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Microsatellites Reveal Clonal Growth and Genetically Distinct Groups in *Cryptocarya chinensis* in Fragmented Lower Subtropical Forest, China

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Abstract

The lower subtropical monsoon evergreen broad-leaved forest in South China (about 22° ~ 24° N) has a high conservation status, because of its uniqueness and high biodiversity. During the last few decades, most of these forests have been destroyed, and the remaining are being degraded by fragmentation. However, genetic information concerning the effects of fragmentation is currently lacking for plant species in these forests. In this study, therefore, eight microsatellites were used to study six *Cryptocarya chinensis* fragmented populations in Guangdong Province South China, and the results revealed a complex pattern of genetic variation within and among *C. chinensis* populations. Firstly, genetic variations demonstrate hitherto undetected clonal growth in *C. chinensis*. Secondly, current population structure of *C. chinensis* reflects an interaction between ancient homogeneous level of genetic variation and con-

temporary bottleneck via fragmentation. Small populations maintain substantial genetic variation of the initial populations through clonal growth, and do not show genetic depauperation compared to larger populations. Finally, two genetically distinct groups (West and Middle-East groups) are found in this area, connected by highly mixed contact zone.

Key words: clonal growth, *Cryptocarya chinensis*, habitat fragmentation, microsatellite, genetically distinct groups, contact zone, genetic diversity.

Introduction

The fragmentation of continuous habitat into smaller patches is a world-wide phenomenon. Its effects have been discussed and studied comprehensively (HOBBS and YATES, 2003; HONNAY et al., 2005; REED, 2004). Fragmentation leads to restricted gene flow among populations, leading to loss of alleles, increased inbreeding and genetic drift within populations (HAMRICK and GODT, 1996; YOUNG et al., 1996), and eventually influence species long-term persistence (FRANKHAM, 2005). Sometimes, however, fragmented populations may experience normal or even enhanced gene flow and do not suffer from genetic erosion (YOUNG et al., 1996, references therein, WHITE et al., 2002). Clonal growth is another way that may mitigate the adverse effect of habitat frag-

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mentation by retarding the loss of genetic variability (HONNAY and BOSSUYT, 2005; HONNAY et al., 2005).

Lower subtropical monsoon evergreen broad-leaved forests are found in the tropical-subtropical transition zone in southern China (about 22° ~ 24° N), near the Tropic of Cancer. In contrast to similar latitudinal areas elsewhere, which are almost all dry or semidry lands, unique and luxuriant subtropical forests are well developed in southern China because of their proximity to the Pacific Ocean to the east and the Indian Ocean to the south, which bring abundant rain in spring and summer (DING et al., 2001).

Guangdong Province is the main distribution area of lower subtropical monsoon evergreen broad-leaved forests. Historically, however, there has been severe deforestation in Guangdong Province (GDSQZB, 1991). Instability of the society (like World War II and the following civil war) caused heavy deforestation in the Guangdong Province in the early 1900s (FORESTS OF GUANGDONG, 1990). By 1949, the forest cover in Guangdong Province was only 17.6%. It restored to 27.7% in 1985 gradually, and increased rapidly to 56.6% in 1998 but mainly due to man-made forest plantations. However, even during these years of forest expansion (1949–1998), in 1958, 1968 and 1978, there were three large-scale deforestations (cutting for woods) of natural forests in Guangdong Province (ZHOU and CHOKKALINGAM, 2004). At present, the natural forest cover is only 1.5% (XUE et al., 2005). Current overexploitation and the environmental deterioration that is accompanying economic development in this area have made the problem of destruction even worse. Today, lower subtropical monsoon evergreen broad-leaved forests have been reduced to a few isolated patches, which are currently protected because of their key role in the lower subtropical ecosystem (MA, 1999).

