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A multi-locus molecular timescale for the origin and diversification of eels (Order: Anguilliformes)

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ABSTRACT

Anguilliformes are an ecologically diverse group of predominantly marine fishes whose members are easily recognized by their extremely elongate bodies, and universal lack of pelvic fins. Recent studies based on mitochondrial loci, including full mitogenomes, have called into question the monophyly of both the Anguilliformes, which appear to be paraphyletic without the inclusion of the Saccopharyngiformes (gulper eels and allies), as well as other more commonly known eel families (e.g., Congridae, Serrivomeridae). However, no study to date has investigated anguilliform interrelationships using nuclear loci. Here we present a new phylogenetic hypothesis for the Anguilliformes based on five markers (the nuclear loci Early Growth Hormone 3, Myosin Heavy Polypeptide 6 and Recombinase Activating Gene 1, as well as the mitochondrial genes Cytochrome b and Cytochrome Oxidase I). Our sampling spans 148 species and includes 19 of the 20 extant families of anguilliforms and saccopharyngiforms. Maximum likelihood analysis reveals that saccopharyngiform eels are deeply nested within the anguilliforms, and supports the non-monophyly of Congridae and Nettastomatidae, as well as that of Derichthyidae and Chlopsidae. Our analyses suggest that *Protanguilla* may be the sister group of the Synaphobranchidae, though the recent hypothesis that this species is the sister group to all other anguilliforms cannot be rejected. The molecular phylogeny, time-calibrated using a Bayesian relaxed clock approach and seven fossil calibration points, reveals a Late Cretaceous origin of this expanded anguilliform clade (stem age ~116 Ma, crown age ~99 Ma). Most major (family level) lineages originated between the end of the Cretaceous and Early Eocene, suggesting that anguilliform radiation may have been facilitated by the recovery of marine ecosystems following the KP extinction.

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1. Introduction

Anguilliformes, also known as "true eels", are an ecologically diverse group of predominantly marine fishes whose members are easily recognized by their extremely elongate bodies with reduced cross-sectional areas and universal lack of pelvic fins. Despite a conserved body plan, some anguilliforms exhibit high diversity in cranial morphology and prey capture mode (Mehta, 2009; Mehta and Wainwright, 2007). Traditionally anguilliforms, the largest order of elopomorphs, comprise three suborders (Robins, 1989; Nelson, 2006): the Anguilloidei (freshwater eels, Anguillidae; the mud eels, Heretenchelyidae; spaghetti eels, Moringuidae); the Congroidei (shorttail eels, Colocongridae; conger eels, Congridae; longneck eels, Derichthyidae; pike congers, Muraenesocidae; snipe eels, Nemichthyidae; duckbill eels, Nettastomatidae; snake eels and worm eels, Ophichthidae; sawtooth eels, Serrivomeridae; cutthroat eels, Synaphobranchidae); and the Muraenoidei (false morays, Chlopsidae; myroconger eels, Myrocongridae; and moray eels, Muraenidae). Anguilliforms have traditionally been thought to be closely related to the Saccopharyngiformes (gulper eels and allies), a group formed by four families of deep-sea fishes (bobtail snipe eels, Cyematidae; gulper eels, Eurypharyngidae; onejaw gulpers, Monognathidae; swallowers, Saccopharyngidae) (Nelson, 2006). Both morphological (Forey et al., 1996) and molecular studies of elopomorph interrelationships based on mitochondrial sequences (Wang et al., 2003; Inoue et al., 2004, 2010) suggested that Anguilliformes was paraphyletic without the inclusion of gulper eels. The recently described monotypic Protanguillidae (Johnson et al., 2012) brings the total

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diversity of Anguilliformes to 937 species spread across 20 families (Wiley and Johnson, 2010; Froese and Pauly, 2012).

Previous phylogenetic studies of anguilliform relationships based on morphological data alone (Forey, 1973; Nelson, 1973; Greenwood, 1977; Patterson and Rosen, 1977; Robins, 1989; Forey et al., 1996) have been unable to resolve the relationships among the three anguilliform suborders (Fig. 1a–c), while mitochondrial analyses (Wang et al., 2003; Lopez and Westneat, 2007; Inoue et al., 2010; Johnson et al., 2012) have revealed some of the largest anguilliform groups to be paraphyletic (Fig. 1d). Inoue et al. (2010) showed that the Congroidei are formed by three different lineages: the Synaphobranchidae; a polyphyletic Congridae + paraphyletic Derichthyidae + Colocongridae + Ophichthidae + Muraenesocidae + a paraphyletic Nettastomatidae; and a clade formed by Serrivomeridae and Nemichthyidae. The Muraenoidei were broken into two different subclades: the Muraenidae + Myrocongridae, being closely related to the anguilloid Heterencheleyidae, and the Chlopsidae, sister group to the large congroid clade. The traditional Anguilloidei were also found to be composed of at least three separate lineages: Anguillidae sister to the Serrivomeridae and Nemichthyidae congroid clade; the Moringuidae; and the Heterenchelyidae (Fig. 1d).

More recently, Johnson et al. (2012) erected the new family Protanguillidae on the basis of the species *Protanguilla palau*, a recently discovered, enigmatic anguilliform that presents a number of morphological traits that are absent in most living eels including collar-like gill openings, a pseudobranch, a premaxilla, unfused symplectic, and metapterygoid (Johnson et al., 2012). The position of this taxon is unclear. Although unconstrained maximum likelihood analyses consistently place *Protanguilla* as the sister group of the Synaphobranchidae (Johnson et al., 2012; Tang and Fielitz, 2013), competing topologies which place *Protanguilla* as the sister group to all other anguilliforms, as suggested by some morphological data, could not be rejected. Johnson et al. (2012) also produced the first time calibrated molecular phylogeny of anguilliforms, inferring a relatively ancient divergence for this group (~250–270 Ma for the stem and ~200–220 Ma for the crown of the anguilliforms). These ages are much older than the oldest

