

**ASPECTS OF THE BEHAVIORAL ECOLOGY, LIFE HISTORY,
GENETICS, AND MORPHOLOGY OF THE
HAWAIIAN KUHLIID FISHES**

A Dissertation

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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 and were often used by Hawaiians in traditional ceremonies. Local fishermen have noted the presence of two morphotypes in Hawai‘i, although at the beginning of this study only one species, *Kuhlia sandvicensis*, was identified in the scientific literature. For this dissertation research, morphological and DNA studies of the two morphotypes determined that two species of the genus *Kuhlia* do exist in Hawai‘i. Subsequently, Randall and Randall (2001) published a revision of this genus, which included a description, based on limited meristic evidence, of the “big-eyed” morphotype as *Kuhlia xenura*. This fish is apparently limited to Hawai‘i, and it is the species found commonly in freshwater streams. In addition to the morphological and DNA analysis, I investigated the life history of both Hawaiian *Kuhlia*. Electron microprobe techniques were used to analyze otolith daily increments to answer questions about whether freshwater use was obligate in these species’ life cycle. I found that *K. xenura*’s use of stream habitats is facultative, although all individuals examined had spent time in fresh water at some point during their life. *Kuhlia sandvicensis* individuals also resided in water of decreased salinity at various points in their life cycle. However, there appears to be differences in habitat preferences by the two species, with only *K. xenura* residing in freshwater streams. Future research comparing their ecology will provide a greater understanding of the importance of streams and tide pools as nursery habitats for these fishes. 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 my

results that these two “types” are separate species with genetically distinct populations. 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.

INTRODUCTION

For this dissertation, I investigated selected aspects of the genetics, morphology, habitat, and life history of the genus *Kuhlia* in Hawai‘i. Also called the Hawaiian flagtail or āholehole, these fishes are marine teleosts whose congeners occur in the tropical and subtropical Eastern Pacific and Indo-Pacific. They live in schools on or near coral reefs as adults and are often found in the lower reaches of streams as juveniles. There they feed on aquatic invertebrates, insects, algae, detritus, and stream fishes. Many *Kuhlia* species are tolerant to a range of temperatures and salinities. Āholehole (adults are sometimes called simply āhole) in Hawai‘i grow to about 30 cm total length and are important food and sportfishes in the Hawaiian Islands (Tester and Takata, 1953; Gosline and Brock, 1965).

Kuhlia were once a favorite food for Hawaiian royalty as well; one report describes a chiefess that desired live āholehole to be eaten raw. The fish was wrapped in wet seaweed and kept alive from the Puna coast to be delivered to her in Hilo, a distance of about 30 miles (Pukui, 1893). Āholehole were also important culturally in the Hawaiian Islands, as they were used in a variety of ceremonies. Pigs were important offerings to Hawaiian gods; when they could not be found, āholehole could be substituted because they were one of the fishes considered a “sea pig.” In addition, they were used in rituals to ward off evil spirits, as the word “āhole” means to strip away (Titcomb, 1972). Āholehole were buried under new structures that were being built to deter any evil influences (Pukui, 1865).

Prior to my investigation, only one kuhliid species was reported to exist in Hawai‘i (Tester and Takata, 1952; Gosline and Brock, 1965; Tinker, 1978; Randall, 1985; Randall, 1996; Witte and Mahaney 1998). This species, *Kuhlia sandvicensis*, was described by the German ichthyologist Franz Steindachner (1876) as *Moronopsis argenteus* var. *sandvicensis*.

Many synonyms for this species occur in the literature and include, *Moronopsis sandvicensis*, *Kuhlia malo*, *Dules sandvicensis*, and *K. bilunulata* (Steindachner, 1877; Fowler, 1938; Schultz, 1943; Jordan and Evermann, 1973). *Kuhlia malo* is actually a separate species, found in fresh waters of French Polynesia. Incidentally, an attempt to introduce *K. malo* to Hawaii's streams in 1958 was thwarted when the fish died enroute because they were being transported in a holding tank filled with salt water (Randall and Randall, 2001).

Kuhlia sandvicensis was reported by many ichthyologists to be endemic to the Hawaiian Islands (Randall et al., 1985; Lewis and Hogan, 1987; Randall, 1996; Witte and Mahaney, 1998). However, further investigation of the literature revealed contradictory opinions. For example, many, especially older, references indicated that this species was found elsewhere, including Johnston Island, Palmyra Atoll, the Tuamotu Archipelago, Wake Island, the Society Islands, the Marquesas Islands, Fiji Islands, South Africa, Australia, and the Phoenix and Samoan Islands (Fowler 1938, Schultz 1943, Tester and Takata, 1952; Tinker 1978).

Adding to this taxonomic confusion was the discovery that local fishermen often identified two different Hawaiian morphotypes of *Kuhlia sandvicensis*, based on eye size. This eye-size difference is mentioned in some publications, but the descriptions are inconsistent as to which type of fish lives in which habitat (Titcomb, 1972; Hosaka, 1973). In addition, some older references in the scientific literature referred to the fact that other kuhliid species, *K. taeniura*, *K. marginata*, *K. rupestris*, and *K. nutabunda* (normally associated with South Pacific Islands), were found in Hawai'i (Titcomb, 1952; Titcomb, 1972; Jordan and Evermann, 1973; Tinker, 1978). One of these species, *K. rupestris* from Guam, was

introduced to Hawaiian waters in 1958 as a potential food and sportfish, but it did not colonize (Randall, 1996).

No modern day records indicate separate Hawaiian names that represent the two “types” of āholehole currently recognized by fishermen in Hawai‘i. Ancient Hawaiians had their own taxonomic system for identifying and classifying flora and fauna, especially those species that were edible or had medicinal uses. In fact, their naming system was likely similar to the scientific names we use today. Modern day scientific names are comprised of a genus (a general name) and a specific epithet or descriptor. The ancient Hawaiian names for many species were also binomial, with the second word being more descriptive and specific (Titcomb, 1972). For example, various unicornfish species in Hawai‘i were given the group name “kala,” which means, among other things, “thorn” (Pukui and Elbert, 1992). This designation may be in reference to either the sharp bony projection that these fish have at the base of their tails or the characteristic projection on their forehead. The spotted unicornfish or *Naso brevirostris* has the second Hawaiian name of lōlō, which means lazy; these fish are known to sleep on the bottom at night and are easily approachable at this time. This Hawaiian title is probably a “better” description for the fish than its somewhat inaccurate scientific name, which means “short-horned.” Although juvenile spotted unicornfish lack a horn, the adult fish actually have a very long, prominent one (Hoover, 1993). Unfortunately, many of these Hawaiian fish names, especially the second, more specific descriptor, have been lost and are not recorded in the scientific or Hawaiian literature.

In addition to their binomial naming system, Hawaiians occasionally had different names for the separate stages of a species’ life cycle. For example, the bird wrasse, which has a very long, curved snout, has the Hawaiian name hīnālea ‘i‘iwi for its terminal phase; ‘i‘iwi

is the same name given to a honeycreeper with a long, curved bill. In its initial phase, Hawaiians termed them hīnālea ‘aki-lolo; this means “brain biting” and probably refers to the use of these fish in the treatment of brain diseases (Hoover, 1993). It is not clear whether the Hawaiians recognized these as two different phases of the same fish, or if they thought they were two different species. Hawaiians developed a detailed naming system for the different life history stages and sizes of *Kuhlia*. Young āholehole were termed pua-hole or pua āholehole; the Hawaiian word “pua” means descendant. Half-grown fish were sometimes called ‘apo‘apo meaning “grasp” (Titcomb, 1972). Full grown fish are called simply āhole or āholehole. For other fish species, many of these life stage names were unfortunately lost or forgotten before they could be recorded by biologists or historians.

There are many studies on the life history of Hawaiian marine (especially coral reef) species. In addition, several investigations of the life cycle and habitat of Hawaii’s amphidromous species, which migrate between streams and the ocean, have been conducted. However, there have been relatively few scientific investigations of the biology of the inshore taxa that move between fresh water and the ocean. This paucity of studies is especially strange considering these fishes (āholehole, moi, ‘ama‘ama, etc.) were often reared in ancient Hawaiian fishponds and continue to be popular food fish today. One noteworthy study, “Contribution to the Biology of the Āholehole, a Potential Baitfish,” was published by Albert Tester and Michio Takata (1953) for Hawai‘i’s Industrial Research Advisory Council. Their research concluded that āholehole scales and otoliths could not be used as annular growth indicators, so they conducted laboratory age and growth studies, looking primarily at length frequency modes in different populations. Some aspects of the reproduction of Hawaiian *Kuhlia* species were also examined, and fecundity data were provided. Tester and Takata

(1953) examined recruitment of juvenile āholehole into nearshore habitats and concluded that they spawn year round, but have a peak in spawning during the winter and spring months. Although they did not observe fish spawning in marine habitats, they did collect adults with ripe ova from the ocean. They also collected mature adults from fishponds, but there was no evidence that fish spawned in fresh or brackish water. For Tester and Takata's (1953) extensive studies, it is likely that their report included data collected from both species as they mention that some of the small āholehole have black reticulations on the top of the head. Except for a comment by McDowall (1988) that *K. sandvicensis* in Hawai'i may be catadromous (migrating from fresh to salt water for reproduction), I am not aware of any studies on the reproduction of this species. Regardless, McDowall does not refer to the big-eyed vs. small-eyed morphotype question.

In addition to the aforementioned habitat and life history studies, some other biological information is available for *Kuhlia* in Hawai'i. Tester and Takata (1953) examined the gut contents of fish of varying sizes from several different habitats. They also provided information on rearing fish in captivity. Because they were interested in the potential use of āholehole as bait fish, most research centered around the tolerance of āholehole to different environmental conditions; they were found to be euryhaline at all ages and able to withstand a wide temperature range. Other investigations into the biology of the āholehole include diet (Tester and Trefz, 1954) and laboratory growth studies (Nakamura, 1968). Several physiology, toxicology, metabolism, and oxygen consumption studies were conducted in the 1950s, 1960s and 1970s with *K. sandvicensis* as the model or experimental fish for the lab research (Takata, 1953; Nakamura, 1965; Niimi, 1966; Bridges, 1967; Muir and Niimi, 1972,

Hiatt et al., 1952). It is not possible to identify which morphotype was used in the aforementioned studies.

I sought to investigate the habitat requirements for Hawaiian kuhliids. Much of the basic knowledge of āholehole is not published but based on farming practices by Hawaiians. Ancient and modern Hawaiian people use *Kuhlia* in their fishponds, so these farmers were and are aware of their fishes' needs. Many seaside ponds have brackish water or fluctuating salinity, and fish like āholehole, that could tolerate this variable habitat, were grown in these ponds (Kikuchi, 1976). The Hawaiians used this knowledge of the animals' life history and behavior to collect them from the pond systems. They knew that young āholehole and other marine fish migrate from the ocean into inshore habitats where they are attracted to protected, coastal areas like fishponds. As juveniles, they probably entered the ponds and were trapped. As they matured they, like other fishes, tended to migrate towards the ocean and spawn. As they congregated around the gates (or makahas) that blocked access to the open ocean, they were easy to capture (Titcomb, 1972).

In 1999, I discovered what appeared to be a second morphotype of *K. sandvicensis* in Hawai'i. Since traditional meristic counts overlapped greatly for the two types, I initiated a DNA sequence analysis and morphometric study of these fish. While these studies were being completed, Randall and Randall (2001) published (independently) a review of Central Pacific *Kuhlia* in which the two *Kuhlia* morphotypes in Hawai'i were identified as separate species. The authors used primarily meristic counts to differentiate between the two species. They reported that the small-eyed morphotype resembled most closely the original species description of *K. sandvicensis* by Steindachner (1876) and used the available name *Kuhlia xenura* for the big-eyed type. The research described herein includes a morphometric and

DNA sequence analysis of these two morphotypes and adds further evidence and justification for the recognition of a second *Kuhlia* species in Hawai‘i.

Although āholehole are an important part of stream and reef ecosystems and Hawaiian culture, little is known about these fishes’ life history and specifically whether or not a freshwater phase is obligatory for one or both species to complete development. This question, along with an attempt to confirm the status of the two morphotypes present in Hawai‘i, is the focus of the following research.

NOTE: This dissertation is presented as two scientific papers, one in preparation for publication and the other in press as Benson and Fitzsimons (a portion of Chapter 2). For clarity, some of the material provided in the preceding introduction may be repeated in the individual chapter introductions.

DNA SEQUENCE VARIATION AND MORPHOMETRIC ANALYSIS OF TWO SPECIES OF *KUHLIA* IN HAWAI'I

INTRODUCTION

Kuhlia sandvicensis, commonly called the āholehole or Hawaiian flagtail, is a marine teleost thought, at the beginning of this study, to be endemic to the Hawaiian Islands and the only kuhliid species in Hawai'i. About nine other members of this family occur in the Central Pacific. Local fishermen have noted the presence of two "types" of *Kuhlia* in Hawai'i and have divided them into groups based on eye size. Other morphological differences are noticeable, including the presence of wide black reticulations on the head that extend posteriorly as two black lines along either side of the dorsal fin in the small-eyed morphotype (Figure 1). In addition, during my preliminary observations, I noted that the small-eyed type has a white patch on the posterior section of its soft dorsal fin and appears to be less deep-bodied than the larger eyed animals. In addition, this small-eyed type's color is silver dorsally and whitish below, while the big-eyed type sometimes appears almost olive along its dorsal surface. The big-eyed morphotype also has reticulations on the head, but these are much fainter and very narrow (Benson and Fitzsimons, in press). The eyes of this type reflect red along their upper edge (Figure 2).

Near the completion of this study, a revision of the family Kuhliidae by Randall and Randall (2001) included a description of the big-eyed morphotype as a second species of *Kuhlia* in Hawai'i. The available name assigned to these fish was *Kuhlia xenura*, whereas the small-eyed type retained the name *K. sandvicensis*, based primarily on a lateral line scale count in the original Steindachner (1876) description. The authors noted the eye size difference between the two species and also included meristic data for both

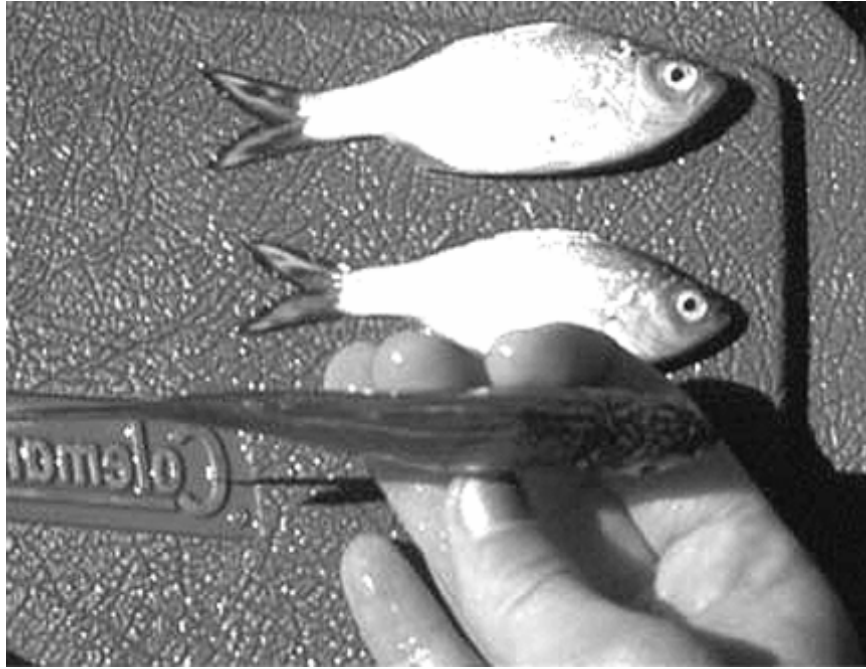


Figure 1. Photograph of *Kuhlia sandvicensis* juveniles showing dorsal head reticulations.



Figure 2. Photograph of a juvenile *Kuhlia xenura* showing large eye with reddish color dorsally.

species, including dorsal, anal, and pectoral ray counts. However, these meristic characters overlap in these two species; the least amount of overlap occurs when counting the gill rakers. *Kuhlia xenura* have an overall lower average gill raker count than *K. sandvicensis*.

Because Randall and Randall (2001) used characters that are highly overlapping to differentiate the two Hawaiian *Kuhlia*, I investigated DNA sequence variation and morphometrics to confirm the validity of recognizing two species in Hawaiian waters. Body measurements were conducted and analyzed by discriminant function analysis (DFA) to determine if the specimens could be classified on the basis of morphometric data. Discriminant function analyses are usually conducted for classification purposes and to examine relationships between and among groups (Pimentel, 1979). DFA is a useful technique because it not only shows how different two or more populations are, but it also helps to maximize the separation between groups (Burbrink, 2000). In addition, it provides an equation that helps to determine the morphotype or species to which an unknown specimen is nearest. Finally, the discriminant function stepwise analyses can reduce the number of variables to those needed to discriminate among groups with the highest percent correctness.

In the last 40 years, techniques that provide data on molecular evolution have become widely used in the field of systematics. Analyses of DNA sequence variation have served as useful tools in understanding population structure and building phylogenies (Moritz and Hillis, 1996). Furthermore, combined molecular genetic, morphological, and behavioral data help to answer basic taxonomic questions. To investigate the genetic relationships between the *Kuhlia* species in Hawai'i and Johnston Island, a phylogenetic analysis of DNA sequences from the mitochondrial cytochrome *b* (*cytb*) gene was conducted.

Cytochrome *b* was chosen because the rate of evolution of this gene is appropriate for measuring divergence between close species and populations (Moritz et al., 1987). In addition, numerous studies using this gene have developed a wealth of primers for *cytb* amplification. Finally, mitochondrial DNA is useful because it is maternally inherited; one can eliminate the confusing effects caused by recombination.

Included in this study were DNA sequences obtained from a similar Johnston Island species (Johnston is the nearest island to the Hawaiian archipelago). A study by Gosline (1955) on the Inshore Fish Fauna of Johnston Island provided information on what he calls the “*Kuhlia sandvicensis-marginata* Complex.” At that time, he considered only one species to exist in Hawai‘i, which he called *Kuhlia sandvicensis*, and a different species to exist in Johnston Island (*K. marginata*). *Kuhlia marginata*, described by Cuvier and Valenciennes (1829), was reputed to be widespread throughout the Indo-Pacific (Tinker, 1978); however *K. marginata* from Johnston Island greatly resembles the small-eyed āholehole from Hawai‘i, *K. sandvicensis*. Gosline (1955) included an in-depth comparison of gill rakers in *Kuhlia* from Hawai‘i and Johnston. He found differences in fish taken from various places throughout the Hawaiian archipelago, including one mention of differences between those taken from fresh water versus fish from salt water. Likely, Gosline was counting gill rakers from both *Kuhlia* species in Hawai‘i, which caused some confusion. Fish from Johnston Island had an overall higher gill raker count (lower limb) than those from Hawai‘i. The aforementioned Randall and Randall study (2001) provided evidence that the species on Johnston is actually *K. sandvicensis*, and this species, both in Johnston and Hawai‘i, has an overall higher gill raker count than *K. xenura*, the species found only in Hawai‘i.

According to Gosline (1955), the earlier identification of *K. marginata* on Johnston Island, but not in the Hawaiian Islands, was somewhat of an ichthyological anomaly. In fact, there are (to date) only 10 fish species found at Johnston Atoll but not in Hawai‘i. This information has been used to refute the idea that fishes arrived in Hawai‘i via a stepping stone pathway from the south through the Central Pacific. Rather, this information supports the hypothesis that the great majority of fishes in the Hawaiian Islands arrived there by way of the Ryuku Islands and southern Japan (Gosline, 1955; Randall et al., 1985). Therefore, the presence of *K. marginata* in Johnston Island was somewhat unlikely and further added to the taxonomic confusion of the kuhliids. Consequently, I selected a combined genetic and morphological approach to answer taxonomic questions about both the kuhliids in Hawai‘i and the species at Johnston Island.

