Multiplexed ISSR genotyping by sequencing distinguishes two precious coral species (Anthozoa: Octocorallia: Coralliidae) that share a mitochondrial haplotype

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Background: Precious corals known as coralliid corals (Anthozoa: Octocorallia), play an important role in increasing the biodiversity of the deep sea. Currently, these corals are highly threatened because of overfishing that has been brought on by an increased demand and elevated prices for them. The deep sea precious corals Pleurocorallium elatius and Pleurocorallium konojoi are co-distributed in Japanese waters and have distinct morphological features: (1) the terminal branches of the colony form of P. elatius are very fine, while those of P. konojoi are blunt and rounded, (2) the autozooids of P. elatius are arranged in approximately four rows, while those of P. konojoi are clustered in groups. However, previous genetic analysis using mtDNA and nuclear DNA did not indicate monophyly. Therefore, it is important to clarify their species status to allow for their conservation.

Methodology: We collected a total of 87 samples (60 of C. japonicum and 27 of P. konojoi) from around the Ryukyu Islands and Shikoku Island, which are geographically separated by approximately 1300 km. We used a multiplexed inter-simple sequence repeat (ISSR) genotyping by sequencing (MIG-seq) and obtained 223 SNPs with which to perform STRUCTURE analysis and principle coordinate analysis (PCoA). In addition, two relatively polymorphic mtDNA regions were sequenced and compared.

Results: Pleurocorallium elatius and P. konojoi share a same mtDNA haplotype, which has been previously reported. However, MIG-seq analysis clearly distinguished the two species based on PCoA and STRUCTURE analysis, including 5% of species-specific fixed SNPs.

Conclusion: This study indicated that P. elatius and P. konojoi are different species and therefore both species should be conserved separately. Our findings highlight the importance of the conservation of these two species, especially P. elatius, whose population has been dramatically depleted over the last 100 years. The study also demonstrated the effectiveness and robustness of MIG-seq for defining closely related octocoral species that were otherwise indistinguishable using traditional genetic markers (mtDNA and EF).
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Abstract

Background: Precious corals known as coralliid corals (Anthozoa: Octocorallia), play an important role in increasing the biodiversity of the deep sea. Currently, these corals are highly threatened because of overfishing that has been brought on by an increased demand and elevated prices for them. The deep sea precious corals Pleurocorallium elatius and Pleurocorallium konojoi are co-distributed in Japanese waters and have distinct morphological features: (1) the terminal branches of the colony form of P. elatius are very fine, while those of P. konojoi are blunt and rounded, (2) the autozooids of P. elatius are arranged in approximately four rows, while those of P. konojoi are clustered in groups. However, previous genetic analysis using
mtDNA and nuclear DNA did not indicate monophyly. Therefore, it is important to clarify their species status to allow for their conservation.

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**Results:** *Pleurocorallium elatius* and *P. konojoi* share a same mtDNA haplotype, which has been previously reported. However, MIG-seq analysis clearly distinguished the two species based on PCoA and STRUCTURE analysis, including 5% of species-specific fixed SNPs.

**Conclusion:** This study indicated that *P. elatius* and *P. konojoi* are different species and therefore both species should be conserved separately. Our findings highlight the importance of the conservation of these two species, especially *P. elatius*, whose population has been dramatically depleted over the last 100 years. The study also demonstrated the effectiveness and robustness of MIG-seq for defining closely related octocoral species that were otherwise indistinguishable using traditional genetic markers (mtDNA and EF).

**Introduction**

The precious corals known as Coraliid (Anthozoa: Octocorallia), play an important role in increasing the biodiversity of the deep sea by providing a complex habitat structure, suitable for other species to live in (Roberts, Wheeler & Freiwald, 2006). Their beautiful red and pink skeleton has drawn great attention and they have been fished for ornaments, jewelry, and currency since ancient times (Chen, 2012; Clark & Rowden, 2009). Recent studies, however, indicated precious corals are vulnerable to overfishing because of their low fecundity and slow growth rate (Nonaka *et al*, 2015; Luan *et al*, 2013; Torrents *et al*, 2005). Consequently, there is an increasing need for the conservation of overexploited precious corals in order to avoid local extinction (CITES, 2007; CITES, 2009).

