Identification of small juvenile scombrids from northwest tropical Australia using mitochondrial DNA cytochrome *b* sequences

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Abstract Small juveniles of the nine species of scombrids in Australian waters are morphologically similar to one another and, consequently, difficult to identify to species level. We show that the sequence of the mitochondrial DNA cytochrome *b* gene region is a powerful tool for identification of these young fish. Using this method, we identified 50 juvenile scombrids collected from Exmouth Bay, Western Australia. Six species of scombrids were apparent in this sample of fish: narrow-barred Spanish mackerel (*Scomberomorus commerson*), Indian mackerel (*Rastrelliger kanagurta*), frigate tuna (*Auxis thazard*), bullet tuna (*Auxis rochei*), leaping bonito (*Cybiosarda elegans*), and kawakawa (*Euthynnus affinis*). The presence of Indian mackerel, frigate tuna, leaping bonito, and kawakawa is the first indication that coastal waters may be an important spawning habitat for these species, although offshore spawning may also occur. The occurrence of small juvenile *S. commerson* was predicted from the known spawning patterns of that species, but other mackerel species (*Scomberomorus munroi, Scomberomorus queenslandicus, Scomberomorus semifasiciatus*) likely to be spawning during the sampling period were not detected among the 50 small juveniles analyzed here.

Key words Scombridae · Juveniles · Northwestern Australia · DNA identification · Cytochrome *b*

Nine species of mackerel (Scombridae) are found in the coastal waters of Australia, and seven of these species are endemic. The other scombrids in Australian waters are nine species of tuna and two species of bonito. Australian scombrid fisheries are economically important for food, bait, and sport fishing. However, apart from some work done in Western Australia on Spanish mackerel (*Scomberomorus commerson*; Cameron and Begg, 2002), little is known about the early life history or timing and location of spawning of Australian scombrids. This information would be useful for the management of scombrid fisheries; for example, juvenile habitat and spawning areas could be protected from exploitation.

Post-larval and early-stage juvenile mackerel, tuna, and bonito can be readily identified as scombrids by their distinctive morphological features (Neira et al., 1998). However, post-larval and early-stage juveniles of the different species of mackerel, tuna, and bonito are morphologically similar to one another. Distinguishing species that belong to the same genus is particularly difficult (Richards, 1989). Although the larvae of most Australian mackerels have been described morphologically (Jenkins et al., 1984; Neira et al., 1998; Leis and Carson-Ewart, 2000), dichotomous keys to identify larvae and juvenile Australian mackerels have not been published. Drawings of larvae and early-stage juveniles may be used to identify these fish, but this requires considerable familiarity with the morphology of these larvae and early-stage juveniles and expert microscopy. Further, use of morphological diagnostic characters such as pigment patterns can be problematical when specimens have deteriorated. DNA-based keys have been used to identify the larvae of crayfish (Booth and Ovenden, 2000), billfishes (McDowell and Graves, 2002), and tunas (Chow and Inoue, 1993; Chow et al., 2003).

We show that the cytochrome *b* gene region from the mitochondrial genome is a suitable marker for eight of the species of mackerel, tuna, and bonito that occur in Australian waters and for identifying post-larval and early-stage juveniles of mackerel from Exmouth Bay, Western Australia.

Materials and Methods

Collection of fish.—Hundreds of post-larval and early-stage juvenile scombrids were caught in light traps around Exmouth Bay, Western Australia, between 1999 and 2001. They were preserved in 70% ethanol soon after capture. The location of the light-trap sampling stations can be seen in Meekan et al. (2001: fig 2, sampling stations A–H). Fifty small juveniles, with head-to-tail fork lengths of 16.1–85.0 mm, were selected for study as representatives of different morphotypes. Adult mackerel were caught by line or trawl in coastal Queensland and Northern Territory and

Table 1. Scombrids found in Australian waters and GenBank accession numbers for the cytochrome *b* sequences (270 base pairs) used in this study

