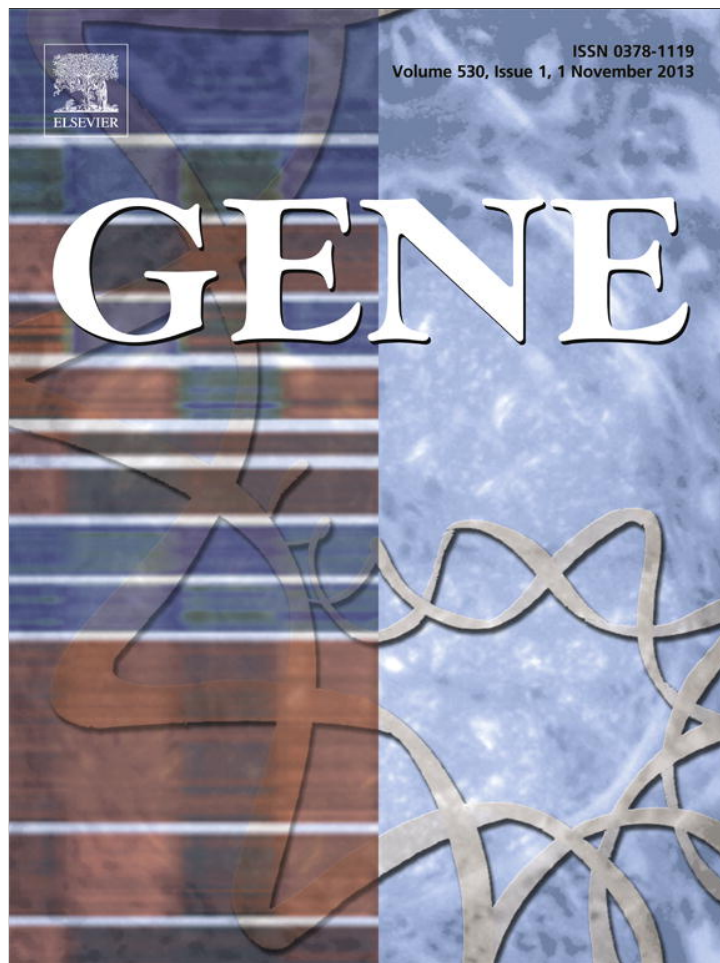


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# Pike and salmon as sister taxa: Detailed intraclade resolution and divergence time estimation of Esociformes + Salmoniformes based on whole mitochondrial genome sequences

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## ABSTRACT

The increasing number of taxa and loci in molecular phylogenetic studies of basal euteleosts has brought stability in a controversial area. A key emerging aspect to these studies is a sister Esociformes (pike) and Salmoniformes (salmon) relationship. We evaluate mitochondrial genome support for a sister Esociformes and Salmoniformes hypothesis by surveying many potential outgroups for these taxa, employing multiple phylogenetic approaches, and utilizing a thorough sampling scheme. Secondly, we conduct a simultaneous divergence time estimation and phylogenetic inference in a Bayesian framework with fossil calibrations focusing on relationships within Esociformes + Salmoniformes. Our dataset supports a sister relationship between Esociformes and Salmoniformes; however the nearest relatives of Esociformes + Salmoniformes are inconsistent among analyses. Within the order Esociformes, we advocate for a single family, Esocidae. Subfamily relationships within Salmonidae are poorly supported as Salmoninae sister to Thymallinae + Coregoninae.

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## 1. Introduction

A consensus on the evolutionary relationships among basal euteleost lineages is emerging as a result of increasing numbers of both taxa and loci represented in molecular datasets. Results from these studies are beginning to identify stable patterns of relationships between a set of lineages whose affinities have been controversial area since the inception of Euteleostei (Greenwood et al., 1966). Protacanthopterygian (Rosen, 1974) relationships have been examined in multiple phylogenetic studies relying on evidence from morphological and molecular traits (Begle, 1991, 1992; Diogo et al., 2008; Fink, 1984; Fink and Weitzman, 1982; Ishiguro et al., 2003; Johnson and Patterson, 1996; Lauder and Liem, 1983; López et al., 2004; Patterson, 1994; Rosen,

1982; Sanford, 1990; Williams, 1987). And, while a sister group relationship between Salmoniformes and Esociformes is broadly supported by analyses based on the suspensorium and associated musculature (Williams, 1987; Wilson and Williams, 2010), mitochondrial genome data (Ishiguro et al., 2003; Li et al., 2010), nuclear sequence data (López et al., 2004; Near et al., 2012; Santini et al., 2009), and combined nuclear and mitochondrial data (Burrige et al., 2012; López et al., 2004), the placement of the Esociformes + Salmoniformes clade among basal euteleost lineages remains problematic.

Mitochondrial genome (mitogenome) sequences from 33 teleost species provide evidence for a sister group relationship between esociforms and salmoniforms; however these two lineages were only represented with one species each in that analysis (Ishiguro et al., 2003). In this study, we expand the sampling of protacanthopterygians to 93 species with the addition of five newly determined mitogenome sequences and a targeted selection of previously published sequences designed to help test existing ideas on basal euteleost relationships. Specifically, we determined mitogenome sequences from two salmoniform and three esociform species. Increased taxon sampling is known to improve phylogenetic inference (Hedtke et al., 2006; Hillis, 1998; Hillis et al., 2003; Pollock et al., 2002), and to enhance the ability to infer macroevolutionary processes from a phylogenetic tree (Heath et al., 2008).

