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POPULATION GENETICS AND EFFECTIVE POPULATION SIZE OF THE CRITICALLY ENDANGERED NIHOA MILLERBIRD (*ACROCEPHALUS FAMILIARIS KINGI*)

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Abstract.—Many species endemic to isolated islands are of conservation interest because of concerns over the potentially devastating effects of environmental stochasticity and the pending threat of anthropogenic influences and invasive species. The effective size (N_e) of these species is a key parameter in their conservation because it predicts the detrimental effects of inbreeding or genetic drift and can be used to inform management plans. We used microsatellite allele frequencies (4 loci) and mtDNA (control region; cytochrome *b*) sequences to assess the genetic diversity and the effective number of breeders (N_b) in the Nihoa Millerbird (*Acrocephalus familiaris kingi*), a critically endangered passerine endemic to the northwest Hawaiian island of Nihoa. Using samples collected in 2007 and 2009, our results reveal extremely low levels of genetic diversity at both microsatellites and mtDNA, and both approximate Bayesian computation (ABC) and sibship methods indicate that the effective number of breeders (N_b) for this species is between 5 and 13 individuals. Our analysis highlights the utility of ABC and sibship methods for estimating N_e in species with low genetic polymorphism or few loci. We compare our results to a recent genetic study of this species and document the loss of alleles at two microsatellite loci and one unique mtDNA haplotype. We discuss our findings in the context of the planned translocation of Nihoa Millerbirds from Nihoa to Laysan Island. *Received 16 June 2010, accepted 17 December 2010.*

Key words: *Acrocephalus*, effective population size, endangered species, Nihoa Millerbird, null alleles, population genetics.

Genética de Poblaciones y Tamaño Poblacional Efectivo del Ave en Peligro Crítico *Acrocephalus familiaris kingi*

Resumen.—Muchas especies endémicas de islas aisladas son de interés para la conservación debido a preocupaciones sobre los efectos potencialmente devastadores de la estocasticidad ambiental y las amenazas potenciales de las influencias antrópicas y de las especies invasoras. El tamaño efectivo (N_e) de estas especies es un parámetro crítico para su conservación porque predice el efecto negativo de la endogamia o de la deriva génica y puede ser usado para guiar los planes de manejo. Empleamos la frecuencia de los alelos de cuatro loci microsatelitales y las secuencias del ADNmt (región control; citocromo *b*) para evaluar la diversidad genética y el número efectivo de reproductores (N_b) en *Acrocephalus familiaris kingi*, un paserino en peligro crítico que es endémico del noroeste de la isla hawaiana de Nihoa. Usando muestras colectadas en 2007 y 2009, nuestros resultados revelaron niveles extremadamente bajos de diversidad genética tanto para los microsatélites como para el ADNmt, y los métodos de cómputo Bayesiano aproximado (ABC) y los basados en grupos de hermanos indicaron que el número efectivo de reproductores (N_b) para esta especie es de 5 a 13 individuos. Nuestro análisis resalta la utilidad del ABC y de los métodos basados en grupos de hermanos para estimar N_e en especies con bajo polimorfismo genético o pocos loci. Comparamos nuestros resultados con un estudio genético reciente de esta especie y documentamos la pérdida de alelos en dos loci microsatelitales y de un haplotipo único de ADNmt. Discutimos nuestros resultados en el contexto del plan de traslocación de *A. f. kingi* desde Nihoa a la isla Laysan.

The concept of effective population size (N_e) is central to our understanding of the mechanisms by which evolutionary forces act on small populations. Wright (1931) defined N_e as being an idealized population with the same genetic parameters (e.g., genetic diversity) as the actual population of interest. Thus, N_e is interpreted as a theoretical population with the same rate of change in allele frequencies or heterozygosity as the observed population. N_e is an important parameter in both population and conservation genetics because it directly relates the genetic diversity within (and among) populations to the relative magnitude of mutation, gene flow, natural selection, and genetic drift. This link between genetic diversity and evolution is used to predict the evolutionary

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response of populations in ways that inform conservation and management programs (e.g., Frankham et al. 2002, Palstra and Ruzzante 2008).

