Phylogeny of deepwater snappers (Genus *Etelis*) reveals a cryptic species pair in the Indo-Pacific and Pleistocene invasion of the Atlantic

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Running Title: Evolution of deepwater snapper Genus *Etelis*

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Abstract

Evolutionary genetic patterns in shallow coastal fishes are documented with dozens of studies, but corresponding surveys of deepwater fishes (> 200 m) are scarce. Here we investigate the evolutionary history of deepwater snappers (genus *Etelis*), comprised of three recognized Indo-Pacific species and one Atlantic congener, by constructing a phylogeny of the genus with two mtDNA loci and two nuclear introns. Further, we apply range-wide Indo-Pacific sampling to test for the presence and distribution of a putative cryptic species pair within *E. carbunculus* using morphological analyses and mtDNA cytochrome *b* sequences from 14 locations across the species range ($N = 1696$). These analyses indicate that *E. carbunculus* is comprised of two distinct, non-interbreeding lineages separated by deep divergence ($d = 0.081$ in cytochrome *b*). Although these species are morphologically similar, we identified qualitative differences in coloration of the upper-caudal fin tip and the shape of the opercular spine, as well as significant differences in adult body length, body depth, and head length. These two species have overlapping Indo-Pacific distributions, but one species is more widespread across the Indo-Pacific, whereas the other species is documented in the Indian Ocean and Western Central Pacific. The dated *Etelis* phylogeny places the cryptic species divergence in the Pliocene, indicating that the biogeographic barrier between the Indian and Pacific Oceans played a role in speciation. Based on historic taxonomy and nomenclature, the species more widespread in the

Pacific Ocean is *E. carbunculus*, and the other species is previously undescribed (referred to here as *E. sp.*). The Atlantic congener *E. oculatus* has only recently (~0.5 Ma) diverged from *E. coruscans* in the Indo-Pacific, indicating colonization via southern Africa. The pattern of divergence at the Indo-Pacific barrier, and Pleistocene colonization from the Indian Ocean into the Atlantic, is concordant with patterns observed in shallow coastal fishes, indicating similar drivers of evolutionary processes.

Key words: Introns, Lutjanidae, marine fishes, mitochondrial DNA, phylogeography, speciation, submesophotic snappers

1. Introduction

Cryptic species are defined as “two or more distinct but morphologically similar species that were classified as a single species” (Pfenninger & Schwenk 2007). Because of this morphological similarity, most cryptic species were initially considered to be a single species until genetic data and rigorous scrutiny of phenotypic characters indicated otherwise. Cryptic species are relatively common across a wide range of taxa and habitats (Knowlton 1993; Beheregaray & Caccone 2007; Bickford *et al.* 2007), and failure to identify these species can have negative consequences for our understanding of evolutionary processes and for the conservation of biodiversity, natural resource management, and disease monitoring.

The evolutionary mechanisms underlying the formation of cryptic species are diverse. In some cases, reproductive isolation is thought to have arisen so recently that most morphological traits have not yet diverged. In these cases, speciation may be driven by reproductive processes

that do not modify external features, including pheromones (e.g. spruce seed moths, Svensson *et al.* 2013) electrical signals (e.g. African weakly electric fishes, Feulner *et al.* 2006), or acoustic signals (e.g. Hawaiian crickets, Mendelson & Shaw 2005). In other cases, genetic analyses indicate ancient speciation events for cryptic species, whereby morphological stasis is usually thought to result from strong stabilizing selection for a particular morphology. For example, morphological similarity across cryptic bonefish species (genus *Albula*) that diverged 3-20 million years ago is thought to result from stabilizing selection in tropical and subtropical sand habitats, which are ecologically homogenous around the world (Bowen *et al.* 2007). In another example, morphological stasis in groundwater dwelling amphipod species that diverged 13 million years ago is thought to result from limitations in adaptive responses to the extreme conditions of groundwater habitat (darkness, anoxia, and limited food resources; Lefébure *et al.* 2006). Alternatively, convergent evolution could be responsible for morphological similarity in cryptic species, irrespective of the length of time since divergence (e.g. *Heliconius* butterflies, Müller 1879; cichlid fishes, Kocher *et al.* 1993; and *Anolis* lizards, Losos *et al.* 1998).

Genetic technologies have proven to be a bonanza for identifying cryptic species, and in many cases have illuminated the evolutionary processes that drive speciation (e.g. Leray *et al.* 2010; DiBattista *et al.* 2011; Gaither *et al.* 2015). However, deep genetic divergences within species do not invariably indicate cryptic species. Many cases are known wherein previously isolated cohorts come back into contact and interbreed, resulting in deep mtDNA lineages co-occurring within the same population (e.g., Horne *et al.* 2008). In contrast, when deep evolutionary lineages are consistent across both mtDNA and nuclear markers, are geographically segregated, or are accompanied by morphological and behavioral differences (Knudsen &

Clements 2012; Knudsen 2013; Victor 2015), then genetic divergences are indeed the harbinger of cryptic species.

