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DNA barcoding reveals the coral "laboratory-rat", *Stylophora pistillata* encompasses multiple identities

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Stylophora pistillata **is a widely used coral "lab-rat" species with highly variable morphology and a broad biogeographic range (Red Sea to western central Pacific). Here we show, by analysing Cytochorme Oxidase I sequences, from 241 samples across this range, that this taxon in fact comprises four deeply divergent clades corresponding to the Pacific-Western Australia, Chagos-Madagascar-South Africa, Gulf of Aden-Zanzibar-Madagascar, and Red Sea-Persian/Arabian Gulf-Kenya. On the basis of the fossil record of *Stylophora*, these four clades diverged from one another 51.5-29.6 Mya, i.e., long before the closure of the Tethyan connection between the tropical Indo-West Pacific and Atlantic in the early Miocene (16–24 Mya) and should be recognised as four distinct species. These findings have implications for comparative ecological and/or physiological studies carried out using *Stylophora pistillata* as a model species, and highlight the fact that phenotypic plasticity, thought to be common in scleractinian corals, can mask significant genetic variation.**

DNA barcoding, usually based on the mitochondrial COI fragment[1], has been extensively used to discriminate between closely related species, to identify new, cryptic or invasive species, and to assess biodiversity across many animal phyla[1]. The rule-of-thumb in DNA barcoding is that interspecific COI divergence is generally $> 2\%$, whereas intraspecific variation is $< 1\%$[1]. However, based on 90 species from 44 genera belonging to 14 families, this criterion has been suggested as not being appropriate for most scleractinian corals[2] (or anthozoans in general) because this region evolves very slowly in these organisms and consequently both

inter- and intra-specific variation are extremely low. We re-evaluated this conclusion by examining COI divergence and phylogeny of the "lab-rat" scleractinian coral, *Stylophora pistillata*, a species, which has been the focus of coral research over the last four decades. *S. pistillata* is a widely-distributed coral species in the Indo-Pacific, with numerous morphological variations (morphotypes) across different habitats, depths, and geographic regions[3]. Revisiting relationships between these morphotypes is necessary in order to ensure the correct taxonomy for comparative studies based on this common coral species[4].

Results

We obtained COI sequences from 241 *S. pistillata* colonies collected from 34 locations across most of its range (Western Pacific Ocean, Indian Ocean, Red Sea and Persian/Arabian Gulf, Fig. 1). Interspecific variation in COI sequences was one of the highest documented among scleractinian genera (sequence diversity (p-distance) = 0.01245 ± 0.00434), only surpassed by *Porites* (0.02686 ± 0.0030) and *Siderastrea* (0.0141 ± 0.0027), which are globally distributed genera consisting of multiple morphologically divergent species (Fig. S1). High variation of the COI gene among these 3 genera suggests very early divergence of species within these genera[5]. *Porites* and *Siderastrea* are extant in the Atlantic, Pacific and Indian Oceans, whereas *Stylophora* is extinct in the Atlantic and only survived in the Indo-Pacific after the Plio-Pleistocene[5].

Phylogenetic construction based on COI sequences found 4 distinct clades of *S. pistillata* (Fig. 2A). Clade 1 is comprised of specimens from the entire Pacific region and East Indian Ocean, ranging from New Caledonia in the southwest Pacific, Shikoku (Japan) in the northwest Pacific, to western Australia in the Indian Ocean (Pacific-Western Australia clade, PWA). Clade 2 includes specimens from Chagos, St. Brandon's Island (Mauritius), La Réunion, Zanzibar, South Africa, and Madagascar (Chagos-Madagascar-South Africa clade, CMSA). Clade 3 includes specimens from the east African coast, the west coast of Madagascar and the Gulf of Aden (Gulf of Aden-Zanzibar-Madagascar clade, AGZM). Clade 4 includes the northwest Indian Ocean (Red Sea-Persian/Arabian Gulf and Kenya, RSPAGK). The genus *Seriatopora* formed a fifth clade embedded at the base of clades 1 and 2.

Intra-clade p-distance based on COI (Table S1) was considerably low (clade 1: 0.00017 ± 0.00009, clade 2: 0.00000 ± 0.00000, clade 3: 0.00460 ± 0.00193, and clade 4: 0.00017 ± 0.00012). Higher intra-clade distance for clade 3 was due to the presence of specimens from Yemen that made a separate sub-clade into clade 3 (Fig. 2A). Inter-clade p-distance (Table S1) was always higher than intra-clade p-distance and ranged from 0.01274 ± 0.00436 (clade 3 vs clade 4) to 0.01592 ± 0.00492 (clade 1 vs clade 3), with the exception for clade 1 vs clade 2 (0.00378 ± 0.00248) and this could be due to the recent split between clade 1 and 2 compared to that between other clades (Fig. 3). The results form p-distance analyses also suggested the presence of 4 clades of *Stylophora* (Table S1).

