Exon probe sets and bioinformatics pipelines for all levels of fish phylogenomics

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Abstract

Exon markers have a long history of use in phylogenetics of ray-finned fishes, the most diverse clade of vertebrates with more than 35,000 species. As the number of published genomes increases, it has become easier to test exons and other genetic markers for signals of ancient duplication events and filter out paralogs that can mislead phylogenetic analysis. We present seven new probe sets for current target-capture phylogenomic protocols that capture 1,104 exons explicitly filtered for paralogs using gene trees. These seven probe sets span the diversity of teleost fishes, including four sets that target five hyper-diverse percomorph clades which together comprise *ca.* 17,000 species (Carangaria, Ovalentaria, Eupercaria, and Syngnatharia + Pelagiaria combined). We additionally included probes to capture exon markers that have been commonly used in fish phylogenetics, despite some being flagged for paralogs, to facilitate integration of old and new molecular phylogenetic matrices. We tested these probes and merged new exon-capture sequence data into an existing data matrix of 1,105 exons and 300 ray-finned fish species. We provide an optimized bioinformatics pipeline to assemble exon capture data from raw reads to alignments for downstream analysis. We show that loci with known paralogs are at risk of assembling duplicated sequences with target-capture, but we also assembled many useful orthologous sequences. These probe sets are a valuable resource for advancing fish phylogenomics because they can be easily extracted from increasingly available whole genome and transcriptome datasets, and also may be integrated with existing PCR-based exon and mitochondrial datasets.

Introduction

Exon markers have played a pivotal role in resolving phylogenetic relationships among ray-finned fishes (Li *et al.* 2007; Near *et al.* 2012; Betancur-R. *et al.* 2013; Betancur-R *et al.* 2017; Hughes *et al.* 2018; Rabosky *et al.* 2018). Identification of these exons has typically involved the comparison of a small number of fish model genomes. For example, a suite of 154 single-copy exons conserved enough for PCR amplification was identified by Li *et al.* (2007). This study used a reciprocal BLAST approach on two genomes, the pufferfish *Takifugu rubripes* and the zebrafish *Danio rerio*, with 10 exons initially optimized with PCR primers for sequencing (Li *et al.* 2007). These exons demonstrated their utility for resolving previously enigmatic relationships among fishes (Li *et al.* 2008), and were the basis for largescale reappraisals of the ray-finned fish Tree of Life (Near *et al.* 2012; Betancur-R. *et al.* 2013), phylogenetic analysis of the large clade of the “spiny-ray” acanthomorph fishes (Near *et al.* 2013b), and new phylogenetic classifications based on sequence data for more than 2,000 fish species (Betancur-R. *et al.* 2013; Betancur-R *et al.* 2017). Most recently, these exons formed a large part of a supermatrix analysis that utilized sequence data for more than 11,000 ray-finned fish species available through GenBank in one of the largest analyses to date (Rabosky *et al.* 2018).

The advent of high-throughput sequencing technologies has drastically increased the number of loci systematists can harness for their groups of interest. Sequence capture based on single-stranded RNA probes that enrich genomic DNA libraries for conserved molecular markers have revolutionized phylogenomics (Faircloth *et al.* 2012; Lemmon *et al.* 2012), allowing cost-effective sequencing of hundreds or thousands of markers for many taxa. Ultraconserved Elements (UCEs) (Faircloth *et al.* 2012) have been used for fish phylogenomics (Faircloth *et al.* 2013, 2020; Harrington *et al.* 2016; Longo *et al.* 2017; Chakrabarty *et al.* 2017; Alfaro *et al.*

2018; Roxo *et al.* 2019; Friedman *et al.* 2019), as have Anchored Hybrid Enrichment (AHE) approaches (Lemmon *et al.* 2012; Eytan *et al.* 2015; Stout *et al.* 2016; Dornburg *et al.* 2017; Irisarri *et al.* 2018). But exon markers have desirable properties for phylogenomics that other approaches may lack. They are relatively easy to align, and a number of software programs have been developed for reading frame-aware alignment (Abascal *et al.* 2010; Ranwez *et al.* 2011, 2018), avoiding potential homology errors with UCE flanking regions that are more difficult to align (Edwards *et al.* 2017). Both protein and nucleotide sequences can be used for phylogenetic inference, making them useful for deep (Hughes *et al.* 2018) and shallow phylogenetic scales (Rincon-Sandoval *et al.* 2019). Exon markers are also easy to integrate with both genomic and transcriptomic data, with sequences being produced and archived for a variety of studies from comparative genomics to gene expression analysis that can provide resources for systematists to increase taxon sampling without incurring additional costs.

Since exon markers tend to be more variable across the target region than UCEs or AHE, two rounds of *in vitro* hybridization are optimal for their sequence capture protocols (Li *et al.* 2013). This improvement in laboratory techniques has resulted in a number of studies using sequence capture to target exons for fish phylogenomics (Ilves & López-Fernández 2014; Li *et al.* 2015; Song *et al.* 2017; Arcila *et al.* 2017; Ilves *et al.* 2017; Kuang *et al.* 2018; Straube *et al.* 2018; Betancur-R. *et al.* 2019; Rincon-Sandoval *et al.* 2019; Yin *et al.* 2019). The increase in genomic resources for fishes has allowed for the comparison of a larger number of genomes for probe design (Li *et al.* 2012), and ultimately eight ray-finned fish genomes were used to identify >17,000 ‘single-copy’ exons (Song *et al.* 2017) using a modification of the reciprocal BLAST approach of Li *et al.* (2007). A subset (4,434) of these were optimized for capture across all ray-

finned fishes (Jiang *et al.* 2019). Other sets of exon probes have been designed to target more specific groups including cichlids (Ilves & López-Fernández 2014), and otophysans (Arcila *et al.* 2017).

