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RESEARCH ARTICLE

Stepping stone speciation in Hawaii's flycatchers: molecular divergence supports new island endemics within the elepaio

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Abstract The elepaio (*Chasiempis sandwichensis*) is a monarch flycatcher endemic to the Hawaiian Islands of Kauai, Oahu, and Hawaii. Elepaio vary in morphology among and within islands, and five subspecies are currently recognized. We investigated phylogeography of elepaio using mitochondrial (ND2) and nuclear (LDH) markers and population structure within Hawaii using ND2 and microsatellites. Phylogenetic analyses revealed elepaio on each island formed reciprocally monophyletic groups, with Kauai ancestral to other elepaio. Sequence divergence in ND2 among islands (3.02–2.21%) was similar to that in other avian sibling species. Estimation of divergence times using relaxed molecular clock models indicated elepaio colonized Kauai 2.33 million years ago (95% CI 0.92–3.87 myr), Oahu 0.69 (0.29–1.19) myr ago, and Hawaii 0.49 (0.21–0.84) myr ago. LDH showed less variation than ND2 and was not phylogenetically informative. Analysis of molecular variance within Hawaii showed structure at ND2 (fixation index = 0.31), but microsatellites showed no population structure. Genetic, morphological, and behavioral evidence supports splitting elepaio into three species, one on each island, but does not support recognition of subspecies within Hawaii or other islands. Morphological variation in elepaio has evolved at small geographic scales within islands due to short dispersal distances and steep climatic gradients. Divergence has been limited by lack of dispersal barriers in the extensive forest that once covered each island, but anthropogenic habitat fragmentation and declines in elepaio population size are likely to decrease gene flow and accelerate differentiation, especially on Oahu.

Keywords *Chasiempis* · Elepaio · Hawaii · Phylogeography · Population structure · Relaxed clock

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Introduction

Islands have long been recognized as natural laboratories for understanding ecological and evolutionary principles (Lack 1976; Mayr and Diamond 2001; Grant and Grant 2007), and the Hawaiian Islands have produced some of the most spectacular and best-known examples of adaptive radiation and diversification. These examples include terrestrial vertebrates such as Hawaiian honeycreepers (Freed et al. 1987; Fleischer and McIntosh 2001), picture-winged flies in the genus *Drosophila* (Carson 1987; Kaneshiro and Boake 1987), tree snails and other terrestrial invertebrates (Roderick and Gillespie 1998; Holland and Hadfield 2004; Cowie and Holland 2008), and several families of flowering plants (Baldwin 1997; Givnish et al. 2008). This capacity for fostering evolution has been facilitated by the remote geographic location of the Hawaiian Islands and a diversity of environmental conditions caused by steep climatic gradients (Simon 1987; Wagner and Funk 1995). The ordered geologic history of the islands often has produced a stepping-stone or conveyor belt pattern of evolution in which new species arise as organisms colonize islands as they are formed (Fleischer et al. 1998; Percy et al. 2008).

The elepaio (*Chasiempis sandwichensis*) is a Passerine bird in the monarch flycatcher family (Monarchidae) and is

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endemic to the Hawaiian Islands. Elepaio occur on Kauai, Oahu, and Hawaii, but are absent from the four islands of the Maui Nui group (Maui, Molokai, Lanai, and Kahoolawe) in the center of the Hawaiian Archipelago (Fig. 1; Pratt et al. 1987; VanderWerf 2007), even in the fossil record (Olson and James 1982; Burney et al. 2001). This disjunct distribution is peculiar given the ordered geologic history of the Hawaiian Islands and raises questions about the sequence and timing of colonization events that lead to the current distribution. Behavioral and biogeographic evidence suggests Elepaio did not go extinct on Maui Nui, but rather that they inadvertently bypassed one of the stepping stones in the Hawaiian chain (VanderWerf 2007). Molecular data indicate elepaio are most closely related to groups of monarchs from the western Pacific and eastern Polynesia in the genera *Monarcha* and *Pomarea*, respectively (Filardi and Moyle 2005), but phylogeographic relationships within elepaio have not been examined using molecular techniques.

Elepaio exhibit substantial morphological variation among and within islands (Pratt 1980; VanderWerf 1998). Body mass ranges from 13.0 ± 0.4 g on Oahu to 16.9 ± 0.5 g on Hawaii (VanderWerf 1998), and the predominant plumage color is gray on Kauai, brown on Oahu, and brown, grayish, or white on Hawaii (Pratt et al. 1987; VanderWerf 1998). Elepaio are sexually monomorphic on Kauai, but on Oahu and Hawaii elepaio are sexually dichromatic in throat color (VanderWerf 1998). Elepaio on all islands are sexually mature and sometimes breed at 1 year of age but exhibit a 2-year delay in plumage maturation in both sexes (VanderWerf 2001, 2004; VanderWerf and Freed 2003).

These complex patterns of morphological variation caused considerable confusion about the systematics of elepaio, and their taxonomy has changed repeatedly. Each island form was originally described as a separate species: *C. sandwichensis* Gmelin 1789 on Hawaii; *C. sclateri* Ridgway 1882 on Kauai; and *C. ibidis* Stejneger 1887 (formerly *C. gayi* Wilson 1891) on Oahu. Various subsequent authors recognized one to five or six species of elepaio, with the subadult plumages sometimes regarded as different species or sexes (Sclater 1885; Newton 1892; Pratt 1980; Olson 1989). These taxa were later reclassified as subspecies by Bryan and Greenway (1944) without explanation, but some authors continue to treat them as species (Olson and James 1982; Conant et al. 1998). The American Ornithologists' Union currently recognizes a single species of elepaio with three subspecies (AOU 1998), but is considering a taxonomic revision that would split the island forms into three species (AOU 2000).