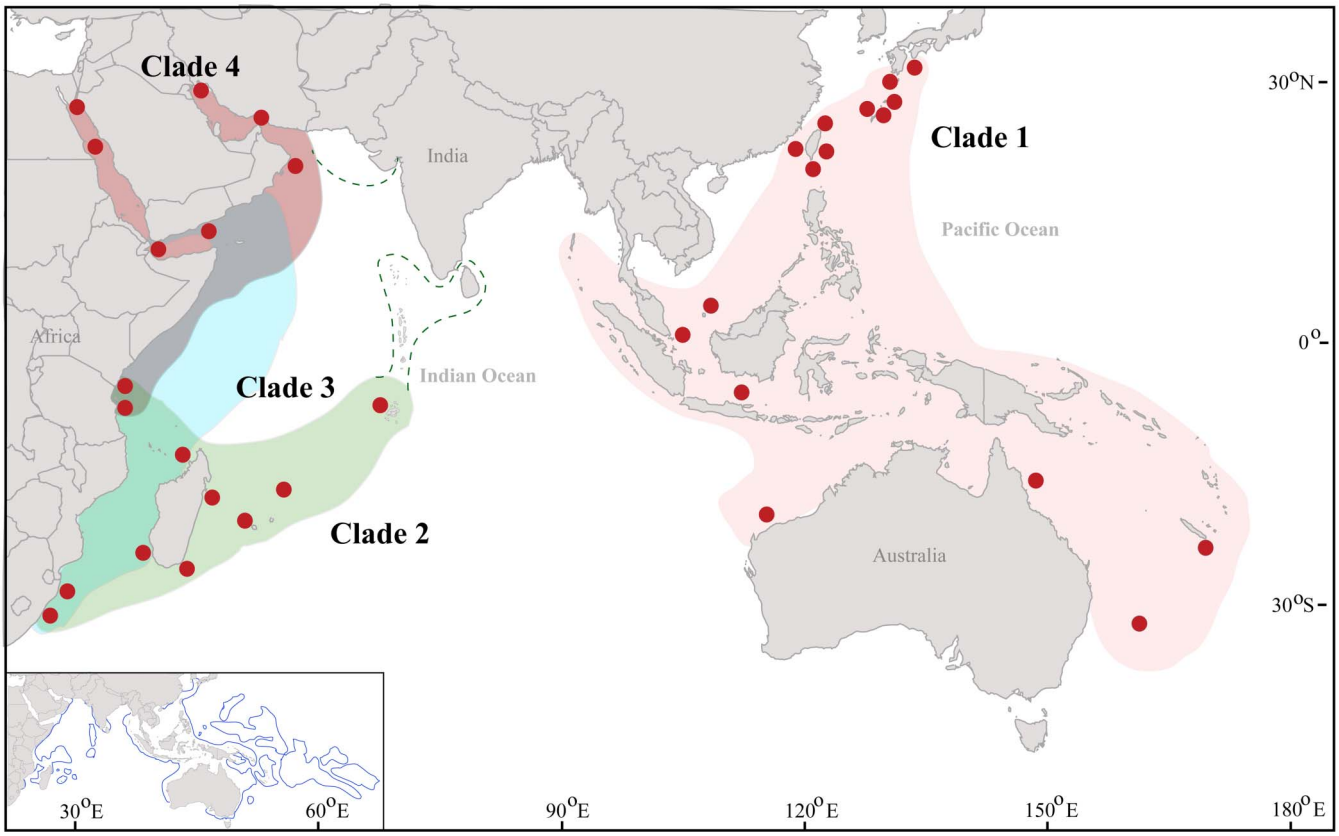


Figure 1 | Map showing the locations from which *Stylophora pistillata* samples were obtained (shown as red dots). The division of four regions based on the four clades of *Stylophora* is marked in colours. Clade 1 - Pacific-Western Australia clade (Pink), Clade 2 - Chagos-Madagascar-South Africa clade (Green), Clade 3 - Gulf of Aden-Zanzibar-Madagascar clade (Blue) and Clade 4 - Red Sea-Persian/Arabian Gulf-Kenya clade (Brown). The samples were obtained from following locations; Japan (Kochi, Okinawa), Taiwan (Beitou, Penghu, Green Island, Kenting), Taiping Island, Tioman, Indonesia, Australia (Lord Howe, Lizard Island, Western Australia), New Caledonia, The Chagos Archipelago, Re Union, Mauritius (St. Brandon's), Madagascar (North and South), South Africa, Kenya (Kanamai), Zanzibar, Yemen, Dijibouti, Saudi Arabia (Arabian Gulf and Gulf of Aqaba), Oman, Iran and Red Sea (Eilat). The dotted green lines represent hypothetical extension of clade 4 and clade 2 to Pakistan-North India (Gulf of Kachhch) and Laccadives (west coast of India), south India and Sri Lanka. Area covered by blue line in the inset map shows the distribution of *Stylophora pistillata* (modified from the map in www.coralgeographic.com). The maps were drawn using the software Magic Maps Ver. 1.4.3 and Adobe Illustrator CS5 (Macintosh version).