The evolutionary history of vertebrate genomes is complicated by two ancient Whole-Genome Duplication (WGD) events, and an additional WGD event in the ancestor to all modern teleosts (Vandepoele *et al.* 2004; Dehal & Boore 2005). While many duplicated gene copies were lost shortly after the teleost WGD (Inoue *et al.* 2015), up to a quarter of genes in teleost genomes have paralogs as a consequence of this event (Braasch *et al.* 2015). Phylogenetic inference relies on the analysis of orthologs, but loci implemented so far for analyses were based on the comparison of a limited number of model genomes and some threshold of similarity to define them as “single-copy” (Li *et al.* 2007). A recent study implementing an explicit tree-based filtering method to test for orthology revealed that one third of the ‘single-copy’ exons >200 bp in length identified by Jiang *et al.* (2019) were affected by paralogy, potentially biasing tree inference (Hughes *et al.* 2018). A set of 1,105 exons free of vertebrate and teleost WGD-derived paralogs identified in this study resolved the phylogeny with confidence for more than 300 species of ray-finned fishes. Other markers used for phylogenomic studies such as UCEs (Faircloth *et al.* 2013), AHE loci (Lemmon *et al.* 2012) and exons (Ilves & López-Fernández 2014; Song *et al.* 2017; Arcila *et al.* 2017; Jiang *et al.* 2019) also have not been explicitly tested for paralogy using approaches based on gene trees.

Here we present a new experimental protocol to obtain sequence data for a set of exons filtered for paralogs (Hughes *et al.* 2018) across the diversity of fishes. We provide probe sets for target-

capture that are designed to enrich genomic libraries for different taxonomic groups, from the early branching teleosts to the major groups within percomorphs, the massive radiation comprising more than 17,000 species. These probe sets target the same loci, but the specific sequences of the probes are tailored to capture more efficiently within taxonomic brackets. We have also included probes for other legacy exon markers (e.g., Lopez *et al.* 2004; Lovejoy *et al.* 2004; Dettai & Lecointre 2005; Li *et al.* 2007) and mitochondrial DNA (mtDNA) loci that have been sequenced for a large number of fishes through PCR-Sanger sequencing methods to facilitate integration of new high throughput sequencing results with existing phylogenetic datasets. We designed a new bioinformatics pipeline that wraps aTRAM 2.0 (Allen *et al.* 2017) with other software packages and python scripts to assemble and filter sequence alignments from Illumina reads.

Methods

Nuclear Exon Probes

Sequences for probe design came from exon alignments derived from a database of 303 bony fish genomes and transcriptomes (Sun *et al.* 2016; Hughes *et al.* 2018). Briefly, the EvolMarkers pipeline (Li *et al.* 2012, 2015; Jiang *et al.* 2019) was used to identify 1,721 single-copy exons in eight ray-finned fish genomes (*Lepisosteus oculatus*, *Anguilla japonica*, *Danio rerio*, *Gadus morhua*, *Oreochromis niloticus*, *Oryzias latipes*, *Tetraodon nigroviridis*, and *Gasterosteus aculeatus*). These exons were mined from 295 other genomes and transcriptomes using nhmmer (Wheeler & Eddy 2013) in HMMER v3.1b2, and exons with paralogs were filtered by testing for duplications in gene trees via topology tests (see Hughes *et al.* 2018 for full details).

A total of 1,105 exons were retained after filtering for loci with paralogs. We generated seven probe sets for these exons based on different underlying references for our target groups, following the classification of Betancur-R. *et al.* (2017). These seven target teleost groups included (i) Elopomorpha (~1000 species, including true eels and tarpons) (Figure 1); (ii) early branching teleosts from Osteoglossomorpha (bonytongues) to Myctophiformes (lanternfishes)—hereafter paraphyletic “Backbone 1” (Figure 1); (iii) Acanthomorphata (from paracanthopterygians (e.g. cods, oarfish) to Anabantaria (e.g. swamp eels, gouramies)—hereafter paraphyletic “Backbone 2”) (Figure 2); and four specific sets aimed for some of the most species-rich clades of Percomorphaceae, including (iv) Carangaria (~1,100 species, including flatfishes and jacks) (Figure 3); (v) Ovalentaria (~5,600 species, including clownfishes, cichlids, flying fishes) (Figure 2); (vi) Eupercaria (~6,800 species, including surgeonfishes, pufferfishes, and groupers) (Figure 3); and (vii) Syngnatharia-Pelagiaria (~1,000 species, including tunas, seahorses, and pipefishes) (Figure 3). The large freshwater Otophysa clade (>10,000 species, including catfishes, knifefishes, and tetras) is not included in Backbone 1 (Figure 1), largely because it was targeted earlier by a more specific probe set designed for the clade by other exon-capture fish studies (Arcila *et al.* 2017; Betancur-R. *et al.* 2019), though 143 exons are shared between the two. We designed probe sets for different subsets of taxa from these 1,105 alignments that initially consisted of 303 species that span the diversity of bony fishes (Hughes *et al.* 2018), as explained above. Each of the seven probe set references were comprised four to eight of the most phylogenetically-distant taxa in the target clade, depending on the phylogenetic breadth the probe set covers (Table 1). We ranked preferred taxa within each of these groups to form the basis for the probe set references (Table 1), and if all preferred taxa were missing from a group, we took the next longest sequence in the alignment for the clade of

interest. This means that some exons may have unique taxa representing them in their reference set. One particularly long exon included highly divergent sequences that were difficult to align and was ultimately excluded from the final probe sets (a total of 1,104 target exons remained).

