Molecular Phylogenetics and Evolution 65 (2012) 727–738

Contents lists available at SciVerse ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Molecular phylogenetics of squirrelfishes and soldierfishes (Teleostei: Beryciformes: Holocentridae): Reconciling more than 100 years of taxonomic confusion

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ARTICLE INFO

Article history:
Received 16 April 2012
Revised 19 July 2012
Accepted 23 July 2012
Available online 3 August 2012

Keywords:
Coral reef
Nocturnal
Phylogeny
Gene-tree species-tree
Taxonomy
Bayesian

ABSTRACT

Squirrelfishes and soldierfishes (Holocentridae) are among the most conspicuous species in the nocturnal reef fish community. However, there is no clear consensus regarding their evolutionary relationships, which is reflected in a complicated taxonomic history. We collected DNA sequence data from multiple single copy nuclear genes and one mitochondrial gene sampled from over fifty percent of the recognized holocentrid species and infer the first species-level phylogeny of the Holocentridae. Our results strongly support the monophyly of the clades Myripristinae (soldierfishes) and Holocentrinae (squirrelfishes). The molecular phylogenies differ with regard to previous hypotheses of relationships within the Myripristinae, resolving a clade of cryptic reef associated and deep water non-reef dwelling lineages (*Corniger* + *Plectrypops* + *Ostichthys*) that is the sister lineage to a monophyletic *Myripristis*. Within Holocentrinae, *Neoniphon* and *Sargocentron* are strongly supported as paraphyletic, while *Holocentrus* is nested within *Sargocentron*. Using Bayesian ancestral state reconstruction methods, we demonstrate the taxonomically diagnostic characters for *Neoniphon* and *Sargocentron* likely represent character states with a complex evolutionary history that is not reflective of shared common ancestry. We propose a new classification for Holocentrinae, recognizing four lineages that are treated as genera: *Sargocentron* Fowler, 1904, *Holocentrus* Scopoli, 1777, *Flameo* Jordan and Evermann, 1898, and *Neoniphon* Castelnau, 1875.

1. Introduction

Holocentridae (squirrelfishes and soldierfishes) comprises a radiation of approximately 84 primarily nocturnal reef-dwelling teleost fishes that are distributed globally throughout tropical and temperate marine habitats (Nelson, 2006). Squirrelfishes and soldierfishes have been important organisms for understanding the evolution of dim light vision in vertebrates (e.g., Yokoyama and Takenaka, 2004; Yokoyama et al., 2008); vocalization in teleosts (e.g., Coombs and Popper, 1979; Carlson and Bass, 2000; Luczkovich and Keusenkothen, 2007; Parmentier et al., 2011), reef fish ecology and behavior (Gladfelter and Johnson, 1983; Golani and Ben-Tuvia, 1985; Tyler et al., 1993) and how pelagic larval dispersal influences gene flow and speciation (e.g., Bowen et al., 2006; Craig et al., 2007; Muths et al., 2011). In addition, the abundance and diversity of fossil holocentrids, with stem lineage representatives that first appear in the Turonian stage of the Cretaceous approximately 93 Ma (e.g., Gaudant, 1978; Gayet, 1980; Gallo-Da-Silva and De Figueiredo, 1999) and crown lineages that dominate the abundance of the Eocene aged Monte Bolca fossil fish fauna (Sorbini and Tirapelle, 1974; Sorbini, 1975, 1979; Bellwood, 1996), suggests that these fishes have also been an important component underlying the evolution and ecology of nocturnal reef communities. Despite the growing use of holocentrids in a variety of biological disciplines, taxonomy that reflects a species-level phylogeny is wanting, leaving a substantial gap in understanding the evolutionary history of Holocentridae.

The monophyly of extant holocentrids is based on a suite of morphological apomorphies including presence of a transverse crest across the supraoccipital, enlargement of the penultimate anal spine, expansion of the haemal and neural spines on either

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http://dx.doi.org/10.1016/j.ympev.2012.07.020

the 4th or 4th and 5th preural vertebral bones, and a hook-like process on the outermost radial of the pelvic fins (Zehren, 1979). In defining Holocentridae, Stewart (1984) included both a single vertical line of scales along the anterior border of the operculum and a medial-lateral expansion of the last pleural rib. Moore (1993a) provided two additional osteological synapomorphies that support the monophyly of the Holocentridae: a rostral spine in larvae and juveniles composed of modified nasal bones supported by an enlarged ethmoidal cartilage, and pelvic bones with a dorsally peaked internal wing, giving the pair of bones a unique shape of an elongate tetragonal disphenoid.

Nelson (1955) classified the Holocentridae in two subfamilies based on the morphology of the swim bladder and auditory bulla: (1) Myripristinae (soldierfishes), which includes *Myripristis*, *Corniger*, *Ostichthys*, *and Plectrypops* and (2) Holocentrinae (squirrelfishes), which includes *Holocentrus*, *Neoniphon*, and *Sargocentron*. This classification was supported by the observation that the ascending process of the premaxilla is long or longer than the alveolar process characterized the Holocentrinae, and a toothed alveolar platform extending laterally and overhanging the lateral side of the dentary characterized the Myripristinae (Stewart, 1984). Moore (1993a) found the Holocentrinae were also characterized by the proximal end of the first dorsal fin pterygiophore inserting into a pocket on the anterior face of the neural spine of the second vertebra. Bacurau and Molina (2004) later provided karyotypic evidence supporting the distinctiveness of the two subfamilies. Subsequent investigations of higher-level relationships of acanthomorph teleosts have not produced results in conflict with the monophyly of Holocentridae, nor the Holocentrinae and Myripristinae (Moore, 1993b; Patterson, 1993a,b; Miya et al., 2005); however, there have been proposals to treat the two major holocentrid subclades as families (Frizzell and Lamber, 1961; Hecht, 1982).

The Myripristinae is characterized, in part, by a constriction in the first third of the swim bladder that creates two distinct chambers (Nelson, 1955), wide mucus head canals compared to the narrower canals found in holocentrines (Randall, 1998) and absence of a spine on the preoperculum in adults, with the exception of *Corniger spinosus*, which has one or two spines at the corner (Woods and Sonoda, 1973). The Myripristinae currently contains *Myripristis* (24 species), *Ostichthys* (11 species), *Plectrypops* (two species), and the monotypic *Corniger* and *Pristilepis* (Randall et al., 1982, 2003; Randall and Myers, 1993; Nelson, 2006). Several potential additional genera with affinities to *Myripristis* were described including: *Rhynchichthys* (Cuvier and Valenciennes, 1831), *Rhinoberyx* (Gill, 1862), *Ramphoberyx* (Gill, 1863), and *Neomyripristis* (Castelnau, 1873). However, all of these genera were based on very small specimens and these were all later found to be larval and prejuvenile forms of *Myripristis* species (Nelson, 1955; Randall and Greenfield, 1996). Agassiz (in von Spix and Agassiz (1831)) described *Corniger spinosus*, and Gill (1862) described *Plectrypops* to contain the Atlantic species *P. retrospinis.* Günther (1874) described *Holotrachys* based on specimens of *H. lima* from the Indo-Pacific, though Lamber (1963) proposed *Holotrachys* was congeneric with *Plectrypops* and synonymized the former. Randall et al. (1982) described *Pristilepis* to accommodate *P. oligolepis*, which was previously classified as *Holotrachys*. Several authors commented on relationships within Myripristinae, specifically that *Plectrypops* and *Corniger* are closely related (Woods and Sonoda, 1973; Greenfield, 1974). Subsequent phylogenetic investigations of Myripristinae have not included more than two genera in their analyses (e.g., Miya et al., 2005; Hubert et al., 2010).

