GENETICS
CHAPTER 16

Using the Genetic Variation of Cycas taitungensis, *an Endangered Island Cycad, to Evaluate Ex Situ Conservation Strategies*

Pei-Chun Liao[1], Li-Ping Ju[2], Ya-Zhu Ko[3], Mei-Hui Chen[4], Yu-Pin Cheng[2], and Yu-Chung Chiang[3, 5]

[1]Department of Life Science, National Taiwan Normal University, Taipei 11676, Taiwan
[2]Botanical Garden Division, Taiwan Forestry Research Institute, Taipei 10066, Taiwan
[3]Department of Biological Sciences, National Sun Yat-sen University, Kaohsiung 80424, Taiwan
[4]Conservation Division, Forestry Bureau, Council of Agriculture, Executive Yuan, Taipei 10050, Taiwan
[5]Author for correspondence; email: yuchung@mail.nsysu.edu.tw

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Abstract

In past decades, infestations of the cycad aulacaspis scale have caused extreme declines in wild populations of *Cycas taitungensis*, an endemic cycad in Taiwan. The Forestry Bureau in Taiwan has considered ex situ conservation management techniques for *C. taitungensis,* such as seed storage or transplanting, to enable the survival of this species if wild populations become extinct. In this study, we use molecular markers, including the inter-simple sequence repeat and simple sequence repeats (SSR), to evaluate genetic variation, identify distinct genetic units, and select individuals for seed storage. In the

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results, half of the SSR loci deviated from Hardy-Weinberg equilibrium and almost all SSR loci showed positive inbreeding coefficient values as a result of heterozygote deficiency. The distinct genetic units for conservation management were evaluated using the assignment test based on the Markov Chain Monte Carlo method, and the grouping was consistent with distinct genetic units for in situ or ex situ conservation management.

Introduction

Comparing the genetics of a population in the past, present, and future is fundamental to conservation genetics (Frankham et al., 2002). Population declines and extinctions caused by human activities are the major force shaping individual distributions (Tatem, 2009) and decreasing gene flow within populations/species (Rago et al., 2012; Welch et al., 2012). Extreme population declines can influence the genetic diversity of a species (Frankham et al., 2002) and increase the risk of extinction (Frankham, 2005). The major impacts of declines in population size caused by human activities are spatially structured into populations, including habitat fragmentation, population isolation, inbreeding, reduced reproductive success, limited evolutionary potential, etc. (Frankham, 2005). Threatened species can be categorized based on their population size and rate of population decline (Mace & Lande, 1991; Mace & Stuart, 1994). In addition, the loss of genetic variation caused by genetic drift in populations with a small effective population size or by extreme population decline can increase the threat of extinction.

Cycads represent an ancient lineage of land plants whose origin can be traced back to the lower Permian period (Zhifeng & Thomas, 1989) and which has recently and rapidly expanded since the late Miocene (Nagalingum et al., 2011). The perennial cycad *Cycas taitungensis* Shen et al. is the only extant and insular endangered species endemic to Taiwan. There are only two remaining populations, with little genetic differentiation (Huang et al., 2001; Huang et al., 2004), along the eastern coast of the island (Osborne et al., 1999). Phylogenetically, *C. taitungensis* and *C. revoluta*, an allopatric species distributed in the Ryukyu archipelagos and the Fujian Province of China, coalesce to a unique lineage, Section Asiorientales, which is basal to other cycads (Hill, 1999). The occurrence of highly diverse organelle DNA across the range of allopatric cycads is due to ancient ancestral polymorphisms (Chiang et al., 2009). These two eastern Asian cycads are inferred to have been stable over the past 5 million years and, according to demographic

scenarios, have been dynamically increasing in population size since approximately 5~3.5 mya (Chiang et al., 2009). The limited seed dispersal across oceans during complex glacial cycles has caused the current restricted habitats of *C. taitungensis* and *C. revoluta*.

Generally, cycads are dioecious plants and require pollination vectors to transfer pollen from male to female cones. There is no direct evidence that identifies the vectors of pollination for *C. taitungensis*; however, cycads are mostly insect pollinated (Pellmyr et al., 1991; Schneider et al., 2002), primarily by beetles, but wind pollination cannot be excluded (cf. Donaldson et al., 1995). Recently, the pollination vectors of *C. revoluta*, the sister species of *C. taitungensis*, were determined to include both insects and wind, but airborne pollens such as those of *C. revoluta* are restricted to within a 2-m radius of male trees (Kono & Tobe, 2007). Gene flow within or between populations that is contributed by seed and pollen movement is thus constrained by the migratory capabilities of pollinators and seed carriers (Huang et al., 2001). Therefore, the range of gene flow between individuals of such species is restricted to short distances (Yang & Meerow, 1996; Huang et al., 2001).

Cycas taitungensis is the only extant and insular species of Taiwan and is restricted to the eastern coast (Osborne et al., 1999). Only hundreds or a few thousands of individuals remain, forming two populations that are restricted to narrow habitats in the Coastal Mountain Range: the Taitung Cycas Forest Reserves and Taitung Hongyeh Village Cycas Nature Reserve (Huang et al., 2001). In 1997, this species was categorized as being at the vulnerable level in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species based on narrow habitats (Walter & Gillett, 1998). In 2000, the invasive cycad aulacaspis scale (CAS), *Aulacaspis yasumatsui*, a native of Thailand, was first detected in Taoyuan County of Taiwan (Bailey et al., 2010). The cycad scale spread throughout all of Taiwan Island, including cultivated *C. revoluta* and native *C. taitungensis* trees. Heavy infestations of CAS due to the introduction of foreign cycads in recent years have infected the native *C. taitungensis* at high levels, causing a high mortality in wild populations of *C. taitungensis*. Thus, *C. taitungensis* are facing the risk of extinction due to the combination of restricted habitats and attacks by herbivorous pests (Bailey & Lai, 2006). In 2010 inventory records, the mortality rate of *C. taitungensis* in the major population located in the Taitung Hongyeh Village Cycas Nature Reserve had reached 62% (Huang, 2010). The IUCN raised the status of this endemic species to the "endangered" level in 2009 in response to the mortality caused by the CAS (Haynes, 2010).