MATERIALS AND METHODS

Specimen Collection

Fishes for this study were collected from several sites on the islands of O‘ahu and Hawai‘i (Figure 3, Table 1). Specimens were collected by a variety of methods, including beach seine, castnet, hook and line, and ‘ōpae net (a small, hand-held seine). Collections took place during March and April 1997, January and May 1998, March and July 1999, January through April 2000, and June and August 2000. Specimens from Johnston Island were provided by the U.S. Fish and Wildlife Service in March 2000. After collection, fishes were immediately frozen for later use in the morphometric study and DNA analysis. In some cases, after being thawed, a fish could not be identified to species because the specimen was in poor condition or the fish was very small. Regardless, some of these “unknown” fish were included in the investigation, and are coded separately in the results below. At the conclusion

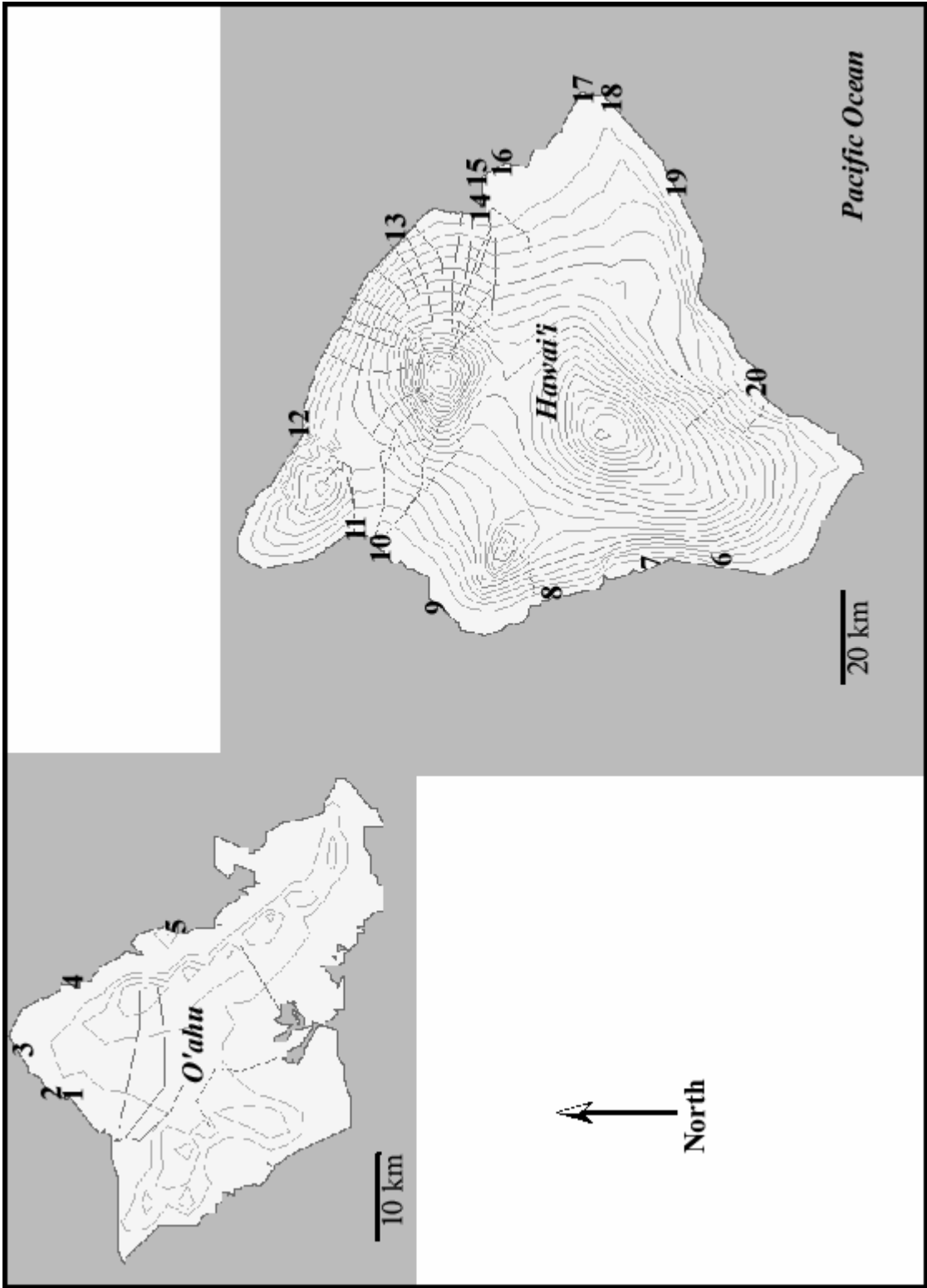


Figure 3. *Kuhlia* collection sites in the Hawaiian Islands for morphometric and DNA study.

Table 1. Locality, habitat and species information for *Kuhlia* collections in Hawai‘i. Species were identified, at time of collection, based on external morphology. (Note: site numbers match map numbers in Figure 1).

Site #	Locality	Habitat	Species Collected
1	Waimea Stream	stream mouth	<i>K. xenura</i>
2	Shark’s Cove	tide pools and reef	<i>K. sandvicensis</i> *
3	Turtle Bay	tide pools	Both
4	Kōloa Stream	stream mouth	<i>K. xenura</i> *
5	Mokoli‘i Island Park	sandy beach	Both *
6	Miloli‘i	tide pools	<i>K. sandvicensis</i>
7	Ho‘okena	tide pools	<i>K. sandvicensis</i>
8	Keahou	tide pools	Both
9	Hualālai	tide pools	Both
10	Kalāhuipua‘a	fishponds	<i>K. xenura</i>
11	Puakō Reef	tide pools	Both *
12	Wailoa Stream	stream lower reach	<i>K. xenura</i>
13	Hakalau Stream	stream mouth	<i>K. xenura</i>
14	Wailoa River	river lower reach	<i>K. xenura</i>
15	Richardson’s Beach Park	tide pools	<i>K. sandvicensis</i>
16	King’s Landing	tide pools	<i>K. sandvicensis</i>
17	Kapoho	surge zone	Both
18	Puna Coast	surge zone	Both
19	Hawai‘i Volcanoes Natl. Park	surge zone	Both *
20	Punalu‘u Beach Park	tide pools	Both

* Specimens used in DNA analysis

of the study, specimens were fixed in 10% formalin for at least one week, and then preserved in 70% ethanol. All fishes will be placed in the Ichthyology Collection at the Louisiana State University Museum of Natural Science.

DNA Extraction

Fishes were thawed and tail muscle was removed. Tissue was either used immediately in the extraction process or placed in 1.5 ml microcentrifuge tubes containing the preservative DMSO (dimethyl sulfoxide-salt solution) for storage (Seutin et al., 1991). About 0.25 g of tissue was ground and homogenized in 1ml of STE buffer (a pH=7.5 solution of 0.1M NaCl, 0.05M tris-HCl, and 0.001M EDTA), by using a sterile micropestle. If tissue was preserved in DMSO, excess solution was blotted off before grinding. For isolation of mitochondrial DNA, the tissue solution was digested with 25 μ l of Proteinase K solution (20 mg/mL) and 25 μ l of 20% SDS (sodium dodecyl sulfate) and incubated for 12-15 hours in a water bath at 55°C. DNA was extracted from the solution by adding 500 μ l of a phenol/chloroform/isoamyl alcohol solution (25:24:1). This mixture was vortexed and spun in a microcentrifuge for two minutes at 14,000 rpm; the aqueous supernatant was removed and transferred to a new microcentrifuge tube. The extraction process was repeated one or two more times before the DNA was precipitated with 1/10 volume of 3M sodium acetate and 1 ml of absolute ethanol. The solution was centrifuged at 14,000 rpm to pellet the DNA sample. Ethanol was decanted, the pellet was dried in a vacuum centrifuge for 15 minutes, and then resuspended in 50 μ l of water. Working samples of the genomic DNA were stored in the laboratory at -4°C.

PCR Amplification

The polymerase chain reaction was used to amplify the entire cytochrome b gene (1140 base pairs). The region was amplified 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 2). Both L-14724 and cyt b-L are general fish primers (Pääbo, 1990; Reed et al., in press), while cyt b-HKu was designed by the author to be more specific for the study taxa.

The amplifications were carried out in 50 µl reactions: 1 or 3 µl purified genomic DNA as template, 5 µl 10X PCR Buffer II or 10X PCR Gold Buffer (Perkin Elmer), 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), 30.75-32.75 µl water, 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). These amplifications began with either a 3 minute or 10 minute (for the AmpliTaq Gold) denaturing step at 94° C, followed by 30 cycles of denaturing at 94° C for 45 seconds, primer annealing at 50° C or 54° C for 45 seconds, and elongation at 72° C for one minute 15 seconds (modified from Palumbi, 1996). These cycles were followed by a final extension at 72° C for 10 minutes, and then storage at 4° C and/or frozen at -4° C. Negative controls were included in most PCR reactions to detect contamination.

Five µl of the PCR reaction mixture was stained with ethidium bromide and loaded into a one percent agarose gel for electrophoresis. Visualization under an ultraviolet light verified the presence or absence of the desired amplified product, and most gels were photographed. 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

Table 2. 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., in press
L-14724	TGA CTT GAA RAA CCA YCG TTG	Pääbo, 1990
L431	GAG GAC AAA TRT CYT TCT GAG G	Reed et al., in press
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

GeneClean® Kit (BIO 101, Carlsbad, CA) to remove incomplete amplification products and unincorporated dNTPs. Purified PCR product was stored in water at -4° C.

DNA Sequencing

Several primers were designed and used for the subsequent cycle sequencing reactions (Table 2). These reactions were performed on the PCR products by using the ABI PRISMBig Dye® kit (PE Applied Biosystems, Foster City, CA). Cyt b-L and L-14724 served as 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 used to modify the L-431 and Cyt b-L primers to be more specific for these specimens; they were 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, including 2 µl or 3 µl PCR product, 3.2 µl of 1µM primer, either 2 µl (light strand) or 3 µl (heavy strand) of Big Dye reaction premix, and 0.8 - 2.8 µl of ddIH₂O or 2.5X sequencing buffer. The reaction was carried out in one of three machines, depending 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°C, 5 seconds at either 48°C or 50°C, 4 minutes at 60°C, and storage at 4°C 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).

Phylogenetic Analyses

The 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). Unreadable portions at the ends of each fragment were trimmed off, and the sequences were visually examined for light and heavy strand discrepancies and reading frame errors. Any mistakes that could be contributed confidently to gel-reading errors were corrected manually. Some specimens heavy strand fragments could not be sequenced successfully; depending on the quality of the light strand sequences, some of these specimens were still included in the study.

This clean-up process resulted in 34 sequences that could be aligned to one another. Seven of the 34 sequences had less than 1140 base pairs; these ranged in length from 1116 to 1136 base pairs. A BLAST search (Altschul et al., 1990) in GenBank was conducted, and the closest sequence in their database was from *Zingel streber*, a percomorph fish in the same suborder as the kuhliids. This sequence was downloaded and used to help align the study specimens. After alignment, all base discrepancies were examined on the original chromatograms to determine if they should be changed. Finally, each aligned sequence was checked for stop codons. Sequences representing each study group will be deposited in Genbank upon publication. Analyses of nucleotide composition and percent informative sites were conducted with the program MEGA version 2.1 (Kumar et al., 2001).

Uncorrected pairwise sequence divergences between all taxa were calculated in PAUP* 4.0 (Swofford, 1998), which is a computer program that analyzes molecular,

morphological, or behavioral data to infer phylogenetic relationships. Based on these results, a neighbor joining tree was built by 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 and Crandall, 1998) to determine a base substitution model and other parameters for a maximum 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 (α) 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 of the optimal branching diagrams was examined by a bootstrap analysis of 100 replicates. Trees were visualized and manually rooted at the midpoint by using the program TREEVIEW (Page, 1996).

Mensural Data Collection

Specimens were thawed and then measured, to 0.1 mm, with hand-held electronic digital calipers (Mitutoyo Plasti-Cal). Fishes too large to be measured with calipers were measured, to the nearest mm, on a measuring board for standard and fork length. A total of 287 *K. xenura* and 132 *K. sandvicensis* (29 from Johnston Island) were analyzed for this study. Both male and female fishes were used in this analysis. There is no external sexual dimorphism in this species, and sex data were not available for all specimens. Measurements included standard length (*L*), fork length (*F*), snout length (*S*), head length (*H*), body depth (*D*), eye diameter (*E*), and interorbital distance (*I*) (Figure 4). These variables were chosen because they are standard classification measurements in ichthyology, and they serve to encompass or summarize the shape of these organisms, as per Pimentel (1979). Standard

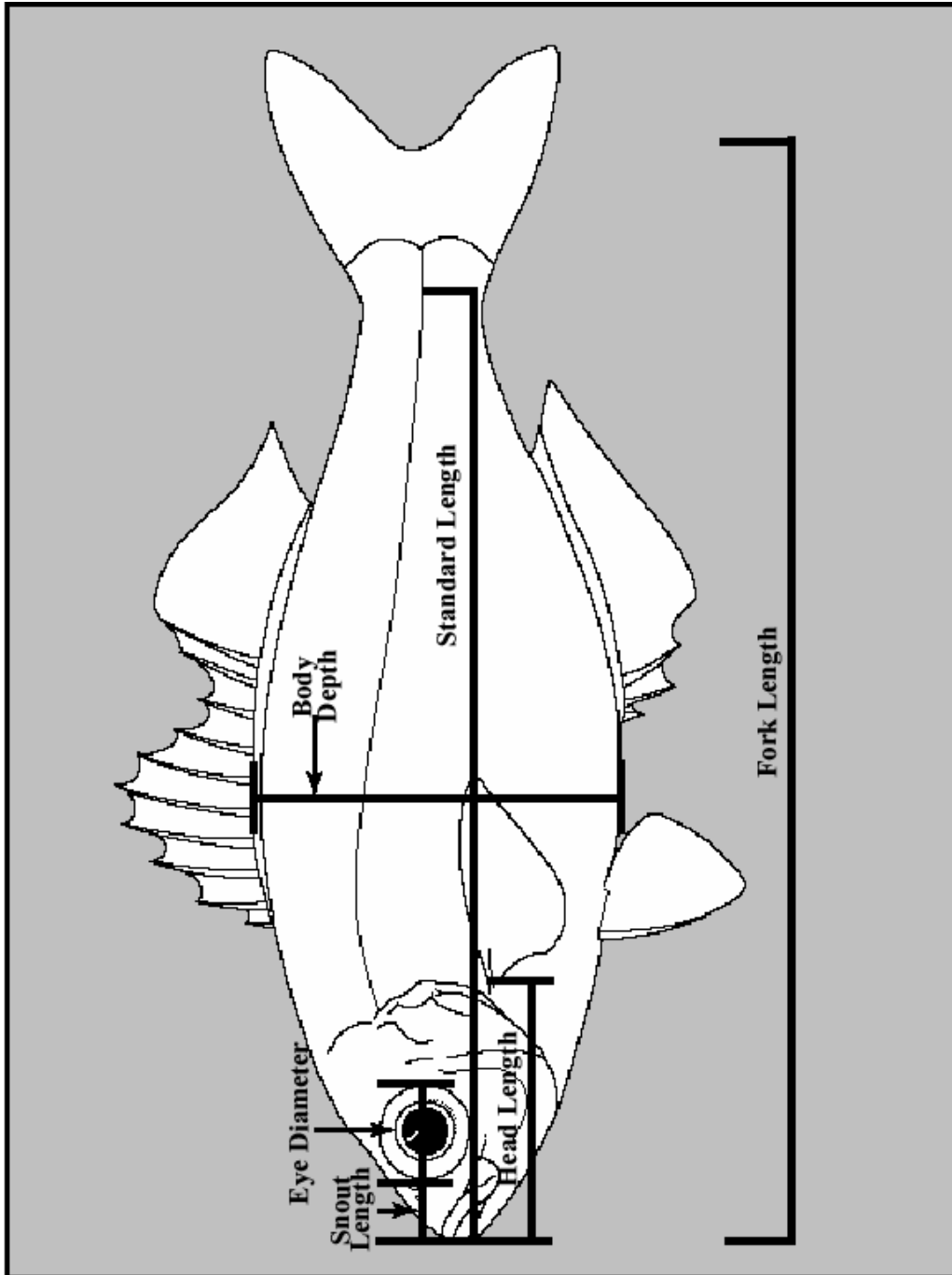


Figure 4. Generalized diagram of *Kuhlia* showing mensural characters for morphometric study. Not pictured is the interorbital distance, which was measured at the narrowest point between the eyes, along the dorsal surface of the head.

length was measured as the straight line distance from the anterior point of the upper lip to the posterior end of the hypural plate; fork length was measured from the same point to the fork of the caudal fin. Snout length was measured from the same anterior point to the fleshy posterior edge of the orbit. Head length was measured from the anterior point of the body to the posterior edge of the opercular membrane. Greatest body depth was measured from the base of the dorsal fin straight to the ventral surface. Eye diameter is the greatest fleshy width of the orbit; interorbital distance was measured at the narrowest point between the eyes, along the dorsal surface of the head.

Morphometric Analysis

Measurement data were analyzed in SigmaPlot version 4.01 (SPSS, 1997) to test for allometric influences, which cause differing patterns of growth between juveniles and adults (Thorpe and 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 (Figures 5 and 6). 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). This procedure resulted in a sample size of 158 *K. xenura* and 100 *K. sandvicensis* (29 from Johnston Island). In a study of *Kuhlia* on Johnston Island, Gosline (1955) also included only fish greater than 40 mm in his meristic 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

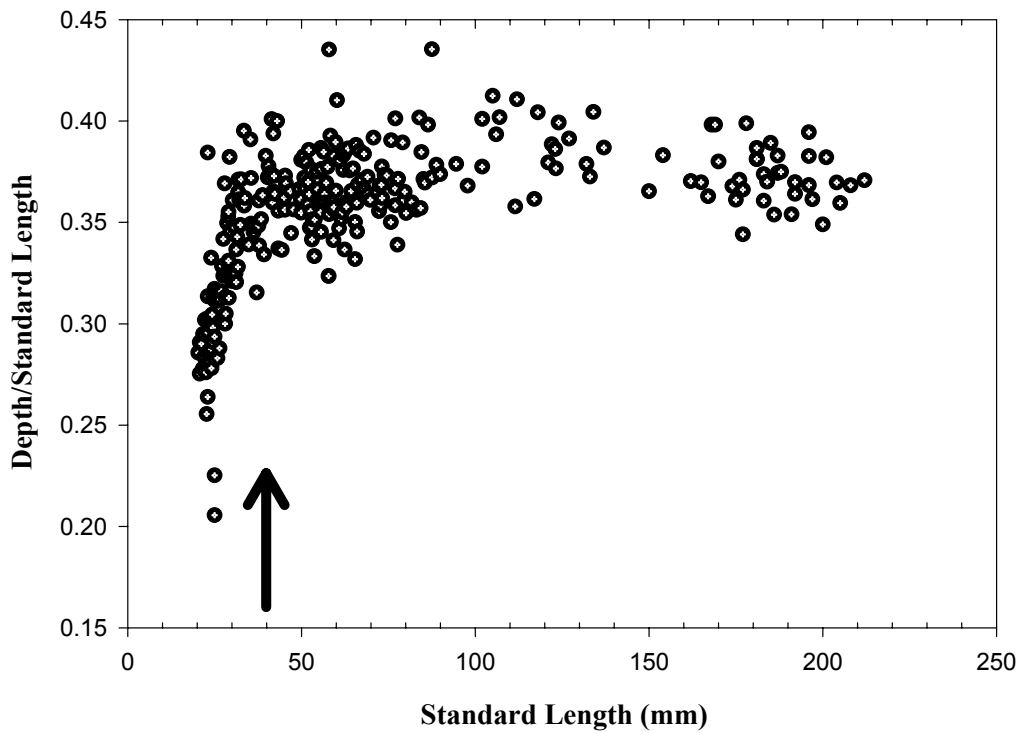


Figure 5. Plot of Body Depth/Standard Length against Standard Length for *Kuhlia xenura*. Arrow indicates where this relationship begins to level off.

