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 debated 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 trans-Arctic northern route, a hypothesis fully compatible with paleogeographic reconstructions.

Keywords: Pleuronectiformes, Bayesian dating, trans-Arctic migration, 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 (Chanet 1997; Friedman 2012), and placed with confidence on the flatfish evolutionary tree (Parham et al. 2012; Campbell et al. 2014a; Harrington et al. 2016). 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 formation of the Isthmus of Panama, which occurred approximately 12 to 3 million years ago [MYA] (Haug and Tiedemann 1998; O'Dea et al. 2016). Also possible is the role played by the opening of the Bering Strait around 5.5-5.4 MYA (Gladenkov et al. 2002), which would have permitted the trans-Arctic migration of ancestral populations

61 from one ocean to the other, as documented in marine invertebrates (*e.g.*, Durham and
62 MacNeil 1967; Reid 1990) or vertebrates (*e.g.*, Grant 1986), but to date, never in flatfish.
63 Our driving hypothesis is then that the sole information relative to the formation of the
64 Isthmus of Panama can be used to calibrate molecular clocks, and allows us to unravel
65 the timing of flatfish evolution, as how rapidly they diversified remains an unsolved
66 question. We show here that the diversification of flatfish in the seas surrounding the
67 Americas followed a complex pattern, related to not only the closure of the Isthmus of
68 Panama, but also to a warming event that opened up a trans-Arctic northern route.

69 Results

70 To test how the evolutionary dynamics of flatfish were affected by a major geological
71 event, the closure of the Central American Seaway (CAS), we reconstructed dated
72 Bayesian phylogenetic trees from a large data matrix under four relaxed molecular clock
73 models, each one of them being based on a different calibration scheme (Fig. 1). Under
74 the first model, no calibration priors were placed on internal nodes. This initial tree, with
75 the rogue sequences removed (data on GitHub: see Methods), was used to identify pairs
76 of sister taxa that are split between the two oceans, with one species in the Atlantic and
77 the other in the Pacific. This led us to single out twelve pairs of such species. These sister
78 species happened not to be evenly distributed on the estimated phylogeny (Fig. 2, top),
79 but they are the only geminate species included in GenBank (as of August 2016). On
80 each pair, we placed calibration date priors corresponding to the closure of the CAS (the
81 'ALL' model). An examination of the posterior distributions of their divergence times
82 suggests that some species have a very narrow speciation window, where all the mass of
83 the posterior distribution is between 5-3 MYA, while others have a wider distribution
84 (Fig. 3A). Closer inspection of these distributions further reveals that most of the species
85 with narrow posterior distributions have a northern range (Fig. 2, and 3A, in blue), while
86 those with the wider posterior distributions have a "southern" distribution, closer to the
87 Isthmus of Panama (Fig. 2, and 3A, in red). To further assess this observation, we first
88 went back to the original clock model, removed all priors initially placed on sister taxa
89 (the 'NONE' model), and were able to validate that even in this case: northern and
90 southern species showed, to one exception each (*Hippoglossus hippoglossus* and *H.*

91 *stenolepis* in the north, and *Poecilopsetta natalensis* and *P. hawaiiensis* in the south; Fig.
92 2), shifted posterior distributions (Fig. 3B). The former pair was actually not estimated as
93 being sister species in any of the four clock models (Fig. 1), while *P. natalensis* and *P.*
94 *hawaiiensis,* although inhabiting the Western Indian and Eastern Pacific oceans, occupy
95 ranges that do not extend to the coasts of the Americas as with the other identified sister
96 taxa. Models with priors placed only on northern (the 'NORTH' model: Fig. 3C) or
97 southern (the 'SOUTH' model: Fig. 3D) species also showed a similar temporal shift.
98 This shift suggested that southern species diverged early, before the complete closing of
99 the CAS, while northern species diverged later, at or possibly after the isthmus was
100 completed. Averaging these posterior distributions for the northern and southern species,
101 to the exception of the two outliers noted above, showed these results more clearly (Fig.
102 3E-H). To assess the robustness of this result to the inclusion of the *P. natalensis* and *P.*
103 *hawaiiensis* sister species, we conducted an additional set of analyses removing all prior
104 information from this pair. The ALL and SOUTH models, which are the only ones where
105 this prior occurs, show that the general pattern that we found above holds: southern
106 species diverged early, before the complete closing of the CAS, and northern species
107 diverged later, at or possibly after the isthmus was completed (Fig. S1).
108 All these results were obtained assuming that Psettodidae is an appropriate outgroup
109 for these analyses. However, the position of Psettodidae in flatfishes is still debated
110 (Betancur-R. and Orti 2014; Campbell et al. 2014a; Campbell et al. 2014b; Harrington et
111 al. 2016), so that their inclusion might affect our results. To assess this possibility, we
112 reran all four models without Psettodidae, letting the molecular clock root the tree (Aris-
113 Brosou and Rodrigue 2012). Figure S2 shows the exact same results as above (Fig. 3), so
114 that our dating results are also robust to the inclusion, or not, of Psettodidae. Our dating
115 results are also robust to the inclusion of internal calibration priors as used in Harrington
116 et al. (2016), and based on the *Scophthalmus rhombus* / *Bothus pantherinus* and the
117 *Crossorhombus kobensis* / *Bothus pantherinus* divergences, dated at 29.62
118 (Vandenberghe et al. 2012) and 11.06 MYA (Carnevale et al. 2006), respectively (Fig.
119 S3).
120 In an attempt to tease out these models (including Psettodidae and without the two
121 internal calibration priors) and their predictions about the exact timing of divergence