To minimize this endangered cycad's risk of extinction, conservation management techniques, an important part of the management strategy for rare and endangered taxa, must be adopted (Sahney & Benton, 2008). The Forestry Bureau and the Conservation Management Office in Taiwan have considered *C. taitungensis* for ex situ conservation strategies such as "seed storage" to enable its reintroduction if the wild population becomes extinct. Molecular markers are used to analyze an endangered species in depth with the aim of applying knowledge of genetic diversity to the conservation and restoration of biodiversity (Frankham, 2003). In this study, dominant molecular markers such as the inter-simple sequence repeat (ISSR) and codominant molecular markers such as simple sequence repeats (SSR) were used to evaluate the population genetics of *C. taitungensis* to better assess the risk of extinction of wild populations, identify distinct genetic units, and select individuals for seed storage. In this study, the analysis of multilocus genome-wide markers was conducted with several specific goals: (1) to evaluate the population genetic variation of the two remaining wild populations, (2) to evaluate the spatial grouping and genetic hotspots of the populations based on the assignment test, and (3) to identify distinct genetic units for in situ and ex situ conservation management.

Materials and Methods

Population Samples

The two remaining populations of *Cycas taitungensis* were sampled: the Taitung Hongyeh Village Cycas Nature Reserve (Red Leaf Conservation Area [RL]), with thousands of individuals growing on a mountain slope along both sides of the Lu-Yeh River, and the Coastal Mountain Range Taitung Cycas Forest Reserves (Coastal Area, [C]), with hundreds of individuals distributed along a coastal ridge. The two populations are 30 to 40 km apart, separated by lowland areas that have been developed into crop fields and fruit orchards by the residents of southeastern Taiwan (Fig. 16-1). The *C. taitungensis* population restricted to the Taitung Hongyeh Village Cycas Nature Reserve can be separated into three subpopulations based on its discontinuous distribution; these subpopulations occur in the 19th, 23rd, and 40th Compartments of Yen-Ping Township (Fig. 16-1). The 23rd and 40th Compartments are located on the banks of the Lu-Yeh River and are considered continuous habitat in the following analyses. Most cycads grow in open land-

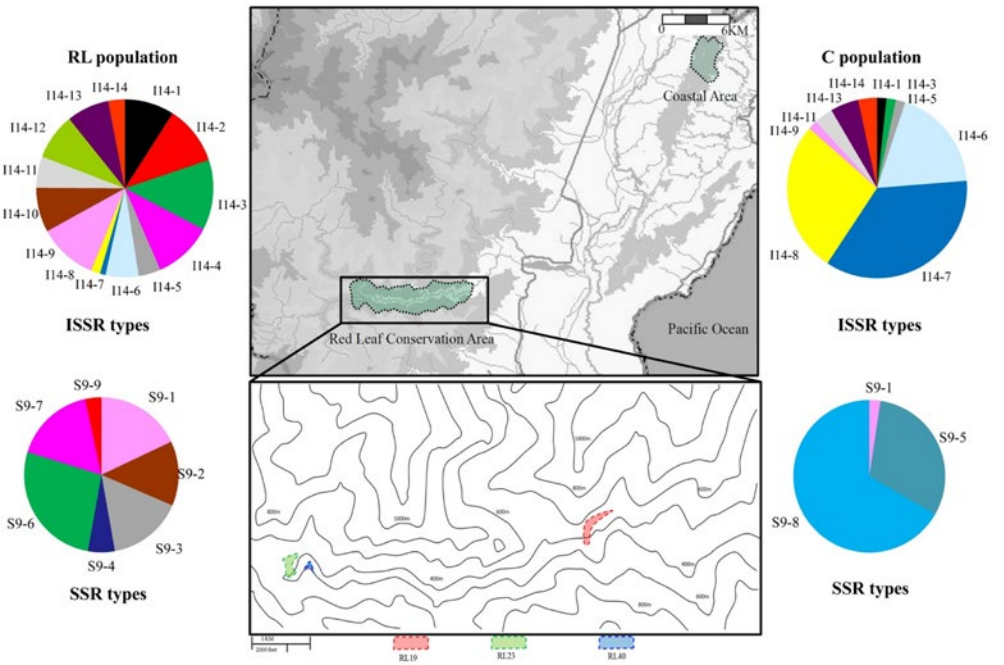

Figure 16-1. Map showing the distribution and genetic composition of populations of *Cycas taitungensis* in Taiwan. Frequencies of inter-simple sequence repeat (ISSR) and simple sequence repeat (SSR) genotypes in populations RL (Red Leaf Conservation Area) and C (Coastal Area) are indicated by pie diagrams. Abbreviations of populations are described in "Materials and Methods."

scapes and sometimes scattered under broadleaf forests as understory plants. In total, 243 individuals from population RL and 59 individuals from population C were sampled. Within population RL, 47, 84, and 112 plants were surveyed from the 19th (RL19), 23rd (RL23), and 40th (RL40) Compartments of Yen-Ping Township, respectively. Subsequently, young and healthy leaves were cleaned with sterile water, quickly dried with tissue paper, and stored in silica gel for DNA extraction. All samples were stored at –70°C before DNA extraction.

DNA Extractions

Dried leaves were ground to powder using liquid nitrogen. Using the cetyltrimethylammonium bromide method (Doyle and Doyle, 1987), total DNA was extracted from the

dried leaves. Ethanol-precipitated DNA was dissolved in 1× Tris-EDTA buffer and stored at –20°C. Qiagen (Valencia, CA) columns were used to clean the DNA samples, which were difficult to amplify using PCR.

