

Cryptic and not-so-cryptic species in the complex “*Holothuria (Thymiosycia) imaptiens*” (Forsskål, 1775) (Echinodermata: Holothuroidea: Holothuriidae)

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Abstract

Identifying accurately species is critical for our understanding of patterns of diversity and speciation. However, for many organisms with simple and variable morphological traits, the characters traditionally used by taxonomists to identify species might lead to a considerable under appreciation of their diversity. Recent advances in molecular-data based computational methods have considerably improved our ability to identify and test species limits. Here, we use an integrative approach to delineate species in a complex of sea cucumbers. We used a three-step approach to show that “*Holothuria imaptiens*”, a common, shallow-water species, occurring across the Indo-Pacific, the Western Atlantic and the Mediterranean Sea, targeted locally by fisheries, is a complex of at least 13 species. (1) We used the Generalized Mixed Yule Coalescent (GMYC) model to identify putative species without *a priori* hypotheses. In the process, we also show that the number of putative species estimated with GMYC can be affected considerably by the priors used to build the input tree. (2) We assessed based on coloration patterns and distributional information, the most relevant hypothesis. This approach allowed us to identify unambiguously 9 species. However, some of the lineages consistently assigned to belong to different species using GMYC, are occurring in sympatry and are not differentiated morphologically. (3) We used Bayes factors to compare competing models of species assignment using the multispecies coalescent as implemented in *BEAST. This approach allowed us to validate that the species identified using GMYC were likely reproductively isolated. Estimates of the timing of diversification also showed that these species diverged less than 2 Ma, which is the fastest case of closely related species occurring in sympatry for a marine metazoan. Our study demonstrates how clarifying species limits contribute to refining our understanding of speciation.

1 Introduction

Despite challenges with definitions, species constitute the biological units used to assess patterns of diversity, identify regions of conservation concern and manage exploited resources [1]. For many

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taxonomic groups, species limits are misunderstood and the level of undescribed diversity debated, making global estimates of diversity poorly constrained [2, 3]. Additionally, conservation efforts aim at preserving the evolutionary potential of the species, but limited understanding of the processes that maintain present and future diversity hinders these goals [4]. Genetic data, and barcoding in particular, are facilitating the documentation of biodiversity not only by providing an inexpensive and efficient way to identify species and resolve complexes, but also by providing insights into the evolutionary history of the species.

Barcoding has revealed that many species, thought to be well-understood, are complexes. In most cases, these “pseudo-cryptic” species differ morphologically but in traits not traditionally considered, or in traits not available to taxonomists because of the preservation methods or the lack of information about the natural history of the species [5]. For instance, differences in coloration [6, 7], habitat [8], or host preference [9], were not considered as indicative of species limits before cryptic lineages were uncovered by molecular data. In these cases, the single-locus approach provided a way to infer species limits using the evolutionary significant unit (ESU) concept [10] that defines species-level units based on reciprocal monophyly in at least one marker (typically mtDNA), and at least another defining attribute (morphology, distribution, reciprocal monophyly in another trait). If this approach has allowed to unravel high levels of unrecognized diversity, when other defining attributes to infer reproductive isolation are lacking, the patterns of genetic differentiation have remained difficult to interpret objectively.

Many species rely on chemical recognition systems to maintain species integrity (e.g., mate recognition, habitat specificity) [5]. Not only these systems are poorly characterized or difficult to investigate in the context of a taxonomic study, they also rarely translate into morphological, behavioral or ecological differences. The absence of defining traits that correlates with genetic clusters identified from single-locus data make these species “true cryptic” complexes. New methods based on the multi-species coalescent are emerging as powerful ways to investigate species limits in these complexes when evidence is equivocal. By using multi-locus datasets, these methods overcome some of the shortcomings of single-locus analyses that were unable to distinguish between different processes leading to identical patterns (e.g., incomplete lineage sorting and introgression), and do not depend on arbitrary threshold of genetic divergence. Instead, they account for the stochastic process associated with the independent sorting of genealogies along the species tree, and use it to estimate the species tree and the demographic history of the species [11]. These methods also provide a statistical framework to test competing species delineation hypotheses [12].

Among marine organisms, the discovery of high levels of cryptic diversity has challenged the importance of physical barriers as the primary driver of diversification [13]. Since most marine invertebrates have a potentially highly dispersive larval stage, and the oceans in general, and the Indo-Pacific in particular, lack clear geographical barriers to dispersal, opportunities for allopatric speciation seem limited. Yet, while many closely related species have non-overlapping distributions suggesting that allopatric speciation is prevalent, overlap in the distribution of sister species is common. These observations have led to the realization that (1) selection (along environmental gradients, sexual selection, host specificity) may play an important role in reproductive isolation (reviewed in [14]); (2) changes in species distributions have obscured the geographic context at the time of speciation. By clarifying the identity of the species, by providing more accurate estimates of the timing of speciation, and by estimating populations sizes, the multispecies coalescent may shed light on the relative contribution of these factors in shaping patterns of diversity, reveal new patterns and refine our understanding of speciation in the sea.

In this study, we use coloration patterns, genetic, distributional and ecological data, to unravel at least 13 species within “*Holothuria impatiens*” (see column “consensus” in Table 1). This complex includes both species that can be distinguished relatively easily from their live appearance, and species that can only be identified genetically. The broad geographical distribution of this complex and the elucidation of the phylogenetic relationships of its species provide the opportunity to investigate the spatial and temporal dynamics of this radiation.

Forsskål described posthumously in 1775 *Fistularia impatiens* from material he collected in Suez, Egypt [15]. The description is limited, but indicates that the body wall is gray with dark spot, and with well-developed, lightly colored tubercles. The drawing accompanying the description corroborates these observations. Since then, the species has been attributed to *Holothuria*, the mostly reef-associated and most diverse genus within the Holothuriidae. The range of *H. impatiens* has been extended, and today is recognized as the most widespread species in the genus. Its range extends from the Red Sea through the entire Indo-West Pacific, to the East Pacific, Caribbean and the Mediterranean. Pearson [16] described *Thymiosycia* as one of five subgenera in *Holothuria*, and designated *Holothuria impatiens* as the type species. The modern concept of *Thymiosycia* was proposed by Rowe [17] to include 13 species.

Holothuria impatiens is a common, to locally abundant species found under rocks (in particular in lagoons and back-reef habitats), and is largely restricted to shallow water (< 10 m), although it has been recorded down to 158 m (Y. Samyn, pers. comm.). Because of its ubiquitous distribution, it is one of the more studied species in the family with studies on its reproduction [18]; Cuvierian tubules [19, 20]; toxicity [21]; feeding preferences [22]; parasites [23]; the chemical composition, statistical analysis of the shape, and ontogenic changes in ossicles [24, 25, 26]. It has also been included in molecular phylogenies that investigated relationships among the major groups of holothurians (e.g., [27]) or as an outgroup when studying relationships within a sub-genus (e.g., [28]). *Holothuria impatiens* is a low-value commercial species that is fished in the Eastern Pacific [29], Madagascar [30], and Palau [31].

Despite its relative biological and commercial importance, the variation observed in color patterns reported by previous workers (e.g., [32, p.178], [33]) has yet to be investigated. The goal of this study is to identify cryptic species in the “*Holothuria impatiens*” complex, and to understand the temporal and spatial dynamics of its diversification. To this end, we sampled the entire known geographic range of this complex, and assembled a multi-locus dataset.

To identify species limits in the complex, we used a combination of complementary methods of species delineation following modifications of the approaches outlined by [34, 35, 36]. First, we used the Generalized Mixed Yule Coalescent method (GMYC) on a portion of the mitochondrial locus COI to delineate putative species [37, 38]. We then used independent lines of evidence (color patterns, ecology and geographic information) to assess the validity of these putative species. When no other line of evidence could separate the putative species identified with GMYC, we used model comparison in *BEAST [39, 12] to validate species limits. Our study shows that these methods can be added to the lines of evidence typically used in integrative taxonomy, and provide a powerful tool for evaluating species limits in rapidly evolving groups with limited morphological and genetic differentiation.

2 Methods

2.1 Sampling

Specimens were collected at low tide, on snorkel, on SCUBA or by dredging. Most specimens were photographed while alive *in situ* or in the lab, anesthetized in a 1:1 solution of sea water and 7.5% solution of magnesium chloride hexahydrate, then preserved in 75% ethanol. When possible tentacles were clipped, immediately put in 95-99% ethanol, and later used for DNA extractions.

Specimens were deposited in the Invertebrate Zoology collections of the Florida Museum of Natural History, University of Florida (UF), Gainesville, FL, USA, while tissue samples are stored in the Genetic Resources Repository of this museum. A few additional tissue samples were taken from vouchers housed at other institutions or were obtained through collaborators without vouchers being retained (Table 5 for details).

We examined 284 specimens morphologically and 208 were used for molecular analyses. These specimens were collected across the entire known range of *H. impatiens*: Mediterranean Sea, Caribbean Sea, Red Sea, tropical Indian and Pacific Oceans (Table 5).

2.2 DNA extraction and amplification

DNA was extracted using either Invitrogen[®] DNAzol[®] or Omega Bio-Tek[™] E.Z.N.A[®] Mollusc DNA kit following manufacturer recommendations. DNA was most often extracted from tentacles, sometimes from gonads, longitudinal muscles or body wall. When possible, the extractions were performed on tissue sampled in the field.

In this study we amplified the mitochondrial markers COI, 16S, ATP6 and the nuclear markers histone 3 (H3a), 18S, ITS1-5.8S-ITS2, c0036 and c0775 (Table 1). ATP6 primers were developed in this study based on sequences available for this locus in GenBank. c0036 and c0775 are anonymous markers developed from a 454 run on genomic DNA from *Holothuria edulis*, and additional details will be provided in a future study. Briefly, these loci were identified using blastx on the contigs obtained from the 454 run against the predicted proteins for the sea urchin *Strongylocentrotus purpuratus* genome. c0036 matches a portion of the gene encoding for an histone H3-like centromeric protein A-like (locus XP_003723879), with an e-value of 2.10^{-43} . c0775 matches a portion of the gene encoding for the protein SFI1 (locus XP_792620), with an e-value of 3.10^{-16} .

Primers and PCR conditions used are provided in Table 1. Because of the length of ITS1-5.8S-ITS2, we used the additional sequencing primer fm-5.8S-f. The primer fm-ITS-f is the reverse complement sequence of the primer 18S-1708R (WN-1708R in [27]), and fm-ITS-r is the reverse complement of LSUFW1 (LSU D1,D2 fw1: 56-74 in [40]).

We conducted PCR in 25 μ L reactions using either 15.4 μ L of water, 2.5 μ L of Sigma-Aldrich[®] 10X PCR buffer, 2.5 μ L of dNTP, 2 μ L of MgCl₂, 1 μ L of the forward primer, 1 μ L of the reverse primer, and 0.1 μ L of Sigma-Aldrich[®] Jumpstart[™] Taq DNA polymerase or the Promega GoTaq MasterMix following manufacturer recommendations.

Sequencing of PCR products was performed by the Interdisciplinary Center for Biotechnology Research at the University of Florida. Chromatograms were edited using Geneious [41].

Overall, 84 positions (1.14% of the alignment length, and 0.02% of the total number of nucleotide positions in the alignment) contained heterozygous positions. We used the International Union of Pure and Applied Chemistry (IUPAC) nomenclature codes for these positions in the alignment, which are considered as missing data by all the software used in this study.

Table 1: Primers and PCR conditions for the markers used in this study. Temp: annealing temperature, Cycles: number of PCR cycles used to amplify the locus, Ref.: reference used for the primers.

Marker	Primer	Primer Forward (5' – 3') & Primer Reverse (5' – 3')	Temp	Cycles	Ref.
COI	COIceF	ACT GCC CAC GCC CTA GTA ATG ATA TTT TTT ATG GTN ATG CC	42	40	[42]
	COIceR	TCG TGT GTC TAC GTC CAT TCC TAC TGT RAA CAT RTG			
16S	16SAR	CGC CTG TTT ATC AAA AAC AT	52	35	[43]
	16SBR	GCC GGT CTG AAC TCA GAT CAC GT			
ATP6	ATP6f ATP6r	GGA CAA TTT TCC CCA GAC CT GGT GAA GAG GGT GTT GAT GG	42	40	This study
28S	LSUFW1 LSUREV2	AGC GGA GGA AAA GAA ACT A ACG ATC GAT TTG CAC GTC AG	42	40	[40]
ITS1-5.8S-ITS2	fm-ITS-f	AGG TGA ACC TGC AGA TGG ATC A	45	40	This study, [27, 40]
	fm-5.8S-f	CGT CGA TGA AGA ACG CAG YW			
	fm-ITS-r	TAG TTT CTT TTC CTC CGC T			
c0036	c0036f	TAA CGA CGG ATC TCA CGG AG	42	45	This study
	c0036r	AAT AAT GCT GGC GTG ACG TC			
c0775	c0775f	GCT CTT CGT TCA ATT TAT CTC GC	42	40	This study
	c0775r	GGG ATG CAG TTT GTC GAG TG			

2.3 Definitions

In this paper we use three concepts: “putative species”, “evolutionary significant units (ESUs)”, and “species”:

- “Putative species” describes the lineages identified by the GMYC method and follows the terminology used by the authors of the method [37, 38].
- “Evolutionary significant units (ESUs)” are defined as reciprocally monophyletic lineages in COI that are also characterized by another independent defining attribute (e.g., coloration, morphology, geographical distribution, reciprocal monophyly in another marker). We use this concept as defined by Moritz [10].
- The “species” are the lineages supported by the model of species delineation that best explains the data as assessed by Bayes factors estimated from the multispecies coalescent in *BEAST. They provide an indirect assessment of reproductive isolation and the species are assumed to not exchange genes, forming independent distinct lineages.

