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## From genome wide SNPs to genomic islands of differentiation: the quest for species diagnostic markers in two scleractinian corals, *Pocillopora* and *Porites*

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## Abstract

Coral reefs are of paramount importance in marine ecosystems, where they provide support for a large part of the biodiversity. Being quite sensitive to global changes, they are therefore the prime targets for biodiversity conservation policies. However, such conservation goals require accurate species identification, which are notoriously difficult to get in these highly morphologically variable organisms, rich in cryptic species. There is an acute need for easy-to-use and resolute species diagnostic molecular markers. The present study builds on the huge sequencing effort developed during the TARA Pacific expedition to develop a genotyping strategy to assign coral samples to the correct species within two coral genera (*Porites* and *Pocillopora*). For this purpose, we developed a technique that we called "Divergent Fragment" based on the sequencing of a less than 2kb long diagnostic genomic fragment determined from the metagenomic data of a subset of the corals collected. This method has proven to be rapid, resolvable and cost-effective. Sequencing of PCR fragments nested along the species diagnostic fragment allowed us to assign 232 individuals of the genus *Pocillopora* and 247 individuals of the genus *Porites* to previously identified independent genetic lineages (*i.e.* species). This genotyping method will allow to fully analyze the coral samples collected across the Pacific during the Tara Pacific expedition and opens technological perspectives in the field of population genomics-guided conservation.

## Introduction

Coral reefs are natural structures which, although covering 0.2% of the ocean surface, host more than 25% of marine biodiversity (Chen et al. 2015). They therefore constitute essential marine ecosystems, providing ecological niches for many species. However, they are threatened by the effects of global changes, in particular the warming and acidification of the oceans (Masson-Delmotte et al. 2018).

In this context, the main objective of the Tara Pacific expedition is to analyze the complexity of coral reefs and their modifications in the face of climate change, as well as anthropic pressures (Planes et al. 2019). Thus, a multi-level multi-species dataset of the coral reef ecosystem across the Pacific Ocean is under construction thanks to the samples taken during the Tara schooner campaign which took place from 2016 to 2018 over more than 100,000 km and visited 32 islands over the entire Pacific transect. Three genera of corals were sampled, two scleractinian corals *Pocillopora* and *Porites*, as well as the hydrocoral *Millepora*. These coral samples are analyzed by multi-omics (metabarcoding, metagenomics, metatranscriptomics and metabolomics) and the quantification of molecular phenotypes (telomere DNA length, stress biomarkers). In addition, these samples are associated with a set of environmental physicochemical data recorded on site at the time of sampling and inferred from satellite and other data, including historical climatic parameters (Planes et al. 2019) (Lombard et al. 2022). This warehouse of data should provide a better understanding of the complexity of reef-building corals and their responses to climate change (Planes et al. 2019).

However, the phenotypic response to climate change can only be properly analyzed if the studied individuals are assigned to species, as the species, viewed as a shared gene pool, is what constitutes the adaptive toolbox (see for example Boulay *et al.*, 2014). The initial set of analyses on *Pocillopora* and *Porites* from the first third of the TARA Pacific islands already represents approximately 330 individuals for each genus. The analysis of the samples by 18S barcoding did not provide an assignation beyond the genus level of the colonies collected. Further genetic analyzes are therefore required to determine the genetic structure of the sampled colonies at the species level. Barcoding approaches in corals are notoriously difficult (Shearer and Coffroth 2008), even if mitochondrial diversity may sometimes be used for species delineation ((Flot and Tillier 2007; Paz-García et al. 2016; Johnston et al. 2017). However, in the two genera here studied, previous studies relied on analyses of arrays of dozen microsatellite loci in *Porites* (Baums et al. 2012; Boulay et al. 2014) and *Pocillopora*

(Gélin et al. 2017, 2018; Oury 2020; Oury et al. 2022a) or on RAD seq data (Oury et al. 2022b) to reliably identify species.

Metagenomic data were obtained for about one third of the TARA Pacific individuals (Belser et al. 2022) which allowed for a genome wide analysis of these colonies from the first third of the sampled islands (Hume et al. 2022). This study showed that the *Pocillopora* samples in fact belong to five independent genetic lineages or species (SVD1 to SVD5) and that the *Porites* samples corresponded to three lineages (K1- K3). Taking advantage of this genome wide analysis of diversity, we could also identify genomic islands of differentiation among these species (Hume et al. 2022). However, a genome wide SNP analysis is too expensive and resource consuming to be applied even to the full sampling of even the first TARA Pacific 11 islands and could not be used for field conservation purposes.

