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FULL PAPER

Specific identification using COI sequence analysis of scombrid larvae collected off the Kona coast of Hawaii Island

Melissa A. Paine · Jan R. McDowell · John E. Graves

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Abstract Physical condition and morphological similarity prohibit unambiguous specific identification in many studies of scombrid larvae, often resulting in several larvae that are unidentified or identified only to genus. Recent molecular techniques allow for the unambiguous identification of early life history stages, even of those specimens that may be damaged. Molecular and morphological techniques were used to determine the species composition of scombrid larvae taken in 43 tows in a putative spawning area off the Kona Coast of Hawaii Island, 19–26 September 2004. Most of these tows were taken at night, at depths of 10 or 14 m, for 1 h each at 2.5 knots. All 872 scombrid larvae collected were identified to species, 29% from unambiguous morphological criteria and 71% using a molecular marker [cytochrome *c* oxidase I (COI) gene sequence]. Yellowfin tuna (*Thunnus albacares*) and skipjack tuna (*Katsuwonus pelamis*) dominated the larval composition almost equally, with frequencies of 48 and 45%, respectively. Five percent of the collection was identified as albacore *T. alalunga*, a higher frequency than reported in previous studies of scombrid larval assemblages around the Hawaiian Islands. This COI molecular marker enabled complete description of species diversity in the assemblage of scombrid larvae collected.

Keywords Larvae · Scombrid · Tuna · Identification · *COI*

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M. A. Paine · J. R. McDowell · J. E. Graves (✉)
Virginia Institute of Marine Science,
School of Marine Science, College of William and Mary,
Rte 1208 Greate Rd, Gloucester Point, VA 23062, USA
e-mail: graves@vims.edu

Introduction

Scombrid fishes (e.g., tunas, mackerels, bonitos) are important worldwide for their economic and ecological value. Bigeye tuna (*Thunnus obesus*), yellowfin tuna (*T. albacares*), albacore (*T. alalunga*), and skipjack tuna (*Katsuwonus pelamis*) are central components of pelagic fisheries that operate in Hawaii's exclusive economic zone (Boggs and Ito 1993; Xi and Boggs 1996). Little is known about the distribution, abundance, ecology, and behavior of early life history stages of these species around Hawaii, but it is this early period that is crucial to understanding survival and recruitment to fishable stocks (Sund et al. 1981).

The composition of scombrid larvae in the central Pacific, particularly around Hawaii, has been described as being dominated by yellowfin tuna, skipjack tuna, and frigate or bullet tunas, *Auxis* spp. (Strasburg 1960; Miller 1979; Boehlert and Mundy 1994). The other scombrid larvae that could be encountered around Hawaii are albacore, bigeye tuna, wahoo (*Acanthocybium solandri*), kawakawa (*Euthynnus affinis*), striped bonito (*Sarda orientalis*), and spotted chub mackerel (*Scomber australasicus*) (see Collette and Nauen 1983). Boehlert and Mundy (1994) identified only a few albacore, bigeye tuna, wahoo, and kawakawa larvae among hundreds of scombrid larvae collected in their surveys around the island of Oahu. Many of the *Thunnus* larvae they collected could not be identified to species, and so it is uncertain if only a few bigeye tuna and albacore were found because their larvae were present in the fraction that could not be identified. Although the incidence of mature adult albacore, wahoo, and bigeye tuna would indicate that spawning could occur in this area, there have been few confirmed collections of the larvae of these species. A macroscopic examination of

albacore gonads collected within 20 miles of the Hawaiian Islands suggested that some spawning was occurring during the summer in the vicinity of the islands (Otsu and Uchida 1959). An analysis of bigeye tuna gonads suggests that spawning occurs well offshore, to the southwest of the island of Hawaii (Nikaido et al. 1991), but the presence of larvae, especially those of smaller size, would be a more direct means to show that spawning occurs in the region (Prince et al. 2005).

Larval tuna are found in abundance near landmasses, especially tropical and subtropical islands (Boehlert and Mundy 1994). Gilmartin and Revelante (1974) hypothesized that nutrient-rich waters near the Hawaiian Islands create favorable conditions for spawning and larval survival. Additionally, physical oceanographic features such as eddies may act to retain larvae near the islands in waters that are favorable for growth and survival (Boehlert and Mundy 1993; Seki et al. 2002). Most studies of nearshore abundance of scombrid larvae around Hawaii have taken place around the island of Oahu (Higgins 1970; Miller 1979; Boehlert and Mundy 1994), and few studies of larval scombrids have been conducted specifically off the Kona coast of the Hawaii Island. The studies off Oahu showed a high concentration of scombrid larvae close to land on the leeward side of the island. These observations and the finding that the Kona coast may be a "hot spot" for billfish spawning (Hyde et al. 2005) suggest that, this area may also be a favorable spawning area for scombrids.

