

SCIENTIFIC REPORTS



OPEN

Microsatellite markers for multiple *Pocillopora* genetic lineages offer new insights about coral populations

Yuichi Nakajima¹ , Patricia H. Wepfer¹, Shohei Suzuki², Yuna Zayasu³, Chuya Shinzato^{3,4}, Noriyuki Satoh³ & Satoshi Mitarai¹

Population genetics of the coral genus *Pocillopora* have been more intensively studied than those of any other reef-building taxon. However, recent investigations have revealed that the current morphological classification is inadequate to represent genetic lineages. In this study, we isolated and characterized novel microsatellite loci from morphological *Pocillopora meandrina* (Type 1) and *Pocillopora acuta* (Type 5). Furthermore, we characterized previously reported microsatellite loci. A total of 27 loci (13 novel loci) proved useful for population genetic analyses at two sites in the Ryukyu Archipelago, in the northwestern Pacific. Clonal diversity differed in each genetic lineage. Genetic structure suggested by microsatellites corresponded to clusters in a phylogenetic tree constructed from a mitochondrial open reading frame (mtORF). In addition, we found an unknown mitochondrial haplotype of this mtORF. These microsatellite loci will be useful for studies of connectivity and genetic diversity of *Pocillopora* populations, and will also support coral reef conservation.

The genus *Pocillopora*, especially *Pocillopora damicornis*, is the most studied reef-building coral in terms of population genetics^{1–9}. This genus includes both broadcast-spawning and brooding species¹⁰ and it is distributed from the Indian Ocean to the East Pacific. In *Pocillopora*, 17 species are currently recognized in Veron¹¹ and in Coral of the World (<http://coral.aims.gov.au/>), while 22 species are registered in the World Register of Marine Species (WoRMS) (<http://www.marinespecies.org/>), as of March 2017. However, based on gross morphology, the number of *Pocillopora* species may be overestimated¹², because recent genetic studies have suggested that the traditional classification may not correspond to genetic species^{10, 12–15}. Nevertheless, 16 or more genetic lineages of *Pocillopora* have been considered as putative species based on haplotypes of a mitochondrial DNA region^{10, 12, 15} and genetic structure analyzed using microsatellite markers^{10, 15}.

Approximately 400 reef-building coral species inhabit the Ryukyu Archipelago¹⁶, Japan. A recent phylogenetic approach confirmed six lineages of *Pocillopora* in the northwestern Pacific¹². *Pocillopora* corals have been damaged by various global and local anthropogenic disturbances, and coral populations have not yet recovered from repeated mass bleaching events since 1998 at reefs in the Ryukyu Archipelago¹⁷. Population genetics studies using genetic markers make it possible to distinguish invisible inter- and intraspecific relationships among populations. These studies have estimated genetic diversity, differentiation among locations, reproductive characteristics, and species delimitations for various organisms, including corals. Microsatellites, composed of tandemly repeated regions of nucleotides are highly polymorphic among individuals and chromosomes. Next-generation sequencing technology has facilitated development of microsatellite loci, which have recently been developed for reef building corals^{18, 19}.

In this study, using next-generation sequencing, we developed cross-lineage microsatellite loci in order to facilitate population genetic analyses for multiple lineages of *Pocillopora* in the Ryukyu Archipelago. Although

¹Marine Biophysics Unit, Okinawa Institute of Science and Technology Graduate University, Tancha 1919-1, Onna, Okinawa, 904-0495, Japan. ²Okinawa Marine Science Support Section, Okinawa Institute of Science and Technology Graduate University, Tancha 1919-1, Onna, Okinawa, 904-0495, Japan. ³Marine Genomics Unit, Okinawa Institute of Science and Technology Graduate University, Tancha 1919-1, Onna, Okinawa, 904-0495, Japan. ⁴Atmosphere and Ocean Research Institute, The University of Tokyo, Kashiwanoha 5-1-5, Kashiwa, Chiba, 277-8564, Japan. Correspondence and requests for materials should be addressed to Y.N. (email: yuichi.nakajima@oist.jp)

Locus	Repeat motif (Type 1/Type 5)	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')	Accession No. (Type 1/Type 5)
Psp_01	(AAGT)2AAGC(AAGT)4N22(TAGA)11/(AAGT)2AAGC(AAGT)9N22(TAGA)10	TCGTTCAATCCACTGACTGC	U19- CCTTTGGATGCGATGTAAT	LC222418/LC222431
Psp_02	(GACCC)9/(GACCC)6	CTGTGCTGGAAT'TCCCTTA	U19- AGCCTACGGCGCAATAGTAG	LC222419/LC222432
Psp_10	(TGAG)11(TGGG)3/(TGAG)2TGAA(CGAG)2(TGAG)2(TGGG)4	AGGCGAAGCCATAATGTTGT	U19- CTTCGTTGTGGGCTAAGAGG	LC222420/LC222433
Psp_16	(AAAAC)8/(AAAAC)5	CCCGCTGCTGAGTAAGAATC	U19- AGAGAAACTGCAAAACCGC	LC222421/LC222434
Psp_18	(TGTA)12/(TGTA)9	CACACGTTTATGACAACGGA	U19- ATAAGCCGTAGGCCCTGTCT	LC222422/LC222435
Psp_23	(TGTA)11/(TGTA)5	ACCATTGCCATCACTGTTC	U19- TTCATTTCATTCGTATTGGCG	LC222423/LC222436
Psp_29	(TTTTTC)8/(TTTTTC)5	TTTCGTACCAAAATCCAGGC	U19- TTTTTCAGTCGCAAGAGGC	LC222424/LC222437
Psp_32	(CTAT)12/(CTAT)7	AAGCAGCAATTCAGCCTAT	U19- AGCCTAAGACGAATCGAGCA	LC222425/LC222438
Psp_33	(AATC)10/(AATC)6	CCATTTCGCAATCTCTCTC	U19- CTCGTCGCCAGATATAAA	LC222426/LC222439
Psp_35	(CTAT)3CTAC(CTAT)10TTAT(CTAT)3/(CTAT)16	TGGCTGATGTCTGTGGGTAA	U19- CGCGATTATCGAAAGTTTG	LC222427/LC222440
Psp_39	(AAGTG)8/(AAGTG)4	TCTTACAGCACAGGAGCCA	U19- TTTTTCTTGCGGTCCAATTC	LC222428/LC222441
Psp_41	(ATTT)10/(ATTT)3ACTT(ATTT)3	U19-CGCACAAGGAAAATTTGTT	TTCCACACCAGAAGATGACG	LC222429/LC222442
Psp_48	(CTTT)11/(CTTT)4	TGAAATTC AAGAGAATGGGCA	U19-GTTTCTGATGGTGTCT	LC222430/LC222443

