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EVIDENCE OF LOW GENETIC VARIATION AND RARE ALLELES IN A BOTTLENECKED ENDANGERED ISLAND ENDEMIC, THE LAYSAN TEAL (ANAS LAYSANENSIS)

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ABSTRACT

Genetic diversity is assumed to reflect the evolutionary potential and adaptability of populations, and thus quantifying the genetic diversity of endangered species is useful for recovery programs. In particular, if conservation strategies include reintroductions, periodic genetic assessments are useful to evaluate whether management efforts have resulted in the maximization or loss of genetic variation within populations over generations. In this study, we collected blood, feather, and tissue samples during 1999–2009 and quantified genetic diversity for a critically endangered waterfowl species endemic to the Hawaiian archipelago, the Laysan teal or duck (*Anas laysanensis*; n = 239 individual birds sampled). The last extant population of this species at Laysan Island was sourced in 2004–2005 for a 'wild to wild' translocation of 42 individuals for an experimental reintroduction to Midway Atoll. To inform future management strategies, we compared genetic diversity sampled from the source population (n = 133 Laysan birds) including 23 of Midway's founders and offspring of the translocated population 2–5 years post release (n = 96 Midway birds). We attempted to identify polymorphic markers by screening nuclear microsatellite (N = 83) and intronic loci (N = 19), as well as the mitochondrial control region (mtDNA) for a subset of samples. Among 83 microsatellite loci screened, six were variable. We found low nuclear variation consistent with the species' historical population bottlenecks and sequence variation was observed at a single intron locus. We detected no variation within the mtDNA. We found limited but similar estimates of allelic richness (2.58 alleles per locus) and heterozygosity within islands. Two rare alleles found in the Laysan Island source population were not present in the Midway translocated group, and a rare allele was discovered in an individual on Midway in 2008. We found similar genetic diversity and low, but statistically significant, levels of differentiation (0.6%) between island populations suggesting that genetic drift (as a result of translocation-induced population bottlenecking) has had a limited effect within five years post-release. Our results have utility for informing translocation and genetic management decisions.

INTRODUCTION

Island ecosystems are useful for evolutionary and population genetic studies because of their discrete geography, speciation via small founding populations, colonizing individuals that are isolated from the source, and resultant high prevalence of genetic drift (Fleischer *et al.* 1991). The level of genetic variation in some island species is extremely low in comparison to other related species (Frankham 1996). A fundamental aspect of management action for species conservation should be to preserve patterns of gene variation so that natural selection and other processes have the potential to respond to environmental change (Reed and Frankham 2003, Frankham 2005). The application of molecular techniques to examine levels of genetic variation can provide valuable information to aid in the genetic management of island populations for long-term persistence (e.g., adaptation capacity in the face of new diseases, or other biotic and abiotic environmental changes).

Ancient DNA testing, historical accounts, and sub-fossil remains indicate the Laysan teal (or Laysan duck; *Anas laysanensis*) occurred throughout the Hawaiian archipelago until 800–900 years ago, but for more than 150 years it was isolated on a small remote atoll (412 ha) in the northwestern chain, Laysan Island (Cooper *et al.* 1996). Like other Hawaiian birds, it was extirpated from islands following the arrival of humans and introduced mammals (Olson and James 1991), and declined to near extinction (7–20 individuals) following the intentional

introduction of European rabbits (*Oryctolagus cuniculus*) to Laysan Island around 1905 (Dill and Bryan 1912). After rabbits were eliminated in 1924 the population on Laysan Island began to recover (Ely and Clapp 1973). By 1957 the only remaining Laysan teal population had grown to more than 500 birds (Ely and Clapp 1973), reaching the island's contemporary carrying capacity (USFWS 2004, Seavy *et al.* 2009). However, two recent population declines are documented, first in 1993–1994 when abundance fell to about 150 adults during a severe drought and epizootic, and then in 2011–2012 after the Tōhoku earthquake generated tsunami, a decline of about 40% was estimated at Laysan Island (Work *et al.* 2004, Seavy *et al.* 2009, Reynolds *et al.* 2015). To reduce the risk of extinction inherent to a species confined to a single small island vulnerable to catastrophes such as hurricanes and tsunamis, wild Laysan teal were moved to Midway Atoll to establish a second population on another island in the northwestern chain. Midway Atoll is within the Papahānaumokuākea Marine National Monument and is 600 km from Laysan (Figure 1). Twenty wild Laysan teal were translocated to Midway in October 2004, and another 22 were moved in October 2005. At Midway Atoll, Laysan teal began breeding at less than one year of age, within six months of the translocation, and abundance increased linearly reaching 500 post-fledgling individuals in 2008, but population growth slowed after annual avian botulism type C (*Clostridium botulinum*) epizootics began in 2008 (Work *et al.* 2010, Reynolds *et al.* 2013).