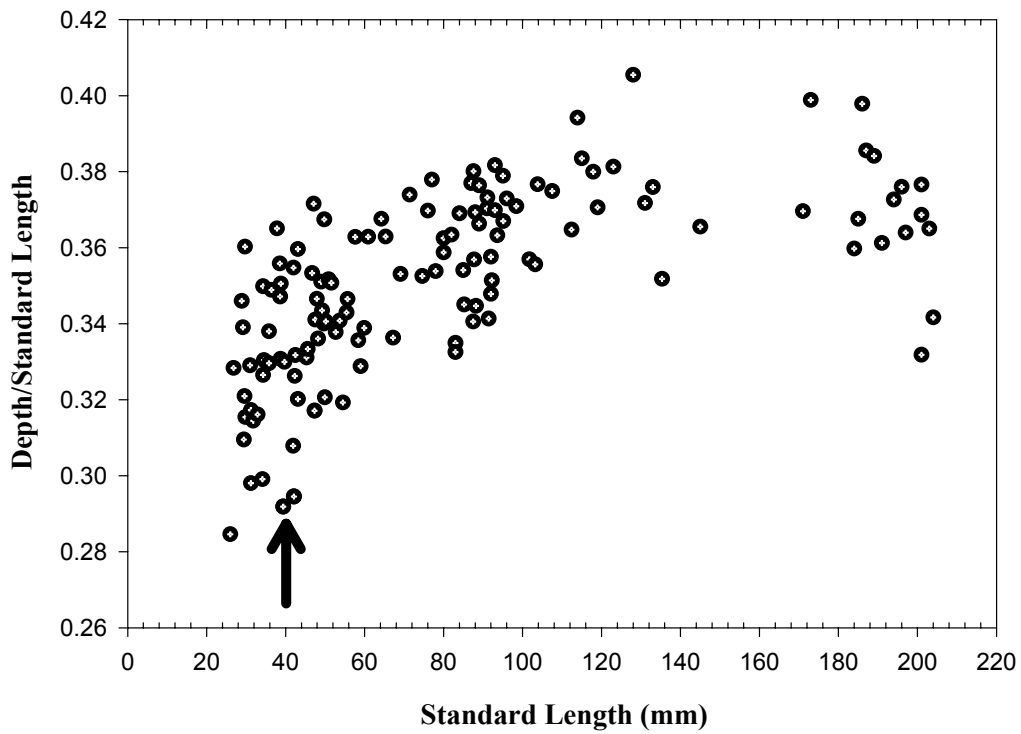


Figure 6. Plot of Body Depth/Standard Length against Standard Length for *Kuhlia sandvicensis*. Arrow indicates where this relationship begins to level off.

five variables were used in subsequent multivariate analyses to eliminate the effect of multicollinearity caused by variables that are correlated to one another. Multivariate statistical analyses were conducted in SYSTAT version 8.0 (SPSS, 1998) and included a MANOVA, by using the Wilks' lambda statistic, to test for a difference between group means. A discriminant function analysis was used 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.

From this output, classification 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. This results in a canonical score or value (C) which can be compared to a cutoff criterion differentiating the two morphotypes. 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.

RESULTS

Sequence Characteristics

Of the 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 are 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). High substitution rates at the third position are expected for protein coding genes. Due to the degeneracy of the genetic code, the third positions of codons evolve at a much more rapid rate than first or second position bases (Swofford et al., 1996). No stop codons were present in any of the *Kuhlia* sequences examined for this study. For these reasons, it is believed that no pseudogenes were sequenced, and all sequences analyzed represent *cytb*. All sequences began with the start codon ATG, which codes for the amino acid methionine. As previously mentioned, 27 of the 34 sequences were 1140 base pairs long (376 amino acids). The seven remaining specimens could not be sequenced to the termination of the gene.

For the *Kuhlia* sequences examined here, nucleotide compositional bias existed, especially at the second and third positions in each codon. The average percent frequencies of T, C, A, and G at the first position were 23.2, 26.3, 23.6, and 26.9. Percentages of these bases at the second position were 41.3, 25.3, 19.7, and 13.7. At the third position, the percent frequencies were 21.8, 41.2, 32.3, and 4.7. 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 *cytb* sequences is reported for at least one other group of teleosts in the Genus *Scomberomorus* (Spanish Mackerels) (Banford et al., 1999).

Phylogenetic Analysis.

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. One *K. sandvicensis* specimen and one fish of unknown morphotype, both from Hawai‘i, shared a haplotype. Finally, the three remaining *K. sandvicensis* specimens (all from O‘ahu) and the five remaining fish of unknown morphotype (all from Hawai‘i) 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 identical haplotypes to 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 are grouped together and compared to *K. xenura*, the average percent sequence divergence is 8.49%. For all of the 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 neighbor joining tree was constructed to visualize these results (Figure 7). This tree revealed a phylogeny with two monophyletic groups, a *K. xenura* clade and a clade containing both Hawaiian *K. sandvicensis* and the fish from Johnston Island. Furthermore, all eight of

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

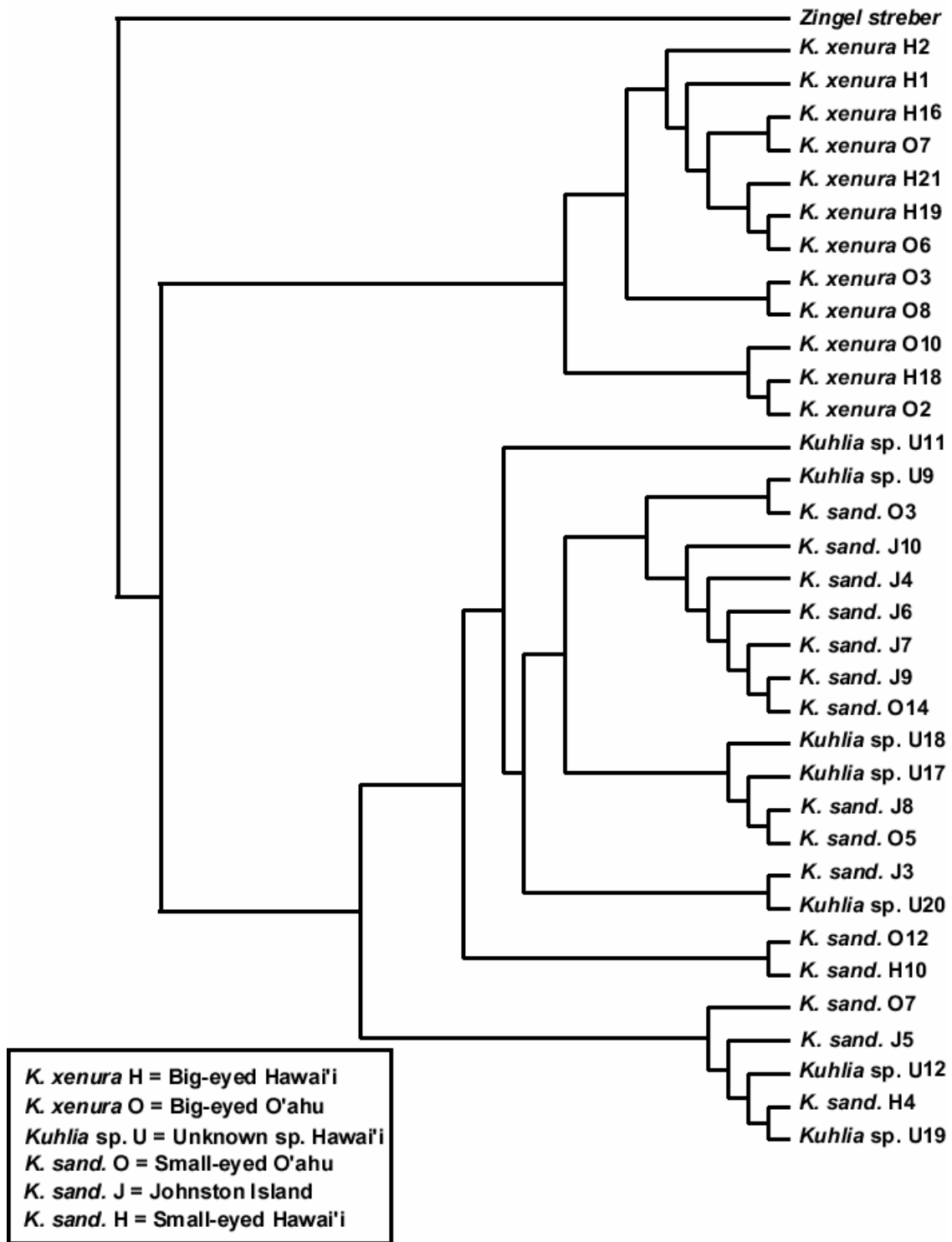


Figure 7. Neighbor Joining Tree based on percent sequence divergences between kuhliids in Hawai'i and Johnston Island. Note: *K. sand.* is short for *Kuhlia sandvicensis*, and "Hawai'i" refers to the island, not the State.

the unknown specimens were grouped within the *K. sandvicensis*/Johnston Island clade. The maximum likelihood analysis yielded identical results, with a bootstrap value of 100% supporting the two aforementioned clades (Figure 8).

Morphometric Analysis

For the discriminant function analysis, the Johnston Island fish were initially coded separately, despite results from the genetic comparisons indicating they were part of the *K. sandvicensis* species complex. By using combinations of the aforementioned morphological variables, the DFA procedure was able to group the three types separately with 84% correctness. There was some overlap between the Johnston Island fish and the *K. sandvicensis* specimens (Figure 9). Nineteen 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 4). Results for the jackknifed classification matrix were identical (Table 5).

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 10. Fourteen of 158 *K. xenura* and six of 100 *K. sandvicensis* were misclassified (Table 6). Again, results for the jackknifed classification matrix were identical (Table 7). From the data output, classification functions were used to derive a discriminant equation that can classify fish of unknown type. The equation for the canonical value, C , is:

$$C = 0.715 + 31.831 \log (D) - 11.655 \log (L) + 41.945 \log (E) - 0.224 \log (S) - 34.78 \log (I)$$

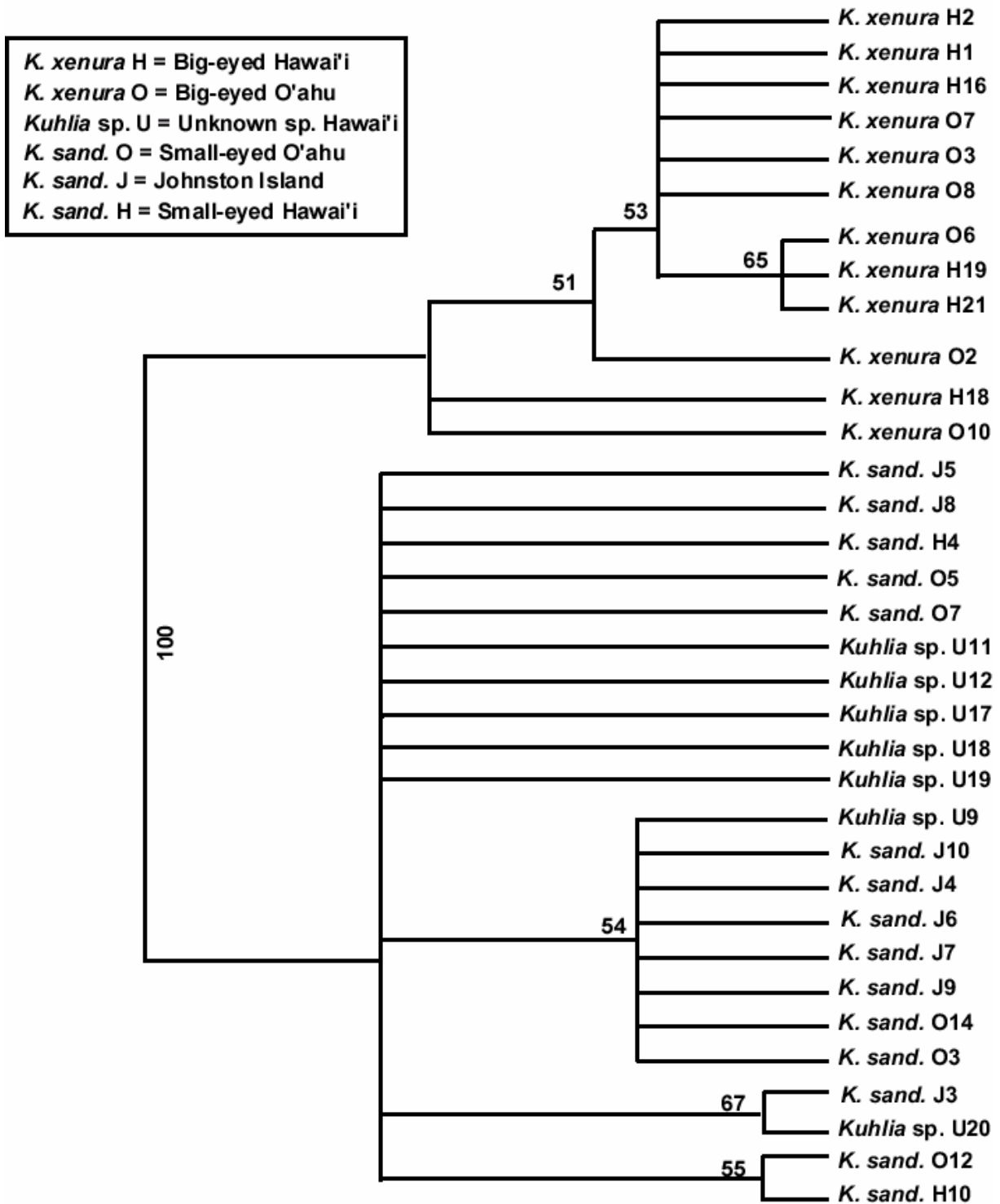


Figure 8. Maximum likelihood tree with bootstrap values (TVM+G model). Numbers at nodes indicate percent bootstrap support (of 100 replicates).

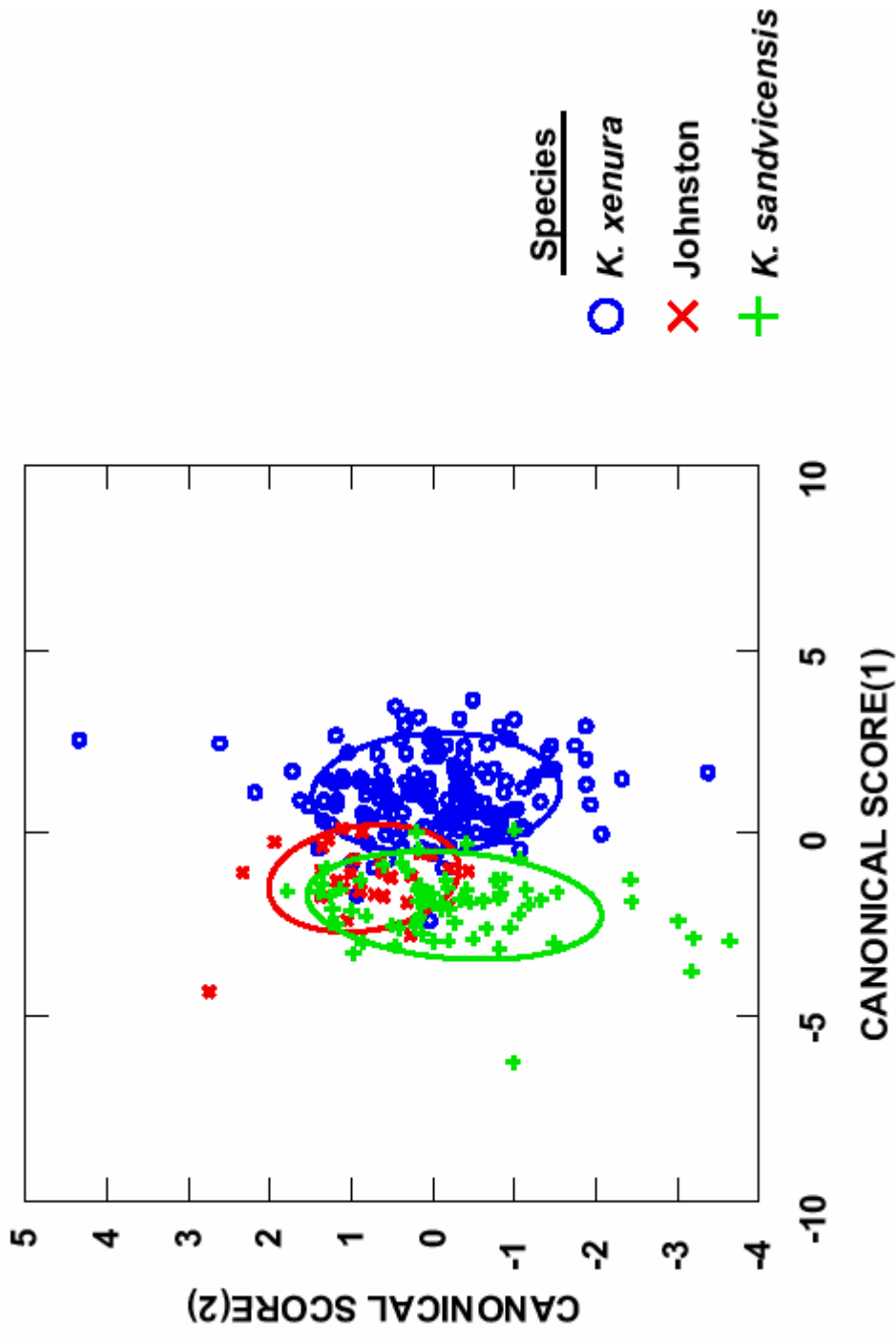


Figure 9. Discriminant function analysis of five morphometric variables used to classify three *Kuhlia* morphotypes. Ellipses represent 95% confidence intervals.

Table 4. Classification matrix for complete discriminant function analysis with Johnston Island fish coded separately.

Type	Number of fish classified as:			%Correct
	<i>K. xenura</i>	<i>K. sandvicensis</i>	Johnston	
<i>K. xenura</i>	141	2	15	89
<i>K. sandvicensis</i>	3	52	16	73
Johnston	0	5	24	83
Total	144	59	55	84

Table 5. Jackknifed classification matrix for complete discriminant function analysis with Johnston Island fish coded separately.

Type	Number of fish classified as:			%Correct
	<i>K. xenura</i>	<i>K. sandvicensis</i>	Johnston	
<i>K. xenura</i>	141	2	15	89
<i>K. sandvicensis</i>	3	52	16	73
Johnston	0	5	24	83
Total	144	59	55	84

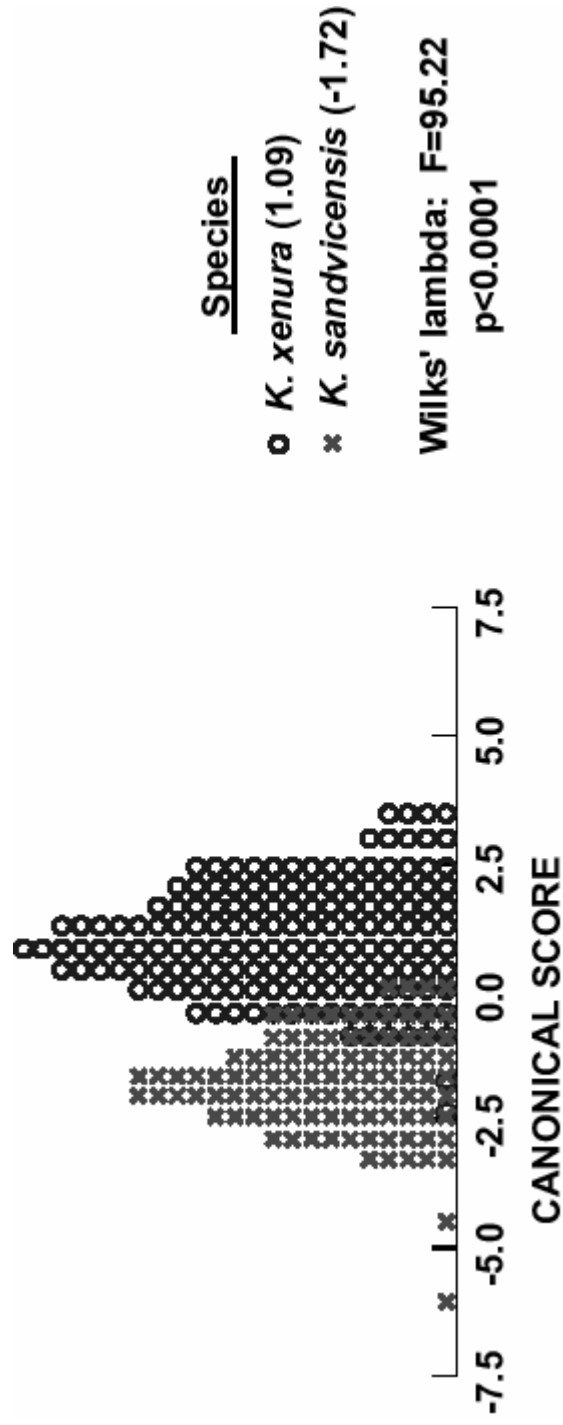


Figure 10. Discriminant function analysis of five morphometric variables used to classify two *Kuhlia* spp. from Hawai'i. Group means are in parentheses.

