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Phylogenetics and phylogeography
of North Pacific bay gobies:
adaptive convergence, relictual endemism,
and climate-driven population structure

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Biology

by

Ryan Ellingson

2012

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ABSTRACT OF THE DISSERTATION

Phylogenetics and phylogeography
of North Pacific bay gobies:
adaptive convergence, relictual endemism,
and climate-driven population structure

by

Ryan Ellingson

Doctor of Philosophy in Biology

University of California, Los Angeles, 2012

Professor David K. Jacobs, Chair

North Pacific bay gobies inhabit bays, beaches, and estuaries of temperate Asia and North America, but are absent from the northernmost latitudes of the central Pacific. Morphological characters have conventionally subdivided the clade into two groups – an elongate infaunal *Astrabe* group, and a deeper-bodied *Chasmichthys* group – each with a disjunct East-West (amphi-) Pacific distribution. In chapter 1, I use multi-locus DNA sequence data to examine phylogenetic relationships of bay gobies. Basal divergence of the tree coincides with a dramatic global cooling event at the Eocene/Oligocene transition, and there is no evidence of subsequent trans-Pacific migration. These results suggest that several morphological characters

previously used to define the *Astrabe* and *Chasmichthys* groups have arisen independently on both sides of the Pacific, revealing convergence of ecologically adaptive characters within a geographically divided clade. Chapter 2 uses inferences of vicariance via biogeographic events to time-calibrate this phylogeny. Divergence time estimates allow me to compare and contrast potential mechanisms of bay goby diversification on either side of the Pacific. Speciation in the West Pacific has been driven largely by interstitial colonization of gravel beaches of varying grain size, and by invasion of freshwater streams around the Sea of Japan. In the East Pacific, diversification appears to be related to an intense upwelling regime combined with isolation in large Miocene-era embayments on the coast of California. These results also provide strong evidence for relictual endemism in the Gulf of California, as the divergence of three out of four Gulf-endemic gobies substantially predates tectonic formation of the Gulf itself. In chapter 3, I use one member of the bay goby clade, *Gillichthys mirabilis*, to investigate population structure within the Gulf and on the adjacent Pacific outer coast. Phylogeography suggests a complex history of extirpation and colonization driven by Pleistocene sea-level fluctuations. A degree of discordance between mitochondrial and nuclear DNA patterns, however, raises the possibility that differential selection may also contribute to genetic subdivision, precluding north-south migration of mitochondrial haplotypes in the face of more extensive nuclear gene flow.

The dissertation of Ryan Ellingson is approved.

Thomas B. Smith

Patrick J. Krug

David K. Jacobs, Committee Chair

University of California, Los Angeles

2012

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VITA

- 2000 B.S., Biology
University of California
Riverside, CA
- 2002-2003 Research Assistant
California Institute of Technology
Pasadena, CA
- 2004 Teaching Assistant
California State University
Los Angeles, CA
- 2006 M.S., Biology
California State University
Los Angeles, CA
- 2007-2012 Teaching Assistant, Dept. of Ecology and Evolutionary Biology
University of California
Los Angeles, CA

AWARDS

- 1995-1999 National Merit Scholarship
University of California
Riverside, CA
- 2004-2006 GK-12 Fellowship, National Science Foundation
California State University
Los Angeles, CA
- 2007-2008, 2010-2011 GAANN Fellowship, US Dept of Education
University of California
Los Angeles, CA
- 2008-2009 RA Mentorship Fellowship, Graduate Division
University of California
Los Angeles, CA
- 2011-2012 Dissertation Year Fellowship, Graduate Division
University of California
Los Angeles, CA

PUBLICATIONS

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CHAPTER 1

Trans-Pacific convergence of ecological adaptations in temperate North Pacific bay gobies

INTRODUCTION

Morphological Classification of North Pacific Bay Gobies

Gobies constitute one of the most speciose and ecologically diverse groups of fishes, with more than 2,000 recognized species in the suborder Gobioidae adapted to a variety of marine, brackish and freshwater habitats (Nelson 2006). Here I focus on a group of ecologically and morphologically diverse estuarine gobies in the North Pacific (bay gobies hereafter; Table 1-1) that fall within the recently proposed family Gobionellidae (Thacker 2009). Bay gobies inhabit temperate estuaries, beaches and coastal streams of the East Pacific on coastal North America and eastern Asian coasts of the West Pacific (Fig. 1-1). Morphology and ecological niche preferences have traditionally divided these gobies into two amphi-Pacific groups, the informal *Chasmichthys* and *Astrabe* (Birdsong et al. 1988). Birdsong et al. (1988) afforded their osteologically-based groupings “no taxonomic status at present,” advising they be examined more closely for monophyly. These groups are placed in quotes hereafter to indicate their unsupported/unresolved taxonomic status.

The “*Chasmichthys*” group (Birdsong et al. 1988) comprises largely benthic, small gobies with a full complement of fins, scales and pigmentation. “*Astrabe*” group taxa are obligatory burrow and crevice-dwelling forms, with pale skin, lost or reduced scales, smaller eyes, loss of lateral cephalic lateral line canals, development of folds on the head, elongate bodies and elevated vertebral counts. Members of each group are found on both sides of the Pacific, exhibiting a disjunct amphi-Pacific distribution. Broad assessments of goby relationships via mitochondrial DNA (Thacker 2003; 2009) place representatives of “*Astrabe*” and “*Chasmichthys*” within a modestly supported monophyletic bay goby clade. It is important to note that *Chaenogobius annularis* in these molecular phylogenies was likely a misidentified

specimen from the genus *Gymnogobius* (see Stevenson 2000).

The small body size of most bay gobies presents challenges for morphological classification, leading to several revisions over the past century. I follow the nomenclature of Akihito et al. (2002) for West Pacific bay goby taxa and of Nelson et al. (2004) for East Pacific forms. Northeastern Pacific bay gobies have long been considered related (Jordan & Evermann 1898; Hubbs 1921, 1926; Ginsburg 1938), and it has been noted that taxonomic study of these gobies should include the northeastern Pacific genera *Lepidogobius*, *Clevelandia*, *Eucyclogobius*, *Gillichthys*, *Typhlogobius*, *Evermannia*, *Ilypnus* and *Quietula* (Ginsburg 1945; Barlow 1961). Birdsong et al. (1988) placed the eastern Pacific blind goby *Typhlogobius* in the “Astrabe” group along with several West Pacific genera, also with reduced eyes. The remaining East Pacific bay goby genera were placed in the “Chasmichthys” group along with the Asian genera *Chasmichthys* (now *Chaenogobius*) and *Chaenogobius* (now *Gymnogobius*; (Stevenson 2000)). Although not examined by Birdsong et al. (1988), *Lethops* was included among “Astrabe” taxa by Akihito et al. (2002) and *Evermannia* had previously been associated with the East Pacific genera of “Chasmichthys” type (Barlow 1961). The species tentatively identified as *E. panamensis* cf. could potentially be *E. erici* (Bussing 1983). This is unlikely, however, given that *E. erici* is not known to have been collected outside of Costa Rica while the samples used here were collected in El Salvador.

Molecular Systematics of Bay Gobies are Poorly Understood

Previous molecular phylogenetic studies that include bay goby taxa have either been too broad in scope (Thacker 2003; 2009; Chakrabarty et al. 2012) or too narrowly focused (Dawson et al. 2002; Harada et al. 2002; Sota et al. 2005; Yamada et al. 2009) to address systematic

relationships of the group as a whole. Moreover, these studies have relied solely upon mitochondrial markers, with just two exceptions (Yamada et al. 2009). Here I use both nuclear and mtDNA sequences to test monophyly of all genera traditionally included within the bay gobies, and to address whether the most ancestral divergence within the group was driven by ecology (adaptation to infaunal habitat) or geography (trans-Pacific isolation). I focus more intently on lineage divergence within the clade, as it addresses fundamental issues regarding adaptation and historical biogeography of the group. If the oldest split was ecologically driven, multiple trans-Pacific migrations events must be invoked to explain the occurrence of each group on the coasts of both North America and Asia. Alternatively, if vicariance divided the clade, then ecological adaptations such as reduced eyes and elongated bodies in the “Astrabe” group are homoplasious, having arisen independently on each side of the Pacific. Other ecological and physiological adaptations to various habitats in both the East and West Pacific, such as fresh water in members of the “Chasmichthys” group, would also be convergent.

Habitat Diversity and Species Richness within the Bay Gobies

Ecological speciation has been implicated in parallel radiations of bay gobies on both sides of the Pacific, illustrating that the group is an excellent object of evolutionary study. In the Sea of Japan, multiple invasions of brackish and freshwater drainages have generated species in the genus *Gymnogobius* ($n \geq 15$ species), where mitochondrial sequence data suggest convergent evolution of freshwater forms (Harada et al. 2002; Sota et al. 2005). On the shores of Taiwan and the Japanese archipelago, microhabitat partitioning within gravel beaches of varying sediment sizes has led to a radiation of species with elongate bodies and elevated vertebral counts in *Luciogobius* spp. ($n \geq 12$; Yamada et al. 2009). Members of the genus *Astrabe* ($n = 3$) similarly

occupy, and have diversified within, pebbly beaches of the intertidal (Akihito et al. 2002). In the eastern Pacific, bay goby diversity is associated with structurally isolated features such as the Gulf of California and the Colorado River Delta (Swift et al. 2011). Seasonal lagoon closure that limits larval dispersal has made the federally endangered tidewater goby, *Eucyclogobius newberryi*, the most locally differentiated vertebrate on the Pacific Coast of North America (Dawson 2001; Earl et al. 2010). Conversely, the tidewater goby's closest relative, the arrow goby *Clevelandia ios*, inhabits tidal flats open to the sea and shows no detectable genetic structure in mtDNA sequence across a comparable coastal distance of ~850 km (Dawson et al. 2002). The monotypic genera *Lepidogobius* and *Lethops* can be found in the deepest waters of larger bays and subtidal kelp forests, respectively. Other bay gobies of the East Pacific occupy invertebrate burrows across distinct parts of the intertidal such as muddy channels (*Gillichthys mirabilis*), tidal flats (*Quietula y-cauda* and *Ilypnus gilberti*) and mixed sandy/rocky beaches (*Typhlogobius californiensis*).

Gulf of California Endemism

Several bay goby genera in the East Pacific (*Ilypnus*, *Quietula*, *Evermannia* and *Gillichthys*) contain species that are currently endemic to the Gulf of California. Moreover, each of these Gulf endemics has a more widely distributed congener with range extension beyond the Gulf, making these northeastern Pacific bay gobies ideal for studying the evolution of the high degree of marine endemism found in the northernmost Gulf (Walker 1960; Brusca et al. 2005; Hastings 2008; Palacios-Salgado and Burnes-Romo 2012). This Gulf-associated endemic diversity that has likely arisen via small-scale geographic subdivision stands in contrast to the ecologically-driven speciation observed in the West Pacific. Temporal calibration of the bay

goby phylogeny presented here will be the subject of chapter 2. In that chapter, I will investigate contrasts in the rates and ecological drivers of diversification between the East and West Pacific, and examine the origins of Gulf of California endemism in the context of tectonics and paleoclimate.

Objectives

This chapter aims to elucidate phylogenetic relationships and evaluate alternative evolutionary histories of bay gobies with comprehensive sampling of taxa and multiple, independent genetic loci. A molecular phylogenetic approach to establishing bay goby relationships is necessary to test whether ecological divergence has occurred repeatedly and in parallel, leading to convergent adaptations that were previously misinterpreted by conventional morphology-based systematics. To this end, I use five protein-coding genes (one mitochondrial and four nuclear) and a 104-character morphological character matrix to accomplish the following: 1) test whether partitioning of DNA sequence data by relative per-site substitution rates, as opposed to the typical approach of partitioning by gene and/or codon position, allows for increased efficiency in the estimation of parameters and improved resolution of short internal branches near the base of the tree; 2) test for monophyly of all nominal North Pacific bay goby genera; and 3) determine whether presumptively adaptive characters are synapomorphic or have independently converged. Results are discussed in the broader context of amphi-Pacific marine biogeography, paleoclimate in the North Pacific and elevated levels of endemism in the Gulf of California.

METHODS

Taxon Sampling

Whole specimens, tissue samples and DNA extracts were obtained via seine collection, donation or loan as described in Table A-1. Several taxa thought to be closely related to North Pacific bay gobies (*Acanthogobius*, *Awaous*, *Ctenogobius*, *Dormitator*, *Eutaeniichthys*, *Everthodus*, *Gobiomorus*, *Microgobius*, and *Mugilogobius*) were included in initial analyses to test monophyly of the group and to determine the most appropriate outgroup for subsequent analyses. Specimens and tissue samples were preserved in 70-95% EtOH and stored at -20°C.

DNA Extraction, Amplification and Sequencing

Genomic DNA was extracted from muscle tissue using the DNeasy Animal Blood & Tissue DNA Kit (Qiagen, Inc., Valencia, CA) and stored in extraction buffer at -20°C prior to amplification. Polymerase chain reactions (PCR) were used to amplify the mitochondrial cytochrome *b* gene (*cytb*) and four independent nuclear loci (substantial fragments of RAG1, RAG2, *myh6* and RYR3). All products were amplified using illustra Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, England) or PCR Master Mix (Promega, Madison, Wisconsin), 1 µL each primer (10 mM), and 1 µL of DNA extract. The genes *myh6* and RYR3 were amplified using a 2-step PCR reaction as described by Li et al. (2007) where 1 µL of PCR product from the first reaction is used as DNA template for a second, more specific PCR reaction at a higher annealing temperature. All reactions were performed under the following thermal cycler conditions: denaturation at 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 52-62°C for 30 s and 72°C for 90 s, with a final extension at 72°C for 10 min. A negative control (no

template) was included in each run. Primer pairs, annealing temperatures, and final sequence length for each target gene are shown in Table A-2.

PCR products were visualized by electrophoresis on 1.5% agarose to confirm amplification of a single product of desired length. To remove excess dNTPs prior to cycle sequencing, PCR products (3 μ L per sequencing reaction) were incubated at 37°C for 15 min with 0.5 μ L Shrimp Alkaline Phosphatase (SAP), 0.25 μ L Exonuclease I and 0.25 μ L dilution buffer (USB Corporation, Cleveland, OH), immediately followed by 15 min at 80°C to inactivate enzymes. Purified products were directly cycle-sequenced in both directions using PCR primers and Big Dye Terminator v3.1 Cycle Sequencing chemistry. Excess dye terminators were removed using Sephadex (Sigma-Aldrich, St. Louis, MO) before samples were electrophoresed on an ABI 3100 Avant Capillary Sequencer (Applied Biosystems, Foster City, CA). Chromatograms were basecalled and aligned by eye using GENEIOUS software (Biomatters Ltd., Auckland, New Zealand).

Data Partitioning

All sequences were unambiguously aligned by eye and concatenated in GENEIOUS. An initial tree, which included all possible outgroup taxa, was built with the concatenated matrix and a separate partition for each gene. All outgroup taxa except for the most proximal species to the ingroup were removed from subsequent analyses. Poor resolution at the base of preliminary trees and extremely high variability at the *cytb* locus led us to suspect that partitioning by gene and/or codon position was making it impossible to account for among-site rate variation using available substitution models. A k-means algorithm was used to group sites into 5 partitions according to their relative rates. This allowed us to compare four distinct partitioning strategies for phylogeny

construction: 1) by gene, 2) by gene + codon position, 3) combined gene + codon position partitions with similar rate properties via PARTITIONFINDER (see below) and 4) by relative per-site rate as estimated from the concatenated dataset of all 5 genes.

Gene + codon position partitions were analyzed with the software PARTITIONFINDER to find the most efficient partitioning scheme for phylogenetic analysis (Lanfear et al. 2012). PARTITIONFINDER uses a hierarchical clustering method to combine user-specified data partitions based on their likelihood fit to nucleotide substitution models, resulting in a “scheme” that reduces the total number of distinct partitions. In this case, the program reduced 15 partitions (one for each codon position within each gene) into 7 by concatenating partitions assigned to the same model (Table A-3).

The relative rate of each site across the concatenated data matrix was estimated in the HYPHY software package (Pond et al. 2005) under the GTR substitution model with local rate variation. Rates were calculated on a neighbor-joining tree. It should be noted that this tree did not share an identical topology with the final tree and thus it is unlikely that it led to rate estimations that would bias final tree construction. All 5058 sites were then separated into 5 bins according to their relative rates using a k-means clustering approach with an in-house R script (J. Chang, *unpub.*). Details about this partitioning method and a demonstration of its utility on various classes of data will be the focus of future work.

To assess the relative phylogenetic utility of alternative partitioning strategies, phylogenetic informativeness (PI) profiles were plotted via the PhyDesign website (<http://phydesign.townsend.yale.edu/>) for each set of partitions (Townsend 2007). PI profiles visually represent the ability of character partitions to resolve nodes along the entire length of a phylogenetic tree. Since the area under each curve is additive, profiles of the same data

partitioned under different strategies can be compared directly. Because sites of highest rate class (n=10) were most likely to exhibit homoplasy and provided relatively scant PI values for any part of the tree, those characters were removed from subsequent analysis. The final data matrix included 5048 sites, 1346 of which were variable.

Phylogenetic Analyses

Bayesian trees were constructed in MRBAYES v3.2.1 (Ronquist et al. 2012). Analyses were run for 5,000,000 MCMC generations, with the first 25% of trees discarded as burnin. For all datasets, the entire GTR model space was sampled using the mixed model option, and a gamma distribution of 4 rate categories was specified to account for rate heterogeneity within each partition. Maximum likelihood trees were constructed in GARLI v2.0 (Zwickl 2006) for 100 bootstrap replicates under the GTR substitution model with a gamma distribution of 4 rate categories for each rate partition.

Morphological Character Evolution

A data matrix of morphological characters was collected from museum specimens by C. C. Swift from 1987-1992, and reviewed from 2009-2012 for this study. Each character state was coded 0-4 (Table A-4), for a total of 104 characters, and compiled in NEXUS format (Table A-5). A maximum parsimony analysis was conducted in PAUP* v4.0a125 (Swofford 2002) with ordered character states and 1000 bootstrap replicates. Character evolution was reconstructed on both the morphological and molecular topologies using the “list of apomorphies” option under the Describe Trees function to identify convergent characters and synapomorphies. When plotting morphological characters onto the molecular tree, both datasets were trimmed so that

only taxa with both types of data were analyzed. Consistency indices (CI) of morphological characters were tabulated for each tree. The highest CI scores on the morphology tree and molecular tree were used to help identify potentially convergent and synapomorphic characters.

RESULTS

Sequence Data

A total of 269 gene sequences were generated with 15 additional sequences downloaded from GenBank. In some cases, multiple individuals were sampled per species. These sequences were largely invariant at nuclear loci between individuals of the same species and were removed from the final analyses to improve efficiency of phylogenetic analyses. Exceptions include a second *Eucyclogobius newberryi* individual thought to represent a cryptic species, and multiple individuals from *Gymnogobius castaneus/taranetzi*, as these two taxa are known to represent a species complex (Sota et al. 2005). Unambiguous alignment revealed the following indels: 1) A 9-bp deletion in *Ilypnus luculentus* at position 615 of the RYR locus, 2) a 3-bp deletion in *Luciogobius ryukyuensis* at position 868 the RAG2 locus and 3) a 3-bp deletion in *Typhlogobius californiensis* at position 988 of the RAG2 locus.

Partitioning of Nucleotide Data

The entire data set was composed of 1346 variable sites, 936 of which were parsimony informative. Using a k-means clustering algorithm where $k = 5$ to create partitions of sites with similar rates, the number of nucleotides in each partition was 10, 30, 105, 249 and 4664, in order from the fastest rates to the slowest relative rates. It should be noted that the slowest rate class

included 3702 invariant sites. PI profiles indicated that all three partitioning strategies based on typical gene and/or codon position loaded an excessive amount of informativeness onto a single partition at the terminal branches of the tree, with very little PI under the curves at more basal nodes (Fig. 1-2a-c; Townsend 2007). In contrast, partitioning by rate distributed PI more evenly across the tree, with the mid- to high-rate classes providing information near the tips of the tree while the slowest partitions provided power to resolve basal relationships (Fig. 1-2d).

Phylogenetic Analyses

Monophyly of all taxa traditionally included in the bay goby group was strongly supported by molecular analyses, with two exceptions (Fig. 1-3). Both *Eutaeniichthys gilli* and the ice goby *Leucopsarion petersii* have been included among *Astrabe* group species (Birdsong et al. 1988; Akihito et al. 2002), but my results suggest they do not belong within the bay gobies. Instead, *L. petersii* is sister to the yellowfin goby *Acanthogobius flavimanus*, a species of Asian origin that has invaded North America and has never been associated with this bay goby group. The most proximal taxon to all bay gobies, *E. gilli*, was used as the outgroup for subsequent analyses. It should also be noted that my tree indicates that *A. flavimanus* is more closely related to the bay goby clade than is *Mugilogobius rivulus*. This conflicts with the most recent mitochondrial-based topology of Thacker (2009), but is consistent with an earlier study using less data and fewer taxa (Thacker 2003). *Inu koma*, often placed within the genus *Luciogobius*, forms a clade with *Astrabe* and *Clariger* that is sister to a clade containing all other *Luciogobius* species in this study. This result mirrors that of a relatively recent phylogeny of *Luciogobius* with largely overlapping gene sampling (Yamada et al. 2009).

Results from analyses using the various gene/codon-partitioning strategies described above and my rate-partitioning scheme all yielded reciprocally monophyletic East and West Pacific clades, although rate partitioning provided substantially higher Bayesian posterior probabilities and maximum likelihood bootstrap support values for two short internal branches near the base of the tree (not shown). Analyses partitioned by rate also reached convergence much more quickly than those partitioned by gene, likely due to an increase in the efficiency of modeling parameters within each rate partition compared to gene and/or codon partitions. Multiple runs using this rate-partitioning strategy in both Bayesian (MRBAYES) and maximum likelihood (RAxML) frameworks resulted in the highly-supported, congruent topology presented here.

Informative characters ($n = 936$) in the molecular dataset greatly outnumbered informative morphological characters ($n = 97$). In addition, phylogenetic analysis combining both classes of data (Fig. A-1) produced a topology that was congruent with molecular-only analyses, albeit with lower support for the most basal East Pacific node likely due to morphological convergence. Thus, I was confident in making interpretations about bay goby systematics, biogeography and morphological convergence within the molecular framework. The morphological tree alone shows relatively strong support for the traditional “Astrabe” group (Fig. A-2). It also showed some support linking the eastern Pacific genus *Gillichthys* with the western Pacific *Chaenogobius* (Fig. A-2), suggesting the possibility of additional convergent traits beyond those used to characterize the “Chasmichthys” and “Astrabe” groups. The entire “Chasmichthys” group was recovered as a clade, but with very low bootstrap support. While many nodes on the morphological tree have bootstrap support values that would be unacceptable for credible phylogeny reconstruction, this topology is presented because it used to identify

individual convergent characters and synapomorphies on the well-supported molecular phylogeny.

Morphological Character Evolution

A potential synapomorphy of the bay gobies as a whole is the absence of nasal bone ossification [character 98], though it also absent in *Leucopsarion pertersii*, which is otherwise excluded from the bay goby clade by molecular data. Monophyly of the bay gobies is also supported by a distinct double lip condition on both upper and lower jaws [char. 67, 68], one or a few dorsal bony projections on the proximal upper surface of the inner half of the second uppermost pectoral ray [char. 31], and fleshy papillae or ridges on the anterodorsal surface of the cleithrum under the operculum [char. 78]. A loss of scales on the cheek and opercula was also a shared feature of bay gobies, though partially regained in *Lepidogobius lepidus* [char. 85]. All members of the bay goby group are united by “the insertion of the first spinous dorsal-fin pterygiophore in interneural space 4 or 5...” (Birdsong et al. 1988). While this is apparently a rare condition in gobioid fishes, it has no obvious adaptive value beyond general body elongation along with posterior displacement of dorsal fins.

Convergent characters shared across East and West Pacific members of the “Astrabe” group include a thread-like extension of the sphenotic bone into the orbital region [char. 19], attachment of the proximal end of Beaudelot’s ligament to the first vertebrae [char. 20], loss of cephalic lateral line canals [char. 81] and a sinuously curving pelvic spine associated with a rounding and thickening of the pelvic fin [char. 99]. *Typhlogobius* is a well-known obligate of callianassid shrimp burrows and is completely blind as an adult, with skin developing over eyes that are present only in its larval stage. *Lethops* is rarely encountered as an adult, but its pale

coloration [characters 1-10, 13-17], reduced eyes, scales [char. 85-88, 101], development of head folds [char. 73-77] and complete loss of pored lateral line canals [char. 81-84] strongly suggest a subterranean or confined existence consistent with members of the “Astrabe” group. The 22 described species in the western Pacific genera *Astrabe*, *Clariger*, *Inu* and *Luciogobius* show a similar but more extreme reductive trend including reduction and/or loss of eyes, scales, spinous dorsal fin [char. 89], pigmentation [char. 1-10, 13-17, 95-97], and size of the gill opening [char. 69, 94]. These West Pacific members of the “Astrabe” group also share free and filamentous lower pectoral rays [char. 35]. The eastern Pacific *Lethops* and western Pacific *Clariger* and *Luciogobius* species share features that strengthen the skeleton (increasing overlap of bones of the suspensorium [char. 57-60]) and streamline the body (small, compact and rounded pelvic disc [char. 70]).

The “Chasmichthys” group can roughly be divided into two ecomorphs as described in the Discussion section, one small-bodied and one larger-bodied. Convergent characters of the small-bodied form (*Lepidogobius lepidus* and all species descended from its most recent common ancestor in the east + *Gymnogobius* spp. in the west) include parallel reduction in squamation, with overlapping ctenoid scales covering the body and head in *Lepidogobius* and slightly or non-overlapping weak cycloid scales restricted to the posterior in *Clevelandia* and *Eucyclogobius* [char. 86-88]. The remaining species are intermediate in these features. In the West Pacific, most *Gymnogobius* species have weakly ctenoid scales and those in the lowest salinity also have the least developed squamation [char. 86-88]. Most species in this group on both sides of the Pacific have exceptionally long maxillary bones, which is more pronounced in males and related to breeding behavior. This dimorphism is very slight or lacking in the brackish *Eucyclogobius* and in some of the western Pacific brackish and freshwater *Gymnogobius*

(Akihito et al. 2002). Dark melanophores cover the testes in *Clevelandia ios*, *Eucyclogobius newberryi*, *Ilypnus gilberti* and *Quietula y-cauda* in the east and some *Gymnogobius* species in the west [char. 10]. The second, larger-bodied ecomorph of the “Chasmichthys” group (*Gillichthys* spp. and *Chaenogobius* spp. in the East and West Pacific, respectively) expectedly revealed convergent characters related to robust bodies (thickened lateral lobes on pelvic disc [char. 70]), and relatively large heads [char. 18, 25, 29]. A strengthening of skeletal muscles conferred by overlapping bones [char. 57] may also be associated with large bodies, while a low-angled mouth [char. 63] and prolonged maxillary [char. 64, 65] are characters consistent with a large head.