In the Indo-Pacific, genetic partitions for shallow-water fishes usually align with biogeographic provinces, those regions defined by >10% endemism (Briggs & Bowen 2013; Bowen *et al.* 2016). The biogeographic provinces peripheral to the Western Pacific, including Hawai‘i and the Red Sea, are now known to be evolutionary incubators that can export species (Eble *et al.* 2011; DiBattista *et al.* 2013; Fernandez-Silva *et al.* 2015). In the center of this range, an intermittent barrier between the Indian and Pacific Oceans can also serve as a starting point for speciation (Gaither & Rocha 2013). These generalizations apply to a suite of reef fishes in shallow habitats; however, corresponding patterns for deepwater fishes are almost unknown. The snappers of the genus *Etelis* (family Lutjanidae) provide an appropriate forum to test shallow-water phylogenetic patterns in bottom-dwelling fishes with a depth range below 200 m, the recognized limit for shallow-water fishes (Randall 2007). First, *Etelis* is comprised of four recognized species, three with overlapping ranges across the Indo-Pacific, and one in the Atlantic. Second, *Etelis* snappers support recreational, artisanal, and commercial fisheries throughout their range (Williams *et al.* 2013; Newman *et al.* 2015), so that expensive expeditions are not necessary to obtain specimens. Third, there is some question about whether the widespread *Etelis carbunculus* Cuvier (Cuvier & Valenciennes 1828) comprises one or two species (Wakefield *et al.* 2014; Williams *et al.* 2015). Fourth, fisheries that target *Etelis* spp. would benefit from phylogenetic resolution because cryptic species may be mismanaged if treated as a single stock (Williams *et al.* 2012).

We tested for the presence of a putative cryptic species pair in *E. carbunculus* (Ruby Snapper) using phylogenetic and morphological analyses. *Etelis carbunculus* inhabits rocky

substrate in the vicinity of crevasses, ledges, pinnacles, and slopes, typically at depths of 200-400 meters (Gomez *et al.* 2015). This species was historically divided into two species, *E. carbunculus* and *E. marshi*, with the holotypes for the two species collected in Seychelles (western Indian Ocean) and Hawai‘i respectively. However, these species were subsequently synonymized after morphological analyses of the holotypes indicated no differences (Anderson 1981).

Despite the morphological similarities reported for the *E. carbunculus* holotypes, several lines of evidence indicate that *E. carbunculus* may actually include two species. The maximum body size attained by this species varies substantially between regions (Smith 1992). In the southwest Pacific, *E. carbunculus* exceeds 110 cm fork length (FL) (Fry *et al.* 2006; Williams *et al.* 2012; Williams *et al.* 2015) whereas in the Commonwealth of the Northern Mariana Islands (CNMI), Hawai‘i, and French Polynesia, the maximum size is typically <65 cm FL (Smith & Kostlan 1991). These differences in maximum size indicate either geographically structured intra-specific variation in growth patterns, or the presence of different species in different regions. In some locations, a bimodal distribution of lengths has been observed for this species, indicating strong inter-annual recruitment variability or the presence of two species (Lokani *et al.* 1990). Otolith microincrement data also indicate regional variation in growth rates (Smith & Kostlan 1991), with fish in Vanuatu and the CNMI growing faster than those in Hawai‘i or French Polynesia. Variation in otolith morphology between some locations also hints at the presence of two species: specimens from Hawai‘i, French Polynesia, and CNMI differ from Fiji and Vanuatu (Smith 1992; Wakefield *et al.* 2014). In addition, a recent population genetics study of *E. carbunculus* in New Caledonia found two distinct mitochondrial DNA (mtDNA)

control region lineages with deep divergence (11-18% divergent), warranting further taxonomic and phylogeographic investigations (Loeun *et al.* 2013).

To determine whether *E. carbunculus* is composed of two cryptic species, we conducted a combination of genetic and morphological analyses. We initially examined DNA sequences from the mtDNA gene cytochrome *b* (*cytb*) for 1,696 specimens collected across the Indo-Pacific range of this species. We chose this locus because it is widely used for phylogeographic assessments in vertebrates (Johns & Avise 1998). These analyses revealed two highly divergent mitochondrial lineages with partially sympatric geographic ranges. To investigate whether these genetically divergent lineages were also morphologically distinct, we conducted external morphological analyses for specimens from each of these two lineages. Finally, to investigate whether strong mtDNA divergence was accompanied by nuclear divergence, and to gain insight into the evolutionary mechanisms driving speciation, we conducted phylogenetic analyses including specimens from all four described species within the genus *Etelis* using two mtDNA markers (*cytb* and cytochrome oxidase I, COI) and two nuclear introns (Alpha amylase intron 2, Am2B-2; and Gonadotropin-releasing hormone 3 intron 3, GnRH3-3).

2. Methods

2.1. Sample collection

A total of 1,696 tissue specimens were obtained from the nominal *E. carbunculus* from 14 locations across the Indo-Pacific (Table S1, Fig. 1). A previous assessment of the Hawaiian population used 770 of these specimens (Andrews *et al.* 2014). Specimens were also obtained

from each of the three additional described species within the *Etelis* genus: six specimens of *E. coruscans* (Hawaiian Archipelago, $N=3$; Sāmoa, $N=1$; Seychelles, $N=1$; Northwest Australia Clerke Reef, $N=1$), five specimens of *E. oculatus* from Puerto Rico; and seven specimens of *E. radiosus* (Northwest Australia Scott Reef, $N=4$; Clerke Reef, $N=1$; and the Red Sea, $N=2$). Two additional deepwater snapper species were used as outgroups: *Pristipomoides filamentosus* ($N=6$) and *Aphareus rutilans* ($N=5$) collected from the Hawaiian Archipelago. Tissue specimens consisted of fin clips or muscle tissue and were purchased through commercial fishers or fish markets, or were collected through research cruises, and were stored in salt-saturated DMSO buffer (Seutin *et al.* 1991). All specimens were collected between 1997 and 2012.

