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Gene trees, species trees, and morphology converge on a similar phylogeny of living gars (Actinopterygii: Holostei: Lepisosteidae), an ancient clade of ray-finned fishes

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ABSTRACT

Extant gars represent the remaining members of a formerly diverse assemblage of ancient ray-finned fishes and have been the subject of multiple phylogenetic analyses using morphological data. Here, we present the first hypothesis of phylogenetic relationships among living gar species based on molecular data, through the examination of gene tree heterogeneity and coalescent species tree analyses of a portion of one mitochondrial (*COI*) and seven nuclear (*ENC1*, *myh6*, *plagl2*, *S7* ribosomal protein intron 1, *sreb2*, *tbr1*, and *zic1*) genes. Individual gene trees displayed varying degrees of resolution with regards to species-level relationships, and the gene trees inferred from *COI* and the *S7* intron were the only two that were completely resolved. Coalescent species tree analyses of nuclear genes resulted in a well-resolved and strongly supported phylogenetic tree of living gar species, for which Bayesian posterior node support was further improved by the inclusion of the mitochondrial gene. Species-level relationships among gars inferred from our molecular data set were highly congruent with previously published morphological phylogenies, with the exception of the placement of two species, *Lepisosteus osseus* and *L. platostomus*. Re-examination of the character coding used by previous authors provided partial resolution of this topological discordance, resulting in broad concordance in the phylogenies inferred from individual genes, the coalescent species tree analysis, and morphology. The completely resolved phylogeny inferred from the molecular data set with strong Bayesian posterior support at all nodes provided insights into the potential for introgressive hybridization and patterns of allopatric speciation in the evolutionary history of living gars, as well as a solid foundation for future examinations of functional diversification and evolutionary stasis in a "living fossil" lineage.

1. Introduction

Living fossils are species belonging to ancient lineages from which most species are now extinct, and which have undergone relatively little evolutionary change (Gould, 2002, p. 815). These lineages offer opportunities to glimpse morphologies that were more common in time periods vastly predating the present, and the relative lack of morphological divergence in living fossil lineages over long evolutionary time scales presents an interesting challenge to ideas regarding the phenotypic changes due to natural selection that form one of the cornerstones of modern evolutionary theory (Gould and Eldredge, 1977; Avise et al., 1994). Living fossil lineages are particularly well represented among among non-tetrapod vertebrates, or "fishes" (Nelson, 2006), a fact that is not terribly surprising when viewed in the context of their deep-time evolutionary history and current species diversity. Fossils identifiable as "fishes" date to the Cambrian (Shu et al., 1999, 2003) and extant species diversity of ray-finned fishes represent approximately half of the planet's current vertebrate species diversity (Stiassny et al., 2004). With such a long time span in which to diversify and the great extent to which they have done so, chance alone would dictate that many present day fish species should represent the remnants of formerly species-rich radiations (Stanley, 1979).

Investigations into the evolutionary origin and diversification of ancient "fishes" cannot proceed without the presence of well-supported phylogenies of extant species. Molecular phylogenetic studies have recently been undertaken for nearly all of the best known lineages of piscine living fossils, including hagfishes (Myxini: Myxiniformes) (Kuo et al., 2003), bichirs and ropefish (Actinopterygii: Polypteriformes) (Suzuki et al., 2010), sturgeons (Actinopterygii: Acipenseriformes) (Birstein and DeSalle, 1998;

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Birstein et al., 2002), bonytongues (Actinopterygii: Osteoglossiformes) (Kumazawa and Nishida, 2000; Lavoué and Sullivan, 2004), and lungfishes (Sarcopterygii: Dipnoi; Zardoya and Meyer, 1996). Coelacanths (Sarcopterygii: Coelacanthiformes) and bowfin (Actinopterygii: Amiidae) are not included in this list because they contain only two and one extant species, respectively, but they have nonetheless been the focus of studies incorporating molecular data to examine aspects of their evolutionary history (Holder et al., 1999; Venkatesh et al., 2001; Sudarto et al., 2010). Conspicuous in their absence from this list are the gars (Actinopterygii: Lepisosteiformes), which have been included in molecular studies to determine their phylogenetic relationships within actinopterygians (Normark et al., 1991; Lê et al., 1993; Venkatesh et al., 2001; Inoue et al., 2003; Kikugawa et al., 2004) and to examine genetic variation within single species (Barrientos-Villalobos and Monteros, 2008; Moyer et al., 2009), but which currently lack phylogenetic hypotheses for all extant species based on comparative DNA sequence data.

Extant gars (Family Lepisosteidae) comprise seven species classified in two genera, *Lepisosteus* and *Atractosteus* (Fig. 1). These fishes are easily recognized by their elongated body and jaws (filled with many needle-like teeth), heterocercal tails, ganoid scales, and posteriorly positioned dorsal and anal fins, along with many other internal, mainly osteological, diagnostic characters (Wiley, 1976; Grande, 2010). Both *Lepisosteus* and *Atractosteus* contain large, mainly piscivorous species that inhabit the freshwaters of central and eastern North America, Cuba and Central America, with some species occasionally venturing into brackish or marine habitats. In addition to these extant species, the Lepisosteidae contains a number of fossil species and genera (e.g., †*Cuneatus*, †*Masillosteus*), with the oldest fossil material assignable to *Lepisosteus* dating to the late Cretaceous, approximately 75 million years ago (Mya). The oldest known fossil *Atractosteus* species is †*A. falipoui*, which dates to the early/late Cretaceous, approximately 100 Mya (Grande, 2010). Lepisosteidae is itself contained within Lepisosteiformes, which also includes the extinct "spiny gars" (Family †Obaichthyidae) (Grande, 2010). When lepisosteid fossils are considered (including those which belong to indeterminate genera and species), the historical geographic distribution of the clade is significantly expanded to include Europe, the Middle East and Central Asia, northern and central Africa, Madagascar, South America, and west and southwestern North America (Wiley, 1976; Grande, 2010).

