

Genetic Differentiation and Relationships of Populations in the *Cycas balansae* Complex (Cycadaceae) and its Conservation Implications

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• **Background and Aims** The *Cycas balansae* complex is arguably a controversial group with regard to species delineation. Some taxonomists recognize a single polymorphic species while others distinguish five narrowly defined ones. The unresolved taxonomy has the potential to bring about significant problems for species conservation. Thus, an investigation to examine the genetic diversity and differentiation in the *C. balansae* complex was performed to determine the relationship of populations and to test whether the morphologically defined segregations represent genetically distinct units.

• **Methods** Inter-simple sequence repeat (ISSR) markers were employed to assess the genetic diversity in the *C. balansae* complex with a sample of 158 individuals from all extant populations in China.

• **Key Results** ISSR markers revealed low genetic diversity in all populations studied (H_E and H_O averaged 0.0639 and 0.0798 at the population level, respectively). Phenetic analysis showed that the *C. balansae* complex grouped into five clusters closely corresponding to the narrowly defined *C. balansae*, *C. parvula*, *C. shiwandashanica*, *C. tanqingii* and *C. simplicipinna*.

• **Conclusions** ISSR data suggest that the *C. balansae* complex has evolved into five genetically distinct units. These might be derived from a relatively widespread common ancestor through multiple vicariant events including geographical isolation resulting from the collision of the Indian plate with the Eurasian plate and from Pleistocene glaciations. In conservation, attention should be paid to each genetic unit.

Key words: Conservation, *Cycas balansae* complex, ISSR, genetic differentiation, genetic units.

INTRODUCTION

Knowledge about taxonomy and genetic variation patterns within and between populations are basic requirements in conservation biology (Franke and Soule, 1981). Species circumscription may have a strong impact on the estimate of genetic diversity within a species (Hedrén, 2004), the extent and structure of which is a prerequisite for evaluating their survival ability in a changing environment, and for establishment of conservation practices. With the goal to preserve species evolutionary processes, taxonomic uncertainty could result in negative impacts on biodiversity conservation, such as genetic contamination of local populations during *in situ* management activities (Moritz, 1994). Furthermore, *ex situ* conservation also requires an advanced understanding of the phylogenetic relationship and distinctiveness of the taxa in order to avoid interspecific hybridization, which is a major threat to the integrity of species as distinct breeding groups (Furman *et al.*, 1997).

Cycads (Cycadaceae, Stangeriaceae, Zamiaceae) are the most primitive living seed plants with over 250 million years of history (Gao and Thomas, 1989; Norstog and Nicholls, 1997; Hill, 2003). Although extremely dominant during the Mesozoic, they are now largely relictual in highly fragmented habitats in the tropics and subtropics. Most cycad species are endangered and face the threat of extinction in the wild (Osborne, 1995). All species of cycads have been listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora. In China,

all cycads have also been given First Grade conservation status. Cycads have recognizable morphological characteristics intermediate between less advanced plants such as ferns and more derived plants including the angiosperms (Brenner, 2003). Efforts must be undertaken to avoid further decline and extinction of cycads, which constitute a key node in plant evolution.

Members of the *Cycas balansae* complex are distributed in south-west China through Yunnan and Guangxi provinces into adjacent northern Indo-China, including Vietnam, Laos and northern Thailand. Their distribution is characterized by their geographically restricted and historically fragmented pattern. Like the *Macrozamia pauli-guilielmi* complex (Zamiaceae) (Sharma *et al.*, 1998) and the *Macrozamia plurinervia* complex (Zamiaceae) (Sharma *et al.*, 2004), the plants in the *C. balansae* complex lack distinct morphological characteristics and their taxonomic and phylogenetic relationships are problematic and controversial. Chen and Stevenson (1999) regarded the complex as one species, *C. balansae*, characterized by acaulescent (versus caulescent) caudex and wide (versus narrow) leaflets. However, most researchers recognize five separate species, i.e. *C. parvula*, *C. tanqingii*, *C. balansae*, *C. shiwandashanica* and *C. simplicipinnata*, separated by characters such as the number and size of ovules (Wang and Liang, 1996; Huang, 2001). Of these species, *C. parvula* and *C. tanqingii* are restricted to south-west China, whereas the others, *C. balansae*, *C. shiwandashanica* and *C. simplicipinnata*, are mainly distributed in northern Indo-China, but with a few populations extending to

