

When North and South don't mix: genetic connectivity of a recently endangered oceanic cycad, *Cycas micronesica*, in Guam using EST-microsatellites

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Abstract

Subject to environmental changes and recurrent isolation in the last *ca.* 250 Ma, cycads are often described as relicts of a previously common lineage, with populations characterized by low genetic variation and restricted gene flow. We found that on the island of Guam, the endemic *Cycas micronesica* has most of the genetic variation of 14 EST-microsatellites distributed within each of 18 genetic populations, from 24 original sampling sites. There were high levels of genetic variation in terms of total number of alleles and private alleles, and moderate levels of inbreeding. Restricted but ongoing gene flow among populations within Guam reveals a genetic mosaic, probably more typical of cycads than previously assumed. Contiguous cycad populations in the north of Guam had higher self-recruitment rates compared to fragmented populations in the south, with no substantial connection between them except for one population. Guam's genetic mosaic may be explained by the influence of forest continuity, seed size, edaphic differences, and human transport of cycads. Also important are the extent of synchrony among flushes of reproductive female seed-bearing sporophylls and restricted pollen movement by an obligate mutualist and generalist insects. An NADH EST-locus under positive selection may reflect pressure from edaphic differences across Guam. This and three other loci are ideal candidates for ecological genomic studies. Given this species' vulnerability due to the recent introduction of the cycad aulacaspis scale, we also identify priority populations for *ex situ* conservation, and provide a genetic baseline for understanding the effects of invasive species on cycads in the Western Pacific, and islands in general.

Keywords: cycad, EST-microsatellites, Guam, invasive species, islands, oceanic islands

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Introduction

Oceanic islands are amongst the most biodiverse and evolutionary intricate ecosystems (MacArthur & Wilson 2001; Caujape-Castells *et al.* 2010). They are also more susceptible than their continental counterparts to human-induced habitat changes, including the introduc-

tion of invasive species (Frankham 1998; Francisco-Ortega *et al.* 2000; Parmesan 2006; Reaser *et al.* 2007; Hellmann *et al.* 2008; Kueffer *et al.* 2009). The island of Guam is one of the most transformed island ecosystems, mostly due to invasive species (Rogers 1995). In recent years the introduction of invasive insects in Guam have impacted the island's flora and triggered a cascade of invasive species interactions (Marler *et al.* 2006). In particular, the introduction of the cycad aulacaspis scale (*Aulacaspis yasumatsui* Takagi; Hemiptera:

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Diaspididae) into Guam in 2003 has caused extensive damage to natural populations of the cycad *C. micronesica* K.D. Hill (Cycadaceae), one of the few remaining endemic plants in Guam (Marler & Muniappan 2006). Here, we describe the extent of standing genetic variation and gene flow in natural populations of *C. micronesica* and their interplay with putative historical and contemporary geographic barriers in this island.

The genus *Cycas* is one of the oldest living lineages of gymnosperms (ca. 250 my) with ca. 90 species distributed from the Western Pacific to coastal regions of Madagascar (Hill 2004; Hill *et al.* 2004). Phylogenetically sister to all other cycads (Nixon *et al.* 1994), *Cycas* has provided critical insights into the evolution of basic developmental and functional traits in early seed plants (Norstog & Nicholls 1997). *C. micronesica* is a plant up to 12 m tall, with megasporophylls not gathered in cones, usually spine-free petiole, and one of the nine *Cycas* species with a seed flotation layer that allows them to disperse and survive through ocean currents (Dehgan 1983; Hill 1994; Hill 2004; Hill *et al.* 2004). Its name is derived from the endemic occurrence of this species in Rota, Guam, Yap and Palau in Micronesia (Hill 2004; Hill *et al.* 2004).

Since its introduction into Guam 2003, the cycad aulacaspis scale has spread throughout all of Guam's forests and recently into the nearby islands of Rota, Yap and Palau, threatening *C. micronesica* regionally and all other Pacific *Cycas* species (Marler & Muniappan 2006). In a few months after it arrives at a site, the cycad aulacaspis scale (phloem-feeder) causes mortality of cycad adults by triggering repeated leaf flushes (Marler 2009). Non-native cycad blue butterflies (*Chilades pandava* Horsfield), yellow crazy ants (*Anoplolepis gracilipes*), and feral pigs (*Sus scrofa*) cause secondary structural damage to plants already injured by the insect scale, elevating mortality up to 90% (Marler 2009).

Local extinction of *C. micronesica* populations could lead to the erosion of genetic diversity in other organisms. Cycads are adapted to long-term turnovers of pathogens and symbionts (Whiting 1963; Mamay 1969; Stevenson 1990; Taylor & Taylor 1993; Norstog & Nicholls 1997; Jones 2002; Schneider *et al.* 2002). They are the oldest known living gymnosperms with true root structures that house nitrogen-fixing symbionts as part of a tripartite symbiotic system inclusive of mycorrhizae and nitrogen-fixing cyanobacteria (Norstog & Nicholls 1997) which maintain soil communities via rhizosphere modification (El-Shatnawi & Makhadmeh 2001). *C. micronesica* interacts with a range of newly discovered specialist insect pollinators in cycads, which offers insights into previously unexplored pollinator complexes (Terry *et al.* 2009). It is also a suspected cause of the neurodegenerative disease ALS-PDC (Spencer *et al.*

1987; Cox & Sacks 2002; Borenstein *et al.* 2007) although this remains controversial (Duncan *et al.* 1988; Steele & McGeer 2008). These intimate associations between cycads and other organisms add to the urgency of conserving cycads in general. Cycads are currently restricted to areas in the tropics and subtropics, with 82% of the species considered threatened (Donaldson 2003; Norstog 2003). *Cycas micronesica*'s dire conservation status, coupled with its ecological, evolutionary, and potential medical importance, makes the study of its current population genetic structure critical.

Given the longevity of adult cycads on Guam (up to ca. 100 years of age) (Hirsh & Marler 2002) and the recent introduction of the cycad aulacaspis scale, it is unlikely to detect a genetic signature correlated to patterns of infection in the island. However, a description of standing variation of remaining populations is critical for management, and important to understand the evolutionary history of *C. micronesica* in Micronesia. Previous to the invasion of the scale, *C. micronesica* was present throughout most of Guam's native habitats. Different environments within Guam have likely shaped patterns of genetic variation of *C. micronesica* by acting as barriers or facilitators to seed and pollen dispersal. Most of the island is a mosaic of patches of secondary forests with grasslands shaped by humans over the past ca. 3000 years (Spoehr 1955; Rogers 1995). The presence of human settlements, an east and west gradient of salt spray and wind exposure along the coasts, and the north and south differences in forest extent and water-availability within Guam, could translate into populations with unique levels of genetic variation and varying degrees of genetic connectivity. Furthermore, most of the north of Guam is comprised of limestone substrates of marine origin, while volcanic substrates of the south are highly acidic, poorly drained, and vulnerable to erosion (Young 1988). *C. micronesica* is one of very few plant native species common to these highly contrasting substrates (Marler 2002). Such native plants must have the genetic resources to contend with calcium deficiency and aluminum toxicity, as these are the main chemical pressures in these substrates (e.g. Marler and Lawrence 2004).