ISSR Genotyping

ISSR analysis was based on the DNA bands, which were amplified by polymerase chain reaction (PCR) using ISSR primers. The 100 commercial ISSR primer sets were purchased directly from the University of British Columbia (UBC, Vancouver, Canada). Of the ISSR primers, 8 primers (Table 16-1) were selected for the following experiments based on the clear and reproducible amplicons in the preliminary test. PCR amplification was performed in a total volume of 20 μL, consisting of 5 ng of DNA template, 0.3 mM ISSR primer (UBC), 0.5 units of *Taq* polymerase (Promega, Madison, WI.), 2.0 μL 10× PCR buffer, 2.0 mM MgCl2, 100 mM of each dNTP, and double-distilled water. PCR was programmed on a Labnet MultiGene™ 96-well Gradient Thermal Cycler (Labnet, Woodbridge, NJ). PCR cycling conditions were as follows: 94°C for 5 minutes for preheating; 30 cycles of 94°C for 40 seconds; 48.0–57.5°C for 60 seconds; 72°C for 60 seconds; and a final extension of 72°C for 10 minutes. Amplicons were electrophoresed on 1.5% Nusieve® 3:1 agarose gels (FMC Bio Products, Rockland, ME) and stained

Table 16-1 List of selected ISSR primers, the sequences used in this study, and the number of reliable bands that each primer generated.

Primer code	Nucleotide sequence[a] 5′ to 3′	Annealing temperature (°C)	No. of analyzed bands
UBC807	$(AG)_7T$	52.0	12
UBC811	$(GA)_8C$	54.0	1
UBC812	$(GA)_8A$	48.0	15
UBC823	$(TC)_8C$	55.0	9
UBC827	$(AC)_8G$	50.0	10
UBC846	$(CA)_8$ RT	52.0	7
UBC868	$(GAA)_6$	57.5	14
UBC 880	$(G(GA)_2)_3$	57.0	10
Total	78		

[a]R = A and G residues.

by ethidium bromide, and the sizes of the products were analyzed using Quantity One Ver. 4.62 (Bio-Rad Laboratories, Hercules, CA). A DNA ladder was applied as a size marker (100–2500 base pairs). Each reaction was repeated three times for repeatable amplicons.

SSR Genotyping

Eight and four of the 12 and 16 primer sets developed from Cibrián-Jaramillo et al. (2008) and Ju et al. (2011), respectively, were successfully amplified in all samples of this study, and eight loci from Cibrián-Jaramillo et al. (2008) were confirmed by cloning and sequencing. Of the SSR primers, 12 primers (Table 16-2) were carefully chosen for the following experiments based on one or two clear and reproducible DNA bands in the preliminary test. To screen for all individuals, 12 primer pairs were labeled with 6-FAM, HEX, NED, PET, or VIC fluorescent dyes (Applied Biosystems, Carlsbad, CA), and all samples from the two populations were examined using 12 polymorphic primers. PCR was performed using the Labnet MultiGeneTM 96-well Gradient Thermal Cycler (Labnet, Woodbridge, NJ). PCR conditions were as follows: 94°C for 5 minutes; 30 cycles of 94°C for 40 seconds; 54–63°C for 60 seconds; 72°C for 60 seconds; and a final extension of 72°C for 10 minutes. Amplicons were verified on 1.5% agarose gels, microsatellite fragments labeled with fluorescent dyes were detected by an ABI 3730 for genotyping, and GeneMapper 3.7 software (Applied Biosystems) was used for fragment analysis.

Data Scoring and Data Analyses

Genetic Diversity and Genetic Polymorphism

ISSR bands with the same molecular weight were considered homologous loci and scored manually as present (1) or absent (0). SSR bands with the same number of base pairs were considered homologous alleles and recorded manually as the length in base pairs. Both types of data were manually entered into an excel worksheet using the GenAlEX Ver. 6 format (Peakall & Smouse, 2006) and converted to Arlequin Ver. 3.5 (Excoffier et al., 2005) and STRUCTURE Ver. 2.3.3 formats (Pritchard et al., 2000; Falush et al., 2003, 2007). The degrees of genetic diversity and polymorphism, including the number of alleles (*Na*); the number of effective alleles [*Ne; Ne* = 1 / (p2 + q2), where p = band frequency and q = 1 − p]; the observed heterozygosity (*Ho*); and the expected

Table 16-2 Characteristics of the 12 microsatellite loci for *C. taitungensis* (Cycadaceae) used in this study.

Locus	Primer sequence (5′→3′)	Repeat motif	Allelic size (bp)	*Ta* (°C)[a]	Fluorescent dyes labeled	Reference
Cy-Tai EST-SSR06	F: CGTCATCAAATTCTGTGCCC R: GCTGAATAGATGTTGATTTG	$(TA)_{17}$	83–89	57	FAM	Ju et al., 2011
Cy-Tai EST-SSR08	F: GAAAATGCTTTGATGTTCCC R: TGGGCCAACTTTAAGCACAC	$(ATGT)_4(TA)_{10}(CA)_9$	170–177	60	FAM	Ju et al., 2011
Cy-Tai EST-SSR13	F: CACCATCTGGCAGTCATGAT R: CCCCTGAACTGTCAAACAGG	$(TA)_{26}(TTTTC)_3$	193–230	60	FAM	Ju et al., 2011
Cy-Tai Genomic-SSR2	F: AGCTTACAGCACCACCGCCAA R: TCAAGCTATGCATCCAACG	$(GAG)_{11}$	144–153	54	FAM	Ju et al., 2011
Cy272	F: TGGTGTGTATTTTGCATTTTCA R: TGGGCATGGAAAACAAGTTAG	(TC)11	266–271	55	FAM	Cibrián-Jaramillo et al., 2008
Cy280	F: CAGAGACTATTCGGGCCAAG R: TCAAACCCTTCCACACATCA	(TA)25	214–221	57	FAM	Cibrián-Jaramillo et al., 2008
Cy284	F: TTTGGTCCACGTTACCATGA R: TCAACGGCGTCTAGTTGTTG	(TA)16	182–196	61	HEX	Cibrián-Jaramillo et al., 2008
Cy226	F: ACAGGGCATCGGAACACTAC R: CTACTCTTCGGCTTCCAACG	(TA)9	245–282	63	PET	Cibrián-Jaramillo et al., 2008
Cy240	F: ATTGCGGAACGAATATCGAC R: TATCGCGAGGCCATAGGTAG	(AT)9	167–168	61	NED	Cibrián-Jaramillo et al., 2008
Cy250	F: ATGAACAAGCGGCTGAGTCT R: CCCACCCTCTTTCTCTCTCC	(AT)8	210–266	59	VIC	Cibrián-Jaramillo et al., 2008
Cy266	F: AAATGCTTTGATGTTCCCAAA R: ATGCAATGCTCAACAAGCTG	(AT)9	246–253	54	FAM	Cibrián-Jaramillo et al., 2008
Cy270	F: CGGATTTGGAGGTTCAAAGA R: CAGTTTGTATAGCTGAACAAGAATAGA	(TG)11	148–156	58	FAM	Cibrián-Jaramillo et al., 2008

[a]*Ta*, optimized annealing temperature; FAM, 6-fluorescein amidite; HEX, hexachloro-fluorescein.

heterozygosity [*He*; *He* = (N / (N − 1)) × h, where N is sample size and heterozygosity (h) = 1 − (p2 + q2)] were computed using GenAlEX Ver. 6 (Peakall & Smouse, 2006).

