



From genomics to integrative species delimitation? The case study of the Indo-Pacific *Pocillopora* corals



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ABSTRACT

With the advent of genomics, sequencing thousands of loci from hundreds of individuals now appears feasible at reasonable costs, allowing complex phylogenies to be resolved. This is particularly relevant for cnidarians, for which insufficient data is available due to the small number of currently available markers and obscures species boundaries. Difficulties in inferring gene trees and morphological incongruences further blur the study and conservation of these organisms. Yet, can genomics alone be used to delimit species? Here, focusing on the coral genus *Pocillopora*, whose colonies play key roles in Indo-Pacific reef ecosystems but have challenged taxonomists for decades, we explored and discussed the usefulness of multiple criteria (genetics, morphology, biogeography and symbiosis ecology) to delimit species of this genus. Phylogenetic inferences, clustering approaches and species delimitation methods based on genome-wide single-nucleotide polymorphisms (SNP) were first used to resolve *Pocillopora* phylogeny and propose genomic species hypotheses from 356 colonies sampled across the Indo-Pacific (western Indian Ocean, tropical southwestern Pacific and south-east Polynesia). These species hypotheses were then compared to other lines of evidence based on genetic, morphology, biogeography and symbiont associations. Out of 21 species hypotheses delimited by genomics, 13 were strongly supported by all approaches, while six could represent either undescribed species or nominal species that have been synonymised incorrectly. Altogether, our results support (1) the obsolescence of macromorphology (i.e., overall colony and branches shape) but the relevance of micromorphology (i.e., corallite structures) to refine *Pocillopora* species boundaries, (2) the relevance of the mtORF (coupled with other markers in some cases) as a diagnostic marker of most species, (3) the requirement of molecular identification when species identity of colonies is absolutely necessary to interpret results, as morphology can blur species identification in the field, and (4) the need for a taxonomic revision of the genus *Pocillopora*. These results give new insights into the usefulness of multiple criteria for resolving *Pocillopora*, and more widely, scleractinian species boundaries, and will ultimately contribute to the taxonomic revision of this genus and the conservation of its species.

Abbreviations: BF, Bayes factor; dAPC, discriminant analysis of principal components; FAMD, factorial analysis of mixed data; GSH/PSH/SSH, genomic/primary/secondary species hypothesis; HPD, highest posterior density; ITS, internal transcribed spacer; ML, maximum likelihood; MLE, marginal likelihood estimation; MST, minimum spanning tree; Mya, million years ago; NMDS, nonmetric multidimensional scaling; ORF, open reading frame; OTU, operational taxonomic unit; RADseq, restriction-site associated DNA sequencing; SEM, scanning electron microscopy; SEP, south-east Polynesia; SNP, single-nucleotide polymorphism; TSP, tropical southwestern Pacific; UCE, ultra-conserved element; WIO, western Indian Ocean.

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1. Introduction

Efficiently protecting species implies understanding their life history traits and functioning, thus requiring accurate species delimitation. This is particularly crucial for scleractinian corals, the cornerstone of coral reefs, which are experiencing critical decline worldwide (Heron et al., 2018; Hughes et al., 2019, 2018, 2017), due both to local (e.g., coastal development, over-fishing, pollution) and global (e.g., climate change) threats.

Coral taxonomy initially relied on skeleton morphological traits (i.e., *corallum* macromorphology and coralite microstructure; e.g., Dana, 1846; Ehrenberg, 1834; Ellis and Solander, 1786), but phenotypic plasticity and convergence hamper reliable species delimitation on this basis (see Todd, 2008). More recently, molecular approaches have been used to explore species boundaries, revealing the incongruences of conventional systematics within many scleractinian genera (e.g., Arrigoni et al., 2020; Cunha et al., 2019; Gélin et al., 2017b; Keshavmurthy et al., 2013; Schmidt-Roach et al., 2014). Nuclear internal transcribed spacers (ITS) and mitochondrial markers have been extensively used in phylogenetic inferences (e.g., Benzoni et al., 2007; Gélin et al., 2017b; Nakajima et al., 2017). However, intra-individual and intra-specific variations for the formers (Chen et al., 2004; van Oppen et al., 2000; Vollmer and Palumbi, 2004), and relatively slow evolutionary rates for the latter (Hellberg, 2006; Shearer et al., 2002; van Oppen et al., 1999), make these markers usually not informative for species delimitation in most genera (e.g., Forsman et al., 2009; Terraneo et al., 2016). The resulting polyphylyies were in turn attributed to hybridisation (Combosch et al., 2008; Richards et al., 2008; Willis et al., 2006), introgression (Combosch and Vollmer, 2015; Hellberg et al., 2016) or incomplete lineage sorting (Fukami et al., 2008; van Oppen et al., 2001). However, the poor performance of the genetic markers or the incorrect species identification could be the main reasons for the observed inconsistencies (see Ramírez-Portilla et al., 2022 and references therein).

The recent development of high-throughput sequencing technologies now enables the cost-effective target of large numbers of loci from hundreds of individuals from virtually any species (Metzker, 2010). These methods appear particularly promising to resolve complex phylogenies such as those involving scleractinian corals (e.g., Arrigoni et al., 2020; Cunha et al., 2019; Forsman et al., 2017). In particular, restriction-site associated DNA sequencing (RADseq; Baird et al., 2008) and target-capture (also called target-enrichment sequencing or sequence-capture; Gnarke et al., 2009; Hedges et al., 2007) are increasingly used in both population genetics and phylogenetic studies (see Quek and Huang, 2022 for a review in anthozoans). While RADseq typically generates datasets of anonymous loci, target-capture enables the deep sequencing of previously identified loci of interest, but needs existing genomic resources to design probes (Davey et al., 2011; Harvey et al., 2016). When such genomic resources are unavailable for the species of interest, probes from genomic regions that are conserved across divergent taxa [e.g., ultraconserved elements (UCE); <https://www.ultraconserved.org/>] can be used (Cowman et al., 2020; Quattrini et al., 2018).

Additionally, scleractinians host diverse symbiont communities (Brener-Raffalli et al., 2018; Cunning et al., 2017; Li et al., 2021; Rabani et al., 2021). Metabarcoding approaches can thus be used to characterise these communities and refine species boundaries. In particular, some coral species (including *Pocillopora* spp.; Harii et al., 2002; Hirose et al., 2001; Sier and Olive, 1994) transmit Symbiodiniaceae maternally (vertical transmission). This could result in species-specific associations and co-evolutions (Johnston et al., 2022; Pinzón and LaJeunesse, 2011; Schmidt-Roach et al., 2012), and could also be responsible for habitat specialisations (driven by symbiont thermotolerance and photosynthetic needs; Baker et al., 2013; Brener-Raffalli et al., 2018; Jokiel and York Jr., 1982; Ros et al., 2021). Characterising associated Symbiodiniaceae communities can therefore bring additional elements to the delimitation of scleractinian species (e.g.,

Arrigoni et al., 2016; Bongaerts et al., 2010; Forsman et al., 2020; Keshavmurthy et al., 2013; Warner et al., 2015).

The coral genus *Pocillopora* Lamarck, 1816 (Scleractinia, Pocilloporidae) represents a key component of coral reef ecosystems from the Indo-Pacific and the Red Sea (Veron, 2000). Its branching colonies are abundant and sometimes the main bio-constructors (e.g., Benzoni et al., 2003). However, its taxonomy remains challenging, and the extraordinary range of morphological diversity among its colonies has led to the description of >40 nominal species over the past two centuries (Hoeksema and Cairns, 2022). Later, based on morphological characters (shape and organisation of branches and verrucae for *Pocillopora*) and ecological evidence (e.g., depth, swell exposure), Veron (2000) recognised only 17 *Pocillopora* species, assuming that some of the species previously described actually represent ecomorphs of the same species. Recent genetic studies (see Gélin et al., 2017b for a review) revealed the presence of distinct genetic lineages within Veron (2000) morphospecies, indicating that much of this lumping was incorrect and does not reflect the taxonomic diversity within *Pocillopora*. The so-called *P. damicornis* was for example disentangled in five genetic lineages (Schmidt-Roach et al., 2012), *a posteriori* defined as five distinct species, some having been resurrected (Schmidt-Roach et al., 2014). Following this last taxonomic revision of the genus, 21 *Pocillopora* nominal species are currently considered valid according to the world register of marine species (WoRMS; Hoeksema and Cairns, 2022).

Using species delimitation methods based on sequence data from colonies sampled in three marine provinces (western Indian Ocean, tropical southwestern Pacific and south-east Polynesia), Gélin et al. (2017b) defined 16 primary species hypotheses (PSH sensu Pante et al., 2015) within the genus *Pocillopora*. Some of these PSHs corresponded to species currently considered valid, but others did not and would therefore represent either undescribed species or nominal species that have been synonymised incorrectly. Species boundaries were then refined using microsatellite markers and genetic assignment tests performed at the genus level, leading to the definition of secondary species hypotheses (SSH sensu Pante et al., 2015). Some PSHs were thus partitioned into several SSHs, themselves partitioned into several divergent but sympatric genetic clusters when performing genetic assignment tests at the SSH level (Gélin et al., 2017a, 2017b, 2018a, 2018b; Oury et al., 2020a, 2021, 2022). This genetic partitioning questions species boundaries and shelves taxonomic uncertainties for which traditional genetic markers do not provide enough resolution. So far, only two studies (Johnston et al., 2017, 2022) have inferred phylogenetic relationships among *Pocillopora* species using high-throughput sequencing data (ezRAD). They resolved clear monophyletic groups coinciding with previously published mitochondrial clades based on the so-called open reading frame marker (mtORF; corresponding to the *tmp362* gene; Banguera-Hinestrosa et al., 2019). Species-specific associations with Symbiodiniaceae of the genus *Cladocodium* were also revealed (Johnston et al., 2022). However, the sampling size was relatively small (13 and 55 samples from seven morphospecies, respectively), such as the sampling area (restricted to the Pacific), thus missing a huge part of the taxonomic diversity of this genus.