Although N_e is a critical parameter in evolutionary biology, it is virtually impossible to collect enough demographic data from most natural populations to calculate it directly. However, there are several indirect genetic approaches to estimate N_e on the basis of either temporal genetic changes in a population between two or more well-spaced samples (temporal methods), or methods based on the statistical properties of a single sample (for a review, see Luikart et al. 2010). The performance of many of these methods has been evaluated using both simulations and empirical data, and there is sufficient information to select an appropriate N_e estimator (Luikart et al. 2010). Many of these estimators are ideally suited for data sets with a large number of highly polymorphic markers (e.g., Waples and Do 2010), but some perform well in studies with limited numbers of genetic markers or low allelic variability (Beebee 2009). However, if these methods are to be applied broadly to systems of evolutionary significance or conservation interest, their utility must be assessed in empirical populations with extremely low polymorphism.

The Hawaiian Island of Nihoa, located 250 km northwest of Kauai, is ~63 ha in area (~1.3 km long and ~0.5 km wide) and is characterized by steep slopes, rocky outcroppings, and a coastline defined by sheer cliffs ranging from 10 to 265 m high (Latchininsky 2008). Nihoa's vegetation is dominated by two grasses at higher elevations (e.g., *Eragrositis variablis*), and the valleys are densely carpeted with dry, scrub-type low shrubs (*Chenopodium oahuense*, *Solanum nelsonii*, *Sida fallax*, and *Sesbania tomentosa*). The island is home to one species of millerbird, the endangered Old World warbler *Acrocephalus familiaris*. The population of *A. familiaris* on Nihoa was recently designated as a subspecies (*A. f. kingi*); the closest subpopulation of *A. familiaris* (the nominate species *A. f. familiaris*) was, prior to its extinction sometime before 1923, found only on the island of Laysan (Fleischer et al. 2007). With an estimated population of 641 ± 295 individuals (95% CI; Morin et al. 1997, Latchininsky 2008, M. MacDonald unpubl. data), Nihoa Island is the entire remaining range of this species of millerbird. The Nihoa Millerbird is listed as a critically endangered species (IUCN 2010), and plans to increase its population size and reduce the potential for extinction include the translocation of individuals from Nihoa to nearby Laysan Island (Fleischer et al. 2007). Detailed knowledge of the genetic diversity in this species will help inform the translocation effort by providing both an estimate of N_e and spatial variation of alleles in the source population.

As part of an effort to assess the taxonomic status of the Nihoa Millerbird, Fleischer et al. (2007) conducted a genetic analysis of individuals collected in 1992. This study revealed low genetic diversity within the Nihoa Millerbird, with only 4 of the 14 microsatellites being polymorphic and a single variable nucleotide observed in ~3,000 base pairs (bp) of mitochondrial DNA (Fleischer et al. 2007). Here, we conduct an in-depth genetic survey to characterize this diversity more fully by exploring the possibility that the limited sample size ($n = 15$) may have underestimated levels of polymorphism by missing rare alleles at the four microsatellite loci or in the mtDNA. To minimize the potential for the confounding effects of sampling social groups composed of related individuals that occur in other reed warblers (e.g., Hansson et al. 2001, Richardson et al. 2005), we sampled across broad spatial and temporal scales by using individuals collected from several locations in two different years. Our objectives were to calculate contemporary N_e from both point samples (2007 and 2009) using the approximate Bayesian computation (ABC) method implemented by the program ONESAMP (Tallmon et al. 2008) and the sibship method implemented in COLONY2 (Wang 2009). Here, we examine these data for evidence of population substructure and discuss the importance of these findings to both the proposed conservation plan and the evolutionary significance of species with extremely small N_e.

Methods

Natural history.—The Nihoa Millerbird is a small, insectivorous songbird that nests in the low shrubs of Nihoa Island's valleys. Age at first breeding and longevity in Nihoa Millerbirds are not known, but reproduction is expected to begin around 1 year of age, and individuals likely live up to 10 years (e.g., Conant and Morin 2001). Clutch size is 2 or 3 eggs; pairs remain on their territories throughout the year, are socially monogamous, and retain pair bonds from year to year (Morin et al. 1997). Conant et al. (1981) estimated that 40 ha of Nihoa's 63 ha offer suitable habitat for Nihoa Millerbird territories, which range in area from 0.2 to 0.4 ha. On the basis of 21 years of census data, Conant and Morin (2001) estimated the carrying capacity (K) of the island as 380 individuals. Nothing is known about the vagility of this species except that Nihoa is 250 km from the nearest land and the Nihoa Millerbird has not been recorded anywhere else.

