Development of Microsatellite Markers for the Hermatypic Coral Porites lutea

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Abstract By mining the EST and genomic microsatellite sequence resources of Porites species in GenBank, and using interspecific amplification and resequencing, we developed 20 microsatellite markers for the stony coral Porites lutea, meanwhile, validated and excluded 43 microsatellite markers of its symbiotic zooxanthella. These markers of P. lutea were genotyped in one wild population (n=16) from the southwest of Hainan Island fringing reefs. Nineteen of the 20 markers showed polymorphism. The number of alleles was 2~7 (mean 3.5±1.8), and the observed heterozygosity (Hs) and expected heterozygosity (He) were 0.06~0.94 (mean 0.34±0.29) and 0.18~0.83 (mean 0.57±0.20), respectively. Four markers remained deviated from the Hardy-Weinberg Equilibrium (HWE) after multiple comparison correction for Bonferroni (P_{HWE}<0.05). This is the first set of microsatellite markers of P. lutea. They should be useful for the research on the genetic diversity and connectivity of this species and the conservation of endangered coral reef ecosystems and reef-building coral communities in the Indo-Pacific region.

Keywords Porites lutea; Stony coral; Microsatellite; Genetic connectivity; Symbiotic zooxanthellae

Coral reef ecosystems contain the highest density of marine biodiversity (Carpenter et al., 2008) and are highly productive. Annual fishery production per square kilometer of healthy coral reef reaches 35 tons, accounting for 10% (Smith, 1978) of global fishery production, which is described as “tropical rain forest” in the ocean. Coral reefs in tropical oceans have been playing an important role in the global carbon cycle and are simultaneously involved in organic carbon metabolism (photosynthesis/respiratory action) and inorganic carbon metabolism (calcification/dissolution) processes (Yan et al., 2009). The growth of coral reefs can efficiently and stably precipitate carbonate (calcification), making it an important component of the global carbonate reservoir. The cumulative CaCO₃ amount accounts for 23%~26% worldwide (Suzuki and Kawahata, 2004), which is an important factor affecting atmospheric CO₂ concentrations. In addition, the complex reef structure formed by the reef-building corals not only provides habitats for a wide variety of reef organisms, but also has an important effect of wave protection, providing a safe ecological environment for seaweed, mangroves and humans (Moberg and Folke, 1999). Therefore, although coral reefs cover only 0.063% of the earth's surface, they have important effects on the shape of the earth's surface, the geographical distribution of organisms, ocean chemistry and the atmosphere (Birkeland, 2015). However, in recent years, due to persistent global warming (Hughes et al., 2017), pollution from human activities (Lapointe et al., 2019), ocean acidification and overexploitation, the species and number of corals have sharply decreased, resulting in a marked decline in the structure and function of coral reefs (Hoegh-Guldberg et al., 2007; Hughes et al., 2018). Global overall coral coverage has fallen by about 50% (Birkeland, 2015) in the last 30-40 years. China's situation is particularly acute, with the number of reef-building corals of the continent and Hainan Island having fallen by 80% (Hughes et al., 2013). Protection and restoration of coral reefs are imminent.

A reef-building coral remains in its adult form and changes the size and genetic structure of its aggregate populations by the migration of planktonic larvae along with the current (Polato et al., 2005). This brief diffusion period plays an important role in the maintenance of its population and the expansion of its habitat (Gaines and Bertness, 1992). Nowadays the preferred strategy for coral reef protection is to protect the original habitat and
genetic diversity of stony corals, and set up a network of marine protected areas to protect the diffusion path of larvae, waiting for their natural recovery (Barber et al., 2002; Almany et al., 2009). Whether it is to monitor the genetic diversity of reef-building corals, or to detect the diffusion path of reef-building coral larvae by analyzing the gene flow of representative coral aggregate populations and their genetic connectivity, molecular markers are needed, especially microsatellite markers with high diversity and convenient and accurate detection (Pritchard et al., 2000; Magalon et al., 2004).

