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Source: The Wilson Journal of Ornithology, 126(3):562-568. 2014.
Published By: The Wilson Ornithological Society
DOI: http://dx.doi.org/10.1676/13-054.1
URL: http://www.bioone.org/doi/full/10.1676/13-054.1

Short Communications

The Wilson Journal of Ornithology 126(3):562–568, 2014

First Record of Hybridization in the Hawaiian Honeycreepers: ʻIʻiwi (*Vestiaria coccinea*) × ʻApapane (*Himatione sanguinea*)

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ABSTRACT.—The adaptive radiation of the Hawaiian honeycreepers is the largest ever recorded for birds on an oceanic archipelago. Despite including >50 species in 21 genera, no hybridizations across honeycreeper species have ever been confirmed. Here, we report genetic and morphological analyses that verify the first hybrid between two Hawaiian honeycreeper species: the ʻIʻiwi (*Vestiaria coccinea*) and ʻApapane (*Himatione sanguinea*). This hybridization is notable given that the parental species diverged ~1.6 mya and show distinct morphological differences. Further, this discovery is important in light of recent evidence that hybridization plays an important role in speciation and genetic diversity in both plants and animals. *Received 2 April 2013. Accepted 5 April 2014.*

Key words: adaptive radiation, Big Island of Hawaii, Drepanididae, intergeneric hybrid, microsatellites, 454 sequencing.

The adaptive radiation of the Hawaiian honeycreepers (Fringillidae: Drepanidinae), with 21 genera and more than 50 species, is the largest avian radiation on an oceanic archipelago (Pratt et al. 2009). New volcanic islands produced empty habitat for colonization; adaptation and competition likely enabled the honeycreepers to rapidly evolve a diversity of bill morphologies, plumages, and feeding techniques (Lerner et al. 2011). Intense specialization and concurrent evolution of pre and post-zygotic reproductive isolation may partly explain the lack of a documented honeycreeper hybrid to date (Grant 1994, Grant and Grant 2009), although it is still surprising given the rate of hybridization in related species (McCarthy 2006). The Galápagos finches, for instance, hybridize frequently both within and across genera (Grant et al. 2004, Grant and Grant 2008). Here, we report evidence supporting the first case of hybridization between two honeycreeper species: the ʻIʻiwi (*Vestiaria coccinea,* Fig. 1A) and ʻApapane (*Himatione sanguinea*, Fig. 1C, hybrid shown in Fig. 1B) and discuss possible reasons for the lack of hybridization evidence in the honeycreepers as well as the circumstances that may have led to this exceptional hybrid bird. Our discovery is important in light of recent evidence that introgression and hybridization play important roles in speciation, maintenance of genetic diversity, and the movement of advantageous alleles within and between species (Mallet 2007, Schwenk et al. 2008, Rheindt and Edwards 2011).

METHODS

DNA Laboratory Analyses.—An unusual honeycreeper was captured on 28 May 2011 on Hawaii Island (19° 40′ 20.17″ N and 155° 20′ 21.00″ W and 1,565 m elevation in the Upper Waiakea Forest Reserve), measured, banded (band 2551-51657), and the right-most retrix collected and sent to the Center for Conservation and Evolutionary Genetics Laboratory in Washington, D.C. Unfortunately, the color-banded individual was never seen or caught again during the subsequent 2 years of field data collection. DNA was extracted from two small (<1 mm in length) sections cut from the feather′s rachis using a DNeasy kit (Qiagen). We used three different types of DNA markers to genetically characterize bird 2551-51657: mitochondrial DNA (to identify the species that was the dam of 2551-51657), nuclear introns (genotypes determined via both Sanger and 454 sequencing), and nuclear microsatellite loci (to ascertain whether the bird showed a genetic signature of interspecific hybrid origin). We determined the gender of 2551-51657 using a standard CHD amplification (Griffiths et al. 1998).

