

# Genetic variation in the endangered Rutaceae species *Citrus hongheensis* based on ISSR fingerprinting

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**Abstract** *Citrus hongheensis* is a critically endangered species endemic to the Honghe river region in southeastern Yunnan, China. Its genetic diversity and differentiation were investigated using Inter-Simple Sequence Repeat (ISSR) markers. One hundred primers were screened, and a total of 245 loci were amplified from seven natural populations by 13 informative and reliable primers. Of these 245 ISSR loci, 233 were polymorphic and the detected variations revealed a relatively high level of intraspecific genetic diversity. At the population level, the mean percentage of polymorphic loci (*PPB*) was 36.50%, while the average expected heterozygosity (*He*) and Shannon diversity index (*Ho*) were 0.1327 and 0.1972, respectively. At the species level (across all populations), *PPB* was 95.10%, while *He* and *Ho* were 0.3520 and 0.5195, respectively. A high *Gst* value (0.6247) indicated that there is significant differentiation among populations, which was confirmed by AMOVA analysis ( $\Phi_{st} = 0.6420$ ). Pairwise genetic identity (*I*) values among populations ranged from 0.6341 to 0.7675, with a mean of 0.7008. We propose that the

high level of genetic differentiation may be the result of habitat fragmentation and limited gene flow ( $Nm = 0.1502$ ). For effective *in situ* conservation and population restoration of *C. hongheensis* it will be important to maintain historical processes, including high outbreeding rates, sufficient gene flow, and large effective population sizes.

**Keywords** *Citrus hongheensis* · Conservation · Differentiation · Genetic diversity · ISSR fingerprinting · Rutaceae

## Introduction

*Citrus hongheensis* Ye et al. is an endemic species of the hot, dry Honghe river watershed in southeastern Yunnan, China (Sheng et al. 1997; Ye et al. 1976). It is a long-lived tree that reaches heights up to 10 m. It has been considered as a primary lineage of the genus *Citrus* (Sheng et al. 1997) based on its morphological characteristics; for example, its petioles are 2–3 times longer than its leaves, and it produces racemes with 5–9 large flowers. It flowers from March to April, producing large white flowers, with 4 or 5 petals, that are approximately 3–3.5 cm in diameter. Fruits have a thick pericarp and mature in October (Huang 1997); the seeds usually lose their vitality after the fruit becomes bletted.

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Long before *C. hongheensis* was systematically described, it was widely cultivated by the local people of Hani. Young leaves are used as flavoring, while fruits are used as a substitute for another Chinese herbal medicine ‘Zhike’ (fruit from *Poncirus trifoliata* (L.) Raf.). The trees have religious significance and are thought to ward off evil. Although local people cultivated *C. hongheensis* around their villages and houses, its origin and initial distribution are still unknown. Some researchers consider *C. hongheensis* to be extinct in the wild, because known populations only occur around villages. According to our investigations, there are approximately 10 remaining *C. hongheensis* populations in the Honghe river watershed, all with less than 50 individuals. It is unknown whether the extant populations of *C. hongheensis* are cultivated or relic populations. Further, if the extant populations are cultivated, it is unclear if their utilization by local people plays a role in conserving or damaging its genetic diversity. Most individuals in the Honghe river watershed are mature, reflecting both the difficulty of reproduction and the general decline of this species. *C. hongheensis* has been listed as ‘Second Grade’ in the Red-Data Book of China.

There are two aspects of the consequences of habitat fragmentation on plant populations that are particularly important for conservation: a demographic aspect and a genetic aspect (Lande 1988; Schemske et al. 1994). Habitat fragmentation causes genetic erosion as a result of founder effects, and subsequent genetic drift and inbreeding (Barrett and Kohn 1991; Ellstrand and Elam 1993; Young et al. 1996). The genetic erosion induced by fragmentation may inhibit population persistence, because reduced heterozygosity may lead to decreased individual fitness (Rajimann et al. 1994; Oostermeijer et al. 1995) and the loss of allelic richness may reduce opportunities for future adaptation (Frankel et al. 1995). The forest in the Honghe river watershed has been almost completely destroyed and the extant *C. hongheensis* populations are isolated by farmland. However, neither the patterns of genetic diversity and structuring of *C. hongheensis* nor the consequences of past habitat fragmentation on its population structure have been previously examined.