Table 1. Characteristics of novel microsatellite loci developed for *Pocillopora* in this study: locus name, repeat motif, forward and reverse primer sequences, and GenBank accession number for each lineage used for isolation of genomic DNA.

microsatellites have been already reported in *Pocillopora*, we developed additional markers to widen the choice of markers for population genetics analyses between and within lineages. We developed 13 additional microsatellite markers from morphological *Pocillopora meandrina* and *Pocillopora acuta* colonies. In addition, we validated and characterized microsatellites previously reported from *Pocillopora* for populations in the Ryukyu Archipelago. Those were developed by Magalon *et al.*²⁰, Magalon *et al.* unpublished, and Starger *et al.*²¹ and recently, more loci have been isolated by Pinzón & LaJeunesse¹³ and Torda *et al.*⁸. Gorospe & Karl²² re-designed primers for two loci, PV7 from Magalon *et al.*²⁰ and Pd3-010 from Starger *et al.*²¹ and designated them as Pd2-AB79 and Pd3-EF65, respectively. Using all of these validated microsatellite markers, we genetically characterized populations from multiple lineages of *Pocillopora* from Ueno and Yoshino, Miyako Island, in the Ryukyu Archipelago.

Results and Discussion

We successfully merged single sequences (2,523,457 reads comprising 1,034,311,164 bp for *P. meandrina* and 7,021,083 reads comprising 2,056,539,596 bp for *P. acuta*) from sequence data derived from genomic DNA. These were used for microsatellite detection and primer design. MISA and Primer3 were employed to identify 1,597 reads with primer sequences, including repeats under the following conditions (4 mer: 10 repeats or more; 5 mer: 8 repeats or more). After removing incomplete or extremely long repeats to facilitate fragment analysis, 752 reads remained (4 mer: 10 to 15 repeats; 5 mer: 8 to 12 repeats). Of these reads, we selected 50 with homologous repeat sequences and flanking regions, from *P. meandrina* and *P. acuta*, to be employed as cross-lineage microsatellite loci. Based on the genetic delimitation and definition by Pinzón *et al.*¹⁰, these morphological colonies displayed Type 1 and Type 5 mitochondrial open reading frames (mtORF), respectively. The nuclear ribosomal internal transcribed spacer 2 (ITS2) sequence from the Type 1 colony was ITS2 type T (see below).

We confirmed that 13 non-overlapping loci could produce sufficient PCR amplification in all genetic lineages, based on mitochondrial haplotypes derived from fragment analyses (Table 1). In our analyses using previously developed microsatellites, we could not confirm the proper amplification for genotyping of PV3 (Magalon *et al.* unpublished), PV5, PV6²⁰, Pd3-002, Pd3-010²¹, or Pd13⁸, which have been widely used in other population genetics studies. However, redesigned primer sequences targeting flanking regions identified in databases, such as Gorospe & Karl²² may have improved amplification of the loci, PV7 and Pd3-010. Actually, Pd3-010 was not useful, but Pd3-EF65 by virtue of nested primer sets, was useful even though it targeted the same locus as Pd3-010. Previously reported loci appear to be effective for populations in the Ryukyu Archipelago (Table 2). Clonal replicates were detected at both sites in Type 5 (Table 3). In addition, the population size of Type 5, based on genetic analysis appears to be large in Miyako Island, as in the Yaeyama Islands, where *P. acuta* exists abundantly²³. Mean values of genetic indices across loci for each lineage/site with large numbers of multilocus genotypes (Types 1, 3, 5 at Ueno, Type 5 at Yoshino) are shown in Table 3. Detailed genetic indices for each locus are in Supplementary Table S1. The P_{ID} ranged from $3.3e^{-26}$ to $1.5e^{-20}$ (Table 3); therefore, tandem use of these loci allows identification of genets with high resolution. Linkage disequilibrium was not significant ($p > 0.05$) in any microsatellite locus pairs after removal of replicate loci (PV7 is the same locus as Pd2-AB79). Novel microsatellite loci developed here are derived from the nuclear region since it exhibits only moderate heterozygosity and one or two peaks in

