



A multilocus molecular phylogeny of combtooth blennies (Percomorpha: Blennioidei: Blenniidae): Multiple invasions of intertidal habitats



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ABSTRACT

The combtooth blennies (f. Blenniidae) is a diverse family of primarily marine fishes with approximately 387 species that inhabit subtidal, intertidal, supralittoral habitats in tropical and warm temperate regions throughout the world. The Blenniidae has typically been divided into six groups based on morphological characters: Blenniini, Nemophini, Omobranchini, Phenablenniini, Parablenniini, and Salariini. There is, however, considerable debate over the validity of these groups and their relationships. Since little is known about the relationships in this group, other aspects of their evolutionary history, such as habitat evolution and remain unexplored. Herein, we use Bayesian and maximum likelihood analyses of four nuclear loci (ENC1, myh6, ptr, and tbr1) from 102 species, representing 41 genera, to resolve the phylogeny of the Blenniidae, determine the validity of the previously recognized groupings, and explore the evolution of habitat association using ancestral state reconstruction. Bayesian and maximum likelihood analyses of the resulting 3100 bp of DNA sequence produced nearly identical topologies, and identified many well-supported clades. Of these clades, Nemophini was the only traditionally recognized group strongly supported as monophyletic. This highly resolved and thoroughly sampled blenniid phylogeny provides strong evidence that the traditional rank-based classification does not adequately delimit monophyletic groups with the Blenniidae. This phylogeny redefines the taxonomy of the group and supports the use of 13 unranked clades for the classification of blenniids. Ancestral state reconstructions identified four independent invasions of intertidal habitats within the Blenniidae, and subsequent invasions into supralittoral and freshwater habitats from these groups. The independent invasions of intertidal habitats are likely to have played an important role in the evolutionary history of blennies.

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1. Introduction

The diverse fish assemblages found in intertidal habitats are thought to be products of multiple invasions by subtidal ancestors (Chotkowski et al., 1999). As such, many of the fish families with representatives in intertidal habitats are comprised of both intertidal and subtidal species. Tests of the subtidal ancestor hypothesis are limited to investigations of transitions to intertidal habitats in the cryptobenthic, North American cottids (Oligocottinae). These studies confirmed invasions of intertidal habitats by subtidal ancestors and also implicated intertidal invasion in the further diversification of the group (Ramon and Knope, 2008; Knope and Scales, 2013). Other groups of fishes may exhibit a similar pattern,

but to our knowledge, none have been investigated. An ideal group for the study of invasions of intertidal habitats is the combtooth blennies (family Blenniidae), one of the most diverse percomorph families (Nelson, 2006), consisting of approximately 58 genera and 387 species (Hastings and Springer, 2009; Smith-Vaniz and Rose, 2012).

Combtooth blennies are small (most <100 mm total length) scaleless fishes common to a diverse range of shallow, mostly marine, communities such as coral reefs, tidepools, mangroves, lower reaches of rivers, oysterbeds, and even supralittoral environments throughout tropical and warm temperate waters (Hastings and Springer, 2009). The common name is derived from the unique dentition of these fishes, consisting of a single, comb-like, row of teeth on the dentary and premaxilla and an absence of teeth on other bones throughout the oral cavity, although in some species teeth are present on the vomer (Springer, 1968). In addition to comb-like teeth, most combtooth blennies also possess an

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enlarged recurved caniniform tooth at the rear of each dentary, thought to be used in interspecific combat and intraspecific defense (Kotrschal and Goldschmid, 1992). Most combtooth blennies also share a similar general appearance: elongate body, short and rounded snout with eyes situated high on the head, long dorsal and anal fins, and often presence of cirri on the head (reviewed in Hastings and Springer (2009); Supplementary Fig. 1).

The taxonomy of combtooth blenny groups has a complex and inconsistent history, likely due to the absence of morphological synapomorphies for certain groups (e.g. Blenniini and Parablenniini) or competing hypotheses of relationships and rank (i.e. Bath, 1977, 2001; Williams, 1990). The first modern, comprehensive taxonomic revision divided blenniids into three groups: Ophioblenniinae, Blenniinae, and Salariae (Norman, 1943). Springer (1968) provided the basis for the current combtooth blenny taxonomy and subdivided the blenniids into four major groups, including the currently recognized Omobranchini, Salariae, Nemophini (referred to as Nemophidinae), and Blenniini (at the time included the monotypic genus *Medusablennius*, which was later moved to Salariae in Smith-Vaniz and Springer (1971)). In the most recent taxonomic revision Williams (1990) presented a classification with six major groups: Blenniini, Nemophini, Omobranchini, Phenablenniini, Parablenniini, and Salariae, recognizing two subgroups within the Salariae, the *Rhabdoblennius* and *Salarias* groups (Fig. 1).

The relationships of the major groups of blenniids have not been extensively assessed in a phylogenetic framework. Williams (1990) proposed the relationships of the currently recognized groups (Fig. 1A) to assist character state delimitation during a study investigating the relationships of the salarine blenny *Scartichthys* and several other genera. The group formed by Salariae and Parablenniini (Fig. 1A) was supported by two hypothesized morphological synapomorphies: ascending process of premaxilla weakly attached to body of premaxilla and the relative insertion points of the radials on the scapula and coracoid (Springer, 1968; Williams, 1990). The group formed by the remaining four groups, Nemophini, Blenniini, Phenablenniini, and Omobranchini, are united by one morphological synapomorphy, an interdigitated suture

between the dentaries (Springer and Smith-Vaniz, 1972). The proposed hypothesis of group relationships in Williams (1990) is not based on phylogenetic analysis of a character matrix, rather it represents a synthesis of many previous studies.