Here, we used target-capture of UCEs and exon loci to collect single-nucleotide polymorphisms (SNP) from 356 *Pocillopora* colonies corresponding to a subset of Gélin et al. (2017b) sampling. These colonies represent the totality of the PSHs, SSHs and clusters previously identified (see Gélin et al., 2017a, 2017b, 2018a, 2018b; Oury et al., 2020a, 2021, 2022), as well as all morphotypes sampled. Maximum-likelihood and Bayesian phylogenetic inferences, clustering approaches and species delimitation methods based on SNP data were applied to resolve the *Pocillopora* phylogeny and define genomic species hypotheses, which were compared to previous genetic partitioning of the genus (i.e., the PSHs, SSHs and clusters previously defined based on the mtORF marker and microsatellites). Genetic evidence was then compared to other lines of evidence (macro- and micromorphology, biogeography and associated Symbiodiniaceae communities), to propose an integrative

delimitation of *Pocillopora* species. The usefulness of each criterion and its integration were then discussed.

2. Materials and methods

Detailed materials and methods, including sampling, sequencing and analytical methods, are available in Appendices A-D.

2.1. Sampling

The sampling was the same as in Gélin et al. (2017b) and comprised ca. 9,000 *Pocillopora* colonies from various habitats, depths and morphotypes, from three marine provinces: the western Indian Ocean (WIO; 10 localities and > 80 sites prospected for ca. 5,000 colonies sampled), the tropical southwestern Pacific (TSP; 5 localities; 50 sites; ca. 4,000 colonies sampled) and the south-east Polynesia (SEP; 3 localities; 6 sites; ca. 250 colonies sampled). The sampling of the *Pocillopora* species diversity was as exhaustive as possible in the WIO and the TSP, without any *a priori* neither on morphology nor on the ecology of the colonies collected. Conversely, sampling in the SEP was intended for studying genetic connectivity (see Magalon et al., 2005) and targeted *P. meandrina*-like colonies. Fragments were preserved in 90% ethanol at room temperature, and deposited at Reunion Island University (Saint-Denis, Réunion Island).

All colonies were previously genotyped with 13 microsatellites. The mitochondrial ORF locus (mtORF) was also sequenced for a subset of all colonies (ca. 10%; see Gélin et al., 2017b for more details). Based on these genetic data (see, for example, Oury et al., 2021), each colony was assigned beforehand to a primary and a secondary species hypotheses (PSH and SSH, respectively; *sensu* Gélin et al., 2017b), and a cluster when appropriate. For this reason, we retained the nomenclature of Gélin et al. (2017b) and subsequent papers (Gélin et al., 2017a, 2017b, 2018a, 2018b; Oury et al., 2020a, 2021, 2022), including the numbering of mtORF haplotypes. However, hereafter to simplify the reading, PSHs that were not subdivided into several SSHs were designated SSHs, keeping their corresponding number (e.g., PSH01 switches to SSH01). These SSHs remain easily recognisable as no lowercase letter follows the number (the lowercase designating the different SSHs found within a subdivided PSH).

Out of this exhaustive sampling, we considered here a subset of 356 *Pocillopora* colonies (194 from the WIO, 152 from the TSP and 10 from the SEP; Fig. 1; Table A.1 in Appendix A). We aimed to maximise the genetic diversity explored by covering the totality of the localities, SSHs and clusters, independently from colonies morphology (a non-discriminant character in this genus). Four *Seriatopora hystrix* and four *Stylophora pistillata* colonies were also included as outgroups in the phylogenomic analyses [both species are Pocilloporidae, diverging from the genus *Pocillopora* in the middle-end Paleogene (42.7–28.4 Mya; Simpson et al. 2011)].

2.2. Molecular analyses

2.2.1. Sequencing and bioinformatics processing

All 364 colonies, plus eight sequencing replicates, were sequenced following a target enrichment protocol of 1,248 ultraconserved elements (UCE) and 1,385 exon loci (Cowman et al., 2020; see Appendix B for more details). The bioinformatics pipeline, from demultiplexed reads to final SNP datasets, including read mapping on *de novo* constructed reference sequences, is detailed in Appendix B. Three individuals were discarded due to too many missing data (>60%).

2.2.2. Phylogenomic analyses

All following analyses (detailed in Appendix B) were performed on two datasets, one keeping all filtered SNPs (hereafter, all-SNP dataset) and the other keeping one randomly chosen SNP per locus (hereafter, one-SNP dataset) to reduce the effect of linkage disequilibrium. Available published *Pocillopora* genomes [i.e., the *P. acuta* from Indonesia (Vidal-Dupiol et al., 2019), the *P. damicornis* from Panama (Cunning et al., 2018) and the *P. verrucosa* from central Red Sea (Buitrago-López et al., 2020)] were also included by retrieving the genotypes of the SNPs corresponding to each dataset. Phylogenetic relationships were investigated using maximum likelihood (ML) and Bayesian inferences with RAxML-NG v0.9.0 (Kozlov et al., 2019) and BEAST v2.6.3 (Bouckaert et al., 2019), respectively, both using the GTR + G model. To support the phylogenomic analyses and further explore the genetic partitioning of the datasets, several clustering approaches were used. First, assignment tests were performed with STRUCTURE v2.3.4 (Pritchard et al., 2000), sNMF (Frishot et al., 2014) and discriminant analyses of principal components (dAPC; Jombart et al., 2010). Signals of admixture were further investigated with NewHYBRIDS v1.1 (Anderson and Thompson, 2002). Second, Nei (1972) individual genetic distances were computed with the R v4.0.4 (R Core Team, 2021) library ‘StAMPP’ (Pembleton et al., 2013), and then used to build a minimum spanning tree (MST) and an unrooted equal-angle split network using EDENetworks v2.18 (Kivelä et al., 2015) and SplitsTree v4.15.1 (Huson and Bryant, 2006), respectively. Finally, the groups identified with all above analyses were named hereafter genomic species hypotheses (GSH). GSHs were compared to SSHs and clusters retrieved from microsatellite data (Gélin et al., 2017a, 2017b, 2018a, 2018b; Oury et al., 2020a, 2021, 2022), allowing to number the GSHs according to SSHs when congruent. F_{ST} (Weir and Cockerham, 1984) values were computed with ‘StAMPP’ (Pembleton et al., 2013) for each pair of GSHs, and the resulting matrix was clustered using the heatmap.2 function from the R library ‘gplots’ (Warnes et al., 2020).

We completed the set of mtORF by sequencing some of the colonies assigned to GSHs for which data were lacking. We further sequenced a subset of colonies for the PoCHistone, a recently discovered marker partly mapping to partial histone 3 genes from other cnidarians, which has previously enabled the identification of *P. grandis* (the senior synonym of *P. eydouxi*; Johnston et al., 2018). The same laboratory protocol and analyses as for the mtORF in Gélin et al. (2017b) were used for both markers (Appendix B).

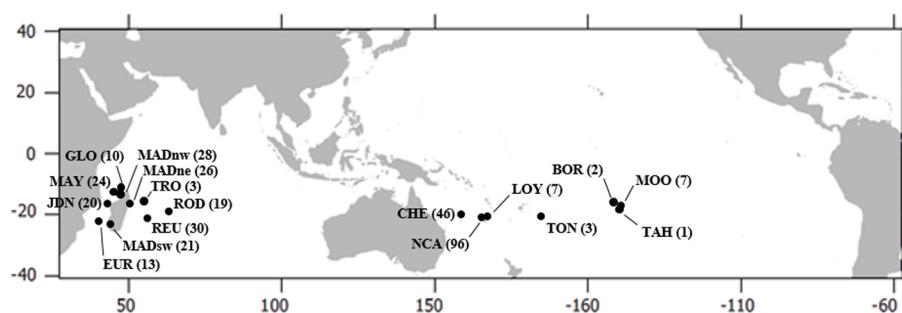


Fig. 1. Sampling localities of *Pocillopora* colonies (number of colonies considered in this study in parentheses). MAY: Mayotte, GLO: Glorioso Islands, JDN: Juan de Nova Island, EUR: Europa Island, MADne: northeastern Madagascar, MADnw: northwestern Madagascar, MADsw: southwestern Madagascar, REU: Reunion Island, ROD: Rodrigues Island, TRO: Tromelin Island, CHE: Chesterfield Islands, NCA: Grande Terre (New Caledonia), LOY: Loyalty Islands (New Caledonia), TON: Tonga Islands, BOR: Bora-Bora, MOO: Moorea and TAH: Tahiti.

2.2.3. Species delimitation analyses and divergence time estimation

Bayes factor delimitation with genomic data (BFD*; Leaché et al., 2014) was used to test several possible species delimitation models using the SNAPP package (Bryant et al., 2012) implemented in BEAST v2.6.3 (Bouckaert et al., 2019; details in Appendix B). To deal with computationally intensive demands from SNAPP, we first tested a batch of species delimitation scenarios to confirm the four main clades from the phylogeny. These were arbitrarily defined as monophyletic groups of individuals separated by branches of at least 0.4 nucleotide substitution per site on the ML tree. Three individuals per clade were sampled, considering only unlinked biallelic SNPs from the one-SNP dataset. We run for each scenario a path sampling method with 48 steps, each one consisting of 10^5 MCMC generations. We stored one generation every 10^3 , after a pre-burn-in of 10^3 , and discarded the first 80% of saved data. Model convergence was assessed with Tracer v1.7.1 (Rambaut et al., 2018) and models were compared and ranked using the Bayes factor (BF; Kass and Raftery, 1995). Once this first batch of scenarios tested and the four clades confirmed (see section 3.1.3), several possible species delimitation models were tested within each clade separately, from one single species to the number of GSHs found for each clade (Table B.3). SNAPP was run as above, and the best-supported model was retained for each clade.

