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Zamia (Cycadales: Zamiaceae) on Puerto Rico: Asymmetric genetic differentiation and the hypothesis of multiple introductions[1]

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- *Premise of the Study:* This study of *Zamia* in Puerto Rico is the most intensive population genetics investigation of a cycad to date in terms of number of markers, and one of few microsatellite DNA studies of plants from the highly critical Caribbean biodiversity hotspot. Three distinctive *Zamia* taxa occur on the island: *Z. erosa* on the north coast, and *Z. portoricensis* and *Z. pumila*, both in the south. Their relationships are largely unknown. We tested three hypotheses about their genetic diversity, including the possibility of multiple introductions.
- *Methods:* We used 31 microsatellite loci across 10 populations and analyzed the data with AMOVA, Bayesian clustering, and ABC coalescent modeling.
- *Key Results:* Puerto Rican zamias exhibit an amalgam of patterns of genetic differentiation that have been reported for cycads. Overall, the taxa are slightly inbred, with high infra-populational variation and little evidence of recent bottlenecks. *Zamia erosa* exhibits a more than threefold greater degree of population differentiation than the other two taxa. Admixture is evident only between *Z. portoricensis* and *Z. pumila*. *Zamia portoricensis* is inferred to be the youngest taxon on the island, on the basis of estimates of coalescence time and effective population size. A selective sweep may be underway in a small population of *Z. erosa* in a saline environment.
- *Conclusions: Zamia erosa* may represent an independent introduction into Puerto Rico; *Z. portoricensis* and *Z. pumila* fit a scenario of allopatric speciation. This will be explored further in the context of genetic analysis across the entire Caribbean region.

Key words: Caribbean biogeography; coalescence; cycads; microsatellite DNA; population genetics.

The Caribbean Basin is considered one of the five "hottest" of global biodiversity hotspots (Myers et al., 2000; Mittermeier et al., 2004; Shi et al., 2005), despite a reduction to 11.3% of its original vegetation (Brooks et al., 2002; Maunder et al., 2008). In a relatively small area (ca. 229–550 km^2), the islands support a native flora of ~12 000 species, of which ~8000 are endemic (Francisco-Ortega et al., 2007). As critical an area of endemic biodiversity as the Caribbean region is, research on the population genetics of its constituent taxa, especially the plants, has hardly begun (Francisco-Ortega et al., 2007, 2008; Namoff et al., 2011).

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The *Zamia pumila* L. complex (Cycadales: Zamiaceae) is a distinctive, monophyletic, and diploid ($2n = 16$) assemblage of populations restricted to the West Indies and Florida (Stevenson 1987a, b; Caputo et al., 2004) that is currently considered to encompass either a single polymorphic species (Eckenwalder 1980) or as many as nine distinct species (Stevenson 1987a, b; Sabato, 1990; González Géigel, 2003). If one rejects Eckenwalder's (1980) treatment of the Caribbean clade of *Zamia* as a single polymorphic species, three distinct taxa can be recognized in Puerto Rico (Acevedo-Rodríguez and Strong, 2005): *Zamia erosa* O. F. Cook and G. N. Collins (syn. *Z. ambliphyllidia* D. W. Stev.; Fig. 1A, B), *Z. portoricensis* Urb. (Fig. 1C), and *Z. pumila* (Fig. 1D). The three are distinguished primarily by the width of the leaflets (Stevenson, 1987a, b), with *Z. portoricensis* having the narrowest and *Z. erosa* the broadest. Stevenson (1987a, b) argued for the further recognition of *Z. integrifolia* L. f. on the island, but consistent morphological discontinuities between *Z. integrifolia* and *Z. pumila* are ambiguous at best (Negrón Ortiz and Breckon, 1989; Acevedo-Rodríguez and Strong, 2005). The most recently described of the three is *Z. erosa* (see Calonje et al., 2010), notable for its large, broad, and very deeply notched to almost forked leaflets. It has also been reported from Jamaica and western Cuba, purely on the basis of leaflet morphology, a character of unknown plasticity. On Puerto Rico, *Z. erosa* is restricted to the northern coast (Fig. 2); *Z. portoricensis* and *Z. pumila* are found in the drier south, distributed to the west (*Z. portoricensis*) and east (*Z. pumila*) of Bahia Tallaboa (Fig. 2). There is a tenfold difference in the amount of annual

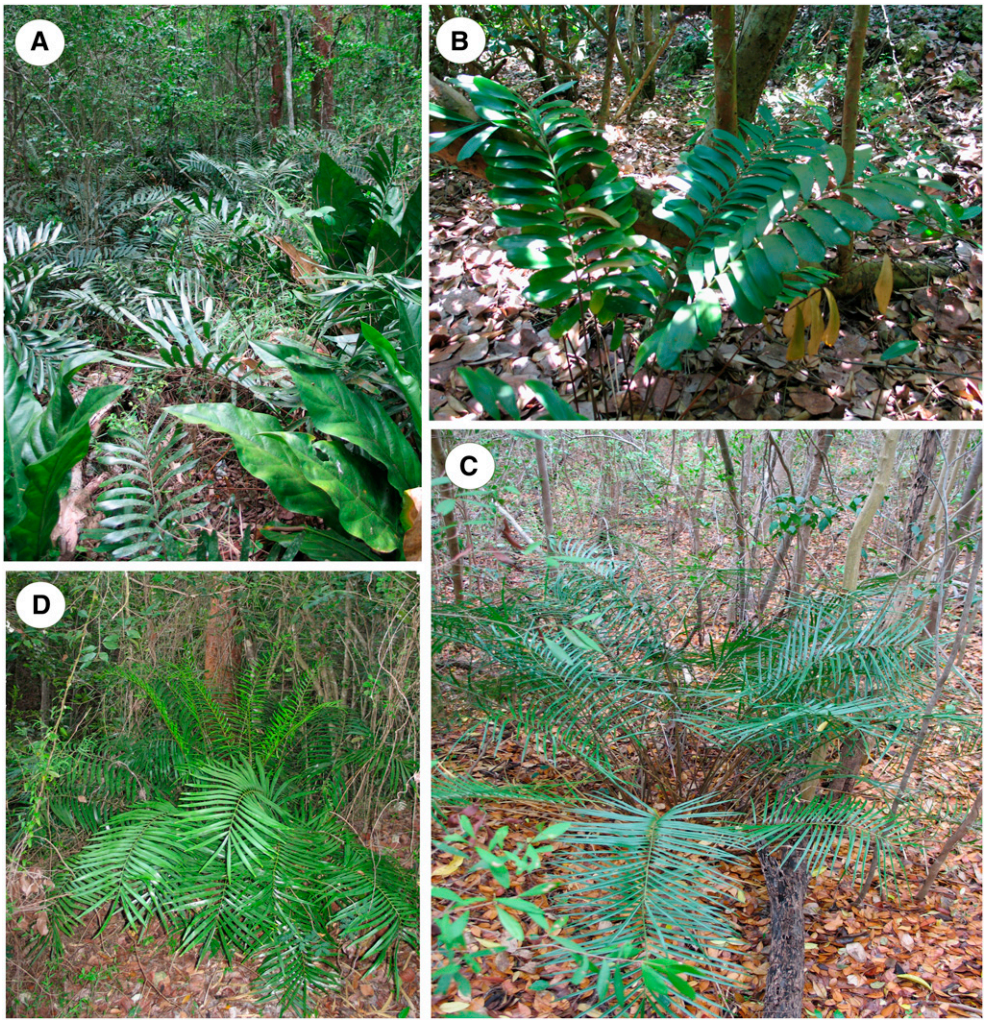

Fig. 1. *Zamia* in Puerto Rico. (A) *Zamia erosa*, Cambalache Forest Reserve (EROSA2). (B) *Z. erosa*, seaside population, Quebradillas (EROSA1). (C) *Z. portoricensis*, Guánica Dry Forest Reserve (PORTO3). (D) *Z. pumila*, Peñuelas (PUM1).