Locus	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Accession No.	Reference
Pd2-001	U19-CAGACTTGTCGGAATGAAAGC	TTTTGTTTATAAGTCGATACAATGCA	DQ684672	Starger <i>et al.</i> ²¹
Pd3-002*	U19-ATCCGAATACAAGCGAAACG	CAAAGCTTCTATCAGAAAATGCAA	DQ684673	Starger <i>et al.</i> ²¹
Pd2-003*	U19-CCTCTTCTGTTGGGCTCT	TCTGCATTACGTTTGTGGTACACA	DQ684674	Starger <i>et al.</i> ²¹
Pd3-004	U19-ACCAGACAGAAACACGCACA	GCAATGTGTAACAGAGGTGGAA	DQ684675	Starger <i>et al.</i> ²¹
Pd3-005	U19-AGAGTGTGACAGCGAGGAT	GTTCCTTCGCCTTCGATTTT	DQ684676	Starger <i>et al.</i> ²¹
Pd2-006	U19-ATCTCCATGTGATCGGCATT	GTCCCCAGCTGAGAAGTT	DQ684677	Starger <i>et al.</i> ²¹
Pd2-007	U19-AAGAAGGTGTGGTATTTTCAGAGGG	GGTGGATAAAGTATTCTCACTCTGG	EF120462	Starger <i>et al.</i> ²¹
Pd3-008	AGTTGAGGTTGTGAAACATG	U19-TCCATGCAGAACCCC	EF120463	Starger <i>et al.</i> ²¹
Pd3-009	U19-CCAATGCGTCCGTAGCTCTC	ATCACCTAAAAATTCAGTCCCTTACC	EF120464	Starger <i>et al.</i> ²¹
Pd3-010*	U19-CTGATCAACAACTGGGAGGC	TCATTAGAAATCATCTTGATTTGATAAGG	EF120465	Starger <i>et al.</i> ²¹
PV2	U19-CCAGGACCCATTATACTCC	TGCAGTGTCTACTTGTCACTGC	AY397777	Magalon <i>et al.</i> ²⁰
PV3*	U19-TGAAACAGGATTGACGACGA	AACCCGAATGATCCACAAT	AY397778	Magalon <i>et al.</i> (unpublished)
PV5*	U19-GTCATCACGCAAAGTTCC	GAATAGCTGCGTTTATTTGG	AY397780	Magalon <i>et al.</i> ²⁰
PV6*	U19-CTTCCCGACCGTTTAGGG	AGCCGTTCACTACCTATGG	AY397781	Magalon <i>et al.</i> ²⁰
PV7	U19-GAGATGGATGGAGACTGC	GGTATCTCTGTCTCAGTTCTTTG	AY397782	Magalon <i>et al.</i> ²⁰
Pd2-AB79 (from PV7)	GGAGATGGATGGAGACTGCT	U19-AGTGCACGCACTAGATAGA	AB214379	Gorospe & Karl ²²
Pd3-EF65 (from Pd3-010)	U19-TGTGCAAGTGTGTGACTGA	TGCTTTTTCACTTTTGCTTCAA	EF120465	Gorospe & Karl ²²
Pd4	U19-ACGCACACAACCAACAAAC	TAATTCATCAACTCAAAGGGG	Not found	Torda <i>et al.</i> ⁸
Pd11	TCGTTTGAAGGAAATGCTC	U19-GCATGCTATGTATGCGAGA	Not found	Torda <i>et al.</i> ⁸
Pd13	TGTTCTCTCTTTCTCTCTTCCA	U19-CATTTATGTTCCTTTCACGGC	Not found	Torda <i>et al.</i> ⁸
Poc40	U19-TTATTATATGGGTGATGC	CTCAAAGTGCAGTAAAGCC	Not found	Pinzón & Lajeunesse ¹³

Table 2. Characteristics of previously developed microsatellite loci for *Pocillopora*: locus name, forward and reverse primer sequences, and GenBank accession number. *We could not confirm the proper amplification for genotyping.

Site	Lineage	N	G	N _{MLL}	N _A	H _O	H _E	F _{IS}	P _{ID}
Ueno	Type 3	1	1	1	—	—	—	—	—
	Type 5	29	16	10	4.48	0.524	0.584	0.103	1.5e ⁻²⁰
Yoshino	Type 1 (ITS2 type T)	11	11	11	6.07	0.530	0.595	0.107	3.3e ⁻²³
	Type 1 (ITS2 type C)	11	11	11	5.85	0.519	0.615	0.170	1.8e ⁻²³
	Type 3	20	20	20	7.82	0.539	0.648	0.172	3.3e ⁻²⁶
	Type 4	1	1	1	—	—	—	—	—
	Type 5	10	8	8	4.67	0.526	0.596	0.106	3.0e ⁻²¹
	Type 8	1	1	1	—	—	—	—	—
	Unknown	6	5	3	—	—	—	—	—

Table 3. The number of samples (N), the number of multilocus genotypes (G), the number of multilocus lineages (N_{MLL}), the mean number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities, and the deviation index from Hardy-Weinberg equilibrium (F_{IS}) across 27 loci for each lineage or site. The probability of identity (P_{ID}) for 27 loci was determined with GenAlix. For lineages or sites with low numbers of MLLs, N_A, H_O, H_E, F_{IS}, and P_{ID} were not calculated in this table. Values of N_A, H_O, H_E, and F_{IS} for each locus are shown in Supplementary Table S1. PV7 was removed from this analysis because it was identical to Pd2-AB79.

fragment analysis, excluding scoring errors. Also, polymorphism of zooxanthella loci was confirmed in the same multilocus lineage (MLL). These loci will facilitate combining multiple loci for effective multiplex PCR.

