

Cycad biodiversity in the Bahamas Archipelago and conservation genetics of the threatened *Zamia lucayana* (Zamiaceae)

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Abstract A conservation assessment for the three cycad species native to the Bahamas Islands is presented. Results are based on field surveys on all islands where these species occur. *Zamia angustifolia* is native to Eleuthera, *Zamia integrifolia* is native to Abaco, Andros, Eleuthera, Grand Bahama and New Providence, and *Zamia lucayana* is endemic to Long Island. *Z. angustifolia* is of the highest conservation concern because of the small number of adult plants, its restricted distribution and the extensive development occurring within its habitat. *Z. integrifolia* also has a restricted distribution on Eleuthera and Grand Bahama and, although threatened by urban development in New Providence, it is relatively common on Abaco and Andros. *Z. lucayana* comprises three populations within a narrow strip of land of c. 1 km²; we propose a reassignment of its current conservation status from Endangered to Critically Endangered. We assessed the genetic structure of *Z. lucayana* based on 15 polymorphic microsatellite DNA loci; this indicated that the three known populations should be considered a single management unit. However, the high number of private alleles suggests that genetic drift, indicative of recent fragmentation, is progressing. We propose in situ conservation strategies, and we also collected germplasm from a total of 24 populations of these three cycad species, for ex situ conservation.

Keywords Biodiversity hotspot, Bahamas, Caribbean, conservation management, cycad, genetic structure, Red List, *Zamia lucayana*

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Introduction

There are c. 331 species of cycads in 10 genera (Osborne et al., 2012) and they are often referred to as living fossils because they first appeared in the Triassic (Norstog & Nicholls, 1997). These gymnosperms are rapidly disappearing because of habitat loss and their popularity in the ornamental plant trade, which has led to drastic declines in population size (Osborne, 1995). Most cycad species are currently on the IUCN Red List (IUCN, 2011) and their trade is regulated by CITES (CITES, 2011). All cycads are dioecious and their pollination and dispersal biology constrain both gene flow and population recruitment to relatively small geographical areas (Norstog & Nicholls, 1997).

There are two cycad genera in the Caribbean island biodiversity hotspot: *Microcycas* and *Zamia*. The former is a monotypic Critically Endangered genus restricted to Cuba (Pinares et al., 2009). *Zamia* (c. 71 species) has a wider distribution from Bolivia to Central America, Mexico, Florida and the West Indies. The Caribbean island species form a monophyletic group with c. nine recognized species, known as the *Z. pumila* complex (Stevenson, 1987). Three of these taxa occur in the Bahamas archipelago: *Z. angustifolia* (also present in Cuba), *Z. integrifolia* (also occurring in Cuba and Florida), and *Z. lucayana* (Fig. 1). The latter (Plate 1) is endemic to Long Island. *Z. tenuis* is another species reported for the Bahamas but it is only known from a single specimen collected in the early 19th century from a plant cultivated at Berlin Botanical Garden. *Z. tenuis* was formerly considered a synonym of *Z. angustifolia* by Britton & Millspaugh (1920) and is currently treated as a synonym of *Z. integrifolia* (Osborne et al., 2012).

The Commonwealth of the Bahamas consists of 22 inhabited islands, c. 700 cays and nearly 2,400 small islets (Albury, 1975). This archipelago has a fast growing population and a strong tourism industry, which represent challenges to the protection of its unique environment (Anonymous, 2002). It is estimated that this archipelago has 110 endemic seed plant species (c. 9% of the native flora; Acevedo-Rodríguez & Strong, 2012). As far as we are aware no Bahamian endemic species has been the subject of a comprehensive study focusing on (1) its conservation status, (2) challenges for management, (3) conservation genetics, (4) ex situ conservation, and (5) current threats. Such studies are particularly relevant to the Bahamas as this archipelago

