How the Central American Seaway and an ancient northern passage affected Flatfish diversification

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Abstract

While the natural history of flatfish has been contentious for decades, the mode of diversification of this biologically and economically important group has never been elucidated. To address this question, we assembled the largest molecular data set to date, covering > 300 species (out of *ca*. 800 extant), from 13 of the 14 known families over nine genes, and employed relaxed molecular clocks to uncover their patterns of diversification. As the fossil record of flatfish is contentious, we used sister species distributed on both sides of the American continent to calibrate clock models based on the closure of the Central American Seaway (CAS), and on their current species range. We show that flatfish diversified in two bouts, as species that are today distributed around the Equator diverged during the closure of CAS, while those with a northern range diverged after this, hereby suggesting the existence of a post-CAS closure dispersal for these northern species, most likely along a northern route, a hypothesis fully compatible with paleogeographic reconstructions.

Keywords: Pleuronectiformes, molecular clock, Bayesian dating, vicariance, Central American Seaway, Isthmus of Panama

The Pleuronectiformes, or flatfishes, are a speciose group of ray-finned fish, containing 14 families and over 800 known species (Munroe 2015). Flatfish begin life in the pelagic zone, but undergo a larval metamorphosis in which one eye, either left or right, depending on the species, migrates to the other side of the cranium. The adult fish then adopts a benthic lifestyle. Flatfish have asymmetric, laterally-compressed bodies, and have lost their swim bladders during transformation. With eyes facing upwards, flatfish are also capable of protruding them. This singular morphology long puzzled taxonomists (Norman 1934), and the phylogeny of this group remains poorly resolved.

At the highest taxonomic level, flatfishes are generally considered to be monophyletic, based on both morphological (Chapleau 1993), and molecular evidence (Berendzen et al. 2002; Pardo et al. 2005; Azevedo et al. 2008; Betancur-R et al. 2013; Harrington et al. 2016). All these studies also support the monophyletic status of most families within the order, to the exception of the Paralichthyidae. As all molecular studies to date have essentially focused on the monophyletic status of the order, they were based on as many representative species of each order. As a result, intra-ordinal relationships, among genera and even families, are still debated. It can therefore be expected that taking advantage of both species- and gene-rich evidence, while incorporating paleontological and/or geological data in the framework of molecular clocks, should help clarify not only the phylogenetic status of this family (dos Reis et al. 2015), but also – and more critically here – their evolutionary dynamics.

However, very few flatfish fossils are known, and placed with confidence on the flatfish evolutionary tree (Parham et al. 2012). As a result, calibrating a molecular clock becomes challenging. On the other hand, a dense species sampling may enable us to take

advantage of a singular feature of flatfishes: some extant species are found both in the Pacific and Atlantic oceans. Furthermore, the existence of geminate species pairs of flatfishes, where sister taxa have one member in each ocean, suggests a speciation event pre-dating the closure of the Isthmus of Panama, which occurred approximately 12 to 3 million years ago [MYA] (Haug and Tiedemann 1998; ODea et al. 2016). Our driving hypothesis is then that this information can be used to calibrate molecular clocks, and allow us to unravel the timing of flatfish evolution, as how rapidly they diversified remains an unsolved question. We show here that the diversification of flatfish in the sea surrounding the Americas followed a complex pattern, related to not only the closure of the Isthmus of Panama, but also to a warming event that opened up a northern route.