As a successional climax species, *Cryptocarya chinensis* (Hance) Hemsl. (Lauraceae) is the main component of typical monsoon evergreen broad-leaved forest in lower subtropical China (PENG, 1996; SUN, 1998; ZHENG, 2002). *C. chinensis* is a forest-dwelling species and occupies the second or third level of the tree layer. As a result of the destruction of the monsoon evergreen broad-leaved forest, it is now patchily distributed and an ideal system to study the influence of forest fragmentation on the population genetics of forest species. In fact, common species, alike to rare and endemic species, could also greatly suffer from habitat fragmentation (HOOFMAN et al., 2003; JUMP and PEÑUELAS, 2006).

Therefore, the main aim of the present study is to examine levels of genetic variation within and among six *C. chinensis* fragmented populations in lower subtropical China in order to determine whether any genetic effects of habitat fragmentation are detectable. Given the severe deforestation in this area, we might expect to detect evidence of severe bottlenecks within populations, as well as genetic differentiation among populations due to restricted gene flow resulting from forest fragmentation. In addition, we also report the genetic characteristic of clonal growth in *C. chinensis*. That is, strong negative F_{IS} , high occurrence of identical genotypes and nearly complete and significant multilocus

linkage disequilibrium for clonal species (BALLOUX et al., 2003; DE MEEÛS and BALLOUX, 2004; HALKETT et al., 2005).

Materials and Methods

Study species

To date, the biology and ecology of *C. chinensis* is poorly characterised. *C. chinensis* is a common evergreen tree, widely distributed in southern China, growing up to 20 meters high. It can live for 50 years and more (LIU S-Z, personal communication). *C. chinensis* is a half-shade tolerant species: high dense canopy will prevent its regeneration. Hermaphroditic flowers form panicle inflorescences and are pollinated by insects. Seeds of *C. chinensis* are dispersed by vertebrates (e.g. birds and small mammals) and gravity (WANG et al., 2003).

Samples collection

Six fragmented populations were selected for leaf sample collection between 2003 and 2004 from the lower subtropic, Guangdong Province, China (Fig. 1, Table 1). HSD, DWL, DHS, GT are natural reserves where *C. chinensis* is currently relatively well preserved. RP and GT are small patches near villages, newly cutting of *C. chinensis* are seen throughout these two sampling areas, indicating intensive human disturbance. There are only five and four large trees remaining in LG and RP populations, respectively. These six *C. chinensis* populations are considered to occur via natural regeneration rather than through human mediated planting.

Leaf samples were randomly collected with each at least 30 m apart and to cover the whole area in populations. Because LG and RP are small patches with area small than 1 ha, for these two populations, samples were collected without considering the distance between them.

Leaf samples were placed in sealed plastic bags containing silica gel until DNA extraction. Eight microsatellite loci (GenBank accession number: DQ679889-DQ679896) were analysed using the amplification procedures previously published by WANG et al. (2007). Individuals with missing data due to amplification failure were excluded for subsequent “genet-level” analyses.

Data analysis

All data format conversions were aided with software CONVERT 1.3 (GLAUBITZ, 2004).

Clonal growth

Wright's F -statistics (F_{IS} , F_{IT} and F_{ST}) (WEIR and COCKERHAM, 1984) (with and without multilocus genotype repeats) were calculated and tested for significance using SPAGeDi 1.2e (HARDY and VEKEMANS, 2002). Linkage disequilibrium was estimated using the correlation coefficient (R_{GDD}) and multilocus linkage disequilibrium (r_D) as DE MEEÛS and BALLOUX (2004) suggested. R_{GDD} was calculated using LINKDOS program incorporated in GENEPOP 3.3 (RAYMOND and ROUSSET, 1995), r_D was calculated with MULTILOCUS 1.3b (AGAPOW and BURT, 2001). Genotypic diversity (the occurrence of identical genotypes) was estimated with G/N , where G was num-