Species	Common name	Collection location	GenBank accession number
Mackerel			
Acanthocybium solandri	Wahoo	QLD	AY390595[a]
Grammatorcynus bicarinatus	Shark	QLD	AY390594[a]
Grammatorcynus bilineatus	Double lined	Not available	
Scomber australasicus	Slimy	QLD	AY390593[a]
Scomberomorus commerson	Narrow-barred Spanish	NT, QLD	AY390589[a]
Scomberomorus munroi	Spotted	QLD	AY390591[a]
Scomberomorus queenslandicus	School	QLD	AY390590[a]
Scomberomorus semifasciatus	Broad-barred Spanish	NT, QLD	AY390592[a]
Rastrelliger kanagurta	Indian	BRU	AY390596[a]
Tuna			
Auxis rochei	Bullet	Pacific[b]	AB098082
Auxis thazard	Frigate	BRU	AY390599[a]
Euthynnus affinis	Kawakawa	QLD	AY390598[a]
Katsuwonis pelmanis	Skipjack	UKN	L11539
Thunnus alalunga	Albacore	UKN	L11556
Thunnus albacares	Yellowfin	UKN	L11557
Thunnus maccoyii	Southern bluefin	UKN	L11558
Thunnus obesus	Bigeye	UKN	L11559
Thunnus tonggol	Northern bluefin	UKN	AF239964
Bonito			
Cybiosarda elegans	Leaping	QLD	AY390597[a]
Sarda orientalis	Oriental	Pacific[b]	AB098098

BRU, Brunei; NT, Northern Territory; QLD, Queensland; UKN, unknown

[a] Sequences generated in this study

[b] See Chow et al. (2003)

identified to species level. Fin-clips and pieces of flesh from adults were preserved in 20% DMSO (dimethyl sulfoxide) saturated with NaCl. Eight of the nine species of mackerel occurring in Australian waters were sampled: only the double-lined mackerel, *Grammatorcynus bilineatus*, was not available (Table 1). Adult mackerel tuna (*Euthynnus affinis*), frigate tuna (*Auxis thazard*), and leaping bonito (*Cybiosarda elegans*) were also collected because these species have small juveniles that are morphologically similar to mackerel. They are also common in the inshore waters around Exmouth Bay, but little is known about their spawning habits. One adult sample from each species was analyzed, as it is expected that interspecific sequence divergence should exceed intraspecific divergence (Ward et al., 2005) for mitochondrial protein gene regions such as cytochrome *b* and cytochrome oxidase subunit I.

Extraction, PCR, purification, and sequencing.—Genomic DNA was extracted from juvenile and adult tissue samples with a Qiagen DNeasy Tissue Kit (Qiagen Pty, Doncaster, VIC, Australia) standard protocol. A fragment of cytochrome *b* was amplified using the polymerase chain reaction (PCR) with the primers CB1 L (5′-CCATCCAACATCT CAGCATGATGAAA-3′) and CB2H (5′-CCCTCAGAAT GATATTTGTCCTCA-3′) (Palumbi and Benzie, 1991). Reaction mixtures consisted of 100 µl containing 8 mM dNTPs, 10X buffer, 10 µM primer, 1.5 mM MgCl, and *Taq* DNA polymerase. The cycling conditions were an initial 90 s denaturation step at 94°C, followed by 35 cycles of 5 s at 94°C, 30 s at 45°C, and 30 s at 72°C, followed by a final extension step of 5 min at 72°C. Amplified DNA fragments were purified with Qiagen QIAquick PCR Purification kit. Five microliters (ca. 20 ng) DNA, 0.3 µl (10 µM) forward or reverse primer, and 6 µl fluorescent dye-labeled premix (ABI Big Dye terminator v 2.0; Applied Biosystems, Foster City, CA, USA) were used for each sequencing reaction. Cycling conditions for sequencing were denaturation for 10 s at 96°C, 5 s annealing at 50°C, and then 2 min extension at 60°C, for 25 cycles. Sequencing products were ethanol precipitated, then separated using an ABI 337 capillary electrophoresis setup. Both strands of DNA were sequenced. Sequencing and PCR reactions were performed on a GeneAmp PCR 2400 System thermocycler (Applied Biosystems).

Genetic analyses.—The forward and reverse sequences from each adult and larval sample were aligned with Sequencher (v 4.6; Gene Codes, Ann Arbor, MI, USA) and a consensus nucleotide sequence was generated. These consensus sequences were then aligned with Sequencher. The first nucleotide of the fragment was at position 98 of the human cytochrome *b* gene (GenBank NC001807). Nucleotide sequences of cytochrome *b* for seven tuna and one bonito species from GenBank (see Table 1) were added to our alignments for reference. The computer program BioEdit (Hall, 1999) was used to calculate the nucleotide composition of the sequences. Nucleotide difference between sequences was calculated by dividing the total

number of polymorphic sites by the total number of base pairs (270 bp; Table 2). The shark mackerel (*Grammatorcynus bicarinatus*) sequence had three ambiguous nucleotides, which were ignored in these paired-sequence calculations.