2.2. DNA extraction, PCR, and sequencing

Genomic DNA was extracted from fin or muscle tissue using a phenol chloroform method (Cummings & Thorgaard 1994), DNEasy extraction kits (Qiagen, Valencia CA), or the Hotshot method (Meeker *et al.* 2007). A 524 bp portion of the mtDNA cytochrome *b* (*cytb*) gene was PCR amplified for all nominal *E. carbunculus* specimens using primers EhucybF and EhucybR and following the protocol in Andrews *et al.* (2014). We PCR-amplified approximately 560 bp of *cytb* for each of the other *Etelis* species, as well as the outgroup species *A. rutilans* and *P. filamentosus*, using primers Cyb-05 L15020 and Cyb-07 H15573 and following the protocol in Andrews *et al.* (2014). In addition, we PCR-amplified portions of the mtDNA gene cytochrome oxidase I (COI) and two nuclear introns, Alpha amylase intron 2 (Am2B-2), and Gonadotropin-releasing hormone 3 intron 3 (GnRH3-3). Primers for COI were FishF2 and FishR2 (Ward *et al.* 2005); for Am2B-2 were Am2b2F and Am2b3R (Hassan *et al.* 2002); and for GnRH3-3 were

GnRH3F and GnRH3R (Hassan *et al.* 2002). All PCRs used 15µl reactions containing 1X Mango Mix (Bioline, Taunton MA, USA) and 0.2µM each primer. Thermal cycle profiles were as follows: 94°C for 1 min; 35 cycles of 94°C for 30 sec, annealing temperature for 30 sec, and 72°C for 30 sec; and a final 72°C extension for 10 min. Annealing temperatures were 68°C for Ehucyb, 55°C for Cyb-05/Cyb-07, 56°C for COI, 58°C for Am2B-2, and 58°C for GnRH3-3. PCR products were cleaned by adding 7.5 units of Exonuclease I and 0.75 units of FastAP alkaline phosphatase (Fermentas Life Sciences, Ontario, Canada) to 7.5ul of PCR product, and incubating samples at 37°C for 60 minutes and 85°C for 15 minutes.

PCR products were sequenced in both directions for Am2B-2 and one direction for all other loci using an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA). Questionable and low-quality reads were resequenced. Sequences were edited and aligned using GENEIOUS PRO 5.6.2 (Biomatters, LTD, Auckland, NZ), and alignments were checked visually. For nuclear introns, alleles were resolved using Bayesian methods implemented in the program Phase 2.1 (Stephens *et al.* 2001; Stephens & Donnelly 2003).

2.3. Phylogenetic analyses

The possibility of a cryptic species pair within *Etelis* was first investigated with a phylogenetic tree using *cytb* sequences from all nominal *E. carbunculus* specimens across the geographic range ($N=1,696$) using default settings for the Neighbor-Joining method in GENEIOUS PRO 6.1.6 (Kearse *et al.* 2012). This analysis provided preliminary evidence for the presence of a cryptic species pair (see Results), and the large sample size provides an indication of the range of these two forms. Population genetic analyses of this dataset will be

explored in a companion paper. To investigate evolutionary relationships of this putative cryptic species pair in the context of all other *Etelis* species, we estimated phylogenetic trees for concatenated mtDNA markers (*cytb* and COI) using specimens from each of the two divergent lineages ($N=6$ per lineage, using specimens from across the geographic range), the other three *Etelis* species, and the two outgroups (deepwater snappers *P. filamentosus* and *A. rutilans*). Phylogenetic trees were estimated for each nuclear intron and concatenated mtDNA using best-fit nucleotide substitution models determined with AIC in jMODELTEST 2.1.3 (Darriba *et al.* 2012). Sequence data were partitioned by locus, with parameters and overall rates allowed to vary across partitions. Maximum Likelihood analyses were conducted using RAxML-HPC2 on XSEDE (Stamatakis 2014) on the CIPRES Science Gateway (Miller *et al.* 2010) using 1000 bootstrap replicates with the GTR-GAMMA model. Bayesian analyses were conducted using MRBAYES 3.2.1 (Ronquist *et al.* 2012) using four independent chains with the default heating scheme. A total of 10 million MCMC generations were run, sampling every 1000 generations, with the first 25% discarded as "burn-in." Stationarity of parameter values was evaluated using TRACER 1.6 (Rambaut & Drummond 2007). Evolutionary relationships within *Etelis* were further examined by creating median-joining allele networks for nuclear introns (Am2B-2 and GnRH3-3) using NETWORK 4.6.1.3 (Bandelt *et al.* 1999) for the same specimens used in the mtDNA phylogenies.

To gain further insight into the geographic distribution of the putative cryptic species, we downloaded all *E. carbunculus* DNA sequences available on GenBank at the time of this study ($N=5$ COI sequences from French Polynesia, West Australia, Japan, and Pumula, South Africa; Accessions JQ431727, EF609353, JF952731, KF489587, KF489588; Ward & Holmes 2007,

Hubert *et al.* 2012). We created a Neighbor-Joining tree using default parameters in GENEIOUS PRO with these sequences and the COI sequences generated in our study.