Sampling.—Nihoa Millerbirds were sampled by mist net from July through September 2007 ($n = 75$) and in September 2009 ($n = 64$) at several locations on the island (Fig. 1); two or three breast feathers were plucked from each individual caught. Sampling focused on areas of high bird activity and abundant habitat and covered nearly the entire island. Three individuals captured in 2007 were recaptured incidentally in the same locations in 2009. Breast feathers were again taken from these individuals and

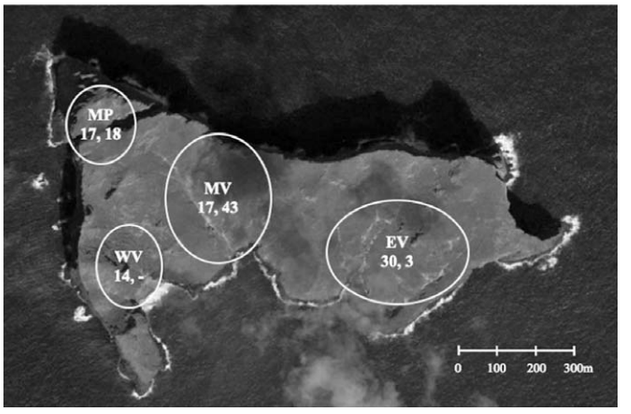

Fig. 1. Nihoa Island. Sample sizes of Nihoa Millerbirds collected in 2007 and 2009 from four habitat patches on the island. MP = Miller's Peak; WV = West Valley and West Palm Valley; MV = Miller's Valley and Middle Valley; EV = East Valley and East Palm Valley.

subjected to the same molecular protocols as in 2007, but the data were included in the statistical analysis only as part of the 2007 sample. All feathers were stored in paper envelopes at room temperature prior to DNA isolation.

DNA isolation and genotyping.—Genomic DNA was isolated from the pulp of two or three feathers, following the general protocol of Devlin et al. (2004). Approximately 1 mm of the calamus was removed using a razor blade and placed in 10 µL of extraction buffer (0.1 M NaCl, 0.05 M Tris-HCl, 0.01 M Na_2EDTA, pH 8.0), 1 µL of 10% Tween-20 and 1µL of Proteinase K (20 mg mL^{-1}). Samples were incubated in a thermal-cycler (C1000, Bio-Rad) at 65°C for 120 min (mixed and spun at 30-min intervals), followed by a 10-min hold at 95°C to denature the Proteinase K. DNA isolations were diluted 1 in 10 with sterile water.

To maximize our potential for finding sufficient variation to calculate N_e and inform the conservation and management protocol for the Nihoa Millerbird, we used primers described by Richardson et al. (2000) to amplify the four microsatellite loci (Ase11, Ase44, Ase48, and Ase57) reported to be polymorphic in 15 birds sampled in 1992 (Fleischer et al. 2007). Amplifications were performed in 10-µL volumes consisting of 1× GoTaq Flexi Buffer (Promega, Madison, Wisconsin), 0.2 mM each of dNTP, $MgCl_2$ (1.5 mM for Ase11; 2.5 mM for Ase44, Ase48, and Ase57), 1 µL of the diluted DNA, 1 pmol of each primer, and 0.25 units (U) of GoTaq polymerase (Promega). The amplifications were performed using a BioRad C1000 thermal-cycler. All cycling protocols began with an initial denaturing step at 94°C for 180 s, followed by 35 cycles of amplification at 94°C for 30 s, annealing temperature for 30 s (see below), and extension at 72°C for 45 s, and finished with a final extension at 72°C for 120 s. Annealing temperatures and touchdown profiles varied for each locus. For Ase44 and Ase57, we used a touchdown protocol of 5 cycles (–2°C $cycle^{-1}$) beginning at 64°C, 10 cycles at 48°C, and 20 cycles annealing at 50°C. For Ase48, we used 5 cycles (–2°C $cycle^{-1}$) beginning at 66°C, followed by 30 cycles of 45 s at an annealing temperature of 56°C. For Ase11 we used 5 cycles (–1°C $cycle^{-1}$) beginning at 68°C, followed by 30 cycles of 45 s at and annealing temperature of 60°C. The amplified products were resolved in 6% (25 cm, 0.2 mm thick) denaturing polyacrylamide gels on a LICOR 4300L DNA Analyzer (LI-COR Biosciences, Lincoln, Nebraska).