122 between northern and southern species, we assessed model fit by means of the modified
123 Akaike's Information Criterion (AICM; Baele et al. 2013), which accounts for
124 uncertainty in the MCMC sampling (Raftery et al. 2007). Even if model ranking based on
125 this measure is known to be unstable (Baele et al. 2013), it is clear that the models with
126 priors only on the northern or on the southern species perform significantly (> 200 AIC
127 units) worse than the two other models, which may be difficult to tease apart (Table 1).
128 The predictions of the best models suggest that *H. hippoglossus* and *H. stenolepis*, both
129 northern species, consistently diverged before the complete closure of the seaway, in
130 tandem with the average southern species, and that the average northern species diverged
131 in tandem with *P. natalensis* and *P. hawaiiensis* (Fig. 3E-F).
132 One intriguing result from these analyses is that the northern sister species seem to
133 come from a single clade (Fig. 2), which would suggest that it is possible to identify the
134 direction of the trans-Arctic migration. For this, we retrieved the current species range of
135 all flatfish analyzed here (Fig. S4), and indicated their average latitude on the
136 phylogenetic trees (Fig. S5). This northern clade, highly supported (posterior probability
137 > 0.9; see asterisk in Fig. S5) even in the analyses including the two additional internal
138 priors (Fig. S6), is mostly comprised of species currently found in the Pacific (*e.g.*,
139 *Reinhardtius evermanni*), suggesting that the northern sister species that we identified
140 originated from the Pacific, and that their trans-Arctic migration was from the Pacific into
141 the Atlantic in a northern direction.

142 Discussion

143 Our results show that flatfish underwent a first speciation at the time when the CAS
144 closed, which led to the species that, today, have an equatorial range (Fig. 2), as the
145 formation of the Isthmus of Panama resulted in a barrier to gene flow leading to their
146 speciation. Our results also show that species that today have a northern range (Fig. 2)
147 either emerged at the closure of the seaway, or after its closure. These timing estimates
148 imply that this second bout of speciation was not caused by gene flow impeded by the
149 closure of the seaway, but demand an interpretation involving trans-Arctic gene flow,
150 through a northern route, before being interrupted, most likely this time by a climatic
151 event. Critically, these conclusions are robust to the inclusion of outgroup species