We therefore developed a single species-diagnostic marker approach to assign the non-sequenced TARA Pacific samples to the species detected in (Hume et al. 2022). This “Divergent Fragment” approach consists in identifying and using a single diagnostic short genomic sequence containing sufficiently divergent linked SNPs to efficiently discriminate the species in large samples.

## **Material & Methods**

### Sampling:

On the 32 islands visited in 2016-2018 by the TARA Pacific expedition, coral colonies were sampled based on morphology, targeting the focal species *Pocillopora meandrina* and *Porites lobata*, as described in Lombard *et al.* (Lombard et al. 2022). On each island, when possible, ten colonies of each species were sampled for each of three different sites. Samples were shipped and stored at Genoscope. Total genomic DNA was extracted from all samples as described in Belser *et al.* (Belser et al. 2022). A third of these samples (3 colonies per site) were sequenced at Genoscope, producing for each colony  $1.10^8$  100bp paired end reads of metagenomic data (MetaG dataset) (Belser et al. 2022). The present study focuses on the analysis of the 11 islands from Eastern to Central Pacific of this sampling scheme, which are Las Perlas (I01), Coiba (I02), Malpelo (I03), Easter (I04), Ducie (I05), Gambier (I06), Moorea (I07), Aitutaki (I08), Niue (I09), Samoa (I10), Guam (I15).

### Identification of divergent genomic fragments

The analysis of the MetaG dataset allowed us to genetically identify 5 and 3 species in respectively *Pocillopora* and *Porites* samples (Hume et al. 2022). This result was based on the

coalescent analysis of thousands of unlinked genome-wide SNPs and was therefore not easily transposable to the full TARA dataset. The species being identified, the idea was then to track the parts of the genome that diverged the most among them, using this time the full, unfiltered MetaG dataset of linked genome-wide SNPs. The raw MetaG dataset used in Hume *et al.* (2022) was then only filtered on SNP quality, missingness (no missing data) and minimum allele frequency (0,05) using vcfTools (Danecek et al. 2011). Genetic differentiation among the species (Weir's  $F_{st}$ ) was then computed in a 500bp sliding window using vcfTools (Danecek et al. 2011). The 1% most divergent 500bp bins were then extracted and sorted to identify the 1 to 2kb long tracts of adjacent bins that contained the highest number of SNPs (this search was limited to the longest genomic contig for *Pocillopora*). The species resolution power of these fragments was tested *in silico* by sNMF clustering (Frichot et al. 2014) of the species assigned, MetaG analyzed individuals using only the SNPs within these genomic fragments. For each coral genus, two to three of these genomic islands of species differentiation were picked as potential targets for amplification.

#### PCR set up *in silico*

The first stage for the selection of the divergent fragment was performed *in silico* by extracting from the MetaG dataset the full fasta sequence of the targeted 1 to 2kb fragments. For this, we used the vcfprimers function of vcflib (Garrison et al. 2022), asking to extract the 500bp flanking each SNP included in the genomic island targeted. The full fragment sequence was then reconstructed by assembling these fragments with mafft (Katoh and Standley 2013). All the SNPs contained in the targeted fragment were then placed by hand on this sequence in order to identify conserved tracts that could be used for PCR primers. Potential 25bp primer pairs were then tested using Primer3 (<https://primer3.ut.ee/>).

#### PCR set up *in vitro*

Optimal amplification conditions were tested for each primer pair in a two steps approach. First, a gradient PCR was performed on a single individual to identify the cycling and annealing conditions resulting in the higher possible amplification yield of a single specific fragment. Then, the primer pair conservation was tested by trying to amplify the fragment at these conditions in a sample of one individual per species.

#### Nested PCR set up

The amplified divergent fragments were 1,117 and 2,054 bp long for *Pocillopora* and *Porites*

respectively. The Illumina sequencing technology we chose allowed for the production of paired ends 250bp reads. We therefore had to set up 500bp long nested PCR along these fragments to prepare the sequencing libraries. 18(+/-2) bp PCR primers were then tested in conserved regions within each of the amplified fragments. 3 and 5 nested PCR products were thus defined for the *Pocillopora* and the *Porites* divergent fragments respectively (Table 1 and Figure 1).