In contrast to the dearth of molecular phylogenetic analyses of living gars, there have been several studies utilizing morphological characters to infer their species-level relationships (Fig. 2A and B; Suttkus, 1963; Wiley, 1976; Grande, 2010). These investigations all resulted in highly similar hypotheses regarding the relationships of extant gars, universally resolving *Atractosteus* and *Lepisosteus* each as monophyletic, as well as agreeing on the interrelationships of the three species of *Atractosteus*, and a sister species relationship between *L. oculatus* and *L. platyrhincus* (Fig. 2A and B). The only difference between the results of any of these studies involves the phylogenetic placement of *L. osseus* and *L. platostomus* (Fig. 2A and B), with the two explicit phylogenetic studies agreeing on the phylogenetic relationships of these two species (Wiley, 1976; Grande, 2010).

These previous morphological phylogenetic studies were able to incorporate fossil taxa in cases where material was complete enough to allow evaluation of pertinent characters (Figs. 2C and D). However, in the case of groups that exhibit low rates of phenotypic evolution, efforts using morphological characters to reconstruct phylogenetic relationships would be expected to suffer from a relative lack of character state changes with which to infer well-resolved phylogenetic hypotheses, although the argument could conceivably be made that in the face of such stasis, any putatively synapomorphic character state transformations would offer relatively stronger support for the relationships of the species sharing them due to a reduced frequency of homoplasy. An example is clearly seen in Grande's (2010) recent monograph on extant and fossil gars, where the phylogenetic relationships of fossil *Atractosteus* and *Lepisosteus* species were largely unresolved, and each of the nodes within *Lepisosteus*, which included both fossil and extant species, were supported with a single character state change. While there is little hope for resolving the relationships of these

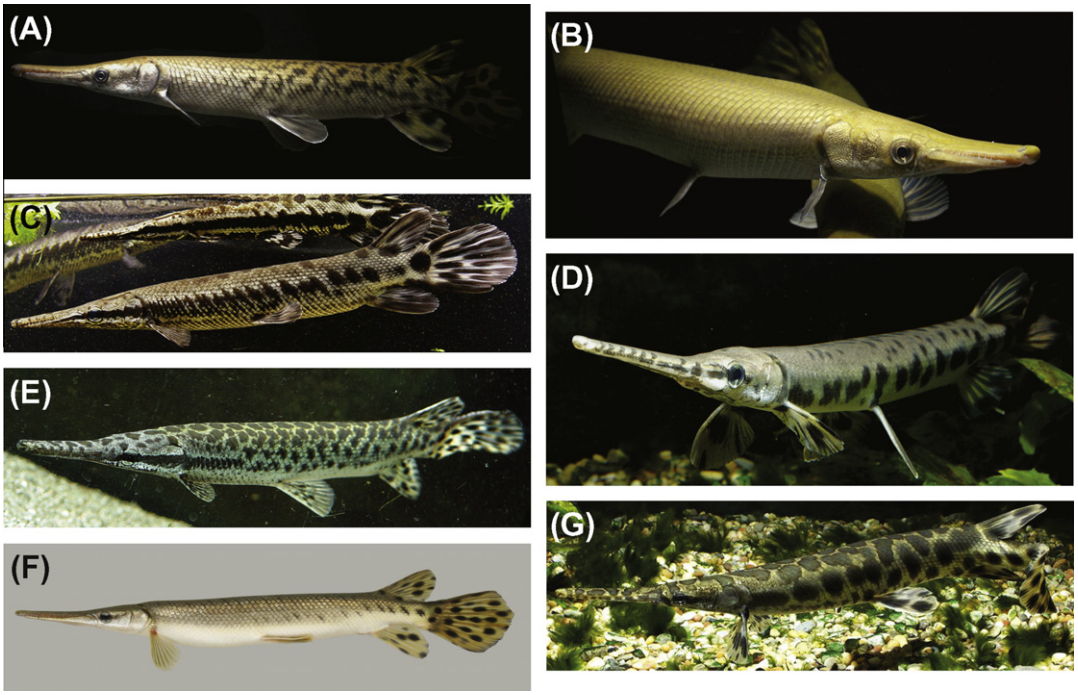

Fig. 1. The seven living gar species examined in this study. (A) *Atractosteus spatula* (Alligator gar). (B) *A. tristoechus* (Cuban gar). (C) *A. tropicus* (Tropical gar). (D) *Lepisosteus osseus* (Longnose gar). (E) *L. platyrhincus* (Florida gar). (F) *L. platostomus* (Shortnose gar). (G) *L. oculatus* (Spotted gar).