We included baits for nuclear markers popular in fish phylogenetics (hereafter referred to as ‘legacy’ markers) to better connect sequence datasets produced by targeted amplicon sequencing approaches (Bybee *et al.* 2011). Several of these widely used markers were already included as part of the “paralogy-tested” 1,105 exons from Hughes *et al.* (2018), including RAG1 (Lopez *et al.* 2004), RAG2 (Lovejoy *et al.* 2004), FICD (Li *et al.* 2011), PANX2, GCS1, GLYT (Li *et al.* 2007), VCPIP (Betancur-R. *et al.* 2013), and MLL (Dettai & Lecointre 2005). A total of 19 additional legacy markers that did not meet the paralogy filtering requirements were nonetheless included in the probe sets, mainly markers developed by the Euteleost Tree of Life Project: TBR1, MYH6, KIAA1239, PLAGL2, BTCHD1, RIPK4, SH3PX3, SIDKEY, SREB2, ZIC1, SVEP1, GPR61, SLC10A3, UBE3A, and UBE3A-like (Li *et al.* 2007, 2011; Betancur-R. *et al.* 2013). Additionally, baits were designed for the markers Rhodopsin (Chen *et al.* 2003), IRBP (Dettai & Lecointre 2008), and RNF213 (Li *et al.* 2009a), which have been widely used in fish systematics. Due to the long sequences of MYH6 and KIAA1239 (>3,000 bp), references for these markers were shortened to the region typically amplified by PCR primers. The reference sequences used in bait design are available on GitHub (<https://github.com/lilychughes/FishLifeExonCapture/tree/master/ProbeSets>).

Probe sequences of 120 bp in length were initially generated with the py_tiler.py script packaged in PHYLUCe for each of the four to eight taxa selected for probe design (Table 1) (Faircloth *et*

al. 2012; Faircloth 2015). Probes were mapped against the consensus sequences of the alignments from Hughes *et al.* (2018) in Geneious Pro v8.1 (<http://www.geneious.com>) to examine the distribution of probes across loci. Visual inspection of the distribution of probes initially revealed highly uneven coverage of probes across longer loci. To have the probes cover the reference alignments more evenly, we applied a staggering strategy by tiling probes every 20 bp across the locus for each of the four to eight taxa (Table 1), so that probes from the first species spanned the first 0-120 bp, and probes from the second species spanned from 20-140, and so on. This strategy ultimately improved tiling density and resulted in more even coverage for longer loci *in silico*. The probe staggering design was generated via custom scripts (Jake Enk, Arbor Biosciences). Probes that had more than 25% repeats detected on the RepeatMasker.org database were eliminated. Probes were filtered for potential self-hybridization. Each of our eight probe sets was designed with a MyBaits1 custom probe set with approximately 20,000 biotinylated probes for each set (Arbor Biosciences, Ann Arbor, Michigan).

Mitochondrial Probes

In addition to exon probes, we designed and synthesized a separate, fish universal bait set to capture four of the most popular mitochondrial DNA (mtDNA) gene markers used in fish systematics: COI (cytochrome c oxidase subunit I), CYTB (cytochrome *b*), and 12S and 16S ribosomal DNA. The goal of maintaining separate mtDNA and nDNA probe sets is to equilibrate nDNA/mtDNA sample ratios by applying spiking dilutions of the mtDNA probe set after capturing the exon markers (mtDNA:nDNA dilution ratios = 1:1000). Probes for these four mtDNA genes were individually designed using mtDNA genomes or single sequences from NCBI that span the diversity of ray-finned fishes (*Amia calva*, *Danio rerio*, *Elops saurus*,

Harengula clupeola, *Harengula jaguana*, *Oryzias latipes*, *Osteoglossum bicirrhosum*, *Polypterus ansorgii*, *Salmo trutta*, *Takifugu vermicularis*, and *Zeus faber*). A total of 7,000 oligonucleotide baits (120 bp long) tiling over the mtDNA genes with 2x density were designed using the py_tiler.py script (Faircloth *et al.* 2012; Faircloth 2015). We did not target other high-copy genes like 28S rDNA, which may have required an additional probe set and spiking dilution.

Library Preparation and Sequencing

Eight fish species were newly sequenced for each bait set (Table 2; 56 total species sequenced). DNA extractions were performed on the GenePrep (Autogen Inc.) following manufacturer's instructions at the Laboratory of Analytical Biology at the Smithsonian Institution National Museum of Natural History in Washington, D.C. DNA was eluted in 50 µL of Autogen R9 Buffer. Quality control was performed by running 1 µL of eluted DNA on a 1.0% agarose gel stained with GelRed (Biotium) and visually inspecting whether bands of high molecular weight DNA were visible. Library preparation was performed at Arbor Biosciences in Ann Arbor, Michigan, using a dual-round capture protocol (Li *et al.* 2013), with an 8-plex capture design. Paired-end sequencing of 100 bp reads was performed at the University of Chicago Genomics Facility on a HiSeq 4000. Samples were multiplexed with 192 in a lane, with sequencing runs containing samples for other projects not used here.

Bioinformatics Pipeline for Exon Assembly

We developed a bioinformatics pipeline based around the software aTRAM 2.0 (Allen *et al.* 2017) with five major steps before multiple sequence alignment (Figure 4). Raw FASTQ files were quality trimmed with Trimmomatic v0.36 (Bolger *et al.* 2014), removing low quality

sequences and adapter contamination with the parameters “ILLUMINACLIP:TruSeq3-
PE.fa:2:30:10 LEADING:5 TRAILING:5 SLIDINGWINDOW:4:15 MINLEN:31”. Trimmed
reads were then mapped against a master file containing all sequences used for bait design for
any of the seven probe sets using BWA-MEM (Li & Durbin 2009). SAMtools v1.8 was used to
remove optical PCR duplicates and separate the reads that mapped to each of the exons (Li *et al.*
2009b). Mapped reads were then assembled individually by exon using Velvet (Zerbino &
Birney 2008), and the longest contig produced by Velvet was used as a reference sequence for
aTRAM v2.0 (Allen *et al.* 2017) to extend contigs, using Trinity v2.8.5 as the assembler
(Grabherr *et al.* 2011). Redundant contigs with 100% identity produced by aTRAM were
removed with CD-HIT v4.8.1 using CD-HIT-EST (Li & Godzik 2006; Fu *et al.* 2012). Open
reading frames for remaining contigs were identified with Exonerate (Slater & Birney 2005),
using a reference sequence checked by eye for each exon, and any contigs that did not contain
the open reading frame were filtered out. If only a single contig contained the open reading
frame, the exon passed all filters and was used for multiple sequence alignment. If multiple
contigs contained the open reading frame, the reading frames were compared with CD-HIT-EST,
using a 99% identity threshold to account for potential allelic variation. If the comparison with
CD-HIT-EST resulted in a single contig, that contig passed filters and was used for phylogenetic
analysis; more divergent sequences were flagged and excluded from downstream analysis.
Unlike another tool recently published to assemble exon-capture data, ASSEXON (Yuan *et al.*
2019), our pipeline is fully automated and does not require Geneious as part of the assembly
(<https://github.com/lilychughes/FishLifeExonCapture>).

