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Morphometric and Genetic Confirmation of Two Species of *Kuhlia* (Osteichthyes: Kuhliidae) in Hawai‘i

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Abstract

Flagtails, members of the Genus *Kuhlia*, are Indo-Pacific fishes found in marine and freshwater habitats. Known locally as *āholehole*, they are important food fishes in the Hawaiian Islands. Local fishermen have noted the presence of two morphotypes occurring sympatrically in Hawai‘i, although at the beginning of this study only one species, *Kuhlia sandvicensis*, was identified in the scientific literature. During the course of this study, Randall & Randall (2001) published a revision of this genus, which included a description, based on meristic evidence, of the “big-eyed” morphotype as *K. xenura*. A small-eyed species with dark reticulations on the dorsal surface of the head and nape of living fish retains the name *K. sandvicensis*. For this study, genetic and morphometric comparisons confirm the presence of two species of *Kuhlia* in Hawai‘i. Also, *K. sandvicensis*, rather than *K. marginata*, is confirmed herein as the species present on Johnston Island. A classification equation was developed and can be used to identify preserved fish from past studies and to aid fishery managers in identifying fish whose reticulations have faded or whose eyes appear to be intermediate in size. The variables that best discriminate between the two species are body depth, eye diameter, and interorbital distance. Genetic data concur with morphology in the recognition of two species of Hawaiian kuhliids; DNA sequences reveal significant distinctions between the two types of *Kuhlia*. In addition to the morphological and DNA analysis, the ecology of juveniles of both Hawaiian *Kuhlia* was investigated. Preliminary analysis of data indicates overlap of juveniles in certain tidepool habitats, but *K. xenura* appears to be the only species utilizing the lower reaches of freshwater streams as nursery habitat. Due to the former recognition of Hawaiian *Kuhlia* as one species, management strategies currently in place are possibly more relevant for one species than the other. Thus, conservation plans for both *āholehole* should be reconsidered in light of these and Randall and Randall’s findings that these two “types” are separate species with genetically distinct populations and different nursery habitat preferences.

Introduction

The monogeneric family Kuhliidae contains approximately 10 species of fishes found in subtropical and tropical fresh, estuarine and marine waters of the Pacific (Randal & Randall, 2001). *Kuhlia* are important fishes in Hawai‘i, both for their popularity as sportfish and because they are culturally important species that were once used by Hawaiians in traditional ceremonies. Until recently, *K. sandvicensis* was the only species listed in the scientific literature for the Hawaiian Islands, and it was believed to be endemic to Hawai‘i. However, local fishermen have long recognized two types of *Kuhlia* or “*āholehole*” from differences in eye size. During underwater observations for doctoral research on the behavioral ecology of Hawaiian kuhliids, I noted other varying external features among small and large-eyed animals. The small-eyed fish have wide black reticulations on the head that extend posteriorly as two black lines along either side of the dorsal fin, a white patch on the posterior section of the soft dorsal fin, a more brightly marked black and white tail, and are less deep-bodied than the larger eyed animals. The small-eyed types are silver dorsally and whitish below, and the big-eyed types are often pale olive along the dorsum. The big-eyed fish also have reticulations on the head, but these are much fainter and very narrow (Benson & Fitzsimons, 2002) (Fig. 1), and their eyes are iridescent red along their upper edge. Near the completion of this study, a revision of the family Kuhliidae by Randall & Randall (2001) included a description of the big-eyed morphotype as a second species of *Kuhlia* in

Figure 1. Dorsal view of head reticulations for big-eyed *Kuhlia xenura* (**A**) and small-eyed *Kuhlia sandvicensis* (**B**) in Hawai‘i.

Hawai‘i. The available name for these fish is *Kuhlia xenura*, and the small-eyed type retains the name *K. sandvicensis*, based primarily on a lateral line scale count in the original description (Steindachner, 1876). The authors noted the eye size difference between the two species and included meristic data for various morphological characters. Because these characters overlap somewhat in the two species, additional morphometric features and DNA sequence variation are employed here to confirm the validity of recognizing two species in Hawaiian waters.

