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RESEARCH ARTICLE

# Patterns of chloroplast DNA variation in *Cycas debaoensis* (Cycadaceae): conservation implications

**Qing-Qing Zhan · Jin-Feng Wang · Xun Gong · Hua Peng**

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**Abstract** Climate changes during glacial periods have had significant effects on the current distribution of plant species. Palaeontologial data suggest that modern cycads originated in southwest China. *Cycas debaoensis* (Cycadaceae) is an endangered species restricted to a small area of southwest China. This species has been classified into two types: sand and karst, according to the soil matrix they grow on. To determine the locations of its glacial refugia and its genetic structure, we examined chloroplast sequence variation of the *atp*B-*rbc*L and *psb*A-*trn*H intergenic spacers. Four chloroplast DNA haplotypes were obtained from 120 individuals collected from 11 populations covering the entire extant distribution range of the species. Significant population subdivision was detected ($G_{ST} = 0.684$ and $F_{ST} = 0.74160$), suggesting low levels of gene flow between regions and populations. There was marked haplotype differentiation between populations in the sand and karst regions, with only one haplotype being present in both. The molecular phylogenetic data, together with the geographic distribution of the haplotypes, suggest that *C. debaoensis* experienced range contraction during glacial periods, and that the current populations are still confined to the areas of the original refugia. These results implied that isolated refugia might have maintained in both sand and karst regions during the last glacial maximum and even earlier glaciations. The low within-population diversity of *C. debaoensis* suggested that there were strong bottleneck events or founder effects within each separate region during the Quaternary climatic oscillations. These findings are important for the conservation of this endangered species.

**Keywords** Chloroplast DNA *atp*B-*rbc*L and *psb*A-*trn*H · *Cycas debaoensis* · Glacial refugia · Range contraction · Population structure · Conservation

## Introduction

Cycads represent an ancient lineage, whose origin can be dated to the late Paleozoic era (Hendricks 1987; Gao and Thomas 1989). The earliest fossil evidence in Gansu province from the Carboniferous era reveals that cycads may originate from northern China (Li et al. 1976; Li 1982). The hitherto most completely preserved cycad specimen is also collected from northeast China (Liaoning) and can be traced back to the Upper Triassic (Wang et al. 2009; Zhang et al. 2010). These discoveries contribute much to our understanding of the morphology and evolution of cycads. Abundant fossil evidence indicates that after the Mesozoic, the distribution of cycads gradually became centered on southwest China where stable environmental conditions since the Mesozoic promoted their development (Guan and Zhou 1996; Hu et al. 1999). This ancient area, the so-called Kang-Dian Old land, included current Sichuan, Guizhou, Yunnan and Guangxi provinces and is where

Qing-Qing Zhan and Jin-Feng Wang contributed equally to this work.

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Q.-Q. Zhan · J.-F. Wang · X. Gong (✉) · H. Peng
Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, 132 Lanhei Road, Kunming 650204, People's Republic of China
e-mail: gongxun@mail.kib.ac.cn

Q.-Q. Zhan · J.-F. Wang
Graduate School of the Chinese Academy of Sciences, Beijing 100049, China

modern cycads are thought to have originated (Zhai and Yang 1986; Wu 1990). It is likely that the present-day distribution of the vegetation of southwest China is the result of both past and present ecological events, with the Pleistocene glacial-interglacial cycles having had a profound influence on shaping the phylogeographic patterns and genetic structure of plant species there.

Cycads are found in tropical and subtropical regions of Asia, Oceania and America. Only one genus of one family occurs in China, namely *Cycas*; this is considered to be the oldest genus of cycads (Hill et al. 2004). *Cycas debaoensis* Zhong and Chen is endemic to southwest China, mainly occurring along the border between Guangxi and Yunnan provinces. It's a distinctive cycad species with dichotomously divided pinnae. As one of the most endangered cycad species, with fewer than 800 individuals surviving in the wild, *C. debaoensis* has been referred to as a 'living fossil' of the plant kingdom (Ma et al. 2003). This species has been subdivided into sand and karst types according to the soil matrix upon which it grows (Ma et al. 2003; Xie et al. 2005; Wang 2007). The populations of the sand type are continuously distributed along the sandy areas of the Gula River between Guangxi and Yunnan provinces, while the populations of the karst type are scattered on isolated limestone hills. The distinctly different environments in these regions suggest that the groups are probably ecotypes. Furthermore, molecular analysis has revealed considerable intraspecific divergence between them. For example, Xie et al. (2005) identified two distinct genetic groups of *C. debaoensis* based on ISSR data, partitioned between the sand and karst regions. None of the populations of both regions is morphologically distinct from the type specimen, which originated from Fuping village, Debao County. They all have tripinnate leaves and 17–25 megasporophyll lobes on each side. It is worth mentioning that in 2007 Wang discovered three new populations (NY, CY and BM) in the northern Gula River area (Wang 2007); these have a few characteristics, including bipinnate (rarely simply pinnate) leaves and fewer megasporophyll lobes (11–19 on each side), distinguish them from the type specimen. However, all other floristic characteristics confirm that these individuals belong to *C. debaoensis.*

Like other cycads, *C. debaoensis* is a unisexual, dichogamous, mainly entomophilous species. The fleshy outer seed coat of cycads attracts rodents and small fruit-eating bats, which serve as dispersal agents (Norstog and Nicholls 1997). The level of gene flow via seed is thus constrained by the limited range of the seed carriers. Gene flow distance between local populations of cycads has been estimated to be only 2–7 km (Yang and Meerow 1996). Hence, low intra-population genetic variation with relatively high spatial differentiation might be expected to be a biological and evolutionary characteristic of cycads (Walters and Decker-Walters 1991). Such characteristics of the population genetic structure have been detected in many cycads based on allozyme and ISSR analyses (Ellstrand et al. 1990; Yang and Meerow 1996; Lin et al. 2000; Keppel et al. 2002; Xiao et al. 2004).

