

# 1 **Hybridization and cryptic speciation in the Tropical Eastern Pacific octocoral genus *Pacifigorgia***

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## 13 **Abstract**

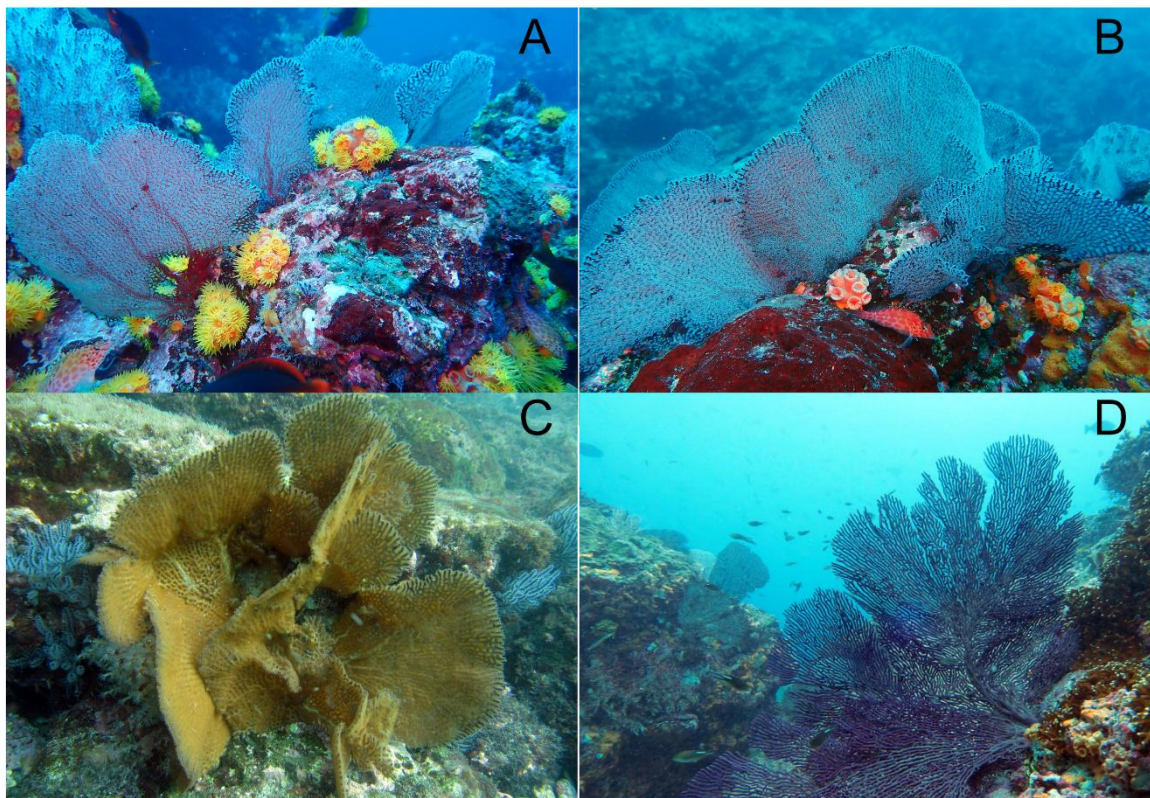
14 The shallow waters of the Tropical Eastern Pacific (TEP) harbor a species-rich octocoral fauna, with  
15 seven genera and 124 octocoral species described to date for the region. Of these lineages,  
16 *Pacifigorgia*, with 35 species, is by far the most speciose and abundant shallow-water octocoral  
17 occurring in the region. The speciation mechanisms resulting in this remarkable diversity remain  
18 speculative, despite the extensive taxonomic and molecular systematic research conducted so far in  
19 the TEP. Using genome-wide SNP markers, we provide evidence for hybridization and extensive  
20 cryptic speciation in *Pacifigorgia*, suggesting that the genus' diversity has been underestimated by  
21 traditional and molecular systematic research. Our study highlights the difficulties faced by both  
22 traditional taxonomy and single-marker based molecular approaches to characterize octocoral  
23 diversity and evolution, and the role genome-wide molecular studies coupled to morphological  
24 research play to advance our understanding of this group.