2.4. Divergence dating

We used BEAST 2.1.3 (Bouckaert *et al.* 2014) to estimate divergence times within the *Etelis* genus based on concatenated *cytb* and COI sequences. Sequence data were partitioned by locus, with site models unlinked across partitions. GTR substitution models were used for both partitions, with uniform relative rate priors. The Yule Model was used for the tree prior, and a strict molecular clock was used based on 1% divergence per MY within lineages for *cytb* and COI for marine fishes (Bowen *et al.* 2001; Lessios 2008; Reece *et al.* 2010). While this clock is not calibrated for snappers, it is consistent across a wide range of teleost fishes. Nonetheless, we caution against strict interpretation of the proposed dates. We conducted two independent runs, each consisting of 10 million generations, sampling every 1,000 generations. Convergence was evaluated based on ESS values and visual inspection of trend lines using TRACER. Tree files from independent runs were combined after removing 10% burn-in using LogCombiner 2.1.3. A maximum clade credibility tree was generated with median ages and 95% highest posterior density intervals using TreeAnnotator 2.1.2.

2.5. Morphological analyses

To investigate whether morphological differences are present between the two putative cryptic species, initial assessments were conducted for 110 whole fish specimens collected on research cruises in New Caledonia ($N=95$) and NW Australia (Montebello Islands $N=7$; Ningaloo Reef

N=8). The NW Australia samples are deposited in the California Academy of Science (Accession 2013-XII:26); the remaining samples were not archived. Specimens were sorted into two putative species based on divergent *cytb* lineages (see Results). For the New Caledonia specimens, morphological observations and photographs were obtained shortly after the fish were collected, and for the NW Australia samples, observations and photographs were obtained after the fish had been shipped frozen to the Hawai‘i Institute of Marine Biology. We initially identified two diagnostic characters: 1) shape of opercular spine; 2) presence or absence of black coloration on the upper-caudal fin lobe (see below). To test our ability to distinguish the putative cryptic species in the field based on the two diagnostic morphological characters, we used these characters to make field assignments for 227 specimens obtained across five South Pacific locations (New Caledonia, Fiji, Tonga, Sāmoa, Wallis & Futuna), and later compared these morphological assignments with genetic assignments based on *cytb* sequences.

To investigate whether the two putative cryptic species differed in other morphometric parameters, we measured morphological characters for 385 specimens from 11 locations, including 21 museum specimens (Tables S2, S3, S4). Species designations were based on *cytb* sequences for all specimens except those from museums, for which species designations were based on opercular spine shape due to the inability to generate *cytb* sequences from formaldehyde-preserved specimens. Morphological measurements included: Fork length (*FL*) measured from the median anterior point of the upper lip to the fork of the tail; body depth (*BD*), measured vertically from the base of the 1st dorsal spine where it emerges from the body (not the internal base); head length (*HL*), measured from the anterior point of the maxilla to the posterior end of the opercular membrane; and eye diameter (*ED*), as the greatest fleshy diameter of the left

eye orbit. To allow comparisons among individuals, these measurements were standardized as ratios to fork length (*BD/FL*, *HL/FL*, *ED/FL*).

The statistical package JMP 11.2.0 (SAS Institute Inc., Cary, NC) was used to plot the frequency distribution of weight and fork length of individuals within each lineage. A two-tailed *t*-test was used to compare the mean values of *BD/FL*, *HL/FL*, and *EL/FL* between both lineages. We investigated whether any of the variables were allometric by regression analyses of log transformed variables,

$$\text{Log}[EL] = a + b * \text{Log}[FL]$$

where $b > 1$ indicates positive allometry.

In light of concordant genetic and morphological divergence, we concluded that *E. carbunculus* is a complex of two cryptic species (see below). To help determine the taxonomic identity of the two species, the lead author inspected the *E. carbunculus* holotype (located at the Muséum National D'Histoire Naturelle in Paris, MNHN 6603, 248 mm SL), focusing on the opercular spine shape and upper-caudal fin lobe coloration for species diagnosis. Genetic analysis was not possible for this holotype due to formaldehyde preservation. *E. marshi* was designated a junior synonym of *E. carbunculus* by Anderson (1981), with its holotype located at the National Museum of Natural History in Washington, D.C. (USNM 50714, ca 318 mm SL). We did not examine this specimen because it was collected from the Hawaiian Archipelago, where only one of the two cryptic species occurs (based on a genetic survey of 770 specimens collected throughout the Hawaiian Archipelago, Andrews *et al.* 2014, see below).

3. Results

3.1 A cryptic species pair

We resolved 436 bp of *cytb* for 1,696 nominal *E. carbunculus* specimens collected across the Indo-Pacific. A Neighbor-Joining tree for these sequences revealed two reciprocally monophyletic lineages separated by 8.1% divergence (Fig. S1), providing evidence for a species pair. Our large sample size allowed a preliminary assessment of the ranges of the two species (Fig. 1), indicating overlapping Indo-Pacific distributions: one species is widespread in the Indo-Pacific, whereas the other species is documented from the Indian Ocean and Western Central Pacific (Fig. 1).