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south-west China. The choice of taxonomic treatment can profoundly affect not only the number of rare and endemic species within cycads in this region but also the establishment of reasonable and effective conservation strategies. In view of the controversy on the *C. balansae* complex, morphological information alone is inadequate to evaluate their conservation status. Traditional taxonomy based on morphological characters may unite cryptic species that are morphologically similar but genetically divergent, which could lead to gene contamination in the course of breeding programmes and reintroduction (Hammond, 2001). Taxon-specific molecular markers that measure the degree of differentiation between taxa can serve as a way to overcome the limitations of traditional morphology-based taxonomy (Hahn and Grifo, 1996). Inter-simple sequence repeats (ISSRs) are markers composed of a microsatellite sequence plus a short arbitrary, adjacent sequence primed by a single oligonucleotide. ISSR primers amplify the region between two closely spaced and oppositely oriented simple sequence repeats. ISSR technologies can reproducibly produce large numbers of polymorphic fragments at low cost (Fang *et al.*, 1997; Moreno *et al.*, 1998). The literature reveals that ISSR markers have a great potential for inferring genetic relationships among closely related species (Zietkiewicz *et al.*, 1994; Salimath *et al.*, 1995; Fang *et al.*, 1998; Martin and Sanchez-Yelamo, 2000; Benharrat, 2002). ISSR markers have also been used to estimate genetic diversity within and between populations of *C. guizhouensis* (Xiao *et al.*, 2004). Here ISSRs are used to analyse genetic diversity and differentiation in the *C. balansae* complex with the goal to understand genetic relationships between populations and their evolutionary history, and whether the morphologically defined segregated species represent genetically distinct units, and to provide information for species conservation.

MATERIALS AND METHODS

Sample collection

Leaf samples were obtained from 158 individuals from eight natural populations representing the five species proposed by Wang (2000) in the *C. balansae* complex (Table 1). The species were not compared with an out-group species since the purpose of this investigation was to establish inter-genetic relationships within the complex and not strictly phylogenetic relationships. Fresh leaflets were dried with silica gel and stored at 4 °C until the DNA extraction. Vouchers were collected from each population and deposited at the herbarium of Kunming Institute of Botany, Chinese Academy of Science (KUN).

DNA extraction, PCR amplification and electrophoresis

Genomic DNA was extracted from leaves of single individuals following the CTAB protocol (Doyle, 1991). Extracted DNA was quantified using a spectrophotometer comparing band intensities with known standards of lambda DNA on 1% agarose gel. The working solution of DNA (approx. 10 ng μ L) was in sterile double-distilled water.

In a preliminary study, 100 ISSR primers, designed by the University of British Columbia (UBC), Vancouver, Canada, were tested in PCR-amplification on a subset of three random individuals. Twelve fragments [USB # 807 (AG)₈T, # 808 (AG)₈C, # 810 (GA)₈T, # 811 (GA)₈C, # 815 (CT)₈G, # 835 (AG)₈YC, # 836 (AG)₈YA, # 840 (GA)₈YT, # 841 (GA)₈YC, # 842 (GA)₈YG, # 843 (CT)₈RA and # 857 (AC)₈YG] were selected for further analysis on all individuals.