Population Differentiation Tests Using Analysis of Molecular Variance and Principle Coordinate Analysis

The genetic distinction and population differentiation between the two wild populations of *C. taitungensis* were evaluated using principle coordinate analysis (PCoA; Gower, 1966) and analysis of molecular variance (AMOVA; Excoffier et al., 1992). PCoA involves calculating a principal coordinate analysis of any symmetric distance matrix following the method of Gower (1966). In this study, we used PCoA to evaluate genetic distinction using the ISSR and SSR loci separately as symmetric distance matrixes in GenAlEx Ver. 6.3 (Peakall & Smouse, 2006). AMOVA is a method of evaluating population differentiation based on molecular data and testing that differentiation directly (Excoffier et al., 1992). We used the AMOVA method in Arlequin Ver. 3.5 (Excoffier et al., 2005) to assess the significance of the geographical divisions among both populations and regions. The statistics of molecular variants F_{ST} (among populations) and F_{SC} (among subpopulations within a population) and inbreeding coefficients, including F_{IS} (inbreeding coefficients for each population) and F_{IT} (inbreeding coefficients at the individual level), were estimated. The significance of these *F*-statistic analogues was evaluated using 1,000 random permutations of sequences among populations.

Population Genetic Structure Analyses Using InStruct and Geneland

Two Bayesian Markov Chain Monte Carlo programs, InStruct (Gao et al., 2007) and Geneland (Guillot et al., 2005a, 2005b) were used to conduct the assignment test using Markov Chain Monte Carlo (MCMC) simulation for the population structure of the studied accessions. In the results of the analyses of genetic polymorphism and *F*-statistics, some SSR loci deviated from Hardy-Weinberg equilibrium and showed inbreeding based on their *F*-statistics. Thus, we chose InStruct analysis to conduct the assignment test because InStruct does not assume Hardy-Weinberg equilibrium and allows simultaneous inference of the selfing rate and the number and admixture of historical lineages (Gao et al., 2007). In addition, Geneland can detect population structure based on deviations

from Hardy-Weinberg and linkage equilibrium and can make use of both genetic and geographic information to evaluate the number of populations in a dataset and delineate their spatial organization (Guillot et al., 2005a, 2005b).

For the InStruct Ver. 1.1 program, we set the mode to 5, inferring population structure with admixture and inbreeding coefficients at the individual level. For each *K* value, 10 independent chains were executed, ranging from 1 to 20. Each chain was iterated 400,000 times after a burn-in period of 40,000 iterations. To estimate best K, the deviance information criterion (DIC) statistic was calculated (Gao et al., 2011) and the minimum value of the DIC across chains was used for inference. Afterwards, a graphical representation of the clustering results was completed using DISTRUCT software (Rosenberg, 2004).

To evaluate the number of clusters by integrating genetic and geographic information, we used Geneland Ver. 4.0 to perform a spatial genetic analysis based on Bayesian MCMC analysis (Guillot et al., 2005a, 2005b). To evaluate the best *K*, 10 independent replications of 500,000 iterations after a burn-in period of 30,000 iterations were executed, ranging from 1 to 20. The MCMC thinning was set to 100, the maximum rate of the Poisson process was fixed at 100, the maximum number of nuclei in the Poisson-Voronoi tessellation was fixed at 300, the correlated frequency model was selected as mixture models for genetic data, and the spatial model was set to correspond to the spatial patterns. The mean log posterior probability for each of the 10 runs was estimated. Then, the runs were sorted in accordance with their mean posterior density and only the best ten runs were considered in the analysis.

Results and Discussion

Genetic Diversity of ISSR and SSR Fingerprints in *Cycas taitungensis*

The eight ISSR primers that were chosen and screened generated a total of 78 clear ISSR loci (Table 16-1). Of those, only 34 ISSR loci were present in all individuals, and the percentage of polymorphic loci was 67.84%. The percentage of polymorphic loci averaged 72.55% in population C and 70.59%, 78.43%, and 49.02% in subpopulations RL19, RL23, and RL40, respectively. From the 12 polymorphic SSR loci in *C. taitungensis*, the number of alleles per locus (*Na*), the number of effective alleles (*Ne*), the observed heterozygosity

Table 16-3 Estimates of genetic diversity based on 12 polymorphic SSR loci for 4 populations of *Cycas taitungensis*.