In the past, cycads were used as sources of food and medicine by many native people in China, particularly during periods when their usual supplies were restricted. In recent decades, with the rapid development of the economy, the native habitat of cycads in China has experienced widespread destruction. As more and more wild cycad populations are either endangered or on the brink of extinction, all cycads in China have been given First Grade Conservation Status (Fu and Jin 1992). In recent years, with the development of commercial floristry, wild *C. debaoensis* have been gathered on a large scale and sold illegally because of the species' peculiar appearance and high value as an ornamental plant. The number of wild individuals of *C. debaoensis* has, therefore, decreased dramatically. Long-term conservation polices and strategies based on information about the population history and dynamics of *C. debaoensis* are urgently needed.

Molecular techniques have provided many tools for studying the phylogeography or migratory footprints of species (Avise 2000). Moreover, phylogeographic studies could provide a basis for conservation biology. In plants, chloroplast DNA (cpDNA) evolves slowly, with genetic recombination occurring at low frequencies. It is, therefore, useful for testing phylogeographical hypotheses related to the presence of isolated glacial populations and refugia (Taberlet et al. 1998; Provan et al. 2001; Hewitt 2004). Although chloroplasts are paternally inherited in many gymnosperms (Reboud and Zeyl 1994), cycad cpDNA is maternally inherited, as confirmed by restriction fragment length polymorphism analysis (Cafasso et al. 2001). Only Xie et al. (2005) have examined the population genetic variation of *C. debaoensis* previously; they investigated five populations using ISSR markers. Our study examined almost all the extant populations of *C. debaoensis*; we concentrated, in particular on comparison research of populations in karst and sand regions and the three newly found, morphologically distinct populations. We examined the sequence variation of chloroplast *atp*B-*rbc*L and *psb*A-*trn*H spacers in 11 populations of *C. debaoensis* in order to identify historical processes reflected in the current population structure. Our analyses suggest a past fragmentation of this species, during the Quaternary glacial periods, into two separate refugia: the sand region and karst region. These results have significant implications for the management and recovery of this endangered species.

## Materials and methods

### Population sampling

*Cycas debaoensis* was formally described in 1997 (Zhong and Chen 1997). As a drought-tolerant perennial, it is characterized by a low rate of seedling survival and a long lifecycle. In our study, leaf tissue was collected from 120 plants, representing 11 populations of *C. debaoensis*, *C. micholitzii* was chosen as outgroup. These samples were collected from populations of the sand region (populations BY, BM, NY, CY, LW, XP and BW) and karst region (populations GC, NH, DY and FP). Fresh and healthy leaflets were dried with silica gel and stored at 4°C whilst awaiting DNA extraction. Voucher specimens were obtained from the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN). Information about each sampling location is presented in Fig. 1 and Table 2.

### DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted following the CTAB protocol (Doyle and Doyle 1987). After preliminary screening of a range of nucleic DNA and organelle DNA in 30–50 individuals, at least 3–5 individuals from each population (Table S, Supplementary material), we chose cpDNA *atp*B-*rbc*L and *psb*A-*trn*H intergenic spacers for the full survey because they contained the highest number of polymorphic sites. PCR reactions were carried out in a total volume of 20 μl, containing 10 ng template DNA, 2.0 μl 10 × *Taq* Buffer with $(NH_4)_2SO_4$, 2.0–2.5 μl $MaCl_2$ (25 mM/l), 1.0 μl dNTP (2.5 mM each), 0.10 mM each primer, 1.0 unit of *Taq* polymerase and double-distilled water. DNA amplification was performed in a T1 thermocycler (Biometra), with an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 1.5 min, and a final extension cycle of 7 min at 72°C. All PCR products were purified directly using a PCR product purification kit (Shanghai Sangon). Purified plasmid DNAs were sequenced in both directions with the same primers for the amplification reactions, using an ABI 3770 automated sequencer, by the Shanghai Sangon Biological Engineering Technology and Services Company.

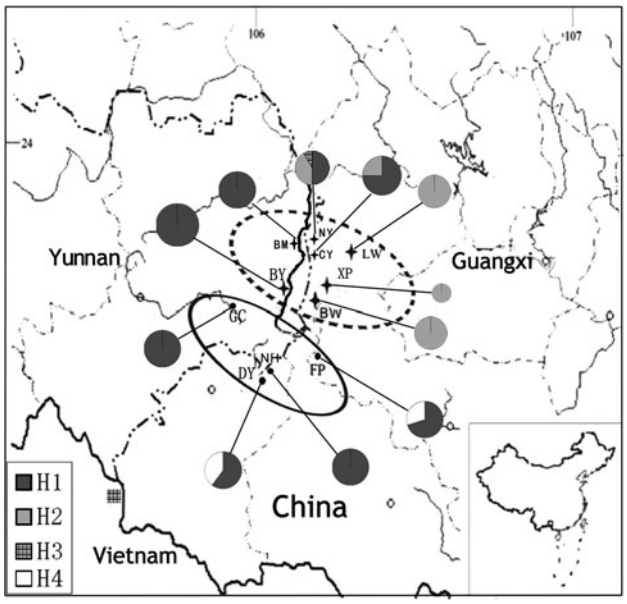


**Fig. 1** Map showing locations of the sampled populations of *C. debaoensis* and the distribution of cpDNA haplotype in the species. The pie sizes of sampled populations are proportional to their sample sizes. The *dashed circle* indicates the sand region; the *solid circle* indicates the karst region; the *bold irregular curve* presents the Gula River

