

Original article

Systematics of Clupeiformes and testing for ecological limits on species richness in a trans-marine/freshwater clade

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Clupeiformes (herring, sardines, shad, anchovies and allies) are a globally distributed clade with nearly 400 marine, freshwater, and diadromous species. Although best known as filter feeding fishes that form large schools, this group occupies a diverse array of trophic guilds and habitats. Theory suggests that species richness in clades is modulated by ecological limits, which results in diversity-dependent clade growth, a pattern that most clades exhibit. As a trans-marine/freshwater clade that has undergone repeated transitions between marine and freshwaters, Clupeiformes are an excellent system for investigating the interplay between ecological diversity and macroevolutionary dynamics. In this study we review the systematics of Clupeiformes and explore discordance in phylogenetic relationships and divergence times between mitochondrial and nuclear loci. We then use comparative methods to test whether ecological limits regulate diversity in Clupeiformes. We find discordance in phylogenetic relationships at various taxonomic scales, but also considerable agreement between genomes. Our results suggest that trans-marine/freshwater clades are able to circumvent ecological limits on clade growth at regional, but not on local scales. Our study demonstrates that phylogenies are a critical link between ecology and macroevolutionary dynamics, and suggests habitat transitions can play a key role in shaping diversity patterns, particularly in the neotropics.

Keywords: Congruence, Comparative methods, Discordance, Diversification, Diversity-dependent.

Clupeiformes (apapás, sardinhas e manjubas) são um clado globalmente distribuído com quase 400 espécies marinhas, de água doce e diádrimas. Embora mais conhecida pela presença de peixes filtradores formadores de cardumes, este grupo apresenta uma diversidade de guilda e habitats tróficos. A teoria sugere que a riqueza de espécies em clados é modulada por limites ecológicos, o que resulta em um crescimento dependente da diversidade, um padrão que a maioria dos clados exibem. Como um clado que sofreu repetidas transições entre águas marinhas e as águas doces, os clupeiformes são um excelente grupo para investigar a interação entre a diversidade ecológica e a dinâmica macroevolutiva. Neste estudo, revisamos a sistemática de Clupeiformes e exploramos a discordância nas relações filogenéticas e os tempos de divergência entre loci mitocondriais e nucleares. Em seguida, utilizamos métodos comparativos para testar se os limites ecológicos regulam a diversidade em Clupeiformes. Encontramos discordância nas relações filogenéticas em várias escalas taxonômicas, mas também considerável concordância entre os genomas. Nossos resultados sugerem que clados que sofreram sucessivas transições entre águas marinhas e águas doces são capazes de contornar os limites ecológicos do crescimento durante a sua diversificação em escala global, mas não localmente. Nosso estudo demonstra que as filogenias apresentam um vínculo crítico entre a ecologia e a dinâmica macroevolutiva, e sugere que as transições de habitats podem desempenhar um papel fundamental na modelagem dos padrões de diversidade, particularmente no neotrópico.

Palavras-chave: Congruência, Discordância, Diversificação, Diversidade-dependente, Métodos comparativos.

Introduction

Understanding the processes that determine spatial and phylogenetic diversity patterns is a fundamental goal of ecology and evolutionary biology. The rise of phylogenetic comparative methods has led to the recognition that diversity patterns are the outcome of the interplay between ecology

and evolutionary dynamics. A central idea that has emerged from the integration of ecology and evolutionary biology is the concept of ecological limits on clade diversity (Rabosky, Lovette, 2008; Rabosky, 2013). The ecological limits hypothesis suggests that equilibrium processes, such as availability of resources and competition, determine clade richness (Rabosky, 2009a; Moen, Morlon, 2014). A prediction of the

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ecological limits hypothesis is that as a clade diversifies resource availability decreases due to competition, and clade growth slows over time. If a clade is bounded by ecological limits it is best explained by a diversity-dependent model and the resulting pattern is a logistic curve in a lineage through time plot (LTT). Alternatively, diversity may be unregulated and determined by non-equilibrium processes, such as vicariance and dispersal (Harmon, Harrison, 2015). Under this scenario clade growth is constant and unbounded over time, resulting in an exponential curve in a LTT plot. Clade dynamics are not static and may shift from diversity-dependent to diversity-independent when environmental conditions change (Moen, Morlon, 2014). In some cases, the environmental conditions may change because a lineage undergoes a habitat transition, which can alter clade dynamics by shifting rates of lineage diversification (speciation and extinction) and morphological evolution, which in turn can help shape diversity patterns (Collar *et al.*, 2010; Betancur-R *et al.*, 2012; Bloom *et al.*, 2013).

Habitat transitions from marine to freshwaters represent a profound shift in environmental conditions (Lee, Bell, 1999; Vermeij, Dudley, 2000). There is generally a mutually exclusive aquatic community composition between adjacent marine and freshwaters, likely due to strong biotic and abiotic barriers. Thus a lineage that transitions between marine and freshwater habitats may be released from diversity-dependent ecological limits on clade growth (Schluter, 1996; Yoder *et al.*, 2010). If habitat transitions circumvent ecological limits, then clades that repeatedly undergo transitions would continue to experience exponential clade growth. The Neotropics harbor the greatest freshwater fish diversity in the world (Lundberg *et al.*, 2000; Albert, Reis, 2011), which would predict this fauna may have reached diversity equilibrium (López-Fernández *et al.*, 2013). Yet, Neotropical freshwaters are a hotspot for trans-marine/freshwater lineages – clades that repeatedly transitioned between marine and freshwaters over macroevolutionary timescales – some of which have invaded freshwaters in the recent past (Bloom, Lovejoy, 2017). The repeated invasions of Neotropical freshwaters, and reversals to marine waters (Betancur-R, 2010; Bloom, Lovejoy, 2012, 2017) suggest that ecological limits have not been reached. This raises a number of questions about the genesis of diversity in the Neotropics. Are there ecological limits on clade diversity in trans-marine-freshwater fishes in general? Do Neotropical lineages exhibit different lineage accumulation patterns than clades in other biogeographic areas? Understanding the role of ecological limits in structuring Neotropical fish diversity will help provide answers to these urgent questions.

Clupeiformes (herring, sardines, shad, anchovies and allies) are best known as near-shore, schooling and filter-feeding fishes (Whitehead, 1985b). However, the ecological diversity of the group is often underappreciated (Whitehead, 1985a). Clupeiformes display a wide range phenotypic diversity, including miniaturized species that achieve a maximum size of 2 mm and large species reaching 300 mm

TL (Whitehead, 1985b; Bloom *et al.* in press). The group occupies an array of trophic guilds, such as planktivores, algivores, piscivores, molluscivores, and more (Egan *et al.*, 2018). Clupeiformes also inhabit a broad spectrum of habitats, including open ocean, coastal areas, estuaries, and freshwater rivers and lakes. While most vertebrate clades are restricted to either marine or continental waters, Clupeiformes are a trans-marine/freshwater clade, yielding nearly 400 marine, freshwater, and diadromous species recognized today (Bloom, Lovejoy, 2014). The ability to traverse the marine/freshwater boundary is shared with groups such as stingrays, needlefishes, silversides, drums and pufferfishes (Lovejoy *et al.*, 2006; Bloom, Lovejoy, 2017). While most trans-marine/freshwater groups have colonized the freshwaters of two or fewer continents, freshwater Clupeiforms are found on every continent except Antarctica and in many cases have colonized freshwaters multiple times in the same geographic area (Bloom, Lovejoy, 2012; 2014).