	Red Leaf Conservation Area												Coastal Area			
	RL19				RL23				RL40				C			
SSR	*Na*	*Ne*	H_O	H_E	*Na*	*Ne*	H_O	H_E	*Na*	*Ne*	H_O	H_E	*Na*	*Ne*	H_O	H_E
CycasR43 F33-FAM	7	4.49	0.61	0.78**	6	3.68	0.57	0.73***	8	5.88	0.55	0.83***	8	4.84	0.50	0.79***
CycasR63 F1-FAM	4	2.36	0.47	0.58*	4	2.09	0.45	0.52***	4	1.66	0.21	0.40**	5	2.58	0.33	0.61***
CycasR60 F63-FAM	5	3.41	0.33	0.71***	7	3.08	0.51	0.67	7	2.86	0.50	0.65	3	2.53	0.31	0.61***
CycasR63 F31-FAM	6	2.92	0.72	0.66***	5	3.10	0.62	0.68***	5	2.43	0.79	0.59***	7	3.96	0.64	0.75***
Cycas272-FAM	5	1.83	0.36	0.45***	3	2.04	0.47	0.51	3	1.90	0.48	0.47	2	1.95	0.50	0.49
Cycas280-FAM	3	2.42	0.39	0.59	4	2.11	0.45	0.53	4	1.81	0.33	0.45***	3	2.24	0.40	0.55***
Cycas284-HEX	3	2.01	0.31	0.50***	4	1.98	0.45	0.50	4	1.85	0.19	0.46**	4	2.92	0.45	0.66***
Cycas226-PET	1	1.00	0.00	0.00	3	1.04	0.04	0.04	1	1.00	0.00	0.00	1	1.00	0.00	0.00
Cycas240-NED	1	1.00	0.00	0.00	1	1.00	0.00	0.00	1	1.00	0.00	0.00	1	1.00	0.00	0.00
Cycas250-VIC	2	1.21	0.08	0.18**	3	2.04	0.62	0.51	2	1.75	0.33	0.43	4	1.81	0.38	0.45
Cycas266-FAM	3	2.08	0.64	0.52*	4	2.67	0.62	0.62	4	3.59	0.71	0.72	2	1.93	0.52	0.48
Cycas270-FAM	2	1.56	0.36	0.36	2	1.67	0.34	0.40	2	1.93	0.48	0.48	2	1.10	0.05	0.09**
Mean	3.50	2.19	0.36	0.44	3.83	2.21	0.43	0.48	3.75	2.31	0.38	0.46	3.50	2.32	0.34	0.46

*Deviation from Hardy-Weinberg equilibrium: $P<0.05$.

**Deviation from Hardy-Weinberg equilibrium: $P<0.01$.

***Deviation from Hardy-Weinberg equilibrium: $P<0.001$.

Na = number of different alleles; *Ne* = number of effective alleles; *HO* = observed heterozygosity; and *HE* = expected heterozygosity; SSR = simple sequence repeats.

(H_O), the expected heterozygosity (H_E), and the inbreeding coefficient (*Fis*) averaged 3.646, 2.256, 0.377, 0.458, and 0.18656, respectively. As shown in Table 16-3, at the population and subpopulation levels, the number of alleles per locus (*Na*) and the number of effective alleles (*Ne*), respectively, ranged from 1 to 7 and 1 to 4.49 in subpopulation RL19, 1 to 7 and 1 to 3.68 in subpopulation RL23, 1 to 8 and 1 to 5.88 in subpopulation RL40, and 1 to 8 and 1 to 4.84 in population C. The observed and expected heterozygosity (H_O and H_E, respectively) varied from 0 to 0.64 and 0 to 0.78 in subpopulation RL19, 0 to 0.62 and 0 to 0.73 in subpopulation RL23, 0 to 0.71 and 0 to 0.83 in subpopulation RL40, and 0 to 0.64 and 0 to 0.79 in population C, respectively. Significant deviations in the Hardy-Weinberg equilibrium (HWE) (Table 16-3) were detected in eight, three, five, and seven of the 12 microsatellite loci and were attributed to the heterozygote deficiency of the subpopulation RL19, RL23, and RL 40, and population C, respectively.

In a previous study using allozyme analysis, the detection rate for loci polymorphism was 10.7%, and the mean observed heterozygosity (H_O) and expected heterozygosity (H_E) were 0.021 ± 0.009 and 0.039 ± 0.008, respectively (Huang et al., 2004). However, the detected genetic variability of *C. taitungensis* was extremely low compared to SSR analysis. The different genetic markers have different resolutions, but nevertheless, the trends of genetic diversity should be similar (Fernandez I Marti et al., 2012). In comparing genetic diversity using two different markers, the observed heterozygosity (H_O) was lower than the expected heterozygosity (H_E), indicating a heterozygote deficiency caused by population isolation, inbreeding, or both. The genetic variability, including the observed and expected heterozygosity, of *C. taitungensis* was low compared with that of other cycad species such as *C. revoluta* (0.438 and 0.540; Ju et al., 2011) and *C. micronesica* (0.349 and 0.545; Cibrián-Jaramillo et al., 2010), but was higher than that of *C. panzhihuaensis* (0.239 and 0.357; Zhang et al., 2010) and *C. debaoensis* (0.163 and 0.175; Ju et al., 2011). Unlikely *C. taitungensis*, *C. revoluta*, and *C. micronesica* are wide distribution species and numerous individuals grow on several habitats to keep high heterozygosity. For *C. revoluta*, this species is widely distributed through the southern parts of Kyushu Island to the Ryukyu Islands and natural occurrences in the coastal Fujian, China (Chiang et al., 2009), and was categorized as being at the least concern level in the IUCN Red List of Threatened Species based on large and stable populations (Hill, 2010). *Cycas micronesica* occurs in different islets of Micronesia, dues to a seed flotation layer that lets them disperse through ocean currents (Dehgan, 1983; Hill et al., 2004). Species with narrow distribution, like *C. panzhihuaensis*

(Zhang et al., 2010) and *C. debaoensis* (Zhan et al., 2011), show an extremely low level of genetic diversity at both population and species levels, as evidenced by low heterozygosity. It may due to isolated small populations, limited dispersal of seeds, recent rapid habitat destruction, and human disturbance (Zhan et al., 2011). In addition, the observed heterozygosity (H_O) is higher than the expected heterozygosity (H_E) in all of the cycad species above, which could be caused by the restricted distributions of these species.

Regional Differentiation

The AMOVA results showed that most of the genetic variation in the species was either within-subpopulation/population variation (65.42% and 69.68% for ISSR and SSR, respectively) or variation among subpopulations within a population (17.66% and 15.98% for ISSR and SSR, respectively) (Table 16-4). However, the genetic variation among populations represented 16.93% and 14.34% of the variation in ISSR and SSR, respectively,

Table 16-4 Analysis of molecular variancee (AMOVA) by Arlequin base on (a) SSR loci and (b) ISSR loci in *Cycas taitungensis*.

