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A New Record of *Pinctada fucata* (Bivalvia: Pterioida: Pteriidae) in Mischief Reef: A Potential Invasive Species in the Nansha Islands, China

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Abstract: Mischief Reef is located in the eastern Nansha Islands of the South China Sea. With increasingly intense anthropogenic disturbance, *Pinctada fucata*, a previously unrecorded species in the reef, has occurred in the region. In this study, we identified and described the occurrence of *P. fucata* in Mischief Reef based on morphology and molecular markers. Furthermore, we performed a population genetics analysis of seven *P. fucata* populations of the South China Sea. All *P. fucata* populations showed significant high-level genetic diversity, but the differentiation among *P. fucata* populations was small. There was an F_{ST} value close to zero (−0.0083) between the Lingshui and Mischief Reef populations. Our results hint that Lingshui may be one of the potential sources of *P. fucata* to Mischief. In addition, we discussed the possible cause of the mass occurrence of *P. fucata*. The present study serves as a warning that anthropogenic disturbances have disrupted the local ecosystem in Mischief Reef.

Keywords: pearl oyster; South China Sea; cytochrome c oxidase subunit I; internal transcribed spacer 2; population genetics

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1. Introduction

The redistribution of species is a significant outcome of anthropogenic disturbance, whether intentional or unintentional, and may occur at any scale. This process can result in the disruption of local ecosystems and even pose a threat to economies [1]. Over the last five centuries, the number of species that have been introduced to new habitats through human activities has grown at an exponential rate, with a particularly notable surge in the past two centuries [2]. The introduction of invasive aquatic species into new environments has emerged as a pressing concern for the world's oceans, representing one of the most substantial risks and ranking among the top four threats [3]. Invasive alien marine species pose a threat to both marine biodiversity and industries, such as fishing and tourism. Unlike oil spills, this situation is likely to exacerbate over time, making it an increasingly pressing issue [4]. However, due to the accidental nature of many introductions, invasion events may be linked to significant data gaps and, in some cases, can remain undetected for extended periods, ranging from years to decades or even centuries [5,6].

The genus *Pinctada* Röding, 1798, a group of pearl oysters in the class Bivalvia and family Pteriidae, is found in a broad range of environments spanning from the Indo-Pacific to Western Atlantic tropical and subtropical regions. They are predominantly associated

with shallow-water habitats, particularly reef environments [7]. *Pinctada fucata* (A. Gould, 1850), also known as *Pinctada fucata martensii* (Dunker, 1880) or *Pinctada martensii* (Dunker, 1880) (https://www.marinespecies.org/aphia.php?p=taxdetails&id=397170, accessed on 8 April 2023), is an economically valuable bivalve species that is endemic to the coastal waters of the Pacific Ocean between the Tropic of Cancer and the Tropic of Capricorn [8–10]. This species is mainly cultivated for pearl production in Asia, especially in Korea, China and Japan [9,11,12]. Mischief Reef is located in the eastern Nansha Islands of China (Figure 1). In recent decades, with increased development in Mischief Reef (reclamation, aquaculture and fishery), the species of *Pinctada* have changed in this area. According to previous research by Wang and Chen [13], *P. maculata* was the sole species of the genus *Pinctada* found in the sea area surrounding the Nansha Islands. However, a recent sampling conducted at Mischief Reef revealed that *P. fucata* was also present (Figure S1). We found that *P. fucata* tended to aggregate in groups at coral reefs and attach to the nets of aquaculture cages. According to the observations of local fishermen, the presence of *P. fucata* in Mischief was initially recorded in 2016. While no substantial ecological or economic issues have been reported due to the presence of *P. fucata* in the Mischief Reef area to date, it remains unclear whether the introduction and potential proliferation of this species may cause any detrimental effects to the reef's ecosystem in the future. Because it may be considered a potential invasive species to the Nansha Islands, it is necessary to describe the new record of *P. fucata* in Mischief Reef and estimate the population genetic diversity and structure of the *P. fucata* populations.

Figure 1. Sites sampled in the present study, and additional sites from which *Pinctada fucata* sequences were obtained from GenBank based on previous study [8]. For more detailed information, please refer to Supplementary Table S1. The base map is Bing Virtual Earth.

