Genome - wide SNP genotyping reveals hidden population structure of an acroporid species at a subtropical coral island: Implications for coral restoration

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RESEARCH ARTICLE

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Genome-wide SNP genotyping reveals hidden population structure of an acroporid species at a subtropical coral island: Implications for coral restoration

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Abstract

1. It is essential to consider genetic composition for both conventional coral restoration management and for initiating new interventions to counter the significant global decline in living corals. Population genetic structure at a fine spatial scale should be carefully evaluated before implementing strategies to achieve self-sustaining ecosystems via coral restoration.
2. This study investigated the population genetic structure of two acroporid species at Kume Island, Okinawa, Japan. There were 140 colonies of *Acropora digitifera* collected from seven study sites, and 81 colonies of *Acropora tenuis* from six sites. In total, 384 single nucleotide polymorphism (SNP) loci for *A. digitifera* and 470 SNPs for *A. tenuis* were obtained using a comparatively economical technique, Multiplexed ISSR Genotyping by sequencing.
3. Observed heterozygosity was significantly lower than expected heterozygosity at all SNP sites in both acroporid species, suggesting deficient genetic diversity possibly caused by past massive coral bleaching. Even though both species are broadcast spawners, the population structure was different in the two species. No detectable structure was evident in *A. digitifera*, but two distinct clades were found in *A. tenuis*. The genetic homogeneity of *A. digitifera* at Kume Island suggests that this species could be used as a focal species for active restoration in terms of genetic differentiation at this island. By contrast, *A. tenuis* unexpectedly included two distinct clades with little or no admixture within a small study area, possibly representing two reproductively isolated cryptic species. Thus, when using *A. tenuis*, it would be prudent to avoid disturbing the genetic composition of wild populations until this question is answered.

Yuna Zayasu and Takeshi Takeuchi contributed equally to this work.

KEYWORDS
biogeography, conservation genetics, corals, genetic diversity, MIG-seq, population genetic structure, SNPs

1 | INTRODUCTION

Coral-zooxanthellae holobionts engineer ecosystems in tropical to temperate coastal waters (Wild et al., 2011). The heterogeneous structures of coral calcium carbonate exoskeletons create habitat complexity on which entire reef ecosystems depend (Fisher et al., 2015). However, due to global warming, increasing light intensities and warming water temperatures are inducing the collapse of this mutualistic relationship in a process known as coral bleaching (Glynn, 1996; Baker & Cunning, 2015). In recent decades, repeated coral bleaching and subsequent coral mortality have led to a serious global decline in living coral coverage (Pandolfi et al., 2003; Bruno & Selig, 2007; Hughes et al., 2018a; Hughes et al., 2018b). Various restoration measures, such as coral nurseries and removal of corallivorous crown-of-thorns seastars, are being rapidly initiated worldwide in an attempt to protect coral reef ecosystems (Omori, 2019; Anthony et al., 2020; Boström-Einarsson et al., 2020).

The stony coral genus, *Acropora*, is a fast-growing, keystone reef-building species globally (Bellwood & Hughes, 2001). In addition to global threats, diseases, predation, sedimentation, coastal development, and intensifying storms cause local damage (Connell, 1997; Knowlton, 2001). These repeated, frequent stressors are prompting restoration efforts using this threatened keystone taxon.

Acropora is a hermaphroditic, sexually reproducing, broadcast spawning taxon. Although motile *Acropora* larvae have the potential to disperse long-distances, many recent population genetic studies support infrequent high gene flow between islands in archipelagos (Underwood, 2009; Shinzato et al., 2015; Cros et al., 2016; Drury et al., 2016; Zayasu et al., 2016; Nakabayashi et al., 2019). In other words, most acroporid larvae settle in their own and neighbouring reefs (Jones et al., 2009; Figueiredo, Baird & Connolly, 2013).

Various coral restoration techniques have been tested in Okinawa Prefecture (Omori, 2011). In contrast, studies of population genetic structure have a shorter history than restoration efforts (van Oppen & Gates, 2006). Consequently, insufficient attention has been given to genetic variation (Shafer et al., 2015; Taylor, Dussex & van Heezik, 2017). To achieve the goal of self-sustaining ecosystem restoration both short and long terms, it is essential to consider genetic composition (Frankham, 2005; Jenkins & Stevens, 2018).

In Onna Village, Okinawa, coral gardening methodology, in which coral fragments are grown in underwater nurseries and are out-planted back to degraded reefs, has been employed since 1998 (Omori et al., 2016; Higa et al., 2018; Okaji et al., 2020). Based on knowledge developed at Onna Village, coral gardening using *Acropora* has become one of the most utilized techniques for reef restoration in the Ryukyu Archipelago. However, population genetic structure of acroporid corals at a very fine spatial scale (<10 km) has not been