Results

To test how the evolutionary dynamics of flatfish were affected by a major geological event, the closure of the CAS, we reconstructed dated Bayesian phylogenetic trees from a large data matrix under four relaxed molecular clock models, each one of them being based on a different calibration scheme (Fig. 1). Under the first model, no calibration priors were placed on internal nodes. The initial data survey tree, with the rogue sequences removed (data on GitHub) was used to identify pairs of sister taxa that are split between the two oceans, with one species in the Atlantic and the other in the Pacific. This led us to single out twelve pairs of such species. These sister species happened not to be evenly distributed on the estimated phylogeny (Fig. 2, top), but they are the only geminate species included in GenBank (as of August 2016). On each pair, we placed calibration date priors corresponding to the closure of the CAS. An examination of the

75 posterior distributions of their divergence times suggests that some species had a very
76 narrow speciation window, where all the mass of the posterior distribution is between 5-3
77 MYA, while others have a wider distribution (Fig. 3A). Closer inspection of these
78 distributions further reveals that most of the species with narrow posterior distributions
79 have a northern range (Fig. 2, and 3A, in blue), while those with the wider posterior
80 distributions have a "southern" distribution, closer to the Isthmus of Panama (Fig. 2, and
81 3A, in red). To further assess this observation, we first went back to the original clock
82 model, with no priors on sister taxa, and were able to validate that even in this case,
83 northern and southern species showed, to one exception each (*Hippoglossus hippoglossus*
84 and *H. stenolepis* in the north, and *Poecilopsetta natalensis* and *P. hawaiiensis* in the
85 south; Fig. 2), shifted posterior distributions (Fig. 3B). The former pair was actually not
86 estimated as being sister species in any of the four clock models (Fig. 1), while *P.*
87 *natalensis* and *P. hawaiiensis,* although inhabiting the Western Atlantic and Eastern
88 Pacific oceans, occupy ranges that do not extend to the coasts of the Americas as with the
89 other identified sister taxa. Models with priors placed only on northern (Fig. 3C) or
90 southern (Fig. 3D) species also showed a similar temporal shift. This shift suggested that
91 southern species diverged early, before the complete closing of the CAS, while northern
92 species diverged later, at or possibly after the isthmus was completed. Averaging these
93 posterior distributions for the northern and southern species, to the exception of the two
94 outliers noted above, showed these results more clearly (Fig. 3E-H).
95 In an attempt to tease out these models and their predictions about the exact timing of
96 divergence between northern and southern species, we assessed model fit by means of
97 AICM. Even if model ranking based on this measure is known to be unstable (Baele et al.

2012), it is clear that the models with priors only on the northern or on the southern species perform significantly (> 200 AIC units) more poorly than the two other models, which may be difficult to tease apart (Table 1). Their predictions suggest that *H. hippoglossus* and *H. stenolepis*, both northern species, consistently diverged before the complete closure of the seaway, in tandem with the average southern species, and that the average northern species diverged in tandem with *P. natalensis* and *P. hawaiiensis* (Fig. 3E-F).

Discussion

Our results show that flatfish underwent a first speciation at the time when the CAS closed, which led to the species that, today, have an equatorial range (Fig. 2), as the formation of the Isthmus of Panama resulted in a barrier to gene flow leading to their speciation. Our results also show that species that today have a northern range (Fig. 2) either emerged at the closure of the seaway, or after its closure. These timing estimates imply that this second bout of speciation was not caused by gene flow impeded by the closure of the seaway, but demand an interpretation involving gene flow through a different route, a northern route, before being interrupted, most likely this time by a climatic event. Strikingly, the geological evidence is directly in line with our date estimates. The fossil record suggests that the first aquatic connection between the Pacific and Arctic (and Atlantic) oceans through the Bering Strait occurred approximately 5.5-5.4 MYA (Gladenkov et al. 2002) due to a rise in sea levels, linked to tectonic activity (Marincovich and Gladenkov 2001). This would have permitted the migration of populations ancestral to today's northern species from one ocean to the other through this

120 ancient "northern passage." This global warming event, between the late Miocene to
121 early Pleiocene, was then followed by a significant period of cooling during the Pleiocene
122 into the Pleistocene (Zachos 2001), leading to periods of repeated glaciations and a
123 subsequent ice age. These cold events would have resulted in the closure of this ancient
124 "northern passage," hereby stopping gene flow between the two oceans, and leading to
125 the more recent speciation of the northern taxa.