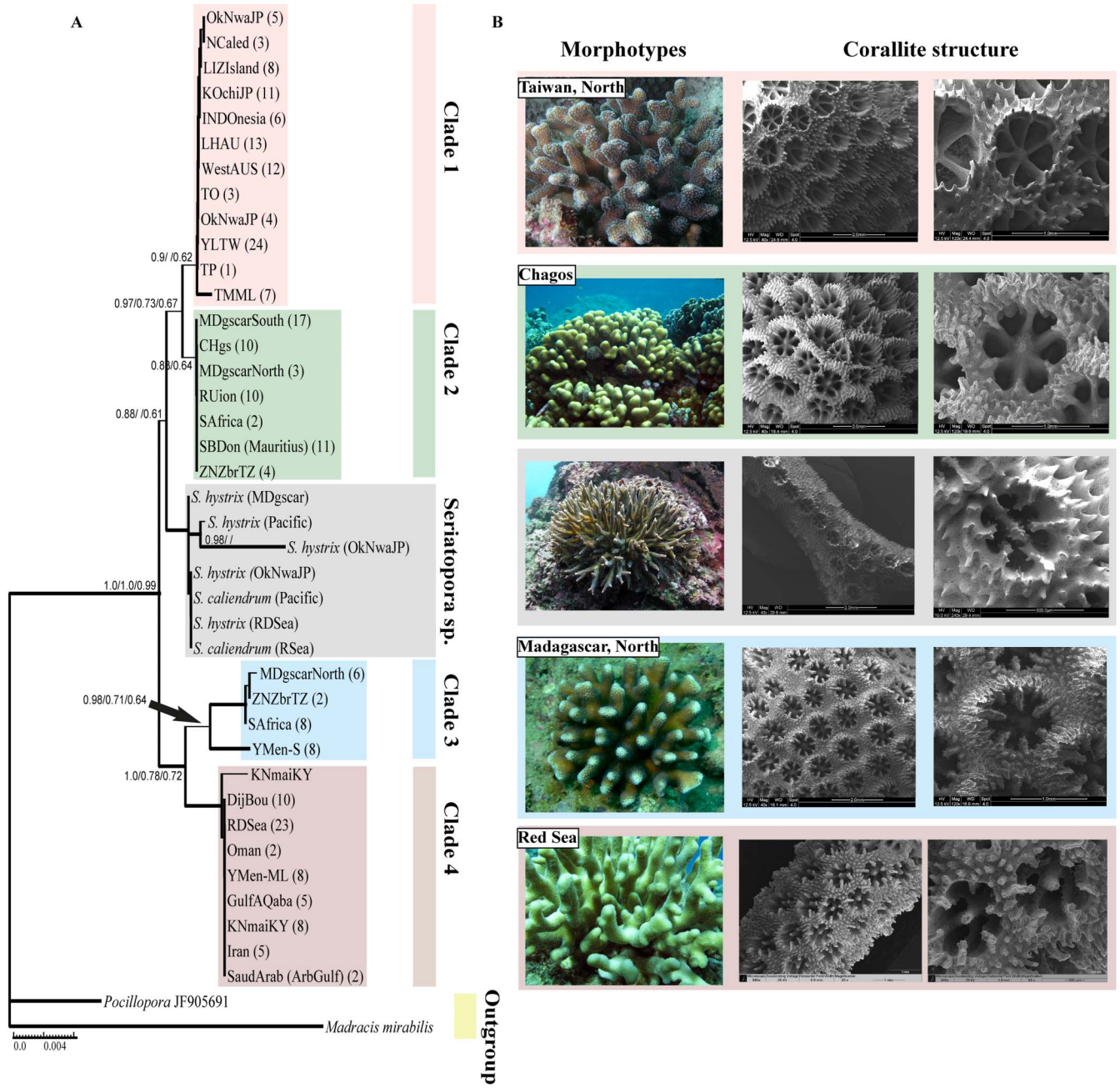
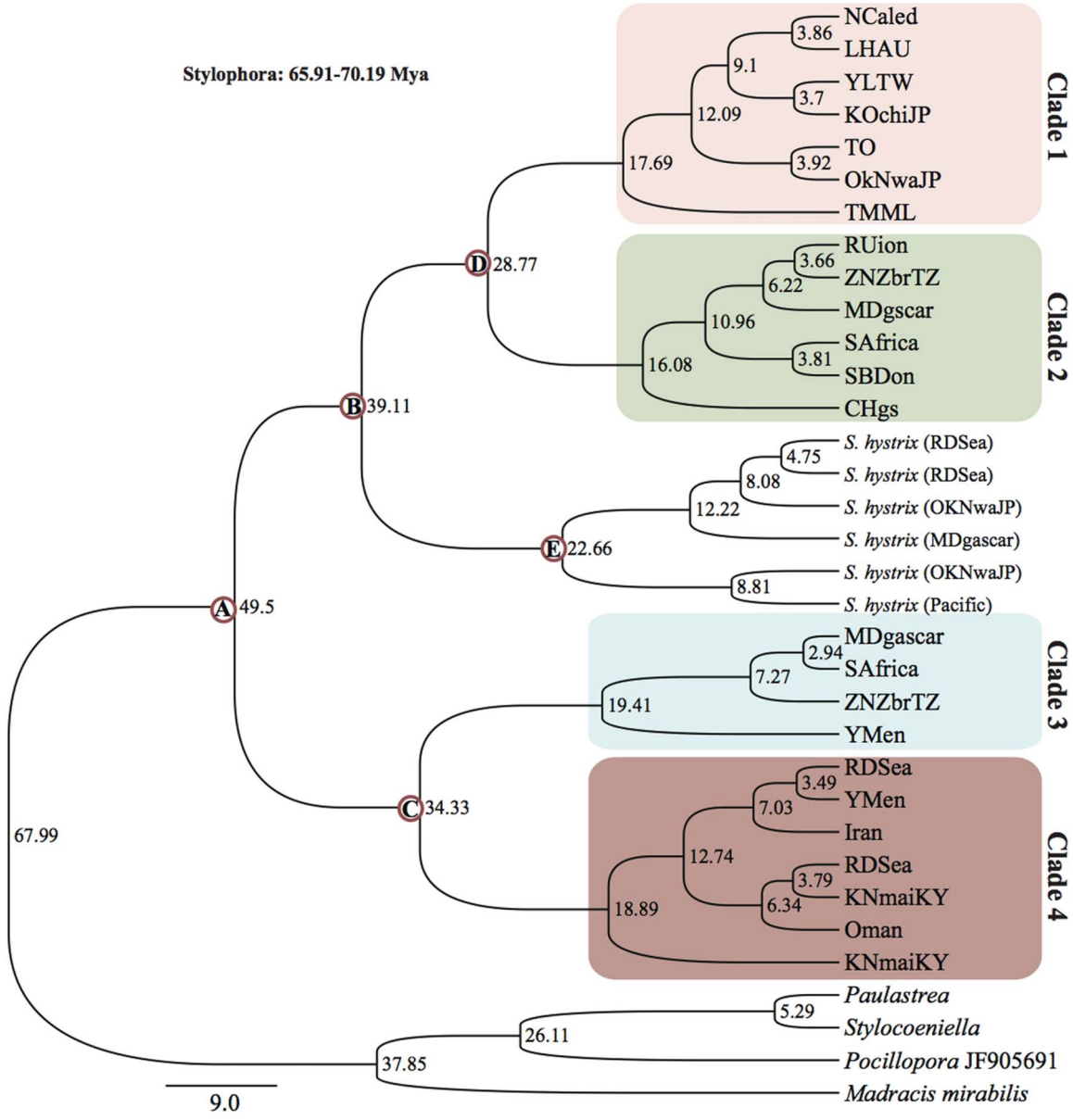