rainfall between the mesic northern and the more xeric southern coast (Helmer et al., 2002). The Cordillera Central (highest elevation at Cerro Punta: 1,338 m) functions as a barrier for dispersal between the northern and southern lowlands of the island (López-Marrero and Villanueva-Colón, 2006). On the basis of herbarium records (UPRRP, MAPR), we estimate the historical range of each taxon as extending the following linear distances in the island: *Z. erosa*, ~120 km; *Z. portoricensis*, ~35 km; and *Z. pumila*, ~45 km (though many historical localities have been extirpated). There is no evidence of sympatric occurrence, even for *Z. portoricensis* and *Z. pumila*, populations of which are presently found no closer than ~19 km.

The fossil record of *Zamia* in Puerto Rico reportedly extends to the mid-Oligocene; Hollick (1928) described two distinct fossil species in the genus based on macrofossil remains, which would date the history of the genus on the island to ≥30 Ma BP. However, the determination of the two Hollick fossils as *Zamia* is currently being questioned (B. Erdei, personal communication), and Van Gestel et al. (1998, 1999) presented evidence that Puerto Rico was below sea level between the Late Oligocene and the Early Pliocene. Therefore, the current presence of *Zamia* on this island may not be older than ~5 Ma BP.

The relationships among these three taxa are largely unknown. Meerow et al. (2012) presented a preliminary analysis of seven populations using 10 simple-sequence-repeat (SSR) loci, which included only a single population of *Z. pumila*. We concluded little from this smaller study concerning the genetic relationships of the three taxa, beyond noting lower genetic distance between *Z. portoricensis* and *Z. pumila* than between either of the former and *Z. erosa*. However, those preliminary data suggested several hypotheses that could be tested with the larger number of loci and populations used here: (1) that genetic divergence between north and south is greater than genetic divergence among populations in either region because of the geographic barrier to gene flow across the center of the island (asymmetric differentiation); (2) that each recognized taxon can be uniquely differentiated by microsatellite data; and (3) that each taxon dispersed separately into Puerto Rico.

This study represents the most intensive population genetics investigation of a cycad to date in terms of number of markers, and one of only a few microsatellite studies of plant populations from the Caribbean biodiversity hotspot (Namoff et al., 2011; Calonje et al., 2012; Geiger et al., 2012) or of cycads in general (Meerow et al., 2007, 2012; Lopez-Gallego and O'Neil, 2009; Cibrián-Jaramillo et al., 2010).

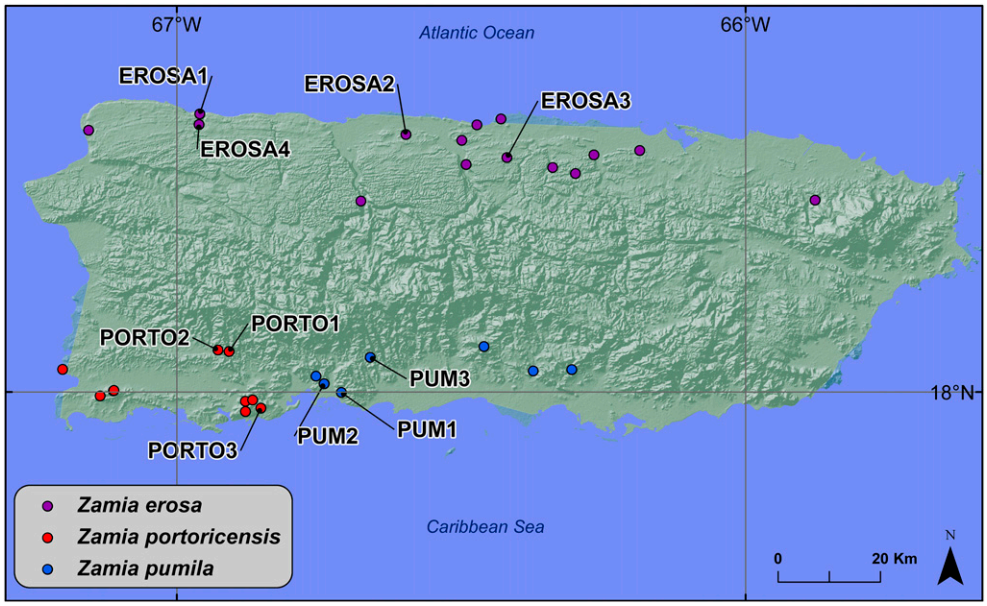

Fig. 2. Map of historical distribution of three *Zamia* in Puerto Rico, compiled from herbarium records, and approximate locations of the 10 populations sampled for this study. Many of the historical localities are now extirpated.

MATERIALS AND METHODS

***Sampling, DNA extraction and amplification*—**Leaflet samples from seven *Zamia* populations were collected in May 2005 (Table 1, Fig. 2), representing all three taxa reported from the island, and from the region where the three occur in closest geographic proximity. Samples were collected randomly but across the geographic breadth of the population, avoiding only very young seedlings (<2 yr old) in our sampling as estimated by plant size and numbers of leaves and leaflets. Three additional populations were collected in 2006 and 2007. Leaflet samples were placed in silica gel for later DNA extraction. Geographic positioning system coordinates are withheld from the population data (Table 1) in the interest of preventing exploitation; requests for this information by researchers or land managers should be sent to the first author. Vouchers are deposited in the herbarium of Fairchild Tropical Botanic Garden (FTG; Table 1).

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 and Nakamura, 2007; Meerow et al., 2007, 2012). We also data-mined the *Zamia* EST databases available in GenBank using the Simple-Sequence Repeat Identification Tool (SSRIT; www.gramene.org/db/searches/ssrtool). Six of the 31 (Zam28, Zam33, Zam34, Zam39, Zam40, Zam45) were previously published (Meerow and Nakamura, 2007), and the remaining 25 are described in Appendix S1 (see Supplemental Data with the online version of this article).

DNA was extracted, PCR amplified using 31 SSR primer pairs (Appendix S2, see Supplemental Data with the online version of this article), and visualized on an ABI 3730 Capillary DNA Analyzer (Applied Biosystems, Foster City, California) as described in Meerow and Nakamura (2007) and Meerow et al. (2007, 2012). Raw data were analyzed using GeneMapper version 4.0 (Applied Biosystems). We did a second round of amplifications for a sampling of homozygotes at each locus from each population using Phusion Hot-Start polymerase (Thermo Scientific, Waltham, Massachusetts). If a locus amplified a second allele with the higher-fidelity enzyme, we then reassayed all homozygotes from the samples for that locus.