To assign post-larval and early-stage juveniles samples to a species, we compared their nucleotide sequence to sequences from adult and subadult mackerel, which had been identified to species by their morphology. A neighbor-joining tree was constructed from pairwise Kimura two-parameter nucleotide sequence distances (Kimura, 1980) using MEGA 3.1 (Kumar et al., 2004). Bootstrapping was performed 1000 times to evaluate the validity of the identifications by resampling all characters in the data set.

Results and Discussion

Cytochrome *b* sequence characteristics. There were no insertions or deletions of nucleotides in our alignment of scombrid sequence from the cytochrome *b* gene region. Interspecific sequence variation ranged from 1.7% to 19.5% (Table 2). The mean nucleotide composition of the sequences was A, 22%; C, 32%; G, 17%; and T, 29%. The A + T content across species varied from 50.3% to 58.0%. The 13 amino acids that are critical to the function of this part of cytochrome *b* were present in eight of the mackerel species studied (Esposti et al., 1993; Finnerty and Block, 1995). This, together with the lack of insertions and deletions, indicated that the nucleotide sequences represented functional mtDNA genes, not pseudogenes of cytochrome *b*. We judged that the degree of nucleotide variation among the scombrid species was suitable for distinguishing these species from their nucleotide sequence. Eight mackerel, nine tuna, and two bonito species, represented by adult and subadult fish that were identified to species by morphology, had 105 polymorphic sites across 270 base pairs of aligned sequence. They differed from one another at 26 (8.7%; *S. commerson* and *Scomberomorus queenslandicus*) to 58 (19.5%; *Scomberomorus australasicus* and *G. bicarinatus*) nucleotide positions (Table 2).

Identification of post-larvae and early-stage juvenile scombrids from their cytochrome *b* sequence. Sixteen different sequence haplotypes were found among the 50 post-larvae and early-stage juveniles. These sequence haplotypes are labeled Juv-1 to Juv-16 in Fig. 1. Two of these haplotype sequences were indistinguishable to sequences from known species of scombrid: the narrow-barred Spanish mackerel (*S. commerson*, Juv-1) and leaping bonito (*C. elegans*, Juv-2) (Fig. 1). The remaining 14 sequence haplotypes were not identical to any of the adult sequences, but they were similar to our reference sequences, differing by one to four base pairs. Provisional identifications for these sequence haplotypes were made from the phylogenetic tree (Fig. 2). The identifications were *Auxis rochei* (2 individuals), *A. thazard* (10), *Cybiosarda elegans* (11), *Euthynnus affinis* (15), *Rastrelliger kanagurta* (10), and *Scomberomorus commerson* (2). Among the ten post-larval and early-stage juveniles that were provisionally identified as *R. kanagurta*, there were five different sequence haplotypes. Likewise, there were five sequence haplotypes among the ten *A. thazard* individuals, two sequence haplotypes for the two individuals of *A. rochei*, and two different sequence haplotypes in the 15 *E. affinis* specimens (Fig. 2). This information gives us a measure of intraspecific variation, which was 0.33% to 1.33%. As the interspecific variation was significantly higher than this (8.7%–19.7%; Table 2), it is likely that this part of the cytochrome *b* gene provides accurate identifications of these larval and early-stage juvenile mackerel.

Implications for ecology and management of Australian mackerel. Information on the temporal and spatial distribution and abundance of *Scomberomorus commerson* larvae may provide insight into patterns of recruitment for this species (Ward and Rogers, 2003). Mackie et al. (2003) found that *S. commerson* juveniles with a fork length of 100 mm were 50 days old based on microincremental analysis of otolith rings. The early-stage *S. commerson* juveniles analyzed here were all less than 85 mm and thus were probably less than 50 days old. Mackie et al. (2003) also examined 237 female *S. commerson* from northwestern Western Australia for evidence of reproductive activity. All the reproductively active females in this sample were from north of Exmouth Bay, and they found that the peak reproductive period was October to January. Our identification of two *S. commerson* juveniles in these waters in October 1987 and March 1998 confirms this pattern of reproductive activity and emphasizes the importance of inshore habitat as nursery areas. Other mackerel species (*Scomberomorus munroi*, *S. queenslandicus*, and *S. semifasciatus*) may have been spawning (October to February; Cameron and Begg, 2002) at the time of collection of the juvenile sample, but these species were not represented among the juveniles identified by us.