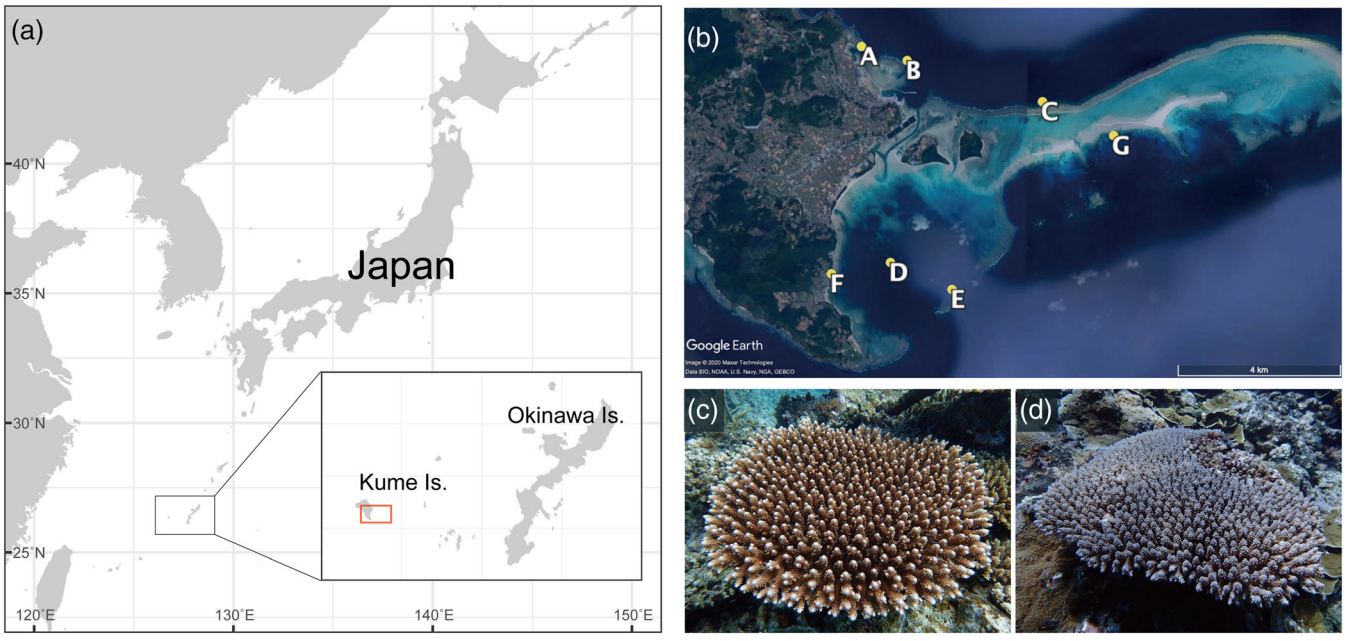

FIGURE 1 Sample localities and target species. (a) Kume Island is located approximately 90 km west of Okinawa Island in the Nansei Archipelago, in south-western Japan. (b) Sample localities (A–G) at Kume Island. Locality details are shown in Table 1. The satellite image was retrieved from Google Earth (https://earth.google.com/web). (c) *Acropora digitifera*. (d) *Acropora tenuis*

investigated until recently in Okinawa. Corals in Okinawa have a more complex population structure and recruitment than simply following the strong northward Kuroshio current, as once thought (Shinzato et al., 2015; Zayasu et al., 2016). Hence, a more fine-grained analysis of population structure is needed for actual management action.

This study investigated the population genetic structure of *Acropora digitifera* and *Acropora tenuis*, two focal species of on-going restoration management at Kume Island, Okinawa. For *A. digitifera*, previous studies detected very little genetic structure using both microsatellite markers (Nakajima et al., 2010) and SNPs (Shinzato et al., 2015) in the Nansei Islands, but these studies did not include the samples from Kume Island. For *A. tenuis*, there are at least two populations reported in the Nansei Islands (Zayasu et al., 2016). The corals in Kume Island should be studied more carefully before an actual intervention because it is located near the contact point of the genetic subdivision of coral populations, and we expected that this island maintains a more complicated structure. To support long-term success of conservation scientifically, i.e. re-create a self-sustaining coral assemblage without genetic disturbance, we carefully evaluated population genetic structure of corals in this study area before initiating management action.

Kume Island (Figure 1) possesses moats within fringing reefs and a lagoon enclosed by barrier reefs (Hasegawa, 1984) (Figure 1). Thus, results of this study should be applicable to many similar subtropical and tropical coral islands. Kume Island is also biologically important, and it has been designated as a Prefectural Natural Park (1983), a Ramsar site (2008), and a Biodiversity Priority Area (2009) by the World Wildlife Foundation Japan (Yasumura, 2010), because of its high biodiversity and important habitats for endemic and endangered species. The coral restoration plan being used by the Okinawa Prefectural Government involves obtaining coral gametes from colonies in outer reefs (hard coral cover 10–20%), fertilizing, and growing the larvae and fragments in nurseries, before out-planting them into degraded reefs inside the lagoon (hard coral cover less than 10%), together with counter measures to eliminate local stressors, such as red soil runoff.

Most local restoration programmes have limited budgets; however, analyses of local-level genetic variation in marine animals require fine-scale data sets, which usually cost more to obtain. Here, we provide an example of a population genomic study using a comparatively economical technique, Multiplexed ISSR Genotyping by sequencing (MIG-seq), to describe the population structure of these target species at very fine spatial scale.