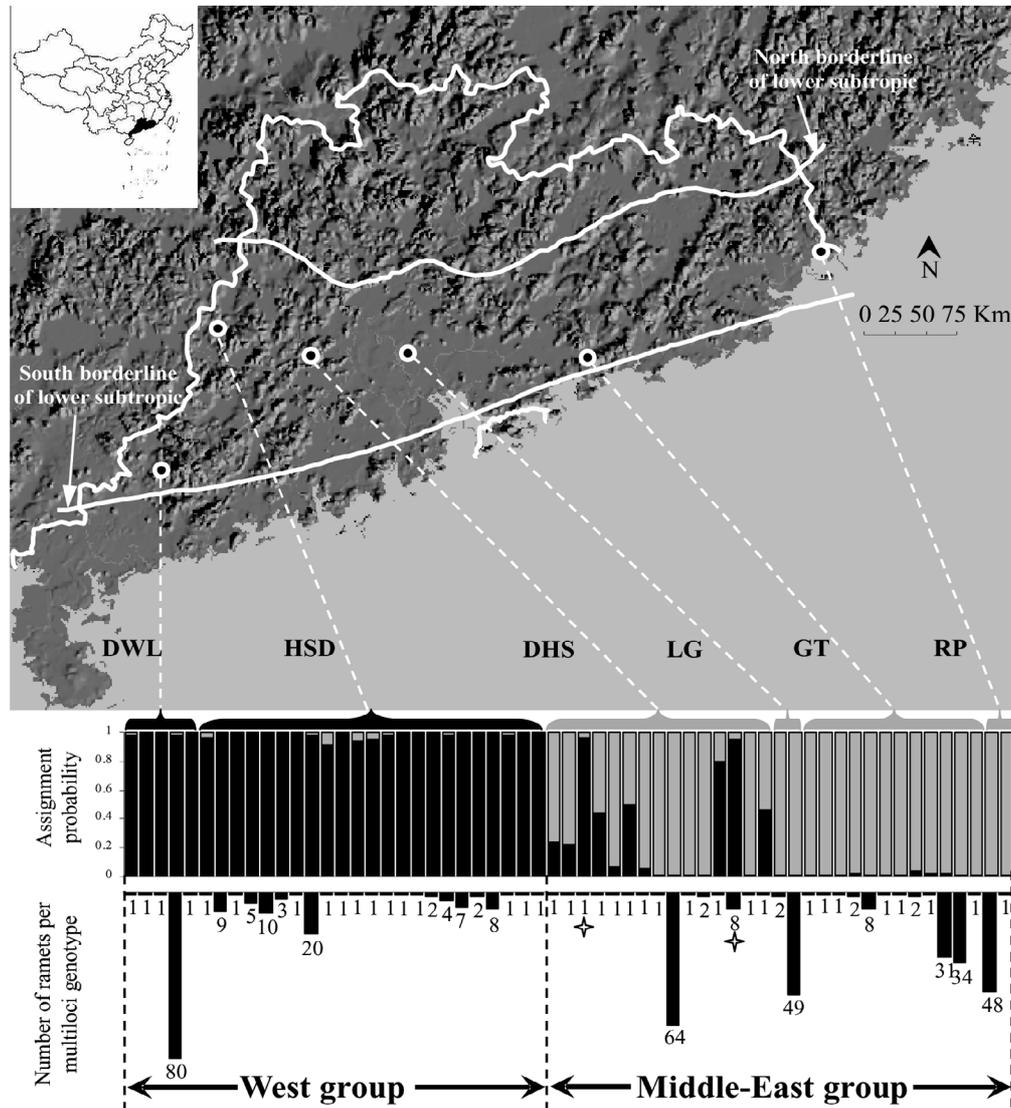


Figure 1. – Sampling location of six *Cryptocarya chinensis* populations in Guangdong Province China, combining with the results of STRUcTURE analysis using 59 multilocus genotypes. Lower subtropical boundaries are according to GDSQZB (1991) and abbreviations of sites are as described in Table 1. STRUcTURE analysis recovers two groups ($K = 2$, West and Middle-East groups). The bars directly below sampling maps represent the assignment probability that each multilocus genotype belongs to a particular group (illustrated by black and grey colours). The bar under each “assignment probability” bar indicates the number of ramets for each multiloci genotype. Two “mis-assigned” multilocus genotype to West group in DHS population are indicated by stars.

ber of observed multilocus genotypes, N was number of sample size.