Species delimitation is important for conserving precious corals due to the fact that the species is the most fundamental unit for preservation (De Queiroz, 2007). However, species identification of the precious corals is sometimes difficult, especially for closely related species. The use of morphological characteristics in determining the species identification of octocorals, including precious corals, is the typical method, (Luan *et al*, 2013) however, variations, and high frequency of homoplasia sometimes hinder identification (Figueroa & Baco, 2014). More recently, molecular and phylogenetic approaches using partial mitochondrial DNA (mtDNA) have emerged as useful tools to identify octocoral species (McFadden *et al*, 2011; Pante *et al*, 2012; Quattrini *et al*, 2014). In Coralliidae, the intergenic region 1 (IGR1) in mtDNA has a relatively high intra-species variation and is proposed as a useful marker for distinguishing between precious coral species (Tu, Dai & Jeng, 2015). Although mtDNA is widely used for species delimitation, discords with the morphological characteristics of species still remain.
For example, morphologically distinct species in *Hemicorallium* cannot be delineated using mtDNA (Ardila *et al.*, 2012; Tu, Dai, & Jeng, 2015). Indeed, the slow mtDNA evolution has long been recognized for anthozoans (Hellberg 2006; France & Hoover, 2002). Octocoral species in particular have a slow mutation rate of mtDNA, due to octocoral-specific DNA repairing enzymes (Bilewitch & Degnan, 2011; France & Hoover, 2002). These facts indicate that a higher resolution genetic marker is required to elucidate the species status of closely related precious coral species.

The advent of high-throughput sequencing technology has rendered a large number of loci of non-model organisms accessible in a short time period. For example, the restriction site associated DNA sequence (RADseq) (Baird *et al.*, 2008) has been applicable in non-model organisms to delimit closely related species of many reference taxa. In deep sea octocorals, only a few studies have applied RADseq for species delimitation. RADseq has helped fine-tune the species status of genus *Chrysogorgia* (Pante *et al.*, 2015) and revealed robust species boundaries in the genus *Paragorgia* using the Bayesian model based-method (Herrera & Shank 2016). Additional studies using the high-throughput technology to delineate closely related precious coral species would be helpful to reveal species boundaries. Multiplexed inter simple sequence repeat (ISSR) genotyping by sequencing (MIG-seq) is an easy, cost-effective, novel method to obtain a moderate number of single nucleotide polymorphisms (SNPs) of non-model organisms using polymerase chain reaction (PCR) and high-throughput technology. The number of available SNPs from MIG-seq analysis is generally less than those using other techniques such as RAD-seq (Miller *et al.*, 2007). However, MIG-seq has several advantages, namely that putatively neutral loci adjacent to microsatellite regions can be obtained and the method can be performed with small amounts and/or low-quality DNA, and is relatively easy to perform cheaply. The ISSR regions are first amplified by PCR using universal primer sets, indexed by a second PCR, and then sequenced to obtain up to a few thousand SNPs. Closely related species of *Heliopora* were successfully delineated using MIG-seq (Richards *et al.*, 2018), implying the effectiveness of this method for other closely related octocoral species.

Our target species, deep sea precious corals *Pleurocorallium elatius* and *Pleurocorallium konojoi* are co-distributed in Japanese waters at depths of 50 to 330 m (Nonaka & Muzik, 2009). *P. elatius* and *P. konojoi* can be distinguished by at least two morphological features: (1) terminal branches of the colony form of *P. elatius* are very fine, while those of *P. konojoi* are blunt and rounded, (2) autozooids of *P. elatius* are arranged in approximately four rows, while those of *P. konojoi* are clustered in groups (Nonaka *et al.*, 2012). Nevertheless, a previous molecular analysis suggested the two species shared a major haplotype at eight mtDNA regions and EF-1, indicating that the species status of these two is unclear (Tu, Dai & Jeng, 2015). Because these two species, especially *C. elatius*, are facing the risk of local extinction due to overfishing (Nonaka & Muzik, 2009), clarifying whether or not they are the same species with gene flow occurring between them is important for devising conservation strategies.
In this study, we applied MIG-seq analysis to clarify the species status of the two closely related precious corals, *P. elatius* and *P. konojoi* in order to obtain basic information for the conservation of precious coral species. We also sequenced widely used mtDNA regions for comparison with the MIG-seq results.