We used the control-region primers L434 and H1248 (Fleischer et al. 2007) to amplify part of the mitochondrial genome (mtDNA) in all the samples collected in both sampling years. We also targeted a 689-bp fragment of cytochrome *b* (cyt *b*) using primers CytBL (5′-AGCCATGCACTACACAGCAG-3′) and CytBR (5′-GGATGGCGTATGCAAATAGG-3′) engineered from the Nihoa Millerbird cyt *b* gene-bank accession (EU119965). However, we sequenced this fragment only in the 2009 samples. Amplifications were performed in 15-µL volumes containing 1× ThermoPol Reaction Buffer (New England Biolabs, Ipswich, Massachusetts), 0.2 mM each dNTP, 2.5 mM $MgSO_4$, 1.5 µL of the diluted DNA, 0.25 µM of each primer, and 1.5 U of LongAmp *Taq* DNA polymerase (New England Biolabs). The cycling protocol consisted of an initial 180-s denaturation step at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 47.1°C, and 48 s at 72°C for the control region, and 35 cycles of 30 s at 95°C, 30 s at 50°C, and 45 s at 72°C for cyt *b*. The final extension at 72°C was held for 180 s. Amplicons were checked by agarose gel electrophoresis. All PCR products were treated with Exonuclease 1 and Antarctic Phosphatase (New England Biolabs) prior to sequencing. The purified product was used as the template DNA for cycle sequencing reactions (Centre for Applied Genomics, Toronto, Ontario) using BigDye chemistry and resolved on an ABI 3730XL automatic sequencer (Applied Biosystems, Carlsbad, California). Sequences were edited using SEQUENCHER (Gene Codes, Ann Arbor, Michigan), aligned in CLUSTALX using the default settings (Thompson et al. 1997), and trimmed to a standard length (Genbank accession numbers HQ880003–HQ880206).

Statistical analysis.—For the microsatellites, we used GENEPOP, version 3.4 (Raymond and Rousset 1995, Rousset 2008) to estimate allele frequencies, observed (H_O) and expected heterozygosity (H_E), and to perform exact tests for the conformance to Hardy-Weinberg expectations (HWE) and llinkage disequilibrium between pairs of loci. Because the presence of null alleles can affect estimates of population differentiation (Chapuis and Estoup 2007) and, thus, estimates of N_e (Chesser et al. 1993, Waples 2010), we tested our microsatellite data for scoring errors, large allele dropout, and the presence of null alleles using MICROCHECKER, version 2.2.3 (Van Oosterhout et al. 2004). For the mtDNA sequence data, haplotype diversity (*h*), number of segregating sites (*S*), and nucleotide diversity (π) were calculated using DNASP, version 4.10 (Rozas et al. 2003).

We assessed the effect of sampling the population 2 years apart by computing all the statistics and measures of genetic diversity both independently for each sampling year and combined as a single sample. We tested for temporal stability in allele frequencies between the habitat patches with repeated samples (MP and MV; see Fig. 1) and between the pooled 2007 and 2009 data using Fisher's exact tests computed in GENEPOP for the microsatellites and in ARLEQUIN, version 3.11 (Excoffier et al. 2005), for the mtDNA sequences. We explored the possibility that habitat patches from which specimens were collected might represent genetically distinct assemblages of birds by computing the pairwise F_{ST} between individuals grouped into 4 clusters (Fig. 1). Significance of all pairwise comparisons was assessed by 10,000 permutations of the data.

Because both population subdivision (e.g., Waples 2010) and genetic structure arising from overrepresenting family groups can lead to gametic disequilibrium and bias estimates of N_e (Luikart et al. 2010), we used the Bayesian model-based clustering algorithm implemented by STRUCTURE, version 2.2.3 (Pritchard et al. 2000), to test for cryptic family and spatial structure in the microsatellite data. The number of genetic clusters (*K*) among our samples was estimated from 1 to 10 using the admixture model with allele frequencies correlated among populations and ignoring prior population information. We ran six Bayesian Markov-chain Monte Carlo searches of between 150,000 and 1 million steps with a burn-in of 10%, and considered the ln $P(X|K)$ when selecting the best-fit value of *K*.