Microsatellites, also known as simple sequence repeats (SSRs), are short (2–5 bp) tandem repeats widely distributed in the eukaryotic genome (Toth et al., 2000). Microsatellite markers are codominant markers with high polymorphism, which can be readily amplified by polymerase chain reaction (PCR) and can be accurately automated detected (e.g., using ABI genetic analyzer). They are the most commonly used molecular markers for analysis of non-model organism (Wang et al., 2010). The traditional strategy of developing microsatellite markers, which establishes microsatellite enrichment libraries by selective hybridization, is time-consuming (Zane et al., 2002; Wang et al., 2009). In addition, the content of microsatellites in coral genome is low so that the enrichment is difficult (Liu et al., 2005). With the rapid development of sequencing technology, more and more coral genome DNA sequences and expressed sequence tag (EST) have been accumulated in GenBank database. By mining microsatellite resources in these sequences, the time and material cost of development can be significantly reduced (Sharma et al., 2007; Qiu et al., 2013), meanwhile, microsatellite markers often have universal potential in genus related species (Cruz et al., 2007; Chu et al., 2010). Therefore, by mining of the GenBank sequence resources of the related species, there is a fast and efficient way for the development of microsatellite markers (Ringler, 2012). However, in reef-building coral cells, single-celled dinoflagellates (zooxanthellae) are commonly found (except for sperm and some types of eggs during sexual reproduction), which make it difficult to avoid zooxanthella DNA contamination in coral genome DNA and transcriptome sequence. Therefore, during the developing of reef-building coral microsatellite markers, it must identify the microsatellite markers and remove the microsatellite markers of zooxanthella (Chen et al., 2013). Magalon et al. (2004) established an exclusion strategy for reef coral microsatellite development: microsatellite markers were first enriched and separated by the DNA sequence library of coral symbionts, and then identified by pure zooxanthella DNA, which is the key step in this scheme.

The stony coral Poretis lutea (Milne Edwards and Haime, 1851), a large massive reef-building coral (Hirose et al., 2001) composed of small polyps, is gonochoristic and gametes-releasing. Widely distributed in Indo-Pacific, it is one of the dominant reef coral species in the South China Sea, and is also the most widely distributed reef coral species on the fringing reefs of Hainan Island (Wang et al., 2013), which makes it an ideal model to study the genetic connectivity of reef-building corals in the South China Sea and Hainan Island. In this study, using the EST and genomic microsatellite sequences of the Porites species in GenBank, 20 P. lutea and 43 zooxanthella microsatellite markers were developed, and confirmed by interspecific amplification and re-sequencing. These markers could be powerful tools for fine analysis of population genetic structure and connectivity of the species. The genetic diversity and larval migration path can be revealed, which can provide scientific basis for the development of the protection strategy of coral reefs.

1 Results
1.1 Identification of pure zooxanthella DNA
The results of amplification of 28S rDNA for Poretis lutea (PL) symbiont and its symbiotic zooxanthellae DNA are shown in Figure 1. Two bands (662 and 595) were obtained from PL symbionts, while only band 595 was shown for symbiotic zooxanthella (obtained from heat stress treatment), which confirmed the reference DNA was only derived from zooxanthellae and there is no coral DNA contamination.

1.2 Development and identification of microsatellite markers
183 microsatellite loci of Poretis species were amplified in 6 PL coral symbionts from different sites, and then confirmed by re-sequencing and pure zooxanthella DNA identification. Microsatellite loci of PL coral and its zooxanthellae were shown in Table 1. Of the 100 P. astreoides EST microsatellites, 35 (35%) were able to amplify
in PL symbionts and were identified as homologous microsatellite sequences by re-sequencing. Among them, 12 (12.0%) and 23 (23.0%) microsatellite markers were identified as PL coral and zooxanthella, respectively. Of the 83 *P. lobate* genomic microsatellites, 28 (33.7%) were able to amplify in PL symbionts, 8 (9.6%) and 20 (24.0%) microsatellite markers were identified as PL coral and zooxanthella, respectively. A total 20 PL coral microsatellite markers were therefore identified, with an output rate of 10.9% (Table 1); 43 zooxanthella microsatellite markers were identified, with an output rate of 23.4%, which was more than double that of PL corals. On the other hand, the proportion of Poreites species EST-SSR completely unamplified in PL symbionts was 23% (23/100), which was much lower than that of genomic-SSR 55.4% (46/83), indicating that EST sequences, which originate from mRNA, are more conserved than genomic sequences. Information and characteristics of 20 microsatellite loci in a wild *P. lutea* population were shown in Table 2.