126 For approximately a million years after the first opening of the Bering Strait, water
127 flowed in a southern direction, until the formation of the Isthmus of Panama occurred
128 close to the equator (Berta 2012). With the formation of the Isthmus, and the closing of
129 the CAS, the ocean currents reversed due to a change in global ocean circulation (Haug
130 and Tiedemann 1998; De Schepper et al. 2015), and have since flowed from the Pacific
131 to the Arctic (Marincovich 2000). Dispersal or migration from the Pacific into the
132 Atlantic in a northern direction through this strait is known as the trans-Arctic
133 interchange (Vermeij 1991). Fossil data also show that the Bering land bridge has been
134 exposed and submerged on multiple occasions since the Pleistocene (Gladenkov and
135 Gladenkov 2004). These openings and closings of the Bering Strait could have provided a
136 mechanism for divergence and the evolution of sister taxa (Taylor and Dodson 1994;
137 Väinölä 2003).

138 Our results also have implication at the family level of flatfish. Further significant
139 global cooling during the Pleistocene resulted in major glaciation events (Zachos et al.
140 2008) that could be responsible for creating barriers that isolated populations. All of the
141 remaining sister taxa in our analysis, who have divergence estimates of less than 2 MYA
142 in our study belong to the family Pleuronectidae (*sensu* Cooper and Chapleau 1998). The

143 Pleuronectidae are the predominant flatfish family found in cold and temperate seas of
144 the northern hemisphere (Norman 1934; Cooper and Chapleau 1998). There are far more
145 Pleuronectidae species in the Pacific Ocean, most of them endemic to the north Pacific
146 Ocean off of north America and Asia in the region extending from the Bering Strait to the
147 gulf of California (Norman 1934). None of the arctic species are restricted solely to arctic
148 waters (Munroe 2015). Munroe also noted that Cooper (in an unpublished manuscript)
149 identified areas of endemism among the current distribution of the Pleuronectidae. It has
150 been shown that during the trans-Arctic interchange, there was a far higher number of
151 species (up to eight times higher) migrating to the Atlantic than to the Pacific (Vermeij
152 1991). Fossil and phylogenetic evidence suggest the Pacific Ocean as the origin for
153 diversification of the Pleuronectidae (Munroe 2015) and our phylogenetic results are
154 highly congruent with this hypothesis.

155 It is possible that the outliers, the Atlantic and Pacific halibut (*H. hippoglossus* and *H.*
156 *stenolepis*, respectively), diverged during one of the first openings of the Bering Strait.
157 The estimated dates from the molecular clock analysis are approximately 5.5 MYA, in
158 accordance with the hypothesized dates for the first aquatic connection (Marincovich and
159 Gladenkov 2001). The remaining sister taxa have a younger age estimate of ~1-2 MYA,
160 corresponding with global cooling during the Pleistocene and repeated glaciations
161 (Zachos 2001) which likely formed more barriers to genetic flow. In the second pair of
162 outliers, *P. hawaiiensis* inhabits waters of the Eastern Central Pacific to the Hawaiian
163 Islands, while *P. natalensis* inhabits coastal waters of Eastern Africa (Fig. 2). Due to the
164 far reaching range of *P. natalensis*, and the relatively younger age estimates for
165 divergence (1-2 MYA), these results beg for future research into other vicariant

166 hypotheses, or dispersal routes, as these two members of *Poecilopsetta* diverged long
167 after the Isthmus had closed.
168 Based on the most extensive multigene sequence alignment available to date across
169 all flatfish species, we have showed here that the evolutionary dynamics of sister species
170 that are distributed across the two oceans strongly supported the existence of two bouts of
171 speciation: one triggered by the closure of the CAS 12-3 MYA, and a second one due to
172 the closure of an ancient northern passage 5-0.01 MYA. The most intriguing implication
173 of our results is that other sea critters than the flatfish should have been affected by the
174 two same geological processes, the closure of the CAS (Bacon et al. 2015) and, most
175 intriguingly, of the ancient northern passage, so that future studies should be able to
176 extend our findings to other living forms inhabiting these two oceans.