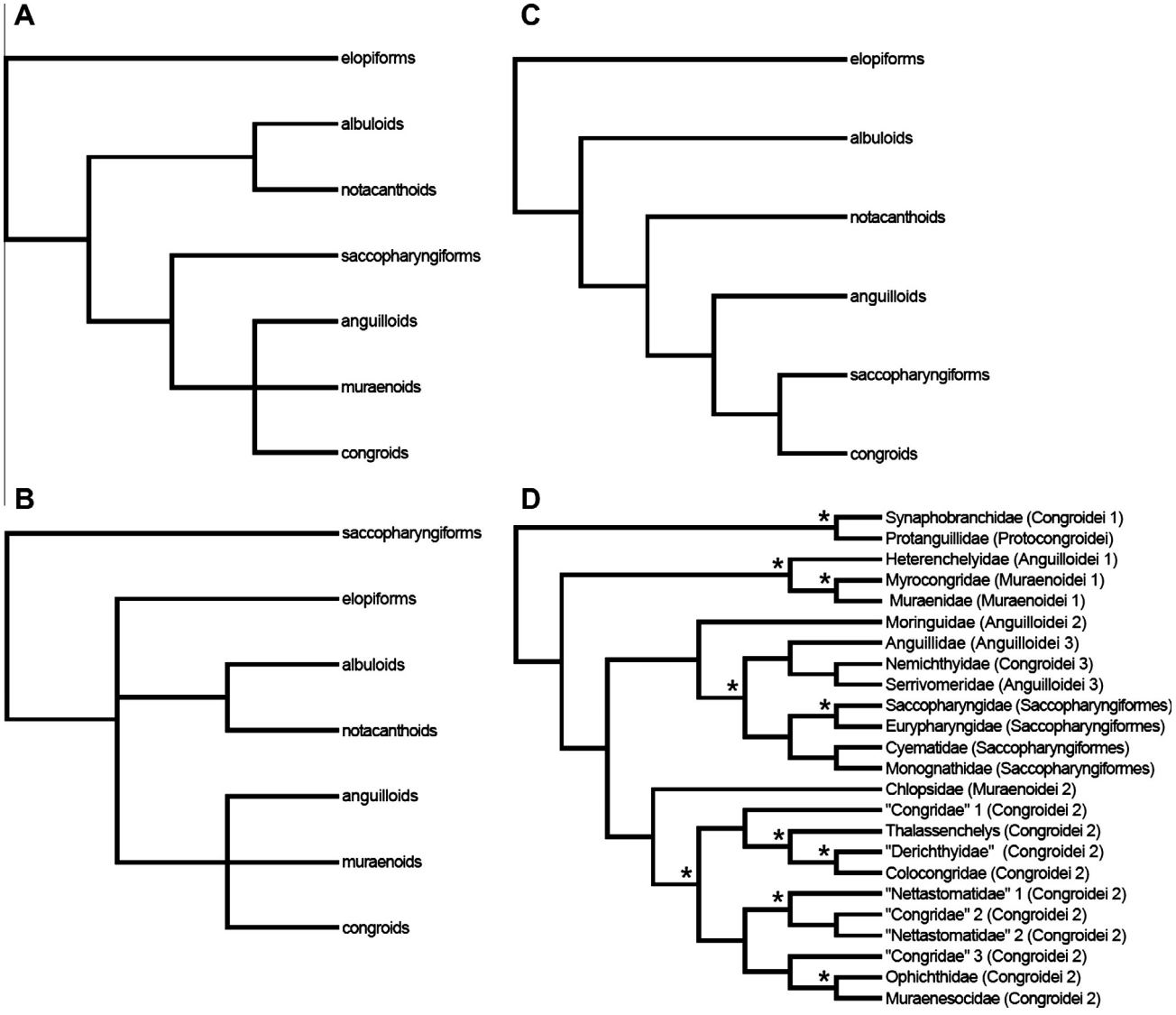

Fig. 1. (a–d) Outline of previous hypotheses of relationships among the major lineages of anguilliforms and their allies. (A) Morphological phylogeny of Patterson and Rosen (1977); (B) morphological phylogeny of Robins (1989); (C) combined morphological and molecular (*12S*, *16S*, *28S*) of Forey et al. (1996); mitogenomic phylogeny of Inoue et al. (2010) and Johnson et al. (2012). Star symbol above branches indicates nodes supported by bootstrap proportions (BSP) greater than 80% in all unconstrained analyses of both studies, other nodes all have BSP of 55% or less, with the exception of node supporting the clade of Congridae 3 + Ophichthidae + Muraenesocidae (BSP = 72% in Inoue et al., 2010).

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known anguilliform fossils (~98 Ma and ~83 Ma of the oldest stem and crown anguilliform fossils) and exceed those from recent large-scale time-calibrated teleost molecular phylogenies for the elopomorphs (Santini et al., 2009; Near et al., 2012; Betancur-R et al., 2013)

To date, no molecular systematic study of interfamilial relationships based on nuclear loci exists, and the largest molecular study published to date (Inoue et al., 2010) only included 56 anguilliform species (including 18 from the freshwater genus *Anguilla*). This sampling represents less than 6% of the extant anguilliform diversity, and likely limits our ability to infer the extent of non-monophyly among the anguilliform subclades currently recognized. Furthermore, no existing time-scale of anguilliforms integrates the rich fossil record of this group (Patterson, 1993; Young, 1993; Taverne, 2002, 2004; Carnevale, 2007), instead relying on the use of mostly external calibration points in addition to a single fossil anguilliform (Johnson et al., 2012).

To test the monophyly of anguilliform families, and to investigate the tempo of evolution of true eels and their allies, we assembled a large dataset of anguilliform tissues for molecular analyses. Here we present the result of a phylogenetic study based on five loci (three nuclear and two mitochondrial genes) and 148 species of anguilliforms and outgroups. We time-calibrate this new phylogeny with seven fossil calibrations (six anguilliforms and one outgroup), most of which have not been used previously in molecular clock studies, to present a new timescale for anguilliform evolution. Our new phylogeny corroborates earlier findings of non-monophyly for many traditional anguilliform groups, places *Protanguilla* as nested within crown anguilliforms, and reveals that eels have experienced a much more recent radiation than previously suggested.

2. Materials and methods

2.1. Sampling

Tissue samples for 113 species of anguilliforms and four outgroups were obtained through tissue loans from university or museum collections, purchases through the pet trade, and field collections by RSM or PCW (Table S1). We downloaded sequences from GenBank, mostly for the mitochondrial loci, for 30 additional anguilliform species, including *Protanguilla*, and one outgroup for which we were unable to obtain tissues. This increased the number of taxa in our study to 148 (143 anguilliforms and five outgroups: three Elopiformes, one Notacanthiformes, one Albuliformes) (Table S1).

