Genomics of population differentiation in humpback dolphins, *Sousa* spp. in the Indo-Pacific Ocean

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Abstract

Speciation is a fundamental process in evolution and crucial to the formation of biodiversity. It is a continuous and complex process, which can involve multiple interacting barriers leading to heterogeneous genomic landscapes with various peaks of divergence among populations. In this study, we used a population genomics approach to gain insights on the speciation process and to understand the population structure within the genus *Sousa* across its distribution in the Indo-Pacifc region. We found 5 distinct clusters, corresponding to *S. plumbea* along the eastern African coast and the Arabian Sea, the Bangladesh population, *S. chinensis* off Thailand and *S. sahulensis* off Australian waters. We suggest that the high level of differentiation found, even across geographically close areas, is likely determined by different oceanographic features such as sea surface temperature and primary productivity.

Keywords: Speciation, Marine Mammals, Delphinids, Genotyping-by-sequencing

Introduction

Understanding drivers of population divergence and speciation is a central question in evolutionary biology. This is especially true in the marine environment where barriers to dispersal are not as obvious as in the terrestrial environment. A central paradigm in marine systems is that populations are typically characterized by weak genetic differentiation due to the potential for long-distance dispersal favouring high levels of gene flow (Palumbi, 1992). However, several studies have shown that marine megafauna show high levels of genetic differentiation (e.g. Hess et al, 2013), as is the case for inshore populations (e.g. Tezanos-Pinto et al, 2009). There are neutral and adaptive processes that

63 can lead to higher than expected differentiation in the marine environment. Neutral
64 processes include population dynamics caused by birth, death and dispersal of organisms
65 through different regions and environments, causing genetic drift. Adaptive processes
66 include local adaptation, where organisms have higher average fitness in their local
67 environment when compared to individuals elsewhere.
68 Cetaceans are a unique taxonomic group in that species underwent drastic evolutionary
69 transitions from terrestrial to marine environments (Steeman et al, 2009). Delphinids, in
70 particular, have radiated very recently (at around 10-12 Ma, McGowen et al. 2009) and
71 have populated many different habitats and environments, providing a unique opportunity
72 to study the role of different evolutionary processes in shaping population structure and
73 genetic diversity at large spatial, but relatively short temporal scales.
74 Several factors and mechanisms have been suggested as likely to influence and drive
75 genetic differentiation and speciation in cetacean species. Despite being marine predators
76 with high mobility and few obvious barriers to dispersal, environmental factors like sea
77 surface temperature, salinity and ocean currents have been shown to influence patterns of
78 population structure, as these dictate prey dispersal and availability (e.g. Amaral et al,
79 2012; Mendez et al, 2011). Other mechanisms such as social interactions, behaviour and
80 culture, have also been suggested to shape population structure and genetic diversity
81 (Alexander et al, 2016; Carroll et al, 2015; Kopps et al, 2014; Riesch et al, 2012).
82 Humpback dolphins (*Sousa* spp.) are distributed discontinuously in coastal waters of
83 West Africa and in the Indian and Western Pacific Oceans and all populations are
84 currently facing anthropogenic pressures, raising conservation concerns (Braulik et al,
85 2015; Jefferson and Smith, 2016b; Parra and Cagnazzi, 2016). This genus comprises four
86 species: *S. teuszii* in the Eastern Atlantic Ocean along the west African coast, *S. plumbea*