Understanding the genetic variation within and among populations is essential for the establishment of effective and efficient conservation practices for rare plants. The genetic diversity and structure of

plant populations reflect the interaction of many factors, including the long-term evolutionary history of the species (e.g. shifts in distribution patterns, habitat fragmentation, and/or population isolation), mutation, genetic drift, mating system, gene flow, and selection (Slatkin 1987; Schaal et al. 1998). All of these factors can lead to complex genetic structuring within populations, and losses of genetic diversity, with severe potential consequences since genetic variation at the intraspecific level is a prerequisite for future adaptive change or evolution (Schaal et al. 1991). Thus, understanding the genetic variation within and among populations is essential for the establishment of effective and efficient conservation programs for rare plants (Fritsch and Rieseberg 1996). Several key aspects, such as the loss of genetic diversity and the restoration of threatened populations, can only be addressed by detailed population genetic studies (Hamrick and Godt 1996). Hence, the study of *C. hongheensis* presented here was undertaken to address the following questions using ISSR molecular markers:

- (1) What is the level of ISSR variation in *C. hongheensis*?
- (2) What is the degree of inter-population differentiation in this species?
- (3) Are the extant populations of *C. hongheensis* relic or cultivated populations?
- (4) How can population genetic information be applied to develop an effective and efficient conservation program for this species?

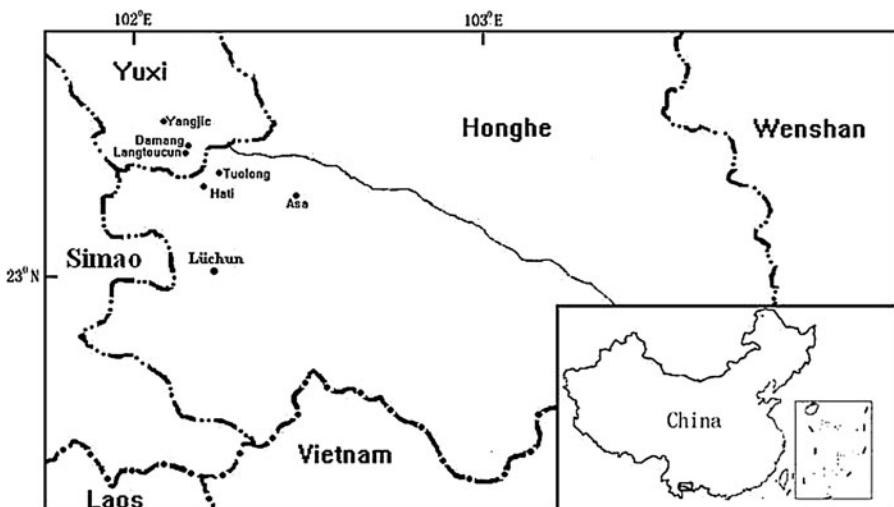
The answers to these questions have important implications for the design, effectiveness and efficiency of any program intended to conserve this species.

## Materials and methods

### Sample collection

Leaf samples were collected from 138 individual *Citrus hongheensis* trees (20 randomly selected from each of six populations, and 18 from another population) in the Honghe river watershed (Fig. 1; Table 1). The samples were dried with silica gel and stored at room temperature prior to DNA extraction.’