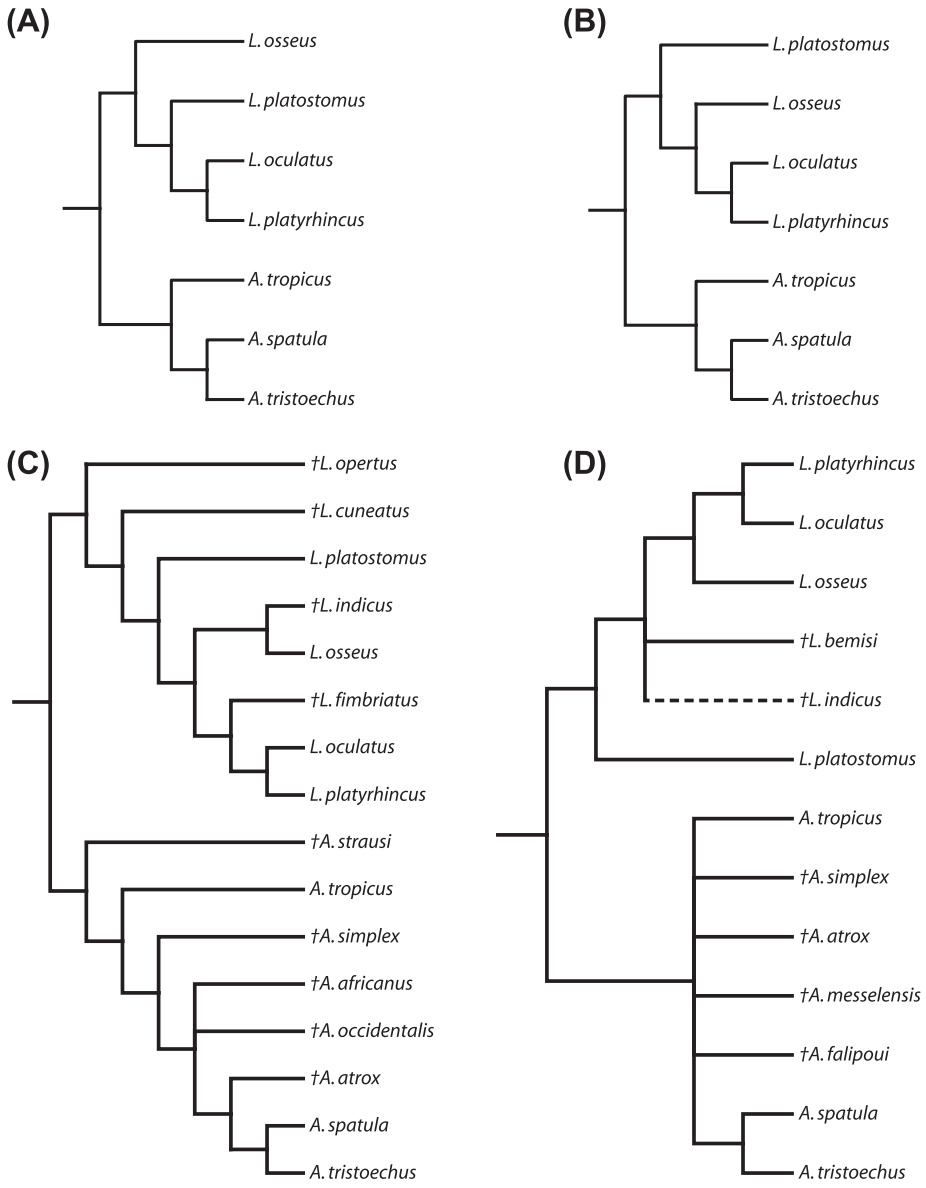

Fig. 2. Previously published gar phylogenies based on morphological data. (A) Phylogeny of living gars only, according to Suttkus (1963). (B) Phylogeny of living gars as determined by Wiley (1976) and Grande (2010). (C) Phylogeny of the Lepisosteidae (including fossil taxa) according to Wiley (1976). (D) Phylogeny of the Lepisosteidae (including fossil taxa) according to Grande (2010).

fossil gar species, barring re-evaluation by future morphological analyses that may include more complete material, additional support for the phylogenetic hypotheses of extant gar species relationships, particularly for those nodes supported by a single putative synapomorphy, is desirable.

Molecular data have the potential to provide this support by offering a much larger pool of characters from which to draw, one that increases rapidly as additional loci are sampled. In many cases the trees resulting from analyses of these data represent hypotheses of species' relationships that are incongruent with previous morphological studies, with abundant examples coming from varied lineages of ray-finned fishes (e.g., Near et al., 2000; Moyer et al., 2004; Miya et al., 2010), as well as a broad range of lineages across the Tree of Life (e.g., Poe, 1996; Graham et al., 1998; Williams et al., 2003). Such disagreement between data sets is not limited to comparisons of morphological and molecular phylogenies. Phylogenies based on individual nuclear (nucDNA) and mitochondrial genes (mtDNA) can differ from one another, as well as the overall species tree for a number of reasons, including incomplete lineage sorting, saturation of nucleotide substitutions, nonstationarity of base composition, skewed substitution rates, horizontal gene transfer, and introgressive hybridization. The expected incongruence among individual gene trees due to different coalescent histories has led to the development of methods that aim to estimate the "species tree" from a set of gene trees estimated from individual gene region (Edwards, 2009). Instances of such phylogenetic incongruence among gene trees are well represented in ray-finned fishes (e.g., Hardman and Page, 2003; Schelly et al., 2006; Egger et al., 2007), with hybridization being of particular interest as a possible source of hybrid recombinant (homoploid) speciation in a few select lineages (e.g., Dowling and Demarais, 1993; Nolte et al., 2005; Meyer et al., 2006); however, these burgeoning species methods have seen little application to questions of ray-finned fish phylogenetics (Keck and Near, 2010; Hollingsworth and Hulsey, 2011; Hulsey et al., 2011).

Here, we present the first set of molecular phylogenetic analyses to infer the relationships of all seven extant gar species. Our datasets comprise DNA sequences from a single mitochondrial

gene and seven nuclear genes. In addition to comparing the gene trees inferred from the mitochondrial and nuclear datasets for discordance, we present a coalescent based species tree analysis that infers the containing phylogeny of gar species relative to the distribution of gene tree topologies and coalescent depths. The molecular phylogenetic inferences are compared to a recently published phylogeny of gars inferred from 105 discretely coded morphological character state transitions (Grande, 2010). In cases where incongruence between molecular and morphological topologies are observed, we identify possible strategies by which such differences are reconciled, thus establishing a strongly supported phylogenetic framework that will serve as the basis for future studies investigating the evolutionary biology of this distinctive lineage of ancient ray-finned fishes.