DNA amplification was performed in a 20- μ L reaction volume containing about 20 ng of template DNA, 2.0 μ L 10 \times PCR buffer, 2.0–2.5 mM MgCl₂, 0.1 mM dNTPs, 2% formamide, 200 nM primer, 1.0 unit of *Taq* polymerase and double-distilled water. Amplification was performed in a PTC-100 thermal cycler (MJ Research, Inc.) programmed for an initial step of 1 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at 52 °C (or 50 °C) and 1.5 min at 72 °C, and a final 7-min extension at 72 °C. A negative control reaction in which the template DNA was replaced by water was also included to verify for absence of contamination.

Amplification products were electrophoretically separated in 1.5% agarose gels buffered with 0.5 \times TBE. A 100-bp DNA ladder (New England Biolabs) was used as size marker. DNA fragments were identified by image analysis software for gel documentation (LabWorks Software Version 3.0; UVP, Upland, CA 91786, USA) following staining with ethidium bromide.

Data analysis

ISSR bands were scored as present or absent. Only those bands that showed consistent amplification were considered. Smear and weak bands were excluded. The resulting presence/absence data matrix was analysed using POPGENE v. 1.31 (Yeh *et al.*, 1999) to estimate four genetic diversity parameters: percentage of polymorphic loci (P), effective number of alleles (A_e), Shannon's Information index (H_o) and Nei's gene diversity (H_E) (Nei, 1973). To examine population genetic structure the proportion of genetic divergence was estimated within and among populations following Nei (1973) and G_{ST} as the coefficient of gene differentiation among the populations. The genetic identity (I) and the genetic distance (D) between populations were also computed using the model presented in Nei (1972). Gene flow estimates (Nm) were calculated as $Nm = (1 - G_{ST})/4G_{ST}$ (Slatkin and Barton, 1989). An unweighted pair group method using an arithmetic average (UPGMA) dendrogram was performed on the data matrix of mean character difference between pairs of samples with software Mvsp3.13b (downloaded from the website at <http://www.kovcomp.com>) and matrix of ISSRs.

An analysis of molecular variance, AMOVA Version 1.5 (Excoffier, 1993), was also used to analyse the proportion of the genetic differentiation among populations.

RESULTS

The 12 primers chosen for final analysis yielded a total of 103 clear ISSR markers. Of those, only 41 markers were

TABLE 1. Populations studied and sample size

| Species | Pop. ID | Locality* | Latitude (N) | Longitude (E) | Altitude (m) | Sample size | Voucher |
|---------------------------|---------|------------------|--------------|---------------|--------------|-------------|------------|
| <i>C. shiwandashanica</i> | FC | GX Fangchenggang | 21°45' | 108°05' | ? | 20 | Gong 03136 |
| <i>C. parvula</i> | YJ1 | YN Yuanjiang | 23°42' | 101°47' | 1454 | 18 | Gong 03105 |
| | YJ2 | YN Yuanjiang | 23°40' | 101°50' | 1445 | 20 | Gong 03106 |
| <i>C. balansae</i> | JP | YN Jinping | 22°41' | 103°00' | 389 | 20 | Gong 03110 |
| | MG | YN Maguan | 22°51' | 104°07' | 610 | 20 | Gong 03111 |
| | PB | YN Pingbian | 23°03' | 103°43' | 745 | 20 | Gong 03112 |
| <i>C. tanqingii</i> | LC | YN Lvchun | 22°37' | 102°19' | 640 | 20 | Gong 03118 |
| <i>C. simplicipinna</i> | ML | YN Mengla | 21°56' | 101°15' | 570 | 20 | Gong 03124 |

*GX is the code for Guangxi Province and YN for Yunnan Province in China.