2.2. DNA extraction, PCR amplification, and sequencing

DNA was extracted from muscle or fin clips previously stored in 70% ethanol using the Qiagen DNEasy kit (Qiagen, Valencia, CA, USA), following the protocol suggested by the manufacturer. Two mitochondrial genes, Cytochrome Oxidase I (*coxI*) and Cytochrome b (*Cytb*), and three nuclear genes, Early Growth Hormone 3 (*EGR3*), Myosin Heavy Polypeptide 6 (*myh6*) and Recombinase Activating Gene 1 (*Rag1*) were amplified using the polymerase chain reaction (PCR). One to two microliters of genomic template was used per 25-μL reaction, containing 5 μL of Go-Taq Flexi PCR buffer (Promega), 2 μL $MgCl_2$ (25 mM), 0.5 μL dNTPs (8 μM), 1.25 μL of each primer, and 0.125 μL of Promega GoTaq Flexi DNA polymerase (5 U/μl). Primers and PCR conditions were obtained from the literature (*coxI*: Ward et al., 2005; *Cytb*: Sevilla et al., 2007; *EGR3*: Chen et al., 2008; *myh6*: Li et al., 2007; *Rag1*: Lopez et al., 2004). We used ExoSap (Amersham Biosciences) to remove the excess dNTPs and unincorporated primers from the PCR products and cycle-sequenced the purified products using the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems) with each gene's primers used for amplification. The cycle sequencing protocol consisted of 25 cycles with a 10-s 94 °C denaturation, 5-s of 50 °C annealing, and a 4-min 60 °C extension stage. Sequencing was conducted at the Yale University DNA Analysis Facility using an ABI 3730xl DNA Genetic Analyzer (Applied Biosystems).

2.3. Phylogenetic analysis

The chromatograms were checked and assembled into contigs using Geneious 5.3 (Drummond et al., 2010). The consensus sequences for each individual gene were aligned in Geneious using the Muscle software (Edgar, 2004), and the alignments were subsequently checked by eye for accuracy. The sequences were trimmed to minimize missing characters, and our final data matrix consisted of 690 bp for *coxI*, 1128 bp for *Cytb*, 830 bp for *EGR3*, 806 bp for *myh6*, and 1331 bp for *Rag1*, for a total of 4785 nucleotides used in the concatenated analyses. All sequences generated for this study were deposited in GenBank (Table S1).

The individual gene datasets were subject to maximum likelihood analyses using RAxML (Stamatakis, 2006) in order to test for incongruence between the phylogenetic signal of the different loci and identify potentially contaminated or mislabeled sequences. We used jModelTest (Posada, 2008) to select the most appropriate model of sequence evolution based on AIC scores. The GTR + G model (with or without the proportion of invariant sites) was selected as the most appropriate for all loci. Due to the fact that the gamma parameter already takes into consideration the sites that are invariants (Stamatakis, 2006), we assigned a GTR + G model to each individual gene partition and ran 500 fast bootstrap replicates. The five individual gene datasets were then concatenated in Mesquite 2.75 (Maddison and Maddison, 2011), and the full dataset was partitioned by gene and subject to maximum likelihood analyses with RAxML (Stamatakis, 2006). We assigned to each partition its own GTR + G model, and ran 1000 fast bootstrap replicates.

We used MrBayes 3.2 (Ronquist et al., 2012) to perform Bayesian inference analyses; we partitioned the concatenated dataset by locus and assigned each gene the GTR + G model selected by JModelTest. We ran two analyses for 20 million generations, with four chains (one cold, three heated) and sampling every 1000 generations. Tracer 1.5 (Rambaut and Drummond, 2009) was used to check the trace files and ensure that the chains had reached convergence. The first 25% of trees was discarded as burnin and the remaining post-burnin trees were combined to obtain a 50% majority rule consensus tree.

2.4. Gene tree, species tree analysis

No phylogenetic analysis of eel relationships based on gene trees, species trees methods has so far been attempted, in order to test for contrasting signal among the various loci. For this study we estimated a species tree based on a reduced dataset of 125 species in *BEAST (Heled and Drummond, 2010) using the program BEAST 1.7.4 (Drummond et al., 2012) We reduced the amount of missing data by removing most species of the genus *Anguilla* as well as the moray eels from the Reece et al. (2010) study for which only GenBank mitochondrial sequences were available. Nuclear loci were partitioned individually, while the mitochondrial *cox1* and *Cytb* loci were linked; each partition was assigned the GTR + G model selected by jModelTest. We applied a local molecular clock model and a birth–death prior. The analysis was run for 100 million generations, sampling every 2500 generations. Chain mixing and convergence was ensured by inspecting trace files in Tracer 1.5 (Drummond and Rambaut, 2007). The first 20% of trees were

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removed as burnin, and we generated the species tree using TreeAnnotator (Drummond and Rambaut, 2007).

2.5. Timetree inference

We identified six nodes within anguilliforms, as well as the split between anguilliforms and elopiforms, that could be time-calibrated using the fossil record. Informations about the stratigraphic origin and phylogenetic placement of the fossils are provided in Appendix A. We assigned exponential priors to these constrained nodes and analyzed the concatenated dataset under a model of uncorrelated, lognormally distributed rates in BEAST 1.7.4 (Drummond and Rambaut, 2007), with a GTR + G model assigned to each individual gene partition. A birth–death prior was assigned to rates of cladogenesis. We ran analyses of 100 million each, with sampling every 10,000 generations discarding the first 10% as burnin. The trace files were checked in Tracer 1.5 (Drummond and Rambaut, 2007) to ensure that the chains had reached convergence. The remaining trees were then combined in LogCombiner, and a timetree was obtained with the use of TreeAnnotator (Drummond and Rambaut, 2007).

2.6. Testing the position of Protanguilla

To determine if improved taxonomic sampling in our study could help resolve the position of *Protanguilla* we performed an SH test (Shimodaira and Hasegawa, 1999) using RAxML (Stamatakis, 2006). We generated a constraint tree using RAxML which forced *Protanguilla* to be the sister group to all other anguilliforms (the preferred 'morphological' position of Johnson et al., 2012) and compared the likelihood of this tree to the best topology found in our unconstrained analysis.

3. Results

3.1. Phylogenetic analyses

Maximum likelihood (ML) and Bayesian analyses of the concatenated dataset produced fairly congruent topologies with regard to the relationships among the anguilliform families and their major subclades. For this reason we will present only the ML topology (Fig. 2), with indication of both the bootstrap proportion (BSP) over 50% and the posterior probability (PP) over 0.80 for each node. The major disagreements between the ML and the Bayesian analyses will also be discussed.