### Data analysis

Sequences were aligned using ClustalX version 1.81(Thompson et al. 1997). Indels were treated as the fifth character. Phylogenetic trees were reconstructed using maximum likelihood (ML) analyses of the nucleotide sequences with software PHYML v2.4.5 (Guindon and Gascuel 2003) and bootstrap consensus values calculated using 1,000 replicates. The general time reversible (GTR) model was determined to be the most suitable model by Model test v3.6 (Posada and Crandall 1998) and was used for all subsequent nucleotide analyses. A parsimony network of chloroplast haplotypes was drawn using TCS version 1.13 (Templeton and Sing 1993; Clement et al. 2000), in which haplotypes were organized into a system of nested clades where a higher nesting level corresponded to longer evolutionary time (Templeton and Sing 1993). Levels of inter- and intra-population genetic diversity were quantified by calculating Nei's nucleotide diversity ($P_i$) and haplotype diversity ($H_d$) indices using DnaSP 4.00 (Rozas et al. 2003). We calculated within-population diversity ($H_S$), total diversity ($H_T$), and two measures of population differentiation $G_{ST}$ and $N_{ST}$ using the Permut (Pons and Petit 1996) (available at http://www.pierroton.inra.fr/genetics/labo/Software/Permut/). We used the program Arlequin version 3.11 (Excoffier et al. 2005) to conduct an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) and, thus, to estimate genetic variations within and among populations, and between geographic regions.

For estimating coalescent time between lineages within populations or species, a well-documented evolutionary rate is needed. In seed plants, evolutionary rates are estimated at $1.01 \times 10^{-9}$ substitutions per site per year for synonymous sites of cpDNAs (Graur and Li 2000). These values approximate the evolutionary rates of introns and

noncoding spacers of organelle DNAs (Chiang et al. 2009). BEAST v.1.6.1 (http://beast.bio.ed.ac.uk) was used to estimate the mutation rates and the ages of the most recent common ancestor (TMRCA) (Drummond and Rambaut 2007). We used the GTR model and a strict molecular clock with uncorrelated log normal distribution of branch lengths. Posterior estimates of the mutation rate and age of the TMRCA were obtained by Markov Chain Monte Carlo (MCMC) analysis, with samples drawn every 500 steps over a total of 25,000,000 steps. To present the demographic scenarios for populations, the BEAST program was also used to create a Bayesian skyline plot with ten steps. The analysis was run for $10^7$ iterations with a burn-in of $10^6$ under the GTR model a strict molecular clock with uncorrelated log normal distribution of branch lengths. Genealogies and model parameters were sampled every 1,000 iterations. All operators were automatically optimized. Convergence of parameters and mixing of chains were followed by visual inspection of parameter trend lines and checking of effective sampling size (ESS) values by three pre-runs. The ESS parameter was found to exceed 200, which suggests acceptable mixing and sufficient sampling. Adequate sampling and convergence to the stationary distribution were checked using TRACER v. 1.5 (Rambaut and Drummond 2004). Posterior estimates of parameters were all distinctly unimodal (although with wide 95% highest posterior densities), and all parameters were identifiable, despite the relatively low information content in the sequences and the small age range of the sequences.

We used a pairwise mismatch distribution to test for population expansion (Rozas et al. 2003) using the DnaSP program. The sum-of-squared deviations (SSD) between observed and expected mismatch distribution were computed and *P*-values were calculated as the proportion of simulations producing larger SSD than the observed SSD. The raggedness index and its significance were also calculated to quantify the smoothness of the observed mismatch distribution. We also conducted neutrality tests, with Tajima's *D* (Tajima 1989) and Fu & Li's $D^*$(Fu 1997), using Arlequin version 3.11(Excoffier et al. 2005), to detect departures from population equilibrium.

## Results

### Genetic composition and diversity

Of seven DNA regions surveyed, totaling 7631 bp in length, only two (*atp*B-*rbc*L and *psb*A-*trn*H) exhibited polymorphisms in *C. debaoensis* populations. The aligned sequences of the *atp*B-*rbc*L spacer were 723 bp long. Three substitutions (178, A/C; 248, C/A; 364, C/A) and poly-T repeats, which varied in length between 15 and 19 bp (positions 380–383), were found. Since mononucleotide repeats (such as poly-A and poly-T) have been shown to provide valuable information for intraspecies studies (Rendell and Ennos 2003; Walter and Epperson 2005; Naciri and Gaudeul 2007; Artyukova et al. 2009), all polymorphism sites were analyzed and three haplotypes were identified. The length of the aligned sequences of the *psb*A-*trn*H spacer was 526 bp. Nucleotide substitutions (319, G/A) and a 27 bp indel resulted in two haplotypes. A total of four haplotypes (hap1–hap4) were identified when *atp*B-*rbc*L and *psb*A-*trn*H sequences were combined (Table 1).

Haplotype frequencies in each population and geographical distribution are presented in Table 2 and Fig. 1. Haplotype 1 was the most dominant haplotype. Haplotype 2 was only located in the eastern populations of the Gula River. Haplotype 4 occurred disjunctly in population DY and FP. Unique haplotype within population was only detected in population NY (haplotype 3). Among the four haolotypes, only haplotype 1 was shared by both sand and karst regions. Haplotype 2 and 3 were private in sand regions while haplotype 4 was only present in karst regions. The relatively higher haplotype richness in northern-edge populations (NY and CY) and the clear differentiation between eastern and western populations of Gula River were also reflected.

### Population and phylogeographic analysis

The average population genetic diversity values ($H_S = 0.179$) are considerably lower than total genetic diversity ($H_T = 0.564$). The comparison between $N_{ST}$ and $G_{ST}$ showed a significant difference ($N_{ST} = 0.816$; $G_{ST} = 0.684$; $P < 0.001$) at the species level, but this pattern is not found in each region (Table 3).