2 | METHODS

2.1 | Sample collection

All samples were collected on 8–9 July 2019 under a permit from the Okinawa Prefectural Government (No. 31–32). Identification of *A. digitifera* (Figure 1c) and *A. tenuis* (Figure 1d) was based on Nishihira & Veron (1995) and Wallace (1999). Samples were taken from 221 colonies, including 81 colonies of *A. tenuis* from six sites and 140 of *A. digitifera* from seven sites (Figure 1; Table 1). Samples were

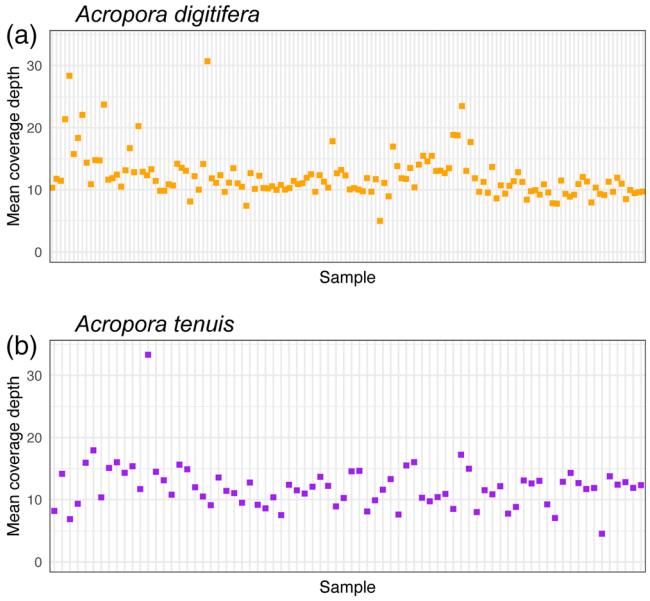

FIGURE 2 Sequence coverage depth for each sample of *Acropora digitifera* (upper) and *Acropora tenuis* (lower)

TABLE 1 Summary of sample collection data

			Acropora digitifera		*Acropora tenuis*	
Locality	Longitude	Latitude	Sampling depth (m)	Number of colonies	Sampling depth (m)	Number of colonies
A	126.812E	26.362N	1–7	20	5–8	20
B	126.823E	26.359N	1–6	20	5–10	20
C	126.857E	26.350N	2–3	20	1–5	7
D	126.819E	26.314N	1–3	20	Not found	
E	126.834E	26.308N	2–5	20	3–13	9
F	126.805E	26.312N	1–3	20	1–3	12
G	126.874E	26.342N	2–8	20	5–17	10

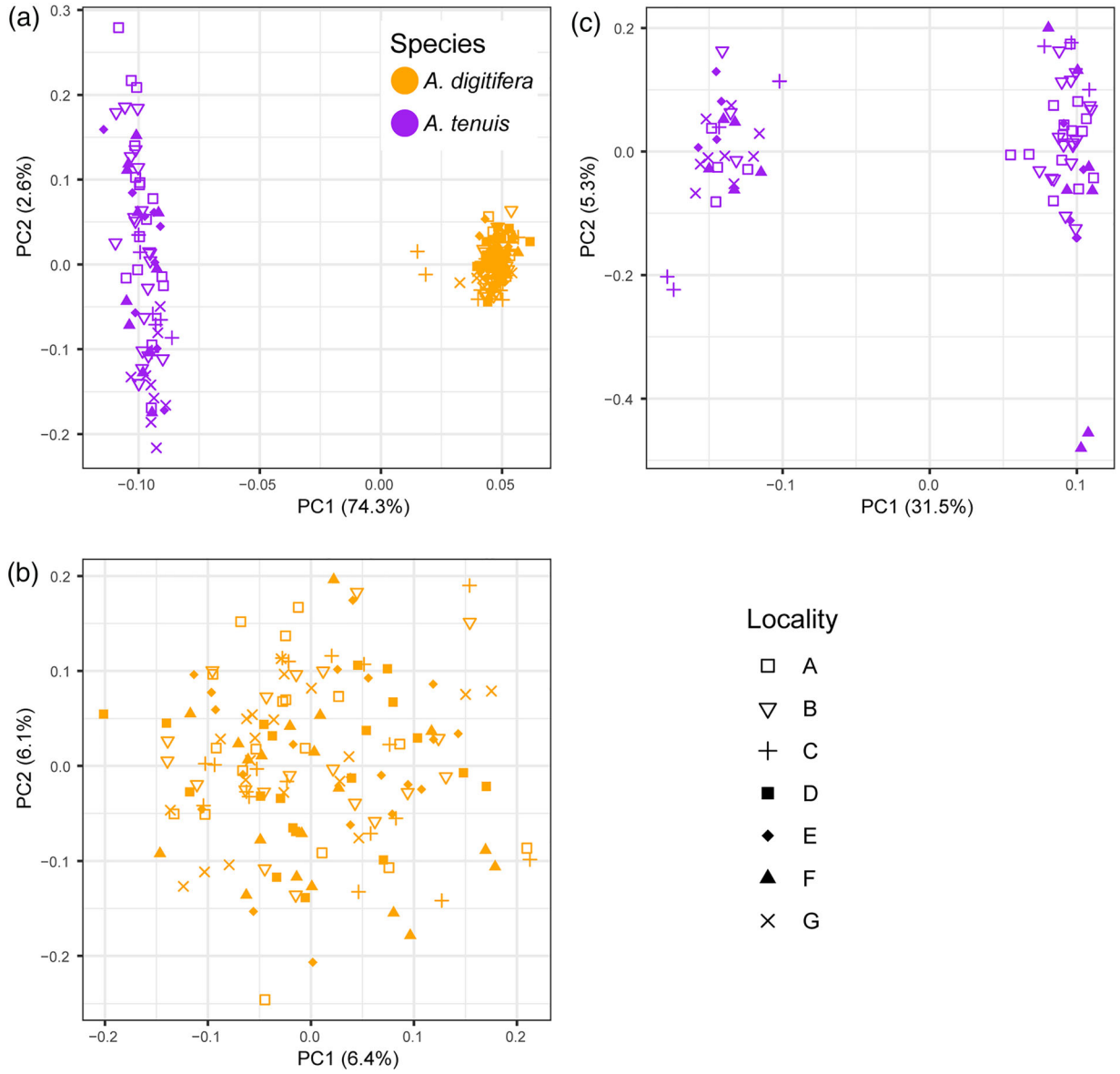

FIGURE 3 Genetic clusters are detected in *Acropora tenuis*, but not in *Acropora digitifera*. Principal component (PC) analysis of (a) all samples, (b) *A. digitifera*, and (c) *A. tenuis*. Each dot indicates one individual. Sampling localities are shown at the bottom, right