Our goals are to test possible placements of the Esociformes + Salmoniformes clade among basal euteleost lineages and to generate a hypothesis of intra-ordinal relationships within the Esociformes

*Abbreviations:* CAT-GTR, Dirichlet process mixture of profiles of equilibrium frequencies combined with general exchange rates; DNA, deoxyribonucleic acid; ESS, effective sample size; HKY, Hasegawa, Kishino, and Yano model of nucleotide evolution; HPD, highest posterior density; I, proportion of invariant sites;  $\Gamma$ , four-category gamma distributed rate variation among sites; GTR, general time reversible model of nucleotide evolution; Ma, million years ago; MCMC, Markov chain Monte Carlo; MRA, most recent common ancestor; ML, maximum likelihood; n, nucleotide; ND6, NADH-ubiquinone oxidoreductase chain 6; PCR, polymerase chain reaction; RNA, ribonucleic acid; rRNA, ribosomal RNA; RY, purine and pyrimidine recoding; TE, tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetic acid; tRNA, transfer RNA.

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**Table 1**

Taxa included in this study and corresponding GenBank accession numbers. Classification follows Nelson (2006) except Esociformes follow López et al. (2004).

		Order or suborder	Family or subfamily	Organism	Accession number
Division	Teleostei				
Subdivision	Osteoglossomorpha	Hiodontiformes	Hiodontidae	<i>Hiodon alosoides</i>	AP004356
		Osteoglossiformes	Osteoglossidae	<i>Osteoglossum bicirrhosum</i>	AB043025
Subdivision	Elopomorpha	Elopiiformes	Elopiidae	<i>Elops hawaiiensis</i>	AB051070
		Anguilliformes	Anguillidae	<i>Anguilla japonica</i>	AB038556
Subdivision	Ostarioclupeomorpha	Clupeiformes	Denticipitidae	<i>Denticeps clupeoides</i>	AP007276
			Pristigasteridae	<i>Pellona flavipinnis</i>	AP009619
			Engraulidae	<i>Engraulis japonicus</i>	AB040676
			Chirocentridae	<i>Chirocentrus dorab</i>	AP006229
			Clupeidae	<i>Sardinops melanostictus</i>	AB032554
		Gonorynchiformes	Chanidae	<i>Chanos chanos</i>	AB054133
			Gonorynchidae	<i>Gonorynchus greyi</i>	AB054134
				<i>Gonorynchus abbreviatus</i>	AP009402
			Kneriidae	<i>Cromeria nilotica</i>	AP011560
				<i>Grasseichthys gabonensis</i>	AP007277
				<i>Kneria sp.</i>	AP007278
				<i>Parakneria cameronensis</i>	AP007279
			Phractolaemidae	<i>Phractolaemus ansorgii</i>	AP007280
		Cypriniformes	Cyprinidae	<i>Cyprinus carpio</i>	AP009047
				<i>Sarcocheilichthys variegatus</i>	AB054124
			Gyrinocheilidae	<i>Gyrinocheilus aymonieri</i>	AB242164
			Catostomidae	<i>Catostomus commersonii</i>	AB127394
			Cobitidae	<i>Lefua echigonia</i>	AB054126
		Characiformes	Balitoridae	<i>Schistura balteata</i>	AB242172
			Distichontidae	<i>Distichodus sexfasciatus</i>	AB070242
			Chilodontidae	<i>Chilodus punctatus</i>	AP011984
			Alestiidae	<i>Phenacogrammus interruptus</i>	AB054129
			Characidae	<i>Chalceus macrolepidotus</i>	AB054130
			Lebiasinidae	<i>Lebiasina astrigata</i>	AP011995
		Siluriformes	Diplomystoidea	<i>Diplomystes nahuelbutaensis</i>	AP012011
			Amphiliidae	<i>Amphilius sp.</i>	AP012002
			Callichthyidae	<i>Corydoras rabauti</i>	AB054128
			Loricariidae	<i>Pterygoplichthys disjunctivus</i>	AP012021
			Bagridae	<i>Pseudobagrus tokiensis</i>	AB054127
			Pimelodidae	<i>Pimelodus pictus</i>	AP012019
		Gymnotiformes	Gymnotidae	<i>Electrophorus electricus</i>	AP011978
			Hypopomidae	<i>Brachyhypopomus pinnicaudatus</i>	AP011570
			Sternopygidae	<i>Eigenmannia virescens</i>	AB054131
			Apterontidae	<i>Apterontus albifrons</i>	AB054132
Subdivision	Euteleostei				
Superorder	Protacanthopterygii	Argentiformes	Argentiniidae	<i>Glossanodon semifasciatus</i>	AP004105
		Argentinoidei	Opisthoproctidae	<i>Opisthoproctus soleatus</i>	AP004110
			Microstomatidae	<i>Nansenia ardesiaca</i>	AP004106
			Bathylagidae	<i>Bathylagus ochotensis</i>	AP004101
		Alepocephaloidei	Platyroctidae	<i>Platyroctes apus</i>	AP004107
				<i>Maulisia maui</i>	AP009404
			Alepocephalidae	<i>Alepocephalus tenebrosus</i>	AP004100
				<i>Narceus stomias</i>	AP009585
		Osmeriformes	Osmeridae	<i>Plecoglossus altivelis</i>	AB047553
		Osmeroidei		<i>Salangichthys microdon</i>	AP004109
				<i>Salanx ariakensis</i>	AP006231
			Retropinnidae	<i>Retropinna retropinna</i>	AP004108
			Galaxiidae	<i>Galaxias maculatus</i>	AP004104
				<i>Galaxiella nigrostriata</i>	AP006853
				<i>Lepidogalaxias salamandroides</i>	HM106490
		Salmoniformes	Salmonidae	<i>Coregonus lavaretus</i>	AB034824
			Coregoninae	<i>Prosopium cylindraceum</i>	This study.
				<i>Thymallus arcticus</i>	FJ872559
			Thymallinae	<i>Thymallus thymallus</i>	FJ853655
			Salmoninae	<i>Hucho bleekeri</i>	HM804473
				<i>Oncorhynchus clarkii</i>	AY886762
				<i>Oncorhynchus gorbuscha</i>	EF455489
				<i>Oncorhynchus kisutch</i>	EF126369
				<i>Oncorhynchus masou</i>	DQ864465
				<i>Oncorhynchus mykiss</i>	DQ288268
				<i>Oncorhynchus nerka</i>	EF055889
				<i>Oncorhynchus tshawytscha</i>	AF392054
				<i>Parahucho perryi</i>	This study

