CSIRO PUBLISHING
www.publish.csiro.au/journals/ajb *Australian Journal of Botany*, 2005, **53**, 219–223

Comparison of the genetic diversity in two species of cycads

Longqian Xiao[A,B], *Xun Gong*[A,D], *Gang Hao*[C], *Xuejun Ge*[C], *Bo Tian*[A] *and Sixiang Zheng*[B]

[A]Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, P.R. China.
[B]Yunnan Agriculture University, Kunming 650201, P.R. China.
[C]South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, P.R. China.
[D]Corresponding author. Email: gongxun@mail.kib.ac.cn

Abstract. Inter-simple sequence repeat (ISSR) markers were used to examine the level and distribution of genetic diversity in two cycad species: *Cycas parvula* S.L.Yang and *C. balansae* Warburg. The former is found in only two adjacent populations and the latter in a relatively wider distribution. Although genetic diversity in *C. balansae* ($H_e = 0.1301$) is higher than that in *C. parvula* ($H_e = 0.0538$), both are still low in comparison with the mean value ($H_e = 0.169$) in gymnosperms. This confirms the general opinion that cycads are genetic relics. The genetic differentiation in both species, however, presents a striking contrast: G_{st} is 0.0978 in *C. parvula*, but 0.4003 in *C. balansae*, which may be ascribed to the difference in distances between their populations.

Introduction

Genetic diversity of species is a prerequisite for their future adaptive change and evolution in the long term (Hamrick and Godt 1996). Since electrophoretic techniques were first applied to examine isozyme variation in populations (Lewontin 1974), genetic studies, with various objectives such as genetic conservation and breeding programs, have documented the level and distribution of genetic diversity in a wide range of species, especially those that are rare or highly restricted distributed.

Cycads (Cycadaceae, Stangeriaceae, Zamiaceae) are a unique group of primitive seed plants. Their origin goes back to the Lower Permian (Zhifeng and Thomas 1989). Although once extremely abundant across the globe in the Mesozoic, today cycads have a limited range, with relict and isolated populations in tropical and subtropical regions, and most species endangered and facing possible extinction in the wild (Osborne 1995). These living survivors with over 250 million years of history may serve as a possible window to address the origin and evolution of all other extant seed plants.

Because of the antiquity and rarity of cycads, biologists have become more interested in them recently. Previous studies have concentrated on ecology and taxonomy (Jones 1993; Guang 1996; Wang and Liang 1996; Huang 2001), although genetic-diversity studies have also been conducted on a very small number of cycads. Most genetic-diversity studies have shown that cycads are characterised by low genetic variation within populations and high genetic differentiation among populations. However, the opposite has been also found in *Macrozamia riedlei*, *M. parciflia* and *M. heteromera* (Table 1). Thus, the rule of genetic diversity in cycads, which will facilitate the effective conservation measures for this endangered group, is not clear-cut.

In this study, we present the results of ISSR variation and analyses of two cycad species, *Cycas parvula* and *C. balansae* (Cycadaceae). The former species is found in just two adjacent regions in Yuanjiang county of south-western China, and the distance between the two extant populations is only 7.3 km. The latter species has a wider range, which is highly fragmented in south-western China, North Vietnam, Thailand, Burma and Laos (Huang 2001), with populations being separated by distances of more than 30 km. The main aims of this study are to (i) document the levels and distributions of genetic diversity in these two cycad species, (ii) find possible factors which influence the distribution of genetic variation and the rule of the genetic diversity in cycads and (iii) present reasonable genetic conservation measures for cycads.

Materials and methods

Leaf samples were obtained from 98 individuals representing two and three extant populations of *C. parvula* and *C. balansae*, respectively (Fig. 1, Table 2). Fresh leaflets were dried with silica gel and stored at 4°C until DNA extraction. Vouchers were collected from each population and deposited at the herbarium of Kunming Institute of Botany, Chinese Academy of Science (KUN).