Mitochondrial data showed two haplotype groups (Types 3 and 5) at the Ueno site, and haplotype groups of mtORF (Types 1, 3, 4, 5, 8, and unknown) at the Yoshino site. Furthermore, Type 1 was separated into two haplotype groups based upon ITS2. At least six lineages of *Pocillopora* inhabit the northwestern Pacific¹². This study confirmed that all of these, plus one more, occur in the Ryukyu Archipelago. One unknown haplotype sequence was related to a sequence from *Pocillopora brevicornis* (GenBank accession No. KR919858, Mayfield *et al.* unpublished; see also Schmidt-Roach *et al.*¹² about *Pocillopora* cf. *brevicornis*), which differed by 5 bp. A maximum likelihood phylogenetic tree based on mtORF haplotypes was constructed (Fig. 1).

Genetic structure among genetic lineages separated on the basis of mtORF and based on 27 microsatellite loci (Fig. 2), supported the results of Pinzón *et al.*¹⁰. Using the method of Evanno *et al.*²⁴ to determine the most

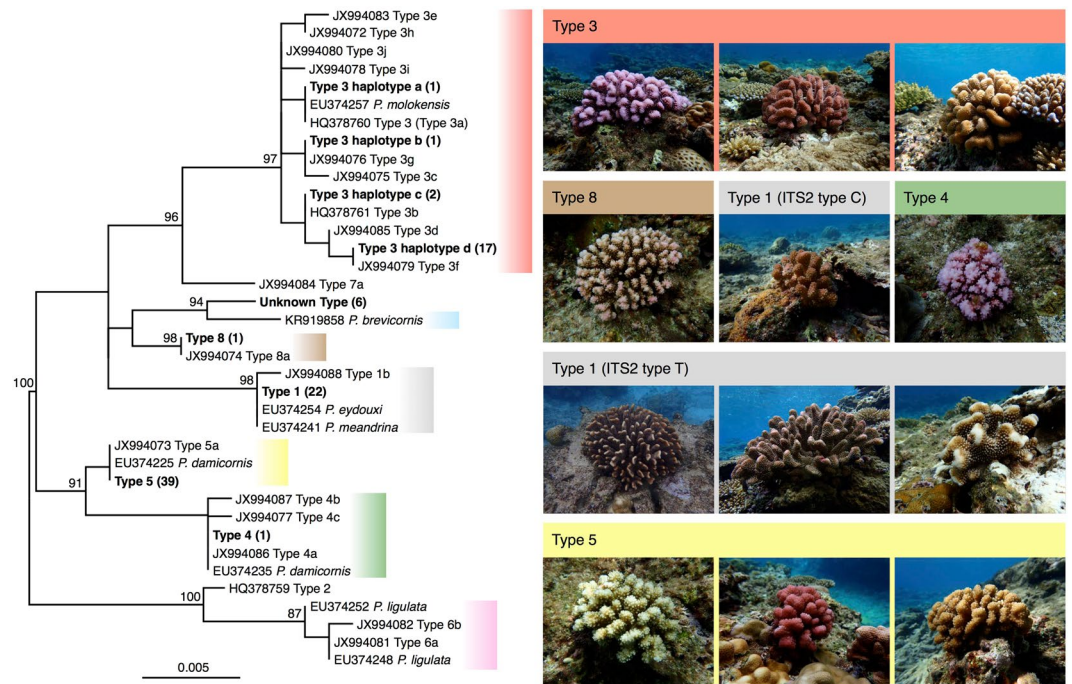


Figure 1. Maximum likelihood phylogenetic tree based on the mtORF comparing the variety of haplotypes with the variety of morphological types of *Pocillopora*. Immense diversity is evident even within lineages in the Ryukyu Archipelago. Boldface indicates haplotypes that were isolated during this study (GenBank accession No. LC222444–LC222452). Values in parentheses indicate the number of colonies collected from two sites at Miyako Island. Values in the tree indicate maximum likelihood bootstrapping (over 75%). Cluster colors correspond to genetic lineages reported by Schmidt-Roach *et al.*¹².

probable number of genetic populations (K), the largest peak of ΔK was $K = 3$ (Fig. 2). Otherwise, ΔK values at $K = 5$ and $K = 8$ also showed small peaks; therefore, we showed genetic population structure for values of K ranging from 3 to 8 (Fig. 2). Genetic structure among Type 1 mtORFs was reflected in different ITS2 haplotypes, as reported by Pinzón *et al.*¹⁰. We defined ITS2 types T and C within mtORF Type 1, based on their haplotype groups (see registered sequences: Genbank accession No. LC222453–LC222460). Some MLLs in mtORF Type 5 were assigned to the cluster containing Type 4. However, there was only one mtORF Type 4 sample. Further studies with additional samples are needed to fully distinguish these two lineages. Also, when $K \geq 6$ in STRUCTURE analysis, definite genetic structure was detected, even between sites within mtORF Type 5, despite the close geographic proximity of these populations, ~ 17 km along the coast. Thus, genetic structure even in the same genetic lineage at fine geographic scale could be confirmed in a brooding species²⁵. In part, genetic differentiation within a given lineage may reflect differences of water depth ($< \sim 17$ m) between sites.

In conjunction with primers developed previously, these new primer pairs also may be used for a variety of *Pocillopora* lineages (including various morphological species) and may provide useful information about coral population genetics in general. By sampling a greater number of specimens across a larger geographic area, these microsatellite loci will be beneficial for population genetics within and between *Pocillopora* lineages in various localities. They will be helpful to understand genetic structure and population dynamics at contemporary and historical time scales, and for parentage analysis, identification of evolutionary lineages, and genetic diversity to understand maintenance of coral populations.