The systematic relationships among Clupeiformes have received increasing attention, but higher-level relationships remain unresolved. Clupeiformes is divided into two suborders, Denticipitoidei, which includes a single species, *Denticiceps clupeioides*, and Clupeioidi, which is comprised of all other extant Clupeiforms. Clupeioidi has traditionally been divided into five families: Clupeidae (herrings, shads, sardines), Engraulidae (anchovies), Pristigasteridae (longfin herrings), Chirocentridae (wolf herrings) and Denticipitidae (denticle herrings). A sixth family, Sundasalangidae, was described in 1981 and later placed in Clupeiformes by Siebert (1997); Sundasalangids have since been included in Clupeidae (Lavoué *et al.*, 2014). Early morphological work by Grande (1985) hypothesized Clupeidae as the sister to Chirocentridae, with the position of Engraulidae and Pristigasteridae unresolved. More recently, Di Dario (2009) found evidence for Chirocentridae as sister to Engraulidae. Di Dario (2009) also supported Clupeidae as the closest relative to the Chirocentridae+Engraulidae clade, with Pristigasteridae the earliest branching lineage within Clupeioidi. Using mitogenomics Lavoué *et al.* (2013) supported Clupeidae+Pristigasteridae, but also found that Clupeidae was not monophyletic because *Spratelloidiini* fell outside of a clade that included Clupeidae, Pristigasteridae and Chirocentridae. Studies that incorporated both mitochondrial (mtDNA) and nuclear DNA (nDNA) also failed to recover a monophyletic Clupeidae, but differed from mitogenomic results because Chirocentridae was sister to Engraulidae (Li, Orti, 2007; Bloom, Lovejoy, 2014). Combined mtDNA and nDNA analyses suggested Pristigasteridae was sister to the Chirocentridae+Engraulidae, but with no statistical support (Bloom, Lovejoy, 2014). Most recently, using six nuclear genes, Egan *et al.* (2018) supported Chirocentridae as sister to Engraulidae, but also recovered Pristigasteridae as nested within Clupeidae.

The phylogenetic uncertainty at higher taxonomic levels is also prevalent at lower taxonomic levels. Grande (1985) proposed five subfamilies within Clupeidae (Alosinae, Clupeinae, Pellonulinae, Dorosomatinae and Dussumieriinae),

but noted these taxa were mostly out of convenience rather than supported by data. Indeed molecular studies have demonstrated that none of the five subfamilies of Clupeidae proposed by Grande (1985) are monophyletic. Lavoué *et al.* (2014) revised the composition of these five subfamilies based in part on recent molecular studies (Li, Orti, 2007; Lavoué *et al.*, 2010, 2013; Bloom, Lovejoy, 2012, 2014); herein we used this revised taxonomy. Engraulidae (anchovies) is divided into a two subfamilies: Engraulinae is a largely New World clade, and Coilinae is restricted to the Old World. Engraulinae is comprised of eight genera, six of which are polytypic and two are monotypic. Of the six polytypic New World anchovy genera, only *Lycengraulis* and *Cetengraulis* have been recovered as monophyletic (Bloom, Lovejoy, 2012, 2014). However, the most taxon rich study (Bloom, Lovejoy, 2014) had a large amount of missing data, and relied heavily on mtDNA data. Both missing data and mtDNA are known to mislead both tree topology (Leaché, 2010; Wiens *et al.*, 2010; Platt *et al.*, 2018) and divergence time estimates (Zheng *et al.*, 2011; Dornburg *et al.*, 2014). The widespread taxonomic incongruence with phylogeny and dependence on mitochondrial data calls for an evaluation of discordance between mitochondrial and nuclear topologies and divergence times in Clupeiformes.

In this study we investigated clupeiform phylogenetics and patterns of lineage accumulation across different phylogenetic scales in Clupeiformes. Our objectives were 1) to evaluate differences in tree topology and divergence times between mitochondrial and nuclear datasets and 2) test the hypothesis that habitat transitions have allowed Clupeiformes to circumvent ecological limits on clade growth. We reviewed the state of systematics in Clupeiformes and proposed that this group is good model for investigating how habitat transitions can influence clade dynamics. More generally, we demonstrated that clades with unresolved systematics can be used to understand the genesis of diversity in Neotropical fishes.

Material and Methods

Taxon sampling. Previous studies that employed both mtDNA and nDNA had large amounts of missing nDNA, which can be problematic for phylogenetic inference (Thomson, Shaffer, 2010; Roure *et al.*, 2013). Because we wanted to focus our efforts on identifying incongruence between loci we used the Bloom and Lovejoy data matrix as a starting point and pruned the dataset to remove all taxa missing more than a single locus. The original Bloom and Lovejoy dataset included DNA sequences from two nuclear (*rag1*, *rag2*) and two mitochondrial (*cytb*, *16s*) genes and 152 terminals. Our pruned data set included 98 in-group taxa representing all clupeiform families (*sensu* Lavoué *et al.*, 2014) and 36 clupeiform genera (S1 – available only as online supplementary file accessed with the online version of the article at <http://www.scielo.br/ni>). Sequences were aligned using the MUSCLE algorithm (Edgar, 2004) in Geneious v. 6.0.3 (www.geneious.com;

Biomatters Ltd., Auckland, New Zealand). We verified the quality of alignments by visual inspection of sequences and their amino acid translation and comparison to previously published alignments (Bloom, Lovejoy, 2014), resulting in sequences of the following lengths (in base pairs): *rag1* 1491, *rag2* 1237, *cytb* 1130, and *16s* 1353.

Phylogenetic analyses. We assembled three concatenated datasets for phylogenetic analysis: (1) mitochondrial genes, (2) nuclear genes, (3) and a combined dataset containing all four loci. We used PartitionFinder v.1.01 (Lanfear *et al.*, 2012) to select partitioning schemes and nucleotide substitution models using Bayesian information criterion (BIC) scores. We did not implement the invariant sites parameter because it is redundant with the gamma distribution parameter (Yang, 2006). The best fitting partitioning scheme for all datasets partitioned by gene and codon position and assigned GTR + gamma nucleotide substitution models to all partitions.

We inferred maximum likelihood phylogenies using all three datasets with RAxML v.8.2.4 (Stamatakis, 2014) via CIPRES. Tree searching and non-parametric bootstrap estimation of node support was conducted simultaneously using the rapid bootstrapping algorithm. We used the bootstopping option, which determines the number of bootstrap replicates required to obtain stable support values and stops analyses automatically.

To time-calibrate our phylogeny we used six exponential calibration priors based upon priors previously used in clupeiform systematics (Bloom, Lovejoy, 2014; Lavoué *et al.*, 2017b):

- (1) Most recent common ancestor (MRCA) of Clupeoidei: The crown clupeoid †*Cynoclupea nelsoni* (Malabarba, Di Dario, 2017) set a minimum age of 125 Ma and the absence of Jurassic Clupeomorpha fossils set a soft 95% maximum age of 145 Ma.
- (2) MRCA of *Dorosoma*: A *Dorosoma petenense* fossil (Miller, 1982) set a minimum age of 2.5 Ma for the MRCA of *Dorosoma*. We set a soft 95% maximum MRCA age of 86.3 because most crown clupeoid fossils are younger.
- (3)–(5) MRCA of three sister pairs of anchovies separated by the Isthmus of Panama (*Cetengraulis edentulus/C. mysticetus*, *Anchovia macrolepidota/A. clupeoides*, and *Lycengraulis grossidens/L. poeyi*): We set a minimum age of 3.0 Ma and soft 95% maximum age of 86.3 (Bloom, Lovejoy, 2014). Our maximum age calibration does not exclude a later separation of the Isthmus of Panama (Montes *et al.*, 2015).
- (6) MRCA of Engraulidae: †*Eoengraulis fasolo* (Marramà, Carnevale, 2017) set a minimum age of 50 Ma and we set a soft 95% maximum age of 86.3 Ma (Bloom, Lovejoy, 2014).