The application of N_e estimators to populations with overlapping generations provides an estimate of the effective number of parents that produced the sample; estimates are best interpreted as the effective number of breeders (N_b) of the parental generation (Waples 2005). We used complete multilocus genotypes from Nihoa Millerbirds collected in 2007 ($n = 64$) and 2009 ($n = 62$) to estimate N_b independently from each sample with two single

sample methods (as suggested in Beebee 2009). First, we used the approximate Bayesian computation (ABC) method developed by Tallmon et al. (2008) and implemented by the online program ONESAMP. This method uses parameters from the empirical data to generate 50,000 simulated populations that reproduce for two to eight generations, after which they are sampled and eight summary statistics are calculated that have been closely related to the N_e of a population through either theory or computer simulations. We set the prior for N_b estimates between 2 and 100 and ran the analysis for the 2007 and 2009 samples independently, and again for the combined data set. Second, we used the sibship assignment (SA) method implemented by the program COLONY2 (Wang 2009) with the assumption of (1) strict monogamy, (2) a polygamous breeding system for males but not for females, or (3) a polygamous system for both sexes (e.g., Richardson et al. 2005). This method infers N_b from the sibship frequencies (full sibs, half sibs, or nonsibs) estimated from a sibship assignment analysis using multilocus genotypes of a sample of offspring taken at random from a single cohort. Searches were conducted using the full likelihood model with medium precision and no prior information. Although our sample design violated the single cohort assumption of this method (temporally spaced samples, multiple age classes of birds), the samples collected in 2007 and 2009 were genetically indistinguishable and we repeated all three of these analyses on the pooled data.

Although our data represent temporal samples, we did not calculate effective population size using temporal methods for two reasons. First, unless samples are collected between 5 and 10 generations apart, the estimated standardized variance in allele frequencies (F) is more strongly influenced by the noise associated with sampling a finite population than it is by genetic drift (Waples and Yokota 2007). Second, the bias in the estimate of N_e associated with sampling the same population at short time intervals is unpredictable and depends largely on the age structure of the sample (Waples and Yokota 2007). Because we are unsure whether our samples were composed mainly of juveniles belonging to one or two cohorts (underestimates N_e) or an abundance of older individuals who actually contribute less to the simulations of reproductive output (overestimates N_e), we would be unsure of the proper interpretation of the results.

RESULTS

Of the 139 individuals collected, we obtained 126 complete multilocus genotypes, and 90–100% successful amplification for all four microsatellite loci (Table 1). In three of the microsatellites (Ase44, Ase48, and Ase57) we detected two alleles, but Ase11 was monomorphic in all samples from both collection years (Table 1). We failed to recover the same number of alleles at Ase44 ($N_A = 3$) and Ase11 ($N_A = 2$) that were present in the 15 individuals sampled in 1992 (Fleischer et al. 2007). Because the probability of detecting an allele with frequency p in a sample of n individuals ($2n$ chromosomes) was calculated as $1 - (1 - p)^{2n}$ (see Glatt et al. 2001), we calculate that in a sample of 64 individuals, the probability of detecting a second allelic variant at Ase11 with a population frequency of 0.036 or higher (calculated based on $H_O = 0.07$; see Fleischer et al. 2007: Table 3) was 0.991. Observed heterozygosity ranged from 0.047 to 0.532, and these values fluctuated among loci between sampling years (Table 1). Exact tests for departures from HWE revealed an excess of homozygotes for Ase44 and Ase57 in both the 2007 and combined data sets, and an excess of homozygotes at Ase48 in the 2009 sample. We also observed more heterozygotes than expected at Ase44 in 2009 and Ase48 in 2007, but these departures from HWE were not statistically significant. There was a significant global departure from HWE in both the 2007 and combined data sets (Table 1). Analysis with MICROCHECKER indicated that null alleles at Ase44 (frequency = 0.1005) were the most likely cause of heterozygote deficiency in the combined data set. The only significant linkage disequilibrium that we detected across all loci and sampling years was between Ase48 and Ase57 ($P = 0.028$), but only in the 2007 sample, and this difference was not significant after a Bonferroni correction for multiple tests (Rice 1989).