Table 1 (continued)

	Order or suborder	Family or subfamily	Organism	Accession number
			<i>Salmo salar</i>	U12143
			<i>Salmo trutta</i>	AM910409
			<i>Salvelinus alpinus</i>	AF154851
			<i>Salvelinus fontinalis</i>	AF154850
	Esociformes	Umbridae	<i>Umbra pygmaea</i>	This study.
		Esocidae	<i>Dallia pectoralis</i>	AP004102
			<i>Esox lucius</i>	AP004103
			<i>Esox niger</i>	This study.
			<i>Novumbra hubbsi</i>	This study.
Neoteleostei				
	Stomiiformes	Diplophidae	<i>Diplophos taenia</i>	AB034825
		Gonostomidae	<i>Sigmops gracile</i>	AB016274
		Stomiidae	<i>Chauliodus sloani</i>	AP002915
	Ateleopodiformes	Ateleopodidae	<i>Ijimaia doefleini</i>	AP002917
			<i>Ateleopus japonicus</i>	AP002916
	Aulopiformes	Synodontidae	<i>Harpadon microchir</i>	AP002919
			<i>Saurida undosquamis</i>	AP002920
		Chlorophthalmidae	<i>Chlorophthalmus agassizi</i>	AP002918
	Myctophiformes	Neoscopelidae	<i>Neoscopelus microchir</i>	AP002921
		Myctophidae	<i>Myctophum affine</i>	AP002922
			<i>Diaphus splendidus</i>	AP002923
	Lampridiformes	Lampridae	<i>Lampris guttatus</i>	AP002924
		Trachipteridae	<i>Trachipterus trachipterus</i>	AP002925
			<i>Zu cristatus</i>	AP002926
Superorder	Polymixiomorpha			
		Polymixiiformes	Polymixiidae	<i>Polymixia japonica</i>
Superorder	Paracanthopterygii			
		Gadiformes	Gadidae	<i>Lota lota</i>
Superorder	Acanthopterygii			
		Beryciformes	Holocentridae	<i>Myripristis berndti</i>
		Perciformes	Zanclidae	<i>Zanclus cornutus</i>
		Pleuronectiformes	Pleuronectidae	<i>Hippoglossus stenolepis</i>
		Tetraodontiformes	Tetraodontidae	<i>Takifugu rubripes</i>
		Stephanoberyciformes	Cetomimidae	<i>Cetostoma regani</i>
		Zeiformes	Zeidae	<i>Zeus faber</i>

and Salmoniformes. Within esociforms we test whether the family Umbridae (Nelson, 2006) is a monophyletic group containing the genera *Umbra*, *Novumbra*, and *Dallia*; and within salmoniforms we examine alternative arrangements of the relationships between the three salmonid subfamilies and among the genera of Salmoninae. Finally, we also estimate the timing of major cladogenetic events in the history of the esociform + salmoniform group. We use a maximum likelihood (ML) framework to infer a mitochondrial genome phylogeny for the 93 taxa considered here and a Bayesian-based joint tree inference and divergence time estimation procedure on a 34 species taxonomic subset to focus on the intra-ordinal history of the esociform + salmoniform clade.

## 2. Materials and methods

### 2.1. Taxonomic sampling

Sampling for novel mitogenome sequence determinations targeted unrepresented lineages within Salmoniformes and Esociformes (Table 1). Species were selected to divide long branches to reduce possible long branch generated artifacts in the phylogenetic inference (Hillis, 1998). We newly determined five mitogenomes for this study: *Novumbra hubbsi*, *Umbra pygmaea*, and *Esox niger* (Esociformes) and *Prosopium cylindraceum* and *Parahucho perryi* (Salmoniformes). The newly determined mitogenome sequences are available on GenBank as accessions AP013046–AP013050. Additional mitogenome sequences were obtained from GenBank guided by the goal of testing the placement of Salmoniformes and Esociformes among basal euteleost lineages.

### 2.2. DNA extraction, PCR amplification and sequencing

We extracted DNA from ethanol-preserved fin clips using QiaGen DNEasy or QIAamp tissue kits following the manufacturer's instructions.

Mitogenome sequences were determined using a combination of long and short PCR amplifications (Miya and Nishida, 1999). Briefly, whole mitogenomes of target organisms were first amplified using long PCR (Cheng et al., 1994). Long PCR amplicons were diluted in TE buffer and used as templates for a series of short PCRs that produced a set of overlapping fragments covering the mitochondrial genome. Short PCR products were purified using the ExoSAP protocol and sequenced with ABI Big-Dye v1.1 chemistry on an ABI 3130XL automated sequencer.