### PCR amplifications

A two step amplification protocol was then performed on each individual total DNA extract. First, the full length divergent fragment was amplified from gDNA samples using the Fidelio® Hot Start PCR kit (OZYA010, Ozyme) with its HF buffer according to the manufacturers' recommendations and the following PCR programs. 98°C 3 min, 20 cycles of 95°C 30s/58°C 30s/72°C 1 min , and final extension step 72°C 2 min for *Pocillopora* specific primer pairs. 98°C 3 min, 20 cycles of 95°C 30s/57.5°C 30s/72°C 2 min , and final extension step 72°C 2 min for *Porites* specific primer pairs. The full length PCR product thus obtained was then purified using paramagnetic beads (AMPure XP – Agencourt A63880), and used as matrix for the nested PCR following the same protocol described above but with a 30s elongation steps at 72°C and 40 cycles (Figure 1).

Nested PCR products were then purified using paramagnetic beads, quantified with a Fluoroskan instrument and validated using a high-throughput microfluidic capillary electrophoresis LabChip GX system (Perkin Elmer, Waltham, MA, USA).

### Library preparation and sequencing

All libraries were prepared using the NEBNext DNA Modules Products and NextFlex DNA barcodes with 100 ng of purified PCR product as input. Purified PCR products were normalized at 2.5 ng / µl. Depending on the size of PCR products, 2 strategies were followed to decrease the number of libraries to prepare. For PCR products of around 500 bp (*Poc\_7F/Poc\_7.1R*, *Poc\_7.1F/Poc\_7.2R* for *Pocillopora* and *Por\_239F/Por\_239.1R*, *Por\_239.1F/Por\_239.2R* , *Por\_239.2F/Por\_239.3R*, *Por\_239.3F/Por\_239.4R* for *Porites*) an equimolar pool of purified PCR products generated from one colony was prepared in order to have a total of 100 ng of amplicons in a total volume of 50 µl. For PCR products around 150 bp (*Poc\_7.2F/Poc\_7R* for *Pocillopora* and *Por\_239.4F/Por\_239R* for *Porites*) 100 ng of PCR product generated from one colony were used directly as input for the library. 100 ng of pooled PCR products (230 pools for *Pocillopora* and 229 pools for *Porites*) or 100 ng of



direct PCR products (229 for *Pocillopora* and 232 for *Porites*) were then end-repaired, A-tailed at the 3'end, and ligated to Illumina compatible adaptors using NEBNext DNA Modules (New England Biolabs, MA, USA) and NextFlex DNA barcodes (BioO Scientific Corporation, Austin, TX, USA) with Genoscope in-house-developed 'on beads' protocol as described in Alberti et al. (Alberti et al. 2017). A liquid handler, the Biomek FX Laboratory Automation Workstation (Beckman Coulter Genomics, Danvers, MA, USA), was used to perform up to 96 reactions in parallel. After two consecutive 1x AMPure XP clean-ups, the ligated products were amplified using Kapa Hifi HotStart NGS library Amplification kit (Kapa Biosystems, Wilmington, MA, USA), followed by 0.6x AMPure XP purification. All libraries were quantified first by PicoGreen in 96-well plates. Library profiles were assessed using a high throughput microfluidic capillary electrophoresis LabChip GX system (Perkin Elmer, Waltham, MA, USA) and qPCR with the KAPA Library Quantification Kit for Illumina Libraries on an MXPro instrument. Libraries were then sequenced on Illumina sequencer (Illumina, San Diego, CA, USA) in order to obtain an average of 30 to 100 000 of useful paired-end reads.

#### Data analysis & species assignment

Sequencing results (fastq files) along with MetaG sequences from individuals of known species assignment (at least 2 per species) were aligned on the reference sequence of the divergent fragment used for PCRsetup using BWA-mem v0.7.15 (Li and Durbin 2009). The obtained sam files were then translated into bam format by samtools v1.10.2 (Li et al. 2009), and the bam files were subsequently sorted and duplicate reads were marked by sambamba (Tarasov et al. 2015). Variable positions were then called by samtools mpileup (Danecek et al. 2021) from all the obtained bam files for each genus. The obtained vcf files were then further filtered for SNP only (no indels), missingness (no missing data) and minimum allele frequency (0,05) with vcftools (Danecek et al. 2011). This file was then converted to plink format with vcftools, and a sNMF hierarchical clustering analysis of this dataset was then performed using the LEA R package (Frichot et al. 2014).