152 (Psettodidae), of outliers species (*P. natalensis* and *P. hawaiiensis*), and are consistently
153 found across the four clock models implemented here, including the one (NONE) relying
154 on no other fossil information than the 47.8-42.1 MYA ancestor to this order (Chanet
155 1997; Friedman 2012) – hereby indicating that our data are highly informative with
156 respect to the timing of the divergence of these sister species (and rejecting our initial
157 hypothesis) following their trans-CAS and trans-Arctic migrations.
158 Trans-Arctic migrations from the Pacific to the Atlantic are not new, and have been
159 described in both invertebrates (*e.g.*, Durham and MacNeil 1967) and vertebrates (*e.g.*,
160 Grant 1986), but never using a relaxed molecular clock relying on as little information as
161 a single fossil and a large multigene data set covering an entire order (Pleuronectiformes).
162 While seminal work was usually based on paleontological (Durham and MacNeil 1967),
163 or on morphological evidence (Reid 1990), subsequent work started using allozymes and
164 other proteins (Grant 1986; Zaslavskaya et al. 1992), followed by genetic evidence,
165 mostly at the population level using mitochondrial DNA (Rawson and Harper 2009).
166 However, most of the recent work that relies on molecular clocks either makes strong
167 assumptions on substitution rates (Rawson and Harper 2009; Liu et al. 2011), uses
168 divergences that are vicariance-motivated but not supported by any fossil information
169 (McCusker and Bentzen 2010), or posit in which direction the trans-Arctic migration
170 took place (Dodson et al. 2007) to infer trans-Arctic migrations. Our work is the first to
171 use sister species to demonstrate the existence and elucidate the timing of both the trans-
172 CAS and the trans-Arctic events, while also inferring in which direction the latter took
173 place, without making too many assumptions.
174 Strikingly, the geological evidence is directly in line with our date estimates. The
175 fossil record suggests that the first aquatic connection between the Pacific and Arctic
176 (and Atlantic) oceans through the Bering Strait occurred approximately 5.5-5.4 MYA
177 (Gladenkov et al. 2002) due to a rise in sea levels, linked to tectonic activity
178 (Marincovich and Gladenkov 2001). This would have permitted the trans-Arctic
179 migration of populations ancestral to today's northern species from one ocean to the other
180 through this ancient "northern passage." This global warming event, between the late
181 Miocene to early Pleiocene, was then followed by a significant period of cooling during
182 the Pleiocene into the Pleistocene (Zachos et al. 2001), leading to periods of repeated

183 glaciations and a subsequent ice age. These cold events would have resulted in the
184 closure of this ancient "northern passage," hereby stopping the trans-Arctic gene flow
185 between the two oceans, and leading to the more recent speciation of the northern taxa.
186 For approximately a million years after the first opening of the Bering Strait, water
187 flowed through the strait in a southern direction, from the Arctic to the Pacific ocean,
188 until the formation of the Isthmus of Panama occurred close to the equator (Berta 2012).
189 With the formation of the Isthmus, and hence the closing of the CAS, the ocean currents
190 reversed due to a change in global ocean circulation (Haug and Tiedemann 1998; De
191 Schepper et al. 2015), and have since flowed in a northern direction through the Bering
192 Strait, from the Pacific to the Arctic (Marincovich 2000). This again is absolutely
193 consistent with our results (Fig. S4), with dispersal or migration from the Pacific into the
194 Atlantic in a northern direction through this strait, which is known as the trans-Arctic
195 interchange (Vermeij 1991). Fossil data also show that the Bering land bridge has been
196 exposed and submerged on multiple occasions since the Pleistocene (Gladenkov and
197 Gladenkov 2004). These openings and closings of the Bering Strait could have provided a
198 mechanism for divergence and the evolution of sister taxa (Taylor and Dodson 1994;
199 Väinölä 2003).
200 Our results also have implication at the family level of flatfish. Further significant
201 global cooling during the Pleistocene resulted in major glaciation events (Zachos et al.
202 2008) that could be responsible for creating barriers that isolated populations. All of the
203 remaining sister taxa in our analysis, who have divergence estimates of less than 2 MYA
204 in our study belong to the family Pleuronectidae (*sensu* Cooper and Chapleau 1998). The
205 Pleuronectidae are the predominant flatfish family found in cold and temperate seas of
206 the northern hemisphere (Norman 1934; Cooper and Chapleau 1998). There are far more
207 Pleuronectidae species in the Pacific Ocean, most of them endemic to the north Pacific
208 Ocean off of North America and Asia in the region extending from the Bering Strait to
209 the gulf of California (Norman 1934). None of the arctic species are restricted solely to
210 arctic waters (Munroe 2015). Munroe also noted that Cooper (in an unpublished
211 manuscript) identified areas of endemism among the current distribution of the
212 Pleuronectidae. It has been shown that during the trans-Arctic interchange, there was a
213 far higher number of species (up to eight times higher) migrating to the Atlantic than to