### 2.3. DNA sequence assembly and alignment

DNA sequences were examined and edited using EditView version 1.0.1, AutoAssembler version 2.1 and DNASIS ver. 3.2. Existing mitogenome sequences were retrieved from GenBank (Benson et al., 2005). Protein coding and RNA loci were extracted from GenBank flatfiles with GenBankStrip.pl versions 2.0 (Bininda-Emonds, 2005). Two separate alignments were generated. An alignment with 93 species including thirteen salmoniform and five esociform representatives was generated to estimate the phylogenetic placement of Esociformes and Salmoniformes among basal Euteleost lineages. To generate this alignment, protein-coding genes were each imported into MacClade version 4.08 (Maddison and Maddison, 2000) and translated to amino acids. The amino acid sequences were aligned using MAFFT version 6.814 (Katoh and Toh, 2008; Katoh et al., 2002) then merged with nucleotide sequence files in MacClade and gaps removed to produce a statistically consistent alignment. The mitochondrial gene NADH-ubiquinone oxidoreductase chain 6 (ND6) was excluded due to heterogeneous base composition. 12S and 16S ribosomal RNA (rRNA) sequences were aligned using ProAlign version 5.3 (Löytynoja and Milinkovitch, 2003) with a 70% posterior probability limit on site homology. Additional gaps were removed by hand from the rRNA alignments, which were subsequently concatenated. Transfer RNA (tRNA) sequences were individually aligned with MUSCLE version 3.8.31 (Edgar, 2004a, 2004b),



**Table 2**  
Fossil calibrations used in divergence time estimation. Taxonomic order to which calibration point is assigned, taxa included in the analysis of which the most recent common ancestor (MRCA) is dated, and priors assigned to the calibration point are shown. Additional information and source details are also included.

Taxonomic group	Dating MRCA of which taxa	Prior			95%	Source and additional information
		Offset	Log (mean)	Log (SD)		
Esociformes	Esocoidae	85.0	1.0	1.00	99.1	From Masstrichian of Cretaceous (Wilson et al., 1992).
	<i>Esox</i> and <i>Kenoza</i> subgenera of <i>Esox</i>	42.0	1.0	0.85	53.0	The first record of <i>Kenoza</i> from the Eocene (Grande, 1999).
Salmoniformes	All Salmonine taxa	51.8	1.618	0.80	70.6	<i>Eosalmo driftwoodensis</i> as stem salmonine (Wilson, 1977; Wilson and Williams, 1992). Calibrated as Near et al. (2012).
Aulopiformes	<i>Saurida</i> , <i>Diaphus</i> , and <i>Lampris</i>	96.0	1.5	1.20	128.3	Santini et al. (2009).
Lampriformes	<i>Diaphus</i> and <i>Lampris</i>	70.0	1.2	1.32	99.1	Santini et al. (2009).

then imported into Mesquite version 2.71 (Maddison and Maddison, 2009) and edited by hand.

A second alignment for evaluating intraordinal relationships and divergence times was generated by excluding some outgroup taxa and increasing Esociformes + Salmoniformes representation. The reduced alignment consisting of five esociforms, seventeen salmoniforms and twelve euteleost outgroups (34 taxa) was generated following the alignment procedure described above.

#### 2.4. Phylogenetic placement of Esociformes + Salmoniformes

Phylogenetic placement of Salmoniformes and Esociformes was estimated by a maximum likelihood (ML) search implemented in RAxML version 7.3.0 (Stamatakis, 2006). The general time reversible model (GTR) with a four-category gamma distributed rate variation among sites ( $\Gamma$ ) model of DNA evolution was used. 1000 bootstrap replicates were used to evaluate the support for different aspects of the optimal topology. In this analysis, third codon position sites were recoded as purines and pyrimidines (RY) to reduce the potential effect of substitution saturation on phylogenetic inference. This coding scheme is noted as  $1_{-n}2_n3_{RY}R_nT_n$ , where subscripts indicate RY or nucleotide (n) coding for each category of sites, numbers denote codon positions for sites within protein-coding regions, R refers to ribosomal RNA coding sites and T indicates transfer RNA coding sites. To characterize the effect of variations in mutation rate among sites, the CAT-GTR model (Lartillot and Philippe, 2004) as implemented in PhyloBayes version 3.3b (Lartillot et al., 2009) was used on the 93-taxon alignment with three coding schemes ( $1_n2_nR_nT_n$ ,  $1_n2_n3_nR_nT_n$ ,  $1_n2_n3_{RY}R_nT_n$ ).

#### 2.5. Simultaneous Bayesian phylogenetic inference and divergence time estimation

We performed Bayesian phylogenetic inference and divergence time estimation on the 34-taxon four dataset with five data partitions ( $1_n2_nR_nT_n$ ), and a Bayesian relaxed clock with uncorrelated lognormal rate heterogeneity as implemented in BEAST version 1.7.4 (Drummond et al., 2006, 2012). An input tree was generated from a partitioned alignment using the HKY +  $\Gamma$  model of sequence evolution with a proportion of invariant sites. We calibrated the root of the tree using the known appearance of euteleost and ostariophysan fish in the fossil record at a minimum of 149.85 million years ago (Ma). Strong evidence exists to constrain this node at 165.2 Ma (Benton et al., 2009). A strict molecular clock was used to generate the input tree with a Markov chain Monte Carlo (MCMC) chain of 50 million generations sampled every 5000 generations. We applied a 10% burnin and used Tracer v 1.5 to examine MCMC output and quality of parameter sampling (Drummond et al., 2012). Subsequently the input tree was used to initialize the divergence time analysis. We used lognormal fossil constraint distributions which produce more conservative estimates of divergence times due to the underlying assumption that the fossil record can inform maximum and minimum divergences of some clades in the analysis (Lavoué et al., 2011).

For each calibration point, a fossil record was used as a hard minimum bound, with upper bounds considered and applied on a case by case basis (Table 2). Fossil aulopiforms provide well supported constraints with both stem and crown representations, constraining the age of this node to between 96 and 128 Ma (Benton, 1993; Kriwet, 2003; Santini et al., 2009). Based on age of crown representatives, the origin of Acanthomorpha and Beryciformes was constrained to between 70 and 99 Ma, respectively (Benton, 1993; Dirk, 2004).