Genetic variation and structure

In order to provide a full and unbiased characterization of the genetic variation and clonal structure of a species the data should be analysed using all ramets (with multiloci genotype repeats) as well as all genets (without multiloci genotype repeats) (DE MEEÛS et al., 2006; IVEY and RICHARDS, 2001; SUZUKI et al., 2004). Therefore, we used both data sets to conduct the subsequent analyses. If the calculation with and without multiloci genotype repeats gave similar results we only reported the latter unless otherwise stated. Within the text, genetic diversity parameters subscripted with an “s”

indicate species values, while those subscripted with a “p” indicated population means.

Genetic diversity

Genetic diversity was described by observed heterozygosity (H_o), the unbiased expected heterozygosity (H_e , NEI, 1978). H_o and H_e were calculated using GDA 1.1 (LEWIS and ZAYKIN, 2001).

Genetic differentiation

Pairwise F_{ST} values between populations using data set with multiloci genotype repeats were calculated according to WEIR and COCKERHAM (1984) and tested for significance using SPAGeDi 1.2e (HARDY and VEKEMANS, 2002).

Table 1. – Description of populations studied for *Cryptocarya chinensis* including locations and their descriptions, population sizes, altitude we collected samples, sample sizes (*N*), number of multiloci genotypes (*G*), genotypic diversity (*G/N*).

Population	Name	Latitude and longitude	Site description (established year)	Area (ha)	Population size	Altitude (m)	<i>N</i> ^a	<i>G</i> ^b	<i>G/N</i> ^c
HeiShiDing	HSD	23°27'N, 111°53' E	Guangdong Natural Reserve (1979)	4200	>5000	300~600	86	23	0.277
DaWuLing	DWL	22°15'N, 111°10'E	Guangdong Natural Reserve (1996)	3534	>2000	600~800	88	5	0.060
DingHuShan	DHS	23°08'N, 112°35'E	National Natural Reserve (1956)	1133	0.13 individuals/m ² (WANG et al. 2003)	100~400	89	15	0.174
LuoGang	LG	23°08'N, 113°19'E	Small patch near village	<1	≈300 (WANG et al. 2005)	50	49	2	0.041
GuTian	GT	23°06'N, 114°47'E	Guangdong Natural Reserve (1984)	2189	>5000	300~500	87	12	0.143
RaoPing	RP	23°49'N, 117°00'E	Small patch near village	<1	<200 (WZF, personal observation)	100	49	2	0.041
								Total	0.136

^a We do not differ “ramet” and “genet” in field.

^b Individuals with missing data are excluded.

^c Individuals with missing data are excluded.

Bottleneck test

Wilcoxon test for heterozygote excess under Infinite Allele Model (IAM) were performed to exam population bottleneck using BOTTLENECK 1.2.02 (CORNUET and LUIKART, 1996; LUIKART et al., 1998). Interrupted or compound microsatellites in our analysis (WANG et al., 2007) were likely to fit the IAM better (CORNUET and LUIKART, 1996).

Substructure test

To test if the six populations represented genetically distinct groups (*K*), a Bayesian cluster analysis was performed using the program STRUCTURE 2.1 (FALUSH et al., 2003; PRITCHARD et al., 2000). Using a model assuming admixture of source populations and correlated allele frequencies between groups, twenty independent

runs were performed for each *K* (from 1 to 6) for 10⁶ iterations after a burn-in period of 10⁶ on total multiloci genotypes without prior concerning of their origin populations. The choice of the appropriate *K* value was conducted as recommended in STRUCTURE user’s manual. Preferred *K* was also examined by Δ*K* (EVANNO et al., 2005). In most case, the maximal value of Δ*K* might indicate the correct estimation of *K*.