We generated two time-calibrated phylogenies using the nuclear and mitochondrial datasets via Bayesian phylogenetic analyses in BEAST v.2.4.5 (Bouckaert *et al.*, 2014) via the CIPRES Science Gateway portal (Miller *et al.*, 2010). For all

analyses we implemented a birth-death speciation prior because extinction has occurred in clupeiformes (Grande, 1985; Grande, Nelson, 1985), an uncorrelated lognormal clock model of molecular evolution, set Markov chain Monte Carlo (MCMC) lengths of 100 million generations, and logged results every 5,000th generation. We conducted five independent BEAST runs for both datasets. We visualized results in Tracer v.1.6.0 (Rambaut *et al.*, 2014) to confirm that MCMC runs reached stationarity, sufficient effective sample sizes of parameters (>200), and convergence of independent MCMC runs. We combined trees and removed burnin in LogCombiner v.2.4.5 and used TreeAnnotator v.2.4.5 to generate maximum clade credibility trees (Bouckaert *et al.*, 2014).

Comparative methods. Testing for slow-downs in diversification rates requires a time-calibrated phylogeny and is sensitive to taxon sampling. For our comparative analyses we used the phylogeny from Bloom, Lovejoy (2014), which represents the most taxon rich time-calibrated phylogeny available for this group. The Bloom, Lovejoy (2014) phylogeny was a multi-gene tree that included 152 of the approximately 400 species of Clupeiformes. We restrict our comparative analyses to Clupeoidei, which includes all Clupeiformes except *Denticeps clupeoides* because this species was recovered as sister to Ostariophysii rather than a member of Clupeiformes. Habitat transitions were previously estimated using maximum likelihood ancestral character reconstruction (Bloom, Lovejoy, 2014).

We tested for a slow-down in cladogenesis rates using models of speciation and lineages through time within Clupeoidei and several subclades that we selected according to biogeographic region and across multiple phylogenetic scales. We visualized lineage accumulation patterns by generating lineage through time plots (LTT), which plot the log number of lineages through time (Nee *et al.*, 1992). Lineages that experience a slow-down in diversification over time are expected to show a downturn (logistic curve), while lineages that accumulate constantly are expected to show a linear pattern (exponential). We computed the gamma-statistic (Pybus, Harvey, 2000) to test for a signal of decreased diversification over time for all Clupeoidei and several subclades, respectively. A gamma value <0 indicates decelerating diversification through time and a gamma value >0 indicates increasing diversification rates through time. Incomplete taxon sampling can incorrectly infer a decline in diversification rates, and even completely sampled phylogenies may fail to include cryptic or undescribed species. To incorporate known missing taxa we used the Monte Carlo Constant Rates (MCCR) test, which examines gamma values for simulated trees that include a known percentage of missing taxa to generate a critical value (0.05) for rejecting constants rates. We implemented the MCCR test for all clades that returned a negative gamma value without accounting for missing species. Because the portion of missing species is unknown, we tested for a significant slow-down using the MCCR test for a proportion missing taxa from 0.25 to 1.0.

If habitat transitions allow a lineage to circumvent ecological limits we expect clades that have experienced numerous transitions between marine and freshwaters will show patterns of constant diversification (Rabosky, 2013; Moen, Morlon, 2014). We fitted five diversification rate models, including two constant rate and three rate variable models of lineage diversification. These models included: exponential (DDX) and linear (DDL) diversity dependent models, a non-diversity dependent variable rate model (Yule-2-rate), and constant rate models (pure-birth and birth-death). Diversity dependent models are consistent with ecological limits on clade growth because speciation rates depend on the number of extant lineages at a given time (Rabosky, Lovette, 2008). We estimated the maximum likelihood of model parameters using the R package LASER (Rabosky, 2006) and determined the best-fit model for each clade using sample-size corrected Akaike Information Criterion (AICc), where the best model has the lowest AIC values.

Results

Phylogenetic relationships. Our concatenated Bayesian and maximum likelihood analyses based upon the combined nDNA and mtDNA dataset resulted in largely congruent topologies (Fig. 1; S2 – available only as online supplementary file accessed with the online version of the article at <http://www.scielo.br/ni>). Both analyses produced phylogenies with poor support for higher-level relationships. In both phylogenies, Clupeoidei (all clupeiforms except the monotypic clupeiform family Denticipitidae containing *Denticeps clupeoides*) was monophyletic. *Denticeps clupeoides* was consistently placed among the outgroup taxa, rendering Clupeiformes paraphyletic. Anchovies (Engraulidae) and longfin herrings (Pristigasteridae) were recovered as monophyletic. The herring and sardine family (Clupeidae) was not recovered as monophyletic in either our Bayesian or likelihood analyses. Our Bayesian analysis placed Clupeinae in a lineage with Dussumieriinae that was sister to a lineage containing Pristigasteridae+Chirocentridae+Engraulidae+the remaining clupeid lineages. Our maximum likelihood phylogeny placed Clupeinae as sister to a lineage containing Pristigasteridae+Chirocentridae+Engraulidae in the maximum likelihood phylogeny. Both analyses recovered Sprattelloidinae, a subfamily of Dussumieriidae (*sensu* Lavoué *et al.*, 2014) as sister to all remaining clupeoids.

We found discordance between mtDNA and nDNA in tree topology, particularly at the highest taxonomic levels (Figs. 1-2). We only discuss maximum likelihood and Bayesian results separately if they produced conflicting results. The nDNA dataset was largely consistent with the combined nDNA + mtDNA topology with a few notable exceptions. The nDNA Bayesian phylogeny resolved Chirocentridae as sister to Pristigasteridae (PP = 0.70) and the maximum likelihood analysis resolved Chirocentridae as sister to a lineage containing Pristigasteridae, Clupeinae, and Dussumieriinae (BS = 0.82). Bayesian analyses of nDNA placed Clupeinae