Source of variation	Sum of squares	Variance components	Variation (%)	Fixation indexes
(a) SSR analysis				
Among populations	126.725	0.46755	14.33683	F_{ST}=0.14337★
Among subpopulations within populations	540.311	0.52117	15.98100	F_{SC}=0.15980★
Within sub/population	379.500	2.27246	69.68218	F_{IT}=0.30318★ F_{IS}=0.18656★
(b) ISSR analysis				
Among populations	531.726	1.03614	16.92800	F_{ST}=0.23089★
Among subpopulations within populations	400.664	1.08072	17.65639	F_{SC}=0.21254★
Within sub/population	2830.823	4.00399	65.41561	F_{IT}=0.55450★ F_{IS}=0.38720★

The results include degrees of freedom (d.f.), sum of squares, variance components, percentage of variation, and fixation indexes: F_{ST} (among populations); F_{SC} (among subpopulations within a population); F_{IS} (inbreeding coefficients for each population); and F_{IT} (inbreeding coefficients at the individual level).
Significant ($P<0.05$) values are indicated with an asterisk (★).

which indicates that the genetic differentiation between the two populations is significant, with significant fixation index values ($Fst = 0.23089$ and 0.14337, $p < 0.05$ for ISSR and SSR, respectively). This was confirmed with the results of the analysis of *F*-statistics using allozyme data, which indicated slight but significant genetic differentiation among populations (Huang et al., 2004). The statistics of the fixation indices of inbreeding coefficients (F_{IS} and F_{IT}) resulted in values of 0.387 and 0.555 based on ISSR data and 0.187 and 0.303 based on SSR data, respectively. The positive fixation indices of the inbreeding coefficients suggest moderate to high levels of inbreeding. Similarly high levels of inbreeding with positive fixation indices, including F_{IS} and F_{IT}, were detected for other cycad species such as *C. micronesica* (0.370 and 0.525; Cibrián-Jaramillo et al., 2010) and *C. seemannii* (0.165 and 0.661; Keppel et al., 2002). Moderate to high levels of inbreeding and significant genetic differentiation between subpopulations/populations of cycads based on ISSR and SSR data, even though the *Fst* values estimated from different data sets did not match (Huang et al., 2001, 2004), suggest some distinct genetic units among the two extant populations. Thus, a certain amount of inbreeding has occurred in this species due to its restricted habitats and isolated populations in addition to the limited dispersal ability of its large and heavy gravity-dispersed seeds and its short pollen dispersal distance (Ellstrand and Elam, 1993; Xiao et al., 2004; Xiao & Gong, 2006).

Genetic distinction between the two wild populations of *C. taitungensis* was evaluated using PCoA. In a comparison of the two wild populations based on 71 ISSR loci, the genetic compositions of the RL and C populations cannot be separated using the first axis (explained 44.59% of variation) or the first two axes (explained 64.05% of variation; Fig. 16-2A). Within population RL, the genetic compositions of the three subpopulations are obviously distinct based on the first axis and the first two axes, excepting some individuals from subpopulation RL19. In contrast, the genetic compositions of neither the RL and C populations nor the subpopulations within the RL population could be separated well based on the first axis (explained 25.13% of variation) or the first two axes (explained 47.82% of variation) of the PCoA comparing 12 SSR loci among wild populations (Fig. 16-2B). The first two axes of the PCoA revealed a low resolution of grouping and mixture patterns of individuals between the population and subpopulation levels, explaining 64.05% and 52.81% of the variance in the ISSR and SSR matrixes, respectively (44.59% and 25.13% for the first axis and 19.46% and 22.69% for the second axis based on ISSR and SSR data, respectively; Fig. 16-2). This result suggests that there