In this study we describe the genetic connectivity of natural populations of *C. micronesica* throughout Guam using microsatellites developed from Expressed Sequence Tags (EST-STRs). This is the first study to apply genomic tools to enable rapid characterization of genetic variation in natural populations of an endangered cycad. We focus on the impact of potential barriers to gene flow throughout Guam, as well as discuss the implications of genetic patterns in the context of the reproductive biology of this species. We provide a basic guideline for management and identify sites that are

priorities for conservation. Overall, we seek to add to the general understanding of the combined historical and contemporary effects of geography, the environment, and human-induced habitat changes on the life history and genetic patterns of cycad populations, and of oceanic plants in general.

Materials and methods

Sampling

We sampled from most of the surviving stands of *C. micronesica* in Guam, which range throughout Guam's edaphic and environmental gradients—in terms of rainfall, salt spray exposure and soil-type, for a total of 321 individuals in 24 localities (Fig. 1). We used 100–300 m transects and sampled at least one plant every 10–30 m in each locality; however, in some cases the terrain was impossible to access, plants were dead, no leaflet tissue was available due to scale infestation, or the physiognomy of the habitat did not allow for a straight transect. The highest number of individuals were sampled in

Racetrack, $N = 17$. In contrast, we could only sample seven individuals in Cetti and Madofan. On average there were 11 individuals per locality (Table S1, Supporting Information). To varying degrees, the insect scale was present in all sites where cycads were sampled.

Genetic variation

QIAGEN DNEasy Mini kits (Valencia, CA, USA) were used to isolate DNA from leaf tissue. We used 14 of the recently developed nuclear EST-microsatellites (EST-STRs) to measure genetic variation following protocols described previously (Cibrián-Jaramillo *et al.* 2008). The availability of ESTs for a related species enabled the use of hypervariable markers without the need of developing a microsatellite library *de novo*. Dataset editing and formatting was done in the Excel Microsatellite Toolkit (Park 2001). Linkage disequilibrium (Weir 1996) and deviations from Hardy-Weinberg equilibrium could indicate the presence of population structure or inbreeding (Hartl & Clark 1997) therefore the presence of Linkage Disequilibrium was investigated at the 5%

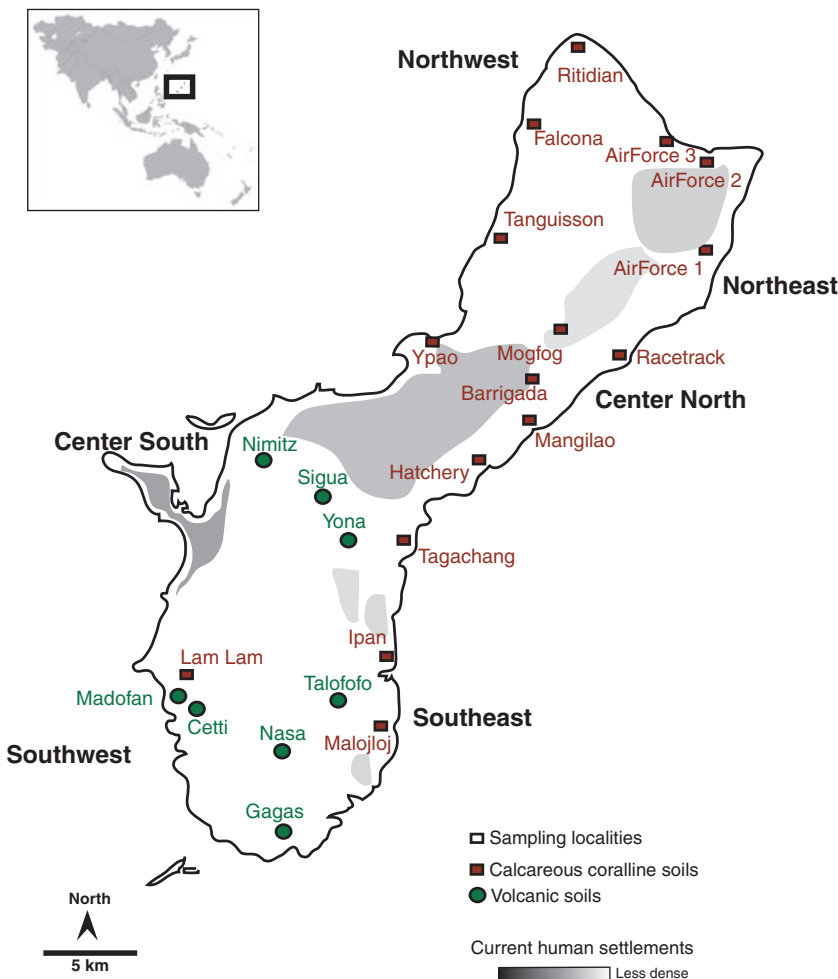


Fig. 1 Sampling sites for *Cycas micronesica* in Guam. Shown are the type of soils for each locality, the distribution of contemporary human settlements and six regions that represent environmental gradients within Guam (Northeast, Northwest, Center East, Center West, Southeast, Southwest).

statistical significance level among loci per population based on sampling sites with 10 000 permutations. Departure from Hardy-Weinberg equilibrium expectations was tested for each locus with default parameters; both analyses were carried out in Arlequin v3.11 (Excoffier *et al.* 2005).

Estimates of mean number of alleles per locality, number of private alleles, information index (I), Fixation index (F), were calculated with GenAIEx v6 (Peakall & Smouse 2006). Heterozygosity was measured as the unbiased expected heterozygosity (H_E), and observed heterozygosity (H_O) corrected for sample size (Nei 1978) using Genetix v4.05 (Belkhir *et al.* 2004). We used rarefaction statistics implemented in HP-Rare to test the influence of different sample sizes across localities (Kalinowski 2005). The presence of null alleles and allelic dropout was tested using Micro-checker v2.2.1 (Van Oosterhout *et al.* 2004) within each subregion. This implementation calculates the probabilities for the observed number of homozygotes within homozygote classes using a cumulative binomial distribution (Weir 1996).