Table 6. Classification matrix for complete discriminant function analysis.

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

Table 7. Jackknifed classification matrix for complete discriminant function analysis.

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

Values of $C < 0$ were classified as *K. sandvicensis*, whereas fish with values of $C > 0$ were classified as *K. xenura*.

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 (Tables 8 and 9). 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 (Tables 8 and 9).

Classification functions provided with the DFA output provide a classification function coefficient for each of the variables (see previous classification equation). The variables' coefficients indicated that the head length to eye diameter ratio and fork length to depth ratio were larger in *K. sandvicensis*. These fish also have a smaller ratio of head length to interorbital distance. In other words, *K. sandvicensis*, the small-eyed type of aholehole, 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*.

DISCUSSION

The DNA sequencing study indicated high percent sequence divergences between the two proposed *Kuhlia* species in Hawai'i. Uncorrected *cytb* divergences, which averaged

Table 8. Classification matrix after backward and forward stepwise discriminant analysis (final model has three of the five original variables).

Type	Number of fish classified as:		%Correct
	<i>K. xenura</i>	<i>K. sandvicensis</i>	
<i>K. xenura</i>	147	11	93
<i>K. sandvicensis</i>	5	95	95
Total	152	106	94

Table 9. Jackknifed classification matrix after backward and forward stepwise discriminant analysis (final model has three of the five original variables).

Type	Number of fish classified as:		%Correct
	<i>K. xenura</i>	<i>K. sandvicensis</i>	
<i>K. xenura</i>	146	12	92
<i>K. sandvicensis</i>	5	95	95
Total	151	107	93

8.52%, suggest species level differences. According to Johns and Avise (1998), 90% of sister species pairs show at least 2% sequence divergence in their *cytb* genes. Furthermore, the Johnston Island specimens (previously identified as *K. marginata*) 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 phylogeny 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 *Kuhlia sandvicensis*, whereas the *K. xenura* specimens were reciprocally monophyletic. At this level of analysis using the *cytb* gene, no island structuring between O‘ahu and Hawai‘i was found. Perhaps meta-populations would be more likely detected by a microsatellite analysis. Recently, DNA microsatellites were used as “population-specific natural tags” to answer questions about reproductive isolation in salmon (Hendry et al., 2000). These highly variable markers have been found to be informative in populations which had otherwise low levels of genetic variation (Paetkau and Strobeck, 1994).

Based on percent sequence divergence, all fish designated as “unknown type” appear to be *K. sandvicensis*. These specimens were interspersed within the *K. sandvicensis* clade on the neighbor joining and maximum likelihood trees. Presumably the dorsal head stripes and brightly colored tail common in these specimens had faded by the time I examined the fish (post-freezing). In addition to color patterns fading in frozen or preserved fish, these specimens did not possess strikingly big or small eyes which further complicated their identification pre-DNA analysis. According to Randall and Randall (2001), one of the characters that differentiates between the two Hawaiian *Kuhlia* is eye diameter; in fact, this is

the only nonoverlapping morphological character listed by these authors. However, use of this character would not have identified at least one of the unknown fish as *K. sandvicensis*, because its eye diameter is intermediate between the two types. In my examination of 270 individuals (of known species) from Hawai'i and Johnston Island, just over 10% were misclassified when using the eye size character alone.

Despite overlap in meristic and morphometric data, discriminant analysis proved to be an effective procedure for distinguishing morphotypes/species. The resulting analysis grouped the two *Kuhlia* species with 94% correctness based strictly upon morphometric data. For both stepwise procedures, eye diameter, depth, and interorbital distance proved important characters for discriminating the two species. For the *K. sandvicensis* from Hawai'i and Johnston Island, fork length to eye diameter ratio and fork length to depth ratios are larger than in *K. xenura*. Overall, the eyes and body depths of *K. sandvicensis* are smaller than in *K. xenura* specimens of the same length. Conversely, the smaller-eyed fish have a larger interorbital distance and a smaller ratio of fork length to interorbital distance.

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 and Randall's 2001 conclusion that the "big-eyed" morphotype is the renamed as *Kuhlia 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 and Randall, 2001; personal observation). Use of a classification equation, which is less time

consuming and costly than DNA sequence analysis, is advantageous. Since 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 ones 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.

The Biological Species Concept emphasizes reproductive isolation as a criteria for differentiating species. Although this research did not specifically address that issue, some observations and anecdotal evidence from fishermen and past studies may provide clues as to whether or not these species are reproductively isolated. Premating isolating mechanisms can be ethological, as in the courtship behavior of goodeid fishes (Fitzsimons, 1976), as well as a combination of spawning habitat, behavior, and morphology in redhorses (various species of *Moxostoma*) (Kwak and Skelly, 1992). Unfortunately, as adults, the two species of *Kuhlia* in Hawai‘i do not lend themselves to classic aquarium studies. They have only very recently spawned in large tanks at aquaculture facilities in Hawai‘i, and they are not known to spawn in fishponds. Thus, observations to determine behavioral premating isolating mechanisms involving mate choice and recognition have not been conducted. In addition, *Kuhlia* species show no external sexual dimorphism, which would provide some evidence of premating isolation via visual cues.

No one, to my knowledge, has observed spawning in either species in the wild, so it is hard to comment on possible spatial or temporal isolating mechanisms. However, it is assumed that both species of āholehole are marine spawners, as fish that have ripe or recently spent gonads have been collected only from the ocean (personal observation, Tester and

Takata 1953). In addition, Tester and Takata collected adults that were ripe or nearly ripe from Diamond Head reef on O‘ahu, and it is known that adult āholehole migrate to the outer edge of reefs in schools (Tinker, 1978). However, evidence in the literature for Hawaiian *Kuhlia* is always suspect, because one does not know to which species the author is referring, as the species were formerly considered conspecific.

Other evidence of marine spawning within this genus, but outside Hawai‘i, is reported in the literature (Oka and Tachihara, 2001), and it is suspected to be a characteristic of all members of the family. One way to verify spawning habitat would be to perform simple laboratory experiments to determine the motility or survivability of sperm from *K. xenura* (the species often found in fresh water) at varying salinities. This test might provide physiological evidence that they actually are marine spawners, because sperm that are active in salt water for a long duration indicate salt water spawning (Ginzburg, 1972). Furthermore, such a study might elucidate whether or not subtle differences in sperm salinity preferences exist between these two species. In the past, these types of studies have provided evidence that both *Kuhlia rupestris* and *K. marginata*, which live primarily in fresh water, spawn in brackish waters or waters of elevated salinities (Hogan and Nicholson, 1987; Oka and Tachihara, 2001). Possibly one of the two kuhliid species in Hawai‘i spawns in slightly lower salinity waters, near estuaries or in areas of freshwater runoff, and, if confirmed, this observation would indicate a spatial or habitat difference that provides a premating reproductive isolating mechanism. Finally, if it is possible to obtain ripe eggs and viable sperm from both sexes of each species at the same time, it would be instructive to determine whether cross-fertilization is possible. Whether the sperm and eggs of the two species are compatible and whether gamete recognition occurs in a natural setting would be worthwhile

questions to explore. Tester and Takata (1953) were unable to obtain ripe eggs from females, and all attempts at artificial fertilization were unsuccessful.

Although the two species are observed together as juveniles, and adults of both species are occasionally caught together by fisherman, there still may be some habitat segregation. This topic will be discussed further in the next chapter, but to summarize, *Kuhlia xenura* commonly enters estuaries, streams, fishponds, and tidal creeks, whereas *K. sandvicensis* does not. Both species occur in tide pools as juveniles, but subtle differences exist in the type of tide pool habitat in which they are most commonly found. Furthermore, adult *K. sandvicensis* have been observed feeding near the surface in spur-and-groove coral habitats during the day when *K. xenura* are often found in caverns within the reef (personal observation). Although nocturnal behavior needs to be observed more, these preliminary habitat observations could be further evidence of a spatial premating isolating mechanism.

Differences in time of spawning may exist between these two species and may serve as a temporal premating isolating mechanism; their gametes simply may not come in contact with one another if they spawn at different times of day or times of the year. Personal observation and anecdotal evidence from fishermen indicate that both species feed nocturnally as adults. However, adult *K. sandvicensis* have also been observed feeding during the day; the possibility exists that they may also spawn during the day or only on nights with a full moon. Perhaps the “big-eyed” *K. xenura* is more nocturnal and spawns at night.

In regard to spawning season, preliminary analysis of my length data for juvenile *K. xenura* indicate that young fish are recruiting from the plankton year round, an observation that indicates year round spawning. This evidence is corroborated by Tester and Takata’s report (1953), which states that fish less than 26 mm were present during all months of the

year. They did see a peak in recruitment that would coincide with a predominantly December to June spawning, and their investigation into gonad maturation throughout the year supports this seasonality as well. Again, one must remember that their data are only somewhat useful since they were unknowingly lumping both species into one population. An intensive study for both species is needed in respect to spawning seasonality. Revisiting the same sites each month and examining the sizes of both species at each site will help elucidate whether there is an interspecific difference in spawning activity with respect to time of year. In addition, otolith data could be used to age the new recruits; then, the spawning dates for both species could be back calculated.

Karyological studies are another traditional approach in examining whether or not postmating isolating mechanisms exist or when studying hybrids (e.g. Fitzsimons, 1974). Many researchers have studied cytological characteristics to solve taxonomic problems and have observed different karyotypes in fish that were suspected of being two species (Cuevas et al., 1999). Both kuhliid species in Hawai'i show high overlap in meristic counts and morphological characters, which is not surprising for two closely related teleosts. However, it is possible that the intermediate morphotypes are actually hybrids formed by matings of the two species. Karyotype analysis could be an effective way to detect hybrids within my study group, as both authors above mention intermediate karyotypes being present in the hybrids of the taxa examined.

In addition, other types of genetic analysis could detect if hybridization is occurring in the populations being sampled. A study of hybridization in sunfish, possible near-relatives to the Kuhliidae, used mitochondrial DNA and allozyme markers to demonstrate low-frequency hybridization in nature (Avisé and Saunders, 1984). If hybridization is occurring, this type of

study should be able to characterize the parentage of the F₁ hybrids, as well as analyze whether introgression has occurred. In addition to DNA sequence analysis, allozyme electrophoresis has also been used to identify hybrids (Wayne et al., 1994).

Hybridization in tropical marine waters is rare and, according to Hubbs (1955), there is an “amazing” lack of hybrids in the “swarming reef fauna of the Indo-Pacific region.” This observation can be explained by a variety of factors: for example, in broadcast spawners, like *Kuhlia*, there are more chance meetings of egg and sperm in freshwater environments. Furthermore, marine environments are more stable than temperate or fresh waters; in environments with much “flux,” hybridization is much more frequent (Hubbs, 1955). If these two species of *Kuhlia* represent two separate colonization events by the same or similar species, only a small amount of time in allopatry is necessary for genetic changes driven by selection or drift. Reproductive isolation in these two species could have occurred after the first wave of *Kuhlia* colonizers experienced new selective pressures that lead to the evolution of reproductive isolation as a byproduct of their localized adaptations (Hendry et al., 2000).

Randall and Randall (2001) recognized a second species of *Kuhlia* in Hawai‘i. However, their evidence was based on often overlapping meristic characters. In some cases, use of external morphology alone may not be acceptable in delineating species boundaries. The Hawaiian *Kuhlia* complex is probably one of those cases, as described by Baverstock and Moritz (1996). These species are sympatric and are so similar morphologically that their specific status went undetected (at least by the scientific community) until very recently. This genetics study addressed the question of whether or not these are just two polymorphic forms within a single interbreeding population; this information, combined with the morphometrics study, supplies compelling evidence that these are two species.

Because both Hawaiian *Kuhlia* were, until recently, classified as one species, management strategies currently in place are likely more appropriate for one species than the other. Thus, conservation and management strategies should be reconsidered in light of the results presented, which indicate that these two morphotypes represent distinct lineages. Their status as popular food fishes, coupled with the evidence that the big-eyed morphotype appears to be endemic to the Hawaiian Islands, make correct identification, classification, and management practices essential for their conservation.

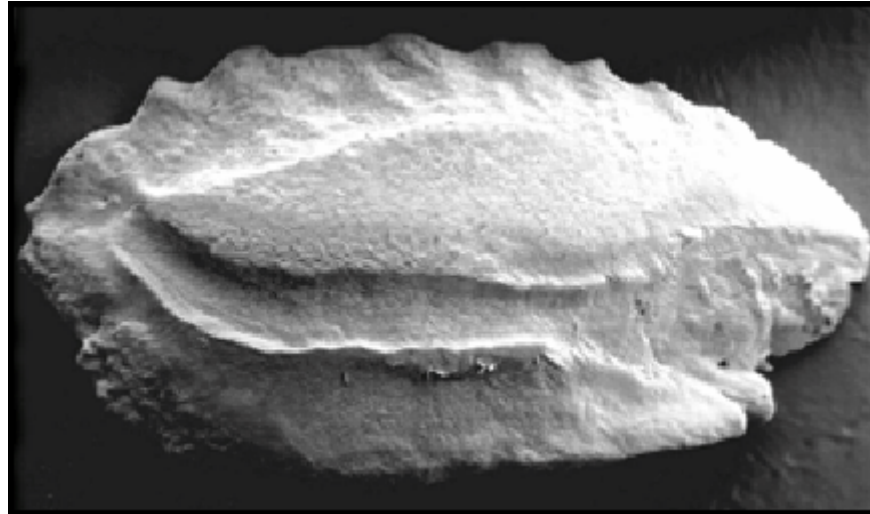
LIFE HISTORY AND HABITAT OF HAWAIIAN KUHLIIDS

INTRODUCTION

Despite the fact that āholehole are relatively important sport and food fishes, little is known about the life history requirements of *Kuhlia* in Hawai'i. This paucity of knowledge is further confounded by the fact that there are now two species recognized in the Hawaiian Islands, rather than one (see Chapter 1). They are believed to spawn in marine waters and live out their lives in marine or estuarine waters. However, the species now recognized as *K. xenura* is also commonly found as juveniles in freshwater streams. All members of the Kuhliidae are believed to spawn in marine or estuarine waters, but, at least one kuhliid species, *K. rupestris*, requires fresh water to complete its life cycle (Lewis and Hogan, 1987). The goal of this research was to investigate the life history of both *Kuhlia* species in Hawai'i and to determine if freshwater or brackish water habitats are an obligate part of the life cycles of *K. xenura* or *K. sandvicensis*.

One research approach commonly used to elucidate fish life history information is otolith analysis. Otoliths are the inner ear bones of fishes used to detect sound in water and to aid in balancing and orientation. Fishes have three pairs of otoliths; the sagitti are the largest pair and are most commonly used for life history investigations (Figure 11). Otolith studies have been conducted to gain information on fecundity and spawning season and frequency of commercially important, and possibly overfished, species like the red drum (*Sciaenops ocellatus*) (Wilson and Nieland, 1994). In studying fishes in temperate regions, annular rings on the otolith have traditionally been counted and measured to provide age and growth information. Under microscopic investigation, the otolith sections have wide translucent zones that represent fast growth in warmer months and thinner opaque bands that represent

A



B



Figure 11. Sagittal otolith from *Kuhlia xenura*; A is medial view and B is distal view (SEM). (A: 22X magnification; B: 24X magnification).

slow growth in the winter. In the tropics, because there are no well-defined growing seasons, it is more useful to count daily increments that are formed in otoliths (Pannella, 1971 and 1974). These increments are formed by a cyclic deposition of calcium and protein, and they are associated with the circadian or endocrine rhythms of fishes (Campana and Neilson, 1982). However, experimental evidence indicates that environmental factors, such as light and temperature, may cause or possibly interfere with the formation of daily increments (Taubert and Coble, 1977; Brothers, 1978). Thus, for a given species, it is important to validate the daily nature of these microincrements before using them as aging tools. Provided that they are formed daily, otolith microincrements are an especially useful ageing tool for young fishes.

Otolith formation and chemical composition are affected by a fish's surrounding environment, and trace elements in the otolith can be analyzed quantitatively via electron microprobe techniques. Otolith microprobe analysis has been used to distinguish the progeny of sympatric anadromous and nonanadromous salmonids, and has proved to be a useful tool in understanding the dynamics of these two types of populations for management purposes (Kalish, 1990; Rieman et al., 1994). Migratory schedules and habitat utilization patterns in anadromous striped bass have been investigated successfully by electron microprobe techniques (Secor et al., 1995). Researchers also recently discovered, via microprobe analysis of otoliths, that certain Atlantic and Pacific anguillid eels were not catadromous (Tsukamoto et al., 1998). This technique has been used to demonstrate the life cycle of the indigenous amphidromous fishes of Hawai'i's streams, as well as to document the length of the marine larval phase for some of these Hawaiian diadromous gobies (Radtke et al., 1988; Radtke and Kinzie, 1996).

The theory behind microprobe analysis of otoliths relies on the assumption that the calcium carbonate in the otoliths can be partially replaced by certain ions that are present in surrounding waters at the time a given increment is being laid down. The ion strontium (Sr) has the same valence (2+) as calcium (Ca), as well as a similar ionic radius; thus, Sr is substituted for Ca during deposition in the otolith. It has been demonstrated that the ratio of Sr to Ca in the otolith reflects the relative amounts of these ions in the surrounding waters (Casselman, 1982; Radtke, 1989). The relative amounts of Sr and Ca are useful indicators of habitat type at a given point in a fish's life history because the Sr to Ca ratio changes with salinity. Marine waters around Hawai'i have a Sr concentration around 8mg/l (Broecker, 1974), whereas Hawaiian fresh waters have a relatively small amount of Sr, 40-90 µg/l (USGS, 1984). Thus, a high Sr to Ca ratio in a particular region of the otolith indicates residence in, or contact with, higher salinity waters, whereas a low Sr to Ca ratio indicates that a fish was living in fresh water at the time when that daily increment was laid down.

For this study, I sought to validate the daily nature of microincrements formed in Hawaiian *Kuhlia* by using an otolith marking technique. In addition, an electron microprobe analysis provided information about the environment in which an individual fish was living at certain points in its life. This approach provided the approximate age of the fish being examined, and the Sr:Ca data proved useful when trying to elucidate whether or not fresh water is an obligate component of the life cycle of Hawaiian *Kuhlia*. In addition, a habitat profile was compiled, with an emphasis on salinity and temperature preferences for the various size classes and morphotypes (species). This information will help not only to differentiate between the two *Kuhlia* species in Hawai'i, but also to shed light on important details of their life cycles. This information will be necessary if either of these species are to

be utilized in aquaculture enterprises in Hawai'i. Furthermore, conservation, and management plans must encompass all life history stages when protecting habitats for these apparently euryhaline fishes.