Figure 2 | Phylogenetic analysis of 241 COI sequences of *Stylophora pistillata*. (A) Bootstrap values are based on Baysian/ML/NJ analyses. The numbers in the brackets stand for the total number of samples obtained form each location. Morphotypes and corallite structure of the four clades of *Stylophora*. (B) Based on SEM photos, in clade 1, six regular primary septa that are confluent to the center, fusing with the columella and corallites, are separated by the cœnosteum covered with sharp, fine and thin spinules. In clade 2, the arrangement of primary septa is similar to clade 1, but the spinules in the cœnosteum are more rounded, and fused corallites appear immersed in the cœnosteum. In clade 3, there are six poorly-developed primary septa with a rudimental second cycle of septa visible in some corallites. Septa remain separated and not confluent with the center. A columella is absent and the corallites are separated by poorly developed walls that raise to the surface of the cœnosteum. Spinules are rounded and poorly developed. Finally, in clade 4, there is one cycle of six poorly-developed septa that is continuous with the hoods in the cœnosteum; the columella is well developed but is not fused with septa. Hoods are rounded and regularly distributed. Seriatopora morphotype and corallite structure is shown between clade 2 and clade 3.

The four *Stylophora* clades were also supported by phylogenies based on nuclear ITS and calmodulin intron 3 (CAD3) (Fig. S2, Table S2). The deletion in CAD3 and duplication of tRNAW support the separation of the clades. In case of CAD3, complete deletion occurred in the samples from Pacific (clade 1), mixture of deletion and non-deletion in the central Indian Ocean (clade 2 and 3) and complete non-deletion in the Red Sea (clade 4). Similar results were also seen from the analysis of tRNAW duplication events (Table S2, Fig. S3). Comparison of tRNAW sequences from four clades showed duplication event only in clade 1 and clade 2 (Table S2). Sequence results of tRNAW were also supported by restricted fragment length polymorphism gel pattern using RsaI restriction enzyme (Fig. S3). While clade 1, 2 and 4 showed distinct patterns, clade 3 had mixture of clade 2 and 4. The results from the analysis of the *Symbiodinium* clade association showed that *Stylophora* in the pacific (clade 1) associated with *Symbiodinium* clade C and in the Red Sea (clade 4)



	mean (mya)	std. error of mean	95% HPD lower	95% HPD upper	effective sample size
A	51.46	0.63	32.3	70.38	295.6
B	41.65	0.61	22.94	68.09	360
C	34.55	0.46	15.84	55.5	527.06
D	29.59	0.58	11.23	51.09	330.94
E	22.66	0.33	6.23	42.18	888.16

Figure 3 | Results from molecular dating on the phylogenetic tree of *Stylophora*. Five major ancestral nodes (A, B, C, D and E) to the clade relating to the four described clades of *Stylophora* were labeled to represent the major divergence events in the evolutionary history of *Stylophora*. Numbers on nodes are divergences time for each clades. The table shows the divergence time of each clade on the phylogenetic tree of *Stylophora*. Time of first appearance of *Stylophora* was used as reference time on the Bayesian evaluation of divergence on Beast (65–70 mya). Results of molecular dating are listed in table which includes means of divergence time, standard error of means, 95% of highest posterior density intervals (HPD), effective sample sizes (ESS) and posterior probability of each clades.

associated with *Symbiodinium* clades A, C or A + C combinations. In case of samples from the Indian Ocean *Stylophora* clades associated with *Symbiodinium* clades C, D or C + D. However, *Stylophora* samples from Yemen (clade 3) associated with *Symbiodinium* clade A and C similar to their clade 4 counterparts. This is also clear from the results of phylogenetic analysis in which the samples form Yemen (clade 3) form their own separate sub-clade. Differential *Symbiodinium* association could be due the presence of corals in different regional environments, which can significantly influence the ecology and evolution of this relationship[6].

The COI phylogeny of *S. pistillata* is corroborated by corallite structure (Fig. 2B), although extensive morphological variation still exists[7]. Generally, corallites are round in shape and regularly distributed along the branches. However, corallite arrangement along the

branches varies among the 4 clades (Fig. 2 for detailed description of the skeletal properties in 4 clades).