177 Methods and Materials

178 *Data retrieval and alignment*

179 Prior to retrieving sequence data, GenBank was surveyed to identify all the genes
180 belonging to species of Pleuronectiform families (as of August 2016), based on
181 GenBank's taxonomy browser. DNA sequences for a total of 332 flatfish species (out of
182 over 800 species in the order) were identified and downloaded for five nuclear genes
183 (KIAA1239, MYH6, RIPK4, RAG1, SH3PX3), and four mitochondrial genes (12S, 16S,
184 COX1 and CYTB). These represented all the taxa having at least one of these six gene
185 sequences in GenBank; see Table S1 for the corresponding accession numbers. Diversity
186 was richly sampled as species from 13 of the 14 families in the order Pleuronectiformes

187 were included in our catchment. In line with current consensus in flatfish systematics, the
188 family Psettodidae was chosen as the outgroup to all other taxa (Chapleau 1993).
189 These sequences were aligned using MUSCLE ver. 3.8.31 (Edgar 2004) on a gene-
190 by-gene basis. Each alignment was visually inspected with AliView ver. 1.18 (Larsson
191 2014), and was manually edited where necessary. In particular, large indels (> 10 bp)
192 were removed prior to all phylogenetic analyses. The 5' and 3' ends of sequences were
193 also trimmed. The aligned sequences were then concatenated into a single alignment
194 using a custom R script.

195 ***Data pre-processing***

196 To gauge the phylogenetic content of our data set, we performed a first series of
197 molecular clock analyses on the concatenated data matrix, with all the nine gene
198 sequences obtained above (12S, 16S, COI, Cyt-b, KIAA1239, MYH6, RIPK4, SH3PX3
199 and RAG1), and all the 332 taxa representing all families of flatfish. To account for rate
200 heterogeneity along this alignment, partitions were first identified with PartitionFinder 2
201 (Lanfear 2012), selecting the best-fit model of nucleotide substitution based on Akaike's
202 Information Criterion (AIC). This optimal partition scheme was then employed in a first
203 Bayesian phylogenetic analysis, conducted with BEAST ver. 1.8.3 (Drummond and
204 Rambaut 2007). This program implements a Markov chain Monte Carlo (MCMC)
205 sampler, which co-estimates both tree topology and divergence times. As determined by
206 PartionFinder, a GTR+I+Γ model was applied to each data partition. These models of
207 evolution across partitions were assumed to be independent (*unlinked*, in BEAST
208 parlance), while both clock model and tree model partitions were shared (*linked*) by all
209 partitions. An uncorrelated relaxed clock was employed with a lognormal distribution

210 prior on rates, and a Yule speciation prior (Drummond et al. 2006). Due to the paucity of
211 reliably placed fossils on the flatfish tree, a unique calibration point was placed on the
212 most recent common ancestor (MRCA) of the ingroup, as a mean-one exponential prior,
213 with an offset of 40 million years reflecting the age of 47.8-42.1 MYA (Chanet 1997). To
214 stabilize the estimate, a lognormal ln(0, 1.5) prior with a 40 MYA offset was also placed
215 on the root of the tree (root height). Two separate MCMC samplers were run, each for
216 10,000,000 generations. Trees were sampled every 5,000 generations, and convergence
217 was checked visually using Tracer ver. 1.6. (Rambaut et al. 2014). Tree log files from
218 each run were combined in LogCombiner, after conservatively removing 10% of each
219 run as burn-in. The resulting maximum a posteriori (MAP) tree was generated with
220 TreeAnnotator (Drummond and Rambaut 2007).
221 As the topology of this resulting MAP tree was unconventional, we suspected the
222 presence of rogue taxa, *i.e.* species evolving either much faster or much slower than the
223 majority, which can contribute negatively to consensus tree support (Aberer et al. 2013).
224 Rogue taxa were identified using the RogueNaRok (Aberer et al. 2013) webserver
225 (http://rnr.h-its.org). The consensus trees from the preliminary analysis using 10,000
226 iterations were used as the tree set for rogue taxon analysis. A total of 22 rogues were
227 identified and pruned from the analysis, leaving 310 species.