**Table 1** Variable sites of the aligned sequences of two cpDNA fragments in four haplotypes of *C. debaoensis*

| Haplotype | Polymorphic sites | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *atp*B-*rbc*L | | | | | | | *psb*A-*trn*H | |
| | 1 7 8 | 2 4 8 | 3 6 4 | 3 8 0 | 3 8 1 | 3 8 2 | 3 8 3 | 3 1 9 | 4 1 6 |
| H1 | A | C | C | T | – | – | – | G | + |
| H2 | C | A | A | T | T | T | T | A | – |
| H3 | A | C | C | T | – | – | – | A | – |
| H4 | A | C | C | – | – | – | – | G | + |

+ indicates presence of insertion (TAAAGAAGAGTACCAAACCT TTCTTTT)

– indicates deletions

**Table 2** Sample sizes and locations of the 11 *C. debaoensis* populations studied, along with cpDNA haplotype distribution, nucleotide diversity ($P_i$) and haplotype diversity ($H_d$) of cpDNA

| Region | Population code | Population | Latitude (N) | Longitude (E) | Altitude (m) | Haplotype | | | | Total | $H_d$ | $P_i$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | H1 | H2 | H3 | H4 | | | |
| Sand | BY | Baiyang, Yunnan | 23°43′ | 106°07′ | 260 | 18 | | | | 18 | 0.00000 | 0.00000 |
| | LW | Liuwang, Guangxi | 23°44′ | 106°10′ | 400 | | 10 | | | 10 | 0.00000 | 0.00000 |
| | XP | Xiapan, Guangxi | 23°42′ | 106°09′ | 410 | | 4 | | | 4 | 0.00000 | 0.00000 |
| | BW | Baiwei, Guangxi | 23°40′ | 106°08′ | 503 | | 11 | | | 11 | 0.00000 | 0.00000 |
| | CY | Cewai, Guangxi | 23°43′ | 106°08′ | 340 | 12 | 3 | | | 15 | 0.34286 | 0.00223 |
| | NY | Nayan, Guangxi | 23°47′ | 106°09′ | 297–519 | 6 | 5 | 1 | | 12 | 0.62121 | 0.00348 |
| | BM | Baiming, Guangxi | 23°47′ | 106°05′ | 319–406 | 10 | | | | 10 | 0.00000 | 0.00000 |
| | Subtotal | | | | | 46 | 33 | 1 | | 80 | 0.50538 | 0.00320 |
| Karst | FP | Fuping, Yunnan | 23°30′ | 106°14′ | 780 | 7 | | | 3 | 10 | 0.46667 | 0.00038 |
| | GC | Guichao, Yunnan | 23°37′ | 105°57′ | 1054 | 10 | | | | 10 | 0.00000 | 0.00000 |
| | NH | Longhuai, Guangxi | 23°28′ | 106°03′ | 1034 | 10 | | | | 10 | 0.00000 | 0.00000 |
| | DY | Dingye, Guangxi | 23°24′ | 106°01′ | 760 | 6 | | | 4 | 10 | 0.53333 | 0.00043 |
| | Subtotal | | | | | 33 | 0 | 0 | 7 | 40 | 0.29615 | 0.00024 |
| Total | | | | | | 79 | 33 | 1 | 7 | 120 | 0.49160 | 0.00132 |

**Table 3** Estimates of average gene diversity within populations ($H_S$), total gene diversity ($H_T$), interpopulation differentiation ($G_{ST}$), and the number of substitution types ($N_{ST}$) (mean ± SE in parentheses) within the karst region, the sand region and the entire range, calculated with permut, using a test with 1,000 permutations

| Region | $H_S$ | $H_T$ | $G_{ST}$ | $N_{ST}$ |
|---|---|---|---|---|
| The sand region | 0.138 (0.0939) | 0.575 (0.0204) | 0.760 (0.1702) | 0.778(0.1580) |
| The karst region | 0.250 (0.1450) | 0.310 (0.1340) | 0.194 (0.0961) | 0.194 (0.0961) |
| Entire range | 0.179 (0.0772) | 0.564 (0.0719) | 0.684 (0.1420) | 0.816 (0.1194) |

The AMOVA analysis showed that around 23% of the variation could be attributed to differentiation between the sand and karst regions and that between-population variation accounted for just over half (57%) of the total variation (Table 4), indicating very strong differentiation and little gene flow between regions and populations. Variation among populations was more significant in the sand region than in the karst region ($F_{ST} = 0.73556$ vs. $F_{ST} = 0.19355$, Table 4).

## Population relationships

Phylogenetic relationships were reconstructed among cpDNA haplotypes of *C. debaoensis*. Rooted at one sequence of *C. micholitzii*, maximum likelihood tree was obtained based on the genetic variation of the cpDNA sequences (Fig. 2a). Two clusters (I and II) were identified in the ML tree and supported with 78 and 84 bootstrap values. Clade I included two haplotypes (Hap1, Hap4) distributed in karst regions, clade II comprised two haplotypes (Hap2, Hap3) from sand regions. The haplotype network analyses also clustered all haplotypes into two groups with the same topological relationships (Fig. 2b). In general, the haplotypes differed by short mutations, suggesting that long distance migratory events have not occurred. Combined with the interior position in the haplotype network, the geographically widespread haplotype 1 might be ancient. Unfortunately, because of the limited genetic polymorphism, the phylogenetic relationship between the haplotypes was not resolved.