In contrast with the relatively stable taxonomy and predicted relationships among the Myripristinae, difficulties in finding apomorphies for major lineages, nomenclatural inconsistencies, and the loss of holotype materials has complicated the taxonomy of Holocentrinae (Shimizu and Yamakawa, 1979). Scopoli (1777) described *Holocentrus* designating *H. adscensionis* as the type species, and for nearly a century Holocentrinae was monogeneric. Castelnau (1875) described a genus within the holocentrinae, *Neoniphon*, based on the presence of an elongate body, a large anal spine, and the last spine of the dorsal fin being in close association with the soft dorsal fin in *N. armatus* (=*N. sammara*). Subsequently, generic names were rapidly added to the Holocentrinae. Jordan and Evermann (1898) described *Flammeo* based on the presence of a long projecting lower jaw. Fowler (1904) described *Sargocentron* as a subgenus of *Holocentrus* to accommodate the morphologically divergent *S. spiniferum* based on serrations of the preopercle and large body size. *Sargocentron* was later elevated as a genus without comment (Fowler, 1944). Starks (1908) recognized distinct differences in the development of the swim bladder in relation to the back of the skull in several holocentrids and therefore separated *Holocentrus adscensionis* from *H. suborbitalis*, placing the latter into a new genus, *Adioryx*. Whitley (1933) added two more subgenera to *Holocentrus*: *Faremusca* for the Indo-Pacific *H. punctatissimus* and *Cephalofarer* for the western Atlantic species *H. vexillarium*. This proliferation of generic and subgeneric names combined with an increasing number of species descriptions complicated the taxonomy of the Holocentrinae.

Woods (1955) attempted to reconcile the profusion of group names in Holocentrinae by classifying species into four subgenera: *Holocentrus*, *Flammeo*, *Sargocentron*, and *Adioryx*. *Flammeo* was recognized over *Neoniphon*, due to the uncertain status of the type species for *Neoniphon* (Woods, 1955). Subsequently, *Adioryx* was elevated as a genus (Woods, 1965). However, Randall and Heemstra (1985, 1986) recognized *Flammeo* as a junior synonym of the *Neoniphon* based on the identification of the type species of *Neoniphon* as *N. sammara*. Similarly, Matsuura and Shimizu (1982) argued that the type specimens of *Adioryx* and *Sargocentron* could not be diagnosed by features of the swim bladder and auditory bulla, and subsequently classified *Adioryx* as *Sargocentron*. Li et al. (1981) separated *Sargocentron rubrum* into a new genus *Dispinus* due to the presence of two haemal spine-like processes on the second abdominal vertebra; however, no subsequent authors have adopted *Dispinus* as a valid holocentrid group name. In the most recent classifications, species of the Holocentrinae are classified among three genera, *Holocentrus* (two species), *Sargocentron* (33 species), and *Neoniphon* (five species) (Randall, 1998; Randall and Greenfield, 1999; Greenfield, 2003; Nelson, 2006).

With the exception of *Holocentrus*, many of the characters proposed as diagnostic for the three genera of Holocentrinae include overall body size, fin morphology, body shape, and degree of jaw protrusion. All of these characters have been invoked as important ecomorphological components in teleosts (e.g., Westneat et al., 2005; Westneat, 2006; Holzman et al., 2012; Dornburg et al., 2011), indicating that the currently recognized taxonomy may reflect ecotypes and not evolutionary relationships. This hypothesis has been supported by molecular phylogenetic investigations that sampled multiple holocentrine genera. Hubert et al. (2010) sampled eight species of *Sargocentron* and one species of *Neoniphon* for the mtDNA *COI* gene and resolved *Sargocentron* as paraphyletic with respect to *Neoniphon.* Additionally, phylogenetic analyses of rhodopsin amino acid sequences resulted in a paraphyletic *Sargocentron* with respect to *Neoniphon* (Yokoyama and Takenaka, 2004; Yokoyama et al., 2008).

In this study we investigate the species-level phylogeny of the Holocentridae using DNA sequence data. We sample several nuclear loci and a mitochondrial gene for all but one of the holocentrid genera and over 50% of the species diversity. Our phylogenetic analyses of the nucleotide data include several Bayesian and maximum likelihood methods, and a Bayesian framework that allows for the possibility of incongruent gene histories for our inference of relationships within the Holocentridae. The results of our phylogenetic

analyses are not congruent with the current taxonomy of the Holocentridae. We demonstrate that several characters used to diagnose currently recognized genera are homoplastic and propose appropriate taxonomic revisions that reflect the evolutionary history of Holocentridae, providing a critical foundation for subsequent investigations into the evolutionary history of this clade.

2. Methods

2.1. Sequence data acquisition

Samples were obtained through museum collections, commercial wholesalers, and field collections with all voucher specimens deposited into the fish collection at the Yale Peabody Museum of Natural History (Table 1). Additional sequences for the mtDNA cytochrome oxidase subunit 1 (*COI*) gene were downloaded from Genbank and the United States Food and Drug Administration Seafood Identification Sequence Library (Table 1). The phylogenetic relationship of the Holocentridae within Acanthomorpha is unclear (Moore, 1993a,b; Johnson and Patterson, 1993; Colgan et al., 2000; Miya et al., 2003). Holocentrids are considered closely allied with beryciforms and most molecular studies suggest beryciforms and stephanoberyciforms form a clade (e.g., Miya et al., 2003), therefore we included two species, one beryciform (*Beryx decadactylus*) and a percopsiform (*Percopsis omiscomaycus*) with the expectation that these will be resolved as successive outgroups in our phylogenetic analyses. Our sampling of the Holocentridae includes 43 of the 84 recognized species and all genera with the exception of the rare myripristine genus *Pristilepis* (Randall et al., 1982).

Muscle tissue biopsies were stored in 95% ethanol or RNAlater (Qiagen, Valencia, CA) and DNA was extracted using Qiagen DNAeasy Tissue Extraction Kits (Qiagen, Valencia, CA). We used polymerase chain reaction (PCR) to amplify one mitochondrial gene, (COI ~ 661 bp) and six protein coding nuclear genes: myh6 (~700 bp), Ptr (~705 bp), ENC1 (~786 bp), rag1 (~1407 bp), sreb (~878 bp), and Glyt (~882 bp). All nuclear genes were amplified using nested PCR. Conditions for the first round of PCR utilize the primers and the protocol described by Li et al. (2007). For each nested round, we used custom primers (Table 2) and a touchdown PCR strategy (Don et al., 1991), with an initial denaturing step of 94 °C for 2 min; 11 cycles with a 30 s. 94 °C denaturation, 1 min, annealing beginning at 67 °C and lowering 1.5 °C per cycle, and a 1 min. 72 °C extension. This was followed by an additional 19 cycles with a 30 s. 94 °C denaturation, 1 min. 57° annealing, and 1 min. 72 °C extension, followed by an additional 10 min. 72 °C final extension. Amplification of the mitochondrial COI gene fragment

Table 1
List of Taxa examined in this study, voucher numbers, and NCBI Genbank accession numbers. Tissue samples were.