**MATERIALS AND METHODS**

**Sample collection and DNA extraction**

In total, 87 samples of *P. elatius* and *P. konojoi* (27 *P. elatius* and 60 *P. konojoi*) were collected from depths of 100 to 330 m using either a traditional coral net, underwater remotely operated vehicles (ROV), or submarines off Ryukyu, Kyushu, and Shikoku, Japan (Table 1). We collected some samples under the Kochi Prefecture sampling permit number Sa 401, Sa 412, and Sa 423 in Kochi. Samples were preserved in 90% ethanol and genomic DNA was extracted using the hot alkaline solution method (Meeker *et al*, 2007) followed by ethanol precipitation (Richards *et al*, 2018).

**MtDNA analysis**

Two mtDNA regions were sequenced for *P. elatius* and *P. konojoi*: IGR1 region using primers (IGR1-Co-F and IGR1-Co-R, Tu, Dai & Jeng, 2015) and putative mitochondrial DNA mismatch repairing gene (mitochondrial mutS-like protein) regions (MSH-Co-F and MSH-Co-R, Tu, Dai & Jeng, 2015) following the original protocols. Two regions were directly sequenced from both directions using Big Dye v 3.1 and the Abi3730 sequencer. All of the sequence data were manually checked using Bioedit ver. 7.0.9.0 (Hall, 1999) and then all the sequences were aligned on GENETIX ver. 12. A maximum likelihood tree using MEGA 7.0 (Kumar, Stecher & Tamura, 2016) was constructed using concatenated mtDNA sequences. The best model was estimated using MEGA 7.0 based on the corrected Akaike Information Criterion and the Tamura 3-parameter model (Tamura, 1992) was used to construct a phylogenetic tree. Confidence values for phylogenetic trees were inferred using 1000 bootstrap replicates. All mtDNA sequences obtained in this study were deposited (DDBJ Accession number: LC464485-LC464516, LC475110-LC475134).

**MIG-seq analysis**

We performed a MIG-seq analysis to detect genome-wide SNPs following the protocol by Suyama & Matsuki (2015). Briefly, MIG-seq amplifies putatively neutral, anonymous genome-wide ISSR regions (Gupta *et al*, 1994; Zietkiewicz, Rafalski & Labuda, 1994), including a few hundred to a few thousand SNPs, using 8 pairs of multiplex ISSR primers (MIG-seq primer set 1) for the first PCR. Then the DNA libraries from each sample with a different index were pooled and sequenced using MiSeq (sequencing control software v2.0.12, Illumina) with the MiSeq Reagent v3 150 cycle kit (Illumina). Image analysis and base calling were performed using real-time analysis software v1.17.21 (Illumina). We analyzed a total of 87 individuals with 27 *P. elatius* and 60 *P. konojoi* individuals collected from the Ryukyu Islands to Shikoku Island, geographically separated by approximately 1300 km (Table 1).
To eliminate low-quality reads and primer sequences from the raw data, we used the FASTX-toolkit version 0.0.14 (fastaq_quality_filter) (Gordon & Hannon, 2012; http://hannonlab.cshl.edu/fastx_toolkit/index.html) with a fastq-quality-filter setting of –Q 33 –q 30 –p 40. We removed adapter sequences for the Illumina MiSeq run from both the 5’ end (GTCAGATCGGAAGAGCACACGTCTGAACTCCAGTCAC) and the 3’ end (CAGAGATCGGAAGAGCGTCGTGTAGGGAAAGAC) using Cutadapt version 1.13 (Martin 2011), and then we excluded short reads less than 80 bp. The quality-filtered sequence data were demultiplexed and filtered through the software Stacks v1.46 (Catchen et al, 2011; Catchen et al, 2013). We used Stacks v. 1.4 (Catchen et al, 2013) to stack the reads and extract SNPs. First, we used the U-stacks with the option settings of ‘minimum depth of coverage required to create a stack (m)’ = 3, ‘maximum distance allowed between stacks (M)’ = 1, ‘maximum distance allowed to align secondary reads to primary stacks (N)’ = 1, and the deleveraging (d) and removal (r) algorithms enabled. Secondly, we used the C-stacks with the option ‘number of mismatches allowed between sample loci when build the catalog (n)’ =4, followed by the S-stacks. To confirm the consistency of the results using different sets of SNPs with different amounts of missing data, we first created three different SNP sets using population software implemented in Stacks v 1.4 by restricting the data analysis to different criteria: (i) the minimum percentage of individuals required to process a locus across all data (r) was set at 70% and restricting data analysis to a single SNP per locus, (ii) r was set at 50% and a single SNP per locus was used, and (iii) r was set at 50% and all SNPs per locus were used. Confirming that all the results were consistent irrespective of the data sets, we only showed the results using (i) r, 70%, and single SNP per locus. For all of the above analyses, we set the following parameters: the minimum number of populations that a locus must be present in to process a locus (p)’ = 1, the minimum minor allele frequency required to process a nucleotide site at a locus (min_maf) = 0.01, the maximum observed heterozygosity required to process a nucleotide site at a locus (max_obs_het) = 0.9. BayeScan v 2.0 was used to detect possible SNPs under natural selection assuming two morphological species with a default setting.