Both concatenated analyses support the sister group relationship between Notacanthiformes + Anguilliformes (Fig. 2). This clade is sister to Albuliformes, and subsequently to Elopiformes. Monophyly of the Anguilliformes is moderately supported (79% BSP, 0.80 PP). Within the Anguilliformes, four main clades can be identified (Fig. 2, clades A–D). Clade A comprising the monotypic *Protanguilla* + Synaphobranchidae, and it appears as the sister taxon to all remaining eels. While monophyly of the Synaphobranchidae is strongly supported (100 bsp), that of the *Protanguilla* + Synaphobranchidae clade is not (47% bsp in the RAxML analysis, but a PP of 0.92 in the BEAST analysis). In the Bayesian phylogeny *Protanguilla* appears to be the sister group to all Anguilliformes, rather than the Synaphobranchidae, but with low support (PP 0.79).

Clade B, poorly supported in the ML analysis (BSP < 50%) but strongly supported in the Bayesian tree (PP 0.99), includes a number of deep-sea forms, including the Saccopharyngiformes, which appear to be a polyphyletic group. Within this clade the Moringuidae are sister to a clade formed by Nemichthyidae + (Cyaematidae + Serrivomeridae) and all remaining Saccopharyngiformes (minus Cyaematidae) + Anguillidae. The relationships among all family-level lineages appear to be poorly supported in the ML tree, but receive high posterior probabilities in the Bayesian phylogeny (PP of 0.99 or higher). Clade B is sister to the remaining two major anguilliform subclades.

The first of these (Clade C) exhibits the greatest diversity both in terms of taxonomy (it includes over 50% of eel species), ecology, and distribution. It includes groups that are predominantly reef-associated, such as the Ophichthidae, as well as deep-sea lineages such as Derichthyidae and Nettastomatidae. Our tree shows that several traditional taxonomic groups are not valid: the family Congridae appears to be composed of at least three different lineages (one includes the rare *Scalanago*, a second is composed of the coastal benthic *Conger* as well as *Gnathophis*, a third includes the garden eels (*Ariosoma*, *Paraconger* and *Heteroconger*), and a fourth includes the genus *Rhyncoconger*. The monophyly of the Nettastomatidae is also not supported, with *Nettastoma* and the remaining nettastomatids originating within two different clades of "congrids". The "chlopsid" *Thalassenchelys* is also found in this clade, and does not appear to be closely related to the other members of its current family. While a number of strongly supported groups can be identified within this large clade (e.g., Muraenesocidae, Ophichthidae, Muraenesocidae + Ophichthidae, some subgroups of Congridae and Nettastomatidae, such as *Faciolella* + *Hoplunnis*, *Conger, Heteroconger* + *Paraconger*, all have BSP > 85 and PP > 0.95), the interrelationships among these lineages remain poorly supported in both likelihood and Bayesian trees.

The last anguilliform subclade (D) includes the remaining Chlopsidae (minus *Thalessenchelys*), the Heterenchelyidae, the Myrocongridae and the Muraenidae (these last two being sister taxa). While the monophyly of clade D is not well-supported (37% bsp), all subclades within it corresponding to families show high bootrstrap support (>90%). The extant morays are divided into two clades, currently assigned subfamilial status (Uropteryginae and Muraeninae); within the Muraenidae, the two large genera *Echidna* and *Gymnothorax* shows major non-monophyly.

The gene-tree, species-tree analysis with *BEAST of the reduced dataset of 125 species produced a rather different topology (Fig. S2). Only two major anguilliform clades are recovered. The first clade shows the Heterenchelyidae to be sister to Myrocongridae + Muraenidae (PP 0.82). The monophyly of this group, as well as that of the Muraenidae and Heterenchelyidae, are strongly supported (PP 1.0), but most of the relationships within it are not (PP < 0.80). The second clade includes most of the same named groups found in the concatenated analyses (Fig. 2), although many of the relationships among these differ. Protanguillidae still appear as the sister group to Synaphobranchidae, but this group is nested deeply within this second anguilliform clade. The Serrivomeridae and Nemichthyidae also appear as sister taxa, but they are then found to be the sister group to a monophyletic Saccopharyngiformes, while the Anguillidae are nested within a large subclade that includes two lineages of congrids (Congridae II and *Ariosoma*), the Nettastomatidae, the Ophichthidae and the Muraenesocidae. Most nodes in this species topology are poorly supported (PP < .80).

Although the *Protanguilla* was nested within anguilliforms in Bayesian and likelihood analyses, the SH test revealed that support for this position is weak. Forcing *Protanguilla* to be the sister group to all other anguilliforms produced a topology with an almost identical log-likelihood ($-\ln L_{best} = 121725.28$, $-\ln L_{constrained} = 121725.76$, $\Delta_{lnL} = -0.48$; $P > 0.99$).

3.2. Divergence time estimation

The topology of the BEAST analysis is highly congruent with that of the maximum likelihood tree, the only major difference being the "Chlopsidae", minus *Thalassenchelys*, being sister group to the clade containing Derichthyidae + Colocongridae +

Fig. 2. Maximum likelihood phylogeny of Anguilliformes based on analysis of the concatenated dataset. Values above branches indicate BSP over 50% (1000 replicates) and posterior probabilities (PP) from MrBayes analysis over 0.80 for clades recovered in the Bayesian tree.

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Congridae + Ophichthidae, and not to the clade formed by Heterenchylidae + Myrocongridae + Muraenidae (Fig. 3). The split between Elopiformes and the remaining elopomorphs is recovered to be 168 My old, while the split between the Albuliformes and the Anguilliformes + Notacanthiformes clade is 129 Ma (95% Highest Posterior Density: 106–153 Ma). The stem age for the Anguilliformes is 116 Ma (95% HPD: 98–134 Ma), while the crown anguilliform age is 99 Ma (95% HPD: 84–116 Ma). *Protanguilla* splits from its sister taxon, the Synaphobranchidae, in the Campanian, 84 Ma (95% HPD: 55–108 Ma), while the radiation of the synaphobranchids appears to be a much more recent event, dating to 33 Ma (95% HPD: 19–49 Ma). The crown age for clade B is 81 Ma (95% HPD: 67–96 Ma), that of clade C is 86 Ma (95% HPD: 74–101 Ma) and that of clade D is 76 Ma (95% HPD: 63–90 Ma), revealing a Late Cretaceous origin for all major anguilliform lineages. Most of the major families date to the early Paleogene: the crown Ophichthidae date to at least 57 Ma (95% HPD: 52–65 Ma), the Muraenidae to 60 Ma (95% HPD: 50–62 Ma), the Nettastomatidae + "Congridae I" subclade to 61 Ma (95% HPD: 49–73 Ma), and the *Thalassenchelys* + Derichthyidae + Colocongridae + *Scalanago* subclade to 59 Ma (95% HPD: 44–73 Ma). The diversity of the extant freshwater eels, while originating from a very ancient lineage with a stem age of 60 Ma (95% HPD: 48–73 Ma), only dates to 17 Ma (95% HPD: 12–23 Ma).