Given that our microsatellites were developed from expressed sequence tags (derived from expressed genes) we tested for evidence of selection on each locus. We tested for signs of positive and balancing selection using the F_{st} -outlier approach (Beaumont & Nichols 1996; Beaumont 2005) implemented in LOSITAN (Antao *et al.* 2008). This method uses the expected distribution of Wright's inbreeding coefficient F_{st} vs. H_E to identify outlier loci that have excessively high or low F_{st} compared to neutral expectations, under an island model of migration and with neutral markers. As recommended by Antao *et al.* (2008) we ran LOSITAN once to identify the set of loci under selection. All loci outside a 99% confidence interval were removed and the mean F_{st} was calculated again. A final run included all loci. We used the infinite alleles model and 10, 000 simulations.

Regional differentiation

We estimated an overall measure of differentiation across all sampling localities using θ_{ST} (Weir and Cockerham 1984) and pairwise frequency based estimates of F_{st} , the inbreeding coefficient within subpopulations, relative to the total (Wright 1951; Hartl & Clark 1997) using Arlequin v3.11. We compared estimates of F_{st} to G_{st} (Nei 1973) to the actual differentiation D and the estimator of actual differentiation (Jost 2008) using SMOGD v2.6 (Crawford 2009). We used a locus-by-locus AMOVA (Excoffier *et al.* 1992) to measure the extent of hierarchical genetic differentiation among and within six regions, Northeast, Northwest, Center North, Center South, Southeast, and Southwest, which represent different environments (Fig. 1). AMOVA was calculated using Arlequin v3.11.

Genetic differences between populations based on genetic distance were estimated with Nei's chord distance D_a , with no assumptions of a Stepwise Mutation Model for microsatellite evolution (Nei *et al.* 1983). A Neighbor Joining phenogram was created in Neighbor of the Phylip v3.6 package (Felsenstein 2006). Bootstraps were calculated using POPULATIONS (Langella, Populations 1.2.30).

Limited dispersal can result in a pattern of genetic differentiation that increases with geographic distance. Isolation by distance was tested with a Mantel test for matrix correspondence (Smouse *et al.* 1986; Smouse & Long 1992). GenAIEx v6 (Peakall & Smouse 2006) was used to produce a geographic and genetic distance matrix using pairwise individual comparisons, following Peakall *et al.* (1995) and Smouse & Peakall (1999). Given the varying environmental conditions, we first tested sampling localities from North to South followed by localities along each coast, separately.

Genetic assignments

Bayesian methods have provided tools for different levels of spatial and temporal resolution to population-level analyses without pre-assumptions of population structure (Storz & Beaumont 2002; Heuertz *et al.* 2003; Coulon *et al.* 2004; Rowe & Beebe 2007). The Bayesian clustering approach in BAPS v4.14 was used to infer the number of genetic groups using individual assignments and spatial clustering of individuals using geographic coordinates based on sampling sites (Corander *et al.* 2008). Five independent runs were carried out to test for congruence among runs. STRUCTURE v3.1 (Pritchard *et al.* 2000) was used for comparison with BAPS. All individuals were clustered with and without providing a priori information on their population of origin. Ten independent runs were performed for each set, with values of K ranging from 1 to 20, a burn-in of 200 000 generations and a subsequent 300 000 MCMC steps. We assumed that allele frequencies were correlated among populations (prior mean = 0.01, prior SD = 0.05, $\lambda = 1.0$) and that each individual draws a fraction of its genome from each of the K clusters, and we thus set a uniform prior on admixture and α (initial value = 1.0, max = 10.0, SD = 0.025). The second-order rate of change of the log probability of the data with respect to the number of clusters (ΔK) was used as an additional estimator of the most likely number of genetic clusters (Evanno *et al.* 2005).

Genetic signature of a population decline

We tested for evidence of historical human invasions in Guam that have transformed the island's landscape.

The last major human-induced transformation occurred 400 years ago (Rogers 1995) after an initial human settlement 3 kya (Spoehr 1955). If the effective population size of *C. micronesica* was significantly reduced in size, alleles at low frequency (<0.1) should be less abundant, and few rare alleles should be present, often resulting in an excess of heterozygosity (Cornuet & Luikart 1996; Luikart & Cornuet 1998). Allelic richness was measured based on the number of alleles per locus and population (A_T), the number of alleles with a frequency greater than 5% ($A_{5\%}$), less than 50% ($A_{<50\%}$) (Maguire *et al.* 2002), and the number of private alleles (A_P). The distribution of allele frequencies was estimated following the graphical method of Luikart *et al.* (1998). The program BOTTLENECK (Cornuet & Luikart 1996) was used to calculate significant deviations of the distribution of the heterozygosity expected from the observed number of alleles (k), given the sample size (n), under the assumption of mutation-drift equilibrium. We calculated these distributions for each sampling locality ($N = 24$) and for each locus using the IAM model with 10,000 replicates.

Recent immigration among regions

We tested the hypothesis of an abrupt, recent population subdivision possibly caused by human influence on Guam's forest landscape, as well as the impact of edaphic substrates between north and south of the island on genetic structure, using BayesAss+ v1.3 (Wilson & Rannala 2003). This method estimates a matrix of pairwise recent immigration rates (m) among populations using a coalescent approach. The default settings for burn-in and number of MCMC iterations were enough to reach convergence based on visual inspection of likelihood scores. We employed the genetic clusters identified with BAPS as our populations, as these are the areas we are interested in evaluating for conservation purposes. Five independent runs were performed to test for congruence, and a likelihood ratio test was then employed to determine whether the prior and posterior probabilities of migration rates are significantly different from each other (Wilson & Rannala 2003).

Identifying key populations for conservation

Given the conservation status of *C. micronesica*, we were interested in identifying critical populations for management. Small populations are typically considered the most vulnerable to detrimental impacts of high inbreeding and low genetic variation (DeSalle & Amato 2004; Kramer & Havens 2009). The extent of gene flow is also important factor in determining vulnerability, especially when there is rapid local extinction as it is the case for *C. micronesica* in Guam (Allendorf & Leary 1986;

Bohonak 1999; Epperson 2003; Manel *et al.* 2003). Critical populations in this context are those that are small and that are mostly self-seeding and are effectively isolated from the rest of the island. Equally important are populations that disproportionately contribute with migrants to other populations (via pollen or seed) maintaining gene flow at a regional scale ('sources' *sensu lato*; Sork *et al.* 1999). Collection for *ex situ* conservation should include seeds from both of these outliers in order to represent the spectrum of genetic patterns of *C. micronesica*. In particular, seeds from 'source populations' can be used to re-establish natural populations from nurseries, minimizing changes in assortative mating that could lead to outbreeding depression (Epinat *et al.* 2009).