**Fig. 1** Map showing the locations of the sampled *Citrus hongheensis* populations



**Table 1** Information on the seven populations sampled for ISSR analysis

Code	Population	Sample size	Latitude (N)	Longitude (E)
A	Asa	20	23°25'	102°42'
D	Damang	20	23°39'	102°11'
H	Hati	20	23°30'	102°15'
L	Langtoucun	20	23°43'	102°11'
T	Tuolong	20	23°34'	102°19'
Y	Yangjie	20	23°41'	102°04'
LC	Lüchun	18	23°03'	102°18'

#### DNA extraction and PCR amplification

Total genomic DNA was extracted from dried leaves using a modified CTAB method (see Doyle 1991). One hundred ISSR primers were obtained from the University of British Columbia (UBC Primer Set #9, Biotechnology Laboratory) and were tested for their ability to amplify loci by PCR. Thirteen primers (UBC # 810, 813, 842, 844, 846, 847, 848, 855, 859, 867, 868, 880 and 881), which generated clear reproducible banding patterns, were chosen for the final analysis. Reactions were carried out in total reaction volumes of 20 µl consisting of 1.0 µl 10 ng/µl template DNA, 2.0 µl 10× PCR buffer (Mg + Free), 2.0 µl 25 mM MgCl<sub>2</sub>, 1.2 µl 2.5 mM dNTPs, 0.4 µl high purity grade (>99.0%) formamide, 0.4 µl 10 nM primer, 1.0 unit of *Taq* polymerase and double-distilled water. Polymerase Chain Reactions (PCR)

were performed using a GeneAmp® PCR System 9700 with the following cycle profile: initial denaturation at 94°C for 5 min, followed by 45 s at 94°C, 1 min annealing at 52–54°C, and a 2 min extension step at 72°C for 36 cycles, then a final extension step for 7 min at 72°C. Amplification products were electrophoretically separated in 1.5% agarose gels buffered with ×0.5 TBE. A 100 bp DNA ladder (New England Biolabs, Beverly, MA, USA) was used as a size marker. A negative control, in which the template DNA was replaced by water, was included to verify that there was no contamination. DNA fragments were identified by LabWorks Software Version 3.0 image analysis software for gel documentation (UVP, Upland, CA, USA) following staining with ethidium bromide (EB). Only those bands that showed consistent amplification were considered. Smeared and weak bands were excluded. Only bands that showed consistent amplification patterns, and could be unambiguously scored across all the population samples, were used for subsequent analysis (see below); smeared and weak bands were excluded.

#### Data analysis

ISSR profiles were scored for each individual as the presence (1) or absence (0) of specific bands. A set of measures of intra- and inter-population genetic statistics were then calculated by the program POPGENE 1.31 (Yeh et al. 1999) including: Nei's (1973)

gene diversity, the percentage of polymorphic loci ( $PPB$ ), expected heterozygosity ( $He$ ), total genetic diversity ( $Ht$ ), intra-population genetic diversity ( $Hs$ ) and the relative magnitude of genetic differentiation among populations ( $Gst = Ht - Hs/Ht$ ). Gene flow was estimated using the formula:  $Nm = (1 - Gst)/4Gst$  (Slatkin and Barton 1989). Nei's (1972) genetic identity ( $I$ ) and genetic distance ( $D$ ) were calculated for all pairwise population combinations. The POPGENE software package was also used to calculate Shannon's index of phenotypic diversity for ISSR diploid data according to  $Ho = -\sum p_i \log_2 p_i$  (Lewinton 1972), in which  $p_i$  is the frequency of a given ISSR fragment.  $Ho$  was calculated at two levels: the average diversity within populations ( $Hpop$ ) and the total diversity across all populations ( $Hsp$ ). The proportion of diversity distributed among populations was estimated as  $(Hsp - Hpop)/Hsp$ .

Components of variance partitioned within and among populations were also estimated using AMOVA. Input data files for the AMOVA v. 1.55 program (Excoffier et al. 1992) were generated by AMOVA-PREP (Miller 1998). The number of permutations for significance testing was set at 1000. AMOVA variance components were used as estimates of the genetic diversity partitioning within and among populations.