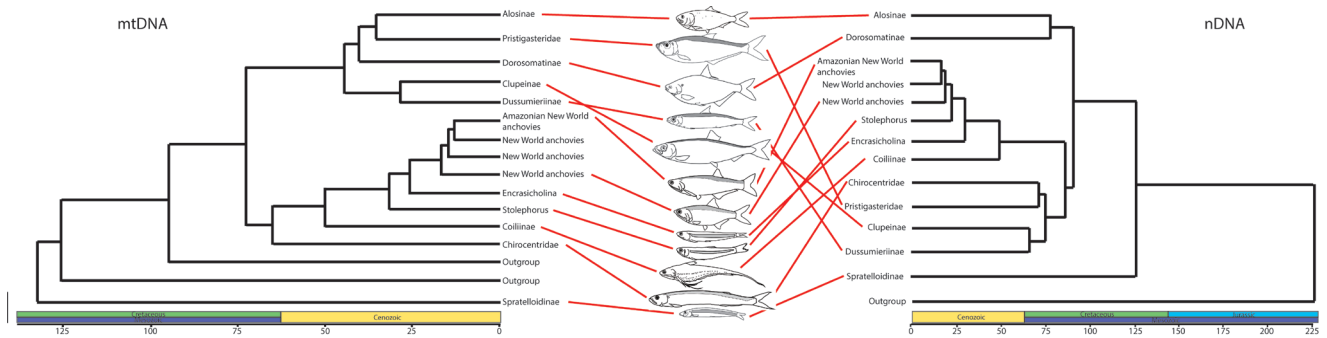


Fig. 1. Clupeiform phylogenies pruned to only show major lineages estimated using concatenated Bayesian analysis of the mtDNA dataset (left) and nDNA dataset (right) in BEAST v.2.4.5. Red lines illustrate similarities and differences in the placement of major lineages by mtDNA versus nDNA. Time, in millions of years, is shown along the x-axis. Line drawings depict representative species from clupeiform lineages: *Brevoortia tyrannus*, *Ilisha elongata*, *Dorosoma cepedianum*, *Etrumeus sadina*, *Clupea harengus*, *Pterengraulis atherinoides*, *Cetengraulis edentulus*, *Encrasicholina heteroloba*, *Stolephorus* sp., *Coilia dussumieri*, *Chirocentrus dorab*, and *Spratelloides gracilis* (from top to bottom).

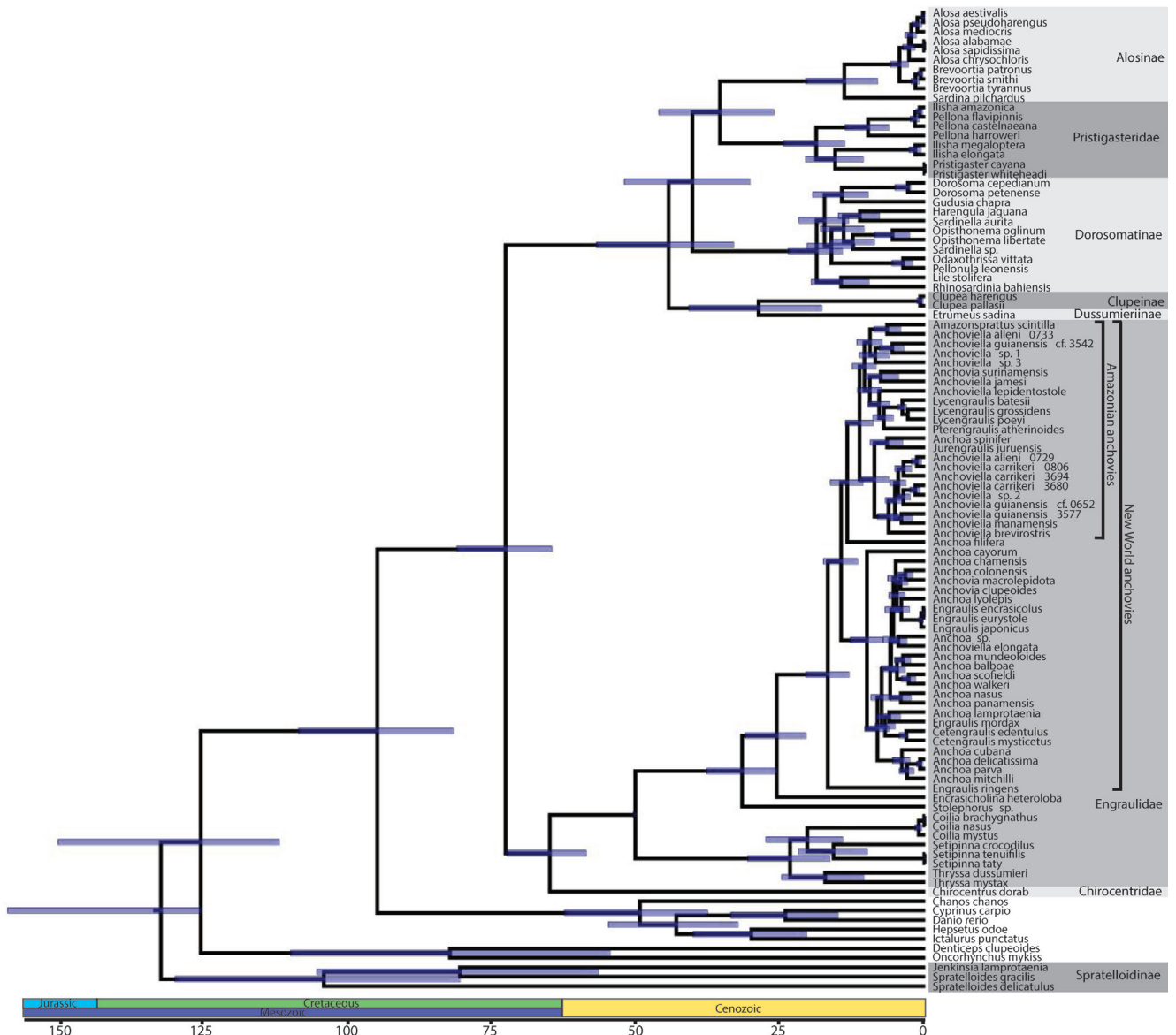


Fig. 2. Time-calibrated clupeoid phylogeny resulting from Bayesian analysis of the mtDNA dataset in BEAST v.2.4.5. Time, in millions of years, is shown along the x-axis. Node bars show the 95% highest posterior density interval of divergence time estimates.

sister to Pristigasteridae + Chirocentridae (PP = 1.0) and maximum likelihood analyses of nDNA placed Clupeinae sister to Dussumieriinae (BS = 0.67). mtDNA placed Pristigasteridae within Clupeidae sister to Alosinae (PP = 1.0, BS = 0.33), but analyses of nDNA recovered pristigasteridae as sister to a clade including Engraulidae, Chirocentridae, Clupeinae, and Dussumieriinae. In contrast to the nDNA, our mtDNA phylogeny placed Alosinae, Pristigasteridae, Dorosomatinae, Clupeinae, and Dussumieriinae in a single clade. nDNA placed Spratelloidinae sister to all remaining Clupeoidei, but mtDNA placed Spratelloidinae outside of Clupeiformes. There was also incongruence between nDNA and mtDNA at lower taxonomic levels. For example, in New World anchovies *Anchoa spinifer*+*Jurengraulis juruensis* were either sister to all other members of the Amazonian anchovy clade (nDNA) or nested well within this clade (mtDNA). The placement of a clade that included *Engraulis encrasicolus*, *E. eurystole*, and *E. japonicas* was resolved as sister to all other New World marine anchovies except *Anchoa filifera* (nDNA) or nested deeply within New World marine anchovies (mtDNA).

Divergence times. The combined nDNA+mtDNA, the nDNA, and the mtDNA datasets all estimated an early to middle Cretaceous MRCA of Clupeoidei (Figs. 1-4). Branching events between major clupeiform lineages were estimated to occur during the late Cretaceous and early Cenozoic: Spratelloidinae (nDNA: MRCA = 100 Ma; mtDNA: MRCA = 109 Ma), Clupeidae (excluding Clupeinae) (nDNA: MRCA = 79 Ma; mtDNA: MRCA = 33 Ma), Engraulidae (nDNA: MRCA = 50 Ma; mtDNA: MRCA = 51 Ma), and Pristigasteridae (nDNA: MRCA = 42 Ma; mtDNA: MRCA = 21 Ma).