![Figure 1 The capillary electrophoretogram (QIAxcel) of 28S rDNA fragment of *P. lutea* symbiont and its symbiotic zooxanthellae DNA](image)

| The source Loci | Amplification/resequencing in *P. lutea* symbiont | Validated SSR marker |
|-----------------|-----------------------------------------------|------------------|
|                 | No/weak/multiple amplicon | No SSR sequence | SSR sequence | *P. lutea* | Zooxanthella |
| **pasE001-100** | 23/12/22                      | 8                | 35 (35.0%)  | 12 (12.0%) | 23 (23.0%)  |
| **plo1-83**     | 46/2/7                        | 0                | 28 (33.7%)  | 8 (9.6%)   | 20 (24.1%)  |

1.3 Polymorphism of PL coral microsatellite sites

Of the 20 microsatellite markers of PL corals, 19 were polymorphic in BS populations (Table 2), with the allele number ranging from 2~7 and average of 3.5±1.8 alleles per marker. The observed heterozygosity (*H*o) and expected heterozygosity (*H*e) were 0.06–0.94 (mean 0.34±0.29) and 0.18–0.83 (mean 0.57±0.20), respectively. Six markers (*plo10, plo37, plo42, plo68, plo74, pasE042*) deviated to Hardy-Weinberg equilibrium (HWE), of which, 4 still remain deviated after Bonferroni correction (*p*<0.05), mainly due to the presence of null alleles, since all deviated loci showed evidences of null alleles (Table 2). These markers should be used with caution in population genetic structure analysis.

Of the 6 deviated markers, 5 were genomic-SSR sources, accounting for 62.5% (5/8) and 1 (1/12, 8.3%) from EST-SSR. Of the 4 markers who still remain deviated after Bonferroni correction, genomic-SSR and EST-SSR source markers were 3 (37.5%, 3/8) and 1 (8.3%, 1/12), respectively. The proportion of null alleles deviating from HWE in the marker of EST-SSR sources is significantly lower than that of the genomic-SSR sources.
### Table 2 Information and characteristics of 20 microsatellite loci in a wild P. lutea population (n=16)