Alignment and Phylogenomic Analysis

Target-capture data were combined with genomic and transcriptomic data from Hughes *et al.* (2018), and 36 additional recently published genomes that were mined for orthologous exon sequences using nhmmer (Wheeler & Eddy 2013). Sequences for each exon were aligned with MACSE v2.03 (Ranwez *et al.* 2018) after cleaning out potentially non-homologous fragments with the *-cleanNonHomologousSequences* option. Alignment edges composed of more than 60% missing data as well as insertions that occurred only in a single taxon were removed with custom scripts (AlignmentCleaner.py, <https://github.com/lilychughes/FishLifeExonCapture>). A total of 1,104 nuclear exons filtered for paralogs were concatenated using geneStitcher.py (<https://github.com/ballesterus/Utensils>).

A concatenated protein matrix was analyzed under maximum likelihood (ML) with IQ-TREE v1.6.9 (Nguyen *et al.* 2015), using the best-fitting model for the entire matrix as determined using ModelFinder (Kalyaanamoorthy *et al.* 2017). A concatenated nucleotide matrix was partitioned into first, second, and third codon positions, with the best-fitting model applied to each partition. Concatenated matrices used contained only the 1,104 loci that have been filtered for paralogy; the legacy markers that failed to pass this filter in Hughes *et al.* (2018) were excluded from these analyses.

Paralogs in Legacy Markers

Eighteen nuclear markers that have been commonly used with Sanger sequencing methods for fish phylogenetics were re-included in our probes to better connect novel sequence capture data with extensive existing datasets, but these genes had been previously filtered out for suspected paralogs. For the 18 legacy markers that were re-included (TBR1, MYH6, KIAA1239, PLAGL2,

PTCHD1, RIPK4, SH3PX3, SIDKEY, SREB2, ZIC1, SVEP1, GPR61, IRBP, RNF213, Rhodopsin, SLC10A3, UBE3A, and UBE3A-like), we integrated our newly sequenced data with the matrices from Betancur-R. *et al.* (2013) for TBR1, MYH6, KIAA1239, PLAGL2, PTCHD1, RIPK4, SH3PX3, SIDKEY, SREB2, ZIC1. For the remaining genes, we pulled a selection of sequences from GenBank for SVEP1, GPR61, IRBP, RNF213, Rhodopsin, SLC10A3, UBE3A, and UBE3A-like to align with our new data. All GenBank accession numbers can be found on the sequence labels of these gene trees available on FigShare (Hughes *et al.* 2020). We inferred gene trees in IQ-TREE 1.6.9, partitioning by codon position and using the ModelFinder algorithm to determine the best-fitting sequence model. Target-capture-derived sequences falling out in unexpected positions or clades were BLASTed against the NCBI nucleotide database to determine their identity.

Results

Capture Efficiency of Nuclear Exons

The number of reads per sample varied substantially, from 216,271 to 7,017,309 (Table 2). However, the number of reads per sample was not correlated with the number of loci assembled per species ($r^2=0.0043$; $p=0.27$). Capture efficiency (measured as the average number of exons assembled per species) varied across probe sets and across samples (Figure 5A), showing a strong negative correlation with clade (or paraphyletic “grade”) age ($r^2=0.82$; $p=0.003131$; Figure 5B). Two probe sets designed to capture markers in paraphyletic groups that include more anciently diverging lineages (~251-144 Ma) with larger phylogenetic diversity (Backbone 1 and 2; Figures 1-2) tended to have lower capture efficiency (Table 2; Figure 5A), with an average of 52% of the loci captured for Backbone 1 and 61% on average captured for Backbone 2. This was

also the case for the rather ancient Elopomorpha clade (196 Ma), for which samples sequenced had only a 60% capture rate. Probe sets designed for more recently diverged percomorph clades (~105-66 Ma) had much higher numbers of loci assembled on average, with 83% for Carangaria, 82% for Ovalentaria, 81% for Eupercaria, and 89% for the Syngnatharia + Pelagiaria clade. Probe sets are publicly available at Arbor Biosciences, Ann Arbor, MI. Exon alignments ranged from a minimum of 60% up to 93.9% sequence identity across the eight model genomes originally used to discover single-copy markers, but the legacy markers tended to have >80% sequence identity (Figure 5C).

Mitochondrial Gene Capture

Mitochondrial genes for which we designed probes (12S, 16S, COI, ATPase6, and CYTB) tended to have the best rate of capture. Complete sequences of 12S, 16S, and COI assembled for all taxa, while ND6, for which we did not design probes, was only represented for 29 taxa, the lowest of any mitochondrial coding gene. CYTB, which was included in the probe set, assembled for 56 of 58 total taxa, but ATPase6 had a relatively poor capture rate, only assembling for 36 species.

Phylogenomic Analysis

Combining the new data for 56 samples collected through exon-capture plus 38 additional recently published fish genomes with the dataset of Hughes *et al.* (2018) generated a matrix with 394 taxa representing all major groups of ray-finned fishes with three lobe-finned (sarcopterygian) outgroups, with a final length of 549,861 bp (183,287 amino acid sites). The entire matrix had 72% present data, excluding loci suspected of having paralogs (Table 3). The

average locus alignment length for genes included in the matrix was 499 bp (range: 129-5,055 bp), and the average number of parsimony-informative sites per locus was 340 (range: 75-3,435).