2. Materials and methods

2.1. Specimen acquisition

Live gars were captured using electrofishing, gill and dip nets, and hook and line fishing, with additional specimens being acquired through aquaculture facilities and the aquarium trade (see Table 1). In some cases, small tissue samples were taken from living fish and preserved in 95% ethanol, while the fish were then used in other studies or released alive. In these cases, photographic vouchers of the specimens were acquired (voucher photographs available upon request to corresponding author). The remaining individuals were euthanized using either an overdose of Tricaine methanesulfonate (MS-222) or clove oil according to a protocol (UCUCA 10228-1) approved by the University of Michigan Committee on Use and Care of Animals. Samples of fin tissue were preserved in 95% ethanol for use in DNA preparations. Ethanol preserved tissues were deposited in the Yale Fish Tissue Collection (YFTC; Table 1). The remaining bodily specimens were deposited in the fish collection of the University of Michigan Museum of Zoology (UMMZ) after fixation in a 10% formalin solution, followed by transfer to 70% ethanol.

2.2. DNA isolation, PCR, and DNA sequencing

Frozen or ethanol preserved tissues were used to extract DNA using standard phenol–chloroform extraction with ethanol precipitation protocols or Qiagen DNAeasy Tissue Extraction Kits (QIAGEN, Valencia, CA). A portion of the mitochondrial gene *COI* was PCR amplified using standard barcoding primer sequences and an annealing temperature of 50 °C in thermal cycling. Seven nuclear genes (*ENC1*, *myh6*, *plagl2*, *S7* ribosomal protein intron 1, *sreb2*, *tbr1*, and *zic1*) were amplified using PCR with primers and cycling conditions reported in previous studies (Chow and Hazama, 1998; Li et al., 2007). Six of the seven sampled nuclear genes were exon regions from protein coding genes. Amplified PCR products were cleaned using a Qiagen Qiaquick PCR Purification Kit or with enzymatic purification using exonuclease 1 and shrimp alkaline phosphatase that was incubated at 37 °C for 15 min followed by 80 °C to inactivate the enzymes.

Purified PCR products were used as templates for Big Dye (Applied Biosystems, Foster City, CA) cycle sequencing. Sequencing reactions were visualized on an ABI 3100 automated sequencer at the Molecular Systematics and Conservation Genetics Laboratory at Science Hill (Yale University, New Haven, CT). In most cases the primers used for PCR were also used in the sequencing reactions. The computer program Sequencher (GeneCodes, Ann Arbor, MI) was used to build contiguous sequences from the individual DNA sequencing chromatograms. The seven protein coding genes, *COI*, *ENC1*, *myh6*, *plagl2*, *sreb2*, *tbr1*, and *zic1* were aligned by eye using the inferred amino acid sequences as a guide. The *S7* intron was aligned using the computer program Muscle version 3.6 (Edgar, 2004).

2.3. Phylogenetic and multi-species coalescent species tree analyses

All phylogenetic analyses of the individual genes used DNA sequences of *Amia calva* as the outgroup, except the *S7* intron locus because the gene sequences from gars were too divergent to align reliably with those sampled from *A. calva*. The optimal molecular evolutionary model for each gene was determined through model fitting and using the Akaike Information Criterion (AIC) as executed in the computer program MrModeltest 2.3 (Nylander, 2004). Optimal molecular evolutionary models were set in Bayesian phylogenetic analyses of each gene and were used when performing the multi-species coalescent species tree analysis.

The posterior set of gene trees was inferred from each of the eight sampled loci using a parallel version of the computer program MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003; Altekar et al., 2004) on a Linux cluster. For each analysis MrBayes was run three separate times for 5.0×10^7 generations with two simultaneous runs each with four chains (one cold and three heated chains with a heating parameter = 0.02 to ensure appropriate mixing). The cold chain was sampled once every 5000 generations. An additional analysis of a concatenated data set containing all sampled genes was performed (1.0×10^7 generations), with the respective optimal evolutionary models determined by MrModelTest being applied to each gene. Stationarity of the chains and convergence of the Metropolis-coupled Markov chain Monte Carlo algorithm were assessed by plotting the likelihood score and all other parameter values against the generation number to determine when there was no increase relative to the generation number using the computer program Tracer version 1.5 (Rambaut and Drummond, 2009). Measuring the average standard deviation of the split frequencies between those runs also assessed convergence; it was assumed that the chains had reached stationarity when this value was less than 0.005. The first 30% of the sampled generations were discarded as burn in and the set of posterior phylogenies were summarized in 50% majority-rule consensus trees. The posterior probability for a given clade was the frequency that the clade was present among the posterior trees, which translates to the probability that the lineage is monophyletic given the model and the data (Larget and Simon, 1999; Huelsenbeck and Rannala, 2004). We followed the standard practice in Bayesian phylogenetics of interpreting a given node in the summarized posterior phylogeny as strongly supported if the clade is present in 95% or greater in the posterior distribution of trees (e.g., Alfaro et al., 2003).