87 in the Western Indian Ocean, *S. chinensis* distributed in the Eastern Indian and Western
88 Pacific Oceans and *S. sahulensis* in Northern Australia and New Guinea (Jefferson and
89 Rosenbaum, 2014) (Figure 1). However, the exact eastern limit of *S. plumbea* in the Bay
90 of Bengal and the western limit of *S. chinensis* are poorly known. In terms of external
91 appearance,; *S. plumbea* has a darker coloration with little spotting and a prominent dorsal
92 fin hump; *S. teuszii* has a similar appearance to that of *S. plumbea* but with significantly
93 shorter rostra and lower tooth counts; *S. chinensis* has light adult coloration, often with
94 bluish gray spotting and lacks the prominent dorsal hump; *S. sahulensis* has no visible
95 dorsal fin hump and the dorsal fin is low and triangular, with adults having a dark grey to
96 grey back and a lighter belly (Jefferson and Rosenbaum 2014). Analyses conducted to
97 date suggest high levels of population genetic structure within both *S. plumbea* and *S.*
98 *chinensis* and a highly differentiated population in the Bay of Bengal (Amaral et al, 2017;
99 Mendez et al, 2013). Oceanographic features such as sea surface temperature and primary
100 productivity have been suggested as important drivers of population differentiation in
101 these animals (Amaral et al., 2017; Mendez et al., 2011).
102 The Bay of Bengal is a marine region in the Northern Indian Ocean that supports an
103 impressive variety of cetaceans, but with little knowledge on the evolutionary processes
104 acting on those species (Mansur et al, 2012; Smith et al, 2008). The extreme infusion and
105 redistributive dynamism of biological productivity in this region is a rare ecological
106 condition that supports cetaceans in numbers generally much larger than other
107 populations in the region (Mansur et al., 2012). While little is known about the
108 morphological differences in the highly-differentiated humpback dolphin population
109 occurring in this region, it has been hypothesized that the relatively rare environmental
110 conditions in the Bay of Bengal explains its genetic distinctiveness (Amaral et al,. 2017).

111 Other marine species occurring in this area have also shown high levels of genetic
112 differentiation (e.g.Li et al, 2015).
113 In this study we aim to build on our previous work that used mtDNA and three nuclear
114 markers to investigate genetic connectedness of Indo-Pacific humpback dolphin
115 populations. Using a population genomics approach, we aim to investigate patterns of
116 genome wide differentiation in Indo-Pacific humpback dolphins across the Indian and
117 West Pacific Oceans.
118
119 **Material and Methods**
120
121 **Sample collection and Sequencing**
122 Our total data set consisted of 30 samples obtained from stranded or biopsied humpback
123 dolphins, which were selected from a set of samples already used in previous studies
124 (Mendez et al., 2013, Amaral et al., 2017). Representing the entire distribution range of
125 the *Sousa* genus in the Indo-pacific region, our data set contains samples from Southeast
126 Africa (SEA - South Africa and Mozambique n=6), Arabian Sea (OM - Oman, n=8), Bay
127 of Bengal (BAN - Bangladesh, n=10), Indo-China (CHI - Thailand, Hong Kong and
128 Taiwan, n=4) and Northern Australia (AUS, n=2) (Figure 1).
129 The genomic DNA from tissues samples already preserved in ethanol (96% v/v) or in
130 sodium chloride-saturated 20% dimethyl sulphoxide (DMSO) solution, was extracted
131 using QIAamp Tissue Kit (QIAGEN, Valencia, CA, USA) and its concentration
132 measured using a Qubit Florometric Quantitation (ThermoFisher). The samples were then
133 shipped to the Cornell University Institute of Biotechnology's Genomic Diversity Facility
134 (http://www.biotech.cornell.edu/brc/genomic-diversity-facility) where the GBS

135 (genotyping-by-sequencing) data was generated. Sequencing libraries were constructed
136 using the restriction enzyme *Pst*I (CTGCAG) by a genotype-by-sequencing protocol
137 (Elshire et al, 2011). Unique oligonucleotide barcodes were added to each sample for
138 multiplexed sequencing on an Illumina HiSeq 2000 (Illumina, San Diego, CA, USA).
139 Template-controls were included with the batch of samples and 100 bp single-end reads
140 were generated.
141
142 **Data processing**
143 Demultiplexing, initial quality control, assembly, and SNP discovery were completed in
144 the TASSEL pipeline v3.0.174 (Glaubitz et al, 2014), which was specifically designed
145 for GBS datasets. The killer whale genome was used as a reference to identify single
146 nucleotide polymorphisms (SNPs) (*O. orca*, Oorc_1.1, 200.0x coverage, (Foote et al,
147 2015; Morin et al, 2010) using bwa (v0.7.8-r455; Li and Durbin, 2009).
148 The TASSEL pipeline relies on the number of times a given tag has been observed as an
149 indicator of sequence quality, and not quality scores, as these are frequently not indicative
150 of sequence quality in short reads as those obtained in a GBS approach (Dohm et al, 2008;
151 Eren et al, 2013; Glaubitz et al, 2014). The first step of the pipeline consists in processing
152 and collapsing all barcoded reads into a set of unique sequence tags, with one TagCounts
153 file produced per input FASTQ file. These separate files are then merged into a single
154 master file and the tag list is aligned to the reference genome. The barcode information
155 in the original FASTQ files is used to infer the number of times each tag in the master
156 file is observed in each sample and these counts are stored in a different TagsByTaxa file.
157 This information is then used to discover SNPs at each set of tags with the same genomic
158 position and filter the SNPs based upon the proportion of taxa covered, minor allele