ModelFinder selected JTT+I+F+G for the protein matrix, and GTR+I+F+G for the first two codon positions, with TVM+I+F+G for the third codon position. Both topologies inferred with IQ-TREE matched previous results obtained by Hughes *et al.* (2018), with newly added taxa placed in their expected phylogenetic placements (Figures 1-3). Tree files and phylogenomic matrices are available on FigShare (Hughes *et al.* 2020).

Paralogs in Legacy Markers

We examined gene trees by eye for evidence paralogs had been assembled for 19 markers that were included in our probe set, despite failing previous tests to exclude loci with paralogs. Nine of these loci assembled one or more paralogs when we applied our new pipeline on the raw reads obtained with sequence capture instead of a single orthologous locus (Table 3). One locus (PTCHD1) could only be assembled for seven of the 56 newly sequenced samples, which made the paralogy assessment difficult. Annotation of the paralogous sequences was determined by blasting assembled contigs against the NCBI nucleotide database.

Discussion

Deep Probe Sets Capture Across Deep Phylogenetic Divergences

We present resources for capturing conserved exon sequences for all groups of teleost fishes, including probe sets for early branching lineages (“Backbone 1”), Elopomorpha, Acanthomorphata (“Backbone 2”), and multiple major percomorph radiations including

Syngnatharia + Pelagiaria, Carangaria, Ovalentaria, and Eupercaria. The exon markers presented here have been explicitly tested for orthology using a large database of 303 bony fish species (Hughes *et al.* 2018), and have been screened for paralogs derived from ancient vertebrate or teleost-specific whole genome duplication events. Capture efficiency is strongly correlated with the phylogenetic span of taxa used to design the probes, with probe sets designed more specifically for younger (~105-66 Ma) percomorph clades (Syngnatharia-Pelagiaria, Carangaria, Ovalentaria, Eupercaria) capturing 200-300 more loci on average than those designed for more ancient (~251-144 Ma) and/or taxonomically disparate clades (Elopomorpha and Backbones 1 and 2; Figure 5B). Estimates for the divergence times of major percomorph series vary, but the youngest estimates place their origin near the Cretaceous-Paleogene boundary, 66 million years ago (Alfaro *et al.* 2018). Conversely, the paraphyletic taxonomic groups spanned by Backbone 1 diverged in the Permian or Triassic, and the late Jurassic to early Cretaceous for Backbone 2 (Betancur-R. *et al.* 2013; Near *et al.* 2013a; Hughes *et al.* 2018). The larger number of nucleotide substitutions accumulated across older clades causes the probes to have less affinity for the targeted DNA regions *in vitro*, and we noticed a substantial increase in the number of loci captured for those clades younger than 100 million years (Figure 5B). One way to improve capture efficiency for particular projects would be to select more closely related taxa to design a new (more specific) probe set.

Despite the variation in the number of loci assembled, all samples with exon-capture data were resolved in their expected clades, and the ML topologies at major nodes matched that of the ML topologies of Hughes *et al.* (2018). One family, Clupeidae, was not monophyletic, but this result has been reported before in previous phylogenetic analyses (Betancur-R *et al.* 2017), and may

reflect the need of taxonomic revision or poor taxonomic sampling rather than underlying phylogenetic estimation error arising from the exon-capture data. These markers appear to be informative for deep divergences in fishes, and the backbone of the ray-finned fish tree largely matches inferences based on legacy gene markers (Near *et al.* 2012; Betancur-R. *et al.* 2013), though many areas of the tree have only been investigated with sparse taxonomic sampling and will require more thorough investigation with additional sequencing. While deep divergences are the focus of this paper, conserved exon markers have also been shown to contain information appropriate for shallower divergences at the phylogeographic level (Rincon-Sandoval *et al.* 2019). The flanking intron regions, which are highly variable, have been removed for the analyses presented here, but we include a branch of our bioinformatic pipeline to additionally use the flanking intron sequences for projects with a more recent evolutionary focus.

Exon markers can be integrated with existing and future datasets

Taxonomic sampling is critical for accurate phylogenetic analysis (Heath *et al.* 2008; Betancur-R. *et al.* 2019), and sequence capture methods are a cost-effective approach for increasing taxonomic sampling across a large number of loci (Lemmon & Lemmon 2013). But both whole-genome sequences (Malmstrøm *et al.* 2016; Musilova *et al.* 2019) and transcriptome sequences (e.g. Hughes *et al.* 2018; Dai *et al.* 2018) are becoming available for an increasing diversity of fish species. These exon markers can be easily mined from public transcriptome or genome data as they become available, increasing taxonomic sampling for the group of interest without duplicating sequencing effort. Taxonomically dense super-matrix approaches in fishes (e.g. Rabosky *et al.* 2018) primarily rely on exon sequences deposited in NCBI. Currently, there are more than 20,000 sequences of RAG1 for teleosts available in NCBI (as of March 20, 2019),

more than 35,000 teleost rhodopsin sequences, and even larger numbers for mitochondrial genes like CYTB (>130,000 sequences). This is a rich resource that can be combined with exon-capture data for the probe sets described here to reduce missing data that are often rampant in super matrix approaches but still produce taxonomically dense trees.