2.4 Species limits with GMYC

To delineate putative species based on molecular data, we fit the generalized mixed Yule coalescent (GMYC) model ([37, 38, 44]) to the COI sequence data. This approach attempts to detect transitions in the branching rate of an ultrametric tree corresponding to the expected increase in lineage accumulation resulting from the shift between speciation and population-level coalescent events. To determine the location of the threshold, the GMYC model is fit at different nodes along the tree, and the one that provides the best likelihood for the location of the switch is selected. Lineages found beyond this threshold are considered different species.

To obtain the ultrametric trees required by this method, we used BEAST as it provides a powerful statistical framework to interpret the chronological context of molecular variation [45]. Despite some investigations of the effects of priors on the number of species estimated by GMYC ([38, 44, 46]), we here revisit the impact of specifying a strict or a log-normal relaxed clock, as well as the type of tree prior used (coalescent with constant population size [47], coalescent with exponentially growing population size [48], and Yule [49]). The type of clock used will affect branch lengths, it is however difficult to predict *a priori* how the misspecification of this parameter will influence the results. Monaghan et al [38] indicate that using a coalescent constant tree prior is a more conservative approach compared to using a Yule prior, as GMYC uses the coalescent as the null model to explain the branching pattern. Their results validated their predictions and the GMYC method detected more species on trees estimated with a Yule prior. However, these results might be influenced by the nature of the data, in particular by the amount of variation observed in the sequences, and deserve further testing.

To our knowledge few studies have investigated the effect of retaining identical haplotypes in the analysis (but see [46]). In other studies, identical sequences were removed as they were thought to make the task of estimating the transition problematic [38], as the GMYC method cannot accommodate for the null branch lengths associated with identical sequences [44]. Contrary to other programs, BEAST does not assign zero edge length to terminal branches for identical sequences, and thus allows retaining them in the analysis. There are obvious theoretical advantages for retaining identical sequences: BEAST assumes that the sequences represent a random sample of the population when estimating effective population sizes. Removing identical sequences will lead to spuriously high levels

of genetic diversity, in turn leading to overestimating population sizes [45, p.98]. As population sizes directly influence the probability that lineages will coalesce, removing identical sequences will lead to longer branches in the trees, blurring the distinction between interspecific and intraspecific coalescent events. We can therefore predict that removing identical sequences of the analysis will lead to higher number of species detected by GMYC and higher uncertainty, both of which are undesirable for the purpose of the analysis.

We performed the GMYC analyses using only COI sequences as the method was designed to be used on single locus datasets, and we wanted to compare how our conclusions might differ if the entire dataset was considered. COI genealogies were estimated using BEAST 1.8.0 [50] and BEAGLE 2.1 [51]. To determine the optimal partition scheme, we used PartitionFinder [52] on the dataset including all sequences as well as on the dataset limited to distinct haplotypes only. We performed a complete search using each codon position as a partition, and limiting the search to the JC, HKY and GTR models with or without a proportion of invariant sites (+I) and/or gamma-distributed rates of evolution (+G) (12 different models *in toto*). Both datasets (all sequences, only haplotypes) favored to assign each codon position to independent partitions. The partition scheme selected by PartitionFinder led to poor mixing due to over-parametrization in initial test runs. We modified the partition scheme slightly, to use: HKY+I+G, GTR+G and HKY for each codon position respectively for the dataset including all sequences; and HKY+G, HKY+G and HKY for the analyses on unique haplotypes. In all analyses, base frequencies were estimated.

We used a log-normal distribution as the prior for the mean rate of substitution for the log-normal relaxed clock and the strict clock, with a $\log(\text{mean})$ of -4 and a $\log(\text{standard deviation})$ of 1.

As no fossils are known in *Holothuria*, we used the closure of the isthmus of Panama to calibrate the clock. The isthmus of Panama was completely closed about 3 Ma but most species were probably isolated earlier [53]. We chose a conservative estimate to account for the fact that the sister species lineages found across the isthmus are the lineages found in the Galapagos and the Caribbean (the coastal Eastern Pacific lineage is sister to the one found in the Galapagos, Fig. 7). We constrained the age of the node corresponding to the most recent common ancestor of the individuals found in the Galapagos archipelago, the coast of the Eastern Pacific and in the Caribbean using a log-normal prior with a $\log(\text{mean})$ of 1.5, a $\log(\text{standard deviation})$ of 0.75 and an offset of 2.5. This distribution translates into a mean age of divergence of 6.98 millions years ago (Ma), (95% confidence interval of [3.53, 21.99]).

We set the MCMC chain lengths at $5 \cdot 10^7$, sampling every $5 \cdot 10^3$ generations. All analyses were run twice from independent starting points and were checked for convergence. From visual inspection of the values sampled by the chains with Tracer 1.6, we determined that a burnin of 10% was sufficient for all runs. ESS values for all parameters were above 350 in each independent run, and the samples from the posterior from each runs were combined.

The trees sampled from the posterior were summarized with treeannotator using the common ancestor tree method, as this method provides a more accurate estimate of the divergence times from the trees sampled from the posterior compared to the other methods available in treeannotator [54].

These trees were used to fit the single-threshold ([37]) and the multi-threshold [38] GMYC model using the R package splits 1.0-19 [55]. We considered the number of “entities” returned by GMYC as the estimated number of species in our analyses. The confidence interval for the number of species corresponds to the minimum and maximum numbers of species for threshold values found within 2 log-likelihood units of the threshold associated with the highest likelihood.

2.5 Species limits with coloration and geography

To determine whether the putative species delineated with GMYC were biologically sound, we investigated color patterns, geographical distribution and ecology.

Coloration information was obtained from the photographs of the live specimens and from field observations. Ecological and geographical coordinates were retrieved from the collecting information associated with the specimens.

2.6 Species limits with *BEAST

Some of the putative species delineated by GMYC form well defined but shallow clusters, have geographically defined ranges, but cannot be differentiated morphologically. Their ranges overlap (Hawaii, Wpac) or border (RedSea) the widespread ESU1, bringing into question the reproductive isolation of these lineages. To test whether these clusters are reproductively isolated, we compared competing hypotheses of species delineation with *BEAST v 1.8.0 [39] using Bayes factors (BF) estimated by the stepping-stone (SS) and the path sampling (PS) methods [12].

*BEAST infers the species trees from a multi-locus dataset using the multispecies coalescent in a Bayesian framework to account for intraspecies polymorphism and incomplete lineage sorting. This method assumes reproductive isolation (absence of gene flow) among species and requires the assignment of individuals to species *a priori*.

Bayes factors (BF) provide a powerful framework for model selection by comparing marginal likelihoods. The models do not have to be nested and the complexity of the models is directly accounted for by the marginal likelihoods [12]. Kass & Raftery [56] developed guidelines to interpret BF values such that $2 \ln(B_{01})$ (where $B_{01} = \frac{p(Y|M_1)}{p(Y|M_0)}$ with Y is the observed data and M_i the model under consideration) must be > 6 to consider the evidence “strong” for (or against) the null hypothesis, and > 10 to consider it “very strong”. Here, we used PS and SS methods to estimate and compare the marginal likelihoods $p(Y|M)$ of alternative assignments of individuals to species in *BEAST. With this approach, we tested whether we could detect the signature of reproductive isolation among individuals assigned to different putative species by GMYC but that cannot be teased apart based on color patterns or other evidence.

We investigated whether *BEAST favored models where all or some of the following ESUs were considered distinct species: WPac, Hawaii and RedSea, or were panmictic with ESU1 (Table 2). We included ESU3 and *gracilis* in our analysis to allow the estimation of the topology when all individuals for the species tested where attributed to ESU1. The null hypothesis was that all putative species were reproductively isolated.

In these analyses, we selected individuals for which we had at least one mitochondrial marker and one nuclear marker (43 individuals: 9 of ESU1, 7 of Hawaii, 7 of Wpac, 5 of RedSea, 7 of *gracilis*, 8 of ESU3). We used data from the following markers: 16S, COI, ATP6, c0036, c0775, ITS, LSU and H3a; and the same approach as for the phylogeny of the entire complex, using Gblocks to remove ambiguous parts of the alignment in ITS, PartitionFinder to find the most appropriate partition scheme and models of molecular evolution. We unlinked clocks for all markers, used strict molecular clocks, fixing the COI clock at $1\% \text{million years}^{-1}$ which is the rate estimated from the analyses on COI used for the GMYC analyses. The rate for the other clocks were estimated relatively to the COI clock. We ran at least two independent runs for $50 \cdot 10^6$ generations for each group.

For the estimation of the marginal likelihoods, we used the settings recommended by Baele et al [12] with path steps of 100, and sampling along evenly spaced quantiles of a $Beta(0.3, 1)$ distribution, but increased the chain lengths to $3 \cdot 10^6$. We interpreted an absolute difference between the marginal

Table 2: Species groupings used in models of species delineation.

Model	Number of species	ESUs attributed to ESU1
M0	5	–
M1	2	Wpac, Hawaii, RedSea
M2	3	Wpac, RedSea
M3	3	Wpac, Hawaii
M4	4	Wpac
M5	4	Hawaii
M6	4	RedSea

likelihoods estimated with the stepping stone and the path sampling methods higher than 1 as a failure of the estimate to converge. In these cases, we repeated the analysis from a different starting point until the difference between the likelihoods was lower than 1.

To rule out that this approach did not favor models that included more species, we added a “ghost” species in the analysis. This “ghost” species was comprised of individuals that formed a shallow reciprocally monophyletic group in COI within ESU1, but that was not identified as a putative species in any of the sGMYC analyses.

To confirm that COI, which was used to delineate putative species with GMYC, did not drive the differences in marginal likelihoods among the models tested with *BEAST, we repeated some of the analyses omitting COI (models M0 and M4-M6 in Table 2). We also repeated the analyses for these models using only nuclear data to assess the influence of mitochondrial data on the results.

2.7 Phylogeny and divergence time estimation

Sequences obtained for each marker were aligned using MUSCLE [57] with the default settings. All markers led to unambiguous alignments except for ITS. For this locus, we used Gblocks [58] to remove parts that were difficult to align using a minimum length of a block of 5 ($-b4=5$), and the option “all gap positions can be selected” ($-b5=a$). We used the resulting alignment to estimate the phylogenetic relationships and the timing of the divergence among the different lineages of *Holothuria impatiens* using RAxML 8.0.1 [59] and BEAST 2.1.2 [60] (with BEAGLE 2.1.2 [51]).

To determine the optimal partition scheme and models of molecular evolution, we analyzed the alignment using PartitionFinder [52]. We defined the data blocks such that the three codon position for ATP6, COI, H3a, and each of 16S, c0036, c0775, ITS and LSU were individual partitions. We used the greedy algorithm, with linked branch lengths, no user tree, and models of molecular evolution available by default in BEAST (JC69, HKY, GTR, without or with a proportion of invariant sites [+I] and/or Gamma distributed rates across sites [+G]). We selected the best-fit models and partition schemes based on the Bayesian Information Criterion. The best fit model had 9 partitions.

For the BEAST analysis, following some initial testing runs, we modified the partition scheme slightly to improve mixing of the chains as the partition scheme selected by PartitionFinder led to over-parametrization (Table 4).

We used three independent strict molecular clocks (one for each of mitochondrial markers, protein-coding nuclear markers, nuclear ribosomal markers) [61]. The tree prior was set to a Yule process, and a random starting tree. The Markov chains were run for 185.10^6 generations (and sampled every 5.10^3 generations). The analysis was repeated twice from independent starting points.

We used Tracer 1.6 to check that the MCMC chains had reached stationarity, that mixing was ad-

equate, and that the two independent runs were consistent. We ensured that a representative sample of the posterior distributions was sampled for each independent runs, by checking that the ESS values for all parameters were above 200. The samples from the posterior distribution from both runs were combined using logcombiner after removing a 20% burnin, resulting in 29601 states sampled. The ESS values obtained by combining the runs were all above 440. We summarized the trees sampled with treeannotator using the common ancestor tree method [54].

3 Results

3.1 Sequence data

The complete sequence data set was 5966 bp (with 2505 bp from mitochondrial genome, and 3461 bp from the nuclear genome, Table 3). Of the 1220 parsimony-informative sites, 69% were from the mitochondrial loci (Table 3).

Table 3: Characteristics of the loci used for the phylogenetic analyses. N : number of individuals sequenced, K : number of unique sequences, bp: length of the aligned (unaligned) sequences, S : number of segregating sites, S_i : number of parsimony informative sites. The statistics given for ITS are for the ones used in the analysis (i.e., after using Gblock).

	mtDNA			nucDNA				
	16S	COI	ATP6	c0036	c0775	H3a	ITS	LSU
N	94	203	64	70	59	39	61	6
K	87	128	60	57	27	19	58	6
bp	1179 (1117)	673 (655)	653 (653)	637 (624)	282 (282)	334 (334)	1085 (1040)	1123 (1072)
S	479	219	330	140	47	46	419	201
S_i	383	198	265	97	28	5	237	7

We obtained sequence data from 207 individuals covering the entire known geographical range of *Holothuria impatiens*. All of these individuals were sequenced for at least one mitochondrial locus, and 36% were sequenced for at least 1 nuclear locus and 34% for 2 or more (Fig. 11).