DISCUSSION

Bay Goby Systematics

This work confirms precisely which genera are members of the North Pacific bay goby clade and the phylogenetic relationships among them, questions that had thus far received minimal treatment in a molecular framework. First, two monotypic genera typically associated with the bay gobies, *Eutaeniichthys gilli* and the ice goby *Luecopsarion petersii*, fell outside of the clade containing the rest of the bay gobies (Fig. 1-3). This result was not entirely unexpected as the placement of *L. petersii* within the *Astrabe* group was tentative, and *E. gilli* was not treated by Birdsong et al. (1988). Second, neither the *Astrabe* nor *Chasmichthys* group as defined via morphological analysis (Birdsong et al. 1988) represents a valid phyletic clades (Fig. 1-4). Instead, bay gobies are divided into two lineages by geography, one in the West Pacific and one in the East Pacific. The short branch connecting this initial split to the divergence of the genus

Gillichthys from the rest of the North American bay gobies suggests that the two most ancestral splits within the North American clade occurred in fairly rapid succession. Monophyly of all recognized bay goby genera received high support, with the exception of *Evermannia* and *Ilypnus*. These two genera form a paraphyletic species complex; clarification of species relationships and taxonomic revision of the currently recognized genera in this subclade is in order but is beyond the scope of this research.

Climate-Induced Amphi-Pacific Distributions of Coastal Marine Fauna

Biogeographers have long recognized amphi-Pacific distributions of coastal temperate marine fauna (Andriashev 1939; Ekman 1953; Briggs 1974), where taxa found on the Pacific coasts of Asia and North America are absent from higher-latitude coasts of the central part of the ocean. This temperate phenomenon should not be confused with the lower latitude Eastern Pacific Barrier (Ekman 1953), first postulated by Darwin to be ‘impassable’ by tropical organisms (Darwin 1859) and given much attention since (e.g. Collin 2003; Robertson et al. 2004; Lessios and Robertson 2006; Baums et al. 2012). Despite a relatively shallow and continuous shelf connecting the West and East Pacific across the Bering Strait, a diversity of temperate taxa exhibit disjunct amphi-Pacific distributions (Ilves and Taylor 2008), including polychaete worms (Uschakov 1971), sardines (Bowen and Grant 1997), surfperch (Bernardi and Bucciarelli 1999), decapod crustaceans (Schweitzer 2001), gastropod molluscs (Amano and Vermeij 2003), pinniped mammals (Demere et al. 2003) and smelt (Ilves and Taylor 2008). These examples involve closely related species that either dispersed across the Pacific relatively recently or were isolated by Plio-Pleistocene Northern Hemisphere glacial cooling (e.g. Andriashev 1939). One possible exception involves two Japanese genera of the surfperch family

Embiotocidae that may have migrated across the Pacific as early as the Late Miocene (Bernardi and Bucciarelli 1999). In contrast, bay gobies likely represent a climate-induced trans-Pacific split that appears to have persisted since global cooling at the Eocene/Oligocene transition (Zachos et al. 2001; Oleinik and Marinovich 2003). In addition to cold temperatures, the limited duration of their larval dispersal stage (Brothers 1975), relatively small body size and consequent low fecundity (Blueweiss et al. 1978; Waples 1987), and the potential for retention of larvae in estuaries (Dawson et al. 2002) likely limit dispersal in the group. These larval characteristics, combined with the coastally fragmented and ephemeral nature of their estuarine habitats, may help to explain the unique persistence of this climatic migratory barrier to bay gobies in the northernmost Pacific.

Timing of Trans-Pacific Isolation

The timing of the basal trans-Pacific divergence of bay gobies likely coincided with a sharp decline in global temperatures that occurred at the Eocene-Oligocene transition ~34 million years ago (Ma; Zachos et al. 2001). Bay gobies are restricted to continental coasts of temperate and sub-tropical latitudes, with northern limits in British Columbia of the East Pacific and the southern Sea of Okhotsk of the West Pacific (Fig. 1-1). Since all extant bay gobies have largely overlapping ranges, their common ancestor would have likely had a similar temperature tolerance. The global cooling phenomenon that affected both marine and terrestrial biota (e.g. Wolfe 1995; Katz et al. 2008) would have thus made the northernmost Pacific uninhabitable for the ancestor of these gobies.

Convergence of Morphology and Ecology across the Pacific

A relatively elongated body form has been one feature used to characterize the “Astrabe” group, but it is likely the ancestral condition for all North Pacific bay gobies and appears to be a critical aspect of repeated evolution in the clade. Elongation in ray-finned fishes has evolved numerous times and can be accomplished by lengthening or increasing the number of either abdominal or caudal vertebrae (Ward and Brainerd 2007). The number of vertebrae in most gobioids is typically in the mid twenties, while counts for bay gobies range from 30-40+, including the outgroup *Eutaeniichthys gilli* (Table A-6). In addition, body length appears to be a remarkably malleable trait among lineages within the bay goby clade, almost certainly playing a critical role in the colonization of interstitial habitat by species of the genus *Luciogobius* (Yamada et al. 2009).

While molecular data indicate that the geographic split between East Pacific (EP) and West Pacific (WP) species reflects the true phylogenetic history of bay gobies, morphology reveals a high degree of repeated convergence throughout the clade. Three ecomorphs can be considered to generally follow the “Astrabe” and “Chasmichthys” group distinctions, while further dividing the latter into two subgroups as described below. Each exhibits an amphi-Pacific distribution and has independently evolved morphological and ecological similarities on each side of the ocean. These three analogous groups are best described as: 1) moderate to large-sized crevice-dwelling, burrowing or commensal marine species of the “Astrabe” group (*Typhlogobius*, *Lethops* - EP; *Astrabe*, *Clariger* and *Luciogobius* - WP); 2) small, generalized microphagous estuarine and freshwater species inhabiting soft substrates (the *Lepidogobius* clade *Lepidogobius*, *Clevelandia*, *Eucyclogobius*, *Ilypnus*, *Quietula*, and *Evermannia* - EP;

Gymnogobius - WP); and 3) large-bodied, large-mouthed predators of intertidal estuaries and rocky coasts (*Gillichthys* - EP; *Chaenogobius* - WP).

Members of the infaunal “Astrabe” ecomorph are obligate inhabitants of various recessed or subterranean environments such as under rocks in tide pools (*Astrabe* and *Clariger*; (Akihito et al. 2000)), interstices of gravel beaches (*Luciogobius*; (Yamada et al. 2009)), ghost shrimp burrows (*Typhlogobius californiensis*; MacGinitie 1939) and kelp holdfasts (*Lethops connectens*; Stephens et al. 2006, C. Pierre and S. Anderson, *pers. comm.*). West Pacific species are more elongate than the East Pacific forms, and have retained thicker skin and robustly built skeletons corresponding to their habitat within the cobble and gravel environment of the intertidal zone (Yamada et al. 2009).

Smaller-bodied species of the “Chasmichthys” group exhibit a gradient of salinity tolerances on both sides of the Pacific. The eastern Pacific *Lepidogobius* clade inhabits soft substrates with habitats ranging from marine to nearly fresh water. *Lepidogobius lepidus* is marine and low intertidal, *Quietula* spp. and *Ilypnus* spp. are found in the low to high intertidal of estuaries, *Evermannia* spp. and *Clevelandia* spp. inhabit mid to high intertidal beaches, and *Eucyclogobius newberryi* is confined to small seasonally-closed estuaries with brackish water that can occasionally fall to very low salinities. This osmotic gradient parallels a reduction in size with *Lepidogobius* the largest (85 mm SL), and *Clevelandia* and *Eucyclogobius* the smallest (45 mm SL).

Species of the robust-bodied, large-mouthed *Gillichthys-Chaenogobius* ecomorph are found in estuaries and sometimes rocky shores. Some species in this group have moderate ctenoid squamation, which spans a gradient in the smaller “Chasmichthys” species and is entirely absent in the infaunal ecomorph species. A low-angled mouth and prolonged jaws allow for

consumption of relatively large prey, primarily macroinvertebrates such as crustaceans and annelids.

Effects of Habitat on Diversification Patterns

Parallels as well as contrasts can be drawn between East and West Pacific gobies with respect to isolating effects of habitat preference and resulting patterns of diversification. For example, infaunal cobble beach habitats are associated with a radiation of at least 11 distinct species in the western Pacific genus *Luciogobius* (Yamada et al. 2009), but this contrasts with the lack of diversification in the monotypic eastern Pacific genera *Typhlogobius* and *Lethops*, despite similar infaunal occupation of beaches or kelp beds, respectively. The distinction here may be a function of geophysical differences between beach settings in Japan and the Pacific coast of North America. A dramatic volcanic/tectonic history has created extensive cobble shorelines in Japan, providing a heterogeneous suite of interstitial habitats (Yamada et al. 2009). This is not the case on American beaches where, even in cobble settings, an abundance of fine-grained material limits the amount of available interstitial habitat. Whereas *Luciogobius* lives interstitially among the variety of grain sizes that compose these beaches, *Typhlogobius* lives commensally in burrows constructed and maintained by the ghost shrimp *Neotrypaea affinis* (MacGinitie 1939). In the genus *Gymnogobius*, multiple independent invasions of fresh water streams by at least two lineages have led to increased diversification in the West Pacific as compared to congeneric marine and brackish water lineages (Sota et al. 2005). Small and seasonally-closed estuaries provide similarly locally-isolated habitats for the tidewater goby *Eucyclogobius newberryi* in the East Pacific, yielding highly structured yet comparatively shallow genetic divergence among populations (Dawson 2001; Earl et al. 2010). This may be

explained by more acute isolation being imposed by the fresh water streams inhabited by *Gymnogobius* species compared to the brackish estuarine habitats of *Eucyclogobius*.

Endemism in the Gulf of California

The remarkable species richness of the West Pacific genera *Luciogobius* and *Gymnogobius* is not apparent in East Pacific gobies. Estuaries currently isolated from the Pacific by the Baja California Peninsula, however, contain six Gulf of California-endemic species distributed across four genera. Moreover, endemism in the Gulf has long been recognized across a range of taxa (Hubbs 1960; Walker 1960; Brusca et al. 2005). The scenario of repeated and independent speciation of gobies in the Gulf can provide insight into the broader origins of elevated Gulf endemism. The aforementioned Eocene/Oligocene vicariant event provides the basis for reconstructing the evolutionary history of bay gobies, and will be used along with two additional biogeographic events to time-calibrate the phylogeny in chapter 2. Particular emphasis will be placed on the temporal and ecological origins of Gulf of California endemism in the context of tectonic formation of the Baja California peninsula.

CONCLUSIONS

Mitochondrial and nuclear DNA markers confirmed monophyly of the temperate North Pacific bay goby group, excluding *Leucopsarion petersii* and *Eutaeniichthys gilli*, which had been tentatively associated with these gobies. Partitioning sequence data by relative per-site rate for phylogenetic analysis allowed resolution of two basal divergences that occurred in fairly rapid succession near the earliest Oligocene. The resulting amphi-Pacific disjunction appears to

have persisted since this initial split and is much older than similar biogeographic distributions in other temperate marine taxa. This geographic division of the bay goby clade also revealed convergent adaptations on each side of the Pacific Ocean. At least three convergent ecomorphs roughly following former “Astrabe” and “Chasmichthys” group designations are found in both the East and West Pacific. While ecological adaptations for salinity gradients and infaunality have converged on each side, disparate speciation times and rates of diversification appear to be driven by differences in geomorphology on the North American and Asian coasts. Current work using divergence time estimation and other phylogenetic methods provide more detailed insight into these aspects of North Pacific bay goby evolution.

Table 1-1. Asian genera and North American species sampled for this study. Total number of known species in incompletely sampled Asian genera in parentheses. *Evermannia* n. sp. is an undescribed species.

Region	Genus/species	Location	Habitat description
"Astrabe" Group			
Asia	<i>Astrabe flavimaculata</i> (3 spp.)	Japan	Rocky tide pools
	<i>Clariger cosmurus</i> (6 spp.)	Japan	Rocky and gravelly tide pools
	<i>Eutaeniichthys gilli</i> (monotypic)	Japan, Korea	Estuarine tide pools under stones
	<i>Luciogobius</i> (5/14 spp. sampled)	Japan, Korea	Shallow marine, interstitial in gravel, rocks and soft substrate
	<i>Inu (Luciogobius) koma</i>	Japan, Korea	Tide pools and among intertidal pebbles
	<i>Leucopsarion petersii</i> (monotypic)	Japan	Pelagic to mid-water
North America	<i>Lethops connectens</i> (monotypic)	Central to southern California	Subtidal marine - associated with kelp beds, likely in holdfasts
	<i>Typhlogobius californiensis</i> (monotypic)	Central California to Magdalena Bay, Mexico	Intertidal ghost shrimp (<i>Neotrypaea</i>) burrows near rocks and cobble on exposed beaches
"Chasmichthys" Group			
Asia	<i>Chaenogobius</i> (2/3 spp. sampled)	Japan, Korea and Russia	Rocky tide pools
	<i>Gymnogobius</i> (7/15 spp. sampled)	Japan, Korea and Russia	Estuaries, coastal streams and lakes
N. American outer coast	<i>Clevelandia ios</i> (monotypic)	British Columbia to Magdalena Bay, Mexico	Tidal mud flats, retreats to invertebrate burrows at low tide or to escape predation
	<i>Eucyclogobius newberryi</i> (monotypic)	California endemic	Small seasonally closed estuaries, varied substrates, brackish to fresh water
	<i>Lepidogobius lepidus</i> (monotypic)	British Columbia to Magdalena Bay, Mexico	Sand or mud burrows of the intertidal in the north to 60 m depth in the south
Gulf endemic	<i>Evermannia</i> n. sp.	Eastern and western Gulf	Sandy protected habitat
	<i>Gillichthys detrusus</i>	Colorado River Delta	Channels of very fine-grained mud/silt with tides up to 10 m

	<i>Gillichthys seta</i>	Central and northern Gulf	High intertidal rocky and sandy pools, tides to 10 m, often hyperthermal and/or hypersaline
	<i>Ilypnus luculentus</i>	Rare Gulf endemic	Brackish, soft-bottomed estuaries
	<i>Quietula guaymasiae</i>	Abundant throughout Gulf	Coarse-grained estuarine channels and sand flats, moderate to high salinity
N. American outer coast	<i>Gillichthys mirabilis</i>	Tomales Bay to Magdalena Bay and throughout Gulf	Muddy estuarine channels, retreats to crab burrows at low tide, hypersaline tolerant
	<i>Ilypnus gilberti</i>	Tomales Bay to Magdalena Bay and throughout Gulf	Constructs burrows in sandy substrate of estuarine bays
	<i>Quietula y-cauda</i>	Morro Bay to Magdalena Bay and throughout Gulf	Constructs burrows in mud or muddy sand of estuarine bays
Gulf and southward	<i>Evermannia zosterura</i>	Tropical Eastern Pacific	Constructs burrows in sandy beaches
Central America	<i>Evermannia panamensis</i>	Tropical Eastern Pacific	Constructs burrows in sandy beaches

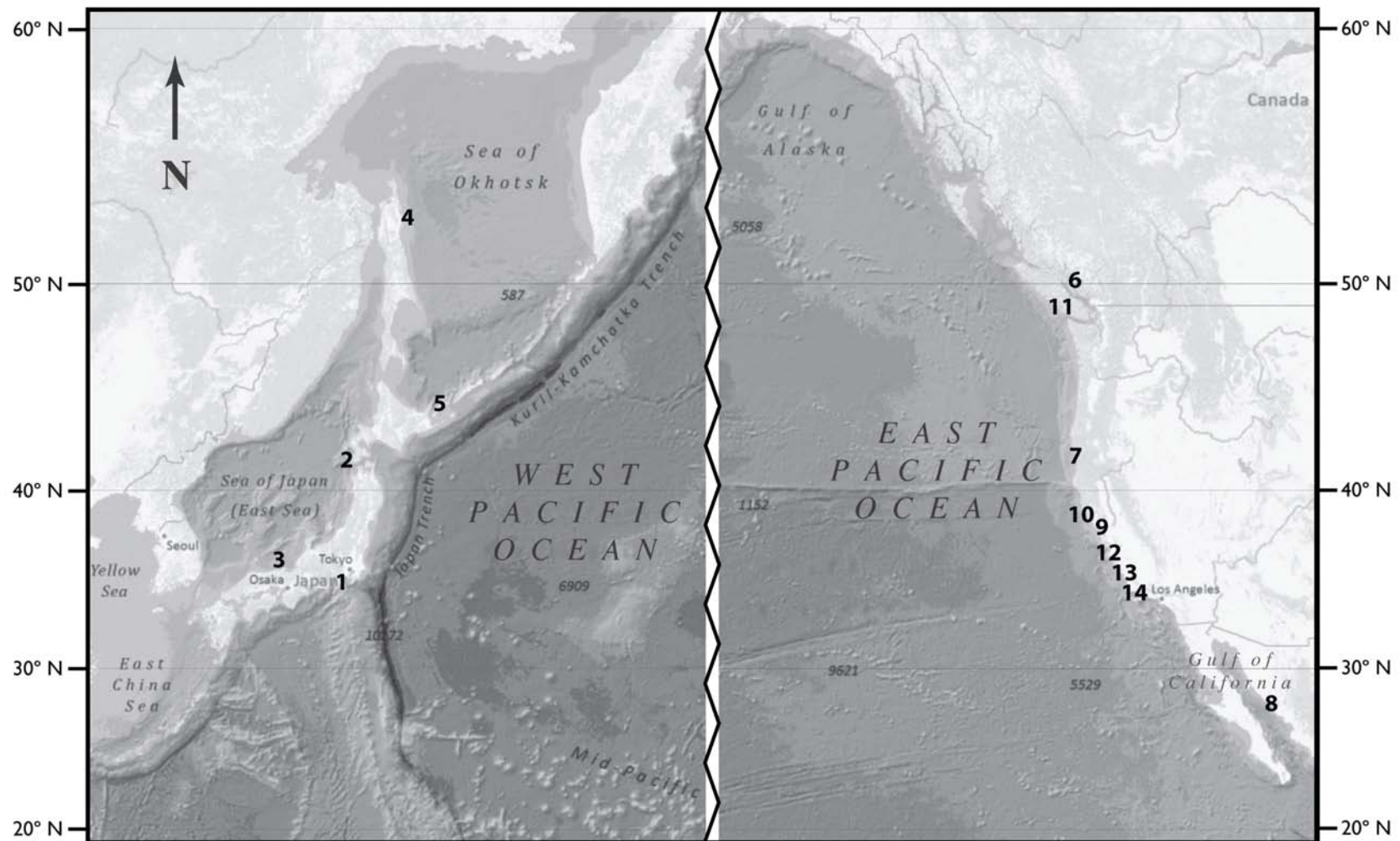
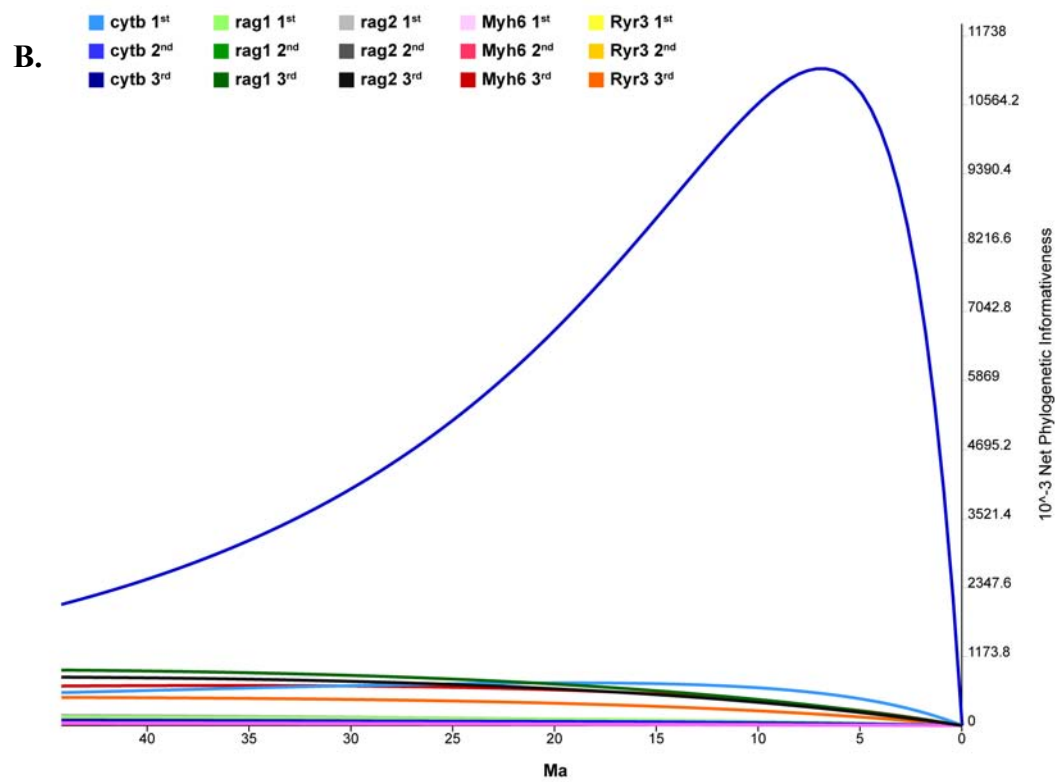
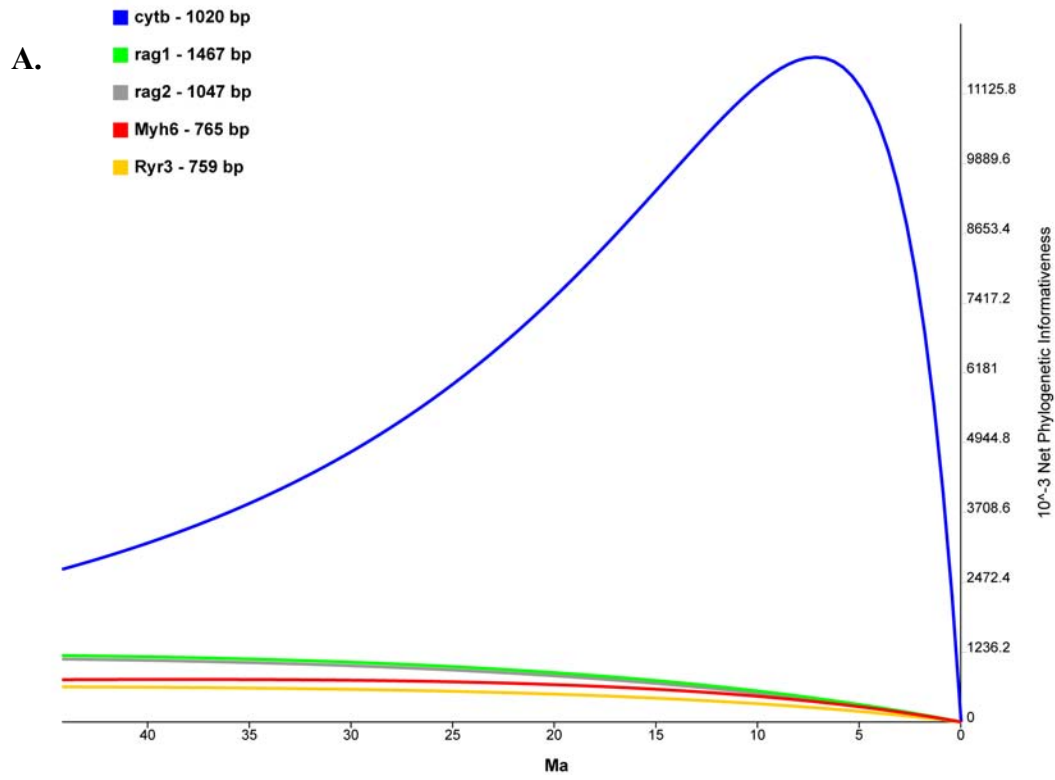


Figure 1-1. Map of the North Pacific Ocean showing bay goby coastal habitat in the west, including the Sea of Japan, and in the east, including the Gulf of California. The bay goby clade exhibits an amphi-Pacific distribution, where species are found on both sides of the Pacific but are absent along coasts of the Bering Sea. The central Pacific (~20° of longitude) has been cropped from the image where gobies do not occur. Northern range limits of each genus are indicated as follows: 1=*Astrabe*, 2=*Chaenogobius*, 3=*Clariger*, 4=*Gymnogobius*, 5=*Luciogobius*, 6=*Clevelandia*, 7=*Eucyclogobius*, 8=*Evermannia*, 9=*Gillichthys*, 10=*Ilypnus*, 11=*Lepidogobius*, 12=*Lethops*, 13=*Quietula*, 14=*Typhlogobius*.