TABLE 2. Genetic diversity in *C. balansae* complex detected by ISSR analysis

| Species | Pop. ID | A_e (s.d.) | H_O (s.d.) | H_E (s.d.) | P (%) |
|---------------------------|---------|-----------------|-----------------|-----------------|-------|
| <i>C. shiwandashanica</i> | FC | 1-1179 (0-2831) | 0-0982 (0-2232) | 0-0669 (0-1548) | 17-48 |
| <i>C. parvula</i> | YJ1 | 1-0852 (0-2137) | 0-0833 (0-1920) | 0-0643 (0-1751) | 24-65 |
| | YJ2 | 1-0880 (0-2242) | 0-0843 (0-1942) | 0-0546 (0-1301) | 17-48 |
| <i>C. balansae</i> | JP | 1-2278 (0-3581) | 0-1921 (0-2783) | 0-1013 (0-2154) | 34-95 |
| | MG | 1-1730 (0-3263) | 0-1491 (0-2529) | 0-0996 (0-1755) | 29-13 |
| | PB | 1-1124 (0-2614) | 0-1031 (0-2141) | 0-0675 (0-1458) | 21-36 |
| <i>C. tanqingii</i> | LC | 1-1058 (0-2681) | 0-0890 (0-2137) | 0-0606 (0-1475) | 15-53 |
| <i>C. simplicipinna</i> | ML | 1-0768 (0-2222) | 0-0690 (0-1852) | 0-0458 (0-1260) | 13-59 |
| Total complex | | 1-3615 (0-3814) | 0-3166 (0-2837) | 0-2110 (0-1995) | 60-19 |

A_e , the effective number of alleles per locus; H_O , Shannon's information index; H_E , Nei's genetic index; P, percentage of polymorphic loci.

present in all individuals and the percentage of polymorphic markers was 60.19%. Table 2 describes the polymorphism in this complex revealed by ISSR in detail. Assuming Hardy–Weinberg equilibrium, Nei's genetic diversity (H_E) and Shannon indices (H_O) were estimated to be 0.2110 and 0.3166, respectively, in the total complex, while both indices averaged 0.0639 and 0.0798, respectively, at the population level.

Generally, there is significant differentiation among the populations in this complex. The level of gene flow between populations (Nm) was estimated to be only 0.3303 individuals per generation, indicating that there is a low migration rate between populations. The coefficient of genetic differentiation between populations (G_{ST}) was 0.6022, of which only 41% (0.2486) contributed to the differentiation between populations within the defined species. This finding is corroborated by AMOVA analysis showing that 67.83% of the total variation was partitioned between populations, of which, 50.60% was between the defined species (Table 3). Table 4 shows an estimate of Nei's genetic identities (I) and genetic distance (D) for every pairwise comparison between both populations and the defined species. The inter-specific genetic distances were 0.2286 on average, ranging from 0.1282 to 0.3610, which were higher than the intra-specific genetic distances with a mean value of 0.0691, and ranging from 0.0110 to 0.0791. A UPGMA dendrogram based on mean character difference visualized that this complex is grouped into five clusters corresponding to the five species, *C. tanqingii*, *C. parvula*, *C. simplicipinna*, *C. shiwandashanica* and *C. balansae*. It also showed that the genetic relationship

among the three species, *C. balansae*, *C. shiwandashanica* and *C. simplicipinnata*, with main distribution in Indo-China, is closer than the other two species in south-west China, *C. parvula* and *C. tanqingii*.

DISCUSSION

Genetic diversity

Walters and Decker-Walters (1991) hypothesized that low within-population genetic variation with relatively high between-population genetic differentiation is a biological and evolutionary characteristic of cycads, a pattern that has been verified by most researchers (Barrett and Kohn, 1991; Ellstrand and Elam, 1993; Xiao *et al.*, 2004). In the present study, every population in the *C. balansae* complex harbours rather low genetic diversity. This has been ascribed to genetic drift and inbreeding effects due to their small and isolated populations, as well as limited dispersal ability of their large, heavy gravity-dispersed seeds, and beetle-dispersed pollen (Barrett and Kohn, 1991; Ellstrand and Elam, 1993; Xiao *et al.*, 2004). However, the genetic differentiation between populations of different species within the complex presents a striking contrast: G_{st} in *C. parvula* is 0.0978, whereas it is 0.4003 in *C. balansae* (no G_{st} values were obtained from the remaining species as they were only represented by a single population each). The difference in distances between populations of either species is postulated to be the main reason for the contrast. Gene flow distance between local populations is 2–7 km in cycads (Yang and Meerow, 1996;