Paralogs in sequence capture datasets

Many nuclear exon fragments that have been in wide use in fish phylogenetics for more than a decade do not appear to have paralogs, and new sequence capture data could be easily integrated from genes like RAG1, RAG2, PANX2, MLL, VCPIP, GLYT, GCS1, and FICD. Many of these exons were defined as ‘single-copy’ based on the comparisons of the relatively few fish genomes available at the time (Li *et al.* 2007). But the specificity of primers designed for nested PCR approaches to amplify and sequence these loci has been a successful strategy to obtain orthologous genes for phylogenetic inference in fishes (Li *et al.* 2007, 2008, 2010; Near *et al.* 2012, 2013b; Wainwright *et al.* 2012; Betancur-R. *et al.* 2013). Shotgun sequencing of enriched libraries, in contrast, is a more challenging approach for assembling orthologous genes, since sequence reads of two or more paralogous copies may be sequenced by this approach and need to be separated using bioinformatic pipelines. Nineteen legacy markers included in our probe set previously had been excluded from downstream phylogenetic analyses due to paralogy issues detected either by comparing additional genomes or by topology tests of gene trees (Table 3). Due to high similarity in certain parts of the coding region to the reference coding sequence used in Exonerate, more than half of these assembled paralogous loci passed through to the alignment stage. Often it was only the paralog that was assembled, and the assembly of multiple contigs was not a reliable way to flag paralogy. The pipeline implemented here (Figure 4) attempts to

remove redundant contigs with CD-HIT at a 99% similarity threshold across the reading frame when multiple contigs assemble, but exons that fail this test are not passed onto the alignment stage. Paralogs of ENC1, MYH6, ZIC1 and other genes known to be duplicated (Table 3) passed on to the alignment stage, and do not appear to have assembled the orthologous sequence. However, a majority of the sequences assembled orthologous exons. With additional scrutiny for paralogs using gene trees, these data are still quite useful for integration with older datasets.

Acknowledgements

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Data Accessibility

Raw reads for newly sequenced exon-capture data are archived on NCBI under Bioproject number PRJNA605876. Newick files and phylogenomic matrices are available on FigShare (doi: 10.6084/m9.figshare.11844783), and pipeline scripts to analyze data and a tutorial are available on GitHub (<https://github.com/lilychughes/FishLifeExonCapture>). The protein tree topology will be made available on Open Tree of Life.

Author Contributions

L.C.H., R.B-R., G.O., K.A.C, C.L., and D.A. contributed to the design of the study. R.B-R., C.C.B., and W.W. provided tissues. L.C.H., H.S., C.L., and R.B-R. analyzed the data. L.C.H., G.O., and R.B-R. wrote the paper with input from all authors.

Table 1. Seven probe sets designed for 1,104 conserved exon markers across teleost fishes.

Probe Set Name	Lineages Included (Preferred lineage in bold)
Elopomorpha	1. Megalopidae 2. Muraenidae 3. Congridae , Chlopsidae 4. Anguillidae
Backbone 1	1. Osteoglossidae , Pantodontidae 2. Notopteridae , Mormyridae

	3. Engraulidae , Clupeidae 4. Galaxiidae 5. Argentinidae 6. Stomiidae , Osmeridae, Plecoglossidae, Salangidae 7. Synodontidae , Chlorophthalmidae 8. Myctophidae
Backbone 2	1. Zeidae , Parazenidae 2. Berycidae , Stephanoberycidae, Rondeletiidae 3. Holocentridae 4. Ophidiidae 5. Apogonidae 6. Gobiidae 7. Synbranchiiformes , Anabantiformes
Syngnatharia-Pelagiaria	1. Syngnathidae , Callionymidae 2. Mullidae , Aulostomidae 3. Scombridae 4. Nomeidae , Stromateidae
Carangaria	1. Coryphaenidae , Carangidae 2. Cynoglossidae , Paralichthyidae, Pleuronectidae, Scophthalmidae, Soleidae 3. Centropomidae 4. Polynemidae , Toxotidae
Ovalentaria	1. Pseudomugilidae , Melanotaeniidae, Atherinopsidae 2. Aplocheilidae , Nothobranchiidae, Rivulidae, Cyprinodontidae, Fundulidae, Poeciliidae 3. Tripterygiidae , Blenniidae, Chaenopsidae 4. Gobiesocidae 5. Pomacentridae
Eupercaria	1. Anoplopomatidae , Channichthyidae, Cottidae, Gasterosteidae, Nototheniidae, Bathydraconidae, Percidae, Sebastidae 2. Gerreidae , Labridae, Scaridae, Pinguipedidae, Lateolabracidae, Epigonidae 3. Tetraodontidae , Molidae, Chaunacidae, Caproidae, Diodontidae, Antennariidae, Balistidae, Acanthuridae 4. Lutjanidae , Haemulidae, Chaetodontidae, Moronidae, Datnioididae, Ephippidae, Sciaenidae

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634 **Table 2.** Species sequenced for each probe set and the number of reads and loci assembled.
635 Estimated clade ages (Hughes *et al.* 2018) are indicated.

Probe set	Family	Taxon	Collection No.	Paired-end Reads	Loci
Elopomorpha (196 Ma)	Albulidae	<i>Albula cf. vulpes</i>	^a USNM 421848	1,801,676	642
	Congridae	<i>Conger cinereus</i>	^b CSIRO GT7882	3,427,134	731
	Elopidae	<i>Elops hawaiiensis</i>	USNM 403422	3,020,903	759
	Halosauridae	<i>Halosaurus johnsonianus</i>	USNM 405058	2,034,292	606
	Halosauridae	<i>Halosaurus ovenii</i>	USNM 407039	4,743,893	719
	Nettastomatidae	<i>Nettastoma parviceps</i>	CSIRO GT6156	1,127,769	631
	Ophichthidae	<i>Myrophis microchir</i>	USNM 435225	1,362,661	586