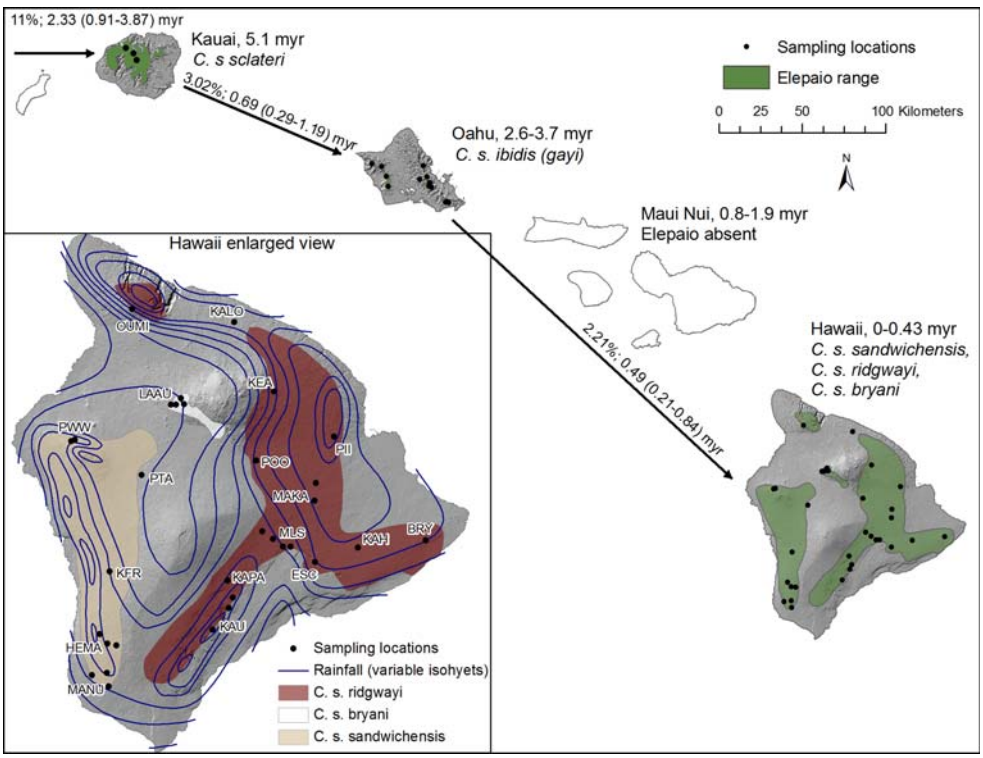

Fig. 1 Map of Hawaiian Islands showing current range of elepaio, sampling locations in this study, and age of islands in millions of years (myr). *Arrows* between islands show most likely colonization events. *Numbers* above *arrows* are divergence in ND2 sequence and divergence times in myr (95% CI) estimated with relaxed clock models. Codes for sampling locations on Hawaii correspond with Appendix

Variation in morphology of elepaio within islands may represent local adaptation to varying environmental conditions. Intra-island plumage color variation is most pronounced on Hawaii, where three subspecies are recognized by some authors (Henshaw 1902; Pratt 1979, 1980). Elepaio in humid forests on the windward, eastern side of the island (*C. s. ridgwayi*) tend to be darker and more red in color, those in dry forests on the leeward, western side (*C. s. sandwichensis*) tend to be paler and more gray, and those in high elevation forest on Mauna Kea (*C. s. bryani*) are paler still and have more white on the head. This correlation of coloration and humidity is often known as Gloger's Rule, and has been described in a variety of birds and mammals in continental areas (Zink and Remsen 1986; Burtt and Ichida 2004).

Elepaio are fairly common and widespread on Kauai and Hawaii (Scott et al. 1986; Foster et al. 2004), but the Oahu elepaio has declined seriously and the population of less than 2,000 birds is highly fragmented (VanderWerf et al. 2001). The Oahu elepaio was listed as endangered under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service 2000), and status of the elepaio as a whole was recently elevated to endangered by the International Union for the Conservation of Nature (IUCN 2007). The primary threats to elepaio are nest predation by alien mammals, diseases carried by alien mosquitoes, and loss and degradation of habitat caused by feral ungulates, invasive alien plants, human development, and wildfires (VanderWerf and Smith 2002; U.S. Fish and Wildlife Service 2006; VanderWerf et al. 2006; VanderWerf 2008, 2009). Information about phylogeography and population structure of elepaio is needed to inform management decisions and help develop the best possible conservation strategy (U.S. Fish and Wildlife Service 2006).

We used mitochondrial and nuclear DNA sequences and microsatellites to examine phylogeography and genetic population structure of elepaio. Specific goals were to (1) determine the relationship and colonization history of elepaio among islands, (2) measure divergence among taxa and estimate divergence times, (3) relate patterns of genetic variation to patterns of morphological variation, (4) assess the classification and validity of current taxa, and (5) make recommendations for conservation and management.

Materials and methods

Sample collection

Elepaio were captured in mist nets on Hawaii, Oahu, and Kauai from 1995 to 2008 and a blood sample was collected from the brachial vein of each bird. Whole blood was stored in Queen's Lysis buffer (Seutin et al. 1991). Samples used in this study were collected from three sites on Kauai, 13 sites on Oahu, and 18 sites on Hawaii (Fig. 1; Appendix). Samples were collected from multiple sub-sites at different elevations in some sites that encompassed large geographic areas, including Hawaii Volcanoes National Park, Manuka Natural Area Reserve, Kau Forest Reserve, Mauna Kea Forest Reserve, and The Nature Conservancy's Kona Hema Preserve. The 18 sampling locations on Hawaii encompassed all portions of the current range of elepaio on the island and the complete breadth of climatic variation within that range, with multiple sites representing each subspecies. The range of the endangered Oahu elepaio is small and highly fragmented, and sampling locations represented almost all areas on the island in which elepaio still occur. Blood samples from the Tinian monarch (*Monarcha takatsukasae*), used as one of three outgroups (see below), were collected from birds caught in mist nets on the island of Tinian in the northern Marianas in 2006 using similar methods.

Laboratory procedures

Genomic DNA (gDNA) was extracted from blood samples using ID Labs IDetect^{TM} DNA purification kit for whole animal blood following manufacturers protocols. Regions of mtDNA and nuclear DNA were amplified using polymerase chain reaction (PCR) with positive and negative controls. We chose the mtDNA gene NADH dehydrogenase subunit 2 (ND2) because it was useful in elucidating phylogeny of other Pacific island monarchs (Filardi and Moyle 2005; Filardi and Smith 2005). We chose the nuclear gene lactate dehydrogenase (LDH) because it was useful in distinguishing population-level variation in Hawaii amakihi (*Hemignathus virens*; Foster et al. 2007).

We amplified the ND2 gene using primers L5215 forward (Hackett 1996) and H1064 reverse (Drovetski et al. 2004). Reactions were carried out in 25 µl volumes containing 40–100 ng gDNA, 1× NH_4 PCR buffer (Bioline, Taunton, MA, USA), 0.8 mM BSA, 2 mM $MgCl_2$, 0.02 mM dNTP, 0.12 mM forward and reverse primers, and 1.5 U *Taq* DNA polymerase (Bioline). The cycling parameters were 10 min denaturation at 94°C, followed by 35 cycles each of 94°C for 30 s, 50°C for 40 s, and 72°C for 1 min, and a final extension at 72°C for 4 min.

We amplified the LDH gene using primers Z15016 forward and reverse (Friesen et al. 1999). Reactions were carried out in 25 µl volumes containing 40–100 ng gDNA, 1× NH_4 PCR buffer (Bioline), 0.8 mM BSA, 2 mM $MgCl_2$, 0.02 mM dNTP, 0.12 mM forward and reverse primers, and 1.5 U *Taq* DNA polymerase (Bioline). The cycling parameters were 10 min denaturation at 94°C, followed by 30 cycles each of 94°C for 30 s, 48°C for 40 s, and 72°C for 1 min, and a final extension at 72°C for 4 min.

All ND2 and LDH amplifications were verified via agarose gel eletrophoresis, and single products were purified prior to sequencing using the ExoSAP-IT® kit (USB Corporation, Cleveland, OH, USA) following the manufacturer's protocol. Cycle sequencing reactions were performed using an ABI BigDye terminator kit (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA, USA) and sequences were electrophoresed and analyzed on an ABI 3730XL (Perkin-Elmer Applied Biosystems, Inc.).

Twelve microsatellite loci were screened from a library developed for Oahu elepaio by Burgess and Fleischer (2006). Six of these loci amplified in samples from Hawaii and five were polymorphic, and those were five used for genotyping. Amplifications were carried out in 11 µl volumes containing 40–100 ng gDNA, 1× NH_4 PCR buffer (Bioline), 1 mM $MgCl_2$, 0.008 mM dNTP, 0.08 mM each of forward and reverse primers, and 0.75 U *Taq* DNA polymerase (Bioline). Loci were multiplexed on an ABI 3730 XL automated sequencer with the forward primer of each primer pair fluorescently labeled. The cycling parameters were 10 min denaturation at 94°C, followed by 40 cycles each of 94°C for 30 s, locus specific TA for 40 s (Table 1), and 72°C for 1 min, and a final extension at 72°C for 10 min. Ten percent of samples were amplified and genotyped twice, and all genotypes were scored independently at least twice with GENEMAPPER (Applied Biosystems).