The two most thoroughly sampled molecular phylogenetic studies that included blenniids focused on resolving relationships within the Blennioidei (Families Blenniidae, Chaenopsidae, Clinidae, Dactyloscopidae, Labrisomidae, and Tripterygiidae). The first study sampled broadly across Blennioids, and included 12 combtooth blenny species (Stepien et al., 1997). Phylogenies resulting from neighbor-joining and parsimony analyses of the mitochondrial locus 12s included a blennioid clade (Stepien et al., 1997). The most thorough phylogenetic analysis to date sampled one mitochondrial and four nuclear markers from 48 combtooth blenny species, representing groups Nemophini, Omobranchini, Parablenniini, and Salariae (Lin, 2009). Bayesian analysis recovered each major group as monophyletic (Fig. 1B), but found different relationships than those hypothesized by Williams (1990; Fig. 1A). Lin (2009) presented a novel clade containing Parablenniini, Nemophini, and Omobranchini and found no support for the division of Salariae into *Rhabdoblennius* and *Salarias* groups (Lin, 2009; Fig. 1B).

The goals of our study were to further resolve relationships within the Blenniidae and investigate evolution of transitions between subtidal, intertidal, supralittoral, and freshwater habitats by providing a thoroughly sampled and well-resolved molecular phylogeny upon which to conduct ancestral state reconstructions. We used maximum likelihood and Bayesian analysis of four protein coding nuclear loci, sampled from a diverse set of blenniids, to address the following questions: (1) Is Blenniidae monophyletic? (2) Are the currently recognized groups monophyletic? (3) If groups are indeed monophyletic, do the relationships between clades support previous hypotheses (i.e. Williams, 1990; Lin, 2009) and does the currently recognized taxonomy of Blenniidae effectively identify monophyletic groups and describe phylogenetic relationships within the Blenniidae? (4) What is the ancestral habitat of blenniids and have there been multiple habitat transitions?

2. Material and methods

2.1. Sampling

The data analyzed in this study included sequences from 102 species of combtooth blennies, representing 41 genera, and 11 outgroup species (Supplementary Table 1). Tissues were collected in the field or obtained as gifts from voucher specimens preserved in museum collections. Sequences from two additional combtooth blennies and eight outgroup species were downloaded from GenBank. The blenny species in this study include representatives from all groups proposed by Williams (1990) (Blenniini – 1 of 2 genera, Nemophini – 5 of 6 genera, Omobranchini – 3 of 7 genera, Parablenniini – 10 of 14 genera, and Salariae – 22 of 28 genera), with the exception of the monotypic Phenablenniini as specimens were not available. The outgroup contains at least one representative from each of the following blennioids: Chaenopsidae, Clinidae, Dactyloscopidae, Labrisomidae, and Tripterygiidae (Lin, 2009) and four other relatively closely related groups, including Gobiesocidae the putative sister clade to Blennioidei (Lin, 2009; Wainwright et al., 2012).

2.2. DNA extraction, amplification, sequencing, and alignment

Total genomic DNA was extracted from muscle or fin clips using Qiagen® DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA),

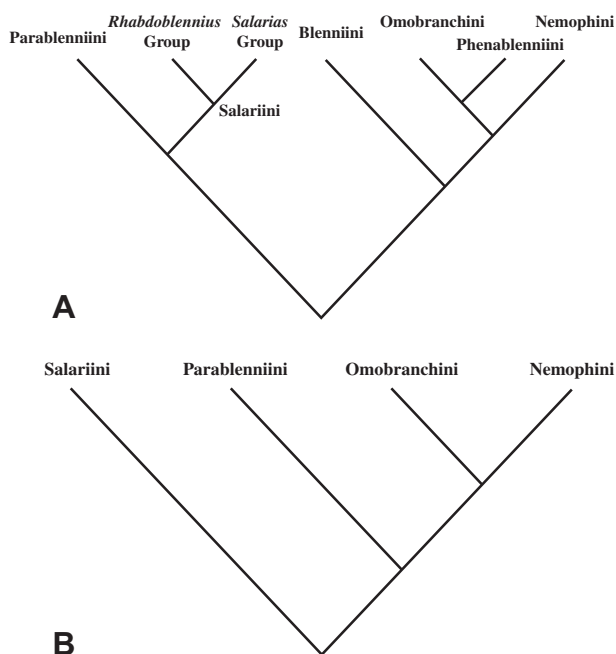


Fig. 1. (A) Hypothesized relationships based on morphological evidence of the six traditionally recognized blennioid groups (including division of Salariae) from Williams (1990). (B) Molecular phylogeny inferred using Bayesian and maximum likelihood methods from Lin (2009).

according to manufacturer suggested protocol. Polymerase chain reaction (PCR) was used to amplify four exons, *ENC1*, *myh6*, *ptr*, and *tbr1*, thought to be well-conserved, single-copy markers (Li et al., 2007), and are widely used in fish systematics (e.g. Dornburg et al., 2012; Li et al., 2010; Wainwright et al., 2012). PCR reactions contained 1.5 µl template DNA, 2.75 µl water, 6.25 µl GoTaq® Green Master Mix (Promega, Madison, WI), 1.0 µl forward primer, and 1.0 µl reverse primer. Forward and reverse primer designs and PCR cycling protocols followed Li et al. (2007). Exonuclease 1 and shrimp alkaline phosphatase were added to PCR products for enzymatic purification at manufacturer suggested thermal profiles. Automated Sanger sequencing of purified PCR products was performed using ABI Prism® BigDye Terminator version 3.1 chemistry (Applied Biosystems, Foster City, CA) at the Biomedical Genomics Center DNA Sequencing and Analysis Facility at the University of Minnesota. Complementary heavy and light strands were aligned into contiguous sequences (contigs) and edited in Geneious v. 6.0.3 (www.geneious.com; Biomatters Ltd., Auckland, New Zealand). Consensus sequences of contigs were aligned to blenniid references from GenBank using the MUSCLE (Edgar, 2004) clustering algorithm as implemented in Geneious v. 6.0.3. Alignments were visually inspected for potential misalignments, verified by viewing amino acid translations, and trimmed to the following lengths: *ENC1* 808 bp, *myh6* 785 bp, *ptr* 738 bp, and *tbr1* 769 bp.