For each of the best-supported model (except for Clade 1, constituted of a single GSH), a coalescent-based tree was calculated with SNAPP, keeping only two randomly drawn individuals per GSH to reduce the complexity in species tree estimation and to increase convergence. Chains were run for 10^7 iterations, sampling every 10^3 iterations. Convergence was checked with Tracer. Species trees that were contained in the 95% highest posterior density (HPD) set were then identified with SNAPP-TreeSetAnalyser v2.6.3 (Bryant et al., 2012). DensiTree v2.2.7 (Bouckaert, 2010) was used to visualise the posterior distribution of topologies as cladograms, hence allowing for a clear depiction of uncertainties in the topology.

Finally, GSH divergence times were estimated with the BEAST package SNAPPER v1.0.1 (Stoltz et al., 2021; Appendix B). The divergence between *Pocillopora* and outgroups (i.e., *S. hystrix* and *S. pistillata*; merged into one group) was constrained to the middle-end Paleogene (28.4–42.7 Mya; Simpson et al., 2011) with a lognormal distribution (offset = 0, $\mu = 36$, $\sigma = 0.1$). The most probable species tree identified with TreeSetAnalyser was used as starting tree. Chains were run for 10^7 iterations, sampling every 10^3 iterations, and checked for convergence and mixing with Tracer. TreeAnnotator v2.6.3 (Bouckaert et al., 2019) was used to generate the maximum clade credibility tree with median node heights.

2.3. Macro- and micromorphological analyses

In order to compare previously described morphospecies to GSHs (defined above), each colony was attributed a morphotype (or several when morphology was unclear), determined only by its *corallum* macromorphology [branch shape and thickness, size and uniformity of verrucae, and overall growth form as described in Veron (2000) and Schmidt-Roach et al. (2014)]. Morphotype identification was further assessed by sending a subset of photographs to three coral specialists (F. Benzoni, G. Faure and D. Obura).

A subset of 10 colonies per GSH were also randomly selected for micromorphological observations of the bleached skeletons (particularly of the corallite structures) using scanning electron microscopy (SEM). A collection of skeleton images was thus obtained for each specimen, and multiple measurements of seven quantitative variables (e.g., corallite and columella diameters; see Appendix C for details) were done with ImageJ2 (Rueden et al., 2017; <https://imagej.nih.gov/ij/>). A non-parametric permutational multivariate ANOVA (PERMANOVA) was then performed using the R library 'RVAideMemoire' (Hervé, 2021) with the GSHs as factor. Each metric was analysed separately using a non-parametric permutational ANOVA. Two additional categorical

variables were also considered, and a factorial analysis of mixed data (FAMD) was performed for all nine variables using the R library 'FactoMineR' (Lé et al., 2008). A reference specimen representative of each species enclosed in the latest *Pocillopora* taxonomic revision (Schmidt-Roach et al., 2014) was included by measuring the variables on the images incorporated.

2.4. Characterisation of associated Symbiodiniaceae

Symbiodiniaceae communities were characterised for a subset of colonies (ca. 15 per GSH, when available; including three replicates) by high-throughput sequencing the ribosomal RNA internal transcribed spacer 2 (ITS2; see Appendix D for details). Reads were processed with the SAMBA v3.0.1 workflow (<https://github.com/ifremer-bioinformatics/samba>). Resulting operational taxonomic units (OTU) were taxonomically assigned by querying a custom reference database of Symbiodiniaceae ITS2 adapted from the one available in SymPortal (downloaded on 13/01/2022; Hume et al., 2019). Taxonomic affiliations of the OTUs were confirmed by reconstructing the phylogenetic relationships among them using MAFFT v7.713 (Katoh and Standley, 2013) and FastTree v2.1.11 (GTR + CAT model; Price et al., 2009). OTUs and individuals with <10 and 500 sequences, respectively, were then removed to reduce possible sequencing errors. Alpha diversity metrics (Chao1 and Shannon) were computed at the OTU level with the R library 'vegan' (Oksanen et al., 2020) and compared using non-parametric permutational ANOVA performed with the R library 'RVAideMemoire' (Hervé, 2021), with the GSHs or the localities as factor. Finally, a nonmetric multidimensional scaling (NMDS) using Bray and Curtis (1957) dissimilarity index was performed to assess community similarity.

Species hypotheses delimited with each criterion separately (genomics, genetics, macro- and micromorphology and symbiosis ecology) were then compared in an integrative species delimitation context. Sampling sites (biogeography criterion) were also integrated to identify sympatric or allopatric GSHs. We followed the unified species concept from De Queiroz (1998) and the consensus protocol for integrative taxonomy (using the GSHs as starting evidence; see Padial et al., 2010) to define species, adopting a conservative approach (i.e., trying to suggest changes in taxonomy only when strong lines of evidence support them). We then discussed the usefulness of each criterion and its integration (either by cumulation or congruence; Padial et al., 2010) for the delimitation of *Pocillopora* species.

3. Results

3.1. Molecular analyses

3.1.1. Sequencing and bioinformatics processing

A total of 1.6×10^9 reads (2.5×10^{11} bp) were produced, with a highly variable number per individual [varying from 9.1×10^3 to 8.2×10^6 reads; mean \pm s.e. = $(4.4 \pm 0.1) \times 10^6$ reads]. Three individuals had less than a million reads and were removed. Quality controls and adapter trims led to the removal of 3.0% of the bases. From the resulting trimmed reads, between 41.0% and 86.2% reads per individual were successfully mapped on the reference sequences (mean \pm s.e. = $78.3 \pm 0.4\%$), with a mean coverage depth (\pm s.e.) of $60.2 \times (\pm 0.1)$. Finally, SNPs calling and filtering (Table B.2) led to two datasets: one including all SNPs (all-SNP dataset: 361 individuals \times 17,465 SNPs; 5.8% missing data) and the other keeping one randomly chosen SNP per locus (one-SNP dataset: 361 individuals \times 1,559 SNPs; 6.0% missing data), with mean SNP coverage depths (\pm s.e.) of $85.8 \times (\pm 0.4)$ and $76.1 \times (\pm 1.3)$, respectively.

3.1.2. Phylogenomic analyses

All results were consistent between both datasets. We thus present below only results from the one-SNP dataset, but results keeping all

SNPs are provided in Appendix B. The phylogenetic trees inferred both with RAxML and BEAST gave similar tree topologies and recovered four strongly supported clades (Clades 1–4; i.e., monophyletic groups of individuals chosen here as separated by at least 0.4 nucleotide substitution per site on the ML tree; Fig. 2). These clades (except Clade 1) split in turn into a total of 21 genomic species hypotheses (GSH). Eighteen GSHs were restricted to a single marine province (Table 1 & Fig. 2) and several GSHs were thus sympatric (12 in the WIO, 11 in the TSP and 2 in the SEP; Table B.4). Moreover, most of the GSHs (see below for the exceptions) roughly corresponded to previously defined secondary species hypotheses on the basis of microsatellites (SSH *sensu* Gélin et al., 2017b). Therefore, to avoid introducing a new nomenclature and to ease comparison with earlier works, the GSHs were named according to the corresponding SSH (e.g., the GSH corresponding to SSH01 was named GSH01).

SSH06, SSH07, SSH08 and SSH16, previously defined from a few individuals (Gélin et al., 2017b), were not retrieved here as the corresponding individuals were grouped with those from SSH09a, SSH09b-1 or SSH13c. Similarly, SSH09b-2 was grouped with SSH10 and was very genetically distant from the rest of SSH09 *sensu lato*, suggesting that individuals from SSH09b-2 correspond to GSH10 (as observed with the mtORF). GSH09b, therefore, corresponds to only SSH09b-1. SSH12 and SSH15, previously grouped with SSH13a and SSH13c, respectively, using microsatellites (Gélin et al., 2017b), were retrieved, confirming the distinction between them. Conversely, the over-partitioning previously found with microsatellites inside SSH04a (Oury et al., 2020a), SSH05d (Clusters 1 and 4 in Gélin et al., 2018b), SSH09a, SSH09c (Gélin et al., 2018a) and SSH13c (Oury et al., 2021) was not retrieved, contrary to the one found within SSH05c (Clusters 2 and 3 in Gélin et al., 2018b; *a posteriori* named SSH05c-1 and SSH05c-2 in Oury et al., 2020b). Finally, SSH05a was split into three new groups (GSH05a-1, GSH05a-2 and GSH05a-3), and SSH09c split into two new groups (GSH09c_{WIO} and GSH09c_{TSP}) restricted to the WIO or the TSP, respectively.

The three assignment methods gave consistent results. Almost all 21 GSHs were retrieved, although optimal K and admixture rates were slightly different between methods (Fig. B.2 & Fig. B.3). In particular, sNMF and STRUCTURE highlighted introgression signals among several GSHs which were further investigated with NEWHYBRIDS. This was notably the case within (1) GSH05 *sensu lato*, but also with (2) GSH12 as hybrids between GSH13a and GSH13c, (3) GSH15 as hybrids between GSH13c and GSH14, and (4) GSH09c_{WIO} as hybrids between GSH09a and GSH09c_{TSP} (Fig. B.2 & Fig. B.3). No individual was assigned to a hybrid class (i.e., F1, F2 or backcrosses), except all GSH09c_{WIO} individuals that were assigned as F2 hybrids from GSH09a and GSH09c_{TSP} (data not shown). The two network clusterings also retrieved the 21 GSHs (Fig. B.4). Thus, whichever the datasets and the analyses, each available published genome was assigned to a same GSH (Fig. 2 & Appendix B): two [*P. acuta* (Vidal-Dupiol et al., 2019) and *P. verrucosa* (Buitrago-López et al., 2020)] were assigned to GSH13a (currently considered as *P. verrucosa*; Gélin et al., 2017b; Pinzón et al., 2013) and the third [*P. damicornis* (Cunning et al., 2018)] to GSH09c_{TSP} (*P. grandis*).