## 25 **Introduction**

26 Among Tropical Eastern Pacific (TEP) octocorals, *Pacifigorgia* (Fig. 1) represents the most species-rich  
27 genus with about 35 valid species described to date (Breedy and Guzman, 2003, 2002; Guzman and  
28 Breedy, 2012). About two-thirds *Pacifigorgia* species, including at least six endemic species restricted  
29 to the shallow waters of the Gulf of Chiriquí, Panama, occur in the Panamic province (from ~16° N to  
30 ~3° N) (Guzman et al., 2004; Vargas et al., 2008). This marked increase in species number toward  
31 lower latitudes in *Pacifigorgia* is consistent with the latitudinal diversity gradient reported for  
32 shallow-water eastern Pacific octocorals (Núñez-Flores et al., 2019) and likely drives it. Thus,  
33 clarifying the mechanisms of speciation that resulted in the high diversity of *Pacifigorgia* in the  
34 Panamic province is pivotal to understand how evolutionary processes shaped the octocoral diversity  
35 patterns observed in the region.

36 In contrast to most octocoral genera, *Pacifigorgia* has been the subject of intense morphological and  
37 molecular research. Breedy and Guzman (2002) thoroughly revised the genus and later described  
38 many new species (Breedy, 2001; Breedy and Guzman, 2004, 2003; Breedy and Guzmán, 2003;  
39 Guzman and Breedy, 2012). Molecular phylogenetic studies of *Pacifigorgia* are also available, yet  
40 species-level relationships within *Pacifigorgia* remain poorly resolved due to the lack of resolution of  
41 both mitochondrial (i.e., mtMutS) and nuclear markers (e.g., 28S rDNA) at this level (Ament-  
42 Velásquez et al., 2016; Soler-Hurtado et al., 2017; Vargas et al., 2014).

43 Despite the difficulties faced studying the diversification process in *Pacifigorgia* and other eastern  
44 Pacific octocorals (e.g., *Leptogorgia* and *Eugorgia*), some patterns arise from the molecular  
45 phylogenies available for the group. For instance, Ament et al. (2016) and Soler-Hurtado et al. (2017)  
46 proposed that hybridization could explain the mito-nuclear conflicts found in several eastern Pacific  
47 octocoral (holaxonian) genera. However, those authors' inability to exclude other processes resulting  
48 in similar branching patterns, such as incomplete lineage sorting after rapid diversification events,  
49 left those claims mostly speculative. Similarly, hypotheses on cryptic speciation within *Pacifigorgia*  
50 are most likely affected by the resolution and the number of phylogenetic markers used, and by the  
51 differences in taxon-sampling across phylogenies inferred using different markers (Ament-Velásquez  
52 et al., 2016; Soler-Hurtado et al., 2017; Vargas et al., 2014). Thus, the contribution of these processes  
53 to the diversification of eastern Pacific octocorals remains to be determined.



54

55 **Fig. 1:** *In situ* photographs of A) *Pacifigorgia cairnsi*, B) *Pacifigorgia rubicunda*, C) *Pacifigorgia firma*, and D)  
56 *Pacifigorgia stenobrochis*. Photo credits: *P. cairnsi* and *P. rubicunda* Kike Ballesteros, *P. firma* Jaime Nivia, *P.*  
57 *stenobrochis* Kevan Mantell.

58 Here, we use genome-wide, Single Nucleotide Polymorphisms (SNPs) and a collection of widespread  
59 and locally restricted *Pacifigorgia* species from the Gulf of Chiriquí, Panama, a biodiversity hot-spot  
60 for this genus (Guzman et al., 2004; Guzman and Breedy, 2008), to assess the contribution of  
61 hybridization and cryptic speciation to the diversification process in *Pacifigorgia*. We detected  
62 hybridization events and several instances of cryptic speciation among the *Pacifigorgia* species  
63 sampled. Our results provide conclusive evidence for reticulation among eastern Pacific octocorals  
64 and pose new challenges for better studying the diversity and distribution of these organisms.

## 65 **Materials and Methods**

66 We collected by SCUBA diving 82 specimens belonging two genera and ten species (*P. bayeri*, *P.*  
67 *cairnsi*, *P. eximia*, *P. ferruginea*, *P. firma*, *P. rubicunda*, *P. smithsoniana*, *P. stenobrochis*, and  
68 *Leptogorgia pumila* and *Leptogorgia taboguillae*) from six different localities in the Coiba National

69 Park, Panama (S. Fig. 1). Sampling depths ranged from 8 m to 24 m. Upon sampling, we sorted and  
70 morphologically identified all specimens in the field before preserving them in absolute ethanol until  
71 further processing.