228 ***Molecular Dating***

229 To assess the impact of the closure of the Central American Seaway (CAS; forming the
230 Isthmus of Panama) on flatfish evolutionary dynamics, a second set of partitioned relaxed
231 molecular clock analyses was performed (without the rogue sequences). The timing of
232 the closure of the CAS is estimated to have occurred between 12 and 3 MYA (Duque-

233 Caro 1990; Coates et al. 1992; Haug and Tiedemann 1998; ODea et al. 2016), and we
234 used this time window as a prior to inform the relaxed molecular clock-based
235 phylogenetic reconstructions. The analyses were performed on the same concatenated
236 data set, with the same single fossil calibration, but we also placed a lognormal prior
237 ln(3,1.5), that has most of its mass on the 12-3 MYA window, on the MRCA of each pair
238 of sister taxa. From the initial BEAST analyses, twelve pairs of taxa were selected based
239 on two criteria: (i) being sister species on that initial tree, (ii) with one species distributed
240 in the Pacific and one in the Atlantic ocean (Fig. 2). Again, two independent MCMC
241 samplers were run each for 100 million iterations, with samples taken every 5000 step.
242 Because these pairs of sister species show a contrasted geographic distribution,
243 having either a southern (equatorial) or a northern range (Fig. 2), two additional sets of
244 analyses were performed. In a first set, calibration priors (ln(3,1.5), as *per* above) were
245 placed only sister taxa that had a geographic range in the southern hemisphere, while in a
246 second set, identical calibration priors were placed only on sister species with a northern
247 range. Finally, a set of analyses was performed using no sister taxa calibrations at all. For
248 each analysis, results from the two MCMC runs were combined using LogCombiner after
249 removing an even more conservative burn-in of 50%. The final MAP tree was generated
250 with TreeAnnotator.
251 In an attempt to rank these different models (priors on all sister taxa; only on southern
252 taxa; only on southern taxa; no "CAS" priors), a modified Akaike's Information Criterion
253 (AICM) that accounts for uncertainly in the MCMC sampling (Raftery et al. 2007) was
254 computed for each model (Baele et al. 2013). These computations were performed in
255 Tracer for each of the four different calibration models, based on 100 replicates.

Availability of Computer Code and Data

Accession numbers, sequence alignments, and BEAST models (as xml files) used in this study are available from https://github.com/sarisbro.

Acknowledgments

This work was supported by the University of Ottawa (LB), and the Natural Sciences Research Council of Canada (FC, SAB). We are grateful to Jonathan Dench for discussions and comments, as well as to Compute Canada and Ontario's Center for Advanced Computing for providing us access to their resource.

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365 **Table 1**. AICM values for the phylogenetic analyses using four different calibration
366 schemes. SE: standard error.

Calibration Model	AICM value	SE
All	374,082.199	+/-1.579
None	374,110.488	+/-3.789
South	374,367.749	+/-0.696
North	374,746.628	+/-3.789