For phylogenetic analyses using all species, we resolved 974 bp for the two mitochondrial markers (375bp for *cytb* after removing sequence data that did not overlap across the fragments amplified for different species, 599bp for COI) for 5-7 individuals of each species (Table S5). The best-fit model identified by AIC for *cytb* was TPM3uf + gamma = 0.104 and for COI was TPM2uf + gamma = 0.187. The number of parsimony-informative sites across concatenated *cytb* and COI sequences was 229. ML and MrBayes trees produced identical topologies for all loci and supported reciprocal monophyly between the putative cryptic species (Fig. 2). Divergence between the two cryptic species within the *E. carbunculus* complex was greater than divergence between other *Etelis* species that are morphologically and geographically distinct, including the Indo-Pacific/Atlantic sister species *E. coruscans* and *E. oculatus*. For nuclear marker analyses, we resolved 258bp for Am2B-2 and 214bp for GnRH3-3 (Table S5). Alleles from the cryptic species pair clustered separately in networks, with no shared alleles (Fig.

3). In contrast, alleles were shared between some other *Etelis* species, e.g. *E. coruscans* and *E. oculatus* for both Am2B-2 and GnRH3-3. Combined phylogenetic results from mtDNA and nuclear loci provide strong support for the presence of a non-interbreeding species pair within *E. carbunculus*. Based on the morphological species designation of the *E. carbunculus* holotype (see below), here we label the species with the Indian Ocean-Western Central Pacific distribution as a new species (referred to here as *E. sp.*). A formal description is in preparation (K.R. Andrews, unpublished data).

A Neighbor-Joining tree generated using COI sequences from our dataset and the five *E. carbunculus* COI sequences available on GenBank at the time of this study indicated the presence of both cryptic species in Pumula, South Africa (GenBank Accessions KF489587, KF489588) and the presence of the widespread Indo-Pacific species in French Polynesia (Accession JQ431727, Hubert *et al.* 2012). Another GenBank sequence confirmed the presence of the Indian-Western Central Pacific species in Western Australia (*E. sp.*, see below, Accession EF609353, Ward & Holmes 2007), and the GenBank sequence from a specimen collected in Japan clustered with *A. rutilans* and is therefore likely a misidentification (Accession JF952731).

DNA sequences generated for phylogenetic analyses in this study were deposited in GenBank (Accessions KU954116-KU954288, Table S5). *Cytb* sequences for specimens from the Hawaiian Archipelago were reported previously (Andrews *et al.* 2014, GenBank Accessions KF920530–KF920552).

3.2 Evolutionary history of *Etelis*

Trend lines and ESS values indicated adequate mixing for BEAST analyses using mtDNA. Results of these analyses inferred an initial diversification within *Etelis* about 4-5 million years ago (Ma) (95% HPD 3.5 – 5.7), into three lineages (Fig. 4). These three lineages include the two cryptic species and a third lineage that later diversified into three species (Fig. 4). The branching order for the initial diversification is unresolved, with low ML bootstrap support and Bayesian posterior probabilities (Fig. 2), and a different branching order in the BEAST tree (Fig. 4). These results indicate that the three lineages diverged so close in time that our analytical tools are unable to distinguish the branching order. The next divergence within *Etelis* occurred approximately 3 Ma (95% HPD 2.4 – 3.7), resulting in the *E. radiosus* lineage and a lineage that later diverged into two extant species: the Indo-Pacific *E. coruscans* and the Atlantic *E. oculatus* about 0.5 Ma (95% HPD 0.32 – 0.78) (Fig. 4).

3.3 Morphological differences between cryptic species

In the initial morphological investigation of 96 whole fish specimens of *E. sp.* (New Caledonia $N = 81$; W. Australia $N = 15$) and 14 specimens of *E. carbunculus* (all from New Caledonia), we identified two diagnostic characters: 1) shape of opercular spine (Fig. 5c,d); 2) presence (*E. sp.*) or absence (*E. carbunculus*) of black coloration on the upper-caudal fin lobe (Fig. 5e,f). The diagnostic resolution of these two characters to identify species in the field was verified by a 98.7% match rate of genetic (*cytb*) and morphological species assignments across 227 specimens (New Caledonia $N = 53$; Fiji $N = 54$; Tonga $N = 51$; Sāmoa $N = 7$; Wallis & Futuna $N = 62$). Of the mismatched assignments, *cytb* sequences indicated two were *E. sp.* and one was *E. carbunculus*. We believe it is more likely that the mismatched identifications resulted from

mislabeling of sample tubes rather than incorrect morphological identifications in the field, but we did not have the logistical resources to voucher 227 specimens to evaluate this possibility. Therefore we did not include the three individuals with mismatched identifications in subsequent morphological analyses.

Measurements of body size and shape parameters for 385 specimens (Tables S2, S3, S4, Fig. 6) confirmed that *E. sp.* attains a larger adult size than *E. carbunculus*. This is confirmed in comparisons of specimens from across the range as well as in comparisons of samples from the same location (Fig. 6, Fig. S2). *E. sp.* has relatively smaller eyes ($EL/FL = 0.056 - 0.129$, mean $EL/FL = 0.084 \pm 0.015$) than *E. carbunculus* ($EL/FL = 0.072 - 0.113$, mean $EL/FL = 0.097 \pm 0.009$), as indicated by eye length:fork length ratios. However, the size of the eye relative to the fish length decreases as the fish grow (Fig. 7 and Fig. S3), with allometric scaling being more marked in *E. sp.* (allometric index $b = 0.6$) than *E. carbunculus* ($b = 0.8$). Comparison with a conventional *t*-test of eye length:fork length ratios (EL/FL) between groups that differ in size is not appropriate. However, when we compared eye length:fork length ratio values among specimens of the same class size, we did not observe differences between the two species (Fig. 7).