The taxonomy of pearl oyster species is primarily established based on their soft tissue and shell characteristics, including shape and colour, as outlined in previous studies [14,15]. However, the taxonomy of pearl oyster species is complex because their shells are quite similar [16] and there are not many morphological diagnosable characters avail-

able for species determination [17]. In recent years, the application of molecular sequence data has provided valuable insights into the taxonomic classification of numerous bivalve species [9,18,19]. The incursion of *P. imbricata radiata* (Leach, 1814) into the coastal waters of the eastern Adriatic Sea was reported by Gavrilović et al. [20], who relied on the analysis of the cytochrome c oxidase subunit I (COI) gene sequences. Somrup et al. [21] distinguished a new species in the genus *Pinctada* collected from Phuket, Thailand based on both morphological and COI gene sequence data. Furthermore, molecular markers also provide valuable information on nonindigenous species, facilitating the estimation of relationship between introduced populations and other geographically distinct populations [22,23]. In the present study, morphology and molecular markers were used to confirm the identity of *P. fucata* samples collected on Mischief Island. In addition, a population genetics analysis was conducted to compare the Mischief Reef population with other geographically distinct populations in the South China Sea, to estimate the potential geographic origin of the *P. fucata* introduced to Mischief Reef.

2. Materials and Methods

2.1. Sampling and DNA Extraction

Twenty *Pinctada fucata* specimens were collected in the coral reefs from Mischief Island (9°57′ N, 115°44′ E) in December 2018. Furthermore, we also sampled *P. fucata* specimens from Haiphong, Lingshui and Zhanjiang (ten individuals per population). The specimens were kept in a freezer after collection (Figure 1). Next, genomic DNA was isolated from a 50-mg sample of the adductor muscle using a phenol-chloroform extraction method following standard protocols.

2.2. Amplification and Sequencing

The specific primers PMCOI-F: 5′-TTT CTT ATC CGA ATG GAGCT-3′ and PMCOI-R: 5′-TGT ATT AAA ATG CCG ATC CG′ [24] were used to amplify a fragment of approximately 500 bp of the COI gene sequence. Internal transcribed spacer 2 (ITS2) was also amplified using the primer pair 5.8S-F and 28S-R from the study of Yu and Chu [25]. Polymerase chain reaction (PCR) amplifications were carried out in 25 reactions using the following mix: DNA template (1 μL), forwards primer (1 μL, 10 μM/L), reverse primer (1 μL, 10 μM/L), dNTPs (2 μL, 2.5 mM/L), EasyTaq DNA Polymerase (0.15 μL, 5 U/μL), and 10 × PCR buffer (2.5 μL, 25 μM/L). The PCR amplification protocol consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min 45 s. A final extension step was performed at 72 °C for 10 min. The resulting PCR products of the COI gene were used as the template DNA for cycle sequencing reactions using the Big Dye Terminator Cycle Sequencing Kit. Sequencing was conducted bidirectionally on an ABI Prism 3730 automatic sequencer (Applied Biosystems, Foster City, CA, USA). The amplified products of ITS2 were purified and cloned into *Escherichia coli* using the pGEM-T Easy vector (Promega, Madison, WI, USA). The plasmids were sequenced using universal primers M13-47 and RV-M. The sequencing products were alcohol-precipitated and subsequently sequenced on an ABI 3730 automatic sequencer (Applied Biosystems, CA, USA).

2.3. Statistical Anazlysis

The obtained sequences were subjected to revision using DNASTAR software (DNASTAR, Madison, WI, USA). To confirm the classification status of our samples, eight COI sequences of *Pinctada* species were downloaded from GenBank and included in the phylogenetic tree study (Table 1). To root the tree, *Pteria penguin* (Röding, 1798) was selected as the outgroup. The neighbour-joining (NJ) tree was constructed using MEGA 5.0 [26] under the Kimura 2-parameter (K2P) model [26,27].

For population genetics analysis, definition of haplotypes was carried out using DnaSP v. 5.00, and gaps were not considered during the analysis of the sequence data [28]. To quantify the genetic diversity in each population, various measures were employed,

including the number of polymorphic sites, number of haplotypes, haplotype diversity, nucleotide diversity, and mean number of pairwise differences. These parameters were calculated using Arlequin v. 3.0 [29]. By employing these measures, a comprehensive analysis of genetic variation within populations was conducted, providing valuable insights into the evolutionary processes shaping genetic diversity.