The following fossil calibrations specific to the Esociformes and Salmoniformes were used: (1) *Esteesox*, a stem esociform from the late Cretaceous (Wilson et al., 1992) as the minimum age of Esociformes at 85 Ma; and (2) *Esox kronneri*, the first record of the subgenus *Kenoza* from the late early Eocene (Grande, 1999) as a minimum bound for the divergence between *Esox lucius* and *E. niger* at 42 Ma. The genus *Novumbra* was present by the Oligocene (Cavender, 1969) however, because this first appearance is much more recent than the evidence for *Kenoza*, it was not used as a minimum bound for the divergence of *Novumbra* from *Esox*. The taxonomic affinities of older fossils associated with Umbridae such as *Boltyshia* from the Ypresian (Benton, 1993; Syševskaâ and Daniltšenko, 1975) remain poorly resolved (Nelson, 2006). Due to that uncertainty, those records are not included in this analysis.

The earliest definitive fossil evidence of a salmoniform comes from fossils of *Eosalmo driftwoodensis* from middle Eocene lacustrine deposits (Wilson, 1977). *Eosalmo* is considered a stem salmonin (Wilson and Li, 1999; Wilson and Williams, 1992). We constrained the minimum date of the origin of Salmonidae at 51.8 Ma (Greenwood et al., 2005; Near et al., 2012). Alternate placements for this fossil exist, such as dating the most recent common ancestor of Coregoninae and Salmoninae (Crête-Lafrenière et al., 2012). Therefore the effects of the *Eosalmo* calibration were examined through an alternative analysis with this calibration point omitted.

For the four data partitions ( $1_n2_nR_nT_n$ ) we used the GTR +  $\Gamma$  + I model of nucleotide evolution. Three independent runs of 100 million generations sampled every 10000 generations were generated. After verifying adequate sampling (ESS > 200) and convergence with Tracer, we applied a 10% burnin and combined the tree files with LogCombiner. Finally, we used TreeAnnotator to calculate a maximum clade credibility tree, mean values of divergence times, posterior probabilities, and bounds for the 95% highest posterior density (HPD) interval.

### 3. Results

#### 3.1. Sequencing

We sequenced complete or nearly complete mitochondrial genomes of *P. cylindraceum*, *P. perryi*, *N. hubbsi*, *Umbra krameri*, and *E. niger*. The mitochondrial control regions contained repeating motifs and were not sequenced completely in some taxa. Gene content and order in the newly determined mitochondrial genomes follow the standard arrangement found in most vertebrates.