collected at least 3 m apart within a 60–100 m radius *for A. digitifera*, and owing to its lower density, within a 60–400 m radius for *A. tenuis*. At each study site, fragments were collected at depths of 1–15 m, using scuba diving for searches <40 min. Colonies were chosen randomly, and approximately 2 cm branch fragments were stored in absolute ethanol in the dark at ambient temperature until DNA extraction.

2.2 | DNA extraction, library preparation, and sequencing

Genomic DNA was extracted from coral fragments using a Maxwell® RSC Blood DNA Kit (Promega Corporation) on a Maxwell® RSC Instrument (Promega Corporation), following the manufacturer's standard protocol, except for 4-hours incubation at 56°C for sample lysis. Then, the quantity and quality of the DNA were checked using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc.).

For MIG-seq library preparation, we adopted the method of Suyama & Matsuki (2015) with some modifications. For the first PCR, we prepared a 20 μl reaction mixture including 2 μl of template DNA, 0.2 μl of each first PCR primer ('primer set-1'; Suyama & Matsuki, 2015), 10 μl of 2× Multiplex PCR buffer, and 0.1 μl of Multiplex PCR Enzyme Mix (Multiplex PCR Assay Kit Ver.2, Takara Bio Inc.). To optimize ISSR amplification for our samples, the annealing temperature was set at 38°C. The first PCR was performed using a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc.), and the PCR product was checked using a TapeStation 4150 (Agilent

Technologies, Inc.). The first PCR product was diluted 100 times and applied to a second PCR as a template. Second PCR conditions were the same as the original protocol of MIG-seq (Suyama & Matsuki, 2015), except for running 10 cycles of denaturation, annealing, and extension. The concentration of each second PCR product was measured using an Infinite M1000 PRO Microplate Reader (Tecan Group Ltd.) with a Quant-iT dsDNA High-Sensitivity Assay Kit (Thermo Fisher Scientific Inc.). The pooled library was purified and size-selected at 300–1,500 bp using Sera-Mag Select (Cytiva, Tokyo, Japan), and sequenced using the Illumina MiSeq platform (Illumina, Inc.).

2.3 | Data processing and genotyping

Raw reads were processed using Trimmomatic (version 0.36) (Bolger, Lohse & Usadel, 2014) to remove low-quality sequences (average quality below 20 with a three-base sliding window). Reads shorter than 35 bases were removed, and 75 bases from the 5′ end were retained. Processed reads were mapped to the reference genome assemblies of *A. digitifera* or *A. tenuis* (Shinzato et al., 2020) using BWA (version 0.7.15) (Li & Durbin, 2009). The genotype of each individual was retrieved using BCFtools (version 1.6) mpileup option (Li, 2011). Variant calling was performed if a locus had a sequencing coverage depth ≥5, and a global minor allele frequency rate of at least 10%, reducing the impact of sequencing errors. We removed single nucleotide polymorphism (SNP) loci present in fewer than 60% of all individuals. If more than one SNP locus was found within a 75 bp window, the locus closest to the 5′ end of the window was retained. Then, we used samples that retained more than 50% of all loci.

2.4 | Population analyses

Principal component analysis (PCA) was performed on all SNP datasets, as well as species-specific SNP subsets (see results for detail) using PLINK 1.9 (Chang et al., 2015). The first 20 eigenvectors and eigenvalues were extracted, and the first two components (PC1 and PC2) were visualized to explore genetic differentiation.

We further investigated the population structure of *A. tenuis* with a model-based approach using a Bayesian clustering method implemented in fastSTRUCTURE (Raj, Stephens & Pritchard, 2014). We tested K = 2 to K = 5 clusters using the simple prior model. Then we chose the most appropriate number of clusters (K = 2) that minimized the marginal likelihood, using the 'chooseK.py' script provided in fastSTRUCTURE.

A neighbour-joining tree was generated based on Nei's distance matrix in R with the nj function from ape (Paradis, Claude & Strimmer, 2004). Cluster support of the tree was estimated using the 'aboot' function in poppr (Kamvar, Tabima & Grunwald, 2014) with 1,000 bootstrap replicates.

To evaluate the genetic diversity of each species, the inbreeding coefficient (F_{IS}), observed heterozygosity (*Ho*) and expected heterozygosity (*He*) were calculated in R using the adegenet library (Jombart, 2008). Paired t-tests were run in R to evaluate whether *Ho* is significantly lower than *He*.