Materials and Methods

Collection methods and disposition of specimens

Fishes were taken by a variety of methods (seines, castnets, and hook and line) from sites on the islands of O‘ahu and Hawai‘i from 1997 to 2000. For specific collection sites, see Benson’s doctoral dissertation (2002). The U.S. Fish and Wildlife Service provided specimens collected in March 2000 from Johnston Island, 724 km WSW of Hawai‘i. Johnston is the nearest island group to Hawai‘i, and prior to this study the fishes there were listed in the scientific literature as *K. marginata*. After collection, all fishes were frozen immediately for later morphometric and DNA analysis. At the conclusion of the study, specimens were fixed in 10% formalin, placed in 70% ethanol as the final preservative, and catalogued into the Ichthyology Collection at the Louisiana State University Museum of Natural Science (LSUMZ 12307-12332, 12334-12341).

Morphometric analysis

Specimens were sexed (whenever possible) and measured using either hand-held electronic digital calipers (Mitutoyo Plasti-Cal) or a measuring board. Measurements included standard length (*L*), fork length (*F*), snout length (*S*), head length (*H*), body depth (*D*), eye diameter (*E*), and interorbital distance (*I*). These variables were chosen because they are standard classification measurements in ichthyology (e.g., Hubbs & Lagler, 1958), and they are the characters that appear most different in these two morphotypes. A small amount of characters was preferred so that field identification and discriminant equation use would be as easy as possible for fishermen and biologists.

Measurement data were examined in SigmaPlot version 4.01 (SPSS, Inc. 1997. SigmaPlot 4.01. SPSS, Inc., Chicago, Illinois.) to test for allometric influences, which cause differing patterns of growth between juveniles and adults (Thorpe & Leamy, 1983). The character chosen to detect allometry was the ratio of body depth to standard length, as this character seems to change the most with increasing size for these fishes. Ratios were plotted against standard length, and a linear regression was conducted. The steep slope due to changes in depth to standard length ratio began to flatten out at lengths of 40 mm, so only specimens 40 mm standard length or greater were used in the multivariate analysis (Burbrink, 2000). While some fish used in these analyses are technically juvenile fish and could not be sexed, their body shape and proportions are consistent with those of adults. In a study of *Kuhlia* on Johnston Island, Gosline (1955) also included only fish greater than 40 mm in his meristic research, further supporting this size cutoff. The final sample size for *K. xenura* was 158; *K. sandvicensis* were harder to collect and less abundant, and the final sample size for this group was 71. Twenty-nine fish from Johnston Island were included in this study.

Data were log transformed prior to further analysis to stabilize variances in specimens of different sizes. A linear regression was then performed to plot depth and standard length against fork length, and the remaining variables against head length. The residuals for these five variables were used in subsequent multivariate analyses to eliminate the effect of multicollinearity and to better express the fishes’ shape without the effects of body size (as per Freund & Wilson, 1997). Multivariate statistical analyses were conducted in SYSTAT version 8.0 (SPSS, Inc. 1998. Systat 8.0 Statistics. SPSS, Inc., Chicago, Illinois) and included a MANOVA, by using the Wilks’ lambda statistic, to test for a difference between group means. In addition, a principal components analysis was conducted on the five remaining log residual variables to examine if the specimens readily fall into separate groups. A t-test on these factor scores was employed to determine the statistical significance of their morphological differences. A discriminant function analysis was employed to calculate the probability of correctly classifying each fish by type. In addition, a jackknifed classification matrix was provided; this resulted from a DFA which used functions computed from all data except the case being classified. Analyses not including all fish were also conducted, and the percentage that the unclassified fish were grouped correctly was noted. Automatic backward and forward stepwise analyses were used to determine which morphometric variables best discriminate between the two groups. The discriminant function analysis was conducted once again and used only the variables selected by the stepwise analyses. Percent correctness data from this output are also provided. Finally, a discriminant analysis was performed on the raw measurement data. From this output, clas-