Divergence time between main clades was inferred using relaxed molecular clock based on COI sequences obtained from this study. Molecular clock calculation was calibrated using the earliest fossil record of *Stylophora* (*S. octophylla*, also see 'Divergence time' section in the Material and Methods for explanation of fossil record), found in Oman and dated around 65–70 Mya[8]. Mean divergence time of clades 1 and 2 from clades 3 and 4 is estimated to be > 51.46 mya (node A), followed by the divergence of clades 3 and 4 from each other (~34.55 mya, node C), the split of clades 1 and 2 from *Seriatopora* (~41.65 mya, node B), and finally the divergence of clades 1 and 2 from each other (~28.77 mya, node D) (Fig. 3). These changes correspond to significant changes in marine connectivity during the middle Eocene (54–38 mya) and Oligocene (38–23 mya)[5,9,10] in which the circumtropical Tethys seaway began to fragment, leading to the isolation of Europe from the Indian Ocean, and the relocation of the centre of diversity for shallow marine species (including corals), from the west Tethys to the Middle East/East-African region[11,12]. Fragmentation of the Tethys seaway may therefore have driven the isolation of *S. pistillata* and the emergence of these different clades[12,13].

Discussion

These results highlight a number of issues in the evolution and taxonomy of this very common coral species. First, our results support the view that, for some marine taxa, the highest biodiversity is recorded in the Indian Ocean[10], particularly the seas around Arabia, rather than the west Pacific. While three distinct clades overlap along the Red Sea, Arabian Peninsula, African coast, Madagascar, and the oceanic islands in the west-central Indian Ocean, the entire Pacific Ocean is dominated by a single clade. Second, the genus *Stylophora* appears to be paraphyletic, as *Seriatopora hystrix*/*caliendrum* are embedded at the base of the PWA and CMSA clades. This supports similar findings for many other conventional taxonomic groups of corals, and further indicates that the present classification of corals, even at the family and genus level, needs major revision[14–16]. Morphological convergence, phenotypic plasticity, recent speciation, and hybridization are likely causes of such patterns/phylogenies. Third, we show that the *Stylophora* "*pistillata*" complex consists of at least four clades. Corroborating this with a detailed morphological and microstructural characterization will be an important step towards formally resolving the taxonomy of this common coral species. In the Gulf of Aden, a recent genetic study[4] discriminated between two *Stylophora pistillata* clades, with a third clade including specimens from the western Pacific[4]. Moreover, morphologic and morphometric data showed the clades to be two distinct species[4], *Stylophora pistillata* and *Stylophora madagascarensis* Veron, 2000. In the latter, strong genetic divergence was noted between the Indian and western Pacific populations, despite no difference in skeletal morphology. We suspect that two of the four clades evident in the present study could correspond to the above-mentioned species, with the other two clades comprising undescribed *Stylophora* species. These results demonstrate that the widely used binomen *Stylophora pistillata* actually encompasses several identities.

Four distinct clades of *S. pistillata* highlight uncertainty in both the taxonomy[3,4] and systematics of corals[14,15]. This inhibits potential action at the species level[16,17] to conserve corals and coral reefs, such as assisted migration[18] which depends on a definitive understanding of biogeographic ranges in order to avoid unintended species introductions, as well as policy action to identify species which are critically endangered[19]. Multiple identities in *S. pistillata* cast doubt on prior research based on a single species[7], and challenge the paradigm of coral as phenotypically plastic organisms with limited genetic variation[20,21].

Methods

Collection of coral samples. *Stylophora pistillata* samples was collected from 34 locations in reefs of Kochi and Okinawa (Japan), Tioman Island (Malaysia, West Pacific), Indonesia, Lord Howe-Australia, Western Australia, Lizard Island-Australia, Taiwan, New Caledonia, Chagos archipelago, Mauritius, Réunion, Madagascar (North and South), South Africa, Zanzibar, Kenya, Dijbouti, Yemen, Oman, Eilat (Israel, Red Sea), Saudi Arabia (Arabian Gulf, Gulf of Aqaba) and Iran. A small fragment of coral was clipped from each colony, placed in a labeled polyethylene bag, and preserved in 70% (v/w) ethanol.

Molecular analyses. DNA extraction was conducted using the protocols described previously[22]. Genetic analyses of coral host were using two mitochondrial DNA region (Mitochondrial cytochrome oxidase I and tRNAW) and two nuclear DNA regions (ITS and Calmodulin intron 3). *Symbiodinum* clades of *Sytlophora* hosts were identified using two ribosomal DNA regions. Finally, the molecular divergence time of *Sytlophora* was estimated using relaxed molecular clock method.

Mitochondrial cytochrome oxidase I (COI). A fragment of COI was initially amplified from *S. pistillata* using the primers, LCO1490 and HCO2198, of[23]. PCR procedure was following[24]; the products were directly sequenced from both ends using the same primers. All the sequencing was carried out by Mission Biotech Co., Ltd, Taipei, Taiwan. The sequences obtained were deposited in the Dryad Repository: http://dx.doi.org/dryad.n2fb2.