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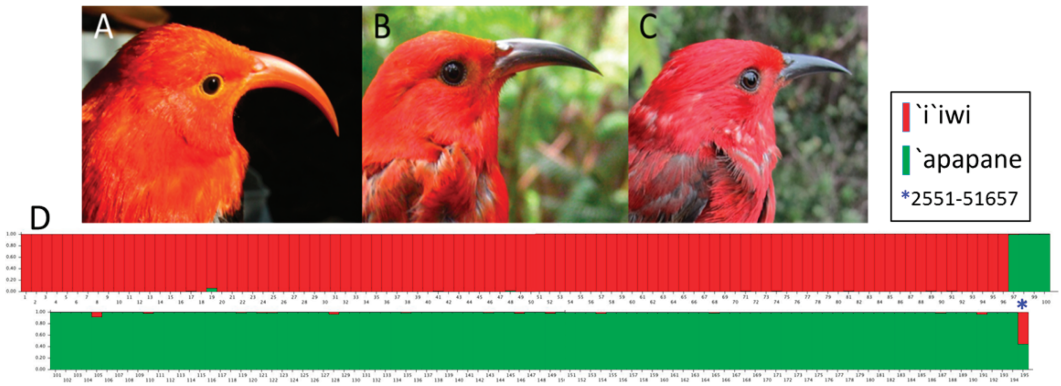

FIG. 1. Images of ʻIʻiwi (A), the putative hybrid bird band number 2551-51657 (B), and ʻApapane (C). A Structure analysis identified $K = 2$ as the optimal number of clusters (Table 2), each cluster corresponding to ʻIʻiwi (96 red bars, D) and ʻApapane (98 green bars, D), with very strong separation of the species. The last bar in D, 2551-51657, is roughly half red and half green, indicating that it is not assignable to either species directly, and has a mixed genetic constitution expected from an F1 hybrid of the two species. (Photo credits: O. Lansdorp).

A 307 bp piece of the mtDNA cytochrome b gene was amplified using primers Cytb1 and Cytb2 (Kocher et al. 1989), and 324 bp of control region was amplified and sequenced using primers Lgl2 and H417 (Tarr 1995). We also amplified with 12 sets of nuclear intron primers (Foster et al. 2007, Reding et al. 2010, Lerner et al. 2011), and following amplification conditions in those references. Amplification products were directly Sanger sequenced using BigDye Terminator v3.1 (Applied Biosystems) on an ABI 3130XL DNA sequencer. In addition, PCR products were ligated to individually tagged adaptors for 454 pyrosequencing. These ligated products were quantified and pooled for emulsion PCR and sequencing on a 454 FLX pyrosequencer (Lerner et al. 2011). We also amplified 12 microsatellite loci that had been used in a previous study on honeycreepers sampled from as close as 20 km from the locality in which 2551-51657 was caught on Hawaii Island (Eggert et al. 2008). We included one individual ʻApapane and one individual ʻIʻiwi in the laboratory analysis, both of whose microsatellite genotypes had been obtained as part of the previous study. These samples served as positive controls for the feather amplifications, and also provided a means to calibrate allele calls for 2551-51657 to the Eggert et al. (2008) analysis. Given the general lack of structure within islands for both ʻIʻiwi and ʻApapane, and the major differences in allele distributions between the two species, it is unlikely that use of a slightly geographically disjunct population would compromise use of the dataset for comparison to 2551-51657. Lastly, we used primers P2 and P8 to amplify genes in the CHD sexing system of birds (Griffiths et al. 1998). The P2 primer was labeled with HEX and the product was run on an ABI 3130XL sequencer in order to determine whether it had two fragments of appropriate size (i.e., sex was female) or only one (i.e., was male).