## **Results/Discussion**

### *In silico* species diagnostic validation

To test which divergent genomic fragment could be species diagnostic, the SNPs contained within each putative genomic fragment were extracted from the whole genomic



datasets on which the species delineation was performed (Hume et al. 2022), and a species assignment was performed on these SNPs only. The results of these assignments for two candidate fragments (one from *Pocillopora* contig 1, the other from *Porites* contig 239), are compared to the genome wide SNP based assignments from the same individuals in Tables 2a and 2b for *Pocillopora* and *Porites* respectively. As can be seen, in both genera, for these two fragments, the two assignments were concordant for more than 97% of the samples (3 colonies over 105 tested were mistyped in *Pocillopora* and 2 in 109 for *Porites*). Considering that in each genus, one of the very few mistyped colonies was detected as an hybrid in the genome wide SNP analysis, this result clearly validated these two genomic fragments as potential species diagnostic divergent fragments.

### Sequencing results

The careful *in silico* selection of PCR primers allowed for successful amplification of the first divergent fragments tested. Sequences were obtained for 225 and 241 individual colonies respectively. After SNP calling and quality filters, the final vcf file contained 26 SNPs in the 1117kb long divergent fragment in *Pocillopora* and 59 SNPs in the 2054kb long *Porites* divergent genomic fragment. This resulted in 25 unique genotypes among 235 *Pocillopora* individuals and 28 unique genotypes among 241 *Porites* individuals.

### Species assignment

As shown in Figure 2a, sNMF analyses stated that the optimal number of ancestral populations (*i.e.* genetic clusters) to assign the individuals to was  $K=5$  in *Pocillopora*. Each pair of reference individuals was unambiguously assigned to a different cluster, which allowed to assign to each of these clusters one and only one species identified by genome wide analyses. As shown in Table 3, among the 235 sequenced individuals, 219 were unambiguously assigned to only one of the five clusters/species (Assignment Q score  $> 0.80$  in one cluster). 16 individuals were detected as hybrids by sNMF (Assignment Q score  $< 0.80$  in all clusters, but  $> 0.25$  in some), and this hybridization was confirmed by the polymorphic positions in their genotypes (Table 4). In *Porites*, the sNMF analysis of the divergent fragment genotypes pointed to an optimal number of ancestral populations of  $K=4$  (Figure 2b), one more cluster than the three species detected by genome wide analysis (Hume et al. 2022). However, the reference individuals were all unambiguously assigned to only three clusters (data not shown). We therefore forced a  $K=3$  assignment in sNMF, and all the individuals of the previous fourth cluster were then assigned to the same cluster as the K3

reference individuals. The  $K=3$  assignment results are shown in Table 5. As in *Pocillopora*, 13 hybrid individuals could be detected by both sNMF and genotype compositions (Table 6).

The Tables 3 and 5 also show the assignment results previously obtained by genome wide analysis (Hume et al. 2022). Though using a different and more numerous set of individuals, the biogeographic distribution of the species obtained with the divergent fragment is highly comparable with these previous results. The accrued sample size in the divergent fragment analysis also probably allowed for the detection of a fourth species in *Porites*, that could correspond to the two individuals from I15 that could not be clearly assigned to any of the three clusters. The genome wide analysis of the full 32 islands samples should confirm the existence of this fourth species, if it becomes more frequent in Western Pacific.

This work made it possible to assign individual coral colonies to species in two genera of corals (*Pocillopora* and *Porites*) collected from 11 islands from eastern to central Pacific during the Tara Pacific expedition, by only amplifying and sequencing a single short genomic sequence of less than 2kb. This simple species assignment tool has thus the same resolution as the genome wide analyses (Hume et al. 2022, Oury et al. 2022b) or as the microsatellite arrays (Boulay et al. 2014; G  lin et al. 2017) which were used so far in these genera. It should however be noted that the identification of these diagnostic divergent fragments stemmed from a previous full-fledged genome wide analysis. The diagnostic value of these fragments outside of the geographic zone here sampled still also needs to be tested. The upcoming analysis of the full set of TARA Pacific 32 islands will show if these divergent fragments are still diagnostic at the full Pacific basin scale.

The taxonomic information provided here has already proved an essential asset for crossover analyzes of phenotypic in this geographical area, allowing for a better understanding of the impact of environmental variation on coral reefs.