Comparisons between both groups indicated that *E. sp.* has a deeper body ($BD/FL = 0.24 - 0.34$, mean $BD/FL = 0.29 \pm 0.02$, $N = 86$) than *E. carbunculus* ($BD/FL = 0.24 - 0.31$, mean $BD/FL = 0.28 \pm 0.02$, $N = 31$) ($t = 2.812$, $P = 0.006$). We also observed significant differences ($t = 3.63$, $P = 0.0004$) in the length of the head, being smaller in *E. sp.* ($HL/FL = 0.22 - 0.32$, mean $HL/FL = 0.29 \pm 0.02$, $N = 90$) than in *E. carbunculus* ($HL/FL = 0.25 - 0.33$, mean $HL/FL = 0.30 \pm 0.02$, $N = 44$). Neither the head length nor the body depth scaled allometrically with fork length, indicating that these characters scale proportionately with body length (Fig. S3).

The opercular spine shape and upper-caudal fin lobe coloration of the *E. carbunculus* holotype was consistent with that of the species more widespread in the Pacific Ocean. Notably, this holotype was collected in Seychelles, whereas our genetic sampling from Seychelles had only revealed *E. sp.* (Fig. 1). It is unclear why *E. carbunculus* was absent from our Seychelles samples. It is possible that the abundance of *E. sp.* is far greater than that of *E. carbunculus* in Seychelles, and that our relatively small sample size from this region was insufficient to detect *E. sp.* This highlights the caution required when inferring species distributions and relative abundance based on Figure 1.

4. Discussion

4.1 Geographic distribution and taxonomy of cryptic species

Genetic and morphological analyses collectively provide strong evidence that the nominal *E. carbunculus* is composed of two non-interbreeding species. Phylogenetic analyses using mtDNA indicated two reciprocally monophyletic lineages within this nominal species, with divergence between lineages dating to approximately 4-5 Ma. Individuals from separate mtDNA lineages also had divergent nuclear alleles, providing evidence that there is no interbreeding. Individuals from separate lineages had extensive morphological similarity, but we identified consistent differences between lineages in adult body length, body depth, head length, coloration of the upper-caudal fin lobe, and shape of an opercular spine. These morphological differences were consistent even when both species occurred at the same collection site. This concordance between diagnostic differences in morphology and multiple genetic loci, along with the depth of

separations at *cytb* ($d = 0.081$) strongly supports a species partition rather than a phylogeographic break (Carstens *et al.* 2013)

The two species co-occurred at collection sites in NW Australia (Heywood Shoal), Cocos (Keeling) Island, New Caledonia, Tonga, Wallis and Futuna, Fiji, and Sāmoa. It is likely that both species are present at more of our study sites, but were not sampled during this study. Therefore, the relative proportions of each species collected at our sampling sites may not represent the true proportions at those sites, and further knowledge of the ecology and life history of each species is needed before species distributions and abundances can be accurately assessed. However, our extensive sampling across the Hawaiian Archipelago (770 specimens across 16 locations), and the absence of any records of *E. carbunculus* >65 cm FL in Hawai‘i, provides strong evidence that only one species is present in this archipelago.

Historically, the nominal species *E. carbunculus* was divided into two species, *E. carbunculus* (holotype from Seychelles, Indian Ocean) and *E. marshi* (holotype from Hawai‘i, Pacific Ocean), but *E. marshi* was later classified as a junior synonym of *E. carbunculus* due to morphological similarity of the holotypes (Anderson 1981). Our morphological examination of the holotype of *E. carbunculus*, type locality Seychelles, confirms it as the species with the widest distribution across the Indo-Pacific, and thus the name *E. carbunculus* is retained for this species. The name *E. marshi* remains a junior synonym of *E. carbunculus*, and the species with a limited Indian Ocean-western central Pacific distribution (*E. sp.*) is a unique species that will shortly be described by us (in preparation). This new nomenclature replaces that used in recent studies, which incorrectly assigned the name *E. carbunculus* to the undescribed species with the Indian Ocean-Western Central Pacific distribution (now *E. sp.*) and *E. marshi* to the species that is widespread across the Indo-Pacific (now *E. carbunculus*) (Andrews *et al.* 2014; Wakefield *et*

al. 2014; Williams *et al.* 2015). This study endorses the conclusion of Anderson (1981), with *E. marshi* confirmed as a junior synonym of *E. carbunculus*.

4.2 Evolutionary history of the deepwater snapper genus *Etelis*

The deepest branches in the *Etelis* phylogeny indicate an initial diversification of three lineages about 4-5 Ma. The branching order for these divergence events is unresolved in the phylogenetic analysis, indicating that the three lineages diverged around the same time. Two of the three lineages were the predecessors of the cryptic species, *E. carbunculus* and *E. sp.* These two lineages show no subsequent diversification. In contrast, the third lineage yielded two subsequent speciation events, one about 3 Ma and another about 0.5 Ma, resulting in the formation of three morphologically unique species, *E. radiosus* and the sister species *E. oculatus* (Atlantic) and *E. coruscans* (Indo-Pacific).