In this study, we examined microsatellite, mitochondrial, mtDNA, and intron variation of Laysan teal from the source island population of Laysan and from the reintroduced population on Midway Atoll. We compared genetic diversity of the two populations and tested for evidence of allelic loss at Midway Atoll two to five years after the translocations. Since recovery strategies emphasize translocation (in effect a management-induced bottleneck; USFWS 2009), often with a small effective population size of founders, periodic genetic assessment of populations are valuable to understand how genetic variation changes over time (i.e., genetic drift) and to inform decisions likely to influence retention of genetic diversity within the translocated population.

METHODS

On Laysan Island (25º46' N, 171º44' W; Figure 1) during 1999–2009, we caught Laysan teal and collected blood (n = 72) or feathers, including rectrices or body feathers (n = 50). Samples were preserved in lysis buffer (0.1 M Tris, pH 8.0, 0.1 M EDTA, 2% SDS [sodium dodecyl sulfate]) and frozen. Additionally, we recovered carcasses on Laysan Island and froze tissue samples (n = 21). The 143 teals sampled from Laysan Island (62 females, 60 males, and 21 juveniles of unknown sex) included 23 of the 42 original founders of Midway Atoll (28°11'–28°16' N and 177°18'–177°25' W; Figure 1). At Midway Atoll during 2007–2010, we also trapped birds and collected feathers from five individuals and tissue samples from 91 carcasses recovered at wetlands during avian botulism surveillance representing the F_1–F_5 generations (38 females, 47 males, and 11 juveniles of unknown sex). The source population size varied from about 380–680 birds, and the translocated population varied from about 250–475 during our study, so our population sampling approached approximately 20–40% of the populations. We excluded any sample that could not be verified as a unique bird.