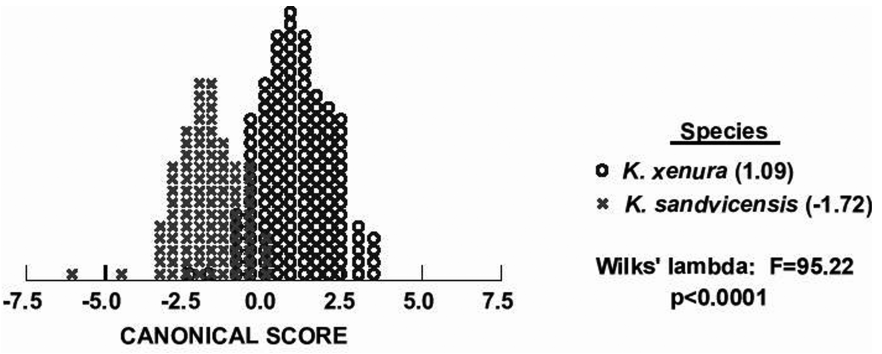

Figure 2. Discriminant function analysis of five morphometric variables used to classify two *Kuhlia* spp. from Hawai'i.

sification functions were used to derive a linear discriminant function equation for future use in classifying Hawaiian *Kuhlia* of unknown species. This equation consists of weighted coefficients that are multiplied by the morphological variables measured for each fish. The procedure results in a canonical score or value (*C*) that can be compared with a cutoff criterion differentiating the species.

DNA sequencing and analysis

DNA (from tail muscle) was isolated from frozen or DMSO preserved muscle tissue via Proteinase K digestion and phenol-chloroform extraction. The polymerase chain reaction (PCR) was used to amplify the entire cytochrome *b* gene (1140 base pairs) in two fragments that overlapped partially by using one primer for the light strand (L-14724) and one for the heavy strand (either cyt b-L or cyt b-HKu) (Table 1). Amplifications were carried out in 50 µl reactions which included 1 or 3 µl purified genomic DNA as template, 5 or 6 µl of MgCl2 Solution (25mM), 1 µl of 10mM dNTP's (Perkin Elmer), 0.25 µl AmpliTaq DNA Polymerase or AmpliTaq Gold Polymerase (5U/µl), and 2.5 µl each of the primers (all 10µM in concentration). The PCR was carried out in either a GeneAmp PCR System 2400 oil-free thermal cycler (Perkin Elmer) or in a PT C-200 Peltier Thermal Cycler (MJ Research). Methods were modified from Palumbi (1996) and included 30 cycles of amplification with primer annealing at 50 °C or 54 °C for 45 seconds. Five µl of the PCR reaction mixture was stained with ethidium bromide and loaded into a one percent agarose gel; electrophoresis was performed in order to visualize and verify the presence of the desired amplified product.

For specimens that exhibited successful amplifications, the remaining PCR product was then loaded into a one percent agarose gel (stained with ethidium bromide) for electrophoresis. The visualized bands were excised out of the gel and purified by using the BIO 101 GeneClean® Kit (BIO 101, Carlsbad, CA). Several primers were designed and used for the subsequent cycle sequencing reactions (Table 1). These reactions were performed on the PCR products by using the ABI PRISMBigDye® kit (PE Applied Biosystems, Foster City, CA). Cyt b-L and L-14724 served as

Table 1. **Primers Used in PCR Amplification and Cycle Sequencing**.

Primer	Nucleotide Sequence (5′to 3′)	Source
Cyt b-L	TGG RAC TGA GCT ACT AGT GTC	Reed *et al*., 2002
L-14724	TGA CTT GAA RAA CCA YCG TTG	Palumbi *et al*., 1991
L431	GAG GAC AAA TRT CYT TCT GAG G	Reed *et al*., 2002
L431-Ku	GAG GAC AAA TRT CAT TTT GAG G	designed by author
H520	TGA GAG TGG CGT TGT CTA CT	designed by author
Cyt b-HKu	GAG CTA CTA GTG CAS CTT CAT T	designed by author