The computer program ∗BEAST version 1.6.1 (Drummond and Rambaut, 2007) was used to perform a set of multispecies coalescent analyses to estimate a species tree for the seven extant gar species (Heled and Drummond, 2010). Two different species tree analyses were performed, one that included all sampled loci and the other that was limited to the seven sampled nuclear genes. Table 1 shows the number of gene copies sampled for each locus. All loci were sampled with at least two copies, except *Atractosteus spatula*, where only one specimen was sequenced for the *S7* intron. Because extinction is documented in Lepisosteidae, as both Wiley (1976) and Grande (2010) have shown that fossil species of both *Lepisosteus* and *Atractosteus* are phylogenetically nested in the gar crown clade, a birth–death speciation branching prior was used for the species tree inference. The uncorrelated log normal model of molecular evolutionary rate heterogeneity was used for all loci and the molecular evolutionary rate for each locus was scaled to the gene with the highest rate (Drummond et al., 2006), the mtDNA encoded *COI* in the all locus analysis and the *S7* intron in

Table 1
Specimens sampled, geographic locality, Yale Fish Tissue Collection (YFTC) numbers, and Genbank accession numbers for each sampled locus. NA indicates specimens not sequenced for a particular locus.

Species	Locality	YFTC	*COI*	*ENC1*	*myh6*	*plagl2*	*S7*	*sreb2*	*tbr1*	*zic1*
Atractosteus spatula	Mississippi River, Missouri, USA	11565	JN853324	JN853362	JN853399	JN853567	NA	JN853507	JN853433	JN853469
Atractosteus spatula	Aquarium trade	21063	JN853325	JN853363	JN853400	JN853568	NA	JN853508	JN853434	JN853470
Atractosteus spatula	Aquarium trade	21064	JN853326	JN853364	JN853401	JN853569	JN853537	JN853509	JN853435	NA
Atractosteus spatula	Barataria Estuary, Louisiana, USA	21065	JN853327	JN853365	JN853402	JN853570	NA	JN853510	NA	JN853471
Atractosteus spatula	Barataria Estuary, Louisiana, USA	21066	JN853328	JN853366	JN853403	JN853571	NA	JN853511	NA	JN853472
Atractosteus tristoechus	Zapata Swamp, Center for Native Ichthyofauna Reproduction, Ciénega de Zapata, Cuba	21067	JN853329	JN853367	JN853404	JN853572	JN853538	JN853512	JN853436	JN853473
Atractosteus tristoechus	Zapata Swamp, Center for Native Ichthyofauna Reproduction, Ciénega de Zapata, Cuba	21068	JN853330	JN853368	JN853405	JN853573	JN853539	JN853513	JN853437	JN853474
Atractosteus tristoechus	Zapata Swamp, Center for Native Ichthyofauna Reproduction, Ciénega de Zapata, Cuba	21069	JN853331	JN853369	JN853406	JN853574	JN853540	JN853514	JN853438	JN853475
Atractosteus tropicus	Otat-Ibam Aquaculture farm, Tobasco, Mexico	21070	JN853332	JN853370	JN853407	JN853575	JN853541	JN853515	JN853439	JN853476
Atractosteus tropicus	Otat-Ibam Aquaculture farm, Tobasco, Mexico	21071	JN853333	JN853371	JN853408	JN853576	JN853542	JN853516	JN853440	JN853477
Atractosteus tropicus	Otat-Ibam Aquaculture farm, Tobasco, Mexico	21072	JN853334	JN853372	JN853409	JN853577	JN853543	JN853517	JN853441	JN853478
Atractosteus tropicus	Otat-Ibam Aquaculture farm, Tobasco, Mexico	21073	JN853335	JN853373	JN853410	JN853578	JN853544	JN853518	JN853442	JN853479
Lepisosteus platyrhincus	Lake Washington, Florida, USA	11453	JN853319	JN853357	JN853394	JN853562	JN853534	JN853502	JN853428	JN853464
Lepisosteus platyrhincus	Lake Okeechobee, Florida, USA	21059	JN853320	JN853358	JN853395	JN853563	JN853535	JN853503	JN853429	JN853465
Lepisosteus platyrhincus	Caloosahatchie River, Ft. Meyers, Florida, USA	21060	JN853321	JN853359	JN853396	JN853564	JN853536	JN853504	JN853430	JN853466
Lepisosteus platyrhincus	Everglades, Florida, USA	21061	JN853322	JN853360	JN853397	JN853565	NA	JN853505	JN853431	JN853467
Lepisosteus platyrhincus	Everglades, Florida, USA	21062	JN853323	JN853361	JN853398	JN853566	NA	JN853506	JN853432	JN853468
Lepisosteus oculatus	Big Sandy River, Tennessee, USA	2701	JN853310	JN853348	JN853385	JN853555	NA	JN853493	JN853422	JN853455
Lepisosteus oculatus	Loon Lake, Michigan, USA	21051	JN853311	JN853349	JN853386	NA	NA	JN853494	NA	JN853456
Lepisosteus oculatus	Lake Pleasant, Michigan, USA	21052	JN853312	JN853350	JN853387	NA	NA	JN853495	NA	JN853457
Lepisosteus oculatus	Barataria Estuary, Louisiana, USA	21053	JN853313	JN853351	JN853388	JN853556	JN853528	JN853496	JN853423	JN853458
Lepisosteus oculatus	Barataria Estuary, Louisiana, USA	21054	JN853314	JN853352	JN853389	JN853557	JN853529	JN853497	JN853424	JN853459
Lepisosteus oculatus	Choke Canyon Reservoir, Texas, USA	21055	JN853315	JN853353	JN853390	JN853558	JN853530	JN853498	JN853425	JN853460
Lepisosteus oculatus	Choke Canyon Reservoir, Texas, USA	21056	JN853316	JN853354	JN853391	JN853559	JN853531	JN853499	JN853426	JN853461
Lepisosteus oculatus	Horseshoe Lake, Illinois, USA	21057	JN853317	JN853355	JN853392	JN853560	JN853532	JN853500	NA	JN853462
Lepisosteus oculatus	Horseshoe Lake, Illinois, USA	21058	JN853318	JN853356	JN853393	JN853561	JN853533	JN853501	JN853427	JN853463
Lepisosteus osseus	Little Wabash River, Illinois, USA	1347	NA	JN853337	JN853375	NA	NA	JN853481	JN853412	JN853444
Lepisosteus osseus	Muddy Boggy Creek, Oklahoma, USA	2823	JN853300	JN853336	JN853374	JN853546	NA	JN853480	JN853411	JN853443
Lepisosteus osseus	Green River, Kentucky, USA	10072	JN853305	JN853342	JN853380	NA	JN853522	JN853486	JN853417	JN853449
Lepisosteus osseus	Illinois River, Illinois, USA	21043	JN853301	JN853338	JN853376	JN853547	JN853519	JN853482	JN853413	JN853445
Lepisosteus osseus	Muskegon River, Michigan, USA	21044	JN853302	JN853339	JN853377	JN853548	JN853520	JN853483	JN853414	JN853446
Lepisosteus osseus	Muskegon River, Michigan, USA	21045	JN853303	JN853340	JN853378	JN853549	JN853521	JN853484	JN853415	JN853447
Lepisosteus osseus	Huron River, Michigan, USA	21046	JN853304	JN853341	JN853379	JN853550	JN853522	JN853485	JN853416	JN853448
Lepisosteus platostomus	Mississippi River near Cassville, Wisconsin, USA	21047	JN853306	JN853344	JN853381	NA	JN853524	JN853489	JN853418	JN853451
Lepisosteus platostomus	Horseshoe Lake, Illinois, USA	21048	JN853307	JN853345	JN853382	JN853552	JN853525	JN853490	JN853419	JN853452
Lepisosteus platostomus	Kansas River, Wyandotte Co., Kansas, USA	21049	JN853308	JN853346	JN853383	JN853553	JN853526	JN853491	JN853420	JN853453
Lepisosteus platostomus	Aquarium trade	21050	JN853309	JN853347	JN853384	JN853554	JN853527	JN853492	JN853421	JN853454