72 We extracted gDNA using a standard CTAB protocol (Porebski et al., 1997), and quality controlled the  
73 extracts on 1.5% agarose gels. We checked the yield and purity of the extracts using a NanoDrop  
74 2100. If needed, we digested RNA with RNase A and cleaned the resulting RNA-free extracts using a  
75 standard sodium acetate-ethanol precipitation. Of the 82 specimens extracted, only 40 yielded high  
76 molecular weight DNA. We used these specimens to prepare reduced representation libraries  
77 following the Genotyping-by-Sequencing (GBS) protocol of Elshire et al. (2011). Briefly, for each  
78 specimen, we digested ~150 ng of gDNA with ApeKI for two hours at 75°C and ligated the resulting  
79 fragments (one hour at 22°C) to one “common” and one barcoded adapter. We stopped the ligation  
80 reaction by heating the samples at 65°C for 30 minutes and we amplified (15 cycles; annealing  
81 temperature of 65°C and extension time of 30s) the adapter-ligated fragments using a universal non-  
82 barcoded primer (GBS\_PrimerA) and a different barcoded primer for each sample. We purified the  
83 PCR products using 1.1 volume Agencourt AMPure XP beads (Beckman Coulter, Inc.) and quantified  
84 them using a Qubit® 2.0 fluorometer with a dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA). Before  
85 sequencing, we pooled the libraries at equimolar concentrations and performed a final quality check  
86 using a Bioanalyzer 2100 (Agilent, Santa Clara, CA). The library pool was adjusted at a concentration  
87 of 10nM and sequenced on two lanes of an Illumina HiSeq 2500 (Illumina, San Diego, CA) using 100-  
88 bp single-end chemistry. The raw sequence reads are available under study accession PRJEB44220.

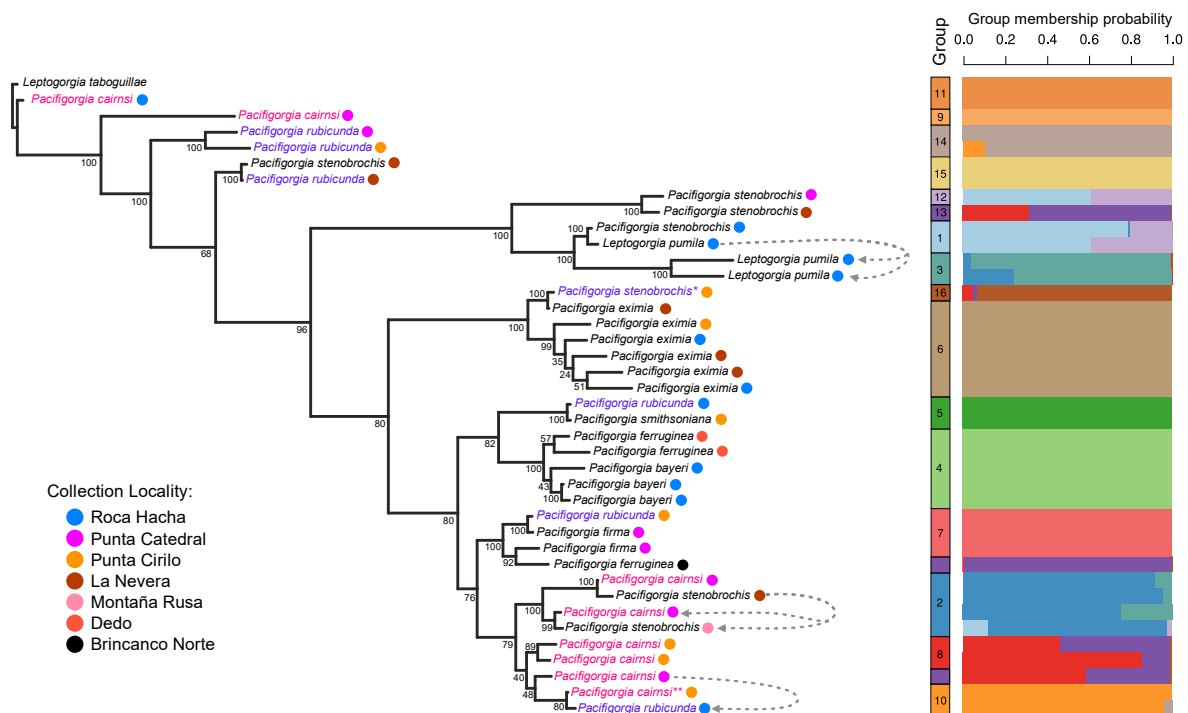
89 We demultiplexed, quality controlled, filtered, and trimmed to 80-bp the ~300x10<sup>6</sup> sequence reads  
90 obtained from the two HiSeq lanes. We determined SNPs *de novo* with IPyRAD (Eaton and Overcast,  
91 2020) using a clustering threshold of 0.85, maximum 20 SNPs and eight gaps per loci, and a minimum  
92 depth of six reads for base calling. These parameters have been successfully used in previous studies  
93 using RAD-Seq on octocorals (Herrera and Shank, 2016; e.g., Pante et al., 2015; Quattrini et al., 2019).  
94 For phylogenetic inference, we first produced an alignment containing loci present in at least 10% of  
95 the taxa (i.e., four specimens) and then discarded columns with >20% gaps to produce an alignment  
96 containing 122,464 sites present in at least 32 of 40 specimens. We used this alignment to infer a  
97 maximum likelihood phylogeny in the program RAxML v8.2.12 (single partition GTRGAMMA+F, 1000  
98 fast bootstrap replicates, Stamatakis, 2014). We estimated a SNP matrix using the same parameters  
99 described above but including only those SNPs present in at least 50% of the taxa (i.e., 20  
100 specimens). We used this matrix to find specimen groups in a phylogenetic independent way using  
101 Discriminant Analysis of Principal Components (DAPC) analyses (Jombart et al., 2010). We used the  
102 package *adegenet* (Jombart, 2008), 10 Principal Components, and the method *find.clusters* to select  
103 the most probable number of species groups via BIC. For each specimen, we also calculated its group  
104 assignment probability. To test for hybridization in *Pacifigorgia*, we used IPyRAD to conduct ABBA-  
105 BABA tests on the best maximum likelihood phylogeny and the SNPs dataset estimated for  
106 phylogeny-independent analyses. The data matrices are publicly available at  
107 <https://gitlab.lrz.de/palmuc/pacifigorgia-gbs>