To assess the genetic differentiation between different populations, the *F*-statistic (*F*st) was calculated using on Arlequin v. 3.0 software [30]. The *F*st value was computed using the K2P method, considering different substitution rates between transitions and transversions [27]. The probability pertaining to the *F*st values was assessed using random permutation techniques, wherein a minimum of 10,000 permutations were performed. Significance tests to determine differentiation between samples were carried out using exact *P* tests with a Markov chain procedure executed in Arlequin. Moreover, for a more comprehensive and straightforward illustration of population differentiation, we used IQ-TREE version 1.6 (http://www.iqtree.org/#download, accessed on 13 January 2022) to select the best-fit model of nucleotide substitution based on the Akaike information criterion (AIC), and we built the maximum likelihood (ML) tree based on the best-fit model [31]. Furthermore, we drew a heatmap of *F*st values and exact *P* tests between different populations.

Table 1. Species and the GenBank accession numbers of the COI sequences used in this study.

Species	GenBank Accession No.	Sample Site
Pinctada fucata martensii *	KX669229	Sanya, China [32]
Pinctada fucata	DQ299941	China
Pinctada albina	AB261165	Kagoshima, Japan [33]
Pinctada martensii *	AB076915	Okinawa, Japan [18]
Pinctada imbricata	KX713492	Florida Keys, USA [34]
Pinctada maculata	AB076928	Okinawa, Japan [18]
Pinctada margaritifera	HM467838	China
Pinctada persica	AB777263	Hendurabi, Iran [35]
Pinctada radiata	KF284062	Ras Al Khaimah, UAE [36]
Pinctada maxima	NC018752	Not mentioned [37]
Pteria penguin	KU552127	Sanya, China [32]

* *Pinctada martensii*, *P. fucata martensii* and *P. fucata* were conspecific, the *P. martensii* and *P. fucata martensii* are not accepted.

3. Results

3.1. Morphological Characteristics and DNA Barcoding Identification

The body and shell are asymmetrical, the left valve is deeper than the right valve, and there is a byssal notch on the anterior side. The average length/height ratio was 0.86. Anterior and posterior auricles are located at the ends of the hinge line, and the former is larger. The hinge line is longer than the shell height. The ligament connects the two valves at the centre of the hinge line. The hinge teeth are well developed, and the posterior tooth of the left valve is above that of the right. The external colour is brown, green, red, yellow, or white, and the shell's internal surface is nacreous; the nacre is of a hard-white metallic lustre and yellow, silver, gold, or pink (Figure 2). Muscular scars are visible. According to the key characteristics for species identification based on the shell morphology of the genus *Pinctada*, the distribution pattern of processes (scales) on the external shell is different between *P. maculata* and *P. fucata* [38,39]. The processes (scales) of *P. fucata* are densely distributed, but those of *P. maculata* are sparsely distributed at regular intervals [39].

The COI gene (500 bp) was sequenced from six individuals. Two haplotypes were found. The accession numbers for the two haplotype sequences submitted to GenBank are MK748604 and MK748605. Sixteen COI sequences of *Pinctada* species, including our haplotypes, were downloaded and analysed (Table 1). The mean genetic distance between all species was calculated as 22.63%, while the genetic distance between our six specimens and *P. martensii* was only 0.26%. Furthermore, the genetic distances between our six

specimens and the other *Pinctada* species (exclude the upper clade) ranged from 9.81% to 44.25%. An NJ phylogenetic tree was constructed using MEGA 5.0, with *P. penguin* chosen as the outgroup. The tree showed that our specimens, *P. fucata*, *P. fucata martensii* and *P. martensii* clustered in the same group (Figure 3).

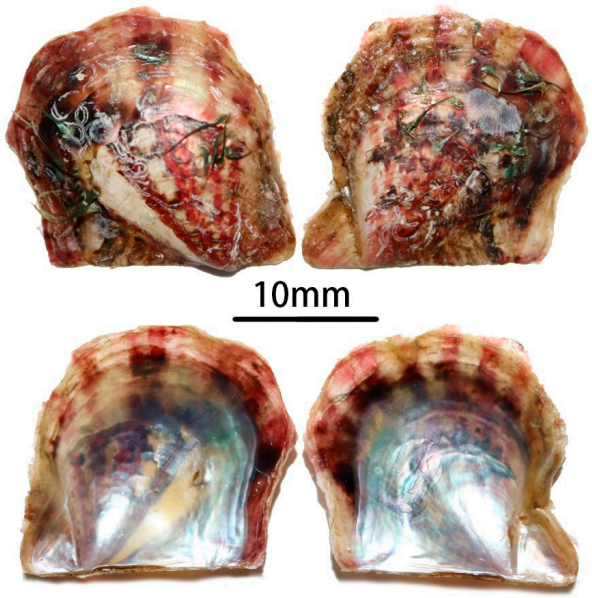

Figure 2. *Pinctada fucata* collected from in the coral reefs from the Mischief Reef.