Table 2
Molecular evolutionary models selected for each gene from comparison of maximum likelihood scores using Akaike Information Criterion.

Gene	Model
COI	HKY + G
ENC1	HKY
myh6	HKY
plagl2	HKY
S7 intron 1	HKY
sreb2	HKY
tbr1	HKY
zic1	HKY

the analysis that only included the nuclear genes. The chain lengths were 10^8 generations with parameters sampled every 10^3 generations. Convergence of parameters values in the Markov chain Monte Carlo were assessed by the effective sample sizes that were calculated using Tracer version 1.5 and visualizing the cumulative split frequencies in the set of posterior trees using AWTY (Nylander et al., 2008). Generations sampled before convergence was attained were discarded as burn-in.

3. Results

The optimal molecular evolutionary model for each sampled gene is presented in Table 2. The phylogenetic resolution of relationships among extant gar species varied among the sampled mtDNA and nuclear genes (Fig. 3). The mtDNA *COI* gene and the *S7* intron exhibited the greatest resolution, where *Atractosteus* and *Lepisosteus* were each monophyletic and relationships among species in each clade were resolved. Two nuclear genes, *tbr1* and *zic1*, resolved *Atractosteus* and *Lepisosteus* as monophyletic, but relationships among species in these lineages were wholly unresolved. Other gene trees resolved either *Atractosteus* (*myh6* and *plagl2*) or *Lepisosteus* (*sreb2*) as monophyletic, but not the other clade. There was variation among the gene trees with regards to the monophyly of the sampled individuals of each species, with only the mtDNA *COI* gene resolving each species as monophyletic (Fig. 3). There was no obvious incongruence among the mtDNA and nuclear inferred gene trees.

The posterior parameter value estimates from the ∗BEAST multicoalescent species tree analysis were characterized by high (>200) effective sample sizes and convergence of the individual runs was confirmed from assessments using both Tracer and AWTY. The maximum clade credibility trees from the posterior sets of species trees inferred from all sampled genes and only the nuclear genes were identical and presented in Fig. 4. All nodes in the species tree inferred using both mtDNA and nuclear genes were supported with strong (>0.95) Bayesian posterior values, while the common ancestor of *Lepisosteus osseus* and *L. platostomus* was supported with a posterior value of 0.60 when the analysis was restricted to the nuclear genes. All other nodes in the nuclear gene species tree were supported with Bayesian posterior values of 1.00 or 0.99 (Fig. 4). Results of the Bayesian analysis of the concatenated data set were congruent with those of the species tree analysis, with the exception that *L. oculatus* was resolved as paraphyletic with respect