Proper specific identification is essential for early life history studies. Although identification of scombrid adults is unambiguous (Collette and Nauen 1983), specific identification of early life history stages is problematic as many morphological characters are difficult to interpret. Scombrid eggs are very similar in appearance and can only be separated by pigment characters that become lost after preservation (Richards 2006). Larvae of the genus *Thunnus* are especially challenging to identify. Specific identification requires clearing and staining to determine the position of the first closed hemal arch for vertebral precaudal/caudal count, but yellowfin tuna and bigeye tuna can only be separated by the presence or absence of certain pigment characters (Richards 2006). It is also not possible to separate larvae of yellowfin tuna from albacore before the appearance of black pigment cells at the tip of the lower jaw in yellowfin tuna at 4.5 mm standard length (Matsumoto et al. 1971). Juvenile stages (15–60 mm SL) of *Thunnus* species are difficult to identify because the development of body pigmentation obscures diagnostic larval characteristics, and meristic counts are broadly overlapping (Nishikawa and Rimmer 1987).

Previous work on scombrid larval distributions has been limited by dependence upon morphological identification. These types of analyses typically have many larvae that cannot be identified to species level because they are too small to have developed distinguishing morphological characteristics or are too disfigured (Strasburg 1960; Leis et al. 1991; Beckley and Leis 2000). Only half the 227 *Thunnus* larvae that Boehlert and Mundy (1994) collected in their September surveys were large enough to be identified to species, and the remainder had to be classified as *Thunnus* spp. Also, bullet and frigate tuna larvae generally are not distinguished and are grouped together as *Auxis* spp. (Higgins 1970; Boehlert and Mundy 1994). To fully utilize information from all scombrid larvae, a more reliable method of identification is necessary to accurately describe the early life history characteristics of these species.

Molecular markers can provide a means for positive identification when morphological identification is uncertain or impossible (Morgan 1975; Graves et al. 1988; Bartlett and Davidson 1991; McDowell and Graves 2002; Hyde et al. 2005; Perez et al. 2005). Techniques such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis have been used to identify species of the scombrid tribes Thunnini and Sardini (Chow et al. 2003) as well as the species of the genus *Thunnus* (Chow and Inoue 1993; Takeyama et al. 2001). In addition, sequencing of a mitochondrial gene region has been used to identify *Thunnus* species (Bartlett and Davidson 1991; Ram et al. 1996; Quintero et al. 1998; Terol et al. 2002). Previously, a method for the identification of all scombrids occurring in the western Atlantic Ocean was developed that utilizes sequence information from the mitochondrial cytochrome *c* oxidase I (COI) gene region (Paine et al. 2007). The high level of conservation of COI allowed the design of a unique primer pair that successfully amplifies the same fragment across the diverse members of the Scombridae. Although the conservation of COI results in fewer differences between species than a more variable gene region, the differences detected in that study were fixed and therefore sufficient for species identification. That study demonstrated that the COI marker worked well in other ocean basins to identify those scombrids with circumtropical distributions. When morphological identification is limited because of a damaged sample or morphological characters are ambiguous, this molecular marker provides a means for unambiguous identification. In the present study, the COI molecular marker was used in concert with morphological identification to describe species composition of scombrid larvae taken off the leeward coast of Hawaii Island in September 2004.

Materials and methods

Sample collection. Ichthyoplankton sampling was conducted off the Kona coast of Hawaii Island (Fig. 1) aboard NOAA R/V *Oscar Elton Sette*, 19–26 September 2004, using a 1.8-m Isaacs-Kidd Trawl with 0.5-mm mesh. A total of 43 tows was taken at 2.5 knots for 1 h each, with an estimated average 12,580 m^3 of water filtered in each tow. Of these, 31 tows were performed at night, 27 of which were taken at a depth of 10 m and the other 4 at 14 m. The nine preliminary daytime collections were stepped oblique tows at various two-step intervals of 14/10 m, 8/14 m, 30/20 m, and 20/10 m. One tow was taken at 20 m and another was just below the surface (<1 m) in the early morning. One scombrid larva (21 mm) was taken from a midwater trawl (50 m) performed at dusk on September 20. Total collections spanned a distance of 80 km along the leeward coast over 2 days and subsequently five nights. Tows were carried out in all directions in an area from 1 to 16 km offshore. Bottom depth averaged 2,000 m. Temperature of the top 50 m layer was at least 27.5°C, and the wind speed during sampling was very light, never exceeding ten knots.

Plankton samples were preserved in 95% ethanol, and putative scombrid larvae were removed. Each sorted larva was given a unique identifying number, stored in 95% ethanol, and analyzed individually. Individual larvae were photographed using a digital camera attached to a stereoscope via a phototube capturing as much detail as possible for future morphological and meristic analysis. Total length was measured using a ruler placed below the larva and approximated to the nearest 0.5 mm.