MATERIALS AND METHODS

Daily Increment Validation

Juvenile *K. xenura* were seined about 140 m upstream from the mouth of Hakalau Stream, Island of Hawai'i. The fish were exposed for 16 hours to a 100 ppm solution of Alizarin complexone, a bone stain, that was dissolved in water from nearby Wailoa River. Fish were removed from the Alizarin bath and allowed to grow in unstained water for 23 days. They were held under natural light/dark conditions and fed to satiation twice daily (as per Ralston and Miyamoto, 1983). Fish were measured and otoliths removed. At the time of otolith removal, fish ranged from 28 to 41 mm standard length.

The sagittal otoliths were rinsed with deionized water to remove any adhering tissue and allowed to air dry. They were then embedded in a two part Araldite epoxy resin and sectioned as close to the core region as possible. Sectioning was conducted with a Buehler Isomet saw along a transverse plane. Thin sections containing the otolith core were mounted on glass slides with thermoplastic cement. 600 and 1200 grit wet-dry sandpaper was used to grind the otolith section down to the core on both sides. The samples were finished by polishing with 0.3 micron alumina paste. Samples were viewed with a compound microscope, and the increments laid down beyond the stained area were counted to the edge of the otolith section. Counts were conducted four times for each specimen, and a mean number of days of growth were calculated for each fish.

Microprobe Analysis

Juvenile fish, 21 - 42 mm SL, were collected about 125 m upstream from the mouth of Hakalau Stream (*K. xenura*) with seine and with dip nets in tide pools at Puakō Reef, Island of Hawai'i (*K. xenura*) and Shark's Cove, O'ahu (*K. sandvicensis*). Two additional *K. xenura* specimens (standard lengths of 123 and 98 mm) were collected via hook and line from Wailoa Stream in Waipi'o Valley also on the Island of Hawai'i. Adult specimens of both species (192 - 204 mm SL) were caught from the ocean, via hook and line, at the Hawai'i Volcanoes National Park (Figure 12). Otoliths from additional adult specimens from this site were photographed whole. The shape of these whole sagittal otoliths from the two species was compared.

All fishes were frozen until measurement and otolith removal. Otoliths were embedded and prepared as described above. Otolith sections from both juvenile and adult fishes were hand-ground, while sections from adult fish were also ground with a lapping wheel and 400 grit wet/dry sandpaper. The sections were viewed with a compound microscope, and, for each juvenile specimen, daily increments were enumerated three or four times and then averaged (Figure 13). The thin sections were photographed, mounted on a glass slide with a two-part epoxy resin, and then coated in a high vacuum evaporator with a 200 Å carbon layer for surface conductivity.

The elemental analysis was performed on a JEOL 733 Scanning Electron Microscope (SEM), which was equipped with four wavelength dispersive X-ray spectrometers (WDS). Strontianite and Calcite were used as standards to calibrate the spectrometer. Coral standards (which are similar in composition and crystal formation to otoliths) were analyzed to confirm that the electron microprobe was calibrated properly. Strontium and calcium were analyzed at

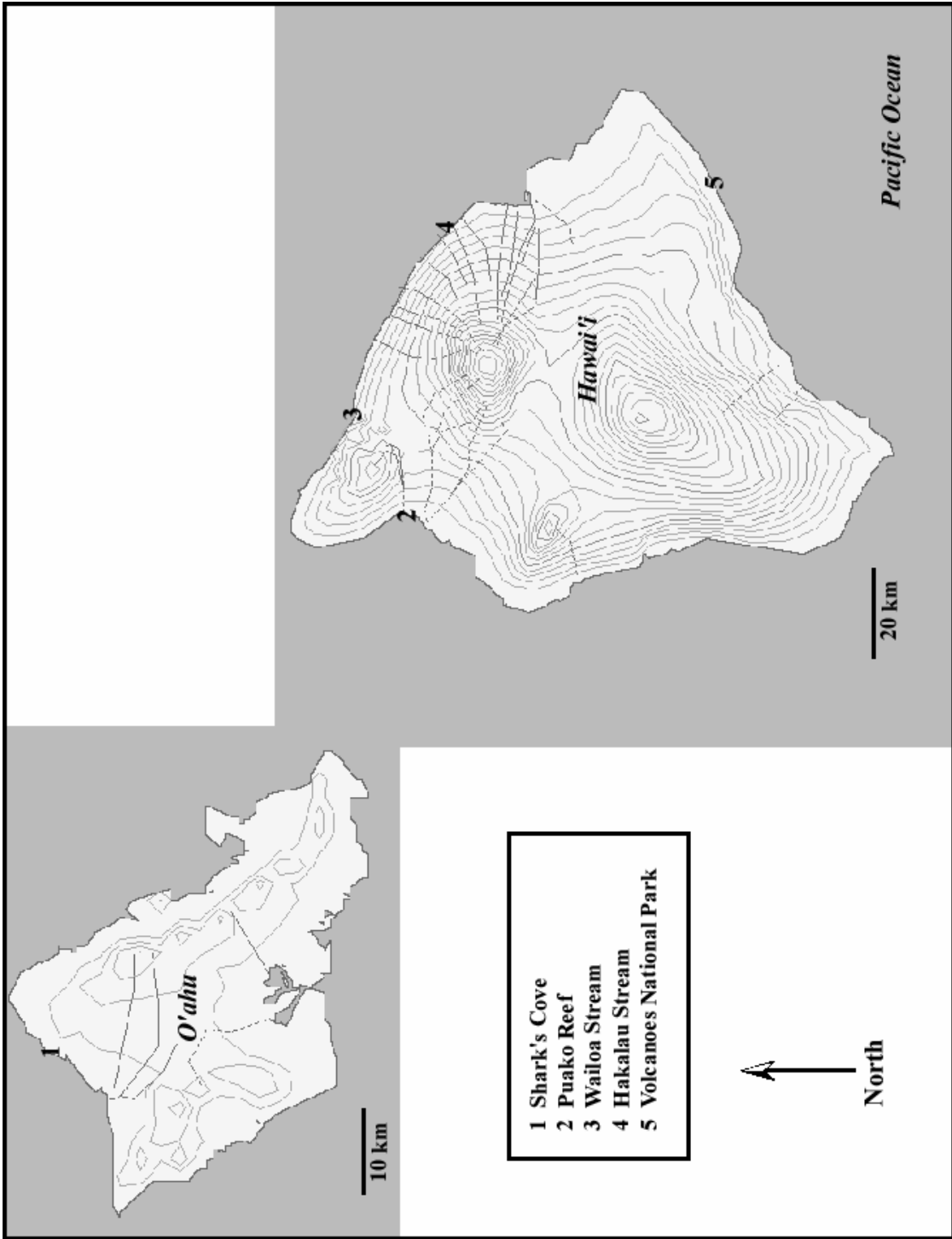


Figure 12. *Kuhlia* collection sites, in the Hawaiian Islands, for otolith microprobe analysis.

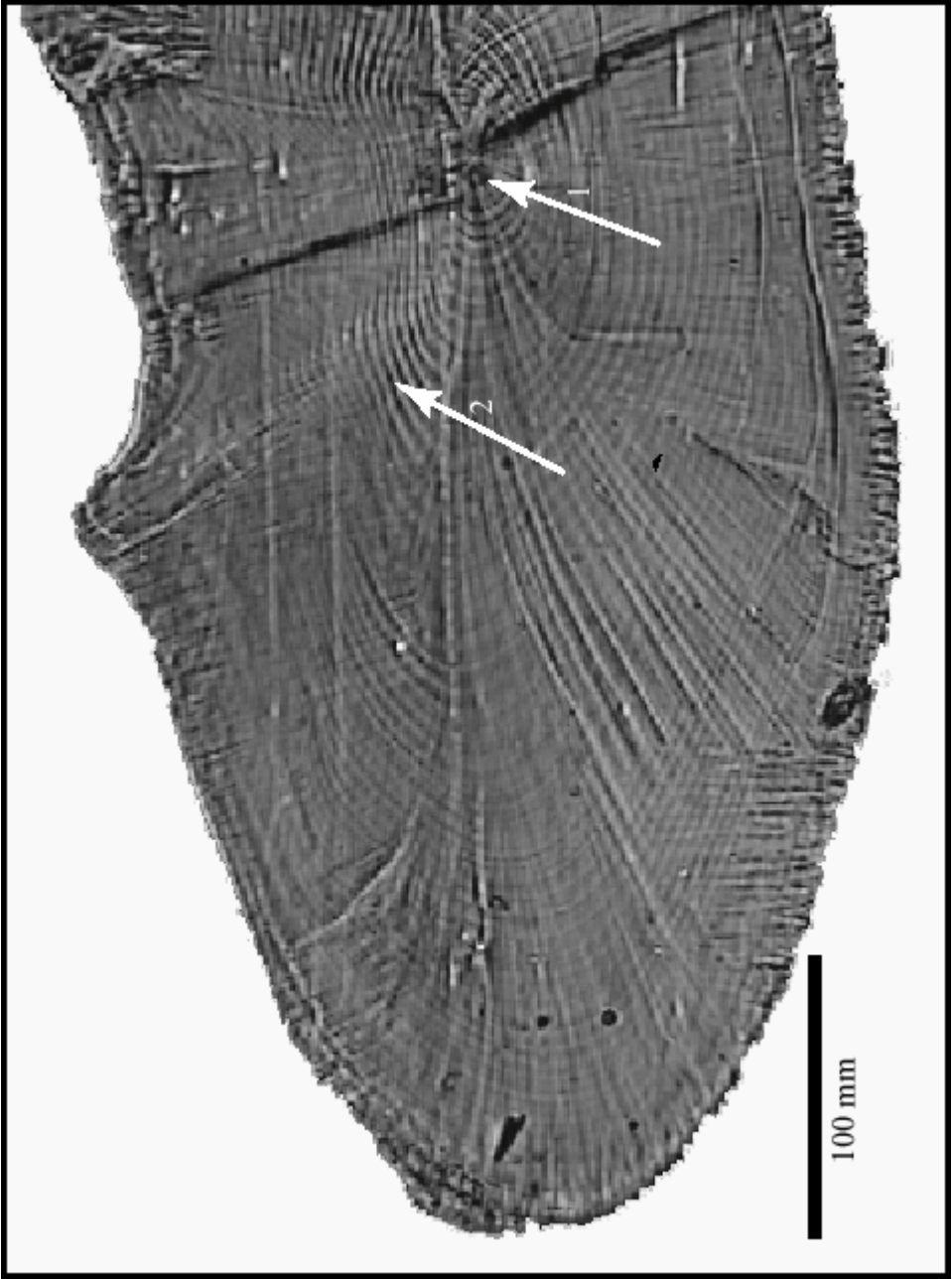


Figure 13. Light micrograph of one half of a *Kulhia xenura* otolith section showing core (1) and increments (2).

sample points 10 μm in diameter, with an accelerating voltage of 15kV and a beam current of 5nA. Counting times for Sr and Ca were 80 and 30 seconds respectively. The analysis was conducted along several transects from the otolith core to the edge, to follow temporal life history profiles. Transect length and the number of points assayed differed according to placement of the microprobe scan and otolith size. Between 17 and 97 equally spaced points were assayed, with distances between points varying according to the size of the otolith. For the juvenile specimens, the transects analyzed were those that could best be overlapped with those transects that had been used to estimate the fish's age. Thus, a rough age estimate at various microchemical analysis points could be obtained. Finally, Sr:Ca ratios were calculated at each analysis point; these ratio values were multiplied by 10^3 for comparison and presentation.

Habitat Observations

From March 1997 to August 2000, *Kuhlia* of both species were observed on the islands of O'ahu and Hawai'i. Whenever possible, both salinity (measured with a refractometer) and temperature measurements were taken at the sites. The type of habitat and a general description of the area were recorded. In addition, if fish were found in tide pools, it was noted whether or not open connections to the ocean existed. Other species found in the same habitat unit were noted as well.

RESULTS

Daily Increment Validation

Twenty-three of 24 fish survived the marking experiment. For three of the specimens, otoliths were either unreadable or destroyed in the preparation process. All readable otolith preparations had distinct increments in the sectioned sagitti. Increments consisted of a pale,

translucent zone followed by a dark, opaque line. There was a pale purple stain, caused by the Alizarin, which extended from the core to about two thirds distance to the edge (Figure 14). Counts in the area beyond the stain ranged from 18 to 25 increments, and the mean of the averages was calculated at 22.4 (± 1.3 standard deviations) for the 20 specimens. The fish were allowed to grow in ambient water for 23 days; a mean increment count of 22.4 validates daily increment formation.

Microprobe Analysis

Fishes (N=15) representing four habitat types (stream mouth, upstream, tide pools, and ocean surge zone) and two species (identified by eye size) were used for electron microprobe analysis. Comparisons of multiple transects within the same otolith section showed similar Sr to Ca ratio trends (Figure 15). Age estimates of several of the juvenile fish examined were overlaid on the microprobe transects, and provided salinity information at specific points in their life history. Sr:Ca values obtained here are comparable to those obtained for salmonids (Kalish, 1990; Rieman et al., 1994) and Hawaiian amphidromous gobies (Radtke and Kinzie, 1996).

(1) Stream mouth—*K. xenura*. Juvenile fish (n=4) (23-25 mm SL) collected from the lower reach of Hakalau stream ranged in age from 75 to 93 days. Although microprobe results differed slightly between these four fish, some general trends are evident. All fish had spent time in salt water, based on Sr:Ca values >2.7 , during the first or second week. Beyond 15 days, Sr concentration dropped, indicating exposure to lower salinity water, as is reflected by Sr:Ca values ranging from one to 2.5 (Figure 16). The profiles for fish collected from fresh water were relatively constant; they had average Sr:Ca values of 2.00 to 2.23. For these fish, no prolonged reentry into salt water after the first two weeks was evident from the life

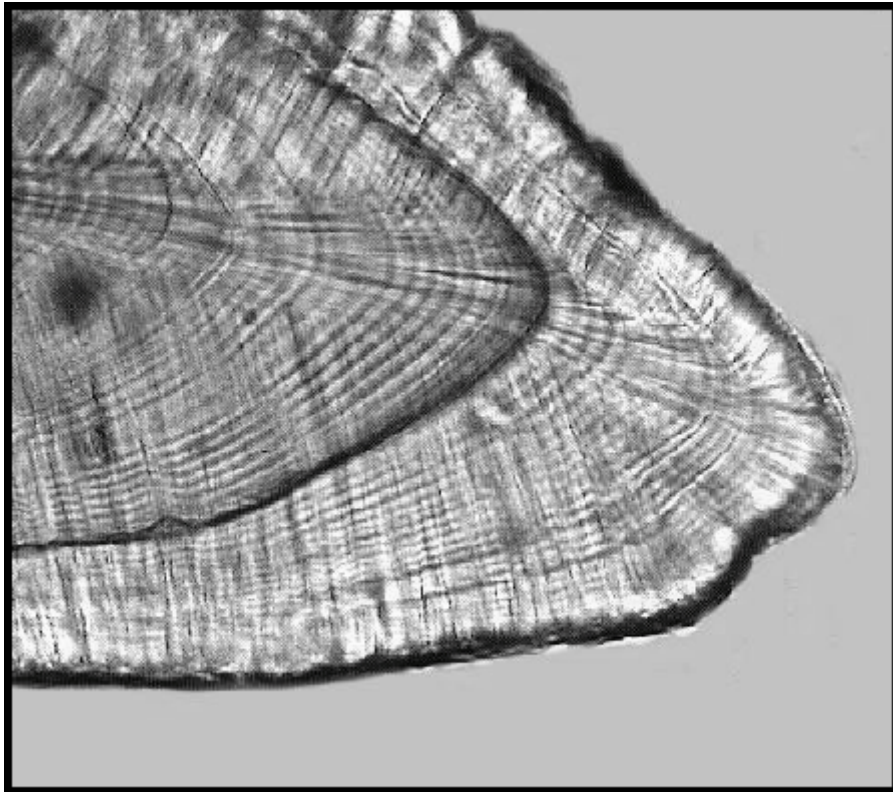


Figure 14. Edge of otolith section showing increments stained darker by Alizarin stain and unstained increments.

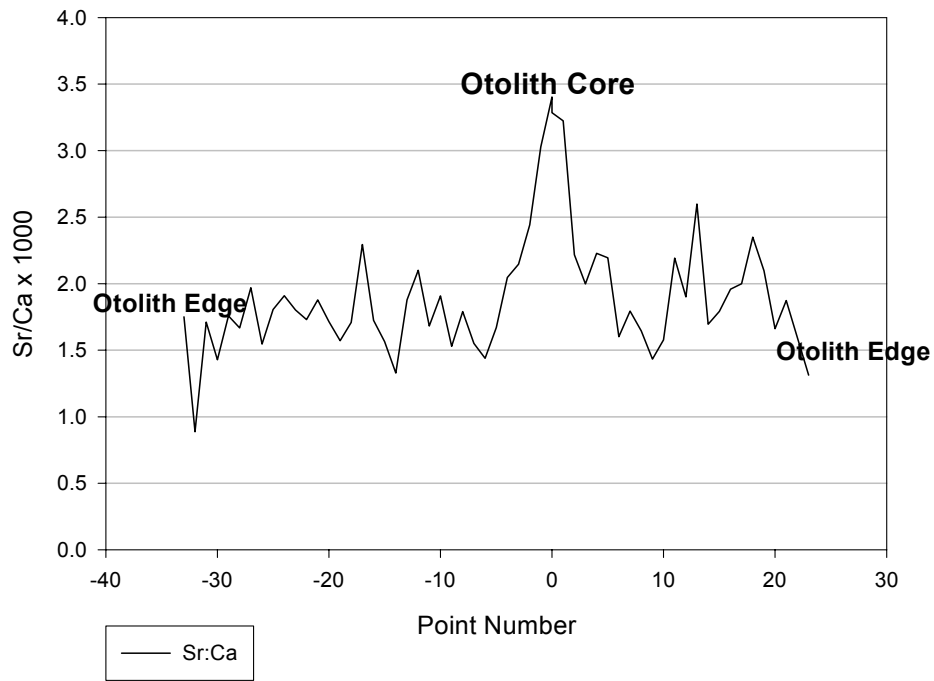
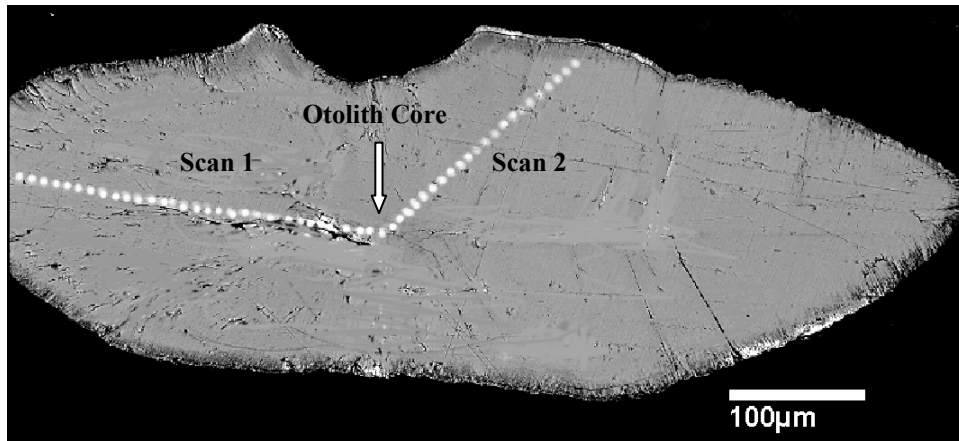
A**B**

Figure 15. (A) Sr:Ca ratios across two life history transects for a Hawaiian *Kuhlia* specimen. Figure shows similar Sr:Ca ratio trends across two scans within the same otolith section. (B) Electron micrograph showing placement of the two scans is included.