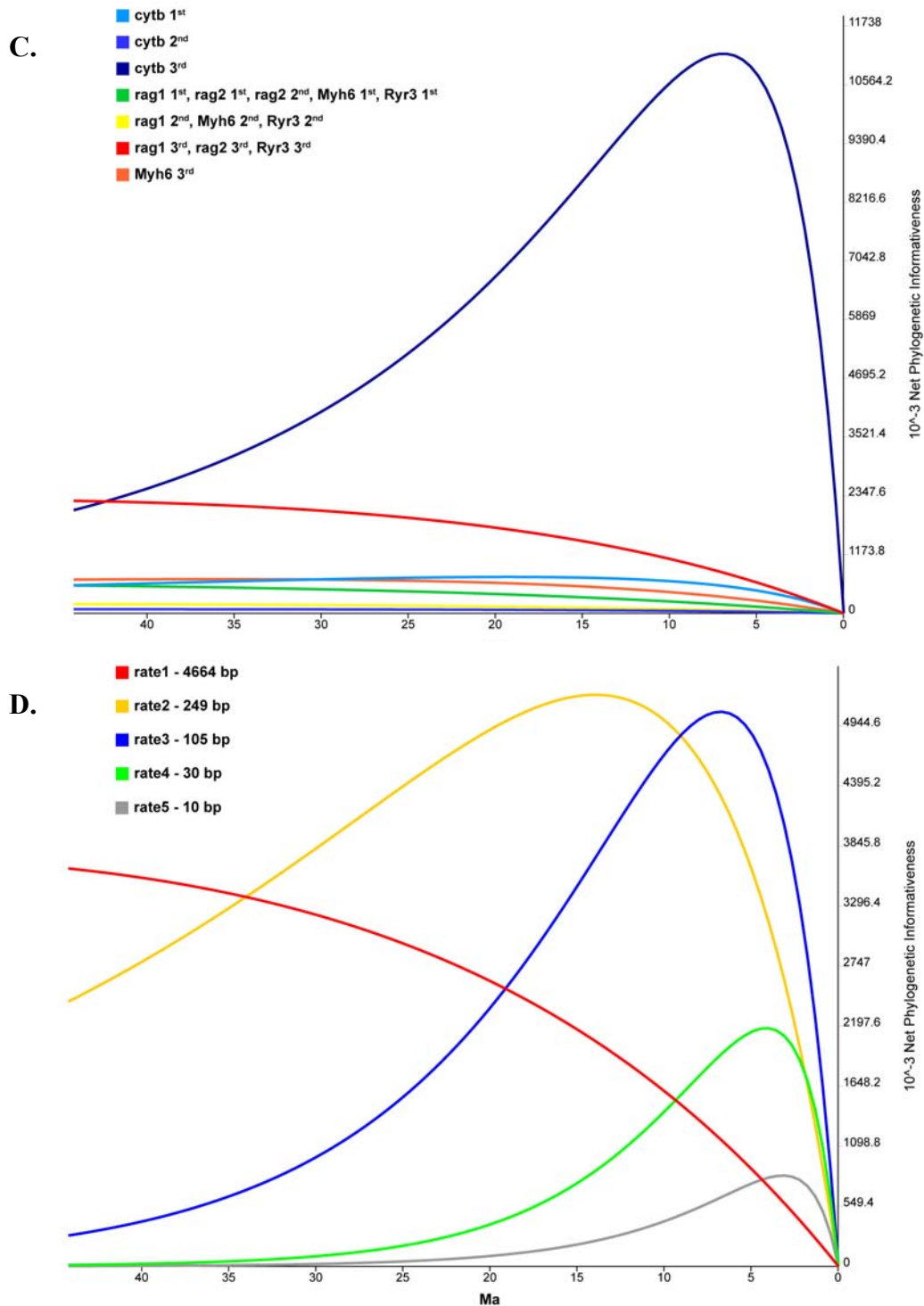


Figure 1-2. Phylogenetic Informativeness plots (Townsend 2007) of the following partitioning schemes: A) Five genes, B) gene + codon position, C) PARTITIONFINDER subsets and D) per-site relative rate as described in Methods. The last plot shows that binning sites by relative rate distributes PI more evenly across the tree.

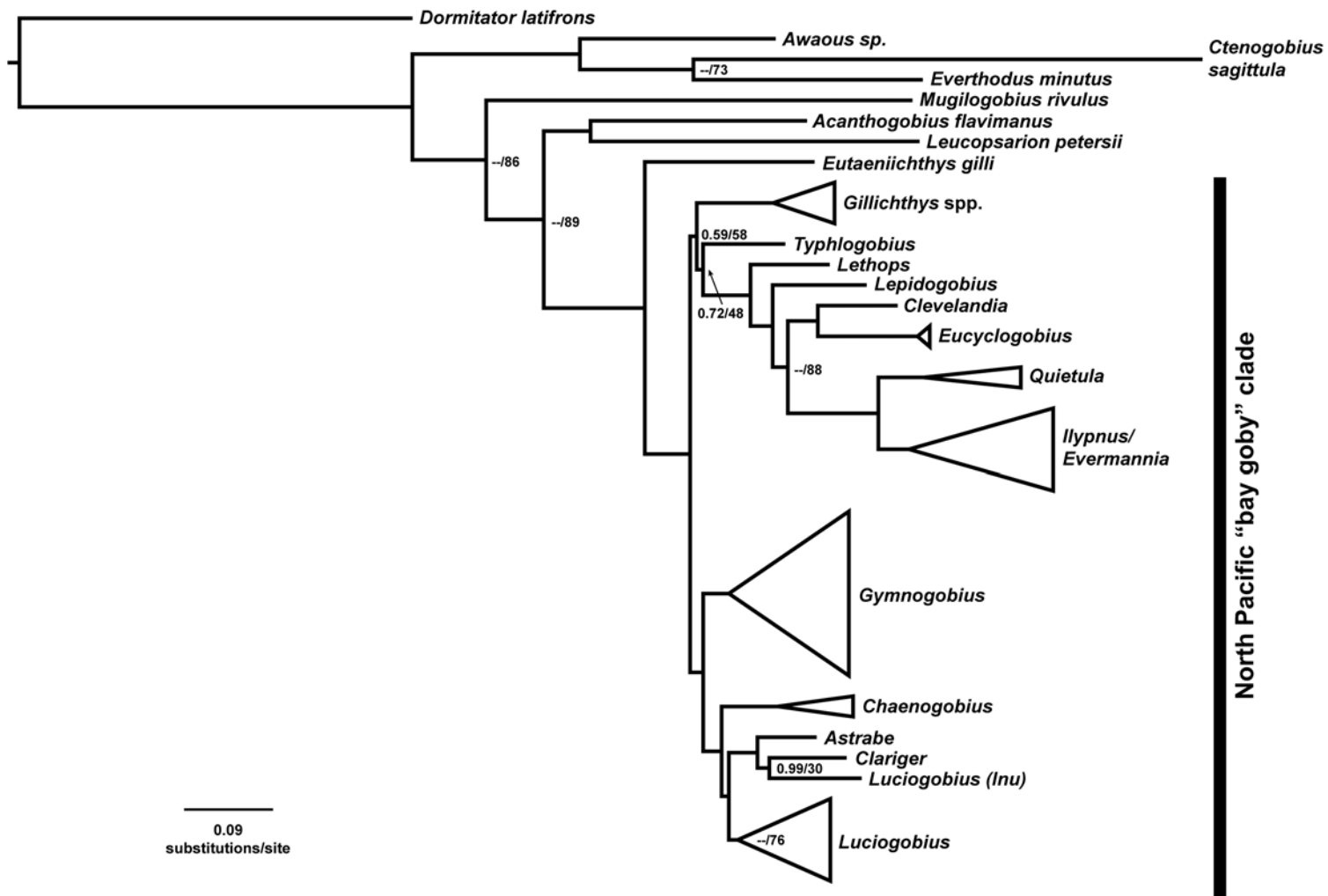


Figure 1-3. Bayesian phylogram (MRBAYES v3.2.1) of North Pacific bay gobies including all genera nominally placed in the group, plus several outgroup taxa. Triangles represent collapsed congeneric species. Number-labeled nodes indicate where posterior probabilities are lower than 1.0 and maximum likelihood bootstrap support values are lower than 90 (RAxML v7.3.2).

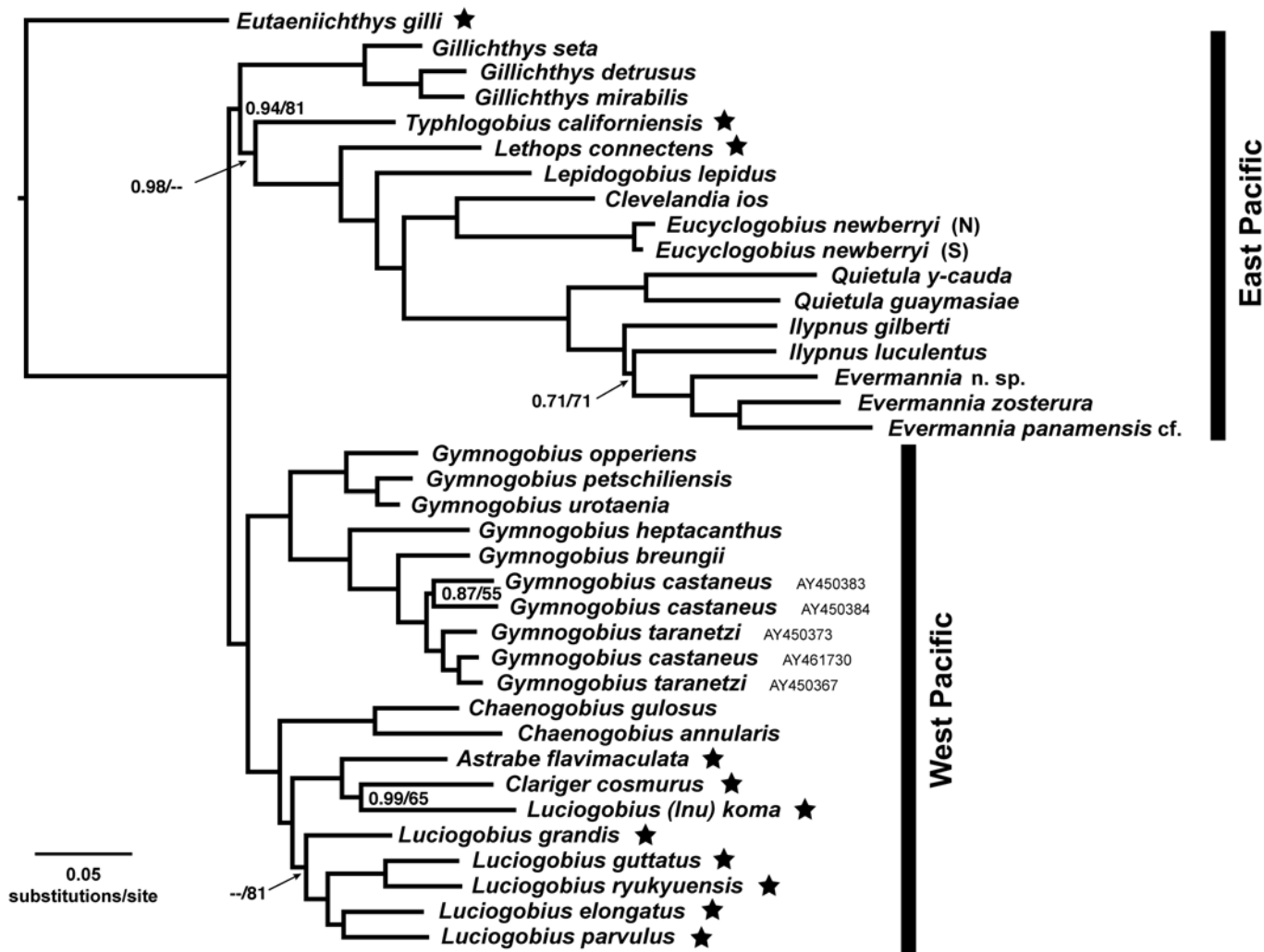


Figure 1-4. Bayesian phylogram (MRBAYES v3.2.1) of the bay goby clade showing trans-Pacific subdivision. ★ indicates species placed in the "Astrabe" group by Birdsong et al. (1988). They placed all other taxa in the "Chasmichthys" group. While they did not examine *Lethops* and *Evermannia*, others authors subsequently associated them with Astrabe and Chasmichthys, respectively. Numbers on nodes represent posterior probabilities lower than 1.0 and maximum likelihood bootstrap support values lower than 90 (RAxML).

Table A-1. Species used for this study, nature of the genetic sample and sources from which they were obtained.

Taxon	Sample obtained	Source
Outgroups		
<i>Acanthogobius flavimanus</i>	whole specimen	O. Miura – Kochi University, Japan
<i>Astrabe flavimaculata</i>	published sequences	NCBI GenBank - Yamada et al. 2009
<i>Awaous</i> sp.	whole specimen	D.K. Jacobs
<i>Ctenogobius sagittula</i>	whole specimen	D.K. Jacobs
<i>Dormitator latifrons</i>	whole specimen	D.K. Jacobs
<i>Eutaeniichthys gilli</i>	published sequences	NCBI GenBank - Yamada et al. 2009
<i>Everthodus minutus</i>	whole specimen	D.K. Jacobs
<i>Gobiomorus maculatus</i>	whole specimen	D.K. Jacobs
<i>Leucopsarion petersii</i>	whole specimen	T. Kokita – Fukui Prefectural University, Japan
<i>Microgobius miraflorensis</i>	whole specimen	D.K. Jacobs
<i>Mugilogobius rivulus</i>	EtOH-preserved tissue	C. Thacker – Natural History Museum, Los Angeles
East Pacific Taxa		
<i>Clevelandia ios</i>	whole specimen	D.K. Jacobs
<i>Eucyclogobius newberryi</i>	whole specimen	D.K. Jacobs/C. Swift
<i>Evermannia panamensis</i> cf.	whole specimen	J. Van Tassell/F. Pezold/L. Tornabene – Texas A&M University
<i>Evermannia</i> sp.	whole specimen	D.K. Jacobs
<i>Evermannia zosterura</i>	whole specimen	D.K. Jacobs
<i>Gillichthys detrusus</i>	whole specimen	D.K. Jacobs/K. Flessa – University of Arizona
<i>Gillichthys mirabilis</i>	whole specimen	D.K. Jacobs
<i>Gillichthys seta</i>	DNA/whole specimen	G. Bernardi – UC Santa Cruz/D.K. Jacobs
<i>Ilypnus gilberti</i>	whole specimen	D.K. Jacobs
<i>Ilypnus luculentus</i>	EtOH-preserved tissue	P. Hastings – Scripps Institute of Oceanography
<i>Lepidogobius lepidus</i>	whole specimen	K. Hieb – California Dept. of Fish and Game
<i>Lethops connectens</i>	whole specimen	C. Pierre – UC Santa Barbara
<i>Quietula guaymasiae</i>	whole specimen	D.K. Jacobs
<i>Quietula y-cauda</i>	whole specimen	D.K. Jacobs
<i>Typhlogobius californiensis</i>	whole specimen	C. Pierre – UC Santa Barbara

West Pacific Taxa

Chaenogobius annularis
Chaenogobius gulosus
Clariger cosmurus
Gymnogobius sp.
Inu koma
Luciogobius parvulus
Luciogobius ryukyuensis
Luciogobius sp.

EtOH-preserved tissue
EtOH-preserved tissue
whole specimen
EtOH-preserved tissue
whole specimen
published sequences
EtOH-preserved tissue
whole specimens

T. Mukai – Gifu University, Japan
T. Mukai – Gifu University, Japan
T. Yamada – Kyoto University, Japan
T. Sota – Kyoto University, Japan
T. Yamada – Kyoto University, Japan
NCBI GenBank - Yamada et al. 2009
T. Mukai – Gifu University, Japan
T. Yamada – Kyoto University, Japan

Table A-2. Loci with primer sequences, annealing temperatures and final sequence length used for this study.

Locus	Primers	Primer Sequences (5' -> 3')	Annealing Temp (°C)	Sequence Length
<i>cytb</i> (Akihito et al. 2000)	AJG15	CAAAAACCATCGTTGTAATTCAACT	50	1020 bp
	H5	GAATTYTRGCTTTGGGAG		
RAG1 (Lopez et al. 2004)	RAG1F1	CTGAGCTGCAGTCAGTACCATAAGATGT	50	1467 bp
	RAG1R1	CTGAGTCCTTGTGAGCTTCCATRAAYTT		
RAG2 (Yamada et al. 2009)	Rag2F2	GCTATCTYCCCCATTACGGTGCCC	50	1047 bp
	Rag2intR*	CAGACTCASAGTAAGGDTTTTCC	50	
	Rag2intF*	GAACGCAARGCAAATGAMAGAAAG		
	Rag2R2	TTGGATCAATTTGACAACCAAGGCA		
<i>myh6</i> ** (Li et al. 2007)	<i>myh6_F459</i> (1 st PCR)	CATMTTYTCCATCTCAGATAATGC	53	765 bp
	<i>myh6_R1325</i> (1 st PCR)	ATTCTCACCACCATCCAGTTGAA	62	
	<i>myh6_F507</i> (2 nd PCR)	GGAGAATCARTCKGTGCTCATCA		
	<i>myh6_R1322</i> (2 nd PCR)	CTCACCACCATCCAGTTGAACAT		
RYR3** (Li et al. 2007)	<i>RYR3_F15</i> (1 st PCR)	GGAAGTATYGGTAAGCARATGG	55	759 bp
	<i>RYR3_R968</i> (1 st PCR)	TGGAAGAANKCCAAAKATGATGC	62	
	<i>RYR3_F22</i> (2 nd PCR)	TCGGTAAGCARATGGTGGACA		
	<i>RYR3_R931</i> (2 nd PCR)	AGAATCCRGTGAAGAGCATCCA		

*RAG2 was amplified and sequenced in two short fragments using internal primers

**Nested PCR design, where product from 1st PCR reaction was used as a template for 2nd reaction

Table A-3. PARTITIONFINDER results show best partitioning schemes as determined by a hierarchical clustering method. A total of 15 *a priori* partitions (one for each codon position within each gene) were reduced to 7.

Subset	Subset partitions	Best Model
1	<i>cytb</i> -1 st	SYM+I+G
2	<i>cytb</i> -2 nd	HKY+I
3	<i>cytb</i> -3 rd	GTR+I+G
4	Myh6-1 st , Ryr3-1 st , rag1-1 st , rag2-1 st , rag2-2 nd	GTR+I
5	Myh6-2 nd , Ryr3-2 nd , rag1-2 nd	HKY+I
6	Ryr3-3 rd , rag1-3 rd , rag2-3 rd	HKY+G
7	Myh6-3 rd	GTR+G

Table A-4. List of morphological characters showing position in data matrix (Table A-5), description of character, and data scoring scheme.

Position	Description, numerical state-scoring scheme
1	Caudal spot obsolescent, 0; round, 1; or vertically oval, 2.
2	Postlarvae with caudal base pigmented, 0; partially pigmented, 1; or distinctly depigmented, 2.
3	Juveniles with caudal base pigmented, 0; partially pigmented, 1; or distinctly depigmented, 2.
4	Vertical rows of black dots or spots of melanophores on caudal fin membranes in juveniles, none, 0; three to five, 1; six to ten, 2.
5	Vertical rows of black dots or spots of melanophores on caudal fin membranes in adults, none, 0; three to five, 1; six to ten, 2.
6	Pale dusky and/or pale lower margin to caudal fin lacking, 0; or present 1.
7	Narrow pale, unpigmented or white or pale yellow or pale orange margins to median fins lacking, 0; present, 1.
8	Horizontal elongate mid-lateral spots or pairs of spots lacking, 0; four to six, 1; seven to ten, 2.
9	Dorsal saddles of melanophores lacking, 0; two to four, 1; five to nine, 2.
10	Black or dark melanophores covering of testis lacking, 0; partially covered, 1; mostly or completely covered, 2.
11	Dorsal snout arteries unpigmented, 0; or darkly lined with melanophores, 1.
12	Roof of mouth arteries unpigmented, 0; darkly lined with melanophores, 1.
13	Melanophore pigmentation on fleshy pectoral base lacking or obsolescent on uppermost edge, 0; on upper half of pectoral base, 1; or base mostly or all covered, 2.
14	Vertical or dorso-ventral extent of longitudinal black or dusky stripe on anal fin in adult males, none to one third, 0; on third to two thirds, 1; more than two-thirds, 2.
15	As 14, but females.
16	White spotting or mottling on blackened median fins, 0; or not 1.
17	Mid-lateral myosepta lined with melanophores for 10% or less of height, 0; 11-50%, 1; more than 50%, 2.
18	Lateral flanges on frontals limiting posterior margin of orbit, none or slight, 0; moderate, 1; strong, 2.
19	Thread like extension of sphenotic extending into orbital region, absent, 0; present, 1.
20	Proximal end of Beaudelot's ligament attached to basioccipital only, 0; up to one third extending posteriorly on to first vertebrae, 1; half to first vertebrae 2; three quarters to first vertebrae, 3; and totally on first vertebrae, 4.
21	Epipleural rib on first vertebrae lacking, 0; floating, unattached, or only attached ligamentously, 1; attached via bony articulation, 2.

- 22 Grooves, feathering, or fimbriation on lateral ethmoid lacking, 0; moderate, 1; deep, 2.
- 23 Medial notch in glossohyal lacking or slight, 0; moderate, 1; or deep, 2.
- 24 Percent of glossohyal notch spanned by thin bridge of bone, 25% or less, 0; 26-75%, 1; more than 75%, 2.
- 25 Degree that anterior edge(s) of glossohyal diverge laterally, none to 15%, 0; 16-35%, 1; 36-90%, 2; lateral edge angular, from 30-40% anteriorly to more than 90% laterally on each side, 3.
- 26 Dorsal longitudinal crest on frontals from interorbital region back to supraoccipital lacking, 0; low, 1; high and well developed, 2. [not including any crests on supraoccipital]
- 27 Gill rakers low and blunt, 0; moderately projecting, 1; long and slender, 2.
- 28 Ossified teeth on intra-gill bar rakers present, 0; absent, 1.
- 29 Process on second hypobranchial present, 0, absent, 1.
- 30 Medial notch along inner or medial edge of lower, fifth pharyngeal tooth plate absent, 0; slight, 1; well-developed, 2.
- 31 Dorsal projection on proximal end of inner half of second upper pectoral ray lacking, 0; weak, 1, strong, 2.
- 33 One or two anterior spinous dorsal fin rays elongated, 1, or not, 0.
- 34 Upper pectoral rays free and filamentous, none, 0; one, 1; more than one, 2.
- 35 Lower pectoral rays free and filamentous, none 0; one, 1; more than one, 2.
- 36 Pelvic spine position relative to rays. Lateral to all rays or slightly overlapping outermost, or first ray only, 0; lying ventral to rays one and two, 1; partially medial to rays two or further medially, 2.
- 37 Shape of pelvic disc an elongate oval, 0; rounded, 1; circular, 2.
- 38 Pelvic fin length, close to cloaca or vent, 0; remote, 1.
- 39 Anal spine or unsegmented ray present, 0; absent, 1.
- 40 Procurrent caudal rays (total or upper?), five to seven, 0; eight or more, 1.
- 41 Caudal rays thin, 0; gradually thicker posteriorly, 1; abruptly thicker posteriorly, 2.
- 42 Modal caudal segmented ray count, upper, six or less, 0; seven, 1; eight or more, 2.
- 43 Modal caudal segmented ray count, lower, six or less, 0, seven, 1; eight or more, 2.
- 44 Position of first dorsal pterygiophore; the number here is one more than in Birdsong et al. (1988); we counted the space in advance of the first neural spine as number one as for percomorphs (three, 0; four, 1; five, 2; six or higher, 3.
- 45 Two or more pterygiophores in front of first haemal spine, 0; first pterygiophore coincides with first haemal spine, 1; or first pterygiophore falls behind two or more haemal spines, 2.

- 46 Number of vertebrae anterior to preural 1 with expanded neural and haemal arches, one, 0; two, 1; three or more, 2.
- 47 Diameter of anterior centrum surface relative to posterior in first vertebra, same, 0; about one third smaller, 1; one half or more reduced, 2.
- 48 Outer upper jaw teeth larger than inner, no or only slightly, 0; one and one third to one and a half times inner, 1; twice or more, 2.
- 49 Outer lower jaw teeth larger than inner, no or slightly, 0; one and one third to one and a half larger, 1; more than one and one half, 2.
- 50 Anterior upper teeth largest, no, 0; yes, 1.
- 51 Anterior lower teeth largest, no, 0; yes, 1.
- 52 Medial ventral projection of pelvic girdle, long and slender, 0; robust, width one sixth to one fourth of length, 1; very robust, width one third or more of length, 2.
- 53 Lateral ventral projection of pelvic girdle lacking, 0; moderately developed, 1; well-developed, 2.
- 54 Lateral flange on pelvic girdle lacking, 0; moderately developed, 1; well-developed, 2.
- 55 Anterodorsal extension of anterior end of pelvic girdle lacking, 0; slightly developed, 1; strongly developed, 2.
- 56 Lachrymal (first circumorbital) ossified, 0; obsolescent, 1; or cartilaginous, 2.
- 57 Pterygoid-metapterygoid articulation dorsally over quadrate, meet and overlapping, 0; closely approximating, 1; widely separated, 2.
- 58 Preopercular extension to posterodorsal end of symplectic lacking, 0; partially closing gap, 1; meeting, 2.
- 59 Proportion of anterior vertical surface of quadrate articulating with pterygoid, up to one third, 0; one third to three quarters, 1; more than three quarters, 2.
- 60 Overlap of palatine and pterygoid, none, 0; up to one third of total length of combined bones, 1; more than one third, 2.
- 61 Calcified patch dorso-medially on rostral cartilage lacking, 0; present, 1.
- 62 Posterior pair of palatine-rostral cartilage ligaments absent, 0; present, 1.
- 63 Angle of mouth, horizontal or raised up to 10 degrees, 0; 11-30 degrees, 1; over 30 degrees.
- 64 Maxillary prolonged in males, no, 0; slightly, 1; considerably, 2.
- 65 Maxillary prolonged in females, no, 0; slightly, 1; considerably, 2.
- 66 Adult size, 30-50 m SL, 0; 51-100 mm, 1; over 100 mm SL, 2.
- 67 Upper double lip lacking or slight, 0; one sixth to one half of toothed margin, 1; more than half, 2.
- 68 Lower double lip lacking or slight, 0; one sixth to one half of toothed margin, 1; more than half, 2.
- 69 Upper limit of gill opening extends above upper pectoral base, 0; equal to it, 1; below it, 2.