***Data integrity*—**We used the program Microchecker version 2.2.3 (van Oosterhout et al., 2004) to identify putative errors of three types in our data (DeWoody et al., 2006): stuttering patterns, large-allele dropout, and null alleles. Putative null alleles were detected in no more than two loci in six populations (EROSA1, PORTO1, PUM1, PUM2, PUM3), in only one in four populations (EROSA2, EROSA3, PORTO2, PORTO3), and in none in a single population (EROSA4). No other genotyping errors were inferred. Because these loci did not manifest any previously undetected alleles with the higher-fidelity polymerase, their excess of homozygotes was interpreted as genuine and no adjustments were made to the data.

Polymorphic loci in each taxon were tested for positive or balancing selection using the programs LOSITAN (Antao et al., 2008), which implements the F_{ST}-outlier detection method (Beaumont, 2005; Vitalis et al., 2001), and BayeScan (Foll and Gaggiotti, 2008), a Bayesian approach that uses differences in allele

TABLE 1. Localities, populations, sample sizes, and voucher specimens for the 10 Puerto Rican *Zamia* analyzed across 10 SSR loci. Localities are deliberately vague to discourage illegal collection of plants. Bona fide researchers should contact the senior author for locality details. All vouchers are deposited in the herbarium of Fairchild Tropical Botanic Garden (FTG).

	Pop. ID	N	Locality	Voucher
Zamia erosa	EROSA1	17	Isabella, near Quebradillas border, understory of coppiced *Coccoloba uvifera*	Meerow and Ayala-Silva 3100
Z. erosa	EROSA2	45	Arecibo, Cambalache Reserve, semimoist forest	Meerow and Ayala-Silva 3101
Z. erosa	EROSA3	38	Vega Baja, semimoist *mogote* woods	Meerow and Ayala-Silva 3102
Z. erosa	EROSA4	8	Isabella, Quebradillas; on a banana farm, close to Hwy 2	Turnbull 21 (NY)
Z. portoricensis	PORTO1	39	Sabana Grande, Susua Forest reserve	Meerow and Ayala-Silva 3103
Z. portoricensis	PORTO2	28	Yauco, just outside Susua Forest reserve	Meerow and Ayala-Silva 3104
Z. portoricensis	PORTO3	30	Guánica, Dry Forest Reserve	Meerow and Ayala-Silva 3105
Z. pumila	PUM1	37	Peñuelas, dry, semideciduous ravine forest understory	Meerow and Ayala-Silva 3106
	PUM2	44	Guayanilla, nearest town Tallaboa Poniente.	Turnbull 1 (NY)
	PUM3	40	Ponce, vic. Marueno.	A. Calonje s.n. (No voucher)

frequencies between populations based on the multinomial Dirichlet model (Foll and Gaggiotti, 2008). The results of the two programs were not congruent, either in the category of selection for which a given locus tested, or the number and identity of loci under selection (Appendix S3, see Supplemental Data with the online version of this article). These discrepancies between the two approaches support the recent assertion that false positives are a consideration with these methodologies (Narum and Hess, 2011). Because BayeScan is the more conservative of the two programs, we experimented with dropping those loci identified by this program as non-neutral. We found very little change to population descriptive statistics and, thus, opted to not exclude them from other analyses.

Linkage disequilibrium (LD), the nonrandom pairwise association of loci, was tested for significance in each population with Arlequin version 3.5 (Excoffier et al., 2005), using a likelihood-ratio test (Slatkin and Excoffier, 1996), and is reported as percentage of total pairwise associations testing positively per population. A Monte Carlo Markov Chain (MCMC) method was applied with 100 000 iterations, 10 000 burn-in runs, and significance level set at $P < 0.001$. Little significant LD was observed. The highest percentage was found in EROSA1 (2.8%), but for all other populations, the percentage was <1.5%, with three populations showing no LD at all (EROSA3, PUM2, and PUM3).

The allele-size permutation test of Hardy et al. (2003) was used with the program SPAGeDi version 1.2. (Hardy and Vekemans, 2002) to assess whether a stepwise mutation process (SMM; Kimura and Otha, 1978) contributed to population differentiation. The maximum allowable number of permutations (20 000) was conducted on the matrix transformed from fragment size in base pairs (bp) to number of repeat units. The test allows the comparison of observed (i.e., nonpermutated) R_{ST}, an analog of Wright's (1965) F_{ST} that incorporates allele size and thus assumes the SMM (Slatkin, 1995) with the value of R_{ST} after permutation (pR_{ST}). A nonsignificant probability that $R_{ST} > pR_{ST}$ supports the null hypothesis that the stepwise increase in allele size is not a significant factor in population differentiation (Hardy et al., 2003). Although stepwise increase in allele size tested significantly in population differentiation across all 10 populations and all loci ($P < 0.001$), only 5 of the 31 loci supported this hypothesis. Tested by taxon, in *Z. portoricensis* there was no contribution of stepwise increase, whereas in *Z. erosa* and *Z. pumila*, the hypothesis was validated at $P < 0.05$ (*Z. erosa*: 3 loci, *Z. pumila*: 2 loci), but the low number of conforming loci suggests that a strict SMM is not applicable to the data.

***Data analyses*—**Descriptive statistics (number of alleles per locus, N_a; number of private alleles; observed (H_o), and expected heterozygosity (H_e), and fixation index or inbreeding coefficient, f (for populations) and F_{IS} (for loci), were generated with GenAlEx version 6.41 (Peakall and Smouse, 2006). Tests for Hardy-Weinberg equilibrium (HWE), the *U* test (Rousset and Raymond, 1995) for heterozygote excess or deficiency, were run with GenePop version 4.0 (Raymond and Rousset, 1995) using 10 000 MCMC iterations (Guo and Thompson, 1992). Analysis of molecular variance (AMOVA) and calculation of pairwise F_{ST} was conducted in GenAlEx with 10 000 permutations of the data. We estimated pairwise number of migrants (N_m) between populations by the private allele (PA) method, after using the Slatkin (1985) correction for sample size. A value of $N_m > 1.0$ was considered evidence of effective gene flow (Wright, 1951; Maruyama, 1977; Nagylaki, 1980).

Structure version 2.3.3 (Pritchard et al., 2000) was employed to evaluate population structure and admixture among the populations. Twenty replicates each of 1 million MCMC iterations were run for $K = 1$–30 on the University of Oslo Bioportal (www.bioportal.uio.no). Our strategy for determining the optimal value of K (number of populations) with Structure is the ΔK method of Evanno et al. (2005), implemented in the program Structure Harvester (Earl and vonHoldt, 2011). A consensus Q-matrix was constructed from the 20 replicates at optimal K using the program CLUMPP (Jakobsson and Rosenberg, 2007) for visualization with Distruct (Rosenberg, 2004).