2.3. Phylogenetic analyses

Best fitting partitioning scheme and nucleotide substitution models for each gene were determined using PartitionFinder v. 1.01 (Lanfear et al., 2012) based on Bayesian information criterion (BIC) scores. The proportion of invariant sites parameter (+I) was not considered for nucleotide substitution models as it is already accounted for by the gamma distribution parameter (+Γ) (Yang, 2006). The same partitioning scheme and nucleotide substitution models were used in all phylogenetic analyses, unless noted otherwise.

Bayesian analysis of the partitioned concatenated dataset was conducted using MrBayes v. 3.2 (Ronquist et al., 2012) on the CIPRES Science Gateway portal (Miller et al., 2010). The Metropolis coupled Markov chain Monte Carlo (MCMCMC) command was used for two simultaneous runs with four chains (three heated chains, one cold) per run, for 30,000,000 generations sampling every 1000 iterations. Preliminary Bayesian analysis runs did not converge so we adjusted the branch length prior to an unconstrained exponential prior with mean 0.01, rather than default value 0.1. This adjustment reduces erroneous estimates of unreasonably long branch lengths which can attract and trap MCMC searches in local optima (Brown et al., 2010; Marshall, 2010). Log files were checked in Tracer v. 1.5 (<http://beast.bio.ed.ac.uk/Tracer>) to assess convergence of runs and burnin was set to remove the first 10% of sampled trees.

Phylogenetic analysis of the partitioned concatenated dataset was also conducted in a maximum likelihood (ML) framework in the program Garli v. 2.0 (Zwickl, 2006) on the CIPRES Science Gateway portal (Miller et al., 2010). A tree with the best likelihood score was determined from five search replicates using strict thresholds for generation termination (5000 generations) and maximum score improvement over recent generations required for termination 0.001 (0.05 default). A bootstrapped search with 1000 replicates using lower thresholds for termination was optimized for search speed by setting the threshold for termination of generations to 2500 generations and maximum score improvement over recent generations required for termination to the default setting 0.05. The nodes of the best ML tree found by the aforementioned five search replicates were annotated with the proportion of nodes

found by 1000 bootstrap replicates using SumTrees v. 3.3.1 in the DendroPy v. 3.11 package (Sukumaran and Holder, 2010).

In order to obtain an ultrametric tree for ancestral state reconstruction, an additional Bayesian analysis of the partitioned concatenated dataset was conducted in the program BEAST v. 1.7.4 (Drummond et al., 2012). A birth–death speciation tree prior was applied to all tree models. We implemented a relaxed clock by using an uncorrelated lognormal (UCLN) model of molecular evolutionary rate heterogeneity. Six independent BEAST analyses ran for 50 million generations, sampling every 1000 generations. The MCMC log files were analyzed in the program Tracer v. 1.5 (<http://beast.bio.ed.ac.uk/Tracer>) to assess convergence of the runs, ensure proper mixing, and determine an appropriate burnin. Models of nucleotide substitution parameters exhibited low Effective Sample Sizes (ESS) and poor mixing was observed in initial BEAST runs, so models of nucleotide substitution were simplified for final analyses. Resulting trees had burnin removed and were combined in LogCombiner v. 1.7.4 (<http://beast.bio.ed.ac.uk/LogCombiner>). TreeAnnotator v. 1.7.4 (<http://beast.bio.ed.ac.uk/TreeAnnotator>) produced a maximum clade credibility tree from the resulting 10,000 trees from LogCombiner.

2.4. Phylogenetic classification

In an effort to stabilize the naming of blenniid subgroups and as a means to discuss our resulting phylogeny, we adopted a rank free, phylogeny based classification system to name clades. Our conservative approach to naming select clades utilized the following guidelines: (1) create new names only for clades highly supported by Bayesian and ML approaches, (2) preserve traditional taxonomy by continued use of names of monophyletic groups even if not strongly supported, and (3) redefine groups to maintain monophyletic groups and consistency with phylogeny.

2.5. Habitat designation and ancestral state reconstruction

Blennies were separated into one of four habitat types (subtidal, intertidal, supralittoral, and freshwater) based on a combination of data gathered from FishBase (Froese and Pauly, 2013), literature (Almada and Santos, 1995; Crabtree and Middaugh, 1982; Domingues et al., 2008; Duci et al., 2009; Griffiths et al., 2003; Nakabo, 2000; Springer, 1967, 1976; Springer and Spreitzer, 1978) and personal observations made by the authors. Species recorded from both subtidal and intertidal habitats were placed in the intertidal category. This decision reflects the physiological challenges, such as frequent osmotic, and pH changes, posed by life in intertidal habitats. Ancestral state reconstructions of habitat type were conducted in a maximum likelihood framework on the maximum clade credibility tree from BEAST using the Markov k-state 1 (Mk1) parameter model in the program Mesquite v. 2.75 (Maddison and Maddison, 2011).

3. Results

3.1. Partitioning scheme and nucleotide substitution model selection

The favored partitioning scheme for each gene was by codon position. The following nucleotide substitution models, plus gamma distribution parameter, were selected for each gene (in order of codon position in alignment): *ENC1* (HKY, SYM, JC), *myh6* (GTR, K80, SYM), *Ptr* (GTR, GTR, SYM), and *tbr1* (SYM, GTR, HKY). For the analysis in BEAST all GTR and SYM models of nucleotide substitution were changed to TN93 and the remaining models were set to HKY.

3.2. Relationships of traditionally recognized groups

The phylogenies resulting from Bayesian (MrBayes) and ML analyses produced nearly identical topologies; only the phylogeny resulting from Bayesian analysis (MrBayes) was reported (Fig. 2). There were two minor differences between the Bayesian (MrBayes) and ML phylogenies: ML analysis recovered *Chasmodes bosquianus* as sister to *C. saburrae* + *C. longimaxilla* and the position of the *Salarias* species were switched in respect to the paraphyletic relationship with *Scartella*. Clades supported by ≥ 0.95 Bayesian posterior probability (pp) and $\geq 70\%$ bootstrap replicate support (bs) were considered to have strong support (Fig. 2). Analyses of a concatenated data set recovered Blenniidae as a monophyletic group (pp = 1.0, bs = 87%; Fig. 2).