ITS rDNA. Targeted segments containing the ITS1-5.8S-ITS2 region were amplified using the "anthozoan-universal" primer pairs, 1S: 5′-GGTACCCTTTGTACAC-ACCGCCCGTCG CT-3′ and 2SS: 5′-GCTTTGGGCGGCAGTCCCAAGCA-ACCCGACTC-3′, as described in[25]. PCR was performed using the following thermal cycle: 1 cycle of 95°C for 4 min; 4 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min; and 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. The amplification reaction used 50 ~ 200 ng of template DNA and BRL Taq polymerase in a 50 μl reaction volume, using the buffer supplied with the enzyme, under conditions recommended by the manufacturer. The PCR products were electrophoresed in a 1% agarose (FMC Bioproduct, Rockland, ME, USA) gel in 1 × TAE buffer to assess the yield. PCR products were cloned using the pGEM-T system (Promega, Madison, WI, USA) according to the manufacturer's recommendations. Nucleotide sequences were determined for complementary strands of at least 3~5 clones from each sample. The sequences obtained were deposited in the Dryad Repository: http://dx.doi.org/dryad.n2fb2.

Calmodulin intron 3. Primers for calmodulin intron 3 (CAD3) were developed in the laboratory of C. A. Chen. A calmodulin-encoded cDNA clone was isolated from the egg-derived cDNA library of *Acropora muricata*[26]. This cDNA clone was characterized and compared to calmodulin genes available in GenBank. One primer was designed specifically for *S. pistillata*, CADStyl-F (5′-ACAAATGAAGTT-GGTGCTGATGGTAGGAGT-3′)(Chiu et al. unpublished data) and the universal primer, CADCORAL-R (5′CTCTGGGAAGTC AATAGTGCCGTTTCC-3′). PCR, cloning, and DNA sequencing were conducted following the conditions described for ITS. The length of CAD3 from different locations was compared. The sequences obtained were deposited in the Dryad Repository: http://dx.doi.org/dryad.n2fb2.

Phylogenetic analyses. COI, ITS1 and CAD3 DNA sequences were aligned using ClustalW algorithm implemented in MEGA 5.0[27]. Alignments were then manually adjusted to increase the overall similarity. COI sequences (546 bp) were aligned and translated to amino acid codes using MEGA 5.0 across all samples. A deletion of ca. 400 bp in one group of CAD3 sequence that was present from 177 bp to 593 bp was adjusted to final 380 bp after alignment. The ITS1 sequences with ~1200 bp length were used in the analysis.

Phylogenetic analyses of COI, ITS and CAD3 based on the Bayesian analysis were performed using MrBayes[28] and Maximum-Likelihood (ML) using PhyML 3.0[29] (http://www.atgc-montpellier.fr/phyml/). For Bayesian analysis, the optimal molecular evolution model was determined using the Akaike Information Criterion (AIC)[30] performed in MrModeltest 2.3[31]. The most suitable models selected by the AIC for the analysis were HKY+I, GTR+I+G, and HKY for COI, ITS, and CAD3 respectively. Markov Chain Monte Carlo (1,000,000 cycles) parameters used to run MrBayes were; 1 cold chain and 3 heated chains with 2000 for Burnin. Model selection for ML was run using the program jModeLTest2[32] within time-reversible (GTR) model under AIC. Models selected were TrN with rate variation among sites TrN+G (nCat=4, gamma shape parameter=0.5960) for COI, GTR+G (gamma shaper parameter=0.5310) for ITS, and HKY (transition/transvertion ratio=1.9428) for CAD3. The levels of robustness were assessed by 1000 bootstrap replicates for COI and CAD3, 500 for ITS for both Bayesian and ML analyses. Only COI sequences were analyzed using Neighbor-joining (NJ) (performed using MEGA 5.0), with 1000 bootstrap and Kimura p-distance parameter was selected as the substitution model.

tRNAW. A section of mtDNA, from NAD5 gene to partial COI gene[33] was amplified with primer sets FNAD5.2deg (5′-GCCYAGRGGTGTTGTTCAAT-3′) and RCOI3deg (5′-CGCAGAAAGCTCBARTCGTA-3′) and followed[34]. One of the genes, tRNAW, was found to have duplicated in some of the regions. PCR products were sent for direct sequencing then compared for duplication. To clarify different patterns for tRNAW in 4 different *Stylophora* clades, representative DNA samples from 4 clades were amplified using the primer set FNAD5.2deg and RCOI3deg and

were subjected to restriction enzyme digestion using RsaI (Thermo Scientific, UK). The reaction mixture was according to the manufacturers instructions. Amplified PCR products were incubated overnight at 37°C with RSAI enzyme. The digested samples were then run on 3% agarose gel (1% normal agarose + 2% low melting agarose) for 3 hours at 50V. The restriction pattern on the gel was photographed using gel documentation system (Vilber Lourmat, France) and visually analysed.

Molecular phylotyping of the Symbiodinium clades. Molecular phylotyping of the *Symbiodinium* clades was conducted using 28S rDNA region and ITS2-DGGE. The protocol for 28S rDNA was modified from methods described by[35]. The variable domains, D1 and D2, of the 28S rDNA of *Symbiodinium* were PCR-amplified using a host-excluding primer set of D1 (5′-CCCGCTGAATTT AAGCATATAAGTA-AGCGG-3′) and D2 (5′-GTTAGACTCCTTGGTCCGTGTTTC AAG A-3′), and then digested with the restriction enzyme, Rsa I, to produce restricted fragment length polymorphism (RFLP) patterns, followed the protocol of[36]. The All enzymes were purchased from MBI (Fermantas). ITS2 region were amplified using primer set 'ITSintfor2' (5′-AAT TGC AGA ACT CCG TG-3′) and 'ITS2 clamp' (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GGG ATC CAT ATG CTT AAG TTC AGC GGG T-3′) from[36] and using touch-down PCR[31]. PCR products of ITS2 were electrophoresed using 45–80% denaturing gradient gels for 16h on CBS Scientific system (Del Mar, CA, USA). Gels were stained with 1 × SYBR Gold (Life Technologies, Invitrogen, U.S.A.) for 20 min, and were photographed using gel documentation system (Vilber Lourmat, France). Method used for assigning the ITS2-DGGE fingerprint was followed as per[36]. Prominent bands of each fingerprint were sent for direct sequencing then match with the sequences from Genbank.