Total DNA was extracted according to the modified CTAB protocol (Doyle 1991). DNA was then PCR-amplified by using ISSR primers obtained from the University of British Columbia. Reactions were carried out in a total volume of 20 μL consisting of 20 ng of template DNA, 2.0 μL 10 × PCR buffer, 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 2%

 10.1071/BT04052 0067-1924/05/030219

Table 1. The level and distribution of genetic diversity in cycads

N, number of studied populations; *P*, percentage of polymorphic loci; A_e, effective number of alleles per locus; *I*, Shannon's information index; H_e, Nei's genetic index; G_{st}, genetic differentiation based on H_e

Species	*N*	*P*	A_e	*I*	H_e	G_{st}	Method	Reference
Macrozamia communis	5	50.0	1.61	0.045	–	0.270	Allozyme	Ellstrand *et al*. (1990)
Macrozamia riedlei	15	93.0	2.43	0.274	0.263	0.092	Allozyme	Byrne and James (1991)
Zamia pumia	2	16.7	1.75	0.041	0.047	–	Allozyme	Walters and Decker-Walters (1991)
Cycas pectinata	11	58.5	1.82	0.076	0.066	0.387	Allozyme	Yang and Meerow (1996)
Cycas siamensis	13	58.9	1.48	0.134	0.114	0.291	Allozyme	Yang and Meerow (1996)
Macrozamia parcifolia	2	–	–	0.172	0.155	0.09	Allozyme	Sharma *et al*. (1998)
Macrozamia heteromera	–	58.0	1.30	0.060	–	0.100	Allozyme	Sharma *et al*. (1999)
Cycas panzhihuaensi	5	–	–	–	0.049	0.345	Allozyme	Yang and Meerow (1999)
Cycas seemannii	5	50.0	1.80	0.138	0.048	0.418	Allozyme	Keppel *et al*. (2002)
Cycas guizhouensis	12	35.9	–	0.169	0.108	0.432	ISSR	Xiao *et al*. (2004)
Cycas parvula	2	25	1.09	0.083	0.054	0.098	ISSR	Present study
Cycas balansae	3	40.0	1.23	0.192	0.130	0.400	ISSR	Present study

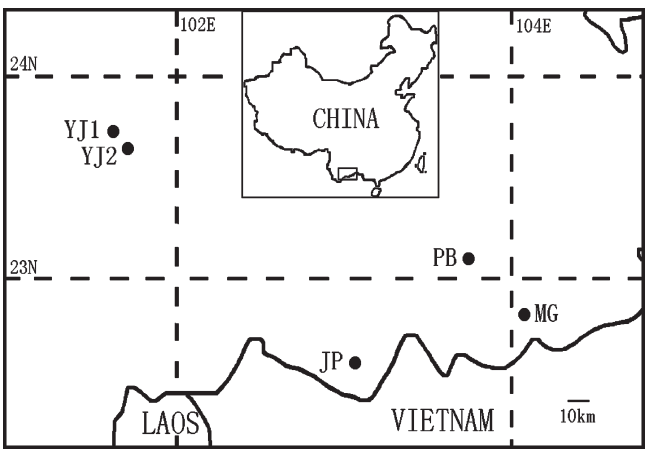

Fig. 1. Map showing locations of the sampled populations of *Cycas parvula* and *C. balansae*.

formamide, 200 nM primers, 1.0 unit of *Taq* polymerase and double-distilled water. PCR was performed with an MJ Research 96-well thermal cycler with a hot bonnet according to the method of Ge and Sun (1999). Amplification products were electrophoretically separated in 1.5% agarose gels buffered with 0.5 × TBE. A 100-bp DNA ladder (New England Biolabs) was used as a size marker. A negative-control reaction in which the template DNA was replaced by water was performed alongside every PCR amplification in order to verify the absence of contamination. DNA fragments were identified by image analysis software for gel documentation (LabWorks Software Version 3.0; UVP, Upland, CA 91786, USA) after staining with ethidium bromide.

Following an initial screen of 100 primers, 12 primers (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.