TABLE 3. Nei's (1972) original measures of genetic identity (above diagonal) and genetic distance (below diagonal)

| Pop. ID | <i>C. shiwandashanica</i> FC | <i>C. parvula</i> | | <i>C. balansae</i> | | | <i>C. tanqingii</i> LC | <i>C. simplicipinna</i> ML |
|---------|---------------------------------|-------------------|--------|--------------------|--------|--------|---------------------------|-------------------------------|
| | | YJ1 | YJ2 | JP | MG | PB | | |
| FC | – | 0.8345 | 0.8363 | 0.8783 | 0.8665 | 0.8797 | 0.6970 | 0.8489 |
| YJ1 | 0.1809 | – | 0.9890 | 0.8371 | 0.8036 | 0.8309 | 0.7238 | 0.7689 |
| YJ2 | 0.1787 | 0.0110 | – | 0.8295 | 0.7970 | 0.8294 | 0.7148 | 0.7662 |
| JP | 0.1298 | 0.1779 | 0.1870 | – | 0.9239 | 0.9124 | 0.7252 | 0.8466 |
| MG | 0.1433 | 0.2186 | 0.2269 | 0.0791 | – | 0.9100 | 0.7068 | 0.8606 |
| PB | 0.1282 | 0.1853 | 0.1871 | 0.0917 | 0.0944 | – | 0.7367 | 0.8676 |
| LC | 0.3610 | 0.3233 | 0.3357 | 0.3214 | 0.3470 | 0.3055 | – | 0.7421 |
| ML | 0.1638 | 0.2629 | 0.2664 | 0.1665 | 0.1501 | 0.1420 | 0.2983 | – |

TABLE 4. AMOVA for the *Cycas balansae* complex

| Source of variance | d.f. | Sum of squares | Mean squares | Variance component | % total variance | P value |
|----------------------------------|------|----------------|--------------|--------------------|------------------|---------|
| Among species | 4 | 904.3267 | 226.082 | 6.109 | 50.60 | <0.0010 |
| Among populations within species | 3 | 134.3020 | 44.767 | 2.081 | 17.23 | <0.0010 |
| Within populations | 150 | 582.5611 | 3.884 | 3.884 | 32.17 | <0.001 |

Xiao *et al.*, 2004). The distance between *C. parvula* populations is just 7.3 km, while those of *C. balansae* populations are >30 km, which implies that much gene exchange occurs between the former populations, but is unlikely between the latter populations.

Genetic relationship

ISSR analysis is helpful to understand the taxonomy and phylogenetic relationships within the *C. balansae* complex. ISSR banding profiles showed that species-specific markers were detected in each defined species as indicated in Fig. 1. Tables 3 and 4 indicate that the majority of genetic differentiation (59%) exists among the defined species in this complex. A UPGMA dendrogram, based on mean character difference, showed that the populations clustered into five distinct branches (Fig. 2), which correspond well with the defined species, *C. tanqingii*, *C. parvula*, *C. simplicipinna*, *C. shiwandashanica* and *C. balansae*. These indicate that the *C. balansae* complex has formed five genetically distinct units. The analyses of sclerotesta morphology (D. Y. Wang and J. P. Liao, unpubl. res) and isozyme data (Yang and Meerow, 1999) also indicate that five genetically divergent entities may be separated. Gottlieb (1977) and Crawford (1983, 1990) found that mean genetic identities of conspecific populations were usually above 0.90, whereas the genetic identities of congeneric taxa were usually below 0.7. In the present study, genetic identities of populations in each defined species were higher than 0.90, ranging from 0.9100 to 0.9890, but those between the recognized species were much lower than the conspecific threshold of 0.90 (average 0.8040) (Table 4). Evidence available from the ISSR data

show that *C. balansae* complex is genetically divergent and has evolved into five genetically distinct units, corresponding well to the defined species. Accordingly, a taxonomy is accepted that separates out the five narrowly defined species, *C. tanqingii*, *C. parvula*, *C. simplicipinna*, *C. shiwandashanica* and *C. balansae*, at species level. The monophyly of these entities needs to be verified by character-based phylogenetic analyses.