159 frequency (MAF = 1%), linkage disequilibrium (minimum median population LD(R^2)
160 was set to 0.1) and inbreeding coefficient (F = 1-Ho/He, where Ho- observed
161 heterozygosity and He – expected heterozygosity) (Glaubitz et al, 2014).
162 After the SNP calling obtained with the TASSEL pipeline, blank-controls and 3
163 individuals were excluded due to missing data, producing a final data set of 27 individuals
164 (Table S1). For these individuals, we applied additional filters to further reduce false
165 positive SNPs for subsequent analysis. Firstly, limits for the genomic depth of coverage
166 were calculated and applied for each individual in RStudio (v1.0.136; RStudio Team
167 (2016); R Core Team (2016)) using a custom script (V. Sousa). The calculation
168 corresponded to 1/3 of the mean-depth for the minimum limit and the double of the mean-
169 depth for the maximum limit. This calculation was applied because it considers the
170 average coverage of each individual. Secondly, we performed the Hardy-Weinberg
171 Equilibrium test using the hardy option in VCFtools v0.1.15 (Danecek et al, 2011). The
172 sites with *P*-values significant at the 0.01 level were excluded. Non bi-allelic sites as well
173 as sites with missing data higher than 50% were also removed using VCFtools.
174 A MAF filter was also applied, as the initial filter of MAF = 1% applied in TASSEL
175 seemed very conservative. We used two different values of MAF (2 and 5%) to
176 understand how this choice would affect subsequent analyses, since rare variants could
177 be false positives of the sequencing protocol but could also be important genetic variation
178 that can have true genetic effects in the population (Nielsen et al, 2012; Whitlock and
179 Lotterhos, 2015).
180 We used two different datasets in all the population structure analyses described in the
181 next section. After this step, each data set was converted to various formats using
182 PGDSpider (v2.1.1.3; Lischer and Excoffier, 2012) for subsequent analyses. The

183 application of the MAF filter greatly reduced the number of SNPs to analyze, but had no
184 effect on the patterns obtained, therefore we chose to use the dataset with the highr
185 number of SNPs (19 462) to generate the results presented in Figures 2-5.
186 In order to measure the genetic differentiation between populations, pairwise F_{ST} values
187 between populations were estimated using Arlequin v.3.5.2.2. (Excoffier and Lischer
188 2010). The significance of these estimates was evaluated with 10,000 permutations. We
189 consider these results to be preliminary due to low sample sizes.
190
191 **Population structure**
192 To infer population structure in the genus *Sousa*, we first used a discriminant analysis of
193 principal components (DAPC) to identify genetic clusters. DAPC is a multivariate
194 approach that transforms individual genotypes using principal components analysis
195 (PCA) prior to a discriminant analysis (DA) (Jombart *et al*, 2010). This maximizes the
196 differentiation between groups while minimizing variation within groups and was
197 conducted using the dapc function in the *Adegenet* package (v2.1.1; Jombart et al, 2008).
198 Since DAPC requires group assignment *a priori*, we employed a K-means clustering
199 algorithm implemented in *Adegenet* to identify the optimal number of clusters from K =
200 1 to K = 10. Different clustering solutions were then compared using Bayesian
201 Information Criterion (BIC), and to avoid over-fitting of discriminant functions, we used
202 Alpha-score optimization to evaluate the optimal number of principal components (PCs)
203 to retain in the analysis, as described in Jombart et al, (2010).
204 Second, we estimated individual genetic ancestry using sNMF (Frichot et al, 2014)
205 through snmf function in the *LEA* package (v1.6.0; Frichot and François 2015), and the
206 program STRUCTURE (v2.3.2) (Pritchard et al, 2000). Both programs compute

proportion quantities called ancestry coefficients that represent the proportion of an individual genome that originate from multiple ancestral gene pools (Pritchard et al., 2000; Frichot et al., 2014). While sNMF generates comparable results to those obtained from STRUCTURE, it does not require Hardy-Weinberg equilibrium assumptions (Frichot et al., 2014).