In order to test for a correlation between genetic distances ( $D$ ) and geographical distances (in km) between populations, a UPGMA (unweighted pair-group method using arithmetic averages) dendrogram was constructed based on the matrix of Nei's genetic distances using MEGA 4.0 software (Tamura et al. 2007).

## Results

### Genetic diversity

A total of 245 ISSR bands were scored, corresponding to an average of 18.85 bands per primer. Among the 245 loci, 233 were polymorphic at the species level. The percentage of polymorphic loci ( $PPB$ ) was 95.10% for all seven populations. Assuming Hardy–Weinberg equilibrium, the average gene diversity was estimated to be  $0.3520 \pm 0.1505$  within populations ( $He$ ), and  $0.3535 \pm 0.0222$  at the species level ( $Ht$ ). The average Shannon indices ( $Ho$ ) were  $0.5195 \pm 0.1954$  at the population level ( $Hpop$ ) and

$0.1972 \pm 0.0283$  at the species level ( $Hsp$ ). Among the seven populations, the Lüchun population contained the highest level of genetic diversity ( $He = 0.1683$ ;  $Ho = 0.2528$ ), while the Tuolong population exhibited the lowest level ( $He = 0.1097$ ;  $Ho = 0.1616$ ) (Table 2).

### Genetic differentiation

There was significant differentiation among the *C. hongheensis* populations. The coefficient of overall genetic differentiation among populations ( $Gst$ ) was 0.6247, which was estimated by partitioning the total gene diversity. The Shannon's diversity index analysis ( $Ho$ ) indicated that 51.95% of the total variation was among-populations. Pairwise inter-population genetic identity values ( $I$ ) ranged from 0.6341 to 0.7675 with a mean of 0.7008. Genetic distances ( $D$ ) between populations ranged from 0.2646 to 0.4555, with a mean of 0.3601 (Table 3). The level of gene flow ( $Nm$ ) was estimated to be 0.1502 individuals per generation among populations. According to the AMOVA analysis, there was also significant genetic differentiation ( $P < 0.001$ ) among the seven *C. hongheensis* populations. Of the total genetic diversity, 64.18% was partitioned among populations and 35.82% within the populations (Table 4).

### Cluster analysis

The UPGMA dendrogram obtained from the 245 ISSR markers scored for the 138 individuals showed seven well-defined groups, corresponding to the

**Table 2** Genetic variability within populations of *Citrus hongheensis* shown by ISSR markers

Population	$P$ (%)	$He$	$Ho$
A	37.55	0.1307 (0.1867)	0.1961 (0.2705)
D	36.73	0.1391 (0.1929)	0.2061 (0.2800)
H	31.43	0.1236 (0.1945)	0.1806 (0.2784)
L	37.55	0.1303 (0.1892)	0.1948 (0.2725)
T	28.57	0.1097 (0.1840)	0.1616 (0.2652)
Y	34.69	0.1269 (0.1885)	0.1886 (0.2728)
LC	48.98	0.1683 (0.1966)	0.2528 (0.2826)
Mean	36.50 (6.4534)	0.1327 (0.0181)	0.1972 (0.0283)

$P$  percentage of polymorphic loci,  $He$  expected heterozygosity,  $Ho$  Shannon's diversity index

Standard deviations are in parentheses

**Table 3** Nei's (1972) original measures of genetic identity (above diagonal) and genetic distance (below diagonal) of *Citrus hongheensis*

Population	A	D	H	L	T	Y
A	—	0.7609	0.7187	0.6652	0.7041	0.6758
D	0.2733	—	0.7342	0.7146	0.7371	0.7396
H	0.3303	0.3090	—	0.7473	0.7056	0.7186
L	0.4077	0.3361	0.2912	—	0.7168	0.7675
T	0.3508	0.3050	0.3487	0.3329	—	0.7250
Y	0.3918	0.3016	0.3305	0.2646	0.3215	—
LC	0.4373	0.4555	0.4290	0.4420	0.4159	0.3678

**Table 4** Results of AMOVA analysis of six *Citrus hongheensis* populations

Source of variation	df	Sum of squares	Mean squares	Variance component	Total variance (%)	P-value
Among populations	6	3716.5486	619.425	30.56	64.18	<0.001
Within population	131	2234.0167	17.054	17.05	35.82	<0.001

seven populations (Fig. 2), confirming that significant genetic differentiation has occurred among populations.