We estimated the influence of each population on recent immigration rates throughout the island. As suggested by Baums *et al.* (2005), we sequentially omitted each of the sampling localities from the immigration matrix estimated by BayesAss (for a total of 24 matrices). This approach is based on the notion that each population can have a different effect on the immigration rates of the rest of the localities (Wilson & Rannala 2003; Baums *et al.* 2005). If a population contributes with migrants, its exclusion will increase its mean self-recruitment rates. In contrast, if its contribution is minimal for the rest of the populations, its exclusion will decrease mean self-recruitment rates. We did three independent BayesAss runs using the same matrix, and another run excluding loci under positive selection (224, 250, 268) to test for congruence and reliability of this method. We developed a series of three Java scripts (available at <http://genomics.amnh.org> or by the authors upon request) to produce the 'jackknifed self-recruitment' matrix, and the difference between the overall self-recruitment rate and each of the jackknifed self-recruitment estimates.

Results

We used 14 EST-microsatellites to describe the levels of genetic variation and genetic connectivity of 321 individuals of *C. micronesica* in 24 localities in Guam. We detected balancing selection on locus 270 (with no known protein function), and positive selection on three others—loci 224 and 250, which correspond to a sugar transporter and NADH dehydrogenase subunit 5 respectively, and locus 268, which has no known protein function (Cibrián-Jaramillo *et al.* 2008; Fig. 2). There were no differences in estimates of heterozygosity or average number of alleles, or estimates of population structure (BAPS) if these loci were removed or maintained and thus they were kept in all of the analyses (Fig. S1, Supporting Information). Locus 280 exceeded the allowed number of alleles for BayesAss (max. 40

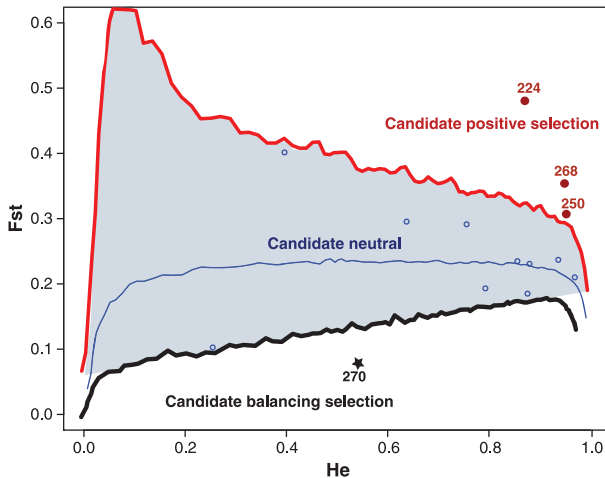


Fig. 2 Test for selection on EST-STR loci. Four loci were detected as subject to selection –270, subject to balancing selection (green points), in which multiple alleles can be maintained in the gene pool of a population by selection favoring heterozygotes; 224, 250, 268 subject to positive selection (red points), where new alleles are fixed following selective sweeps. The remaining loci were within neutral expectations (blue circles). Neutral F_{ST} with 15 000 runs and a confidence interval of 99%.

alleles) and was removed from those calculations. Given that microsatellites were developed from *Cycas rumphii*, there is a possibility of null alleles accounting for the lack of heterozygosity. There was no evidence for large allele dropout, but the general excess of homozygotes in some allele size classes suggested that null alleles might be present, albeit with no significant pattern specific to a site. Likewise, significant deviations from Hardy–Weinberg equilibrium expectations and Linkage disequilibrium ($P < 0.01$) were detected but showed no consistent patterns across loci or across populations.

Overall, there is an average of 4.2 alleles per locality across Guam (Table S1, Supporting Information and Fig. 3). The minimum average number of alleles per site was found in Madofan ($N_a = 3.5$) and the maximum in Talofoto ($N_a = 5.8$; Fig. 3). Private alleles, or alleles found only within a particular locality, were found throughout Guam in low frequency. Talofoto had the highest number of private alleles ($A_p = 2.1$). The average expected and observed heterozygosity across sites were $H_E = 0.545$ (SD = 0.014) and $H_O = 0.349$ (SD = 0.015), respectively. The highest unbiased expected heterozygosity was found in Sigua $H_E = 0.68$ (SD = 0.077) and the lowest in Ritidian $H_E = 0.45$ (SD = 0.08). In terms of observed heterozygosity, Falcona $H_O = 0.268$ (SD = 0.03) and Airforce 3 $H_O = 0.437$ (SD = 0.05) had the lowest and highest, respectively. We found a weak correlation between sample size and expected heterozygosity (Spearman's rank correlation

corrected coefficient = -0.12 , $P = 0.6$) and observed heterozygosity (-0.37 , $P = 0.08$; Table S1, Supporting Information). Fixation indices suggest that there is moderate to high levels of inbreeding, with the variation within individuals relative to the subpopulations as $F_{IS} = 0.370$ ($P < 0.01$) and within individuals relative to the total as $F_{IT} = 0.525$ ($P < 0.01$; Table S1, Supporting Information).

Using all 24 sampling localities as putative populations, genetic differentiation ($\theta_{ST} = 0.245$) was significantly different from zero ($P < 0.01$), indicating low gene flow among localities. Pairwise F_{ST} estimates show that most of the variation is indeed found within localities (Table S2, Supporting Information). D estimates ($D = 0.55$, $D_{est} = 0.52$) are higher than F_{ST} , which is closer to $G_{ST} = 0.287$. This was expected given that G_{ST} and F_{ST} are sensitive to gene diversity and to subpopulation differentiation (Jost 2008). However, all three estimates unequivocally suggest low (historical) genetic connectivity *Cycas micronesica*.

We measured hierarchical genetic differentiation among and within six regions, Northeast, Northwest, Center North, Center South, Southeast and Southwest (from Fig. 1). AMOVA showed that overall, 41% of the variation is found within populations, 33% is distributed among individuals within populations, 26% is distributed among populations either within the north region or within the south region, and almost none of the variation is distributed among the six different regions in the island.