Comparative analyses. The LTT plot for Clupeoidei shows constant lineage accumulation through time (Fig. 5). The calculated gamma value was -0.61 but not significant if missing taxon sampling is as low as 25% (P = 0.89); the Bloom, Lovejoy (2014) dataset included approximately 37% of described Clupeoidei. The best-supported model of diversification was a pure birth model, in which lineages accumulate constantly over time regardless of the number of species in the clade. Clupeidae LTT plots show a constant accumulation of lineages over time. The MCCR test showed the negative gamma value of -1.34 for this clade was not significant (P = 0.93) at threshold of 67% missing data, which represents described clupeid diversity and is likely an underestimate of the actual number of species in this clade. The chosen diversification model was a pure birth model, followed closely by a diversity-dependent model. The LTT plot for the subclade Dorosomatinae (*sensu* Lavoué *et al.*, 2014) showed a slow-down in lineage accumulation and a corresponding highly negative gamma value (-3.08). The negative gamma value for Dorosomatinae was significant at a threshold of 50% missing data (P = 0.01) but not when missing data was set at 75% (P = 0.13). The best-fit model for Dorosomatinae was a diversity dependent logistic growth

model in which lineages accumulate as a function of clade diversity. The LTT plot of the New World marine anchovy clade showed a slow-down in lineage accumulation rate and had a gamma value of -2.51. The negative gamma is significant with 25% missing taxa (P = 0.02), but only marginally significant with 50% missing data (P = 0.51). Our taxon sampling (28 species) likely represents more than 50% of the species in this clade, thus we interpret this clade as experiencing a slow-down. The chosen diversification model for New World marine anchovies was diversity dependent growth. The LTT plot for South American freshwater anchovies reveals a slow-down in lineage accumulation over time and the gamma value of -3.59 is highly negative and significant at a threshold of 75% missing taxa (P = 0.01). The best-fit model for New World freshwater anchovies was a diversity dependent model.

Discussion

Concordant and conflicting phylogenetic relationships.

The phylogenies resulting from our concatenated Bayesian and maximum likelihood analyses based upon the combined nDNA+mtDNA dataset yielded topologies largely congruent with previous molecular studies (Lavoué *et al.*, 2007, 2010, 2013, 2017a,b; Li, Orti, 2007; Bloom, Lovejoy, 2012, 2014), and the Lavoué *et al.* (2014) classification of Clupeoidei. However, relationships among major clupeiform lineages remain unresolved. Our separate analyses of the nDNA and mtDNA datasets revealed discordance between the mitochondrial and nuclear genomes among major clupeiform lineages.

Genome and gene discordance has long been recognized as a challenge for inferring phylogenetic relationships, including in ray-finned fishes (Waters *et al.*, 2010; Toews, Brelsford, 2012; Betancur-R *et al.*, 2013), and can be caused by interspecific hybridization and incomplete lineage sorting (Pamilo, Nei, 1988; Maddison *et al.*, 2006; Wallis *et al.*, 2017). Therefore, it is not surprising that our separate analyses of the nDNA and mtDNA datasets revealed discordance between the mitochondrial and nuclear genomes. In our dataset, most disagreement between genomes occurred in early branching events, with notable conflicts concerning the placement of Spratelloidinae, Chirocentridae, Pristigasteridae, Clupeinae, and Dussumieriinae. Both genomes found Clupeidae and Dussumieriidae (*sensu* Lavoué *et al.*, 2014) to be non-monophyletic because they placed Clupeinae sister to Dussumieriinae and did not place Spratelloidinae sister to Dussumieriinae. Like previous mitogenomic studies (Lavoué *et al.*, 2013), our mtDNA analyses suggest a close affiliation between Pristigasteridae and Clupeidae, although our mtDNA tree recovered Pristigasteridae nested within Clupeidae, a result that seems tenuous. Previous studies that included nDNA support Pristigasteridae, Engraulidae, and Chirocentridae as a clade, however our analysis of nDNA does not support this topology. Previous mitogenomic studies placed Chirocentridae in a clade sister to Pris-