## Discussion

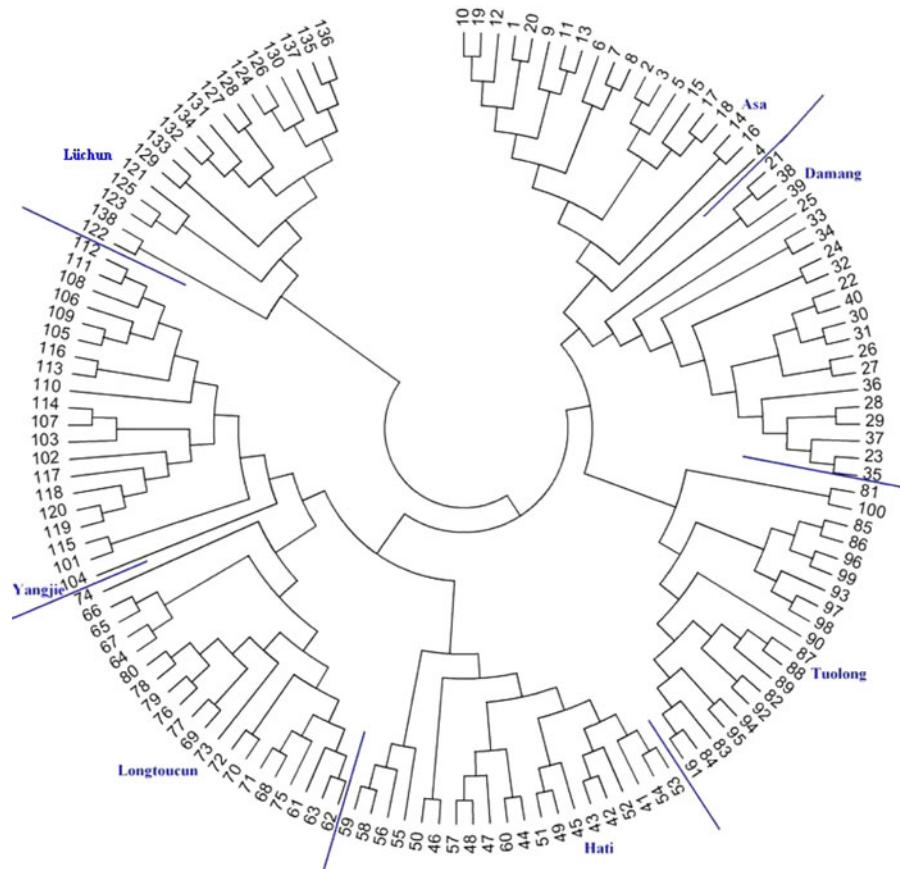
### High genetic diversity

According to Hamrick and Godt (1989), there are strong associations between geographical range and genetic diversity. Allozyme analyses have shown that endemic and geographically limited plant species generally have lower levels of genetic variation than more widely distributed species, due to genetic drift and restricted gene flow (Karron 1991; Hamrick and Godt 1996). Consequently, a low level of diversity was expected for *C. hongheensis*, based on both its limited distribution and small population sizes. However, the analyses of the ISSR markers using several approaches (i.e. Popgene, AMOVA, Shannon's diversity indices, and cluster analysis) all revealed high levels of genetic diversity and significant genetic structuring of *C. hongheensis* populations (Table 2). The percentage of polymorphic loci and genetic diversity ( $Ps = 95.10\%$ ,  $Hes = 0.3520$ ,  $Pp = 36.50\%$  and  $Hep = 0.1327$ ) maintained in this species are more than twice the mean values