Most localities are genetically different based on Nei's chord distance (D_a) but there is no clear pattern of differentiation among the six subregions nor in areas with various degrees of insect scale abundance (Fig. 4). Bootstrap values were low (< 50) for all nodes except for (Ritidian, Racetrack) which had high support (> 80). This was expected from the topology and congruent with the Mantel test. In the latter, the null hypothesis of no correlation between geographic and genetic distance throughout the island and along the coasts was not rejected.

BAPS showed that the optimal partitioning was $K = 18$, revealing the highest gene flow in the center north of the island (Fig. 5). This was similar to the estimate from STRUCTURE of $K = 15$ when *a priori* information is provided on the geographic origin of each population using the ΔK method (Evanno *et al.* 2005). STRUCTURE also estimated $K = 5$ if no information was provided on the origin of populations (Fig. S2, Supporting Information). In the north of the island, Ritidian is the most isolated population geographically, with almost no estimates of immigrants from nearby populations in any direction. Talofoto and Nasa are among the most diverse sites in the south.

We estimated the proportion of migrants among the inferred BAPS genetic clusters using BayesAss. The

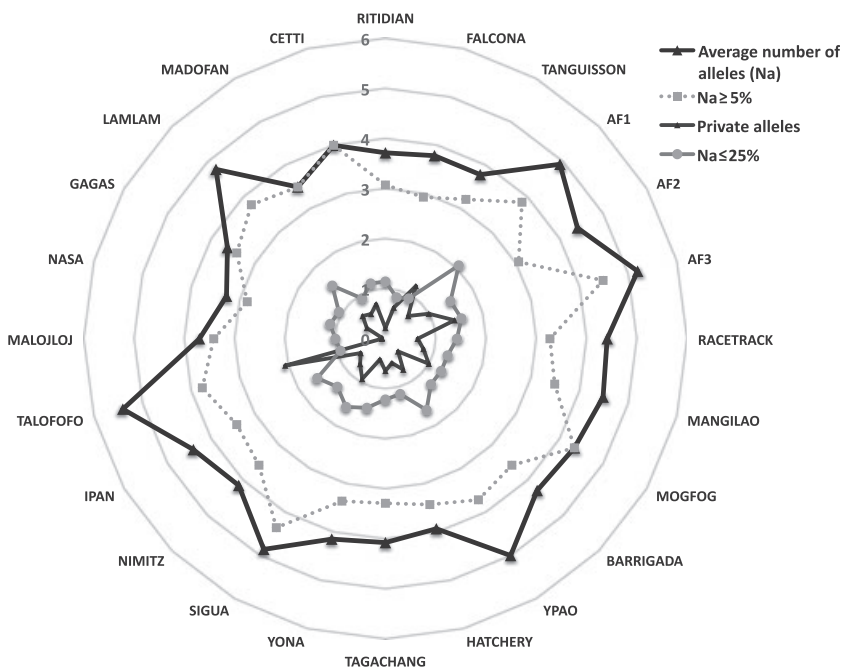


Fig. 3 Average number of alleles per population; $N_a \geq 5\%$ Number of locally common alleles occurring in 50% or less of the populations; $N_a \leq 25\%$. Number of locally common alleles (Freq. > 5%) occurring in 25% or less of the populations; Private alleles are equivalent to the number of alleles unique to a single population in the data set.

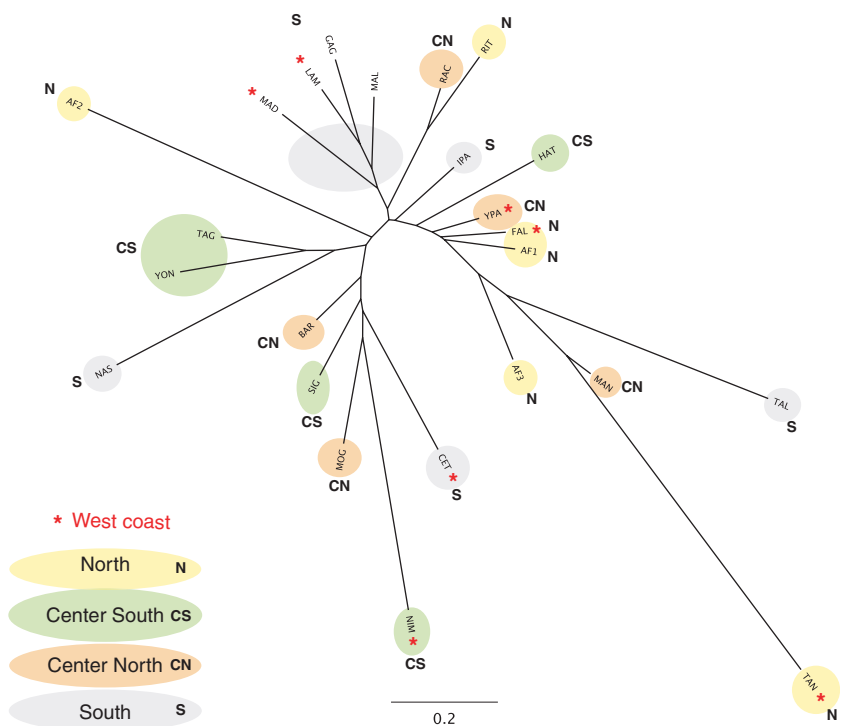


Fig. 4 Genetic subdivision based on a neighbor-joining tree from Nei's chord distances among populations. Island regions are highlighted as well as populations on the East coast, those without a star are found on the West coast.

significant χ^2 values and log-likelihood ratio tests ($P < 0.001$) excluded the possibility of a lack of sufficient microsatellite polymorphism to detect migration rates between sampled localities in BayesAss. There was higher self-recruitment within the north of Guam than the south. For the most part individuals were assigned to the sampling locality from which they origi-

nated (Table 1). Pairwise comparisons across localities with BayesAss show that Racetrack is the highest contributor with migrants to other populations.