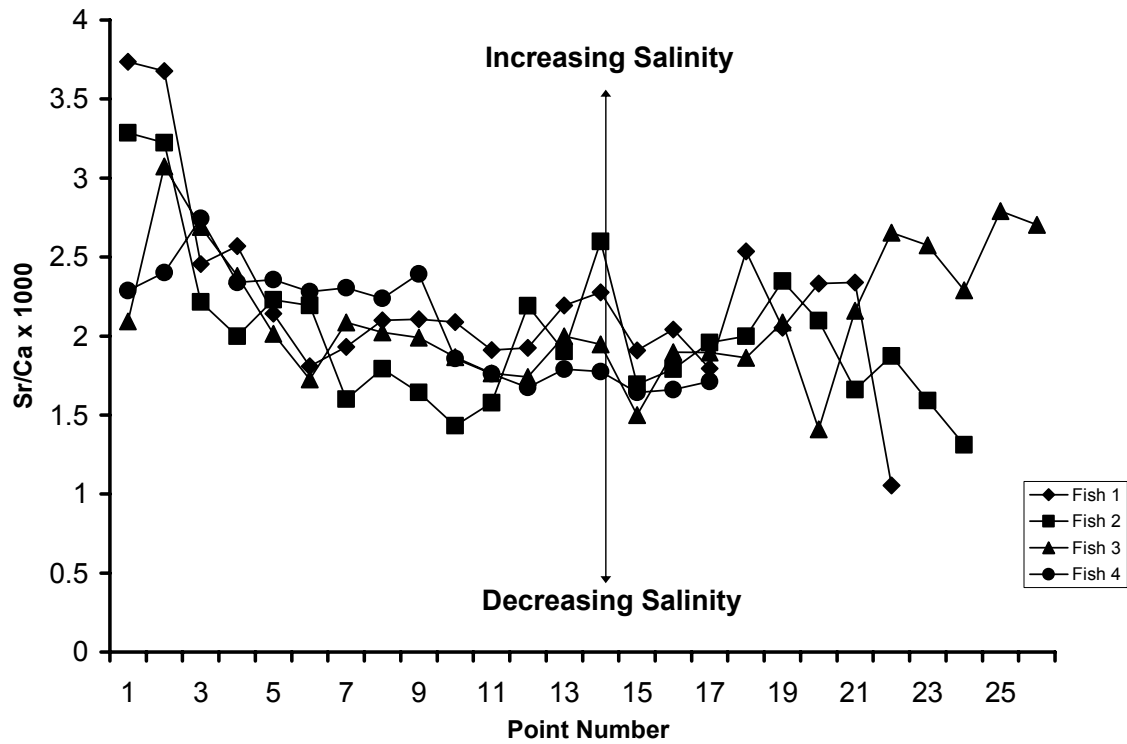


Figure 16. Sr:Ca ratio at ordinal point values along a transect from the core to the edge of juvenile *Kuhlia xenura* otoliths collected from the mouth of Hakalau Stream, Hawai‘i.

history transects. In fact, three of the four fish exhibited no Sr:Ca values higher than 2.59 beyond the first few analysis points. For two fish, it was possible to overlay daily increment counts with microprobe transects and obtain Sr:Ca value information at specific ages (Figures 17 and 18).

(2) Upstream in Lower Reach—*K. xenura*. Two additional specimens (123 and 98 mm SL) collected from fresh water (Wailoa Stream, Island of Hawai‘i) were analyzed on the WDS microprobe. These specimens were from further upstream than those analyzed from the mouth of Hakalau Stream (approximately 3.34 and 1.79 km inland vs. 125 m for the Hakalau specimens). The two fish could not be aged because of the difficulty and inaccuracy of counting daily increments in fish older than six months; based on sizes, they were most likely in their second year (Tester and Takata, 1953). Several zero values in this transect indicated that Sr concentrations were below the detection limit of the microprobe. These values may reflect the high amounts of rainfall that occur in this valley (personal observation). The first specimen (W1) exhibited Sr:Ca values that were indicative of hatching in marine waters (Figure 19). The mean Sr:Ca value for this life history profile was 1.97; the standard deviation of 1.76 illustrates the fluctuation in Sr:Ca values for this fish. The range in Sr:Ca ratio values for this specimen was from zero to 6.55, and no periodicity or pattern was evident in this life history transect. More than half the points represented a surrounding environment of fresh or brackish water, and the median Sr:Ca value of 1.70 reflects this.

The second specimen collected from Wailoa Stream (W8) also exhibited much fluctuation in Sr:Ca values and no distinguishable pattern across its life history transect (Figure 20). The mean Sr:Ca value for this fish was 2.48, with a range in values from zero to 7.88. Again, time early in life was spent in salt water; in fact, most of the Sr:Ca values in the

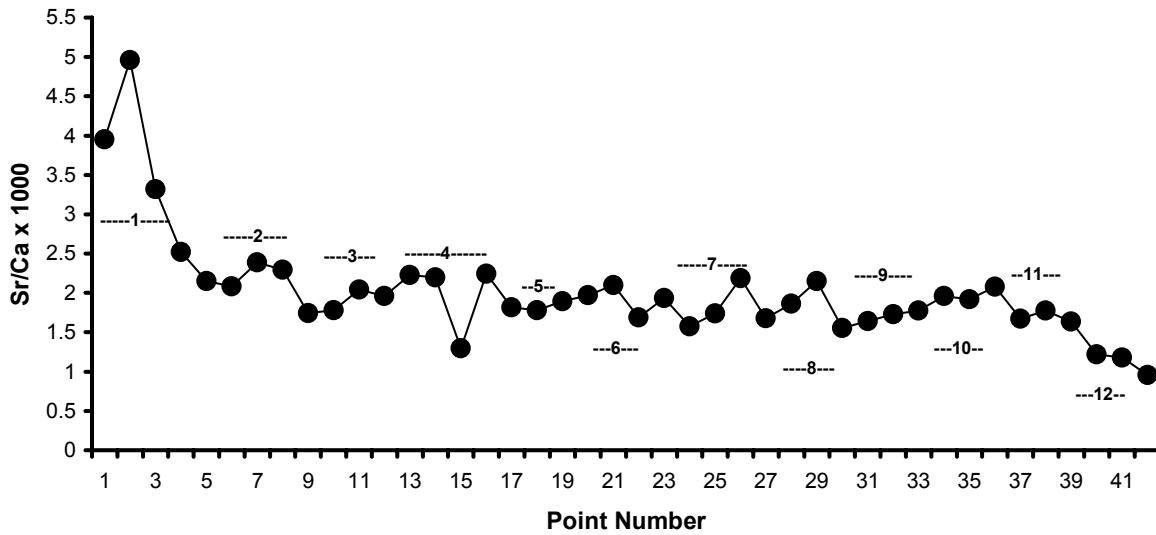


Figure 17. Sr:Ca ratio profiles for a juvenile *Kuhlia xenura* collected from Hakalau Stream mouth, with age information overlaid (numbers in body of graph indicate age in weeks).

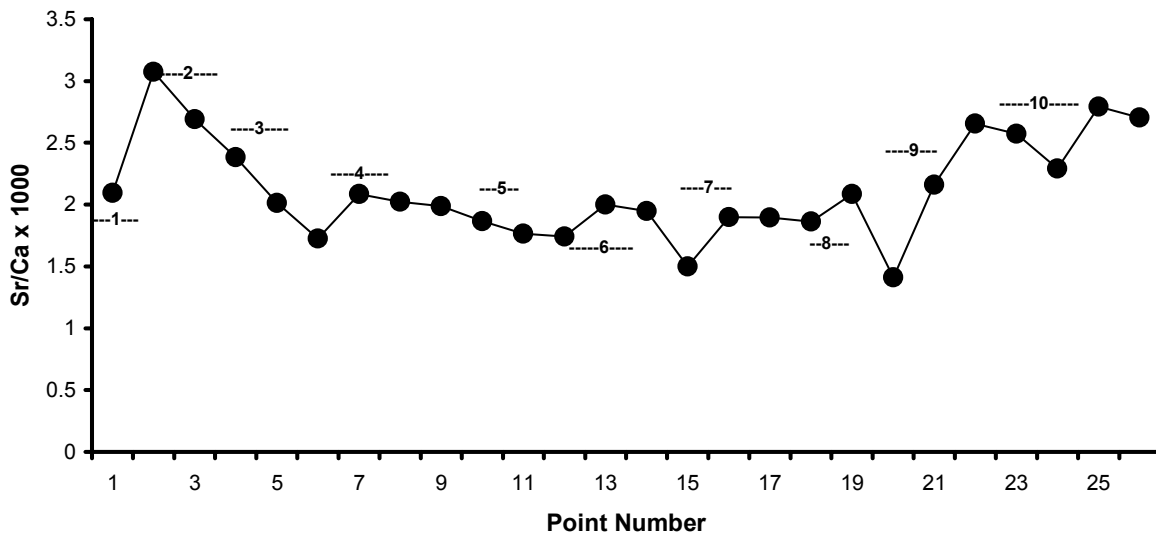


Figure 18. Sr:Ca ratio profiles for a juvenile *Kuhlia xenura* collected from Hakalau Stream mouth, with age information overlaid (numbers in body of graph indicate age in weeks).

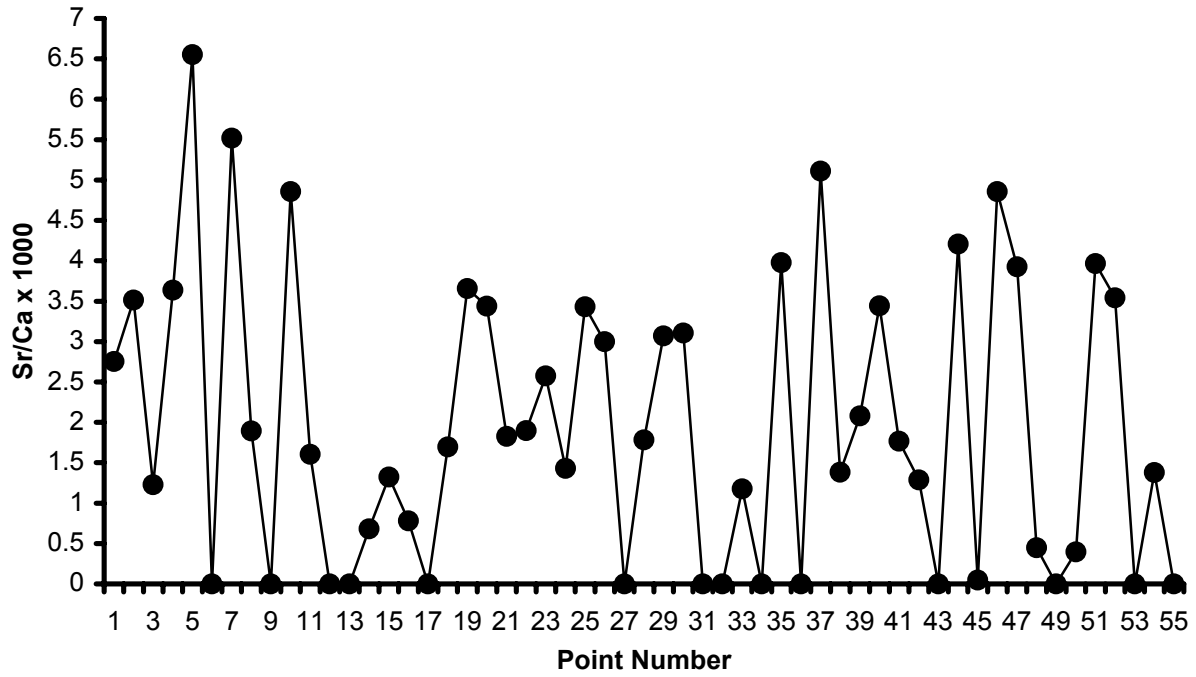


Figure 19. Sr:Ca ratio profiles for a mid-sized *Kuhlia xenura* collected upstream from mouth in Wailoa Stream.

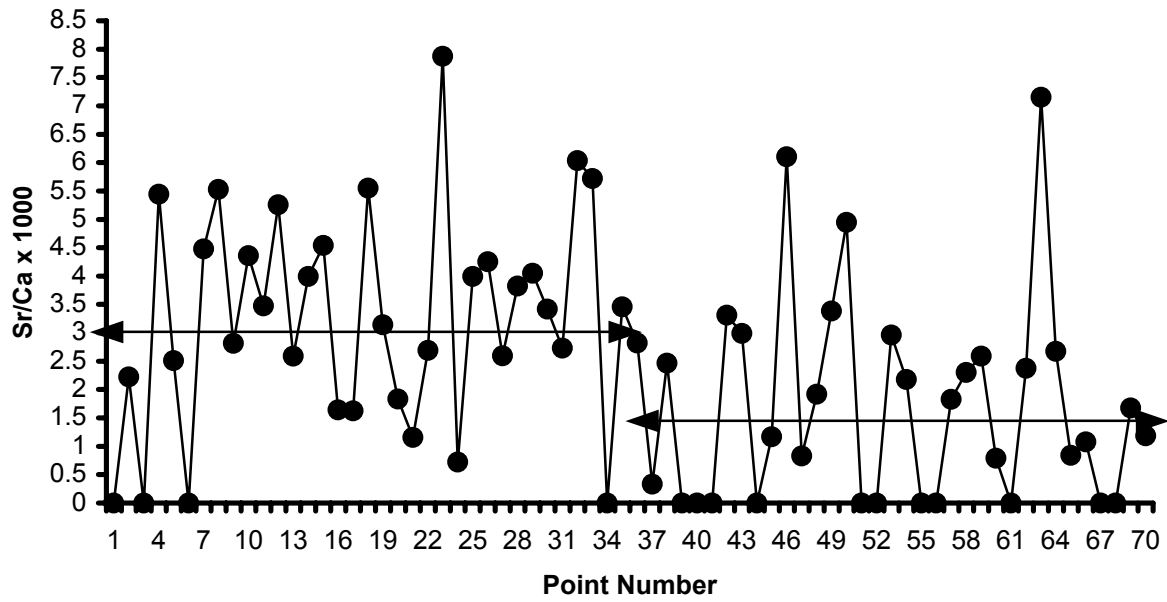


Figure 20. Sr:Ca ratio profiles for a mid-sized *Kuhlia xenura* collected upstream from mouth in Wailoa Stream. Arrowed bars indicate Sr:Ca means for first and second half of this fish's life history.

first half of its life were indicative of a surrounding marine environment (first half mean = 3.24). For the second half of its life, however, most points analyzed indicated existence in fresher water (second half mean = 1.71).

(3) Tide pools—*K. xenura*. Based on daily increment counts, two juvenile fish (21 and 30 mm SL) collected from tide pool habitats were 56 (P5) and 73 (P6) days old. Mean Sr:Ca ratios for specimens P5 and P6 were 2.54 and 3.14 respectively. Sr:Ca values were more variable for these specimens than those for similar sized juveniles collected from Hakalau Stream (Figure 16). Hakalau juveniles exhibited standard deviations around the mean of 0.42 – 0.69, whereas the tide pool fish have higher standard deviations of 1.27 and 1.63. The range of Sr:Ca values for these two fish was from zero to 5.6 (with one, possibly anomalous point of 9.76).

Similar to the freshwater juveniles, these fish had spent time, early in life, in waters of higher salinity (reflected by multiple Sr:Ca values greater than 2.75). In addition, values indicated time spent in waters of lowered salinity (Sr:Ca values ranging from zero to 2.5). Specimen P6 exhibits some of its highest Sr:Ca values during week one, similar to the pattern exhibited for the fish collected from fresh water (Figure 21). Specimen P5 also showed high Sr:Ca values (greater than three) early in its life history, with fluctuating high and low Sr:Ca levels throughout the rest of its life history transect. No clear periodicity or regular pattern exists across these transects.

(4) Tide pools—*K. sandvicensis*. Based on an average of daily increment counts, the two fish (39.4 and 42.1 mm SL) collected from Shark's Cove tide pool habitats on O'ahu were 65.75 (P4) and 86.5 (P5) days old. There was less variability in Sr:Ca values across the life histories of these specimens than for similar sized *K. xenura* juveniles. The mean Sr:Ca

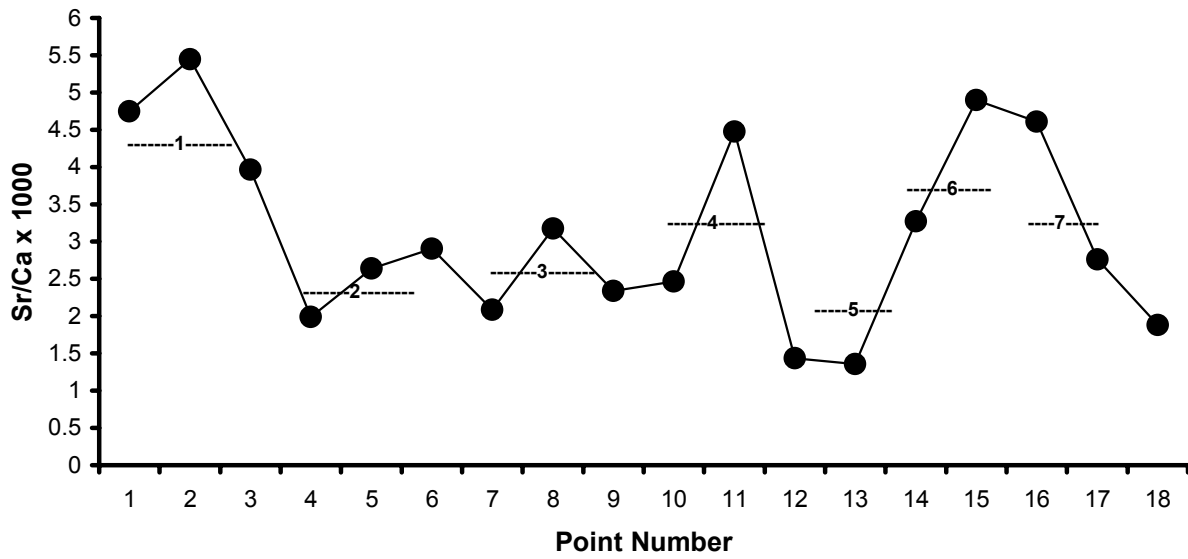


Figure 21. Sr:Ca ratio profiles for a juvenile *Kuhlia xenura*, with age information overlay, from Puakō tide pool (numbers in body of graph indicate age in weeks).

value for both fish was 2.43, and the standard deviations around those means were 0.41 (P4) and 0.54 (P5). Similar to the specimens reported above, fish P4 had spent time early in life in waters of higher salinity (first two data points were 3.92 and 2.83) (Figure 22). In addition, time was spent in brackish waters during most of its life (Sr:Ca values ranging from 1.85 to 2.6). As seen in Figure 23, specimen P5 exhibited an unusual Sr:Ca profile, as there is a sudden upward trend of increasing salinity at the end of its life. The only Sr:Ca values greater than 2.74 are the last five points analyzed. The range in values for this specimen were similar to the other *K. sandvicensis* juveniles examined (1.74 to 3.92).

(5) Ocean surge zone—*K. xenura*. Adult specimens (204 and 192 mm SL) collected from marine waters along the cliffs of the Island of Hawai‘i also exhibited Sr:Ca fluctuations across their life histories; one example is provided (Figure 24). Mean values for Sr:Ca ratios for the two specimens were 3.69 and 3.56, with standard deviations of 1.72 and 1.37. Sr:Ca values ranged from zero to 7.73 for these two specimens. Again, the zero values in this transect indicated that strontium concentrations at those points were below the detection limit of the microprobe.

Both specimens exhibited values characteristic of a marine environment early in their lives. In addition, Sr:Ca values indicated that fresh water was either sought out or encountered frequently. There was a trend in the later half of the life histories of increasing salinities (see trendline on Figure 24), although they continued to make forays into waters of reduced salinities later in their lives.