Possible scenarios of population divergence through time were evaluated with DIYABC version 0.7.3 (Cornuet et al., 2008), which applies approximate Bayesian computation, or ABC (Beaumont et al., 2002), using a similarity criterion between simulated and observed data sets, measured by a distance between summary statistics computed on both data sets. The approach allows the posterior probability (PP) of different scenarios applied to the same data set to be estimated (Miller et al., 2005; Pascual et al., 2007), with the assumption that the scenario with highest PP is the most accurate based on a polychotomic logistic regression of each scenario probability on the deviations between simulated and observed summary statistics (Fagundes et al., 2007; Beaumont, 2008; Cornuet et al., 2008). In setting priors for effective population sizes, we used N_e estimates generated with the program LDNe (Waples and Do, 2008). A generalized step mutation (GSM) model (Fu and Chakraborty, 1998; Estoup et al., 2002) was imposed, wherein a mutation increases or decreases the number of repeats by one or several units, the latter derived from a geometric distribution, and no single nucleotide allele differences were recognized. Statistics selected upon which the simulations were based were F_{ST}, shared allele distance, index of classification (Rannala and Mountain, 1997; Pascual et al., 2007), and the Garza and Williamson (2001) M coefficient. The latter is a measure of whether a recent bottleneck was experienced in a population and is calculated as the mean ratio of the number of alleles to the range in allele size. An M value <0.68 is the estimated threshold below which bottleneck is considered to have occurred (Garza and Williamson, 2001). It is less sensitive to bottleneck duration than alternative tests (Williamson-Natesan, 2005).

RESULTS

***Descriptive statistics and private alleles*—**Analyzed together across all 31 loci, the mean percentage of polymorphic loci was 87.7% (SE 1.25), and the range was 80.7–93.6% (Table 2). Across all three taxa, the loci uniquely genotyped 320 of 328 individuals (97.5%). Although the 31 SSR loci were polymorphic across all three taxa analyzed together, two, four, and four loci were monomorphic (including loci that exhibited a second allele in only a single individual) within *Z. erosa*, *Z. portoricensis*, and *Z. pumila*, respectively (Table 2; Appendix S2, see Supplemental Data with the online version of this article). Consequently, analyses of each taxon alone were conducted with 29 (*Z. erosa*) or 27 (*Z. portoricensis* and *Z. pumila*) loci. The average number of alleles per locus in the 10 populations ranged from 4.5 to 7.0 (Table 2). Overall levels of heterozygosity were similar, ranging from 0.53 to 0.58 (Table 2). When each taxon was analyzed separately (monomorphic loci dropped in each taxon), 4 of the 10 populations were significantly in heterozygote deficit (Table 2): 2 of *Z. erosa* (EROSA2, $P < 0.001$; EROSA4, $P < 0.01$) and 2 of *Z. portoricensis* (PORTO1, $P < 0.05$; PORTO3, $P < 0.05$). The highest number of private alleles was found in *Z. erosa* regardless of whether the three taxa were analyzed together or separately (Table 2); *Z. portoricensis* had the least.

***Effective population sizes*—**Estimates of N_e from the ABC analysis (Table 3) were largest for *Z. erosa* and *Z. pumila* and lowest for *Z. portoricensis*. The largest single estimate was for PUM2 and the lowest for PORTO2. Second lowest was EROSA1, a small seaside population restricted to the understory of a coppiced *Coccoloba uvifera*-covered low bluff within a few meters of the open ocean.

***Genetic structure and population differentiation*—**The AMOVA (Table 4) indicated that most of the genetic variation in Puerto Rican *Zamia* is partitioned within the populations (87%), with 6% among the three taxa and 7% among the populations. Analyzed by taxon, the percentage within populations rose to 96% for *Z. portoricensis* and *Z. pumila* but remained at 87% for *Z. erosa* (Table 4). Mean F_{ST} across all loci and populations was 0.129 ($P < 0.0001$), and F_{ST} values for each taxon (all $P < 0.0001$) were 0.131 for *Z. erosa*, 0.036 for *Z. portoricensis*, and 0.042 for *Z. pumila*. The highest pairwise F_{ST} values within taxa were between populations of *Z. erosa* (Appendix S4, see Supplemental Data with the online version of this article), and the lowest were between populations of *Z. portoricensis*, followed by *Z. pumila*. F_{ST} values between populations of *Z. portoricensis* and *Z. pumila* ranged from 0.057 to 0.116. The pairwise F_{ST} values for *Z. erosa* populations were mostly greater than 0.115 and were generally 2–4 times greater than F_{ST} values between populations of either *Z. portoricensis* or *Z. pumila* (Appendix S4, see Supplemental Data with the online version of this article).

TABLE 2. Descriptive statistics of genetic variation across 10 Puerto Rican populations of *Zamia*, each analyzed separately. % PL = percent polymorphic loci, N_a = number of alleles per locus, PA = private alleles, H_o, H_e = observed and expected heterozygosity, f = fixation index, M = Garza and Williamson (2001) coefficient. Values without standard errors (SE) reported are not means.

Population	% PL		N_a	Total PA across all loci (all spp. together, 31 loci)	H_o	H_e	f [a]	M
				Zamia erosa (29 loci)				
EROSA1	100	Mean	4.8	21 (13)	0.558	0.532	–0.037	0.9412
		SE	0.6		0.058	0.051	0.042	
EROSA2	100	Mean	6.9	34 (12)	0.525	0.540	0.027***	0.9333
		SE	0.9		0.051	0.052	0.020	
EROSA3	100	Mean	6.0	28 (14)	0.555	0.562	0.015	0.9231
		SE	0.7		0.051	0.050	0.024	
EROSA4	100	Mean	4.5	13 (6)	0.543	0.560	0.027**	0.9775
		SE	0.5		0.055	0.050	0.042	
				Zamia portoricensis (27 loci)				
PORTO1	100	Mean	6.9	26 (5)	0.519	0.534	0.014*	0.6929
		SE	1.0		0.054	0.055	0.019	
PORTO2	100	Mean	5.7	11 (2)	0.507	0.491	–0.042	0.6652
		SE	0.8		0.059	0.057	0.021	
PORTO3	100	Mean	6.3	18 (5)	0.524	0.539	0.016*	0.6813
		SE	0.8		0.054	0.055	0.021	
				Zamia pumila (27 loci)				
PUM1	100	Mean	6.9	19 (7)	0.548	0.565	0.011	0.7460
		SE	0.9		0.051	0.052	0.026	
PUM2	100	Mean	7.0	26 (7)	0.535	0.551	0.021	0.7015
		SE	1.0		0.055	0.055	0.019	
PUM3	100	Mean	6.6	16 (5)	0.564	0.579	0.024	0.7417
		SE	0.8		0.055	0.052	0.029	

[a] Significant departures from HWE at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, based on the U test (Rousset and Raymond 1995) for heterozygote excess or deficiency.

Structure analyses—The highest modal value for ΔK indicated that $K = 4$ is most likely the "true" number of genetic clusters within our sample of 10 geographic populations (Fig. 3A). All three *Z. portoricensis* populations constituted one cluster, and most of *Z. pumila* formed a second cluster, although a small but significant degree of admixture between PUM2 and *Z. portoricensis* was indicated. *Zamia erosa* was almost exclusively assigned to the last two clusters. EROSA1 and EROSA4 individuals were wholly assigned to one of those clusters, and EROSA3 individuals were assigned to the other, whereas EROSA2 is almost evenly admixed between the two.