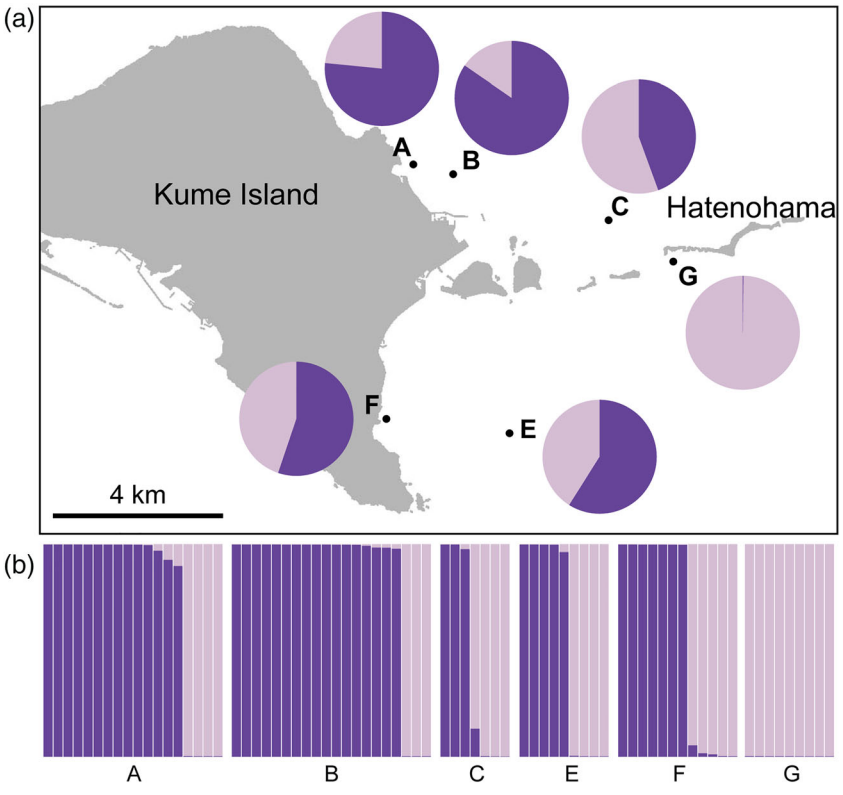

FIGURE 4 Sympatric distribution and reproductive isolation of *Acropora tenuis* clusters suggested by STRUCTURE analysis. (a) Geographic distribution of the associated probability of assignments (K = 2) for each locality. (b) Each vertical column represents individual and associated probabilities of assignments to genetic clusters (purple and pink). The Y-axis represents the probability that a given individual belongs to the cluster(s) indicated

3 | RESULTS

In total, 221 individual colonies were sampled from Kume Island, including 81 colonies of *A. tenuis* from six sites and 140 of *A. digitifera* from seven sites (Figure 1; Table 1). Two runs of sequencing were performed with the MiSeq platform, yielding 81.9 million reads or 6.34 billion base pairs in total. After quality trimming and filtering, approximately 29.6 million base pairs per sample were retained, corresponding to approximately 0.07× coverage depth of *A. digitifera* (420 Mb; Shinzato et al., 2011; Shinzato et al., 2020) and *A. tenuis* (408 Mb; Shinzato et al., 2020) genomes. Despite the relatively small amount of sequence data, mean sequence coverage depths at the SNP sites were >7× for most samples (Figure 2; 137 out of 138 samples for *A. digitifera* and 74 out of 76 for *A. tenuis*), demonstrating that targeted sequencing using MIG-seq increased the sequence coverage ≥100× compared with random shotgun sequencing. In addition, standard deviations of mean sequence coverage were 3.7 (*A. digitifera*) and 3.6 (*A. tenuis*), indicating that indexed sequencing produced a consistent quantity of data for each sample.

Initially, PCA was performed using *A. digitifera* and *A. tenuis* data collectively (Figure 3a). To identify shared SNP sites between *A. digitifera* and *A. tenuis*, all quality-filtered reads of *A. digitifera* were mapped to the *A. tenuis* genome assembly, and 123 possible common SNP sites were obtained. As expected, PCA using the common SNPs showed that *A. digitifera* and *A. tenuis* were clearly separated by PC1, which explained 74.3% of the total variance (Figure 3a). *Acropora tenuis* colonies showed greater dispersion in terms of PC2, indicating higher genetic diversity of this species.

Next, species-specific SNP datasets were prepared for downstream analyses by mapping reads to their respective species. The result of PCA using a dataset for *A. digitifera*, including 384 SNP loci for 136 colonies, did not show detectable population structure (Figure 3b). In contrast, two distinct clusters without overlap were found in *A. tenuis* (Figure 3c, 470 SNPs among 75 colonies) along the PC1 axis. In addition, there were a pair of outliers from each cluster in terms of PC2, presumably clonal colonies (discussed in detail below).

Population ancestry of each *A. tenuis* colony was inferred using fastSTRUCTURE (Figure 4). The results suggest two genetically differentiated clusters in *A. tenuis*, as seen in the PCA (Figure 3). These two clusters co-existed at each locality, except Site G. Notably, 72 out of 75 colonies showed high posterior probability (>95%) of belonging to one of the two clusters. The remaining colonies also