3.2 Species limits with GMYC

The maximum-likelihood estimates for the number of putative species varied widely and ranged from 14 to 20 for the single-threshold method (sGMYC), and from 17 to 23 for the multi-threshold method (mGMYC) (Fig 2).

Species estimates from sGMYC were more conservative and with narrower confidence intervals (CI) than the mGMYC approach. The maximum-likelihood estimates for the sGMYC were in most cases at the lower limit of the CI, while the estimates from the mGMYC were at the upper limit. For the analyses performed on all the sequences, the CI for the estimated number of species using sGMYC and mGMYC did not overlap.

On the genealogies estimated with a strict clock, sGMYC was less sensitive to the type of tree prior used, as the estimated number of species was identical for the three priors tested with 16 and 17 estimated species for analyses on all haplotypes and on unique haplotypes respectively. The Yule prior produced equal or more conservative estimates of the number of species compared to the two types of coalescent priors.

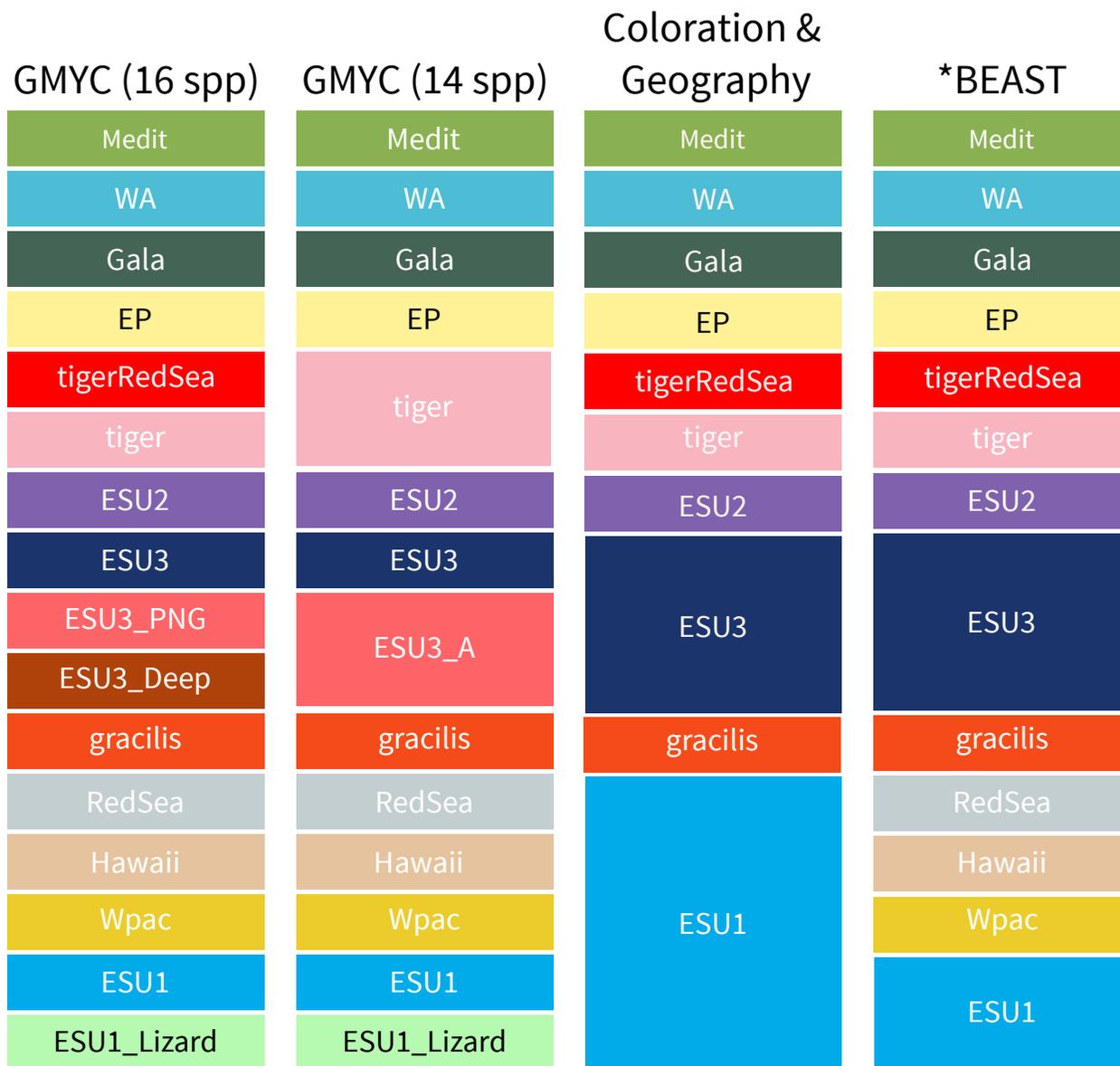


Figure 1: Correspondence between the different criteria used to delineate the species of the “*Holothuria impatiens*” complex. Medit: Mediterranean, WA: Western Atlantic, Gala: Galapagos, EP: Eastern Pacific; PNG: Papua New Guinea; Wpac: Western Pacific.

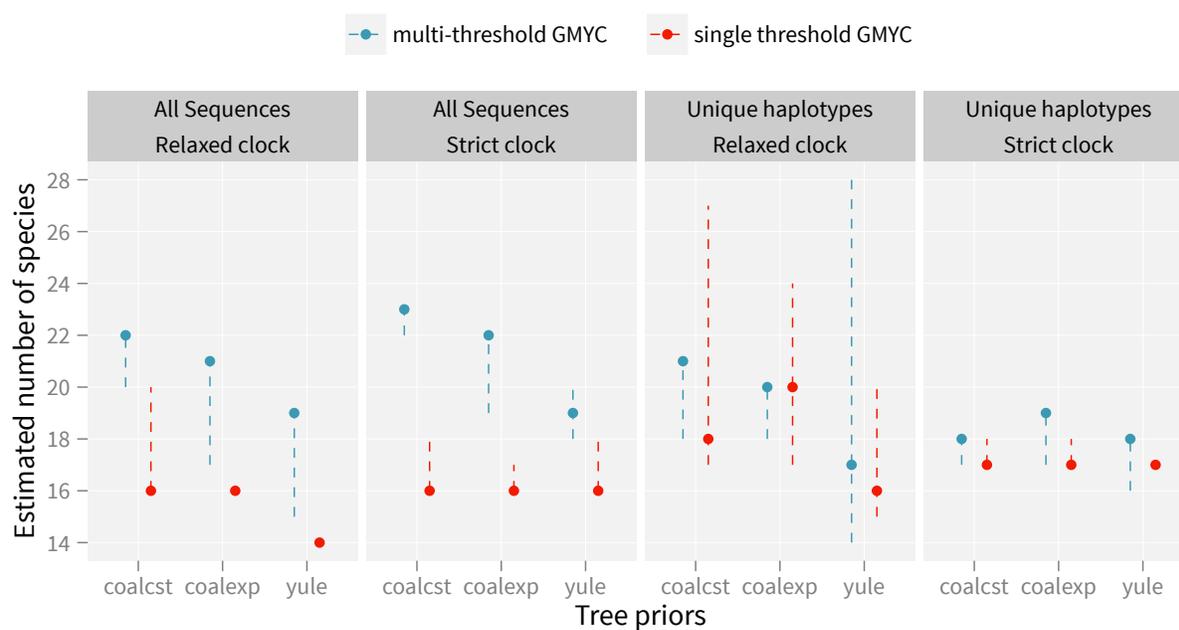


Figure 2: Number of species estimated by single and multi-threshold GMYC methods on COI phylogenies. The dots represent the number of species associated with the node(s) that correspond(s) to the highest likelihood(s) for the location of the shift(s), the dotted lines represent the range for the estimated number of species for nodes within 2 likelihood units of the maximum-likelihood estimate.

With sGMYC, the estimated number of species was more conservative and less sensitive to the priors used to reconstruct the genealogy when all sequences were included.

Overall, the putative species delineated with sGMYC were consistent across the different priors used. For the analyses based on all sequences, the difference between the 14 species estimated with the Yule prior and the 16 species estimated with the other priors was caused by (1) the lumping of *tigerRedSea* and *tiger*; (2) the later time to coalescence for divergent haplotypes related to ESU3 with the Yule prior.

For the analyses based on unique haplotypes and a relaxed clock, with the Yule prior, the coalescence of the haplotypes happened later in the tree leading to lower estimated number of species compared to the coalescent priors. With the relaxed clock, many coalescent events were reconstructed in the vicinity of the threshold leading to broad confidence intervals.

With the strict clock analyses, the additional species delineated between the unique haplotypes and all sequences corresponds to the recognition of both *tiger* and *tigerRedSea*.

For all analyses, at least one putative species was represented by a single sequence. A divergent lineage related to ESU1, collected in Lizard Island, Australia, was assigned to its own species (ESU1_Lizard in Fig. 1). In all analyses (except with the most conservative estimate, i.e., using all sequences, a relaxed clock and a Yule prior), two sequences related to ESU3 (from the Ryukyus, Japan, and from Papua New Guinea), were each assigned to their own species (ESU3_Deep and ESU3_PNG respectively in Fig. 1).

3.3 Species limits with coloration and geography

Members of the “*H. impatiens*” complex are characterized by two dominant colors. The dark coloration is typically solid towards the anterior end, forms bands in the middle of the body, and then spots that become rare towards the posterior end. This pattern varies considerably across species and across individuals. The dark color is typically brown but varies in lightness and can take a red or purple tint. The lighter color is typically gray but varies in lightness from light brown to yellow or even white. The light-colored areas typically harbor a “salt-and-pepper” pattern with small dark spots on the light background. Despite these common characteristics, the combination of color patterns and geographical distribution allows us to distinguish unambiguously at least ten species (Fig. 1, 3, 4).

Medit, *WA*, *Gala*, *EP*, *ESU1* have similar color patterns but subtle differences, and the restriction of their ranges to an oceanic basin or an archipelago allow teasing them apart. *ESU2* is the only species with bright yellow papillae and tentacles (Figure 4). *tiger* and *tigerRedSea* are also very distinctive with sparse, dark, tubercles that barely raise from the body wall, giving them a smoother appearance than the other species. They are also much larger than the other species and specimens reach regularly more than 30 cm. *gracilis* has very large, conical, typically red tubercles, and a very distinctive “gritty” texture. *ESU3* has elongated, pyramidal tubercles with white papillae. However, *ESU1*, *Hawaii* and *Wpac* have broadly overlapping distributions and are morphologically indistinguishable (Figure 3).

3.4 Species limits with *BEAST

Both path sampling (PS) and stepping-stone sampling (SS) gave almost identical estimates of the marginal likelihoods (Fig 6). The values reported for the Bayes factors (BF) are the means of the two independent replicates. Here, the more negative the BF, the stronger the statistical evidence in favor of the null hypothesis specifying that *Wpac*, *Hawaii*, *RedSea* and *ESU1* are all reproductively isolated species.

The model that included putative species identified by GMYC represented by more than one in-

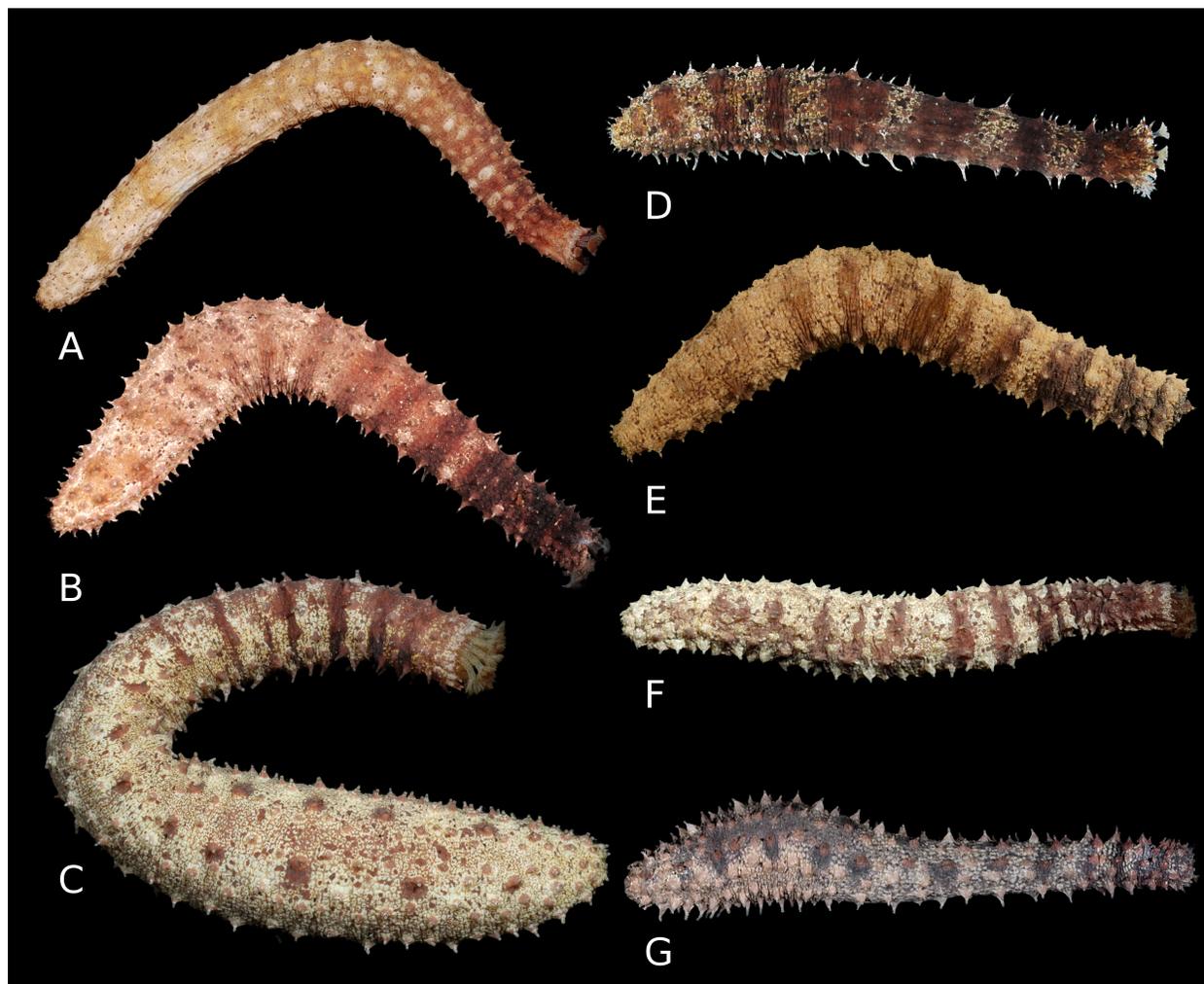


Figure 3: Dorsal view of members of the “*Holothuria impatiens*” complex. A. ESU1, Réunion Island, UF6487; B. ESU1, Réunion Island, UF6588; C. ESU1, Majuro, Marshall Islands, UF6748; D. ESU1, Okinawa, Japan, UF10950; E. RedSea, Djibouti, UF12070; F. Wpac, Majuro Marshall Islands, UF6771; G. Hawaii, French Frigate Shoal, Hawaiian archipelago, UF6243.