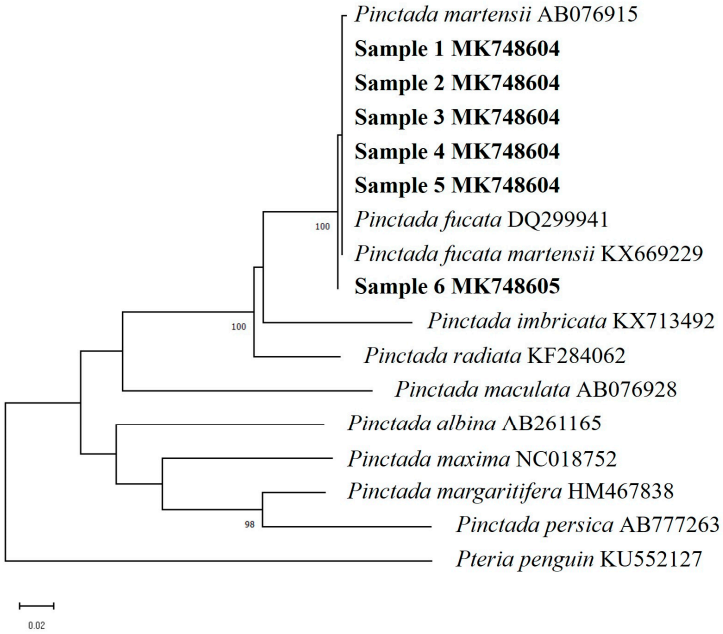

Figure 3. Neighbour-joining phylogenetic tree based on COI sequence data. The regular texts refer to sequences downloaded from GenBank, while the bold texts represent sequences that were sequenced in the present study. *Pteria penguin* was designated as the outgroup and used to root the tree.

3.2. Population Genetic Diversity

In this study, we obtained 40 ITS2 sequences with a length of 548 bp (OQ629249-OQ629288). Among these sequences, we identified a total of 31 haplotypes. Among the 31 haplotypes identified, 26 were found singly in the four locations sampled, representing 83% of the total (Supplementary Table S2). The Lingshui and Mischief populations exhibited the highest number of haplotypes with 10 each, while the Zhangjiang population had the lowest eight haplotypes (see Table 2). Notably, out of the 10 haplotypes detected in the

Mischief Reef population, only two were shared with other populations (Supplementary Table S2). Additional genetic diversity parameters are presented in Table 2, where the gene diversity ranged from 0.93 to 1.00, indicating that all *P. fucata* populations demonstrated a high level of genetic diversity.

Table 2. Population genetic diversity parameters.

	Zhanjiang	Lingshui	Mischief	Haiphong
Numbers of haplotypes	8	10	10	9
Haplotype diversity	0.9333	1.0000	1.0000	0.9778
Nucleotide diversity	0.0129	0.0148	0.0151	0.0142
Mean number of pairwise difference	7.1556	8.1556	8.3111	7.8222

3.3. Population Genetic Structure and Differentiation

Additionally, we downloaded 25 ITS2 sequences from previous study [8], and their corresponding sampling sites are shown in Figure 1. Supplementary Table S1 provides further information on these samples. The subsequent analysis was performed on a total of 65 sequences. Pairwise *F*st were calculated based on the ITS2 sequences, and the values ranged from −0.0700 to 0.1101 (Figure 4). The Beihai and Mischief Reef populations exhibited the highest F_{ST} values, while the Shenzhen and Lingshui populations had the lowest *F*st values. Notably, the *F*st values between the Mischief Reef and Lingshui populations were negative (−0.0093), indicating a closer relationship between these populations than within populations. Among the populations, the Beihai population displayed greater genetic differentiation (0.0400–0.1101) than the other populations. The optimal model of nucleotide substitution was found to be HKY + F + R2. The ML tree was constructed based on 1000 replicates of ultrafast bootstrap approximation. The ML tree had a shallow topology, and there were no significant genealogical branches or sample clusters corresponding to the sampling sites (Figure 5). However, the topological configuration of the phylogenetic tree showed that the majority of individuals from Mischief Reef displayed a greater similarity to the Lingshui population in comparison to other populations, thus indicating a strong connection between the Lingshui population and the *P. fucata* population of Mischief Reef.