Our sequence alignments included 672 bp for the control region ($n = 136$; Table 2) and 608 bp for cyt b ($n = 68$). We detected two haplotpyes at the control region that differed by a two-base insertion (GC) in a $(GC)_4$ repeat region. The frequency of this haplotype varied between sampling years, from 0.092 in 2007 to 0.175 in 2009 (Table 2). Haplotype diversity in the combined 2007 and 2009 data was low ($H_d = 0.220$), but it was nearly twice as high in

TABLE 1. Genetic diversity at four microsatellite loci in Nihoa Millerbirds sampled on Nihoa Island in 1992, 2007, and 2009 (n = sample size; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = inbreeding coefficient.

		Ase44	Ase48	Ase57	Ase11	Mean
1992[a]	n	15	15	15	15	15
	N_A	3	2	2	2	2.75
	H_O	0.53	0.13	0.27	0.07	0.25
2007	n	75	68	71	75	71.3
	N_A	2	2	2	1	2
	H_O	0.280	0.147	0.324	0	0.250
	H_E	0.503	0.137	0.464	—	0.361
	F_{IS}	0.455***	–0.065	0.238*	—	0.321**
2009	n	62	64	63	64	63
	N_A	2	2	2	1	2
	H_O	0.532	0.047	0.460	0	0.347
	H_E	0.503	0.104	0.479	—	0.362
	F_{IS}	–0.120	0.549**	0.115	—	0.043
2007 and 2009	n	137	132	134	139	135.5
	N_A	2	2	2	1	2
	H_O	0.394	0.098	0.388	0	0.293
	H_E	0.502	0.121	0.470	—	0.364
	F_{IS}	0.218*	0.185	0.183*	—	0.153**

[a]Data from Table 3 in Fleischer et al. (2007); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

TABLE 2. Sample size (n), haplotype diversity (H_d), and the frequencies (f) of two mtDNA haplotpyes (A and B) detected in the control-region sequences of Nihoa Millerbirds sampled on Nihoa Island in 2007 and 2009.

Sample	n	H_d	f(A)	f(B)
2007	73	0.153	0.918	0.082
2009	63	0.293	0.825	0.175
2007 and 2009	136	0.220	0.875	0.125

Table 3. Effective number of breeders (N_b) with 95% confidence intervals for Nihoa Millerbirds sampled on Nihoa Island in 2007 and 2009, estimated from three-locus microsatellite genotypes using sibship or approximate Bayesian computation (ABC) methods.

		Sample			
Estimator	Parentage	2007	2009	2007 and 2009	Adjusted[a] 2007 and 2009
Sibship[b]	Strict monogamy	12 (6–30)	13 (7–30)	11 (6–26)	15 (8–30)
	Male polygamy	9 (4–24)	7 (4–21)	9 (4–24)	12 (6–27)
	Male and female polygamy	6 (2–21)	5 (2–20)	9 (4–24)	7 (4–21)
ABC[c]	Random mating, monogamy	8.5 (4.5–16.1)	9.0 (5.2–15.4)	8.7 (4.9–14.4)	8.0 (4.8–13.1)

[a]Allele frequencies at Ase44 were adjusted to reflect the presence of a null allele as suggested by MICROCHECKER (see text).
[b]COLONY2 (Wang 2009).
[c]ONESAMP (Tallmon et al. 2008).

the 2009 sample as in 2007 (Table 2). Even though we sequenced mtDNA from 136 individuals, we did not detect the variable site (a T–C transition in the 5′ end of the control region) reported by Fleischer et al. (2007). Cyt *b* was monomorphic in all 68 individuals we sequenced from 2009.

There was no evidence for temporal or spatial variation in microsatellite alleles. Pairwise F_{ST} for the microsatellites computed between sample clusters ranged from <0.0 to 0.014, but none of these values approached significance. Exact tests indicated that microsatellite allele frequencies did not differ significantly between the 2007 and 2009 samples, either within the same habitat patch (MP: $P = 0.513$; MV: $P = 0.821$) or pooled across the entire island ($P = 0.974$). Genetic diversity at the mtDNA control region was not significantly different between temporal samples at MV (exact $P = 0.449$), but the samples collected at MP were significantly different between 2007 and 2009 (exact $P = 0.045$). This difference was driven by the absence of haplotype B in this region of the island in the 2007 sample, and its presence at low frequency in the same location (0.278) in 2009. There was no evidence for temporal variation between the pooled mtDNA sequences collected in 2007 and 2009 (exact $P = 0.123$), and pairwise comparisons between habitat patches were not significant, indicating no spatial variation ($F_{ST} < 0.0$ to 0.071).