Taxon	Tissue source	Genbank gi numbers						
		COI	*ENC1*	*Glyt*	*myh6*	*Ptr*	*ragl*	*sreb*
Beryx decadactylus	YFTC 13625	AB679219	JX390750	JX390790	JX390829	JX390869	JX390903	JX390936
Holocentrus adscensionis	KU114/YFTC 21876	JX390748	JX390751	JX390791	JX390830	JX390870	JX390904	JX390937
Holocentrus rufus	YFTC 16195	JQ840116	JX390752	JX390792	JX390831	JX390871	JX390905	JX390938
Neoniphon argenteus	KU4917	JX390737	JX390767	JX390807	JX390846	–	JX390916	JX390953
Flammeo marianus	YFTC 21765	JX390734	JX390768	JX390808	JX390847	JX390886	JX390917	JX390954
Neoniphon opercularis	YFTC 16276	JX390740	JX390769	JX390809	JX390848		JX390918	JX390955
Neoniphon sammara	KU4921	JQ350142	JX390770	JX390810	JX390849	JX390887	JX390919	JX390956
Sargocentron caudimaculatum	KU 4485	JQ350308	JX390774	JX390813	JX390853	JX390891	JX390923	JX390960
Sargocentron cornutum	YFTC 13564	FJ237589	JX390776	JX390814	JX390854	–	JX390924	JX390961
Neoniphon coruscum	KU 210/YFTC 21822	JQ840674	JX390777	JX390815	JX390855	JX390892	JX390925	JX390962
Neoniphon diadema	ASIZP0910955	JQ350312	JX390778	JX390816	JX390856	–	–	JX390963
Neoniphon inaequalis	YFTC 16279	JX390738	JX390779	JX390817	JX390857	JX390893	JX390926	JX390964
Neoniphon ittodai	ASIZP0910670	GU207342	JX390780	JX390818	JX390858	JX390894	–	JX390965
Sargocentron melanospilos	KU 6987	HM034260	JX390781	JX390819	JX390859	JX390895	JX390927	JX390966
Neoniphon microstoma	KU 5567	HM034264	JX390782	JX390820	JX390860	JX390896	JX390928	JX390967
Sargocentron praslin	YFTC 16282	JX390735	JX390783	JX390821	JX390861	JX390897	JX390929	JX390968
Neoniphon punctatissimum	ASIZP0911452	JQ350316	JX390784	JX390822	JX390862	JX390898	–	JX390969
Sargocentron rubrum	YFTC 22126	IQ623980	1X390785	1X390823	1X390863	–	1X390930	1X390970
Sargocentron seychellense	KU 6908	JX390736	JX390786	JX390824	JX390864	JX390899	JX390931	JX390971
Sargocentron spiniferum	KU753	JQ350318	JX390775	JX390825	JX390865	JX390900	JX390932	JX390972
Neoniphon suborbitalis	KU1155	JX390739	JX390787	JX390826	JX390866	JX390901	JX390933	JX390973
Sargocentron tiere	KU 5702	JQ350322	JX390788	JX390827	JX390867	JX390902	JX390934	JX390974
Sargocentron tiereoides	Genbank	HM034280	–	–	–	–	–	–
Neoniphon vexillarium	YFTC 16179	JQ840676	JX390789	JX390828	JX390868		JX390935	JX390975
Neoniphon xantherythrum	Genbank	DQ521006	–	–	–	–	–	–
Corniger spinosus	FDA	CFSAN18511	–	–	–	–	–	–
Myripristis adusta	KU 5598	FJ583669	JX390753	JX390793	JX390833	JX390872	–	JX390939
Myripristis amaena	KU 5565	HM034218	JX390754	JX390794	JX390834	JX390873	JX390906	JX390940
Myripristis berndti	KU 5043	HM034161	JX390755	JX390795	JX390835	JX390874	–	JX390941
Myripristis botche	YFTC 22141	JX390744	JX390756	JX390796	JX390836	JX390875	JX390907	JX390942
Myripristis chryseres	ASIZP0911002	JX390746	JX390757	JX390797	JX390837	JX390876	–	JX390943
Myripristis hexagona	KU 4597	JX390749	JX390758	JX390798	JX390838	JX390877	–	JX390944
Myripristis jacobus	YFTC 21825	JQ842253	JX390759	JX390799	JX390839	JX390878	JX390908	JX390945
Myripristis kuntee	YFTC 16277	HM034232	JX390760	JX390800	JX390840	JX390879	JX390909	JX390946
Myripristis leiognathus	YFTC 22200	JX390743	JX390761	JX390801	JX390841	JX390880	JX390910	JX390947
Myripristis murdjan	YFTC 12631	FJ459546	JX390762	JX390802	JX390842	JX390881	JX390911	JX390948
Myripristis pralinia	KU4018	HM034233	JX390763	JX390803	JX390843	JX390882	JX390912	JX390949
Myripristis randalli	KU670	JX390745	JX390764	JX390804	JX390832	JX390883	JX390913	JX390950
Myripristis violacea	YFTC 12630	HM034163	JX390765	JX390805	JX390844	JX390884	JX390914	JX390951
Myripristis vittata	KU 4220	JX390747	JX390766	JX390806	JX390845	JX390885	JX390915	JX390952
Ostichthys trachypoma	KU1155	JX390742	JX390771	–	JX390850	JX390888	JX390920	JX390957
Plectrypops lima	FMNH 119175	JQ350231	JX390772	JX390811	JX390851	JX390889	JX390921	JX390958
Plectrypops retrospinus	KU123	JX390741	JX390773	JX390812	JX390852	JX390890	JX390922	JX390959

Table 2
Primers used for nested PCR and sequencing.

Gene	Primer	Sequence 5′–3′
ENC1	H_ENC1F	ACA TCC GDG ATG CCT GCG CT
	H_ENC1R	GGC TGG GAG GCA GCC AGT TG
Glyt	H_GLYTF	CCA CAG GGD CCC AAA GCC AA
	H_GLYTR	GCC TCR TTG GYG GCT TGG ACT G
myh6	H_MYH6F	ACG AGC AAG GGA ACA CTG GAG GA
	H_MYH6R	VCC TTG GCC TTT GGT GAC ATA TTC ATT
Ragl	H1_RAGF	GGG GCT GCC TCG GTG GAT GA
	H1_RAGR	GAG GCC TCT GCC CGN CTG GA
	H2_RAGF	NCG TGA GAT GGA GGG CCT GG
	H2_RAGR	NCA GGG ACA TGG GCC AGG GT
PTR	H_PTRF	TCA TAC NCT CTG ACC CAG CAG
	H_PTRR	ACC CAA GAC DCC CAG CTC CA
ube3a	H_UbeF	AGC AAY CTY CAC AGC CCA GAG T
	H_UbeR	AGT CBT TGT CCA TCT CCA GCA CCT

was conducted using the primers in Eytan et al. (2009) with an initial denaturing step conducted at 94 °C for 2 min; 10 cycles with a 1 min. 94 °C denaturation, 1 min. 60 °C annealing that decreased 1 °C per cycle to 50 °C, and a 1 min. 72 °C extension. This was followed by an additional 28 cycles with a 1 min. 94 °C denaturation, a 1 min. 50 °C annealing step, and a 1 min. 72 °C extension, that were followed by an additional 5 min. 72 °C final extension and a 5 min 23 °C cool down. PCRs were purified using Qiagen Qiaquick PCR Purification Kits and sequenced using Applied Biosystems Big Dye chemistry and 3730xL DNA analyzers (Applied Biosystems) at the Keck Biotechnology Resource Laboratory (Yale University).

2.2. Alignment and model selection

Alignment of all genes was conducted using MUSCLE v3.7 (Edgar, 2004), then refined by eye using the translated amino acid sequences. Individual alignment files were concatenated using Phyutility (Smith and Dunn, 2008). Gene matrices were edited in Geneious (Drummond et al., 2010) and TextWrangler (BareBones Software). We trimmed sequences to the size of the smallest fragment for each gene to minimize missing characters in the data matrix. Our aligned nucleotide data matrix consisted of 5929 base pairs used in analysis with all sequences deposited in GenBank (Table 1).

Models of nucleotide substitution and optimal partitioning strategies were chosen simultaneously under the Bayesian Information Criterion (BIC) using heuristic search algorithms in PartitionFinder (Lanfear et al., 2012). For each gene, all five combinations of codon positions were considered as candidate partitions, spanning the range from a single partition for the entire gene to each codon position treated as a partition. All 56 available nucleotide substitution models were included in the candidate pool, though this was repeated with a restricted candidate pool of models ($Model_{rcp}$) to mirror the nucleotide substitution model restrictions in MrBayes 3.2 (Ronquist and Huelsenbeck, 2003). Additionally, this procedure was repeated to assess optimal partitioning strategies when gene matrices were concatenated. Each gene was given a maximum of three partitions, so the final candidate pool of partitioning strategies spanned the entire possible range of partitioning strategy combinations between a single partition and a maximum of 21 partitions. This strategy was replicated with the *COI* data excluded for subsequent phylogenetic analyses restricted to the sampled nuclear genes.

2.3. Likelihood and Bayesian phylogenetic inference

We analyzed a total of nine datasets: each of the seven genes independently, the concatenated matrix containing all genes, and a concatenated matrix of the six sampled nuclear genes to account for the possibility of the mtDNA having an undue influence on the analyses. We used the Randomized Accelerated Maximum Likelihood (RAxML) software v. 7.3.0 to infer phylogenies from each of the sampled genes and the concatenated gene datasets (Stamatkis, 2006). As RAxML has a restricted selection of nucleotide substitution models, we used the GTRGAMMA model to model each partition identified using PartitionFinder (Lanfear et al., 2012). Although this potentially over parameterizes each dataset, Dornburg et al. (2008) demonstrated that nucleotide substation model over parameterization has a negligible effect on topology estimation as well as the calculation of both bootstrap support values and Bayesian posterior probabilities. For each analysis, 200 maximum likelihood searches were conducted using a random starting tree (-d setting) and a random search convergence criterion based on Robinson–Foulds distances (-D setting). Two thousand bootstrap replicates were conducted using the thorough bootstrap search (option –f i) for each dataset and confidence values were mapped onto the tree topology with the highest maximum likelihood inferred from the random searches.