## 108 Results and Discussion

109 Previous molecular phylogenetic studies of eastern Pacific octocorals recovered *Pacifigorgia*  
110 monophyletic but could not resolve its species-level relationships (Ament-Velásquez et al., 2016; Soler-  
111 Hurtado et al., 2017; Vargas et al., 2014). In contrast, we recovered a maximum likelihood phylogeny  
112 generally showing branch support values >75% (Fig. 2) and clades corresponding to recently revised  
113 and described *Pacifigorgia* species, such as *P. eximia* and *P. bayeri*. These species are morphologically  
114 well-defined and can be accurately identified in the field and laboratory (Breedy and Guzman, 2004,  
115 2003). Accordingly, these species belonged to clearly defined DAPC groups, and we could not detect

116 introgression between these species and specimens of other *Pacifigorgia* clades. Thus, our genome-  
 117 wide GBS data further support their species-level status.

118 Our phylogeny also recovered well-supported clades grouping individuals assigned to different  
 119 morphologically defined species. For instance, we detected a group composed of individuals assigned  
 120 to *P. stenobrochis* and *Leptogorgia pumila* (Fig. 2). Ament et al. (2016) also found this same grouping  
 121 using partial mtMutS and 28S rDNA sequences and proposed to transfer *L. pumila* to *Pacifigorgia*.  
 122 However, such taxonomic decision requires the amendment of *Pacifigorgia*'s diagnosis to include non-  
 123 anastomosing or loosely anastomosing species, an act that will let the genus so poorly defined that it  
 124 could, in principle, include all *Leptogorgia* species; hitherto described. Within this clade, DAPC assigned  
 125 *L. pumila* to groups one and three and *P. stenobrochis* specimens to groups one, twelve, and thirteen  
 126 (Fig. 2). Groups one and twelve likely are in admixture; *P. stenobrochis* specimens have high group  
 127 membership probability for either group. Group thirteen appears to encompass specimens with high  
 128 levels of missing data in the SNP matrix (S. Fig 2) and should be taken cautiously. We detected  
 129 introgression between all specimens identified morphologically as *L. pumila* (Fig. 2 and S. Fig. 3) despite  
 130 the phylogenetic tree pointing to a closer phylogenetic relationship between some *P. stenobrochis* and  
 131 *L. pumila* specimens and the assignment of *L. pumila* specimens to different DAPC groups.