214 the Pacific (Vermeij 1991). Fossil and phylogenetic evidence suggest the Pacific Ocean
215 as the origin for diversification of the Pleuronectidae (Munroe 2015), and our
216 phylogenetic results are highly congruent with this hypothesis.
217 It is possible that the outliers, the Atlantic and Pacific halibut (*H. hippoglossus* and *H.*
218 *stenolepis*, respectively), diverged during one of the first openings of the Bering Strait.
219 The estimated dates from the molecular clock analysis are approximately 5.5 MYA, in
220 accordance with the hypothesized dates for the first aquatic connection (Marincovich and
221 Gladenkov 2001). The remaining sister taxa have a younger age estimate of ~1-2 MYA,
222 corresponding with global cooling during the Pleistocene and repeated glaciations
223 (Zachos et al. 2001), which likely formed more barriers to genetic flow. In the second
224 pair of outliers, *P. hawaiiensis* inhabits waters of the Eastern Central Pacific to the
225 Hawaiian Islands, while *P. natalensis* inhabits coastal waters of Eastern Africa (Fig. 2).
226 Due to the far reaching range of *P. natalensis*, and the relatively younger age estimates
227 for divergence (1-2 MYA), these results beg for future research into other vicariant
228 hypotheses, or dispersal routes, as these two members of *Poecilopsetta* diverged long
229 after the Isthmus had closed. One candidate would be the Indo-Pacific barrier, which
230 formed an almost continuous land bridge between Australia and Asia, and could have
231 hence limited dispersal between the Pacific and the Indian oceans, hereby promoting
232 speciation (Gaither et al. 2011). This barrier formed during the Pleistocene glacial cycles
233 (2.58-0.01 MYA; Voris 2000), which is, again, fully consistent with our date estimates
234 (Fig. 3, S1).
235 Based on the most extensive multigene sequence alignment available to date across
236 all flatfish species, we have showed here that the evolutionary dynamics of sister species
237 that are distributed across the two oceans strongly supported the existence of two bouts of
238 speciation: one triggered by the closure of the CAS 12-3 MYA, and a second one due to
239 the closure of an ancient northern passage 5-0.01 MYA. Our work adds to a growing
240 body of evidence pointing not only to the role of geological processes in shaping biotic
241 turnovers (Bacon et al. 2015), but also on the consequences of climate change that can
242 either trigger speciation events (Reid 1990), or promote faunal interchanges whose
243 ecological and economic impacts are difficult to predict (Wisz et al. 2015).

244 # Materials and Methods

245 ## *Data retrieval and alignment*

246 Prior to retrieving sequence data, GenBank was surveyed to identify all the genes
247 belonging to species of Pleuronectiform families (as of August 2016), based on
248 GenBank's taxonomy browser. DNA sequences for a total of 332 flatfish species (out of
249 over 800 species in the order) were identified and downloaded for five nuclear genes
250 (KIAA1239, MYH6, RIPK4, RAG1, SH3PX3), and four mitochondrial genes (12S, 16S,
251 COX1 and CYTB). These represented all the taxa having at least one of these six gene
252 sequences in GenBank; see Table S1 for the corresponding accession numbers. Diversity
253 was richly sampled as species from 13 of the 14 families in the order Pleuronectiformes
254 were included in our catchment. In line with current consensus in flatfish systematics, the
255 family Psettodidae was chosen as the outgroup to all other taxa (Chapleau 1993).
256 These sequences were aligned using MUSCLE ver. 3.8.31 (Edgar 2004) on a gene-
257 by-gene basis. Each alignment was visually inspected with AliView ver. 1.18 (Larsson
258 2014), and was manually edited where necessary. In particular, large indels (> 10 bp)
259 were removed prior to all phylogenetic analyses. The 5' and 3' ends of sequences were
260 also trimmed. The aligned sequences were then concatenated into a single alignment
261 using a custom R script.

262 ## *Data pre-processing*

263 To gauge the phylogenetic content of our data set, we performed a first series of
264 molecular clock analyses on the concatenated data matrix, with all the nine gene
265 sequences obtained above (12S, 16S, COI, Cyt-b, KIAA1239, MYH6, RIPK4, SH3PX3
266 and RAG1), and all the 332 taxa representing all families of flatfish. To account for rate
267 heterogeneity along this alignment, partitions were first identified with PartitionFinder 2
268 (Lanfear 2012), selecting the best-fit model of nucleotide substitution based on Akaike's
269 Information Criterion (AIC). This optimal partition scheme was then employed in a first
270 Bayesian phylogenetic analysis, conducted with BEAST ver. 1.8.3 (Drummond and
271 Rambaut 2007). This program implements a Markov chain Monte Carlo (MCMC)
272 sampler, which co-estimates both tree topology and divergence times. As determined by
273 PartionFinder, a GTR+I+Γ model was applied to each data partition. These models of