Results

Clonal growth

Estimates of *F*_{IS} indicated a general excess of heterozygotes, seven of eight loci showed highly significant negative of *F*_{IS} (Table 2). There was a significant excess of heterozygotes with a mean *F*_{IS} over all loci of –0.596.

Table 2. – Summary of *F*-statistics at eight microsatellite loci with and without multiloci genotype repeats for *Cryptocarya chinensis*.

Loci	With multiloci genotype repeats			Without multiloci genotype repeats		
	<i>F</i> _{IT}	<i>F</i> _{IS}	<i>F</i> _{ST}	<i>F</i> _{IT}	<i>F</i> _{IS}	<i>F</i> _{ST}
Cch01	-0.191**	-0.626**	0.268**	-0.055	-0.239**	0.148**
Cch02	-0.214**	-0.763**	0.311**	-0.184**	-0.364**	0.132**
Cch03	0.524**	-0.190**	0.600**	0.420**	0.257*	0.220**
Cch04	-0.054	-0.579**	0.333**	-0.096	-0.250*	0.124**
Cch05	-0.257**	-0.754**	0.283**	-0.159*	-0.413**	0.180**
Cch06	0.913**	0.743**	0.660**	0.753**	0.653**	0.289**
Cch07	-0.178**	-0.687**	0.302**	-0.087	-0.252**	0.132**
Cch08	-0.531**	-0.902**	0.195**	-0.480**	-0.772**	0.165**
Overall loci	-0.016	-0.596**	0.364**	0.006	-0.201**	0.172**

* *P* < 0.05, ** *P* < 0.01 (after 5000 permutations).

76% of the pairwise correlation coefficient (R_{GGD}) were significantly greater than zero after Bonferroni correction ($\alpha < 0.05$), 91% of the multilocus linkage disequilibrium values (r_D) between loci were positive (most of them greater than 0.5) and significant after Bonferroni correction ($\alpha < 0.05$), indicating high LD between the loci.

Among 448 individuals that were genotyped, a total of 59 different multiloci genotypes could be identified, and no population shared the same multiloci genotypes (Fig. 1). There were only two multiloci genotypes for the LG and RP populations, and one or two multiloci genotypes dominated the other four populations. Overall genotypic diversity (G/N) was 0.136.

Genetic variation and structure

Genetic diversity

All estimates of genetic diversity for HSD population were highest compared to other populations. Two small populations, LG and RP, did not show decreased genetic diversity. RP population displayed lowest genetic diver-

sity in H_E with multiloci genotype repeats. On the contrary, LG population showed moderate genetic diversity in general (Table 3).

Genetic differentiation

Significantly higher genetic differentiation was found between West and Middle-East populations compared to the differentiation within West or Middle-East populations (Table 4).

Bottleneck test

Wilcoxon test for population bottleneck revealed significant bottleneck in all tested populations ($P < 0.05$) under IAM (Table 3).

Substructure test

Mean Log-likelihood increased greatly from $K=1$ to $K=2$, and began to plateau until $K=4$ (Fig. 2). According to Pritchard and Wen (2004)'s "more-or-less plateaus" statement, $K=2$ was then most probable. ΔK was highest at $K=2$, confirming that two groups was

Table 3. – Observed heterozygosity (H_O), Expected heterozygosity (H_E) and BOTTLENECK test for *Cryptocarya chinensis*, and comparing with other *Cryptocarya* species and species having similar life-history traits. Numbers in grey shading indicate the highest, boxed the lowest of H_O or H_E in populations. LG and RP populations were excluded from H_O and H_E calculation and BOTTLENECK test due to their multiloci genotypes as low as two. Abbreviations of sites are as described in Table 1.