The phylogeny relationships between the five species in the *C. balansae* complex have been reconstructed with a set of quantitative morphological traits (Wang and Liang, 1996; Hill and Stevenson, 1998, 1999; Huang, 2001). The UPGMA dendrogram presented here is largely congruent with the phylogeny proposed by Hill and Stevenson, in which, however, *C. parvula* was mistaken as *C. diannanensis*, a widely accepted species with tomentose ovules (versus glabrous ovules in *C. parvula*). *Cycas tanqingii* is hypothesized to have diverged from the others first, followed by *C. parvula*. The species mainly distributed in Indo-China, including *C. simplicipinna*, *C. shiwandashanica* and *C. balansae*, were more closely related to each other.

All investigated individuals in the *C. balansae* complex shared a considerable number of markers (41) indicating that these species form a genetic assemblage. Due to slow reproduction rates (Tang, 1990) and absence of a long-distance dispersal mechanisms for seeds (Bank *et al.*, 2001), it seems reasonable to assume that the species in this complex have been derived from a common ancestor through multiple vicariant events, which arose from geographical isolation resulting from the collision of the Indian plate with the Eurasian plate and from glaciations in the Pleistocene. Morphological cladistics and biogeography indicate that a primary split occurred during cycad evolution between populations in south-west China and those in Indo-China (Hill, 1999) with *C. tanqingii* and *C. parvula* in south-west China separating away from the others species mainly in Indo-China. *Cycas tanqingii* has a characteristic of only two ovules (versus more than two ovules in the remaining species); and *C. parvula* of small ovules (versus big ovules). *Cycas simplicipinna*, which is distributed mainly in northern Indo-China and Thailand, has leaflets that become silvery-grey when dry (as opposed to blackish-brown in the other species). *Cycas balansae* (in north Vietnam) and *C. shiwandashanica* (in north-east Vietnam), which have a sister

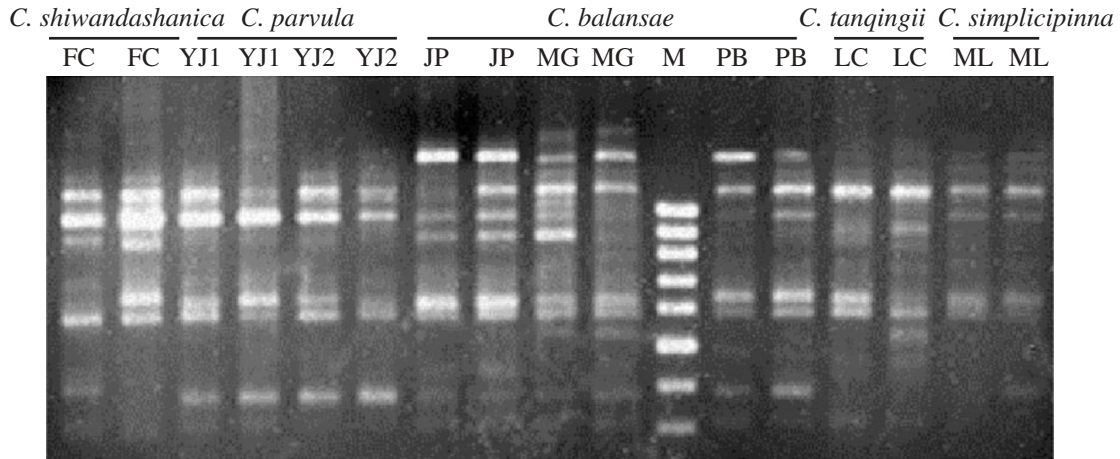


FIG. 1. ISSR banding profiles obtained on 1.5% agarose gels with primer USB 843 for 16 individuals representative of eight populations of the five species in *C. balansae* complex. M = molecular size marker, 100-bp ladder.