Nemophini was the only traditionally recognized group strongly supported as monophyletic in both analyses (pp = 1.00; bs = 100%). Omobranchini (*sensu* Springer, 1968) was recovered in both analyses, but only strongly supported by Bayesian methods (pp = 0.99; bs = 62%). Salariini (*sensu* Smith-Vaniz and Springer, 1971) was paraphyletic with respect to all other blenniids and Parablenniini (*sensu* Williams, 1990) was paraphyletic with respect to Blenniini (*sensu* Smith-Vaniz, 1976), with strong support in both analyses (pp = 1.00; bs = 98%; Fig. 2).

Monophyly of almost all genera was well supported, with the exception of *Omobranchus*, *Parablennius*, *Salarias*, and *Entomacrodus*, which were all recovered as paraphyletic. One set of sequences downloaded from GenBank, was referred to *Meiacanthus grammistes*, but likely represents a misidentification. We suggest these sequences come from *Petroscirtes* sp. (see Fig. 2), known to be a Batesian mimic of *M. grammistes* (Smith-Vaniz, 1976).

3.3. Phylogenetic classification

Our phylogenetic analyses provided strong evidence that the traditional rank-based classification does not adequately delimit monophyletic groups. Many of the relationships recovered by our analyses, especially deep in the phylogeny, represent novel relationships previously obscured by the traditional rank based taxonomic system. To accommodate these changes we propose four new group names and redefine Salariini, Parablenniini, and Blenniini. Thus, we recommend the use of 13 unranked clade names based on the following phylogenetic definitions (Fig. 2):

Blenniidae

Regan (1912, p. 266) Definition – The clade originating with the most recent common ancestor of *Ecsenius yaeyamaensis* and *Omobranchus punctatus*. Synapomorphies – Blenniidae is diagnosable based on the combination of eight morphological character complexes as reviewed in Hastings and Springer (2009): (1) scaleless, (2) coracoid reduced and fused to cleithrum (Springer, 1968), (3) reduced interopercle that does not extend beyond the posterior end of posterior ceratohyal (except Omobranchini (*sensu* Springer, 1968)), (4) palatine teeth absent, (5) Premaxillar and dentary bones with a single row com-like row of teeth (6) premaxilla not protractile, (7) adult males with rugosities associated with anal fin spines, and (8) two lateral projections from urotyls attached via ligaments to hypohyals.

Ecsenius

McCulloch (1923, p. 121) Definition – The clade originating with the most recent common ancestor of *Ecsenius aroni* and *Ecsenius namiyei*. Synapomorphies – Monophyly of *Ecsenius* is supported by at least two morphological synapomorphies reviewed in Smith-Vaniz and Springer (1971): (1) absence of an ossified

median ethmoid bone and (2) dorso-medial extensions of the proximal end of epipleural ribs (of at least of the anteriormost) from their point of contact with pleural ribs. Smith-Vaniz and Springer (1971) suggested a third synapomorphy: presence of anterior and posterior canines on dentary, rather than an anterior or a posterior in all other blenniids. Type species – *E. mandibularis* (McCulloch, 1923).

Springerichthys

Definition – The clade originating with the most recent common ancestor of *Blennius ocellaris* and *Plagiotremus tapeinosoma*. Etymology – This clade honors Dr. Victor G. Springer, an ichthyologist at the Smithsonian National Museum of Natural History who has made considerable contributions to the study of blenniids, specifically descriptions of osteology and classifications. Synapomorphies – No morphological synapomorphies have been described. Species in this clade have an interdigitated suture of right and left dentaries, except those of Parablenniini (*sensu* Williams, 1990).

Nemophini

Kaup (1858, p. 169) Definition – The clade originating with the most recent common ancestor of *Petroscirtes breviceps* and *Plagiotremus tapeinosoma*. Synapomorphies – There are five morphological synapomorphies; two with exceptions (Smith-Vaniz, 1976): (1) only blenniid to have cirri associated with symphyseal mandibular sensory canal, (2) supracleithrum articulates with posttemporal and epiotic (other blenniids just posttemporal), (3) absence of basisphenoid, (4) absence of intercalar bone (except some species of *Petroscirtes*), and (5) all adults of this clade have depressions or deep pits in their cranial bones (except *Xiphasia*).

Plagiotrematinae

Gill (1865, p. 138) Definition – The clade originating with the most recent common ancestor of *Xiphasia setifer* and *Plagiotremus tapeinosoma*. Comments on name – Plagiotrematinae was a subfamily applied to a group containing *Plagiotremus* and *Nemophis* (= *Xiphasia*) (Gill, 1865). Subsequently not used, but resurrected here to preserve nomenclature. Synapomorphies – No morphological synapomorphies have been described. Comments – Both genera of Plagiotrematinae have noticeably more elongate bodies, especially *Xiphasia* which can be greater than 500 mm standard length (Smith-Vaniz, 1976).

Smithvanizichthys

Definition – The clade originating with the most recent common ancestor of *Petroscirtes breviceps* and *Aspidontus taeniatus*. Etymology – This clade honors Dr. William F. Smith-Vaniz, an ichthyologist at the Academy of Natural Sciences of Philadelphia and Florida Museum of Natural History who has made considerable contributions to the study of blenniids, specifically to the clade Nemophini. Synapomorphies – No morphological synapomorphies have been described.