4. Discussion

4.1. Phylogenetic analysis

Our results based on the concatenated dataset analyses are largely in agreement with the previous molecular studies of anguilliform relationships regarding the number and composition of the major anguilliform lineages. All major clades retrieved in this study are congruent with the mitogenomic studies of Inoue et al. (2010) and Johnson et al. (2012) in their composition, although some of the relationships within these differ. Our greater taxonomic sampling corroborates the earlier finding that Anguilliformes, the three traditionally recognized suborders and several currently recognized eel families (e.g., Congridae) are not monophyletic (Inoue

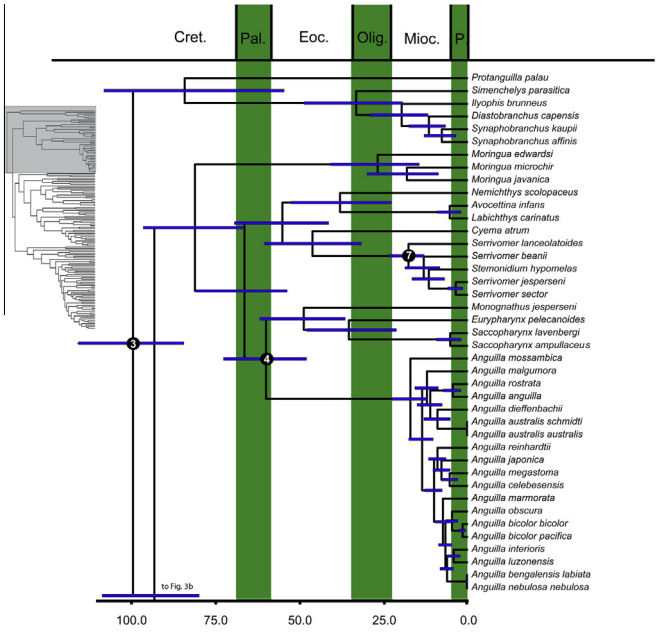

Fig. 3. Timetree of Anguilliformes obtained from Bayesian relaxed clock analysis of the concatenated dataset using seven fossil calibration points. Blue lines indicate 95% posterior probability densities (HPD) around point estimates. Numbers in black circles indicate fossil calibration points from Appendix A. Calibration points 1 (crown Elopiformes) and 2 (Anguilliformes vs Notacanthiformes) not shown. All nodes that were assigned a calibration point had their monophyly constrained in the BEAST analysis.

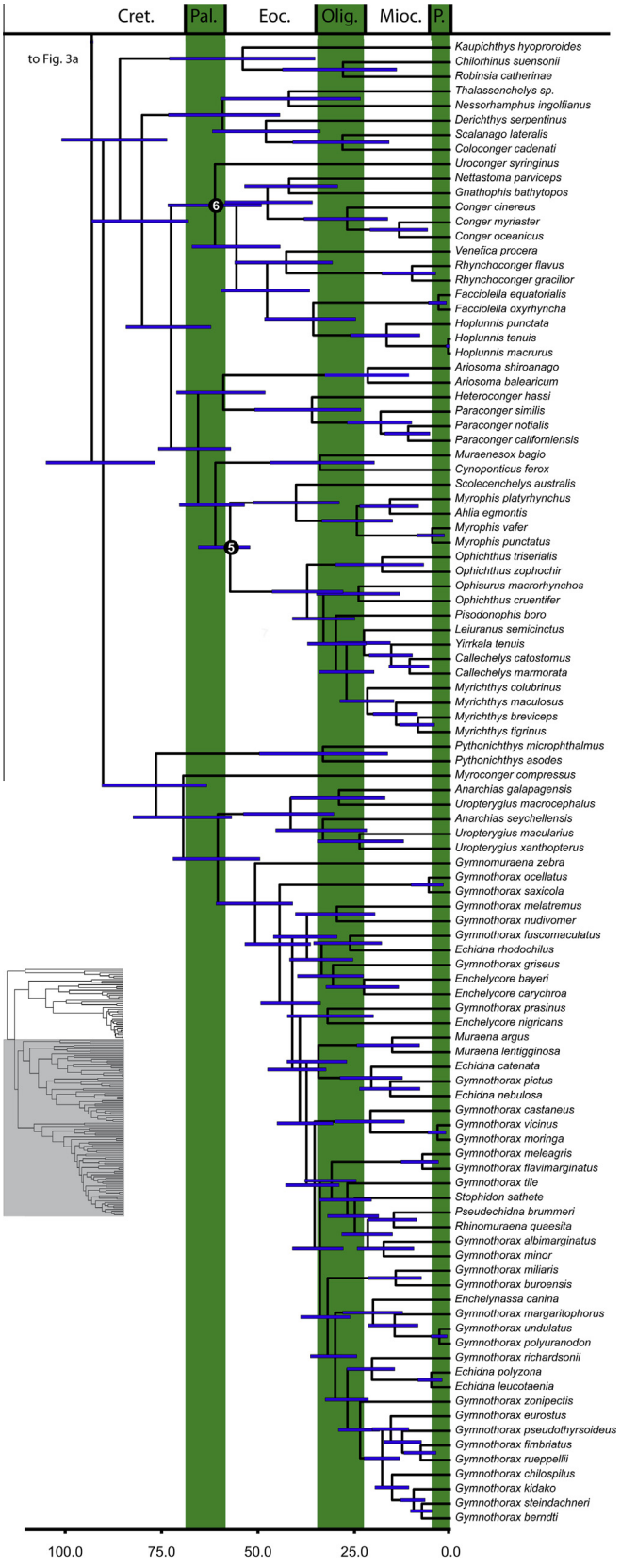

Fig. 3b.

et al., 2010). It also reveals non-monophyly of several other traditional taxonomic groups (Derichthyidae and Chlopsidae). Our inference of a sister group relationships between Anguillidae and Saccopharyngiformes (minus Cyematidae) conflicts with the mitogenomic data (Inoue et al., 2010; Johnson et al., 2012), which inferred serrivomerids and nemichthyids to be the sister groups to anguillids, albeit with BSP below 50%.