The ancestry coefficients were estimated from a specified number of ancestral populations (K). For sNMF, the ancestry coefficient was calculated for K 1 to 10 using 100 replicates for each K. The preferred number of K was chosen using a cross-entropy criterion based on the prediction of masked genotypes to evaluate the error of ancestry estimation. For STRUCTURE, a correlated allele frequency model with no admixture was used (Hubisz et al, 2009). We conducted 20 runs for each K value (1-6) with a burn-in of 10,000 repetitions for each value of K followed by 100,000 MCMC repetitions. To determine the best value of K we employed two approaches. We used an iterative approach based on the ΔK statistic (Evanno et al, 2005) and also used the ln(Pr(X|K) values in order to identify the K for which Pr(K=k) is highest, as described in Pritchard et al. 2000. Both approaches were conducted using CLUMPAK (Kopelman et al, 2015) and STRUCTURE HARVESTER (v0.6.94; Earl and vonHoldt, 2012).

A maximum-likelihood framework was also applied to infer phylogenetic relationships between populations. The analysis was implemented using RAxML (v8.2.11; Stamatakis, 2014) in which we carried out 1,000 inferences using the GTR model with no rate heterogeneity modelled (ASC_GTRCAT). The branch support was estimated using bootstrap by a majority-rule criteria as implemented in RAxML and visualized simultaneously in a single consensus tree (Holland et al, 2005) in Figtree (v1.4.3; Rambaut 2016). The consensus tree was set at 0.1, which means that bipartitions that

appeared in at least 200 of the 2,000 bootstrap trees participated in network construction. RAxML was run using the two data sets (Table 1).

Results

We generated genome-wide SNPs for 30 individuals, 3 of which were excluded due to high levels of missing data (higher than 90% of missing SNPs - CHI12,14, 13), producing a final data set of 27 individuals (Table S1) that were used for the downstream analysis: Southeast Africa (SEA - South Africa and Mozambique n=6), Arabian Sea (OM - Oman, n=8), Bay of Bengal (BAN - Bangladesh, n=10), Thailand n=1 and Northern Australia (AUS, n=2) After the TASSEL pipeline, 55615 SNPs were obtained, and this number was reduced to a range of 11591 – 19 462 SNPs, depending on the value of MAF used.

Population structure and differentiation

No differences were found in the initial exploratory analyses using the two datasets obtained using different MAF filters. All the results presented below correspond to the results obtained with 19 462 SNPs.

The clustering analysis performed in STRUCTURE resulted in the best value of K=3 with both the Evanno method and the highest value of ln(Pr(X|K) (Table 2). The results obtained using sNMF showed K=4 as the best fitting number of clusters (Figure 2). The overall pattern obtained in these two clustering methods corresponds to the separation of the three species, *S. sahulensis*, *S. plumbea* and *S. chinensis* (Figures 2, 3, 4 and 5) and a fourth cluster including the subdivision within *S. plumbea* separating the populations from the African coast and the Arabian Sea. The *S. chinensis* population of Bangladesh is clearly separated from all other populations. In addition, both STRUCTURE and

255 SNMF analyses showed the individual from Thailand as an individual with a mixed
256 ancestry from Bangladesh, Oman, East African coast and Australia (Figure 2). The DAPC
257 results show five clearly separated clusters (Figure 3). For this analysis, 5 PCs were
258 retained as indicated by the a-score (Figure S1) and the best-fitting value of K was chosen
259 according to the BIC plot (Figure S2).
260 The preliminary F_{ST} analysis show results consistent with those described above, with
261 high levels of genetic differentiation found between the Bangladesh population and the
262 Arabian Sea and the African coast populations. The lowest value of differentiation is seen
263 between the Arabian Sea and the African coast populations (Table 1).
264
265 **Phylogenetic relationships**
266 Using the ML method, the phylogenetic tree showed the same pattern mentioned above.
267 Three main and highly supported clusters, corresponding to the three described species
268 are seen. The subdivision within *S. plumbea* is also identified and supported with
269 bootstrap values of 100 (Figure 5). The individuals from Bangladesh and the individual
270 from Thailand are also found in separate highly supported clusters.
271
272 **Discussion**
273 In this study, we conducted for the first time a genome-wide population analysis of
274 humpback dolphins occurring in the Indo-Pacific Ocean. We found high levels of species
275 and within-species divergence consistent with previous studies using mitochondrial DNA
276 and five nuclear loci, that support the currently recognized species of *Sousa* as well as
277 strong genetic subdivisions within species.
278