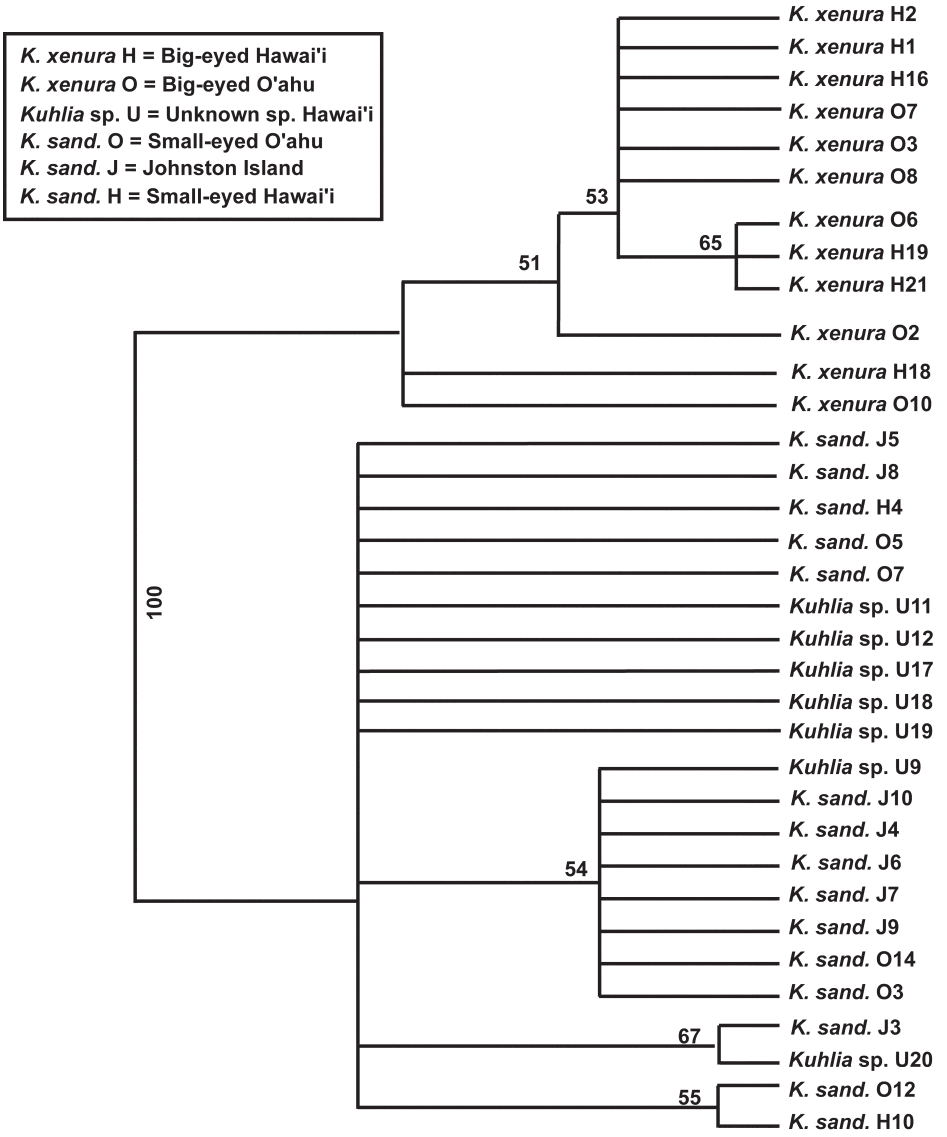

Figure 3. Maximum likelihood tree (manually rooted at midpoint) with bootstrap values (TVM+G model). Numbers at nodes indicate percent bootstrap support (of 100 replicates).

external primers for the heavy and light strands respectively; another general fish primer, L-431, was used to sequence the light strand internally. Initial sequences were examined and further primers were designed and renamed L431-Ku and Cyt b-HKu. A specific internal primer for the heavy strand was designed as well (H520). Subsequent sequencing used the L-14724 primer along with the three primers designed by the author. Cycle sequencing was conducted in 10 µl reaction volumes, which included 2 µl or 3 µl PCR product, 3.2 µl of 1µM primer, and either 2 µl (light strand) or 3 µl (heavy strand) of Big Dye reaction premix. The reaction was carried out in one of three machines, depend-

Table 2. **Classification matrix and jackknifed classification matrix for complete discriminant function analysis with Johnston Island fish coded separately (A) and with Johnston Island fish grouped with *K. sandvicensis* from Hawai'i (B)**. Numbers under the species/location names represent fish classified as each type by the DFA analysis.