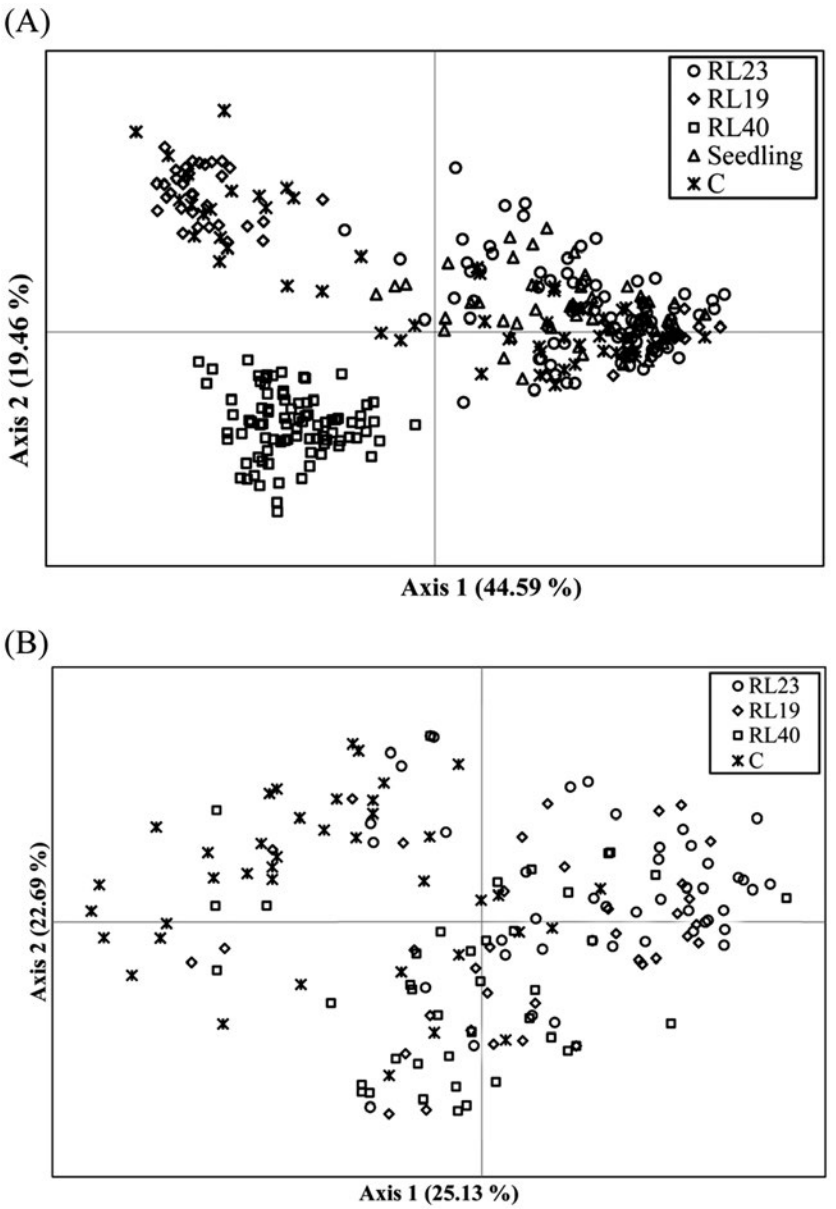

Figure 16-2. Plots of the first two axes of principle coordinate analysis (PCoA) calculated using (A) inter-simple sequence repeat (ISSR) data and (B) simple sequence repeat (SSR) data in *Cycas taitungensis*. The abbreviations RL19, RL23, RL40, and C indicate the subpopulations of the 19th, 23rd, and 40th Compartments of Yen-Ping Township from Taitung Hongyeh Village Cycas Nature Reserve and the population of the Coastal Mountain Range Taitung Cycas Forest Reserves, respectively.

is low genetic differentiation with certain admixture between groups at the population or subpopulation levels, which is consistent with the results of the AMOVA and *F*-statistics.

Genetic Assignments

To understand whether there are cryptic groupings and distinct genetic units of conservation value in the wild populations of *C. taitungensis*, we performed an assignment test based on genetic composition with the assistance of InStruct Ver. 1.1 based on deviations away from Hardy-Weinberg equilibrium and positive fixation indices of the inbreeding coefficients. In the InStruct analyses, the assignment test based on Bayesian clustering analysis of *C. taitungensis* using ISSR and SSR loci showed the best clustering results at $K=14$ and $K=9$ (Fig. 16-3) based on the minimum value of DIC (Gao et al., 2011). Depending on Bayesian clustering, each individual can be identified to a specific SSR and ISSR genotype (Fig. 16-1). Only one SSR genotype was shared between the RL and C populations, but six and two unique SSR genotypes, resprctively, belonged to the RL (S9-2, S9-3, S9-4, S9-6, S9-7, and S9-9) and C (S9-5 and S9-8) populations, (Fig. 16-1). In contrast, the two populations share 10 ISSR genotypes, but only four ISSR genotypes belong to the RL population alone (I14-2, I14-4, I14-10, and I14-12). This result indicates that the genetic composition of SSR loci is not dispersed as well as the grouping of ISSR loci and infers resolution of different molecular markers to genetic admixture. For the independent Bayesian clustering analyses for each restricted habitat, the best clustering results were $K=13$, 5, and 6 and $K=7$, 7, and 6 for subpopulations RL23/RL40 and RL19 and population C using ISSR and SSR data, respectively. Combining the ISSR and SSR results, the genetic composition for each individual can result in cryptic groupings, and distinct genetic units for conservation are proposed in the two extant wild populations. The results of the assignment test confirmed the effects of restricted habitats and isolated populations on inbreeding. The large seed of this species, with a dry weight of 9.27 ± 1.39 g (Huang et al., 2004), limits dispersal distance. Almost all related individuals are restricted to within a short distance in the field (Snow & Walter, 2007; Cibrián-Jaramillo et al., 2010).

A model-based Bayesian clustering analysis integrating genetic and geographic information was executed in Geneland Ver. 4.0. The best grouping inferred four clusters using both ISSR and SSR data that corresponded, with the exclusion of a few individuals, to the subpopulations of the 19th, 23rd, and 40th compartments and the population of the

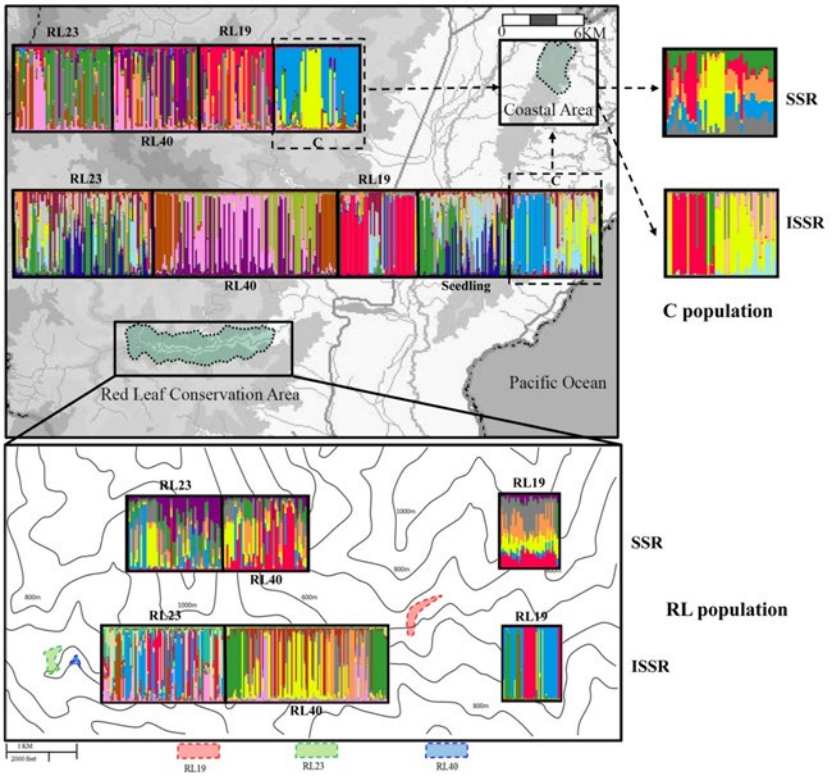

Figure 16-3. Assignment test using Bayesian clustering analysis of two wild populations of *C. taitungensis* based on ISSR and SSR results at clustering numbers $K=14$, 13, 5, and 6 and $K=9$, 7, 7, and 6 for all populations, RL23/RL40, RL19, and C, respectively. The patterns of genetic composition of each individual are detected using 78 neutral ISSR loci and 12 polymorphic SSR loci. The abbreviations RL19, RL23, RL40, and C indicate the subpopulations of the 19th, 23rd, and 40th Compartments of Yen-Ping Township from Taitung Hongyeh Village Cycas Nature Reserve and the population of the Coastal Mountain Range Taitung Cycas Forest Reserves, respectively.