274 evolution across partitions were assumed to be independent (*unlinked*, in BEAST
275 parlance), while both clock model and tree model partitions were shared (*linked*) by all
276 partitions. An uncorrelated relaxed clock was employed with a lognormal distribution
277 prior on rates, and a Yule speciation prior (Drummond et al. 2006). Due to the paucity of
278 reliably placed fossils on the flatfish tree, a unique calibration point was placed on the
279 most recent common ancestor (MRCA) of the ingroup, as a mean-one exponential prior,
280 with an offset of 40 million years reflecting the age of 47.8-42.1 MYA (Chanet 1997;
281 Friedman 2012). To stabilize the estimate, a lognormal ln(0, 1.5) prior with a 40 MYA
282 offset was also placed on the root of the tree (root height). Two separate MCMC samplers
283 were run, each for 10,000,000 generations. Trees were sampled every 5,000 generations,
284 and convergence was checked visually using Tracer ver. 1.6 (available at:
285 http://tree.bio.ed.ac.uk/software/ tracer/). Tree log files from each run were combined in
286 LogCombiner, after conservatively removing 10% of each run as burn-in. The resulting
287 maximum a posteriori (MAP) tree was generated with TreeAnnotator (Drummond and
288 Rambaut 2007).
289 As the topology of this resulting MAP tree was unconventional, we suspected the
290 presence of rogue taxa, *i.e.* species evolving either much faster or much slower than the
291 majority, which can contribute negatively to consensus tree support (Aberer et al. 2013).
292 Rogue taxa were identified using the RogueNaRok (Aberer et al. 2013) webserver
293 (http://rnr.h-its.org). The consensus trees from the preliminary analysis using 10,000
294 iterations were used as the tree set for rogue taxon analysis. A total of 22 rogues were
295 identified and pruned from the analysis, leaving 310 species.

296 ***Molecular Dating***

297 To assess the impact of the closure of the CAS on flatfish evolutionary dynamics, a
298 second set of partitioned relaxed molecular clock analyses was performed (without the
299 rogue sequences). The timing of the closure of the CAS is estimated to have occurred
300 between 12 and 3 MYA (Duque-Caro 1990; Coates et al. 1992; Haug and Tiedemann
301 1998; O'Dea et al. 2016), and we used this time window as a prior to inform the relaxed
302 molecular clock-based phylogenetic reconstructions. The analyses were performed on the
303 same concatenated data set, with the same single fossil calibration, but we also placed a
304 lognormal prior ln(3,1.5), that has most of its mass on the 12-3 MYA window, on the

305 MRCA of each pair of sister taxa (the 'ALL' model). From the initial BEAST analyses,
306 twelve pairs of taxa were selected based on two criteria: (i) being sister species on that
307 initial tree, (ii) with one species distributed in the Pacific and one in the Atlantic ocean
308 (Fig. 2). Again, two independent MCMC samplers were run each for 100 million
309 iterations, with samples taken every 5000 step.

310 Because these pairs of sister species show a contrasted geographic distribution,
311 having either a southern (equatorial) or a northern range (Fig. 2), two additional sets of
312 analyses were performed. In a first set, calibration priors (ln(3,1.5), as *per* above) were
313 placed only sister taxa that had a geographic range in the southern hemisphere (the
314 'SOUTH' model), while in a second set, identical calibration priors were placed only on
315 sister species with a northern range (the 'NORTH' model). Finally, a set of analyses was
316 performed using no sister taxa calibrations at all (the 'NONE' model). For each analysis,
317 results from the two MCMC runs were combined using LogCombiner after removing an
318 even more conservative burn-in of 50%. The final MAP tree was generated with
319 TreeAnnotator.

320 In an attempt to rank these different models (priors on all sister taxa; only on southern
321 taxa; only on southern taxa; no "CAS" priors), the AICM was computed for each model
322 (Baele et al. 2013). These computations were performed in Tracer for each of the four
323 different calibration models, based on 100 replicates.

324 *Species distribution data*

325 In order to map the distribution of each species of flatfish, we resorted to the Global
326 Biodiversity Information Facility (GBIF: https://www.gbif.org/) database, accessed with
327 the R library rgbif (Chamberlain 2017). Up to 1,000 records were retrieved for each
328 species, and plotted on a geographic map based on GBIF observational records. Where
329 needed, species locations were summarized by mean latitude and longitude (Fig. S4).
330 Approximate attribution to a particular ocean was based on species-specific mean
331 longitudes (Atlantic: between 20˚ and 120˚; Indian: 120˚ and -100˚; Pacific otherwise).