- 70 Posterior margin of fleshy skirt on anterior margin of pelvic disc entire, 0; frilled, 1; with slight lateral lobes, 2; with prominent thickened lateral lobes, 3.
- 71 Posterior nostril round, 0; elongate oval and/or with truncated posterior edge, 1; slit-like, 2.
- 72 Rounded dorsal flange on posterolateral end of premaxillary absent, 0; low, height one third or less length, 1; well-developed, height more than one third, 2.
- 73 Fleshy fold below eye and nasal capsule lacking, 0; present, 1.
- 74 Fleshy fold below lachrymal lacking, 0; present, 1.
- 75 Fleshy fold medial to nasal capsule and eye lacking, 0; present, 1.
- 76 Fleshy fold on ventral dentary lacking, 0; present, 1.
- 77 Fleshy fold posteromedial to eye lacking, 0; present, 1.
- 78 Fleshy cleithral ridge and/or papillae lacking, 0; one to two papillae, 1; three to four papillae, 2; continuous ridge possibly undulating but not broken into separate papillae, 3.
- 79 Preopercular and mandibular rows of papillae (neuromasts), inner (medial) row equal in size than outer, 0; up to twice as large, 1; more than twice as large, 2.
- 80 Longitudinal rows of neuromasts below the eye, three, 0; four, 1.
- 81 Cephalic lateral line canals developed precociously (early), 0; or delayed, 1.
- 82 Supraorbital canal in adults lacking, 0; only developed posterolateral to eye, 1; anterolateral also, 2, antero- and posterolateral of each side joined, 3; both sides joined over top of head with or without median pore, 4.
- 83 Temporal canal lacking, 0; present, 1; and joined to supraorbital canal, 2;
- 85 Scales on cheek and opercle absent, 0; one third to one half covered, 1; more than half covered, 2.
- 86 Scales on nape absent, 0; one third to one half covered, 1; more than one half scaled, 2.
- 87 Scales on belly and chest absent, 0; one third to one half covered, 1; more than one half covered, 2.
- 88 Scales on body absent, 0; few mid-lateral rows only, 1; one third to one half covered, 2; more than half of area scaled, 3.
- 89 Dorsal spine count, zero, 0; three, 1; four to five, 2; five to six, 3; seven or more, 4.
- 90 Black “cheek spot” absent or incipient, 0; distinct, but poorly defined, 1; intense and sharp, 2.
- 91 Ascending process of posterior dentary lower than length of dentary, 0; about as high as length, 1; height more than twice length, 2.
- 92 Dorsal epipleural rib on second vertebrae present, 0; or lacking, 1.
- 93 Depressed haemal spines, none, 0; one, 1; two, 2; three, 3.
- 94 Lower gill membrane attachment near lower end of pectoral fin (pectoral fin insertion), 0; extended more anteriorly, 1; far forward on to the isthmus, 2.
- 96 Posteriormost roof of mouth and gill chamber white, 0; largely black, 1.

- 97 Median gular area mostly white or dusky, 0; blackened, 1.
- 98 Nasal bone not ossified, 0; yes, 1.
- 99 Sinuously curving pelvic spine lacking, 0; slight, 1; extensive, 2.
- 100 Blackened tips on vomerine and lingual lobes absent, 0; present, 1.
- 101 Ctenoid or ciliated scales present at some life stage, 0; absent, 1.
- 102 Neuromast organs posterior to mental frenum, two, 0; five or more, 1.
- 103 Front rostral cartilage ligament attached to ascending processes of the premaxillary, 0; or more posterior to rostral cartilage, 1.