Figure 4: Dorsal view of members of the “*Holothuria impatiens*” complex. A. ESU2, Nosy Bé, Madagascar, UF7287 (juvenile); B. ESU2, Guam, UF6729; C. ESU2, Lizard Island, Australia, UF8355; D. ESU2, Okinawa, Japan, UF11012; E. *gracilis*, Lizard Island, Australia, UF8288; F. *tigerRedSea*, Djibouti, UF11956; G. *tiger*, Kosrae, Federated States of Micronesia, UF6929; H. ESU3, Nosy Bé, Madagascar, UF7469.

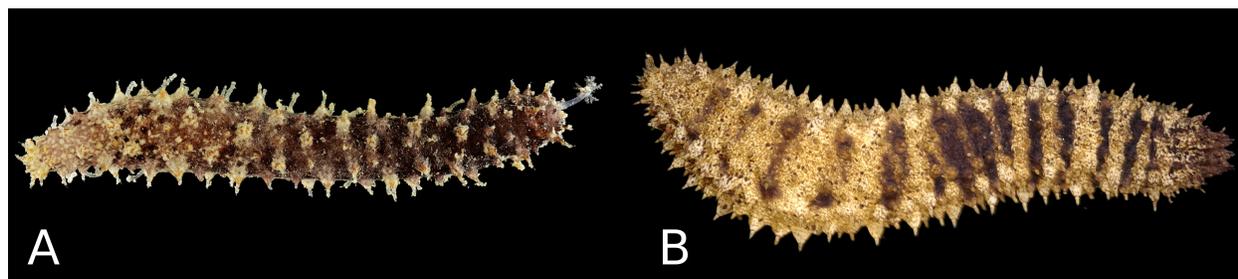


Figure 5: Dorsal view of members of the “*Holothuria impatiens*” complex. A. WA, St Martin, UF11790; B. EP, Panama (no Voucher).

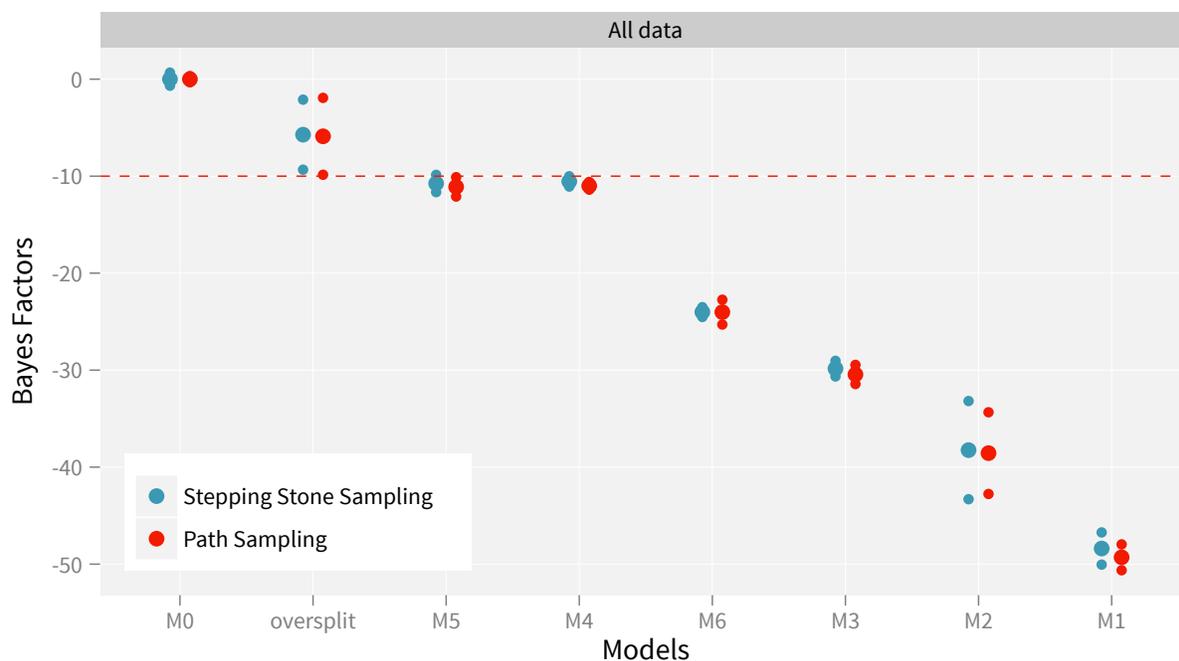


Figure 6: Bayes factors comparing the null hypothesis (M0: ESU1, RedSea, Wpac, Hawaii are all different species) to alternate models of species assignment: additional “ghost” species added (oversplit), no Hawaii (M5), no Wpac (M4), no RedSea (M6), no Wpac and no Hawaii (M3), no noWpac and no RedSea (M2), all individuals attributed to ESU1 (M1), see also Table 2. The small dots indicate the values obtained for independent replicated estimations, the large dots the mean of these values. Estimates obtained using the stepping-stone sampling method (on the left in blue) are consistent with the estimates obtained using the path sampling method (on the right in red). The horizontal dashed red line represents the value considered as “very strong evidence” in favor of the null hypothesis according to Kass & Raftery [56].

dividual, that do not differ morphologically (Hawaii, Wpac and ESU1) had the highest marginal likelihood (Figure 6). The Bayes factors for the models that considered Hawaii, Wpac and RedSea as being panmictic with ESU1 lent “very strong” support in favor of the null hypothesis (BF = -10.8, -10.5, and -24 respectively). The model with an additional “ghost” species also indicated a poorer fit (BF = -5.7), indicating that this approach did not favor a model that included more species. The model with a random assignment of the species had the worst fit (BF = -243.5).

Similar results were obtained when COI was omitted. The differences in likelihood were however smaller leading to $-10 < \text{BF} < -6$ for models that considered Hawaii (BF = -8.2) and Wpac (BF = -9.4) as panmictic with ESU1 indicating “strong” support in favor of the null hypothesis. The BF was still below -10 for the model that considered RedSea as panmictic with ESU1 (BF = -19.9).

Analyses conducted on nuclear data alone led to low BF that do not support the null model (BF = -0.2 for no Hawaii, and BF = -4.9 for no RedSea). For the model that considered Wpac as the same species as ESU1, the BF was positive suggesting a better fit to the data than the null hypothesis, but below the threshold to consider the evidence “strong” (BF = 2.8).

3.5 Phylogenetic relationships among the ESUs

The phylogenetic relationships recovered with BEAST and RAxML of the species included in the “*H. impatiens*” complex on the concatenated alignment were identical (Fig. 7 and Fig. 12). The topology was highly supported, and the nodes corresponding to the most recent common ancestors of the ESUs had high posterior probabilities (> 0.99) and bootstrap values (10 out of 13 ESUs > 0.9 , others > 0.8) for the Bayesian and maximum-likelihood tree respectively.

Two main clades can be distinguished that diverged approximately 10 Ma (Fig. 7). The first clade (Medit, WA, EP, Gala, ESU2, tiger, tigerRedSea) is circumtropical and is found in the Mediterranean Sea, the Caribbean basin, the Eastern-Pacific and across the Indo-West Pacific, including the Red Sea. The other clade (ESU1, Hawaii, Wpac, ESU3, gracilis) is restricted to the Indo-West Pacific (including the Red Sea).

The geographical ranges of the species vary considerably. Some are restricted to an ocean basin (Medit, WA, RedSea, tigerRedSea) or an archipelago (Hawaii, Gala) but others have ranges that encompass large sections of the Indo-West Pacific (ESU1, ESU2, ESU3). This diversity of distributions results in the co-occurrence of several species of the complex across most of the Indo-West Pacific, and culminates in the Philippines where at least 5 species are found. More generally, at least 3 species are co-occurring in the Northern part of the Western Pacific (the Philippines, Micronesia, Hawaiian archipelago).

Overall, species found in the Indo-Pacific have overlapping ranges, even for species that diverged recently. For instance, Wpac and Hawaii co-occur with ESU1 and diverged most recently (1.8 Ma and 2.2 Ma respectively, Fig. 7 and Fig. 9).

4 Discussion

4.1 Species limits in the “*Holothuria impatiens*” complex

Species delineation is a complex issue and the conclusions will depend of the characters and methods chosen. Here, using complementary approaches, combining discovery (GMYC) and testing methods (*BEAST), we unraveled at least 13 species within the complex “*Holothuria impatiens*” supported by multiple lines of evidence.

The discovery methods, based on genetic differentiation in the mitochondrial locus COI, found

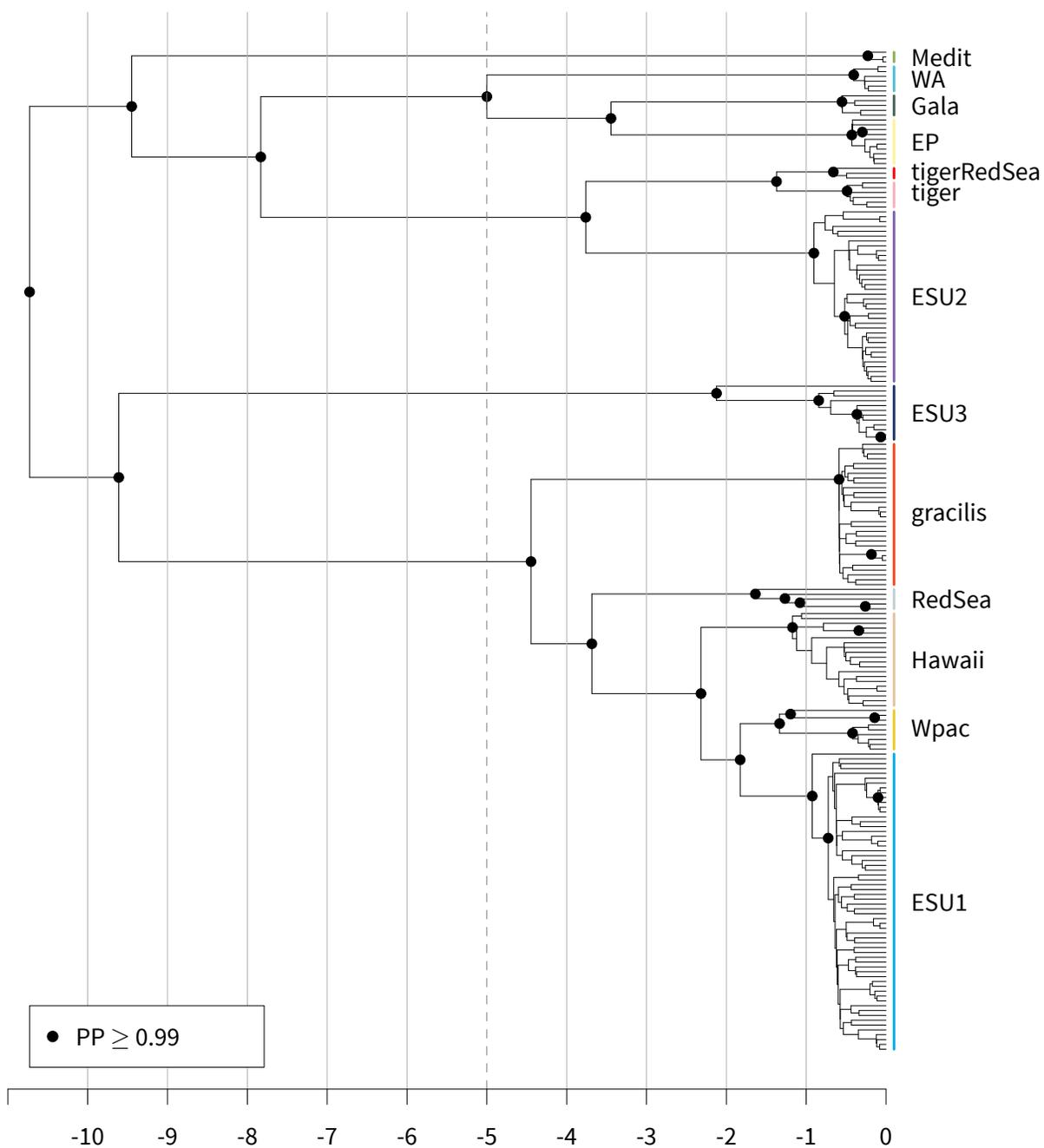


Figure 7: Bayesian phylogenetic tree of the *Holothuria impatiens* complex. Vertical bars along the tips of the tree show the different ESUs found in the complex. Axis on the bottom in million years.