(6) Ocean surge zone—*K. sandvicensis*. Three adult specimens (V4, V5, and V6) were collected at the same locality as the *K. xenura* specimens above. Their standard lengths ranged from 194 mm to 204 mm. As with the *K. xenura* specimens reported earlier, data from

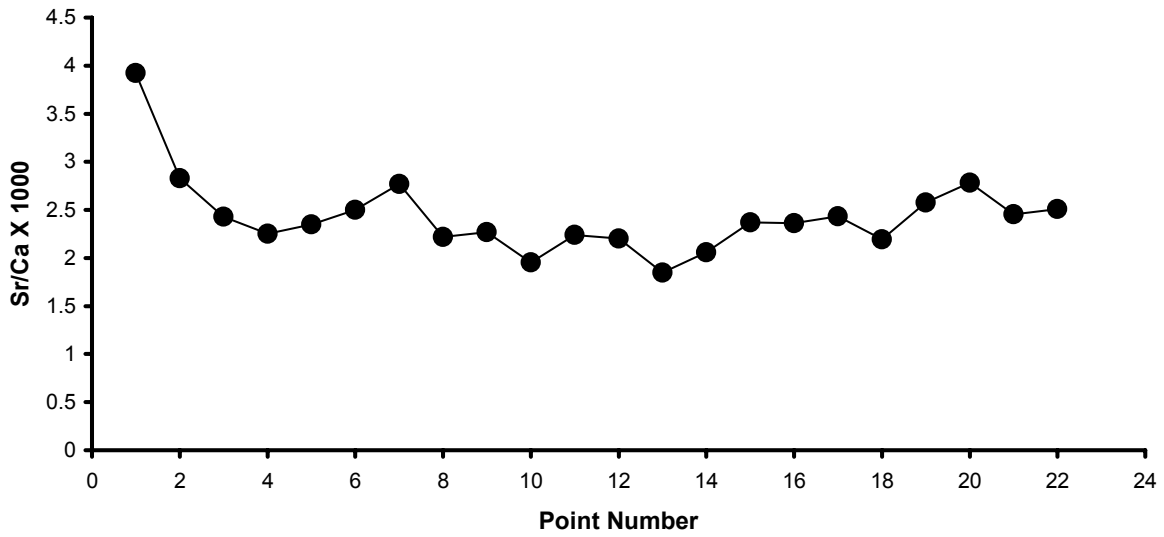


Figure 22. Sr:Ca ratio profile for a juvenile *Kuhlia sandvicensis* collected from Shark's Cove tide pool.

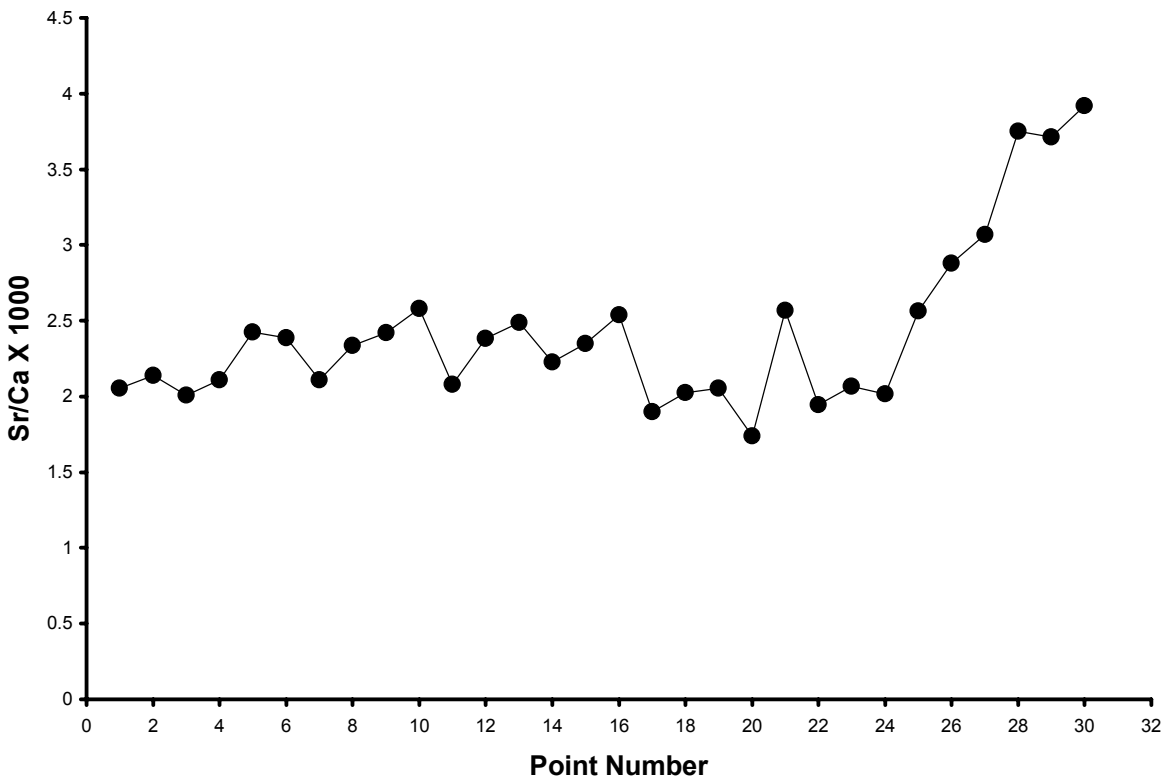


Figure 23. Sr:Ca ratio profile for a juvenile *Kuhlia sandvicensis* collected from Shark's Cove tide pool.

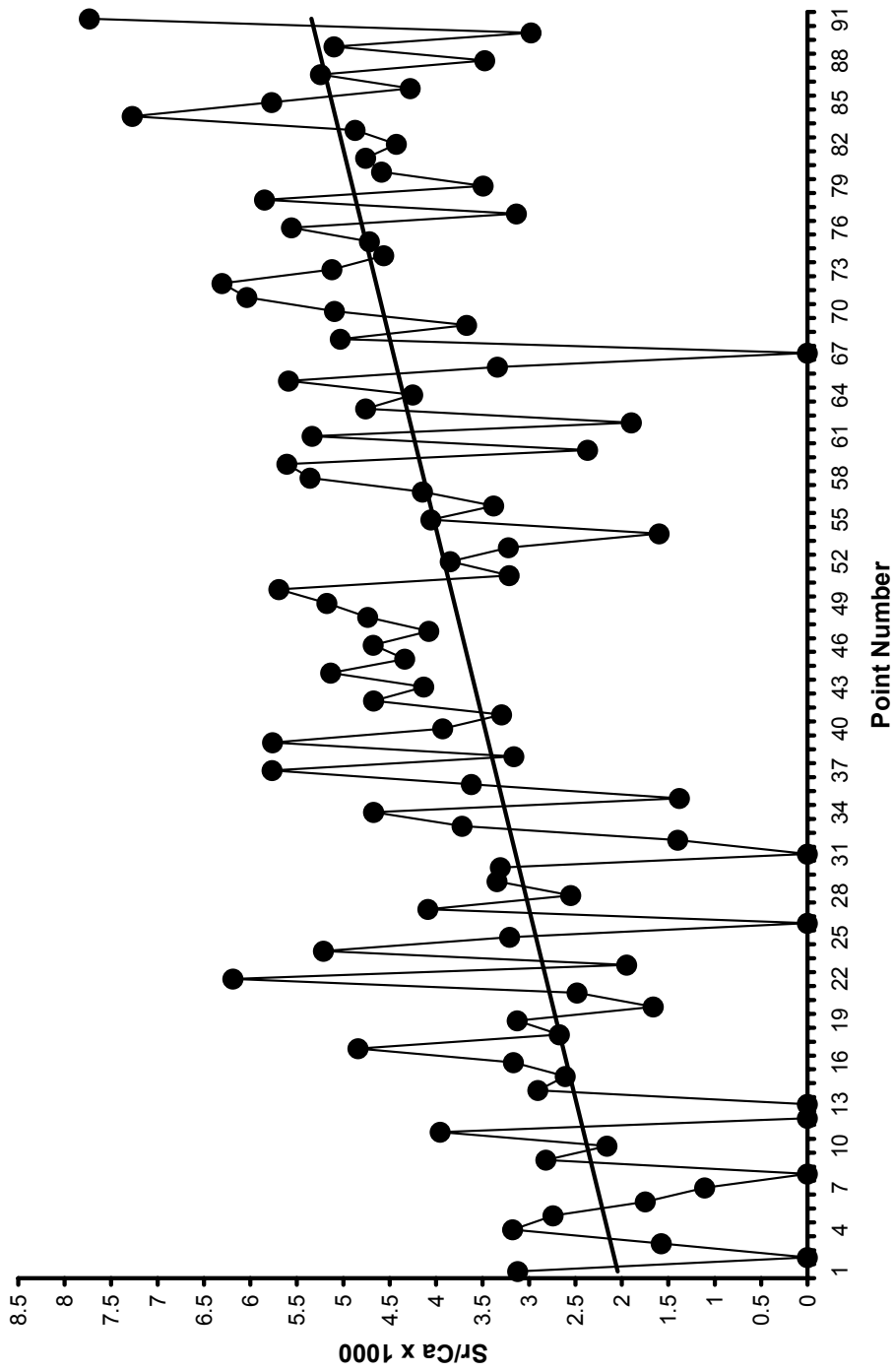


Figure 24. Sr:Ca ratio profile for an adult *Kuhlia xenura* collected from the ocean surge zone along Hawai'i Volcanoes National Park; trendline shows an increase in average salinity with increasing age.

specimen V6 indicated that fresh water was either sought out or encountered in tide pools often in its life. There was a trend in the latter half of its life toward increasing salinities (Figure 25). Specimens V4 and V5 exhibited this same pattern of increasing salinities, but differed from V6 in having less Sr:Ca fluctuations across their life histories; one example is provided in Figure 26. The mean (with standard deviation) for specimen V6 was 3.29 ± 1.36 ; the range of values was from zero to 6.07. For specimens V4 and V5, Sr:Ca values ranged from 1.56 to 4.68, with the mean values for the two fish being $3.07 (\pm 0.798 \text{ standard deviations})$ and $3.10 (\pm 0.6218)$. Again, there was a trend of increasing salinity in the latter half of their life histories (see trendline on Figure 26). Despite more contact with fresh water early in their life histories, all three specimens still exhibited values characteristic of a marine environment early in their lives.

Habitat Description

Kuhlia xenura were found in almost every aquatic habitat on the Islands of O‘ahu and Hawai‘i. This species proved to be especially common in the lower reaches of streams on the windward or rainy side of the islands. Smaller individuals were usually observed in streams, with the largest individuals always observed in the ocean. Occasionally, individuals were found above the lower reaches, upstream of the first true waterfall. *Kuhlia xenura* juveniles were also found in a variety of other coastal habitats, including mixohaline tide pools, tidal creeks, and around forests of exotic mangrove (*Rhizophora mangle*) and hau (*Hibiscus tiliaceus*). Finally, juvenile fish were also identified in shallow ocean waters along sandy beaches and in brackish water fishponds.

In contrast, juvenile *K. sandvicensis* seem to be restricted to tide pools; more specifically, they are found most often in tide pools that retain open connections to the ocean,

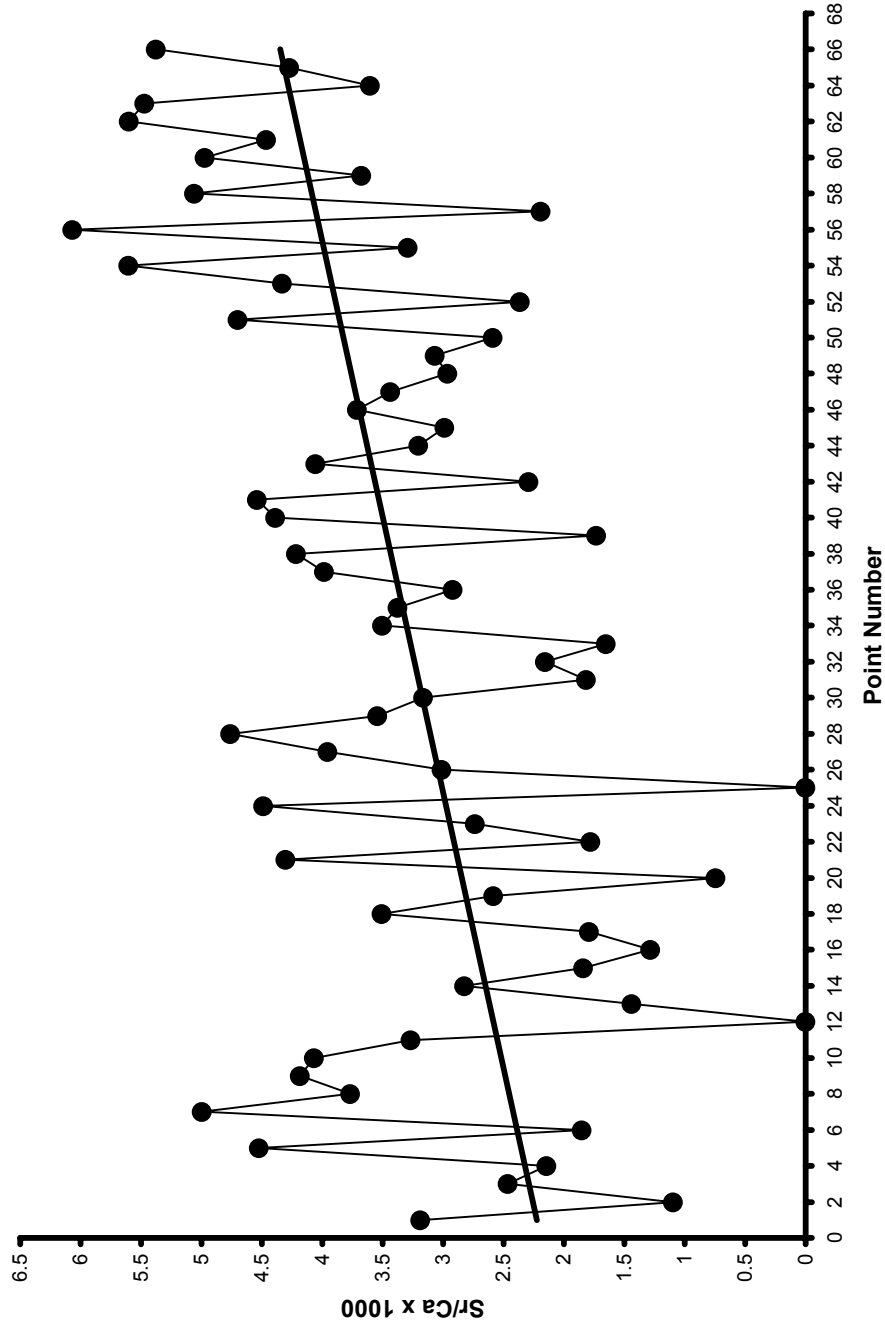


Figure 25. Sr:Ca ratio profile for an adult *Kuhlia sandvicensis* collected from the ocean surge zone along Hawai'i Volcanoes National Park; trendline indicates an increase in average salinity with increasing age.

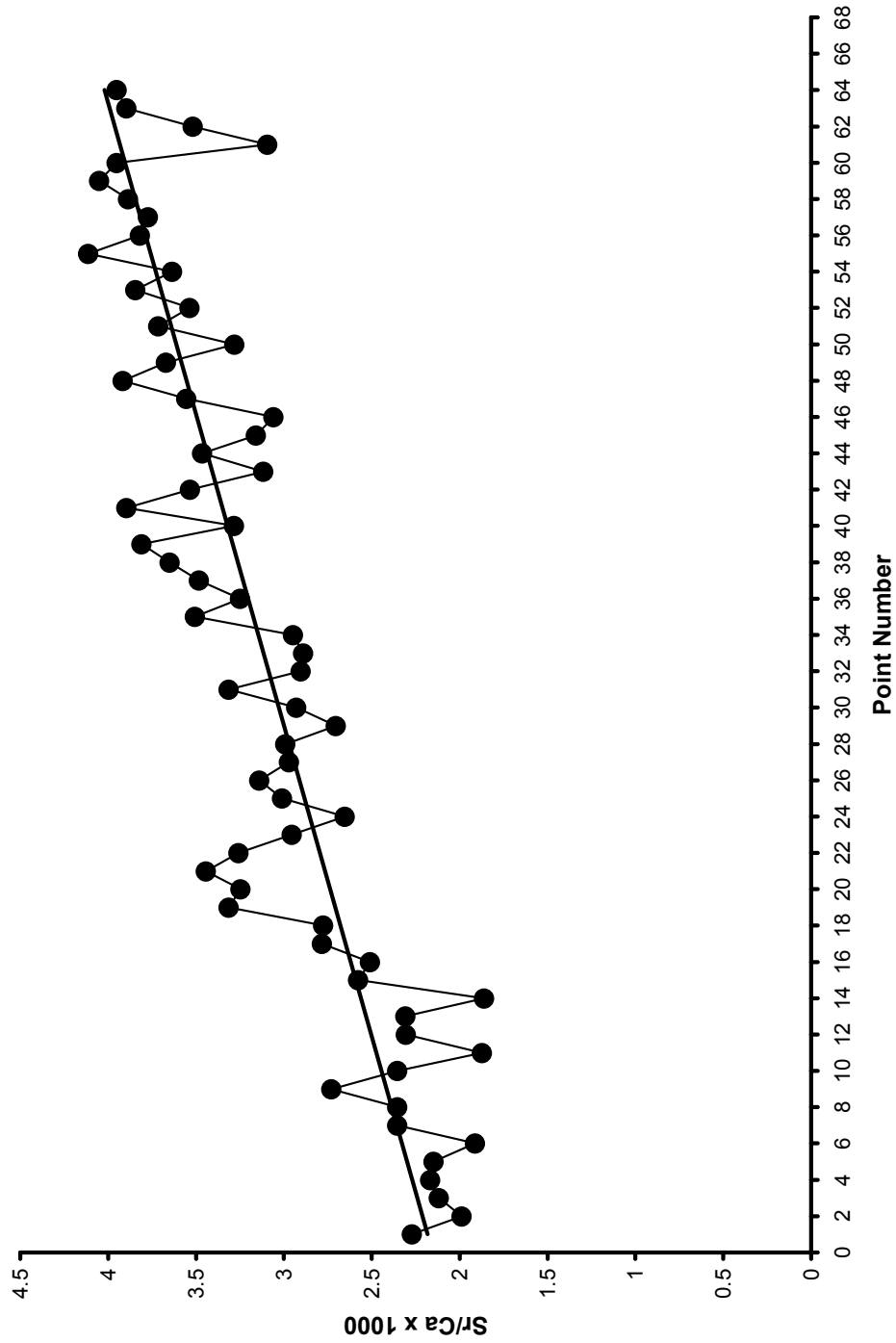


Figure 26. Sr:Ca ratio profile for an adult *Kuhlia sandvicensis* collected from the ocean surge zone along Hawai'i Volcanoes National Park; trendline indicates an increase in average salinity with increasing age.

even at low tide. Unlike *K. xenura*, they are very rarely found in isolated tide pools at low tide; they tend to leave tide pool habitats at a falling tide or when threatened (such as being approached by a diver). In addition, a few observations of juveniles were made in waters along sandy beaches. This species was never observed in the lower reaches of streams. However, specimens of *K. sandvicensis* were observed in tide pools with salinities as low as five ppt. Both species were found in waters of salinities as high as 39 ppt. In addition, both were found to be tolerant of a large temperature range; the maximum water temperature observed for each type was 30.1° C. *Kuhlia sandvicensis* were observed in waters as cold as 19.9° C, whereas *K. xenura* were found in water temperatures as low as 18° C in freshwater stream habitats.

Both types of āholehole are known to live in schools on or near coral reefs as adults; also, some of my observations included adult individuals in caverns or caves. Mature individuals are reported to reside in these types of protected areas during the day and then venture out at night to feed (Hosaka, 1973). However, adult *K. sandvicensis* were also observed and collected during the day as they were feeding in spur-and-groove coral reef habitat. Regardless, fishermen seem to target āholehole at night because they believe that they feed more actively then. Both species of *Kuhlia* are usually fished along coastlines in the ocean surge zone at this time.