Analyses

Sequences were trimmed, edited, and aligned using Sequencher 4.8 (Gene Codes Corp. Ann Arbor, MI, USA), and unique haplotypes were selected using DAMBE (Xia and Xie 2001). Sequences were unambiguously aligned without having to add gaps. We used the auto-trimming function of Sequencher to trim ends with many ambiguous bases, resulting in a 699 bp segment of ND2 and a 504 bp segment of LDH that allowed inclusion of maximum numbers of individuals.

Several individuals were found to be heterozygous at one site in LDH using forward and reverse sequences. In those individuals, alleles were separated into two haplotypes for inclusion in analyses. Two individuals were found to be heterozygous at two sites in LDH. The maximum-likelihood haplotypes of these individuals were determined with Arlequin (Excoffier et al. 2005) using an iterative expectation-maximization algorithm based on the observed haplotype frequencies. Splitting of heterozygotes resulted in a total of 151 sequences. The potential problem of nuclear copies of mitochondrial genes (NUMTs; Sorenson and Quinn 1998) was addressed by comparing forward and reverse ND2 sequences for the presence of double peaks and by comparing divergence rates in mitochondrial and nuclear genes. NUMTs usually evolve more slowly than their mitochondrial counterparts, so lower than expected rates of divergence in mitochondrial sequences may indicate the presence of NUMTs. Divergence was substantially higher in ND2 than in LDH (see Results) and there was no evidence of nuclear duplication.

The best-fit nucleotide substitution model for each marker was determined by hierarchical likelihood ratio tests with Modeltest 3.06 (Posada and Crandall 1998). Phylogenetic relationships were reconstructed using maximum likelihood and parsimony methods performed with PAUP* 4.0 (Swofford 2002) and using Bayesian analysis performed with Mr. Bayes 2.01 (Huelsenbeck and Ronquist 2001). For parsimony, all characters were unordered and equally weighted, and a heuristic search was performed with 100 random sequence addition replicates and 500 bootstrap replicates. For maximum likelihood, a heuristic search was performed with tree-bisection-reconnection branch-swapping and 100 bootstrap replicates. For Bayesian analyses, four Markov chains were run for 11 million generations with a burn-in of 1 million generations, and

Table 1 Summary of diversity in ND2 (699 bp) and LDH (504 bp) sequences in elepaio taxa

Island	Kauai		Oahu		Hawaii		Hawaii		Hawaii	
Subspecies	*C. s. sclateri*		*C. s. ibidis*		*C. s. sandwichensis*		*C. s. bryani*		*C. s. ridgwayi*	
Marker	ND2	LDH	ND2	LDH	ND2	LDH	ND2	LDH	ND2	LDH
No. of Sequences	28	26	47	35	42	39	14	13	35	38
No. of Haplotypes	11	7	6	3	6	10	2	4	15	7
% Variable sites	1.6	1.0	1.3	0.4	1.0	1.6	0.1	0.6	2.8	1.4
No. of Transitions	9	4	8	1	6	8	1	3	19	7
No. of Transversions	2	1	1	1	1	0	0	0	1	0
Haplotype diversity (h)	0.802	0.683	0.759	0.213	0.415	0.692	0.264	0.654	0.897	0.643
Nucleotide diversity (π)	0.0022	0.0018	0.0033	0.0004	0.0014	0.0019	0.0004	0.0016	0.0040	0.0017

trees were sampled every 1,000 generations. All priors were set according to the chosen substitution model. Phylogenies were visualized using MEGA 4.1 (Tamura et al. 2007) and FigTree 1.2.1 (Rambaut 2009).

We used three outgroups to root the trees: the Tinian monarch from the Marianas in the western Pacific, the Iphis monarch (*Pomarea iphis*) from the Marquesas in eastern Polynesia, both of which shared a recent common ancestor with elepaio (Filardi and Moyle 2005), and the shining flycatcher (*Myiagra alecto*), a more distantly related species of continental origin that is basal to the clade including *Pomarea*, *Monarcha*, and *Chasiempis* (Filardi and Smith 2005). ND2 sequences for *Pomarea* and *Myiagra* were obtained from GenBank (accession numbers DQ084097 and DQ468934, respectively). For LDH, no *Pomarea* or *Myiagra* sequences were available from GenBank, so the Tinian monarch was the only outgroup used.

We tested whether application of a strict molecular clock was appropriate with Tajima's relative rate tests (Tajima 1993) using MEGA 4.1 (Tamura et al. 2007). These tests indicated there was heterogeneity in ND2 mutation rates and that application of a strict molecular clock was not appropriate. Instead, we estimated divergence times using relaxed clock models in BEAST 1.4.8 (Drummond and Rambaut 2007). We used a relaxed clock uncorrelated lognormal model and a speciation birth–death process tree prior. Priors for model parameters and statistics were set using two approaches. First, we used the age of Kauai, 5.1 million years (myr), as a calibration point by setting the prior for time to most recent common ancestor of the Kauai–Tinian monarch node to normal with a mean of 5.1 myr and standard deviation (SD) of 0.1 myr. Second, following Ho (2007), we allowed the substitution rate to vary among branches with a normal distribution by setting the ucld.mean prior to normal with a mean of 0.023 and SD of 0.0037. The mean was based on average avian ND2 substitution rates reported in the literature (1.79% from Pereira and Baker 2006, 2.76% from Drovetsky et al. 2004) and the SD was based on the value required to produce 5 and 95% quantiles encompassing rates used to calculate the mean. In both cases we used an MCMC chain length of 10,000,000 with a burn-in of 1,000,000 and parameters logged every 1,000 trees. We ran each analysis three times to obtain sufficient effective sample sizes (>200) and ensure that models converged on the same distribution, and combined results from the three runs using the LogCombiner tool. Trees were visualized using FigTree v1.2.1 (Rambaut 2009).

We assessed genetic variation among and within elepaio populations by four methods. First, haplotype diversity measures for ND2 and LDH were calculated with DnaSP 4.50.3 (Rozas et al. 2003), with sequences divided into five groups based on current subspecific designations. Haplotype networks were created using statistical parsimony methods in TCS 1.21 (Clement et al. 2000). Second, analyses of molecular variance (AMOVA) were conducted for ND2 and LDH using Arlequin (Excoffier et al. 2005). For ND2, a one-factor AMOVA was conducted with Hawaii subspecies only because no haplotypes were shared among islands. For LDH, a two-factor AMOVA was conducted among islands and among Hawaii subspecies. Third, genetic differentiation among subspecies was measured with Φ_{ST} for mtDNA and F_{ST} for nuclear data using Arlequin, with 1,000 MCMC simulations. Fourth, microsatellite allele frequencies were used to examine geographic population structure within Hawaii. GENEPOP 3.1b (Raymond and Rousset 1997) was used to calculate microsatellite allelic diversity and observed and expected heterozygosity, and to test loci for departures from Hardy–Weinberg equilibrium and for linkage disequilibrium. Microsatellite diversity was used to examine fine-scale geographic structure within Hawaii and determine the number of effective populations using a Bayesian maximum likelihood analysis performed in STRUCTURE 2.2 (Falush et al. 2007). This analysis assumed no prior knowledge of population structure among the 18 sampling locations, and used a series of simulations with 1–18 inferred populations corresponding to the sampling locations to determine which number of populations was most likely given the observed allele frequencies. Each simulation used an admixture model with correlated allele frequencies and 100,000 iterations after a burn-in of 50,000 iterations. The analysis was run independently three times to test for convergence of the MCMC.