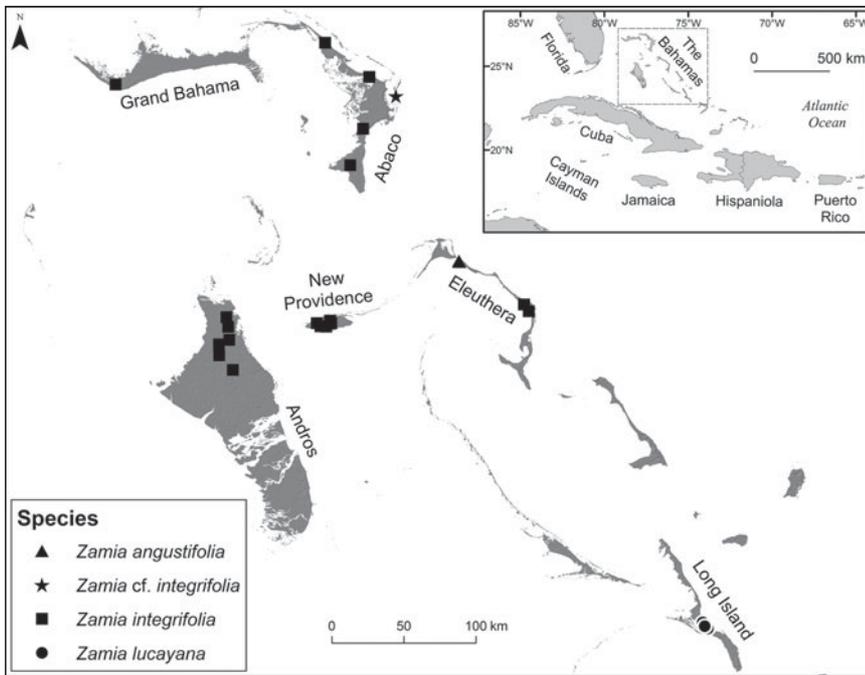


FIG. 1 Distribution of the three species of *Zamia* in the Bahamas Islands. Each point represents a population included in our field studies. Population symbols overlap on Andros, Eleuthera, and New Providence. Plants from Tilloo Cay, Abaco, were identified as *Zamia* cf. *integrifolia* for this study. See Table 1 for number of populations and species studied on each island. The inset indicates the location of the Bahamas in the Caribbean.