In this study, Bayesian estimates of the mutation rates and the age of the most recent common ancestor (TMRCA) of the cycad sequences were obtained using BEAST v. 1.6.1. All the cpDNA haplotypes coalesced at about 2.66 (95% CI 1.07–4.91) million years ago (MYA). Using a coalescence-based approach, times coalesced back to TMRCA were dated to 0.78 (95% CI 0.03–2.16) and 1.46 (95% CI 0.46–2.94) MYA for lineages I and II, respectively (Fig. 2). Almost all coalescences of the major lineages predate the LGM. The demographic scenarios for populations of *C. debaoensis* are represented by the Bayesian skyline plot (Fig. 3). Based on the pattern of

**Table 4** AMOVA analysis of *C. debaoensis* from the 11 populations using *atp*B-*rbc*L and *psb*A-*trn*H intergenic spacers of cpDNA

| Group of regions | Source of variation | d.f. | SS | VC | Variation (%) | Fixation index |
|---|---|---|---|---|---|---|
| Sand | Among populations | 6 | 56.362 | 0.81352 | 73.56 | $F_{ST} = 0.73556$** |
| | Within populations | 73 | 21.350 | 0.29247 | 26.44 | |
| | Total | 79 | 77.713 | 1.10599 | | |
| Karst | Among populations | 3 | 1.275 | 0.03000 | 19.35 | $F_{ST} = 0.19355$** |
| | Within populations | 36 | 4.500 | 0.12500 | 80.65 | |
| | Total | 39 | 5.775 | 0.15500 | | |
| Karst and sand | Among groups | 1 | 18.429 | 0.22637 | 23.00 | $F_{SC} = 0.74160$** |
| | Among populations within groups | 9 | 56.363 | 0.56216 | 57.11 | $F_{ST} = 0.80102$** |
| | Within populations | 109 | 21.350 | 0.19587 | 19.90 | $F_{CT} = 0.22996$** |
| | Total | 119 | 96.142 | 0.98440 | | |

*d.f.* Degrees of freedom, *SS* sum of squares, *VC* variance components, ** $P < 0.001$

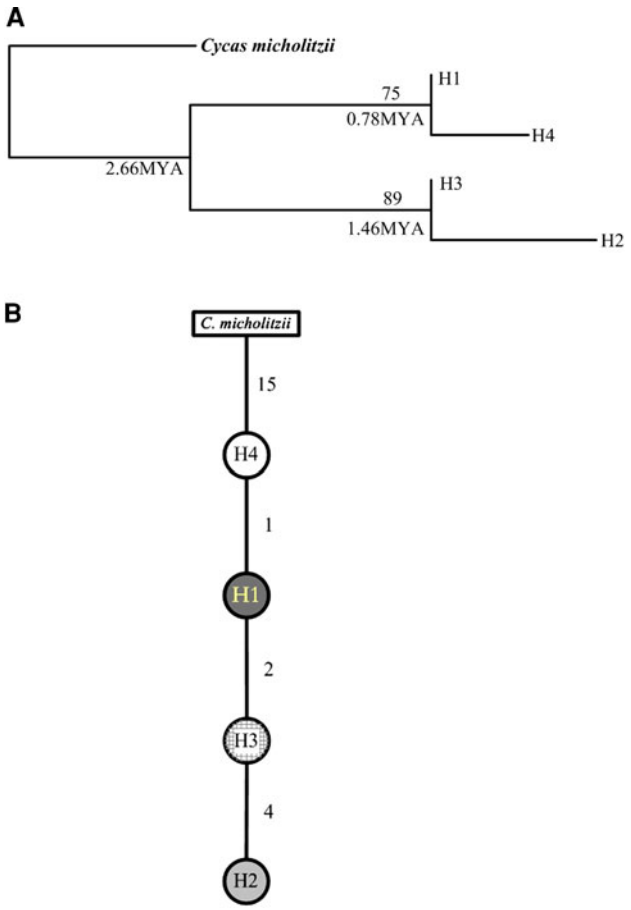


**Fig. 2** (**a**) Maximum likelihood (ML) tree of cpDNA haplotypes. Bootstrap values were indicated at nodes. Major cpDNA lineages are indicated. The divergence time between the two major lineages, and the coalescence time for each lineage are indicated at nodes. (**b**) The network of cpDNA haplotypes. The numbers present the mutation steps in the haplotype network

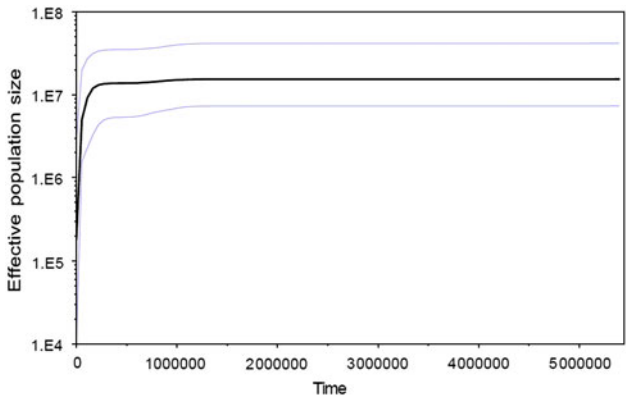


**Fig. 3** Bayesian skyline plot based on the cpDNA *atp*B-*rbc*L and *psb*A-*trn*H intergenic spacers of *C. debaoensis* for the population size fluctuations throughout time. The population size was measured as the effective population size per generation. (*black line*, median estimations; area between *gray lines*, 95% confidence interval)

variation in cpDNA, a long history of constant population size of *C. debaoensis*, followed by a evident decline (bottleneck) in Quaternary glaciations with no subsequent expansion is recovered.