A Bayesian individual-based assignment approach as implemented in STRUCTURE 2.3.4 was used to examine the genetic boundaries between P. elatius and P. konojoi individuals. Twenty independent runs were performed in STRUCTURE using an admixture model and allele frequency correlated model without any morphological priors. Both the length of the burn-in period and the number of Markov Chain Monte Carlo analyses (MCMC) were 200,000. We estimated ∆K (Evanno, Regnaut & Goudet, 2005), the most likely number of clusters using STRUCTURE HARVESTER (Earl & Bridgett, 2012), CLUMPAK (Kopelman et al, 2015), and DISTRUCT (Rosenberg, 2004) to summarize and visualize the STRUCTURE results. In addition, individual-based principle coordinate analysis (PCoA) was performed using GeneAlex ver. 6.5 to visualize the genetic relationship among different individuals in 2 dimensions. Data files were converted to each software using PGDspider ver 2.0.8.3(Lischer & Excoffier, 2011).

RESULTS
**MtDNA analysis**

A total of 961 bp (456 bp IGR1 sequences and 505 bp MutS after trimming unreliable sequences) from 25 individuals (13 *P. elatius* and 12 *P. konojoi*) were obtained and concatenated to reconstruct the phylogenetic tree (Fig. 1). The genetic diversity of the concatenated sequences for *P. elatius* and *P. konojoi* was low, with only 3 haplotypes with 2 polymorphic sites, including 1 gap, uncovered across 25 sequences in the concatenated sequence. The haplotype diversity (h) was 0.453 and nucleotide diversity (π) was 0.00047. The haplotype network analysis revealed *P. elatius* and *P. konojoi* shared a major haplotype (Fig. 1a). Four out of 12 *P. konojoi* individuals shared a major haplotype with *P. elatius* and all the *P. elatius* individuals shared the major haplotype with *P. konojoi*. The reconstructed phylogenetic tree indicated that the two morphological species are not monophyletic (Fig. 1b). *P. elatius* included one polymorphic site and one gap site in IGR1 and no polymorphic sites in MutS regions, while all *P. konojoi* shared a single haplotype at both loci (Table 1).

**MIG-seq analysis**

In total, 25,444,986 raw reads with an average of 292,471 reads per sample were obtained for 87 individuals by MIG-seq analysis, of which 25,241,178 reads remained after filtering out low quality reads. We obtained 16,011,472 reads with an average of 184,039 reads per individual after two step filtering. The three SNP data sets created using different criteria resulted in a different number of loci: (i) r = 0.7 and only a single SNP per locus resulted in 223 loci, (ii) r = 0.5 and only a single SNP per locus resulted in 762 SNPs, and (iii) r was set at 0.5 and all SNPs per locus resulted in 2251 SNPs. All data sets indicated consistent STRUCTURE and PCoA results, regardless of the different ratio of missing data and possible linkage disequilibrium (Suppl. 1).