Our results support these of Reece et al. (2010) in identifying the Myrocongridae as the sister taxon to Muraenidae, which are then composed of two sister lineages, each currently having subfamilial status (Muraeninae and Uropteryginae). Within the Muraeninae we also corroborate Reece et al. (2010) findings of non-monophyly for the two large genera *Echidna* and *Gymnothorax*.

Our analyses revealed *Protanguilla* to be either the sister group of the Synaphobranchidae (Maximum likelihood and BEAST topologies, Figs. 2 and 3) or the sister group to all other Anguilliformes (MrBayes topology). The nested *Protanguilla* hypothesis received low bootstrap but high posterior support (Figs. 2 and 3) while the 'morphological' topology with *Protanguilla* as sister to other eels (i.e. Johnson et al., 2012) was poorly supported. Topological tests comparing our best ML topology to constrained 'morphological' topology reveal that there is almost no difference in the support of the data for either hypothesis ($\Delta_{\ln L} = -0.48$)

This uncertainty in the position of *Protanguilla* is similar to that seen in analyses presented by Johnson et al. (2012), suggesting that the expanded taxonomic sampling of our analysis does not strongly improve the ability of the molecular data to place this taxon. We note that the 'morphological' topology used here and in Johnson et al. (2012) is based upon the apparent distribution of several key morphological traits rather than an explicit phylogenetic analysis of morphological characters of living and fossil taxa. A novel analysis incorporating both morphological and molecular data from both fossil and living taxa (Belouze, 2002; Belouze et al., 2003a,b; Johnson et al., 2012) is needed to further clarify whether *Protanguilla* truly constitutes a 'living fossil' or an unusual synaphobranchid.

4.2. Divergence time estimation

Our Bayesian relaxed clock analysis reveals an early Cretaceous origin for the stem (116 Ma, Albian) and a Late Cretaceous origin for the crown (99 Ma, Cenomanian) of the anguilliforms. All the anguilliform lineages (roughly corresponding to eel families, or main components of these) date to the very end of the Cretaceous or the early to mid Paleogene (until the end of the Eocene, 33.9 Ma). This could potentially suggest that eels experienced a major radiation in the aftermath of the KP extinction, mirroring the explosive diversification already described in acanthomorph fishes (Friedman, 2010). In spite of the ancient age of many stems, several crown families appear to be the product of much more recent diversification dating to the Oligocene or Miocene (33.4–5.3 Ma). The young age of some crown subclades of deep-sea eels (e.g., Serrivomeridae, Synaphobranchidae) could be the result of recent recolonization of deep-sea waters following periods of anoxia (Hallam and Wignall, 1997). The recent radiation of freshwater eels, which appears to have colonized freshwater environments across all the major water basins (including the Atlantic and Pacific Oceans, as well as the Mediterranean Sea), in both the Northern and Southern hemispheres may have been strongly influenced by Miocene and Pliocene events such as the final closure of the Tethys Sea (~14 Ma) and the uplift of the Isthmus of Panama, that led to major rearrangements of the world's ocean currents.

Our BEAST timetree recovers much younger ages for anguilliform diversification than a recently published study based upon mtDNA (Johnson et al., 2012). For example, Johnson et al. (2012) inferred ages of 268 and 220 Ma, respectively, for the stem and crown anguilliforms (their Table S6), *vs* our ages of 116 and 99 Ma. Similarly, while we infer an age of 84 Ma for the split between *Protanguilla* and Synaphobranchidae, Johnson et al. (2012) infer an age of 199 Ma for this split in their unconstrained analyses (their Table S7, Tree 1). This discrepancy is likely caused by a number of factors. Mitochondrial loci tend to evolve at much faster rates than nuclear loci (Lukoschek et al., 2012), and divergence time analyses based upon them often produce ages for most fish lineages that are much older than these produced by studies based on nuclear markers (e.g., Hurley et al., 2007; Santini et al., 2009; Near et al., 2012). Furthermore, Johnson et al. (2012) study included mostly non-anguilliform calibration points. Our study includes a much larger number of fossil calibrations within the anguilliforms (6 vs 1), which should provide more reliable estimates of divergence times for the group. We also followed the published phylogenetic morphological studies and treated *Anguillavus quadripinnis* and *Anaethalion* as, respectively, a stem anguilliform (Belouze, 2002) and a stem elopomorph (Arratia, 2004), thus constraining the age of ancient splits in the anguilliform tree of life. In contrast, Johnson et al. (2012) used these fossils to constrain crown ages within anguilliform and elopomorphs despite the lack of explicit character evidence placing these fossils in the crown group. This difference in fossil constraint likely underlies the much older ages recovered in Johnson et al., 2012.

5. Conclusions

Our study represents the first attempt to infer the phylogeny of anguilliforms using nuclear loci, and to provide a timetree for this group using multiple anguilliform fossil calibration points. Our investigation corroborates previous findings based on mitogenomic datasets of the existence of four major anguilliform clades, and reveals non-monophyly of the anguilliforms (paraphyletic without the saccopharyngiforms), of the three traditional suborders (muraenoids are composed of two distinct lineages, while both anguilloids and congroids split into three lineages), and of several of the traditional families (Nelson, 2006). The position of *Protanguilla* remains unresolved with some analyses supporting a sister group relationship with synaphobranchids instead of the 'morphological' hypothesis of this taxon as the sister group to all other living eels. Support for several key nodes within the eel tree remains low. Our timetree reveals a Late Cretaceous origin of the crown anguilliforms and of the four major eel clades, most eel families had originated by the Eocene, even though a number experienced significant Miocene radiations (anguillids, serrivomerids).

Although our sampling has more than doubled the number of eel species included in studies published to date, we have sampled roughly 15% of extant diversity of this group. The large number of poorly supported nodes at deeper levels in the tree, which also characterized mitogenomic analyses (Inoue et al., 2010; Johnson et al., 2012) could reflect the low degree of taxonomic sampling and/or a large number of rapid and ancient divergence events within anguilliforms. Expanded taxonomic sampling in conjunction with new phylogenomic techniques enabling genome-wide character sampling (Faircloth et al., 2013) will likely be needed to more confidently resolve relationships within this major radiation of fishes. In addition, expanded morphological analyses which explicit incorporate living and fossil anguilliforms will help clarify the position of *Protanguilla* and provide a firmer basis for interpreting the evolutionary history of this fascinating lineage.