( $Ps = 40.0\%$ ,  $Hes = 0.096$ ,  $Pp = 26.3\%$  and  $Hep = 0.063$ ) found for other endemic species (see Hamrick and Godt 1989). It should be noted that although species with small geographic ranges tend to have less genetic diversity than geographically widespread species (Hamrick and Godt 1989), exceptions are not uncommon (Godt and Hamrick 1996; Gitzendanner and Soltis 2000; Lopez-Pujol et al. 2002; Wang et al. 2004). For example, *Castilleja levisecta* Greenm. is a rare species with a distribution restricted to two island populations in British Columbia and nine populations in Washington State. Nevertheless, despite its small range and few extant populations, this species has been found to have high genetic diversity ( $P = 100\%$ ,  $Hep = 0.285$  and  $Hes = 0.213$ ; Table 5) (Godt et al. 2005). However, relatively low genetic diversity was found within populations of this species when examining  $PPB$  values, which ranged from 28.57 to 48.98% (average, 36.50%), implying that a large proportion of variation resided among populations.

The levels and patterns of genetic variation in plants are dynamic, and are influenced by the specific characteristics of the species (Hamrick and Godt 1989), as well as by its evolutionary history. The high genetic variation in *C. hongheensis* indicates that this species might have been much more widespread in its evolutionary history than it is at present, and

**Fig. 2** UPGMA dendrogram (all individuals) based on Nei's (1972) genetic distance



**Table 5** Comparison of genetic diversity between *Citrus hongheensis* and other endemic plants

Species/group	<i>Ps</i>	<i>As</i>	<i>Aes</i>	<i>Hes</i>	<i>Pp</i>	<i>Aep</i>	<i>Hep</i>
<i>C. hongheensis</i>	95.1	1.951	1.6163	0.352	36.50	1.2291	0.1327
Endemic plants	40.0	1.80	1.15	0.096	44.61	1.2884	0.063
<i>Castilleja levisecta</i>	100	2.94	1.56	0.285	65.7		0.213

possessed abundant genetic variation. *C. hongheensis* has a centralized distribution pattern in southeast Yunnan and the suitable climate in this region may have allowed its survival since the Mesozoic Era (Walker 1986); therefore, this area may have been a refuge for *C. hongheensis*. Human activities have resulted in both reductions in size and increased isolation of its populations in recent decades, but its high genetic variation indicates that the sharp reductions in population size have not yet led to similar reductions in genetic variation in this long-lived species. Thus, recent population history better explains the present-day genetic variation of

*C. hongheensis* than actual population sizes. Further, its high level of self-pollination has probably helped to maintain some rare alleles within populations.

#### High genetic divergence

The present study of seven *C. hongheensis* populations revealed a high level of genetic differentiation among populations ( $G_{ST} = 0.6247$ ); much higher than the mean value ( $G_{ST} = 0.073$ ) recorded for 121 woody angiosperm species examined using allozyme markers (Hamrick et al. 1992). Indirect estimates suggest that gene flow ( $Nm = 0.1502$ ) between *C. hongheensis*

populations is limited. Furthermore, the AMOVA analysis indicates that 64.18% of the total variance is apportioned among populations and only 35.82% within populations, hence there is significant differentiation among populations. Mean genetic identity among population pairs ( $I = 0.7008$ ) in *C. hongheensis* was lower than the means ( $I = 0.95$ ) reported for 22 species by Gottlieb (1977) and for 32 species by Crawford (1983), suggesting that gene flow between populations is restricted. This high inter-population genetic differentiation between *C. hongheensis* populations can be attributed to habitat fragmentation, small population sizes, and inbreeding.