Three tuna species (*A. rochei*, *A. thazard*, and *E. affinis*) are known to inhabit oceanic waters as adults, yet this study identified juveniles and larvae in the inshore waters of Exmouth Bay. This observation suggests these species may spawn in coastal waters adjacent to Exmouth Bay, although offshore spawning may also occur. DNA methods provide information otherwise difficult or impossible to obtain. Their application to fisheries species is likely to yield important new data for fisheries management of commercial species and for species biology.

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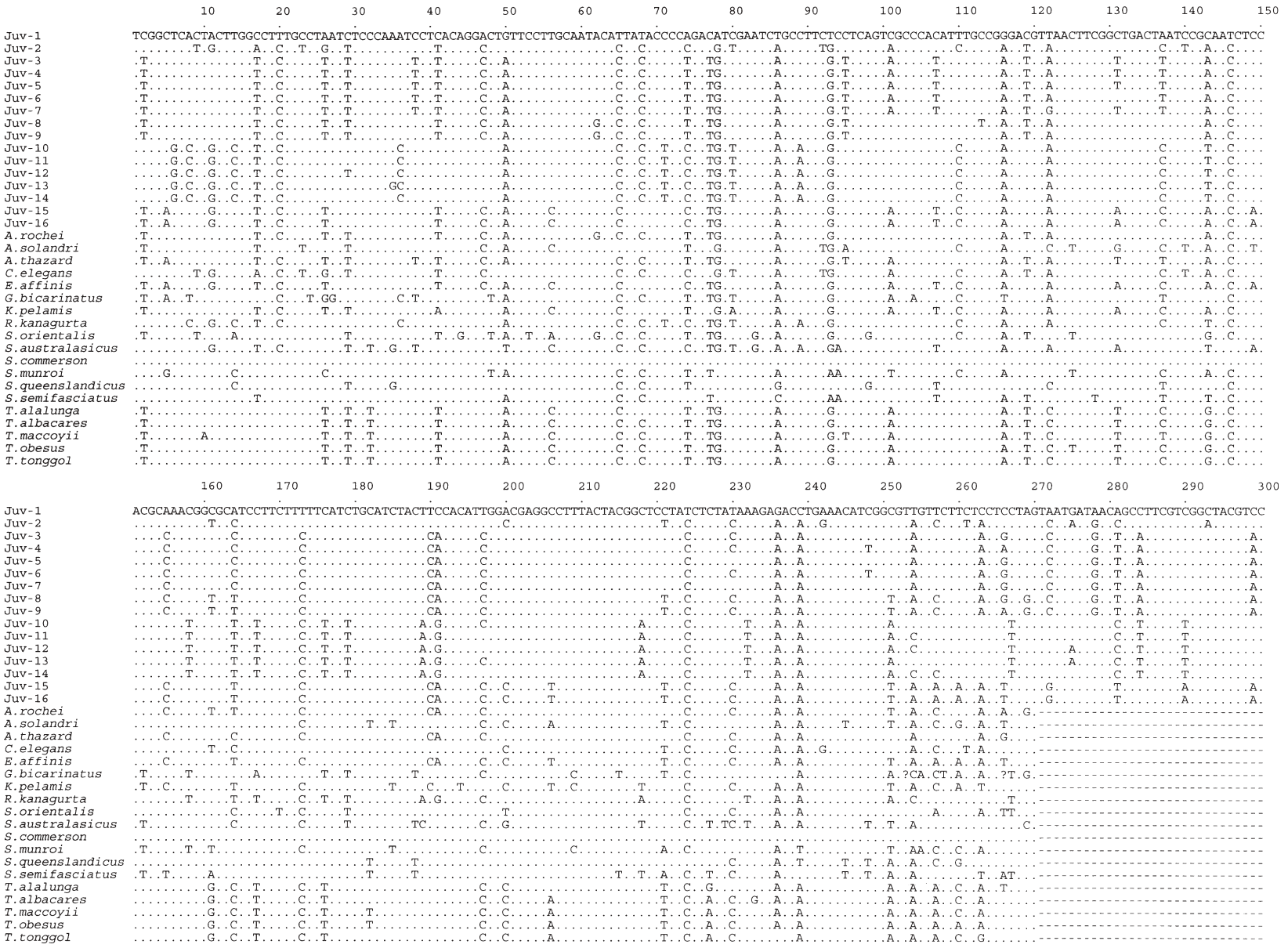