| Locus | GenBank No. | Motif | Primer sequence (5’-3’) | Ta(°C) | Size (bp) | H0 | He | P<sub>HWE</sub> |
|-------|-------------|-------|--------------------------|--------|-----------|----|----|---------------|
| plo7  |             | (TTG)<sub>6</sub> | F: acaacagtctgtaacaacgga | 50     | 4         | 206-225 0.75 | 0.75 | 0.568         |
| HQ435873-HQ435879 | (TTTA)<sub>6</sub> | R: tatgtagagaggttcttaaatgg | 50     | 4         | 204-249 0.06 | 0.63 | 0.004         |
| plo10 |             | (AT)<sub>9</sub>  | F: caagcataacgcatagactaccc | 50     | 4         | 388-423 0.38 | 0.75 | 0.031         |
| HQ435880-HQ435883 | (AGA)<sub>8</sub> | R: ttcgcttcttactaaagtgttg | 55     | 6         | 500-574 0.13 | 0.83 | 0.000         |
| plo37 |             | (ATG)<sub>11</sub> | F: tcctttatcatacaaggaagg | 55     | 6         | 186-202 0.19 | 0.72 | 0.002         |
| HQ435922-HQ435924 |             | (GAC)<sub>4</sub>  | F: taatagctgacagacagagt | 55     | 2         | 0.38 | * | 0.001         |
| plo42 |             | (CAA)<sub>3</sub>  | F: atcctctgtgcgtggagttg | 55     | 2         | 196-199 0.00 | 0.38 | 0.001         |
| HQ435936-HQ435939 | (TGA)<sub>3</sub>  | R: tttggtgtagctgtgtggtg | 55     | 2         | 187-196 0.56 | 0.53 | 0.592         |
| plo66 |             | (TG)<sub>3</sub>   | F: ttcctctctcaaccaaaat | 55     | 7         | 354-433 0.13 | 0.83 | 0.000         |
| HQ435976-HQ435980 | (TCA)<sub>8</sub>  | R: ttcatttctaatcttcggtt | 55     | 7         | 186-202 0.19 | 0.72 | 0.002         |
| plo68 |             | (ATC)<sub>6</sub>  | F: cagcagatgacagagagagag | 55     | 2         | 0.38 | * | 0.001         |
| HQ435985 | (CATC)<sub>16</sub> | R: tcgccatactgctgaaaagagaa | 55     | 5         | 115-124 0.81 | 0.56 | 0.074         |
| plo78 |             | (AC)<sub>6</sub>   | F: acgatgtaataacgaagagga | 50     | 3         | 202-204 0.48 | 0.48 | 1.000         |
| GU137158 | (AT)<sub>6</sub>  | R: gctcgttgaatctgtcttga | 50     | 3         | 202-204 0.48 | 0.48 | 1.000         |
| paeE005 | (AT)<sub>6</sub>  | F: tctgctctctctctctctctt | 48     | 6         | 221-247 0.94 | 0.78 | 0.033         |
| KP407156 | (CT)<sub>10</sub> | R: gctctctctctctctctctct | 48     | 3         | 220-235 0.38 | 0.66 | 0.212         |
| paeE016 | (GA)<sub>12</sub> | F: tctgctctctctctctctctt | 48     | 6         | 220-235 0.38 | 0.66 | 0.212         |
| KP407157 | (CA)<sub>6</sub>  | R: acgacgagacgacgacgacg | 48     | 6         | 220-235 0.38 | 0.66 | 0.212         |
| paeE021 | (AG)<sub>6</sub>  | F: aagcagagacgacgacgagag | 48     | 6         | 220-235 0.38 | 0.66 | 0.212         |
| KP407158 | (CT)<sub>10</sub> | R: gctctctctctctctctctct | 48     | 3         | 226-248 0.13 | 0.67 | 0.002         |
| paeE030 | (CT)<sub>10</sub> | F: gttcccaacgacgacgacg | 50     | 1         | 195     0.00 | 0.18 | - *         |
| KP407159 | (GA)<sub>6</sub>  | R: gctctctctctctctctctct | 50     | 1         | 195     0.00 | 0.18 | - *         |
| paeE041 | (AAACA)<sub>4</sub>(AAC)<sub>4</sub> | F: agtgctggctaacctccctctt | 48     | 3         | 220-235 0.38 | 0.66 | 0.212         |
| KP407160 | (TT)<sub>12</sub> | R: atctctctctctctctctctctt | 50     | 3         | 290-298 0.25 | 0.52 | 0.147         |
| paeE056 | (CAT)<sub>12</sub> | F: gctacagtctgtaacaagag | 50     | 3         | 290-298 0.25 | 0.52 | 0.147         |
| KP407161-KP407162 | (AT)<sub>10</sub> | R: aaggagctagctgtctct | 48     | 4         | 329-342 0.75 | 0.73 | 0.013         |
| paeE060 | (CT)<sub>10</sub> | F: gctacagtctgtaacaagag | 50     | 7         | 272-298 0.50 | 0.81 | 0.012         |
| KP407163-KP407164 | (AG)<sub>6</sub>  | R: gctacagtctgtaacaagag | 53     | 2         | 202-288 0.06 | 0.34 | - *         |
| paeE062 | (AG)<sub>6</sub>  | F: gctacagtctgtaacaagag | 53     | 2         | 202-288 0.06 | 0.34 | - *         |
| KP407165-KP407166 | (AT)<sub>10</sub> | R: gctacagtctgtaacaagag | 53     | 2         | 202-288 0.06 | 0.34 | - *         |
| paeE065 | (TTA)<sub>4</sub>  | F: gctacagtctgtaacaagag | 53     | 2         | 202-288 0.06 | 0.34 | - *         |
| KP407167-KP407168 | (TTA)<sub>4</sub>  | R: gctacagtctgtaacaagag | 53     | 2         | 202-288 0.06 | 0.34 | - *         |
| paeE073 | (AT)<sub>6</sub>   | F: gctacagtctgtaacaagag | 53     | 2         | 202-288 0.06 | 0.34 | - *         |
| KP407169-KP407170 | (AAC)<sub>4</sub> | R: gctacagtctgtaacaagag | 53     | 2         | 202-288 0.06 | 0.34 | - *         |
| paeE099 | (CA)<sub>3</sub>   | F: gctacagtctgtaacaagag | 53     | 2         | 202-288 0.06 | 0.34 | - *         |