Availability of Computer Code and Data

Accession numbers, sequence alignments, BEAST models (as xml files), estimated phylogenetic trees (maximum *a posteriori* trees from BEAST), and the R scripts used to plot the figures in this study are available from https://github.com/sarisbro.

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485
486

487 **Table 1**. AICM values for the phylogenetic analyses using four different calibration
488 schemes. Models are ranked from the best AICM model (top) to the worst model. SE:
489 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

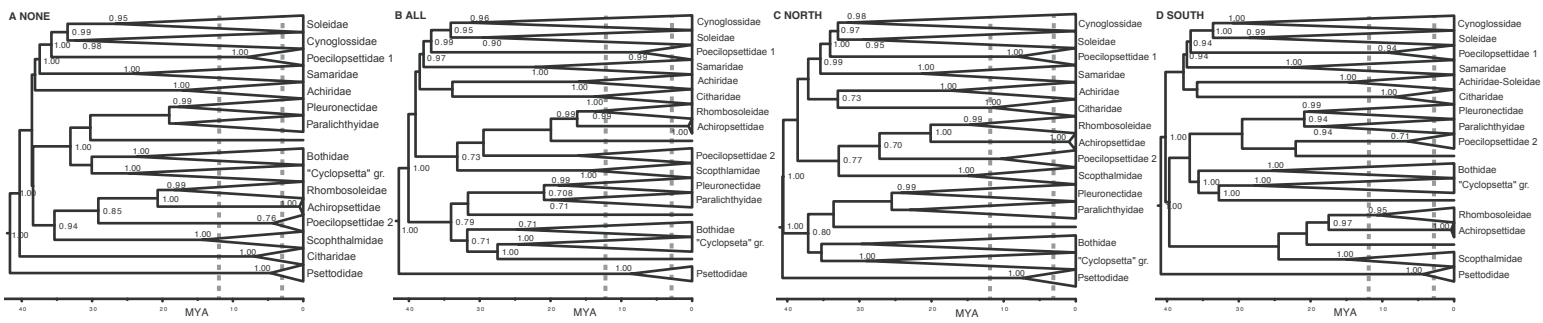
490
491