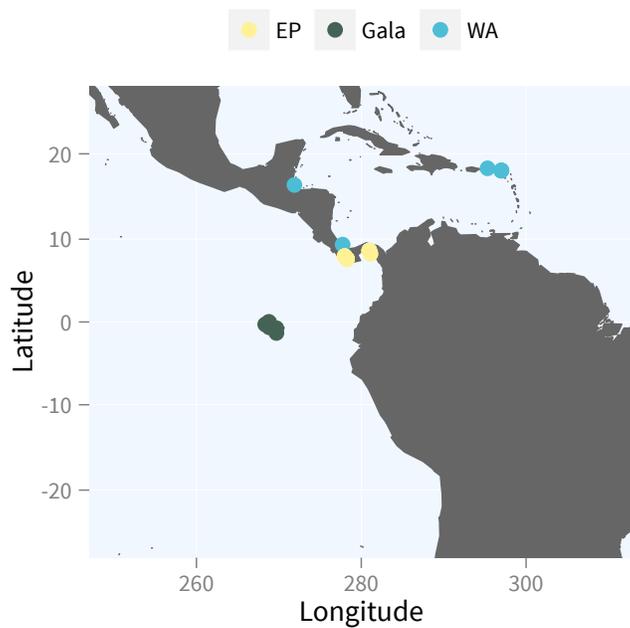


Figure 8: Distribution maps of the ESUs in Eastern Pacific and Caribbean (WA, Gala, EP)

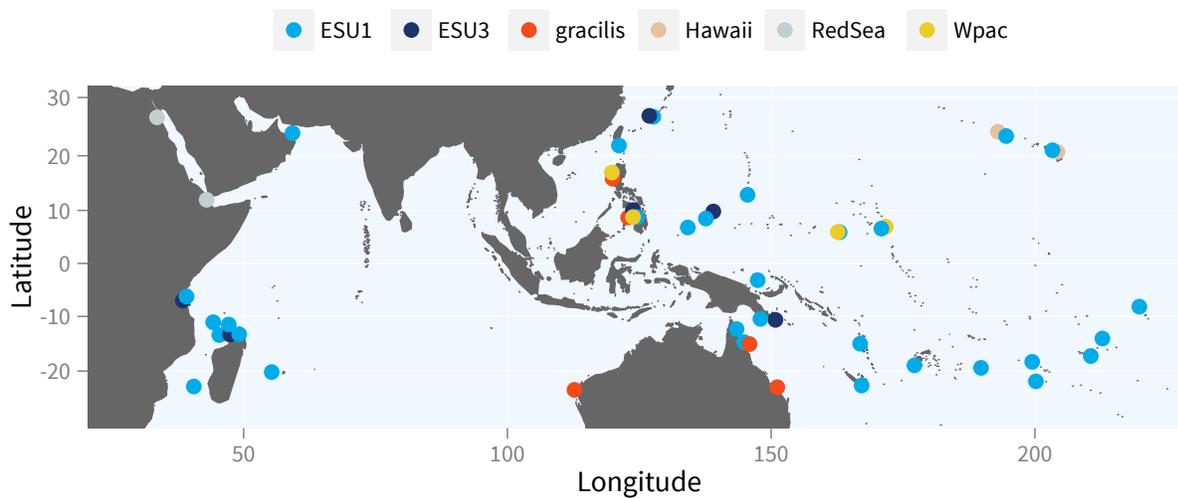


Figure 9: Distribution map for ESU1, ESU3, gracilis, Hawaii, RedSea, Wpac.

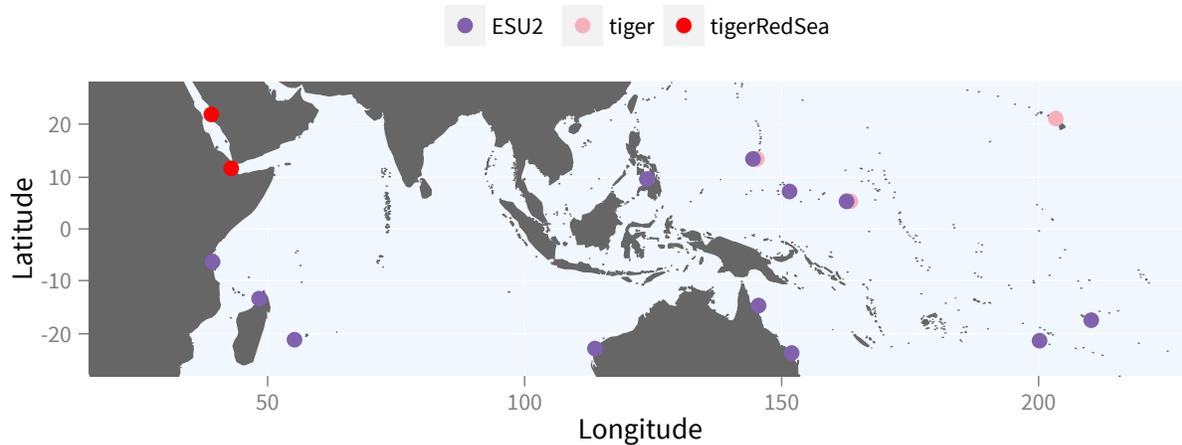


Figure 10: Distribution map for ESU2, tiger, and tigerRedSea.

putative species that require additional material to be evaluated. In particular, three individuals characterized by divergent haplotypes, related to ESU1 for one, and to ESU3 for the others, were consistently recovered as separate species. In both cases, a single individual was available, making it impossible to assess reproductive isolation with *BEAST. The individual related to ESU1 (UF8191) had the typical color pattern of ESU1, with which it co-occurs and no line of evidence seem to indicate that it could be a different species. The same situation occurs with one of the individuals related to ESU3 (N0027) that cannot be differentiated from other individuals from ESU3. The other individual related to ESU3 (RUMF-ZE-0074) is small, and was dredged from 32 m in a soft bottom bay of Kume Island, Ryukyus, Japan, a different habitat from the typically reef-associated ESU3. The distinct ecology and deeply divergent sequences characterizing this individual suggest it could represent a different species but additional material is needed to assess this hypothesis.

The combination of geography and differences in color patterns, in retrospect, allow the differentiation of 10 species in the complex (Figure 1). Taxonomic studies of most marine invertebrates have relied on preserved specimens. Preservation can render delicate morphological characters, including coloration, difficult or impossible to see, and are frequently lost or fade with time. Because hard parts are easily preserved and their form is generally not impacted by preservation, taxonomists often favor them to differentiate species. For instance, in sea cucumbers, species-level taxonomy has almost entirely relied on the shape of ossicles, microscopic calcareous secretions found in many tissues. Because ossicles show substantial diversity and variability, whilst variation in the simple anatomy of these animals is limited at lower taxonomic levels. Even with ossicles, until recent decades, workers have mostly focused on those found in the body wall, ignoring ossicles in internal structures, and have paid little attention to intraspecific variation, typically illustrating the ossicle assemblage of a single individual. This, in combination with the fact that several of these species co-occur and can share the

same habitat, has contributed to the failure to recognize this diversity, and differentiate species in this complex.

Three additional species, that do not seem to be distinguishable morphologically, and that co-occur were validated by the multispecies coalescent statistical framework.

4.2 Species limits with GMYC

Despite being widely used to delineate species, the sensitivity of the GMYC method to the factors affecting the shape of the tree used have not been investigated thoroughly. Tree shape can be affected by biological factors or by the type of analysis used to build the tree. Simulated data have been used to test the influence of diversification rates, effective population sizes, and migration rates, on the accuracy and precision of GMYC estimates [62, 63]. Some of the effects of the methodological choices have been evaluated by the authors of the method [37, 38, 44] and by others [46], but the conclusions might be difficult to generalize as they depend in part on the nature of the dataset. Other species delineation methods using sequence data are also available (e.g., RESL [64], ABGD [65], bPTP [66], bGMYC [67]), and it would be interesting to investigate how they perform compared to the results obtained here.

The multiple-threshold method (mGMYC) led to higher estimates of putative species, and was less accurate than the single-threshold method (sGMYC). We did not find any other line of evidence that could corroborate the delineation proposed by this approach. In particular, some of the putative species proposed by mGMYC would split lineages that were otherwise highly supported by the multi-locus phylogeny. The overall lower accuracy of mGMYC was also observed by the authors of the method using a simulated dataset. They however found that the 95% confidence set for the estimated number of putative species between sGMYC and mGMYC were overlapping [44]. We observed this pattern on the analyses using only unique haplotypes but not in the analysis that included all sequences.

On our dataset, when analyzing all sequences, the number of putative species was consistently 16, except for the relaxed clock with the Yule prior in which case it was 14. The method is therefore relatively robust to prior specification on our dataset. The 16 species delineation is the most realistic as it recovers morphologically differentiated species that were not considered distinct in the 14 species hypothesis (*tiger* and *tigerRedSea*). Additionally, sGMYC recovered more putative species than we can confidently recognize, since they were represented by single individuals. This is a common pitfall of single-locus species delineation methods, where the broad geographical sampling of many individuals will reveal unique, divergent haplotypes that may not represent different species. However, in our analysis, it is worth noting that all our recognized species were apparently reciprocally monophyletic making the GMYC approach suitable.

The combination of priors used in our study led to overestimating the number of species when it was tested by the authors of the method [38]. On the other hand, Talavera et al [46] did not find any notable differences using trees estimated with different sets of priors. Our dataset differs from these studies by including a younger radiation, less species, and more individuals per species.

Talavera et al [46] did not find that using all haplotypes or only the unique haplotypes affected the number of species estimated with the GMYC method. Here, we confirmed the theoretical expectation that removing the duplicated sequences overestimated the number of species and decreased the precision of the estimates when the tree was estimated with BEAST. Removing identical sequences in the analysis overestimates the effective population size parameters that BEAST uses. In turn, these overestimated sizes spuriously increase the time to coalescence for the lineages, making the estimation of the inflection point between species- and population-level coalescent events earlier in the tree, and

more difficult to detect. This bias leads to oversplitting and decreases accuracy. Investigating how many individuals need to be sampled to obtain correct estimates would be worth pursuing.

GMYC was developed with the intent of automating the estimation of the number of species from large-scale single-locus sampling efforts, particularly from environmental samples. In this context, a reasonable amount of inaccuracy or imprecision from the true number of species may be acceptable. The advantage of the method, namely providing a rapid and objective assessment of the number of species, outweighs the possible deviations from the true value. However, in the context of a taxonomic study where the goal is to identify species limits among a few hundred individuals, the factors affecting tree shape need to be considered, and additional lines of evidence need to be included to validate the putative species delineated [68, 63].

4.3 Species limits with *BEAST

The statistical framework offered by *BEAST that compares alternative models of species assignment seems a promising venue to investigate species limits, when other lines of evidence are equivocal (e.g., [36, 35]). The requirement to assign specimens to species *a priori* can however be challenging. When morphological evidence is scarce, and deeply divergent lineages are recovered with genetic data, circularity is introduced when the same data is used to recognize species, and to provide statistical support for these species. Here, COI was used to assign species to individuals for putative species that could not be differentiated morphologically.

The confounding effects of using COI to formulate the species hypotheses and to test species limits can stem from: (1) the information contained in the nucleotides forming the locus, and (2) from the absence of recombination in mitochondrial DNA. We attempted to evaluate the relative contribution of these confounding effects by conducting the analyses without COI and without mitochondrial respectively. The results for the analyses conducted without COI were qualitatively similar to the analyses performed with this locus, but the statistical evidence in favor of the hypothesis considering these closely related lineages as different species, was weaker. There was no statistical evidence supporting this hypothesis when the analysis was conducted on nuclear data alone, owing to the low differentiation in these loci for the putative species tested by the models. These results suggest that the remaining of the mitochondrial loci used in our study also favor the null hypothesis, but additional independent nuclear loci with enough differentiation to tease out these species are needed.

Another potential issue with assigning species to individuals based on their COI sequences, is that they may not reflect species limits because of introgression or incomplete lineage sorting. Recently diverged species might still be poly- or paraphyletic in their COI genealogy making species assignment inaccurate. Inaccurate species assignment can potentially be detected from the population sizes estimated by *BEAST that will be unusually high [69, 45]. This would however require to analyze more loci than in the present study to obtain accurate estimates of population sizes [39, 70]. Determining how the Bayes factors will behave if the null hypothesis is misspecified remains to be investigated.

4.4 Species limits and coloration

Before the confirmation by molecular methods that differences in coloration were relevant to differentiate closely related species, marine invertebrate taxonomists have been reluctant to consider species defined solely by differences in coloration [5]. Although variation in coloration within “*H. impatiens*” has been noticed by previous workers, and some unusual color morphs were described as sub-species by Clark [32, 71], the focus on ossicle shape to distinguish sea cucumber species has led to an underestimation of the diversity in this group.