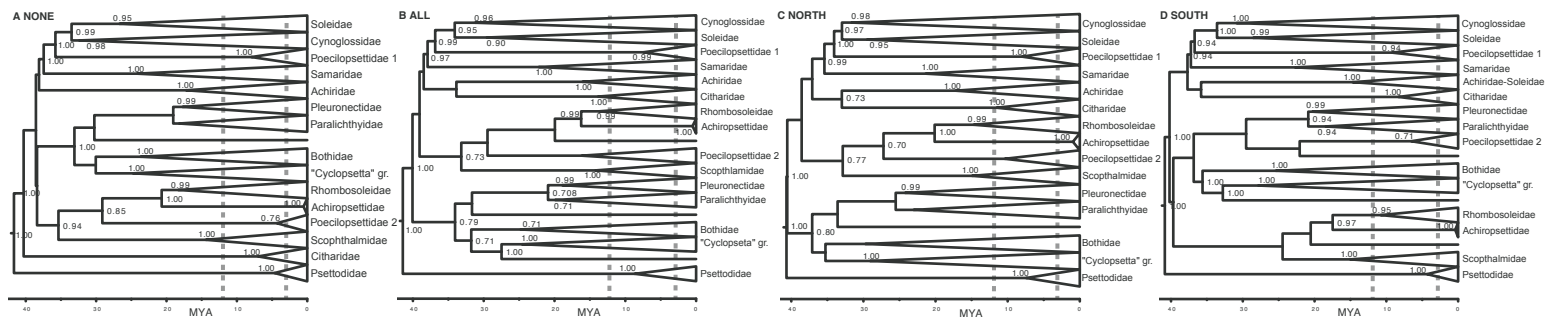
367
368

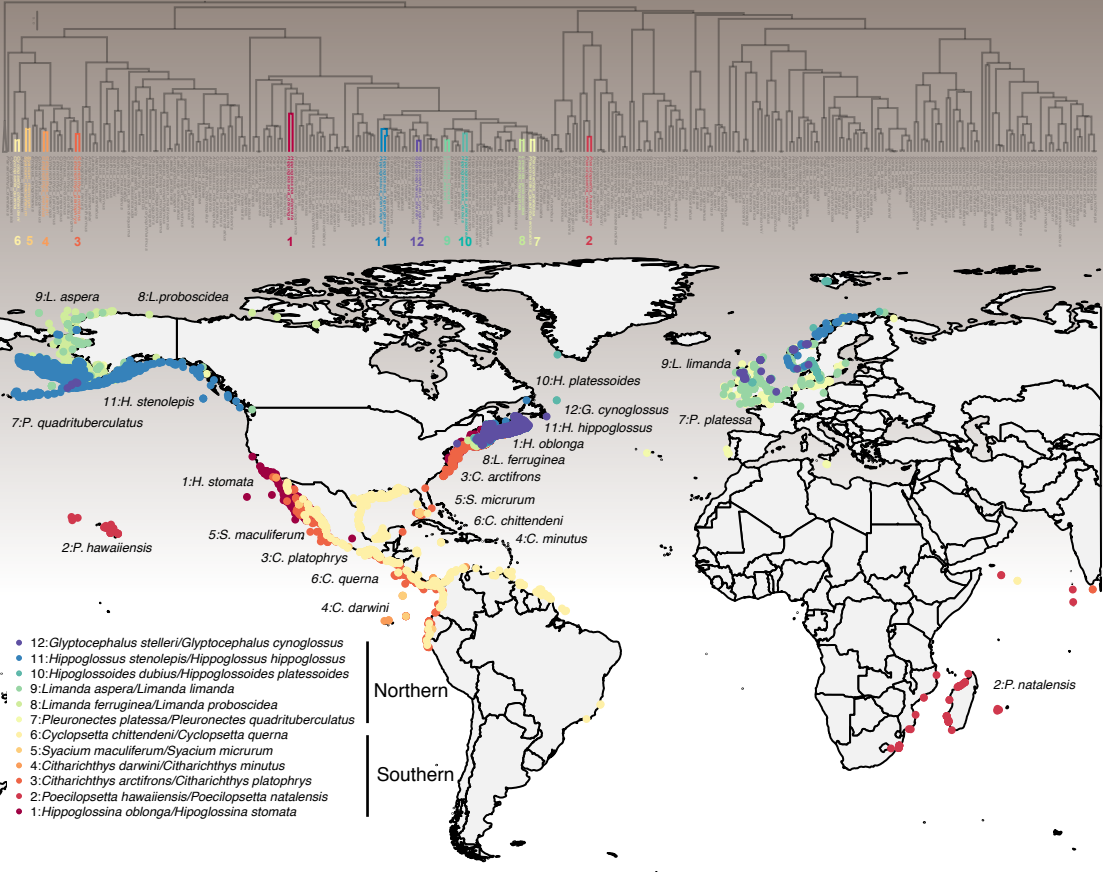
Figure captions

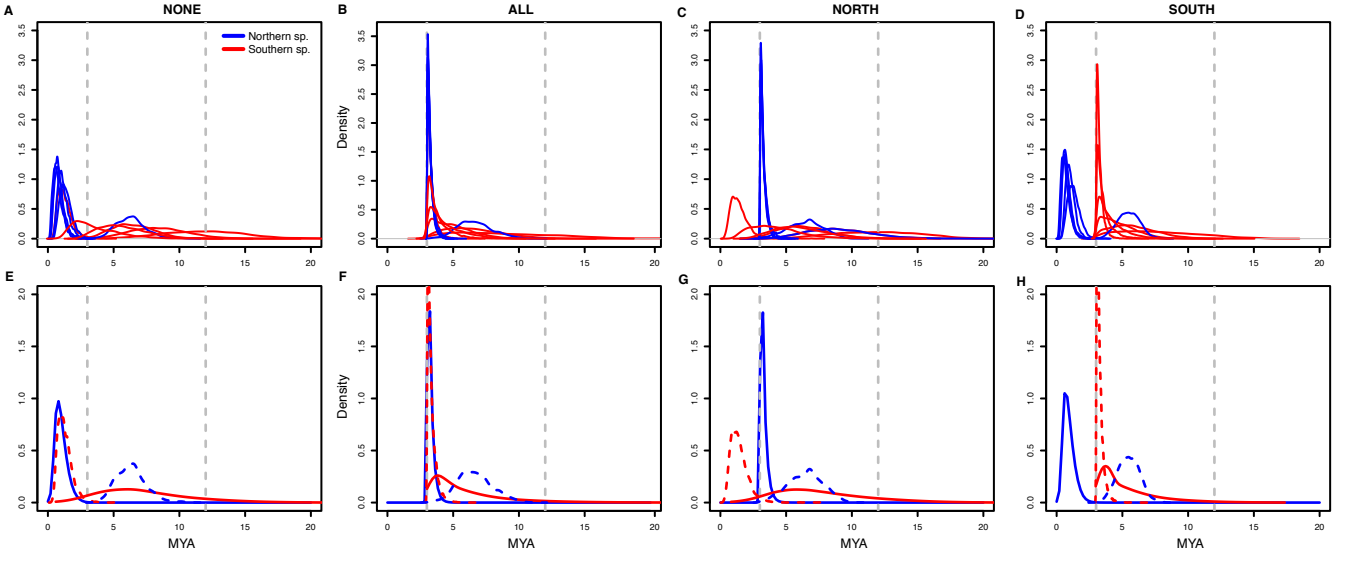
FIG. 1. Phylogenetic trees reconstructed based on relaxed clock models. Four models were employed, representing different specifications of prior distributions set on sister taxa. (**A)** No priors were set on sister taxa. (**B)** Priors were set on all pairs of taxa. (**C)** Priors were set only on sister taxa with a current northern range. (**D)** Priors were set only on sister taxa with a current southern range. Horizontal scale is in million years ago (MYA). The closure of the CAS (12-3 MYA) is shown between vertical gray broken lines.