### Demographic analysis

The mismatch distributions for all the populations combined were multimodal (Fig. 4), indicating a demographic equilibrium. The SSD value (0.03096, *P*-value 0.01091) and raggedness index (0.10126, *P*-value 0.03818) of the overall populations also reject a sudden expansion model (Table 5). Both Tajima's *D* value (2.24110, $P < 0.05$) and Fu & Li's $D^*$ value (1.30715, $P > 0.10$) support the result of the mismatch distribution. The positive values of Tajima's *D* and Fu & Li's $D^*$ statistics for both the sand and karst regions also provided no evidence of population expansion in *C. debaoensis* (Table 5).

## Discussion

### Population structure

As long-lived, dioecious gymnosperms, the levels of genetic variation in cycads should theoretically be

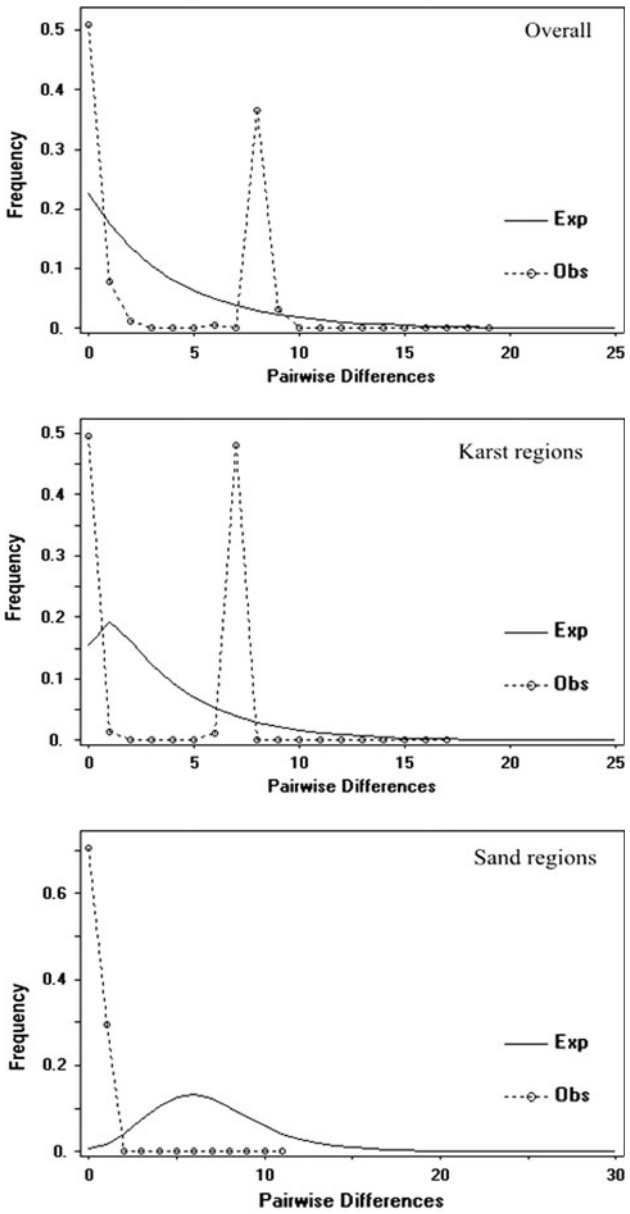


**Fig. 4** Mismatch distribution of the *C. debaoensis* cpDNA *atp*B-*rbc*L and *psb*A-*trn*H intergenic spacers for the sampled populations overall, and for the karst and sand regions separately

relatively high within populations and relatively low between populations (Hamrick et al. 1992). A high level of genetic variation do characterize some of the New World *Dioon* species (González-Astorga et al. 2003; Cabrera-Toledo et al. 2008; GonzáLez-Astorga et al. 2008a), where high levels of genetic variation within populations have been found. However, in contrast to expectations, the genetic structure of *C. debaoensis* found in this study was characterized by low genetic variation within populations and high genetic differentiation between populations ($H_d = 0.49160$, $P_i = 0.00132$; $F_{ST} = 0.80102$), which is similar to that reported in a previous study of *C. revoluta* ($h = 0.641$, $\pi = 0.00071$; $F_{ST} = 0.82724$) based on organelle DNA variation (Kyoda and Setoguchi 2010).

In this study, the total genetic diversity ($H_T = 0.564$) of *C. debaoensis* uncovered by two noncoding regions was relatively low, compared with the mean genetic diversity value estimated from cpDNA-based studies of 170 plant species ($H_T = 0.67$) (Petit et al. 2005). Like most other cycads, the rather limited distribution and small populations of *C. debaoensis* may mainly be the result of Pleistocene glaciations (cf. Cabrera-Toledo et al. 2010; Xiao et al. 2004), Thus, genetic diversity within these populations would have been reduced by severe bottleneck effects and genetic drift. There is distinct reduction in the genetic diversity of *C. debaoensis* from the sand region to the karst region (Table 2). Compared to the continuous distribution of the sand-type populations, the karst-type populations are scattered on isolated limestone hills. Random losses of rare alleles and reduced genetic diversity are more likely in the latter, as a result of the high levels of genetic drift and inbreeding in the smaller and isolated populations (Barrett and Kohn 1991; Ellstrand and Elam 1993).