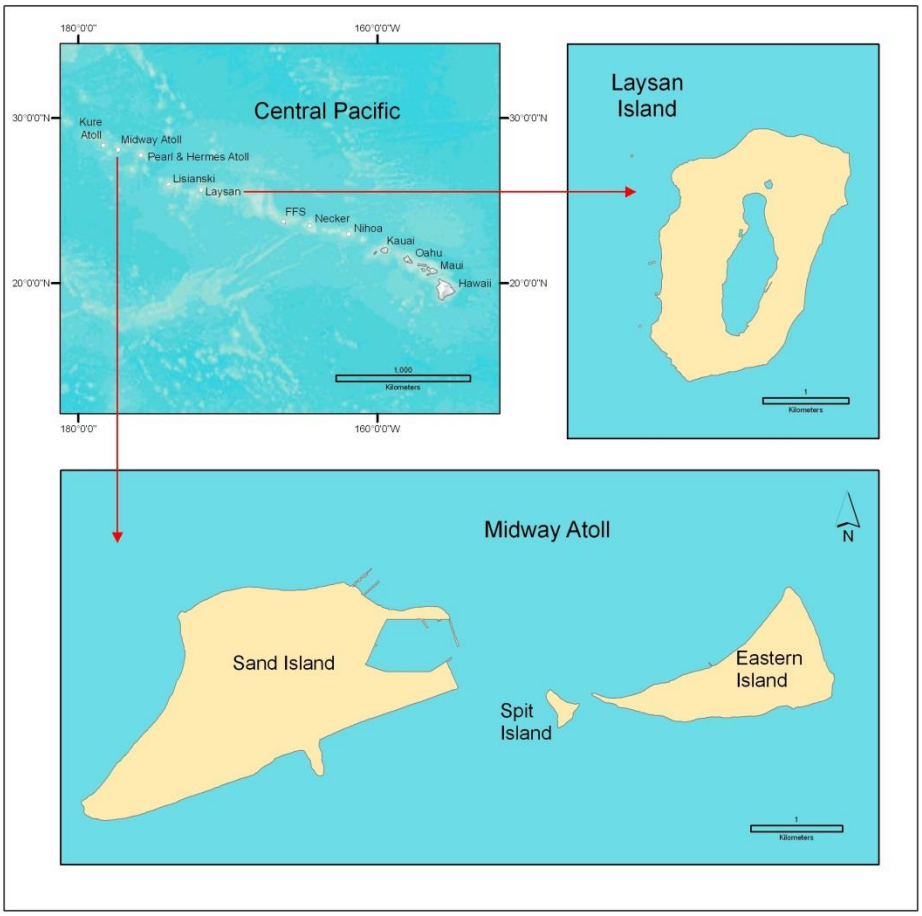

Figure 1. Map of Hawaiian Islands with enlargements of Laysan Island and Midway Atoll. Wild Laysan teal (*Anas laysanensis*) from Laysan Island were translocated to Midway Atoll during 2004–2005.

We used a subset of samples to screen for variation in three classes of genetic markers (microsatellite, mtDNA, and intron variation) with the intent of examining the larger dataset once variable markers were identified. For microsatellite loci, we screened 18–31 samples from Laysan Island for a total of 83 microsatellite primer pairs and examined results for allelic variation. The majority of these primers came from published sources examining variation of microsatellites in waterfowl (Fields and Scribner 1997, Buchholz *et al.* 1998, Cathey *et al.* 1998, Maak *et al.* 2003, Paulus and Tiedemann 2003, Denk *et al.* 2004, and Munoz-Fuentes *et al.* 2005) and other species (Primmer *et al.* 1995). PCR amplification of all nuclear loci during screening and data collection involved identical reagent cocktails as described in Pearce *et al.* (2004), except that all were amplified with the same PCR temperature profile (94° C for 2 min followed by 40 cycles of 94° C for 2 min, 50° C for 1 min, and 72° C for 1 min) by means of an MJ Research PTC-200 thermal cycler. PCR products were visualized on 6% polyacrylamide gels with a LI-COR 4200 DNA sequencer. Genotypes were scored according to allele size on the basis of an initial comparison to an M13 DNA sequence ladder and then to samples established as size standards that were run on each subsequent gel. We obtained sequence data from the

mtDNA control region (645 bp) and 19 nuclear introns (7,146 bp in total; Appendix I) for 21 Laysan teals (Laysan Island [n = 10] and Midway Atoll [n = 11]). Primer sequences and protocols for DNA extraction, PCR, and DNA sequencing are described in detail elsewhere (Peters *et al.* 2012, Lavretsky *et al.* 2014b). Of the 83 microsatellite loci screened, a total of 68 primer pairs yielded a PCR product and of those, nine loci (13.2%) were variable among the test samples. However, three of these nine loci were difficult to reliably score and were subsequently dropped. Thus, we analyzed all 229 samples comprising 133 individuals from Laysan Island and 96 from Midway Atoll with six loci that were polymorphic and reliably scored: Bca3 (Buchholz *et al.* 1998), Smo4, Smo6, Smo11 (Paulus and Tiedemann 2003), Aph21 (Maak *et al.* 2003), and Sifu8 (Fields and Scribner 1997; Table 1). Approximately 25% of genotypes (range per locus 10–50%; 342 samples) were amplified more than once across the six microsatellite loci to verify genotypes. From these data, six discrepancies were observed yielding an error rate of 1.8%. We calculated allelic richness (i.e., the number of alleles per locus), expected (H_e) and observed heterozygosities (H_o), and inbreeding coefficient (F_{IS}) for microsatellite loci in FSTAT 2.9.3 (Goudet 1995). Hardy-Weinberg equilibrium and linkage equilibrium (LE) were tested in GENEPOP 3.1 (Raymond and Rousset 1995; Markov chain parameters: dememorization number 1,000; number of batches 100, and number of iterations per batch 10,000). Population subdivision between Laysan Island and Midway Atoll was assessed by calculating the pairwise fixation index (F_{ST}) in ARLEQUIN version 3.5 (Excoffier and Lischer 2010). Microsatellite data also were analyzed in STRUCTURE 2.1 (Pritchard *et al.* 2000) to test for the occurrence of population structure and to probabilistically assign individuals to putative populations without *a priori* knowledge of island source. Data were analyzed using an admixture model assuming correlated frequencies using a burn-in period of 10,000 steps followed by 100,000 Markov chain Monte Carlo iterations. The number of possible populations (K) ranged from 1–5. The STRUCTURE analysis was repeated five times for each value of K to evaluate consistency across runs.