Application of marker to identify species. The morphological criteria of Nishikawa and Rimmer (1987) and Richards (2006) were used to identify as many of the scombrid larvae as possible. The unique morphological characters used to positively identify larvae from these guides were forebrain pigment and ventral pigment spot in skipjack tuna, lower jaw pigmentation in kawakawa, lateral tail pigmentation in frigate tuna, and snout length in wahoo. Well-preserved larvae of skipjack tuna, kawakawa, wahoo, and, at some sizes, frigate and bullet tunas were distinguished following the aforementioned identification criteria. Larval *Thunnus* are generally problematic and were distinguished following the cytochrome *c* oxidase I (COI) sequence analysis method developed previously (Paine et al. 2007). This molecular identification method was also used for larvae that could not be identified because of physical damage or questionable morphological characters.

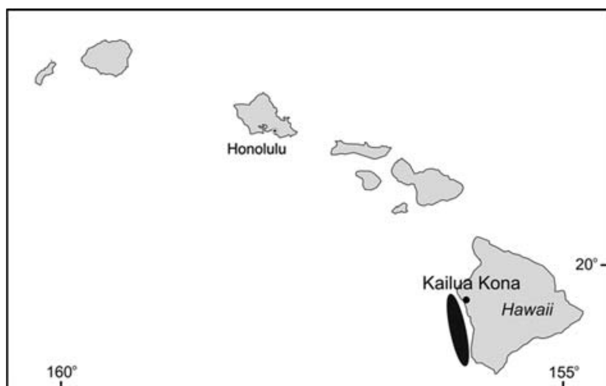

Fig. 1 Map of Hawaiian Islands with scombrid larval collection area shown by *black oval*

DNA was isolated, amplified, and sequenced following the short-fragment COI sequence analysis method (Paine et al. 2007). In cases in which there was no amplicon, the reaction was repeated with both the COI primers and with universal COI primers (Paine et al. 2007). If there was no amplification with the universal primers, then it was inferred that the sample was too degraded for analysis. If amplification resulted from the universal primers but not from the scombrid specific primers, then the sample was inferred to be a non-scombrid. All sequences were edited using Sequencher version 4.2.2 (Gene Codes, Ann Harbor, MI, USA). The species identity was inferred by noting where the sample sequence clustered in an unweighted pair-group method with arithmetic averaging (UPGMA) tree constructed of reference sequences using absolute number of differences in the program MacVector version 7.2 (Accelyrs, San Diego, CA, USA) (Paine et al. 2007). In cases where an unknown sample clustered between two species, informative base positions in the unknown sequence were compared to reference sequences in a molecular key. The positions at which a species has a consistent, unique combination of nucleotide base pairs are indicated in the molecular key shown in Fig. 2.

Preliminary genetic identification of a few larvae showed them grouping between the yellowfin tuna and bigeye tuna reference COI sequences, which only differ by two base position differences. Upon referencing the molecular key, it appeared that only one of the bases was consistently discriminatory between the two species. To refine the key and confirm species identity, part of the mitochondrial cytochrome *b* (cyt *b*) gene was sequenced for four known yellowfin tuna and four known bigeye tuna samples to provide another set of mtDNA reference sequences. The primers used were cytbL686 (5′TCC TTG GTT TCG TGA TCC3′) and cytbH982 (5′GGG TTC AGA ATA GGA ATT GG3′). All PCR and sequencing of cyt *b* was carried out in the same manner as for COI, with a 53°C annealing temperature.

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ALBC CTGCAGGAGGGGGAGACCCAATCCTTTACCAGCATCTATTCTGATTCTTTGGACATCCAGAAGTCTACATT
BLFT ......N.......R................R..C.......K............................
YFT  ...............................A..C.......................R............
BET  ...............................A..C....................Y...............
SKJT .......T..A...........T........A..C..............C..C.....R............
AUXR ......................T........A..C..............Y..T..................
AUXT ......................T........A..C..............C.....................
EUTH .......C........T..............A..C..............C..C..C........A......
ASOL .C........A..T.................A..C.................Y..C..C.....A..T...

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ALBC CTTATTCTTCCCGGATTCGGAATGATTTCCCACATTGTTGCCTACTACTCAGGTAAAAAAGAACCTTTCG
BLFT ...........T..G........K..C...........................................
YFT  ..........................C...........................................
BET  .......................R..C...........................................
SKJT ..A.....A..A..............Y........C............G.C...................
AUXR T.A.....G..A..C.................T..C...........YG.C...................
AUXT T.A.....G..A..C...........C..Y..T..C...........TG.C...................
EUTH T.A.....A..A..............C..T..................G.C...................
ASOL ........C..A.....T.....A.....T..T.....C........T.....C................

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ALBC GCTACATGGGTATGGTATGAGCCATGATGGCCATCGGCCTACTAGGGTTCATTGTATGAGCCCAYCACAT
BLFT ....................................................C.....R.....C.....
YFT  ......................................Y.............C...........T.....
BET  ....................................................C...........C.....
SKJT ....T..........................T....................C...........C.....
AUXR ......................................Y.............C...........C.....
AUXT ..................................T.................C..T..R.....C.....
EUTH ....T........R..G...............................................T.....
ASOL .T..T.....Y....................T....................C...........C.....