Population structure and environmental drivers

Our study supports previous findings that humpback dolphins in the Indo-Pacific region appear to be divided in five main genetic clusters. These correspond to: *S. plumbea* along the East African coast, the Bangladesh population, *S. chinensis* here represented by an individual sampled in Thailand and *S. sahulensis*. Within *S. plumbea*, we further obtained a genetic division, albeit weaker, between the African coast and the Arabian Sea. The Bangladesh population in the Bay of Bengal seems to be genetically more similar to the individual from Thailand and to *S. sahulensis*, even though the dolphin's outer body morphology is similar to the other species, *S. plumbea*. Since this population is located in a transition region between *S. plumbea* and *S. chinensis* and shows morphological characters of both species, hybridization between the two types was hypothesized (Jefferson and Rosenbaum, 2014; Mendez et al., 2013). However, both previously obtained mitochondrial DNA data and the genomic DNA obtained in this study show congruent results, ruling out the hybridization scenario (Amaral et al., 2017). Based on our previous results with the mithochondrial DNA and those obtained in this study, we suggest that this population may constitute a separate taxonomic entity, but additional evidence with samples from surrounding areas is needed. This region seems to harbour a strong potential for endemism and speciation, as seen in the high levels of genetic differentiation obtained for a sympatric dolphin species, the Indo-Pacific bottlenose dolphin (Amaral et al., 2017), as was well as other mobile marine species (e.g. Li et al., 2015). The northern Bay of Bengal is located in an ecological "cul-de-sac" and has extraordinary oceanographic conditions, including intrusion of massive and dynamic freshwater and sediment flow from among the world's largest river systems, leaf litter and other bio-productivity from a large mangrove forest. In addition, this region has an

upwelling from a deep submarine canyon which supports a large sediment fan and a seasonally reversing current gryre with associated meso-eddies that retain and redistribute nutrients (Cheng et al, 2013; Hussain and Acharya, 1994). Together these local conditions are unique in terms of their dynamics and scale, and likely explain the genetic distinctiveness found in marine organisms occurring in the northern Bay of Bengal.

The sample from Thailand showed a mixed ancestry with genetic contributions from *S. sahulensis* and the Bangladesh population, and on a much lower level from *S. plumbea.* This suggests that it could be a hybrid individual and more samples are needed to understand the level of genetic distinctiveness of individuals occurring in this region.

The genetic division obtained with *S. plumbea*, separating the southeast South Africa population from the Arabian Sea population, has already been described using mtDNA and a few nuclear markers (Mendez et al., 2013; Amaral et al., 2017). Both these regions are characterized by unique oceanographic features that could explain this pattern. The coast of Oman is part of the Arabian Sea Upwelling Province, where the annual monsoon influences the system of currents and the occurrence of rich upwelling areas (Longhurst, 2006). The coasts of Mozambique and South Africa are part of the Eastern African Coastal Province, which includes the Mozambique Channel, and is also influenced by a series of gyres and currents, creating unique environmental conditions. Surface currents and other oceanographic variables such as water turbidity and chlorophyl concentration are known to influence and drive distribution patterns in mobile marine species, such as turtles (e.g. Bass et al, 2006), common dolphins (Amaral et al*.,* 2012) and franciscana dolphins (Mendez et al, 2010) and could therefore also determine the patterns of genetic differentiation seen in humpback dolphins.