This study suggested that 13 new and 14 previously reported loci are useful as cross-lineage microsatellites for population genetic analyses of *Pocillopora* in the Ryukyu Archipelago. mtORF Type 5 obtained at two sites for validation of microsatellites indicated large number of clonal replicates, especially in Ueno. We detected distinct genetic structure among lineages, as estimated from mtORF haplotypes (ITS2 was also considered for Type 1). Although Schmidt-Roach *et al.*¹² reported that at least six lineages of *Pocillopora* inhabit the northwestern Pacific region, this study indicated that all six occur in the Ryukyu Archipelago, despite sampling only two sites. We confirmed one more lineage with an unknown haplotype in this region. Additional genetic diversity may be discovered in this region by extending the number and geographical distribution of sampling sites. More extensive analyses of genetic diversity and connectivity in within and between populations will be needed to better understand population maintenance and dynamics for conservation and management of coral reef organisms.

Methods

Next-generation sequencing and isolation of microsatellites for two *Pocillopora* lineages. We collected branches from the morphological species, *P. meandrina* and *P. acuta*, along the western coast of Okinawa Island in the Ryukyu Archipelago, a subtropical area located in the northwestern Pacific Ocean, Japan. These belonged to mitochondrial open reading frame (mtORF) Type 1 and Type 5 (see Results and Discussion).

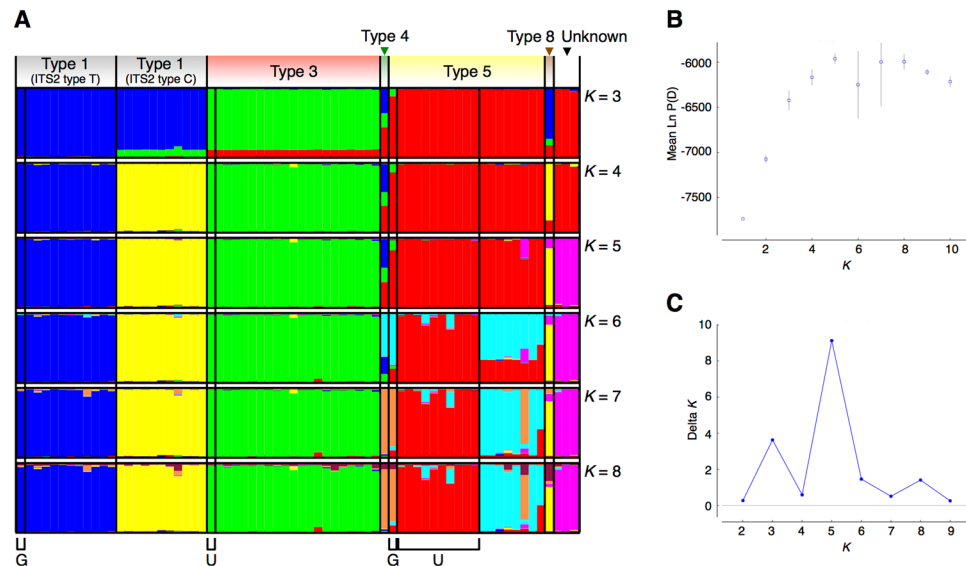


Figure 2. Population genetic assessment using STRUCTURE suggests five probable genetic clusters within just the pair of sites we surveyed. These clusters reflect primarily the mtORF (including ITS2) types. The Evanno method was used to decide the probable number of genetic clusters. (A) Each bar plot shows the possible membership of genetic clusters for each MLL. Those ranged from three to eight ($K=3$ to $K=8$), with $K=5$ being optimal. G: MLLs of samples collected from the western coast of Okinawa Island, used for Illumina sequencing, U: MLLs of samples collected at Ueno, Miyako Island. Samples of other MLLs were collected at Yoshino, Miyako Island. (B and C) The model criterion of choice to detect the most probable number of genetic clusters ($K=1$ to $K=10$) across 10 iterations per K (the assumed number of clusters). Graphs of (B) mean log probability (Ln P(D)) and (C) ΔK values based on the rate of change in Ln P (D) between successive K values ($K=2$ to $K=9$) for detecting the most probable number of K .

Specimens were preserved in ethanol and genomic DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen) following the standard protocol. We sequenced 250-bp paired-end reads using a MiSeq sequencer (Illumina) according to the manufacturer's instructions. After merging single-end sequences using PEAR ver. 0.9.8²⁶, to isolate reads of 100 bp or more having repeat sequences of *P. meandrina* (Type 1), we used MISA (<http://pgrc.ipk-gatersleben.de/misa/>) to detect simple sequence repeats and to design PCR primers using Primer3 (<http://pgrc.ipk-gatersleben.de/misa/primer3.html>). We searched *P. acuta* (Type 5) using BLAST+ ver. 2.3.0²⁷. We confirmed that sequence regions, including microsatellites, were conserved between the two lineages.