The *Etelis* phylogeny provides an evolutionary perspective on the morphological similarity between *E. carbunculus* and *E. sp.* A parsimonious explanation is that the ancestor of all extant *Etelis* species was morphologically similar to the cryptic species pair, as these comprise two of the three deep lineages within *Etelis*. Therefore, the morphological similarity between the *Etelis* cryptic species most likely results from stasis over 4-5 MY rather than convergent evolution. This morphological stasis for *E. carbunculus* and *E. sp.* lineages, when a third *Etelis* lineage was diversifying into three morphologically divergent species, could result from stabilizing selection across a homogenous deepwater habitat. However, it is also possible that ecological or life-history differences between species exist but were not apparent in our

initial studies. For example, the cryptic species could differ in habitat preference or reproductive behaviors.

Insight into the evolutionary mechanisms driving speciation in *Etelis* can be gained from the geographic distributions of the species. Our data regarding geographic distributions of *E. carbunculus* and *E. sp.* are preliminary but indicate a pattern similar to many species pairs of shallow Indo-Pacific fish: one species being potentially more prevalent in the Indian Ocean, the other species more prevalent in the Pacific Ocean, and a region of distributional overlap around the biodiversity hotspot of the Indo-Malay Philippine region (reviewed in Gaither & Rocha 2013). This distributional pattern is thought to be driven by speciation in separate ocean basins during historic periods of reduced water flow between the Indian and Pacific Oceans, followed by range expansions and secondary contact during more recent periods of contact between oceans. Water flow between Indian and Pacific Oceans decreased during the mid-Miocene (16-8 Ma) when the Australian and Eurasian plates collided, and then fluctuated throughout the Pleistocene (2.6-0.12 Ma) as glacial sea level changes caused land barriers to form and then disappear across the Sunda and Sahul shelves of Indonesia, Malaysia, and Northern Australia (the “Indo-Pacific Barrier”) (Kennett *et al.* 1985; Gordon 2005; Naish *et al.* 2009). Species pairs with distributions similar to our preliminary observations for the *Etelis* cryptic species tend to have divergence dates in the Pliocene (5.3-2.6 Ma) or Pleistocene, a signature for the rise and fall of the Indo-Pacific Barrier as a mechanism driving speciation and present-day distributions (Gaither & Rocha 2013). Similarly, the cryptic *Etelis* species pair diverged in the Pliocene, indicating that the Indo-Pacific barrier also played a role in the initial divergence of these species. Under this scenario, reproductive isolation between the cryptic species was likely achieved in allopatry, with sympatric distributions occurring later through range expansions and

secondary contact. An alternative explanation is that *E. sp.* arose in a semi-isolated region within the range of *E. carbunculus*. Archipelago-level divergences are documented for other groups of Indo-Pacific fauna (Meyer *et al.* 2005; Drew *et al.* 2008), but tend to occur in less-dispersive organisms. For this reason we believe the Indo-Pacific barrier is a more likely explanation for *Etelis* evolution.

Geographic distributions and divergence times for *Etelis* species also demonstrate a recent invasion of the Atlantic from the Indo-Pacific. The sole Atlantic member of the genus (*E. oculatus*) diverged from its Indo-Pacific sister species about 0.5 Ma. Invasions of the Atlantic have been relatively rare for tropical and warm-temperate marine species since the uplift of the Isthmus of Panama 3.1-3.5 Ma, which closed off one of the two warm water routes from the Indo-Pacific into the Atlantic Ocean. However, genetic, morphological, and fossil evidence point to recent invasions of the Atlantic for molluscs, sea turtles, and warm-water fishes (Vermeij & Rosenberg 1993; Rocha *et al.* 2005; Bowen *et al.* 2006; Bowen & Karl 2007; Reece *et al.* 2010; Graves & McDowell 2015). These invasions are thought to have occurred via transport of planktonic larvae or juveniles in warm water from the Indian to Atlantic Oceans around the southern tip of Africa via one of two possible mechanisms: (i) cessation of the Benguela cold water upwelling along the coast of southwest Africa during interglacial periods, or (ii) Agulhas gyres that bring pockets of warm water from the Indian Ocean around the southern tip of Africa and through the cold Benguela upwelling zone (Flores *et al.* 1999; Penven *et al.* 2001; Peeters *et al.* 2004). Warm water traversing these paths can return to the Indian Ocean or become entrained in currents moving west across the Atlantic (Gordon 2003). Larvae or juveniles that enter the westward moving South Equatorial Current are expected to traverse the distance from Africa to Brazil in about 70 days (Scheltema 1971).

Inferring historical pathways of colonization can be an uncertain endeavor without complete taxon sampling (Craig et al. 2004). *Etelis* provides a robust example, with complete species sampling and broad geographic coverage within most species. Based on these data, several observations support a recent colonization around the southern tip of Africa to account for the presence of *E. oculatus* in the Atlantic: (i) colonization of the Atlantic by *Etelis* could not have occurred through the Isthmus of Panama, because *E. oculatus* diverged from its sister species more than 2 MY after the closure of this route, (ii) *E. coruscans*, the Indo-Pacific sister species of *E. oculatus*, occurs along the east coast of Africa, supporting the feasibility of dispersal around the tip of Africa, (iii) the pelagic larval duration of *Etelis* species is likely greater than the length of time expected for current-entrained larvae to traverse the distance between Africa and Brazil (Leis 1987; Leis & Lee 1994), and (iv) the West Atlantic distribution of *E. oculatus* aligns with predictions (based on ocean currents) that the first region colonized after an invasion around the tip of Africa would be the western Atlantic (Gordon 2003).