Table A-5. NEXUS-formatted data matrix of morphological characters described in Table A-4.

```

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  matrix

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Gy_heptacanthus             00000102000012201003111110210110000101011101101111101010202100100022000200000000132100133010220000011011
Gy_mororanus                 00000102010002201003212100210220000101011101110000001021200200110022001200000001131101123010220000011011
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Gy_taranetzi[67]            00000002200010000003212011210120000001011101111100002000212200100122000200000201110100133110020000001001
Gy_taranetzi[73]            00000002200010000003212011210120000001011101111100002000212200100122000200000201110100133110020000001001
Gy_urotaenia[63]            10012010001122200003011000210220000101012211101100000000202200100122001200000001041101133110120000001001
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Gill_mirabilis[REF1]        02200010201112110122202111211220000211012211121111101000101200011221021100000311042101133020110000001101
Astrabe_flav                 021000102000022001142021210001100222101121310?000000000000020010002213111111101100100121011010000200001
Chaen_gulosus[KYO1]         200000100010211001021010302112100202210113211012200201101220001122130200000221042101133000110000001101
Chaen_annularis[TSY1]       2002201000??20000?????3?????00002210?????????????????????0112??3??0000??11?411011330?????????1001
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Clevelandia_ios[SQU1]       00022002221011011103002010210020000201012102121010002220101200111022000200000301040100022120210000000001
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Eucycl_newb[CCS01058_20]    0002201222001220010301201021011000000101110212211111121101200100022200200000111130100133120010000000001
Everm_zost[REF1]            00000100200021110203002120210110100011001001122100022220001201100021010100000111040100012020210110000001
Everm_pana[Ever1]           000001002000122102030021202101101000110010011221000222100012011000210101000001?1040100012020210??0000001
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Ilyp_luc[ILA1]              21100012200012210103001010210220100000011101102221021120202200100021010200000111040100022010010100000001
Lepido_lep[BDG1]            200111112000011000003002220110220000101012221002201001120212200100220120200010201040111233010010000000001
Lethops_con[1]              0000000000000001001411000221022000010111221300222010200011210100022100111111301100100000302001000010?001
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Lucio_par                    010000000000200000140020000110100222?10022133012201??011011001000222?001111030110010000000200000?00001
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Typhlo_cal[COP1]            0100000000000001001400110201022000021100021300210000100011120010002212101111031110010000102001000010?001
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end;

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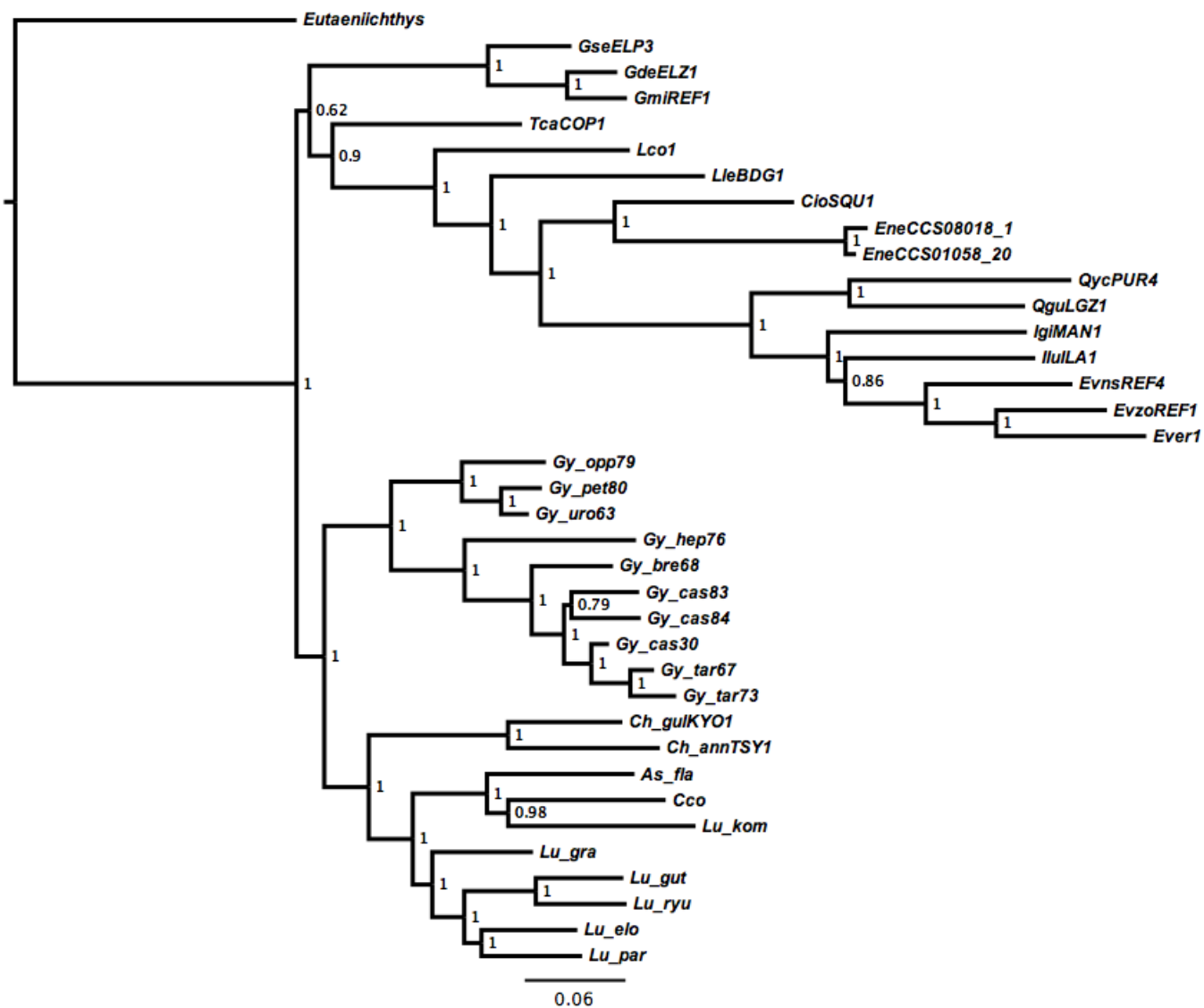


Figure A-1. Phylogram constructed in MRBAYES using combined molecular and morphological data. Molecular data was treated as in Fig. 1-4, with the 10 fastest-rate sites excluded. Node numbers indicate posterior probabilities.

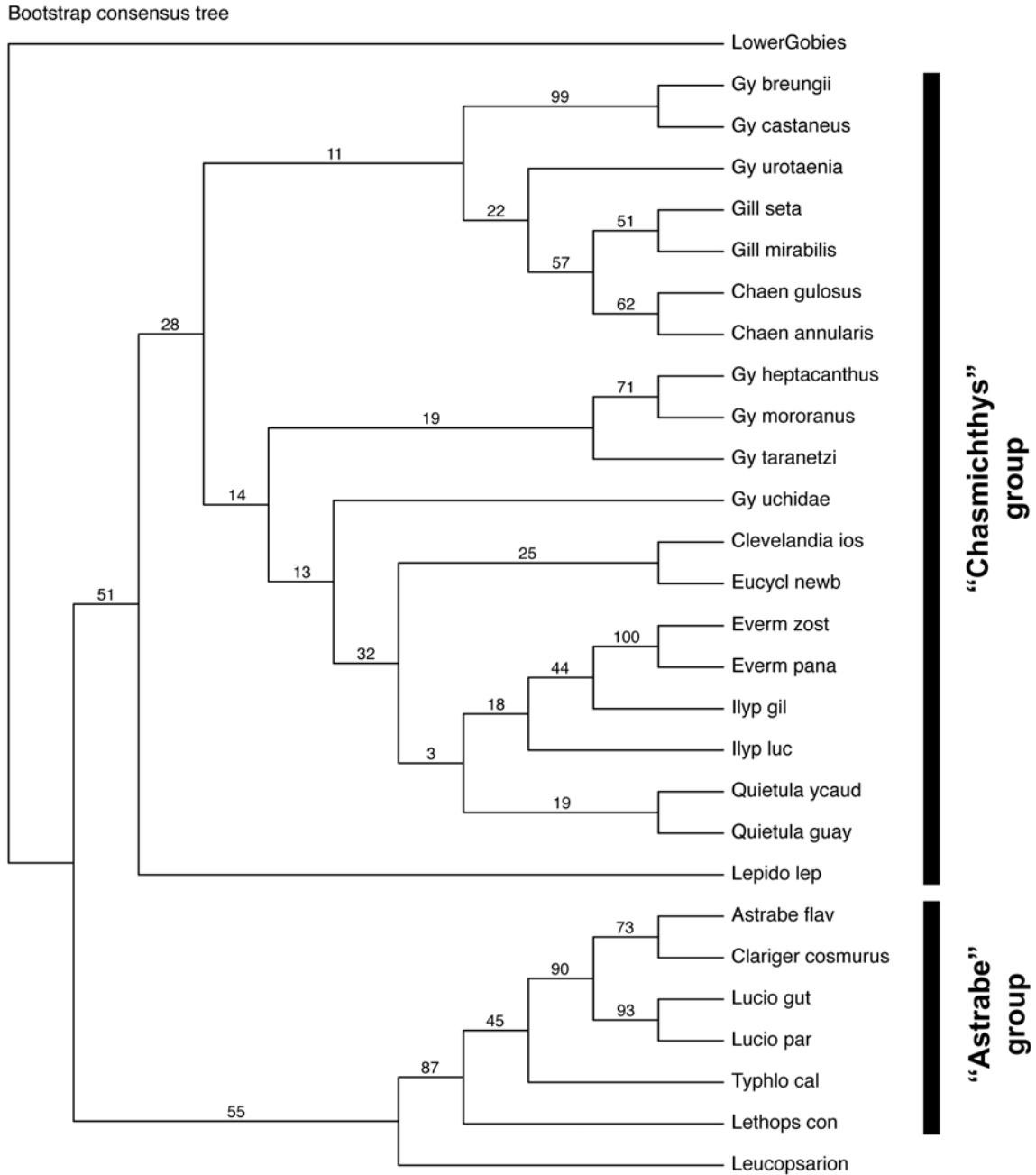


Figure A-2. Cladogram of bay goby morphological characters constructed with the maximum parsimony criterion in PAUP*. Numbers indicate bootstrap support from 1000 replicates.

Table A-6. Vertebral counts of North Pacific bay goby species. Counts are either broken down by abdominal + caudal vertebrae, or a range is given for multiple individuals where possible.

Species	Vertebrae (abdominal + caudal = total)
West Pacific taxa: (abdominal/caudal breakdown from Akihito et al. 1984, others from Yamada et al. 2009)	
<i>Leucopsarion petersii</i>	14 + 20 = 34
<i>Eutaeniichthys gilli</i>	22 + 17 = 39
<i>Chasmichthys dolichognathus</i>	14 + 18 = 32
<i>C. gulosus</i>	14 + 19 = 33
<i>Gymnogobius castaneus</i>	15 + 20 = 35
<i>G. laevis</i>	15 + 18 = 33
<i>G. heptacanthus</i>	17 + 21 = 38
<i>G. mororanus</i>	16 + 22 = 38
<i>G. urotaenia</i>	15 + 17 = 32
<i>G. isaza</i>	15 + 18 = 33
<i>G. cylindricus</i>	15 + 17 = 32
<i>G. macrognathus</i>	16 + 19 = 35
<i>G. uchidai</i>	15 + 19 = 34
<i>Astrabe lactisella</i>	13 + 17 = 30
<i>A. flavimaculata</i>	30
<i>A. sp.</i>	14 + 16 = 30
<i>Clariger cosmurus</i>	15 + 19 = 34
<i>C. exilis</i>	15 + 18 = 33
<i>C. papillosus</i>	14 + 18 = 32
<i>Inu koma</i>	14 + 17 = 31
<i>I. saikaiensis</i>	15 + 17 = 32
<i>L. guttatus</i>	17 + 21 = 38
<i>L. grandis</i>	19 + 22 = 41
<i>L. platycephalus</i>	17 + 24 = 41
<i>L. dormitories</i>	18 + 18 = 36
<i>L. parvulus</i>	20 + 22 = 42
<i>L. pallidus</i>	19 + 18 = 37
<i>L. elongates</i>	38-44
<i>L. adapel</i>	50
East Pacific taxa: (from Miller & Lea 1976, Bussing 1983, Watson 1996, Kindermann et al. 2007, Swift et al. 2011, D. Hoese <i>pers. comm.</i> , and this study)	
<i>Gillichthys mirabilis</i>	30-33
<i>G. detrusus</i>	30-32
<i>Lepidogobius lepidus</i>	37-38
<i>Eucyclogobius newberryi</i>	33-38
<i>Clevelandia ios</i>	36-37
<i>Quietula y-cauda</i>	33-34
<i>Q. guaymasiae</i>	33-34
<i>Ilypnus gilberti</i>	32-34
<i>I. luculentus</i>	36
<i>Evermannia panamensis</i>	30-32
<i>E. erici</i>	30-32
<i>E. zosterura</i>	30
<i>Typhlogobius californiensis</i>	30-32
<i>Lethops connectens</i>	34-36

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CHAPTER 2

Divergence time estimation of North Pacific bay gobies reveals relictual endemism in the Gulf of California

INTRODUCTION

Regions of elevated marine endemism tend to be viewed as engines of speciation (center-of-origin hypothesis; Briggs 2003; Mora et al. 2003). Alternative explanations for concentrated endemism include the center-of-overlap and center-of-accumulation hypotheses, which interpret such areas as convergent biogeographic ranges or refugia for species survival, respectively (Mora et al. 2003; Bellwood and Meyer 2009b). While the relative merits of these models remain contentious (Briggs 2009), they need not be mutually exclusive (Bellwood and Meyer 2009a).

Elevated Endemism in the Gulf of California

The northern Gulf of California (hereafter referred to as the Gulf) has long been recognized as an area of elevated endemism for marine mammals, reptiles, fishes and invertebrates, including the overfished totoaba, *Totoaba macdonaldi* (Guevara 1990; Rowell et al. 2008), and the critically endangered vaquita, *Phocoena sinus* (D'agrosa et al. 2000). Little is known about the geographic and temporal origins of endemism in the northern Gulf, although it has been suggested that the phenomenon is a product of the isolation of populations following formation of the Gulf (Hubbs 1960; Walker 1960; Robertson and Cramer 2009). Relative to the outer coast, the upper Gulf experiences greater tidal flux (Matthews and Mathews 1968), seasonal variation in sea surface temperature (Fig. 2-1), and, historically, high volumes of fresh water runoff from the Colorado River (Fradkin 1996). These factors likely contributed to ecological isolation of Gulf populations since tectonic formation of the Gulf ~6.3 million years ago (Ma). Others have argued that isolation in the Gulf occurred during glacial cycles when cooler temperatures would have forced southern range extensions and allowed northern species into the Gulf (Hubbs 1960; Walker 1960; Robertson and Cramer 2009), a process presumably

restricted to the last 3 Ma. It also appears possible that endemics could be older than tectonic formation of the Gulf given the complexity of tectonic processes and evolution of coastal environments through the Neogene ~25 Ma to the present (Jacobs et al. 2004). In this chapter I use temperate bay gobies as a model to assess the divergence times of Gulf endemics relative to the aforementioned tectonic events and climatic regimes.

Bay Gobies as an Ideal Study System for Gulf Endemism

North Pacific bay gobies inhabit temperate estuaries and lagoons on the coasts of both North America and Asia, but their absence from northernmost latitudes (Fig. 2-2) gives them a disjunct amphipacific distribution (e.g. Andriashev 1939; Ekman 1953; Briggs 1974). A multi-locus phylogeny revealed geographic subdivision and convergence of ecological adaptations across the Pacific (chapter 1). In the West Pacific, ecological radiations are represented by the genera *Luciogobius*, with many infaunal lineages in the beaches of Japan (Yamada et al. 2009), and *Gymnogobius*, which has diversified among coastal streams of East Asia (Sota et al. 2005). While such speciose genera are not found in North America, estuaries in the Gulf of California contain six endemic species distributed across four recognized genera, making this assemblage important for understanding the evolution of endemic diversity in the Gulf. The genus *Gillichthys* comprises three species, two of which are Gulf endemics: *G. seta* is the oldest species in the genus and is endemic to the northern half of the Gulf, and *G. detrusus* is endemic to the relatively small region of the Colorado River delta (Swift et al. 2011). The third species, *G. mirabilis*, is found on the outer coast as well as in the Gulf and is sister to *G. detrusus*. The genus *Quietula* is composed of just two recognized sister species, with *Q. guaymasiae* being endemic to the northern half of the Gulf and *Q. y-cauda* on the outer coast from Morro Bay south and

inside the Gulf. The genus *Evermannia* forms a clade nested within a paraphyletic *Ilypnus* group (chapter 1). This *Evermannia/Ilypnus* clade contains the Gulf-endemic *I. luculentus*, and an undescribed species of *Evermannia* that appears to be endemic to the Gulf, although its southern limit is not well documented. The two youngest species in the clade, *E. zosterura* and *E. panamensis*, are found from the Gulf down to Peru and from El Salvador to Panama, respectively. *Evermannia* is the only genus in the entire amphi-Pacific bay goby clade that extends into the tropics. The independent origination of multiple Gulf-endemic bay goby lineages provides a means to explore the temporal history of the Gulf and comparable environmental conditions in the region.

Onset of Marine Conditions in the Northern Gulf Region

Analyses of various classes of geologic data have resulted in a range of possible dates for Gulf origination. Tectonic plate reconstruction based on paleomagnetism suggests that opening of the Gulf began no more than 8 Ma, and further work involving the offset of dated volcanics and the age of marine rocks of the Imperial Formation constrain formal opening of the Gulf to less than 6.5 Ma (Moore and Curray 1982; Oskin and Stock 2003a). Biostratigraphic examination of cores appears to document marine sediments from ~12 Ma in what is now the northern Gulf (Helenes and Carreno 1999; Helenes et al. 2009) and similar aged sediments are inferred to exist in surrounding low-lying areas (Dorsey et al. 2007). These deposits appear to occupy a “proto-Gulf” that was older than the modern Gulf and seemingly of different tectonic (Basin and Range) origin. Thus, from a paleontological as opposed to tectonic perspective, the exact timing of Gulf inception is complex and endemic speciation that predates tectonic isolation of the Gulf may be possible.

Biogeographic Time-Calibration of Phylogenetic Trees

Interpretation of phylogenetic trees in the context of historical events requires time calibration, which in turn depends upon age constraint of one or more nodes on a tree. The earliest fossil appearances of taxa inferred to be ancestral, or the ages of vicariant biogeographic events, are frequently used for this purpose. Fossil calibration assumes 1) that species duration is known or is negligible, as populations forming new species can separate at any time along a lineage, 2) that species are morphologically distinct and recognizable in the fossil record, and 3) that stem and crown group taxa are adequately sampled such that a phylogeny inclusive of fossil and modern taxa can be determined with confidence (Rutschmann et al. 2007; Donoghue and Benton 2007). Fossil-based approaches may work well at higher taxonomic levels with an exceptionally well-preserved morphologic record, or at lower levels where species are of short duration. However, a dearth of suitable fossils for a given taxonomic group of interest often precludes proper implementation of fossil-based constraints.

Alternatively, node constraints based on vicariant events require accurate dating of a given event in the rock record and confidence that this event produced the lineage split of interest. While there appears to be a prevailing bias toward the use of fossil calibration in modern literature (e.g. Heled and Drummond 2012; Ronquist et al. 2012), biogeographic calibration takes advantage of data from tectonics, climate history and/or paleoceanography without relying on fossil preservation. No known fossils constrain the age of any common ancestors within the North Pacific bay goby clade. Thus, I associate three well-understood abiotic events with vicariant cladogenesis in the tree.

I use an iterative process to time-calibrate a well-supported multi-locus phylogeny of bay gobies with vicariant events at three distinct nodes: 1) The onset of a marine opening between

Korea and Japan constrains the temporal origination of a clade within the West Pacific genus *Gymnogobius*. The *G. castaneus/taranetzi* species complex is largely confined to freshwater streams around the Sea of Japan, and was isolated into two distinct lineages by the onset of marine conditions in the Tsushima Strait beginning 3.5 Ma (Sota et al. 2005; Kitamura and Kimoto 2006). 2) A tree calibrated with the above constraint suggested that east-west division of the clade across the North Pacific occurred near the Eocene/Oligocene boundary, defined by a major cooling event dated at 33.8 Ma (Zachos et al. 2001; Oleinik and Marincovich 2003). I therefore refined calibration by associating the basal trans-Pacific divergence with this rapid global cooling, which likely rendered the northernmost Pacific uninhabitable for the ancestor of these gobies. 3) Finally, I calibrated speciation of the Colorado River Delta endemic, *Gillichthys detrusus*, at the first appearance of deltaic sediments in the region 5.33 Ma (Dorsey et al. 2007), based on its endemism and specialization for the highly turbid and historically fresh deltaic habitat (Swift et al. 2011).

Objectives

The dated phylogeny constructed here will allow me to test three alternative hypotheses to explain the origins of Gulf-endemism: 1) Endemics are the products of relatively recent events, likely related to isolation in the Gulf by climate-induced Pleistocene range shifts; 2) endemics have arisen via geographic isolation caused by tectonic rifting of the Baja California peninsula; 3) endemics are relicts of species that arose prior to tectonic rifting, in similarly warm estuarine habitats of the Miocene-era California coast, and subsequently took refuge in the Gulf (Jacobs et al. 2004). Thus, understanding the temporal origins of Gulf-endemic bay gobies will

provide insight into whether the region has acted as an engine for endemism or an ecological refuge for historically more widespread species.

METHODS

DNA Sequence Generation and Partitioning

Whole specimens, tissue samples, DNA extracts and sequences were obtained via seine collection, donation, loan, or from GenBank (chapter 1). All sequences were unambiguously aligned by eye and concatenated in GENEIOUS v6.0.2 (Biomatters). Sequence data was partitioned according to per-site relative rate as described in chapter 1.

Temporal Calibration Using Biogeographic Data

Paleoceanography, climate history and tectonics were used to calibrate the bay goby phylogeny. The onset of the northeastward Tsushima Current between Japan and the Korean Peninsula 3.5 Ma indicates relatively deep marine conditions that created a probable boundary between populations of *Gymnogobius* (Sota et al. 2005). The *Gymnogobius castaneus/taranetzi* species complex is presently confined to fresh water streams around the Sea of Japan. Before the current began flowing, the common ancestor of these lineages likely moved unabated across the Tsushima Strait, which was narrower, shallower, and less saline than it is today. The periodic presence of warm-water diatoms in the stratigraphic record shows that historical inflow of the Tsushima Current into the Sea of Japan was marked by five intervals from 3.5-1.9 Ma, followed by intervals at each interglacial period starting at 1.7 Ma (Kitamura and Kimoto 2006). While the current was characterized by deeper water and higher salinity during this second set of intervals,

the initial flow indicates a marine presence that was likely sufficient to prevent migration as every extant member of the *G. castaneus/taranetzi* complex spends its entire life cycle in fresh water.

Bay gobies are restricted to temperate and sub-tropical latitudes, with northern limits in British Columbia and the southern Sea of Okhotsk in the East and West Pacific, respectively (Fig. 2-2). Since extant members of the group have largely overlapping ranges, their common ancestor likely had a similar temperature tolerance. Rapid glaciation of Antarctica, as inferred from oxygen isotope ($\delta^{18}\text{O}$) records of benthic foraminifera, is associated with a sharp decline in global temperatures at 33.8 Ma (e.g. Zachos et al. 1996; 2001). This global cooling phenomenon extended from temperate to tropical latitudes, affecting both marine and terrestrial systems (e.g. Wolfe 1995; Lear et al. 2000; Katz et al. 2008). To connect this expansive cooling event to trans-Pacific isolation of bay goby ancestral populations, I point to a contemporaneous shift from temperate to cool-water gastropod faunal assemblages in the North Pacific between 55° and 60° N on the West Coast of the Russian Kamchatka Peninsula and on the Gulf Coast of southeastern Alaska (Oleinik and Marincovich 2003). Taken together with the latitudinal distribution of extant bay gobies, these data support my inference of 33.8 Ma for the deepest split in the phylogeny, separating North American and Asian taxa.