Collection of coral samples, sequencing of mtORF, and phylogenetic analysis of *Pocillopora*. To confirm validation and polymorphism of microsatellite loci, branches from 90 *Pocillopora* colonies were collected at Ueno (30 colonies; 24°43'09" N, 125°20'27" E; <1 m depth, by walking along the seashore) and Yoshino (60 colonies; 24°44'55" N, 125°26'45" E; <18 m depth, by SCUBA diving) at Miyako Island at the Ryukyu Archipelago, Japan. Genomic DNA was extracted from preserved coral branches as described above. Mitochondrial haplotypes were confirmed by sequencing the mtORF region²⁸. PCR reaction mixtures consisted of: (10 μ L) containing <30 ng/ μ L template DNA, AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific), and the primers (final concentration: 2 μ M for each primer) for mtORF: FATP6.1 (5'-TTTGGGSATTCGTTTAGCAG-3') and RORF (5'-SCCAATATGTTAAACASCATGTCA-3')²⁹. The PCR protocol consisted of 94 °C for 1 min, followed by 40 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 75 s, with a final extension at 72 °C for 5 min. After reaction of Exonuclease I (Takara) and Shrimp Alkaline Phosphatase (Takara) to clean up PCR products, sequencing was conducted by MacroGen Japan.

mtORF sequences obtained were aligned with others from previous studies^{10, 13, 28, 29} including unpublished work of Mayfield *et al.*, using MUSCLE in MEGA ver. 6.06³⁰. The molecular evolution model was selected by MEGA, and the best model was determined to be the HKY (Hasegawa-Kishino-Yano) model with a gamma distribution of rate variation across sites (+G). Rapid bootstrap analysis employing the maximum likelihood method for phylogenetic analysis was carried out using MEGA with the model using 1000 bootstrap replicates. The phylogenetic tree was drawn in FigTree ver. 1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

The nuclear ribosomal internal transcribed spacer 2 (ITS2) was used for identification of less differentiated genetic lineages within Type 1^{10, 29}. PCR reaction mixtures comprised: (10 μ L) containing <30 ng/ μ L template DNA, AmpliTaq Gold 360 Master Mix, and primers (final concentration: 2 μ M for each primer) of ITS2: ITS2c-5 (5'-AGCCAGCTGCGATAAGTAGTG-3') and R28S1 (5'-GCTGCAATCCCAAACAACCC-3')²⁹. Conditions for PCR and sequencing were the same as for mtORF.

Genotyping of *Pocillopora* using novel and known microsatellite loci. Together with novel microsatellite loci developed here, we characterized loci for *Pocillopora* previously developed by Magalon *et al.*²⁰,

Magalon *et al.* (unpublished), Starger *et al.*²¹, Pinzón & LaJeunesse¹³, Torda *et al.*⁸, and two loci modified by Gorospe & Karl²². For scoring of microsatellite genotypes of each colony, the PCR reaction mixture (5 µL) contained template DNA (<30 ng/µL), AmpliTaq Gold 360 Master Mix, and three primers for each locus: a non-tailed reverse primer (0.5 µM), a forward primer with a sequence tail of U19 (5'-GGTTTCCCCAGTCACGACG-3') (0.5 µM), and a U19 primer (0.5 µM) fluorescently labeled with FAM, VIC, NED, or PET³¹. The PCR protocol consisted of 95 °C for 9 min, followed by 35 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. We furthermore conducted PCR using microsatellites for zooxanthellae, C1.02³², SymC_3-02, SymC_3-03³³, and D1Sym11³⁴ within the same MLL to confirm that the microsatellites were derived from nuclear loci, since different symbiont genotypes have been detected from the same MLL in *Pocillopora*³⁵. PCR conditions were the same as for microsatellites from colonies, except for the annealing temperature (50 °C for 4 zooxanthellae microsatellites). PCR products were analyzed using an ABI 3730 capillary DNA sequencer (Thermo Fisher Scientific) with the GeneScan 600 LZ size standard (Thermo Fisher Scientific) to identify genotypes by the length of the amplicon. Fragment size and intensity were confirmed using Geneious ver. 9.0.4 (Biomatters).

Data analyses to characterize microsatellites and populations. The concept of multilocus lineage (MLL) was employed to avoid underestimating clonality due to genotyping errors and somatic mutations³⁶. If genotypes of ≤ 3 loci differed and all other loci coincided, a colony was considered to be clonal, derived by asexual reproduction. Either a scoring error or a somatic mutation was assumed to be responsible for the variable locus/loci. The number of different loci was calculated using GenAlEx ver. 6.501³⁷. When different genotypes were determined to belong to the same MLL, the most common genotype was assigned³⁸. However, if the most common genotype could not be determined (*e.g.*, if two genotypes occurred in equal numbers), the genotype was set to zero³⁹. We also calculated P_{SEX} to estimate the possibility of coincidence of multilocus genotypes by sexual reproduction, using GenClone ver. 2.0⁴⁰. After removing clonal replicates from the data set, the number of alleles, values of observed and expected heterozygosity (H_O and H_E , respectively), and deviation index (F_{IS}) from Hardy-Weinberg equilibrium were evaluated for each locus and lineage using GenAlEx. Lineages with few MLLs were not used for calculation of these genetic indices. The probability of identity (P_{ID}) was also calculated using GenAlEx to estimate the resolution of loci for each lineage/site. Linkage disequilibrium was estimated for each lineage using Genepop on the Web^{41, 42}.

Genetic structure based on Bayesian clustering was inferred using STRUCTURE ver. 2.3.4⁴³. A burn-in period of 100,000 followed by 1,000,000 Markov chain Monte Carlo (MCMC) replications was used for population clustering without LOCPRIOR (prior sampling location information) model under the admixture model and assuming independent allele frequencies⁴⁴. After calculation of the mean log probability, $\ln P(D)$, to estimate the most probable number of genetic clusters, the number of clusters was determined using the method of Evanno *et al.*²⁴, as implemented in STRUCTURE HARVESTER⁴⁵. Run data were merged using CLUMPAK⁴⁶.