The within-population diversity of *C. debaoensis* revealed by both cpDNA markers was pretty low, coupled with strong population differentiation ($H_S = 0.179$, $N_{ST} = 0.816$, $G_{ST} = 0.684$). The high level of interpopulation differentiation recorded within this species is probably due to several factors which could either cause inbreeding or slow down the gene flow. First, most of the geographical distances among extant populations of *C. debaoensis* far exceed the limited effective gene flow distance (2–7 km) between local populations of *Cycas* (Yang and Meerow 1996). Second, in cycads there is maternal inheritance of cpDNA (Cafasso et al. 2001). Compared to biparentally and paternally inherited genomes, maternally inherited markers generally exhibit much

**Table 5** Results of the mismatch distribution analysis and neutrality tests in the populations overall, and separately in the karst and sand regions

| Populations | SSD | *P*-value | Raggedness index | *P*-value | Tajima's *D* | Fu & Li's $D^*$ |
|---|---|---|---|---|---|---|
| Overall | 0.03096 | 0.01091 | 0.10126 | 0.03818 | 2.24110** | 1.30715 |
| Sand region | 0.04864 | 0.01429 | 0.15913 | 0.07714 | 3.48471*** | 1.20971 |
| Karst region | 0.01146 | 0.09750 | 0.12778 | 0.13250 | 0.37079 | 0.56369 |

** $P < 0.05$, *** $P < 0.001$

higher population subdivision, since seeds are normally distributed over shorter distances than pollen (Liepelt et al. 2002). In *C. debaoensis*, gravity and rodents may serve as the main dispersal agents for the subglobose seeds. These large, heavy seeds (diameter: 3 cm; weight: 5 g; averagely) are unlikely to be transported far by rodents. This may result in siblings growing in close proximity to each other or to the maternal plant and, consequently, inbreeding. Thus, the low level of gene flow and the high level of interpopulation differentiation were expected. In addition, the gene flow may have been reduced because of human-influenced factors. During the field survey we noticed a clear dominance of young plants in the wild populations of *C. debaoensis*; individuals of flowering age are uncommon. This population pattern is mainly the result of human activity, because local villagers dig up mature, female individuals which are more commercially valuable.

## Phylogeographic analysis

Fossil evidence has provided critical information on the demographic history of plants (Hewitt 1996, 2000). The hitherto most completely preserved cycad specimen is collected from northeast China (Liaoning, 42°N) and can be traced back to the Upper Triassic (Zhang et al. 2010; Wang et al. 2009). Another Fossil cycadalean leaves was recorded from the Turkish (Soma, 39°N) Miocene (Erdei et al. 2010). Analysis on the morphology indicates that these fossils are closely related to living Zamiaceae in Cycadales. In contrast, the north most distribution of living Zamiaceae is seen in southern USA (Georgia, 31°N) (Wang et al. 2009). These evidence supported the hypothesis that cycads might migrate southward gradually since late Triassic as the northern hemisphere became more and more dry (Guan and Zhou 1996).

*Cycas debaoensis* has a limited geographical range along the border between Guangxi and Yunnan provinces (23°N), and exists in rather small and isolated populations. Such geographical isolation of the populations of most cycads results from the climate oscillations during the Pleistocene (González-Astorga et al. 2005; González-Astorga et al. 2008b). According to a palaeovegetation reconstruction based on fossil evidence, the hot-dry valley of southwest China is thought to be the most likely refugium for cycads of East Asia during the Quaternary glaciations (Guan and Zhou 1996). These regions to which current *C. debaoensis* populations are restricted probably represent their former distribution and the existence of several small refugia can be deduced.

Under the single refuge hypothesis, newly originated haplotypes would usually form a star-shaped phylogeny, surrounding the basic haplotype (Avise 2000); however, we failed to detect such a pattern in *C. debaoensis*. In addition, based on mismatch distribution analysis and neutrality tests, we found no evidence of long-distance dispersal or population expansion. As some other ancient and drought-resistant species in southwest China (Opgenoorth et al. 2010; Wang et al. 2010), *C. debaoensis* may have been able to endure cold conditions during the glacial periods and may have remained in multiple microrefugia throughout its current range. The cpDNA haplotypes might represent ancient polymorphism rather than recent gene flow and expansion (Schaal et al. 1998). The phylogeographic structure of cpDNA variation in *C. debaoensis* supports this hypothesis.

Within the sand region, the dominant haplotypes 1 and 2 are associated with populations on western and eastern bank of the Gula River, respectively. Since that the heavy seeds of *C. debaoensis* sink in water, the Gula River might act as a contemporary barrier to gene flow. Relatively high genetic and haplotype diversities were detected in the newly discovered populations (NY and CY), which located at intermediate locality of sand regions; this is probably the consequence of the admixture of these two haplotypes (1, 2) colonizing the area from separate sources (Petit et al. 2003). Moreover, populations NY may have long survived over the periodical glaciations, as supported by the existence of private haplotype (3) within population. In addition, Van Valen (1965) suggested that morphological variation in different populations may be adapted to different environments. Therefore, both the decrease in the number and increase in the width of leather leaves of the newly found populations (NY, CY and BM) can be considered to represent morphological adaptations to the less drought-prone environment provided by the sandy substrate (Cai et al. 1999).

In the karst region, no clear phylogeographical structure was detected. The ancestral haplotype 1 was found in all populations across this area. All populations here have tripinnate leaves and large numbers of megasporophylls lobes, which are considered to be ancient characteristics (Wang 2007). Because of geological changes during the late Triassic, north China became a drought-prone area and cycad distribution became centered on warm-humid southwestern China (Guan and Zhou 1996). This region thus provided sheltering for the surviving species, including *C. debaoensis*, over the periodical glaciations. Thus, we deduce that in the last interglacial period the relatively ancient haplotype 1 tended to inhabit the arid karst region which was similar to the droughty paleoenvironment of cycads in northern China. The morphological characteristics of *C. debaoensis* in the karst region could be regarded as the preservation of ancient morphological characters. In the karst region, private haplotype 4 has a disjunct distribution. The distance between the two locations of haplotype 4 (populations FP and DY) is more than 30 km. This haplotype might have occurred widely in the past, but currently it is only retained in two

larger populations in this area. This suggests that rare haplotypes are being lost as populations decline.