Juvenile *Kuhlia* occasionally were found schooling with young *Mugil cephalus* (striped mullet) in both marine and freshwater habitats. In streams, species associated with *K. xenura* (or found within the same habitats) include the diadromous gobioids *Sicyopterus stimpsoni*, *Eleotris sandwicensis*, *Awaous guamensis*, and *Stenogobious hawaiiensis*. Exotic stream species were numerous in these areas as well, and included *Poecilia mexicana* (molly),

P. reticulata (guppy), *Gambusia affinis* (mosquitofish), *Xipophorous helleri* (green swordtail), and unidentified species of *Tilapia*. In estuarine waters, the introduced blacktail snapper *Lutjanus fulvus* occurs in the same habitat areas as *K. xenura*, along with various jack species, and other marine invaders. Mixohaline tide pools had several characteristic species, in addition to both types of āholehole, including *Abudefduf abdominalis* (Hawaiian sergeant), *A. sordidus* (blackspot sergeant), and *Acanthurus triostegus* (convict tang). These estuarine and tide pool species are, like the kuhliids, known to be euryhaline and appear to be able to tolerate a wide range of temperatures.

While collecting habitat data, preliminary observations indicated behavioral differences between the two species of *Kuhlia*. For example, juvenile *K. xenura* in tide pools tend to hide in caverns or crevices when threatened, whereas *K. sandvicensis* usually flee from the tide pool. In addition, *K. sandvicensis* avoid being trapped in tide pools that are closed off from the ocean at low tide, whereas *K. xenura* are commonly seen in these habitats. Finally, juveniles of both morphotypes seem to feed actively during the day, but adult fish feed, by all accounts, nocturnally. However, adult *K. sandvicensis* were observed and collected actively feeding during the day on at least one occasion.

DISCUSSION

Results of the Alizarin experiment indicate that I was justified in counting each microincrement as one day, and this experiment validated age estimates at various points along the microprobe transects for some of the juvenile fish. However, it is not known how long the yolk sac persists in Hawaiian *Kuhlia* and when the first daily increment is laid down in the otolith. In other species, experimental evidence indicates that first increment formation corresponds to hatching, first feeding, or start of activity (Brothers and McFarland, 1981;

Morales-Nin, 1992). Consequently, additional days may need to be added to the age estimates if it is discovered that daily increments do not form immediately at hatching for these fishes.

With the exception of one juvenile *K. sandvicensis*, all fishes examined in this study had Sr:Ca values indicating high salinity early in their lives. They most likely were hatched in the ocean. It is not known why one specimen's transect did not indicate marine values near the otolith core, because it is believed that *Kuhlia* in Hawai'i spawn in marine waters. Perhaps this fish was spawned and spent its early life in waters very near shore where subsurface or stream runoff occurred. Although Sr:Ca profiles differed, all specimens examined on the microprobe also had encountered fresh water at some point in their lives.

Microprobe and Habitat Studies

(1) Stream mouth—*K. xenura*. Values for the four fish collected from fresh water in Hakalau Stream are somewhat perplexing because Sr:Ca ratios of 1.5 to 2.5 do not indicate pure fresh water. One explanation could be that the fish were migrating in and out of the stream within a given day, and their movements thereby caused these intermediate Sr:Ca values. Another possibility is that their otoliths reflect higher Sr:Ca ratios than those expected for completely fresh water because they were exposed to higher Sr levels at the stream mouth where fresh water and the ocean meet. Although a sand or gravel berm sometimes forms at the mouth of Hakalau Stream, one was not present at the time of specimen collection. Thus, fish had an open corridor to move freely between salt and fresh water, and an intrusion of salt water with rising tides and wave action usually occurs in the absence of a berm. It does appear that once these fish had moved into fresh water early in life, they did not venture far

from it for any extended period of time, as they had Sr:Ca values indicative of brackish water throughout their lives.

(2) Upstream in Lower Reach—*K. xenura*. The two fish collected further upstream, Wailoa Stream specimens W1 and W8, showed much movement between salt and fresh water throughout their lives. Presumably because they were upstream far enough to avoid tidal flux, many of their Sr:Ca values reflected pure fresh water. Both specimens exhibited Sr:Ca values that were indicative of hatching or early life in marine waters. Specimen W8's Sr:Ca ratio pattern shows a decidedly more marine existence in the first half of its life, whereas the second half of its life was spent mostly in fresh or brackish water. Specimen W1 does not show this same pattern, however. Even though this specimen was collected further upstream (approximately 3.34 km vs. 1.79 km inland for W8), Sr:Ca ratio values indicate that it continued to make migrations to salt water on a regular basis. Future work may include tracking individuals to determine if their daily movements are properly reflected in the otolith microelemental ratios. Otolith Sr:Ca values indicated these two fish migrated out of fresh water fairly regularly once they reach the size of these two specimens (98 and 123 mm). It would be informative to determine if these stream residents are actually going to different streams or if they are simply reentering the same bodies of fresh water. Furthermore, Font (personal communication) suggests *K. xenura* migrations from stream to stream may prove to be important factors in distributing freshwater parasites to native stream gobies in previously uninfected areas.

(3) Juveniles of Tide Pools. Specimens of both *Kuhlia* species had some unexpected Sr:Ca values across their life history transects. Fish have little access to freshwater streams on the dry side of the island. However, I found that they were exposed to fresh water, or at

least waters of reduced salinity, on a regular basis. Although these fish could have entered the few intermittent freshwater streams that exist on the dry side of this island, they were more likely just encountering the subsurface freshwater flow that commonly enters tide pools and the ocean among the Hawaiian Islands. It should be noted, however, that there was much less variation in Sr:Ca values across the life histories of *K. sandvicensis*, and no values indicating pure fresh water was present. This discovery is not surprising as *K. sandvicensis* were never observed in freshwater streams or fishponds, and they appear to be almost entirely restricted, as juveniles, to tide pool habitats. These mixohaline tide pools may serve as nursery habitats on the dry sides of the islands, whereas the fresh water streams are possibly providing (for *K. xenura*) refugia from predators on the windward coasts. In both these habitats, large marine predators are less likely to forage due to size or salinity constraints.

(4) Adults of the Ocean Surge Zone. Again, all specimens exhibited values characteristic of a marine environment early in their lives, which indicated a marine spawning and larval phase. In addition, specimens of both *K. xenura* and *K. sandvicensis* had either sought out or somehow encountered fresh water often during their lives. As previously mentioned, there was a trend in the later half of the life histories of increasing salinities for both species. This trend is consistent with personal observations that large adult āholehole are less likely than juveniles to enter fresh water streams and inshore habitats. However, I believe that at least *K. xenura* can tolerate fresh water as adults, because I have observed them in fishpond holding pens. Food constraints or fishing pressure may lead to a more marine existence for adult fish.

Future Research

Representative whole otoliths from both species (specimens of similar length) were viewed for shape differences and photographed (Figures 27 and 28). Otoliths are known to be species specific with respect to shape (Shepherd, 1910). Differences in otolith shape have also been used in the past as an indicator of reproducing populations or stocks (Casselman et al., 1981). Upon cursory examination, a difference in otolith shape existed for the *K. sandvicensis* and *K. xenura* specimens. The *K. sandvicensis* otoliths had a semi-circular indentation along one edge and also appeared narrower in width. This species is also narrower in body depth than *K. xenura*. Even though these are just initial observations, this information adds further evidence to support the conclusions from Chapter One that there are two *Kuhlia* species in Hawai'i. A formal shape analysis and description of their sagittal otoliths would be a helpful addition to this research.

In conclusion, microprobe analysis and personal observations of *K. xenura* indicate that it is euryhaline during all phases of its life cycle. In addition, *K. sandvicensis* apparently experiences fluctuations in salinity throughout their lives from almost pure fresh water to marine waters, although they were never observed in freshwater streams. I found no characteristic salinity profile for these species, thereby indicating that there is no obligate requirement for fresh water at a given stage in their lives. Despite the fact that these species coexist in some habitats, the possibility remains that, given different salinity choices, *K. xenura* and *K. sandvicensis* may have different salinity preferences. In addition, future studies may indicate a salinity level that is optimum for these fishes' growth, thereby facilitating both proper management and aquaculture techniques.

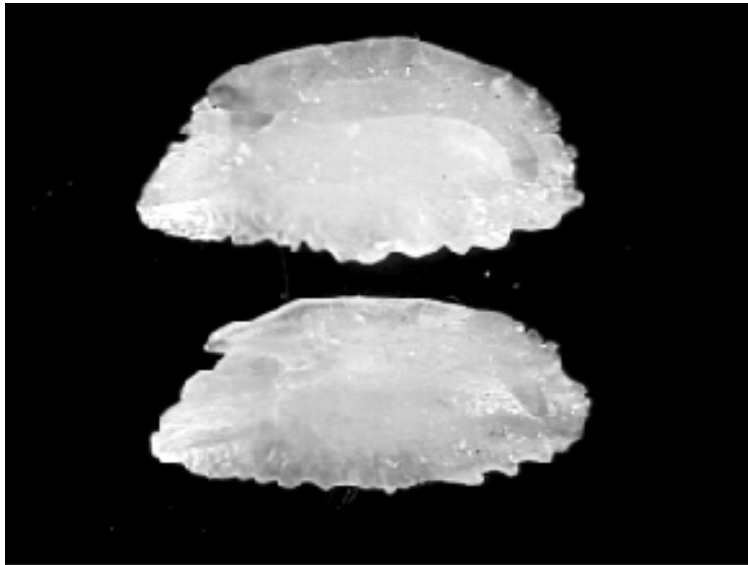


Figure 27. Medial view of *Kuhlia xenura* (top) and *Kuhlia sandvicensis* (bottom) sagittal otoliths.

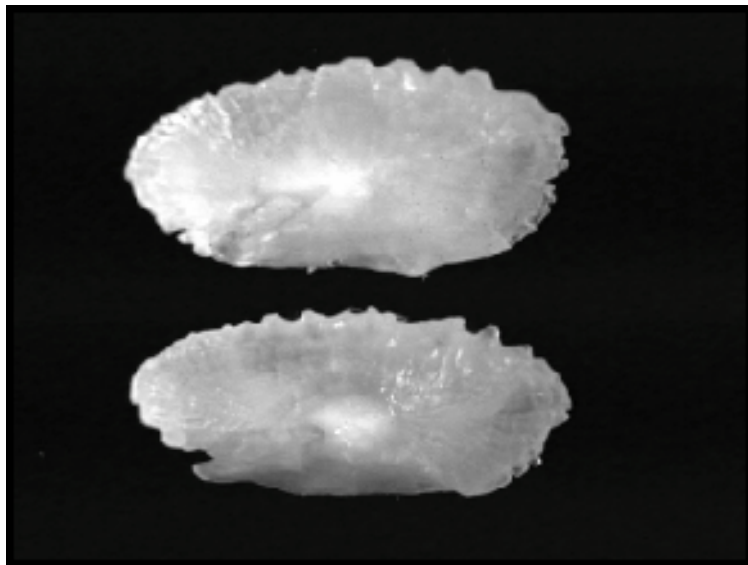


Figure 28. Distal view of *Kuhlia xenura* (top) and *K. sandvicensis* (bottom) sagittal otoliths.

The microprobe analysis provided insight into the natural history of these species. More specifically, this research is leading to an understanding of the diversity of lifestyles that exist within the populations of both species of āholehole in Hawai‘i. Continuing behavioral studies (especially tracking) will enable us to understand the role that *K. xenura* plays in freshwater stream communities and the importance of streams as nursery habitats or essential fish habitat for this species. Similarly, an ecological study investigating the differences in habitat use by Hawaiian *Kuhlia* will shed light on the importance of tide pools for both species. From a conservation perspective, it should be noted that tide pools exposed to increasing human-caused disturbances along the coastlines of Hawai‘i.

Although fresh water may be used as a nursery ground, there does not seem to be a physiological requirement for fresh water at specific points in these two species’ life cycles. This observation is in contrast to the requirements of at least one other kuhliid species, which requires fresh water at a certain point in its life history. Freshwater streams are a critical and possibly endangered habitat in Hawai‘i because of water allocation issues in the islands. Access to fresh water is required by Hawaiian amphidromous gobies to complete their life cycle (Fitzsimons et al., 1996). Maintaining natural stream flow to the ocean may also prove to be a significant advantage for the endemic *K. xenura* (the big-eyed āholehole) as well.

CONCLUSIONS

SUMMARY

This research represents the first attempt at otolith microchemical and DNA sequence analysis for āholehole in Hawai‘i and for fishes in the family Kuhliidae in general. As demonstrated by DNA sequence and morphometric analysis, the *Kuhlia* in Hawai‘i are actually two species. The species that enters freshwater streams and has larger eyes and a deeper body was described by Randall and Randall (2001) as *Kuhlia xenura*. This fish appears to be endemic to the Hawaiian Islands. The small-eyed species has, in life, dark reticulations on the dorsal surface of the head; this species retains the name *Kuhlia sandvicensis*. The aforementioned studies also confirmed that *K. sandvicensis* is the species present on Johnston Island, the nearest island group to Hawai‘i. Meristic counts are highly overlapping in these two species, so a morphometric equation is provided which allows for more accurate discrimination. This classification equation can be used to identify preserved fish from past studies or 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 these two species are the ratios of fork length to body depth, eye diameter, and interorbital distance.

Microprobe analysis and personal observations of *K. xenura* indicate that the species is euryhaline during all phases of its life, although no large adults were observed in freshwater streams. This big-eyed species was found in almost every inshore habitat in Hawai‘i, including freshwater streams and fishponds, tide pools, coral reef habitat, and along sandy beaches. In contrast, *Kuhlia sandvicensis* did not use this variety of habitats; they were found only in tide pools as juveniles. More specifically, they occurred almost exclusively in tide

pools that retained open connections to the ocean, even at low tide. Although few observations were made of adults of *K. sandvicensis*, they appear to occur in the same marine habitats as *K. xenura* (coral reefs and along cliff lines in the surge zone), but may be active diurnally as well as nocturnally. Microprobe studies indicated no characteristic salinity profile for either of these species, but both fresh or brackish water and pure salt water were evident in every life history profile. The tide pools that *K. sandvicensis* frequent experience subsurface freshwater intrusion, and these lowered salinities were reflected in the otolith microchemistry of these fish. With the exception of one fish, all fishes examined had spent time, early in their lives, in marine waters; they were most likely hatched there. Although fresh water in streams or tide pools may be part of their nursery habitat or even used for refugia, there does not seem to be a physiological requirement for fresh water at specific points in these species' life cycles.

IMPLICATIONS FOR CONSERVATION AND MANAGEMENT

Now that a new species of *Kuhlia* has been identified in Hawai'i (and because there appear to be habitat differences for these two species), management strategies currently in place must be examined to assure that they adequately protect both species. The reproductive study conducted by Tester and Takata (1953) is problematic because it is not known which of the two species of *Kuhlia* was used in the study. However, given this caveat, some of their information on size at first reproduction may be useful in determining if current management strategies are sufficient. Tester and Takata (1953) reported mature or maturing fish as small as 145 mm standard length for males and 148 mm for females. Although age at maturity was highly variable and "no exact statement can be made as to the size at which aholehole matured" (Tester and Takata, 1953: 39), it is still noteworthy that immature males as large as

174 mm and females as large as 189 mm were collected by Tester and Takata. Current fishing regulations in Hawai‘i prohibit take below five inches total length (about 125 mm) for both species. Total length encompasses more body size than standard length, so this size regulation may be too small for these species. There is a possibility that most fishes under six inches have not yet reproduced, and it would be beneficial to have the size limit greater than the estimated length at first reproduction. Obviously, larger individuals are more fecund and merely allowing take beyond the minimum reproductive size may not provide enough protection.

Estimating current population sizes for each species is difficult, but *K. sandvicensis* seems to be less common based on preliminary observations. *Kuhlia xenura* juveniles, which are found in so many different habitat types, seem plentiful, but large adults seem harder to locate and catch by fishermen. Although I did not observe fish larger than approximately eight inches in streams, there is anecdotal evidence that large adults entered and lived in streams in the past, possibly before fishing pressure became so heavy and before crossnetting in the lower reaches became common.

Based on otolith results and habitat observations, the lower reaches of streams are now believed to be crucial nursery areas for *Kuhlia xenura*. Obviously, streams and rivers are critical and possibly threatened habitats in Hawai‘i because of freshwater allocation problems in the islands. Other threats to stream ecosystems exist as well. In a study of fishes found in the lower reaches of Wailoa Stream, Hawai‘i, it was determined that high overlap in microhabitat use exists for *K. xenura* and two species of introduced poeciliids (McRae, 2001). Extremely high population densities of these exotic fishes exist in the terminal reaches of many Hawaiian streams (Englund et al., 2000) and, according to McRae (2001), the

introduced poeciliids may serve to displace or exclude āholehole from their preferred microhabitats. In addition to competition issues, this high degree of spatial overlap may put āholehole at risk for parasite transmission from exotic species; it has been determined that non-native, pathogenic parasites (now common in Hawaiian fresh waters) were transmitted to native fishes from the exotic poeciliids (Font, 1998). Five species of Hawaiian diadromous gobioid fishes (four of which are endemic) require fresh water (primarily in streams) for reproduction. This critical resource needs to be closely guarded as it may also serve as an important nursery habitat not just for *Kuhlia xenura* but also for other marine species of commercial importance, like the striped mullet. As for *K. sandvicensis*, the small-eyed āholehole that does not enter freshwater streams, the specific tide pools that juveniles use may also be in danger of disturbance due to commercial and residential developments and pesticide runoff from golf courses.

RECOMMENDATIONS FOR FUTURE RESEARCH

The next study I have planned is a more detailed investigation of the microhabitat preferences of these two fishes. In terms of other research possibilities, it would be informative to investigate why *K. sandvicensis* are more widespread in the Central Pacific than *K. xenura*, which is endemic to Hawai‘i. Is it because they have a longer larval stage, allowing greater distribution as plankton? Otolith analyses of *K. xenura* specimens indicate that they are as young as one to two weeks old when they first show a freshwater signal in their otoliths; maybe their larval stage in the open ocean is relatively short.

In 2001, Randall and Randall’s study cleared up the taxonomic confusion in this family caused by synonyms and mistaken localities in the literature. Now that there is reliable information available about which species occur in specific island groups, one could examine

phylogenetic and geographic relationships within this family around the Pacific. This information could be overlaid with facts about the geologic history of the Indo-Pacific, as well as with ocean current data. This type of analysis could provide knowledge about movement of fishes around the Pacific and possibly lead to a better understanding of how new island groups were colonized by larval fishes. The Kuhliidae is a somewhat unusual family of fishes because even though it contains relatively few species, they run the gamut from freshwater fishes that are catadromous, to euryhaline fishes, as well as true marine species.

Gosline and Brock (1965) state “It is possible that the Hawaiians of Captain James Cook’s time knew more about the fishes of their islands than is known today. Most of this information has now been lost.” Likely, most of their knowledge was restricted to food fishes, as it is known that they had detailed and strongly enforced fishing regulations for these species. It is true that modern-day ichthyologists are still struggling to catalogue and name the World’s fishes, especially in the Indo-Pacific region where biodiversity is high and many places remain relatively unexplored. Biologists have had difficulties just estimating the total number of shore fishes in the Indo-Pacific, as up-to-date checklists from most regions do not exist (Randall, 1998). Obviously, documenting what species are present and identifying cryptic species are necessary for management and conservation purposes in the Hawaiian Islands. This study answered questions not only on the taxonomy of kuhliids in Hawai‘i, but in Johnston Island as well. Initial habitat observations and discoveries about the life history of these two fishes set the stage for future studies that will guide fishery managers when preserving these unique and important species.

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VITA

Lori K. Benson was born in Covington, Kentucky, on May 24, 1973. Her interest in marine biology was developed at a young age, thanks to frequent trips to Florida beaches with her family. In elementary school, one particular trip with her Aunt Ellen and Uncle Hal resulted in a harrowing, but exciting, first open water snorkeling experience, and she has been an avid “fish watcher” ever since. In 1991, Lori moved to Florida to get away from cold winter weather, be closer to the beach, and to attend college. She received, in 1995, a Bachelor of Science Degree in marine science and biology from the University of Tampa. In 1996, she began attending Louisiana State University and working with Dr. Mike Fitzsimons, at the Museum of Natural Science, on various Hawaiian fish projects. She will receive the degree of Doctor of Philosophy in zoology in December of 2002. Lori also enjoys horseback riding, spending time with her family in Kentucky, and Tae Kwon Do. She is currently an Assistant Professor in the Biology Department at her alma mater in Florida.