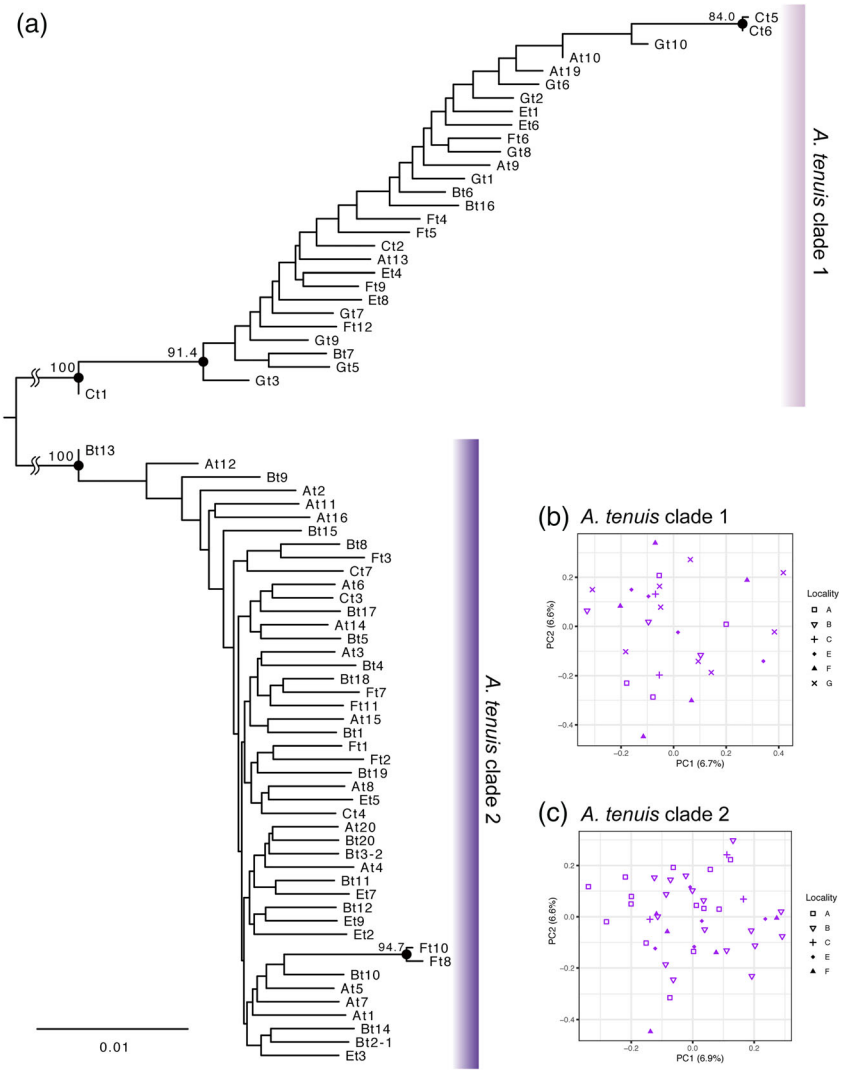

FIGURE 5 Genetic diversification between *Acropora tenuis* clusters and possible clonal colonies. (a) A neighbour-joining tree based on Nei's distances. Nodes supported with bootstrap values higher than 80% are indicated with black circles. Branch lengths between two clades are compressed with wavy lines. (b, c) Principal component analysis for *A. tenuis* clades 1 and 2, indicating no genetic structure in either clade. Clonal colonies were omitted from principal component analyses

exhibit significant posterior probabilities (87–93%). These results indicate that these two genetic clusters are clearly differentiated and that admixture events between them are apparently rare at Kume Island.

To assess population structure in each cluster, PCA was also conducted separately. MIG-seq reads from colonies belonging to the two clusters were mapped to the *A. tenuis* genome, producing clade 1 (579 SNPs for 27 colonies) and clade 2 (453 SNPs for 44 colonies) datasets, respectively. A neighbour-joining tree based on Nei's distance also supported two distinct clades with a bootstrap value of 100% (Figure 5a). Putatively clonal colonies (discussed below) were removed from these datasets. PCA results demonstrated that there were no detectable genetic structures in either clade (Figure 5b,c).

Two pairs of colonies (Ct5/6 and Ft8/10) showed significant links with high bootstrap values (84.0% and 94.7%), suggesting that they could be clonal colonies. To evaluate genetic identity of colonies, pairwise percentage identities of genotypes between all colonies in each *A. tenuis* clade were calculated (Figure 6). Genotypic identity between Ct5 and Ct6 was 96.1% (373 out of 388 SNP loci were