References

- Stoddart, J. A. Genetic differentiation amongst populations of the coral *Pocillopora damicornis* off southwestern Australia. *Coral Reefs* **3**, 149–156 (1984).
- Stoddart, J. A. Genetic structure within populations of the coral *Pocillopora damicornis*. *Mar. Biol.* **81**, 19–30 (1984).
- Adjeroud, M. & Tsuchiya, M. Genetic variation and clonal structure in the scleractinian coral *Pocillopora damicornis* in the Ryukyu Archipelago, southern Japan. *Mar. Biol.* **134**, 753–760 (1999).
- Magalon, H., Adjeroud, M. & Veuille, M. Patterns of genetic variation do not correlate with geographical distance in the reef-building coral *Pocillopora meandrina* in the South Pacific. *Mol. Ecol.* **14**, 1861–1868 (2005).
- Ridgway, T., Riginos, C., Davis, J. & Hoegh-Guldberg, O. Genetic connectivity patterns of *Pocillopora verrucosa* in southern African Marine Protected Areas. *Mar. Ecol. Prog. Ser.* **354**, 161–168 (2008).
- Combosch, D. J. & Vollmer, S. V. Population genetics of an ecosystem-defining reef coral *Pocillopora damicornis* in the Tropical Eastern Pacific. *PLoS One* **6**, e21200 (2011).
- Torda, G., Lundgren, P., Willis, B. L. & van Oppen, M. J. H. Genetic assignment of recruits reveals short- and long-distance larval dispersal in *Pocillopora damicornis* on the Great Barrier Reef. *Mol. Ecol.* **22**, 5821–5834 (2013).
- Torda, G., Schmidt-Roach, S., Peplow, L. M., Lundgren, P. & van Oppen, M. J. H. A rapid genetic assay for the identification of the most common *Pocillopora damicornis* genetic lineages on the Great Barrier Reef. *PLoS One* **8**, e58447 (2013).
- Noreen, A. M. E., Schmidt-Roach, S., Harrison, P. L. & van Oppen, M. J. H. Diverse associations among coral host haplotypes and algal endosymbionts may drive adaptation at geographically peripheral and ecologically marginal locations. *J. Biogeogr.* **42**, 1639–1650 (2015).
- Pinzón, J. H. *et al.* Blind to morphology: genetics identifies several widespread ecologically common species and few endemics among Indo-Pacific cauliflower corals (*Pocillopora*, Scleractinia). *J. Biogeogr.* **40**, 1595–1608 (2013).
- Veron, J. Corals of the World. Australian Institute of Marine Science, Townsville, Queensland, Australia (2000).
- Schmidt-Roach, S., Miller, K. J., Lundgren, P. & Andreakis, N. With eyes wide open: a revision of species within and closely related to the *Pocillopora damicornis* species complex (Scleractinia; Pocilloporidae) using morphology and genetics. *Zool. J. Linn. Soc.* **170**, 1–33 (2014).
- Pinzón, J. H. & LaJeunesse, T. C. Species delimitation of common reef corals in the genus *Pocillopora* using nucleotide sequence phylogenies, population genetics and symbiosis ecology. *Mol. Ecol.* **20**, 311–325 (2011).
- Schmidt-Roach, S. *et al.* Assessing hidden species diversity in the coral *Pocillopora damicornis* from Eastern Australia. *Coral Reefs* **32**, 161–172 (2013).
- Gélin, P., Postaire, B., Fauvelot, C. & Magalon, H. Reevaluating species number, distribution and endemism of the coral genus *Pocillopora* Lamarck, 1816 using species delimitation methods and microsatellites. *Mol. Phylogenet. Evol.* **109**, 430–446 (2017).
- The Japanese Coral Reef Society & Ministry of the Environment. Coral Reefs of Japan. Ministry of the Environment, Tokyo, Japan (2004).
- van Woesik, R., Sakai, K., Ganase, A. & Loya, Y. Revisiting the winners and the losers a decade after coral bleaching. *Mar. Ecol. Prog. Ser.* **434**, 67–76 (2011).
- Shinzato, C. *et al.* Development of novel, cross-species microsatellite markers for *Acropora* corals using next-generation sequencing technology. *Front. Mar. Sci.* **1**, 11 (2014).