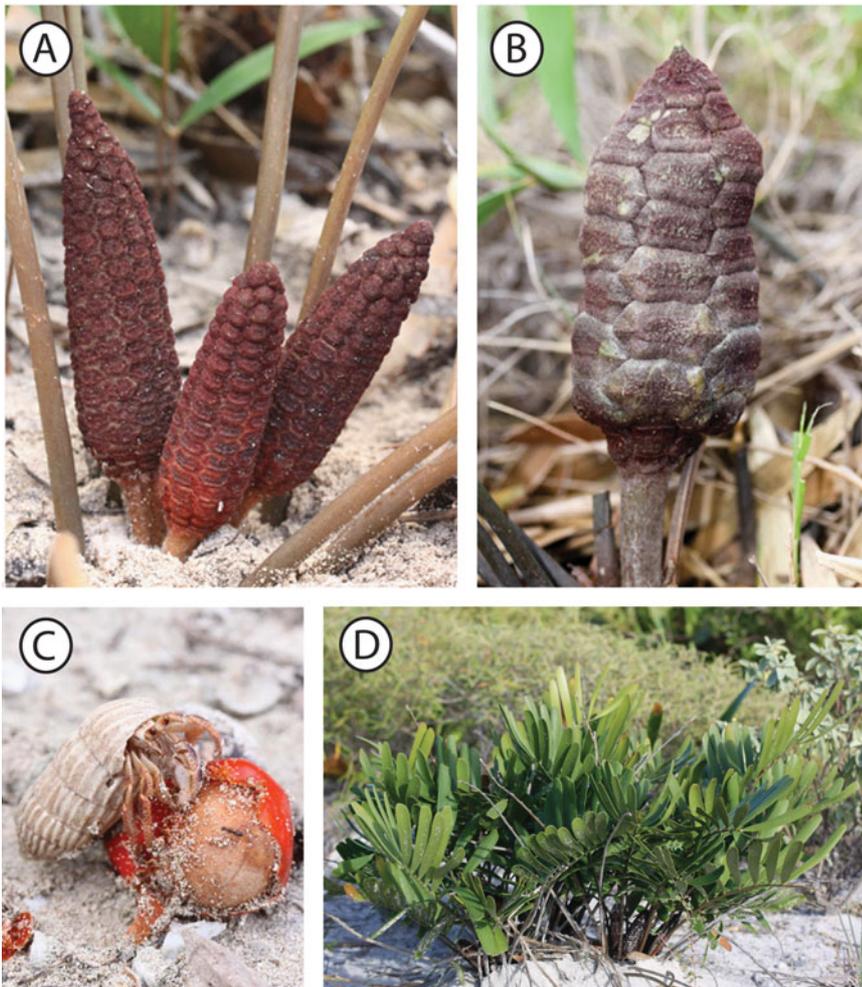


PLATE 1 *Zamia lucayana*: (A) male cones, (B) female cone, (C) hermit crab *Coenobita clypeatus* feeding on the fleshy seed coat, (D) adult male individual.

is part of the Caribbean island biodiversity hotspot (Maunder et al., 2008). Here, we formulate conservation assessments of Bahamian *Zamia* species, with special emphasis on the Long Island endemic *Z. lucayana*.

Methods

Collecting sites

Prior to our field studies we compiled a list of historical sites for the target species as recorded by collections deposited in seven herbaria (BW, BNH, C, FTG, GH, MO, NY; acronyms follow Thiers, 2011). In addition, we consulted floristic and taxonomic treatments that include information concerning the distribution of the genus in the Bahamas (Britton, 1907, 1909; Hill, 1974; Eckenwalder, 1980; Correll & Correll, 1982), and gathered provenance information from living collections at Montgomery Botanical Center and Fairchild Tropical Botanic Garden. Between December 2009 and July 2011 we conducted field studies on the five islands where the three species occur (Table 1, Fig. 1). Each island has only one species, except Eleuthera, where *Z. angustifolia* and *Z. integrifolia* co-occur, although their distribution ranges do not overlap (Fig. 1). We visited a total of 24 populations (Fig. 1, Table 1) and we also explored areas where the species were not previously reported.

Conservation assessments and demographic surveys

For each site and island we gathered information pertinent to the cycads' conservation status and threats. Field studies were more exhaustive for *Z. lucayana*, for which we estimated total distribution area, number of adult and juvenile plants, and the approximate ratio of males to females. Juveniles are readily identified by their overall smaller size, absence of remains of reproductive structures, lower number of leaves (usually < 4) on the stem, and lower number (usually < 5 pairs) of leaflets on the leaves. These demographic data were obtained through a census of 10 × 10 m plots that covered c. 25% of the populations. Locations of individual plants occurring at the edges of the populations were determined with a global positioning system; these points were imported to *ArcMap v. 10* (ESRI, Redlands, USA) and used to determine area of occupancy and extent of occurrence (IUCN, 2011) for the species.

Microsatellite DNA analysis

We used 15 DNA microsatellites to investigate the genetic structure of *Z. lucayana*. The three known populations of *Z. lucayana* were included (Table 2) and we sampled 33–46 individuals per population. DNA was isolated following the protocols described by Meerow & Nakamura (2007). Microsatellite loci were developed in two ways.

A modified version of the enrichment/hybridization method of Edwards et al. (1996) was used with genomic DNA of Florida *Z. integrifolia* (Meerow & Nakamura, 2007; Meerow et al., 2007, 2012a,b). The second methodological approach involved retrieving of microsatellite loci in the *Zamia* expressed sequence tag (EST) databases available in GenBank using the Simple-Sequence Repeat Identification Tool in *GRAMENE* (Ware et al., 2002). Six of the 15 loci (Zam28, Zam33, Zam34, Zam39, Zam40, Zam45) have been previously published (Meerow & Nakamura, 2007). Protocols for PCR amplification and subsequent visualization of SSR fragments follow Meerow & Nakamura (2007).