We also inferred the phylogenetic relationships of Holocentridae using Bayesian methods as executed in MrBayes 3.2 (Ronquist and Huelsenbeck, 2003). Mixed partitioned analyses were performed for each of the sampled genes and on the concatenated matrices, utilizing the optimal $Model_{rcp}$ partitioning strategies identified by PartitionFinder (Lanfear et al., 2012). For each analysis set we assigned default priors to all model parameters (topology: uniform, revmat: Dirichlet (1.0, 1.0, 1.0, 1.0, 1.0, 1.0), statefreq: Dirichlet (5.0, 5.0, 5.0, 5.0) pinvar: uniform (0.0, 1.0), Brlengths: exp (10.0), shape: uniform (0, 1)). We ran all analyses using three heated and one cold Markov Chain. We ran the Markov Chain Monte Carlo (MCMC) sampler for 30 million generations, sampling every 1000, for a total of 30,000 trees. To insure that our samples came from the target distribution, convergence of the chains was assessed by visualizations of the state likelihoods, potential scale reduction factors, and the average deviation of the split frequencies using the MrBayes output and AWTY (Nylander et al., 2007). Majority rule consensus trees were constructed from the remaining post-burn in samples of each data set.

2.4. Species tree inference

To account for the possibility of incongruent gene histories influencing our inference of species level relationships, we simultaneously inferred gene trees and the underlying species tree using the *BEAST package (Heled and Drummond, 2010) within BEAST v.1.6.2. (Drummond and Rambaut, 2007). Trees were unlinked by gene region and each gene was partitioned using the optimal partitioning strategies used in RAxML and MrBayes 3.2 analyses, and allowed to evolve under the best fitting nucleotide substitution model as identified using BIC in PartitionFinder (Lanfear et al., 2012). We ran five independent MCMC analyses for 200 million generations each, sampling every 5000 generations. To allow for the possibility of clade specific rate heterogeneity misleading branch length estimates (Dornburg et al., 2012), analyses were conducted under both a model of uncorrelated rates under a log-normal distribution (UCLN) and a random local clock (RLC) model that allows for clade-specific shifts in the nucleotide substitution rate across the tree. The fit of the two models to the data was compared using Bayes factors (Suchard et al., 2001) using the criteria of 2ln Bayes factor >10 to determine support (Kass and Raftery, 1995). Convergence of the chains was assessed by visualizations of the state likelihoods in TRACER v. 1.5 (Rambaut and Drummond, 2007) and comparisons of the split frequencies using AWTY (Nylander et al., 2008). Effective sample sizes (ESS) for all model parameters were calculated to ensure adequate mixing of each

chain, with ESS values above 200 indicating appropriate sampling. We reran these analyses with the *COI* data removed to determine whether the mitochondrial gene region had an undue influence on our species tree inference. To assess the influence of the prior on our posterior species-tree inferences, we replicated two analyses without sequence data that sampled solely from the prior distribution of parameter estimates (Drummond et al., 2006).

2.5. Ancestral state reconstruction

We compiled a dataset from the literature on discretely coded morphological characters that have been used to diagnose holocentrine genera to test the probability of independent origins or multiple losses of these traits across the posterior distribution of species trees. Characters assessed included components of fin ray morphology, jaw protrusion, body depth, and body color (Supplemental Table 1). Ancestral character states for each putative diagnostic trait were estimated using the methods of Pagel et al. (2004) and Pagel and Meade (2006) using scripts provided by J. Beaulieu (unpublished) in R. Character state reconstructions were conducted for 25 species sampled in the Bayesian-inferred species tree. Species for which there was no character state information were pruned from the phylogeny. Custom R scripts were used to visualize the probabilities of the inferred ancestral reconstructions.

3. Results

3.1. Nucleotide substitution model and partition selection

Selection of a partitioning strategies for the individual genes using BIC determined that all of the genes, except *Rag1* and *COI*, were optimally partitioned with two partitions, one containing the pooled first and second codon positions and a second containing the third codon positions. The optimal partitioning strategies for both the *rag1* and *COI* genes treated each of the codon positions as individual partitions. Best-fitting substitution models varied across the gene partitions. When assessing the best strategy for partitioning the concatenated nuclear gene matrix a six-partition set-up was favored, with combinations of five of the six genes' codon positions overlapping across four partitions, while *sreb* required it's own two partitions that were independent of the other genes. This strategy was replicated with the addition of the *COI* data to the concatenated matrix, with *COI* requiring its own three additional partitions.

3.2. Likelihood and Bayesian phylogenetic inference

The RAxML analysis of the concatenated nuclear gene dataset resulted in a well resolved phylogeny with monophyly of both the Myripristinae and Holocentrinae, supported by bootstrap proportions (bps) of 100% (−Lnl = −18575.10; Fig. 1A), and resolved six major clades: (1) *Ostichthys* + *Plectrypops*; (2) *Myripristis*; (3) *Sargocentron* group 1 (*S. spiniferum* group); (4) *Sargocentron* group 2 (*S. punctassimum* group + *Neoniphon opercularis*); (5) *Holocentrus*; and (6) *Sargocentron* group 3 (*S. suborbitalis* group + remaining *Neoniphon*). The monophyly of each of these subclades was strongly supported with bootstrap proportions ⩾71; however, the resolution of *Holocentrus* relative to the other clades was unclear (bps = 43). The RAxML analyses that included the mtDNA *COI* gene resulted in a phylogeny congruent with that inferred from the nuclear genes, but resolved *Corniger* in the clade containing *Ostichthys* and *Plectrypops* (Fig. 2A). In the RAxML analyses of the two concatenated datasets, species of *Neoniphon* were nested within multiple subclades of the Holocentrinae and *Sargocentron* was paraphyletic (Figs. 1A–2A).

The Bayesian analyses using MrBayes 3.2 reached convergence and appeared to have adequate sampling of the posterior