Jackknifing showed that most populations behave as sources in terms of their own recruitment, if removed from the rest of Guam populations. That is, most populations in Guam are self-seeding, in particular those

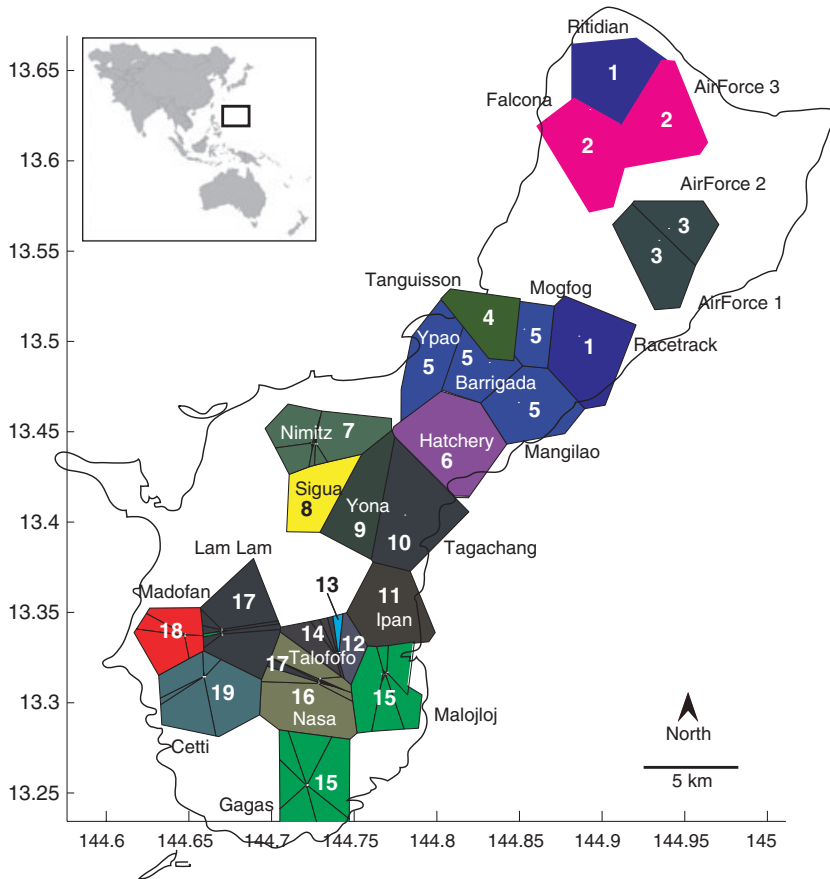


Fig. 5 Genetic subdivision among localities based on Bayesian clustering using BAPS. The most likely number of K given 321 individuals collected from 24 localities is 18. Shown are the proportions of individual multilocus genotypes attributable to genetic clusters, or populations (K). Each color denotes a genetic cluster (similar colors do not necessarily reflect genetic similarity). A polygon surrounds GPS-based records of individual samples (name of locality also shown).

found in the north. This is congruent with the patterns of high differentiation observed throughout Guam, as seed and pollen donors are more likely to originate from the source population, rather than from other populations, diminishing gene flow among geographically distant localities. Tanguisson, the Northeastern cluster (AF1 and AF2), Tagachang, Sigua, and the Southeastern cluster (Gagas and Nasa) behave more like sinks—when removed there is an increase in mean self-recruitment for the remaining populations (Fig. S3, Supporting Information). When a population is near zero, then they are neither sources nor sinks they are near the average for all populations (e.g. Southeast cluster).

We tested the effects of human-induced forest fragmentation in Guam. There was no detectable evidence of a recent bottleneck according to the graphical method (Luikart & Cornuet 1998; Fig. 4). There were no significant P -values for any of the tests of deviations from mutation-drift equilibrium (Cornuet & Luikart 1996; Luikart & Cornuet 1998; Luikart *et al.* 1998) for any sampling locality using *BOTTLENECK*. We did not detect changes in mode-shift that would distinguish bottlenecked populations from stable populations except for localities Madofan and Cetti but this was due to their small sample size ($N = 7$) rather than evidence of a bottleneck.

Discussion

Guam cycads: moderate genetic variation, restricted and asymmetrical gene flow

Population genetics theory predicts that genetic variation in small and isolated populations will erode over time (mostly due to genetic drift), resulting in an isolation-by-distance pattern where individuals at closer geographic distance will have higher chances of being identical by descent (Slatkin 1993; Hedrick 2000; Rousset 2000). Indeed, with only a few exceptions (Xiao & Gong 2006) most cycad species have a highly structured distribution of low genetic variation, with low levels of gene flow and a pattern of isolation by distance even at small geographic distances (Ellstrand *et al.* 1990; Walters & Decker-Walters 1991; Yang & Meerow 1996; Sharma *et al.* 1999, 2004; Lin *et al.* 2000; Keppel 2002; Huang *et al.* 2004; Xiao *et al.* 2004; Jianguang *et al.* 2005). It is thus not entirely surprising that similar to other cycads and in contrast to genetic patterns in most long-lived dioecious gymnosperms (Hamrick *et al.* 1992; Hardy *et al.* 2006; Sork & Smouse 2006), *C. micronesica* has low levels of gene flow among populations, with most recruitment restricted to small

Table 1 Bayesian assessment of migration within and among sampling localities implemented in BayesAss+. For each sampling locality, numbers are the mean proportion of individuals from each source locality. Bold face terms along the diagonal are proportion of non-migrants (self-recruitment). Empty cells represent mean proportions <0.05

Genetic cluster	RIT	NW	TAN	NE	RAC	CN	HAT	TAG	YON	SIG	NIM	IPA	TAL	SE	NAS	LAM	MAD	CET
RIT	0.981																	
NW		0.679			0.264													
TAN			0.970															
NE				0.683	0.055	0.150												
RAC					0.982													
	NORTH continuous forests no surface water calcareous soils																	
CN						0.991												
HAT					0.211		0.688											
TAG								0.686										
YON					0.041	0.112			0.774									
SIG					0.206					0.687								
NIM					0.193						0.688							
IPA						0.175						0.687						
TAL					0.058								0.866					
SE					0.268									0.679				
NAS					0.095	0.122									0.687			
LAM					0.175	0.046										0.686		
	SOUTH fragmented forests surface water volcanic soils																	
MAD					0.109													0.702
CET					0.099													0.705

geographic areas, and most of the variation found within populations.

In contrast to most population genetic studies in cycads however (mostly conducted with allozymes; Yang & Meerow 1996; Lin *et al.* 2000; Keppel 2002; Huang *et al.* 2004; Xiao *et al.* 2004; Xiao & Gong 2006) we found that wild populations of *C. micronesica* in Guam have moderate levels of inbreeding among individuals within populations and high levels of genetic variation in terms of number and distribution of alleles, including private alleles. This is congruent with expectations for long-lived outcrossing tropical species, and similar to other estimates of genetic variation in cycads using hypervariable molecular markers: *Macrozamia riedlei* (Byrne & James 1991), *Cycas debaoensis* (Jianguang *et al.* 2005), *Zamia pumila* (Meerow & Nakamura 2007), and *Dioon edule* (Moynihan *et al.* 2007). Often-cited estimates of the extent of gene flow in cycads are from 2 to 7 km (Ellstrand *et al.* 1990; Walters & Decker-Walters 1991; Yang & Meerow 1996; Lin *et al.* 2000; Huang *et al.* 2001, 2004). This is the case for some populations of *C. micronesica*, such as Ritidian and Tanguisson but our results suggest that to assume restricted gene flow up to 7 km as an *a priori* for cycads may be premature.