Descriptive statistics (Table 2) were generated with *GenAlEx v. 6.41* (Peakall & Smouse, 2005). Tests for Hardy–Weinberg equilibrium and the U test (Rousset & Raymond, 1995) for heterozygote excess or deficiency were run with *GenePop v. 4.0* using 10,000 Monte Carlo Markov chain iterations (Guo & Thompson, 1992). Linkage disequilibrium (LD) was tested for each population with *ARLEQUIN v. 3.5* (Excoffier et al., 2005) using a likelihood-ratio test (Slatkin & Excoffier, 1996). A Monte Carlo Markov chain method was applied with 100,000 iterations, a burn-in of 10,000 and the significance level set at $P < 0.001$.

Analysis of molecular variance (AMOVA) among populations, and permuted calculation of F_{ST} (10,000 permutations) were generated with *GenAlEx*. An additional measure of gene diversity, D_{est} (Jost, 2008), was calculated with *SMOGD* (Crawford, 2010).

Genetic distance among populations and individuals was calculated with *POPULATIONS v. 1.2.31* (Langella, 2010), using D_a (Nei et al., 1983). The population distance matrix was used for permuted (10,000 iterations) Mantel (1967) tests for isolation by distance following the methods of Smouse et al. (1986) and Smouse & Long (1992), as implemented in *GenAlEx*. These distance coefficients were also used in principal coordinate analysis with *GenAlEx*. *BOTTLENECK 1.2.02* (Cornuet & Luikart, 1996) was used to test for recent genetic bottlenecks in the populations under both the infinite allele model (Kimura & Crow, 1964) and the two phase model (Di Rienzo et al., 1994).

The Bayesian clustering programme *STRUCTURE v.2.3.3* (Pritchard et al., 2000) was used to estimate the underlying genetic structure among populations. The *STRUCTURE* analyses were carried out on the University of Oslo Biportal (Kumar et al., 2009). K values of 1–15 were simulated across 20 replicate runs of 1,000,000 iterations after a burn-in of 100,000. The Δk method of Evanno et al. (2005) as implemented in *STRUCTURE HARVESTER* (Earl & vonHoldt, 2012) was used to determine the 'true' value of K across samples. After the likely level of K was estimated, a consensus Q-matrix from the 20 runs was constructed using *CLUMPP* (Jakobsson & Rosenberg, 2007) for visualization with *DISTRUCT* (Rosenberg, 2004).

TABLE 1 The six islands of the Bahamas archipelago (Fig. 1) on which cycads of the genus *Zamia* occur, with the species present, and the number of populations studied, seeds collected, and individuals sampled for DNA studies.

Island	Species	No. of populations studied	No. of seeds collected (no. of mother plants sampled for seeds)/no. of seedlings collected	No. of individuals sampled for DNA studies
Abaco*	<i>Z. integrifolia</i>	5	798 (16)/0	76
Andros	<i>Z. integrifolia</i>	6	497 (8)/0	158
Eleuthera	<i>Z. angustifolia</i>	2	400 (11)/40	37
	<i>Z. integrifolia</i>	2	236 (8)/18	46
Grand Bahama	<i>Z. integrifolia</i>	1	196 (8)/0	45
Long Island	<i>Z. lucayana</i>	3	910 (16)/0	114
New Providence	<i>Z. integrifolia</i>	5	670 (14)/17	125
<i>Total</i>		24	3,707 (81)/75	602

*Includes the Tilloo Cay site

TABLE 2 Main demographic features and descriptive genetic diversity statistics for the three populations of *Z. lucayana*.

	Hamilton's	Buckley's	Petty's
Population code	ZBLI1	ZBLI2	ZBLI3
No. of adults	400	250	300
No. of juveniles	267	643	828
No. of females	120	53	144
No. of males	160	125	120
No. of adults without cones (unknown sex)	120	72	36
Approximate area of occupancy (km ²)	0.45	0.13	0.06
No. of individuals sampled for DNA studies	46	43	33
Average no. of alleles per locus	5.3	4.5	4.3
No. of private alleles	15	15	7
Observed heterozygosity	0.519	0.478	0.454
Expected heterozygosity	0.501	0.486	0.483
Inbreeding coefficient	0.040	0.090	0.053
Percentage of paired loci in linkage disequilibrium	5	0	1

Results

Distribution patterns

With few exceptions (Lubber's Quarters Cay off the coast of Abaco and in the vicinity of Eight Mile Rock, Grand Bahama), we found populations of *Zamia* in the areas recorded on the herbarium specimens and in the relevant literature. In our field surveys beyond the historical distribution ranges we did not find any new localities for the species, with the exception of those located on the cays near Abaco.