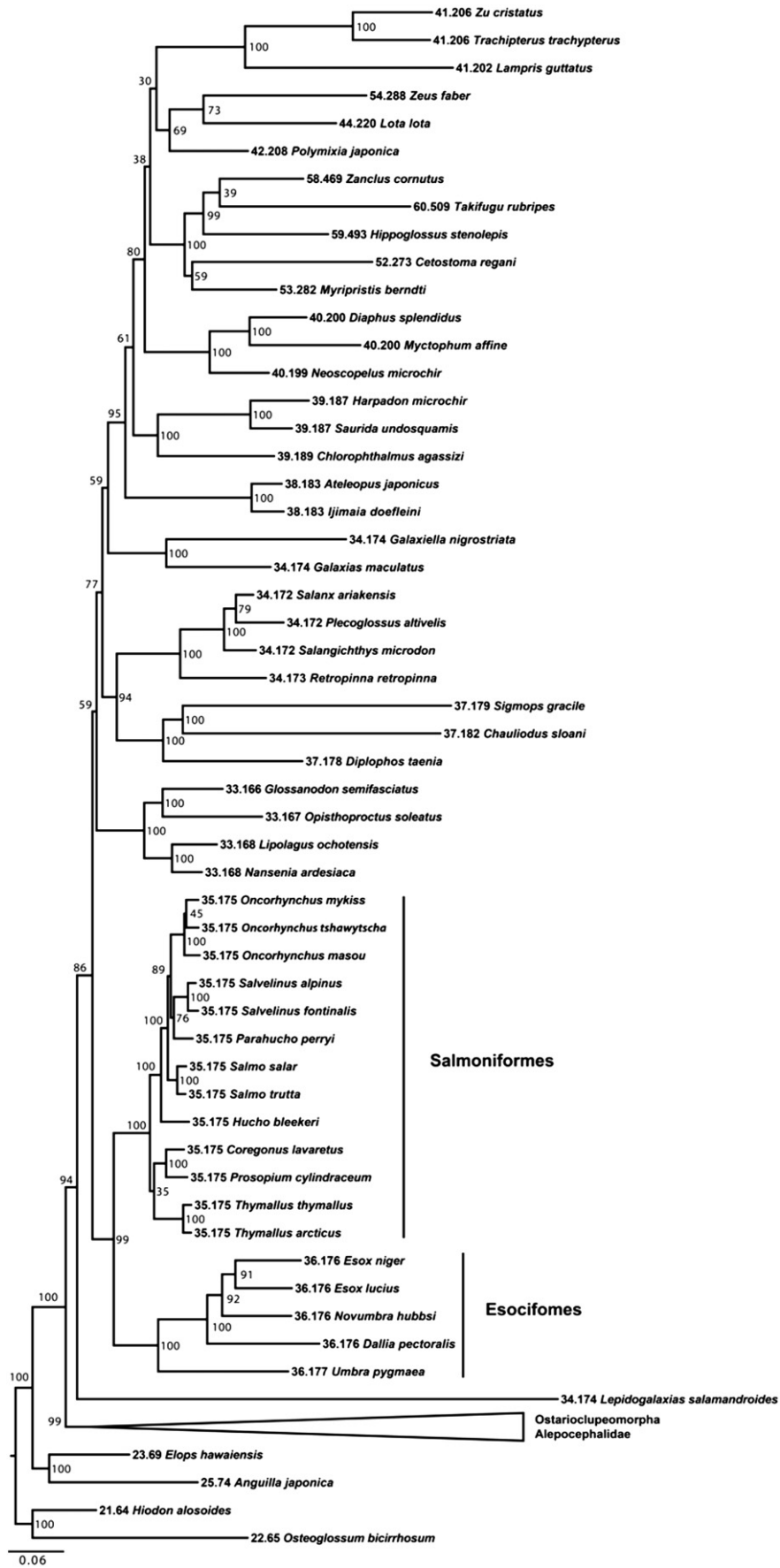
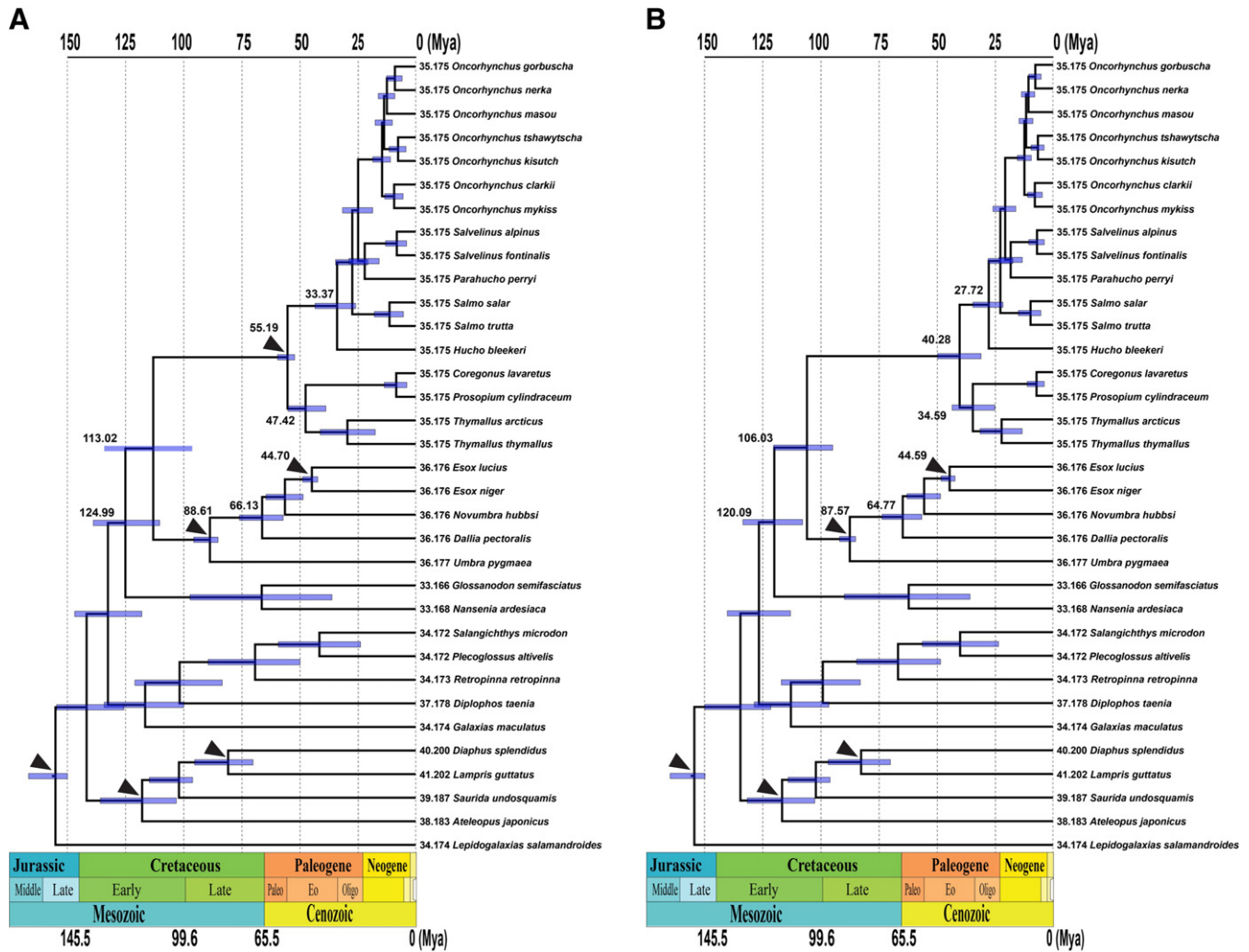


Fig. 1. Maximum-likelihood (ML) phylogenetic tree of 93 actinopterygian taxa. Analysis is based on a 1<sub>n</sub>2<sub>n</sub>R<sub>n</sub>T<sub>n</sub> data partition and coding scheme (details in text). Bootstrap values are shown as node labels.



**Fig. 2.** Fossil calibrated phylogenies of Salmoniformes + Esociformes and twelve outgroup taxa generated using a Bayesian relaxed clock in BEAST. 95% HPD intervals are shown as blue bars at nodes. Fig. 2A contains *Eosalmo* as a calibration point for the origin of Salmonidae. In Fig. 2B, a tree is shown in which there is no calibration in salmonid lineages but the other calibration points are the same. Calibration points are indicated by black triangles.

### 3.2. Esociform and salmoniform phylogenetic relationships

The Esociformes + Salmoniformes clade is supported in the ML topology using the  $1_{n2_n}R_nT_n$  and  $1_{n2_n3_{RY}R_nT_n}$  codings with bootstrap values of 99 and 100 (Fig. 1). *Lepidogalaxias salamandroides* as the most basal Euteleost is supported with a bootstrap value of 93 and 98

using  $1_{n2_n}R_nT_n$  and  $1_{n2_n3_{RY}R_nT_n}$  codings, respectively. Among esociforms, *Umbra* is sister group to a clade formed by the remaining three esociform genera, and *Novumbra* and *Esox* are sister lineages. Among salmoniforms, there is weak support for a sister relationship between Coregoninae and Thymallinae under the  $1_{n2_n}R_nT_n$  coding scheme (35% bootstrap). In contrast, with the  $1_{n2_n3_{RY}R_nT_n}$  scheme,

**Table 3**  
Posterior characteristics of selected nodes from a simultaneous Bayesian divergence time and tree search conducted in BEAST. The results from both the inclusion and exclusion of *Eosalmo* as a calibration point are presented. The time to most recent common ancestor of taxa is present as a mean with 95% highest probability density (HPD) upper and lower bounds. The posterior probability (posterior prob.) of the particular node is also included.