A

Type	*K. xenura*	*K. sandvicensis*	Johnston	%Correct
K. xenura	141	2	15	89
K. sandvicensis	3	52	16	73
Johnston	0	5	24	83
Total	144	59	55	84

B

	Number of fish classified as:		
Type	***K. xenura***	***K. sandvicensis***	**%Correct**
K. xenura	144	14	91
K. sandvicensis	6	94	94
Total	150	108	92

ing on availability: a GeneAmp PCR System 2400 oil-free thermal cycler (Perkin Elmer Applied Biosystems, Norwalk, Connecticut), a PT C-200 Peltier Thermal Cycler (MJ Research), or a HYBAID Omn-E Thermal Cycler. The following sequencing protocol (modified from Hillis *et al*., 1996) was used: 10 seconds at 96 ºC, 5 seconds at either 48 ºC or 50 ºC, 4 minutes at 60 ºC, and storage at 4 ºC or frozen until cleanup. Sequencing products were precipitated by using a sodium acetate/ethanol cleanup protocol, as per the manufacturer's directions. Purified cycle-sequence reaction products were sequenced with an ABI model 377-XL Automated Sequencer (Perkin Elmer Applied Biosystems).

Sequences were visualized, and heavy and light strand fragments were aligned for each specimen by using the program Sequencher 3.1 (Gene Codes Corporation, Ann Arbor, Michigan). After cleanup, 34 useable sequences were analyzed for this study: six big-eyed *K. xenura* from the Big Island of Hawai'i, six big-eyed *K. xenura* from O'ahu, two small-eyed *K. sandvicensis* from the Big Island of Hawai'i, five small-eyed *K. sandvicensis* from O'ahu, eight Johnston Island *Kuhlia* (previously thought to be *K. marginata*), and seven fish of intermediate eye size that were classified as unknown *Kuhlia*. Seven of these 34 sequences had less than 1140 base pairs, and these ranged in length from 1116 to 1136 base pairs. A BLAST search (Altschul et al., 1990) in GenBank was conducted, and the most similar sequence in the database was from *Zingel streber* (Song et al., 1998), a percomorph fish in the same suborder (Percoidei) as the kuhliids. This sequence was downloaded and used to help align the study specimens. Sequences representing each study group were deposited in GenBank (see Acknowledgments). Analyses of nucleotide composition and percent informative sites were conducted with the program MEGA version 2.1 (Kumar, S., T. Koichiro, I. Jakobsen, & M. Nei. 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. Arizona State University, Tempe, Arizona).

Uncorrected pairwise sequence divergences between all taxa were calculated in PAUP* 4.0 (Swofford, D.L. 1998. PAUP*. Phylogenetic Analysis Using Parsimony [* and other methods]. Version 4. Sinauer Associates, Sunderland, Massachusetts). Based on sequence divergence, a neighbor joining tree (Saiton & Nei, 1987) was built using *Zingel streber* as an outgroup to root the tree. Because the outgroup was highly divergent from the study group, it was excluded from further analyses in this study. The sequencing dataset (minus the outgroup taxon) was also run in ModelTest v. 3.06 (Posada & Crandall, 1998) to determine the optimal DNA substitution model for a maximum

Table 3. **Range and average of percent sequence divergences (uncorrected p x 100) among and within all operational taxonomic units (OTUs) involved in this study**. Unless otherwise indicated, *K. sandvicensis* refers to only Hawaiian fish.

Pair of OTUs	Range in % Sequence Divergences	Average % Sequence Divergence
Interspecific Variation		
Kuhlia xenura vs. *K. sandvicensis*	7.982–9.219	8.523
Johnston Island vs. *K. sandvicensis*	0.000–0.360	0.173
K. xenura vs. Johnston Island	7.982–9.045	8.454
K. xenura vs. Johnston and *K. sandvicensis*	7.982–9.219	8.488
Comparisons involving fish of unknown type		
Unknown *Kuhlia* sp. vs. *K. sandvicensis*	0.000–0.618	0.256
Unknown *Kuhlia* sp. vs. *K. xenura*	7.982–9.158	8.489
Unknown *Kuhlia* sp. vs. Johnston Island	0.000–0.527	0.231
Johnston Island vs. Unknown and *K. sandvicensis*	0.000–0.618	0.202
K. xenura vs. Unknown and *K. sandvicensis*	7.982–9.219	8.506
K. xenura vs. three remaining types	7.982–9.219	8.489
Intraspecific Variation		
K. sandvicensis	0.088–0.361	0.213
K. xenura	0.179–1.405	0.705
Johnston Island	0.000–0.263	0.113
Unknown *Kuhlia* sp.	0.000–0.617	0.314
K. sandvicensis, Johnston Island, and Unknown fish	0.000–0.617	0.506
Comparisons involving outgroup		
K. xenura vs. Outgroup *(Zingel streber)*	18.519–19.491	19.155
K. sandvicensis vs. Outgroup *(Zingel streber*)	18.881–19.386	19.208
Johnston Island vs. Outgroup *(Zingel streber)*	19.123–19.386	19.342
Unknown *Kuhlia* sp. vs. Outgroup *(Zingel streber)*	19.211–19.496	19.350
Unknown and *K. sandvicensis* vs. Outgroup	18.881–19.496	19.289
Unknown/*K. sandvicensis*//Johnston vs. Outgroup	18.881–19.496	19.308