326 The overall phylogeographic pattern obtained in this study, with distinct lineages in the
327 east and west of the Indo-Pacific Ocean has also been described in other marine species
328 (Ahti et al, 2016; Bowen et al, 2016; Farhadi et al, 2017; Li et al, 2015). This pattern may
329 have resulted from restricted connectivity of populations across the Sunda shelf
330 (southeast extension of the continental shelf of Southeast Asia comprising the Malay
331 Peninsula, Sumatra, Borneo, Java and Bali) during periods of low sea level in the glacial
332 periods of the Pleistocene (Voris, 2000).
333
334 **Final considerations**
335 In the present study we analysed 19 462s genome-wide SNPs, following a population
336 genomics approach to evaluate the variability and differentiation in Indo-Pacific
337 populations of the genus *Sousa*. Our work supports previous studies where five clusters
338 were observed. The three Indo-Pacific species, *S. sahulensis*, *S. plumbea* and *S. chinensis*
339 were clearly separated from each other with absence of gene flow between them. Genetic
340 segregation within *S. plumbea* was also observed separating the African Coast population
341 from the Arabian Sea and the Bangladesh population was highly differentiated from the
342 other species with little gene flow between them. Oceanographic features have been
343 suggested as important factors driving the divergence of these populations. The
344 discontinuous range resulting from past sea level rise has also likely contributed to
345 population isolation. Future studies need to investigate molecular dating to estimate the
346 time of dispersal events and a biogeographical analysis to study the origin and dispersal
347 of the various populations of *Sousa.* This was the first study to our knowledge, to use
348 genome-wide markers to analyse the population divergence in these dolphin species.

The clarification of the population structure within *Sousa* and the processes involved in this differentiation is extremely important for the conservation of these species. Living in nearshore habitats with freshwater input and in developing nations heavily influenced by human activities, makes the genus extremely vulnerable to fatal entanglements in fishing gear, impacts of vessel traffic and the increasing degradation of their habitat.

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Author contribution

A.R.A. and H.C.R. conceived the study. A.R.A. and C. C. analysed the data and wrote the manuscript. B.D.S., R.M., T.C., R. B., G.M., G.J.P., M.K., T.A.J., L.K., A.G. and R.LB Jr., were involved in sample collection.

Data Availability

All the primary data used in this study is deposited in DRYAD.

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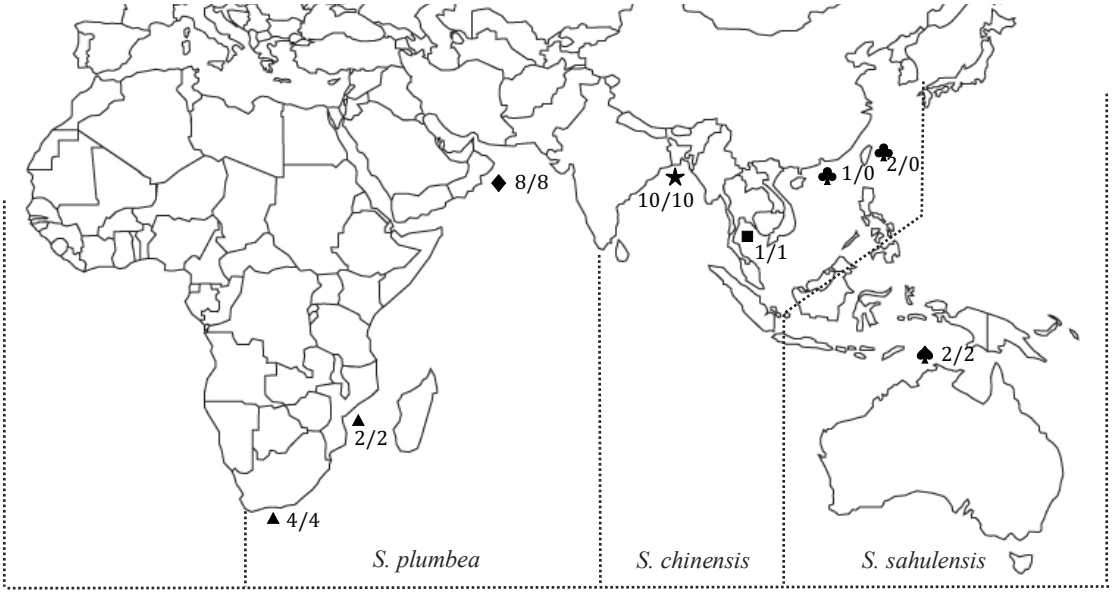