Fig. 1. Alignment of cytochrome *b* gene region sequence for the 16 different sequence haplotypes found in the sample of 50 small juvenile scombrids plus sequences of known species. Sequence *Juv-1* matched with the sequence of *Scomberomorus commerson*. *Juv-2* matched perfectly with *Cybiosarda elegans* sequence and *Juv-3* to *Juv-7* matched closely with *Acanthocybium thazard*. *Juv-8* and *Juv-9* matched closely with *Auxis rochei*, and *Juv-10* to *Juv-14* matched closely to *Rastrelliger kanagurta*. *Juv-15* and *Juv-16* matched closely to *Euthynnus affinis*. Some sequences were not obtained (–)

Table 2. Number of polymorphic sites in the 270-bp alignment of cytochrome *b* sequences (below diagonal) and % nucleotide difference (above diagonal) among 19 species of scombrids

	S.comm	S.semi	S.quee	S.munr	A.sola	G.bica	R.kana	S.aust	C.eleg	A.thaz	E.affi	T.alal	T.alba	T.obes	T.macc	T.tong	K.pela	S.orie	A.roch
S.comm		12.7	8.7	13.3	15.3	16.8	14.7	16.7	14.7	14.7	16.0	14.3	15.3	15.3	15.7	14.7	15.7	14.0	14.3
S.semi	38		11.7	14.3	17.7	18.5	16.7	15.7	18.0	16.7	18.3	16.7	16.7	16.7	16.0	16.7	17.0	17.0	15.3
S.quee	26	35		14.3	14.7	18.2	17.0	17.0	16.3	15.7	17.3	15.3	15.0	14.3	14.3	15.0	15.7	15.7	14.7
S.munr	40	43	43		14.0	14.5	14.7	19.3	16.0	15.3	15.7	13.3	13.7	14.0	15.0	14.0	14.0	15.7	13.7
A.sola	46	53	44	42		18.5	18.3	19.0	13.0	14.0	12.0	11.3	12.3	11.7	12.7	12.7	12.0	15.3	12.7
G.bica	50	55	54	43	55		14.1	19.5	16.5	15.5	16.8	15.2	15.8	15.5	16.5	15.5	17.2	17.5	16.2
R.kana	43	50	51	44	55	42		13.0	15.7	15.0	14.7	14.3	14.7	15.3	15.3	14.7	15.7	16.0	14.3
S.aust	50	47	51	58	57	58	39		17.0	15.7	15.7	16.7	16.3	16.7	16.7	16.3	15.7	17.3	15.0
C.eleg	44	54	49	48	39	49	47	51		11.0	12.0	12.7	12.3	12.3	13.7	12.7	13.7	14.0	11.3
A.thaz	44	50	47	46	42	46	45	47	33		8.0	10.3	10.3	10.3	10.3	10.7	11.0	13.0	5.0
E.affi	48	55	52	47	36	50	44	47	36	24		10.7	11.3	11.0	12.0	11.7	8.3	14.7	9.0
T.alal	43	50	46	40	34	45	43	50	38	31	32		2.0	3.0	3.3	2.3	12.7	12.7	11.0
T.alba	46	50	45	41	37	47	44	49	37	31	34	6		1.7	2.0	1.0	12.7	13.0	11.0
T.obes	46	50	43	42	35	46	46	50	37	31	33	9	5		2.3	2.0	12.3	12.3	11.0
T.macc	47	48	43	45	38	49	46	50	41	31	36	10	6	7		2.3	14.0	13.3	12.0
T.tong	44	50	45	42	38	46	44	49	38	32	35	7	3	6	7		12.7	13.3	11.3
K.pela	47	51	47	42	36	51	47	47	41	33	25	38	38	37	42	38		16.0	9.7
S.orie	42	51	47	47	46	52	48	52	42	39	44	38	39	37	40	40	48		15.7
A.roch	43	46	44	41	38	48	43	45	34	15	27	33	33	33	36	34	29	47	

A.roch, *Auxis rochei*; A.sola, *Acanthocybium solandri*; A.thaz, *Auxis thazard*; C.eleg, *Cybiosarda elegans*; E.affi, *Euthynnus affinis*; G.bica, *Grammatorcynus bicarinatus*; K.pela, *Katsuwonis pelmanis*; R.kana, *Rastrelliger kanagurta*; S.orie, *Sarda orientalis*; S.aust, *Scomber australasicus*; S.comm, *Scomberomorus commerson*; S.munr, *Scomberomorus munro*; S.quee, *Scomberomorus queenslandicus*; S.semi, *Scomberomorus semifasciatus*; T.alal, *Thunnus alalunga*; T.alba, *Thunnus albacares*; T.macc, *Thunnus maccoyi*; T.obes, *Thunnus obesus*; T.tong, *Thunnus tonggol*
All fish were subadults or adults and thus could be identified to species by morphology (Allen, 1997)