DNA Marker Analyses.—Sequences were edited and aligned in Sequencher 4.10 (GeneCodes Corporation). For mtDNA, Sanger sequences were matched to existing mtDNA sequences for Hawaiian honeycreepers to determine the species identity of 2551-51657′s mother. For nuclear introns, Sanger sequences were examined for double or heterozygous peaks, particularly at sites that differed between two honeycreeper species based on previous sequencing (Reding et al. 2010). In addition, for some introns there might be insertion or deletion (indel) differences between the sequences for each parental species that would misalign the reading frame between the two alleles, and such cases could be considered as further evidence that 2551-51657 was derived from hybridization of two species. Intron 454 sequences were assembled and corrected for gaps and singletons in mononucleotide repeat regions, and aligned to ʻIʻiwi, ʻApapane and the 2551-51657 feather sequences. Successful base calls were counted where there was sequence variation (in five loci; Table 1).

The multilocus microsatellite genotype for 2551-51657 was combined with a dataset of 96 ʻIʻiwi and 98 ʻApapane genotypes for the same 12

TABLE 1. (a) Variable sites from 307 bp of mtDNA Cytochrome b sequence and 344 bp of mtDNA control region. Note the match of feather 2551-51657 with ʻIʻiwi. This indicates the mother of bird 2551-51657 was an ʻIʻiwi. (b) Variable sites from five nuclear introns, most differ between ʻIʻiwi and ʻApapane. Genotype call for the feather from Sanger sequencing, and the % of each base and coverage (number of sequences recovered) at that base for the 454 amplicon sequences from the feather. ''?'' indicates that base could not be scored from chromatograms because of indel differences between haplotypes.

a.	Cytb							CR													
ʻApapane:	G	G	C	A	C			A	C	T	C	A	A	T	G	T	T	C	T	T	A
ʻIʻiwi:	A	A	T	G	T			G	A	A	A	T	G	C	A	C	A	A	C	C	C
Feather 2551-51657:	A	A	T	G	T			G	A	A	A	T	G	C	A	C	A	A	C	C	C

b.	GAPD						TROP	ENOL
ʻApapane:	T	G	::	A/G	A	C	C	C
ʻIʻiwi:	G	C	GG	G	G	T	T	G
Feather 2551-51657 (Sanger):	T/G	G/C	GG/::	G	?	?	-	G
Feather 2551-51657 (454, %):	50%T	56%G	100%::	100%G	100%A	100%C	100%T	100%G
Feather 2551-51657 (coverage):	66x	75x	41x	39x	46x	46x	52x	36x
Supports hybrid status	Y	Y	Y?	N	N	N	N	N

	LDH						RP40	
ʻApapane:	A	A	G	A	G	C	C	G/A
ʻIʻiwi:	G	G	A	G	G	T	A	A
Feather 2551-51657 (Sanger):	-	-	-	-	-	C/T	C/A	G/A
Feather 2551-51657 (454, %):	70%A	63%G	66%G	50%A	71%G	56%T	53%A	56%A
Feather 2551-51657 (coverage):	20x	8x	3x	6x	7x	66x	118x	115x
Supports hybrid status	Y	Y	Y	Y	Y	Y	Y	?

loci (Eggert et al. 2008). This dataset was analyzed in Structure 2.3.2 (Evanno et al. 2005), using no location or other prior information to determine the number of clusters (K) in the dataset and the genetic representation of 2551-51657. An initial run from $K = 1$ to $K = 4$ was made, using 100,000 burn-in and 900,000 repetitions. A second run, with five iterations of 50,000 burn-in and 150,000 repetitions was made for $K = 1$ to $K = 3$. Results from both runs were the same, and the results for the second run were evaluated by the Evanno et al. (2005) method to determine the optimal K in StructureHarvester (Earl and Vonholdt 2012). In addition, we ran ten replicates for $K = 2$ (10,000 burn-in and 90,000 repetitions) with loc prior for ʻIʻiwi and ʻApapane, but not for the putative hybrid, and assuming an ancestry model with two prior generations (Gensback = 2 option) to test for backcrossing versus F1 status of the individual. The dataset was also analyzed in the Bayesian program NewHybrids 1.1 (Anderson and Thompson 2002) to test whether 2551-51657 was pure ʻApapane, pure ʻIʻiwi, an F1 or F2 hybrid, or a backcrossed hybrid into either background species. We ran 100,000 post burn-in replicates in two independent chains with no priors.