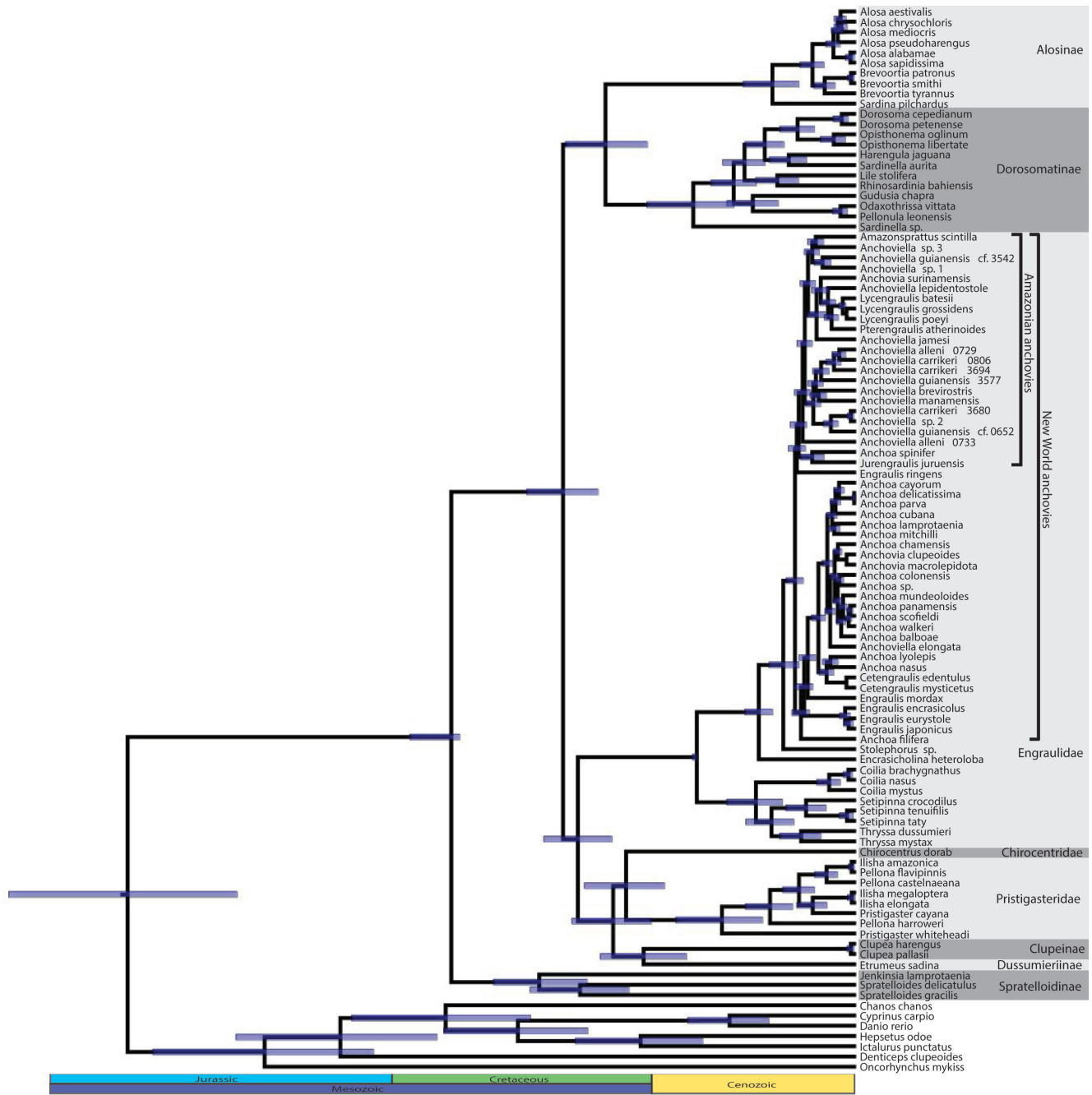


Fig. 3. Time-calibrated clupeoid phylogeny resulting from Bayesian analysis of the nDNA dataset in BEAST v.2.4.5. Time, in millions of years, is shown along the x-axis. Node bars show the 95% highest posterior density interval of divergence time estimates.

tigasteridae+Clupeidae (Lavoué *et al.*, 2013), but previous morphological and molecular studies that included nuclear data have inferred Chirocentridae as sister to Engraulidae (Bloom, Lovejoy, 2014). Therefore, it is surprising that our nDNA dataset placed Chirocentridae sister to Pristigasteridae and our mtDNA dataset placed Chirocentridae sister to Engraulidae. Reconciling incongruence between mitogenomic (Lavoué *et al.*, 2013) and combined mtDNA+nDNA (Li, Orti, 2007; Bloom, Lovejoy, 2014) has been challenging because the combined datasets relied heavily on mtDNA. The short branches and lack of statistical support for any

of these relationships suggest lineage sorting might make resolving these relationships challenging. Unfortunately our pruned dataset, which removed nearly all missing data, does not clarify the unstable higher level relationships of Clupeiformes.

This study and previous studies on clupeiform systematics and biology suggest gene tree and genome discordance result from both incomplete lineage sorting and hybridization (Anderson, Karel, 2007; Jolly *et al.*, 2011; Bloom, Lovejoy, 2012). Hybridization can be difficult to detect (Holder *et al.*, 2001), and the extent that it has occurred in Clupeiformes

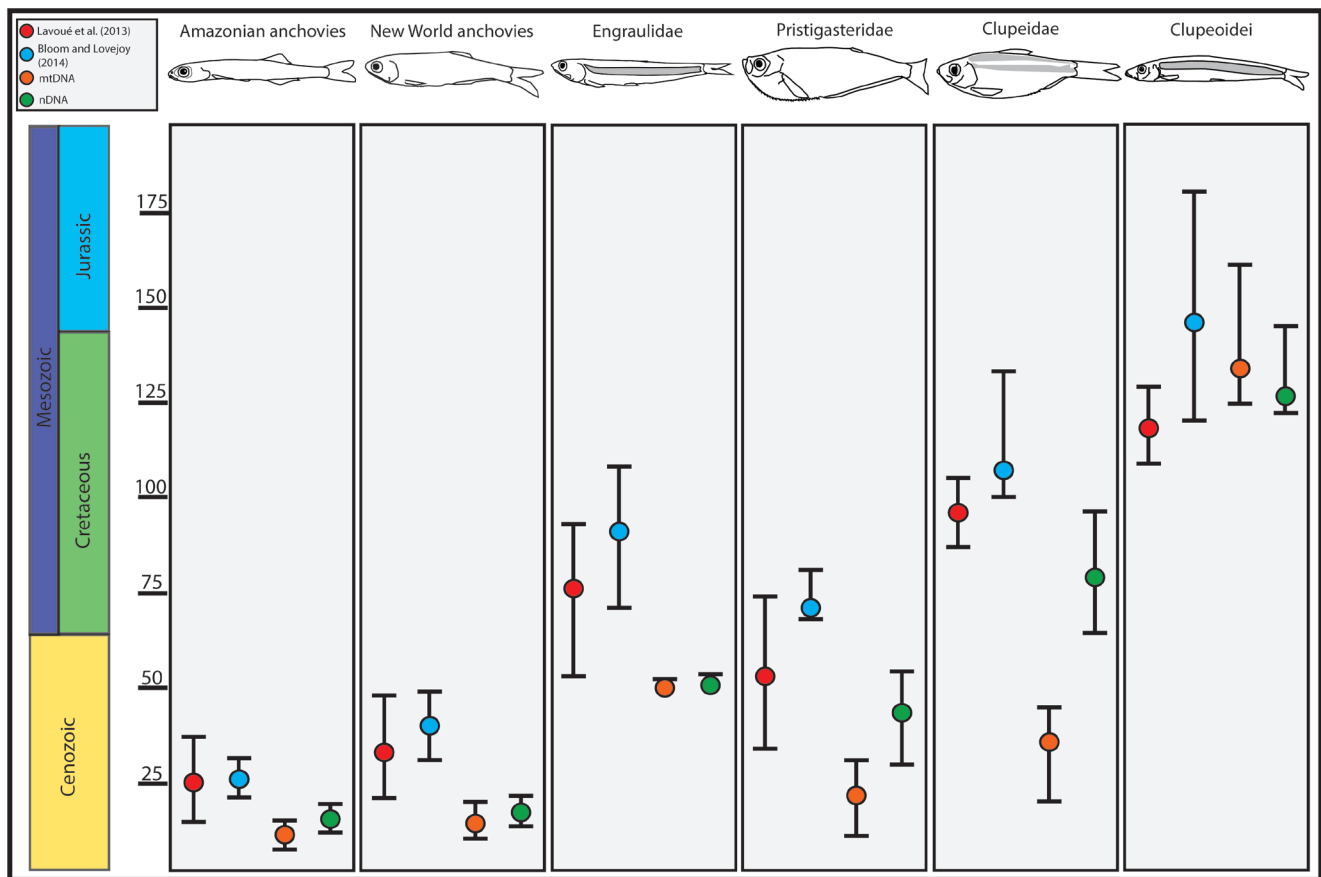


Fig. 4. Divergence time estimates for major clupeiform lineages estimated using nDNA and mtDNA separately by this study, mitochondrial genomes by Lavoué *et al.* (2013) and a combined mtDNA + nDNA dataset by Bloom, Lovejoy (2014). Time, in millions of years, is shown along the y-axis. Circles represent mean age estimates and whiskers delineate the 95% highest posterior density interval of divergence time estimates.

over macroevolutionary time is unclear. However, hybridization is common in fishes and there are instances of contemporary interspecific hybridization within marine and freshwater clupeiforms (Anderson, Karel, 2007; Jolly *et al.*, 2011; McBride *et al.*, 2014). Incomplete lineage sorting is most likely when there are large effective population sizes and short branch lengths (Pamilo, Nei, 1988). There are several regions of the clupeiform phylogeny with very short branches such as New World anchovies and shads (*Alosa* spp.). Consequently, future clupeiform molecular phylogenetics research should use large, multi-locus datasets to build upon our existing knowledge of clupeiform systematics that is based upon mtDNA datasets (Lavoué *et al.*, 2007, 2010, 2013, 2017a,b) and datasets containing small numbers of loci (Li, Orti, 2007; Bloom, Lovejoy, 2012, 2014). We conclude that analyzing individual data partitions is a necessary step in phylogenetic analyses, and that while relying only on mtDNA can be problematic, in combination with nDNA it remains a useful source of data for phylogenetic inference.