Z. angustifolia is extremely rare and occurs only on coastal sand dunes of Eleuthera. We found only 150 plants

in two sites within a small area of 0.34 km² near Gregory Town (Fig. 1).

Z. integrifolia is typically found in the understory of the Bahamian pine forests and occasionally in dry evergreen forests, except on Tilloo Cay and Eleuthera. In these two islands pine forests are not present and the species occurs on limestone bluffs in coastal thicket (Eleuthera) or in sandy coastal scrub (Tiloo Cay). The species is abundant in Abaco, relatively common in northern areas of North Andros and locally abundant in a few areas with unfragmented pine forest on New Providence. However, it is rare on Grand Bahama and Eleuthera (Fig. 1).

Z. lucayana occurs on the eastern coast of Long Island between the settlements of Hamilton's and Buckley's (Fig. 2). The three populations grow exclusively within a narrow strip (c. 6.5 km by 100 m wide) of coastal scrub vegetation on sandy soils in association with sea grape *Coccoloba uvifera*, occupying a total area of c. 1 km². The combined area of occupancy for the surveyed populations is 0.06 km² (Table 2). Approximately 80% of the fertile adult individuals surveyed were male and 20% female (Table 2). Approximately 27% of individuals were juvenile plants (Table 2). All female individuals with mature cones had nearly complete seed set, indicating efficient pollination. We observed hermit crabs *Coenobita clypeatus* feeding on the fleshy seed coats, perhaps accelerating germination and short-range dispersal (Plate 1). We estimated there are c. 980 adult individuals throughout the entire range of this species, with 240–400 plants per population (excluding the two outlier sites; Table 2).

Population genetics of *Z. lucayana*

The 15 polymorphic microsatellite loci uniquely genotyped all 122 individuals assayed; i.e. there were no identical repeated multi-locus genotypes. The mean number of alleles was 4.3–5.3 (Table 2) but the populations had a relatively

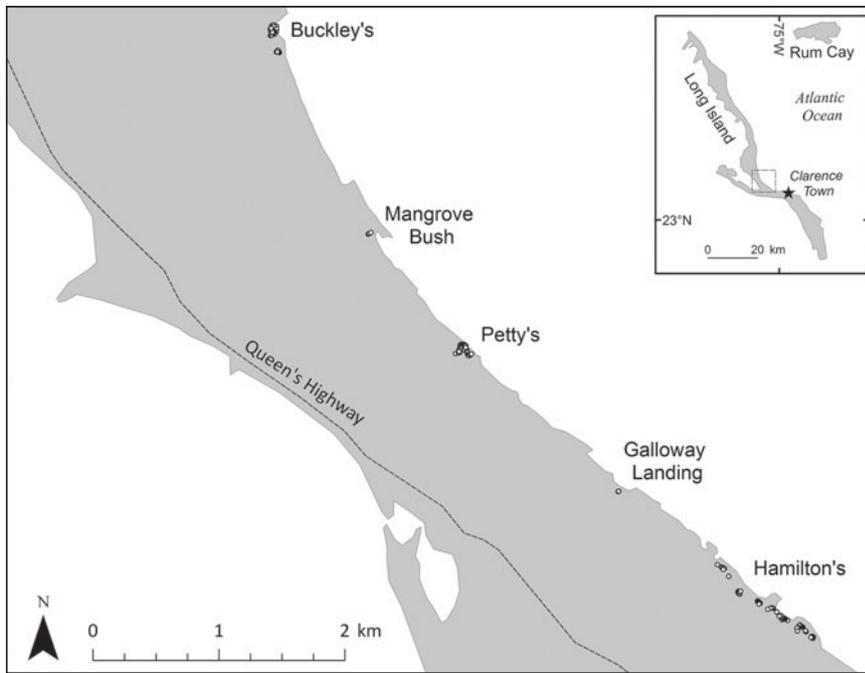


FIG. 2 Distribution of *Zamia lucayana* on Long Island, showing the three major populations included in the conservation genetic study (Buckley's, ZBLI2; Hamilton's, ZBLI1; Petty's, ZBLI3) and two further localities (Galloway Landing, where 10 adult plants were observed, and Mangrove Bush, where 20 adult plants were observed). Each dot represents a site where individual leaflets were sampled for genetic studies (Table 2). No material was sampled at Galloway Landing or Mangrove Bush.