Guam cycad populations are genetically structured (*F_{st}* and BAPS, STRUCTURE) but their genetic relationships

with other populations vary (BAYESASS, IBD, NJ). Environmental and geological conditions on Guam have imparted pressures that can promote genetic disjunction of populations of *C. micronesica* plants (Marler 2002). Despite no clear isolation by distance patterns within the island, there is a genetic division between north and south of Guam in terms of recent immigration rates among populations. In the north, most populations are self-seeding, with the exception of Racetrack, which is common to north and south. In the south all populations contribute with migrants to other populations to a greater extent. Together, our results suggest asymmetrical gene flow where populations can contribute with genetic migrants only to themselves, unidirectionally to only a few others, or to many others. These patterns reveal a more complex population dynamics than previous studies in cycads, and to some extent the influence of human transport in Guam's cycad populations. The north and south immigration rates in Guam can be associated to three main factors: largely contiguous forests in coastal regions in contrast to fragmented forests, seed size differences, and differences in edaphic conditions. The influence of biological, geographic and environmental factors on the genetic connectivity of *C. micronesica* are discussed below.

Most variation found within populations driven by sporadic reproduction and local pollinators

Local assortative mating is a key biological factor that maintains genetic variation within populations in plants (Epperson 2003; Fortuna *et al.* 2008). Given most of the genetic variation concentrated within localities, this factor may play an important role in *C. micronesica* populations. Cycads have many sporadic reproductive events throughout their life but as dioecious species, they are restricted by the number of reproductive females and the availability of pollen donors every year (Terry 2001). On average, female *C. micronesica* plants in natural populations have reproductive events every 63 weeks, while males cone every 44 weeks. Within populations in Guam, only some individuals cone regularly, every several years (T. Marler, University of Guam, Guam). Habitat conditions in the island, and most recently the cycad aulacapsis scale, also affect the amount and regularity of coning episodes in *C. micronesica*, and a low number of seeds produced when plants are under stress (T. Marler, University of Guam, Guam). These patterns could effectively decrease the probability of reproductive events, increasing genetic differentiation among populations and decreasing the effective population size within populations (e.g. Shapcott & Playford 1996; Luna *et al.* 2007).

Within populations and among spatially structured populations, the availability and origin of pollen and seed donors are critical for determining genetic structure (Sork & Smouse 2006; Fortuna *et al.* 2008; Nathan *et al.* 2008). Most cycad pollination is mediated by specialist insects that are dependent on male cones for food and larval development (Terry *et al.* 2009). Possible pollinators of *C. micronesica* are the microlepidopteran *Anatrachyntis* sp. (Lepidoptera: Cosmopterygidae) and several species of *Carpophilus* sp. (Coleoptera: Nitidulidae) (Terry *et al.* 2009), which have low vagility. The leptokurtic curve for the pollen kernel in this species should have a long tail, contributing to most variation found within rather than among populations. In terms of seed-mediated gene flow, bats have been suggested as seed dispersers of cycads, but there is no empirical evidence of this assertion (Pijl 1957; Jones & Lloyd 1993). In Guam the Little Mariana bat (*Pteropus tokudae*) is believed to be extinct, and the Mariana fruit bat *Pteropus mariannus* has declined in the last 50 years (Wiles & Johnson 2004), including colonies that were thought to be stable in the northwest, with only one colony persisting near Air Force 2 in the northeast. If indeed bats played a historical role in seed dispersal, the limited gene flow across Guam supports the notion that populations of *P. mariannus* in the northern Marianas tend to remain close to their colonies, and consume seeds on-site (Wiles & Johnson 2004). It is more likely that rivers and

seed size differences are more important for *C. micronesica*'s seed dispersal within Guam (discussed below).

Higher recent self-recruitment in contiguous (north) than in isolated (south) forests

In Guam, most of the northern populations are self-recruiting, while in the south, a higher number of immigrants from populations other than the one sampled, are required to maintain the observed allele frequencies. The north of Guam has the largest extent of conserved forests (Fosberg 1960). Previous to the cycad aulacapsis scale, densities of 3000 or more plants per hectare were not uncommon in the north of the island, with populations covering up to 15 km along the west coast and 36 km along the east coast (T. Marler, University of Guam, Guam). Cycad populations in a few mixed forests of ravines in southern Guam are considerably smaller. Most of the south is composed of old volcanic material and a complex mosaic of grassland with early succession stages of the forest (Fosberg 1960). It is likely that within contiguous forest in the north, pollinators may have a higher chance of finding synchronized reproductive male and females that are also close geographically (Sork & Smouse 2006; Fortuna *et al.* 2008). An outlier in the north and south patterns is Racetrack. This locality has high levels of self-recruitment, but it is the only population that acts as an important source of migrants towards many other populations. Although the specific role of human movement is difficult to discern, it is possible that seeds from this region may have been transported more frequently by humans than other sites in Guam. *Cycas micronesica* was one of the most common garden plants on Guam in the early 19th century (Barratt 2003) and there was a historical reliance on cycad seeds as food source by the Chamorro people, who made use of the starch-rich gametophyte tissue (Whiting 1963). The history and genetic connectivity of the entire range of *C. micronesica* will help enlighten the role of humans in Guam's cycads.

Smaller seeds in the south associated with higher genetic connectivity

Seed size is an important biological factor in the extent of genetic connectivity (Sork & Smouse 2006). With a few exceptions (Rossetto *et al.* 2009) species with larger seeds tend to be dispersed shorter distances than species with smaller seeds (Muller-Landau *et al.* 2008). Large or heavy seeded plants tend to be clumped (Snow & Walter 2007). In previous work we had observed that seed size is smaller in southern populations in Guam. We measured seed size from a random sample of 100 seeds collected from areas between Air

Force 3 and Ritidian (north), versus areas between Lam Lam and Gagas (south). Fresh weight of these samples was on average 87 g (range from 67 to 128 kg, SD = 12.3) for north populations versus 56 g (range from 47 to 64 kg, SD = 4.3) for south populations. While seed dispersal does not guarantee survival and recruitment, smaller seeds in the south of Guam probably disperse further distances than larger seeds from the north. The presence of rivers in the south also enables them to move more than seeds in the north. Previous to the invasion of the cycad aulacaspis scale, cycad seeds were commonly seen floating in rivers and streams, which are unique to the south. Higher clumping in the north would more often favor mating among available nearby plants, increasing the probability that seeds originate from related mothers, and that pollen donors are also related (Seidler & Plotkin 2006; Fortuna *et al.* 2008). This would result in higher mean self-recruitment rates in the north, when compared to the more open south.