RESULTS

The mtDNA sequence from the feather exactly matched the sequence from ʻIʻiwi and was 23 bp different from ʻApapane (Table 1). Nine of 15 variable sites within five nuclear introns showed evidence of both specific bases in 2551-51657 for ʻApapane and ʻIʻiwi (Table 1). CHD analysis showed a single product, indicating that 2551-51657 is male. Structure analysis of microsatellite genotypes identified $K = 2$ as the optimal number of clusters (Evanno et al. 2005, Table 2), corresponding to ʻIʻiwi and ʻApapane (red and green bars, respectively, in Fig. 1D). The bar for 2551-51657 is roughly half red and half green (Fig. 1D), indicating it has a mixed genetic constitution expected from an F1 hybrid of the two species. No other tissues were collected when the bird was captured, so we were unable to determine if 2551-51657 was reproductively viable.

The 307 bp of cytochrome b sequence from the feather extract matched that of ʻIʻiwi exactly and was five bp different from ʻApapane, while the 324 bp of control region were also identical to ʻIʻiwi (Table 1), but differed by 18 bp from the ʻApapane sequence. No variability at these loci

TABLE 2. Structure results from microsatellites. We used the method of Evanno et al. (2005) to estimate the number of clusters from the dataset testing $K = 1$–3 (with initial run testing $K = 1$–4), and five repetitions. Largest Ln′(K) is for $K = 2$, and Delta K is a very high for $K = 2$, indicating that two is the most likely number of clusters or groups that arise from the dataset. These two groups correspond to ʻIʻiwi and ʻApapane (Fig. 1D).

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln′(K)	|Ln″(K)|	Delta K
1	5	−10443.680	0.576	—	—	—
2	5	−9047.780	0.277	1395.900	1316.700	4745.057
3	5	−8968.580	10.667	79.200	—	—

has been detected among a large sample of ʻIʻiwi from the island of Hawaii (Jarvi et al. 2004, Foster et al. 2007), and ʻApapane also show very low levels of variation (Foster 2007), so these sequences should be stereotypical for each species. We successfully sequenced, with both direct sequencing and 454 pyrosequencing, five nuclear introns for the 2551-51657 sample that showed some variation that might be evidence of ʻApapane and ʻIʻiwi specific alleles (GAPD and RP40, [Reding et al. 2010]; LDH and ENOL, [Foster et al. 2007]; and TROP, [Lerner et al. 2011]; Table 1). Comparisons were made for these five loci with sequences from ʻApapane and ʻIʻiwi (as in Reding et al. 2010, Lerner et al. 2011). The other loci either did not sequence well or did not show variability or consistent differences between the two species and are not discussed further. Average coverage was 47x (range 3x to 118x). There were 15 variable sites across the five loci, 12 of which differed substitutionally between the two sequences of ʻIʻiwi and ʻApapane. There was also a region with two sequential missing bases (gaps) in the GAPD intron sequences, and with offset strand sequences in the Sanger sequence that made reading it difficult. Oddly, in the 454 sequences of the feather, all 41 sequences showed the gap, perhaps caused by a bias in the emulsion PCR. A total of nine of the 12 variable sites showed the appropriate pattern of different bases at a site between ʻApapane and ʻIʻiwi, and both bases found in feather 2551-51657 in the Sanger (when available) and/or the pyrosequences. For three sites with high coverage (36–52x; one each in GAPD, TROP and ENOL; Table 1), only one base of the two alternates was recovered, in one case the base of the ʻApapane and in two cases the ʻIʻiwi. There were two variable sites in ʻApapane (in GAPD and RP40), which showed only one of the bases in one case in the feather Sanger and 454 sequences (GAPD), and matched the ʻIʻiwi. For the feather sample, from either or both Sanger and 454 sequences, we found polymorphic sites at 10 of the 15 sites assayed, while we found zero such cases for ʻIʻiwi and two cases for ʻApapane. We take the above combined evidence as reasonable support for the hypothesis of a hybrid origin of the feather, with ʻIʻiwi and ʻApapane as parents.