BayeScan indicated that all the loci were neutral (q-values > 0.05). Among the 223 SNPs examined, 12 SNPs indicated species-specific alleles (fixed substitution between the species) that could be observed.

Both STRUCTURE and PCoA indicated the same patterns in all of the SNPs sets; *P. elatius* and *P. konojoi* are genetically distinct. Calculation of $\Delta K$ of the STRUCTURE results indicated that $K = 2$ best explained the data with a mean likelihood = -5666.970 and mean similarity score among 10 independent runs = 0.999. Clear genetic differences corresponded with the morphological differences without any genetically admixed individuals (Fig. 2a). PCoA indicated two morphological species are clearly separated by the x-axis, which explains 18.5% of the data (Fig. 2b).

**DISCUSSION**

Japan has been the main exporter of the precious corals to mainland China. The increased demand of the precious corals in mainland China in recent years resulted in elevated prices for raw corals, and promoted illegal fishing and overexploitation. Precise harvest data for precious corals is currently unavailable due to the inconsistent application of CITES listings, unreported
trade as personal or household effects, and illegal and unreported trade. Under these circumstances, having species-specific customs codes for CITES would be necessary to obtain accurate trade data for conservation (Shiraishi, 2018).

Previously, *P. elatius* and *P. konojoi* were considered morphologically different; traditional genetic analysis using mtDNA and nuclear elongation factor region (Tu, Dai & Jeng, 2015) did not indicate their monophyly and thus they have been genetically indistinguishable. In the present study, we confirmed that *P. elatius* and *P. konojoi* share a same haplotype (Fig. 1), which is consistent with the previous study. MIG-seq analysis, however, successfully discriminated *P. elatius* from *P. konojoi*. PCoA and STRUCTURE results indicated that the two species are clearly different with no intermediate genotypes, indicating no current hybridization between the two species. It is possible that extremely slow substitution rates of mtDNA of *P. elatius* and *P. konojoi* resulted in non-reciprocal monophyly due to the maintenance of an ancestral shared variation presented at the time of divergence. On the other hand, it is possible that a strong purifying selection could encourage them to split to reciprocal monophyly in nuclear DNA, as the MIG-seq analysis suggested more than 5% of the loci showed fixed substitution between *P. elatius* and *P. konojoi*, while BayeScan indicated all MIG-seq loci are all neutral.

This study demonstrated that *P. elatius* and *P. konojoi* are indeed different species without any hybridization or on-going gene flow consistent with morphological differences, and therefore require separate conservation management plans since *P. elatius* populations cannot be replenished by *P. konojoi* populations. *P. elatius* made up 20.6% of the total catch of precious corals in Japan from 1904 to 1920. Currently, this has dropped to 2.0% because the biomass of *P. elatius* has been dramatically depleted due to overfishing (Iwasaki, 2018); thus, there is an urgent need to devise and implement conservation strategies for *P. elatius*.

This study highlighted the effectiveness of the MIG-seq analysis using the high throughput sequencing technology for delimiting octocoral species. This study, as well as a previous study on shallow water octocoral species, demonstrated that MIG-seq analysis can uncover genetic lineages that are undetectable when using mtDNA or ITS2 (Richards *et al.*, 2018). MIG-seq provides a time-saving (3 days at shortest), simple (two PCR steps), and economical (15 US dollars per sample) approach that is applicable even for small amounts of degraded or valuable samples for SNP genotyping. Data comparison among different species or genera is easy as the MIG-seq always uses the same multiplex primer sets, which would also be suitable for nuclear barcoding among different species. Further application of MIG-seq analysis to a wider range of octocoral taxa would help to clarify the unsolved status of closely related species.

**CONCLUSIONS**

This study indicated that *P. elatius* and *P. konojoi* are different species and that both species should be conserved separately. The results highlighted the importance of conservation of these two species, especially *P. elatius*, which has been dramatically depleted in the past 100 years.
Again, it does suggest that separate conservation strategies will be required for the two different morphospecies. This study also demonstrated the effectiveness and robustness of MIG-seq for delimiting closely related octocoral species that were previously indistinguishable when using traditional genetic markers (mtDNA and EF). Further application of MIG-seq analysis for a wider range of octocoral or other taxa would aid in verifying the robustness of MIG-seq analysis on species delimitation.