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649 Figure 2

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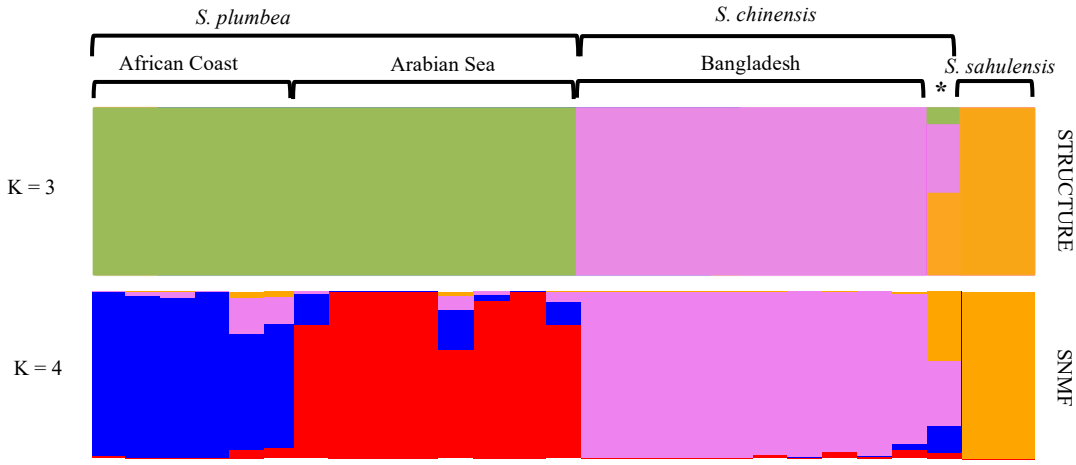

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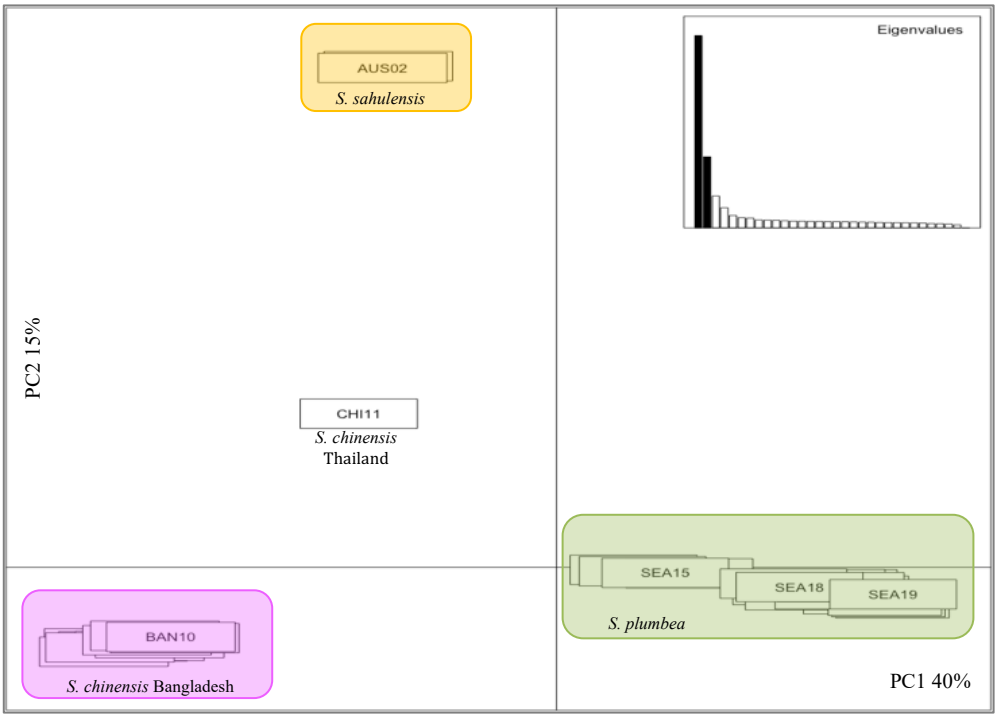