Population	H_O	H_E	F_{ST}	BOTTLENECK Test (P value) ^a
Data with multiloci genotype repeats				
HSD	0.800	0.582		0.0039
DWL	0.747	0.388		0.0117
DHS	0.649	0.455		0.0273
LG	0.745	0.399		0.0078
GT	0.715	0.499		0.0039
RP	0.750	0.380		0.0078
Overall	0.732(H_{os}) 0.734(H_{op})	0.675(H_{es}) 0.450(H_{ep})	0.364	
Data without multiloci genotype repeats				
HSD	0.777	0.626		0.00195
DWL	0.700	0.508		0.00781
DHS	0.658	0.608		0.00977
LG	—	—		—
GT	0.667	0.584		0.00195
RP	—	—		—
Overall	0.714(H_{os}) 0.707(H_{op})	0.687(H_{es}) 0.582(H_{ep})	0.172	
<i>Cryptocarya aschersoniana</i> ^b	0.324(H_{op})	0.365(H_{ep})	0.320(G_{ST}) ^c	
<i>Cryptocarya moschata</i> ^d	0.344(H_{os})	0.531(H_{es})	0.285	
Data from SSR ^e				
Long-lived perennial	0.63	0.68	0.19	
Regional mixed mating ^f	0.65	0.65	0.28	
Late successional status	0.51	0.60	0.26	
	0.66	0.70	0.17	

^a after 10000 permutations.

^b MORAES and DERBYSHIRE (2003).

^c G_{ST} , analogy to F_{ST} .

^d MORAES and DERBYSHIRE (2004).

^e Genetic diversity (H_O and H_E) were population means (NYBOM, 2004).

^f Mixed mating system indicated by other *Cryptocarya* species (MORAES and DERBYSHIRE, 2002).

Table 4. – Pairwise F_{ST} for six *Cryptocarya chinensis* populations. Populations are ordered in the table according to the groups (West and Middle-East groups) they belong to. Abbreviations of sites are as described in Table 1.

	West group population			Middle-East group population			
		HSD	DWL	DHS	LG	GT	RP
West group	HSD	—					
population	DWL	0.2572**	—				
Middle-East	DHS	0.3493**	0.4798**	—			
	group	LG	0.4097**	0.5228**	0.3337**	—	
	population	GT	0.2791**	0.4215**	0.2103**	0.3137**	—
		RP	0.4150**	0.4939**	0.3219**	0.3824**	0.2144**

** $P < 0.01$ (after 5000 permutations).

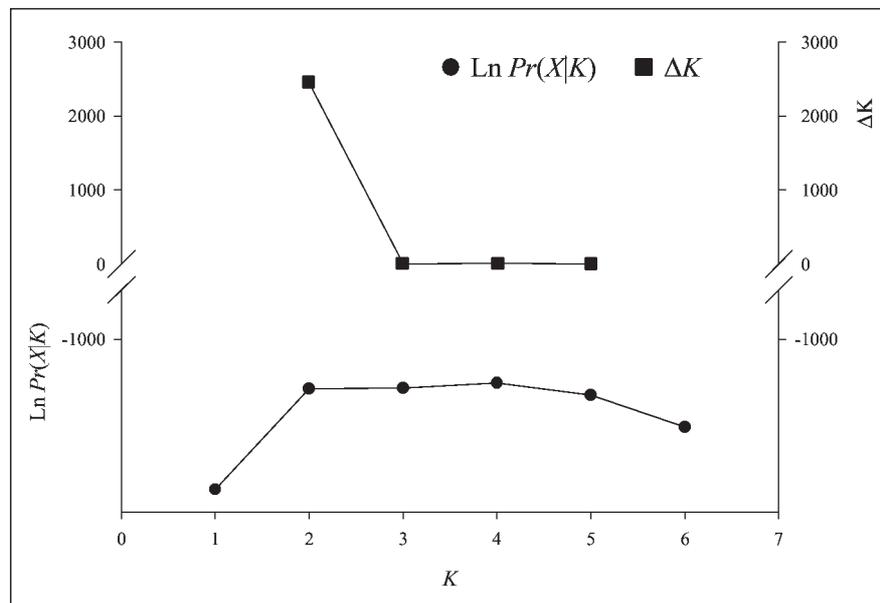


Figure 2. – Magnitude of $\text{Ln Pr}(X|K)$ and ΔK to infer the genetic groups (K) in STRUCTURE analysis. $\text{Ln Pr}(X|K)$ refers to the mean log likelihood estimate using 20 independent runs for each K value. ΔK is calculated according to EVANNO et al. (2005).

the correct estimation (Fig. 2). Two groups also had a clear geographical correspondence, as one group contained HSD and DWL populations (West group) and the other group contained DHS, LG, GT and RP (Middle-East group).