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Appendix A. Description of calibration points

A.1. MRCA of Elopiformes

An indeterminate species of the genus *Elopsomolos* (*Elopsomolos* sp. 1) from the Kimmeridgian Fossil-Lagerstätte of Schamhaupten, Germany documents the Jurassic existence of the Elopiformes (Arratia, 2000a). Both the Lagerstätten date back to the late Kimmeridgian, around 152–151 Ma (Viohl and Zapp, 2007). This Jurassic taxon shares remarkable similarities with the extant genus *Elops* (Arratia, 1997; Arratia, 2000a,b), including: preopercle broadly expanded posteriorly; cleithrum with short and sharp dorsal limb; presence of numerous and ramified tubules of the preopercular sensory canal almost reaching the margin of the bone; opercular and subopercular broadly expanded posteriorly; and dorsal-fin origin opposite or slightly anterior to that of the pelvic fin.

In addition to *Elopsomolos* sp. 1, at least five species of the genus *Anaethalion* and *Eoprotelops vireti* document the occurrence of a diverse assemblage of Elopiformes in the late Kimmeridgian (Arratia, 1997, 2000a), thereby suggesting that the origin of this group is certainly older than 152–151 Ma.

The Norian pholidophorid species *Pholidophorus latiusculus* from the organic-rich facies of the Seefeld Formation, Austria documents the Late Triassic existence of the Teleostei and is used herein to establish an upper boundary. The organic-rich 'Seefelder Fischschiefer' belongs to the middle Norian Alaunian regional stage (Hopf et al., 2001). A recent stratigraphic study (Donofrio et al., 2003) assigned the fish-bearing layers to the *Epigondolella postera* conodont zone corresponding to the upper portion of the *Himavatites hogarti* ammonoid zone, dating back to 208–207.5 Ma (see Gradstein et al., 2004). The inclusion of *Pholidophorus latiusculus* within the Teleostei is supported by at least two features (Arratia, 2000b): (1) possession of an elongated posteoventral process of the quadrate, and (2) presence of a mobile premaxilla. Thus, the prior assumed 151 My as the minimum age, and 207.5 My as the upper boundary.

A.2. Anguilliformes vs Notacanthiformes

At least five taxa (*Abisaadia hakelensis*, *Anguillavus mazeni*, *Anguillavus quadripinnis*, *Luenchelys minimum*, *Urenchelys germanum*) from the lower Cenomanian deposits of the Sannine Limestone exposed at Hajula and Hakel, Lebanon document an earliest radiation of the eel-like body plan (Belouze, 2002; Belouze et al., 2003a,b; Taverne, 2004). The Lower Cenomanian age of these deposits was proposed by Hückel (1970) based on the presence of the benthic foraminifer *Orbitolina concava*; this foraminifer is commonly used as a biostratigraphic marker for the base of the Cenomanian in shelf carbonates of the Tethyan Realm (Gradstein et al., 2004; see also Schroeder and Neumann, 1985); the fish-bearing layers of Hajula and Hakel can thus be assigned to the lower portion of the *Rotalipora truncanoides* zone of planktonic foraminifers corresponding to the *Mantelliceras mantelli* Ammonite zone (about 99–98 Ma). Belouze (2002) and Belouze et al. (2003a,b) investigated the morphology and phylogenetic relationships of these Cretaceous taxa, and evidenced their position on the stem of the anguilliform clade; their inclusion within the Anguilliformes is supported by the possession of several synapomorphies (Johnson et al., 2012), including: ethmoid fused with the vomer; pterotic extends anteriorly above prootic to contact pterosphenoid; dermo- and auto-palatine absent; pectoral gidle displaced posteriorly; opercula series characterized by a distinctive morphology; uppermost branchiostegals curving dorsally behind and often slightly above opercle; posterior ceratohyal almost equal to anterior ceratohyal; posteriormost one to four branchiostegals with spatulate expansions distally; interhyal absent in adults; angular, articular and retroarticular fused into a single bone. Additional synapomorphic features are listed and discussed by Belouze (2002), Belouze et al. (2003a,b) and Taverne (2004).

We used indeterminate species of the genus *Elopsomolos* (*Elopsomolos* sp. 1) from the Kimmeridgian Fossil-Lagerstätte of Schamhaupten (151–152 Ma), the oldest crown elopomorph fossil, to establish the upper boundary for this calibration point. Our prior thus assumes 98 Ma as the minimum age, and 151 Ma as the upper boundary.

A.3. MRCA of Anguilliformes

Nardoechelys robinsi (Taverne, 2002) from the Santonian-Campanian Calcari di Melissano (about 83 Ma; Schlüter et al., 2008) cropping out near the town of Nardò, southern Italy documents the earliest occurrence of the Ophichthidae in the fossil record. The available material consists of a single incomplete skeleton lacking the skull and the anterior part of the axial skeleton. The definition of this family is primarily based on cranial and branchial features (e.g., McCosker, 1977). However, according to Taverne (2002), the inclusion of the fossil within the family Ophichthyidae is justified by the possession of a combination of several features of the axial skeleton (anal-fin length remarkably shorter than dorsal-fin length; neural and haemal spines rudimentary; short and delicate pleural ribs; dorsal- and anal-fin pterygiophores not segmented and ramified; caudal fin pointed; elongated hypural plates; parhypural absent; body naked); of these features only the possession of rudimentary neural and haemal spines is currently considered as synapomorphic of the group (McCosker et al., 1989). Due to the uncertainty regarding the relationships among the major anguilliform subclades in both our analyses (e.g., Figs. 2 and 3) and previous studies (e.g., Inoue et al., 2010; Johnson et al., 2012) we use this calibration point to date the crown anguilliforms, as opposed to the split between ophichthids and their sister taxon.

We used indeterminate species of the genus *Elopsomolos* (*Elopsomolos* sp. 1) from the Kimmeridgian Fossil-Lagerstätte of Schamhaupten (151–152 Ma), the oldest crown elopomorph fossil, to establish the upper boundary for this calibration point. Our prior thus assumes 83 Ma as the minimum age, and 151 Ma as the upper boundary.