The genetic differentiation between the sand type and karst types of *C. debaoensis* was high (23%). The cpDNA haplotypes 2 and 3 were restricted to the sand region, and haplotype 4 was found only in the karst region. Thus, both areas had unique cpDNA haplotypes, which could be considered to be the indicators of refugia (Ikeda et al. 2008). This and the results of the ISSR studies of *C. debaoensis* by Xie et al. (2005), suggest past population fragmentation between sand and karst regions.

It has been known that the Quaternary glaciers largely shaped the distribution of plant species (Hewitt 2000). During glacial population contraction and post-glacial expansions, *C. debaoensis* may have experienced extensive bottleneck or founder effects resulting in a single haplotype becoming fixed in most surviving populations. In the study, the molecular dating showed a coalescence time about 2.66 MYA, while the latest division happened about 0.78 MYA. These molecular dating revealed that the cpDNA polymorphisms may have long been maintained since the Pleistocene. Based on seed dispersal (3–10 m/year on average) from a maternal tree and the age of maturity (ca. 20–50 years old) of *C. debaoensis* (personal investigation), ca. 0.300–0.675 million years would be needed to cover the distribution range of the dominant haplotypes. Combined with the Bayesian skyline plot analysis, it is possible that *C. debaoensis* used to have a wider geographic rang and has recently experienced contraction. Till the time of the Last Glacial Maximum (LGM, 23–19 Ka) or even earlier glaciations, the continuously distributed populations of *C. debaoensis* might have contracted to occupy the current sand and karst regions, representing two isolated refugia.

In conclusion, our results are in accordance with a number of previous palebotanical studies of cycads (Li et al. 1976; Li 1982; Guan and Zhou 1996) as follows. First, Cycads had no mass migration during glacial periods. Second, southwest China provided the refugia for cycads during Quaternary glacial periods. Modern cycads are relicts of ancient cycads that survived in refugia. Besides glacial effects, the current restricted distribution and isolated small populations of *C. debaoensis* may due to recent rapid habitat destruction, limited dispersal of seeds and human disturbance. Rare haplotypes may have been lost with the rapid decline in the number of wild individuals caused by extensive commercial exploitation and habitat fragmentation in recent years.

## Conservation

As a long-lived gymnosperm, *C. debaoensis* has extreme tolerance of harsh climates and less fertile soils (Ma et al. 2003). However, in recent years, this precious species has been seriously affected by anthropogenic activities, resulting in significant losses of wild individuals. An extremely low level of genetic diversity at both population and species levels in *C. debaoensis* was revealed by our data. Although we examined several molecular markers that had been informative in other analogous studies, most of them proved monomorphic or exhibited low variability. Failure to find variability can be considered valuable because it 'confirms' prior expectations that a population or species is impoverished (Amos and Harwood 1998). Maintenance of genetic diversity is crucial to the survival of organisms because it allows them to evolve and adapt to changing environmental conditions (Frankel and Soulé 1981; Lynch 1996; Maxted et al. 1997), so effective measures to protect this species against further loss of genetic diversity are urgently needed.

Considering the high genetic differentiation of *C. debaoensis*, the loss of a single population might significantly reduce overall genetic diversity, so preservation of any one population would be insuffcient to conserve all the variation in the species. Moreover, our field surveys indicated that most populations of *C. debaoensis* were small in size with few mature females. Population size is the most influential factor of the five criteria for listing species as endangered under the International Union for the Conservation of Nature (IUCN) system (Frankham et al. 2002). Almost all extant populations of *C. debaoensis* face a serious threat of extinction due to stochastic processes because of low genetic diversity and small population sizes. Hence, all populations should be protected in situ from further human disturbances or other damage (Jian et al. 2006). Populations that have the significant distinct haplotypes need to be carefully protected (Liao et al. 2007). Our results reveal that the highest haplotype diversity of *C. debaoensis* is in the newly discovered populations (NY and CY) which exhibit morphological variation. Thus, to protect them in situ and prevent anthropogenic destruction is of great importance.

Populations that show haplotype frequency differences have been termed Management Units, or MUs (Moritz 1994) because they are assumed to be demographically independent with a limited amount of gene flow and hence should be managed separately. In our study, AMOVA analysis indicated the sand and karst regions account for 23% of genetic variance, however, it is also difficult to determine the suitability of these regions as conservation management units based on these data. Although there is a lack of remarkable genetic differences, ecological isolation may exist in between these regions. That is, priority should be given to conserving areas of connecting habitat to promote population connectivity and maintain adaptive diversity and evolutionary potential (Burns et al. 2007).

Other main threats to the species are habitat deterioration and loss that may result from global warming (Root et al. 2003; Franco et al. 2006). For the sake of preserving the genetic resources of *C. debaoensis*, *ex situ* conservation strategies, based on germplasm collections, in nature reserves or botanical gardens would be of practical value. Self-sustaining wild populations should be reestablished via reintroductions and introductions, and then be extended into suitable habitats within the previous range of the species (Frankham et al. 2002). If *ex situ* conservation is put in practice, samples should be collected from as many populations as possible, especially from those harbouring relatively high genetic diversity, such as population CY, NY, DY and FP.

Despite the fact that all cycads in China are protected by law, it is essential that the population be made aware of the importance of the preservation of this 'living fossil'. Effective education and information dissemination are needed. More genetic and field studies are required to ensure better protection of this rare species. Further work on the role of selection and potential breeding barriers acting between ecotypes, and the extent to which environment differences are affecting contemporary differentiation in this rare species, are recommended. Additionally, to obtain a better understanding of the factors that have influenced the evolutionary history of flora in the Southwest China, studies on a wide range of different species endemic in this region are urgently needed.

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