132  
 133 **Fig. 2:** Maximum likelihood phylogeny of *Pacifigorgia* based on 122,464 genome-wide SNP markers. Numbers below  
 134 the branches represent bootstrap support values. *Pacifigorgia cairnsi* and *Pacifigorgia rubicunda*, two widespread  
 135 species, are highlighted in light red and purple blue, respectively. Gray dashed arrows indicate introgression events  
 136 detected using ABBA-BABA tests on the maximum-likelihood topology. On the right, group assignments and group  
 137 membership probabilities of different *Pacifigorgia* specimens obtained using DAPC of the SNPs matrix. \**P.*  
 138 *stenobrochis* and \*\**P. cairnsi* colonies with anomalous sclerome and DAPC group assignments.

139 Similarly, we detected a well-supported clade grouping specimens of *P. cairnsi* and *P. stenobrochis* (Fig.  
 140 2). DAPC assigned all four specimens included in this clade to the same group, and we detected  
 141 introgression between them (S. Fig. 4). These results indicate that this group's specimens, although  
 142 assigned to different morphological species, are in admixture. Previous DNA barcoding studies also

143 revealed multiple *P. stenobrochis* clades in the eastern Pacific (Vargas et al., 2014). We recovered four  
144 not closely-related clades and an equal number of DAPC groups including *P. stenobrochis* specimens,  
145 further pointing to the existence of cryptic lineages within this species. Some of these clades and  
146 groups include “typical” *P. stenobrochis* individuals while other consist of specimens differing in colony  
147 shape or spiculation from this species’ type (e.g. DAPC Group 16 *P. stenobrochis*). Despite its very  
148 characteristic colony morphology, *P. stenobrochis* displays some variation in the mesh, and two  
149 recognized color morphs can be observed in the same colony. The species’ sclerome is also variable,  
150 with either spindles or blunt spindles as the dominant sclerite form (Breedy and Guzman, 2004, 2002).  
151 However, in phenotypically very plastic organisms like octocorals (Calixto-Botía and Sánchez, 2017;  
152 Prada et al., 2008; West et al., 1993), it is hard to justify solely based on morphology the segregation  
153 of *P. stenobrochis* into multiple species with slightly different coloration, sclerome or colony shapes,  
154 or based on the occasional collection of specimens not fitting perfectly the *P. stenobrochis* gestalt. Our  
155 results indicate that a taxonomic reevaluation of *P. stenobrochis*’ different morphs is warranted and  
156 provide an integrative framework to morphologically describe new species leveraging high-resolution,  
157 genome-wide markers.

158 Our phylogenetic results supported clades including specimens of *P. rubicunda* and *P. cairnsi*, and of *P.*  
159 *rubicunda* and *P. stenobrochis* (Fig. 2). These clades also corresponded to DAPC groups (i.e., groups 10  
160 and 15). The morphology of the specimens included in these groups was typical; the specimen of *P.*  
161 *cairnsi* had a somewhat divergent sclerome dominated by spindles. *Pacifigorgia rubicunda* is  
162 morphologically diverse, with colonies consisting of single fans or forming rosettes. This species  
163 coexists with *P. cairnsi* and *P. stenobrochis* throughout its geographic range (Breedy and Guzman,  
164 2003, 2002). We found evidence for introgression between *P. cairnsi* and *P. rubicunda* (Fig. 2 and S.  
165 Fig. 5), which are morphologically different. However, this hybridization event involves one *P. cairnsi*  
166 specimen assigned to group thirteen, a group joining mainly specimens with many missing SNPs, and  
167 a clade with internal low bootstrap support values (Fig. 2). Therefore, this introgression event should  
168 be taken cautiously. Beside these mixed clades, we found highly supported clades of *P. cairnsi* and *P.*  
169 *rubicunda* specimens collected in different localities interspersed in the tree (Fig. 2). DAPC also  
170 supported these clades (e.g., group 14). We could not detect introgression for these clades, which  
171 likely represent cryptic lineages within *P. cairnsi* and *P. rubicunda*, two species with a wide geographic  
172 distribution (Breedy and Guzman, 2003).