Spratelloidinae is currently either recognized as a subfamily of either Dussumieriidae or Clupeidae (Lavoué *et al.*, 2014). Our analyses found strong support for the recognition

of Spratelloidinae as a distinct lineage. Spratelloidinae did not form a monophyletic group with Clupeidae or the either of the two lineages of Dussumieriidae in any of our analyses. Bayesian mtDNA and nDNA analyses placed Spratelloidinae sister to all remaining Clupeidae and mtDNA analyses placed Spratelloidinae with our outgroup. Previous molecular studies have also recovered Spratelloidinae as a distinct group and have most consistently placed this lineage sister to all remaining Clupeoidei (Lavoué *et al.*, 2013, 2014; Bloom, Lovejoy, 2014; Egan *et al.*, 2018). As such, including Spratelloidinae in either Dussumieriidae or Clupeidae renders these taxa as non-monophyletic. To resolve this taxonomic issue, we propose elevating Spratelloidinae from a subfamily to the level of family and changing the taxon name to Spratelloididae.

Divergence times. Our separate mtDNA- and nDNA-based estimates of divergence times suggest that conflicting results reported by previous mtDNA (Lavoué *et al.*, 2013, 2017b) and mtDNA+nDNA (Bloom, Lovejoy, 2014) studies on clupeiform divergence times are partially due to the different genes in these datasets. We estimated divergence times for major clupeiform clades that were nearly always young

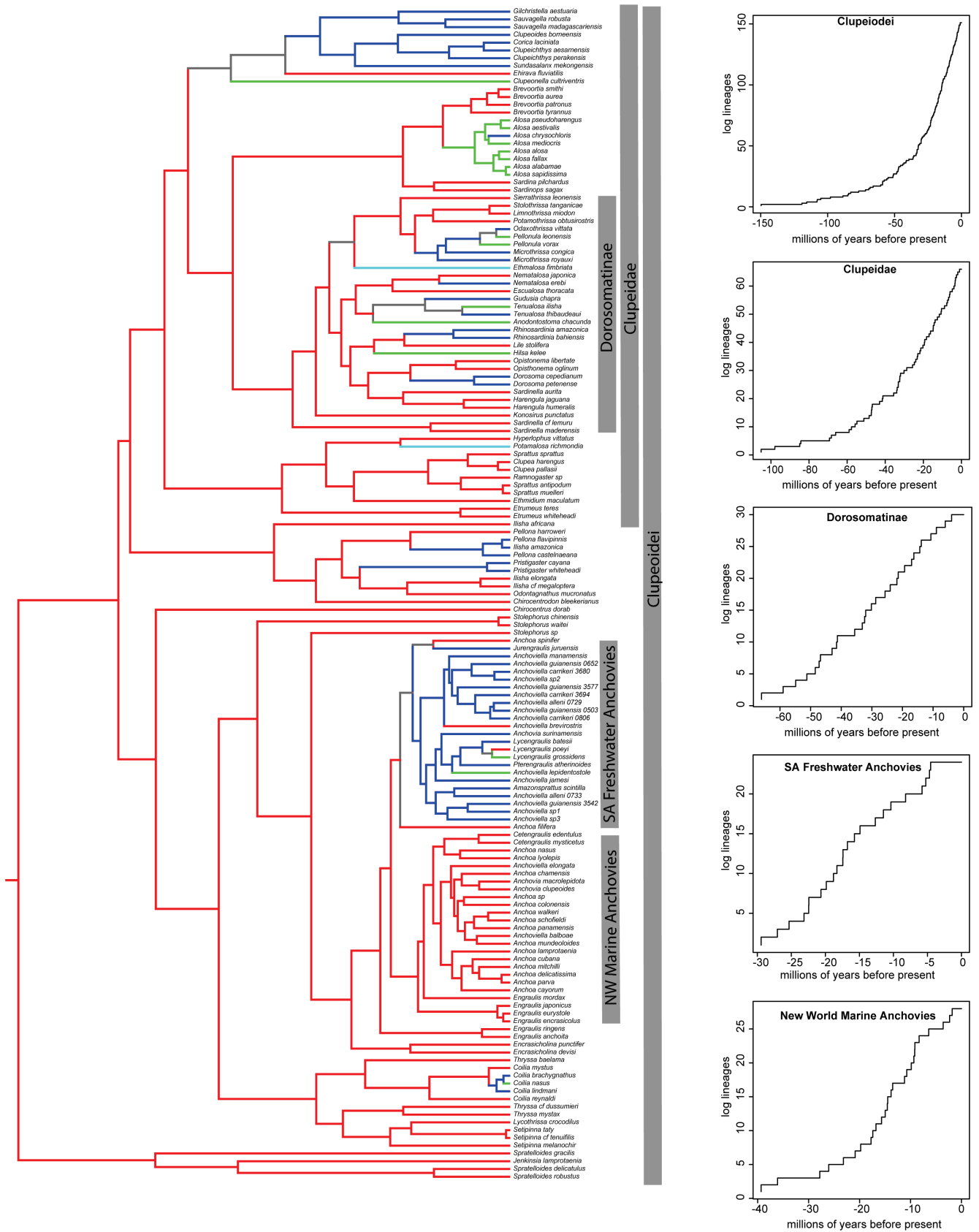


Fig. 5. The left panel shows a phylogeny of Clupeiformes showing ancestral reconstructions of marine (red), freshwater (blue), anadromous (green) and catadromous (light blue) lineages from Bloom, Lovejoy (2014). The right panel shows lineage through time plots for select clades, which are indicated by grey bars.

ger than previous studies. In contrast with previous studies (Zheng *et al.*, 2011; Mulcahy *et al.*, 2012; Dornburg *et al.*, 2014) our mtDNA dataset did not systematically estimate older divergence times than the nDNA dataset (Fig. 4). This result and may be because the nuclear genes in our dataset appear to evolve at a rate similar to the mitochondrial loci. This finding is encouraging because it suggests that including mtDNA in divergence time analyses does not always lead to spurious age estimates. Age estimates for the MRCA of Pristigasteridae and Clupeidae were particularly different between the mtDNA and nDNA datasets. Differences between in our age estimates from previous studies are likely also due to the exclusion of fossils used in previous studies because of uncertainty regarding their placement (Lavoué *et al.*, 2017b): †*Gasteroclupea branisai*, previously used to set a minimum age for the MRCA of Pristigasteridae, †*Nolfia riachuelensis*, previously used to set a minimum age for the MRCA of Clupeidae (Bloom, Lovejoy, 2014), and †*Lecceclupea ehiravaensis* to set a minimum age for the MRCA of the clupeid lineage Ehiravini (*Gilchristella* + *Clupeichthys*; Lavoué *et al.*, 2013).