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## Tables & Figures

**Table 1** external (PCR) and nested (sequencing) primers

Position	Name	Sequence	Size (bp)	Tm (calculated)
<b><i>Porites</i></b>				
External	Por_239F	AATATTTGTTTTATGCAAATGTACG	25	56.65
External	Por_239R	ATCAGATTCATTTTCTTACGTCAAC	25	57.87
Nested	Por_239_1R	TGGGGCTTCTAGACGAGTGT	20	59.87
Nested	Por_239_1F	ACACTCGTCTAGAAGCCCCA	20	59.87
Nested	Por_239_2R	GAAGAATCGCTGATGAAAGC	20	57.64
Nested	Por_239_2F	GCTTTCATCAGCGATTCTTC	20	57.64
Nested	Por_239_3R	CCTATGTGTTGCTGTAAACC	20	53.26
Nested	Por_239_3F	GGTTTACAGCAACACATAGG	20	53.26
Nested	Por_239_4R	GATTAGCGATAGCGGTTTCC	20	58.51
Nested	Por_239_4F	GGAAACCGCTATCGCTAATC	20	58.51
<b><i>Pocillopora</i></b>				
<b><i>a</i></b>				
External	Poc_7F	ACTCTTATCTGTTGGTGCTTAATCT	25	56.94
External	Poc_7R	TACATCTTCACTTCTATTCACAACC	25	56.63
Nested	Poc_7_1R	TGCATTTATTTCTCAGTGGTC	21	55.39
Nested	Poc_7_1F	GACCACTGAGAAATAAATGCA	21	55.39
Nested	Poc_7_2R	TGTTGTGTTTTTCTTGCTTTAT	22	54.46
Nested	Poc_7_2F	ATAAAGCAAGAAAACACAACA	22	54.46



**Table 2 in silico Divergent fragment validation test.** These tables give the species assignment results based on either the full set of genomewide SNPs (from Hume et al 2022) in columns or from the putative divergent fragment SNPs in a) *Pocillopora* (SNPs from contig 1, from bp 7013501 to bp 7014500), b) *Porites* (SNPs from contig 239, from bp 280001 to bp 282000)

a) *Pocillopora*

		Genome wide SNPs						Total
		SVD1	SVD2	SVD2/SVD3	SVD3	SVD4	SVD5	
Fragment	SVD1	12	-	-	-	-	-	12
	SVD2	-	18	1	-	2	-	21
	SVD3	-	-	-	18	-	-	18
	SVD4	-	-	-	-	34	-	34
	SVD5	-	-	-	-	-	20	20
Total		12	18	1	18	36	20	105

b) *Porites*

		Genome wide SNPs				Total
		K1	K2	K2/K3	K3	
Fragment	K1	23	-	1	-	24
	K2	-	35	-	-	35
	K3	-	1	-	49	50
Total		23	36	1	49	109

**Table 3 Species assignments from the divergent fragment and genome wide SNPs in *Pocillopora*.** For each island, the number of individual colonies assigned to a given species is given in italic for the genome wide SNP analysis (from Hume *et al.* 2022), and in bold for the divergent fragment analysis

Species assignment	Island											Total
	I01	I02	I03	I04	I05	I06	I07	I08	I09	I10	I15	
<b>SVD1</b>	-	-	-	-	<i>4 / 8</i>	<i>7 / 11</i>	<i>1 / 1</i>	-	-	-	-	<i>12 / 20</i>
<b>SVD2</b>					<i>- / 1</i>	<i>- / 1</i>	<i>1 / 5</i>	<i>4 / 14</i>	<i>3 / 8</i>	<i>7 / 16</i>	<i>3 / 8</i>	<i>18 / 53</i>
<b>SVD2/SVD3</b>	-	-	-	-	-	-	-	-	<i>1 / -</i>	-	-	<i>1 / -</i>
<b>SVD2/SVD4</b>	-	<i>- / 2</i>	-	-	-	<i>- / 1</i>	-	-	-	-	-	<i>- / 3</i>
<b>SVD2/SVD5</b>	-	-	-	-	<i>- / 1</i>	<i>- / 1</i>	-	<i>- / 3</i>	-	<i>- / 2</i>	-	<i>- / 7</i>
<b>SVD3</b>	-	-	-	-	-	-	-	<i>5 / 1</i>	<i>5 / 14</i>	<i>2 / 5</i>	<i>6 / 14</i>	<i>18 / 34</i>
<b>SVD3/SVD5</b>	-	-	-	-	-	-	<i>- / 1</i>	-	-	-	-	<i>- / 1</i>
<b>SVD4</b>	<i>11 / 24</i>	<i>10 / 25</i>	<i>10 / -</i>	-	<i>3 / 5</i>	<i>2 / 2</i>	-	-	-	-	-	<i>36 / 56</i>
<b>SVD4/SVD2/SVD5</b>	<i>- / 1</i>	-	-	-	<i>- / 1</i>	-	-	-	-	-	-	<i>/ 2</i>
<b>D5</b>												
<b>SVD5</b>	-	-	-	<i>12 / 23</i>	<i>2 / 7</i>	<i>- / 5</i>	<i>7 / 17</i>	<i>/ 4</i>	-	-	-	<i>21 / 56</i>
<b>SVD5/SVD2/SVD4</b>	-	-	-	<i>- / 1</i>	-	-	-	-	-	-	-	<i>- / 1</i>
<b>D4</b>												
<b>SVD5/SVD4</b>	-	-	-	<i>- / 2</i>	-	-	-	-	-	-	-	<i>- / 2</i>
<b>Total</b>	<i>11 / 25</i>	<i>10 / 27</i>	<i>10 / -</i>	<i>12 / 26</i>	<i>9 / 23</i>	<i>9 / 21</i>	<i>9 / 24</i>	<i>9 / 22</i>	<i>9 / 22</i>	<i>9 / 23</i>	<i>9 / 22</i>	<i>106 / 235</i>