$K = 2$ was the optimal number of clusters in the Structure analysis based on the method of Evanno et al. (2005, Table S2). As expected, the clusters corresponded to ʻIʻiwi and ʻApapane (red and green filled bars, respectively, in Fig. 1D), with very strong separation of the species. The last individual in Fig. 1D is 2551-51657. The bar for this individual is roughly half red and half green, indicating that it is not assignable to either species directly, and that is has a mixed genetic constitution expected from an F1 hybrid of the two species. In addition, proportional representation of ʻIʻiwi genes was 0.538, very close to the expected 0.5 for an F1 hybrid, and this value did not vary regardless of whether we ran the program with expectations of backcrossing. Our analysis with NewHybrids (Anderson and Thompson 2002) showed strong support for purity of ʻIʻiwi and ʻApapane samples (likelihoods averaging 0.9997 and 0.9993, respectively), and for an F1 status for 2551-51657 (likelihood of 0.9185), versus pure ʻIʻiwi (0), pure ʻApapane (0), F2 (0.0316), or either backcross with ʻIʻiwi (0.0418) or ʻApapane (0.0082). Thus, we conclude that 2551-51657 is an F1 hybrid of an ʻIʻiwi and ʻApapane.

Morphological measurements of the hybrid bird were closer to average measurements of male ʻApapane taken at the same site, with the exception of bill length, which was close to the mid-point of male ʻApapane and ʻIʻiwi average bill measurements (Table 3).

TABLE 3. Average, standard deviation, maximum, and minimum measurements for female (first number) and male (second number) ʻApapane and ʻIʻiwi caught in the same year and at the same field site as the hybrid individual 2551-51657, whose measurements are also given. Bill measurement is the exposed culmen.

	Wing (mm)	Tarsus (mm)	Bill (mm)	Weight (g)
*ʻApapane (*females *n = 118,* males *n = 174)*				
Average	69.5, 75.5	23.0, 23.8	16.1, 17.2	13.9, 15.3
SD	2.2, 2.0	0.9, 0.9	0.8, 0.8	1.5, 1.3
Max	74.5, 80.0	25.3, 27.3	17.6, 22.7	19.0, 20.0
Min	64.0, 68.0	21.3, 21.4	13.2, 15.4	9.5, 10.0
Hybrid	74.0	23.9	22.7	17.0
*ʻIʻiwi (*females *n = 65,* males *n = 88)*				
Average	73.4, 80.4	23.9, 25.4	24.5, 27.4	16.7, 19.5
SD	2.8, 2.2	0.9, 1.0	1.4, 0.9	2.1, 3.6
Max	80.0, 86.0	26.0, 28.4	27.6, 29.2	25.0, 25.5
Min	66.5, 73.0	21.8, 22.8	20.7, 25.2	12.5, 15.5

DISCUSSION

Our genetic analyses show that 2551-51657 is a male F1 hybrid resulting from a cross between a female ʻIʻiwi and a male ʻApapane. This individual is the first hybrid ever confirmed for Hawaiian honeycreepers, despite ongoing study of these species for >40 years with thousands of individuals captured and banded and many thousands of specimens collected for museums (Banko 1979). There are only three cases of individual birds whose appearance suggested they could be hybrids (Lepson and Woodworth 2002), but two were not genetically tested and the third was tested with mtDNA and nuclear intron sequencing and was determined not to be a hybrid (RCF and S. L. Olson, unpubl. data). Hybridizations between honeycreeper species are not necessarily unexpected; in the family Fringillidae, 27% of non-Hawaiian species are known to hybridize in the wild (McCarthy 2006). Further, in the closely related families Emberizidae, Estrilididae, and Thraupidae, 31%, 40%, and 37% of species, respectively, are known to hybridize in the wild (McCarthy 2006). What makes the Hawaiian honeycreepers different?