Finally, all pairwise F_{ST} values were significantly positive ($P < 0.001^{***}$) and the dendrogram topology obtained from their clustering was comparable to the reconstructed phylogenies (Fig. B.5). Intra-clade F_{ST} ranged from 0.092*** to 0.689*** [mean (\pm s.e.) = 0.332 ± 0.011], while inter-clade ones ranged from 0.420*** to 0.795*** [mean (\pm s.e.) = 0.551 ± 0.004 ; Table B.5].

We sequenced the mtORF in 59 additional colonies, but no new haplotype was found. All haplotypes (except ORF09, ORF18 and ORF27) were restricted to a single GSH (Table 1), confirming previous results from Gélin et al. (2017b). In particular, ORF27 was found in GSH09 *sensu lato*, thus corresponding to *P. grandis* and/or *P. meandrina*. To distinguish the two species, we sequenced the PocHistone in 10 colonies

of each of the five GSHs from Clade 4. Among the 43 successfully sequenced colonies, no heterozygote was found and eight novel 588 bp-haplotypes were identified (Hist01–08; GenBank accession numbers ON155826-ON155833; Table B.7), to which we added the two available from GenBank (MG587096 and MG587097, corresponding to *P. grandis* and *P. meandrina*, respectively; Johnston et al., 2018; Table B.7). All but one (Hist07) haplotypes were restricted to a single GSH, and up to four different haplotypes were found within a single GSH (Table 1). Hist07 and Hist08 had the *P. grandis* diagnostic SNP, suggesting that GSH09c corresponds to *P. grandis* (Table 1 & Table B.7). The reconstructed PocHistone phylogeny consistently grouped these two haplotypes with the *P. grandis* from Johnston et al. (2018), but all other haplotypes were grouped inconsistently with the defined GSH (Fig. B.6).

3.1.3. Species delimitation analyses

Among the scenarios tested to delimit the four main clades (Clades 1–4; Table B.3), the best-supported model was the one separating those four clades (model 4: MLE = -11,868.07). The three other models were ranked with a decreasing number of clades (i.e., from three clades to a single one; $1,294.87 < BF < 4,706.03$; Table B.3). Within each clade, the model with the lowest MLE was the one separating colonies according to the different GSHs previously identified based on phylogenomic and clustering analyses. The best-supported model for Clade 1 (composed of one GSH) was therefore the 1-species model (GSH01; model 1.1: MLE = -1,112.45), followed by the 1-species-per-ocean model (model 1.2: MLE = -1,381.70, BF = 538.49). For Clade 2 (10 GSHs), analyses supported the 10-species model (model 2.17: MLE = -20,955.08), followed by the models lumping GSH05a-1 and GSH05d (model 2.16: MLE = -21,098.78, BF = 287.39) or GSH05a-2 and GSH05c-2 (model 2.14: MLE = -21,408.68, BF = 907.18). Finally, for Clades 3 and 4 (five GSHs each), the best supported models were the 5-species ones (model 3.8: MLE = -10,529.96; model 4.11: MLE = -12,717.20). However, the 5-species model for Clade 4 was closely followed by the model lumping GSH09c_{WIO} and GSH09c_{TSP} (model 4.10: MLE = -12,763.15, BF = 91.89; Table B.3). In brief, BFD* supported the 21 GSHs identified with the phylogenomic analyses.

A total of four species trees were estimated (i.e., one for the best-supported model in the initial batch of scenarios, and then one for each best-supported model for scenarios within Clades 2–4 separately; Fig. 3a). For the initial batch of scenarios, three (out of three) consensus tree topologies were identified in the 95% HPD set, and all grouped Clades 1 and 4 together, whereas Clade 1 was the most distant group according to all previous analyses. The three topologies differed in whether Clades 2 or 3 shared a direct common ancestor with Clades 1 and 4 (59.1% and 22.0% of the trees, respectively), or together (18.9%; Fig. B.7). For Clade 2, two (out of nine) consensus tree topologies were identified in the 95% HPD set. Both topologies were very similar and consistent with previous analyses, except that GSH05a-3 was alternatively grouped with or without GSH05a-2 and GSH05c-2 (52.1% and 44.3% of the trees, respectively; Fig. 3a & Fig. B.7). Only one (out of four) consensus tree topology was identified in the 95% HPD set for Clade 3 (representing 98.4% of the trees). This topology was consistent with previous analyses, i.e. grouping GSH12 and GSH13a on one side, GSH13c and GSH15 on the other side, and GSH14 being the most distant species (Fig. 3a). Finally, for Clade 4, a total of 15 consensus tree topologies were found, of which five were in the 95% HPD set. All topologies identified GSH09c_{WIO} and GSH09c_{TSP} as sister species, but then differed in whether GSH09a shared a direct common ancestor with them (83.9% of the trees) and whether GSH09b and GSH13b were sister species (17.8%) or progressive outgroups (65.4%; Fig. 3a & Fig. B.7).

The time-calibrated phylogeny indicated a first divergence within *Pocillopora* 20.4 Mya (95% HPD interval = [15.6–26.1] Mya), separating on one side Clades 1 and 4, and on the other side Clades 2 and 3. Clade pairs then diverged 17.4 Mya (95% HPD interval = [12.6–22.4]

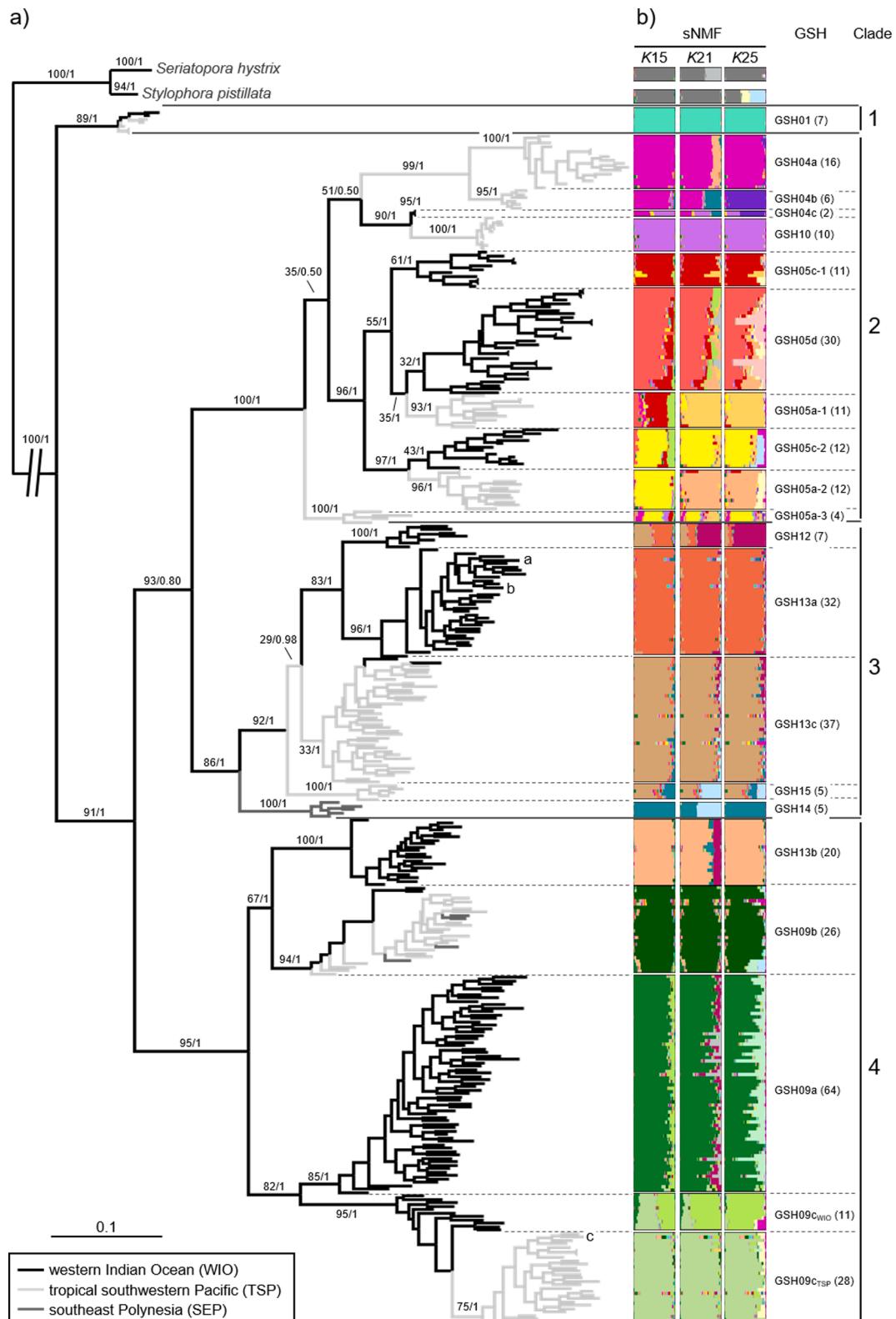


Fig. 2. *Pocillopora* phylogeny reconstructed with the one-SNP dataset (361 individuals \times 1,559 SNPs). (a) maximum likelihood (ML) phylogenetic tree. Branches are coloured according to marine provinces and branch support, based on ML bootstrap analyses (first number) and Bayesian posterior probabilities (second number), is indicated for the main branches. Published genomes are indicated by lowercase letters [a: *P. verrucosa* (Buitrago-López et al., 2020); b: *P. acuta* (Vidal-Dupiol et al., 2019); c: *P. damicornis* (Cunning et al., 2018)]. (b) sNMF assignments at $K = 15$, $K = 21$ and $K = 25$, genomic species hypotheses (GSH; delimited by dashed lines; number of colonies in parentheses) and clades (delimited by full lines). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Summary of the different approaches exploring *Pocillopora* species boundaries: genetics [genomic species hypotheses (GSH), **mtORF** (mitochondrial open reading frame) and **PocHistone** (**PocHist**) **haplotypes**; values in bold are retrieved in several GSHs], micro- and macromorphological, **symbiosis** (Symb; each colour denotes distinct dominant Symbiodiniaceae; see Fig. D.4) and biogeographic (Bioge; marks denote GSH presence in each of the three marine provinces; WIO: western Indian Ocean; TSP: tropical southwestern Pacific; SEP: south-east Polynesia) lines of evidence. Lineage current names (according Schmidt-Roach et al., 2014) and other corresponding lineages from previous studies are also indicated [arabic numerals correspond to types from Pinzón et al., 2013, roman numerals to clades from Martí-Puig et al., 2014 and greek letters to types from Schmidt-Roach et al., 2012, 2014; lineages in parentheses were extrapolated from Gélin et al. (2017b) secondary species hypotheses (SSH)]. (For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)