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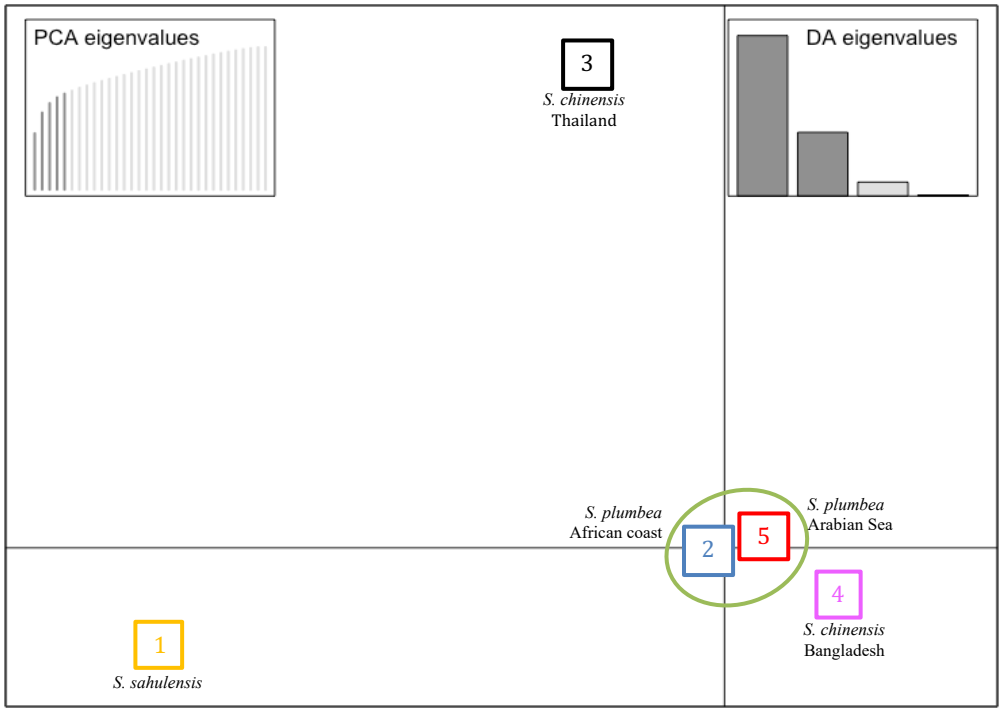

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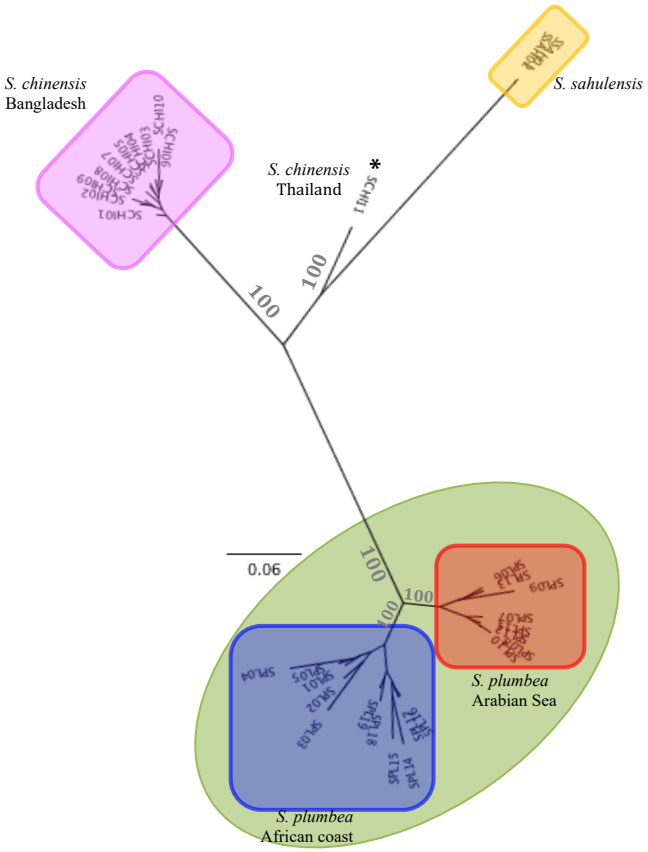

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662 Table 1

	Bangladesh	African Coast	Arabian Sea
Bangladesh	-	0.699	0.549
African Coast	-	-	0.302
Arabian Sea	-	-	-

663 Note: All values were significant (P<0.001).

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669 Table 2

K	Reps	Mean LnP (K)	Delta K
2	20	-142077.4400	-
3	20	-124200.8100	12.7604
4	20	-124508.5200	0.4863
5	20	-124925.4600	0.2613
6	20	-124940.8000	-

670 *K – number of clusters tested; Reps – number of repetitions.*