Edaphic stress as a driving force for different north-south genetic patterns

Positive selection detected in locus 250, which encodes NADH dehydrogenase, suggests that the genetic disjunction observed in Guam's cycad populations is, at least in part, directly associated with edaphic pressure. Limestone soils impose extreme iron deficiency in plants that do not have the genetic capability to cope with calcareous substrates that hinder iron availability (e.g. Marler *et al.* 2002). NADH-dependent metabolic activity (e.g. Fe³-citrate reduction) is important in plants exposed to iron-poor soils (Buckhout *et al.* 1989; Wolfgang Bruggemann 1989; Marler *et al.* 2002; Durrett *et al.* 2007). Most of the north of Guam is comprised of limestone substrates of marine origin while volcanic soils of the south are highly acidic, poorly drained, and vulnerable to erosion (Young 1988). Soil composition may also be an important component of seed germination and recruitment in cycads (Snow & Walter 2007). Furthermore, differences in edaphic conditions also correlate to cycad chemistry. Sterols and derived glucosides vary among locations throughout Guam (Marler *et al.* 2005, 2007; Marler & Shaw 2010). In particular, cycads on sandy soils from the littoral habitat have higher seed concentrations of steryl glucosides than plants on upland regions of Guam (Marler 2002). The large genetic cluster detected in the center-south of the island connecting Ypao, Mogfog, Barrigada and Mangilao may be associated to the distinct terrain in this part of the island, composed of old karst (argillaceous limestone), rounded hills and marshy sinks. The correlations of edaphic differences in north and south of Guam with standing and adaptive genetic variation, and their rela-

tionship to cycad chemistry are worth investigating in this system. Locus 250 is a good a particularly good candidate for future ecological genetic studies in *C. micronesica*.

Critical populations for conservation

We have focused on an oceanic island species recently endangered by an invasive insect, first to elucidate the evolutionary history of its wild populations, and second to contribute with basic guidelines for its management and conservation. As with all comprehensive conservation strategies, use of genetic guidelines should be taken in context with ecological and demographic observations when available. Guam cycads are a clear example of the rapid decline of oceanic species when invasive species are introduced, including the detrimental repercussions to the island's ecology, and to the plant's economic value in the ornamental market. For *C. micronesica* it is important to conserve as many plants originating from the genetic populations (clusters) identified here (Fig. 5). North and south populations could be kept separate, as well as Ritidian and Tanguisson, in which most of the individuals are assigned to the population of origin, and have high pairwise differentiation values (*F_{st}*). Ritidian is one of the best-conserved forest regions in Guam, yet its population of *C. micronesica* has among the lowest levels of variation and is the most genetically and geographically isolated, making it a valuable and fragile resource. Seeds from this locality should be maintained for future reintroductions, as habitat availability would ensure its long-term survival despite its low variation. In addition to Ritidian and Tanguisson, and based on pairwise differentiation (*F_{st}*) alone, Talofoto and Nimitz, are probably best if managed as separate genetic entities. Talofoto with the highest number of private alleles should also be prioritized and could be used as a source for seeds. Nasa, although not as variable, could be an additional seed source, given its proximity to Talofoto and the fact that they are located on the same river. If needed, seeds could be moved among the two smaller clusters identified with BAPS concomitant with lower pairwise differentiation when compared to all other populations: Gagas-Malojloj, and Air Force 3-Falcona. Populations from the south could be mixed within nurseries if required, rather than populations from the north, which showed higher self-recruitment rates.

Conclusions

As with most cycads, *C. micronesica* has high-genetic structure, with most of the variation found within populations. This species has moderate to high genetic variation, different recruitment rates across populations,

and varying degrees of genetic connectivity, in some instances beyond the 2–7 km limit to gene flow proposed previously for the cycads. We identified a total of 18 genetic populations from 24 sampling localities in Guam. Patterns of genetic connectivity in *C. micronesica* are partially a result of mating system and geographic and environmental differences within Guam. Genetic patterns within populations are probably a result of localized pollination and a low availability of males and female donors from distant areas within Guam. Human transport and pollinator availability probably contribute to the maintenance of genetic variation within and among populations. Interestingly, there is higher recent self-recruitment in the north than the south of Guam, associated to the extent of forest fragmentation, seed size, and to contrasting soil types. Our study identified an EST-microsatellite coding for NADH dehydrogenase under positive selection in Guam cycad populations, which is probably associated to edaphic stress induced by the iron-deficient soils in the north. Any loss of cycad genetic diversity carries the risk of exponential ecological consequences both from recent adaptations in oceanic islands and from older interactions with other organisms (Schneider *et al.* 2002). Given the current endangerment of *C. micronesica* by an invasive scale, we recommend maintaining separate populations when establishing nurseries or reintroducing seeds, particularly in the north, but we have also identified areas that have local gene flow and thus could be mixed if needed. We identified Ritidian and Talofofu among the highest priority populations for conservation. We are currently investigating the use of these and other EST-microsatellite loci for exploring the ecological genomics of this species within Guam, and extending the study of its populations throughout its purported distribution in Rota, Yap, and Palau.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Sample size and levels of heterozygosity across populations. H_E , average expected heterozygosity; H_O , average observed heterozygosity; N , sample size. Estimates of genetic variation

Table S2 Pairwise population F_{st} (via frequency) values for all localities

Fig. S1 The number 14 indicates all loci included in the analysis, 10 only includes neutral loci (loci 224, 250, 268, 270 excluded). H_E , unbiased expected heterozygosity. H_O , observed heterozygosity; N_a , average number of alleles.

Fig. S2 Most likely number of clusters estimated using STRUCTURE. Shown is the coefficient of membership for individuals assigned to $K = 5, 8, 18$ and 24 clusters.

Fig. S3 Comparison of self-recruitment rates from genetic populations. The influence of each locality on self-recruitment rates of others. Shown is the difference between the self-recruitment rates of the remaining localities when omitting one locality (jackknifing, mean variance).

Fig. S4 Test for recent population bottlenecks based on distortion of allele frequency distribution. There is no evidence of a recent population bottleneck in any of the sampling localities.

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