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     167890123456789023456789012345678901234567890123456789
ALBC GGTTCACGGTAGGAATGACGTAGACACACGGGCATACTTTACATCCGCAACTAT
BLFT .......................................Y..............
YFT  .......A..............................................
BET  .............W........................................
SKJT .......A......................A..C.................Y..
AUXR .......A.........................C.....C...........A..
AUXT .......A......................A..C.....C...........A..
EUTH R......A......................A..C.....C...........A..
ASOL A...T..A......................A..C.................A..
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Fig. 2 Molecular key of interspecific differences in the cytochrome *c* oxidase I (*COI*) fragment between consensus sequences of each scombrid species. The sites that are useful in distinguishing very closely related species (i.e., between *Thunnus albacares* and *Thunnus obesus*; *Thunnus thynnus* from other *Thunnus*) have an *asterisk*. Species abbreviations with number of reference samples represented in each consensus sequence are *ASOL, Acanthocybium solandri* (21); *ALBC, Thunnus alalunga* (17); *BLFT, T. thynnus* (18); *BET, T. obesus* (18); *YFT, T. albacares* (18); *EUTH, Euthynnus alletteratus* (10); *SKJT, Katsuwonus pelamis* (19); *AUXR, Auxis rochei* (16); *AUXT, A. thazard* (10). Note that *Euthynnus alletteratus* occurs in the western Atlantic and is the congener of *E. affinis* that occurs in Hawaii. Because *E. affinis* is the only *Euthynnus* that occurs around Hawaii, except for the rare waifs of *E. lineatus* from the eastern Pacific, the EUTH reference consensus sequence can be used to identify *E. affinis*

Because introgression of albacore mtDNA into Pacific bluefin tuna *T. orientalis* has been reported previously (Chow and Kishino 1995), the possibility exists that bluefin tuna may be misidentified based on mtDNA characters alone. Preliminary mtDNA analyses identified 43 larvae as albacore and, to address the issue of potential misidentification of bluefin tuna as albacore resulting from introgression, the nuclear internal transcribed spacer (ITS)-1 region of these larvae was sequenced using the primers F-ITS-1 (5′GAG GAA GTA AAA GTC GTA ACA AGG3′) and 5.8SR2 (5′GTG CGT TCG AAR KGT CGA TGA TCA AT3′) (K. Johnson, Virginia Institute of Marine Science, unpublished data). This fragment was amplified and sequenced as previously described for the COI fragment, except that a 45° annealing temperature and 5 μl Q solution (Qiagen, Valencia, CA, USA) were used in the 25-μl reaction. Using the program Sequencher version 4.2.2 to find restriction sites, the restriction enzyme *Eag*I (New England BioLabs, Ipswich, MA, USA) was found to distinguish between bluefin tuna and albacore based on sequence information. Samples were subsequently distinguished in a 15-μl restriction digestion reaction using 0.5 μl

*Eag*I, 1.5 μl 10× NEB43 10× reaction buffer, 11 μl deionized sterile water, and 2 μl PCR product, digested at 37°C for 1 h, then at 65°C for 20 min.

Results

Forty-three ichthyoplankton tows yielded a total of 872 scombrid larvae. Scombrids were found in all collections except two of the nine daytime tows. The daytime tows averaged 2.3 ± 2.2 scombrids per tow and those taken at night averaged 24.3 ± 21.2. Morphological characters were used to positively identify 29% of the larvae, and the remaining 71% were identified using the COI marker. All DNA isolations of scombrid larvae were amplified successfully on the first attempt, with the exception of two specimens that amplified on the second effort. All these isolations were amplified with the scombrid COI primers, except four non-scombrids that amplified with the universal COI primers.

Four of the scombrid larvae grouped between the reference bigeye tuna and yellowfin tuna sequences; two of these individuals (OES18-36, OES22-2) are shown in Fig. 3. Sequence information obtained from the mitochondrial cyt *b* gene demonstrated that three of these were yellowfin tuna and one was a bigeye tuna.

Preliminary analyses identified 43 albacore larvae using the COI marker. To exclude the possibility that some of these putative albacore larvae were not bluefin with introgressed "albacore-like" mtDNA, a portion of the nuclear ITS-1 region was sequenced from these larvae and from reference samples of bluefin tuna and albacore. The albacore larvae identified using the COI marker had ITS sequences more similar to the known albacore samples and consequently were inferred to be albacore. The nucleotide base differences in the ITS-1 region for the reference samples of these two species are shown in Fig. 4.

To provide a quick molecular diagnostic tool to discriminate albacore and bluefin tuna in future studies, a restriction fragment length polymorphism was found in the nuclear ITS-1 region that distinguishes the two species. The restriction enzyme *Eag*I does not digest the ~800 bp PCR product in bluefin tuna but does create 350 and 450 bp bands for albacore (Fig. 5). The northern bluefin tuna (six) and albacore (nine) ITS-1 sequences generated in the study by Chow et al. (2006) were also found to have the same *Eag*I restriction site pattern found in this study, and further there were no intraspecific differences at this cut site.