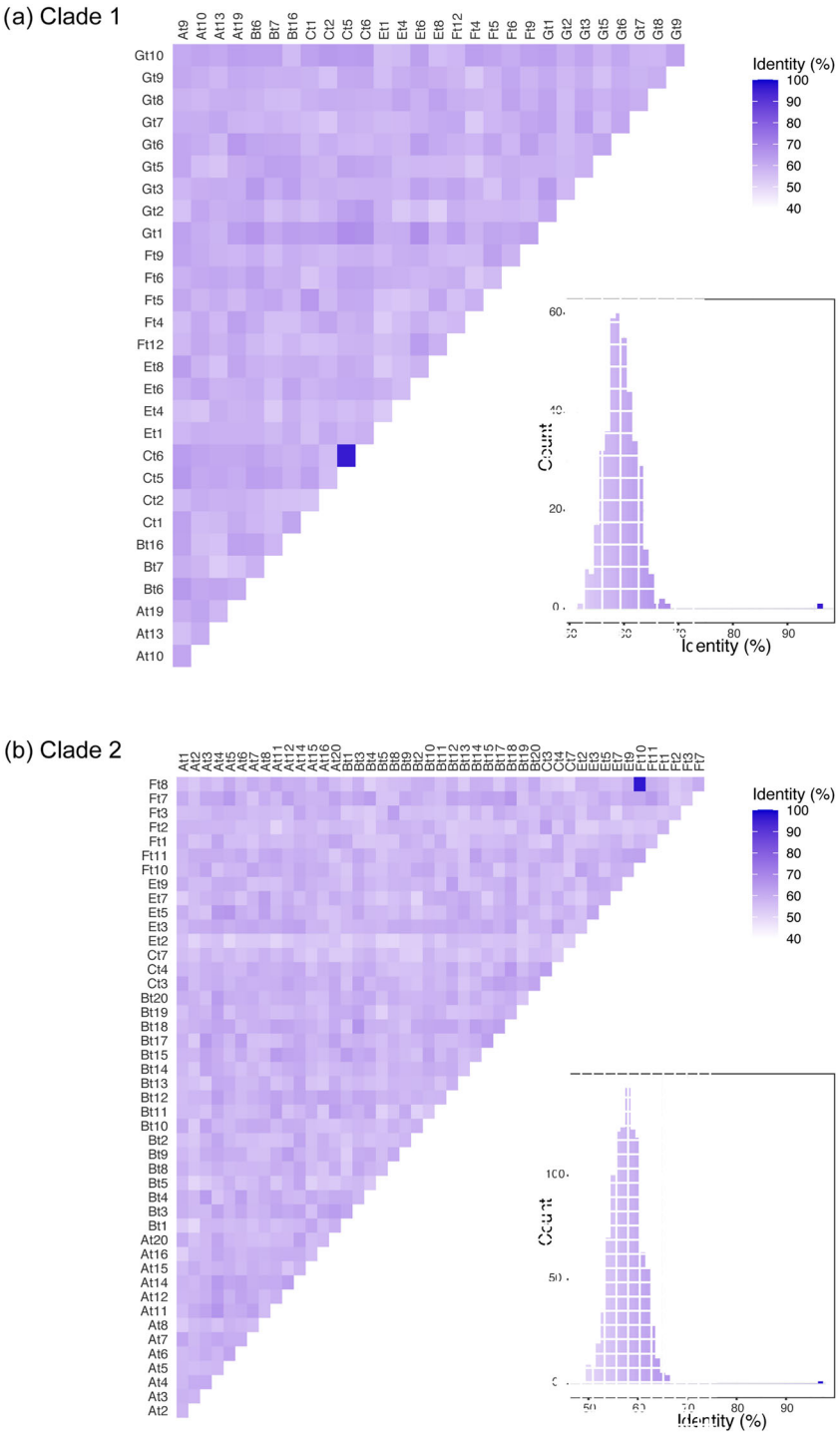

FIGURE 6 Pairwise comparisons of percentage identity of genotypes between *Acropora tenuis* colonies. A pair of clonal colonies was observed in each clade

identical), contrasting with an average value of 59.3% for clade 1 (Figure 6a). Similarly, colonies Ft8 and Ft10 showed significant genotypic identity (96.8%, 301/311) compared to an average value of 57.73% in clade 2 (Figure 6b). In the case of *A. digitifera*, mean genotypic identity was 52.5%, and no probable clonal colonies were observed (Supplementary Figure S1).

Genetic diversity of *A. digitifera* and two *A. tenuis* clades was assessed by estimating inbreeding coefficients (F_{IS}) for all individuals (Figure 7a). Mean F_{IS} values of *A. digitifera* and *A. tenuis* clades 1 and 2 are 0.1666, 0.1027, and 0.11789, respectively. These high F_{IS} values suggest recent reduction of population size. We also calculated expected and observed heterozygosity at all SNP sites (Figure 7b). In all cases, *Ho* was significantly lower than *He*, indicating a deficiency of genetic diversity in these coral populations.

4 | DISCUSSION

Using MIG-seq, we assessed population genetic structure of two acroporid species at Kume Island. *Acropora digitifera* showed no detectable population structure. By contrast, *A. tenuis* included two distinct clades with little or no admixture. Out of 76 *A. tenuis* samples examined, only two pairs of clonal colonies were detected.

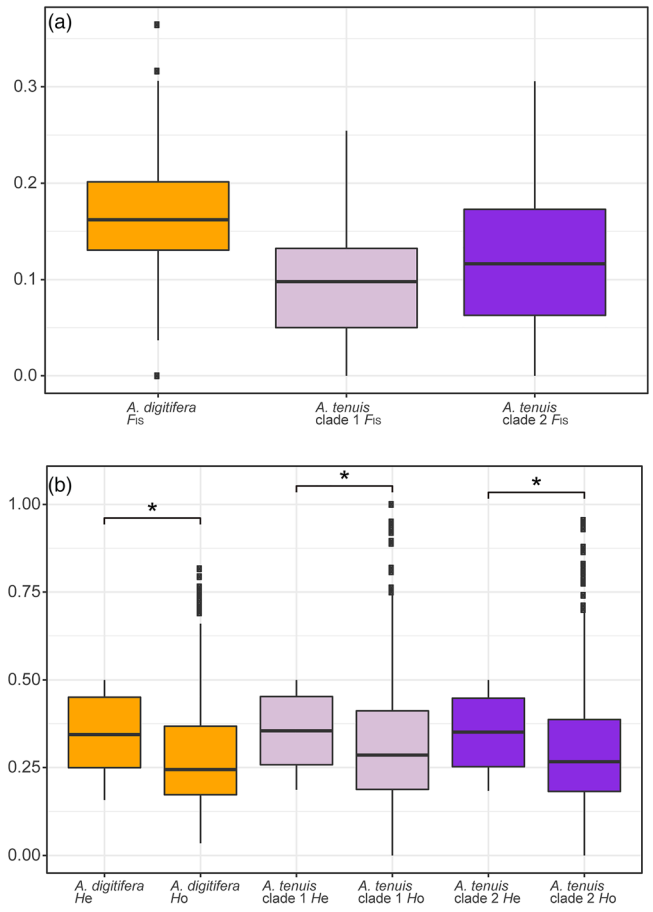

FIGURE 7 Reduced genetic diversity found in each clade. (a) Box plots for inbreeding coefficient (F_{IS}). (b) Box plots for expected (*He*) and observed heterozygosity (*Ho*). Asterisks indicate that *Ho* is significantly lower than *He* ($p < 0.01$)