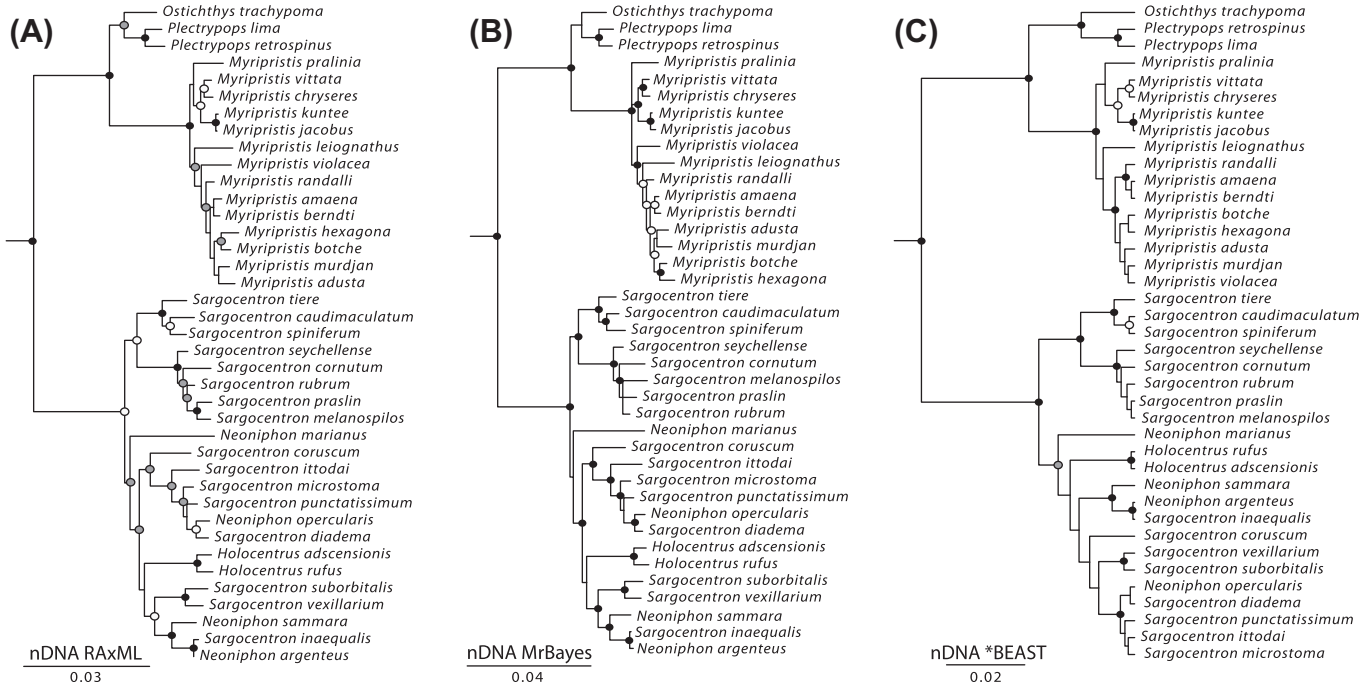

Fig. 1. Phylogenetic relationships of the Holocentridae based on analyses of the nuclear gene dataset. (A) Phylogeny inferred using maximum likelihood (RAxML). Solid black circles at nodes represent bootstrap support values (BS) of 100, open circles represent BS between 90 and 99, and gray circles represent BS of 70–90. (B) Fifty percent majority-rule consensus of the posterior distribution of phylogenies inferred by our Bayesian (MrBayes) analysis of the concatenated nuclear gene dataset. Posterior probability (PP) estimates of 1.00 are indicated by solid black circles, while PP between 0.95 and 0.99 are represented by open circles. (C) Fifty percent majority-rule consensus tree resulting from the multi-species coalescent species tree analysis (*BEAST) using the nuclear genes. Solid black circles represent PP estimates of 1.00, open circles represent PP estimates of 0.95–0.99, and gray circles represent PP estimates of 0.9–0.94.

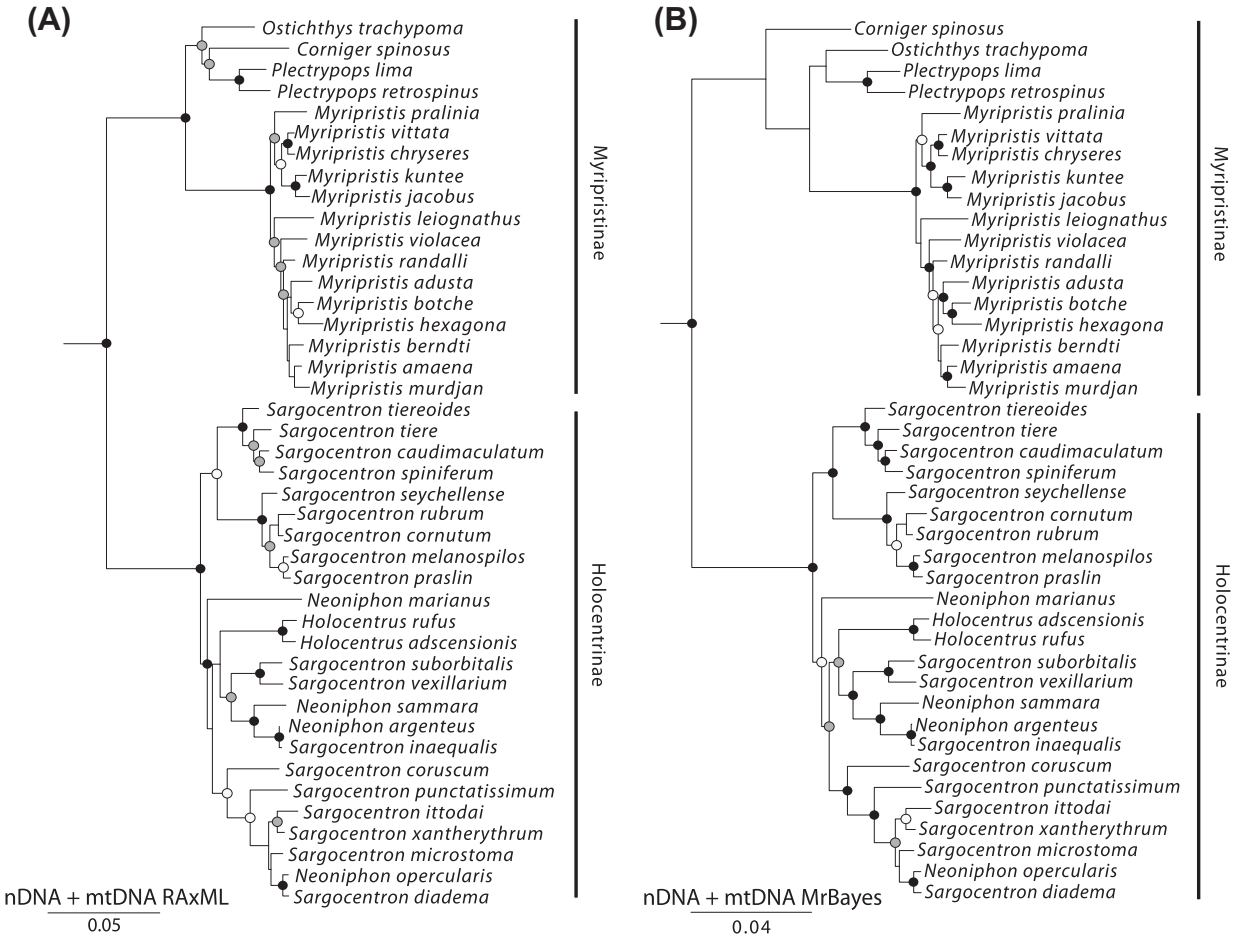

Fig. 2. Phylogenetic relationships of the Holocentridae based on concatenated analyses of the nDNA and mtDNA. Phylogeny inferred using maximum likelihood (RAxML). Solid black circles at nodes represent bootstrap support values (BS) of 100, open circles represent BS between 90 and 99, and gray circles represent BS of 70–90. (B) Fifty percent majority-rule consensus of the posterior distribution of phylogenies inferred by our Bayesian (MrBayes) analysis of the concatenated the nuclear gene dataset. Posterior probability (PP) estimates of 1.00 are indicated by solid black circles, while PP between 0.95 and 0.99 are represented by open circles. All branch lengths are in substitution units.

distribution as assessed using the compare and cumulative functions in AWTY, standard deviations of the clade split frequencies below 0.1%, and potential scale reduction factors of approximately 1.00 for all parameters (Gelman and Rubin, 1992). Results from the concatenated Bayesian inference of all nuclear genes were congruent with concatenated maximum likelihood analyses, resulting in an identical tree topology that was strongly supported (Fig. 1B). The reciprocal monophyly of Holocentrinae and Myripristinae and the monophyly of the six major holocentrid clades observed in the RAxML inferred phylogenies were each supported by Bayesian posterior probabilities (BPP) $\geqslant$ 0.95 with the exception of the clade containing *Ostichthys* and *Plectrypops*, BPP = 0.87). The phylogenetic relationships within Holocentrinae were resolved and supported with strong Bayesian posterior probabilities, with the exception of the placement of *Holocentrus*. As in the RAxML analyses, *Neoniphon* and *Sargocentron* were paraphyletic (Fig. 1B).

The Bayesian analysis of the concatenated nuclear genes and the mtDNA *COI* data resulted in a topology that was largely congruent with the analysis restricted to the nuclear genes (Fig. 2B), with the exception of *Corniger* resolved as sister lineage of all other sampled species of the Myripristinae, though this result was not strongly supported (BPP = 0.74). The only nucleotide data available for *Corniger* is *COI* gene, and its resolution in the concatenated data analysis is incongruent with the *COI* inferred gene tree (Supplemental Figs. 1 and 2). Within *Myripristis*, the phylogenetic relationships are identical to those inferred using the concatenated nDNA dataset. Within the Holocentrinae, there was strong Bayesian posterior support for the paraphyly of both *Neoniphon* and *Sargocentron* (Fig. 2B).

3.3. Species tree inference

Comparisons of analyses conducted using empty alignments to analyses with data indicated the prior settings to not have had an undue influence on resulting tree topologies. Analyses of the clock model-fit to the nDNA dataset strongly favored the UCLN relaxed-clock model (log Bayes Factor = 53.47), though phylogenies inferred under both clock models were identical (Supplemental Fig. 3). Phylogenies inferred using the *BEAST multispecies coalescent species tree method supported the reciprocal monophyly of the Myripristinae and the Holocentrinae with strong Bayesian posterior probabilities (BPP = 1.00; Fig. 1C). The phylogenetic relationships within the Myripristinae were identical to those inferred in the RAxML and Bayesian analyses of the concatenated datasets, with a clade containing *Ostichthys* and *Plectrypops* sister to *Myripristis* (all BPP = 1.00; Fig. 1C). Relationships within *Myripristis* were not fully resolved, though there is strong support (BPP = 0.99) for a clade containing *M. jacobus*, *M. kuntee*, *M. chryseres,* and *M. vittata*. Within the Holocentrinae, the paraphyly of *Neoniphon* and *Sargocentron* was strongly supported (Fig. 1C).