A.4. MRCA of Anguillidae plus "Saccopharyngiformes"

The Eocene eel *Anguilla ignota* from Messel, Germany is the oldest known representative of the genus *Anguilla* (Micklich, 1985). The rich fossiliferous layers of Messel originated in a post-eruptive maar-lake, during the Lutetian, 47.8 Ma (Franzen, 2005). *Anguilla ignota* exhibits a general physiognomy and skeletal structure almost indistinguishable to those of extant congenerics (see, e.g., Ege, 1939; Smith, 1989a), including the typical maxillary and ethmo-vomer complex granular dentition pattern (Micklich, 1985). Smith (1989a) argued that the most remarkable and characteristic feature of the family Anguillidae is its life history, since it repre-

sents the only anguilliform group that also inhabits freshwaters; this ecological trait was also characteristic of *Anguilla ignota*, which as a consequence, provides evidence of the ancient anguillid invasion of freshwater habitats.

We used *Nardoechelys robinsi* from the Santonian-Campanian Calcari di Melissano (83 Ma), the oldest crown anguilliform fossil to establish the upper boundary for this calibration point. Our prior thus assumes 47.8 Ma as the minimum age, and 83 Ma as the upper boundary.

A.5. MRCA of Ophichthidae

The British fossil *Echelus branchialis* from the Eocene London Clay and Barton Clay formations is the oldest crown ophichthid currently known (Young, 1993). Some of the available specimens belonging to this species have been collected from the Ypresian grey silty clay of the London Clay Formation cropping out at Sheppey (Kent). According to King (1981, 1984), the Sheppey outcrops of the London Clay Formation can be assigned to the lower portion of the *Charlesdowniea coleothrypta* dinoflagellate cyst assemblage. The first occurrence of *Charlesdowniea coleothrypta* is correlated to the early part of middle NP12 calcareous nannoplankton Zone (polarity chron C23r), dating back to 52.2–52.1 Ma (Iakovleva, 2011). The known material of *Echelus branchialis* consists of well-preserved three-dimensional skulls and neurocrania that were formerly assigned to the genus *Rhynchorhinus* (see Woodward, 1901). Based on a detailed osteological analysis, Young (1993) demonstrated that this British fossil eel actually is a member of the extant Mediterranean genus *Echelus* because of its possession of numerous characters, including: supraoccipital that contributes to foramen magnum; subopercle and interopercle reduced; foramina for the main branches of the trigeminal nerve of the ramus buccalis, the palatine of the facial nerve, and of the jugular vein opens mainly on the pterosphenoid, and both the prootic and parasphenoid may or may not contribute to the margin of this foramen; foramen for the ramus ophthalmicus of trigeminal nerve situated ventral, or postero-ventral, to the exit foramen for the supaorbital sensory canal of the frontal; transverse commissure of the cephalic sensory canal system runs in a bony tube within the frontal, uniting the supraorbital sensory canal and has a small and medial epiphyseal pore.

We used *Nardoechelys robinsi* from the Santonian-Campanian Calcari di Melissano (83 Ma), the oldest crown anguilliform fossil to establish the upper boundary for this calibration point. Our prior thus assumes 52.1 Ma as the minimum age, and 83 Ma as the upper boundary.

A.6. MRCA of Nettastomatidae

The Eocene *Nettastoma belgica* from the Sables de Lede (Lede Formation), Belgium is the oldest crown nettastomatid known to date (Taverne and Nolf, 1978). The Lede Formation consists of a fine calcareous sand characterized by a rich content of the larger benthic foraminifer *Nummulites variolarius* (Nolf and Steurbaut, 1990); moreover, the larger foraminifer *Nummulites laevigatus* is present in the conglomerates placed at the base of the formation; the concurrent presence of these foraminifers is indicative of the upper portion of the Lutetian SBZ 13 zone of Serra-Kiel et al. (1998), (about 47 Ma; Molina et al., 2011). The known material consists of several elongate ethmovomerine complexes and dentaries that exhibit the typical dentition pattern of the genus *Nettastoma* characterized by a very long vomerine tooth patch with median elements somewhat enlarged but not forming a single row, short patch of intermaxillary teeth, and dentary teeth arranged in numerous narrow bands (see Smith et al., 1981; Smith, 1989b). Due to the uncertainty regarding the monophyly of the Nettastomatidae, and the phylogenetic placement of *Nettastoma belgica* within the most inclusive clade that includes all extant nettastomatids, we use this fossil to calibrate the MRCA of the clade labelled Congridae II + Nettastomatidae in Figs. 2 and 3.

We used *Nardoechelys robinsi* from the Santonian-Campanian Calcari di Melissano (83 Ma), the oldest crown anguilliform fossil to establish the upper boundary for this calibration point. Our prior thus assumes 47 Ma as the minimum age, and 83 Ma as the upper boundary.

A.7. MRCA of Serrivomeridae

Fossil articulated skeletons assigned to *Serrivomer* sp. from the Middle Miocene of Torricella Peligna, central Italy provides a minimum age estimate for the family Serrivomeridae. The fish-bearing layers of Torricella Peligna date back to the Serravallian and belong to the *Dentoglobigerina altispira altispira* planktonic Foraminifera Zone of Foresi et al. (1998), corresponding to the MNN6 calcareous nannoplankton Zone of Fornaciari et al. (1996); based on astronomical calibration of Middle Miocene Mediterranean bioevents, Carnevale (2007) hypothesized that the age of the fossil fishes from Torricella Peligna oscillates between 13.39 Ma and 12.62 Ma. The material reported by Carnevale (2007) consists of partially complete specimens that exhibit a number of diagnostic features of the family Serrivomeridae and, more precisely, of the genus *Serrivomer*, including (e.g., Tighe, 1989): body naked; jaw teeth small, erect, pointed, arranged in two or more rows; vomerine teeth erect, greatly enlarged, laterally compressed, and arranged in two alternating rows; dorsal fin relatively low; anal fin higher than dorsal fin. The possession of the vomerine dentition of the *longidentatus* type seems to support a close relationship of the fossils to the extant species *Serrivomer beanii* and *S. schmidti* (Bauchot-Boutin, 1953).

We used The Eocene eel *Anguilla ignota* from Messel (47.8 Ma), the oldest crown fossil anguilliform that can be assigned to the anguilliform subclade that includes serrivomerids to establish the upper boundary for this calibration point. Our prior thus assumes 13 Ma as the minimum age, and 47.8 Ma as the upper boundary.

Appendix B. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.06.016.

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