiation and structure among populations

Populations of *K. septentrionalis* were significantly differentiated with $F_{ST} = 0.105$, and deviated from zero significantly ($P < 0.05$). Pairwise F_{ST} statistics revealed all population pairs were also differentiated significantly ($P < 0.05$, Table 2). As a consequence, each population existed as an evolutionarily significant unit. No significant

Table 2 Pairwise population F_{ST} values (below diagonal) and geographic distances (above diagonal) for five populations of *Kmeria septentrionalis*

	ML	LB	MLS	MG	LC
ML	0.000	52.972	1.000	397.676	101.900
LB	0.093	0.000	52.972	503.71	120.532
MLS	0.095	0.064	0.000	397.676	101.900
MG	0.118	0.109	0.068	0.000	555.042
LC	0.098	0.141	0.129	0.121	0.000

All pairwise F_{ST} values were significant at 0.01 ($P < 0.01$). The unit of geographic distance is km

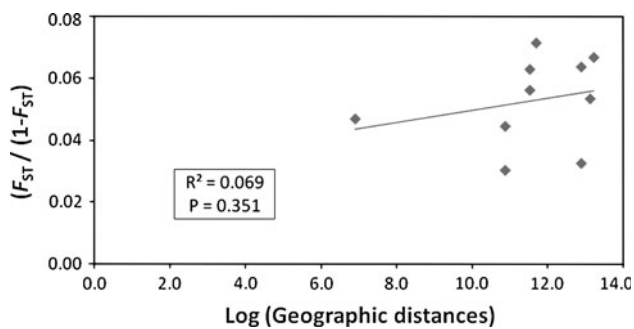


Fig. 2 Plot of geographical distance against genetic distance for five populations of *Kmeria septentrionalis*

effect of isolation-by-distance was detected, as the correlation between geographic and genetic distances (both transformed) was non-significant ($R^2 = 0.026$, $P = 0.221$), which was supported by the result of Mantel test (Fig. 2, $R^2 = 0.069$, $P = 0.351$). A re-analysis of Mantel test after removing the outlier, i.e. the population pair with closest geographical distance, generated a similar result ($R^2 = 0.078$, $P = 0.265$). Although the geographic distance between ML and MLS was the smallest of all population pairs (ca. 1.0 km), the genetic distance between these two populations was not the lowest.

Fine scale genetic structure

A total of 591 loci were used for the spatial autocorrelation analysis of population MLS. The range of geographic distances between individuals was 1.0–97.7 m. There was a positive genetic correlation between individuals, when compared with permuted r , in the smallest distance class of 9 m ($P < 0.05$), and a significant correlation also found in the distance class of 18 m ($P < 0.05$) (Fig. 3). Bootstrapping estimates of the 95% CI about r showed that r values for the distance class 9 and 18 m was greater than 0 ($P < 0.05$). There were no significant correlations for the other distance classes, indicating no genetic structure in these distance classes. The genetic patch size, defined as

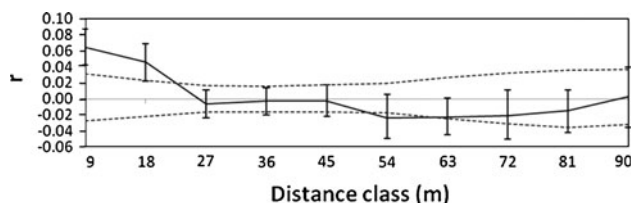


Fig. 3 The genetic correlation coefficient (r) for different distance class sizes. The 95% CI (dashed lines) about the null hypothesis of no spatial structure, and the 95% confidence error bars about r as determined by bootstrapping (with 999 simulations), for 591 amplified fragment length polymorphism (AFLP) markers scored in population MLS of *Kmeria septentrionalis*

where r first passes from significant to non-significant within the correlogram, was 22 m.

Discussion

Contrasting coarse and fine scale patterns of spatial genetic structure have been identified in relic populations of *K. septentrionalis*. Mantel test results showed no pattern of isolation-by-distance at a coarse scale spanning over 500 km, while fine scale spatial patterns of genetic variation within a single population suggested significant isolation-by-distance up of distance of 22 m. These contrasting patterns can largely be explained by the biology of the species, i.e. the limited capacity of dispersing pollen and seeds at a fine scale, and long isolated evolutionary history of *K. septentrionalis* at a coarse scale.