The purpose of coloration in sea cucumbers has not been explored but since species can be distinguished based on their color patterns, natural selection may play a role in its evolution. Color patterns have been documented to be diagnostic of species limits in crustaceans [7], nemerteans [5], fish [72]. In some of these cases, the association between color patterns and species limits is maintained by assortative mating (fish [72], crabs [73, 74]). Given that most sea cucumbers inhabit cryptic habitats and do not possess image-forming eyes, the mechanisms maintaining a tight association between coloration and species identity needs to be investigated.

While some species of "*H. impatiens*" harbor slight variations of the typical color pattern characterizing the complex, others deviate from it and are polymorphic. For instance, ESU2 has two color morphs: a common color pattern that occur throughout the range of the species, and one restricted to the Western Indian Ocean (Nosy Bé, Madagascar and Réunion Island). Both *gracilis* (alternate morph in the southern part of the Great Barrier Reef) and ESU1 (alternate morph found in Lizard Island, Australia; Hawaii; and Guam) are also polymorphic. Interestingly, these alternate color morphs are similar for the three species (uniform chocolate brown background with white to yellowish tubercles), and could result from allelic polymorphism shared across species in genes involved in the pigmentation pathways (as in albinism or melanism), or from convergent selection. These two hypotheses are not mutually exclusive. Furthermore, since coloration seems indicative of species limits, these alternate color morphs could also represent additional cryptic lineages.

Even though, there is no genetic differentiation in the markers used in this study to distinguish individuals exhibiting these alternate color patterns, studies in fish indicate that color differentiation between incipient species might precede any genetic signal (e.g., [72, 75]). Additional genetic data (microsatellites, SNPs) can test whether they represent distinct evolutionary lineages.

4.5 Geographical distributions

The geographical ranges of the species vary considerably, and a diversity of processes seem to best explain their present distributions. Interestingly no speciation along a continental margin is observed in the complex.

The close phylogenetic relationship of WA with EP and Ga1a indicates that the closure of the isthmus of Panama led to the isolation of the species in the two basins.

The estimated ages of *Medit*, and the two Red Sea species are older (8.4-10.5 My, 1.1-1.7 My and 3.2-4.1 My respectively) than the last salinity crises characterizing these seas (*c.* 5 My for the Mediterranean [76] and potentially as early as *c.* 19,000 years ago for the Red Sea). This, in combination with the occurrence of these species outside their basins (*Medit* is known from Portugal and the Canaries; and both Red Sea species from Djibouti) indicate that these species invaded these seas recently and found refugia at the periphery of these basins during the salinity crises [77]. It is also interesting to note that the age of divergence for the two species from the Red Sea do not coincide suggesting that different events were responsible for the geographic isolation of these populations.

tiger and *tigerRedSea* are nocturnal species found on the reef slope. They are very sensitive to light, and retract quickly, deep into the reef matrix if a diver shines light on them. These species are known from a handful of individuals, and it is possible that their ranges are larger than what is reported here.

For species with wide ranges the causes of population divergence are less obvious, and the current distributions may not reflect species distributions at the time of speciation. The two most widespread species (ESU1 and ESU2) are sympatric or parapatric with their closely related species, and both form the terminal clades in their respective radiations. This pattern suggests that these species were the

sequential source of the peripheral species [7].

The lack of genetic differentiation between the Indian and Pacific Oceans for the species of the complex that occur on each sides of the Indo-Pacific barrier (IPB) is noteworthy. The IPB results from the restricted seaway between the Sunda and the Sahul shelves. During glaciation events, when sea levels were 100+ m below present level, only a narrow channel connected Indian and Pacific Oceans. In a recent literature survey, 15 out of 18 species of fish and invertebrate investigated showed genetic differentiation across the IPB associated with these low stands events [78]. Additionally, the genetic diversity of ESU1 is low: among the 61 individuals sequenced, we found a total of 18 haplotypes, with the 3 most common haplotypes found in 40 individuals. Finally, both Marquesas and Oman, characterized by distinct ecological conditions from the rest of the Indo-Pacific hypothesized to increase the speed and probability of divergence, harbor the most widespread haplotype suggesting recent colonization of these peripheral areas to ESU1's range. Together, these lines of evidence suggest a recent geographical expansion for the widespread species in the complex, but more analyses to confirm this hypothesis are needed.

Differences in the geographical ranges for some of the species might be explained by differences in habitat preferences. For instance, tiger is only found in the oligotrophic shallow waters of Micronesia and Hawaii, while ESU3 and *gracilis* appear restricted to continental areas. For instance, ESU3 found in Madagascar and Tanzania, was not encountered in the oceanic Mayotte or Scattered islands. We lack data to evaluate the role played by the ecology of the species in the diversification process and the co-existence of multiple species in sympatry.

4.6 Rates of secondary sympatry

Local diversity results from the accumulation of species that are reproductively isolated. Because reproductive isolation is most commonly initiated in allopatry [79], understanding how quickly species can be found in sympatry after diverging in geographical isolation is key to gain insights into the temporal dynamics of local diversity.

The restriction of marine invertebrates to “oceanic” and “continental” habitats has been documented for a range of organisms (e.g., land snails [80], marine snails [81, 82, 83], hermit crabs [7]), and a variety of biotic and abiotic factors may explain this pattern (reviewed in [7]). Interestingly, clades associated with continental habitats are characterized by much faster rates of secondary sympatry than species associated with insular habitats in both vertebrates (e.g., [84, 85]), and invertebrates (e.g., [86, 7, 83]). As a result, closely related species typically show strict allopatric distributions in oceanic setting (e.g., as in [86]) but often occur in sympatry along continental margins (e.g., [83]). Differences in rates of secondary sympatry vary across taxa but are faster in continental settings. For instance, in the wrasse *Anampes* sympatric sister species in continental setting diverged 600 ka ago, while the most closely related sympatric species in oceanic setting shared a common ancestor more than 10 Ma ago [85]. In the turbinid *Lunella*, none of the 7 lineages inhabiting oceanic islands that have diverged more than 15 Ma occur in sympatry while sister species found along the coastline of Oman have diverged 5 Ma [83].

The “*Holothuria impatiens*” complex shows a strikingly different pattern, and ESU1, Hawaii and Wpac are, to our knowledge, the fastest documented examples of speciation for a broadcast spawner invertebrate in an oceanic setting (ESU1 and Hawaii diverged less than 2 Ma, and ESU1 and Wpac about 2.5 Ma). Thus, species have been able to evolve reproductive isolation rapidly, and differ enough ecologically to allow for co-existence. In other words, the temporal dynamics of diversification in some oceanic species of the “*Holothuria impatiens*” complex is similar to what is observed for species

occurring in continental settings.

Gamete recognition proteins (GRPs) play an important role in driving reproductive isolation in many free spawning organisms, such as sea urchins [87, 88] and gastropods [89]. For instance, strong positive selection has been detected in the GRPs between closely related species of sea urchins in sympatry but not in allopatry [90], suggesting the role of these proteins in maintaining species limits. GRPs have not yet been identified in sea cucumbers but bindin is known from sea urchins [88] and sea stars [91] which indicates that it is plesiomorphic in the clade that sea cucumbers emerged from. They could explain how species in the “*H. impatiens*” complex acquired reproductive isolation quickly.

Hellberg [92] proposed that climatic fluctuations isolate populations temporarily leading to speciation (transient allopatry), and as climatic conditions change, species shift their ranges. In continental settings, these shifts will lead to species coexistence as their distributions are constrained by the one dimensionality of the coastline. In insular conditions, however, these shifts in species distributions could be accompanied with the colonization of new islands and species could remain allopatric. Another difference is that in the continental setting the environmental conditions will vary continuously along the coast. Therefore, if species are adapted to different temperatures, the temperature gradient along the coast will allow species to overlap. However, in an insular setting, the absence of available habitat between the islands restricts the distribution of the species. These hypotheses could explain why most species remain allopatric for long periods of time and are restricted to archipelagos (as observed for Hawaii and Gala): rare migrants fail to colonize as local adaptation to environmental conditions render them maladapted to new islands. This model implies niche conservatism: species are narrowly adapted to a particular environment. The predictions of this model would suggest that ESU1 has broadened its niche to allow the colonization of the range of Hawaii and Wpac.

4.7 Future directions

A shortcoming of the approach used in this study is that the results might be strongly influenced by the loci that harbor the most genetic differentiation, here the mitochondrial data. A recent extension of these methods, using single-nucleotide polymorphisms across the genome [93] would allow the confirmation of the species delineated in this study.

5 Conclusions

This study showed that the widespread, common and well known sea cucumber, *Holothuria impatiens* is actually a complex of at least 13 species. This estimate is conservative as additional lineages that could not be fully evaluated were also unraveled.

The multispecies coalescent framework allows the investigation of species limits in situations where evidence might have been inconclusive in the past. It is now possible to investigate species limits in groups that show some level of genetic differentiation but no morphological differences. The re-evaluation of species limits, and a better accuracy in the estimation of the divergence times, may improve our understanding of the speciation process by revealing new patterns and generating new hypotheses. Here, we showed that some species in this complex are characterized by the most rapid rate of secondary sympatry documented for a broadcast spawner in an oceanic setting. These results would need to be confirmed with genetic data that capture a larger fraction of the genome.

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6 Supplementary materials

Table 4: Partition table. The subscript number corresponds to the to the codon position in the alignment. The “real” codon position is offset by 2 for COI, H3a and ATP6 (i.e., COI₁ is 3rd codon position)

Partition	Model	Loci
1	GTR+I+G	16S, ATP6 ₂
2	HKY+I+G	ATP6 ₁ , COI ₁
3	HKY+I+G	ATP6 ₃ , c0775
4	GTR+I+G	c0036
5	GTR+I+G	COI ₂ , H3a ₂
6	HKY+I	COI ₃
7	HKY	H3a ₁ , LSU
8	JC	H3a ₃
9	HKY+G	ITS

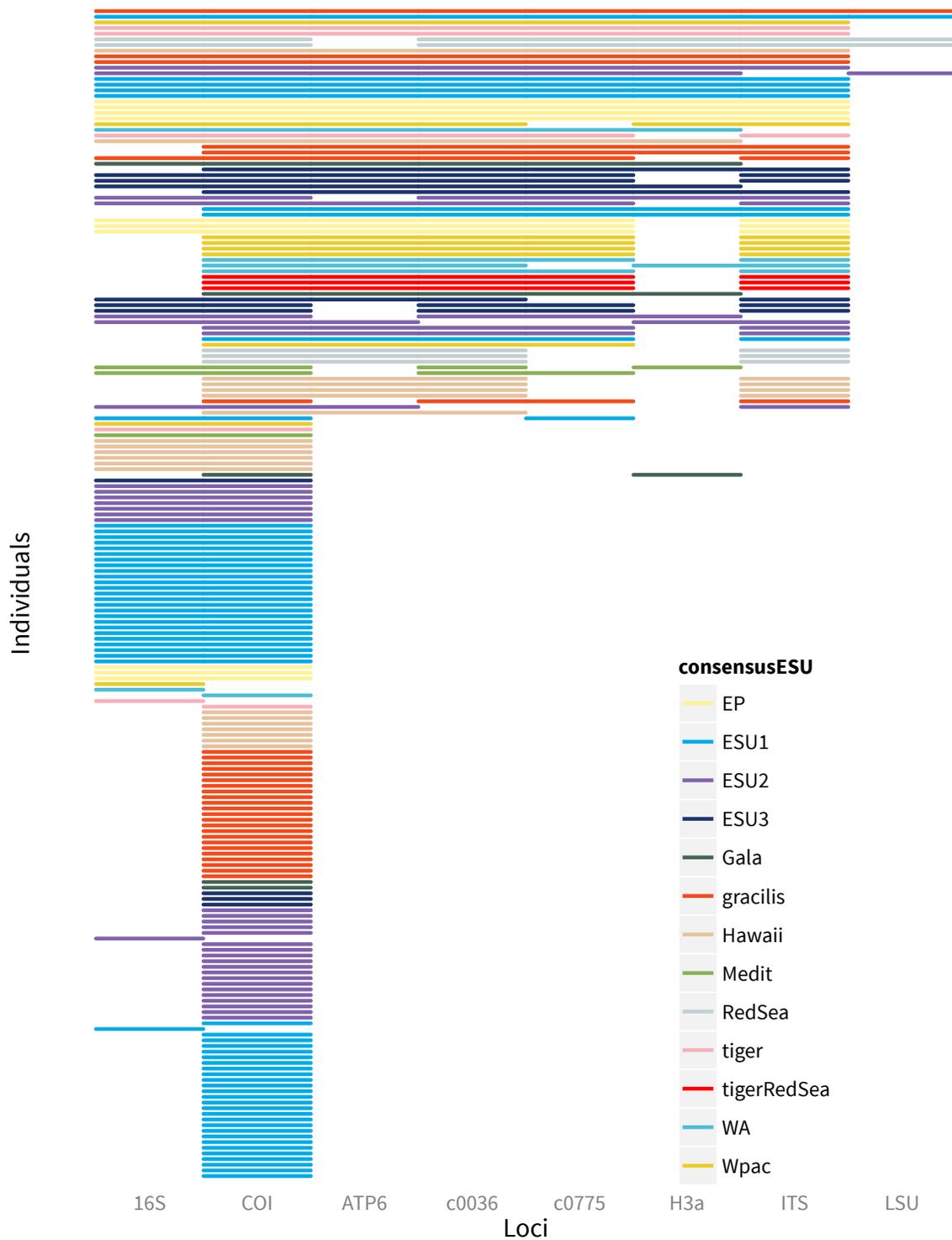


Figure 11: Representation of the loci sequenced for each individual included in the analyses.