Results

Sequence variability

Of the 699 bp of ND2 sequence, 97 sites (13.9%) were variable and 51 (7.3%) were parsimony informative. Thirty-eight unique ND2 haplotypes were recovered in 166 individuals, 11 on Kauai, six on Oahu, and 21 on Hawaii, none of which were shared among islands (Table 1). Of the 504 bp of LDH sequence, 15 sites (3.0%) were variable and 10 (2.0%) were parsimony informative. Twenty-one unique LDH haplotypes were recovered in 151 sequences, seven on Kauai, three on Oahu, and 13 on Hawaii. The two most common LDH haplotypes were shared among 116 sequences on all three islands, but the remaining 19 were private haplotypes found on a single island (Table 1). Most measures of sequence diversity were similar among geographic areas, but Oahu exhibited lower haplotype diversity (0.21) and lower nucleotide diversity (0.0004) at LDH, and areas on Hawaii represented by the *C. s. bryani*

subspecies exhibited lower haplotype diversity (0.26) and lower nucleotide diversity (0.0004) at ND2 (Table 1). All haplotype sequences can be found in GenBank under accession numbers (GQ373322–GQ373380).

Phylogenetic analyses and divergence

The best nucleotide substitution models were the Tamura-Nei model with invariable sites (TrN + I) for ND2 and the Kimura three-parameter model with unequal base frequencies and invariable sites (K81uf + I) for LDH. The average corrected pairwise divergence in ND2 sequence was 3.02 ± 0.03% between Kauai and Oahu, 2.8 ± 0.02% between Kauai and Hawaii, and 2.21 ± 0.03% between Oahu and Hawaii. Corrected ND2 sequence divergence was lower among the three subspecies on Hawaii, ≤0.55% in all cases, and also lower within islands, 0.37 ± 0.02% on Kauai, 0.52 ± 0.07% on Oahu, and 0.57 ± 0.02% on Hawaii. The average corrected divergence in LDH was low in all comparisons, with similar values among islands (range 0.34–0.50%) and within islands (range 0.51–0.54%).

Maximum likelihood, parsimony, and Bayesian ND2 trees had the same topology at all major nodes and showed each island formed a reciprocally monophyletic group, with Kauai ancestral to Oahu and Hawaii (Fig. 2). In contrast, none of the three subspecies within Hawaii formed separate clades. Each subspecies on Hawaii was paraphyletic, with haplotypes from different subspecies mixed throughout the island clade (Fig. 2). Similarly, there was no evidence of distinct geographic clades within Kauai or Oahu, with haplotypes from different locations on each island mixed throughout the island clade. Among the outgroups, *Myiagra* was basal to elepaio and both other outgroups, as expected, and *Pomarea* and *Monarcha* together formed a sister clade to elepaio. For LDH, the maximum

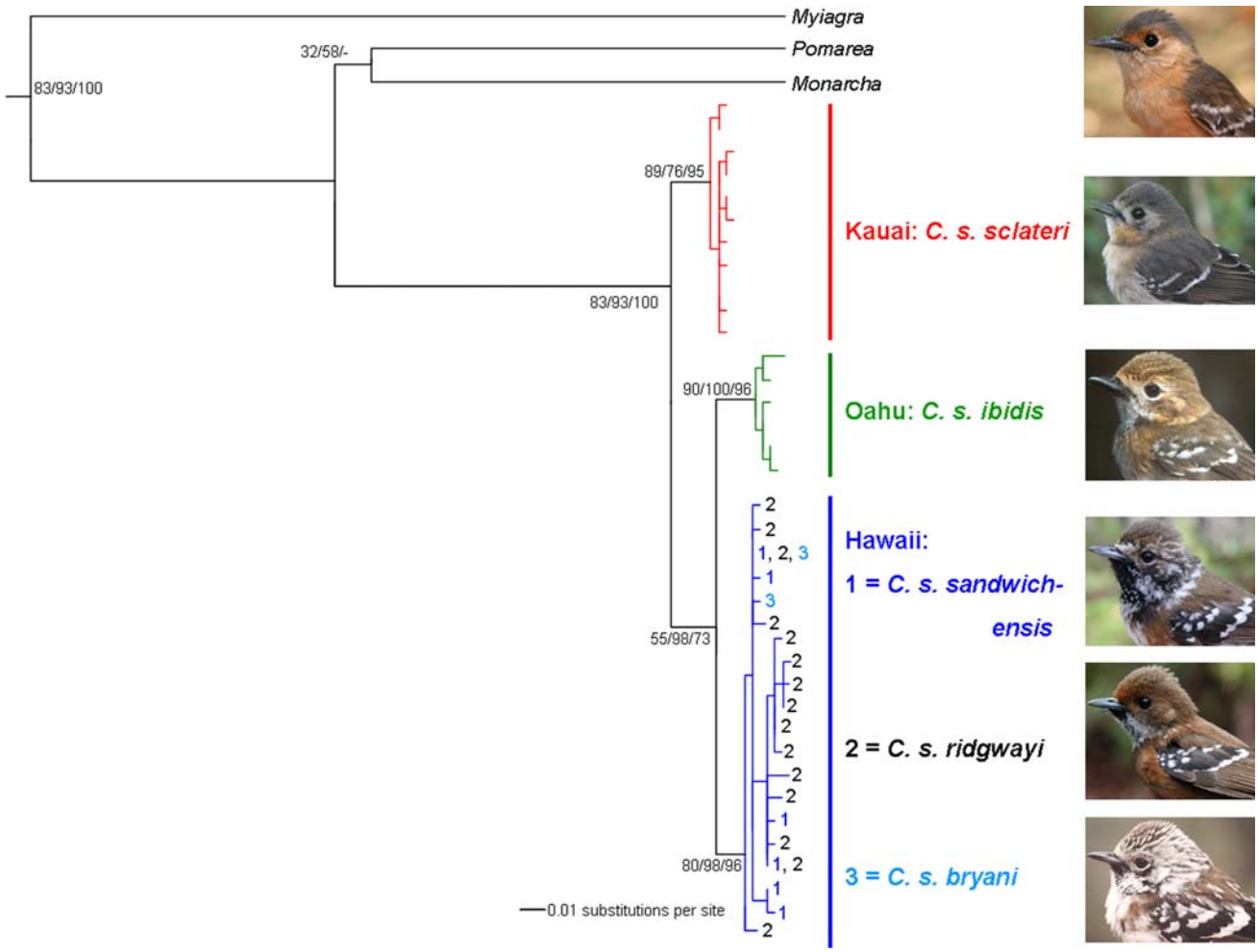

Fig. 2 Elepaio phylogenetic tree based on 699 base pairs of the mtDNA ND2 gene. Maximum likelihood, parsimony, and Bayesian analyses yielded similar topologies. Values at major nodes are percent support from maximum likelihood/Bayesian/parsimony analyses

likelihood, parsimony, and Bayesian trees each resulted in an unresolved polytomy that was uninformative for all ingroup taxa.