Average±SD across loci: 3.5±1.8, 0.34±0.29, 0.57±0.20

Note: Values in bold represent significant (P<0.05) deviation from HWE; * no enough allele information for HWE test; ‡ Loci showing evidences of null; † The P. lutea’s sequences resulted from amplification of primer pair Pl078 were too short to submit to GenBank and get an access number. GU137158 is the GenBank number of original P. lobata microsatellite sequence

### 2 Discussion

#### 2.1 Genetic characteristics of microsatellite markers from different sources

Twenty microsatellite markers of PL corals were identified in this study, most of these markers showed high polymorphism. Six markers (4 corrected by multiple comparisons) deviate from the Hardy-Weinberg equilibrium.
due to the presence of null alleles, showing that PL microsatellite markers also had poor conservation on the flanking sequence, which resulted in the difficulty of binding primers during PCR amplification, and thus the occurrence of null alleles (Wang et al., 2010). The proportion of EST-SSR null allele deviated from Hardy-Weinberg equilibrium was significantly lower than that of genomic-SSR markers, which is consistent with the consensus that EST-SSR flanking sequence is more conservative than that of genomic-SSR due to the selective pressure from the mRNA source (Gadaleta et al., 2011; Qiu et al., 2014). In addition, in this study it was found that the proportion of related species EST-SSR completely unamplified in PL symbionts is much lower than that of genomic-SSR, which also support the above conclusion from another side. Null alleles, which can lead to underestimation of population genetic diversity and even deviation from the Hardy-Weinberg equilibrium (Carlton and Lippe, 2008; Wang et al., 2010), are common problems in microsatellite markers, especially in marine invertebrates such as marine shellfish (Hedgecock et al., 2004; Reece et al., 2004). Therefore, the relevant loci should be carefully used in population analysis.

2.2 Microsatellite markers for symbiotic organisms

The special intracellular symbiotic relationship between reef-building coral and its symbiotic algae (zooxanthella) constitutes the structural and nutritional basis of coral reef ecosystems (Davies, 1993). The genome information carried by zooxanthella is huge (Lin et al., 2015), accounting for more than 90% of the genetic material of the whole symbiont (Spector, 1984; Gregory, 2020). As a result, both genomic and transcriptome data of coral are inevitably mixed with zooxanthella information (Hou et al., 2018). In this study, a remarkable 23.4% of the microsatellite recall rate of the symbiotic zooxanthella was much higher than that of the PL coral (10.9%), indicating that the zooxanthella sequence contamination is quite widespread among those who were claims as the reef coral genetic sequences in GenBank database. For example, Concepcion et al. (2010) confirmed that, the microsatellite markers of four reef-building corals submitted to the GenBank by them, were not identified with pure zooxanthella DNA.