**Table 4 Genotypes obtained in *Pocillopora* and their species assignation.** Each genotype is designated by a number, an \* indicates this genotype was found in at least one reference individual.

Species assignation	Genotypes	
	N°	SNPs
<b>SVD1</b>	<i>I</i> *	AAATCAAGTATTAGATTTGCGGCCGC
	<i>II</i> *	AAATCAAGTATTAGWTTYRCGGCCGY
	<i>III</i>	ARAYYAARTATTAGATTTGCGGCCGC
<b>SVD2</b>	<i>IV</i>	TGACTAAATATAAGTATCATAAATTT
	<i>V</i>	TGACTAAATATAAGTATCATAAATKT
	<i>VI</i>	TGMCTAAATATAAGTATCATAAATKT
<b>SVD2/SVD4</b>	<i>VII</i>	TGACTAAAWRTAAGTWTCAYAAATGT
<b>SVD2/SVD5</b>	<i>VIII</i>	TGACTAAATATAAGTWTCRYAAATGT
<b>SVD3</b>	<i>IX</i> *	AACCCAGAAATAGGTTCCGCAGCCTT
	<i>X</i> *	AACCCMAGAATAGGTTCCGCAGCCTT
	<i>XI</i>	AACCCAGAAATAGGTTYCGCAGCCTT
	<i>XII</i>	AACCCMAGAATAGGTTYCGCAGCCKT
	<b>SVD3/SVD5</b>	<i>XIII</i>
<b>SVD4</b>	<i>XIV</i> *	TGACTAMAAGTAAGTTTCACAAATGT
	<i>XV</i>	TGACTACAAGTAAGTTTCACAAATGT
	<i>XVI</i>	TGAYTAAATACAAGTTTCGCAAATGT
<b>SVD4/SVD5/SVD2</b>	<i>XVII</i>	TGACTAAATRTAAGTTTCACAAATGT
<b>SVD5</b>	<i>XVIII</i> *	TGACTAAATACAAGTTTCGCAAATGT
	<i>XIX</i>	TGMCTAAATAYAARTTTTCGCAAATGT
	<i>XX</i>	TGCCTAAATATAAATTTTCGCAAATGT
	<i>XXI</i>	TGACTAAATAYAAGTTTCGCAAATGT
	<i>XXII</i>	TGACTAAATATAAGTTTCGCAAATGT
	<i>XXIII</i>	TRACTAAATATAAGTTTCGCAAATGT
<b>SVD5/SVD2/SVD4</b>	<i>XXIV</i>	TGMCTAAATATAARTTTTCGCAAATGT
<b>SVD5/SVD4</b>	<i>XV</i>	TGACTAAAWRYAARTTTTCRCAAATGT

**Table 5** Species assignments from the divergent fragment and genome wide SNPs in *Porites*. For each island, the number of individual colonies assigned to a given species is given in italic for the genome wide SNP analysis (from Hume et al. 2022), and in bold for the divergent fragment analysis