When the three taxa were analyzed separately, with taxon-specific monomorphic loci removed, ΔK estimated K = 3, 3, and 2 clusters for *Z. erosa* (Fig. 3B), *Z. portoricensis* (Fig. 3C), and *Z. pumila* (Fig. 3D), respectively. The greatest degree of admixture was among the populations of *Z. portoricensis*, and the least was among the populations of *Z. erosa*. EROSA1 and 4 are still assigned to the same cluster, but EROSA2 is assigned with high proportion to its own.

TABLE 3. Parameter estimates from the coalescent analysis of *Zamia* in Puerto Rico. N_e = mean effective population size, mean μ = mutation rate, mean $4N_e\mu$ = migration rate.

Population	N_e	μ	$4N_e\mu$
Zamia erosa		0.00018	
EROSA1	489		0.31878
EROSA2	5588		3.7397
EROSA3	1510		0.99225
EROSA4	6348		4.39608
Z. portoricensis		0.0006	
PORTO1	2100		4.82418
PORTO2	46		0.11011
PORTO3	1461		3.31399
Zamia pumila		0.00017	
PUM1	5401		3.33655
PUM2	7660		4.99017
PUM3	5509		3.54862

***Gene flow*—**By the PA method, the highest gene flow is among the populations of *Z. portoricensis* and *Z. pumila*, respectively, and the lowest between populations of *Z. erosa* (Table 5). Only a single pairwise N_m estimate in *Z. erosa* is ≥1.0 (EROSA2-3), whereas 7 of 24 pairwise N_m values between *Z. erosa* and the other taxa are >1.0. *Zamia pumila* is intermediate but closer to *Z. portoricensis* (1.8–2.7). A degree of historical gene flow is indicated between *Z. portoricensis* and *Z. pumila*, particularly with PUM2 (1.2–1.8).

***Population history based on coalescent simulations*—**Before attempting ABC coalescent analysis on all 10 populations across the three Puerto Rican taxa, we evaluated each taxon individually. For *Z. erosa*, six scenarios (Appendix S5A–F, see Supplemental Data with the online version of this article) were first evaluated for 500 000 iterations. On the basis of highest PPs in the logistic regression, the two highest scoring scenarios (Appendix S5D and F) were selected for further analysis with 1.5 million iterations. Of these two, the scenario with highest PP (Fig. 4A, B) showed the western populations, EROSA1 and EROSA4, coalescing more recently than the eastern populations, EROSA2 and EROSA3. Nonetheless, there was not tacit rejection of the second scenario, which places the coalescence of the eastern populations more recently than that of the western. The highest migration rate was found in EROSA2 and EROSA4 (low sample size in EROSA4 biases this estimate), and the lowest was in EROSA1. The mean coalescence time for the eastern

TABLE 4. Analysis of molecular variation (AMOVA) across 10 Puerto Rican *Zamia* populations and within each taxon.

Source of variation	df	Sum of squares	Variance components	Percentage of variation
All 10 populations				
Among taxa	2	334.327	0.524	6
Among populations	7	324.379	0.620	7
Within populations	646	4993.274	7.730	87
Zamia erosa				
Among populations	3	204.652	1.225	13
Within populations	216	1752.035	8.111	87
Z. portoricensis				
Among populations	2	48.456	0.266	4
Within populations	191	1372.956	7.188	96
Z. pumila				
Among populations	2	70.917	0.344	4
Within populations	239	1857.748	7.773	96

and western populations was estimated to be 7528 generations. Coalescence of EROSA2 and EROSA3 was estimated at 4422 generations, and coalescence of EROSA1 and EROSA4 was 1525 generations (but see above caveat for latter).

Three scenarios were tested for *Z. portoricensis* (Appendix S5G–I) for 1.5 million iterations. The scenario with the highest PP (Fig. 4C, D) placed the primary divergence between PORTO3 (from the Guánica Dry Forest Reserve) and the ancestor of PORTO1 and PORTO2, which occur in foothills of the Cordillera Central. Migration rate is lowest for PORTO3 (Table 3). The coalescent time between PORTO1 and PORTO2 was very recent, estimated at a mean of 21 generations, whereas the mean estimate for the Guánica (PORTO3) and foothill populations was 392 generations (Table 3). The mean estimated mutation rate for *Z. portoriciensis* was 3 times higher (0.0006) than for either of the other two taxa (0.00018, 0.00017).

Three scenarios were also tested for *Z. pumila* (Supplementary Information Fig. 1J–L). The scenario with highest PP position PUM3 as the earliest divergence (Fig. 4E, F). Mean coalescent times were estimated at 456 generations for PUM1 and PUM2 and at 1109 generations for the basal coalescence (Table 3). Migration rate estimates were >3 migrants per generation for all three populations (Table 3), with the highest estimate for all taxa in PUM2 (4.99).

We then attempted an analysis of the three taxa together, using the best-supported coalescent scenario for each taxon. After numerous attempts, with diverse choices of priors, we found it impossible to get DIYABC to accept a scenario of coalescence for all three taxa when all populations were incorporated in the analysis. We then tried analyzing all combinations of two taxa with the same results. We only succeeded in analyzing coalescent scenarios across the three taxa by combining all populations of each into a single combined taxon-level sample in the data set. Of the three scenarios tested (Appendix S5M–O), the scenario with highest PP resolved the earliest divergence between *Z. erosa* and the common ancestor of *Z. portoricensis* and *Z. pumila* (Fig. 4G, H). However, a number of performance indicators built into DIYABC (Cornuet et al., 2008) suggested that the scenarios did a poor job of explaining the observed data for this last analysis. For example, a direct estimate (the number of times that a given scenario is found in the first *n* simulated data sets once they have been sorted by ascending distance to the observed data set) indicated little differences in PP among the three scenarios (range of only 0.3–0.4, not shown), though the aforementioned scenario had the highest. Moreover, a principal component analysis (PCA) on the table of summary statistics of simulated data did not overlap the observed data set in any of the first four components of the PCA (not shown).

There was little evidence of recent bottlenecks among the 10 populations. The ABC estimates of the *M* coefficient (Table 3) indicate that only *Z. portoricensis* (PORTO2) may have undergone a recent bottleneck ($M < 0.68$), but only *Z. portoricensis* populations had values <0.7.

DISCUSSION

We now address the three hypotheses posed by our study: (1) that genetic divergence between north and south is greater than