p-distance analysis. The *p*-distances (number of base substitution per site ± standard error estimate) based on COI sequences within and between the four clades were determined using MEGA5[27] under Kimura 2-parameter model[37]. Interspecific pairwise distances in *Stylophora* and *Seriatopora* were compared with distances in other Scleractinia genera. COI sequences obtained in the present study were used for *Stylophora* and *Seriatopora* and COI sequences for other genera were obtained from the NCBI GenBank database.

Divergence time. Divergence time of each clade was calculated (based on phylogenetic COI analysis) using Beast[38], which allow relaxed molecular clock among different lineages. Yule birthrate process was chosen as prior, the distribution of divergence on each node was set as normal distribution with 5% of standard error. HKY model (Hasegawa, Kishino, Yano 85 model) with proportion of invariance (HKY + I) was selected as the most appropriate evolutionary model in the evaluation of likelihood ratio tests for molecular clock estimate[39]. 10 million generations were performed and saved every 1000 generations to calculate their phylogenetic relationship. First quarter of 10000 topologies were discarded as burnin while the remaining were saved to calculate the posterior probabilities. Fossils of *Stylophora* can be traced back to late Cretaceous in both the Caribbean and the Indo-Pacific region before it disappeared in the Caribbean during the early Miocene[40]. It appears that *Stylophora* genus was distributed worldwide prior to Cenozoic before becoming extinct in the Caribbean. To give a reliable reference point for molecular dating, wherein present day *Stylophora* occurs only in the Indo-Pacific, we used the information from the fossil record found in Oman (*Stylophora octophylla*), which can be traced back to its first appearance in Santonian and in Oman during Maastrichtian which is around 65.5–70 Mya[8].

Morphology analysis. Microstructure of coral skeleton from Taiwan, Chagos, Madagascar, Red Sea and Yemen was compared using photographs obtained through scanning electron microscopy[4].