Kmeria septentrionalis is dioecious, and mainly pollinated by thrips (Pan 2007). Thrips (Thysanoptera) are tiny, short-lived insects with winged adults. Although thrips are capable of pollinating tree species such as *Shorea leprosula* (Nagamitsu et al. 2001) and a few other species in the Indomalayan region (Corlett 2004), they have weak flying ability and therefore, have a limited capacity to disperse pollen to distant plant individuals. For example, a single species of *Taeniothrips* was the only pollinator of an endangered herbaceous perennial, *Ophiopogon xylorrhizus* (Liliaceae) in the tropical forests of southwest China, where field observations, spatial genetic structure, and paternity analysis of seeds show that pollen is rarely transferred more than 20 m (He et al. 2000; He and Smouse 2002). Seed dispersal in populations of *K. septentrionalis* is also restricted. It has been suggested that birds could disperse the seeds of some magnolia species (Callaway 1994; Setsuko et al. 2004), however, field observations revealed that the seed bank density was greatest within 10 m of the maternal tree, indicating that the majority of seeds were dispersed passively by gravity (Pan 2007). Consequently, a limited dispersal capacity (via pollen and seed) contributes to the significant clustering of genetic variation within populations, with significant spatial genetic structure to a distance of 22 m, and no structure beyond.

The significant genetic differentiation between neighbouring populations separated by a distance of less than 1.5 km, suggesting genetic connectivity among populations is weak or non-existent. Such results could also imply that these relic populations have evolved separately for a long period of time. *Kmeria septentrionalis* is thought to be a relic species of the Tertiary period, and may have originated ca. 30 Myr ago in the middle Oligocene (Law 1984; Nie et al. 2008). The Southeast Asian region has undergone continuous and complicated geological changes due to the extrusion of Indian Plate during the past 30 Myr.

(Tapponnier et al. 1990). A dramatic change in climatic pattern in eastern Asia happened in 8–6 Myr ago due to rapid mountain folding during that period of time (Tapponnier et al. 1990; An et al. 1999). Many evidence have suggested Quaternary glaciations (ca. 2.5 Myr ago) had shaped the distribution of remnant plant species in South of China (Hwang et al. 2003; Gao et al. 2008). Such dramatic and continuous changes could have created a large number of plant species in isolated populations, including *K. septentrionalis*. These physically isolated populations have undergone evolution separately for a long period of time, and subject to genetic drift and selection. Geographical distance between populations had no detectable effect on genetic differentiation since genetic exchanges between populations were weak or non-existent. As a consequent, isolation-by-distance cannot be detected above a limited scale. Moreover, facultative apomixes have been observed in *K. septentrionalis*, which may be a mechanism adapted to isolation and separate evolution over long period of time (Zeng et al. 2003).

Conservation implications

Our results suggest that there is likely adequate variation and adaptive potential to maintain this species. Indeed, genetic diversity revealed in *K. septentrionalis* is not particularly low compared those plant species that were surveyed with AFLP markers (Nybom 2004). However, most populations of *K. septentrionalis* are of small size with rarely more than 300 individuals, partly because of illegal logging. Inbreeding could occur in small isolated populations and potentially decrease the genetic diversity (Frankham and Ralls 1998). Management practices must include, a complete and reinforced ban on logging for this species. For those populations that have declined due to logging, conservation efforts, such as, re-introducing seedlings germinated from seeds from the same population, could provide assistance for a better and quick population recovery. However, introducing genetic material from other populations for restoration should be avoided since each population is likely an evolutionarily significant unit, under separated evolution over a long period of time. Genetic material introduced from other populations could be mal-adapted, and outbreeding depression could occur (McKay et al. 2005).

Due to increasing habitat degradation and an intensifying conflict between alternative land uses, protection of *K. septentrionalis* in situ is inadequate. Looking ahead, ex situ collection for conservation, and establishing seed bank and DNA bank for *K. septentrionalis* should also be in the agenda. The result of significant genetic differentiation among populations and each populations existing as

independent evolutionarily significant unit indicate efforts of collection should be extend to every population, i.e. maximize the number of populations in the collection.

Despite their scientific, cultural and socio-economic importance, many species in the Magnoliaceae genus are considered threatened because of habitat decline and overexploitation. It is broadly recognised that more information is needed on the genetic diversity, conservation status, and the impact of climate change on Magnoliaceae species (Cicuzza et al. 2007). The findings of our work have significant implications for the conservation of *K. septentrionalis*, and other Magnoliaceae tree and plant species with similar distributions and evolutionary histories.

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