Divergence time estimates differed between the two approaches. First, using the age of Kauai, 5.1 myr, to calibrate age of the Kauai elepaio–Tinian monarch node produced a mean substitution rate of 0.85% per myr (95% CI 0.07–1.62) and ages of 2.47 (0.35–5.76) myr for the Kauai–Oahu node and 2.19 (0.30–5.75) myr for the Oahu–Hawaii node. Second, using a relaxed clock model that allowed substitution rate to vary among branches with a normal distribution and a mean of 2.3 ± 0.37% per myr produced younger ages for all nodes, including 2.33 (95% CI 0.92–3.87) myr for Kauai–Tinian monarch, 0.69 (0.29–1.19) myr for Kauai–Oahu, and 0.49 (0.21–0.84) myr for Oahu–Hawaii. Divergence time of elepaio from *Myiagra,* the outgroup of continental origin, was 3.81 (1.04–6.53) myr. Divergence time from the Iphis monarch, 2.42 (0.96–3.97) myr, was slightly older than from the Tinian monarch.

Population variability within Hawaii

Analysis of molecular variance in ND2 showed significant variation on Hawaii, but the majority of variation was partitioned within subspecies, not among them (Table 2a). In pairwise comparisons of Φ_{ST} on Hawaii, the windward subspecies *C. s. ridgwayi* differed from both of the leeward subspecies *C. s. sandwichensis* and *C. s. bryani*, but *C. s. sandwichensis* and *C. s. bryani* did not differ from each other (Table 3). The most common ND2 haplotype on Hawaii was shared by 50 birds representing all three subspecies (Fig. 3a). Nineteen of the 21 ND2 haplotypes were restricted to one of the three subspecies, but distance between these private haplotypes was not related to subspecies. Most individuals from the Kau and Saddle Road areas, which were described as zones of subspecies overlap by Pratt (1980), shared haplotypes with individuals from the eastern side of the island occupied by *C. s. ridgwayi*.

The AMOVA in LDH also showed significant variation that was partitioned largely within subspecies, though the fixation indexes were lower than in ND2 (Table 2b). Most pairwise comparisons of F_{ST} among the five subspecies were significant, though all F_{ST} values were relatively low (Table 3). LDH haplotypes showed no geographic pattern, with the two most common haplotypes shared not only among sites represented by all three subspecies within Hawaii, but also among all three islands (Fig. 3b).

None of the five microsatellite loci exhibited deviations from Hardy–Weinberg equilibrium or evidence of linkage disequilibrium (Table 4). Microsatellites did not show any evidence of population structure within Hawaii. The

Table 2 Analysis of molecular variance (AMOVA) in elepaio markers

Source of variation	*df*	Sum of squares deviations	Variance components	% of variation	Fixation index
ND2					
Among Hawaii subspecies	2	21.15	0.35	30.85	0.31**
Within Hawaii subspecies	89	68.92	0.79	69.15	
LDH					
Among islands	2	2.22	0.005	1.18	0.045*
Among Hawaii subspecies	2	1.70	0.018	4.51	0.057*
Within islands/subspecies	164	53.61	0.367	94.31	0.011

For ND2, a one-factor AMOVA was conducted with Hawaii subspecies only because no haplotypes were shared among islands. For LDH, a two-factor AMOVA was conducted among islands and among Hawaii subspecies. Asterisks (*) indicates values significant at * $P < 0.05$ and ** $P < 0.001$

Table 3 Pairwise population F_{ST} values for LDH (above diagonal) and Φ_{ST} for ND2 (below diagonal) of elepaio subspecies

	C. s. sclateri	*C. s. ibidis*	*C. s. ridgwayi*	*C. s. bryani*	*C. s. sandwichensis*
C. s. sclateri	–	0.077**	0.035*	0.055*	0.065**
C. s. ibidis	NA	–	0.044**	0.250**	0.060**
C. s. ridgwayi	NA	NA	–	0.044	0.003
C. s. bryani	NA	NA	0.320**	–	0.098**
C. s. sandwichensis	NA	NA	0.330**	0.028	–

* $P < 0.05$; ** $P < 0.01$. No ND2 haplotypes were shared among islands, so Φ_{ST} values were not applicable (NA)

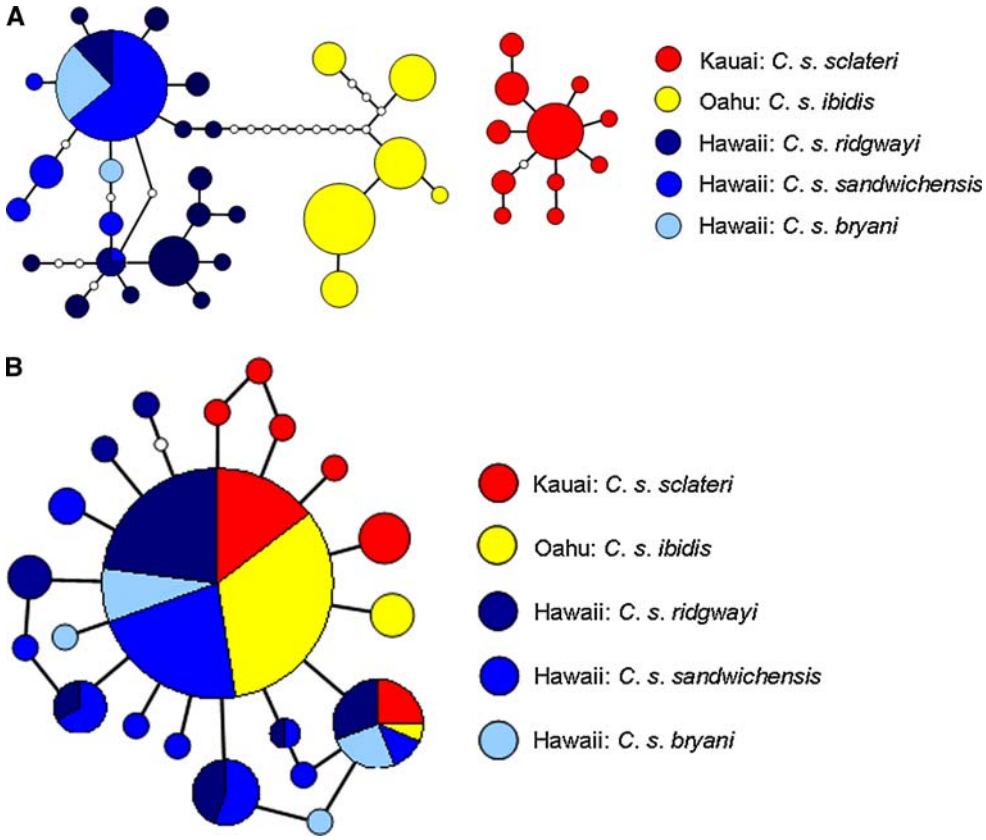

Fig. 3 Elepaio haplotype networks for **a** ND2 and **b** LDH. Unjoined networks differ by >11 substitutions. Area of each circle is proportional to the number of individuals represented by that haplotype. Each branch represents a single substitution and white circles indicate hypothetical haplotypes

Table 4 Characteristics of five dinucleotide microsatellite loci in Hawaii elepaio

Locus ID	No. of alleles	Range	Het(obs)	Het(exp)	T_A (°C)
O26	12	352–374	0.861	0.822	60
O43	24	154–192	0.800	0.929	62
K49	5	297–303	0.382	0.411	63
K91	17	178–204	0.674	0.755	63
K59	11	263–285	0.889	0.827	61

Range is the size in base pairs of alleles, Het(obs) and Het(exp) are the observed and expected heterozygosity, respectively, and T_A is the annealing temperature for each locus

average observed microsatellite allelic diversity and heterozygosity were similar among all three subspecies on Hawaii, and there were no significant pairwise differences in F_{ST} values among subspecies. Bayesian analyses of microsatellite allele frequencies at the 18 sampling locations found no population structure, with posterior probabilities of 0.054–0.067 for all numbers of inferred populations from 1 to 18, though sample sizes from some sites were small (Appendix). Probabilities from the three runs differed by ≤0.01 for all values, indicating the runs were sufficiently long.