Omobranchus

Ehrenberg, in Cuvier and Valenciennes (1836, p. 287) Definition – The clade originating with the most recent common ancestor of *O. elegans* and *O. punctatus*. Comment on definition – This clade contains a proposed synonymy of *Laiphognathus* (Smith, 1955), represented by *L. longispinis* in this study, with *Omobranchus*, as recognition of *Laiphognathus* renders *Omobranchus* paraphyletic. Synapomorphies – No morphological synapomorphies have been

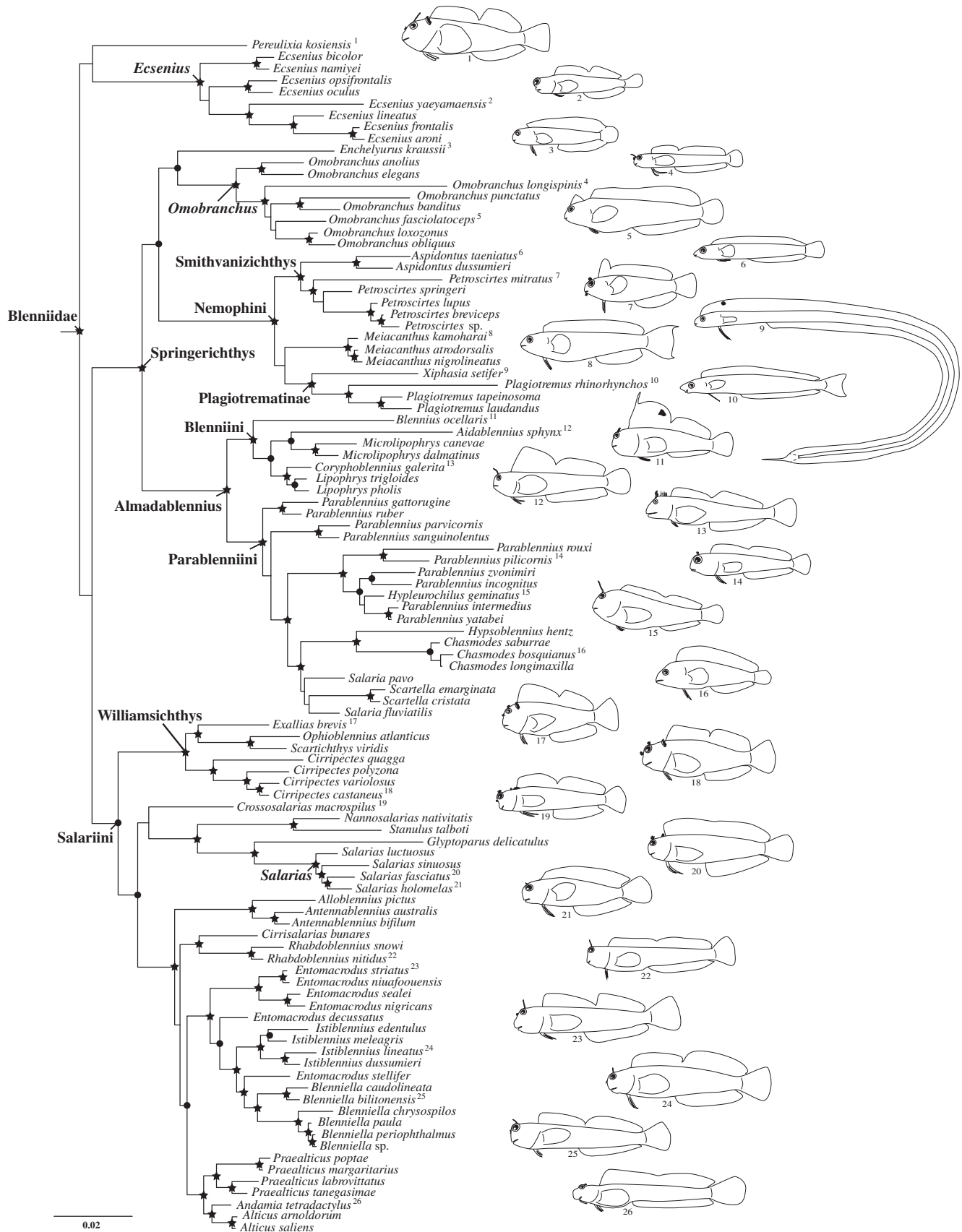


Fig. 2. Phylogeny of combtooth blennies inferred from a partitioned Bayesian analysis of four nuclear loci (*ENC1*, *myh6*, *ptr*, and *tbr1*). Stars indicate clades supported by ≥ 0.95 Bayesian posterior probability and $\geq 70\%$ bootstrap replicate support. Circles indicate clades supported by ≥ 0.95 Bayesian posterior probabilities, but with $< 70\%$ bootstrap replicate support. Thirteen clades labeled in bold indicate a new rank-free phylogenetic classification of blenniids (see Section 3.3. Phylogenetic classification for clade descriptions). Eleven outgroup species not shown.

described. However, species in this clade have labial flaps near the corner of the dentary (except *Omobranchus banditus*) (Springer and Gomon, 1975) and some species in this group, *Omobranchus longispinis* (Hayashi, 2000 in Murase, 2007), *Omobranchus anolius* (Thomson and Bennett, 1953), and possibly others including *Omobranchus fasciolatoceps* (Springer and Gomon, 1975) exhibit mouthbrooding, a trait unique in blennioids. Type species – *O. fasciolatus* (Ehrenberg 1836).

Almadablennius

Definition – The clade originating with the most recent common ancestor of *Blennius ocellaris* and *Hypsoblennius hentz*. **Etymology** – This clade honors Dr. Vítor C. Almada, an ichthyologist in the Eco-Ethology Research Unit at Instituto Universitário who has made considerable contributions to the study of blenniids, specifically in the clade *Almadablennius*. **Synapomorphies** – No morphological synapomorphies have been described.

Blenniini

Definition – The clade originating with the most recent common ancestor of *Blennius ocellaris* and *Aidablennius sphyx*. **Synapomorphies** – No morphological synapomorphies have been described.

Parablenniini

Definition – The clade originating with the most recent common ancestor of *Parablennius gattorugine* and *Hypsoblennius hentz*. **Synapomorphies** – No morphological synapomorphies have been described.

Salariini

Definition – The clade originating with the most recent common ancestor of *Exallias brevis* and *Alticus arnoldorum*. **Synapomorphies** – No morphological synapomorphies have been described. **Comment** – The new definition excludes two genera, *Ecsenius* and *Pereulixia*, which were previously grouped in *Salariini* (sensu Smith-Vaniz and Springer, 1971) based on two morphological characters (reviewed in Williams, 1990): (1) premaxilla forms an open capsule and (2) unique shape of infrapharyngobranchial plate.