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Figure 1

MtDNA haplotype network considering gap regions (a) and maximum likelihood phylogenetic tree excluding gap regions (b).

*P. elatius* (PE) is shown in pink and *P. konojoi* (PK) is shown in black/white. (a) The size of the circle represents the number of haplotypes found in the analysis. (b) Only the nodes with bootstrap values (>50) were indicated.
Figure 2

STRUCTURE (a) and PCoA result (b).

(a) $K=2$ with Mean (LnProb) = -5666.970 and Mean (similarity score) among 10 runs = 0.999 and PCoA results using 223 SNPs data obtained by MIG-seq analysis (b). X axis indicates 18.5% and Y axis indicates 3.52% of the data. PK and PE represent *P. konojoi* and *P. elatius* respectively.
### Table 1 Precious coral samples used in this study

(on next page)
| Location            | Coordinate                  | MIG-seq | mtDNA | Sampling year | Sampling method        |
|---------------------|-----------------------------|---------|-------|---------------|------------------------|
| **Pleurocorallium konojoi** |                             |         |       |               |                        |
| Shikoku Island      | Kouchi          | 32°30'41.87"N 132°49'42.26"E | 47      | 0 Apr.11-Nov.14 | traditional coral net  |
| Ryukyu Islands      | Iou Is.         | 30°47'31.812"N 132°18'13.5"E | 5       | 4 Dec.07-Aug.09 | manned submarine       |
|                     | Take Is.        | 30°48'36.684"N 130°25'43.356"E | 7       | 7 Feb.08-May.09 | manned submarine       |
|                     | Tanega Is.      | 30°36'34.38"N 130°58'43.932"E | 1       | 1 Dec.07-Aug.08 | manned submarine       |
|                     |                 |         | 60    |               |                        |
|                     |                 |         | 12    |               |                        |
| **Pleurocorallium elatius** |                             |         |       |               |                        |
| Shikoku Island      | Kouchi          | 32°30'41.87"N 132°49'42.26"E | 1       | 0 Jul.11      | traditional coral net  |
| Ryukyu Islands      | Iou Is.         | 30°47'31.812"N 132°18'13.5"E | 2       | 4 Jun.05-Apr.09 | manned submarine       |
|                     | Take Is.        | 30°48'36.684"N 130°25'43.356"E | 2       | 0 Sep.07-Oct.08 | manned submarine       |
|                     | Tanega Is.      | 30°36'34.38"N 130°58'43.932"E | 3       | 3 Feb.06-Dec.07 | manned submarine       |
|                     | Yaku Is.        | 30°20'40.524"N 130°30'45.756"E | 4       | 1 Aug.07-Aug.09 | manned submarine       |
|                     | Amami Is.       | 28°16'4.224"N 129°21'43.596"E | 1       | 0 Aug.07      | manned submarine       |
|                     | Tokuno Is.      | 27°47'25.512"N 128°58'0.48"E | 2       | 0 Oct.07-Aug.09 | manned submarine       |
|                     | Izena Is.       | 26°56'2.976"N 127°56'28.932"E | 1       | 1 Jan.06      | ROV                    |
|                     | Hateruma Is.    | 24°11'40.272"N 123°33'45.54"E | 3       | 0 Sep.07-Mar.08 | ROV                    |
|                     | Ikema Is.       | 24°55'49.836"N 125°14'24.216"E | 1       | 1 May.09      | ROV                    |
|                     | Tarama Is.      | 24°39'19.908"N 124°41'48.12"E | 1       | 1 Aug.05-Dec.07 | ROV                    |
|                     | Ishigaki Is.    | 24°24'23.04"N 124°10'31.584"E | 5       | 1 Nov.05-Mar.09 | ROV                    |
|                     | Nakanokami Is.  | 24°11'40.272"N 123°33'45.54"E | 1       | 1 Mar.08-Apr.08 | ROV                    |
|                     |                 |         | 27    |               |                        |
|                     |                 |         | 13    |               |                        |

**total** | 87 | 25 |