Clade/GSH	mtORF	Poc Hist	Micromorphology		Macromorphology	Symb	Bioge			Lineage current name (Schmidt-Roach et al. 2014)	Corresponding lineages	Consensus								
			Columella	Septa			W	T	S											
1 01	01	04a 04b 04c 10 05c-1 05d 05a-1 05c-2 05a-2 05a-3	Styliform Flat and spinulate Weakly developed to flat, covered with spinulae Oval-convex Variable	Robust, with lobes Absent to rudimentary, indicated by small septal teeth Developed (+ long teeth) Often developed, with small ($\approx 50 \mu\text{m}$) teeth Well developed, with long ($\approx 130 \mu\text{m}$) teeth Long and thin teeth Thin teeth (80–120 μm)	Robust and encrusting Slender branches, round to flattened with more or less pointed ends Short verrucose branches Slender and bushy branches, ramified towards terminal ends Robust, short and verrucose branches, with a cauliflower aspect	W I O S E P	x x x x x x x x x x	x x x x x x x x x x	P. cf. <i>effusa</i> <i>P. damicornis</i> n/a <i>P. cf. brevicornis</i> <i>P. acuta</i> <i>P. verrucosa</i> <i>P. meandrina</i> <i>P. grandis</i>	2, IIIa 4a, Ib (4b, 4c, α) n/a (ϵ) 5, Ia, β 7 3c, 3e (3g, 3j) 3b, 3d, 3f, 3h, IIa, γ (3i, χ) n/a n/a	01 04 n/a 10 05 05 05 05									
2	18																			
3	18,19																			
4	18																			
5	34																			
6	36,38,39																			
7	35,42,47, 53,54																			
8	52																			
9	50																			
10	46	01-04																		
11	23,27	05																		
12	06	07-08																		
13	1-3 stylae																			

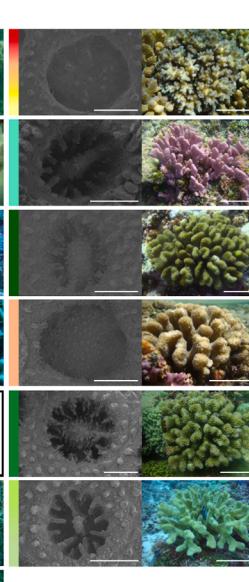
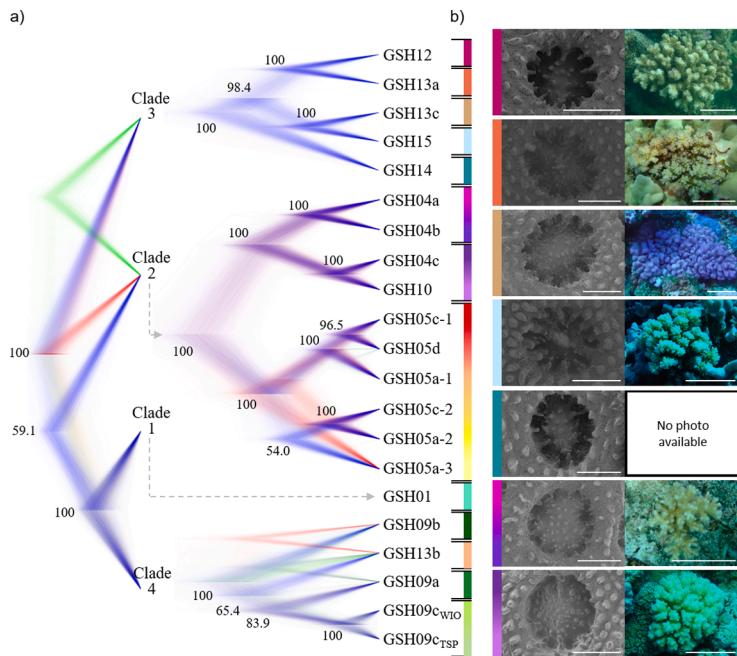


Fig. 3. Species tree estimation for the 21 delimited *Pocillopora* genomic species hypotheses (GSH). (a) complete set of consensus trees visualised with DensiTree for each best-supported model (i.e., for the initial batch of scenarios, and then for scenarios within Clades 2–4 separately). Higher density areas indicate greater topology agreement and different colours represent different topologies (trees with the highest clade credibility in blue). Node supports (Bayesian posterior probabilities) > 50% are indicated. (b) micro- (scale $\approx 500 \mu\text{m}$) and macromorphological (scale $\approx 10 \text{ cm}$) overview of the GSHs (characteristic features only; see Appendix E for more illustrations). Colours refer to GSHs or groups of morphologically indistinct GSHs (indicated alongside the species tree). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

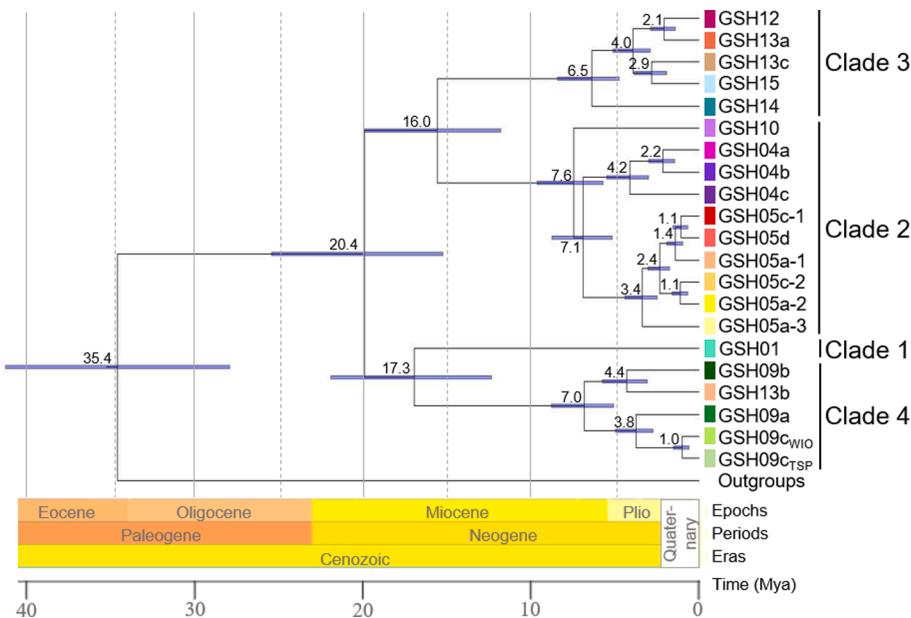


Fig. 4. Time-calibrated phylogeny of *Pocillopora* genomic species hypotheses (GSH). Values above nodes indicate median node ages and blue bars represent the 95% highest posterior density (HPD) interval. Plio: Pliocene. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Mya) and 16.0 Mya (95% HPD interval = [12.1–20.4] Mya), respectively (Fig. 4). Each clade then went through a first diversification period in the late Miocene (6.5–7.5 Mya), followed by a second period in the Pliocene and the Quaternary (i.e., from 4.5 Mya). Thus, almost all *Pocillopora* GSHs appeared relatively recently (Fig. 4).

3.2. Macro- and micromorphological analyses

Morphotypes based on macromorphology were not exclusive of a single GSH. Indeed, each GSH usually grouped colonies with a dominant morphotype, but also included several other morphotypes (e.g., GSH09b mostly grouped *P. meandrina*-like colonies, but also *P. damicornis*-like, *P. grandis*-like or *P. verrucosa*-like). Reciprocally, colonies from different GSHs can share the same morphotype (e.g., *P. damicornis*-like colonies were found in 14 GSHs). Clades 1 and 4 were mostly characterised by robust morphs with large branches, while Clades 2 and 3 grouped more stunted colonies (Table 1, Fig. 3b & Appendix E).

Concerning micromorphology, intraspecific variations were smaller (Fig. 3b & Fig. C.1). In particular, all species from Clades 1 and 4 (except GSH13b) and GSH15 were characterised by a styliform (GSH01, GSH09c_{WIO} and GSH09c_{TSP}) or oval-convex (GSH09a, GSH09b and GSH15) columella, while all other species had a flat, more or less spinulate one. Accordingly, significant differences among GSHs were found for the columella diameter variables ($v6$ and $v7$; non-parametric permutational ANOVA; $v6: F_{(5,46)} = 92.95, P < 10^{-3}***$; $v7: F_{(5,46)} = 98.20, P < 10^{-3}***$), distinguishing three groups: GSH01 + GSH15, GSH09a + GSH09b and GSH09c_{WIO} + GSH09c_{TSP} (pairwise permutational t-tests; $P < 0.05^*$; Fig. C.1). Significant differences among GSHs were also found for the five other numeric morphological variables ($v1-v5$; non-parametric permutational ANOVA; $4.53 \leq F_{(19,150)} \leq 18.96; P < 10^{-3}***$), but no particular pattern was identified, except that GSHs from Clade 2, GSH13a and GSH13b had poorly developed septa (Fig. 3b & Fig. C.1). The PERMANOVA and FAMD (Fig. C.1 & Fig. C.2) also highlighted these differences. Five micromorphological groups were thus distinguished on the first three principal components of the FAMD (explaining 68.4% of the variability): GSH01, Clade 2 + GSH13a + GSH13b, GSH12 + GSH13c + GSH14, GSH09a + GSH09b + GSH15 and GSH09c_{WIO} + GSH09c_{TSP} (Fig. C.2). Detailed macromorphological and micromorphological illustrations of the GSHs are provided in Appendix E.