Sample OES23-73 clustered nearest to EUTH (*Euthynnus alletteratus)*, the only *Euthynnus* included in the original key developed in our previous study of western North Atlantic scombrids. We infer that this specimen is *Euthynnus affinis* as this species is the only congener that is thought to have a persistent population off Hawaii. *Euthynnus lineatus* has been recorded from the Hawaiian Islands twice, but in both cases the single specimens were thought to be vagrants from the eastern Pacific (Matsumoto 1976; Collette and Nauen 1983). Because *E. alletteratus* is the only species in this study without a circumtropical distribution, the reference sequence information of *E. alletteratus* allows for identification of *E. affinis* individuals.

The species composition of scombrids collected in all tows is represented in Fig. 6. Yellowfin and skipjack tuna larvae dominated the collections at frequencies of 48 and 45%, respectively. Albacore were found in 20 tows and comprised 5% of the scombrid larvae. Information on all collections as well as the range of lengths and mean lengths (±SD) of the larvae collected of each species are given in Table 1. Among the commonly encountered larvae, the lengths of skipjack tuna were smaller on average than albacore or yellowfin tuna ($P < 0.05$). The length–frequency distribution of the most common species collected (yellowfin tuna, skipjack tuna, and albacore) at all stations is presented in Fig. 7.

Discussion

Previous studies of scombrid larvae assemblages have been limited by reliance on morphological identification and typically had a fraction of the collection that could not be assigned to a species. The combination of morphological identification and the COI molecular marker used in this study allowed for unambiguous species identification of all scombrid larvae collected off the Kona coast of Hawaii Island. For example, previous larval studies were not able to distinguish past the generic level in *Auxis*, but using sequence information from COI allowed for the distinction between frigate tuna and bullet tuna. Also, previous studies have had a *Thunnus* spp. group or have made tentative specific identifications based on unreliable characteristics, such as one or two very small and inconspicuous ventral pigment spots that separate intact bigeye tuna larvae from yellowfin tuna. In the present study, bigeye tuna and yellowfin larvae were confidently identified using this molecular marker. Additionally, previous studies could not discriminate albacore from yellowfin tuna larvae below 4.5 mm SL. In this study, 46% of the *Thunnus* larvae collected fell into this size range, and all were successfully identified using the COI marker. The use of the COI marker allowed for a complete description of species diversity of the scombrid larval assemblage collected off the Kona coast, which is in contrast to the surveys taken around Oahu by Boehlert and Mundy (1994) in which almost half of the more than 200 *Thunnus* larvae they collected in September could not be identified to species.