Discussion

Phylogeography of elepaio

The elepaio lineage first colonized Kauai, and Kauai was ancestral to elepaio on Oahu and Hawaii. Identity of the immediate ancestor of the elepaio lineage is not entirely clear. Filardi and Moyle (2005) found that elepaio shared a recent common ancestry with a group of closely related *Monarcha* from the western Pacific, including the Tinian monarch, and with several genera endemic to various central Pacific island groups, including the Iphis monarch. The position of elepaio with respect to *Monarcha* and *Pomarea* depended on which marker was used (Filardi and Moyle 2005 supplementary material). In this study, *Monarcha* and *Pomarea* together formed a sister clade to elepaio.

The second and third colonization events in the elepaio lineage were most likely from Kauai to Oahu and from Oahu to Hawaii, respectively. Although Oahu and Hawaii are sister groups and the sequence of colonization cannot be inferred with certainty, it is more likely that Oahu was colonized before Hawaii because the branch length and divergence time were shorter between Kauai and Oahu than Kauai and Hawaii. VanderWerf (2007) used biogeographical and behavioral (song) evidence to suggest

Hawaii was colonized directly from Kauai, but molecular data indicate Hawaii elepaio are closer to Oahu elepaio. Either way, speciation of elepaio was facilitated by colonization of island stepping stones, as observed in many Hawaiian organisms (Wagner and Funk 1995; Fleischer et al. 1998), although elepaio inadvertently bypassed the Maui Nui stepping stone. The palila (*Loxioides bailleui*), a Hawaiian honeycreeper, has a similar distribution, with an extant population on Hawaii and fossils known only from Kauai and Oahu (Olson and James 1982; Burney et al. 2001), indicating other Passerine birds have bypassed Maui Nui.

Estimates of avian mtDNA substitution rate generally cluster around 2% per million years, and this rate has been widely used as a standard method for dating divergence in birds (Lovette 2004). Substitution rates can vary, however, and application of a strict molecular clock is not always appropriate (Arbogast and Slowinski 1998; Garcia-Moreno 2004; Peterson 2006; Ho 2007). Substitution rates can be calibrated using biogeographic information, such as age of an island, but this approach assumes colonization occurred shortly after island formation and development of suitable habitat. This assumption appeared to be true in Hawaiian honeycreepers (Fleischer et al. 1998), and the substitution rate of 1.6% per myr calculated in that group is one of few estimates of mtDNA divergence in Passerine birds.

In elepaio, application of a strict molecular clock was not appropriate, and using the age of Kauai to calibrate mutation rate also was not appropriate because that approach yielded a divergence time for Hawaii elepaio (2.19 myr) substantially older than the age of the island (0.43 myr). That approach also produced a mean substitution rate (0.85% per myr) lower than any previous estimate of mtDNA mutation in birds (Lovette 2004), but given the inaccuracy of the divergence time, that substitution rate seems erroneous. In contrast, allowing mutation rate to vary with a normal distribution based on published estimates ($2.3 \pm 0.37\%$) yielded a mean divergence time for Hawaii elepaio (0.49 myr) much closer to the island age. This approach also indicated Kauai and Oahu were colonized long after these islands became subaerial, about 2.8 and 3.0 myr, respectively, and that the time between colonization of Oahu and Hawaii was relatively short, perhaps only 200,000 years. Filardi and Moyle (2005) estimated age of the elepaio lineage at >1.5 myr using a strict molecular clock with a substitution rate of 2.76% per myr. Results of this study agreed with that age in general, but suggested the elepaio lineage is somewhat older, possibly due to time dependent variation in substitution rate (Ho et al. 2005). Most avian lineages in the Hawaiian Islands colonized the archipelago after formation of Kauai, and many colonizations occurred within the last 1 myr (Fleischer and McIntosh 2001), likely because that period constituted a peak in the number and size of high islands and thus a peak in habitat suitable to terrestrial birds (Price and Clague 2002).

Lack of variation in LDH among islands and low F_{ST} values compared to ND2 are consistent with incomplete lineage sorting. Sister taxa can be expected to exhibit reciprocal monophyly only after about 4N generations (Avise 2004), and times to reciprocal monophyly are fourfold longer in nuclear genes due to the larger effective population sizes of nuclear loci (Nei 1987). The incomplete lineage sorting observed in LDH is not surprising given the relatively recent divergence of elepaio, and that elepaio populations on each island probably have been large over much of their history and elepaio often do not begin breeding until 3 years of age (VanderWerf 2004). Mitochondrial loci have been the most useful molecular markers for examining phylogenetic relationships, in part because of their shorter coalescence times, but it is often desirable to corroborate mtDNA results using nuclear loci (Zink and Barrowclough 2008). LDH was useful in distinguishing population level variation in Hawaii amakihi (Foster et al. 2007), but was not useful for this purpose in elepaio because divergence among elepaio occurred too recently.

Speciation, dispersal, and relation of genetic and morphological variation

Expanses of ocean between islands have been barriers to dispersal by elepaio, leading to divergence and eventually speciation due to drift and differences in selection regimes among islands (Grant 2001). Elepaio are sedentary and rarely cross large areas of unforested habitat. Elepaio dispersal is driven by intraspecific competition and territory availability, and they usually disperse only far enough to find a vacant territory (VanderWerf 2008). Natal dispersal distances observed in elepaio have been less than 1 km and instances of breeding dispersal have been less than 400 m (VanderWerf 2008). Elepaio probably did not deliberately fly between islands but rather were blown from one island to the next during rare storm events (VanderWerf 2007).

Although individual elepaio do not disperse far, few dispersal barriers exist within each of the Hawaiian Islands. Extensive forest habitat allowed gene flow throughout each island and limited divergence. Areas of bare lava from recent volcanic activity would have posed barriers initially, and still do in the geologically youngest areas of Hawaii, which may have imposed some population structure (Vandergast et al. 2004). These barriers slowly subsided with soil development and vegetative succession, and most of each island reached a forest community capable of supporting elepaio or a shrubland community that

facilitated dispersal. Unforested alpine zones on Mauna Kea and Mauna Loa also are barriers to elepaio, but continuous forest encircling these volcanoes at lower elevations provided a pathway, albeit a more circuitous one, around the island. Elepaio on the eastern (*C. s. ridgwayi*) and western (*C. s. sandwichensis* and *C. s. bryani*) sides of Hawaii showed some differentiation, perhaps reflecting lower gene flow around alpine areas and lava fields.