FIG. 2. Distribution of the geminate species used in this study. Geographic distribution of the twelve pairs of sister species of flatfish used to calibrate the relaxed molecular clock models. Original data come from GBIF (www.gbif.org; accessed Nov 9, 2017). The top panel shows the phylogenetic distribution of these species based on the clock model including prior ages on all twelve pairs of species; scale bar: 5 MYA.

FIG. 3. Posterior densities of divergence times for sister taxa used as calibration points in the relaxed molecular clock analyses, under four different calibration schemes. NONE: no priors were placed on sister taxa; ALL: priors were placed on all pairs of sister taxa; NORTH: priors were placed on only pairs of sister taxa with a northern distribution; SOUTH: priors were placed on only pairs of sister taxa with a southern distribution. Top row (**A**-**D**) shows all 17 distributions, while bottom row (**E**-**H**) shows range-averaged distributions (solid) to the exception of outlier pairs (dashed lines). Densities are color-coded for species with northern (blue) and southern (red) range. Dashed vertical gray lines indicate the closure of the CAS (21-3 MYA).


A NONE
Soleidae
Cynoglossidae
Poecilopsettidae 1
Samaridae
Achiridae
Pleuronectidae
Paralichthyidae
Bothidae
"Cyclopsetta" gr.
Rhombosoleidae
Achiropsettidae
Poecilopsettidae 2
Scophthalmidae
Citharidae
Psettodidae
MYA
B ALL
Cynoglossidae
Soleidae
Poecilopsettidae 1
Samaridae
Achiridae
Citharidae
Rhombosoleidae
Achiropsettidae
Poecilopsettidae 2
Scophthalmidae
Pleuronectidae
Paralichthyidae
Bothidae
"Cyclopseta" gr.
Psettodidae
MYA
C NORTH
Cynoglossidae
Soleidae
Poecilopsettidae 1
Samaridae
Achiridae
Citharidae
Rhombosoleidae
Achiropsettidae
Poecilopsettidae 2
Scophthalmidae
Pleuronectidae
Paralichthyidae
Bothidae
"Cyclopsetta" gr.
Psettodidae
MYA
D SOUTH
Cynoglossidae
Soleidae
Poecilopsettidae 1
Samaridae
Achiridae-Soleidae
Citharidae
Pleuronectidae
Paralichthyidae
Poecilopsettidae 2
Bothidae
"Cyclopsetta" gr.
Rhombosoleidae
Achiropsettidae
Scophthalmidae
Psettodidae
MYA


6 5 4 3
1
11 12 9 10
8 7
2
9:L. aspera
8:L.proboscidea
11:H. stenolepis
7:P. quadrituberculatus
1:H. stomata
2:P. hawaiiensis
5:S. maculiferum
3:C. platophrys
6:C. querna
4:C. darwini
10:H. platessoides
12:G. cynoglossus
11:H. hippoglossus
1:H. oblonga
8:L. ferruginea
3:C. arctifrons
5:S. micrurum
6:C. chittendeni
4:C. minutus
9:L. limanda
7:P. platessa
2:P. natalensis
12:Glyptocephalus stelleri/Glyptocephalus cynoglossus
11:Hippoglossus stenolepis/Hippoglossus hippoglossus
10:Hipoglossoides dubius/Hippoglossoides platessoides
9:Limanda aspera/Limanda limanda
8:Limanda ferruginea/Limanda proboscidea
7:Pleuronectes platessa/Pleuronectes quadrituberculatus
6:Cyclopsetta chittendeni/Cyclopsetta querna
5:Syacium maculiferum/Syacium micrurum
4:Citharichthys darwini/Citharichthys minutus
3:Citharichthys arctifrons/Citharichthys platophrys
2:Poecilopsetta hawaiiensis/Poecilopsetta natalensis
1:Hippoglossina oblonga/Hipoglossina stomata
Northern
Southern


A
NONE
Northern sp.
Southern sp.
B
ALL
C
NORTH
D
SOUTH
E
F
G
H
Density
MYA