TABLE 3 Pairwise D_{est} values (above diagonal) and F_{ST} values (below diagonal) for the three populations of *Z. lucayana* (Table 2).

	ZBLI1	ZBLI2	ZBLI3
ZBLI1		0.051	0.045
ZBLI2	0.075		0.040
ZBLI3	0.060	0.062	

high number of private alleles, with seven for ZBLI3 and 15 for both ZBLI1 and ZBLI2 (Table 2). All populations are moderately heterozygous (Table 2). All but ZBLI1 are slightly inbred but variation in F was not significant. ZBLI1 also has the highest mean number of alleles per locus. Exact tests found no significant departure from Hardy–Weinberg equilibrium in any populations. Overall, there was 6% linkage disequilibrium among loci, the majority of which were concentrated in ZBLI2 (5%). ZBLI1 had no loci in LD; ZBLI3 had two.

Mean F_{ST} was highly significant (0.067, $P < 0.0001$) but indicates little differentiation among populations (Table 3). Similarly Jost's D_{est} estimate of differentiation is low, at 0.04–0.051 (Table 3). The mean number of migrants (Nm) determined by the F_{ST} method is 3.6 (range 3.07–3.94; Table 4), indicating gene flow among the populations. There is no significant isolation by distance among the populations. Over 90% of the genetic variation is within populations (Table 5).

The Evanno method of determining the true K identified $K = 2$ as optimal across all *Z. lucayana* populations (Fig. 3). ZBL3 is located between ZBLI1 and ZBL2; it shows a closer genetic relationship to ZBLI1 but with some significant admixture from ZBLI2. Principal coordinate

TABLE 4 Estimates of the number of migrants per generation and significance (P) of the analysis of molecular variation (AMOVA) between the three populations of *Z. lucayana*.

Population comparison	No. of migrants	P
ZBLI1–ZBLI2	3.074	<0.001
ZBLI1–ZBLI3	3.936	<0.001
ZBLI2–ZBLI3	3.778	<0.001

TABLE 5 Analysis of molecular variance for the three populations of *Z. lucayana*. The results indicate that the majority of genetic variation is found within, rather than among, populations.

Source	df	Sum of squares	Estimated variance	%
Among populations	2	51.270	0.273	7
Within populations	239	911.739	3.815	93
Total	241	963.008	100	

analysis indicates much the same as the Bayesian clustering (Fig. 4) and ZBLI3 mostly overlaps with ZBLI2. The first two coordinates of this analysis accounted for 43.44% of the variation.

Across all three *Z. lucayana* populations there is only weak evidence of genetic bottlenecks. ZBLI1 and ZBLI2 only tested at $P < 0.05$ for the Sign test under the infinite allele model. ZPLI3 tested at $P < 0.05$ for both the Sign and Wilcoxon tests under the infinite allele model. With the two phase model imposed there was no evidence of bottlenecks in any of the populations.



FIG. 3 Graphical representation of genetic structure across three populations of *Z. lucayana* using Bayesian clustering of microsatellite frequency data. $K = 2$ was found to be the optimal partition of genetic variation. The individuals of ZBLI1 and ZBLI2 are predominantly assigned to the same cluster (light grey), whereas ZBLI2 is primarily assigned to the second (dark grey). Admixture between the two clusters is indicated by varying proportions of the opposing colour in individuals of each population. ZBLI3 shows a greater proportion of admixture with ZBLI2 than does ZBLI1.