likelihood analysis. The model chosen was "TVM+G" and the parameters specified were: nucleotide frequencies A= 0.2566, C = 0.3058, G = 0.1540, T = 0.2836, and a gamma shape parameter (·) of 0.0702. Transition and transversion rates were specified as follows: A-C = 0.19, A-G = 3.09, A-T = 0.06, C-G = 0.26, C-T = 3.09, and G-T = 1. Support for the internal nodes in the maximum likelihood tree was assessed by a bootstrap analysis using 100 replicates. Trees were visualized and manually rooted at the midpoint by using the program TREEVIEW (Page, 1996).

Results

Morphometric analysis

Using combinations of the aforementioned morphological variables, the DFA procedure was able to group the three types (*K. xenura*, *K. sandvicensis*, and Johnston Island) separately with 84% correctness. There was some overlap between the Johnston Island fish previously identified as *K. marginata* and the *K. sandvicensis* specimens. Sixteen of the 71 *K. sandvicensis* specimens were mistakenly classified as Johnston Island fish; overall percent classification correctness was 73%. Johnston Island and *K. xenura* specimens were correctly classified a higher percentage of the time (Table 2A). Results for the jackknifed classification matrix were identical (Table 2A). Because the Johnston Island fish appear very similar to *K. sandvicensis* individuals, and because genetic evidence indicated that they were the same species, the analysis was repeated with the Johnston Island fish coded as *K. sandvicensis*. With all five variables as part of the MANOVA, the Wilks' lambda statistic revealed

a significant difference in group means for the two types of fish ($F = 95.2187$, $P<0.001$). The DFA grouped the two species of *Kuhlia* with 92% correctness (*xenura* = 91% and *sandvicensis* = 94%). Group means and overlap are shown in Figure 2. Fourteen of 158 *K. xenura* and six of 100 *K. sandvicensis* were misclassified (Table 2B). Again, results for the jackknifed classification matrix were identical (Table 2B). When the same analysis was performed with 30 randomly selected individuals coded with no species designation, the DFA grouped the fishes that were defined a priori with 93% correctness. Twenty-eight of the 30 fishes (93%) that were not coded by species were classified correctly as either *K. xenura* or *K. sandvicensis*.

Automatic backward stepwise discriminant analysis resulted in three variables that could classify the two species with 94% correctness overall and with 93% correctness for the jackknifed classification procedure. The final analysis included eye diameter, interorbital distance, and body depth as variables with discriminatory value; these three variables had the three highest F-to-remove values (all greater than 44.9). The other two variables, standard length and snout length, had low F-to-remove values (below 1.0) and were therefore removed from the DFA. Automatic forward stepwise discriminant analysis added depth to the analysis first, as it had the highest F-to-enter value. Interorbital distance and eye diameter were then added; again the final output did not include standard length or snout length. The number of misclassifications and the percent correctness were identical to that of the automatic backward stepwise procedure. Classification functions provided with the DFA output provide a classification function coefficient for each of the variables. These results indicate that *K. sandvicensis*, the small-eyed type of āholehole, has overall smaller eyes and body depths relative to their length, and they possess a larger distance between their eyes along the dorsal surface than does *K. xenura*.

From the second analysis involving raw measurement data, classification functions were used to derive a discriminant equation that can classify fish of unknown type. The equation for the canonical value, C, is:

$$C = -2.887 + (0.679D) - (0.166L) - (2.406I) + (3.079E) + (0.638S) - (0.73H).$$

Values of $C < 0$ were classified as *K. sandvicensis*, whereas fish with values of $C > 0$ were classified as *K. xenura*. When this equation was applied to fish of known species, 237 of 258 specimens (approximately 92%) were classified correctly.