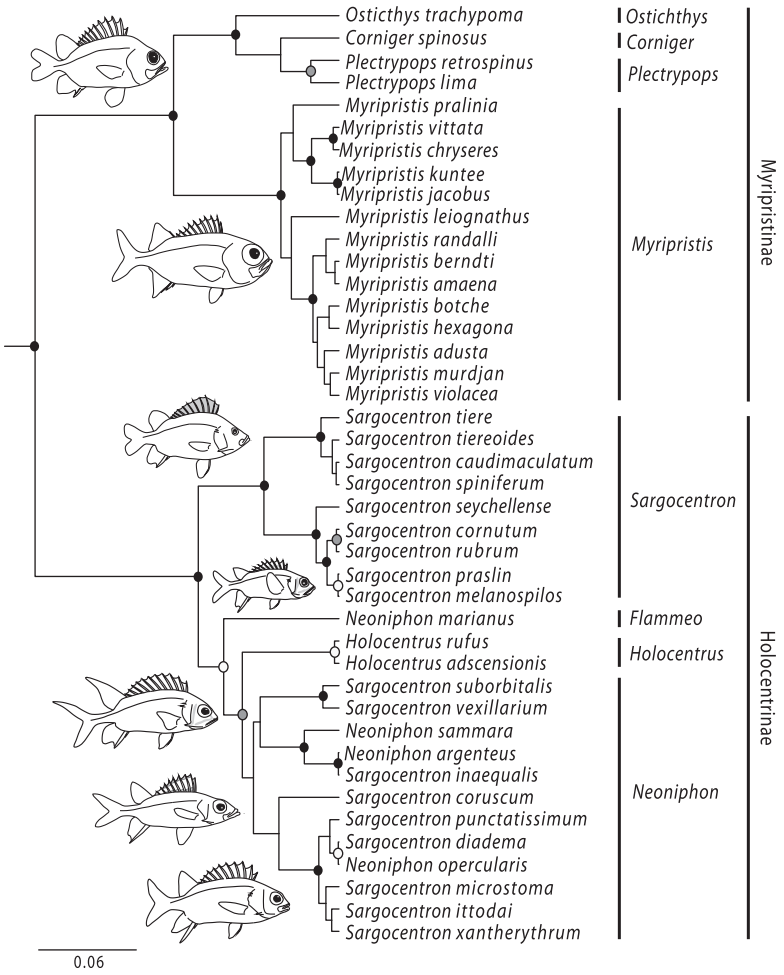

Fig. 3. Fifty percent majority-rule consensus of the posterior distribution of phylogenies inferred by the multi-species coalescent species tree analysis (*BEAST) of the combined nDNA and mtDNA data sets. Solid black circles represent PP estimates of 1.00, open circles represent PP estimates of 0.95–0.99, and gray circles represent PP estimates of 0.9–0.94. All branch lengths are in substitution units.

Analysis of clock model fit with the addition of the mtDNA data still strongly favored the UCLN model (ln Bayes Factor = 55.39), though topologies under both clock models were identical (Supplemental Fig. 3). The monophyly of Myripristinae and Holocentrinae were both strongly supported (BPP = 1.00). Within Myripristinae, there is strong support for a clade containing *Ostichthys, Corniger*, and *Plectrypops* that was resolved as the sister lineage of *Myripristis* with strong support (BPP = 1.00; Fig. 3). Within Holocentrinae, the phylogeny resolved the same subclades as the species tree inferred using the nuclear genes (Fig. 3).

3.4. Ancestral state reconstruction

Reconstructions of the ancestral character states indicated that many of the morphological traits that serve as diagnostic characters for groupings in the accepted holocentrid taxonomy exhibit a complex history of evolution that does not always reflect shared common ancestry. Snout elongation (Fig. 4A) and lower jaw projection (Fig. 4B) are found in both *Neoniphon* and *Sargocentron* with multiple origins or losses of these traits. First dorsal fin traits used to diagnose *Neoniphon* that include the placement (Fig. 4C) and size of the last spine of the first dorsal fin (Fig. 4D) are optimized on the molecular phylogenies as exhibiting multiple evolutionary origins. The silvery body coloration of *Neoniphon* was reconstructed as having multiple evolutionary origins, while uniform red or red bodies with silver stripes have evolved repeatedly in *Sargocentron* (Fig. 5A). The evolution of body depth was also not diagnostic of clades resolved in the molecular phylogeny, as our analyses reconstruct repeated body shape modification throughout the history of the Holocentrinae. *Holocentrus* represents the only example where taxonomically diagnostic characters reflect common ancestry, with both the direct connection of the swim bladder to the skull (Fig. 5B) and the red and white chevron body coloration (Fig. 5C) were each optimized as having a single evolutionary single origin.

4. Discussion

4.1. Relationships of the Myripristinae

We provide an extensive molecular phylogenetic dataset sampled from both mtDNA and nuclear genes that allow the inference of the first species-level phylogeny of Myripristinae. Greenfield (1974) postulated the relationships of myripristine genera, placing *Plectrypops* as sister to *Corniger* in what appears to be a basal trichotomy with *Ostichthys* and *Myripristis*. Analysis of the nuclear gene datasets strongly support a clade containing *Ostichthys* and *Plectrypops* that is sister to *Myripristis*, and our species tree inferences resolve *Corniger* within the clade containing *Ostichthys* and *Plectrypops*. This novel phylogeny indicates that the cryptic reef and non-reef dwelling deep-water soldierfishes represent a clade that is sister to the reef dwelling *Myripristis*, suggesting that