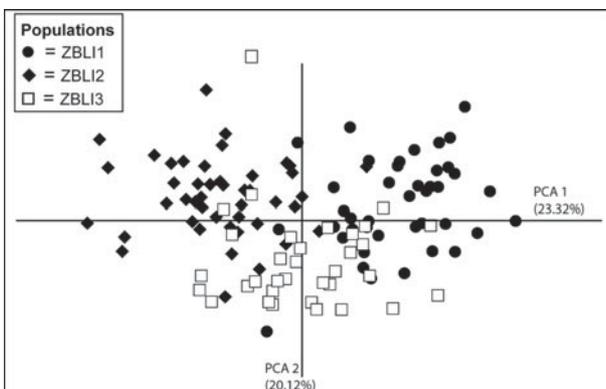


FIG. 4 The first two axes (PCA1, PCA2, with the percentage variation explained by each) of a principal coordinate analysis of DNA microsatellite data for the three main populations of *Z. lucayana* (Table 2, Fig. 2). Each point represents a single individual.

Discussion

Distribution patterns and conservation implications

Z. angustifolia is the species of highest conservation concern because of the small number of adult plants, its limited habitat extent, and the extensive housing development projects occurring in this area. The species also occurs in Cuba, where it is relatively abundant (González Géigel, 2003). However, it is uncertain whether the narrow-leaved morph of Cuba is the same taxon that occurs on Eleuthera. The Bahamian populations are highly threatened and additional studies that include the Cuban populations are needed to determine the conservation status of this species.

Z. lucayana was described by Britton (1907) based on a single individual reported in an unknown locality apparently near Clarence Town (Fig. 2). Later Hill (1974) rediscovered this species, finding a dense population in the vicinity of Hamilton's Settlement (Fig. 2). Our field studies confirmed the existence of this population and we discovered two additional populations near the Settlements of Buckley's and Petty's. We found that human activities

have not yet had a major negative effect on the populations of *Z. lucayana*. The population at Hamilton's Settlement is within an area that has been exploited for sand mining but this activity is localized. All the locations of this species are accessible by road and are on prime ocean-front real estate that has been subdivided for sale, although few houses have been built.

New Providence and Grand Bahama are the two islands where *Z. integrifolia* is at most risk. As urban development of Nassau expands westwards the remaining habitats of this species will be destroyed; its future on the island is thus uncertain. On Grand Bahama the species occurs near Freeport, at a few sites within an area with intense industrial development.

Conservation genetics

Critically Endangered species usually have reduced numbers of populations and individuals per population (IUCN, 2011) and the genetic structure of their populations may therefore be severely influenced by genetic drift and inbreeding (Höglund, 2009). This results in a decrease in heterozygosity, with subsequent risk of inbreeding depression, and loss of potential adaptive alleles (Peterson & McCracken, 2005). Nevertheless, our data show that despite its Critically Endangered status (see below) the populations of *Z. lucayana* are still in Hardy-Weinberg equilibrium and are retaining heterozygosity, and are only moderately inbred and exhibit little differentiation.

Our results indicate that the three populations of *Z. lucayana* on Long Island have been a single panmictic population throughout most of their history. The low levels of genetic differentiation (Table 3) and relatively high migration rate (Table 4) among the three populations indicate that gene flow has been historically high. However, the high number of private alleles (Table 2) indicates that genetic drift is occurring, especially in ZBLI1 and ZBLI2. Given the characteristically local dispersal of both pollen and seeds of *Zamia* (Norstog & Nicholls, 1997) our

expectations are that the populations will continue to fragment further if disturbed, and become more inbred. The genetic data indicate that the three populations of *Z. lucayana* should be considered as a single management unit. The population at Petty's has the highest levels of admixture, and therefore this population would be a good source of material for reintroducing the species to other areas of the island or for ex situ conservation.

Our data contrast with those recently reported for other Critically Endangered plant taxa from the Caribbean islands in which microsatellites have been used to determine genetic structure. Namoff et al. (2011) found strong evidence for genetic drift, inbreeding and moderate gene flow for the Critically Endangered palm *Pseudophoenix ekmanii*, endemic to the Dominican Republic. Geiger et al. (in press) reported that populations of *Ipomoea microdactyla* from highly fragmented and disturbed areas of South Florida exhibited significantly lower levels of genetic variation than those from the contiguous and well preserved pine forest on Andros Island.