For the Bayesian clustering method implemented in STRUCTURE, at $K = 1$ all individuals were assigned a Q value (posterior probability) of 1 for belonging to the same cluster. At $K \geq 2$, all individuals were equally admixed among the clusters, with no individuals being strongly assigned to any one group. On the basis of this pattern and the ln $P(X|K)$, no significant substructure was evident in the Nihoa Millerbirds that we sampled.

Levels of diversity in local populations are strongly influenced by population substructure and migration rates that can severely bias estimates of N_b (Waples 2010). However, our analyses indicate that the population of Nihoa Millerbirds is panmictic, so we do not expect our analyses of N_b to have been influenced by genetic structure and migration among habitat patches. Estimates of N_b calculated using the single-sample methods of Tallmon et al. (2008) and Wang (2009) were very small, ranging from 5 to 13 individuals within and across years (Table 3). Implementing a monogamous breeding system in the sibship analysis returned slightly higher estimates of N_b, whereas considering both sexes polygamous generated estimates in the lower end of the range. The estimates from both methods were remarkably similar, but the 95% confidence intervals were much narrower using ABC than they were for the sibship method. We considered the possibility that a single null allele at Ase44 could have biased our estimates of N_b by adjusting the genotypes using the Oosterhout algorithm in MICROCHECKER. We then randomly assigned the corrected genotypes at Ase44 to our combined (2007 and 2009) multilocus data set and reanalyzed the data. Updated estimates of N_b calculated using the adjusted genotypes were similar to the raw data and ranged from 8 to 15 (Table 3).

Discussion

Our results reveal extensive and strikingly low levels of neutral genetic diversity in the Nihoa Millerbird. Although we sampled about 15–40% of the census population, we failed to identify new alleles at the same polymorphic microsatellite loci studied in 15 individuals collected in 1992 (Fleischer et al. 2007). To our surprise, we report the loss of alleles at two different microsatellite loci and a single nucleotide polymorphism at the mtDNA control region in a span of only 15 years, which suggests a recent decline in genetic diversity of this population. Although our sampling was limited to three polymorphic loci that revealed low allelic diversity and moderate heterozygosity, our estimation of an exceedingly low effective number of breeders ($N_b = 5$–13) is robust to the assumptions of no temporal or spatial genetic subdivision among the samples. Although these results can be used to inform the conservation and management plans for this species (discussed below), our analysis highlights the utility of ABC and sibship methods for estimating N_e in species with low genetic polymorphism or for which few loci are available.

Using both sibship (Wang 2009) and ABC methods (Tallmon et al. 2008), we estimate the effective number of breeders (N_b) to be between 5 and 13 individuals. The estimates from the two different methods were remarkably similar, and the confidence limits were generally superimposed on one another. The presence of null alleles generally had a limited influence on the estimation of N_b, although the confidence intervals for the sibship (monogamy) and the ABC methods did not overlap with each other. This disparity is likely a result of the sensitivity of the sibship analysis to

an increase in the number of alleles (and, thus, an increase in the number of genotypes). Further comparative analyses through simulations are required to better understand the bias that null alleles have in the estimation of N_e.

The choice of breeding system had a large effect on the values generated for each sample by the sibship program, with strict monogamy estimating approximately twice as many breeders as a polygamous male and female breeding system. This results from the fact that in polygamous systems a single female or male can combine their gametes with more than one mate to increase the number of different genotypes in their offspring. However, the N_b estimates from the combined 2007 and 2009 data were slightly larger and less sensitive to the mating system because of the increase in the total number of unique genotypes in the sample. Estimates of the N_e from a single sample more closely reflect the effective number of breeders when only one or a limited number of cohorts are examined rather than a generation as a whole. Thus, we interpret our point estimates for 2007 and 2009 as more closely reflecting the N_b of those samples, but combining the samples from 2007 and 2009, we significantly increase the number of overlapping generations and our estimate of N_b begins to approach the true N_e for the species.