RESULTS

Of the 83 microsatellite loci screened, a total of 68 primer pairs yielded a PCR product and of those, nine (13.2%) loci were variable among 229 Laysan teal samples with six microsatellite loci with dinucleotide repeat motifs suitable for analysis (clear product, no allele fragment sizes that differed from the repeat motif; i.e. one base pair repeat). Sequences from mtDNA and 18 of 19 nuclear introns revealed no genetic variation. Three haplotypes, one common and two rare, were detected in the FGB locus in the 10 samples from Laysan Island. For the six microsatellite loci examined, the number of alleles per locus and allelic richness were nearly the same for the two island groups (Table 1), with an average of 2.58 alleles per locus. Observed heterozygosity ranged from 32–57% across all loci and both island groups (Table 1). The inbreeding coefficient (comparing the heterozygosity within sampled individuals from the population) ranged from 0.009 to 0.020 (mean = 0.002). None of the inbreeding coefficients were significantly different from zero ($P > 0.05$). Both island groups were in Hardy-Weinberg and linkage equilibrium. The overall estimate of F_{ST} between the two island groups was low but statistically significant (F_{ST} = 0.006, P = 0.03). Results from STRUCTURE were similar between islands. Maximum model likelihood was at a single population (K = 1) and the log likelihood (–1788.4) was the same across five runs. Smaller (more negative) and more variable likelihood estimates were observed across the five runs for each value of $K > 1$.

Table 1. Metrics of genetic diversity across six nuclear microsatellite loci for two populations (source and translocated) of the Laysan teal (*Anas laysanensis*) in the Hawaiian archipelago.

Genetic diversity metric	Laysan Island (source) $n = 133$	Midway Atoll (translocated) $n = 96$
No. of alleles	2.67	2.50
Allelic richness	2.64	2.45
Observed heterozygosity (H_o)	0.476	0.442
Expected heterozygosity (H_e)	0.476	0.442
Inbreeding coefficient (F_{IS})	–0.009	0.020
Fixation index (F_{ST}) between populations	0.006 ($P = 0.03$)	

There were three instances of microsatellite alleles being present in only one population. Two alleles were found in the source population, but not in the Midway translocated group, while a third allele was found only in the Midway group (Table 2). This single allele (Aph21 locus, allele 176) was novel in the data set and came from an unmarked juvenile bird in 2008 from Midway Atoll (first generation or fourth generation offspring) that died of avian botulism type C. The presence of this unique allele on Midway could be due to a novel mutation post-translocation or this rare allele was not yet sampled in the source population.

Table 2. Microsatellite allele frequencies for two populations of the Laysan teal (*Anas laysanensis*) in the Hawaiian archipelago. Private alleles present in only one population are shown in bold.

Locus	Allele	Laysan Island (source) *n* = 133	Midway Atoll (translocated) *n* = 96
Bca3	169	0.238	0.244
	173	0.761	0.755
Smo4	279	0.028	0.010
	283	0.580	0.636
	287	0.383	0.352
	291	**0.081**	**0.000**
Smo6	146	0.435	0.542
	151	0.564	0.457
Smo11	199	0.608	0.688
	205	0.392	0.311
Aph21	169	0.647	0.724
	176	**0.000**	**0.052**
	177	0.325	0.255
	181	0.027	0.015
Sfiu8	**144**	**0.018**	**0.000**
	146	0.476	0.533
	152	0.504	0.466