The final age constraint involves the first appearance of the Colorado River Delta 5.33 Ma, which provided the conditions necessary for origination of the delta mudsucker *Gillichthys detrusus*. This recently resurrected species is geographically restricted and ecologically specialized to the deltaic environment at the mouth of the Colorado (Swift et al. 2011). It is found in very close proximity to its wider ranging sister species, *G. mirabilis*, yet each species lives in ecologically distinct habitats. *Gillichthys mirabilis* inhabits marsh-top muddy channels of

estuaries, while *G. detrusus* lives in deep channels produced by up to 10-meter tides in the river delta, where it appears to be highly adapted to fine, silty sediments and heavily turbid waters. Relative to *G. mirabilis*, the delta mudsucker has a depressed, almost shovel-like head with a slightly upturned mouth. This may facilitate movement through the channels where silt is so pervasive that it is difficult to distinguish where water ends and substrate begins. Smaller eyes and drab coloration compared to *G. mirabilis* are likely adapted to the persistent turbidity of *G. detrusus* habitat. It is likely that *G. detrusus* arose coincident with or shortly after emergence of the deltaic environment for which it is specialized. I therefore estimate the age of speciation to coincide with initial deposition of delta-associated sediments from the Colorado River 5.33 Ma (Dorsey et al. 2007). Although the sedimentary outcrop that supports this date is at Split Mountain Gorge in southern California, far north of the current Colorado River mouth, this is due to the mouth having been displaced by substantial tectonic movement as well as progression of the Colorado Delta southward across its alluvial fan.

Divergence Time Analyses

Divergence times were estimated using BEAST v1.7.4. XML input files were created using BEAUti 1.7.4, with the following modifications to default settings. Constraints placed on node ages to time-calibrate the phylogeny are listed in Table 2-1, and the detailed rationale for choosing these dates is presented in the Discussion section. In addition to calibration with all three of these points, each was used as a single calibration for independent runs to rule out substantial discordance among them. An analysis was also performed with a calibration date of 6 Ma for the speciation of Gulf endemics in order to evaluate the common assumption that they must be no older than tectonic formation of the Gulf itself. A normal prior distribution was

specified for each value to account for uncertainty of the exact age of the abiotic event and the assumption that the event coincided with lineage divergence (Ho 2007). The GTR substitution model was applied to each data partition, with a gamma distribution of 4 rate categories. The continuous-time Markov chain prior (CTMC; Ferreira and Suchard 2008) was used to estimate rates under the lognormal relaxed uncorrelated clock model with initial rate values of 0.01 for each partition. Tree topology was linked among partitions, with most clades constrained to the congruent and highly supported topologies constructed in MRBAYES and RAxML (chapter 1).

Two independent runs of 20,000,000 generations each were conducted, with the first 50% of trees and parameter estimates being discarded from each as burnin. To confirm that effective sample size (ESS) values for all parameter estimates were greater than 200, suggesting convergence of both runs, posterior distributions were analyzed in TRACER v1.5. Post-burnin trees from each run were combined in LOGCOMBINER v1.7.4, and a consensus tree was created using TREEANNOTATOR v1.7.4.

RESULTS

Divergence time estimates are illustrated on the tree in Figure 2-3. Confidence intervals of mean divergence times did not exceed ± 5 Ma for any node within the ingroup. The mean timing of speciation events of Gulf of California endemics ranged from 16.0 to 10.7 Ma. Although incompletely sampled here, the West Pacific clade contains more extant species ($n = 37$) than the East Pacific ($n = 16$). Diversification of the West Pacific clade appears to be delayed relative to the East Pacific. Only one speciation event occurs during the Oligocene (spanning ~ 10 million years) in the West Pacific clade, while the East Pacific records five branching events

during the same time span (Fig. 2-3). In the east, diversification appears to begin shortly after the initial trans-Pacific split, with a relatively uniform rate of speciation continuing to the present. This seems to slow somewhat during a period surrounding the Oligocene-Miocene transition, from ~25-20 Ma. The most derived lineages then continue to diversify while the oldest (with the exception of *Gillichthys*) remain static. In the West Pacific, just one speciation event occurs between the clade's origin and the beginning of the Neogene, a span of ~10 Ma. After a few divergences in the Early Miocene, the rate of diversification appears to increase dramatically within the clades comprising the genera *Gymnogobius* and *Luciogobius*. Future work will use quantitative methods to formally test these visually apparent patterns of shifting diversification rates.

Several analyses were run to assess consistency among different calibration points. When the Oligocene global cooling event was omitted from analyses as a time-calibration point, the basal trans-Pacific split was independently estimated to fall within a 5 Ma period following the Eocene/Oligocene boundary (Fig. B-1 – B-3). It should be recognized that global temperature also dropped off, although less dramatically, in the Middle Miocene ~14 Ma. Calibration of the basal divergence at this time, however, yields an unrealistically young tree (Fig. B-4) that conflicts with the calibration points near the crown and requires unusually high rates of nucleotide substitution (~5%/Ma at *cytb* for recently diverged taxa). Uncertainties surrounding node age estimates were substantially smaller when all three calibration points were used as compared to any single point. Assigning a node age deep in the tree, in this case at the earliest split of the bay goby ingroup, had a particularly large influence on the precision of divergence time estimates throughout the tree. Confidence intervals were up to two times wider at some nodes when this basal constraint was omitted from the analysis. An analysis that assumed a

young age of 6 Ma for Gulf endemics reduced the overall age of the tree by more than half (Fig. B-5). Much like constraining the basal split to the Middle Miocene, this “young endemic” calibration requires sequence evolution at approximately twice the typical rate for mtDNA.

DISCUSSION

Most Gulf-Endemic Gobies are Older than the Gulf

Divergence time estimates (Fig. 2-3) for *Ilypnus luculentus* (16.0 ± 2.4 Ma), *Quietula guaymasiae* (13.6 ± 3.1) and *Gillichthys seta* (10.6 ± 2.9) all predate the inferred age of an isolated Gulf at ~6 Ma (Oskin and Stock 2003a,b). Even under the most conservative interpretation (i.e. using the lower bound of the confidence interval surrounding the divergence of *G. seta*), speciation of these three endemics still occurs prior to tectonic formation of the Gulf. This suggests that these taxa likely arose in Gulf-like temperate to subtropical habitats in the Middle to Late Miocene, but not in the tectonically formed Gulf as it is known today. There are two possible scenarios for this evolution: 1) Continuity of habitat through time allowed the fauna to be directly inherited by the modern tectonic Gulf from a pre-existing “proto-Gulf” in the same area, or 2) Gulf endemics evolved in Late Miocene embayments on the outer coast with hot seasonal environments comparable to the northern Gulf (Hall 2002; Jacobs et al. 2004) and were subsequently introduced to the Gulf through a seaway from the north.

The concept of a Middle to Late Miocene proto-Gulf was introduced by Karig and Jensky (1972). Marine rocks of this age would represent marine incursions associated with the end of the Basin and Range tectonic phase of the region. These deposits would likely pertain only to the northern portion of the Gulf and need not be associated with the transform motion

that initiated opening of the modern Gulf ~6.3 Ma (Oskin and Stock 2003a,b). Drill cores with sediments suggestive of a proto-Gulf marine embayment have been dated at 11.2 Ma (Helenes and Carreno 1999; Helenes et al. 2009). Other less temporally-constrained marine sediments of presumptive Miocene age are found below Pliocene marine deposits in the Altair region adjacent to the northern terminus of the Gulf. Additionally, marine deposits likely to be of Miocene origin are found below the Bouse formation in some Basin and Range settings in southeastern California and Arizona (Dorsey et al. 2007). Thus, there is a possibility that northern Gulf endemics evolved in the proto-Gulf context, remained there *in situ* and were inherited by the modern Gulf as it formed. It is possible, however, that a marine environment was not temporally continuous in the region, as some sedimentary sections show evaporates indicative of terrestrial conditions between these older marine deposits and those associated with tectonic opening of the Gulf (Dorsey et al. 2007).

Another, possibly more likely, scenario involves warm tectonically-isolated bay habitats that were clearly present along the California coast in the Middle to Late Miocene (Hall 2002) and are thought to represent Gulf-like environments in other respects due to the absence of summer rain (Jacobs et al. 2004). Many components of these systems have shifted northward in excess of 300km as a consequence of tectonic movement along the San Andreas fault (Oskin et al. 2001; Wernicke 2011), but were at comparable latitudes to the modern northern Gulf region in the Middle to Late Miocene. These coastal embayments represent a likely source of the oldest Gulf-endemic taxa. Prior to dramatic Plio-Pleistocene uplift of the transverse ranges of southern California, continuity between marine and estuarine habitats in the northern Gulf region (contemporary Salton Trough and southwestern Arizona) and the Pacific coast via the Los Angeles basin is evident in the Late Miocene (Moore and Curray 1982; Wernicke 2011).

Continuous marine sedimentary rock units as well as biological affinities between Los Angeles and Colorado basin fishes support this idea. Speckled dace of the genus *Rhinichthys* from the LA Basin were unexpectedly found to be more closely related to congeners of the Colorado drainage than those of the more proximal Owens River and northern California (Smith and Dowling 2008). In addition, Late Miocene to Pliocene marine sediments of the Puente Formation in the LA basin, the Imperial Formation of the Salton Trough (a former extension of the Gulf), and at the base of the Bouse Formation spanning the California-Arizona border all point to a connection from the outer coast through the LA basin (Wernicke 2011) to the Gulf at its tectonic inception ~6.3 Ma (McDougall et al. 1999; Oskin and Stock 2003a). All of this suggests an opportunity for estuarine taxa to enter the northern Gulf at its tectonic inception when deeper marine rocks of the Imperial formation were first deposited. More broadly, further study of the biological consequences of the complex tectonic evolution of the region are in order.

Temporal Disparity between East and West Pacific Diversification

One striking aspect of the time-calibrated phylogeny is that patterns of lineage diversification through time appear to be quite different between the East and West Pacific clades (Fig. 2-3). While cladogenesis begins earlier in the East Pacific, more recent bursts in the West Pacific have generated more than double the number of total species. This pattern may be largely attributable to isolating effects of the habitats of two West Pacific genera. The genesis of *Luciogobius* ~17 Ma represents colonization of interstitial habitat, where the subsequent radiation of no fewer than 12 species was likely driven by heterogeneous grain size among gravel beaches of coastal Japan (Yamada et al. 2009). The earliest divergence within *Gymnogobius* occurred nearly 20 Ma, but the bulk of species diversity within this genus has

occurred over just the past 8 Ma and has largely been generated by invasion of and isolation within fresh water streams draining into the Sea of Japan (Sota et al. 2005). More comprehensive species sampling within the genera will allow for statistical analyses to determine the extent to which diversification rates increase in these two lineages, and how that may relate to geological formation of beaches on the Japanese Archipelago and climatic changes influencing fresh water drainage into the Sea of Japan.

CONCLUSIONS

In the absence of fossils, divergence time estimates were obtained by using a comprehensive mix of paleoclimatic, geological and paleoceanographic data to time-calibrate the multi-locus molecular phylogeny of bay gobies. The independent origination of multiple species endemic to the Gulf of California prior to tectonic formation of the Gulf suggests diversification was driven by pre-Gulf environments comparable to those confined to the northern Gulf today. This suggests that the center-of-origin hypothesis, invoked to explain Coral Triangle endemism and other hotspots of biodiversity (Briggs 2003; Mora et al. 2003), does not sufficiently account for the extent of endemism currently observed within the Gulf. Instead, a substantial fraction of Gulf endemics appears to be relictual (Bellwood and Meyer 2009b). These species likely arose in large Miocene embayments with ecological similarities to modern northern Gulf estuaries (Hall 2002), and migrated to their present location via a marine connection from the Pacific through the Los Angeles basin (Moore and Curray 1982; Present 1987; Wernicke 2011). The northern Gulf then became a refuge for these species when tectonic activity isolated the region as climate change and tectonic activity eliminated comparable large estuarine features from the outer

Pacific coast (Jacobs et al. 2004). Younger endemic species are also present within the Gulf (e.g. Swift et al. 2011), as are intraspecific phylogeographic clades on both sides of the Baja Peninsula (Present 1987; Sandoval-Castillo et al. 2004), suggesting that aspects of tectonic isolation and unique ecological regimes continue to generate local reproductive isolation in the region. The bay goby phylogeny also suggests incongruent diversification rates on either side of the Pacific, warranting further investigation to formalize the apparent pattern of increased rates in the West and to explain their potential causes. Thus, interpretation of the timed phylogeny in the context of Late Cenozoic climate and tectonics reveals a complex history of adaptation and migration in this temperate group of fishes.

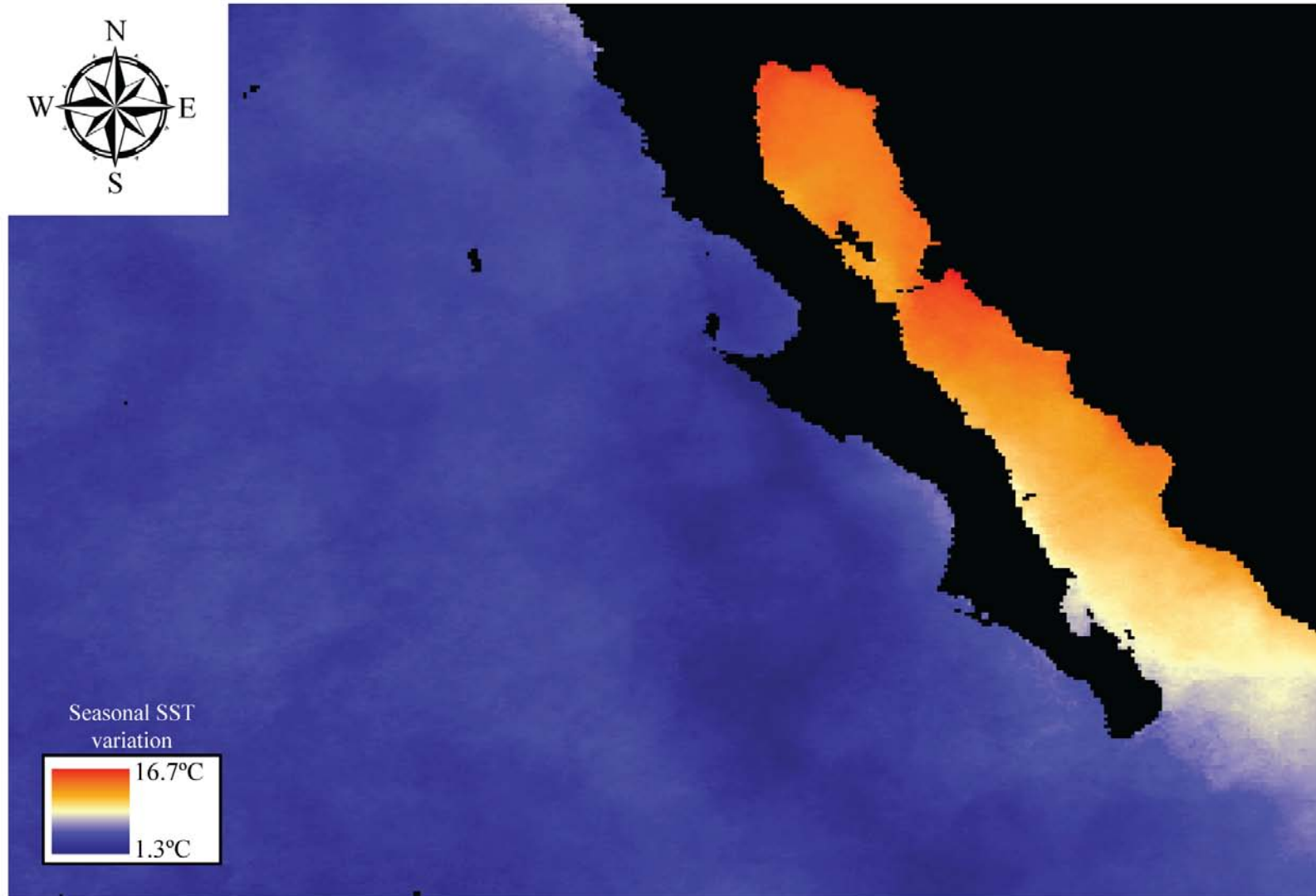


Figure 2-1. Seasonal sea surface temperature (SST) variation in the waters surrounding Baja California. Image illustrates the difference between mean SST, recorded in 2009, for the two hottest months of the year (July and August) and the two coldest months (January and February).

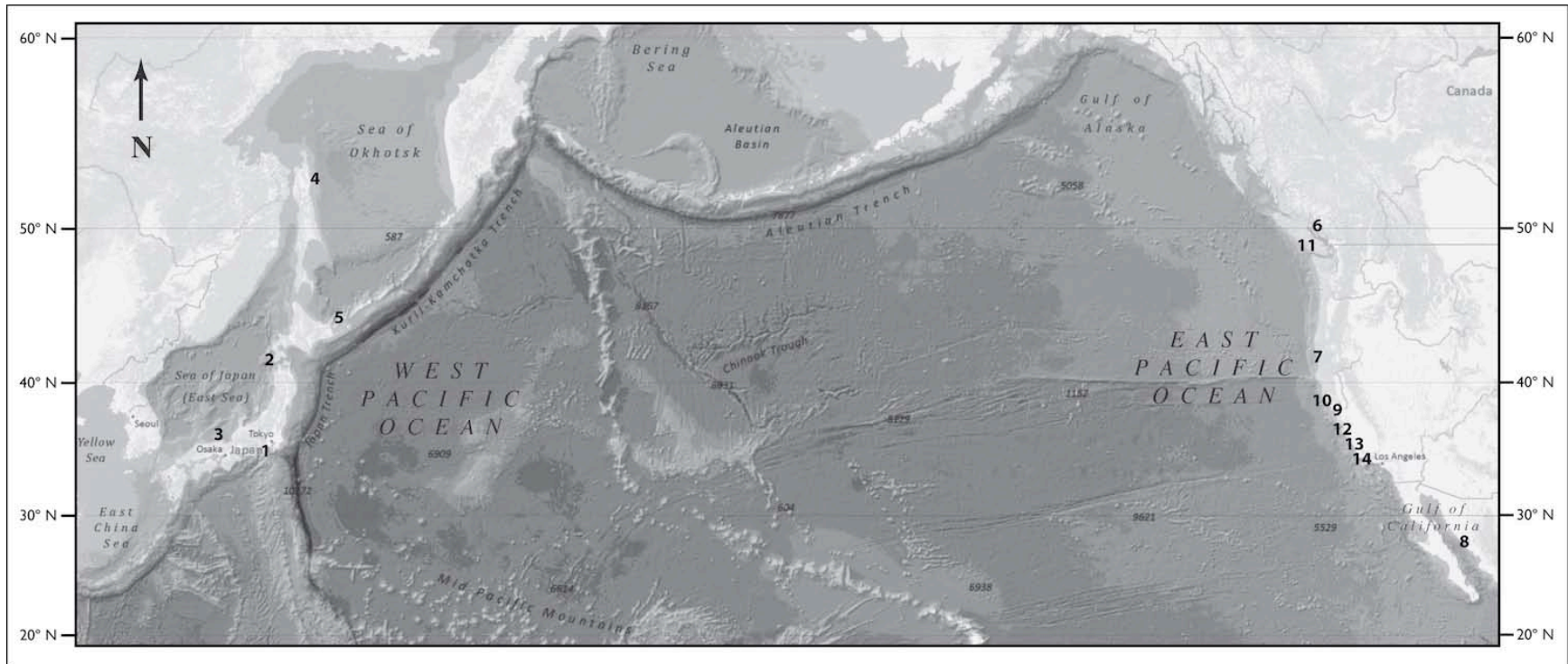


Figure 2-2. Map of the North Pacific Ocean showing the range extent of bay goby habitat on the coasts of Asia and North America. These temperate gobies exhibit an amphi-Pacific distribution, where species are found on either side of the Pacific but are absent along coasts of the Bering Sea and Aleutian Islands. Northern range limits of each genus are numbered as follows: 1=*Astrabe*, 2=*Chaenogobius*, 3=*Clariger*, 4=*Gymnogobius*, 5=*Luciogobius*, 6=*Clevelandia*, 7=*Eucyclogobius*, 8=*Evermannia*, 9=*Gillichthys*, 10=*Ilypnus*, 11=*Lepidogobius*, 12=*Lethops*, 13=*Quietula*, 14=*Typhlogobius*.

Table 2-1. Vicariant events used to time-calibrate the phylogeny of North Pacific bay gobies. Further details supporting the rationale for inferring bay goby vicariance due to these events can be found in the Methods section.

Biogeographic Event	Date of Event (Ma)	Calibration Prior Mean \pm S.D.	Calibration Prior Distribution
Eocene/Oligocene global cooling event is inferred to have pushed the North Pacific bay goby ancestor south along the coasts of North America and Asia, resulting in the most basal lineage divergence within bay gobies and an amphi-Pacific disjunction that persists today.	33.8	33.8 \pm 1.0	normal
First appearance of deltaic sediments at the mouth of the Colorado River (Bouse Formation) indicates inception of the Colorado Delta, the environment where <i>G. detrusus</i> is endemic and for which it is highly adapted.	5.33	5.33 \pm 0.3	normal
Onset of saline Tsushima Current indicative of marine conditions inferred to have isolated freshwater lineages of the <i>Gymnogobius castaneus/taranetzi</i> species complex between Japan and the Korea Peninsula.	3.5	3.5 \pm 0.3	normal

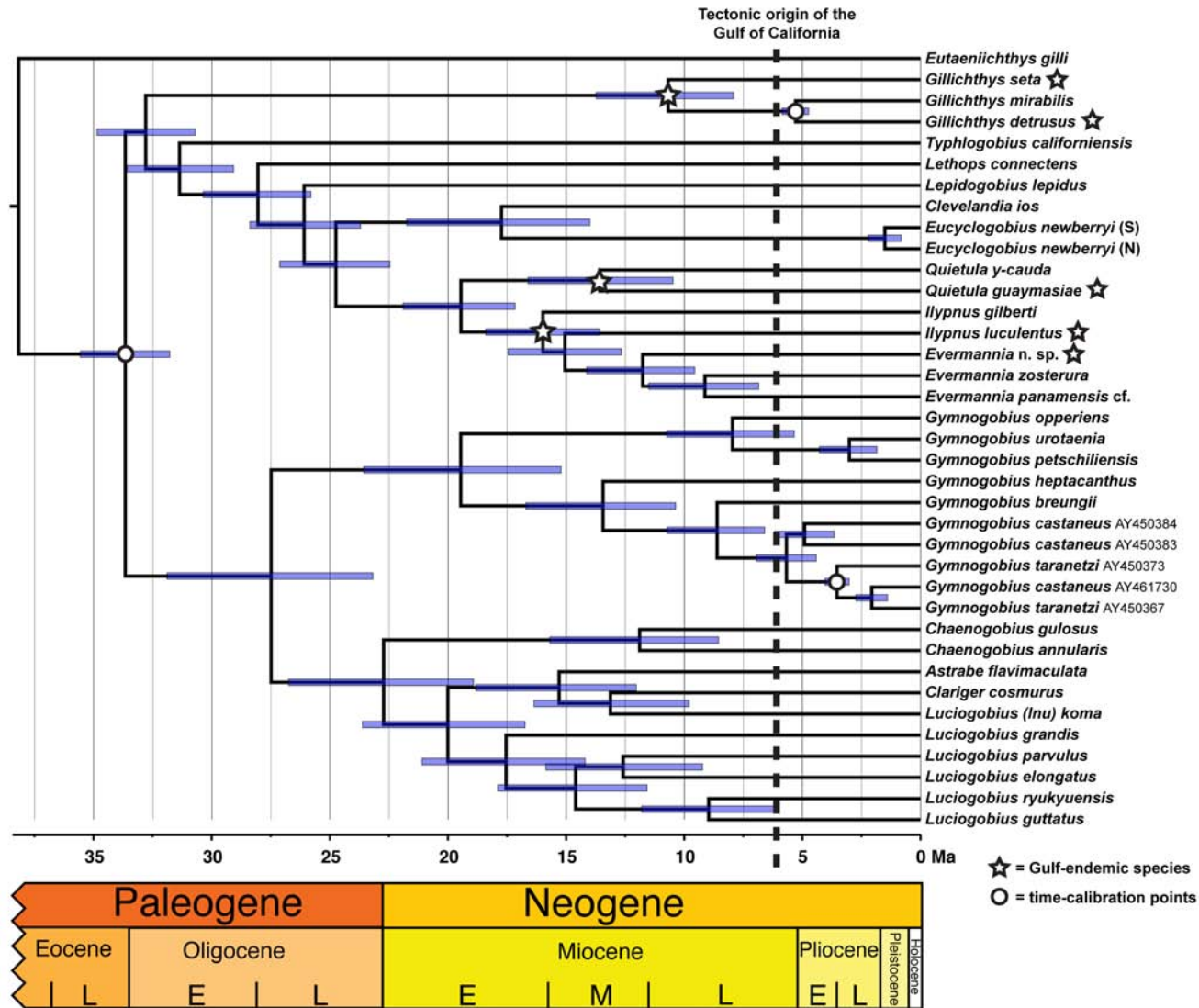


Figure 2-3. Temporal phylogeny of bay gobies representing divergence times as estimated in BEAST. Node bars represent 95% confidence intervals on age estimates. Stars on nodes represent the earliest independent originations of lineages that are currently endemic to the Gulf of California.

Appendix Figures. In all figures, stars represent species endemic to the Gulf of California and open circles indicate calibration point(s) used for that analysis.

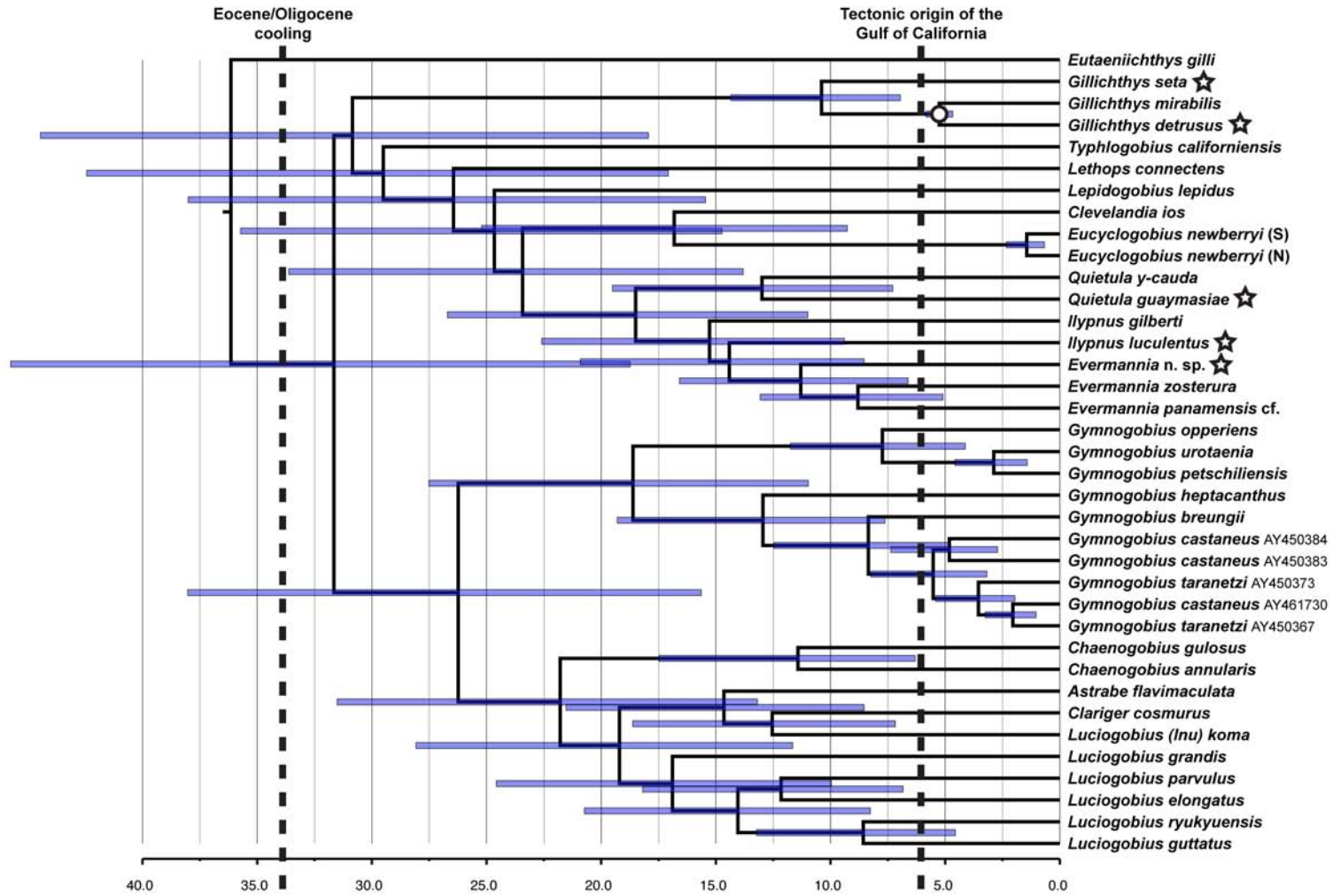


Figure B-1. Chronogram calibrated using only the speciation event of the Colorado Delta Mudsucker *Gillichthys detrusus* at 5.3 Ma.

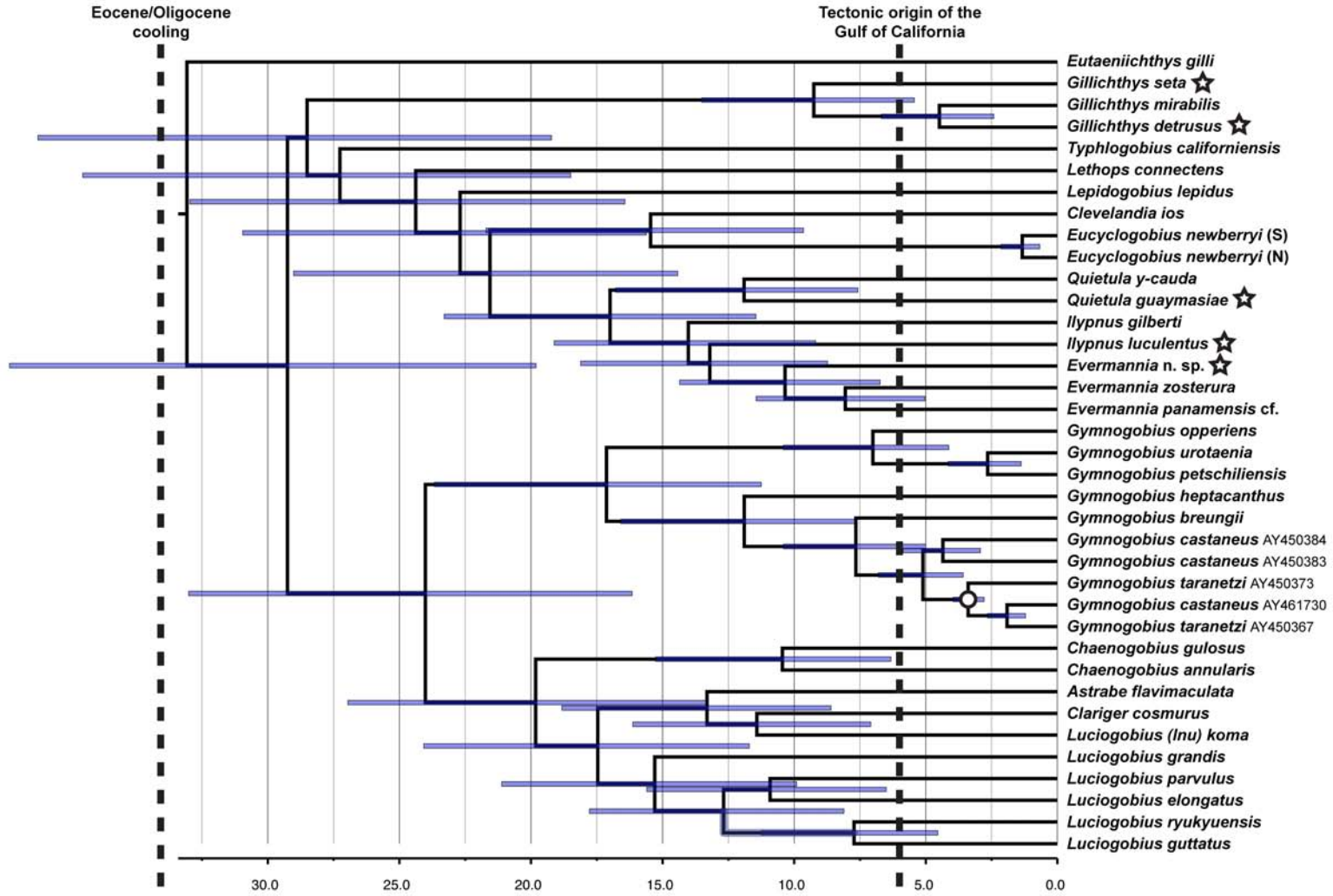


Figure B-2. Chronogram calibrated using only the onset of the Tsushima Current 3.5 Ma.

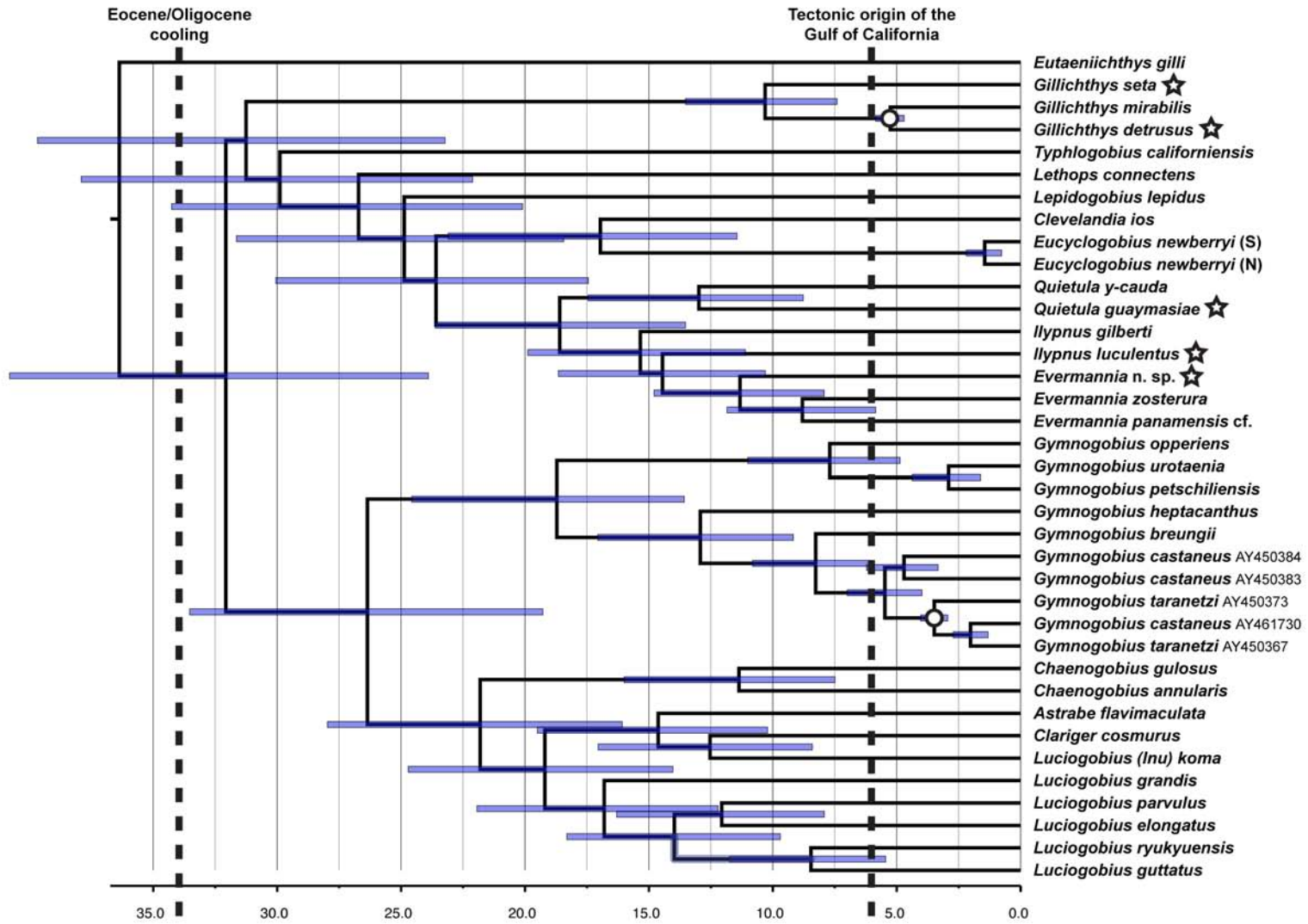


Figure B-3. Chronogram calibrated using the speciation event of the Colorado Delta Mudsucker *Gillichthys detrusus* at 5.3 Ma and the onset of the Tsushima Current 3.5 Ma.

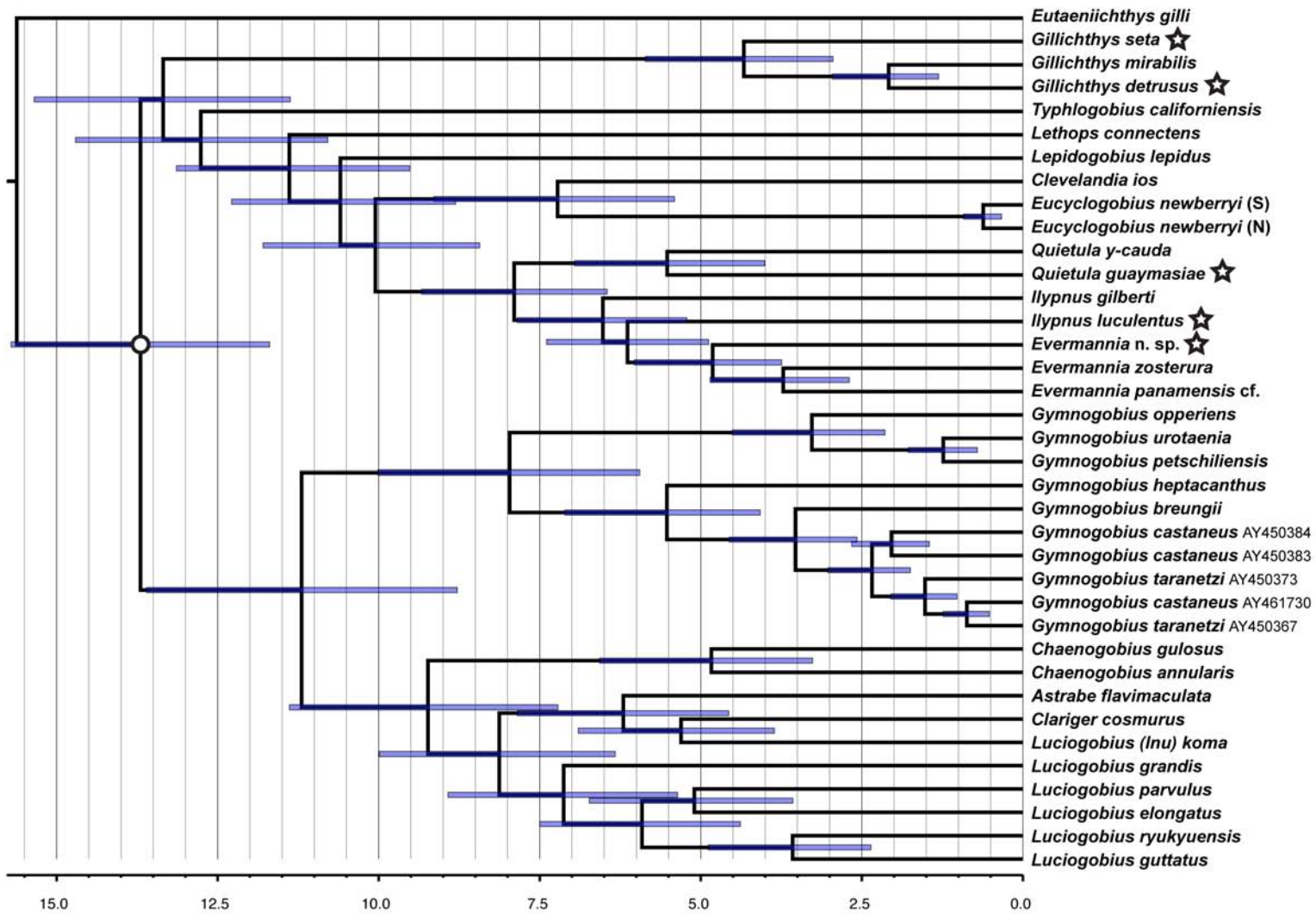


Figure B-4. Chronogram calibrated by associating the most basal ingroup split with a Middle Miocene cooling event ~14 Ma.