173 We observed a similar phenomenon for the specimens of *P. firma* included in the analyses, which did  
174 not form a clade but grouped with specimens of *P. rubicunda* and *P. ferruginea* (Fig. 2). Except for *P.*  
175 *ferruginea*, DAPC assigned this clade’s specimens to group seven. We could not find any evidence for  
176 introgression within this clade, suggesting that *P. ferruginea* and *P. firma* currently include multiple  
177 evolutionary lineages (S. Fig. 6). *Pacifigorgia ferruginea* is endemic to the Gulf of Chiriqui and generally  
178 easy to identify in the field by its characteristic rusty appearance. In our phylogenetic analysis, *P.*  
179 *ferruginea* specimens from Dedo did not group with the single specimen collected in the nearby  
180 Brincanco Norte island, suggesting that cryptic species can occur within potentially very short  
181 geographic scales in *Pacifigorgia*. However, given the assignment of the single specimen of *P.*  
182 *ferruginea* from Brincanco Norte to DAPC group thirteen and a large number of missing SNP loci in this  
183 specimen, this interpretation should be corroborated in future studies. *Pacifigorgia firma* is widely  
184 distributed and morphologically variable (Breedy and Guzman, 2003). Despite its morphological  
185 plasticity and the continuous nature of most characters used to define the species, *P. firma* can be  
186 accurately determined. The finding of specimens morphologically assigned to *P. rubicunda* within a  
187 highly supported *P. firma* clade and DAPC highlights the challenges in establishing a morphological-  
188 molecular classification of *Pacifigorgia*. In particular, in morphologically highly variable and widespread  
189 species such as *P. rubicunda*, defining the extent to which hybridization contributes to the generation

190 of new *Pacifigorgia* lineages and the molecular and morphological boundaries of those species remains  
191 to be determined.

192 Hybridization occurs in several scleractinian coral and octocoral lineages and is thought to be an  
193 important speciation mechanism in these groups (Mao, 2020). In scleractinian corals, hybridization  
194 between species with overlapping distribution ranges explains the large number of morphological  
195 species observed in important West and East Pacific reef-builders such as *Acropora* (Van Oppen et al.,  
196 2002) and *Pocillopora* (Combosch and Vollmer, 2015), respectively. In octocorals, McFadden and  
197 Hutchinson (2004) found evidence for hybridization in *Alcyonium* and Quattrini et al. (2019) found  
198 evidence of both morphological diversification in the absence of molecular divergence and  
199 hybridization in *Sinularia*. In this genus, local hybrids can become highly abundant, replacing their  
200 parental lines and changing community composition overtime (Slattery et al., 2008). Thus, other than  
201 evolutionary dead-ends, coral and octocoral hybrids represent evolutionary experiments capable of  
202 affecting the entire ecosystem. In the specific case of *Pacifigorgia*, the poor congruence between some  
203 morphological species and molecular groups we inferred using an extensive, genome-wide marker set  
204 indicates that genotyping specimens throughout a species' geographic range is necessary to unveil  
205 morphologically cryptic and hybrid *Pacifigorgia* lineages and uncover the "true" diversity of this  
206 species-rich genus. Additionally, identifying differences in the reproductive cycles and strategies  
207 among different *Pacifigorgia* species and their populations is of crucial importance for better linking  
208 the molecular results with the reproductive ecology and natural history of these organisms (Gomez et  
209 al., 2018). In conjunction, our study suggests that the diversity of eastern Pacific *Pacifigorgia* is larger  
210 than currently recognized. Neutral processes such as the mid-domain effect cannot explain the  
211 diversity patterns observed for eastern Pacific octocorals (Núñez-Flores et al., 2019). Our data indicate  
212 that hybridization and cryptic speciation shape *Pacifigorgia's* diversification history and likely are the  
213 drivers of the octocoral diversity patterns observed in the Tropical Eastern Pacific.

214

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227

## 228 **Author contributions**

229 **AP:** Investigation, Formal Analysis, Data curation, Visualization, Writing – Original Draft. **OB:**  
230 Conceptualization, Investigation, Resources, Writing – Review & Editing, Project Administration.  
231 **HMG:** Resources, Funding Acquisition, Writing – Review & Editing, Project Administration. **SV:**  
232 Conceptualization, Methodology, Formal Analysis, Visualization, Writing – Original Draft, Resources,  
233 Supervision, Project Administration, Funding Acquisition.

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