DISCUSSION

The low genetic variation at these loci for Laysan teal is consistent with historical accounts of a dramatic population decline and a genome-wide loss of variation of an extremely isolated island endemic (Table 3). Compared to other island endemic species, the Laysan teal harbors low genetic diversity, likely due to combined archipelago founder effects, its long duration of isolation, and radical range restriction on Laysan Island (Table 3). Because lack of genetic diversity correlates with a species' risk of extinction and disease susceptibility (Spielman *et al.* 2004), the low genetic diversity documented in this study has conservation implications for this species, especially with respect to reducing genetic drift and further losses in remaining genetic diversity, a possible side effect of reintroductions (Reynolds *et al.* 2013). Laysan finches (*Telespiza cantans*) were also restricted to Laysan Island after extirpation from the main Hawaiian Islands and a similar study describes their microsatellite variation on Laysan Island and at Pearl and Hermes Atoll (47 ha) about 20–25 years after translocation. The study revealed dramatic genetic drift between the translocated and source populations (Tarr *et al.* 1998). Populations became established on four islands of Pearl and Hermes Atoll, however, all but the largest one (Southeast Island, 18.4 ha) have been extirpated since 2011, and the last Pearl and Hermes population is at risk of inundation from sea-level rise and sudden inundation events and other catastrophes (Tarr *et al.* 1998, Reynolds *et al.* 2012).

Because lack of genetic diversity correlates with a species' risk of extinction and disease susceptibility, the low genetic diversity documented in Laysan teal in this study has conservation implications for this species, especially with respect to reducing genetic drift, a possible side effect of reintroductions (Speilman *et al.* 2004, Frankham 2005, Bristol *et al.* 2013, Reynolds *et al.* 2013). To improve probabilities of retaining the Laysan teal's limited genetic variation, the number of founders and the frequency and number of immigrants to translocate, could be tailored according to the carrying capacity of and rate of population growth observed at translocation sites (Reynolds *et al.* 2013, Weiser *et al.* 2013). Further, maximizing diversity at genes associated with immune response (i.e. major histocompatibility complex; Lavretsky et al. 2014a) and retention of rare alleles may be important metrics for conserving the limited genetic diversity. Additional molecular tools with higher resolution, such as next-generation sequencing that simultaneously sequence 1000's of loci, will also be useful for future assessments quantifying genomic diversity in Laysan teal and for applying those data to conservation and management practices (Allendorf *et al.* 2010).

Management Implications

At least three management decisions arise for future decision makers regarding the conservation of allelic diversity for the management objective of improving Laysan teal persistence: 1) how many (immigrant) birds should be added to the existing, reintroduced population on Midway Atoll so that it represents most of the genetic diversity found in the original population on Laysan Island? Furthermore, when populations are established on other islands, 2) where should these founders be taken from, and 3) how many birds should be translocated to the new location and for how many years so as to represent and conserve their limited genetic diversity and reduce genetic drift? Species persistence is the management objective and conservation of genetic diversity or allelic retention is a means to help achieve this objective. Effort would need to be invested to keep allelic diversity in a genetically isolated reintroduced population that has likely lost some allelic diversity during a translocation-caused

Table 3. The historical and current range, range contraction and duration of isolation of island endemic waterfowl species and the Laysan finch (*Telespiza cantans*), and indices of genetic diversity present in modern populations.

Species	Historical range (total island area [ha])	Modern range	Extent and time of range contraction (island area [ha])	Genetic diversity indices in modern population: % of loci found to be polymorphic (no. microsatellites screened)	Genetic diversity indices in modern population: Nucleotide diversity observed	Source
Laysan duck (*Anas laysanensis*)	Hawaiian Is.: ≥ 5 Main and ≥ 2 Northwestern (>1.6 million ha)	Hawaiian Is.: 2 Northwestern (Laysan Is. and Midway Atoll [R])	Laysan Is. (415 ha) ca. 800–1500 ybp	13% (83)	None detected (among mtDNA control region, 19 nuclear introns, and Melanocortin receptor)	*this paper, Cooper *et al.* 1996, Olson and Ziegler 1995
Hawaiian duck (*A. wyvilliana*)	Hawaiian Is.: 6 Main (~1.5 million ha)	Hawaiian Is.: 2 Main (Hawai`i [R] and Kaua`i)	Kaua`i (157,400 ha) by 1962	≥ 67% (55)	Unknown	*Fowler *et al.* 2009
Brown teal (*A. chlorotis*)	New Zealand: Widespread on 3 main islands (North, South, and Stewart) and associated islets (~26.7 million ha)	New Zealand: 3 islets (2 reintroductions) and 4 sites on North Is.	One region of North Island and one islet, Great Barrier Is. (~1.1 million ha) Population decline began ca. 1900	100% (5)	Unknown	*Bowker-Wright *et al.* 2012, Worthy 2002