3.3. Characterisation of associated Symbiodiniaceae

ITS2 amplicon sequencing yielded a total of 1.6×10^7 reads (4.0×10^9 bp) with 1.7×10^4 to 1.2×10^5 reads per individual [mean \pm s.e. = $(6.1 \pm 0.1) \times 10^4$ reads]. After merging paired reads and removing chimeras, 9.0×10^6 sequences were retained [from 0 to 7.2×10^4 sequences per individual; mean \pm s.e. = $(3.5 \pm 0.0) \times 10^4$ sequences], corresponding to 1,014 amplicon sequence variants that were clustered in 590 operational taxonomic units (OTU; represented by 1 to 6.1×10^5 sequences). Finally, 534 OTUs (90.5%) were taxonomically assigned, with a majority (511 OTUs, representing 97.6% of the sequences) belonging to *Cladocopium* (formerly *Symbiodinium* clade C), and mostly to clades C1 (173 OTUs and 35.6% of the sequences), C40 (267 OTUs; 56.6% of the sequences) and C42 (45 OTUs; 3.6% of the sequences). The other OTUs were assigned to *Symbiodinium* (clade A1; 6 OTUs; 0.2% of the sequences), *Durisdinium* (clade D1; 2 OTUs; < 0.1% of the sequences), *Gerakladium* (clade G3; 12 OTUs; 0.1% of the sequences) and *Symbiodiniaceae* clade I (clades I1 and I3; 3 OTUs; < 0.1% of the sequences). The reconstructed phylogeny based on these OTUs retrieved the five genera, with largely unresolved polytomies within *Cladocopium*, as previously observed (Brener-Raffalli et al., 2018; LaJeunesse, 2005; Fig. D.1). Nevertheless, such polytomies should not affect subsequent analyses, as performed at the OTU level.

From the remaining 252 individuals and 552 OTUs that passed the filtration steps, OTU richness within colonies varied from 0.14 to 2.67 for Shannon diversity index, and from 2 to 39 for Chao1 index. Both indices were significantly different among GSHs (non-parametric permutational ANOVA; Shannon: $F_{(20,231)} = 3.89$, $P < 10^{-3}***$; Chao1: $F_{(20,231)} = 2.96$, $P < 10^{-3}***$; Fig. D.2), but no significant difference was found in Chao1 post-hoc tests (pairwise permutational t-tests; $P > 0.05^{NS}$), and no obvious pattern was found for Shannon index (Fig. D.2). Differences were clearer when looking at the proportion of each taxon within samples (Fig. D.3). For example, individuals from GSH04c, GSH09a and GSH13a displayed mainly C1ky [$38.8 \pm 2.4\%$ on average ($\pm s.e.$)], while it was almost absent in other GSHs. Similarly, GSH05c-2, GSH13c and GSH15 contained mainly C1ag ($56.5 \pm 4.6\%$) and GSH05c-1, GSH05d, GSH12 and GSH14 mainly C1d ($50.0 \pm 4.4\%$). With the exception of a few individuals, other GSHs contained almost exclusively C40c (Fig. D.3). Accordingly, the NMDS based on Bray and Curtis (1957)

dissimilarity index followed this partitioning with three groups on the first two principal components (explaining 41% of the variability): (1) individuals mostly harbouring C1ky, (2) those mostly harbouring C1ag or C1d (separated with the third principal component) and (3) individuals mostly harbouring C40c (Table 1 & Fig. D.4).

Concerning localities, significant differences were found for Chao1 (again, without any obvious pattern; non-parametric permutational ANOVA; $F_{(13,238)} = 5.94$, $P < 10^{-3}***$), but neither for Shannon (non-parametric permutational ANOVA; $F_{(13,238)} = 1.69$, $P = 0.07^{NS}$; Fig. D.2), nor by looking the individual proportions (Fig. D.3) or the NMDS (Fig. D.4).

3.4. Summary and integration of all lines of evidence

Genomic analyses allowed the definition of 21 GSHs, while Symbiodiniaceae communities distinguished four species hypotheses, and micromorphology up to 10. Thus, integrating all approaches (i.e., genomics, genetics, macro- and micromorphology and symbiosis ecology; Table 1), including biogeography (Fig. 5), and considering that two species can be differentiated only if all lines of evidence support them (=integrative taxonomy by strict congruence), here only a single unambiguous species (corresponding to the entire genus) could be delimited (i.e., no species boundary was supported by all criteria; Table 1). Removing the Symbiodiniaceae criterion, five species, corresponding to Clades 1–3, GSH13b and GSH09 *sensu lato*, are delimited by the strict congruence of all other criteria. Then, sequentially removing the macro- and micromorphology criteria (i.e., considering genetic evidence alone) could lead to nine and 12 species (Table 1). However, three out of the four GSHs within GSH09 *sensu lato* are split by other criteria (morphology and sometimes Symbiodiniaceae). Consequently, integrating criteria only by congruence among all approaches will underestimate the number of species, while integrating them by cumulation will overestimate it (Padial et al., 2010). Following the consensus protocol for integrative taxonomy proposed by Padial et al. (2010), which takes advantage of both the cumulative and congruence approaches, we propose here a conservative (i.e., introducing changes in taxonomy only when strong lines of evidence support them) consensus of 13 species strongly supported by most criteria (Table 1), three of which (*P. acuta*, *P. damicornis* and *P. grandis*) could represent species complexes according to genetic data, and six may represent either undescribed species or nominal species that have been synonymised incorrectly (Table 1). Below, we discuss the usefulness of each criterion and its integration with other lines of evidence.

4. Discussion

Delimiting species remains of particular importance in evolutionary biology and requires integrating multiple criteria to obtain robust conclusions. Indeed, investigated criteria do not necessarily provide the same resolution nor congruent insights. Not all criteria should therefore be considered equally informative in order to define a consensus of the species boundaries as parsimonious as possible. In this study, we focused on the scleractinian genus *Pocillopora* across a wide range of sampled localities (18 islands or regions from three marine provinces) and collected genetic, morphological, geographic and symbiosis data to define robust species boundaries and assess the usefulness of each criterion. The different genetic approaches allowed the delimitation of 21 genomic species hypotheses (GSH). Moreover, 13 species appear strongly supported by all approaches, six of which either undescribed or previously synonymised. Some of the other GSHs were supported by biogeography or symbiotic associations, but additional investigations are needed to firmly conclude on their taxonomic status. In any case, a taxonomic revision of *Pocillopora*, taking into account insights from these results and previous ones, is urgent. This will allow the designation of formal names and thus overcome the multitude of current nomenclatures based on genetic lineages which can be difficult to follow, even

for specialists.

4.1. On the (ir)relevance of symbiosis ecology to define species

Pocillopora corals have been demonstrated to transmit Symbiodiniaceae maternally (vertical transmission; Harii et al., 2002; Hirose et al., 2001; Sier and Olive, 1994) and are thus expected to develop species-specific associations and co-evolutions (Johnston et al., 2022; Pinzón and LaJeunesse, 2011; Schmidt-Roach et al., 2012). This association could also be responsible for habitat specialisations (driven by symbiont thermotolerance and photosynthetic needs; Baker et al., 2013; Brener-Raffalli et al., 2018; Jokiel and York Jr., 1982; Ros et al., 2021). The characterisation of associated Symbiodiniaceae communities can therefore help refining *Pocillopora* species boundaries, as previously shown in other scleractinian genera (Arrigoni et al., 2016; Bongaerts et al., 2010; Forsman et al., 2020; Keshavmurthy et al., 2013; Warner et al., 2015). Unfortunately, this does not guarantee a self-sufficient criterion.

Indeed, our results show that symbiosis ecology alone does not appear sufficiently informative to delimit species. We found a high prevalence of *Cladocopium* C1 (*C. goreau*) and C40, both host-generalists, consistently with other studies on *Pocillopora* (e.g., Armstrong et al., 2021; Brener-Raffalli et al., 2018; Johnston et al., 2022; Magalon et al., 2007; Pinzón and LaJeunesse, 2011; Schmidt-Roach et al., 2012). C1 variants allowed to distinguish four groups of colonies with distinct Symbiodiniaceae communities, but colonies within those groups were very distinct morphologically and genetically. Conversely, colonies from a single GSH generally shared the same communities.