Coastal Mountain Range Taitung Cycas Forest Reserves (Fig. 16-4). For the separated restricted habitats, the best grouping inferred 2 and 6 clusters within subpopulations RL23/RL40, 2 and 2 in subpopulation RL19, and 2 and 4 in population C based on the ISSR and SSR results, respectively (Fig. 16-4). By including both genetic and geographic information, genetic discontinuities in the dataset were clearly recognized as barriers to delineating the groupings that are apparent on a geographic map.

Similarly to most cycads, *C. taitungensis* has an extremely high level of genetic structure, with most of the genetic diversity found within populations (Huang et al., 2001,

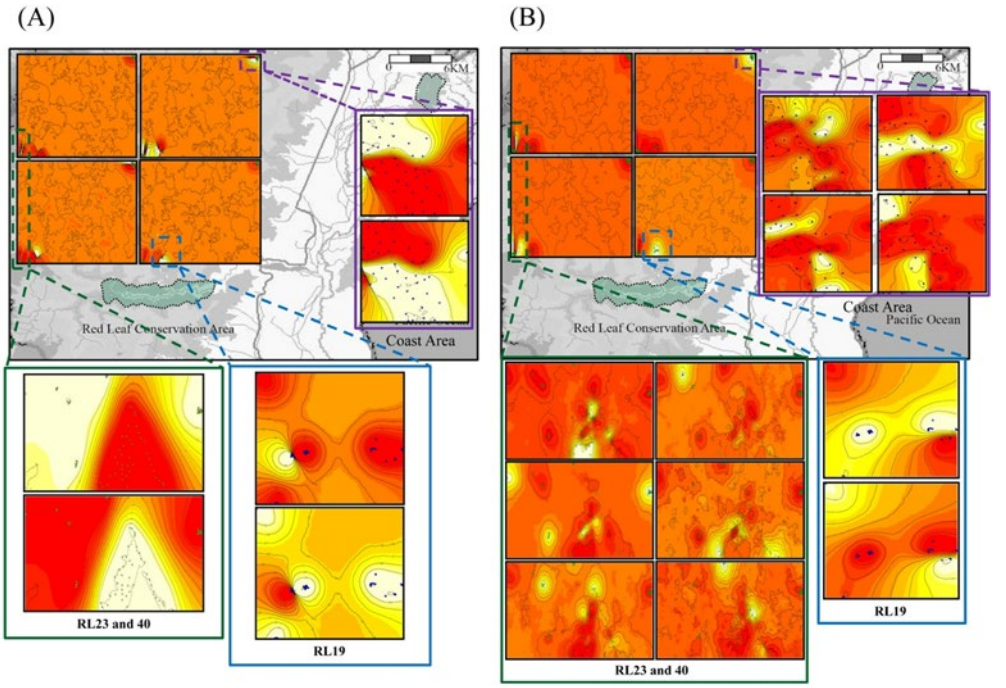

Figure 16-4. Spatial genetic clustering analysis with Geneland Ver. 4.0 using (A) ISSR data and (B) SSR data in *Cycas taitungensis*. The best clustering numbers are *K*=4, 2, 2, and 2, and *K*=4, 6, 2, and 4 for total, RL23/RL40, RL19, and C, respectively. The contour maps show the posterior probability of belonging to each cluster computed with Geneland for the best run (the 10 following runs were highly similar). The abbreviations RL19, RL23, RL40, and C indicate the inclusion of the subpopulations of the 19th, 23rd, and 40th Compartments of Yen-Ping Township from Taitung Hongyeh Village Cycas Nature Reserve and the population of the Coastal Mountain Range Taitung Cycas Forest Reserves, respectively. The assignment results corresponding to these clusters were plotted on the map of the sampling area.

2004; Chiang et al., 2009). Our results have important implications for conservation management planning for the recovery of *C. taitungensis*, the endemic and insular cycad restricted to the eastern coast of Taiwan. Thus, *C. taitungensis* should be considered an evolutionarily significant unit (ESU), defined as a historically isolated taxon or set of populations based on the distribution of alleles in relation to their phylogeny (cf. Moritz, 1994). In addition, genetic evaluation using highly variable DNA-based genetic markers, such as AFLP, SSR, and ISSR, can provide useful information to develop conservation strategies for endangered species such as *C. taitungensis*. Analyses of population genetic

structure can aid in the understanding of the evolutionary histories of cycad species (Chiang et al., 2009) and identify the demographically distinct genetic units for "management units" (Moritz, 1994; Gibbs et al., 1999).

Conclusion

Cyas taitungensis appears to have declined severely during recent decades as a result of infestations of CAS. In 2010 inventory records, the mortality rate had reached 62% (Huang, 2010), and effective conservation management techniques are needed to intervene. The genetic information collected for *C. taitungensis* in this study enables the implementation of a more efficient conservation strategy for this rare and endangered taxon. The loss of genetic diversity brings the risk of serious evolutionary consequences, both from recent adaptations on oceanic islands and from longer-term interactions with other organisms (Schneider et al., 2002). In this study, the assignment test conducted using MCMC simulation to clarify the population structure of the studied accessions, including InStruct and Geneland, could identify distinct genetic units suitable for conservation "management units." By combining the distinct genetic units and spatial information, we can evaluate the value of each individual with a unique genotype as a seed source to maintain the maximum genetic diversity of *C. taitungensis* if the wild populations become extinct. Further work on the ex situ conservation strategies is to build the well-managed seedling orchard and high quality seed storage bank to avoid extinction. Selected male and female individuals from different distinct genetic units are the first step to maintain a high degree of genetic diversity of *C. taitungensis*. Additionally, in order to obtain seeds from different genetic units, artificial pollination between male and female individuals within same conservation management units is urgently needed.

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