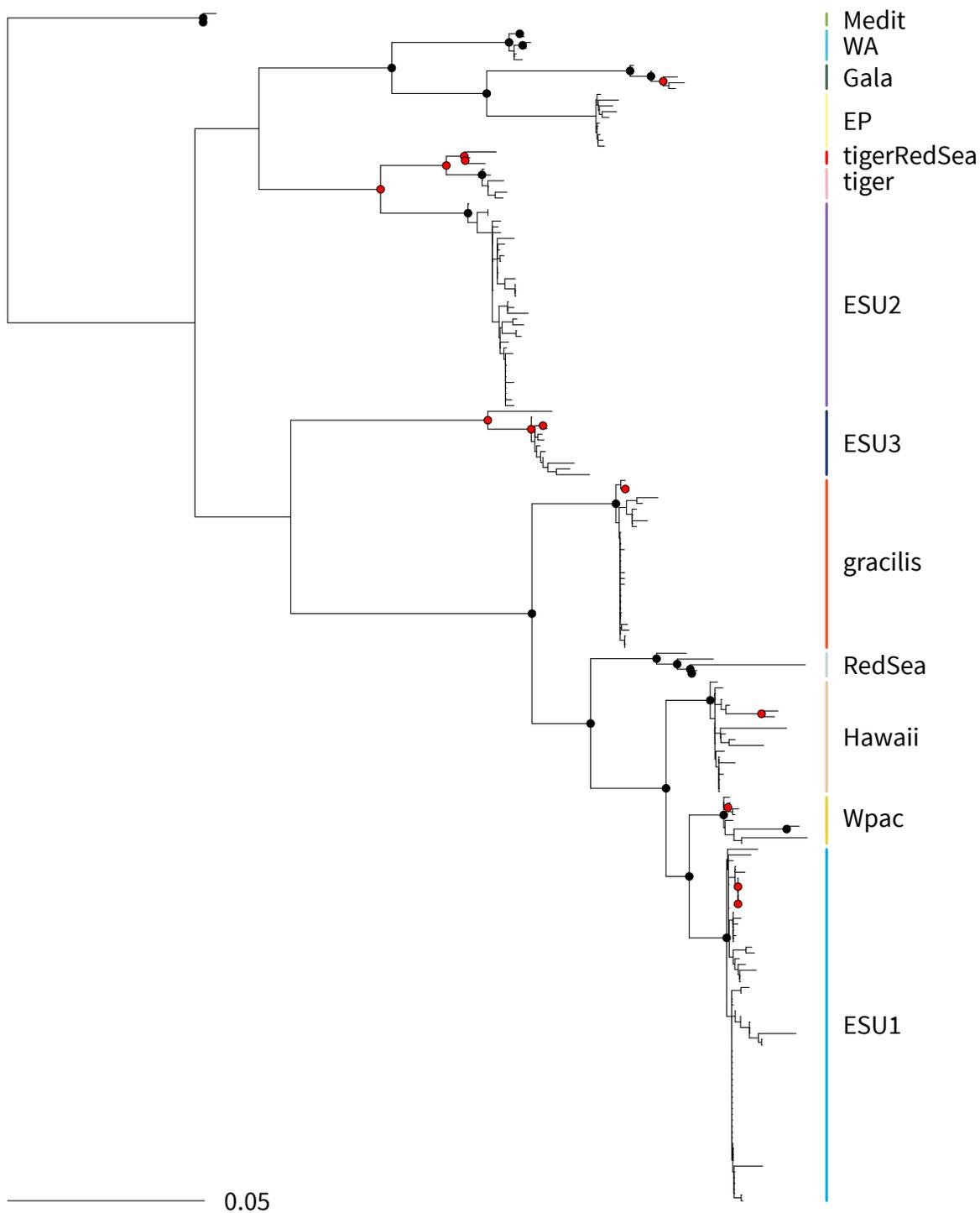


Figure 12: Maximum-likelihood phylogeny for all concatenated loci estimated using RAxML with a GTR+G model for each partition. Black circles represent bootstrap values ≥ 90 , red circles bootstrap values ≥ 80 . Colored vertical bars represent consensus ESUs.

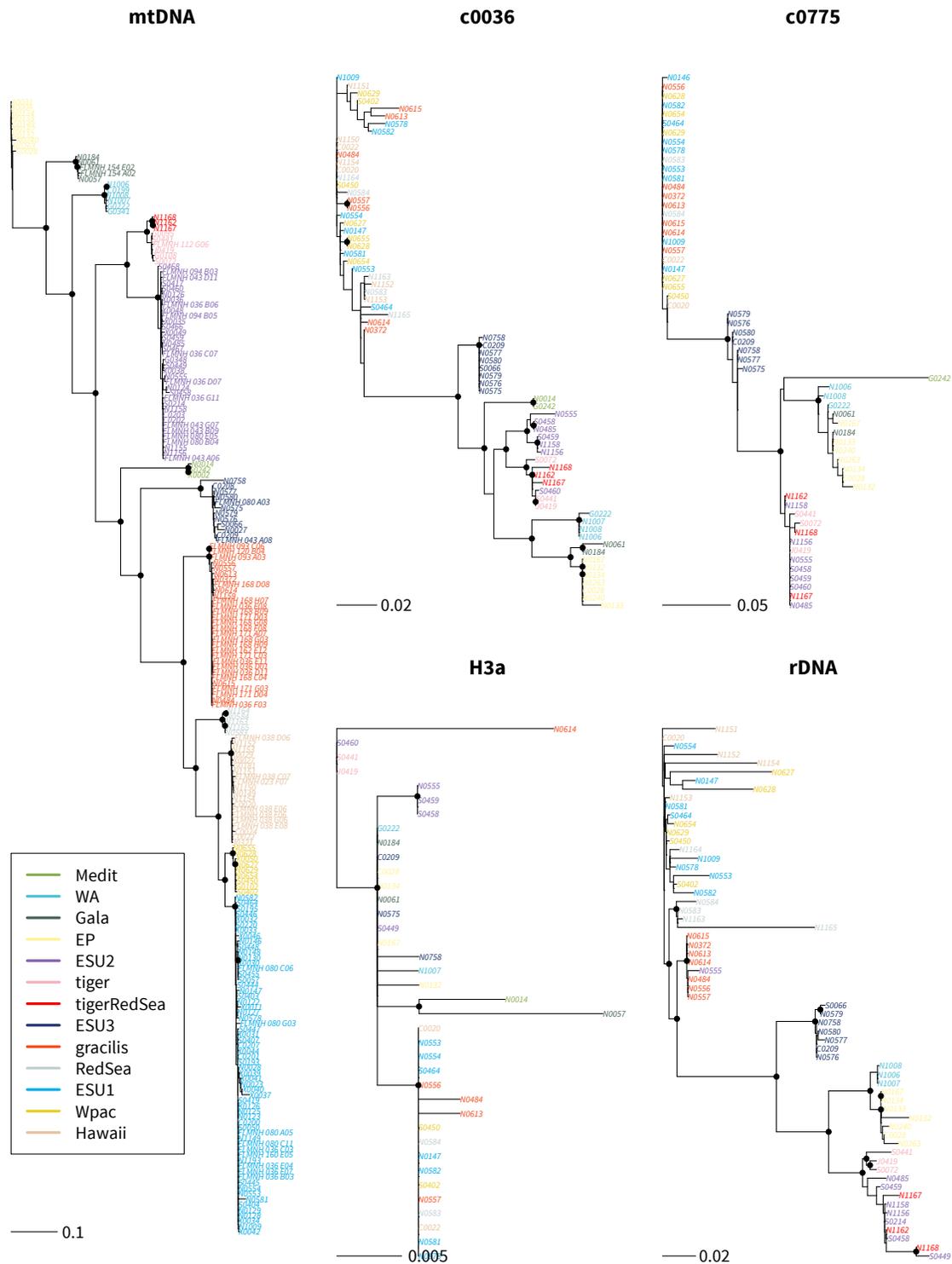


Figure 13: Maximum-likelihood genealogies (unrooted) for each locus reconstructed using RAxML with a GTR+G model of molecular evolution for each partitions. Black circles on the nodes indicate bootstrap values ≥ 80 based on 500 replicates. Terminals are colored based on their ESU. “mtDNA” is the concatenation of ATP6, COI, 16S; “rDNA” is the concatenation of ITS and LSU.

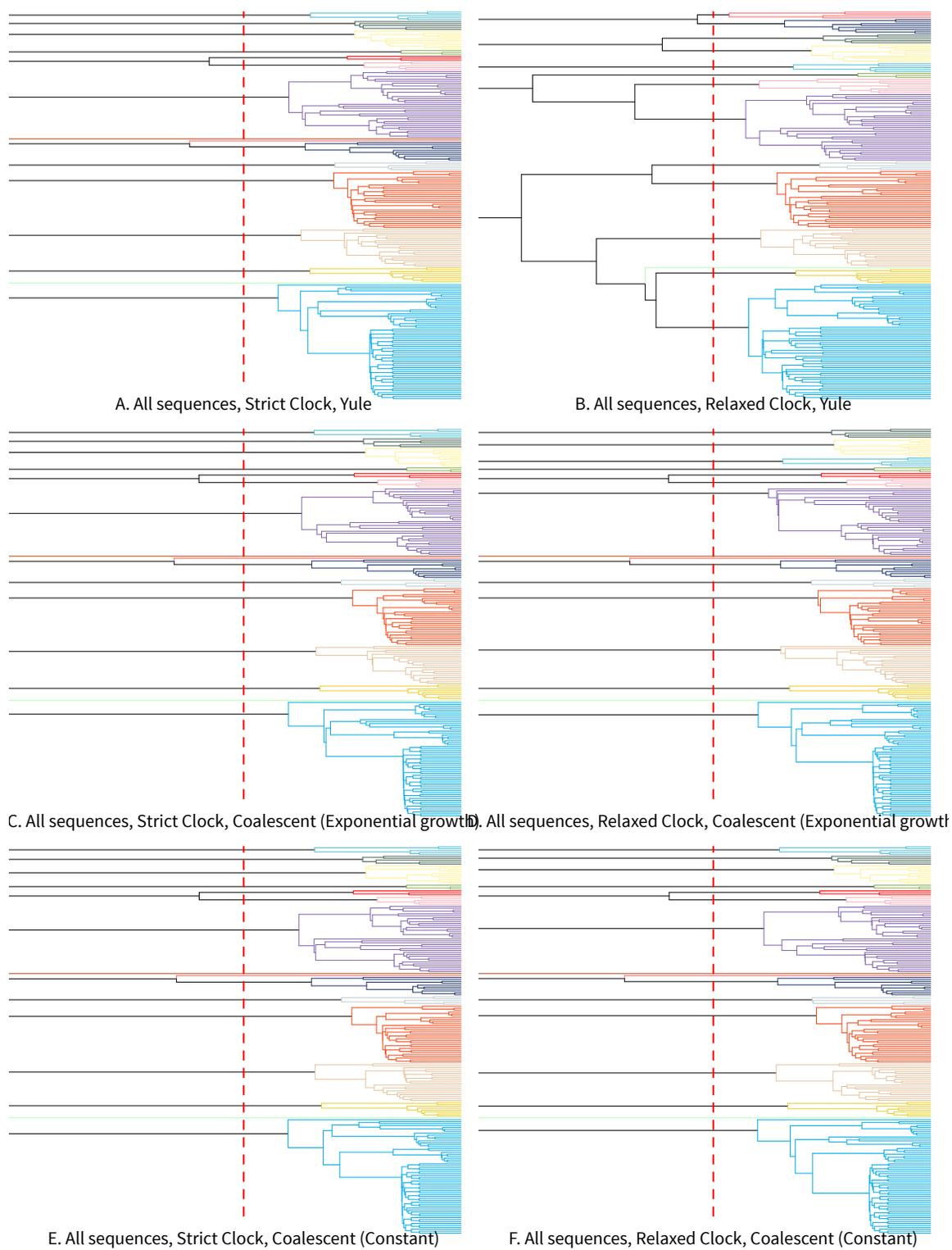


Figure 14: Position of the species delineated with GMYC on COI genealogies estimated using various priors with BEAST. Each color corresponds to a different putative species