These results should nevertheless be considered cautiously since (1) host-symbiont associations vary over time and depth (Cunning et al., 2013) and appear driven by biogeography and environmental parameters (Glynn et al., 2023), and (2) quantitative interpretation of metabarcoding results can be misleading (Lamb et al., 2019). First, Pinzón and LaJeunesse (2011) found that *Pocillopora* type 1 (ORF27; probably GSH09b or GSH09c_{TSP}) was the only type associated to the thermotolerant *Durusdinium glynnii* (D1; Wham et al., 2017) in the tropical eastern Pacific. However, *D. glynnii* was later found in *Pocillopora* types 3a and 3b (ORF46 and ORF47; GSH13b and GSH13c, respectively), with different prevalence among sites (Cunning et al., 2013), suggesting variable host-symbiont associations. In particular, *Durusdinium* would represent an opportunist genus, replacing specialist Symbiodiniaceae in health-compromised (e.g., bleached) corals (Stat and Gates, 2010), potentially explaining these results. In our study, *Durusdinium* was rare (representing ca. 0.5% of the sequences in a single individual), suggesting no recent bleaching event prior to sampling, and thus mature host-symbiont associations. However, horizontal (i.e., from the water column) acquisition of Symbiodiniaceae remains possible, potentially corrupting species-specific associations. More recently, Glynn et al. (2023) also evidenced that sea surface temperatures and geographic isolation drive *Pocillopora*-Symbiodiniaceae associations. Second, PCR inherent biases (reviewed in Lamb et al., 2019) can result in differential sequence amplifications, either quantitatively or qualitatively. This can result in artificial differences in Symbiodiniaceae compositions among individuals and GSHs. Conversely, rare or specific Symbiodiniaceae taxa that could be diagnostic of a GSH might not be amplified, sequenced or detected.

Species boundaries delimited from symbiosis ecology inferred with metabarcoding data should therefore be taken cautiously, and rather used in support of other criteria in an integrative context. Furthermore, this criterion has not been systematically explored in previous taxonomic revisions of scleractinian genera (e.g., Arrigoni et al., 2021, 2020; Benzoni et al., 2010; Wepfer et al., 2020), supporting that it is not the most relevant criterion, particularly for *Pocillopora*.

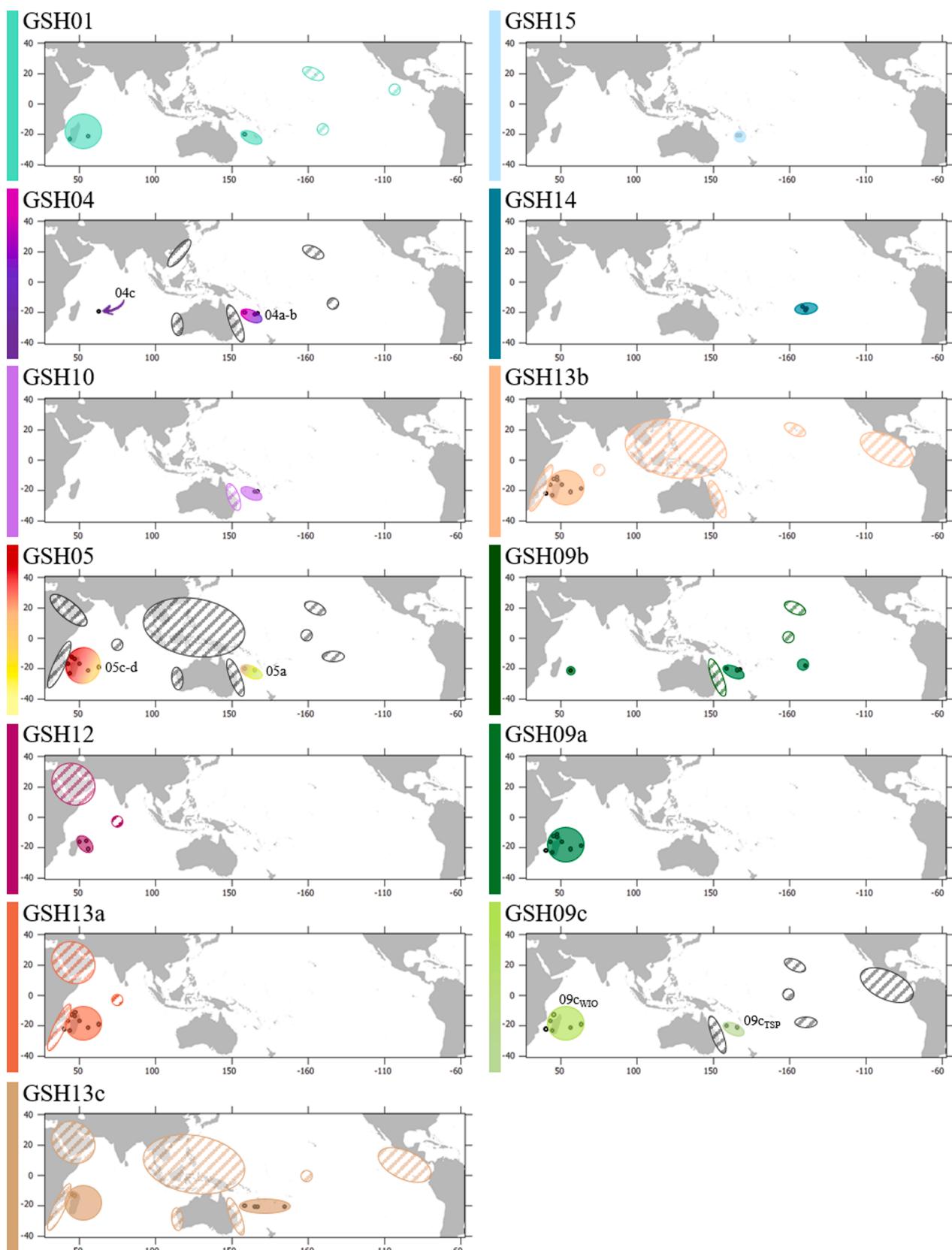


Fig. 5. Geographic distribution of *Pocillopora* genomic species hypotheses (GSH). Filled ellipses represent data from this study (black circles represent localities where GSHs were found) while hashed ones were taken from the literature [based on mtORF identifications; colours refer to GSHs and black denotes ambiguous identifications (several GSHs with the same mtORF haplotype)]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.2. Should we trust morphology?

Most of the delimited GSHs group colonies presenting the same major morphotype (which could be shared among several GSHs) but also harbour high morphotype diversities. This demonstrates, once again (e.g., Gélin et al., 2017b; Martí-Puig et al., 2014; Pinzón et al., 2013), the limitation of using *corallum* macromorphology to define *Pocillopora* species boundaries, as in other scleractinian genera (e.g., Bongaerts et al., 2021; Shimpi et al., 2019; Terraneo et al., 2021; Warner et al., 2015). Indeed, *Pocillopora* corals can display great morphological plasticity mostly driven by light and currents (Paz-García et al., 2015a, 2015b). As an illustration, five morphospecies have been reported in the Gulf of California (Glynn and Ault, 2000), but all have the same mtORF haplotype (Pinzón et al., 2013). Switches from one morphospecies to another have also been demonstrated following shifts in environmental conditions (Paz-García et al., 2015a, 2015b).

Contrary to macromorphology, micromorphology brought additional insights in refining *Pocillopora* species boundaries, as in other scleractinian genera (e.g., Arrigoni et al., 2020; Benzoni et al., 2007; Budd et al., 2012; Budd and Stolarski, 2011; Forsman et al., 2010; Stefanini et al., 2011). Intraspecific variations were smaller, and several differences (especially in the size and shape of septa and columellae; Table 1) allowed to distinguish almost all GSHs. The GSHs within Clade 2 were not separated, but Schmidt-Roach et al. (2014) raised several differences that we could not recover. It is also possible that the morphological characters investigated here were not the most relevant to distinguish these GSHs.

Current morphology-based criteria are thus questionable and subject to subjective interpretation (particularly for the presence/absence of subtle characters). These findings, coupled with morphological plasticity and convergence, make morphology alone inadequate to identify *Pocillopora* species. The incorrect species identification based on morphology of two out of the three currently available *Pocillopora* genomes perfectly illustrates this point. First, the genome from Panama (Cunning et al., 2018), identified as *P. damicornis* by the authors based on morphology, was here assigned to GSH09c_{TSP}, corresponding to *P. grandis*, using genomics. This is not so surprising, as in Panama, Pinzón and LaJeunesse (2011) previously found only two different mtORF haplotypes in colonies morphologically identified to *P. capitata*, *P. damicornis*, *P. elegans* and *P. eydouxi* (the junior synonym of *P. grandis*): types 1a and 3a [sensu Pinzón et al., 2013 and corresponding here to ORF27 (GSH09 sensu lato; i.e., *P. grandis* and *P. meandrina*) and ORF46 (GSH13b; *P. villosa* nomen nudum in Gélin et al., 2017b), respectively]. Therefore, the colony sequenced by Cunning et al. (2018) may have been identified as *P. damicornis* based on morphology but rather corresponds to *P. grandis* (GSH09c_{TSP}). Concerning the genome from Vidal-Dupiol et al. (2019), first assigned to *P. acuta*, while the morphological identification of the colony has been confirmed molecularly using the mtORF, the haplotype was not provided. We retrieved it in scaffold167434_cov1917:1989–1148, corresponding to ORF39 (GSH13a). Concordantly, this genome, together with the genome from central Red Sea (Buitrago-López et al., 2020), were assigned to GSH13a, restricted to the Indian Ocean and the Red Sea and currently considered as *P. verrucosa*. However, the type locality of *P. verrucosa* is Lizard Island (northern Queensland, Australia; Schmidt-Roach et al., 2014), therefore GSH13c most likely corresponds to *P. verrucosa*, and GSH13a either corresponds to an undescribed species (which would be surprising given its abundance and distribution) or to another nominal species that has been synonymised incorrectly with *P. verrucosa* and whose type locality is Indian Ocean or Red Sea (e.g., *P. hemprichii*). Further phylogenetic investigations, including topotypes and an examination of types, are needed to conclude, but our study nevertheless evidenced that none of the three currently available *Pocillopora* genomes corresponds to the species it pretends to be. Consequently, when colonies are used to generate baseline data for other studies, such as reference genomes, type localities should be considered to reduce misidentifications and species

identifications should be systematically confirmed using molecular tools. There is also a need to find (if possible) unambiguous morphological diagnostic characters for species identification in the field and field collectors should be aware that colony morphology does not allow to identify unambiguously the species in *Pocillopora* corals.