Species assignment	Island											Total
	I01	I02	I03	I04	I05	I06	I07	I08	I09	I10	I15	
<b>K1</b>	<i>9/22</i>	<i>2/11</i>	<i>12/9</i>	-	-	<i>-/1</i>	-	-	-	-	-	<i>23/43</i>
<b>K2</b>	-	<b>7/8</b>	<b>-/3</b>	-	<i>2/1</i>	<i>2/4</i>	<i>7/13</i>	<i>7/3</i>	<i>2/-</i>	<i>9/23</i>	<i>4/11</i>	<i>36/66</i>
<b>K2/K3</b>	-	<b>-/4</b>	<b>-/3</b>	-	-	<i>-/2</i>	<i>-/2</i>	-	-	-	<i>1/-</i>	<i>1/11</i>
<b>K3</b>	-	-	<i>-/1</i>	<i>12/29</i>	<i>7/22</i>	<i>7/14</i>	<i>6/2</i>	<i>6/19</i>	<i>7/22</i>	<i>-/1</i>	<i>5/9</i>	<i>49/119</i>
<b>K4 ? (K1/K3/K3)</b>	-	-	-	-	-	-	-	-	-	-	<i>-/2</i>	<i>-/2</i>
<b>Total</b>	<i>9/22</i>	<i>9/23</i>	<i>12/16</i>	<i>12/29</i>	<i>9/23</i>	<i>9/21</i>	<i>13/17</i>	<i>9/22</i>	<i>9/22</i>	<i>9/24</i>	<i>9/22</i>	<i>109/241</i>

**Table 6 Genotypes obtained in *Porites* and their species assignation.** Each genotype is designated by a number, an \* indicates this genotype was found in at least one reference individual.

Species assignation	Genotypes	
	N°	SNPs
<b>K1</b>	I	AAGAGCCTACCCGAGACGCGCAWCACGCATTCTAGRTTAAYTCTCTGCACACATGAGGT
	II	AAGAGCCTACCCGAGACGCGCAWCACGCATTCTAGATTAATTCTCTGCACACATGAGGT
	III	AAGAGCCTACCCGAGACGCGCAWCACGCATTCTAGGTAACTCTCTGCACACATGAGGT
	IV	AAGAGCCTACCCGAGACGCGCAWCACGCAKTCTAGGTAACTCTCYGCACACRTGRRGT
	V*	AAGAGCCTACCCGAGACGCGCAACACGCATTCTAGRTTAAYTCTCTGCACACATGAGGT
	VI*	AAGAGCCTACCCGAGACGCGCAACACGCATTCTAGATTAATTCTCTGCACACATGAGGT
<b>K2</b>	VII*	AAAGAGAAGACTTATGCACGCATTTCGATGGCTTCTGCCGACACACCAGCACCGCGGAGT
	VIII*	AAAGAGAAGACTTATGCACGCATTTCGRTGGCTTCTGCCGACWCACCAGCACCGCGGAGT
	IX	AAAGAGAAGACTTATGCACGCATTTCGGTGGCTTCTGCCGACTCACCAGCACCGCGGAGT
	X	AAAGAGAAGACTTATGCACGCATTMGRTGGCTTCTGCYRRCWYASCRGCCCCGTGGAGT
	XI	AAAGAGAAGACTTATGCACGCATTTCGRTGGCTTCTGCCGACACACCAGCACCGCGGAGT
	XII	AAAGAGAAGACTTATGCACGCATTMGRTGGCTTCTGCYRACACACCAGCMCCGYGGAGT
<b>K2/K3</b>	XIII	AAAGAGAAGAYTKRTRCRYGCRRTTMSRYRKYTYTMKGYRRCWYASCRGCMCYGCKGAKW
	XIV	WRAGAGAAGAYTKRTRMRYRYATTMSATGGCYCTGYRRCWYASCRGCMCYGCKGAKW
	XV	AAAGAGAAGAYTKRTRCRYGCRRTMSGYRKYTYTCTGYRRCWYASCRGCMCYGCKGAKW
<b>K3</b>	XVI*	TGAGAGAAGATTGGTAAGTATATTACATGGCCCCTGTTAGCTTAGCGGCCCTGCTGATA
	XVII	AAAGAGAAGATTGGTACGTGCGTTACGCATTCTATGTTAGCTCAGCGGCCCTGCTGATA
	XVIII	WRAGAGAAGATTGGTAMGTRYRTTACRYRKYCYMTGTTAGCTYAGCGGCCCTGCTGATA
	XIX	WRAGAGAAGATTGRTAMGTRYRTTACRYRKYCYMKGTTAGCTTAGCGGCCCTGCTGATA
	XX	AAAGAGAAGCCTGGTACGTGCATTACGTGGCCCCTGTTAGCTTAGCGGCCCTGCTGATA
	XXI	AAAGAGAAGATTGGTAAGTGCATTACGTGGCCCCTGTTAGCTTAGCGGCCCTGCTGATA