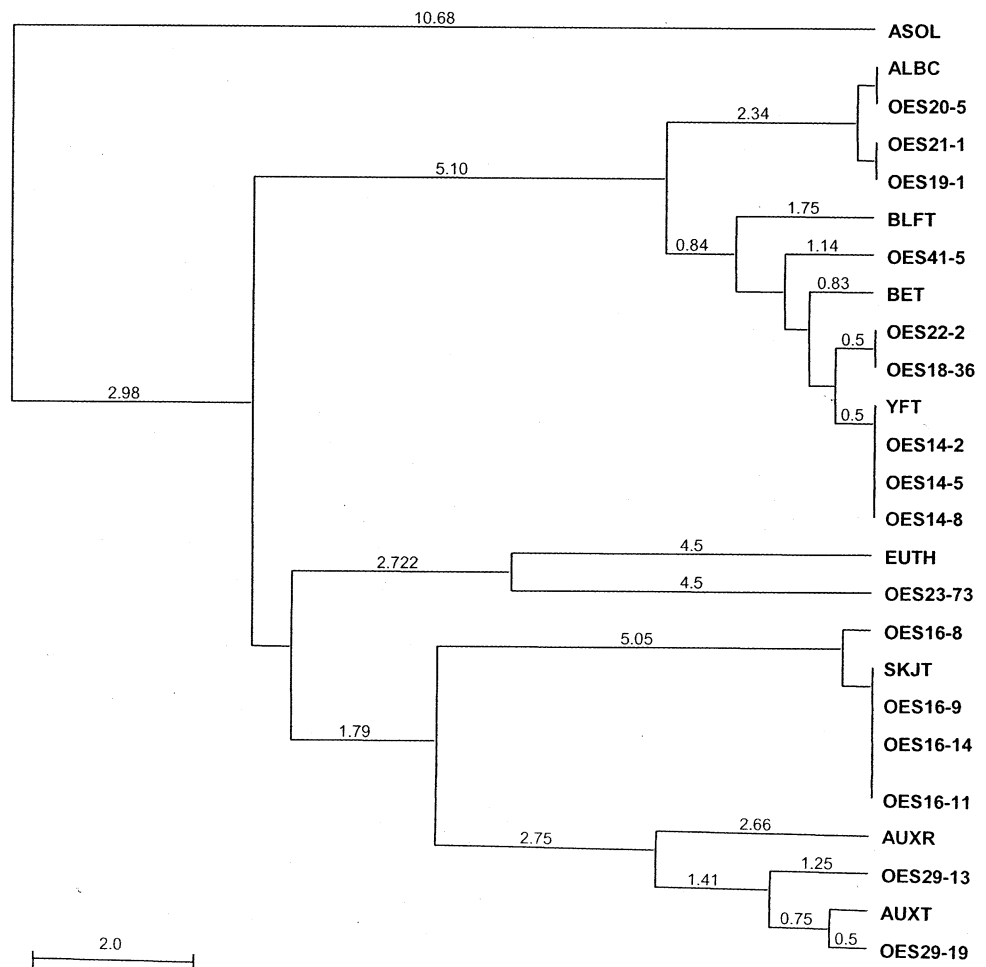

Fig. 3 UPGMA tree constructed based upon absolute number of nucleotide differences between consensus sequences and unknown larval specimens from Hawaii Island. Unknown larvae are designated by an *OES* prefix. Each species group is a consensus sequence of all haplotypes of COI of that given species. Species abbreviations are given in Fig. 2. Samples *OES41-5* (bigeye), *OES22-2* (yellowfin), and *OES18-36* (yellowfin) were compared against the molecular key to verify species assignment. *OES23-73* clusters nearest to EUTH (*E. alletteratus*), and we infer that it is *E. affinis* as this is the only congener that is found persistently off the Hawaiian Islands

Previous studies around Oahu encountered mostly *Thunnus* spp., skipjack tuna, and *Auxis* larvae (Higgins 1970; Miller 1979; Boehlert and Mundy 1994). Our surveys off Kona were dominated by yellowfin and skipjack tuna almost equally, with 421 and 395 larvae, respectively. In comparison, the September surveys of Boehlert and Mundy (1994) were dominated by *Thunnus* larvae (227 of 365 scombrid larvae). In that study, Boehlert and Mundy encountered almost 75 *Auxis* spp. larvae in September, which outnumbered the 50 skipjack tuna they caught. In the present study we found only 9 frigate tuna while encountering almost 400 skipjack tuna. Boehlert and Mundy found *Thunnus* spp. and skipjack tuna larvae when water temperatures were warmest, during September and June. During sampling off Kona, the water temperature was above 27°C, which may account for the increased abundance of larval yellowfin and skipjack tuna.

A surprising number of albacore larvae were collected off the Kona coast. The 43 albacore larvae found comprised 5% of the total sample, more than the 9 (2.5%) found by Boehlert and Mundy in September off Oahu. There is the possibility that some of the unidentified *Thunnus* spp. in their study may have been additional albacore or even bigeye tuna larvae. The number and size of albacore larvae in this study suggests spawning within the region of the Kona coast. Albacore spawning is considered to be centered around 20°N in the North Pacific, with Hawaii located on the northeastern border of this range (Ueyanagi 1969). Albacore are known to spawn in the general vicinity of Hawaii, with limited spawning to the east of the islands and more frequent spawning to the west (Sund et al. 1981; Nishikawa et al. 1985). Identifying larval habitat at the perimeter of the spawning range is important in describing appropriate conditions for spawning (Boehlert and Mundy 1994).

In the larval surveys performed by Boehlert and Mundy (1994) taken around Oahu in September, December, April, and June, bigeye tuna, wahoo, and kawakawa were all uncommon. Similarly, in the present study performed off Kona in September, few larvae of these species were encountered. Many larval surveys around Hawaii have found lower abundance of bigeye tuna compared to other tuna species; this may be the result of interspecific behavioral differences that result in different catchabilities

Fig. 4 Internal transcribed spacer (ITS)-1 sequence alignment of *Thunnus alalunga* and *T. thynnus* showing interspecific nucleotide differences. Insertions and deletions between species are shown by *dash marks*

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                   1111111111222222222233333333334444444444555555555566666666667
          1234567890123456789012345678901234567890123456789012345678901234567890
ALBC5ITS  ACCTGCGGAAGGATCATTACCGGTTTGCCCGCCCTGTCGCAGGGGCGCGCAACAGACGCCTCCGGACA
ALBC11ITS ........-.............................................................
ALBC9ITS  ......................................................................
BLFT17ITS ........-.............................................................
BLFT18ITS ........-.............................................................
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BLFT20ITS ........-.............................................................

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ALBC5ITS  GCTGAGGGCCAGAGGGTAGGTCTCTTCCCCCGCCGGGT-CCCGTCGCGGTCGTCCTCCCGAGGACTTCCC
ALBC11ITS ......................................-...............................
ALBC9ITS  ......................................-...............................
BLFT17ITS ...............................C......T...............................
BLFT18ITS ...............................C......-...............................
BLFT19ITS ...............................C......T...............................
BLFT20ITS ......................................-...............................

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          1234567890123456789012345678901234567890123456789012345678901234567890
ALBC5ITS  CGGCGGGGGGGGGGCGGTGGTCGTTTCCCTCCC-GACGGCCCGAGGCGCGGGGCGGGCGCTTCTCTTCCC
ALBC11ITS ......C..C.C.....................C................-C.......G..........
ALBC9ITS  .....-C..C.C.....................-.................C..................
BLFT17ITS .....-C..C.C.....................GA.G.C..G-.......-C..................
BLFT18ITS .....-C..C.C.....................GA.G.C..G-.......-C..................
BLFT19ITS .....-C..C.C.....................GA.G.C..G-.......-C..................
BLFT20ITS .....-C..C.C.....................C................-C.......G..........