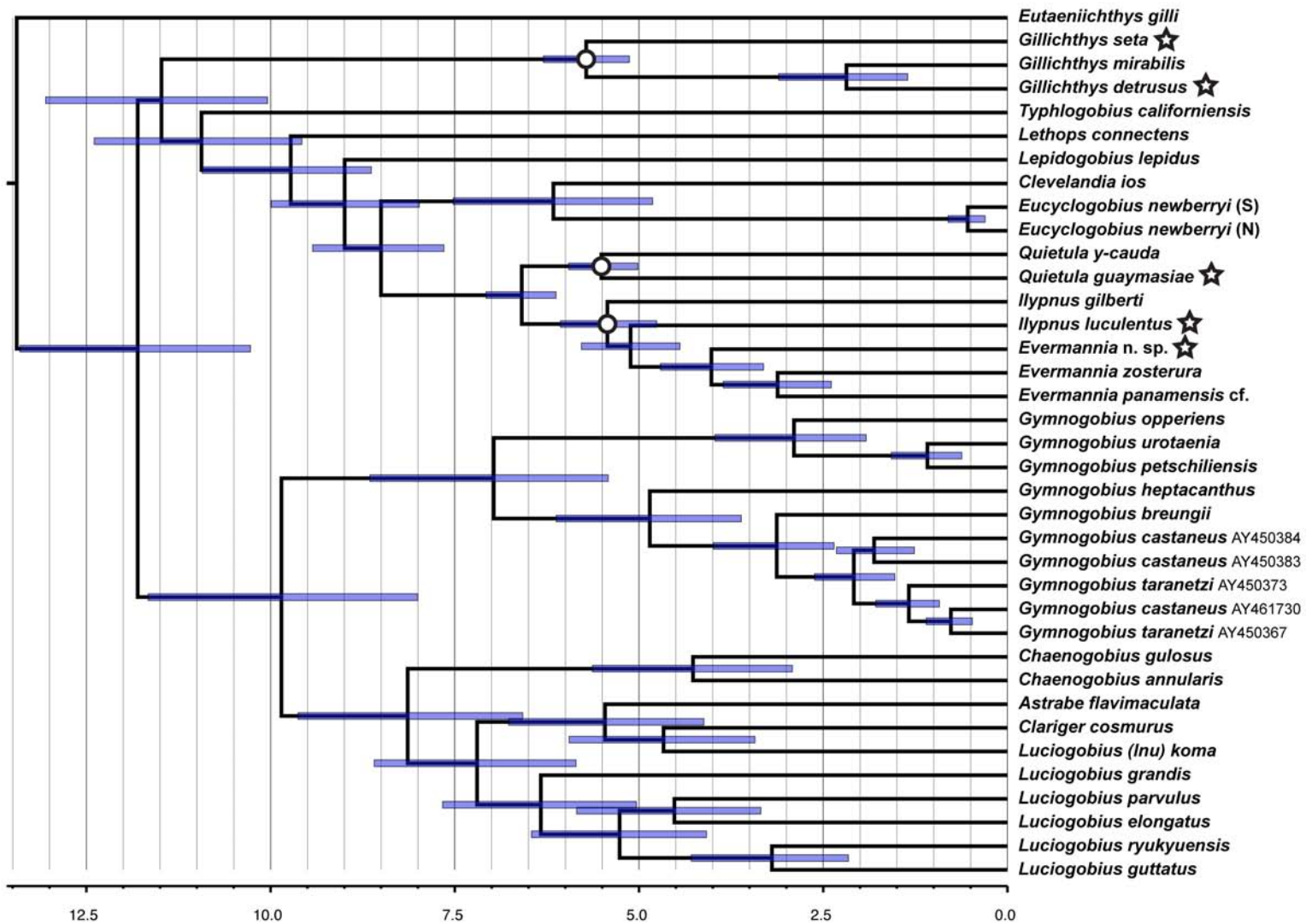


Figure B-5. Chronogram calibrated under a hypothetical assumption that the three oldest Gulf-endemic lineages all diverged coincident with tectonic opening of the Gulf ~6 Ma.

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CHAPTER 3

**Climate, tectonics and habitat fragmentation
drive complex local population structure
in the estuarine goby *Gillichthys mirabilis***

INTRODUCTION

Genetic structure in marine animals with dispersive larvae can be difficult to interpret since few barriers to oceanic dispersal are obvious (Palumbi 1994). While phylogeographic breaks have been correlated with species range limits in coastal species (Avice et al. 1987), biogeography is not always a clear predictor of intraspecific genetic variation, particularly on the southwestern coast of North America (Dawson 2001). In addition, using modern coastlines to elucidate drivers of intraspecific genetic patterns may be only marginally useful, or even misleading, due to relatively recent Pleistocene climatic fluctuations and frequent tectonic activity (Jacobs et al. 2004). The problem becomes even more complicated for estuarine species, whose habitats can be ephemeral even on very short time scales. West Coast estuaries provide ephemeral and spatially discontinuous along an essentially one-dimensional coastline, and can produce varying levels of genetic differentiation among populations of estuarine fishes and invertebrates (Bernardi and Talley 2000; Dawson et al. 2001; 2002; Ellingson and Krug 2006; Earl et al. 2010). Since habitat persistence can vary greatly between estuaries, comprehensive geographic and genetic sampling is critical for phylogeographic studies of estuarine taxa of southwestern North America.

The longjaw mudsucker *Gillichthys mirabilis* is an estuarine goby that inhabits muddy channels of the intertidal. Like many temperate coastal species in the region (Walker 1960; Hubbs 1960), the Baja California peninsula creates a disjunct distribution in *G. mirabilis* where populations are present both within the Gulf and on the outer Pacific Coast, but are absent from the waters of southernmost Baja (Fig. 3-1). The tropical climate of the Cabo San Lucas region is presumed to be largely responsible for this geographic disjunction, and corresponding phylogeographic breaks have been documented in gastropods (Hurtado et al. 2007), isopods

(Hurtado et al. 2010), sea lions (Maldonado et al. 1995; Schramm et al. 2009), marine plants (Muñiz-Salazar et al. 2005) and several fishes (Present 1987; Terry et al. 2000; Stepien et al. 2001; Huang and Bernardi 2001; Bernardi et al. 2003; Bernardi and Lape 2005; Schinske et al. 2010). Of these studies, the most comprehensive geographic sampling across the region revealed highly structured populations in the isopod genus *Ligia* (Hurtado et al. 2010). Phylogeographic patterns and their potential causal mechanisms are tentative, however, as results were based entirely on mtDNA sequences.

Uniparental inheritance, a lack of recombination and high nucleotide substitution rates make mtDNA quite useful for identifying phylogeographic patterns (Avice 2000), but relying on this single class of genetic data can lead to overinterpretation of results (Edwards and Bensch 2009). Multi-locus phylogeographic studies commonly uncover discrepancies between intraspecific patterns of variation in mitochondrial DNA (mtDNA) and nuclear DNA (nuDNA). Such conflicting geographic patterns between mtDNA and nuDNA, often called cytonuclear discordance, have several different causal explanations. Sex-biased dispersal, demographic disparities, adaptive introgression/mtDNA selection, human introduction, hybrid zone movement, and *Wolbachia* infection in insects have all been implicated either empirically or theoretically in generating discordant patterns between mtDNA and nuDNA (Toews and Brelsford 2012). But while identification of incongruence is not uncommon, finding evidence to support a single explanation is not always tenable. The aforementioned characteristics of mtDNA result in a 4-fold reduction in effective population size relative to nuDNA. Incongruent patterns can therefore be produced simply by incomplete lineage sorting of nuDNA relative to mtDNA (Zink and Barrowclough 2008), or conversely from the increased lag time of mtDNA to

homogenize after secondary contact. In such situations, invoking additional arguments for explanations (e.g. selection) may be unnecessary.

One aspect of historical demography that may not receive adequate attention in phylogeographic studies is localized extinction. While the genetic signatures of post-glacial range expansion are well-documented in both marine and terrestrial systems (e.g. Arbogast and Kenagy 2001; Hellberg et al. 2001), finer-scale patterns of extinction and recolonization may often be overlooked in marine phylogeography (Cunningham and Collins 1998). Estuarine systems on the North American West Coast are particularly susceptible to extirpation on relatively short time scales. The potential for low precipitation in California on an annual scale can cause small drainages to disappear following just one or two dry winters. Active West Coast tectonics interrupt the persistence of estuaries on a larger scale, creating high coastal slopes and migrating drainages that likely provided few opportunities for continuous habitats through Pleistocene glacial cycles. This temporal instability of already geographically patchy habitat presumably has a substantial effect on the genetic patterns of estuarine species, particularly on the rapidly evolving mitochondrial genome.

In this chapter, I will present phylogeographic patterns of *G. mirabilis* throughout its range based on mtDNA haplotypes, and then reconcile those patterns with nuDNA microsatellite allele frequencies to examine finer-scale aspects of population dynamics and contemporary gene flow. To that end, the following questions will be addressed. 1) What can mtDNA tell us about the history of extinction and re-colonization of *G. mirabilis* habitats through sea level fluctuations of the Pleistocene? 2) How might nuDNA allele frequencies support or conflict with these interpretations? 3) Should arguments in addition to extinction/colonization (e.g. selection)

be invoked to explain any discrepancies? 4) What kinds of additional data and/or analyses might help discriminate between alternative hypotheses?

METHODS

Population sampling

Specimens of *Gillichthys mirabilis* were collected between 2005 and 2011 from estuaries spanning approximately 4,440 km of the North American Pacific coastline (Fig. 3-1). Thirty-one collection sites ranged from San Francisco Bay in the north to the coast of Nayarit in mainland Mexico (Table 3-1). Fish were collected from muddy estuarine channels using a handheld seine and immediately placed into 90% ethanol in the field. Upon returning to the laboratory, samples were placed in fresh 70% ethanol and stored at -20°C prior to DNA extraction. Specimens of the only two *G. mirabilis* congeners, both endemic to the northern Gulf of California, were also collected. *Gillichthys detrusus*, with its highly restricted range in the Colorado River delta (Swift et al. 2011), was collected from 4 sites. The Bernardi Lab at UC Santa Cruz generously provided DNA extracted from *G. mirabilis* from two additional sites in the northern Gulf of California.

DNA Extraction, Amplification, and Sequencing

Genomic DNA was extracted from muscle tissue of the caudal peduncle using the DNeasy Animal Blood & Tissue DNA Kit (Qiagen, Inc., Valencia, CA) and stored in extraction buffer at -20°C prior to amplification. Polymerase chain reactions (PCR) were used to amplify a variable length fragment of the mitochondrial control region (mtCR), using primers CR-A and CR-M (Lee et al. 1995), and a 1197-bp fragment of the mitochondrial protein-coding gene

cytochrome *b* (*cytb*), with the primers AJG15 and H5 (Akihito et al. 2000). A negative control (no template) was included in each run. PCR conditions were as described in chapter 1, with an annealing temperature of 50°C for each locus.

PCR products were visualized by electrophoresis on a 1.5% agarose gel to confirm amplification. To remove excess dNTPs prior to cycle sequencing, PCR products (3 µL per sequencing reaction) were incubated at 37°C for 15 min with 0.5 µL Shrimp Alkaline Phosphatase (SAP), 0.25 µL Exonuclease I and 0.25 µL dilution buffer (USB Corporation, Cleveland, OH), immediately followed by 15 min at 80°C to inactivate enzymes. Products were then directly cycle-sequenced in both directions using the amplification primers and Big Dye Terminator v3.1 Cycle Sequencing chemistry, and electrophoresed on an ABI 3100 Avant Capillary Sequencer (Applied Biosystems, Foster City, CA). Despite the occurrence of a problematic poly-T region near the middle of the amplified D-loop segment, re-sequencing confirmed the presence of exactly 13 consecutive thymine bases in every individual of the genus *Gillichthys*.

Sequence Alignment

Sequences for *cytb* were obtained from a total of 298 individuals from the genus *Gillichthys* (*G. mirabilis* n=235, *G. detrusus* n=54, *G. seta* n=9). Sequences were trimmed to a length of 1020 bp so that all individuals were truncated at the same position for haplotype analyses. A total of 268 mtCR sequences were generated (*G. mirabilis* n=236, *G. detrusus* n=24, *G. seta* n=8). Both datasets were aligned unambiguously by eye. Since the mitochondrial genome is maternally inherited as a single unit and each locus produced congruent phylogenetic topologies, sequences were concatenated for all analyses.

Microsatellite Discovery and Genotyping

Discovery of microsatellite loci was done via high throughput 454 sequencing. DNA from a single *Gillichthys mirabilis* individual (from Ballona Lagoon, CA) was used to prepare a genomic library for 454 sequencing. The library was tagged with a unique barcode, pooled with DNA from another species and processed at the UCLA Genotyping and Sequencing Core using Roche 454 pyrosequencing on 1/16th of a lane. Automated screening of sequences for microsatellite repeats and primer design was performed in MSATCOMMANDER v1.0.6 (Faircloth 2008). Eleven polymorphic loci amplified across the entire geographic range of *G. mirabilis*. Summary statistics and primers for each microsatellite locus are listed in Table 3-2. Microsatellite peaks were scored in GENEIOUS v6.0 (Biomatters).

Data Analysis

For mtDNA sequences, nucleotide diversity, haplotype diversity, mismatch distributions and other summary statistics were calculated in ARLEQUIN v3.5.1.3 (Excoffier and Lischer 2010). A phylogeny of all concatenated *G. mirabilis* sequences, using a single *G. detrusus* sequence as the outgroup, was constructed in MRBAYES v3.2.1. The mixed substitution model option was used, with a gamma distribution of rate heterogeneity. The analysis was run for 20 million generations with the first 25% of trees discarded as burnin. For divergence time analysis, the rate partitioning strategy described in chapter 1 was employed. All *G. detrusus* individuals for which both mtDNA sequences were available were included, and the origination of *G. detrusus* was used to time calibrate the tree as described in chapter 2. The topology was constrained to include major clades of interest that were highly supported by results of the

MRBAYES analysis. Divergence time estimation was performed in BEAST v1.7.4, with two independent runs of 20 million generations each and the first 50% of trees discarded.

ARLEQUIN was used to test for linkage disequilibrium and loci under selection, and to calculate heterozygosity and analysis of molecular variance (AMOVA) statistics. Population subdivision was inferred using STRUCTURE v2.3.4 (Pritchard et al. 2000). The admixture model with independent allele frequencies was employed for (K) values 2 through 10, using 50,000 burn-in iterations followed by 500,000 MCMC steps. A neighbor-joining tree was constructed in Populations v1.2.30 (Langella 1999) using Nei's D_A distance (Nei et al. 1983).

Direction and magnitude of migration rates were estimated in MIGRATE-N v3.3.2 (Beerli and Palczewski 2010). Populations were defined as the four geographic areas listed in Table 3-3. Separate analyses were run for mtDNA and nuDNA to assess relative differences in migration between the two classes of data.

RESULTS

Mitochondrial DNA Sequences

No indels were present in the alignment of protein-coding *cytb* sequences, and the 809-bp alignment for mtCR had 5 total indels. Molecular diversity summary statistics are shown in Table 3-3, with populations defined according to the geographic structure of the phylogram created using all mtDNA sequences (Fig. 3-2). Nucleotide diversity (π) was higher inside the Gulf relative to the outer coast. Tajima's D statistic suggested that out of the four genetic entities, populations of the northern outer coast depart furthest from neutrality (this metric is sensitive to both selection and changes in population size, but does not distinguish between the two).

Unimodal mismatch distributions suggest population expansion has occurred in three out of the four phylogeographic entities, with the most recent expansion on the northern Pacific Coast (Fig. 3-3, shown in green) and the oldest expansion in the northern Gulf (red). A multimodal mismatch distribution of haplotypes in the southern Gulf (purple) is consistent with a large and stable population.

Phylogenetic Relationships of mtDNA

Phylogenetic analysis recovered three major clades with high support (Fig. 3-2). One clade is almost exclusive to the northern Gulf (clade A) and another was sampled exclusively from the northern outer coast from San Francisco Bay, CA south to San Quintín, Baja California (clade B). The remaining clade contained haplotypes that formed a monophyletic clade representing the southern outer coast of Baja (clade D) nested within haplotypes sampled from the southern Gulf (group C). A few exceptions to this geographic structure can be seen. Six out of nineteen group C haplotypes were sampled from the primarily purple-coded southern Gulf sites at Mojón (MOJ), and one out of 20 individuals sampled at Bahía Kino belongs to clade A. Similarly, a single individual sampled north of Bahía Kino near La Cholla (NWC) fell into the predominantly southern Gulf group C. On the outer coast, a total of seven southern Baja clade D haplotypes were sampled at the northern sites of San Quintín (QTN), Famosa Slough (FAM) and Ballona Creek (BNA). Without exception, however, the clade B represents only green-coded northern outer coast sites, and although the mostly southern Pacific Coast clade D is derived from the paraphyletic southern Gulf group C, no haplotypes were shared between genetic entities of the Gulf and outer coast. Under the assumption that the common ancestor of all extant populations was either in the Gulf or a large panmictic population on both sides of the peninsula,

the two most parsimonious explanations for the given tree, these phylogenetic relationships suggest a minimum of two colonization events from Gulf to outer coast and one event in the opposite direction. A more detailed chronological proposal of colonization events is presented in the Discussion section.

Divergence Time Estimates of mtDNA Clades

The oldest split in the phylogeny suggests that clade A in the northern Gulf split from the rest ~1 Ma (Fig. 3-4). The remaining major splits in the tree also have strong geographic patterns, and the following chronology of divergence: 1) The predominantly northern Pacific Coast clade B diverges at 0.63 Ma; 2) a deep split within the southern Gulf group C haplotypes occurs at 0.46 Ma; and 3) the southern Pacific Coast clade D splits at 0.19 Ma.

Microsatellite Relationships

The most prominent geographic break reflected by nuDNA occurs between the Gulf and outer coast, as seen in both the NJ tree (Fig. 3-5) and Structure plots ($K = 2$; Fig. 3-6). Although populations from either side of Baja California are not strictly reciprocally monophyletic on the mtDNA tree (because clade D is nested within group C), the overall lack of haplotype mixing between the two regions suggests that the peninsula is a barrier to contemporary gene flow. STRUCTURE plots of the microsatellite data reveal genetic subdivision up to $K = 4$ (Fig. 3-6), while the ability for the program to divide the data into discrete clusters deteriorates when $K \geq 5$. On the outer coast, a genetic cline is apparent from north to south, with the steepest part of the cline in the area of Punta Banda (BAN) and San Quintín. While this is geographically proximal to the geographic break in mtDNA (Fig. 3-2), it is spatially distinct in that these two locations

have almost entirely northern haplotypes while showing roughly equal frequencies of northern and southern microsatellite alleles (Fig. 3-6).

A minority of individuals scattered throughout the Gulf cluster with most individuals from Boca Mojón, an estuary at the north end of Bahía Concepción on the Gulf coast of Baja (purple cluster at $K = 4$). Boca Mojón is a small estuary that would be unlikely to persist through a decline in sea level. Thus, the microsatellite pattern suggests that either it was extirpated and subsequently re-colonized by genotypes from the southeastern Gulf that are more rare in the rest of the Gulf, or it experiences a unusually high degree of contemporary isolation from the rest of the sampled Gulf estuaries. A few individuals from Boca Mojón show more genetic affinity for Pacific Coast genotypes than any other Gulf population, suggesting some marginal level of migration from the coast into the southern Gulf. There is no indication that migration is occurring in the opposite direction, from the southern Gulf to the Pacific Coast.

DISCUSSION