Salarias

Cuvier (1817, p. 251) Definition – The clade originating with the most recent common ancestor of *Salarias sinuosus* and *Salarias luctuosus*. **Comment on definition** – This clade contains a proposed synonymy of *Atrosalarias* (Whitley, 1933), represented by *A. holomelas* in this study, with *Salarias*. **Synapomorphies** – No morphological synapomorphies have been described. **Comment** – *Salarias* is closely related to *Glyptoparus delicatulus*, a clade formed by *Nannosalarias nativitatis* and *Stanulus talboti*, and *Crossosalarias macrospilus*, but we refrained from naming any of these clades on the basis of the novel placement of *Stanulus* (Fig. 2), previously thought to be closely related to *Entomacrodus* (Smith-Vaniz and Springer, 1971). Type species – *S. quadripennis* (Cuvier, 1817).

Williamsichthys

Definition – The clade originating with the most recent common ancestor of *Cirripectes polyzona* and *Scartichthys viridis*. **Etymology** – This clade honors Dr. Jeffrey T. Williams, an ichthyologist at the Smithsonian National Museum of Natural

History who has made considerable contributions to the study of blenniids, specifically *Salariini* (sensu Smith-Vaniz and Springer, 1971). **Synapomorphies** – Williams (1990) hypothesized five morphological synapomorphies of this clade: (1) Dorsal fin membrane attached to caudal fin membrane, (2) two distinct process on the fourth epibranchial, (3) Expanded, posteroventrally directed flange on the fifth ceratobranchial, and *Ophioblennius* stage larvae with one or two large recurved canines at symphysis of (4) premaxilla and (5) dentary. **Comment** – All *Scartichthys* and *Ophioblennius* species are restricted to Eastern Pacific Ocean and Atlantic and Eastern Pacific Ocean, respectively. The other species in this clade, *Exallias brevis* and all *Cirripectes* species, are found from the central Pacific Ocean to Indian Ocean and Red Sea (Smith-Vaniz and Springer, 1971). Williams (1990) included *Pereulixia kosiensis* in this clade based on three shared derived characters, including the easily observed transverse row of cirri across the nape. However, this relationship was not recovered in our analysis, which places *Pereulixia* as sister to *Ecsenius* (Fig. 2).

3.4. Habitat designation and ancestral state reconstruction

The topology of the ultrametric tree resulting from analysis in BEAST was nearly identical to that of the analysis in MrBayes. One minor exception was the position of the well supported clade *Meiacanthus*, which was weakly supported as sister to *Smithvanizichthys* in the MrBayes analysis (Fig. 2). This relationship shifted to a weakly supported sister relationship with *Plagiotrematinae* in the BEAST analysis (Fig. 3). This likely had little impact on the ancestral state reconstruction, as all sampled *Nemophini* are associated with subtidal habitats (Fig. 3).

Ancestral state reconstructions revealed a high likelihood for an ancestral blenny associated with a subtidal habitat (Fig. 3). There have likely been four independent invasions of intertidal habitats: *Almadablennius*, *Omobranchus*, *Salarias*, and an unnamed group (UG) in *Salariini* originating with the most recent common ancestor of *Alloblennius pictus* and *Andamia tetradactylus* (Fig. 3). Within each of these intertidal associated groups there is at least one species, which has reverted to living in subtidal habitats (e.g. *Blennius ocellaris* and *Salarias holomelas*). The freshwater blenny species included in our analysis (*Salarias fluviatilis*) and supralittoral species (*Andamia tetradactylus* and *Alticus* sp.) are nested deep within groups of intertidal blennies (Fig. 3).

4. Discussion

4.1. Taxonomy of Blenniidae

This study presents the first phylogeny resolving blennioid relationships using molecular phylogenetic methods and thorough sampling of blennioid generic diversity. Monophyly of the Blenniidae is strongly supported by our findings (Fig. 2) and in other phylogenetic studies focused on resolving the relationships of all six clades in the Blennioidei (Lin, 2009; Stepien et al., 1993, 1997). Additionally, taxonomists have used morphological characters for many years to distinguish combtooth blennies from other blennioids (e.g. Norman, 1943; Smith-Vaniz and Springer, 1971; Springer, 1968; Williams, 1990) and have identified eight morphological character complexes that support the monophyly of Blenniidae (reviewed in Hastings and Springer (2009)).

The relationships recovered from our phylogenetic analyses are a departure from the traditionally recognized major groups based on morphological data and expand the resolution of previous molecular phylogenetic studies. There are two major differences between our findings and the hypothesis of major group relationship based on morphology (Williams, 1990); we found strong