Like other congeners that are monoembryonic (e.g. *C. maxima*), the mating system of *C. hongheensis* and its cultivars (He 1999) involves both cross-pollination and self-pollination. Hamrick et al. (1991) emphasized that wide-spread, long-lived, wind-pollinated outcrossing species maintain more isozymic variation than those with other trait combinations. Moreover, inbreeding or selfing annual species have been shown to maintain much more genetic variation among populations than within populations, contrasting with outcrossing and/or perennial plants. Genetic differentiation estimates among populations for outcrossing species obtained using RAPD markers to calculate Shannon's indices range between 15% and 38%, and when AMOVA is used the values are usually <20%. For inbred species, genetic variation values are usually >50% (reviewed in Bussell 1999). The genetic variation among *C. hongheensis* populations (64.18%) is very high, in accordance with the incrossing nature of the species. Moreover, apomictic seed production has probably played an important role in the genetic differentiation of *C. hongheensis* populations, because apomictic seed production tends to reduce within-population variability and increase genetic differentiation among populations.

For *C. hongheensis*, although the exact time of habitat fragmentation remains unknown, at least several generations have passed since then, according to oral testimony by the Hani villagers. The extant populations of *C. hongheensis* have remained because they have been utilized by the local people. Fragmented populations are expected to experience stochastic loss of rare alleles, because only a small portion of the original gene pool remains after fragmentation (see reviews by Barrett and Kohn 1991; Ellstrand and

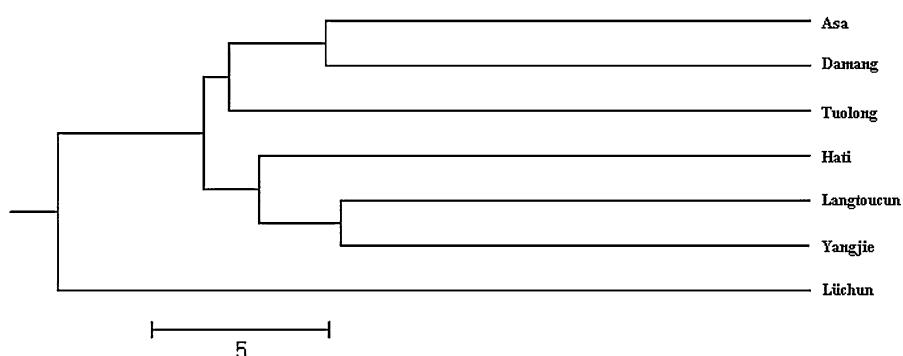
Elam 1993; Young et al. 1996). Further, elevated inbreeding induced by fragmentation can rapidly erode heterozygosity, leading to increased inbreeding coefficients. Subsequently, random genetic drift will reduce allelic richness and increase interpopulation genetic divergence. However, it takes several generations for drift to have significant effects.

The extant *C. hongheensis* populations are very small (each having less than 50 individuals) and are isolated by farmland. The genetic effects of fragmentation are directly related to the stochastic processes associated with small population sizes (Gilpin 1987, 1991). As populations become isolated with small effective sizes, genetic bottlenecks and founder events may reduce the population genetic variability further through extinction and recolonization processes. Such reductions in genetic diversity may result directly from founder events, as well as from the combined effects of genetic drift and inbreeding (Wright 1978).

A possible explanation for some of the observed genetic structure of *C. hongheensis* is that some of the populations may have been through severe bottlenecks in the past. If so, the low effective size of these populations would have increased genetic drift and led to the loss of allelic diversity with the random fixation of some alleles. This would have resulted in a new random allelic composition of populations, independent of their geographical location. The high level of genetic differentiation among populations suggests that if such bottleneck events occurred, they must have happened a long time ago. Whatever the causes of the high levels of population differentiation in *C. hongheensis*, the absence (or the extreme weakness) of gene flow between them explains the maintenance of this structure through time.