Combined with the dramatic temporal variation in the observed heterozygosity and frequency of mtDNA haplotypes, our results indicate variability in the genotypes of the adult breeders each year. This pattern is expected in small finite populations because of deviations from expected genotype and gametic frequencies (i.e., violation of the Hardy-Weinberg assumption of an infinite population size). Linkage-disequilibrium methods have been developed extensively to take advantage of these differences (Luikart et al. 2010), but because we had few loci and low polymorphism, these methods performed poorly on our data (not shown). The single-sample methods that we used do not rely on the average linkage disequilibrium or heterozygosity excess across multiple markers to estimate N_b, and our results are consistent with other studies of species with low genetic diversity (Beebee 2009, Johnson et al. 2009). This suggests that sibship and ABC methods may be broadly applicable to species with small populations for which few genetic markers are currently available.

Recent severe bottlenecks caused by anthropogenic influences or the introduction of non-native plants and insects may have rapidly reduced the levels of genetic diversity on the island. One recent invader to Nihoa is the Gray Bird Grasshopper (*Schistocerca nitens*), first reported on the island in 1977 (Latchininsky 2008 and references therein). In the past, population outbreaks of grasshoppers have denuded ~90% of the island's vegetation, especially the shrubs in which Nihoa Millerbirds nest. A grasshopper outbreak may also indirectly affect Nihoa Millerbirds because defoliation may reduce the population of herbivorous insects that constitute the bird's main prey. Outbreaks such as these may have been responsible for reductions in the bird population to well below 200 individuals in 1994, 1996, and 2005 (Latchininsky 2008).

We conclude that because the Nihoa Millerbird is found only on the small, isolated island of Nihoa, the entire species is extremely vulnerable to extinction through exposure to stochastic factors (e.g., climate and disease). We support the current conservation plan to establish additional populations of Nihoa Millerbirds on the ecologically similar Laysan Island (Kohley et al. 2010). Similar translocation plans have been successful in establishing new populations of the Saddleback (*Philesturnus carunculatus carunculatus*; Taylor and Jamieson 2008), the Laysan Finch (*Telespiza cantans*; Tarr et al. 1998), and the closely related Seychelles Warbler (*A. sechellensis*; Komdeur 1994, Richardson et al. 2006). Although a significant loss of genetic diversity in newly founded populations is not expected when diversity in the source population is high (e.g., Clegg et al. 2002), translocating species with low genetic diversity can generate sampling artifacts and reduce heterozygosity (Taylor and Jamieson 2008). The result can be fewer alleles and lower polymorphism in the newly established population, and an increase in the genetic difference from the source population. Furthermore, consistent with population genetics theory, newly established populations experience less inbreeding when they are founded with more individuals and grow quickly to large population sizes (Biebach and Keller 2010).

Although protocols are currently being developed for the translocation of Nihoa Millerbirds to Laysan Island, few guidelines exist to estimate the number of individuals that should be introduced to buffer against the loss of genetic diversity as a result of subsequent demographic and stochastic processes in the new population. Conant and Morin (2001) simulated the 100- and 1,000-year extinction risks of a new population under several scenarios of introduction. In their simulations, populations had an extinction probability of zero if translocations were supplemented with additional individuals in subsequent years because a much higher level of heterozygosity was maintained. They concluded that a population founded with the introduction of 40 individuals (20 males and 20 females) followed by supplementation with both sexes every 50 years would persist for 1,000 years. Our results indicate that targeting as few as 8 to 13 individuals for translocation to Laysan Island could preserve the genetic diversity in the Nihoa Millerbird. However, because the probability of survival, variance in reproductive success, and mating system are unpredictable in any newly established population and because the remote location and fragile status of the island mean that resolving those uncertainties is not practicable prior to translocation, a sample large enough to mitigate environmental and stochastic uncertainty should be considerably larger than N_e. Using the results of Conant and Morin (2001) as an upper bound, we suggest that the number of individuals targeted for translocation should be between 20 (~$2N_e$) and 40. Consistent with both population genetics theory and the findings of Conant and Morin (2001), the long-term success of establishing a (relatively) genetically diverse and fast-growing population of Nihoa Millerbirds on Laysan Island will benefit from the movement of individuals over the course of several years. The lack of spatial structure that we found in the population also suggests that individuals could be translocated from any convenient part of the island.

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