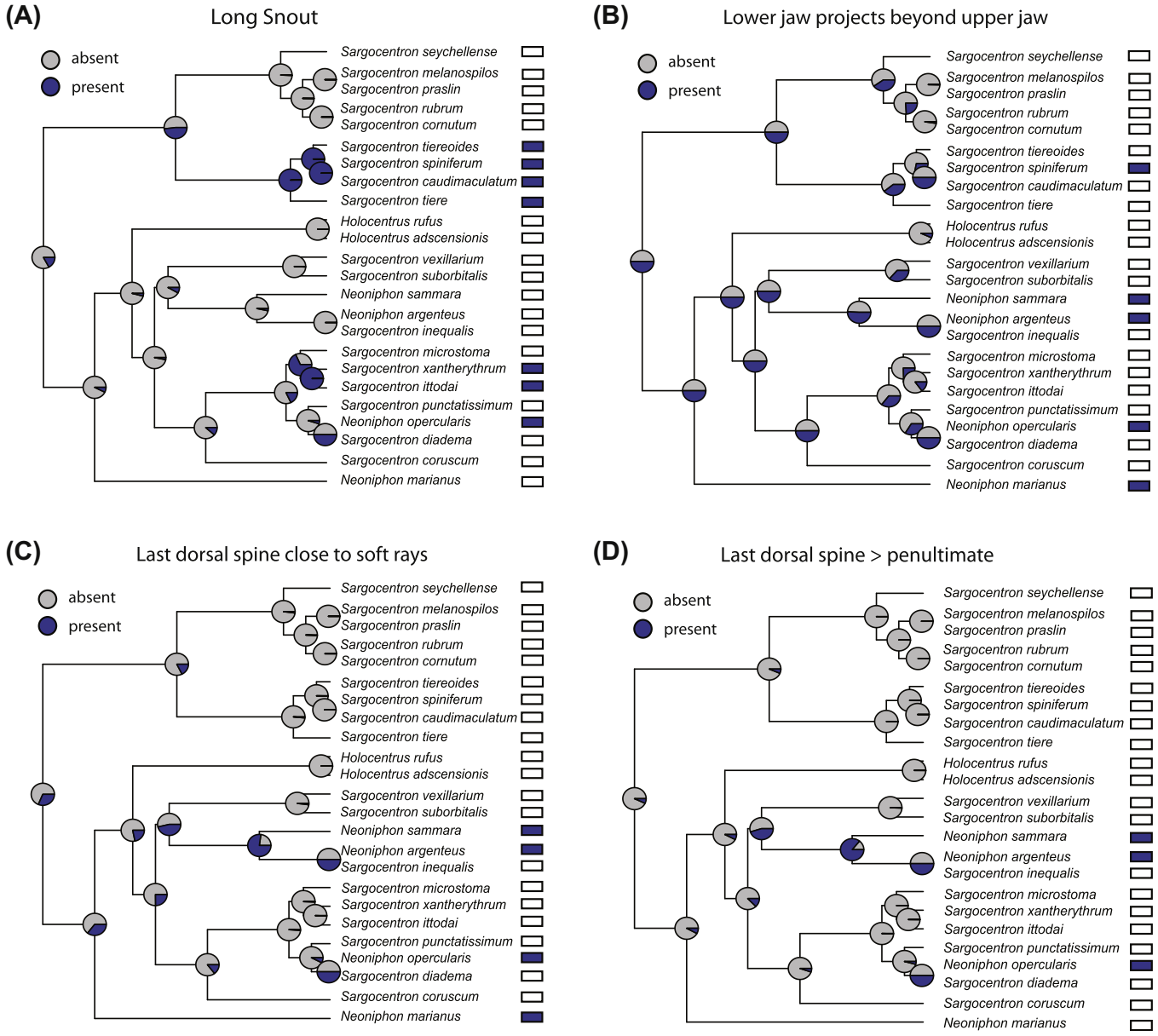

Fig. 4. Ancestral state reconstructions of characters used to inform the taxonomy of the Holocentrinae. Reconstructions are based on the topological estimates that utilized the combined molecular data matrix while accounting for potential gene-tree heterogeneity. Presence of character states are indicated next to each tree and pie charts represent probability of ancestral states. Characters analyzed and corresponding genera were: (A) Snout length (*Sargocentron*); (B) projection of the lower jaw relative to upper jaw (*Neoniphon*); (C) proximity of dorsal fin spines to soft dorsal ray (*Neoniphon*) and (D) dorsal fin morphology (*Neoniphon*).

Plectrypops represents a transition from deeper marine habitats to shallow reef habitats independent of *Myripristis*.

Greenfield (1968) presented the only phylogenetic tree for species of *Myripristis*. In our phylogeny, we resolve two subclades that subtend the common ancestor of *Myripristis*. This differs from Greenfield (1974), who proposed that *Myripristis vittata* and *M. trachyacron* belong to a subgenus, *Archeaomyripristis*, that is the sister lineage of all other *Myripristis*. *Archeaomyripristis* was suggested to represent the transitional form between stem myripristine fossils and extant species of *Myripristis*. In our phylogeny, *M. vittata* was deeply nested within one of the main subclades of the Myripristinae radiation, suggesting the purported primitive conditions of *M. vittata*'s frontal bone morphology to in fact represent a derived character state. Greenfield (1974) also noted that *M. vittata* and *M. chryseres* possess many similarities, most notably characters relating to the morphology of the anal fin spines, and speculated that these species may be closely related. However, given the primitive frontal bone morphology of *M. vittata*, he left the placement of *M. chryseres* as ambiguous. *Myripristis chryseres* is closely related to *M. vittata* in our phylogenetic trees (Fig. 3), validating Greenfield's (1974) hypothesis while not supporting the monophyly of *Archaeomyrpristis*.

The resolution of *M. chryseres* and *M. vittata* in a clade containing *M. jacobus*, *M. kuntee*, and *M. pralinia* suggests that *M. chryseres* and *M. vittata* have secondarily lost the black shoulder bars that characterize the latter three species. Greenfield (1974) suggested *M. jacobus* independently evolved this disruptive coloration pattern under strong selective pressures in the Caribbean. While we cannot rule out selective pressure maintaining this trait, the phylogenetic relationships of these species indicate it more likely that *M. jacobus* retained black shoulder bars (Fig. 3). Black bars near the operculum or black pigmentation of the opercular membrane are

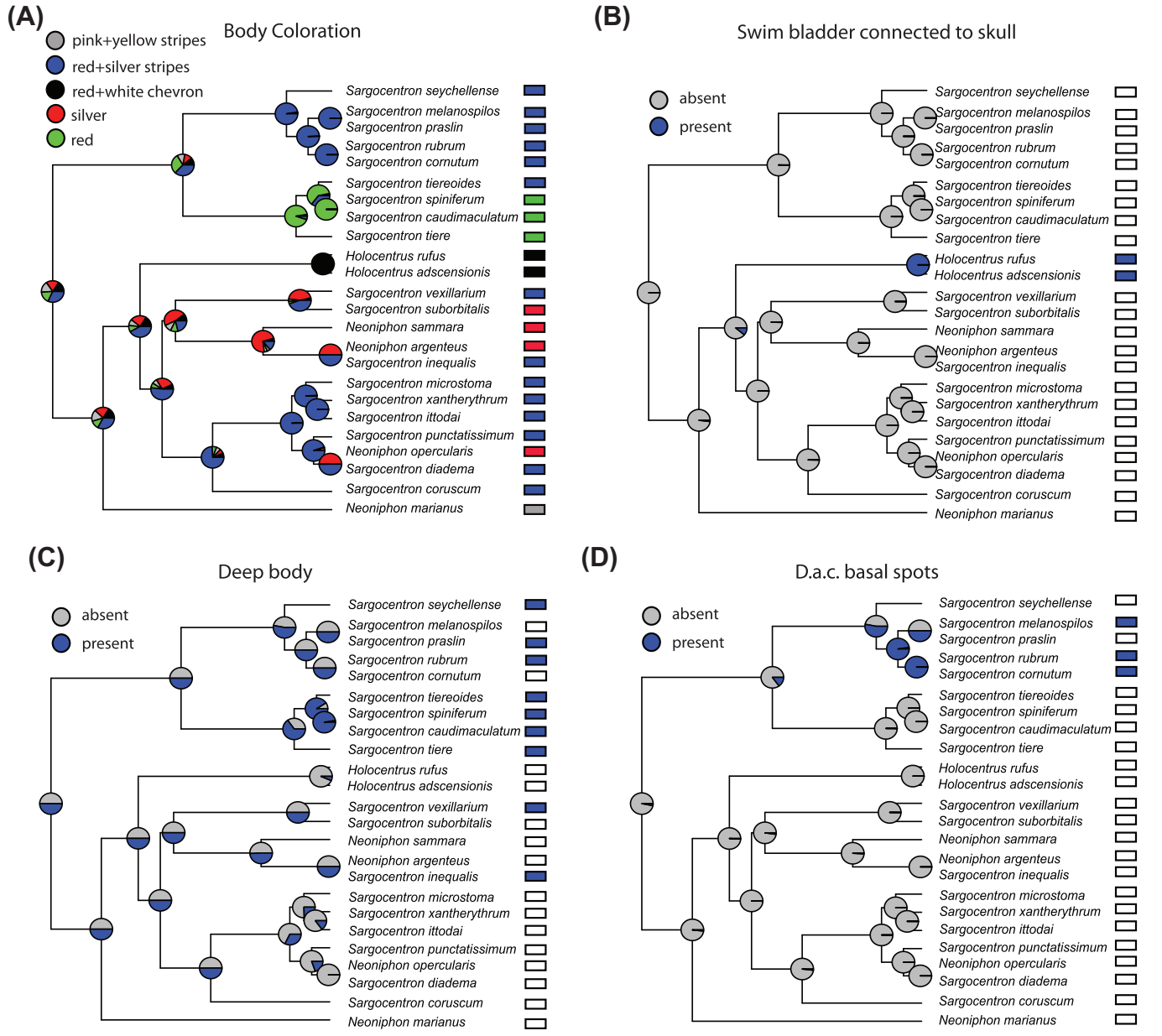

Fig. 5. Ancestral state reconstructions of characters used to inform the taxonomy of the Holocentrinae. Reconstructions are based on the topological estimates that utilized the combined molecular data matrix while taking the potential for gene-tree heterogeneity into account. Presence of character states are indicated next to each tree and pie charts represent probability of ancestral states. Characters analyzed and corresponding genera were: (A) Body coloration (*Neoniphon*); (B) swim bladder morphology (*Holocentrus*); (C) body depth (*Sargocentron*) and (D) spotting of the dorsal, anal, and caudal fins (*Sargocentron*).

also found in various degrees in several other species of *Myripristis* including *M. botche*, *M. amaena*, *M. murdjan*, *M. berndti*, and *M. randalli* (Randall and Greenfield, 1996, 1999). Additionally, Randall and Greenfield (1996) pointed out that several species of *Myripristis* contain various degrees of pigmentation on the peritoneum, including *M. greenfieldi*, *M. randalli*, *M. robusta*, *M. botche*, *M. adusta*, *and M. hexagona.* The phylogenetic hypotheses generated in this study suggest a complex pattern of pigment loss or independent origination.