Our results illustrate a difference in population structure between *A. digitifera* and *A. tenuis* in this study area, consistent with previous studies using whole-genome SNPs (Shinzato et al., 2015) and microsatellite markers (Nakajima et al., 2010; Zayasu et al., 2016; Zayasu, Satoh & Shinzato, 2018). Whole-genome approaches incur greater sequencing costs and effort, and SNPs obtained by MIG-seq yield more statistical power than do microsatellites. Therefore, for studies at regional scale, MIG-seq may be one of the best methods for precisely delineating species with decoded genomes, considering a balance between ease and cost-effectiveness.

Unexpectedly, physical dispersal barriers alone cannot explain the existence of two clades of *A. tenuis*, because they are sympatric everywhere except at Site G. At Site G, Hatenohama, one of the largest sand cays in Japan may have prevented recruitment of one clade. Furthermore, these two clades of *A. tenuis* do not appear to cross even in this small, restricted study area. One possible explanation is that *A. tenuis* represents two cryptic species with some kind of reproductive isolation, although the result is not conclusive. Additional sampling, detailed morphological information, and experimental crossing are required to confirm this finding. The possibility of cryptic species within *A. tenuis* is likely not just a regional characteristic. In Western Australia, Rosser et al. (2017) also reported that *A. tenuis* has genetically polyphyletic lineages. The fact that a phylogenetic tree based on *PaxC* was clearly associated with different spawning seasons, whereas the SNP tree was not, suggested recent polymorphism and incomplete reproductive barriers in *A. tenuis*.

At Kume Island, measures have been implemented against the extreme red soil runoff that was caused by comprehensive land development after 1972 (Yamano et al., 2015). Many corals died after mass bleaching events in 1998 and 2001 (Shimoike, 2004). Coral cover reached its nadir in 2002, and then increased slightly until 2016 (Nature Conservation Division, 2010). Coral cover again decreased significantly after mass bleaching in 2016–2017. The most recent survey showed that the abundance of branching *Acropora* is particularly low (Masucci et al., 2019). Presumably, this massive damage decreased the effective population size and, since then, genetic drift may have affected population structure at Kume Island. Deficits of *Ho* relative to *He* may be explained by the Wahlund effect. Moreover, the density of live *A. tenuis* is lower than at other locations in Okinawa (Zayasu et al., 2016). Therefore, we had to collect branches from all *A. tenuis* colonies that we found in order to achieve an adequate number of samples. In contrast, it was possible to collect *A. digitifera* randomly from many colonies. This difference in sampling intensity may explain the detection of clonal colonies only in *A. tenuis*, and not in *A. digitifera*.

Broad geographic studies are obviously necessary to develop an overview of population structure; however, regional scale information is crucial in conservation management. Such fine spatial scale studies provide important insights into effectiveness of restoration strategies (Cros, Toonen & Karl, 2020; Fuller et al., 2020). We believe that knowledge obtained from SNP genotyping supports not only

conventional management by providing better resolution, but also that it may facilitate development of new conservation approaches, such as molecular breeding, in the future. Our finding of different genetic structures of sympatric congeneric species, sharing the same reproductive strategy, and potentially cryptic species in *A. tenuis* illustrates the need for careful evaluation of each focal species before conducting restoration efforts. Poorly considered conservation strategies may also alter structures in the long term.

The goal of active restoration is to promote self-sustaining coral communities, although coral cover easily garners most of the attention in the short term. Previous studies have investigated the efficacy of increasing larval supply by aggregating reproductive material (Amar & Rinkevich, 2007; Montoya Maya et al., 2016; Zayasu & Suzuki, 2019). Mismatched reproductive material may not promote stable ecosystems. Although neither *A. tenuis* clade has genetic structure at Kume Island, until the presence or absence of reproductive isolation between the two clades is clarified, using *A. tenuis* should be suspended to increase the potential for successful interventions. Additionally, *Acropora* seldom self-fertilize (Heyward & Babcock, 1986; Willis et al., 1997). Excess clonal ramets should be avoided to ensure a broad genetic base, because asexual propagation in the wild is negligible for both of these species.

In summary, genetic homogeneity of *A. digitifera* at Kume Island indicates that there is no problem in using this as a focal species of active restoration in terms of genetic differentiation within the island. By contrast, *A. tenuis* may contain potential cryptic species with reproductive boundaries. Thus, this species should be used prudently to avoid disturbance of wild genetic composition.

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CONTRIBUTIONS

Y.Z. designed the experiments. T.N. and M.K. carried out the fieldwork. M.F., M.K., and W.C. carried out the laboratory work. T.T. carried out the bioinformatics and statistical analyses. T.T., Y.Z., C.S., and N.S. interpreted data. T.T. and Y.Z. wrote the manuscript. All authors read and approved the manuscript.

PERMISSIONS

Coral collection permit from Okinawa Prefecture (No. 31–32).

COMPETING INTERESTS

All authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

Data from the article are publicly available on the Dryad Digital Repository (doi:10.5061/dryad.xgxd254fv).

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