Principal components analysis resulted in three axes that accounted for 94.25% of the variation in the morphometric dataset. Based on factor loading scores, principal component one represented the three head measurement variables, which were strongly and positively correlated to one another. Analysis of the second and third component axes was not straightforward. The second axes indicated that standard length (a strong negative loading score) and body depth (a positive loading score) were negatively correlated. Meanwhile, the third principle component shows a strong positive loading for depth and a weaker positive loading for standard length. A paired t-test was conducted on each of the first three factors to determine if any morphological differentiation, by species, existed. Probability values were, for factors one, two, and three, 0.059, 0.000, and 0.000. With axes two and three statistically significant, and axis one nearly so, differentiation by species is supported by these morphological data.

DNA sequencing and analysis

Among 1140 sites, 127 base pairs were variable (11.1%) and 106 were parsimony informative (9.3%). This corresponds to 16 of 380 variable amino acids (4.2%), five of which were parsimony informative (1.3%). Base substitutions were most frequent at the third position of the codons (109 variable sites), while substitution rates at the first and second positions were nearly identical (8 and 10 respectively). All sequences began with the start codon ATG, and no stop codons were present in any of the *Kuhlia* sequences examined for this study. For the *Kuhlia* sequences examined here, nucleotide compositional bias existed, especially at the second and third positions in each codon.

Overall, the average percent nucleotide composition was 28.7 for T, 31.0 for C, 25.2 for A, and 15.1 for G. This low percentage of guanine in cytochrome *b* sequences is reported for at least one other group of teleosts in the genus *Scomberomorus* (Spanish Mackerels) (Banford *et al*., 1999).

Twenty-five distinct haplotypes were noted from the 34 ingroup taxa. All 12 specimens of *K. xenura* had unique haplotypes. One haplotype was shared by five of the eight Johnston Island fish; this haplotype was also shared by a fish from O‘ahu that was identified as *K. sandvicensis*. In addition, a second Johnston Island haplotype was shared by a *K. sandvicensis* from O‘ahu and two fish of unknown morphotype from Hawai‘i. The remaining two Johnston Island fish each had unique haplotypes. Pairwise comparisons between *K. sandvicensis* and *K. xenura* revealed raw percent sequence divergences between 7.98% and 9.22%, with the average percent sequence divergence at 8.52% (Table 3). Comparisons between *K. xenura* and the Johnston Island *Kuhlia* yielded similar percent sequence divergences, with the mean at 8.45%. However, comparisons between Hawaiian *K. sandvicensis* and the Johnston Island *Kuhlia* yielded very different results, with an average percent sequence divergence of only 0.17%. Several Johnston Island fish had haplotypes identical to those for some of the *K. sandvicensis* specimens, and the range in sequence divergences between these two groups was from zero percent to 0.36%. This is similar to the range and mean for comparisons within the *K. sandvicensis* fish or when comparing the Johnston Island fish to one another. When *K. sandvicensis* and Johnston Island fish were grouped together and compared to *K. xenura*, the average percent sequence divergence was 8.49%. For all fish coded as “unknown type,” their sequences were always highly divergent from the *K. xenura* specimens (mean of 8.49%), and they were either identical to or at the most only 0.62% different from the *K. sandvicensis* and Johnston Island fish. Finally, all *Kuhlia* examined were roughly 19% divergent (uncorrected) from the outgroup *Z. streber*. A maximum likelihood analysis provided a tree with two monophyletic groups, a *K. xenura* clade and a clade containing both Hawaiian *K. sandvicensis* and the fish from Johnston Island. All eight of the unknown specimens were grouped within the *K. sandvicensis*/Johnston Island clade. The maximum likelihood analysis had a bootstrap value of 100% supporting the two aforementioned clades (Fig. 3).

Discussion

Discriminant function analysis proved to be an effective procedure for distinguishing and classifying species. With Johnston Island fish coded as *K. sandvicensis*, the analysis grouped the two *Kuhlia* species with 94% correctness based strictly upon morphometric data. While DFA maximizes differences between species and then classifies unknowns to these groupings, it does not answer the question of whether these groupings should be designated to begin with (Beuttell & Losos, 1999). In this study, genetic analysis supports our groupings, as does principal components analysis, which resulted in statistically significant factor score differences between the two species.