The UPGMA dendrogram (Figs. 2, 3) and the AMOVA analysis (Table 4) showed that there is clear genetic differentiation among populations and that no individuals intersect or intermix between populations (Fig. 2), in accordance with the high  $Gst$  estimate (0.6247). Our findings indicate that the present populations are more likely to be relic than cultivated populations, since gene flow is restricted among the populations by habitat fragmentation and inbreeding, and species with restricted gene flow should theoretically exhibit more genetic differentiation than species with widely dispersed pollen and seeds (Hamrick 1990).

**Fig. 3** UPGMA dendrogram (among populations) based on Nei's (1972) genetic distance



### Conservation implications

Knowledge of the levels and distribution of genetic diversity are important for designing conservation strategies for threatened and endangered species (Hamrick 1983; Hamrick and Godt 1989; Francisco-Ortega et al. 2000). Hence, the information gained from the levels and distribution of ISSR variation in the threatened *C. hongheensis* can be used to devise appropriate management strategies. As the differences among *C. hongheensis* populations are in allele frequencies rather than in gene composition, loss of populations at certain locations would probably cause the immediate loss of genetic diversity. Furthermore, there may be more serious long-term damage to genetic diversity due to a reduced number of populations and a smaller overall population size. The utilization of *C. hongheensis* by the local people has inadvertently protected this species. The mean  $Gst$  value (0.6247) across all the studied populations indicates that 62.47% of the total genetic variation resides among populations of this species. This is insufficient for developing a conservation plan that focuses on representative populations with the greatest genetic diversity, because each population may be an evolutionary significant unit (ESU).

The heterozygote deficiency indicates that inbreeding takes place in most of the sampled populations. Thus, we suggest that, in the context of *in situ* conservation and restoration genetics, the maintenance of historically significant processes, including high outcrossing, is particularly important. Therefore, preserving all populations *in situ* would be valuable since it may maintain the specific genetic structure, which is evident from the high level of genetic differentiation.

The very limited gene flow detected in this study has been an important factor shaping the observed

population structure and will influence future changes in these populations. Therefore, efforts should be made to increase the levels of gene flow among populations to maintain the natural genetic variation within *C. hongheensis* populations. Our results show that habitat fragmentation and small population size, which affect genetic differentiation among populations and the genetic diversity within all the extant *C. hongheensis* populations, must be taken into consideration when conservation strategies are devised. Growth of the severely isolated populations would maintain and accelerate dispersal forces among populations resulting from human activities, and thus enhance interpopulation gene flow. Furthermore, gene flow by transplantation could help to restore the highly isolated populations, but would present some risks. For example, transplantation could cause changes in the genetic composition of the populations, resulting in decreased fitness through outcrossing depression and the disruption of locally adapted gene combinations (Storfer 1999). Therefore, it is not advisable to mix seeds or live plants collected from different populations as a first option. However, our results indicate that the fairly small and fragmented populations may harbor insufficient genetic variation to suit particular environments and to avoid potential outcrossing depression. Thus, artificially propagated plants recruited from local seed sources are likely to exhibit increased fitness over non-local genotypes in particular environments. Consequently, seedlings from different populations should only be reintroduced into their original parental localities to increase their effective population sizes.

Finally, the results of our study highlight the need for further research and here we describe some of the important aspects that warrant further investigation. In the future other populations of this species should

be sampled, and other related species in the genus *Citrus*. This would clarify the probable speciation and sequence of founding events that gave rise to this species, and possibly other relatives. Such information could also better explain why *C. hongheensis* shows a high degree of genetic differentiation and determine its extent of inbreeding. This could be achieved by a combined approach of phylogeography and classical population genetics using other molecular markers, and perhaps DNA sequencing data, which could be used to identify the population phylogenetic histories. The second promising line of investigation would be to determine how crossing may have affected the population structure of this species. It would also be interesting to determine how utilization by the local people has influenced the structure of genetic diversity of this species. The last important line of research would be to study the mechanisms of pollination, seed and clone dispersal, as well as the variation in breeding system for this species. An integrated research program that combines genetic analyses with studies of reproductive biology may provide further valuable data that would greatly extend the present conclusions.

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