Twenty STRUCTURE runs at $K=2$ produced nearly identical individual assignment probability, we just chose the one with the highest log likelihood at $K=2$ to graph the individual assignment probability (Fig. 1). In DHS population, two individuals (indicated with stars in Fig. 1) had a relatively high probability (assignment probability > 0.95) to be migrants or offspring of migrants from the West group, and some individuals showed a fairly high degree of admixture. For the other five populations, however, almost all individuals showed that they were likely to descend from one of “pure” West or Middle-East ancestry.

Discussion

Unexpected clonal growth

Strong negative F_{IS} estimates (meaning excess of heterozygote, Table 2), high occurrence of identical genotypes (G/N values, Table 1) and nearly complete and significant multilocus linkage disequilibrium suggest that current growth mode of *Cryptocarya chinensis* is predominantly clonal (BALLOUX et al., 2003; DE MEEÛS and BALLOUX, 2004; HALKETT et al., 2005). Our data also fit very well with DE MEEÛS and BALLOUX (2005) strictly clonal species criterion, that was $F_{IT}=0$ and $F_{ST} = -F_{IS}/(1-F_{IS})$. Our F_{IT} is -0.01572 , not significantly different from zero (Table 2). The result of $-F_{IS}/(1-F_{IS})$ is 0.3734, very close to the value of F_{ST} (0.3635).

In addition, when the analysis was based on a single representative of each multiloci genotype, the extensive

linkage disequilibrium between loci almost disappeared (decreasing to 8% for R_{GGD} , 9% for r_D after Bonferroni correction), indicating such LD was also mainly caused by multicopy of each multiloci genotype (clonal growth).

Our overall G/N value of *C. chinensis* is 0.136, lower than the previously reported value for clonal species (0.17, 21 studies; 0.27, 47 studies, data from TSYUSKO et al., 2005). However, these values were obtained by allozyme markers, which might resolve lower diversity than DNA-based markers (ARNAUD-HAOND et al., 2005), our G/N value should be even lower when using the similar allozyme markers, and confirmed extensive clonal growth in *C. chinensis*.

However, highly clonal growth in *C. chinensis* has not previously been detected, and it was always considered sexual growth as way of regeneration (Peng, 1996; WANG et al., 2003). Moreover, the other *Cryptocarya* species studies also showed no such phenomenon.

Using allozyme, MORAES and DERBYSHIRE (2002; 2003; 2004) studied two other *Cryptocarya* species' genetic diversities in Brazil, and did not find an excess of heterozygotes as in *C. chinensis*. Recently, WELLS et al. (2007) used eight microsatellites to study 623 seedlings and 99 trees of *Cryptocarya mackinnoniana*, and found no excess of heterozygote within and linkage disequilibrium between loci.

Genetic variation and structure

1. Populations suffer from bottleneck

Compared to "genet-level" genetic variation, clonality does not influence the overall genetic diversity (H_O and H_E) greatly, but causes a two fold increase in genetic differentiation (Table 3). When multiloci genotype repeats are removed, the single "genet" can be reckoned to derive from sexual reproduction (SMITH et al., 1993; VAUGHAN et al., 2007; WALSER et al., 2004), and its genetic variation can reflect the sexual reproductive status in species. Thus, by comparing with the other similar life-history species, the "genet-level" genetic diversity ($H_E = 0.582$) and differentiation ($F_{ST} = 0.172$) of *C. chinensis* are comparable to or lower than those in other studies ("data from SSR", Table 3).