South Georgia pintail (*A.* g. georgica)	Widespread on main island and offshore islands (~375,500 ha)	Widespread on main island and offshore islands	No known range contraction, but population declines through the 1960s due to anthropogenic causes	Unknown	0.0005–0.0004 (among mtDNA control region and 5 nuclear introns [all 6 loci])	Martin 2002, *McCracken *et al.* 2013
Hawaiian goose (*Branta sandvicensis*)	Hawaiian Is: All or most of the 8 Main (>168 million ha)	Hawaiian Is.: 4 Main (Hawai`i, Maui (R), Moloka'i (R), and Kaua'i (R))	Restricted to less than 30 individuals on Hawai`i Is. and 11 birds in captivity by 1950 (~1.05 million ha)	≥ 50% (38)	Unknown	Banko *et al.* 1999, *Veillet *et al.* 2008
Laysan finch (*Telespiza cantans*)	Hawaiian Is.: ≥ 2 Main and ≥ 1 Northwestern (>1.6 million ha)	Hawaiian Is.: 2 Northwestern (Laysan Is. and Southeast Is., Pearl & Hermes Reef [T])	Laysan Is. (415 ha) ca. 800–1500 ybp	100-78% (9)	Unknown	Cooper *et al.* 1996, Tarr *et al.* 1998

R: Reintroduced; T: Translocated *: Modern Genetics Study

bottleneck. Because of small population sizes and the absence of natural dispersal and immigration, reintroduced populations are expected to continue to lose diversity over time. The two populations may retain different rare alleles (by chance), so establishment of additional populations with founders from both Laysan Island and Midway Atoll populations will improve the genetic diversity of additional reintroduced populations. Maximizing the genetic quality of these reintroduced populations is especially important if future translocations will be made to islands with small carrying capacities, or if population demographics and conditions are similar to those observed at Laysan Island (i.e., with lower fecundity and slower population growth).

A new translocation to Kure Atoll was sourced from Midway Atoll in September 2014. Twenty-eight founding birds were released in restored habitat on Green Island (100 ha). Additional founders from Laysan Island and periodic supplementation from the larger population at Midway Atoll are being discussed for the future, if the Kure population becomes established (survive to breed and increase in abundance). Models suggest that periodic translocation of immigrants from the source population may reduce drift and help achieve the ultimate goal of species persistence over the long term, most likely with future reintroduction to a larger higher elevation island after predator removal (USFWS 2009, Reynolds et al 2013).

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APPENDIX I

Appendix I. Characteristics of 19 nuclear DNA loci examined in the Laysan duck (*n* = 21). Bold text denotes the locus with nucleotide polymorphism.

Locus	Abbreviation	Location[1]	Length[2]
Chromo-helicase-DNA binding protein gene 1, intron b	CHD1Z	Z	329
Lactate dehydrogenase 1, intron 4	LDH1	1	559
Ornithine decarboxylase, intron 7	ODC1	3	370
Fibrinogen beta chain, intron 7[3]	**FGB**	**4**	**907**
Serum amyloid A, intron 2	SAA	5	341
Annexin A11, intron 2	ANXA11	6	440
Myostatin, intron 2	MSTN	7	303
Soat1-prov protein, intron 10	SOAT1	8	362
Nucleolin, intron 12	NCL	9	406
Preproghrelin, intron 3	GHRL	12	365
Glutamate receptor, ionotropic, N-methyl D aspartate I, intron 13	GRIN1	17	333
Sex determining region Y-box 9, intron 2	SOX9	18	406
Carboxypeptidase D, intron 9	CPD	19	330
Phosphenolpyruvate carboxykinase, intron 9	PCK1	20	357
Alpha enolase 1, intron 8	ENO1	21	306
Alpha-B crystallin, intron 1	CRYAB	24	355
Growth hormone 1, intron 3	GH1	27	417
Lecithin-cholesterol acyltransferase, intron 3	LCAT	Unk	391
S-acyl fatty acid synthase thioesterase, intron 2	FAST	2	337

[1]Location gives the chromosomal linkage in the chicken (*Gallus gallus*) genome.
[2]Length is number of base pairs sequenced.
[3]Denotes a separate analysis of 10 individuals from Laysan Island