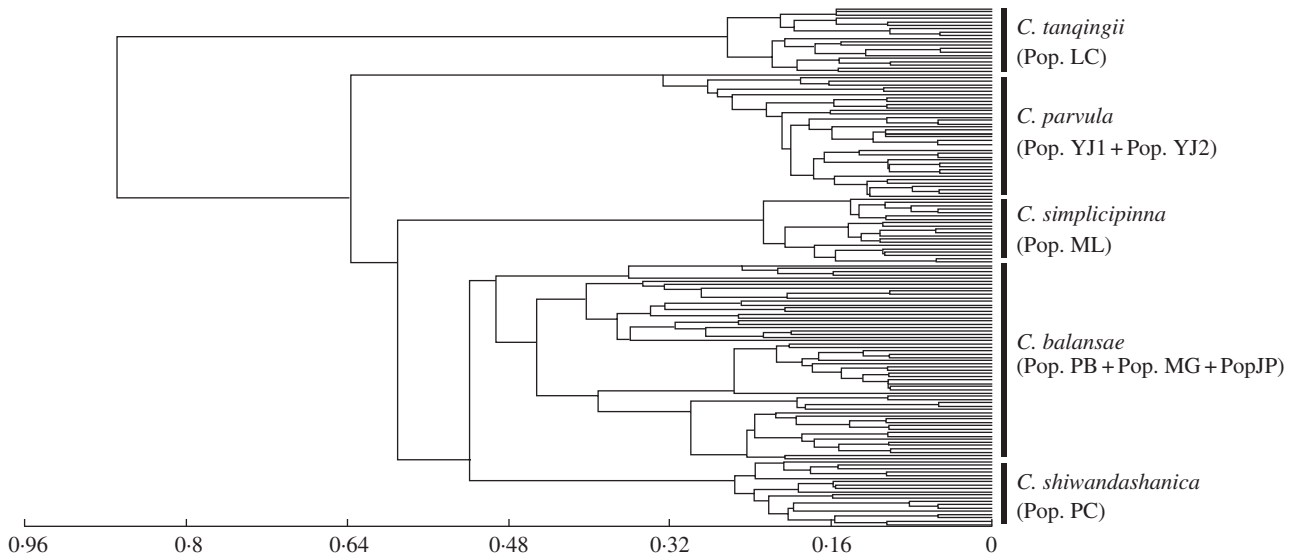


FIG. 2. UPGMA dendrogram based on mean character differences estimated from ISSR data from 158 plants in eight populations in the *C. balansae* complex.

relationship, are separated by the Honghe river. *Cycas balansae* has papery leaflets, whereas those of *C. shiwandashanica* are leathery.

Conservation implications

Populations and individuals of the *C. balansae* complex appear to have declined dramatically during recent decades. The two principal threats to cycads are (a) severe habitat loss and (b) selective removal from the wild for trade or utilization due to their popularity among horticulturalists. Accordingly, it is essential to take action to preserve the species and their habitats, which includes setting up nature reserves and protection stations, education for cycad protection and strengthening the co-operation between governments in this region on wild cycad conservation management. The pronounced spatial genetic structure

suggests that it is necessary to preserve as many populations as possible and that it is not appropriate to preserve only part of populations, since the low genetic diversity in populations implies a reduced ability to survive changing environments and increased susceptibility to diseases in the long term (Ellstrand and Elam, 1993). If methods to enhance genetic diversity, such as transplant programmes and artificial pollination between populations, are required to promote gene exchange and species survival, genetic contamination must be avoided between genetic units.

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