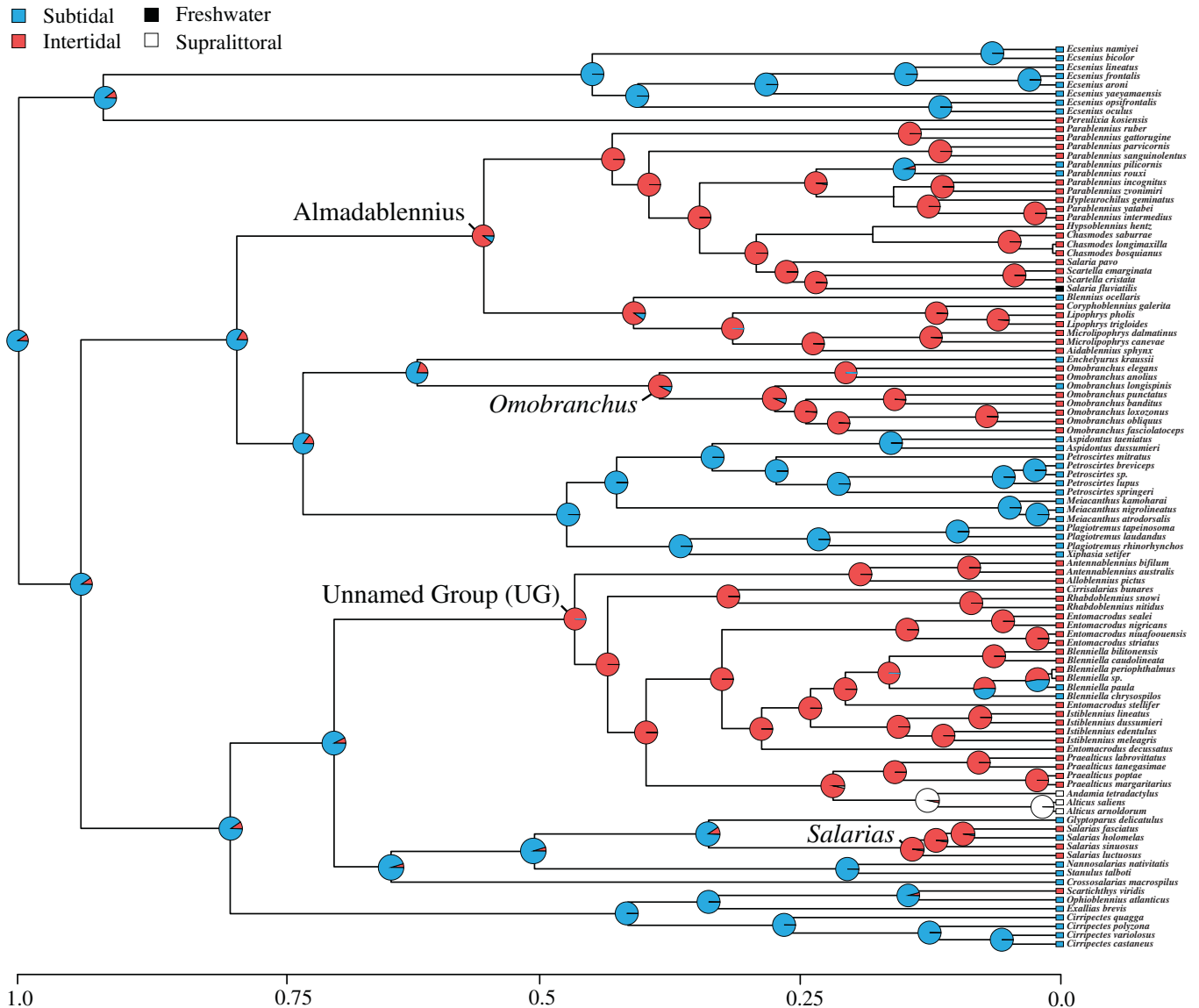


Fig. 3. Ancestral state reconstructions of habitats mapped on the chronogram (relative rates) resulting from partitioned analysis of four nuclear loci (*ENC1*, *myh6*, *ptr*, and *tbr1*) in BEAST. Pie chart at each node represent likelihood of ancestral habitat.

support for a clade Almadablennius and paraphyly of the traditionally recognized group Salariini (*sensu* Smith-Vaniz and Springer, 1971) (Figs. 1 and 2). Lin (2009) recovered similar relationships to those reported here (Fig. 1B); however, Lin's sampling was less extensive.

The proposed new clade, Springerichthys contains the newly named clade Almadablennius, clade Nemophini, and Omobranchini (*sensu* Springer, 1968) (Fig. 2). This clade may also contain the relatively rare, not sampled, monotypic genus *Phenablennius* (Fig. 1A). The potential inclusion of *Phenablennius* is based on two synapomorphies shared with Nemophini and Omobranchini (*sensu* Springer, 1968): unbranched central caudal rays (some other blenniids also exhibit this character) and reduction in number of epurals from two to one (Smith-Vaniz, 1976).

The clade Almadablennius and nested sister clades Blenniini and Parablenniini (Fig. 2) were not identified in previous molecular phylogenetic studies, largely due to insufficient taxonomic sampling. Previous studies have sampled different portions of Almadablennius (Almada et al., 2005; Levy et al., 2013), but none included the overall diversity of our study. Although our sampling is adequate to resolve some of the relationships within

Almadablennius we do not have the sampling necessary to name the remaining clades nested in Blenniini or Parablenniini.

The saber-tooth blenny clade, Nemophini, is well supported by Bayesian and ML analyses (Fig. 2), in previous phylogenetic analyses (Lin, 2009), and also supported by five morphological synapomorphies, two of which are lost in some species (Smith-Vaniz, 1976). Within Nemophini, our findings strongly support *Xiphiasia* sister to *Plagiotremus* (clade Plagiotrematinae) and *Petroscirtes* sister to *Aspidontus* (clade Smithvanizichthys). These relationships are not supported by previous phylogenetic or morphological studies, which each recovered different groups (Lin, 2009; Smith-Vaniz, 1976). Our discordant results with previous molecular phylogenetic studies may be related to taxonomic sampling, as Lin (2009) only included one species of *Meiacanthus* and *Petroscirtes*. *Meiacanthus* was recovered as monophyletic in our analyses, but its position was not well supported (Figs. 2 and 3). It is possible that further sampling of *Meiacanthus* species and other saber-toothed blennies will help fully resolve its position within Nemophini.

The last group in Springerichthys, consists of species from Omobranchini (*sensu* Springer, 1968). Lin (2009) found high support for

the monophyly of this group, but only included two species of *Omobranchus*. Our sampling of this group was only slightly higher, including only three of the described seven genera and nine of the 34 described species. Our reluctance to name this clade is based on relatively low support in ML (Fig. 2), which is likely due to the incongruent placement of *Enchelyurus kraussii* in the *ENC1* and *tbr1* gene trees (not shown). The other species sampled from *Omobranchini* (sensu Springer, 1968), *Omobranchus* spp. form a well-supported *Omobranchus* clade.

The remaining portions of the tree contain taxa traditionally grouped in *Salariini* (sensu Smith-Vaniz and Springer, 1971). Our results show support for the paraphyly of this group (Fig. 2) and we present a new definition of *Salariini* that excludes *Ecsenius* spp. and *Pereulixia kosiensis*. There are many well-supported clades formed by subsets of *Salariini* (Fig. 2), such as *Stanulus* sister to *Nannosalarias*, and the *Andamia*, *Alticus*, *Praealticus* clade, but these groups did not meet our criteria for naming due to limited taxon sampling and desire to limit future renaming of clades.