19. Nakajima, Y., Shinzato, C., Satoh, N. & Mitarai, S. Novel polymorphic microsatellite markers reveal genetic differentiation between two sympatric types of *Galaxea fascicularis*. *PLoS One* **10**, e0130176 (2015).
20. Magalon, H., Samadi, S., Richard, M., Adjeroud, M. & Veuille, M. Development of coral and zooxanthella-specific microsatellites in three species of *Pocillopora* (Cnidaria, Scleractinia) from French Polynesia. *Mol. Ecol. Notes* **4**, 206–208 (2004).
21. Starger, C. J., Yeoh, S. S. R., Dai, C.-F., Baker, A. C. & DeSalle, R. Ten polymorphic STR loci in the cosmopolitan reef coral, *Pocillopora damicornis*. *Mol. Ecol. Resour.* **8**, 619–621 (2008).
22. Gorospe, K. D. & Karl, S. A. Genetic relatedness does not retain spatial pattern across multiple spatial scales: dispersal and colonization in the coral, *Pocillopora damicornis*. *Mol. Ecol.* **22**, 3721–3736 (2013).
23. Kitano, Y. F., Nagai, S., Ueno, M. & Yasuda, N. Most *Pocillopora damicornis* around Yaeyama Islands are *Pocillopora acuta* according to mitochondrial ORF sequences. *Galaxea* **17**, 21–22 (2015).
24. Evanno, G., Regnaut, S. & Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol. Ecol.* **14**, 2611–2620 (2005).
25. Nakajima, Y. *et al.* Elucidating the multiple genetic lineages and population genetic structure of the brooding coral *Seriatopora* (Scleractinia: Pocilloporidae) in the Ryukyu Archipelago. *Coral Reefs* **36**, 415–426 (2017).
26. Zhang, J., Kobert, K., Flouri, T. & Stamatakis, A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* **30**, 614–620 (2014).
27. Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. & Madden, T. L. BLAST+: architecture and applications. *BMC Bioinformatics* **10**, 421 (2009).
28. Flot, J.-F. & Tillier, S. The mitochondrial genome of *Pocillopora* (Cnidaria: Scleractinia) contains two variable regions: The putative D-loop and a novel ORF of unknown function. *Gene* **401**, 80–87 (2007).
29. Flot, J.-F., Magalon, H., Cruaud, C., Couloux, A. & Tillier, S. Patterns of genetic structure among Hawaiian corals of the genus *Pocillopora* yield clusters of individuals that are compatible with morphology. *C. R. Biol.* **331**, 239–247 (2008).
30. Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **30**, 2725–2729 (2013).
31. Schuelke, M. An economic method for the fluorescent labeling of PCR fragments. *Nat. Biotechnol.* **18**, 233–234 (2000).
32. Bay, L. K., Howells, E. J. & van Oppen, M. J. H. Isolation, characterisation and cross amplification of thirteen microsatellite loci for coral endo-symbiotic dinoflagellates (*Symbiodinium* clade C). *Conserv. Genet. Resour.* **1**, 199–203 (2009).
33. Howells, E., van Oppen, M. J. H. & Willis, B. High genetic differentiation and cross-shelf patterns of genetic diversity among great barrier reef populations of *Symbiodinium*. *Coral Reefs* **28**, 215–225 (2009).
34. Pettay, D. T. & Lajeunesse, T. C. Microsatellite loci for assessing genetic diversity, dispersal and clonality of coral symbionts in 'stress-tolerant' clade D *Symbiodinium*. *Mol. Ecol. Resour.* **9**, 1022–1025 (2009).
35. Pettay, D. T., Wham, D. C., Pinzón, J. H. & Lajeunesse, T. C. Genotypic diversity and spatial–temporal distribution of *Symbiodinium* clones in an abundant reef coral. *Mol. Ecol.* **20**, 5197–5212 (2011).
36. Arnaud-Haond, S., Duarte, C. M., Alberto, F. & Serrão, E. A. Standardizing methods to address clonality in population studies. *Mol. Ecol.* **16**, 5115–5139 (2007).
37. Peakall, R. & Smouse, P. E. Genalex 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* **6**, 288–295 (2006).
38. Arnaud-Haond, S. *et al.* Vicariance patterns in the Mediterranean Sea: east-west cleavage and low dispersal in the endemic seagrass *Posidonia oceanica*. *J. Biogeogr.* **34**, 963–976 (2007).
39. Nakajima, Y., Zayasu, Y., Shinzato, C., Satoh, N. & Mitarai, S. Genetic differentiation and connectivity of morphological types of the broadcast-spawning coral *Galaxea fascicularis* in the Nansei Islands, Japan. *Ecol. Evol.* **6**, 1457–1469 (2016).
40. Arnaud-Haond, S. & Belkhir, K. GENCLONE: a computer program to analyze genotypic data, test for clonality and describe spatial clonal organization. *Mol. Ecol. Notes* **7**, 15–17 (2007).
41. Raymond, M. & Rousset, F. Genepop (version 1.2): Population genetics software for exact tests and ecumenicism. *J. Hered.* **86**, 248–249 (1995).
42. Rousset, F. Genepop'007: A complete reimplementation of the Genepop software for Windows and Linux. *Mol. Ecol. Resour.* **8**, 103–106 (2008).
43. Pritchard, J. K., Stephens, M. & Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959 (2000).
44. Falush, D., Stephens, M. & Pritchard, J. K. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics* **164**, 1567–1587 (2003).
45. Earl, D. A. & vonHoldt, B. M. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* **4**, 359–361 (2012).
46. Kopelman, N. M., Mayzel, J., Jakobsson, M., Rosenberg, N. A. & Mayrose, I. CLUMPAK: a program for identifying clustering modes and packaging population structure inferences across K. *Mol. Ecol. Resour.* **15**, 1179–1191 (2015).

Acknowledgements

This study was financially supported by a Japan Society for the Promotion of Science (JSPS) KAKENHI grant (25840152 and 16H05621 to Y.N. and 26290065 to C.S.) and by the Okinawa Institute of Science and Technology Graduate University. We thank Dr. Steven D. Aird (Okinawa Institute of Science and Technology Graduate University) for editing the manuscript. Collection of all corals for this study was approved by Okinawa Prefecture, Japan (permission number: 26–62).

Author Contributions

Y.N. and S.M. conceived and designed the experiments. Y.N., P.H.W., S.S., and Y.Z. collected samples. Y.N. performed the experiments and analyzed the data. C.S. and N.S. contributed reagents and analytical tools. Y.N. wrote the manuscript, and P.H.W. and S.M. edited it.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-06776-x

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or

format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2017