As for body shape differences and characteristics, both stepwise discriminant procedures indicated that eye diameter, depth, and interorbital distance were important characters for discriminating the two species. Overall, the eyes and body depths of *K. sandvicensis* are smaller than in *K. xenura* specimens of the same length. Conversely, the smaller-eyed *K. sandvicensis* have a larger interorbital distance than *K. xenura* specimens of the same size. One aspect of future research could be to add more specimens, of a larger size range, from Johnston Island. Interestingly, *K. xenura* specimens were more likely to be misclassified as Johnston Island fish than as *K. sandvicensis*. A further morphological comparison of the two populations of *K. sandvicensis* might indicate shape differences, although DNA sequence evidence, at least for *cyt b*, indicates that some level of gene flow is occurring between Hawai‘i and Johnston.

The DNA sequencing study indicated high percent sequence divergences between the two proposed *Kuhlia* species in Hawai‘i. Uncorrected *cyt b* divergences, which averaged 8.52%, suggest species level differences. According to Johns & Avise (1998), 90% of sister species pairs show at least 2% sequence divergence in their *cyt b* genes. Furthermore, the Johnston Island specimens (previous-

ly identified as *K. marginata* in Gosline, 1955 and Randall *et al*., 1985) are as closely related to the *K. sandvicensis* specimens as they are to each other. In addition, they are as divergent from the *K. xenura* specimens as are the *K. sandvicensis* individuals (mean of 8.45%). Maximum likelihood analysis revealed a tree with two well-supported clades (bootstrap values of 100), which correspond to the two species. In addition, the Johnston Island individuals were included within the clade containing *K. sandvicensis*, whereas the *K. xenura* specimens were reciprocally monophyletic. These results corroborate Randall & Randall's (2001) assertion that Johnston Island fish, formerly classified as *K. marginata*, a widespread fish throughout much of Oceania, are actually *K. sandvicensis*. Obtaining *K. sandvicensis* specimens for DNA analysis from other island groups besides Johnston and Hawai‘i, and determining definitively their distribution in the process, would be prudent.

Morphological and DNA sequence data provide strong evidence that there are two species present in Hawai‘i, with one of them being the same species that is present in Johnston Island. These data support Randall & Randall's 2001 conclusion that the "big-eyed" morphotype is correctly renamed *K. xenura*, and the small-eyed morphotype and the Johnston Island fish are *K. sandvicensis*. Even though morphological differences between the two species are subtle, it is possible, in some cases, to identify fish based solely on superficial appearance. However, correct identification of specimens is occasionally difficult, as features like reticulations and stripes often fade when the fish are frozen or preserved (Randall & Randall, 2001; pers. observ.). Use of a classification equation, which is less time consuming and costly than DNA sequence analysis, is advantageous. Because traditional meristic counts in the two forms of *Kuhlia* overlap, this morphometric classification equation will be helpful in telling apart fish whose stripes or reticulations may have faded after death or whose eye sizes are intermediate. In addition, it may be possible, by using this equation, to analyze voucher specimens from past *Kuhlia* studies, where investigators did not designate the "type" on which they were working.

Young *K. xenura* are ubiquitous in the lower reaches of Hawaiian streams, but this species' use of fresh water is facultative (Benson & Fitzsimons, 2002). Conversely, *K. sandvicensis* has not been observed in freshwater streams; members of this species likely encounter reduced salinities only in tide pools where there is freshwater input from subsurface runoff. Now that two species of *Kuhlia* have been identified in Hawai‘i and because there appear to be discrete habitat differences for them (Benson & McRae, unpubl. data), management strategies currently in place must be examined to assure that they adequately protect both species. Previous studies upon which management decisions have been based should be reconsidered. The single study on reproduction in Hawaiian *Kuhlia* by Tester & Takata (Tester, A.L. & M. Takata. 1953. A contribution to the biology of the *āholehole,* a potential baitfish. Industrial Research Advisory Council Grant no. 29, 1953. Hawaii Marine Laboratory, 54 pp.), for example, is problematic because it is not known which of the two species of *Kuhlia* was used in the study. Their status as popular food fishes, coupled with the evidence that *K. xenura* appears to be endemic to the Hawaiian Islands, makes proper identification, monitoring, and management practices essential for their conservation.

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