1. Valentini, A., Pompanon, F. & Taberlet, P. DNA barcoding for ecologists. *Trends in Ecology and Evolution* **24**, 110–117 (2008).
2. Shearer, T. L. & Coffroth, M. A. Barcoding corals: limited interspecific divergence, not intraspecific variation. *Molecular Ecology Resources* **8**, 247–255 (2008).
3. Flot, J. F. *et al.* Incongruence between morphotypes and genetically delimited species in the coral genus *Stylophora*: phenotypic plasticity, morphological convergence, morphological stasis or interspecific hybridization? *BMC Ecology* **11**, 22 (2011).
4. Stefani, F. *et al.* Comparison of morphological and genetic analyses reveals cryptic divergence and morphological plasticity in *Stylophora* (Cnidaria, Scleractinia). *Coral reefs* **30**, 1033–1049 (2011).
5. Schettino, A. & Turco, E. Tectonic history of the western Tethys since the Late Triassic. *Geological Society of America Bulletin* **123**, 89–105 (2011).
6. LaJeunesse, T. C. *et al.* Long-standing environmental conditions, geographic isolation and host- symbiont specificity influence the relative ecological dominace and genetic diversification of coral endosymbionts in the genus *Symbiodinium*. *Journal of Biogeography* **37**, 785–800 (2010).
7. Loya, Y. Homage to *Stylophora pistillata*: an important coral in coral reef research (Darwin's Lecture), *9th International Coral Reef Symposium*, October 23–27, Bali, Indonesia (2004).
8. Baron-Szabo, R. C. Corals of the K/T-boundary: Scleractinian corals of the suborders Astrocoeniina, Faviina, Rhipidogyrina and Amphiastraeina. *Journal of Systematic Palaeontology* **4**, 1–108 (2006).
9. Meulenkamp, J. E. & Sissingh, W. Tertiary palaeogeography and tectonostratigraphic evolution of the Northern and Southern Peri-Tethys platforms and the intermediate domains of the African/Eurasian convergent plate boundary zone. *Palaeogeography, Palaeoclimatology, Palaeoecology* **196**, 209–228 (2003).
10. Wallace, C. C. & Muir, P. Biodiversity of Indian Ocean from the perspective of staghorn coral (*Acropora* spp). *Indian Journal of Marine Science* **34**, 42–49 (2005).
11. Renema, W. *et al.* Hopping Hotspots: Global Shifts in Marine Biodiversity. *Science*, **321**, 654–657 (2008).
12. Obura, D. O. Evolutionary mechanisms and diversity in a western Indian Ocean center of diversity. *12th International Coral Reef Symposium*, Cairns, Australia, 9–13 July 2012.
13. Wilson, M. & Rosen, B. Implications of paucity of corals in the Paleogene of SE Asia: plate tectonics or Centre of Origin? In: Hall, R., Holloway, J., editors. Backhuys Publishers, Leiden, The Netherlands: Biogeography and Geological Evolution of SE Asia. pp. 165–195 (1998).
14. Fukami, H. *et al.* Conventional taxonomy obscures deep divergence between Pacific and Atlantic corals. *Nature*, **427**, 832–835 (2004).
15. Fukami, H. *et al.* Mitochondrial and nuclear genes suggest that stony corals are monophyletic but most families of stony corals are not (Order Scleractinia, Class Anthozoa, Phylum Cnidaria. *PLoS ONE* **3**, e3222 (2008).
16. Huang, D. Threatened Reef Corals of the World. *PLoS ONE* **7**, e34459 (2012).
17. Carpenter, K. E. *et al.* One-third of reef-building corals face elevated extinction risk from climate change and local impacts. *Science* **321**, 560–563 (2008).
18. Hoegh-Guldberg, O. *et al.* Assisted colonization and rapid climate change. *Science* **321**, 345–346 (2008).
19. NMFS-NOAA. Management report for 82 corals status review under the endangered species act: Existing regulatory mechanisms. Accessed online form National Marine Fisheries Service webpage on 28th July 2012.
20. Pinzón, J. H. & LaJeunesse, T. Species delimitation of common reef corals in the genus *Pocillopora* using nucleotide sequence phylogenies, population genetics and symbiosis ecology. *Molecular Ecology* **20**, 311–325 (2011).
21. Combosch, D. J. & Vollemer, S. V. Population Genetics of an Ecosystem-Defining Reef Coral *Pocillopora damicornis* in the Tropical Eastern Pacific. *PLoS ONE* **6**, e21200 (2011).
22. Chen, C. A., Wang, J. T., Fang, L. S. & Yang, Y. W. Fluctuating algal symbiont communities in *Acropora palifera* (Cnidaria; Scleractinia) from Taiwan. *Marine Ecology Progress Series* **295**, 343–347 (2005).
23. Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* **3**, 294–299 (1994).
24. Medina, M., Weil, E. & Szmant, A. M. Examination of the Montastraea annularis species complex (Cnidaria: Scleractinia) using ITS and COI sequences. *Marine Biotechnology* **1**, 89–97 (1999).
25. Chen, C. A., Willis, B. L. & Miller, D. J. Systematic relationships between tropical corallimorpharians (Cnidaria: Anthozoa: Corallimorpharia): utility of the 5.8 S and internal transcribed spacer (ITS) regions of the rRNA transcription unit. *Bulletin of Marine Science* **59**, 196–208 (1996).
26. Chiou, C. Y. *et al.* Analysis of *Acropora muricata* Calmodulin (CaM) indicates that Scleractinian corals possess the ancestral exon/intron organization of the eumetazoan CaM gene. *Journal of Molecular Evolution* **66**, 317–324 (2008).
27. Tamura, K. *et al.* MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* **28**, 2731–2739 (2011).
28. Huelsenbeck, J. P. & Ronquist, F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 745–755 (2001).
29. Guindon, S. *et al.* "New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0." *Systematic Biology* **59**, 307–21 (2010).
30. Akaike, H. A new look at the statistical model identification. *IEEE Transactions on Automatic Control* **19**, 716–723 (1974).
31. Posada, D. & Crandall, K. A. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818 (1998).
32. Darriba, D., Taboada, G. L., Doallo, R. & Posada, D. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9**, 772 (2012).
33. Chen, C., Dai, C. F., Plathong, S., Chiou, C. Y. & Chen, C. A. The complete mitochondrial genomes of needle corals, *Seriatopora* spp. (Scleractinian; Pocilloporidae): an idiosyncratic atp8, duplicated trnW gene, and hypervariable regions used to determine species phylogenies and recently diverged populations. *Molecular Phylogenetics and Evolution* **46**, 19–33 (2008).
34. Flot, J. F. *et al.* Mitochondrial sequences of *Seriatopora* corals show little agreement with morphology and reveal the duplication of a tRNA gene near the control region. *Coral Reefs* **27**, 789–794 (2008).
35. Loh, W. K. W., Loi, T., Carter, D. & Hoegh-Guldberg, O. Genetic variability of the symbiotic dinoflagellates from the wide ranging coral species *Seriatopora hystrix* and *Acropora longicyathus* in the Indo-West Pacific. *Marine Ecology Progress Series* **222**, 97–107 (2001).
36. LaJeunesse, T. C. Diversity and community structure of symbiotic dinoflagellates from Caribbean coral reefs. *Marine Biology* **141**, 387–400 (2002).

37. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111–120 (1980).
38. Drummond, A. J. & Rambaut, A. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7**, 214–221 (2007).
39. Hasegawa, M., Kishino, H., & Yano, T. A. Dating of the Human Ape Splitting by a Molecular Clock of Mitochondrial-DNA. *Journal of Molecular Evololution* **22**, 160–174 (1985).
40. Budd, A. Diversity and extinction in the Cenozoic history of Caribbean reefs. *Coral Reefs* **19**, 25–35 (2000).

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Author contributions

C.A.C., D.O. and M.P. designed the research. S.Y., A.A., S.K., S.F., S.D.P. and Y.C. performed all the molecular work and analysis. S.Y., C.A.C., A.A., M.P., D.O., F.S., F.B., A.M., A.N., C.C., C.W., R.M.P., V.D., A.A., J.D., T.M., C.S., Y.L., M.M., A.B., P.G.M. and B.S. sampled *Stylophora pistillata* from different locations. S.K. and C.A.C. wrote the paper in discussion with all authors.

Additional information

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