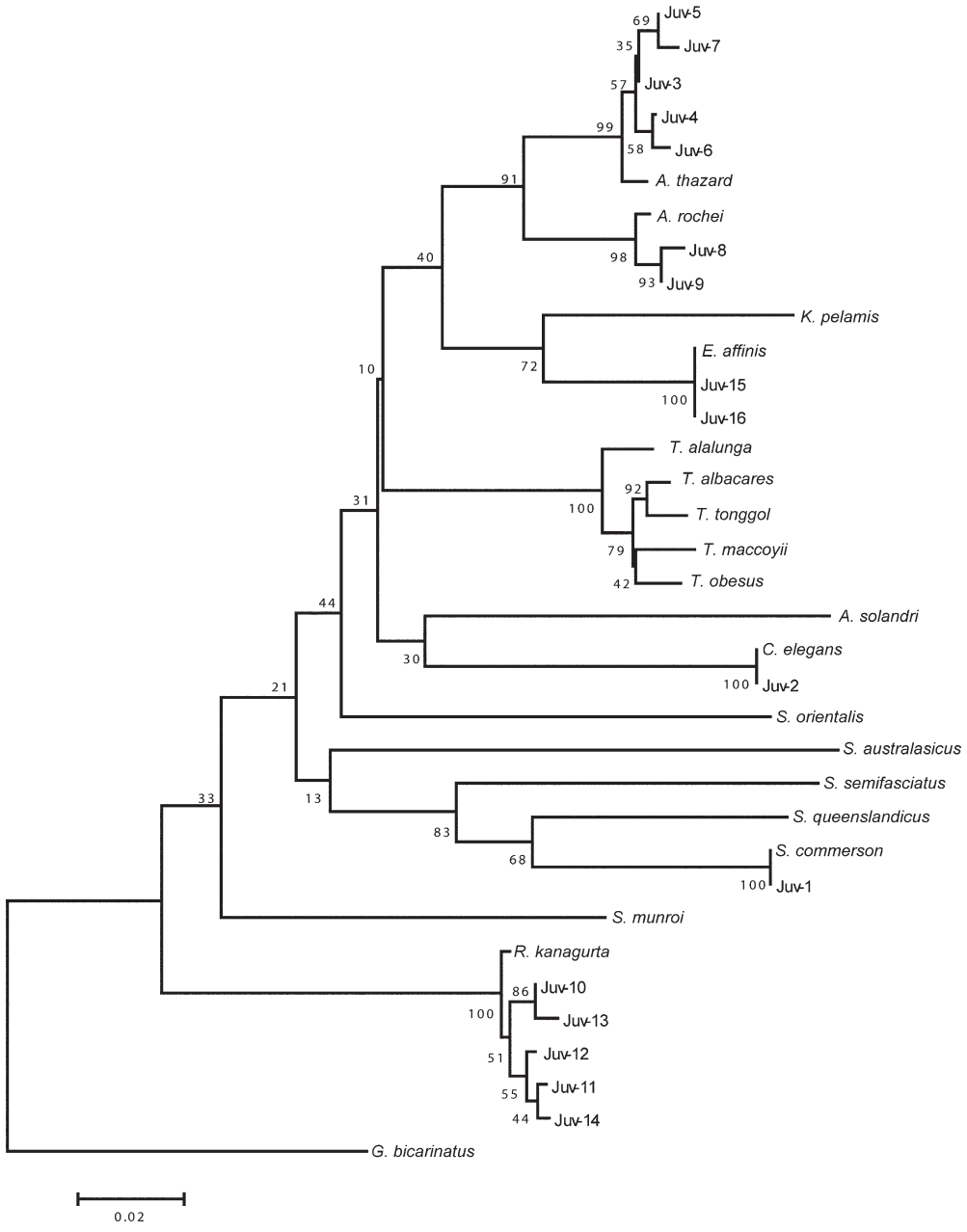

Fig. 2. Neighbor-joining tree showing relationships between scombrid species, and between those species and 16 sequence haplotypes from 50 juveniles. Units of nucleotide divergence were measured by the Kimura two-parameter method. Bootstrap values (%) are shown for branch points

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