ISSR bands were scored as present or absent binary characters, excluding smeared and weak bands. The resulting presence/absence data matrix was analysed by POPGENE Version 1.31 (Yeh *et al*. 1999) to estimate the level and distribution of genetic diversity. Parameters for the level of genetic diversity were the percentage of polymorphic loci (*P*), the effective number of alleles per locus (A_e), Nei's diversity index (H_e) and Shannon's information index (*I*). The distribution of genetic diversity was measured by the coefficient of genetic differentiation (G_{st}), which was measured by following Nei's index (Nei 1973). Gene flow (N_m) was then calculated as $(1 - G_{st})/4G_{st}$ (Slatkin and Barton 1989).

In addition, an analysis of molecular variance, AMOVA Version 1.5 (Excoffier 1993), was used to analyse the proportion of the genetic diversity within and among populations.

Results

Table 3 describes the polymorphism in these two species revealed by ISSR. The 12 primers generated a total of 98 markers for *C. parvula* and 102 for *C. balansae*, with 24.65% and 34.95% polymorphic loci, respectively (evaluated at the 99% level). A_e values of 1.0852 in *C. parvula* and 1.2278 in *C. balansae* are rather similar; however, there is a relatively obvious distinction in H_e and *I* between these two species: at the population level, *I* ranges from 0.0643 to 0.0843 and H_e ranges from 0.0419 to 0.0546 in *C. parvula*, whereas *I*

Table 2. Populations of *Cycas parvula* and *C. balansae* for ISSR analysis

Species	Population code	Locality (county)	Latitude, longitude	Altitude (m)	Est. population size	Sample size
C. parvula	YJ1	Yuanjiang	23°42′N, 101°47′E	1454	30	18
	YJ2	Yuanjiang	23°40′N, 101°50′E	1445	80	20
C. balansae	JP	Jinping	22°41′N, 103°00′E	389	40	20
	MG	Maguan	22°51′N, 104°07′E	610	60	20
	PB	Pingbian	23°03′N, 103°43′E	745	50	20

Table 3. Genetic diversity in *Cycas parvula* and *C. balansae* as detected by ISSR analysis
A_e, effective number of alleles per locus; I, Shannon's information index; H_e, Nei's genetic index; P, percentage of polymorphic loci

Species	Population	A_e (s.d.)	I (s.d.)	H_e (s.d.)	P-value
C. parvula	Total	1.0852 (0.2137)	0.0833 (0.1920)	0.0538 (0.1278)	24.65
	YJ1	1.0690 (0.2092)	0.0643 (0.1751)	0.0419 (0.1183)	13.59
	YJ2	1.0880 (0.2242)	0.0843 (0.1942)	0.0546 (0.1301)	17.48
C. balansae	Total	1.2278 (0.3581)	0.1921 (0.2783)	0.1301 (0.1937)	34.95
	JP	1.1126 (0.2650)	0.1013 (0.2154)	0.0669 (0.1472)	20.39
	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

Table 4. Analysis of molecular variance (AMOVA) for the populations of *Cycas parvula* and *C. balansae* by using ISSR markers

Species	Source of variance	d.f.	SSD	MSD	Variance component	%Total	P-value[A]
C. parvula	Among population	1	12.1	12.102	0.467	12.55	<0.001
	Within population	36	117.2	3.254	3.254	87.45	
C. balansae	Among population	2	122.1	61.050	2.813	37.02	<0.001
	Within population	57	272.8	4.786	4.785	62.98	

[A]Significance tests after 5000 permutations.

ranges from 0.1013 to 0.1491 and H_e from 0.0669 to 0.0996 in *C. balansae*. At the species level, I is 0.0833 and H_e is 0.0538 in *C. parvula*, and 0.1921 and 0.1301 in *C. balansae*, respectively.

The coefficient of genetic differentiation among populations (G_{st}) is 0.0978 for *C. parvula* and 0.4003 for *C. balansae*, and the level of gene flow (N_m) is 2.3058 and 0.3746, respectively. This result is corroborated by AMOVA analysis (Table 4): in *C. parvula*, only 12.55% of the total genetic diversity is distributed among populations; however, in *C. balansae* this value is 37.02%.