Dating MRCA of which taxa	<i>Eosalmo</i> calibration included				<i>Eosalmo</i> calibration excluded			
	Posterior				Posterior			
	Mean	95% low	95% high	Posterior prob.	Mean 95%	Low 95%	High	Posterior prob.
Esociformes + Salmoniformes and Argentiformes	124.99	110.81	138.81	0.65	120.09	107.92	133.63	0.87
All Esociformes and Salmoniformes	113.02	96.24	134.11	1.00	106.03	94.93	120.40	1.00
All Esociformes	88.61	85.09	95.57	1.00	87.57	85.10	92.02	1.00
Esocidae	66.13	57.11	75.88	1.00	64.77	56.49	73.68	1.00
<i>Novumbra</i> + <i>Esox</i>	56.31	48.48	64.44	1.00	55.56	48.52	62.94	1.00
All Salmoniformes	55.19	52.16	59.47	1.00	40.28	31.05	49.80	1.00
Thymallinae and Coregoninae	47.42	38.68	54.97	1.00	34.59	25.07	43.52	1.00
Coregoninae ( <i>Prosopium</i> and <i>Coregonus</i> )	29.40	17.43	41.13	1.00	22.18	13.33	31.44	1.00
Salmoninae	33.87	52.16	59.47	1.00	27.72	21.58	34.55	1.00
<i>Oncorhynchus</i>	14.49	10.88	18.50	1.00	12.36	8.71	14.67	1.00

the Thymallinae + Salmoninae clade is strongly supported (100% bootstrap). Convergence occurred in PhyloBayes using CAT-GTR only when third codon position sites were excluded ( $1_n2_nR_nT_n$ ), and not under any coding schemes that included those sites. In the PhyloBayes analysis, a posterior probability of 0.99 is assigned to the Esociformes + Salmoniformes clade. The topology: (*L. salamandroides*, ((Esociformes + Salmoniformes), (remaining euteleosts))) was supported by this analysis. Strong support for this branching pattern is observed with a posterior probability of 0.96 for the placement of *L. salamandroides*, 1.00 for support of Esociformes + Salmoniformes, and 0.99 for the Esociformes + Salmoniformes as sister clade to all other euteleosts.

### 3.3. Intraordinal relationships and divergence time estimation

The divergence time estimation analysis based on the 34 species alignment with the *Eosalmo* calibration point included yields a divergence time for the Esociformes + Salmoniformes from other euteleost lineages of 124.99 Ma (Fig. 2A, Table 3). The divergence between Esociformes and Salmoniformes is estimated to be 113.02 Ma. As in all other analyses, the Esociformes + Salmoniformes clade is strongly supported (1.00 posterior probability). The mean divergence estimate between *Umbra* and the *Esox* + *Novumbra* + *Dallia* clade is 88.61 Ma. Monophyly of both esociforms (1.00 posterior probability) and the *Esox* + *Novumbra* + *Dallia* clade are strongly supported (1.00 posterior probability). Major salmonid lineages originate within the last 55.19 million years, with a sister Thymallinae and Coregoninae relationship strongly supported (1.00 posterior probability). The estimated divergence between Coregoninae and Thymallinae is 47.42 Ma. The age of Salmoninae is estimated to be 33.87 Ma.

Removing the *Eosalmo* calibration point produced a divergence time of Salmoniformes + Esociformes from other euteleost lineages of 120.09 Ma and a divergence between Esociformes + Salmoniformes of 106.03 Ma (Fig. 2B, Table 3). The mean estimated age for time to most recent common ancestor of salmonids is 40.28 Ma. Thymallinae and Coregoninae are strongly supported as sister taxa (1.00 posterior probability) with a mean estimated divergence time of 34.59 Ma. The origin of Salmoninae is estimated to be 27.72 Ma.

## 4. Discussion

### 4.1. Phylogenetic placement of the Esociformes + Salmoniformes

Results of both full and reduced taxon set analyses reported here further strengthen the case for a sister group relationship between esociforms and salmoniforms (López et al., 2000, 2004). All our analyses invariably support a sister relationship of Esociformes and Salmoniformes. Among the euteleosts, the placement of *Lepidogalaxias* as the sister group of all other euteleost is in agreement with mitogenomic (Li et al., 2010), combined nuclear and mitochondrial data (Burrige et al., 2012), and with multilocus nuclear data (Near et al., 2012). We recover five clades of Euteleosts (excluding *Lepidogalaxias*) with high support: Esociformes + Salmoniformes, Argentiformes, Osmeriformes + Stomiiformes, Galaxiids, and the neoteleosts. Relationships among these five clades are unstable in our analyses, and consequently so is the sister group of the Esociformes + Salmoniformes. The sister of Esociformes + Salmoniformes is inferred to be all remaining euteleost fishes (less *Lepidogalaxias*) in this study with a 93 taxa  $1_n2_nR_nT_n$  data scheme analyzed under both ML and Bayesian frameworks. A similar relationship was demonstrated by Burrige et al. (2012). However, under ML and using a  $1_n2_n3_nR_nT_n$  coding scheme for that same taxon set results in Esociformes + Salmoniformes sister to a clade of Osmeriformes + Stomiiformes and Argentiformes. In the simultaneous Bayesian divergence time estimation and phylogenetic inference of a 34-taxon  $1_n2_nR_nT_n$  alignment, Esociformes + Salmoniformes is sister to the Argentiformes without strong support (posterior probability of

0.65 or 0.85). Stronger support for a sister relationship of Argentiformes to the Esociformes + Salmoniformes was found by Li et al. (2010) and Near et al. (2012).