4.2. Relationships and taxonomy of Holocentrinae

The phylogenetic trees generated in this study, regardless of inference method, resolve the Holocentrinae into two subclades that strongly support the non-monophyly of *Sargocentron* and *Neoniphon* (Figs. 1–3). This finding is not unprecedented as recent DNA barcoding studies have suggested *Neoniphon* is nested within *Sargocentron* (Hubert et al., 2010, 2012). Additionally, examination of morphological characters did not identify any synapomorphies for *Sargocentron*, and silvery coloration was the only putative synapomorphy supporting *Neoniphon* (Stewart, 1984). The Bayesian ancestral state reconstruction of silvery coloration indicated that this trait has multiple origins in the clade (Fig. 5A). The ancestral state reconstructions of the other putative synapomorphies for *Neoniphon* and *Sargocentron* strongly reject a single origin for each of these characters (Figs. 4 and 5), emphasizing the need to revise the taxonomy of the Holocentrinae to reflect inferred phylogenetic relationships.

There are several ways genera could be delimited taxonomically based on the phylogenetic resolution of Holocentrinae. For example, the classification scheme of Scopoli (1777) could be resurrected and classify all species of Holocentrinae in *Holocentrus*.

Table 3
Proposed taxonomic revisions to the holocentrinae. Current taxonomic assignment proposed Taxonomic Assig.

Current taxonomic assignment	Proposed taxonomic assignment
Holocentrus adscensionis	*Holocentrus adscensionis*
Holocentrus rufus	*Holocentrus rufus*
Neoniphon argenteus	*Neoniphon argenteus*
Neoniphon aurolineatus	*Not evaluated*
Neoniphon marianus	*Flammeo marianus*
Neoniphon opercularis	*Neoniphon opercularis*
Neoniphon sammara	*Neoniphon sammara*
Sargocentron bullisi	*Not evaluated*
Sargocentron caudimaculatum	*Sargocentron caudimaculatum*
Sargocentron cornutum	*Sargocentron cornutum*
Sargocentron coruscum	*Neoniphon coruscum*
Sargocentron diadema	*Neoniphon diadema*
Sargocentron dorsomaculatum	*Not evaluated*
Sargocentron ensifer	*Not evaluated*
Sargocentron hastatum	*Not evaluated*
Sargocentron hormion	*Not evaluated*
Sargocentron inaequalis	*Neoniphon inaequalis*
Sargocentron iota	*Not evaluated*
Sargocentron ittodai	*Neoniphon ittodai*
Sargocentron lepros	*Not evaluated*
Sargocentron macrosquamis	*Not evaluated*
Sargocentron marisrubri	*Not evaluated*
Sargocentron megalops	*Not evaluated*
Sargocentron melanospilos	*Sargocentron melanospilos*
Sargocentron microstoma	*Neoniphon microstoma*
Sargocentron poco	*Not evaluated*
Sargocentron praslin	*Sargocentron praslin*
Sargocentron punctatissimum	*Neoniphon punctatissimum*
Sargocentron rubrum	*Sargocentron rubrum*
Sargocentron seychellense	*Sargocentron seychellense*
Sargocentron shimizui	*Not evaluated*
Sargocentron spiniferum	*Sargocentron spiniferum*
Sargocentron spinosissimum	*Not evaluated*
Sargocentron suborbitalis	*Neoniphon suborbitalis*
Sargocentron tiere	*Sargocentron tiere*
Sargocentron tiereoides	*Sargocentron tiereoides*
Sargocentron vexillarium	*Neoniphon vexillarium*
Sargocentron violaceum	*Not evaluated*
Sargocentron wilhelmi	*Not evaluated*
Sargocentron xantherythrum	*Neoniphon xantherythrum*

Alternatively, we could classify each of the two major holocentrine subclades into a single genus, with *Sargocentron* for one and *Holocentrus* for the other. However, both of these options would ignore the anatomical differences that separate the two Atlantic species of *Holocentrus* from other squirrelfishes (e.g., Starks, 1908; Nelson, 1955) and ignore the strongly supported relationships of the holocentrine subclades resolved in the molecular phylogenies (Fig. 3). To continue the delimitation of *Holocentrus* as containing *H. adscensionis* and *H. rufus*, and to reflect the biological and evolutionary diversity of Holocentrinae, we suggest the elevation of several previously described group names as genera to classify the various smaller subclades of Holocentinae resolved in molecular phylogenies (Table 3).

The type species of *Sargocentron* (*S. spiniferum*) is within the first major subclade of the Holocentrinae that includes several of the species recognized by Woods (1955) as the deep-bodied, more uniformly-colored, larger-sized species that he placed in *Sargocentron* (e.g., *S. spiniferum*, *S. tieroides*, and *S. praslin*). As reflected in the results of our phylogenetic analyses, we delimit the genus *Sargocentron* as containing *S. tieroides*, *S. tiere*, *S. caudimaculatum*, *S. spiniferum*, *S. seychellense*, *S. rubrum*, *S. melanospilos*, *S. praslin*, and *S. cornutum*. Although not sampled in our phylogenetic analyses, *S. shimizui*, *S. ensiferum*, and *S. violaceum* likely belong to this clade given their phenotypic similarities to sampled *Sargocentron* species, though this warrants an explicit test in future phylogenetic analyses.

The remaining holocentrines comprise a monophyletic radiation that is resolved as the sister lineage of *Sargocentron sensu stricto*, with the currently classified *Neoniphon marianus* supported as first diverging lineage (Fig. 3). In this instance, the genus *Flammeo* (Jordan and Evermann, 1898) can be resurrected for the single species *Flammeo marianus*. The remaining species currently classified in *Neoniphon* are spread throughout the subclade sister to *Flammeo* and our analyses suggest that *Neoniphon* has most likely been based upon independently derived ecomorphs (Figs. 4 and 5). The species tree supports *Holocentrus* as the sister to the remaining species of *Sargocentron* and *Neoniphon* (Fig. 3). This clade includes several smaller-bodied species, most frequently placed within *Sargocentron* (plus the species *N. opercularis*, *N. sammara*, *N. argenteus*) and a clade that includes *Sargocentron suborbitalis*. Based on the inclusion of *N. Sammara,* we designate *NeoNiphon* (Castelnau, 1875) as a generic name for the species in this clade (Table 3).

With the holocentrid phylogenetic relationships presented in this study, we aim to prompt a reexamination of morphological characters that have been used to traditionally diagnose and delimit taxonomic groups within the clade. Given the dominance of squirrelfishes and soldierfishes in the nocturnal reef-fish community, and their use as an exemplar lineage in studies of teleost vocalization (e.g., Luczkovich and Keusenkothen, 2007; Parmentier et al., 2011), vision (e.g., Yokoyama and Takenaka, 2004), and dispersal (Craig et al., 2007; Muths et al., 2011), our phylogeny provides a basis for subsequent comparative evolutionary analyses of the Holocentridae.

Acknowledgments

We thank Mark Vermeij and the staff at the CARMABI field research station in Curaçao for facilitating fieldwork and the staff of Curaçao Sunshine and Ocean Encounters West for providing field assistance. Greg Watkins-Colwell at the Yale Peabody Museum of Natural History, Mark Westneat at the Field Museum of Natural History, Andrew Bentley at the University of Kansas Natural History Collection, and Tun-Yuan Cheng at the Taiwan Research Center for Biodiversity Academia Sinica generously provided tissue loans. Jeffrey C. Oliver, Jeremy Beaulieu, and Ron Eytan provided helpful discussions of the manuscript. Computational resources were provided by the Yale University Faculty of Arts and Sciences High Performance Computing Facility. Analysis files have been uploaded to the Dryad data repository:doi:10.5061/dryad.3t19n. This is Harbor Branch Oceanographic Institution contribution no. 1866. This work was supported by a National Science Foundation awards ANT-0839007 and DEB-1061806 to TJN, DEB-1061981 to PCW and DEB-1011328 to AD and TJN.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.07.020.

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