Discussion

The effective number of alleles per locus (A_e) and Nei's diversity index (H_e) are two important parameters of the level of genetic diversity. The former reflects the allelic richness, whereas the latter reflects the evenness as well as the richness of alleles, which is more accurate for estimating the genetic variation. In the present study, A_e values in both species are similar, but H_e in *C. balansae* is approximately twice that observed in *C. parvula* (Table 3). This may be related with a severe habitat decline in both extent and quantity in *C. parvula* as well as with overcollection and exploitation. This species has been extinct across most of the known distribution, including Honghe, Shiping and Jianshui counties in Yunnan province (Wang and Liang 1996), for several years and is now represented by two adjacent populations in Yuanjiang county. The remaining few individuals in an extremely narrow range must have been experiencing strong uniform selection effects and genetic drift, resulting in genetic erosion. The similarity and discrepancy in these two species indicate that the allele number of *C. parvula* is in the process of rapid decline, whereas that of *C. balansae* remains relatively stable.

Genetic diversity in *C. balansae* ($H_e = 0.1301$) is higher than that in *C. parvula* ($H_e = 0.0538$), but both are still low in comparison with the mean value ($H_e = 0.169$) of the gymnosperms (Hamrick *et al*. 1992), particularly at the population level (Table 3). This is consistent with the hypothesis that low genetic variation within populations is biologically and evolutionarily typical for cycads (Walters and Decker-Walters 1991). However, the distribution of genetic diversity among populations contrasts dramatically between these two species. Low differentiation exists in *C. parvula* ($G_{st} = 0.0978$), whereas high genetic differentiation occurs in *C. balansae* ($G_{st} = 0.4003$). The

relatively high genetic diversity in *C. balansae* at species level may result from the wider geographic range and more significant genetic differentiation. In conclusion, our findings agree with most previous reported ones. However, the lower genetic differentiation in *C. parvula* is much closer to the few results found in *Macrozamia riedlei*, *M. parciflia* and *M. heteromera* (Table 1).

The level and distribution of genetic diversity in natural populations of cycads are probably a comprehensive consequence of their evolutionary history, reproduction mode and pattern of distribution. Most cycads have a restricted geographical range, with disjunctive populations. The genetic consequences of habitat fragmentation depend on such factors as distance, continuity and remnant size (Saunders *et al*. 1991). Both cycads studied here are distributed patchily, with small and isolated populations, and the estimated number of individuals in each population is no more than 80 (Table 2). This distribution pattern may be an effect of Pleistocene glaciations, which at their maximum would have restricted these species to refugia, and such areas are expected to have been small (Xiao *et al*. 2004). Small population size as well as isolation for long periods of time are usually accompanied by inbreeding and genetic drift. In addition, the reproduction modes of cycads, such as their dispersal of seed and pollen, further contribute to the occurrence of inbreeding (mentioned in detail below). These two factors are likely to contribute to the low level of genetic diversity and high genetic differentiation in *C. balansae*. The same reasons and its much narrower range could also explain the very low genetic diversity in *C. parvula*.

In addition, phylogenetic studies of cycads have shown that the genus *Cycas* diverged from other cycads first in the Mesozoic but genus *Macrozamia* occurred in the Miocene and Pliocene (Norstog and Nicholls 1997; Treulein and Wink 2001). So there has been a much longer time for *Cycas* to diversify prior to the isolation in the Pleistocene than for *Macrozamia*. This may also be another important reason why *M. riedlei*, *M. parciflia* and *M. heteromera* showed low genetic differentiation, whereas the opposite is found in most *Cycas* species (Table 1).