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ALBC5ITS  GAGACGCCCCGCGCACACAACCTCTCCTGGTCAGGACCTCGTCCCGACCGGGCGGGCCTCCCCATCTCTC
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BLFT17ITS ...............A.AC.............................................CT....
BLFT18ITS ...............A.AC.............................................CT....
BLFT19ITS ...............A.AC.............................................CT....
BLFT20ITS ...............A.AC.............................................CT....

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ALBC5ITS  CCCCCC-TGGGCGACACGCCTCGCGATCCCGGCCGGCCAAAGCTCCGCGGGGCTGACGGAGCGGCGGGTC
ALBC11ITS ......-...............................................................
ALBC9ITS  ......-...............................................................
BLFT17ITS ......C...................C.......A...T...............................
BLFT18ITS ......C...................C.......A...T...............................
BLFT19ITS ......C...................C.......A...T...............................
BLFT20ITS ......C...................C.......A...T...............................

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          5555555556666666666777777777788888888889999999999000000000011111111112
          1234567890123456789012345678901234567890123456789012345678901234567890
ALBC5ITS  GGACGTCGCGGGGCGCCCTCGCGGGGGTCGGGGACCCCGTCCGGTGCACCGGGCCTGGGCCGACACTCGG
ALBC11ITS ......................................................................
ALBC9ITS  ......................................................................
BLFT17ITS ........--................GTC...GA....................................
BLFT18ITS ........--................GTC...GA....................................
BLFT19ITS ........--................GTC...GA....................................
BLFT20ITS ........--................GTC...GA....................................

          4444444444444444444444444444444444444444444444444444444444444444444444
          2222222223333333333444444444455555555556666666666777777777788888888889
          1234567890123456789012345678901234567890123456789012345678901234567890
ALBC5ITS  AACCTAAACCCTAAGCGCGGAAGCGGAGGCTTCGCCCCCCGCCGCACGCCGCGCGCCCCCGGGTACCCAA
ALBC11ITS ......................................................................
ALBC9ITS  ......................................................................
BLFT17ITS .....T....A........C..................................................
BLFT18ITS .....T....A........C..................................................
BLFT19ITS .....T....A........C..................................................
BLFT20ITS .....T....A........C..................................................

          4444444445555555555555555555555555555555555555555555555555555555555555
          9999999990000000000111111111122222222223333333333444444444455555555556
          1234567890123456789012345678901234567890123456789012345678901234567890
ALBC5ITS  CTCTCCCCCCTCCTTCGGAGGGAGGAGGGGGGTTCAATGTCTCCCCACCCCGCCTCGGCGGGGTCCGGAG
ALBC11ITS ......................................................................
ALBC9ITS  ......................................................................
BLFT17ITS ................................................................T.....
BLFT18ITS ................................................................T.....
BLFT19ITS ................................................................T.....
BLFT20ITS ................................................................T.....
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Fig. 4 continued

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            5555555555555555555555555555555555555555666666666666666666666666666666
            6666666666777777777788888888888999999999990000000000111111111122222222223
            1234567890123456789012345678901234567890123456789012345678901234567890
ALBC5ITS    CGCCCGGGGGTCCYGTCGTCTTCCCCTTTCCAAACCCGAATTGTCTCTGAACGTTGGCAACCTCTGTGCG
ALBC11ITS   ......................................................................
ALBC9ITS    ......................................................................
BLFT17ITS   .............T.........................C..............................
BLFT18ITS   .............T.........................C..............................
BLFT19ITS   .............T.........................C..............................
BLFT20ITS   .......................................C..............................

            6666666666666666666666666666666666666666666666666666666666666666666667
            3333333333444444444455555555556666666666777777777788888888889999999990
            1234567890123456789012345678901234567890123456789012345678901234567890
ALBC5ITS    GTGTAAAAACCGACAAAAAAGTTGTGACAACTCTTAGCGGTGGATCACTCGGCTCGTGCGTCGATGAAGA
ALBC11ITS   ......................................................................
ALBC9ITS    ......................................................................
BLFT17ITS   ......................................................................
BLFT18ITS   ......................................................................
BLFT19ITS   ......................................................................
BLFT20ITS   ......................................................................