4.3. Exploring species boundaries: Lessons from genomics

Pocillopora species boundaries have been extensively studied using genetic markers over the past decades (e.g., Gélin et al., 2017b; Martí-Puig et al., 2014; Pinzón et al., 2013; Schmidt-Roach et al., 2012), revealing a great diversity within some morphospecies (e.g., *P. damicornis*; Schmidt-Roach et al., 2012). Most of these previous studies used mtDNA and microsatellites to explore species boundaries. Up to now, genomic data have been only employed by Johnston et al. (2017, 2022), who inferred genetic relationships among a few tens of *Pocillopora* colonies from the Pacific. Our study thus represents the most extensive genomic investigation to date of the species boundaries within *Pocillopora*. Noteworthy, only two GSHs (GSH09b and GSH14) were found in the SEP, but several may have not been collected as the sampling focused on *P. meandrina*-like colonies (see Magalon et al., 2005). In Moorea (Society Islands), for example, five distinct species were previously delimited using genomic data (ezRAD; Johnston et al., 2022).

Our genomic analyses based on SNPs collected from the target-capture of UCEs and exon loci provided very congruent results among methods and allowed the robust definition of four main clades comprising 21 GSHs. However, despite thousands of SNPs and loci analysed, we were not able to fully resolve GSH relationships, and multiple species tree topologies were inferred (Fig. 3a & Fig. B.7). Recent species divergences and the presence of several closely related sister species, as well as introgression, could explain unresolved topologies. Indeed, most GSHs diverged <5 Mya, with a substantial number in the Quaternary (i.e., 0–2.6 Mya; Fig. 4). This suggests a recent radiation, probably linked to major geological and climatic events during the Pliocene or the Pleistocene [e.g., changes in currents (Philander and Fedorov, 2003), glacial-interglacial cycles (Adams et al., 1999; Lambek et al., 2002) and formation of the Isthmus of Panama (O'Dea et al., 2016)], as already suggested for this genus (Johnston et al., 2017). Recent divergences also suggest that some sister GSHs might still be actively speciating, being in the grey zone (*sensu* De Queiroz, 1998) where distinctive characters are set up and gene flow is still possible. Some allopatric GSHs may thus still be able to hybridise (e.g., GSH09c_{WIO} and GSH09c_{TSP}). Not all investigated criteria can therefore distinguish these GSHs and the question of their validity as two distinct species arises. Therefore, some of the GSHs should be conservatively considered as a potential species complex (e.g., *P. damicornis* with two GSHs or *P. acuta* with six GSHs), pending further (e.g., ecological or reproductive) evidence to separate them and possibly exclude population structure. One of the limitations of the present study is the absence of intermediate sampled localities between the WIO and the TSP, and future studies should investigate species boundaries across the geographic range of the genus to help sort it out (see for example Huang, 2020; Smith and Carstens, 2022). Process-based approaches (Smith and Carstens, 2020; Sukumaran et al., 2021) to statistically model secondary contacts and other population-level processes could also be used in order to define robust species hypotheses even in the presence of gene flow.

Interestingly, almost all the 21 GSHs corresponded to previously defined genetic species hypotheses or clusters (based on the mtORF marker and microsatellites). Several GSHs had their own mtORF or PocHistone haplotypes, confirming that both can be used as diagnostic markers for some (but not all) *Pocillopora* species. Conversely, the over-partitioning previously found in several SSHs using microsatellites (e.g., Gélin et al., 2018a; Oury et al., 2021) was not retrieved. This could be an effect either of the limited numbers of microsatellite loci, or of phylogenetic inferences at the genus scale masking such genetic patterns. Genetic criteria therefore appear robust to define species boundaries but

present a risk of overestimating their number. BFD*, similarly to other molecular species delimitation methods, has been already suggested to overestimate the number of species (Derkarabetian et al., 2022; Grummer et al., 2014; Hundsdoerfer et al., 2019). This supports the need for integrative approaches where molecular data should be first applied to explore species boundaries robustly and objectively. The definition of genetic species hypotheses should be then confirmed by other criteria (as previously suggested by Pante et al., 2015). In particular, genome-wide data, although not systematically necessary to molecularly identify species, appears fundamental to set robust species boundaries in such taxa for which phylogenetic reconstructions are complex. An exhaustive inventory of *Pocillopora* species using genomics/genetics is recommended to first identify the species present in the field. This will allow careful biodiversity monitoring [e.g., the global coral reef monitoring network (GCRMN)] for each biogeographic region.

4.4. From multiple criteria to integrative species delimitation: Towards a revision of the genus *Pocillopora*

Genomic evidence therefore appears to be the most reliable criterion, if population structure can be ruled out by integrating biogeographic or ecological lines of evidence, then morphological or other lines of evidence to support the delimitation of species hypotheses. Here, from the 21 GSHs that have been delimited within the *Pocillopora* genus, thirteen species appeared strongly supported when combining all lines of evidence (i.e., genetics, morphology, geography and symbiosis ecology), six of which potentially correspond to nominal species that were previously synonymised or to new species that need to be described (Table 1). Clades 1 and 2 support recent taxonomic works, the first consisting of a single species (*P. effusa*, corresponding to GSH01), and the second being consistent with Schmidt-Roach et al. (2014) taxonomic revision [i.e., three species: *P. damicornis* (GSH04 *sensu lato*), *P. brevicornis* (GSH10) and *P. acuta* (GSH05 *sensu lato*)]. Further investigations are nevertheless needed to state whether *P. damicornis* and *P. acuta* each represent a species complex. Indeed, *P. damicornis* was separated into two GSHs and SSHs (04a and 04b) not supported by other non-genetic criteria, but which could be ecologically distinct (Oury et al., 2020a). Similarly, *P. acuta* was partitioned into several GSHs and SSHs/clusters, either sympatric or allopatric, and some associated to distinct Symbiodiniaceae. Multiple genetic entities were previously delimited in this species (Gélin et al., 2018b, 2017a; Torres et al., 2020), questioning its monophyly. All five GSHs within Clade 3 are strongly supported by all criteria, but we were unable to assign a species name to them. One of them, probably GSH13c, should correspond to *P. verrucosa*, but only an examination of all type specimens will confirm this and state whether the other GSHs correspond to nominal species that have been synonymised incorrectly with *P. verrucosa* (Bonito et al., 2021). Finally, four species seemed strongly supported within Clade 4: GSH09a, GSH09b and GSH13b each correspond to distinct species (GSH09b likely corresponds to *P. meandrina*, while GSH13b to *P. villosa nomen nudum*, as previously suggested by Gélin et al., 2017b). Only GSH09cw10 and GSH09cw10 could not be distinguished with certainty and are conservatively considered as different *P. grandis* lineages in allopatry, pending further evidence.

In the light of these results, a new taxonomic revision of the genus *Pocillopora* is urgent. It will be necessary to consider all type specimens previously described in order to distinguish undescribed species from species that have been incorrectly synonymised. This will also allow to tidy up the multitude of current nomenclatures based on genetic lineages (Gélin et al., 2017b; Martí-Puig et al., 2014; Pinzón et al., 2013; Schmidt-Roach et al., 2014) which can be overwhelming, even for specialists.

5. Conclusions

To date, this study is the most extensive exploration of the species

boundaries within the genus *Pocillopora* in terms of both genomic and geographic coverage. The definition of its species boundaries has been challenging for decades, all the more remarkable for a major, even the most important in some places, bio-constructor of Indo-Pacific coral reefs. Several other criteria including morphology, biogeography or symbiosis ecology were also investigated to refine species boundaries and propose consensually and conservatively species hypotheses in the most integrative way possible. Some criteria appeared thus more informative than others, but all provided helpful insights to refine species boundaries. In particular, genomics appears certainly the most resolute form of evidence, and allowed here to delimit 21 genomic species hypotheses from 356 colonies sampled in three marine provinces (western Indian Ocean, tropical southwestern Pacific and south-east Polynesia). Thirteen of these species were strongly supported by all approaches and six might correspond either to undescribed species or to nominal species that have been synonymised incorrectly. We demonstrate once again the limitation of *corallum* macromorphology in identifying most of the species. Conversely, micromorphological diagnostic characters and mtORF and PocHistone diagnostic haplotypes were useful for several species. Our recommendation is therefore to systematically identify *Pocillopora* species using these diagnostic criteria, prior to all types of studies involving the colonies (e.g., biodiversity, ecology, reproduction, adaptation, connectivity, exo- and endo-symbiosis...) in order to reduce misidentifications and erroneous interpretations. As shown by the incorrect identification of the three currently available *Pocillopora* genomes, taking into account type localities also appears fundamental. Finally, our results give new insights into the puzzle of *Pocillopora* species boundaries, highlighting several problems in the taxonomy. Next steps are thus to formally revise the taxonomy of *Pocillopora* with the approach suggested by Bonito et al. (2021) to correct these problems.

CRediT authorship contribution statement

Nicolas Oury: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Cyril Noël:** Formal analysis. **Stefano Mona:** Formal analysis, Writing – review & editing. **Didier Aurelle:** Formal analysis, Writing – review & editing. **Hélène Magalon:** Conceptualization, Resources, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

See the 'Data Availability Statement' section in the manuscript.

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Data availability statements

All data underlying this article are available online or upon reasonable request to the corresponding author. Raw sequencing reads (Bio-Project PRJNA831687; *Pocillopora* target-capture: accession numbers SRR19052129-SRR19052500; Symbiodiniaceae ITS2 metabarcoding: accession numbers SRR19152377-SRR19152635) and new haplotype sequences (GenBank accession numbers ON155826-ON155833) were deposited on the NCBI. Microsatellite genotypes, morphometric data, code used, reference sequences and SNP datasets were deposited on Zenodo: <https://doi.org/10.5281/zenodo.7885458>.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympev.2023.107803>.

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