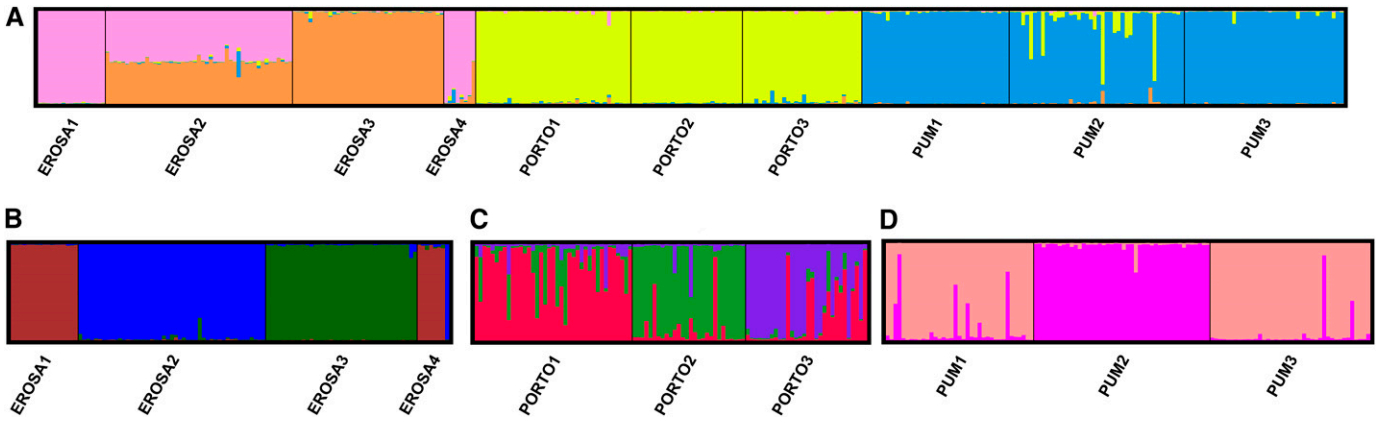

Fig. 3. Structure cluster histograms of 10 *Zamia* populations in Puerto Rico. (A) All three, $K = 4$. (B) *Z. erosa*, $K = 3$. (C) *Z. portoricensis*, $K = 3$. (D) *Z. pumila*, $K = 2$.

TABLE 5. Number of migrants per generation (N_m) estimated by the private allele method (Slatkin, 1985; Barton and Slatkin, 1986; Slatkin and Barton, 1989).

Population	EROSA1	EROSA2	EROSA3	EROSA4	PORTO1	PORTO2	PORTO3	PUM1	PUM2	PUM3
EROSA1	–									
EROSA2	0.8	–								
EROSA3	0.5	1.4	–							
EROSA4	0.8	0.9	0.7	–						
PORTO1	0.7	1.1	1.0	0.8	–					
PORTO2	0.7	0.6	0.5	0.8	2.8	–				
PORTO3	0.9	1.3	0.9	0.9	2.9	2.2	–			
PUM1	0.7	1.0	0.7	0.7	1.3	1.0	1.3	–		
PUM2	0.7	1.0	1.1	0.8	1.8	1.2	1.7	2.7	–	
PUM3	0.6	1.1	0.9	0.7	1.2	0.8	1.0	2.7	1.8	–

genetic divergence among populations in either region because of the geological barrier to gene flow across the center of the island, (2) that each recognized taxon can be uniquely differentiated by microsatellite data, and (3) that each taxon dispersed separately into Puerto Rico.

Is there asymmetric genetic differentiation of Zamia on Puerto Rico?—It has long been thought that species with limited geographic distribution would show lower genetic diversity than congeneric, widespread species (Loveless and Hamrick, 1984; Karron, 1987; Hamrick et al., 1991), but the phylogenetic relationships of the taxa in question have not always been factored into comparison (Gitzendanner and Soltis, 2000; Cole, 2003; Leimu et al., 2006; Cabrera-Toledo et al., 2010). Where these have been considered, some studies have found that congeneric species with differing geographic ranges can share similar genetic structure and diversity (Morjan and Rieseberg, 2004; Duminil et al., 2007). Cabrera-Toledo et al. (2010) compared allozyme diversity patterns across two *Dioon* Lindl. (Zamiaceae) species, *D. caputoi* De Luca, Sabato & Vázq. Torres and *D. merolae* De Luca, Sabato & Vázq. Torres, which are closely related phylogenetically but differ markedly in their geographic range and relative abundance. Although *D. caputoi*, the rarer of the two, had significantly lower genetic diversity, the relative level of population differentiation (assessed with F_{ST}) was not appreciably different between species. By contrast, in the case of Puerto Rican *Zamia*, we find little difference among the taxa in genetic diversity, but very significant differences between the more widespread *Z. erosa* and the more geographically constrained *Z. portoricensis* and *Z. pumila* in patterns of genetic structure and differentiation.

Negrón-Ortiz et al. (1996) studied the reproductive morphology of the Cambalache population of *Z. erosa* (EROSA2) for 3 yr and found that very few adult female plants produced cones in a given year. They concluded that adult survivorship is more crucial to persistence of zamia populations than recruitment of new individuals. This seems particularly true of zamia populations growing under primary forest; Clark and Clark (1987) noted nearly a threefold increase in coning of Costa Rican *Zamia neurophyllidia* D.W. Stev. (as *Z. skinneri* Warsz. ex A. Dietrich) in secondary forest, which they attributed to higher light levels. Lopez-Gallego and O'Neil (2009) reported similar findings for *Z. fairchildiana* L. D. Gómez. If this is a constant component of life history in undisturbed forest populations of *Z. erosa*, it would further reinforce the gene-flow constraints engendered by pollination and dispersal biology, discussed previously, as well as facilitate the accumulation of private alleles.

Evidence for selection in Zamia erosa—Six of the 13 (21 when the taxa are analyzed separately; Table 2) private alleles in EROSA1 have frequencies >10%, two >20% (Appendix S6, see Supplemental Data with the online version of this article). All private alleles in *Z. portoricensis* occur at <4% frequency, and <6.5% in *Z. pumila*. EROSA1 is adapted to a saline environment on bluffs along the shore, whereas EROSA2, EROSA3, and EROSA4 (the population most closely related to EROSA1) occur in semi-evergreen moist forest understory. The high frequency (and high number) of private alleles in EROSA1 may thus have adaptive significance, perhaps even representing signs of a selective sweep within this small population via the hitchhiking effect (Maynard Smith and Haigh, 1974). EROSA1 may be the youngest in our sample of *Z. erosa*, based on estimates of N_e with the coalescent. Percentage LD is highest in this population, and increased LD among neutral loci often initially accompanies ongoing selective sweeps in populations (Hudson et al., 1994; Sabeti et al., 2002). We would argue that the colonization by EROSA1 of a high-saline environment represents true adaptation. On the other hand, the colonization by PORTO3 of xeric dry forest habitat, in contrast to the more mesic habitats of PORTO1 and PORTO2 as well as *Z. pumila*, may represent the consequences of drift. *Zamia portoricensis* has the highest mutation rate in our coalescent estimates, three times that of *Z. erosa* and *Z. pumila*.

Can each taxon be distinguished genetically?—Our data across 10 populations of the *Z. pumila* complex in Puerto Rico support recognition of three distinct taxa, each of which appears to have maintained its own genealogical history for at least hundreds, if not thousands, of generations. Their genetic distinction is supported by Bayesian model-based clustering.