Dispersal ability and the distance between populations may account for the lower genetic differentiation in *C. parvula*. Dispersal ability mainly involves seed and pollen migration. The large seeds, whose diameters are 2.3–3.0 cm in *C. parvula* and 2.0–2.5 cm in *C. balansae* (Wang and Liang 1996), are constrained in movement by their dispersal agents. Field observations suggest that seeds of both species are mainly dispersed within very small circles (radius <5 m) by gravity and rodents (also see Schneider *et al*. 2002) as seedlings often occur in clusters around the mother plant. Thus, dispersal via seed is unlikely to prevent genetic differentiation between populations in either species. The other means of dispersal, by comparative heavy pollen in *Cycas* (Norstog and Nicholls 1997), is mainly by beetles, and its dispersal distance is estimated to be 2–7 km between local populations (Yang and Meerow 1996), which is further verified by the study of *C. guizhouensis* (Xiao *et al*. 2004). The distance between *C. parvula* populations is just 7.3 km, but the distance between *C. balansae* populations is more than 30 km, which implies that dispersal by pollen can happen to great degree between the former species, but is unlikely between the latter populations. This is also supported by the N_m value of these two species derived from the present study: the estimate of N_m was 2.3058 and 0.3746 in *C. parvula* and *C. balansae*, respectively. Theoretically, $N_m > 1$ is enough to prevent genetic differentiation among populations through genetic drift (Wright 1965). The low N_m value in *C. balansae* indicates that genetic drift is likely to occur. However, some gene movement between populations of *C. parvula* makes its gene pool homogenise effectively. Therefore, it is believed that the difference in distances between populations in these two species is responsible for the distinction in their genetic differentiation.

Declining numbers of individuals and low genetic diversity in both *C. parvula* and *C. balansae* show that both of them might be susceptible to environmental change and disease (Ellstrand and Elam 1993). Protection of all existing populations *in situ* against further decrease in population number and size and loss of genetic diversity, is urgent. On the other hand, with different genetic differentiation available in these two species, different strategies for sampling and propagation should be taken when *ex situ* conservation is required. For *C. balansae*, collections should be made from as many populations and mothers as possible, especially from populations harbouring relatively high genetic diversity, such as Population Maguang. But a strategy would be to examine the genetic diversity in these two species from the whole range to target specific populations for germplasm conservation.

Acknowledgments

This work is supported by the National Natural Science Foundation of China (No. 30070081). The manuscript was much improved by comments and suggestions from two anonymous reviewers.

References

Byrne M, James SH (1991) Genetic diversity in the cycad *Macrozamia riedlei*. *Heredity* **67**, 35–39.

Doyle J (1991) DNA protocols for plants—CTAB total DNA isolation. In 'Molecular techniques in taxonomy'. (Eds GM Hewitt, A Johnston) pp. 283–293. (Springer-Verlag: Berlin)

Ellstrand NC, Elam DR (1993) Population genetic consequences of small population size: implication for plant conservation. *Annual Review of Ecology and Systematics* **24**, 217–242. doi: 10.1146/annurev.es.24.110193.001245

Ellstrand NC, Ornduff R, Clegg JM (1990) Genetic structure of the Australian cycad, *Macrozamia communis* (Zamiaceae). *American Journal of Botany* **77**, 677–681.

Excoffier L (1993) 'Analysis of molecular variance (AMOVA) Version 1.5. Genetics and biometry laboratory.' (University of Geneva: Switzerland)

Ge XJ, Sun M (1999) Reproductive biology and genetic diversity of a cryptoviviparous mangrove *Aegiceras corniculatum* (Myrsinaceae) using allozyme and intersimple sequence repeat (ISSR) analysis. *Molecular Ecology* **8**, 2061–2069. doi: 10.1046/j.1365-294x.1999.00821.x

Guang ZT (1996) 'Cycads of China.' (Sichuan Science and Technology Press: Chengdu, P.R. China)

Hamrick JL, Godt MJW, Sherman-Broyles SL (1992) Factors influencing levels of genetic diversity in woody plant species. *New Forest* **6**, 95–124. doi: 10.1007/BF00120641

Hamrick JL, Godt MJW (1996) Conservation genetics of endemic plant species. In 'Plant population genetics, breeding and genetic resource'. (Eds JC Acise, JL Hamrick) pp. 43–46. (Sinauer: Sunderland, MA)