Overall, the geographic patterns of divergence in the genus *Etelis* are very similar to those observed for shallow Indo-Pacific fishes (reviewed in Eble et al. 2015). These patterns include sister species that overlap around the boundary of the Indian and Pacific Oceans (Gaither & Rocha 2013), partitions that coincide with biogeographic boundaries (Briggs & Bowen 2013; Bowen et al. 2016), and rare colonization into the Atlantic (Reece et al. 2010). It could be postulated that the thermal regime and depth occupied by *Etelis* would facilitate connections between the Indian and Atlantic Oceans, but we observe only a single colonization event in the 4 – 5 MY history of *Etelis* resolved here. While few studies exist on the phylogeography of deepwater fishes, the scant literature on this subject indicates high dispersal and biogeographic histories similar to their shallow counterparts (Gaither et al. 2011; Rivera et al. 2011; Andrews et

al. 2014, but see Ovenden *et al.* 2004). One potential point of departure between shallow and deep fishes is the intermittent Indo-Pacific Barrier created by lower sea levels. It is possible that this barrier is more effective for deep species, as many (but not all) of the remaining connections between the Indian and Pacific Oceans are shallow sluices between islands (Gordon 2005).

4.3 Fisheries management

Identification of a cryptic species pair within the genus *Etelis* has strong management implications. *E. carbunculus* and *E. sp.* are important target species in many commercial, recreational, artisanal, and subsistence fisheries across the Indo-Pacific (Dalzell *et al.* 1996; Newman *et al.* 2015). However, catch estimates are uncertain for most fisheries because the social and spatial complexity of artisanal and subsistence fisheries results in poorly reported catch statistics. Reliable estimates of catches are limited to only a few long-established commercial fisheries in Australia, Hawai‘i, and Tonga, where annual catches have ranged from approximately 100-1000 t (Newman *et al.* 2015). *E. carbunculus* and *E. sp.* co-occur across a very wide distribution, and both species are frequently captured on the same line (A.J. Williams, unpublished data). In regions where both species are harvested, management as a single species could result in overexploitation of one or both species, particularly because productivity is likely to vary between the species due to their substantially different growth patterns. Hence it is critical that these species are differentiated in the catches and in subsequent assessments to facilitate effective management. The ability to visually distinguish these two species in the field is particularly important for fisheries management because it eliminates the time and cost of genetic identification. In a companion paper, we will analyze the range-wide population genetic

structure for the widespread *E. carbunculus*, *E. sp.*, and *E. coruscans*, with the objectives of quantifying dispersal and identifying management units for these valuable fisheries.

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Figure Titles

Figure 1. Geographic sampling distribution of two cytochrome *b* haplotype lineages corresponding to a cryptic species pair for the nominal *Etelis carbunculus*. Here we label the species widespread in the Indo-Pacific *E. carbunculus* (purple in the pie charts), and the species present in the Indian Ocean and Western Central Pacific *E. sp.* (yellow). GenBank sequences available at the time of this study also documented the presence of *E. carbunculus* in French Polynesia, and both species in Pumula, South Africa. A museum specimen also documented the presence of *E. carbunculus* in Seychelles.

Figure 2. Phylogeny of the genus *Etelis* inferred using Maximum Likelihood (ML) and Bayesian inference (BI) of concatenated *cytb* and COI sequences. The best ML tree is shown, and branch support is indicated at major nodes when ML bootstrap support >70% (1000 replicates) or BI posterior probability >0.7. ML bootstrap is reported first, followed by BI posterior probability.

Figure 3. Median-joining network of a) Am2B-2 nuclear intron allele sequences, and b) GnRH3-3 nuclear intron allele sequences. Each circle represents an allele sequence, and the size of the circle represents the number of individuals with that allele. Line lengths represent the number of mutational differences between alleles, and colors represent species designations.

833

834 Figure 4. Chronogram of the genus *Etelis* from concatenated *cytb* and COI sequences inferred
835 using BEAST, using a strict molecular clock based on a divergence rate of 1% per million years
836 within lineages for both markers. Maximum clade credibility tree is shown. Values at major
837 nodes represent posterior probabilities, and node bars represent 95% confidence intervals for
838 divergence time.

839

840 Figure 5. Photos of *Etelis carbunculus* (to the left) and *E. sp* (to the right) showing body shape
841 (a, b), black fin tip in *E. sp.* (c, d), and operculum morphology (e, f). a) Specimen of 217 cm
842 from Hawaii (BPBM8474); b) Specimen of 290 cm from Okinawa (BPBM19207). (Photos of
843 voucher specimens by J. E. Randall)

844

845 Figure 6. Frequency distribution of fork length and weight of individuals described in Table S2
846 (for fork length) and Table S3 (for weight), *Etelis carbunculus* to the left (in blue); *E. sp.* to the
847 right (in red).

848

849 Figure 7. Plots of ratios of Head Length: Fork Length, Body Depth:Fork Length, and Eye
850 Length:Fork Length, respectively, against Fork Length of specimens summarized in Table S4;
851 *Etelis carbunculus* in blue; *E. sp.* in red.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

*Graphical Abstract

Etelis sp.

Etelis carbunculus