Because the symbiotic zooxanthella is haploid (Freudenthal 1962), different products amplified by co-dominant microsatellite markers represent different types (often more than two) of zooxanthellae, containing genetic differentiation in a coral individual. A data matrix of haploid genotypes can be used to analyze the diversity and genetic differentiation of zooxanthellae among coral geographic populations (Chen et al., 2013). But if mixed with microsatellite markers of coral, it will cause serious interference and wrong results during population detection (Magalon et al., 2004; Chen et al., 2013). Therefore, the exploitation (whether for coral or zooxanthella) of microsatellite markers with these resources, needs to be confirmed by pure DNA of either side of the symbionts. On the other hand, even using selective hybridization to enrich the microsatellite markers of PL corals, since PL coral eggs released during sexual reproduction contain maternal symbiotic algae (Hirose et al., 2001), unless one can obtain sperm that does not contain algae DNA, the microsatellite markers development must also be identified by “exclusion strategy”.

The key step of the exclusion strategy is to obtain pure in situ symbiotic algae to extract DNA without coral DNA contamination. But PL corals tend to secrete too much mucus at elevated temperatures, blocking the small mouth of the polyp, making it difficult for the discharge of zooxanthellae (Li, 2011). Therefore, the coral sample size needs to be relatively large and vigorous, and the heating must be very gentle and slow.

2.3 Interspecific versatility of reef-building coral microsatellites

In this study, the microsatellite markers of PL corals were developed using the genomic and EST-SSR sequences of related species, obtaining an overall yield of 10.9%. The versatility of EST-SSR (12.0%) was higher than that of genomic-SSR (9.6%), mainly due to the aforementioned conservatism of EST sequences. To test the interspecific versatility, we have amplified 20 pairs of these PL coral and 17 pairs of zooxanthella microsatellite markers in two populations (n=13 and 16) of stony coral Galaxea fascicularis in the same geographical region. The results showed that the microsatellite markers with universal properties were all belong to zooxanthella, none of PL coral microsatellite markers can be amplified. This indicates that the versatility of reef-building coral microsatellite markers is limited to closely related species. Using 5 universal zooxanthella microsatellite markers,
we analyzed the genetic differences in zooxanthellae of different *G. fascicularis* geographic populations. It was found that the differentiation was significantly lower than that of coral *G. fascicularis* (Chen et al., 2013). This observation is consistent with the results of Howells et al. (2016).

The effective technology scheme of microsatellite marker development is verified in this study. The PL coral microsatellite markers developed here, as well as the identified microsatellite markers of zooxanthella, are powerful tool for analysis of population genetic structure and connectivity of the species, which could provide scientific basis for the development of coral reef protection strategy.

3 Materials and Methods

3.1 Samples of PL coral populations and acquisition of symbiotic zooxanthella

PL coral population samples were collected from Basuo reefs in Dongfang City, southwest of Hainan Island (BS, 19°8’25.8” N; 108°39'33.0” E). A small health sample of each colony was collected from the upper part, with a minimum spacing of 5 m between two colonies. The method from Chen et al. (2013) was used to extract the zooxanthellae, in brief, the newly collected, energetic PL corals were put in a 2 L beaker. The seawater covered sample was filtered by 0.2 μm membrane and placed in a water bath pot 2°C above its natural environment. The beakers were slightly shook from time to time. The water samples were collected at 2, 4 and 6 hours, respectively, and filtered with 0.45 μm membrane and vacuum pump. Coral and zooxanthella samples were fixed with 95% alcohol.

3.2 DNA extraction and identification of PL coral and zooxanthella

DNA of each PL coral and zooxanthella sample was extracted using a UNIQ-10 column genomic DNA isolation kit (Shanghai Sangon Biotech) (Chen et al., 2013). The coral symbiont and zooxanthella DNA were examined using PCR amplification of 28S RNA universal primers and the amplified products were detected by QIAxcel capillary electrophoresis (Magalon et al., 2004).

3.3 Microsatellite mining and primer design

All 142 genomic microsatellite sequences of *Porites lobata* (Concepcion et al., 2010) and 11,516 EST of *Porites astreoides* (accessed January 12, 2014) were downloaded from NCBI databases, and then were checked for duplicates using Vector NTI Advance 11.0.0 (http://www.invitrogen.com) and screened for microsatellites (contains at least 6 di-, 5 tri-, 5 tetra-, 4 penta-, and 3 hexa-, hepta-, and octa-nucleotide repeats motifs) using MISA software (http://pgrc.ipk-gatersleben.de/misa/). 183 good sequences (83 from *P. lobata* genomic sequences, and 100 from *P. astreoides* ESTs) with sufficient flanking region were selected for primer design with Primer 3 (http://primer3.ut.ee/) (Wang et al., 2010).