	Synphobranchidae	<i>Meadia roseni</i>	CSIRO GT6877	2,734,565	735
Backbone1 (251 Ma)	Clupeidae	<i>Jenkinsia majua</i>	^c UPR FL0045	5,391,027	407
	Gonostomatidae	<i>Diplophos taenia</i>	USNM 405007	7,017,309	703
	Myctophidae	<i>Myctophum nitidulum</i>	USNM 405014	1,913,709	607
	Neoscopelidae	<i>Neoscopelus microchir</i>	USNM 407030	2,050,980	668
	Osteoglossidae	<i>Arapiama gigas</i>	USNM 440586	3,024,165	742
	Sternoptychidae	<i>Argyripnus atlanticus</i>	USNM 405229	2,328,143	651
	Stomiidae	<i>Chauliodus sloani</i>	USNM 405061	3,058,984	377
	Synodontidae	<i>Saurida gracilis</i>	^d STRI BFT11840	2,623,498	600
Backbone2 (144 Ma)	Apogonidae	<i>Apogon robbinsi</i>	UPR FL0162	818,383	787
	Batrachoididae	<i>Opsanus tau</i>	STRI BFT09764	924,435	633
	Eleotridae	<i>Dormitator latifrons</i>	STRI BFT02768	1,584,877	671
	Gobiidae	<i>Ctenogobius sagittula</i>	STRI BFT18404	1,525,545	467
	Gobiidae	<i>Ginsburgellus novemlineatus</i>	UPR FL0141	1,265,097	597
	Grammicolepididae	<i>Xenolepidichthys dalgleishi</i>	USNM 407099	3,208,789	788
	Holocentridae	<i>Plectrypops retrospinis</i>	UPR FL0166/ MZUPRRP-I-00357	3,237,186	920
	Synbranchidae	<i>Synbranchus marmoratus</i>	STRI BFT05012	1,762,538	681
Carangaria (65 Ma)	Achiridae	<i>Trinectes inscriptus</i>	USNM 414275	979,351	988
	Carangidae	<i>Carangoides armatus</i>	USNM 435427	1,983,024	1034
	Cynoglossidae	<i>Cynoglossus maculipinnis</i>	USNM 437669	1,387,678	868
	Echeneidae	<i>Remora remora</i>	USNM 405009	1,385,700	994
	Pleuronectidae	<i>Microstomus kitt</i>	USNM T5415	597,910	927
	Rachycentridae	<i>Rachycentron canadum</i>	USNM T3521	1,428,271	1026
	Samaridae	<i>Samariscus triocellatus</i>	USNM 391219	216,271	637
	Sphyraenidae	<i>Inegocia japonica</i>	USNM T10332	2,968,482	1012
Ovalentaria (95 Ma)	Ambassidae	<i>Ambassis nalua</i>	USNM 403430	2,666,551	1017
	Atherinidae	<i>Hypoatherina panatela</i>	USNM 437959	945,584	878
	Blenniidae	<i>Exallias brevis</i>	USNM 390993	2,475,300	920
	Opistognathidae	<i>Opistognathus castelnaui</i>	USNM 435841	1,117,986	840
	Plesiopidae	<i>Belonepterygion fasciolatum</i>	USNM 432574	1,453,409	956
	Poeciliidae	<i>Phallichthys fairweatheri</i>	STRI BFT06906	1,984,926	911
	Pomacentridae	<i>Microspathodon chrysurus</i>	STRI BFT13594	2,156,316	985
	Zenarchopteridae	<i>Zenarchopterus dispar</i>	STRI BFT07992	1,744,848	916
Eupercaria (105 Ma)	Acanthuridae	<i>Acanthurus mata</i>	USNM 403159	2,439,554	1005
	Gerreidae	<i>Eucinostomus lefroyi</i>	UPR FL0004 / MZUPRRP-I-	2,257,350	994

	Lutjanidae	<i>Gymnocaesio gymnoptera</i>	00223 USNM 435461	1,944,110	953
	Monacanthidae	<i>Cantherhines pardalis</i>	USNM 435717	3,649,643	788
	Ogcocephalidae	<i>Halieutichthys aculeatus</i>	USNM 433145	930,425	765
	Sciaenidae	<i>Pareques acuminatus</i>	UPR FL0151/ MZUPRRP-I-00347	1,553,389	948
	Serranidae	<i>Hypoplectrus nigricans</i>	UPR FL0324/ MZUPRRP-I-00497	1,632,142	943
	Sparidae	<i>Calamus penna</i>	UPR FL0063/ MZUPRRP-I-00281	3,576,529	929
SynPela (96 Ma)	Bramidae	<i>Brama orcini</i>	USNM 403327	2,359,571	1027
	Centriscidae	<i>Macroramphosus scolopax</i>	USNM 405231	2,823,407	1034
	Chiasmodontidae	<i>Kali macrura</i>	USNM T2229	643,335	973
	Gempylidae	<i>Lepidocybium flavobrunneum</i>	USNM 407069	2,559,203	1065
	Mullidae	<i>Upeneus tragula</i>	USNM 403208	3,042,224	918
	Scombridae	<i>Rastrelliger brachysoma</i>	USNM 409000	2,423,976	1016
	Stromateidae	<i>Peprilus snyderi</i>	USNM 421333	1,581,129	1044
	Syngnathidae	<i>Syngnathus pelagicus</i>	USNM 423115	1,244,405	867

^aUnited States National Museum (Smithsonian Institution), Washington, DC; ^bCommonwealth Scientific and Industrial Research Organisation, Tasmania, Australia; ^cZoological Museum at University of Puerto Rico-Rio Piedras, San Juan, PR. Specimens without a MZUPRRP number have no voucher due to their small size, only a field number beginning with FL; ^dSmithsonian Tropical Research Institute, Panama.

Table 3. Legacy markers and their paralogs assembled from exon-capture data.

Locus ID	Gene Name	Paralogs Assembled
E1541	TBR1	-
E1728	KIAA1239	-
E1730	MYH6	MYH7, MYH6-like, MYHb
E1732	ENC1	ENC2
E1735	PLAGL2	PLAGL1
E1736	PTCHD1	(Failed to assemble)
E1737	RIPK4	-
E1738	SH3PX3 (SNX33)	SNX18
E1739	SIDKEY	-
E1740	SREB2 (GPR85)	GPR173
E1741	ZIC1	ZIC4
E1746	SVEP1	-
E1747	GPR61	-
E1748	IRBP	-
E1749	RNF213	RNF213b-like, RNF213a-like
E1750	RH	EXORH

E1751	SLC10A3	-
E1752	UBEA3	-
E1753	UBEA3-like	UBEA3

Figure Legends

Figure 1: Position of Elopomorpha and Backbone 1 probe sets in the Maximum likelihood analysis of protein from 394 fish taxa, combining genomes, transcriptomes, and exon capture. Newly sequenced taxa are represented with black dots at the tips. This clade has a specific probe set with a different but overlapping set of exons designed in an earlier study, Arcila *et al.* (2017), but is not specifically targeted by Backbone 1.