There is strong support for a clade *Ecsenius*, by far the most species-rich genus of combtooth blennies. Smith-Vaniz and Springer (1971) suggested that *Ecsenius* is unique among the traditionally recognized group *Salariini* (sensu Smith-Vaniz and Springer, 1971) and *Blenniidae* based on several unique morphologies (see description). *Ecsenius* is weakly supported as sister to *Pereulixia kosiensis*, together these form a strongly supported clade sister to all other blennies. Neither of these relationships have been previously reported and the *Pereulixia kosiensis* + *Ecsenius* relationship was not recovered in any phylogenetic analyses of individual genes (gene trees not shown). However, phylogenetic analysis of multilocus molecular data presented in this study and morphological synapomorphies (Smith-Vaniz and Springer, 1971) both support the monophyly of genus *Ecsenius*.

4.2. Multiple invasions of intertidal habitat from subtidal habitats

Ancestral state reconstruction suggests that the family *Blenniidae* is derived from a subtidal ancestor and blenny species have invaded intertidal habitats multiple times. This finding is similar to those of another fairly diverse cryptobenthic percomorph clade, *Cottidae*. Cottids of the subfamily *Oligocottinae* from the north-eastern Pacific Ocean are derived from a subtidal ancestor with a subsequent invasion of intertidal habitat (Ramon and Knope, 2008; Knope and Scales, 2013). It has been suggested complete loss of scales and reduction in body size are adaptive traits that may have played a critical role in the invasions of intertidal habitats by *Oligocottinae* cottids, as it may facilitate life in intertidal habitats characterized by fluctuating environmental conditions (Knope and Scales, 2013). Since all blenniids are scaleless and almost all are quite small (<100 mm) blennies may possess similar physical attributes critical for transitions to and life in intertidal habitats. Studies directly comparing physiological attributes of subtidal, intertidal, and supralittoral blennies are limited. However, a recent study of gill structure and several ion transporters, both critical to maintaining homeostasis, found distinct differences between *Andamia tetradactylus* (supralittoral), *Praealticus tanegasimae* (intertidal) and *Ecsenius yaeyamaensis* (subtidal), likely related to differences in habitat and behavior (Uchiyama et al., 2012).

It appears transitions into intertidal habitats have set the stage for transitions into freshwater habitats in *Almadablennius* and into supralittoral habitats in UG. *Salaria fluviatilis*, the lone freshwater species included in this study, is nested in *Almadablennius* and derived from an intertidal dwelling ancestor. *Lupinoblennius paivai* was not sampled in this study, but it is a freshwater species in *Parablenniini* (sensu Williams, 1990), likely nested in *Almadablennius*, and may also be derived from an intertidal ancestor. Within the clade UG, *Andamia tetradactylus* and *Alticus* spp. live

in supralittoral habitats, likely transitioned from intertidal ancestors. Physiological studies of some species in UG have shown some possess physical attributes for life in a frequently fluctuating environment and even for periods of aerial exposure (reviewed in Hsieh, 2010). The intertidal species in UG consists of amphibious species (*Praealticus*) and some known to occasionally emerge from water (*Entomacrodus*, *Istiblennius*, *Rhabdoblennius*, *Blenniella*). The ability to withstand periods of aerial exposure is exemplified by the mostly terrestrial species of *Andamia* and *Alticus*. These species are highly mobile on land and feed, spawn, and care for eggs in supralittoral habitats (reviewed in Hsieh (2010)). The transition to supralittoral habitats by *Andamia* and *Alticus* was likely setup by intertidal species capable of tolerating extended periods of aerial exposure. More extensive sampling of UG should provide further evidence of the intertidal zone setting the stage for transitions to supralittoral (terrestrial) habitats.

All four of the invasions of intertidal habitats have examples of species, which appear to have moved back to subtidal habitats. Within *Almadablennius*, *Parablennius rouxi* and *P. pilicornis* have both transitioned back to subtidal habitats. Of note in this clade is the transition by *Blennius ocellaris* to one of the deepest subtidal habitats by any blenniid (up to 400 m). In the UG clade, several species have moved back to subtidal habitats (*Blenniella paula* and *B. chrysopilos*). Other possible examples of transitions back to subtidal habitats are in the clades *Salarias* (*S. holomelas*) and *Omobranchus* (*O. longispinis*).

Almadablennius and UG are dominated by intertidal species, but each appears to have a different center of diversity and regions of origins. The novel clade *Almadablennius* is found mostly in the Atlantic Ocean, Caribbean Sea, and Mediterranean Sea, with two species (sampled in this study) found in the Pacific Ocean. A recent study using molecular dating techniques supports an Atlantic ancestor in the *Almadablennius* genus *Parablennius*, followed by more recent dispersal to the Indo-Pacific (Levy et al., 2013). Unlike *Almadablennius*, the diversity of UG is centered in the Indo-Pacific, with only a few species occurring in the Atlantic Ocean (represented by *Entomacrodus nigricans* in this study). This pattern suggests that the independent origins of intertidal invasions occurred in different regions and warrants further investigation with expanded taxonomic and molecular marker sampling.

Transitions from subtidal habitats to intertidal habitats may be widespread throughout fish families that commonly inhabit intertidal habitats, particularly in the extremely diverse clade *Percomorpha*. Other families that warrant similar investigation include *Gobiidae*, *Stichaeidae*, *Zoarcidae*, *Gobiesocidae*, other blennioids, and expanded sampling in *Cottidae*. In *Blenniidae*, transitions between habitats have likely played an important role in the evolution of clade. However, at this time we are not aware of any studies investigating the ecological and biological factors (i.e. competition for space, food availability, predator avoidance) driving transitions to intertidal habitats across blenniids. Future investigations of such factors will be aided by our phylogenetic hypothesis of blenniid relationships and will help to elucidate the evolutionary history of this diverse group.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2013.09.001>.

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