Lack of structure in microsatellites may seem at odds with differentiation in ND2, but this apparent discrepancy between nuclear and mtDNA loci may be caused by sex-biased dispersal. Juvenile male elepaio tend to disperse farther than juvenile females because survival of adult males is higher, requiring young males to move farther in search of a vacant territory (VanderWerf 2008).

Geographic variation, and ultimately speciation, depends on a complex interaction of gene flow, local adaptation, and environmental variation (Endler 1977; Case and Taper 2000; Cicero 2004). Haldane (1956) reasoned that local adaptation caused by environmental variation may be inhibited by gene flow, and that the equilibrium between these factors may determine a species' distribution. Similarly, the steepness of environmental gradients responsible for selection on certain characters can influence the geographic scale over which an optimal phenotype persists (Case and Taper 2000). Although these ideas were originally used as mechanisms to explain the limits of a species' range, they can also be applied to intraspecific variation in locally adapted characters within a species' range.

In elepaio, variation in plumage color and body size has arisen recently and at small geographic scales due to short dispersal distances and steep gradients in rainfall and temperature. The degree of differentiation in elepaio within each island has remained small because there are few barriers to dispersal at a landscape scale and because elepaio adapt readily to areas with a wide range of climates and forest types (VanderWerf 1998; Johnson and Cicero 2002). Plumage characters can exhibit remarkable plasticity, and even large differences in plumage color and degree of sexual dichromatism can arise in a short time and over short geographic distances (Omland and Lanyon 2000; Badyaev and Hill 2003; Cibois et al. 2004; Filardi and Smith 2008).

Taxonomic revision of *Chasiempis*

Elepaio currently are regarded as a single species, with subspecies on Kauai (*C. s. sclateri*), Oahu (*C. s. ibidis*), and Hawaii (*C. s. sandwichensis*), and two additional subspecies on Hawaii recognized by some authorities (*C. s. ridgwayi* and *C. s. bryani*; Pratt 1979, 1980). However, a combination of morphological, behavioral, and molecular evidence indicates elepaio populations on each island represent distinct species in both biological and phylogenetic terms. Variation in elepaio morphology among islands has been used as evidence that each island form should be regarded as a species (Olson and James 1982, Conant et al. 1998). This argument was strengthened by results of song playback experiments that showed elepaio do not respond as strongly to songs from other islands, indicating song could serve as a behavioral isolation mechanism (VanderWerf 2007). Elepaio populations on different islands do not actually interbreed because they are isolated by water barriers, and their potential for interbreeding would be inhibited by lack of song recognition if they came into secondary contact.

Molecular evidence from this study indicates two changes are warranted in order to make taxonomy of *Chasiempis* congruent with phylogenetic units and biologically distinct populations. First, the proposed split of elepaio on each island into separate species is supported by mtDNA variation. Elepaio on each island formed a reciprocally monophyletic group that did not share ND2 haplotypes with other islands. The sequence divergence of elepaio taxa among islands (3.02–2.21%) was similar to that between Kauai and Oahu amakihi (3.7%; *Hemignathus kauaiensis* and *H. chloris*; Tarr and Fleischer 1993), species of *Pomarea* monarchs throughout eastern Polynesia (3.8 ± 1.8%) and within the Marquesas (3.1 ± 1.5%; Cibois et al. 2004), island and western scrub jays (3.14%; *Aphelocoma insularis* and *A. californica*; Delaney and Wayne 2005), masked and Nazca boobies (1.3–2.0%; *Sula dactylatra* and *S. granti*; Friesen et al. 2002), and several other avian sibling species (Avise and Zink 1988). It is also noteworthy that only 6 of 12 microsatellites developed for Oahu elepaio (Burgess and Fleischer 2006) amplified in Hawaii elepaio, indicating divergence in a nuclear marker between islands. The nomenclature of elepaio on each island has already been established because each taxon was originally described as a species and the original epithets still apply: *C. sclateri* Ridgway 1882 on Kauai; *C. ibidis* Stejneger 1887 (formerly *C. gayi* Wilson 1891) on Oahu; and *C. sandwichensis* Gmelin 1789 on Hawaii. Common names for these species would logically be the Kauai elepaio, Oahu elepaio, and Hawaii elepaio, respectively.

Second, molecular evidence does not support recognition of subspecies within Hawaii. There was significant variation in ND2 on Hawaii, but ND2 haplotypes were shared among the subspecies on Hawaii and most variation in ND2 occurred within subspecies, not among them. Microsatellites showed no evidence of geographic population structure on Hawaii.

The subject of avian subspecies has been controversial, with debate about criteria for identifying subspecies and

even validity of the subspecies concept (Mayr 1982; Zink 2004; Phillimore and Owens 2006). Subspecies traditionally have been based on geographical discontinuities in phenotypic traits, and some authors have prescribed a minimum proportion of morphological differentiation to qualify as subspecies (Amadon 1949; Patten and Unitt 2002). Molecular data has often shown a lack of congruence in traditional morphological subspecies and phylogenetic units. In a review of 259 avian subspecies worldwide, Phillimore and Owens (2006) found that 36% of subspecies were distinct phylogenetic units and that island subspecies were more likely to be monophyletic than subspecies in other biogeographic realms. In elepaio, there was partial congruence of morphologically based subspecies and phylogenetic units; subspecies on each island proved to be monophyletic, but subspecies restricted to portions of an island (*C. s. ridgwayi* and *C. s. bryani*) did not.

Conservation and management implications

Status and trend of elepaio populations differ considerably among islands. The Oahu elepaio has declined severely in the past few decades and the population of less than 2,000 birds is restricted to isolated fragments that comprise less than 4% of its original range (VanderWerf et al. 2001). The Kauai and Hawaii elepaio are more widespread and numerous, with populations of >23,000 and approximately 207,000, respectively (Scott et al. 1986; Foster et al. 2004). The Kauai elepaio appears to be increasing (Hawaii Division of Forestry and Wildlife, unpublished data) and the Hawaii elepaio has declined in some areas (Gorresen et al. 2005, 2006). The Oahu elepaio is already listed as endangered under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service 2000), but raising it to species status would increase its recovery priority number from three to two, which in theory should increase allocation of federal funding. The IUCN considers only the status of full species, so splitting elepaio into three species would allow individual assessment and appropriate categorization of the threat level on each island. The rapid rate of decline in Oahu elepaio and small population might warrant classification as critically endangered.

The genetic structure of elepaio we see today still largely reflects prehistoric environmental conditions when much of each island was covered in forest and elepaio populations were largely continuous (VanderWerf 1998; VanderWerf et al. 2001). However, anthropogenic factors are causing rapid environmental changes, and this can be expected to influence the evolutionary trajectory of elepaio populations. Clearing of land for agriculture by Polynesians beginning 1,600 years ago and acceleration of urban and agricultural development in the last 200 years has resulted in extensive loss and fragmentation of forest (Kirch 1982; Scott et al. 2001). Elepaio numbers and population density have declined in many areas, which will diminish intraspecific competition for territories that drives dispersal (VanderWerf 2008). Increasing barriers and decreasing need for dispersal can be expected to result in decreased gene flow and effective population size, accelerated differentiation, and possibly loss of genetic diversity and inbreeding (Keller and Largiadèr 2003; Vandergast et al. 2004; Martinez-Cruz et al. 2007). Changes in dispersal regime and evolutionary trajectory are likely to be most severe on Oahu, where forest fragmentation has been most extensive and elepaio populations are small and isolated by unsuitable habitat (VanderWerf et al. 2001).