Of the three taxa, *Z. erosa* is the most highly differentiated. F_{ST} and Structure analysis denote the greater population differentiation of *Z. erosa* in Puerto Rico compared with *Z. portoricensis* and *Z. pumila*. In some pairwise estimates, there appears to be lower differentiation between populations of *Z. erosa* and either *Z. portoricensis* and *Z. pumila* than among populations of *Z. erosa*. Why is there such a degree of population differentiation in this taxon compared with *Z. portoricensis* and *Z. pumila*? Geographic distance is clearly a factor. Both *Z. portoricensis* and *Z. pumila* appear more constrained in geographic distribution than *Z. erosa*; the greatest pairwise distance between populations of *Z. portoricensis* in our sample is 14 km, in *Z. pumila* 10 km. In comparison, pairwise distances between *Z. erosa* populations range from 19 to 58 km, except between EROSA1 and EROSA4 (2 km). These two populations have the lowest pairwise F_{ST} values for the taxon, and Structure resolves them in a single genetic cluster. Yet F_{ST} values are higher than any pairwise value within the other two taxa. However, the larger factor by far is our first alternative hypothesis: *Z. portoricensis* and *Z. pumila* are

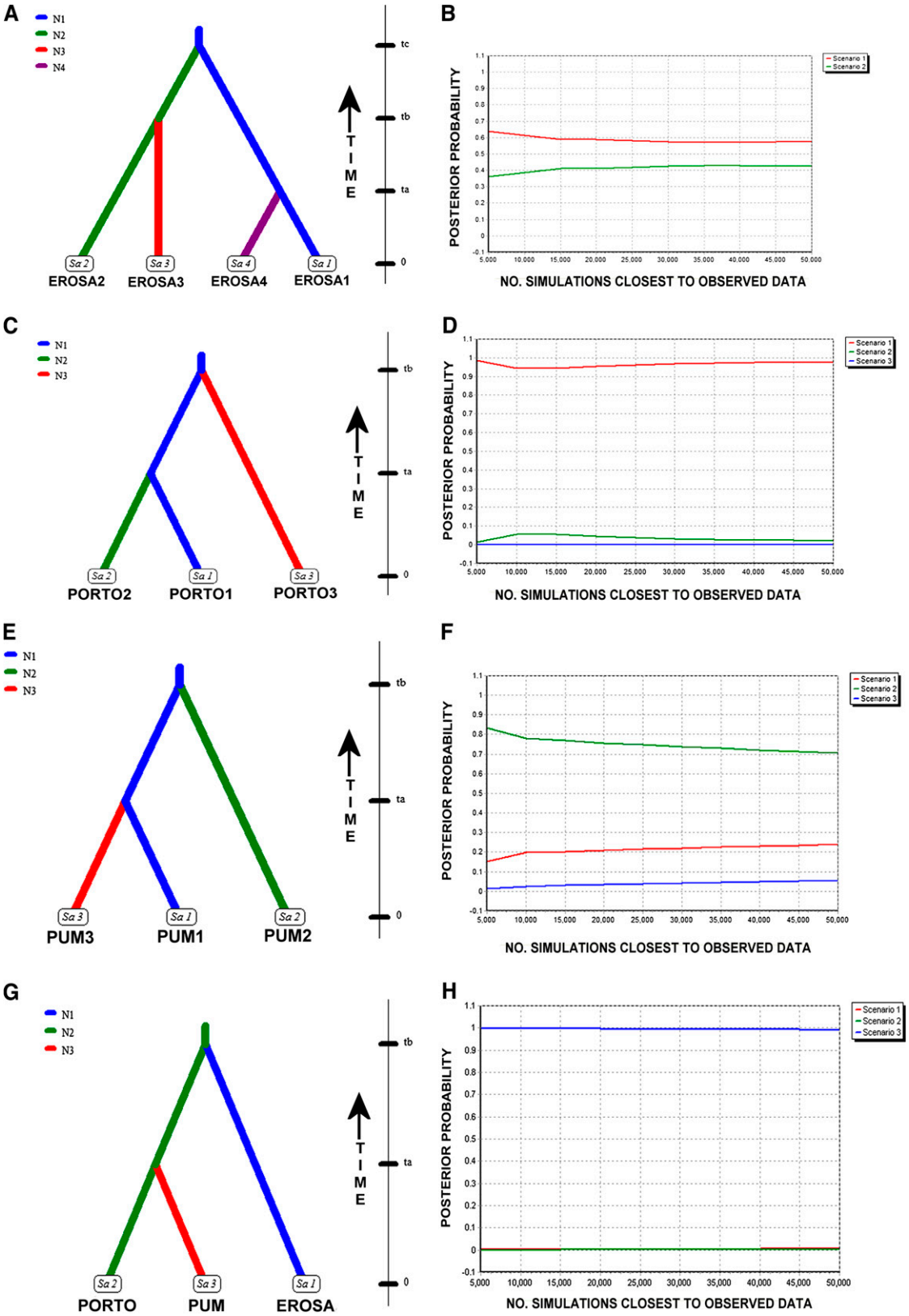

Fig. 4. Coalescent analysis in Puerto Rican *Zamia*. (A, B) *Zamia erosa.* (A) Scenario with highest posterior probability (PP). (B) Logistic regression. (C, D) *Z. portoricensis*. (C) Scenario with highest PP. (D) Logistic regression. (E, F) *Z. pumila*. (E) Scenario with highest PP. (F) Logistic regression. (G, H) Analysis with the population of each with highest N_e (Table 5). (G) Scenario with highest PP. (H) Logistic regression.

allopatrically derived taxa, while we believe that *Z. erosa* represents an independent introduction onto the island.

The hypothesis of multiple introductions—Historical admixture between *Z. pumila* and *Z. portoricensis* is evident in PUM2, the *Z. pumila* population that has the lowest pairwise F_{ST} values with *Z. portoricensis*. This population is found in a more xeric habitat than either PUM1 or PUM3 and is the closest geographically to any population of *Z. portoricensis*. ABC analysis also supports a more recent coalescence between *Z. portoricensis* and *Z. pumila* than between either and *Z. erosa*. Their current distribution suggests a scenario of allopatric speciation. This event may be sufficiently old that mutation and drift have eliminated all but a faint footprint of their origins. Can we discount the possibility that this admixture pattern in PUM2 represents secondary contact between *Z. portoricensis* and *Z. pumila*? PUM2 has the largest N_e of any of the 10 populations in our sample. Long generation times in cycads in general (Treutlein and Wink, 2002), which would tend to maintain small N_e, allow us to infer that this population may also represent the oldest in our sample. Similarly, the low N_e of all three populations of *Z. portoricensis* suggests that it is the youngest taxon of the three. This is further supported by the evidence of a bottleneck in PORTO3 ($M < 0.68$), and M coefficients <0.7 in the other two populations, which may signify the lingering traces of founder effects (Barton and Charlesworth, 1984; Carson and Templeton, 1984; Cornuet and Luikart, 1996). Any bottlenecks associated with founding events of populations of *Z. erosa* and *Z. pumila* have long been obscured by population expansion and differentiation. Neither has loss of populations since European colonization resulted in recent bottlenecks in these two taxa, because gene flow is predominantly local. If, in fact, PUM2 is the oldest population of *Z. pumila* in our sample as N_e suggests, it is not surprising that it harbors evidence of shared alleles with the allopatrically derived *Z. portoricensis*. It is also the population of *Z. pumila* in our sample with closest geographic proximity to a population of *Z. portoricensis*.

The available evidence appears to reject the hypothesis that *Zamia* in Puerto Rico represents a single introduction and subsequent fragmentation of an initial panmictic population. Instead, we suggest that two independent introductions to Puerto Rico took place, on the north and south coasts of the island, respectively. No significant patterns of admixture between *Z. erosa* and the other two taxa are evident. The disjunct distribution of *Z. erosa*, the low dispersal amplitude of cycads in general, and the substantial impediment to migration presented by the Cordillera Central, leads us to consider that *Z. erosa* represents an independent introduction into Puerto Rico, or at least a much earlier divergence from the south-coast Puerto Rican zamias. The difficulty in applying a model of coalescence across all three taxa on the island lends credence to the first possibility. In fact, as we extend these studies throughout the Caribbean clade, preliminary data from both SSRs and single-copy nuclear genes are resolving a much more complex pattern of inter-island relationships than anticipated that may further support a scenario of independent introduction (A. Meerow, unpublished data).