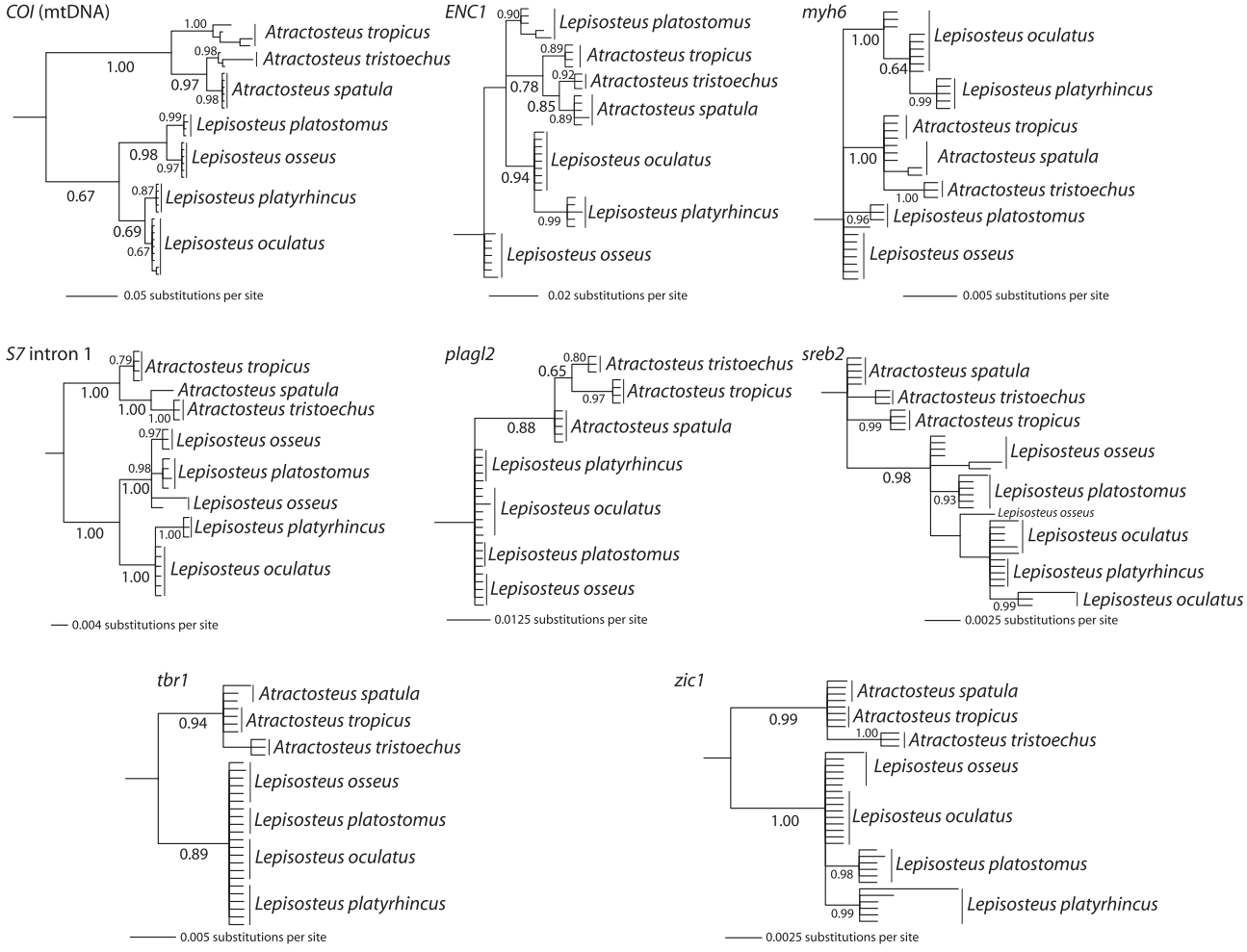

Fig. 3. Gene trees for each of the molecular markers used in the present study. All trees, while varying in their level of resolution, showed very little discordance between themselves in terms of the relationships that were recovered. Note that only the *COI* tree showed complete resolution of all species relationships, while the *S7* intron tree also showed relatively high levels of resolution.

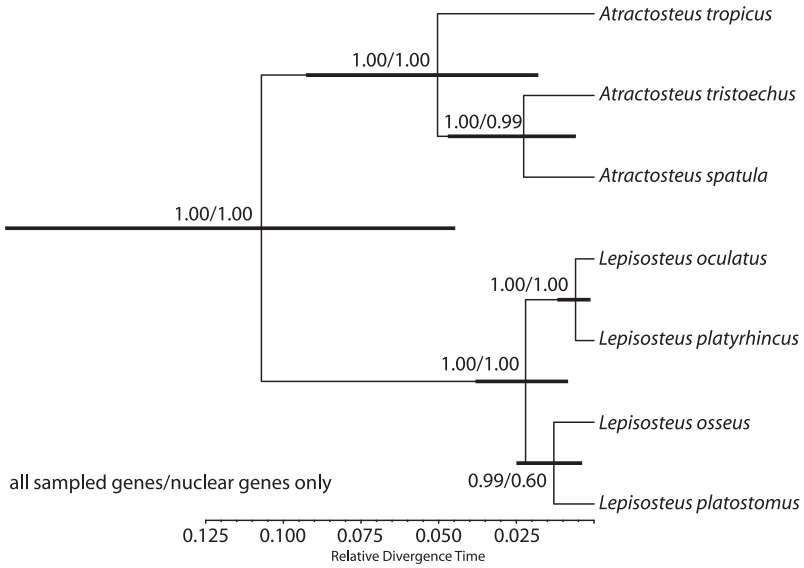

Fig. 4. The species tree of extant gars, resulting from coalescent analyses of both the complete molecular data set, as well as the nuclear genes only. Values at nodes represent Bayesian posterior support values. Bars spanning nodes represent credible intervals for relative divergence time estimates.

to *L. platyrhincus*. This result is most likely due to ancestral polymorphisms of alleles for the sampled nuclear genes, as both species were monophyletic in the mtDNA *COI* gene tree.

4. Discussion

The phylogenetic relationships among living gars presented in this study are based on cutting-edge multi-species coalescent species tree analyses of DNA sequences sampled from multiple loci. The use of species tree methods to accommodate incongruence between gene and species trees is typically applied to the most apical branches in the Tree of Life that have a fairly recent history of diversification (e.g., Kubatko et al., 2011). However, our analysis of individual species trees shows a distinct lack of phylogenetic resolution that is most likely driven by incomplete lineage sorting of ancestral alleles among living gar species (Fig. 3). The fossil record of *Atractosteus* and *Lepisosteus* indicate that the age of the crown lineage of gars is at least 100 million years old (Grande, 2010). Our analyses demonstrate that multi-coalescent species tree methods have substantial utility in resolving the phylogenetic relationships of ancient radiations of ray-finned fishes. Below, we use the molecular phylogenies generated in this study to discuss the congruence between molecular and morphological inference of gar relationships, the potential for hybridization among gar species in generating incongruence among individual gene trees and phylogenetic inferences from autosomal and mtDNA genomes, and patterns of allopatric speciation in a clade of living fossils.