            7777777777777777777
            0000000001111111111
            1234567890123456789
ALBC5ITS    ACGCAGCTAGCTGCGAGAA
ALBC11ITS   ...................
ALBC9ITS    ...................
BLFT17ITS   ...................
BLFT18ITS   ...................
BLFT19ITS   ...................
BLFT20ITS   ...................
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Fig. 5 Restriction digestion pattern of bluefin tuna (*BLFT*) and albacore (*ALBC*) samples using the restriction enzyme *Eag*I. *Lane 1*, 1 kb+ ladder; *lanes 2–5*, digests of bluefin tuna samples; *lanes 6–9*, digests of albacore samples. Bands have been accentuated using Adobe Photoshop, and numbers associated with bands denote the size of the band in number of base pairs

Fig. 6 Species composition of all tows grouped by each night or day in which they were taken, and only showing the most commonly collected species: yellowfin tuna, skipjack tuna, and albacore. The dates of collection are as follows: *Day 1* (Sept. 19), *Day 2* (Sept. 20), *Night 1* (Sept. 21/22), *Night 2* (Sept. 22/23), *Night 3* (Sept. 23/24), *Night 4* (Sept. 24/25), and *Night 5* (Sept. 25/26). One wahoo larva was found in each of the daytime tows, one kawakawa larva was found on the last night, and one bigeye larva was found in *Night 4*

(Nishikawa et al. 1985) or lower levels of spawning in the area. Also, despite the abundance and high fecundity of the cosmopolitan wahoo (Collette and Nauen 1983), their larvae are rarely encountered around Hawaii (B. Mundy, personal communication). Only 2 wahoo larvae were collected in the present study and only 11 were taken in the Oahu study in September and June (Boehlert and Mundy 1994). Additionally, only one kawakawa larva was found off Kona, consistent with the results of Boehlert and Mundy (1994), who only encountered five kawakawa larvae total in their April, June, September, and December surveys.

Although the COI molecular marker offers many advantages for species identification of larvae, one limitation of using only a mitochondrial marker is the possibility that introgression may result in the misidentification of samples. Mitochondrial introgression has been previously reported in the genus *Thunnus*. Historically, hybridization occurred between male bluefin tuna and female albacore, and the maternally inherited albacore mitochondria were subsequently retained in backcrossing between the hybrids and bluefin tuna (Chow and Kishino 1995). The mitochondrial genome of the albacore has introgressed into the bluefin tuna genetic background within the Pacific at a high frequency (98%) (Chow and Kishino 1995), and the possibility exists that a bluefin tuna with albacore-like mtDNA could be incorrectly identified as albacore if only a

Table 1 Description of scombrid larvae collected off the Kona coast of Hawaii Island during 19–26 September 2004

Species	Total number of larvae collected	Percent of collection	Number of tows in which larvae were found of 35 (night) and 8 (day) tows	Range of larvae lengths (mean length ± SD)
Skipjack	395	45	31 (night)	2–9 mm (4.1 ± 1.3 mm)
Albacore	43	5	16 (night); 3 (day)	3–10.5 mm (5.5 ± 1.7 mm)
Frigate tuna	9	1	5 (night)	2.5–6 mm (4.3 ± 1.1 mm)
Wahoo	2	<1	2 (day)	4.5, 9.5 mm (4.1 ± 1.3 mm)
Kawakawa	1	<1	1 (night)	2.5 mm
Bigeye	1	<1	1 (night)	4 mm

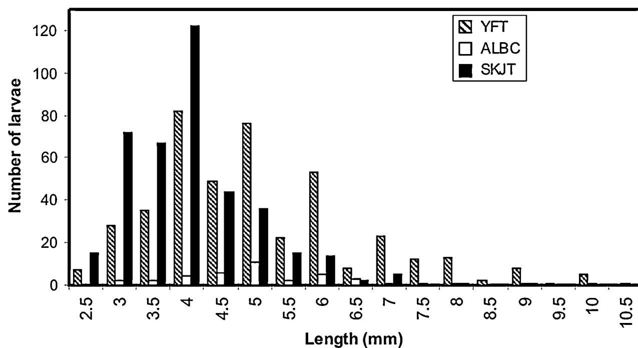

Fig. 7 Length–frequency distribution of the most common scombrid species collected: *Thunnus albacares* (*YFT*), *Katsuwonus pelamis* (*SKJT*), and *T. alalunga* (*ALBC*) from all tows targeting scombrids. Skipjack tuna were on average smaller than albacore and yellowfin tuna ($P < 0.05$)

mitochondrial marker is used. However, bluefin tuna are not commonly encountered around Hawaii (Boggs and Ito 1993; NMFS 1999) and are not known to spawn there (Nishikawa et al. 1985), so there was only a small possibility of misidentification. This possibility of introgression was excluded by sequencing the nuclear region ITS-1 of the 43 larvae that had been initially identified as albacore using the mitochondrial gene COI and confirming their identity as albacore. The nucleotide variation between bluefin tuna and albacore in the ITS-1 region generated different-sized *Eag*I restriction fragment bands between these species, a molecular marker that can be used as a quick diagnostic for introgression in future studies.

The Kona coast of Hawaii Island has not been the focus of many scombrid larval studies, and the information from the present study highlights the importance of further investigations in this area. The successful identification of all scombrid larvae of any size and in any physically damaged condition indicates the potential of this molecular marker as a means for describing putative spawning grounds off the Kona coast of Hawaii Island and elsewhere. This approach would be applicable for use in future ichthyoplankton surveys targeting scombrids, as it is especially useful to distinguish *Thunnus* and *Auxis* early life history stages to species.

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