	XXII	TGAGAGAAGATTGGTAMGTRYATTACATGGCCCCTGTTAGCTTAGCGGCCCTGCTGATA
	XXIII	AAAGAGAAGATTGGTACGTGCGTTACGCATTCTMTGTTAGCTYAGCGGCCCTGCTGATA
	XIV	WRAGAGAAGATTGGTAMGTRYRTTACRYRKYCYMKGTTAGCTTAGCGGCCCTGCTGATA
	XXV	TGAGAGAAGATTGGTAAGTRYATTACATGGCCCCTGTTAGCTTAGCGGCCCTGCTGATA
	XXVI	TGAGAGAAGATTGGTAMGTGCATTACATGGCCCCTGTTAGCTTAGCGGCCCTGCTGATA
<b>K4?(K1/K2/K3)</b>	XXVII	AAGGACAAACCTKRTRMRYRYATTACGCAGCCTCGGTAACTCACCGGCCATGTGGAGT
	XXVIII	AAGGACAAACCTGATACATGCATTACGCAGCCTAGGTAACTCACCGGCCACGTGGAGT

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**Fig. 1** Positions of External and Nested primers and sequences produced for a) *Pocillopora* contig 1 from bp 7,013,343 to bp 7,014,460, b) *Porites* contig 239 from bp 279,979 to bp 282,033. External PCR primers had to be defined slightly out of the genomic zone tested in silico due to the presence of small indels/SNPs close to the extremities of these zones.

a) *Pocillopora*



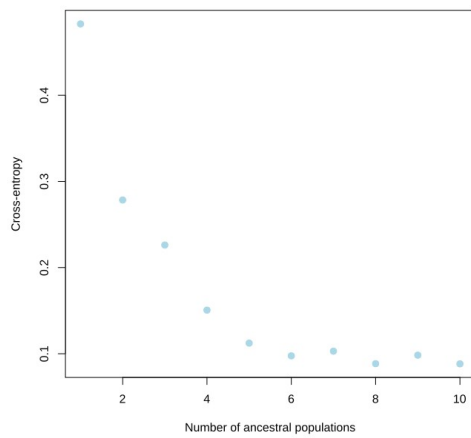
b) *Porites*



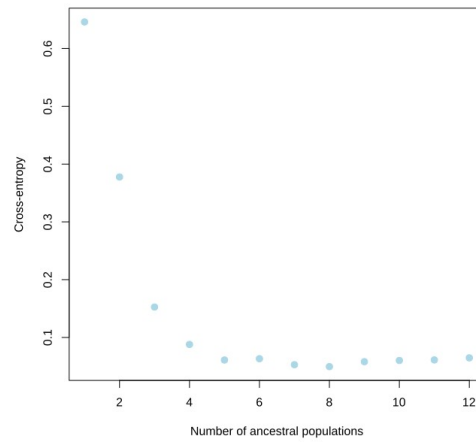


**Fig. 2 sNMF Entropy graphs.** These graphs identify the optimal number of ancestral populations (*i.e.* clusters) to which the individual sequences are assigned. a) *Pocillopora*. The optimal number of ancestral populations was  $K=5$ , which corresponded to the number of species identified by genome wide analysis; b) *Porites*. Here the optimal number of ancestral populations was  $K=4$ , when three species were detected by genome wide analysis. However, the third and fourth clusters were totally merged when forcing a  $K=3$  clustering.

a) *Pocillopora*



b) *Porites*



## Annex

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#### **Colombia :**

We are keen to thank the commitment of the people of Colombia and the following local institutions for their administrative and scientific support that made this singular expedition possible : the Prime Minister Office and their teams; the Ministry of Environment and National Natural Parks of Colombia with Guillermo Alberto Santos Ceballos, Edna Carolina Jarro Fajardo, and their teams; la Fundacion Malpelo y Otros Ecosistemas Marinos with their Executive Director and Founder Sandra Bessudo, with Felipe Orlando Ladino and their teams; el Santuario de Fauna y Flora Malpelo and their teams; la Direccion General Maritima with Ivan Fernando Castro Mercado and their teams; The Pacific Territorial Directorate and their teams; the Colombian biodiversity information system and their teams; and all other locally supports.

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