The species trees inferred from DNA sequences are very similar to the previous phylogenetic hypotheses of gars presented by Wiley (1976) and Grande (2010) that were both based on parsimony optimization of morphological character state changes (Fig. 2B). The only difference between our molecular and these morphological phylogenetic hypotheses involves the relationships of *Lepisosteus osseus* and *L. platostomus*. In the morphology-inferred phylogeny, *L. platostomus* is the sister species of all other *Lepisosteus* with the next node resolving *L. osseus* as the sister species of a clade containing *L. oculatus* and *L. platyrhincus* (Fig. 2B). However, in the molecular-inferred species trees *L. osseus* and *L. platostomus* are resolved as sister species (Fig. 4). Interestingly, in the two gene trees, *COI* and *S7* intron 1, where relationships among *Lepisosteus* species were resolved, *L. osseus* and L. *platostomus* were supported as a clade with strong Bayesian posterior support values, and no gene tree resolved *L. osseus*, *L. oculatus*, and *L. platyrhincus* as a clade (Fig. 3). The characters supporting the clade containing *L. osseus*, *L. oculatus*, and *L. platyrhincus* in the morphological phylogenies were adults with only very small teeth on the dermopalatine (character 66 in Grande, 2010) and the number of teeth in the outer premaxillary tooth row, which was coded as four to 18, or one to four (character 11 in Grande, 2010; Wiley, 1976). However, in an alternate coding of this latter character there are four possible character states. For instance, *Atractosteus tristoechus*, *A. tropicus*, and *L. platostomus* have 10 teeth in the outer premaxillary tooth row (Grande, 2010, Figs. 309, 330 and 119), *A. spatula* has 15 teeth (Grande, 2010, Fig. 227), *L. osseus* has four teeth (Grande, 2010, Fig. 31), and *L. oculatus* and *L. platyrhincus* have a single tooth in this row (Grande, 2010, Figs. 140 and 161). If this character was scored as four character states, then it no longer provides support for the clade containing *L. osseus*, *L. oculatus*, and *L. platyrhincus*. Given that this clade would be supported with a single morphological character state change after the alternative coding of the number of teeth in the outer premaxillary tooth row, there is very little incongruence between the morphological and molecular inferences of phylogenetic relationships among living gar species.

Over the past several years hybridization has been implicated as a mechanism of recombinant hybrid speciation in animals, including some ray-finned fish lineages (Mavarez and Linares, 2008; Larsen et al., 2010). More specific to phylogeny inference, hybridization and associated mtDNA introgression has disrupted efforts to use mtDNA genes to infer relationships among closely related species of ray-finned fishes (e.g., Bossu and Near, 2009). Documentation of hybridization among gar species is limited to observations of F_1 individuals resulting from a *L. osseus* × *A. spatula* hybrid cross under captive conditions (Herrington et al., 2008). A history of hybridization in the diversification of the living gar species that would disrupt phylogenetic inferences from DNA sequence data is not reflected in our set of inferred gene trees (Fig. 3). In addition, there is complete congruence with the phylogenetic tree inferred from the mtDNA *COI* gene and the species phylogeny estimated using the sampled nuclear genes, indicating that mtDNA introgression is not present among extant gar species (Figs. 3 and 4).

The historical biogeography of gar species has been investigated using the fossil record of the clade and morphology inferred phylogenies (Wiley, 1976; Grande, 2010). Living gars are restricted to the Western Hemisphere in North America east of the Rocky

Mountains, Central America, and Cuba, but the fossil record for the clade extends across the Northern Hemisphere (Grande, 2010). In the resolved species tree of gar species there are three sister species clades, and only one of these sister species pairs occur in sympatry. The other two species pairs, *L. oculatus* and *L. platyrhincus*, and *Atractosteus tristoechus* and *A. spatula* are allopatric and in adjacent areas, hinting at a role for geographic isolation in the history of speciation in living gar lineages. Interestingly, one of the two sympatric sister species, *Lepisosteus osseus*, may be more closely related to the western North American Eocene aged fossil species †*L. bemisi* (Grande, 2010). The sympatry exhibited by *Lepisosteus osseus* and *L. platostomus* may reflect range expansion subsequent to speciation, with the most closely related species of either, or both species, lost to extinction.

The phylogenetic relationships of the seven living gar species are highly resolved in the species tree inferred from mtDNA and nuclear gene DNA sequences (Fig. 4). In addition to providing an example of congruence between morphological and molecular inferences of phylogeny and insights into the geographic history of speciation in the clade, this gar phylogeny will find utility in understanding patterns of functional diversification and potentially serve as an example of the integration of paleontological information in time calibration of gene trees and species trees (Kammerer et al., 2006; Grande, 2010). This temporal perspective on the diversification of living gars will facilitate investigations of rates of phenotypic and molecular evolution within extant lepisosteids to assess whether or not expectations of evolutionary stasis are met in this lineage of living fossils. Such investigations have played a central role in theories regarding mode and tempo of evolution as inferred from the fossil record (e.g., Gould and Eldredge, 1977; Avise et al., 1994; Jackson and Cheetham, 1999; Eldredge et al., 2005), and the molecular data generated by this study represent a major resource for the development of additional insights into the factors influencing this perplexing evolutionary phenomenon.

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