Differentiation in elepaio among but not within islands indicates each island should be managed as a separate population. Geographic variation in morphology at small scales within islands suggests there are locally adapted phenotypes. Translocation or release of captive bred birds to areas where elepaio have been extirpated or declined is an appropriate management strategy (U.S. Fish and Wildlife Service 2006), but if such actions are attempted, birds should be selected from areas with habitat and climate similar to the target area to preserve and benefit from local adaptation in plumage color and other characters.

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Appendix

See Tables 5 and 6.

Table 5 Number of genetic samples collected from elepaio at locations on Kauai, Oahu, and Hawaii, and subspecies at each location based on Pratt (Pratt 1980)

Island	Location	Location code	No. of samples	Putative subspecies
Kauai	Halepaakai	HPK	16	*C. s. sclateri*
Kauai	Kawaikoi	KWK	11	*C. s. sclateri*
Kauai	Mohihi	MOH	15	*C. s. sclateri*
Oahu	Aina Haina	AIN	5	*C. s. ibidis*
Oahu	Halawa North	HAL	2	*C. s. ibidis*
Oahu	Halawa South	HAL	3	*C. s. ibidis*
Oahu	Lualualei	LUA	1	*C. s. ibidis*
Oahu	Makua Military Reservation	MMR	1	*C. s. ibidis*
Oahu	Moanalua Valley	MOA	8	*C. s. ibidis*
Oahu	Palehua	PALE	9	*C. s. ibidis*
Oahu	Pia Valley	PIA	3	*C. s. ibidis*
Oahu	Schofield Barracks West Range	SBW	8	*C. s. ibidis*
Oahu	Waihee	WAIH	1	*C. s. ibidis*
Oahu	Waikane	WAIK	7	*C. s. ibidis*
Oahu	Waimano	WAIM	1	*C. s. ibidis*
Oahu	Wiliwilinui	WILI	3	*C. s. ibidis*
Hawaii	Puu Waa Waa Bird Sanctuary	PWW	12	*C. s. sandwichensis*
Hawaii	Kona Hema Preserve	HEMA	10	*C. s. sandwichensis*
Hawaii	Kona Forest NWR	KFR	5	*C. s. sandwichensis*
Hawaii	Manuka NAR	MANU	15	*C. s. sandwichensis*
Hawaii	Pohakuloa Training Area	PTA	4	*C. s. sandwichensis**
Hawaii	Puu Laau, Mauna Kea FR, Kaohe GMA	LAAU	17	*C. s. bryani*
Hawaii	Puu Oumi NAR	OUMI	6	*C. s. ridgwayi**
Hawaii	Kalopa SP	KALO	2	*C. s. ridgwayi*
Hawaii	Keanekolu Road	KEA	1	*C. s. ridgwayi*
Hawaii	Piihonua, Hilo FR	PII	1	*C. s. ridgwayi*
Hawaii	Puu Oo Trail (Saddle Road)	POO	5	*C. s. ridgwayi**
Hawaii	Puu Makaala NAR	MAKA	3	*C. s. ridgwayi*
Hawaii	Hawaii Volcanoes NP Mauna Loa Strip	MLS	13	*C. s. ridgwayi*
Hawaii	Hawaii Volcanoes NP Escape Road	ESC	2	*C. s. ridgwayi*
Hawaii	Kahaualea NAR	KAH	1	*C. s. ridgwayi*
Hawaii	Bryson's (Puna)	BRY	2	*C. s. ridgwayi*
Hawaii	Kapapala FR	KAPA	2	*C. s. ridgwayi**
Hawaii	Kau FR	KAU	5	*C. s. ridgwayi**

Locations of sites are indicated in Fig. 1 by the corresponding codes. *FR* Forest Reserve, *GMA* Game Management Area, *NAR* Natural Area Reserve, *NP* National Park, *NWR* National Wildlife Refuge, *SP* State Park. An asterisk (*) indicates locations where elepaio are intermediate in appearance or from which no previous samples have been collected and where birds have not been assigned to any subspecies; in these cases subspecies were based on similarity of plumage (E. VanderWerf, unpublished data)

Table 6 Elepaio haplotype distribution list for (a) ND2 and (b) LDH

Haplotype ID	Kauai *C. s. sclateri*	Oahu *C. s. ibidis*	Hawaii *C. s. ridgwayi*	Hawaii *C. s. bryani*	Hawaii *C. s. sandwichensis*
(a) ND2					
ND2-K1	12	0	0	0	0
ND2-K2	4	0	0	0	0
ND2-K3	2	0	0	0	0
ND2-K4	2	0	0	0	0
ND2-K5	2	0	0	0	0
ND2-K6	1	0	0	0	0
ND2-K7	1	0	0	0	0
ND2-K8	1	0	0	0	0

Table 6 continued

Haplotype ID	Kauai *C. s. sclateri*	Oahu *C. s. ibidis*	Hawaii *C. s. ridgwayi*	Hawaii *C. s. bryani*	Hawaii *C. s. sandwichensis*
ND2-K9	1	0	0	0	0
ND2-K10	1	0	0	0	0
ND2-K11	1	0	0	0	0
ND2-O1	0	19	0	0	0
ND2-O2	0	10	0	0	0
ND2-O3	0	8	0	0	0
ND2-O4	0	5	0	0	0
ND2-O5	0	4	0	0	0
ND2-O6	0	1	0	0	0
ND2-H1	0	0	9	0	0
ND2-H2	0	0	6	12	32
ND2-H3	0	0	3	0	1
ND2-H4	0	0	2	0	0
ND2-H5	0	0	2	0	0
ND2-H6	0	0	2	0	0
ND2-H7	0	0	2	0	0
ND2-H8	0	0	2	0	0
ND2-H9	0	0	1	0	0
ND2-H10	0	0	1	0	0
ND2-H11	0	0	1	0	0
ND2-H12	0	0	1	0	0
ND2-H13	0	0	1	0	0
ND2-H14	0	0	1	0	0
ND2-H15	0	0	1	0	0
ND2-H16	0	0	0	0	4
ND2-H17	0	0	0	0	2
ND2-H18	0	0	0	0	1
ND2-H19	0	0	0	2	0
ND2-H20	0	0	0	0	2
ND2-H21	0	0	1	0	0
(b) LDH					
LDH-1	14	31	26	7	21
LDH-2	4	1	6	4	2
LDH-3	4	0	0	0	0
LDH-4	1	0	0	0	0
LDH-5	1	0	0	0	0
LDH-6	1	0	0	0	0
LDH-7	1	0	0	0	0
LDH-8	0	3	0	0	0
LDH-9	0	0	4	0	5
LDH-10	0	0	4	0	0
LDH-11	0	0	2	0	4
LDH-12	0	0	0	0	2
LDH-13	0	0	1	0	0
LDH-14	0	0	1	0	0
LDH-15	0	0	0	0	1
LDH-16	0	0	0	0	1
LDH-17	0	0	0	0	1

Table 6 continued

Haplotype ID	Kauai *C. s. sclateri*	Oahu *C. s. ibidis*	Hawaii *C. s. ridgwayi*	Hawaii *C. s. bryani*	Hawaii *C. s. sandwichensis*
LDH-18	0	0	0	0	1
LDH-19	0	0	0	1	0
LDH-20	0	0	0	1	0
LDH-21	0	0	0	0	1

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