One possible explanation for the lack of honeycreeper hybrids to date is that over half of Hawaii′s honeycreeper species have gone extinct; there may have been hybridization in the past. However, no hybrids, other than the two putative ones noted above, have been described thus far in the more than 4,500 honeycreeper specimens from >30 species housed in museums around the world (Banko 1979). Another explanation stems from the geographic distribution of the honeycreepers on the Hawaiian Islands; the great majority of congeneric honeycreeper species occur on different islands, and thus rarely, if ever, come into contact with congeners. The majority of bird taxa that hybridize do so within genera, among sympatric populations. Only 5%, 7%, 1%, and 6% of species in Fringillidae, Emberizidae, Estrilididae, and Thraupidae, respectively, have been documented to hybridize across genera (McCarthy 2006). However, many of the parental species of those intergeneric hybrids have a divergence date much older than the oldest known divergence date for the honeycreepers (Arnaiz-Villena et al. 2001). The two parental species of the intergeneric hybrid we document here, ʻIʻiwi and ʻApapane, diverged around 1.6 mya (Lerner et al. 2011), which is fairly recent. Why these species do not hybridize more frequently is unknown, but may stem from behavioral and morphological differences.

Despite the relatively recent split of ʻIʻiwi and ʻApapane the circumstances that gave rise to a mating between a female ʻIʻiwi and a male ʻApapane are difficult to imagine. ʻIʻiwi are aggressive and socially dominant to ʻApapane, and the average bill length of ʻIʻiwi is more than 10 mm greater than ʻApapane (Table 3). Further, ʻIʻiwi are larger than ʻApapane (Table 3), and it is unusual for a female of a larger species to choose to mate with a male of a smaller species; many studies across a wide range of taxa have documented the opposite pattern (summarized in Grant and Grant 1997). Almost nothing is known about mate choice in the Hawaiian honeycreepers. However, song is known to play a large role in

mate choice in birds (Nowicki and Searcy 2005), and ʻApapane do show wide variation in their song repertoire. Moreover, some hybridizations between finch species on the Galapagos Islands occurred when one species imprinted on the song of another species during the critical learning period (Grant and Grant 1997). ʻApapane and ʻIʻiwi are more similar in courtship behavior to each other than with other honeycreeper species and have overlapping breeding seasons (Fancy and Ralph 1997, 1998). In the fragmented forest where the hybrid was captured, ʻApapane are four times as abundant as ʻIʻiwi (T. J. Kovach, unpubl. data). Therefore, the Hubbs principle may explain this hybridization, whereby interspecific interbreeding results when one of the two species is rare (Hubbs 1955, Grant and Grant 1997). Nevertheless, to date there has been no confirmation of hybridization between any of the endangered honeycreepers and any of the more common species.

The discovery of this intergeneric honeycreeper hybrid raises important questions regarding both the past and future evolution of these species. For example, it will be interesting to note if these hybridizations increase in frequency in areas where ʻIʻiwi populations are declining. ʻIʻiwi are extremely vulnerable to avian malaria (*Plasmodium relictum*) infection (90% mortality rate, Atkinson et al. 1995) while ʻApapane show mixed resistance (25–50%, Atkinson and Samuel 2010). Thus, hybrid offspring may have greater malaria resistance than the pure ʻIʻiwi.

ACKNOWLEDGMENTS

We thank P. LoCicero, N. Lancaster, E. Ames, D. Rankin and O. Lansdorp for assistance in the field and J. Lock, C. Hofman and H. Lerner for information or assistance with data analysis. Funding was provided by NSF grants DEB-1019928 and DEB-0643291, NIFA McIntire-Stennis and the Smithsonian Conservation Biology Institute.

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