Patterns of genetic variation, structure, and gene flow—Having addressed our three hypotheses, we now briefly discuss the patterns of genetic variation in Puerto Rican *Zamia*, with reference to previous population genetic studies of cycads. Pollination and dispersal biology in cycads constrain both gene flow and population recruitment to relatively small geographic areas (Tang, 1987, 1989; Norstog and Nicholls, 1997). Thus, the expectation would be high genetic differentiation between populations, directly proportional to the distance among them, and low intrapopulation differentiation. This is what the majority of the studies of cycad populations have found (e.g., Ellstrand et al., 1990, Jian et al., 2006). Yang and Meerow (1996) estimated gene flow of only 2–7 km between neighboring populations of Chinese *Cycas* species based on allozymes, and high levels of population differentiation. However, Byrne and James (1991) reported low differentiation among populations for *Macrozamia riedlei* L.A.S. Johnson in southern Australia (though no estimates of gene flow distance were provided), similar to what we report for *Z. portoricensis* and *Z. pumila*. They attributed this disparity to the more continuous distribution of *M. riedlei* across a fairly homogeneous area of low relief, thereby promoting high levels of gene flow, relative to the congener *M. communis* (Gaudich.) C.A. Gardner, each population of which represented an isolated element of a long-term fragmentation process initiated by geologic activity (Ellstrand et al., 1990).

During the Early Pliocene, sea levels were ~100 m above the present ones (Haq et al., 1987; Nunn, 1994). During the Pleistocene interglacials, average planetary sea levels changed only an average of ~20 m above present levels (Haq et al., 1987; Carter, 1998; Andersen and Borns, 1997), with lower changes (~8 m above current levels) reported for the Caribbean Islands during this period (Pregill and Olson, 1981; Stienstra, 1983; Nunn, 1994). All three taxa of *Zamia* on Puerto Rico occur above the 100-m threshold; therefore, eustatic sea-level changes would have affected only populations below the threshold. Pleistocene interglacials may have reinforced isolation of *Z. erosa* populations on the north-coast *mogotes* (isolated, steep-sided, limestone hills), by eliminating intervening lowland subpopulations.

We found 87–96% of the total genetic variation partitioned within the individual populations of Puerto Rican *Zamia*, and, consequently, much lower levels among populations. High infrapopulation genetic diversity has also been reported in *Dioon* with allozyme markers (Cabrera-Toledo et al., 2010), and in *Encephalartos* Lehm. with AFLPs (Da Silva et al., 2012), but is very typical of microsatellite data sets (Heller and Siegismund, 2009). High infra-population differentiation in Puerto Rican *Zamia* could also be explained by the suppression of the loss of genetic variation in long-lived species with long generation times (González-Astorga and Castillo-Campos, 2004), high rates of mutation and drift after new populations are founded, as well as the relatively short span of time (ca. 5 Ma BP) that Puerto Rico has been above sea level (Van Gestel et al., 1998, 1999).

As in most previous studies of cycad population genetics (for a summary of genetic diversity and differentiation statistics across 26 cycad species in 5 genera, see González-Astorga et al., 2008), observed heterozygosity is lower than expected in 8 of our 10 populations, although significant heterozygote deficiency occurs in only 4. Sixty percent of our populations may thus be effectively at HWE. Inbreeding coefficient values in the Puerto Rican zamias are similar to those reported by González-Astorga et al. (2006) for *Z. loddigesii* Miq., a Mexican species that has experienced population reductions and fragmentation (Vovides et al., 1983). The values reported here are in marked contrast to the negative values reported for *Dioon* species (e.g., Cabrera-Toledo et al., 2008; González-Astorga et al., 2008), some of which exhibit significant heterozygote excess at allozyme loci (Cabrera-Toledo et al., 2008; 2010). Hedrick (2000) suggested that dioecy favors HWE with low positive to zero values for inbreeding coefficients, which is exactly what we have found for most of the *Zamia* populations in Puerto Rico.

Our results for the three Puerto Rican taxa of *Zamia* on Puerto Rico—low to moderate inbreeding within populations and fairly elevated genetic variation in terms of allelic diversity and private alleles—is similar to what Cibrián-Jaramillo et al. (2010) found for another insular cycad, *C. micronesica* K. D. Hill, on Guam. It thus appears that generalizations regarding population structure and genetic variation across Cycadales are premature, and that choice of marker may contribute to estimates as much as historical demography of the populations under study.

To what extent Pre-Columbian human activity has affected *Zamia* distribution is not clear. *Zamia* was a major source of carbohydrate for Pre-Columbian indigenous Caribbean people (Mickleburgh and Pagán-Jiménez, 2012; Pagán-Jiménez, 2013). There is an association with *Zamia* fossil remains and Amerindian settlements in Aruba, the Bahamas, Cuba, Dominican Republic, Puerto Rico, the Lesser Antilles (Saba), and Trinidad (Veloz Maggiolo and Vega, 1982; Berman and Pearsall, 2000; Mickleburgh and Pagán-Jiménez, 2012; Pagán-Jiménez, 2013), including areas where extant *Zamia* populations have never been reported. Our continuing studies of *Zamia* throughout the Caribbean may help shed light on this controversial but potentially highly significant factor in the history of the *Z. pumila* complex.

***Conclusions*—**The three distinctive taxa of *Zamia* found on Puerto Rico exhibit a combination of the patterns of genetic differentiation that have been reported for cycads based on allozymes and dominant DNA markers. High infra-populational genetic variation is the rule for all three, perhaps a factor of the high degree of polymorphism in the SSR loci. Population differentiation is 3 times greater in *Z. erosa* than in *Z. portoricensis*. Gene flow is much greater in *Z. portoricensis* populations than in the other two taxa. Nine of the 10 populations do not appear to have undergone recent bottlenecks, based on the current sampling. Significant heterozygote deficiency is characteristic of several populations of *Z. portoricensis* and Z. *erosa*, which may be a cause for concern from a conservation perspective.

If all three taxa of *Zamia* on Puerto Rico share immediate common ancestry, the primary divergence between them occurred so long ago as to obscure the threads of common genealogy by isolation, drift, and mutation. Evidence of historical gene flow between *Z. portoricensis* and *Z. pumila* and their geographic distribution suggest allopatric speciation. We found no such evidence of a similar relationship between *Z. erosa* and either of the two other taxa in Puerto Rico. The difficulty in modeling the relationships of the three taxa with coalescent simulation is the best evidence at this time that the genus may have reached the north and south coasts of the island in independent events. Relatively low levels of genetic differentiation across all three as measured by F_{ST} reflect slow rates of molecular evolution in cycads (Walters and Decker-Walters, 1991). Our data do not support any model of recent panmixis among these three taxa. Whether they each deserve recognition as distinct species is a question that remains unresolved until we have a more complete understanding of the genetic and phylogenetic relationships of the entire *Zamia pumila* complex throughout the Caribbean region.

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