Figure 2: Position of Backbone 2 and Ovalentaria probe sets in the Maximum likelihood analysis of 394 fish taxa, combining genomes, transcriptomes, and exon capture. Newly sequenced taxa are represented with black dots at the tips.

Figure 3: Position of Carangaria, Syngnatharia-Pelagiaria (Syn-Pela) and Eupercaria probe sets in the Maximum Likelihood analysis of 394 fish taxa, combining genomes, transcriptomes, and exon capture. Newly sequenced taxa are represented with black dots.

Figure 4: Major steps of bioinformatics pipeline and software used to transform data from Illumina reads to multiple sequence alignments. Scripts and pipeline tutorial are available on GitHub (<https://github.com/lilychughes/FishLifeExonCapture>).

Figure 5: A) Box-and-whisker plots showing the variation in the number of exons captured for each probe set. Eight newly sequenced taxa were used for each probe set. Percentages above each box refer to the average percentage of exons assembled by probe set. B) The average number of loci captured for each of the seven probe sets plotted against the age of the most recent common ancestor of that particular group, showing higher capture of loci for younger clades. C) Percent sequence identity and length across alignments of eight model genomes (*L. oculatus*, *A. japonica*, *D. rerio*, *G. morhua*, *O. latipes*, *O. niloticus*, *G. aculeatus*, and *T. nigroviridis*) for exons presented in this study (pink circles) compared to legacy markers (blue triangles).

CAR.

OVALENTARIA

EUPERCARIA

S-P

BACKBONE 1

OTOPHYSA

BACKBONE 2

Teleostei

BACKBONE 1

ELO.

Acanthomorphata

Percomorphaceae

CAR.

OVALENTARIA

EUPERCARIA

S-P

SYN-PELA

CARANGARIA

EUPERCARIA

BACKBONE 2

Kali macrura
Brama orcin
Lepidocybium flavobrunneum
Nomeus gronovii
Pampus argenteus
Peprilus snyderi
Rastrelliger brachysoma
Scomber scombrus
Scomberomorus regalis
Thunnus thynnus
Thunnus albacares
Thunnus orientalis

Macroramphosus scolopax
Aulostomus maculatus
Aulostomus maculatus
Parupeneus indicus
Upeneus tragula
Foetorepus agassizii
Syngnathoides biaculeatus
Hippocampus comes
Hippocampus erectus
Syngnathus pelagicus
Syngnathus scovelli

Centropomus undecimalis
Remora remora
Rachycentron canadum
Coryphaena hippurus
Trachinotus ovatus
Caranx ignobilis
Carangoides armatus
Selene dorsalis
Seriola dumerili
Seriola rivoliana
Seriola quinqueradiata
Seriola lalandi
Polynemus dubius
Toxotes jaculatrix
Trinectes inscriptus
Samariscus triocellatus
Solea ovata
Cynoglossus maculipinnis
Cynoglossus semilaevis
Scophthalmus maximus
Paralichthys olivaceus
Hippoglossus hippoglossus
Microstomus kitt
Pseudopleuronectes yokohamae

Hypoplectrus nigricans
Chironema squamentum
Perca fluviatilis
Lepidonotothen nudifrons
Notothenia coriiceps
Gymnodraco auliceps
Chaenoccephalus aceratus
Peristedion brevisrostris
Anoplopoma fimbria
Gasterosteus aculeatus
Pungitius pungitius
Cyclopterus lumpus
Cottus rhenanus
Myoxocephalus scorpius
Scorpaenopsis cirrosa
Synanceia verrucosa
Dendrochirus zebra
Pontinus castor
Sebastes nigrocinctus
Sebastes rubrivinctus
Sebastes aleutianus
Sebastes norvegicus
Sebastes minor
Sebastes steindachneri
Epigonus sp.
Lateolabrax maculatus
Gerres filamentosus
Eucinostomus lefroyi
Eucinostomus jonesi
Parapercis xanthozona
Thalassoma bifasciatum
Labrus bergylla
Symphodus melops
Scarus ghobban
Scarus iseri
Terapon jarbua
Oplegnathus fasciatus
Oplegnathus punctatus
Amblycirrhitus pinos
Maccullochella peelii
Micropterus flordianus
Coreoperca whiteheadi
Siniperca scherzeri
Dicentrarchus labrax
Morone chrysops
Morone saxatilis
Drepane punctata
Chaetodipterus faber
Datnioides microlepis
Haemulon chrysargyreum
Haemulon flavolineatum
Pareques acuminatus
Equetus punctatus
Larimichthys crocea
Milichthys miluy
Acanthurus mata
Acanthurus tractus
Pomacanthus paru
Chaetodon auriga
Lutjanus sebae
Gymnoaesio gymnoptera
Lutjanus fulviflamma
Pagrus major
Eyynnus cardinalis
Calamus penna
Spondyllosoma cantharus
Sparus aurata
Acanthopagrus latus
Acanthopagrus schlegelii
Siganus guttatus
Antigonia capros
Priacanthus tayenus
Chaunax pictus
Antennarius striatus
Haliutichthys aculeatus
Lactoria cornuta
Ostracion rhinorhynchus
Cantherhines pardalis
Pseudobalistes fuscus
Mola mola
Diodon holacanthus
Tetraodon nigroviridis
Takifugu flavidus
Takifugu rubripes

Quality trim Reads

Trimmomatic

Map reads to reference
sequences

BWA, Samtools

Assemble reads by
locus & extract longest
contig

Velvet, getLongest.py

Extend initial contigs
into longer contigs

aTRAM (BLAST, Trinity)

Remove redundant
contigs & find reading
frame

CD-Hit, Exonerate, FilterExons.py

Align sequences

MACSE