### 4.2. Relationships within Esociformes and Salmoniformes

Among esociforms, all our analyses support the (*Umbra*, (*Dallia*, (*Novumbra*, *Esox*))) topology with a monophyletic *Esox* previously advanced based on molecular evidence (Burrige et al., 2012; Grande et al., 2004; López et al., 2004). This hypothesis is incongruent with the morphology based hypothesis (e.g. Wilson and Veilleux, 1982) that serves as the basis of currently accepted classification schemes for esociform taxa, but is in agreement with the morphological hypothesis of Wilson and Williams (2010). A classification congruent with relationships based on more recent morphological and molecular evidence would require alteration of the generic composition of the families Esocidae and Umbridae. We propose the redefinition of the Esocidae to be coextensive with the order Esociformes and abandonment of the Umbridae. If taxonomic classification is to reflect best understanding of phylogenetic relationships, no compelling argument remains to preserve current usage of the two esociform families.

Within salmoniforms, some of our analyses yield high support for a sister group relationship between Coregoninae and Thymallinae. Previous analyses based on mitogenomic sequences did not sample the genus *Prosopium*. Li et al. (2010) found with the inclusion of *Thymallus* and *Coregonus*, moderate support for this relationship with ML (76% bootstrap) and high support from Bayesian analyses (1.00 posterior probability). However, in another mitogenomic study with two representatives of *Thymallus*, Thymallinae was found to be more closely related to Salmoninae (Yasuike et al., 2010). Results of a single nuclear locus phylogenetic analysis of the Salmonidae support a Salmoninae + Thymallinae clade (Shedko et al., 2012). Alternatively, multilocus nuclear data and combined mitochondrial and nuclear data support Coregoninae + Salmoninae (Crête-Lafrenière et al., 2012; Near et al., 2012) or Thymallinae + Coregoninae (Burrige et al., 2012). The morphologically-based hypothesis of salmonid relationships (Sanford, 1990; Wilson and Williams, 2010) groups Thymallinae and Salmoninae in a clade that is sister group to the coregonins. If these relationships remain labile under more extensive trait and taxonomic sampling, the lack of agreement may prove to be the result of a rapid salmonid radiation into the three subfamilies.

### 4.3. Divergence time estimation

Living and fossil esociforms and salmoniforms are restricted to northern hemisphere landmasses. Given this distribution it is interesting to ask whether or not the timing of origin of the group or the orders coincides with key events in the evolution of the northern hemisphere geography. The 95% HPD interval for divergence between Esociformes + Salmoniformes and Argentiformes in our study is contained in the early Cretaceous. Our estimate of divergence time between Esociformes and Salmoniformes corresponds to the boundary between the Aptian and Albian of the Cretaceous (Walker and Geismann, 2009). Roughly, the 95% HPD for Esociformes and Salmoniformes divergence spans the younger half of the Early Cretaceous. During that period, the Atlantic Ocean was beginning to form and Eurasia and North America were well separated during the Early Cretaceous (Vullo et al., 2012). It is unlikely that the breakup of Laurasia was a vicariant event marking the split of esociforms and salmoniforms as it happened much earlier than our estimates of this divergence.

Both the ages of Esociformes and Salmoniformes are constrained by fossil calibration points in this study. The age of Salmonidae is constrained by the use of *Eosalmo* to date the MRCA of all three salmonid subfamilies. The characters which support the placement of *Eosalmo* as sister to extant salmonids also support a Thymallinae and Salmoninae sister relationship (Wilson and Li, 1999). The contradictory



molecular support for ((Coregoninae, Thymallinae), Salmoninae) indicates that an alternative placement of the fossil for calibration may be appropriate or that it should be excluded. The age of the origin of Salmonidae is forced by the *Eosalmo* calibration to be at least 51.8 Ma. Alternatively, if *Eosalmo* is used to constrain the age of a subfamily or two subfamilies, the estimated origin of Salmonidae will be older as in Crête-Lafrenière et al. (2012). By excluding the *Eosalmo* calibration point from the analysis we removed the assumptions required to place the fossil. The age of the Salmonidae was estimated to be 27.0% younger without a fossil calibration included for this group. Consequently, a more rapid diversification of salmonid lineages is inferred. Regardless of how the *Eosalmo* evidence is treated, the 95% HPD intervals for the time to MRCA of Esociformes and of Salmoniformes do not overlap and support a smaller time to MRCA for salmoniforms.

The Esociformes and Salmoniformes broadly overlap in distribution and have evolved under similar conditions. A key difference between the two orders is an ancestral polyploidization event in the salmoniform lineage. Salmoniforms also show markedly higher extant species diversity than esociforms. Our data and analyses suggest a markedly higher rate of species accumulation in salmoniforms. Future estimations of age of divergence in the two groups without relying on internal calibration points and incorporating nuclear data will be needed to more precisely compare their diversification rates.

## 5. Conclusion

Our results add to the emerging consensus on basal euteleost relationships in which Esociformes and Salmoniformes are sister lineages. Given the stability of this relationship, it may be appropriate at this time to identify an appropriate name for the Esociformes + Salmoniformes clade. A possible solution is to modify the limits of Salmoniformes to encompass both groups, abandon Esociformes and treat the two major lineages in the newly defined salmoniforms as the families Esocidae and Salmonidae. Regardless of nomenclatural choices, the relevant relationships reported here and elsewhere are backed by ample evidence and are consistently supported thus it is advisable to adopt a classification scheme that accurately reflects them. Concerning intraordinal relationships, our analyses support esociform monophyly and the generic inter-relationships proposed by López et al. (2000, 2004). Among salmoniforms, subfamily inter-relationships remain unresolved using mitogenomic data.

## Conflict of interest

None.

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