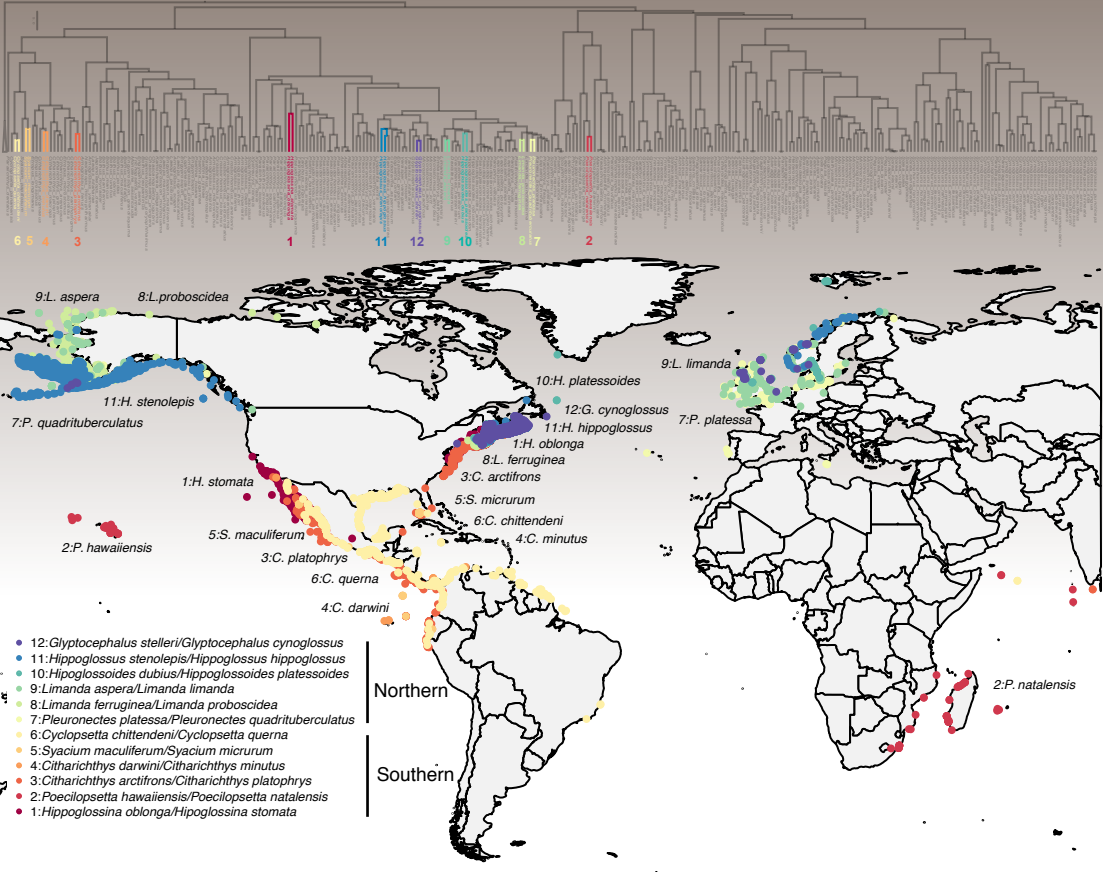
Figure captions

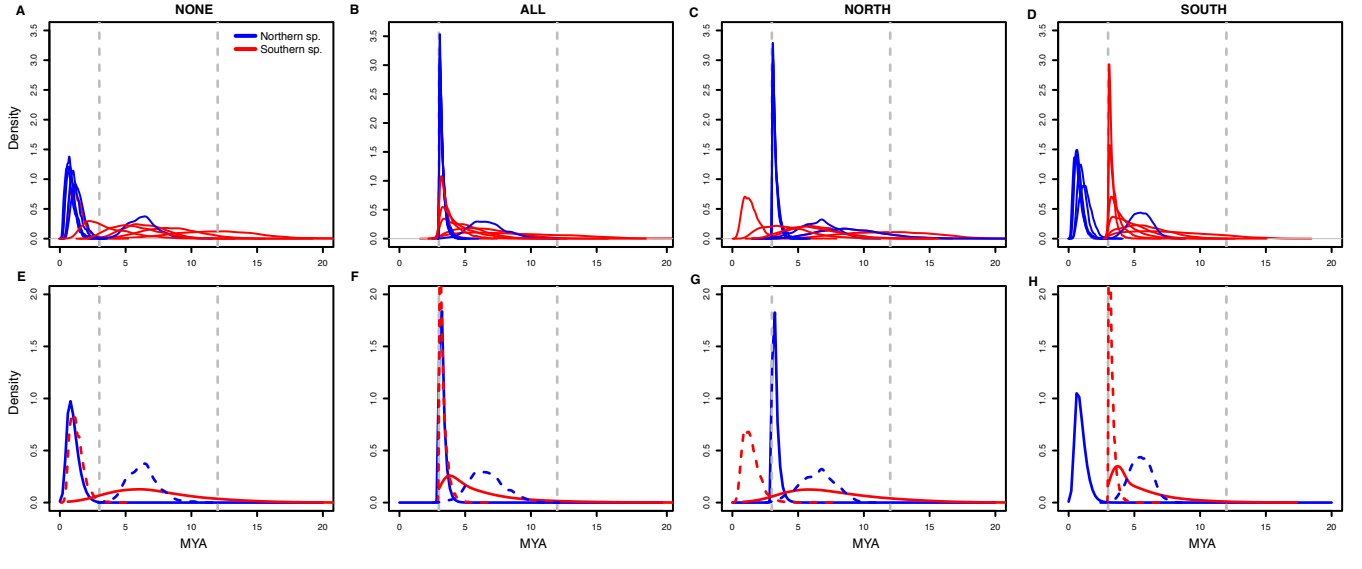
FIG. 1. Phylogenetic trees reconstructed based on relaxed clock models. Four models were employed, representing different specifications of prior distributions set on sister taxa. (**A)** No priors were set on sister taxa. (**B)** Priors were set on all pairs of taxa. (**C)** Priors were set only on 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 only on pairs of sister taxa with a northern distribution; SOUTH: priors were placed only on 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
B
ALL
C
NORTH
D
SOUTH
Northern sp.
Southern sp.
Density
E
F
G
H
MYA