Both mtDNA and nuDNA reveal two obvious and broadly consistent phylogeographic patterns in *Gillichthys mirabilis*. 1) There appears to be very little if any contemporary gene flow between Gulf of California and outer Pacific Coast populations. This pattern is consistent with similarly disjunct distributions observed across a variety of coastal marine animals (e.g. Hubbs 1960; Walker 1960; Present 1987; Maldonado et al. 1995; Muñiz-Salazar et al. 2005; Hurtado et al. 2010). While the majority of the Baja California/Gulf coastline spans temperate to subtropical latitudes, the southern tip of the peninsula extends just beyond the Tropic of Cancer. Thus, warm water likely limits the ability of temperate species on either side of the peninsula to migrate

around the cape in either direction. 2) Genetic diversity is lower on the Pacific Coast in both the mitochondrial and nuclear genomes relative to the Gulf. Diversity is particularly low in the northern Pacific Coast clade A, which displays the shortest branch lengths (Fig. 3-2), lowest measures of nucleotide diversity (Table 3-3) and highest frequency of redundant haplotypes (Fig. 3-3).

These northern populations were likely the most susceptible to extirpation due to the absence of low-slope shelf area that could support estuaries during glacial maxima. While thermal extinction may have limited available refugia for northern Pacific Coast populations, Pleistocene sea level fluctuation likely had a dramatic effect on the persistence of estuarine habitat throughout the range of *G. mirabilis*. A gradually sloping coastline is critical for the building of estuarine habitat, but the tectonically active margin along western North America combined with dramatic cycling of sea level likely left few places where suitable estuarine habitat could persist. Many of the modern estuaries on the Pacific Coast are relatively small and therefore ephemeral on the scale of glacial cycles. However, the Gulf contains a higher abundance of large estuarine systems that would have been more resistant to extirpation via sea level change, particularly on the mainland coast of the southern Gulf (D. Jacobs, *pers. comm.*).

History of Migration and Colonization

Beyond these general patterns, the time-calibrated phylogeny of mtDNA sequences can be interpreted in the context of migration and colonization events from the Middle to Late Pleistocene. The most parsimonious explanation for the current geographic distribution of mtDNA clades in *G. mirabilis* begins with the common ancestor in the northern Gulf of California, and possibly on the outer Pacific Coast with the condition that these populations

would be connected to those inside the Gulf. Given this starting condition, the mtDNA phylogeny is consistent with the following scenario of migration and colonization:

- 1) The common ancestor of all *G. mirabilis* lived in the Gulf of California and subsequently colonized the outer Pacific Coast. Alternatively, the ancestor lived on both sides with high gene flow across the Baja Peninsula. In either case, gene flow was cut off ~1 Ma, resulting in the divergence of the “red” clade from the remaining lineages (Fig. 3-4). This isolation may have been caused by the closing of a hypothesized mid-peninsular seaway connecting the Gulf to the Pacific in the vicinity of Punta Eugenia. While convincing geologic evidence has not yet been found for a seaway of the appropriate age, genetic evidence in both terrestrial (e.g. Riddle et al. 2000; Zink 2002; Montanucci 2004; Lindell et al. 2006; Crews and Hedin 2006) and marine (Terry et al. 2000; Riginos 2005) systems is consistent with a Middle Pleistocene marine connection across the peninsula.
- 2) The next split on the tree occurs between the northern Pacific Coast clade and the clade composed of haplotypes in the south on both sides of the peninsula, coincident with a dramatic rise in sea level (and thus temperature) at 630 ka. This divergence may reflect recolonization of the southern Gulf from the Pacific Coast following extirpation during a previous glacial period.
- 3) The most recent geographically relevant split is defined by the origin of the “gold” southern Pacific Coast clade at 190 ka. The maximum age of this clade coincides with a sharp decline in sea level and may reflect another episode of colonization from the Gulf following local extinction on the Pacific Coast during sea-level highstand.

An additional deep divergence within the southern Gulf “purple” haplotypes dates to 460 ka, but has no clear geographic significance. This subdivision may reflect an intermittent history of isolation and migration between mainland Mexico and the southern coasts of Baja.

Migration of mtDNA Haplotypes

There are a few rare instances of haplotypes primarily restricted to one region being sampled elsewhere. Within the Gulf, several haplotypes from the northern Gulf “red” clade were sampled from Boca Mojón. This estuary appears to be an area of secondary contact between the predominantly northern and southern haplotypes. A single haplotype from the northern clade taken from Bahía Kino (KIN) can likely be similarly explained. Only one haplotype from a northern Gulf population, northwest of La Cholla (NWC), fell into the typically southern Gulf group C.

On the Pacific Coast, a small clade of five haplotypes within the predominantly southern Gulf group C were sampled farther north at San Quintín and Ballona Lagoon. This clade appears to have originated from a single haplotype that migrated slightly beyond the typical range of the southern clade and subsequently diversified in the north. Only clade A was restricted to a single geographic region, suggesting that it is either difficult for gobies of this population to reach southern estuaries or they are not competitively viable when they arrive.

Comparison of Mitochondrial and Nuclear Patterns

Contrasting the mtDNA phylogeny (Fig. 3-2) with the neighbor-joining tree based on microsatellite data (Fig. 3-5) reveals some topological inconsistency in that the mitochondrial

data do not recover reciprocally monophyletic Gulf and Pacific Coast clades. The mtDNA nests the southern Pacific Coast clade within the southern Gulf population, while the nuDNA tree combines northern and southern Pacific Coast populations with no latitudinal resolution. The STRUCTURE plots (Fig. 3-6), however, reveal a degree of subdivision beyond that of the NJ tree. In one sense, all analyses are congruent in that the strongest divide is between Gulf and Pacific Coast populations. This split shows up at $K = 2$ and remains for every analysis of $K > 2$. As K then increases, a genetic cline from north to south along the Pacific Coast is revealed, with the sharpest shift in allele frequencies occurring in the vicinity of Punta Banda (BAN; Fig 3-6). This is just slightly north of the phylogeographic break of the mtDNA tree, between San Quintín (QTN) and Laguna Manuela (MAN). Although the ~300-km coastline between San Quintín and Laguna Manuela is one of the longest gaps in suitable estuarine habitat for *G. mirabilis*, the clustering of microsatellite alleles suggests the distance does not constitute a significant barrier to gene flow. That a very distinct phylogeographic break in mtDNA is shifted slightly southward, along with the fact that the southern Pacific Coast clade is derived from the southern Gulf, raises the possibility that southern haplotypes may be better adapted to warm, tropical conditions. This would allow selection to prevent or at least slow northward migration of mitochondrial haplotypes relative to the presumptively neutral microsatellite loci. It may not be necessary to invoke selection, however, as the slower migration rate of nuDNA combined with presumably frequent local extinction cannot be ruled out as a cause for this pattern. Finally, the low nucleotide diversity (Table 3-3) and exclusively green genotypes (Fig. 3-6) at Hidden Lagoon (HID) likely reflect a recent founder event. This location is an extremely small estuary in San Diego County known to either dry or wash out with variable rainfall resulting in frequent extirpation of estuarine species.

Inside the Gulf, modest cytonuclear discordance is similarly observed between northern and southern populations. The mtDNA phylogeny (Figs. 3-2 and 3-4) suggests that, following initial peninsular divergence ~1 Ma, clade B on the Pacific coast recolonized the Gulf from the south, and these derived haplotypes (group C) have not mixed with the older haplotypes in the northern Gulf (clade A) beyond an apparent area of secondary contact at the mid-Gulf sites of Boca Mojón (MOJ) and Bahía Kino (KIN). The nuDNA NJ tree shows no subdivision within the Gulf (Fig. 3-5), while the STRUCTURE plots distinguish Boca Mojón from all other Gulf populations (Fig. 3-6). Boca Mojón is a relatively small system compared to others in the Gulf, and a low-diversity mtDNA clade suggests a recent bottleneck (Fig. 3-7), likely due to extinction followed by colonization by a single haplotype from the southern mainland Gulf coast. A few haplotypes in the predominantly northern Gulf clade A also collected at Boca Mojón likely reflect southward migration following initial colonization from the east, although this is not reflected in the nuDNA. As on the Pacific Coast, slight discordance in the Gulf leaves open the possibility that selection prevents warm-adapted mitochondrial haplotypes from migrating north in the face of relatively high nuclear gene flow. While these data cannot rule out the slower migration rate of mtDNA as an explanation, relatively old populations would not be subject to the effects of differential migration rate. Future work will include methods to date population divergence using microsatellite data in an attempt to distinguish between hypotheses of selection versus migration rate to explain cytonuclear discordance.

CONCLUSIONS

Phylogeographic patterns in *Gillichthys mirabilis* suggest a history of extirpation and colonization of ephemeral estuarine habitats influenced by tectonics, sea level fluctuation and

possibly by mitochondrial adaptation from the Middle to Late Pleistocene. Additional information might provide more evidence to distinguish among the alternative hypotheses of differential migration rate versus mtDNA adaptation to explain cytonuclear discordance. Future work intended to address this issue will include: 1) accumulation of more microsatellite loci to increase signal and improve resolution of relationships within the Gulf and on the Pacific Coast, 2) population genetic analyses to determine whether more fine-scale subdivision can provide insight into processes of migration, gene flow and extinction/colonization on the scale of individual estuaries, and 3) development of nuclear sequence markers to improve upon the ability to infer common ancestry as compared to microsatellite loci.

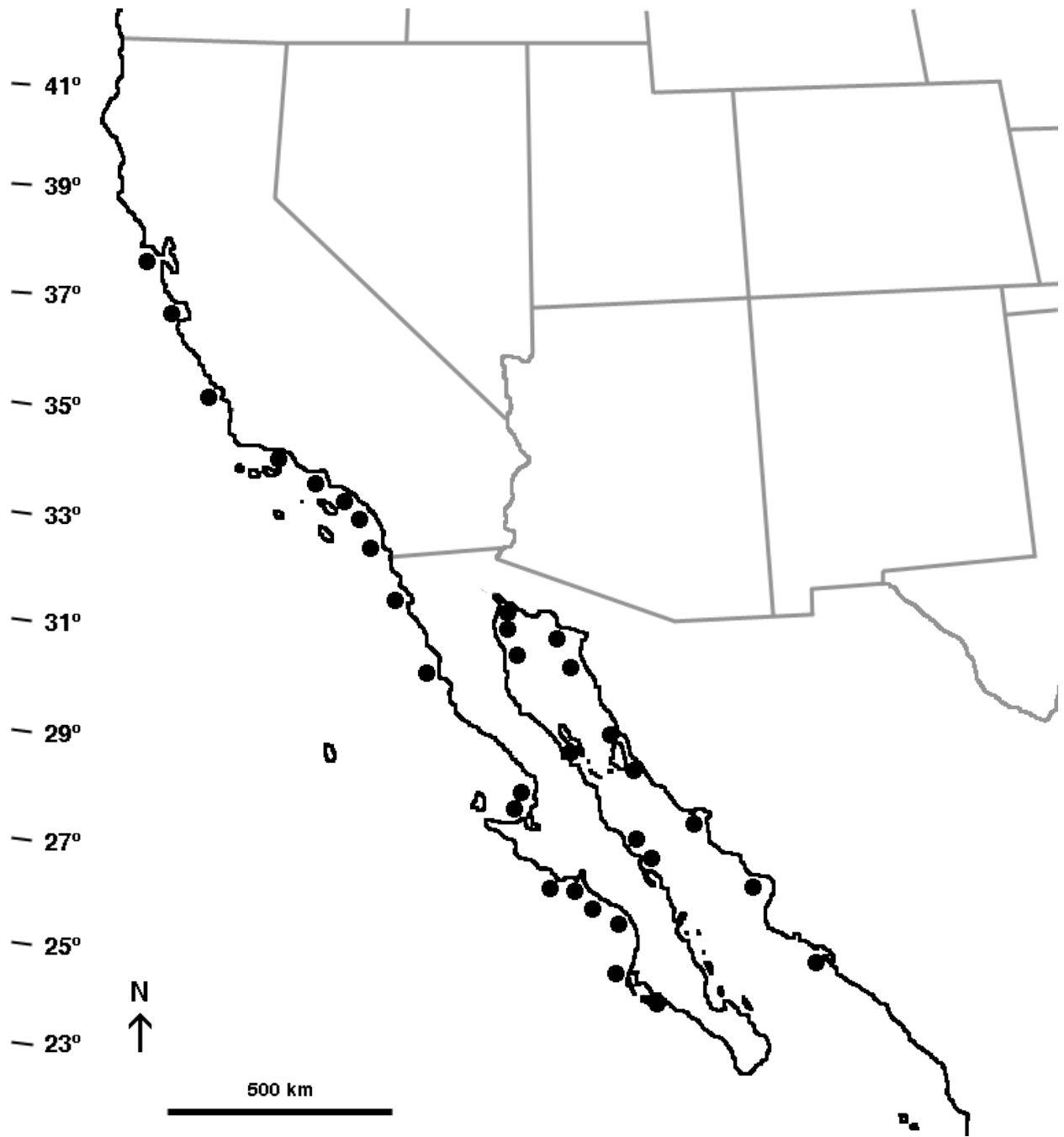


Figure 3-1. Distribution map of *Gillichthys mirabilis* along the North American West Coast, based on museum records and collections for this study.

Table 3-1. Location (with abbreviation), sample size, latitude and longitude of *Gillichthys mirabilis* collections. Where sample size between mtDNA and nuDNA datasets, the nuDNA sample size is in parentheses. The last column lists colors corresponding to geographic regions as coded on maps and trees in the figures (green=northern Pacific Coast, gold=southern Pacific Coast, red=northern Gulf, purple=southern Gulf).

Location	Sample size	Latitude (N)	Longitude (W)	Map color
Albany, San Francisco Bay, CA (ALB)	10	37° 53.36'	122° 18.70'	green
Morro Bay, CA (MOR)	9	35° 20.91'	120° 50.02'	green
Devereaux Slough, CA (DEV)	8(10)	34° 25.04'	119° 52.44'	green
Campus Lagoon, Santa Barbara, CA (USB)	10	34° 24.56'	119° 50.70'	green
Mugu Lagoon, CA (MGU)	3	34° 05.94'	119° 05.04'	green
Ballona Lagoon, CA (BNA)	10	33° 57.77'	118° 26.75'	green
Carlsbad, CA ("Hidden Lagoon" – HID)	10	33° 16.53'	117° 27.11'	green
Famosa Slough, Mission Bay, CA (FAM)	8(12)	32° 45.30'	117° 13.14'	green
Punta Bonda, Ensenada, Baja CA Norte (BAN)	10	31° 45.98'	116° 36.68'	green
San Quintín, Baja CA Norte (QTN)	12	30° 25.92'	116° 01.01'	green
Laguna Manuela, Baja CA Sur (MAN)	4	28° 14.85'	114° 05.13'	gold
Guerrero Negro, Baja CA Sur (GNG)	10	28° 01.30'	114° 06.88'	gold
La Bocana, Baja CA Sur (BOC)	2	26° 47.36'	113° 40.54'	gold
San Ignacio Lagoon, Baja CA Sur (IGN)	10	26° 49.12'	113° 10.89'	gold
Querante, Baja CA Sur (CUA)	2	26° 33.37'	113° 00.17'	gold
La Purisima, Baja CA Sur (PUR)	10	26° 03.76'	112° 16.93'	gold
Bahía Magdalena, Baja CA Sur (GAL)	2(0)	24° 33.45'	111° 44.12'	gold
Los Corrales, Baja CA Norte (PAL)	1	28° 06.56'	112° 48.68'	red
Bahía de las Animas, Baja CA Norte (ANI)	10	28° 47.86'	113° 20.89'	red
La Gringa, Baja CA Norte (GRI)	10	29° 02.38'	113° 32.46'	red
Estero Santa María, Baja CA Norte (MAR)	2	30° 44.73'	114° 42.01'	red
Estero Percebu, Baja CA Norte (PCB)	8(9)	30° 47.29'	114° 42.58'	red
Bahía Adair, Sonora (ADR)	0(10)	31° 32.24'	113° 58.91'	red
Bahía la Cholla, Sonora (NWC+ELC*)	10+6	31° 27.82'	113° 37.90'	red
Estero la Pinta, Sonora (MOI+ELP*)	2+6	31° 17.20'	113° 15.17'	red
Barra San Francisquito, Sonora (GE)	6	30° 57.35'	113° 05.57'	red
Boca Mojón, Baja CA Sur (MOJ)	19(20)	27° 01.42'	112° 00.62'	purple
Estero Santa Cruz, Bahía Kino, Sonora (KIN)	20	28° 47.50'	111° 54.54'	purple
Estero el Ranchero, Guaymas, Sonora (RCH)	1	27° 58.21'	110° 52.19'	purple
Bahía de Yavaros, Sonora (YAV)	1	26° 40.70'	109° 29.60'	purple
La Reforma, Sinaloa (REF)	1	25° 04.23'	108° 03.53'	purple

*ELC and ELP are samples collected by Bernardi Lab, UC Santa Cruz

Table 3-2. Primer sequences, repeat motif, allele size range, number of alleles and expected heterozygosity (H_E) for each microsatellite locus in this study.

Locus	Primer sequences (5'-3')	Motif	Size range (bp)	# alleles	H_E
Gmi2	F: AACTCGAACGCTAATCAGAC R: AGTCTACGGCATGACATCAC	ACT	406-436	10	0.53
Gmi4	F: CTGTCAATCAACACCAGACC R: CAAACAACGTGACTCTCAAG	ACT	192-216	9	0.57
Gmi6	F: CAAGCACGACATTCTACCTC R: GCTCGACCACCACTATTAC	ACT	310-402	13	0.77
Gmi11	F: CATCTCAGTGGGTTGGTATG R: TGAGGATGGACTCAATGATG	AATT	354-366	4	0.57
Gmi13	F: CTCCAGTCCAAACATTTGTC R: ATGCTCCAGTTGCTACAGAG	AAAC	204-228	7	0.59
Gmi16	F: GCCACTGTCCCTTATTACTC R: GGAAACTTTAGACCGAAATG	AACT	146-198	14	0.82
Gmi17	F: CCCTCACAGACCTGTATGAG R: AGGGTAGCCAAAGAACTAC	AGC	124-136	5	0.37
Gmi23	F: TGCATCAAATCATTTGTAGG R: AGGAGAGGACAACCAAAGAC	ATC	123-147	9	0.69
Gmi24	F: CCTTCATTAGCATGCACAC R: TATAATTGGCGAAACACATC	AGC	114-132	7	0.60
Gmi31	F: TGTAGTTGCAAAGCTTGAAG R: CGTGAAAGTGATCAAATGTG	ACTC	144-176	8	0.55
Gmi36	F: GATTTAAAGCCAACAAATGAC R: CTGAGGAAGTTGACGTCAG	AAAC	139-159	6	0.64

Table 3-3. Molecular diversity indices and neutrality tests based on mtDNA haplotypes. Regional groups based on geographic structure of mtDNA tree. Collection sites listed by abbreviations in Table 3-1, with nearby sites grouped together to avoid sample sizes < 5. Haplotypes from the same location that fell into different geographic clades (e.g. clade B and clade C haplotypes sampled from San Quintín) were analyzed as separate populations. Significant departure from neutrality based on simulations of Tajima's D and Fu's F_S indicated by stars (* P <0.05, ** P <0.01).

Region	Sample size (n)	Haplotype diversity (H)	Nucleotide diversity (π)	Tajima's D	Fu's F_S
Northern CA (green)	83	0.868	0.0015	-2.049**	-26.659**
ALB	10	0.378	0.0002	-1.401	-1.164*
MOR	9	0.583	0.0010	-1.189	0.270
DEV	8	0.750	0.0013	0.159	0.522
USB	10	0.200	0.0001	-1.112	-0.339
MGU+BNA	10	0.956	0.0023	0.097	-2.392
HID	10	0.000	0.0000	0.000	0.000
FAM	7	0.714	0.0009	0.239	1.014
BAN	10	0.978	0.0017	-0.643	-5.500**
QTN	9	0.972	0.0022	-1.328	-3.383*
Southern Baja (gold)	47	0.994	0.0036	-1.917*	-25.164**
FAM+BNA+QTN	7	0.857	0.0019	-0.217	-0.385
MAN+GNG	14	1.000	0.0042	-1.345	-8.046**
BOC+IGN+CUA	14	0.978	0.0028	-1.258	-5.478**
PUR+GAL	12	1.000	0.0034	-1.366	-7.041**
Southern Gulf (purple)	36	0.911	0.0083	-0.789	-3.932
MOJ	13	0.295	0.0003	-1.652*	-0.689
KIN+SE Gulf	23	0.996	0.0086	-0.939	-8.586**
Northern Gulf (red)	68	0.989	0.0067	-1.664*	-24.372**
MOJ	6	0.733	0.0034	1.170	3.528
ANI+PAL	11	0.964	0.0050	-0.846	-1.075
GRI	10	1.000	0.0049	-1.239	-3.819*
MAR+PCB+PRI	11	0.727	0.0050	-0.974	2.392
NWC+ELC	15	1.000	0.0075	-0.892	-5.859**
GE+ELP	15	1.000	0.0081	-0.970	-5.499**

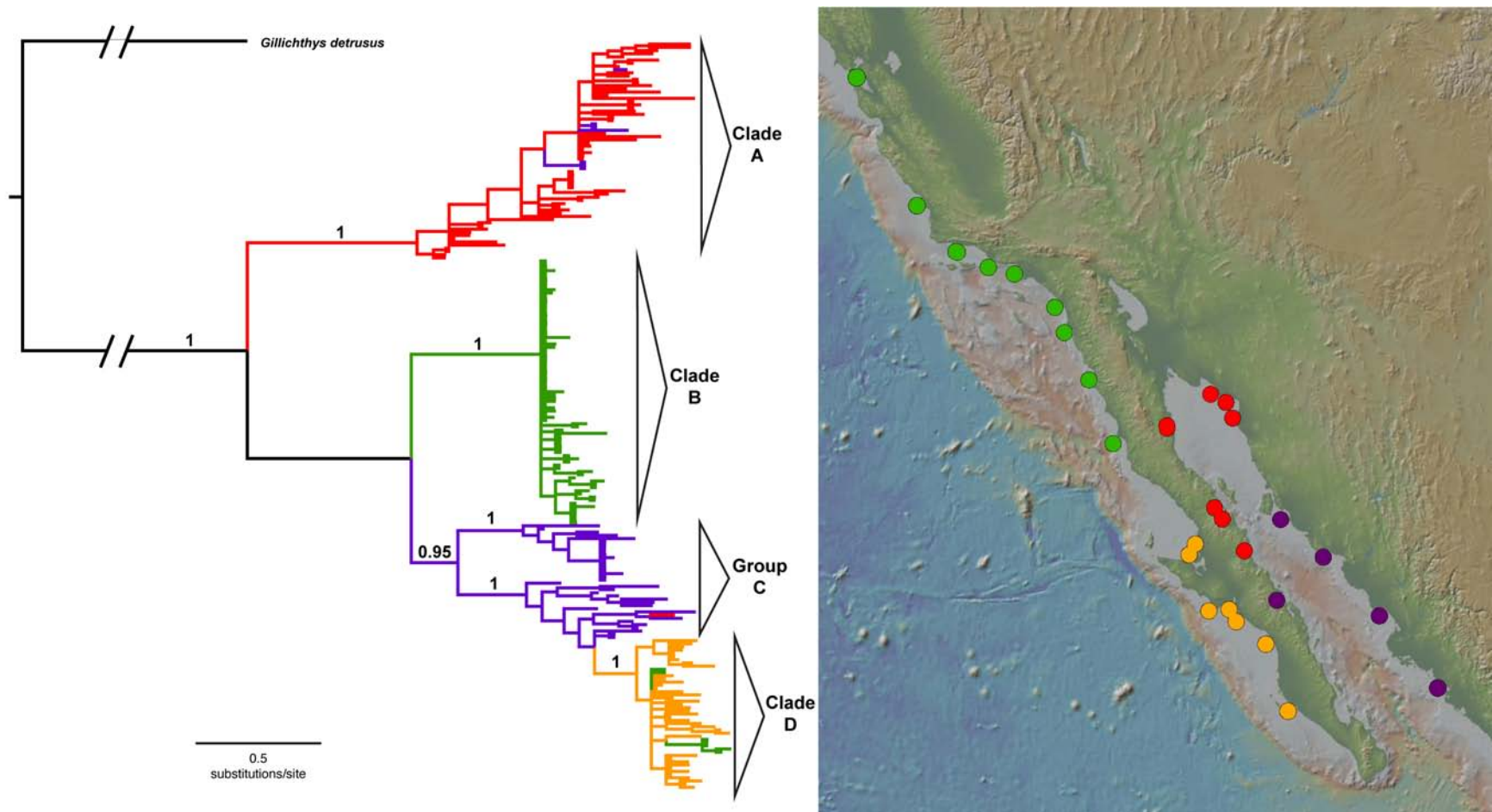


Figure 3-2. (Left) Bayesian phylogeny of all collected *Gillichthys mirabilis* individuals ($n = 234$), constructed in MRBAYES using concatenated cytochrome *b* and mitochondrial control region sequences (1830 bp). Numbers show posterior probabilities for nodes of interest. Branch colors correspond to collection localities. (Right) Map of collection localities colored by geographic region (green=northern Pacific Coast, gold=southern Pacific Coast, purple=southern Gulf, red=northern Gulf).

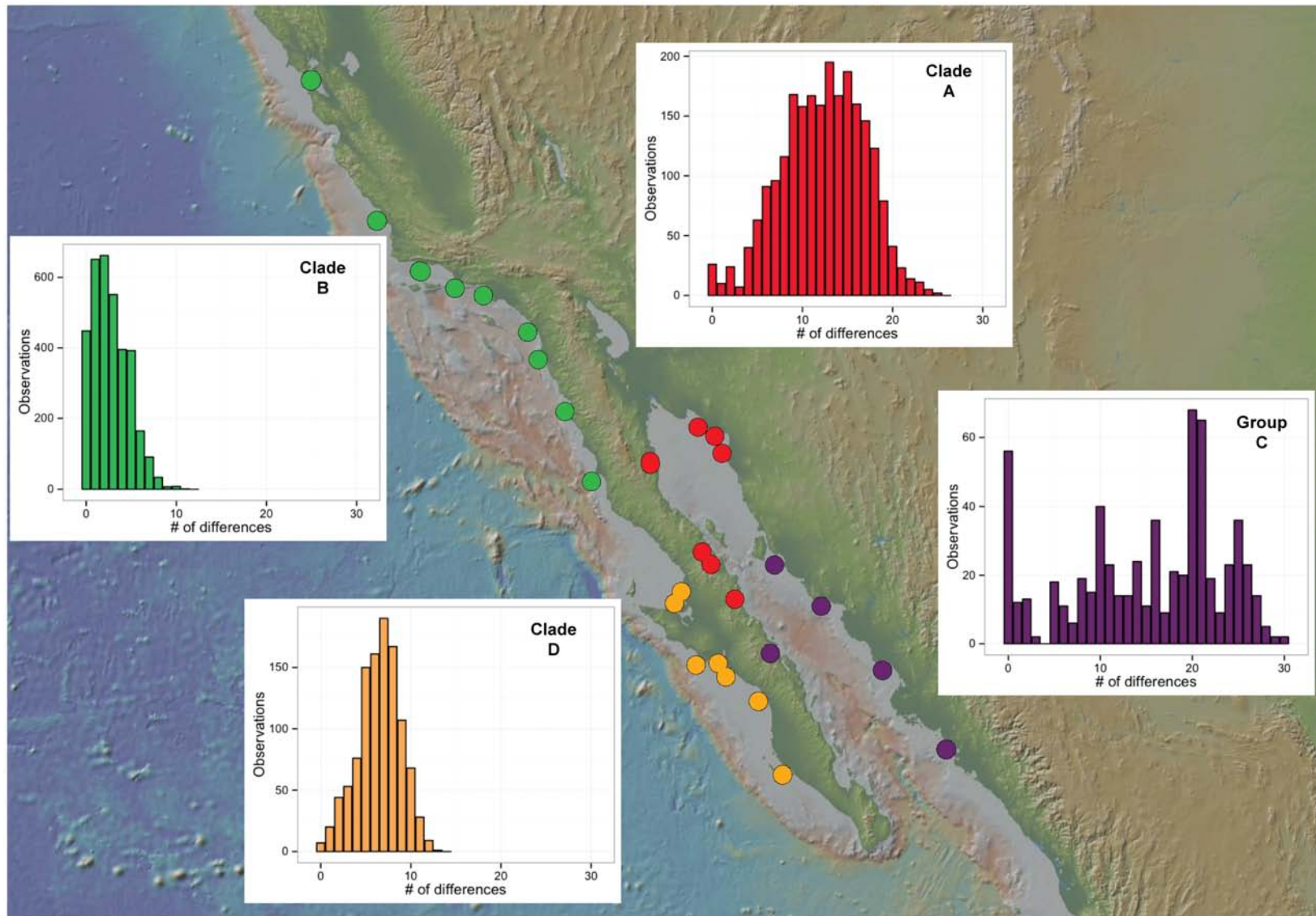


Figure 3-3. Map of collection localities showing mtDNA mismatch distributions for haplotypes in each major geographic group of the mtDNA tree. Three unimodal distributions suggest population expansion, with clade B on the northern Pacific Coast representing the most recently expanded population.

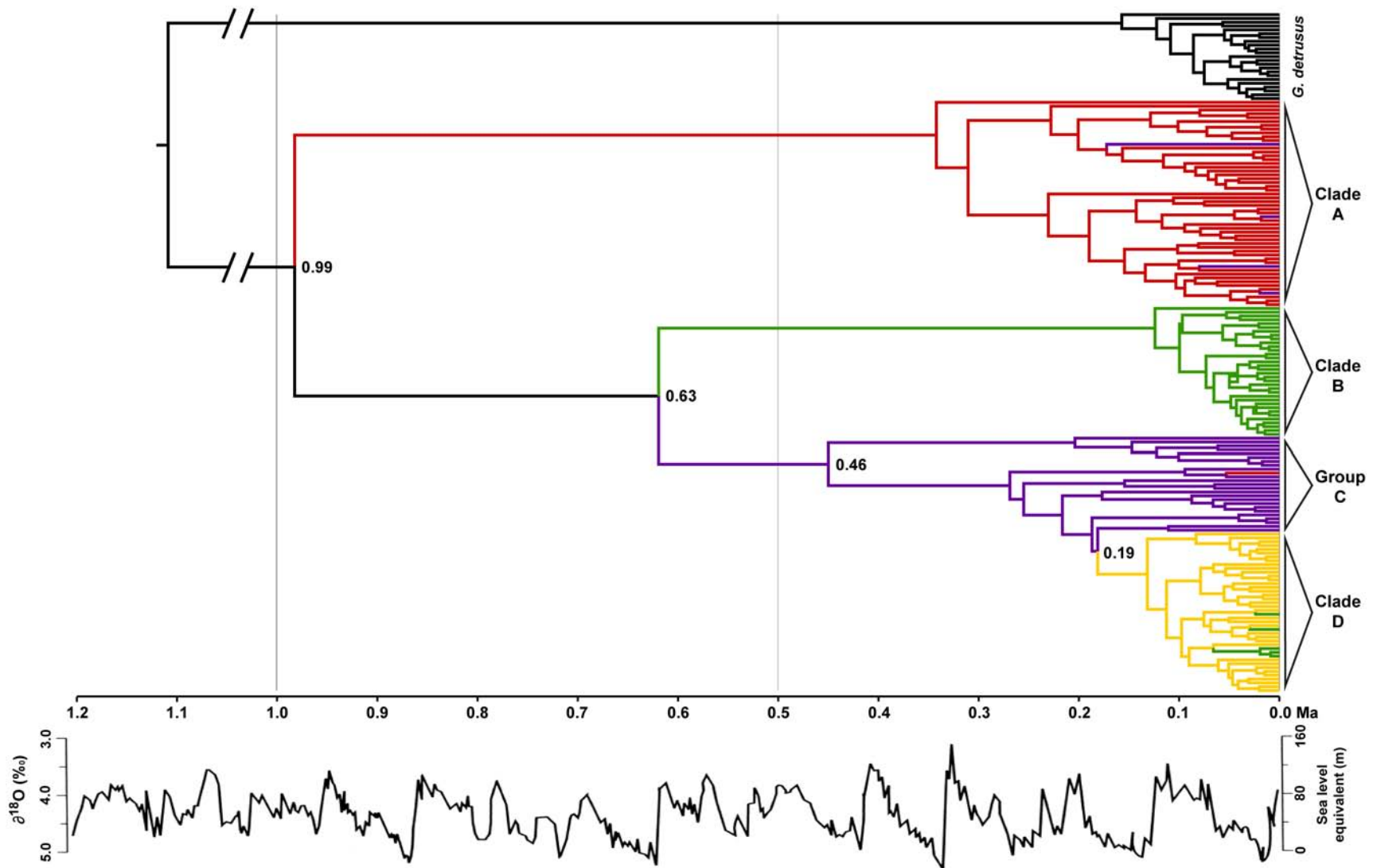


Figure 3-4. Chronogram of concatenated mtDNA haplotypes, generated in BEAST. Mean divergence time estimates are shown at selected nodes. Below the tree is a Pleistocene eustatic sea level curve inferred from benthic $\delta^{18}\text{O}$ isotopes at Ocean Drilling Program site 677 (modified from Pillans et al. 1998).

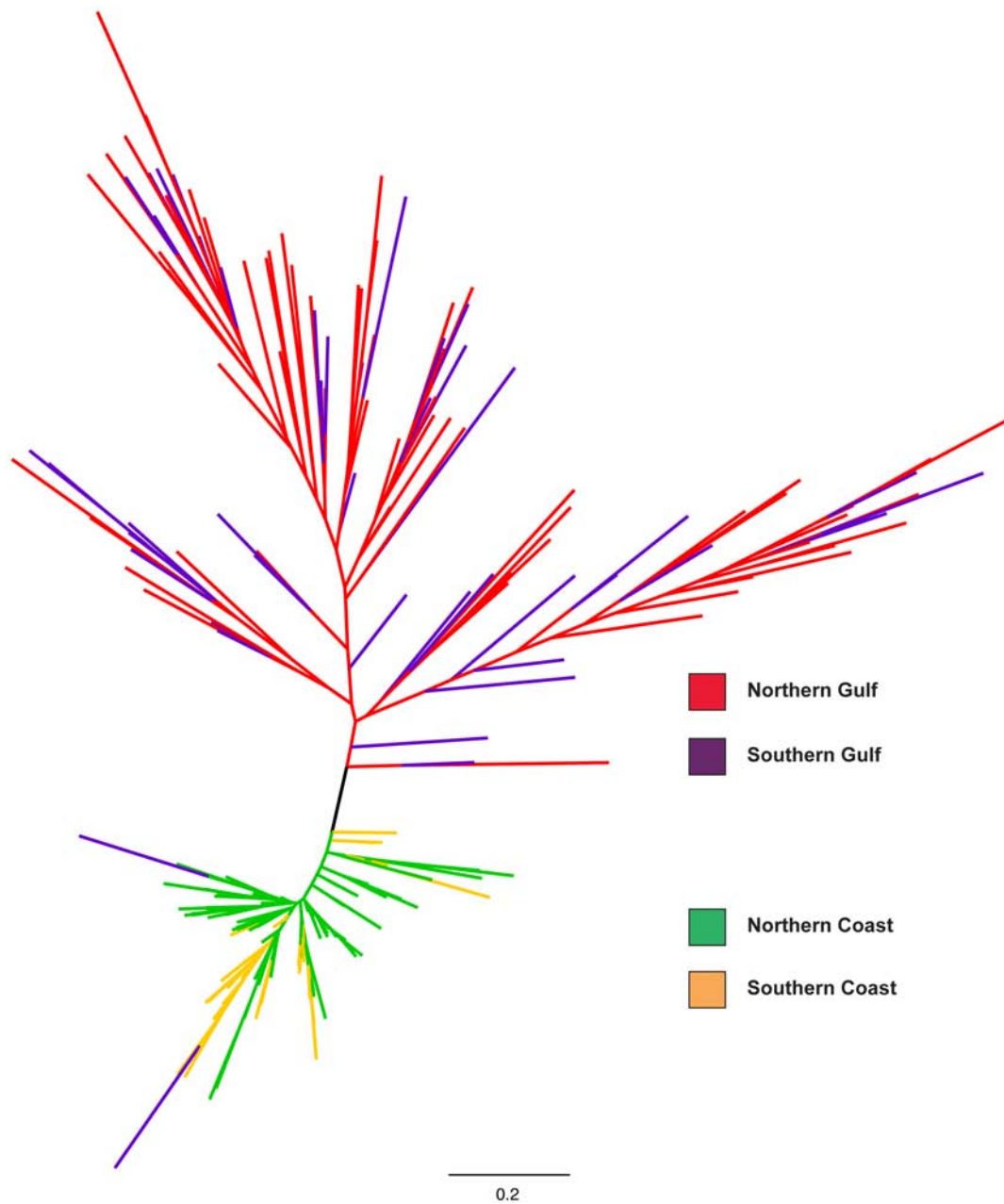


Figure 3-5. Neighbor-joining tree based on distance matrix of microsatellite genotypes of *Gillichthys mirabilis*, constructed in POPULATIONS. Colors correspond to geographic regions on the map in Figure 3-2. The placement of two long purple branches among Pacific Coast genotypes is likely artifactual; preliminary analyses with additional microsatellite loci place these individuals at the center of the tree, near the black branch seen above.

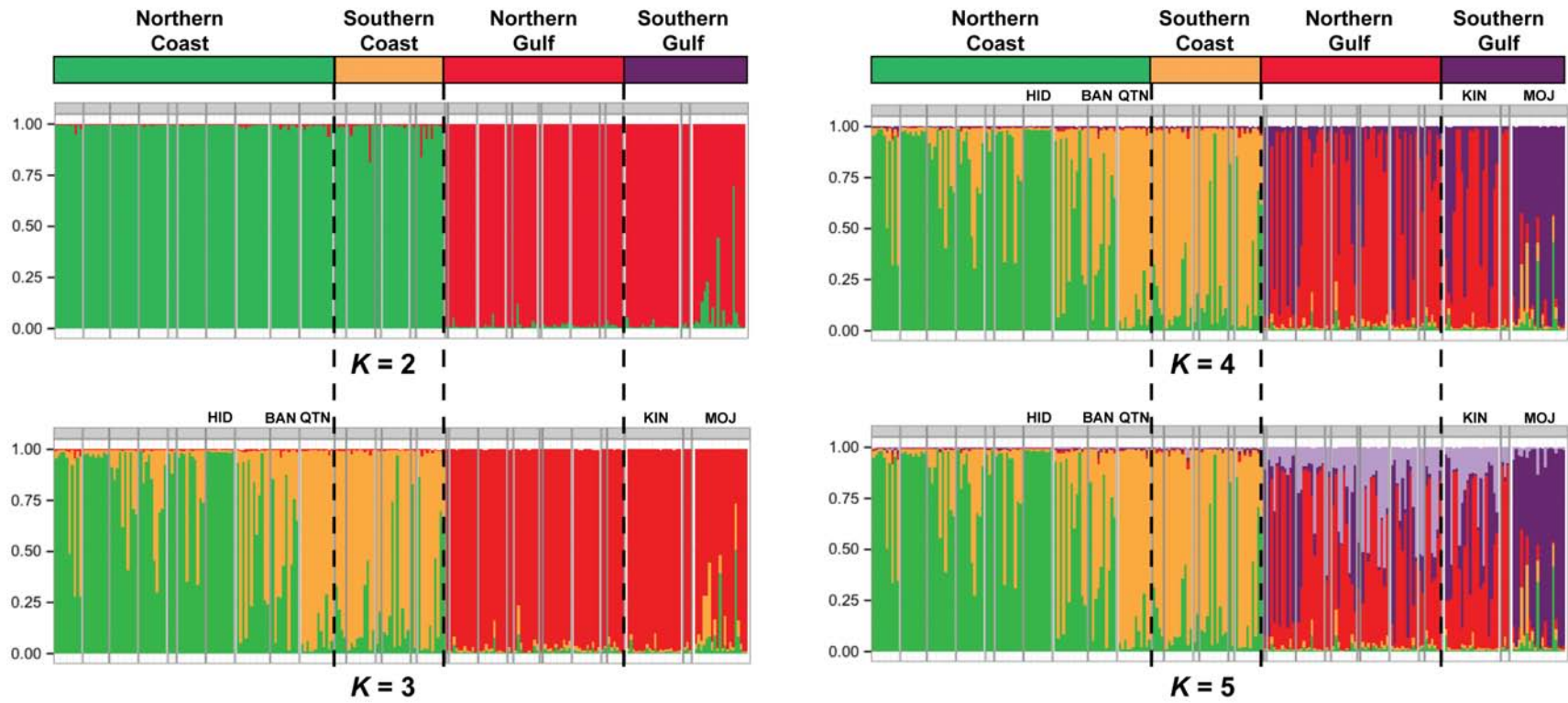


Figure 3-6. STRUCTURE plots of *Gillichthys mirabilis*. Colored bars above plots indicate geographic regions as colored on the map in Figure 3-2. Abbreviations correspond to references to specific collection sites in the text.

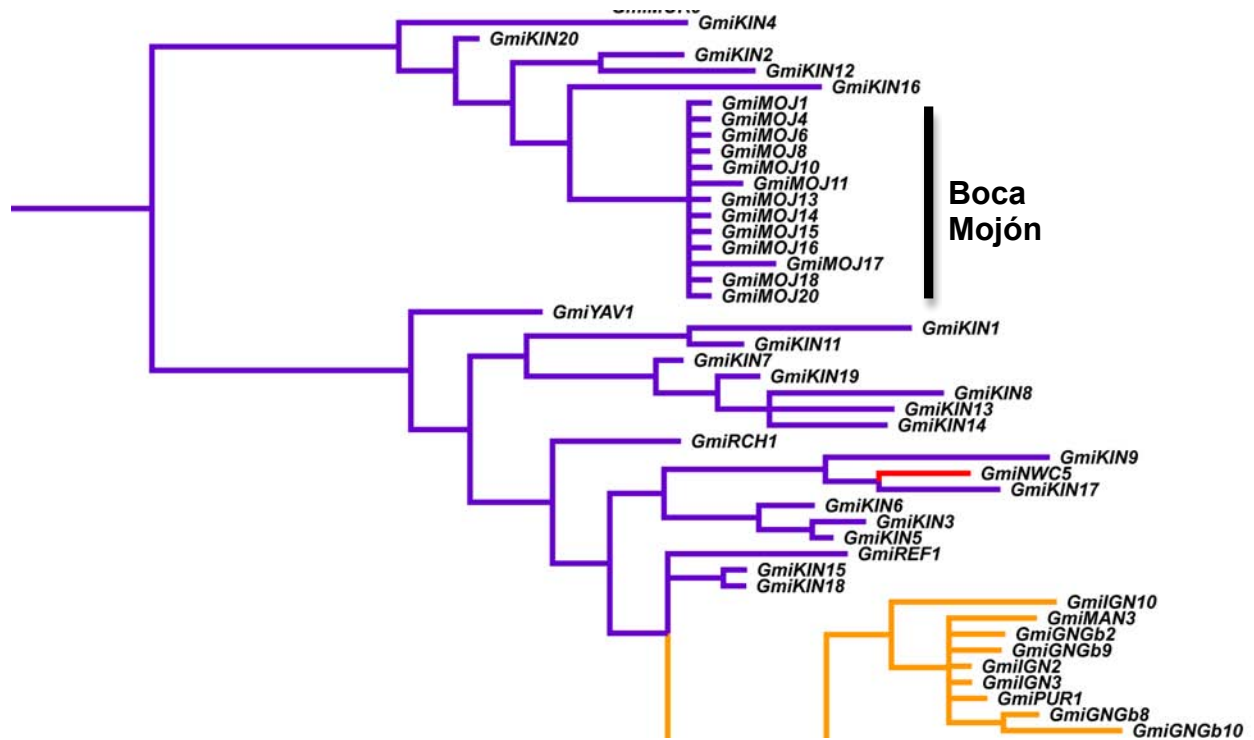


Figure 3-7. Zoomed-in view of southern Gulf sequences from the tree in Fig. 3-2 showing isolation and low diversity of haplotypes sampled at Boca Mojón (MOJ), likely reflecting a founding event from the southeastern Gulf.

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