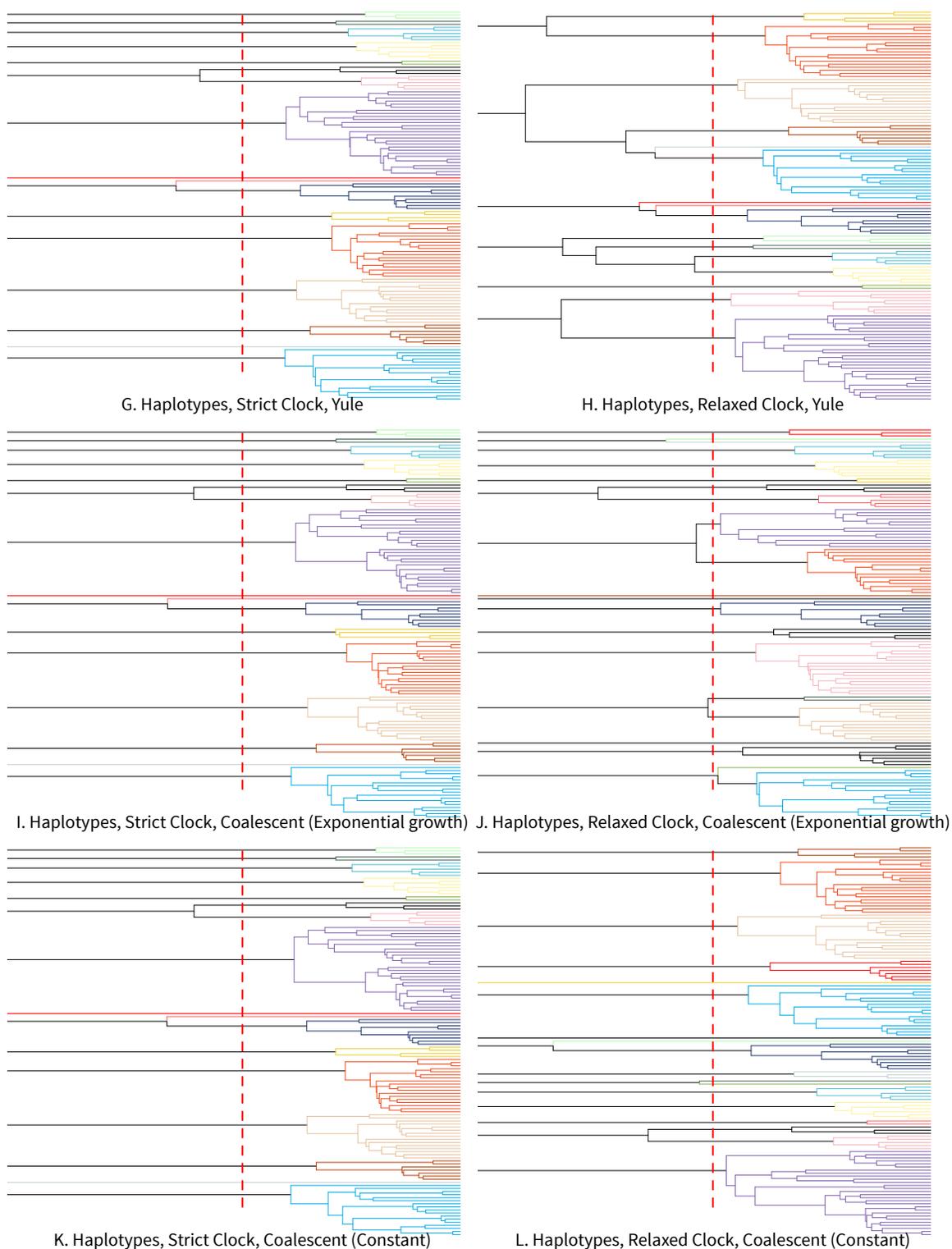


Figure 15: Position of the species delineated with GMYC on COI genealogies estimated using various priors with BEAST. Each color corresponds to a different putative species

Table 5: Specimen information including location, catalog number, and ESU (consensus)

Catalog Nb.	ESU (consensus)	Location	Specimen Nb.
MHE006	EP	Mexico	A0004
MHE048	EP	Mexico	A0031
FRM134	EP	Panama	C0028
3430	EP	Panama	G0149
FRM106	EP	Panama	N0132
FRM107	EP	Panama	N0133
FRM110	EP	Panama	N0134
FRM124	EP	Panama	N0167
FRM124small	EP	Panama	N0240
FRM153	EP	Panama	N0263
7070a	ESU1	Mayotte	C0200
7070b	ESU1	Mayotte	C0201
7055b	ESU1	Mayotte	N1193
7055b	ESU1	Mayotte	C0207
8585	ESU1	Lizard Island	FLMNH 036 B03
8544	ESU1	Lizard Island	FLMNH 036 C03
8459	ESU1	Lizard Island	FLMNH 036 E04
8192	ESU1	Lizard Island	FLMNH 036 F07
8574	ESU1	Lizard Island	FLMNH 080 A05
8604	ESU1	Lizard Island	FLMNH 080 C06
8939	ESU1	Tanzania	FLMNH 080 C11
8603	ESU1	Lizard Island	FLMNH 080 G03
9192	ESU1	Europa	FLMNH 160 E05
Samyn	ESU1	Comoros	K0126
MOLAF125	ESU1	GBR	N0023
MOLAF139	ESU1	PNG	N0028
3625	ESU1	Palau	N0122
4343	ESU1	Vanuatu	N0123
2268	ESU1	Papua New Guinea	N0125
3929	ESU1	Okinawa	N0127
3928	ESU1	Okinawa	N0128
4307	ESU1	Vanuatu	N0129
574	ESU1	Tikehau	N0130
4264	ESU1	Oman	N0146,C0021
1850	ESU1	Oahu	N0147,C0023
596	ESU1	Moorea	N0148
10951	ESU1	Okinawa	N0553
10955	ESU1	Okinawa	N0554
8193	ESU1	Lizard Island	N0578,FLMNH 036 E07
8191	ESU1	Lizard Island	N0581,FLMNH 036 G07
10313	ESU1	Guam	N0582
11210	ESU1	Marquesas	N1009

Table 5: (continued) specimen information

Catalog Nb.	ESU (consensus)	Location	Specimen Nb.
932	ESU1	Hawaii	N1149
5556	ESU1	Vanuatu	S0050
5566	ESU1	Vanuatu	S0057
6485	ESU1	Réunion	S0193
6487	ESU1	Réunion	S0195
REU 172 1	ESU1	Réunion	S0220
6774	ESU1	Majuro	S0403
6748	ESU1	Majuro	S0404
6745	ESU1	Majuro	S0407
6957	ESU1	Kosrae	S0419
6989	ESU1	Kosrae	S0444
6990	ESU1	Kosrae	S0445
6988	ESU1	Kosrae	S0446
6987	ESU1	Kosrae	S0447
6982	ESU1	Kosrae	S0448
6967	ESU1	Kosrae	S0455
7081	ESU1	Guam	S0464
4255	ESU1	Oman	X0011
318	ESU1	Rangiroa	X0030
4258	ESU1	Oman	X0031
4098	ESU1	Taiwan	X0032
4257	ESU1	Oman	X0033
5326	ESU1	Moorea	X0034
5294	ESU1	Moorea	X0037
5339	ESU1	Moorea	X0039
5320	ESU1	Moorea	X0040
5206	ESU1	Moorea	X0041
5290	ESU1	Moorea	X0042
5679	ESU1	Philippines	X0044
5597	ESU1	Bohol	X0046
7163a	ESU2	Nosy Bé	C0202
7163b	ESU2	Nosy Bé	C0203
8406	ESU2	Lizard Island	FLMNH 036 B06
8172	ESU2	Lizard Island	FLMNH 036 C07
8174	ESU2	Lizard Island	FLMNH 036 D07
8355	ESU2	Lizard Island	FLMNH 036 G11
7488	ESU2	Nosy Bé	FLMNH 043 A06
7531	ESU2	Nosy Bé	FLMNH 043 B09
7541	ESU2	Nosy Bé	FLMNH 043 D11
7384	ESU2	Nosy Bé	FLMNH 043 G07
8943	ESU2	Tanzania	FLMNH 080 B04
8944	ESU2	Tanzania	FLMNH 080 E05

Table 5: (continued) specimen information

Catalog Nb.	ESU (consensus)	Location	Specimen Nb.
9628	ESU2	Ningaloo	FLMNH 094 B03
9611	ESU2	Ningaloo	FLMNH 094 B05
BNZ1761	ESU2		G0348
216	ESU2	Guam	N0124
220	ESU2	Guam	N0126
10123	ESU2	Heron Island	N0485,N1161
11012	ESU2	Okinawa	N0555
7221	ESU2	Nosy Bé	N1155
7287	ESU2	Nosy Bé	N1156
7318	ESU2	Nosy Bé	N1158
6371	ESU2	Réunion	S0214
7421	ESU2	Réunion	S0417
6983	ESU2	Kosrae	S0449
6711	ESU2	Guam	S0458
6729	ESU2	Guam	S0459
6712	ESU2	Guam	S0460
7076	ESU2	Guam	S0466
6726	ESU2	Guam	S0467
6722	ESU2	Guam	S0468
5271	ESU2	Moorea	X0035
5340	ESU2	Moorea	X0036
5263	ESU2	Moorea	X0038
5625	ESU2	Bohol	X0048
5608	ESU2	Bohol	X0049
7519a	ESU3	Nosy Bé	C0208
7519b	ESU3	Nosy Bé	C0209
7383	ESU3	Nosy Bé	FLMNH 043 A08
8942	ESU3	Tanzania	FLMNH 080 A03
MOLAF138	ESU3	PNG	N0027
5624	ESU3	Siquijor Prov.	N0575,X0043
7360	ESU3	Nosy Bé	N0576,FLMNH 043 H05
8928	ESU3	Tanzania	N0577,FLMNH 080 H09
8941	ESU3	Tanzania	N0579,FLMNH 080 G01
7469	ESU3	Nosy Bé	N0580,FLMNH 043 D04
RUMF-ZE-00074	ESU3	Okinawa	N0758
5877	ESU3	Yap	S0066
9637	Gala	Galapagos Islands	FLMNH 154 A02
9641	Gala	Galapagos Islands	FLMNH 154 E02
9667	Gala	Galapagos Islands	N0057,N0188,FLMNH 154 G06
9668	Gala	Galapagos Islands	N0061,N0185,FLMNH 154 H06
9655	Gala	Galapagos Islands	N0184,FLMNH 154 G04
8336	gracilis	Lizard Island	FLMNH 036 D01

Table 5: (continued) specimen information

Catalog Nb.	ESU (consensus)	Location	Specimen Nb.
8348	gracilis	Lizard Island	FLMNH 036 D11
8288	gracilis	Lizard Island	FLMNH 036 E08
8354	gracilis	Lizard Island	FLMNH 036 E11
8364	gracilis	Lizard Island	FLMNH 036 F03
9440	gracilis	Ningaloo	FLMNH 093 A03
9544	gracilis	Ningaloo	FLMNH 093 C06
9412	gracilis	Ningaloo	FLMNH 120 B04
10078	gracilis	Heron Island	FLMNH 162 E12
10207	gracilis	Heron Island	FLMNH 168 B09
10145	gracilis	Heron Island	FLMNH 168 C04
10192	gracilis	Heron Island	FLMNH 168 D08
10184	gracilis	Heron Island	FLMNH 168 F08
10146	gracilis	Heron Island	FLMNH 168 G03
10208	gracilis	Heron Island	FLMNH 168 G08
10193	gracilis	Heron Island	FLMNH 168 H07
10206	gracilis	Heron Island	FLMNH 168 H09
10043	gracilis	Heron Island	FLMNH 171 A07
10129	gracilis	Heron Island	FLMNH 171 C03
10130	gracilis	Heron Island	FLMNH 171 D03
10053	gracilis	Heron Island	FLMNH 171 D04
10124	gracilis	Heron Island	FLMNH 171 G03
5620	gracilis	Bohol	N0372
10122	gracilis	Heron Island	N0484
10127	gracilis	Heron Island	N0556,FLMNH 171 E02
10126	gracilis	Heron Island	N0557,FLMNH 171 H01
PH-9	gracilis	Philippines	N0613
PH-10	gracilis	Philippines	N0614
PH-11	gracilis	Philippines	N0615
8346	gracilis	Lizard Island	N1159
4812	Hawaii	Oahu	C0020
1858	Hawaii	Maui	C0022
1842	Hawaii	Maui	C0024
6243	Hawaii	FFS	FLMNH 023 F07
6138	Hawaii	FFS	FLMNH 038 C07
6140	Hawaii	FFS	FLMNH 038 D06
6141	Hawaii	FFS	FLMNH 038 E06
6139	Hawaii	FFS	FLMNH 038 E08
6142	Hawaii	FFS	FLMNH 038 F06
6143	Hawaii	FFS	FLMNH 038 G06
4808	Hawaii	Oahu	G0181
929	Hawaii	Hawaii	J0321
1587	Hawaii	Hawaii	N0149

Table 5: (continued) specimen information

Catalog Nb.	ESU (consensus)	Location	Specimen Nb.
962	Hawaii	Hawaii	N1150
6059	Hawaii	Oahu	N1151
6279	Hawaii	Maui	N1152
6280	Hawaii	Maui	N1153
6281	Hawaii	Maui	N1154
4805	Hawaii	Oahu	X0027
1277	Hawaii	Maui	X0029
MOLAF087	Medit	Sicily	G0242
MOLAF123	Medit	Sicily	N0014
MOLAF124	Medit	Sicily	X0002
9699	RedSea	Egypt	N0583,FLMNH 084 H05
9701	RedSea	Egypt	N0584,FLMNH 084 D05
12030	RedSea	Djibouti	N1163
12049	RedSea	Djibouti	N1164
12051	RedSea	Djibouti	N1165
4713	tiger	Marianas	FLMNH 112 G06
4737	tiger	Guam	G0095
4709	tiger	Guam	G0108
1280	tiger	Maui	J0419
5970	tiger	Maui	S0072
6929	tiger	Kosrae	S0441
11956	tigerRedSea	Djibouti	N1162
12086	tigerRedSea	Djibouti	N1167
12184	tigerRedSea	Saudi Arabia	N1168
7902	WA	Belize	C0199
2100	WA	Virgin Islands	G0222
LACM 2003 76	WA	Bocas	G0341
11817	WA	StMartin	N1006
11743	WA	StMartin	N1007
11790	WA	StMartin	N1008
1611b	Wpac	Palau	G0102
PH-HI-1	Wpac	Philippines	N0627
PH-HI-2	Wpac	Philippines	N0628
PH-HI-3	Wpac	Philippines	N0629
PH-HI-4	Wpac	Philippines	N0654
PH-HI-5	Wpac	Philippines	N0655
6771	Wpac	Majuro	S0402
6809	Wpac	Kosrae	S0450
5610	Wpac	Bohol	X0050