Huang YY (2001) 'Systematics and evolution of cycads in China.' (Meteorology Press: Beijing)

Jones DL (1993) 'Cycads of the world.' (Reed New Holland: Sydney)

Keppel G, Lee SW, Hodgskiss PD (2002) Evidence for long isolation amongst populations of a Pacific cycad: genetic diversity and differentiation in *Cycas seemannii* A.Br. (Cycadaceae). *Journal of Heredity* **93**, 133–139. doi: 10.1093/jhered/93.2.133

Lewontin RC (1974) 'The genetic basis of evolutionary change.' (Columbia University Press: New York)

Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences, USA* **70**, 3321–3323.

Norstog KJ, Nicholls TJ (1997) 'The biology of the cycads.' (Cornell University Press: New York)

Osborne R (1995) The world cycad census and a proposed revision of the threatened species status for cycad taxa. *Biological Conservation* **71**, 1–12. doi: 10.1016/0006-3207(95)91086-D

Saunders DA, Hobbs RJ, Margules CR (1991) Biological consequences of ecosystem fragmentation: a review *Conservation Biology* **5**, 18–32.

Schneider D, Wink M, Sporer F (2002) Cycads: their evolution, toxins, herbivores and insect pollinators. *Die Naturwissenschaften* **89**, 281–294. doi: 10.1007/s00114-002-0330-2

Sharma IK, Jones DL, Forster PI, Young AG (1998) The extent and structure of genetic variation in the *Macrozamia pauli-guilielmi* complex (Zamiaceae). (Zamtaceae). *Biochemical Systematics and Ecology* **26**, 45–54. doi: 10.1016/S0305-1978(97)00094-X

Sharma IK, Jones DL, Forster PI, Young AG (1999) Low isozyme differentiation amongst five species of the *Macrozamia heteromera* group (Zamtaceae). *Biochemical Systematics and Ecology* **27**, 67–77. doi: 10.1016/S0305-1978(98)00065-9

Slatkin M, Barton NH (1989) A comparison of three indirect methods for estimating average levels of gene flow. *Evolution; International Journal of Organic Evolution* **43**, 1349–1368.

Treulein J, Wink M (2001) Molecular phylogeny of cycads referred from rbcL sequening. *Naturewissenschaffen* **89**, 211–215.

Walters TW, Decker-Walters DS (1991) Patterns of allozyme diversity in the West Indies cycad *Zamia pumila* (Zamiaceae). *American Journal of Botany* **78**, 436–445.

Wang FX, Liang HB (1996) 'Cycads in China.' (Guangdong Science and Technology Press: Guangzho, P.R. China)

Wright S (1965) The interpretation of population structure by *F*-statistics with special regard to systems of mating. *Evolution; International Journal of Organic Evolution* **19**, 395–420.

Xiao LQ, Ge XJ, Gong X, Hao G, Zheng SX (2004) ISSR variation in the endemic and endangered plant *Cycas guizhouensis* (Cycadaceae). *Annals of Botany* **94**, 133–138. doi: 10.1093/aob/mch119

Yang SL, Meerow AW (1996) The *Cycas pectinata* (Cycadaceae) complex structure and gene flow. *International Journal of Plant Sciences* **157**, 468–483. doi: 10.1086/297364

Yang SL, Meerow AW (1999) Genetic variation in Chinese cycad population. In 'Biology and conservation of cycads—proceedings of the fourth international conference on cycad biology'. (Ed. CJ Chen) pp. 175–186. (International Academic Publishers: Beijing)

Yeh FC, Yang RC, Boyle T (1999) 'POPGENE. Microsoft Windows-based freeware for population genetic analysis. Release 1.31.' (University of Alberta: Edmonton)

Zhifeng G, Thomas BA (1989) A review of fossil cycad megasporophylls, with new evidence of *Crossozamia pommel* and its associated leaves from the lower Permian of Taiyuan, China. *Review of Paleobotany and Palynology* **60**, 205–223. doi: 10.1016/0034-6667(89)90044-4

Manuscript received 20 April 2004, accepted 23 December 2004