Habitat transitions circumvent ecological limits across large phylogenetic scales. Over time, ecological limits are thought to slow net diversification rates within clades via resource competition (Rabosky, 2009a,b, 2013; Moen, Morlon, 2014). Our results show that some trans-marine/freshwater lineages have likely circumvented ecological limits on clade diversity, while ecological limits have potentially slowed net diversification rates within several other clupeiform clades. For example, diversification patterns across all Clupeoidei and Clupeidae, which are widespread clades containing dozens of marine/freshwater transitions, showed constant diversification rates over time. However, both geographically restricted trans-marine/freshwater clades and entirely marine or freshwater clades showed diversity dependent diversification. For example, two neotropical clades, the new world marine anchovies and South American freshwater anchovies (largely freshwater, but includes at least two reversals to marine) showed diversity dependent diversification. Dorosomatinae, a largely tropical and subtropical trans-marine/freshwater clade showed declining lineage accumulation and the best-fit model was diversity dependent diversification. This suggests that on regional or local scales ecological limits are regulating diversity patterns in Clupeoidei, even in trans-marine/freshwater clades. A large study on diversification in mammals found that medium to large clades (hundreds of species) experienced diversification slow-downs, while small clades experienced constant rates (Machac *et al.*, 2018). The relationship between clade size and diversification dynamics detected in mammals is consistent with theory on ecological limits (Rabosky, 2009b, 2013), and numerous empirical studies (Rabosky, Glor, 2010; Kennedy *et al.*, 2012; López-Fernández *et al.*, 2013; Weir, Mursleen, 2013), which taken with our findings, suggests that trans-marine/freshwater clades may

have unique diversification dynamics. Determining the relationship between phylogenetic and geographic scales and diversification dynamics will be critical step towards understanding the role of ecological limits on diversification (Rabosky, Hurlbert, 2015; Graham *et al.*, 2018).

The link between ecological diversity and diversification rates may be widespread (Ezard *et al.*, 2011). Several other trans-marine/freshwater clades also exhibit constant diversification rates across large phylogenetic scales, such as new world silversides (Atherinopsidae; Bloom *et al.*, 2013) and ariid catfishes (Ariidae; Betancur-R *et al.*, 2012). Both clades experienced repeated transitions between marine and freshwaters and also show patterns of constant diversification over time. Hydrophilid beetles have transitioned between aquatic and terrestrial habitats repeatedly and have a positive relationship between clade age and species richness, which is evidence for constant diversification rates (Bloom *et al.*, 2014). Derryberry *et al.* (2011) found that species rich and ecologically diverse woodcreepers and ovenbirds show constant diversification rates across continental scales and concluded these clades have not reached their ecological limit. Guinot, Cavin (2015) found that freshwater fishes have yet to reach ecological limits, while marine fishes fit an equilibrium model, which suggests in some cases, non-equilibrium dynamics in ecologically diverse clades may be driven by one or few habitats. Together, our results and these studies suggest that clades that undergo evolutionary habitat transitions may avoid ecological limits over deep time.

Most studies to date have detected slowdowns in diversification rates over time (Rabosky, 2013; Moen, Morlon, 2014). Clades revealed to be undergoing constant diversification rates were often young clades that likely have not reached their ecological limit (*e.g.* Weir, 2006). Assuming ecological limits exist, why do Clupeoidei and other trans-marine/freshwater clades show constant diversification rates across large phylogenetic scales? In aquatic communities ecological limits may operate separately in marine and freshwaters because most organisms are restricted to either marine or freshwater habitats (Lee, Bell, 1999; Bloom, Lovejoy, 2012; Grosberg *et al.*, 2012), with near complete turnover in community composition between adjacent marine and freshwaters (Winemiller, Leslie, 1992). Thus a lineage may reach saturation in either a marine or freshwater habitat, but have minimal influence on the dynamics in the other habitat. If a lineage undergoes a macroevolutionary transition to an adjacent habitat (*e.g.* marine to freshwater, or vice versa), it may experience reduced competition, release from pathogens, new ecological opportunity for diversification, or newly imposed ecological limits (Rabosky, 2009a,b, 2013; Ricklefs, 2011; Moen, Morlon, 2014). Repeated exposure to ecological opportunity may maintain diversification rates by increasing the probability of speciation (Barraclough *et al.*, 1998). Alternatively, trans-marine/freshwater clades may have an affinity to avoid extinction. Trans-marine/freshwater clades may have an intrinsic ability to respond to dynamic environmental conditions over geological timesca-

les (Bamber, Henderson, 1988; Lee, Bell, 1999), which may cause extinction in lineages that are unable to respond to environmental change (Jablonski, 2008; Ezard *et al.*, 2011; Moen, Morlon, 2014).

Alternatives to ecological limits. We have argued that lineages that undergo habitat transitions avoid ecological limits on species richness and play an important role in structuring diversity patterns. However, this relies on the assumption that ecological limits exist and regulate clade diversity. Recently, Harmon, Harrison (2015) proposed that species diversity is unbounded by ecological limits at both local and continental scales, and varies dynamically over space and time. It is also possible that diversity patterns are driven by extrinsic factors, such as environmental change and paleogeography rather than diversity dependent interactions (Ezard *et al.*, 2011; Moen, Morlon, 2014). If ecological limits are not regulating species diversity then constant diversification rates are not explained by circumventing ecological limits, but rather driven by neutral processes. Allopatric speciation is the primary mode of speciation in fishes (Coyne, Orr, 2004). The process of vicariance and subsequent speciation generally divides a widespread ancestor into daughter lineages with smaller ranges sizes. As diversification of a clade proceeds, species with smaller ranges are less likely to experience vicariant events, resulting in a slow-down in diversification (Moen, Morlon, 2014). Habitat transitions may result in allopatric speciation; if so, repeated transitions will result in constant diversification over time. Thus if speciation is linked to habitat transitions then ecological limits need not be invoked to explain constant diversification in trans-marine/freshwater clades.

Phylogenetic comparative methods offer a powerful suite of tools to determine the processes underlying diversity patterns. The Neotropics is a biodiversity hotspot, particularly for fishes. However, few studies have tested for ecological limits on clades of Neotropical fishes. This is likely because phylogenetic relationships of many Neotropical clades remain uncertain. However, in this study we demonstrate that Clupeiformes, a clade with unresolved systematics at multiple phylogenetic levels, can be a useful model for testing macroevolutionary hypotheses. Specifically we provide evidence that trans-marine/freshwater clades avoid ecological limits on clade diversity across higher phylogenetic and geographic scales. The neotropics are rich with trans-marine/freshwater clades. If habitat transitions circumvent ecological limits or drive non-diversity dependent processes that yield constant diversification rates, understanding these processes will be important for understanding the origins and maintenance of this region's tremendous diversity. More generally, habitat transitions across other environmental gradients (*e.g.* benthic *vs.* pelagic, riverine *vs.* lacustrine) may also circumvent ecological limits on clade diversification. We hope this study motivates future studies on lineage diversification and trait evolution to assess the role of ecological limits across Neotropical fishes.

Acknowledgments

DDB. was supported by start up funds and a FRACAA award from Western Michigan University. J.P.E. received financial support from a National Science Foundation Graduate Research Fellowship (00039202). DDB. thanks Hernan Lopez-Fernandez for over a decade of discussing neotropical fish evolution, which greatly improved this manuscript. We thank Ricardo Betancur-R, Marina Loeb and an anonymous reviewer for providing helpful feedback that improved this manuscript. M. Loeb graciously helped us revise our Portuguese abstract. We are grateful for the opportunity to participate in the II International Symposium on Phylogeny and Classification of Neotropical Fishes.

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Submitted March 13, 2018

Accepted August 9, 2018 by Brian Sidlauskas