However, due to severe deforestation history in this area and significant bottleneck, we may expect to find much higher genetic differentiation at genet level, if not much lower genetic diversity. Genetic diversity estimated with H_E was less sensitive to bottleneck (SPENCER et al., 2000), and could be influenced by self-incompatible system etc. In Brazil, MORAES and DERBYSHIRE (2003) found forest fragmentation and bottleneck effects caused high genetic differentiation ($G_{ST} = 0.32$) among population in *Cryptocarya aschersoniana*.

One reasonable interpretation is that bottleneck are truly severe, but occurred recently. The fragmented populations still harbor the previous genetic homogeneous variation. Moreover, extensive clonal growth and subsequent long generation time actually help to keep these ancient genetic variations for long time, that is why for small populations (LG and RP) we do not find decreased genetic variation compared to larger populations (Table 3).

2. Two distinct groups and contact zone

Two distinct groups are clearly demonstrated by pairwise F_{ST} (Table 4), substructure test (using STRUCTURE, Fig. 1). Such separation cannot be caused by naturally occurring barriers, or local selection, but is more likely to be due to independent establishment events.

Although there are mountain ranges (above 1,000 meter in elevation) to separate West and Middle-East populations, GT and RP populations (belong to Middle-East group) are also divided by one 1,000-meter-high mountain range (Fig. 1). Therefore, it is hard to say that the ones between West and Middle-East groups serve as barrier to gene flow, but not the other one. In addition, pairwise F_{ST} show that the differentiation between HSD and DWL ($F_{ST} = 0.2572$) is relatively lower than that between HSD and DHS ($F_{ST} = 0.3493$) (Table 4), although the geographic distance between the former two is twice as far as the later two.

Local selection can be unlikely. Firstly, our sampled populations are located in the same climate zone. Secondly, at least two mis-assigned DHS multiloci genotypes (Fig. 1) that are believed to represent migration from the other group have been found, one of them even has the second highest number of duplicate ramets of the same multiloci genotype (8 repeats). If local selection happens, the "migration" may not spread its "ramets" so successfully considering our fully random sampling.

Therefore, the formation of two distinct groups in *C. chinensis* is more likely caused by history independent establishment events. It can also be true, that flora of west lower subtropic is mainly influenced by tropic flora, while the Middle-east flora is mainly influenced by middle subtropic (GDSQZB, 1991). DHS, due to its special geographic location, plays as contact zone to connect the two groups in *C. chinensis*. In addition, according to the results of STRUCTURE, DHS is the only place to find the individuals of admixture because it is geographically close to West group. However, why and when did the westwards or eastwards gene flow cease at the point of DHS? Because of our limited studied populations, we cannot answer this question at present study, and we could also miss some important populations (such as including other relevant westwards or eastwards gene flow) which can provide more information to resolve this problem.

Conclusion

Historical deforestation in lower subtropic China caused severe bottleneck and highly fragmentation even for common species, like *C. chinensis*, which might also trigger its extensive clonal growth. Although the linkage between fragmentation and clonal growth need further confirmation, the clonal growth actually helps to keep the genetic diversity within and among *C. chinensis* populations. However, even *C. chinensis* is not threatened currently with danger, the consequences of the long term clonal growth may increase the risk of its sexual extinction (HONNAY et al., 2005; HONNAY and BOSSUYT, 2005). Therefore, it is still important to maintain the existing levels of genetic diversity to prevent future genetic risks. The distinguished two groups and contact

zone clear demonstrate forest fragmentation may restrict the recently eastwards gene flow because Middle-East area of lower subtropic China has remained free of geographic barriers (*Fig. 1*), though west mountain range could obstacle the westwards gene flow. However, more extensive geographical samplings are required in South China (not just lower subtropic) to make clear the marked separation of West and Middle-East groups, understanding the exact nature in *C. chinensis*.

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