Walters & Decker-Walters (1991) used isozyme data to determine levels of genetic variation within the *Z. pumila* complex but found limited genetic variation with these markers. Our research suggests that microsatellites are more useful than isozymes to understand the population genetic structure of this species complex (Meerow & Nakamura, 2007; Meerow et al., 2007, 2012a,b). This is supported by our results for *Z. lucayana*.

Most population genetic studies of cycads have been based on isozymes (reviewed by Pinares et al., 2009), with only three studies focusing on Critically Endangered species (*Dioon caputoi*, Cabrera-Toledo et al., 2008, 2010; *Microcycas calocoma*, Pinares et al., 2009). *Cycas debaoensis* is the only Critically Endangered cycad (IUCN, 2011) for which conservation genetic studies based on microsatellites are available (Yang et al., 2008).

The unusual patterns of genetic diversity detected in *Z. lucayana* are also exhibited by these three other Critically Endangered species, which also exhibit relatively high levels of heterozygosity. These results appear to provide additional support for the hypothesis (Cabrera-Toledo et al., 2008) that in cycads 'rarity is compatible with high levels of genetic diversity'. Unlike many seed plants, cycads are long lived and allogamous. These life-history characteristics may help reduce inbreeding and genetic drift and subsequent detrimental effects to genetic diversity (Cabrera-Toledo et al., 2008).

Conservation recommendations and conclusions

Z. lucayana is currently categorized as Endangered on the IUCN Red List (IUCN, 2011). We propose that it should be recategorized as Critically Endangered based on criteria

B1ab(i-v) + 2ab(i-v). This recommendation has been submitted to the IUCN Cycad Specialist Group. This evaluation is based on the species' highly restricted extent of occurrence (1 km²) and area of occupancy (0.06 km²). In addition, *Z. lucayana* requires a unique habitat that is already under residential development.

Z. lucayana is one of three single-island endemic plants on Long Island (Taylor, 1921; Correll & Correll, 1982). The others are *Euphorbia longinsulicola* and *Matelea correllii*. The former has a relatively narrow distribution range (Hill, 1976) and partially co-occurs with *Z. lucayana*. Because of the few endemics restricted to Long Island, the protection of *Z. lucayana* and its habitat is clearly a major conservation priority for this island.

We recommend both ex situ and in situ conservation for *Z. lucayana*. The occurrence of the species on private land could impede establishment of an effective management plan. Long-term conservation will therefore depend on whether the Bahamas National Trust and/or the Ministry of the Environment of the Bahamas can purchase the land where this species occurs. Until the required funds are available we recommend the following actions: (1) limit sand mining, (2) plan residential development on central eastern regions of the island that is compatible with the area where *Z. lucayana* occurs, (3) establish national ex situ conservation collections in the Bahamas, with duplicates in other sites, (4) increase conservation awareness for the species through environmental education programmes, and (5) develop voluntary agreements with landowners to limit development on their property.

During our visit we collected a total of 910 seeds for ex situ conservation from 17 individual plants (Table 1) of *Z. lucayana*. In addition, we collected total of 75 seedlings and 2,797 seeds from 65 plants of *Z. angustifolia* and *Z. integrifolia*. The location of each plant was determined and sent to the Ministry of the Environment of the Bahamas. The collections were based on the protocols developed at the Montgomery Botanical Center (Walters, 1999; Namoff et al., 2010). In each population we collected seed from five plants and a minimum of 50 seeds per individual. Progeny from each plant is accessioned separately for subsequent ex situ conservation planting. This germplasm has been distributed to the Bahamas National Trust, Fairchild Tropical Botanic Garden, Montgomery Botanical Center, the National Germplasm Repository of USDA-ARS at Miami, the Jardín Botánico Francisco Javier Clavijero (Xalapa, Mexico), and Fairylake Botanical Garden (Shenzhen, China). Among these institutions, the Montgomery Botanical Center maintains one of the most comprehensive living collections of Cycadales worldwide, especially of *Zamia* (Calonje et al., 2009). The seeds we collected had an 85% germination rate, suggesting that an ex situ conservation programme will be feasible.

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