3.4 Resequencing and elimination of the site

The primers of the above microsatellite regions were amplified by conventional PCR methods in 6 PL coral individuals from different sites. PCR was conducted in a 10 μL solution containing about 100 ng template DNA, and reagents as follows: Taq-HS PCR Master Mix(2×) 5 μL, 5 pmol forward primer and 5 pmol reverse primer. PCR was conducted with the following steps: 94°C for 4 min followed by 30 cycles of 94°C for 30 s, annealing (50/55°C) for 30 s, 72°C for 20 s, and a final extension at 72°C for 10 min. The annealing temperature was first amplified by 55°C, and the primers which could not be amplified were then annealed at 50°C. Amplified products were detected by 1.5% agarose gel electrophoresis, and the amplified products with clear bands and meeting the expected product size were sent to Shanghai Sangon Biotech for sequencing (using ABI 3730xl sequencer). By comparing the obtained sequences with the original sequences, those homologous microsatellite sequences are identified as PL symbiont microsatellite loci. The PL symbiont primers with M13 (−21) leading sequences were synthesized, that is, 18 bp M13 (−21) (5’-TGTAAAAACGACGCTCAGT) sequences were added to the 5’ end of each forward primer, which was used for the subsequent economical fluorescent PCR reactions (Schuelke, 2000). The PL symbiont DNA and its pure zooxanthella DNA were PCR amplified using the above confirmed conditions. The product was detected by 1.5% agarose electrophoresis. Primers capable of simultaneously amplifying in PL symbionts and zooxanthella are considered to be microsatellite sites of zooxanthella, whereas only PL symbiont DNA amplified sites were identified as microsatellite sites of PL corals, which were used for further population genotyping and analysis.
3.5 Genotyping and statistical analysis

The microsatellite loci identified as PL corals were PCR amplified and genetically characterized in a wild population (BS, 16 individuals). The amplification was performed using an economical semi-nested PCR fragment fluorescence labeling method (Schuelke, 2000). PCR was conducted in a 10 μL solution containing about <100 ng template DNA, and reagents as follows: Taq-HS PCR Master Mix (2×) [Mona (Wuhan) Biotechnology Co., Ltd.] 5 μL, 0.8 pmol M13 (-21) tailed primer labeled with fluorescent dyes (FAM, VIC, NED, or PET; Applied Biosystems, Foster City, CA, USA), 1.2 pmol reverse primer and 0.4 pmol forward primer. PCR was conducted with the following steps: 94°C for 5 min followed by 30 cycles of 94°C for 30 s, annealing (temperatures indicated in Table 2) for 30 s, 72°C for 20 s 15 cycles, denaturing at 94°C for 30 s, annealing at 53°C for 30 s, extending at 72°C for 45 s, and a final extension at 72°C for 15 min. Genotyping was performed using ABI 3130 gene analyzer and GeneMapper software 3.7 (Applied Biosystems).

GENEPOP on the web (http://genepop.curtin.edu.au/) was used to identify deviations from Hardy-Weinberg equilibrium (HWE) for each locus as well as for linkage disequilibrium (LD) between all pairs of loci (exact tests, 1000 iterations). The ARLEQUIN 3.0 software was used to calculate observed (H0) and expected (Hg) heterozygosity. The MICRO-CHECKER 2.2.1 software (van Oosterhout et al., 2004) was used for identifying possible null alleles (1000 randomizations).

Authors’ contributions

LF completed the data analysis and article writing of the paper; CD and HJ completed the experimental operation and data collection; LY and LS completed the sample collection and fixation; WD completed the implementation of the research; WY completed the experimental design and the revision and finalization of the paper. All authors read and approved the final manuscript.

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