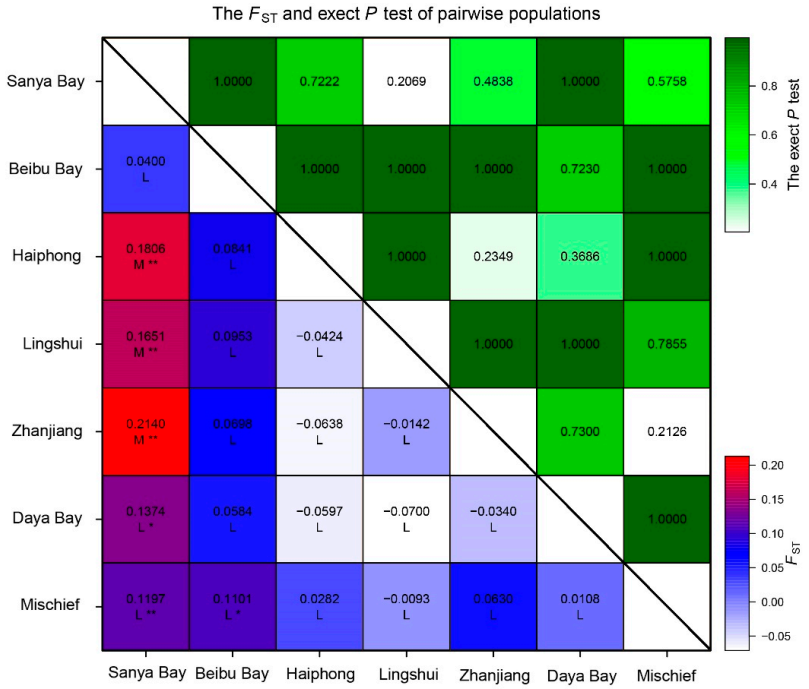

Figure 4. Heatmap of *F*st (**right**, below diagonal) and exact *P* test (**left**, above diagonal) values of *P. fucata*. *: $p < 0.05$, **: $p < 0.01$.

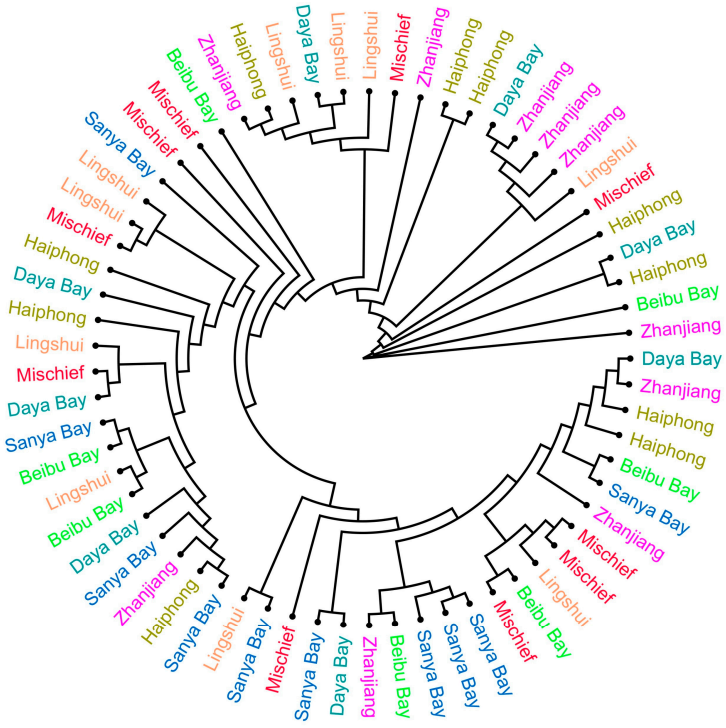

Figure 5. Maximum likelihood tree of all ITS2 sequences, with each sampling site indicated by a distinct colour. The lengths of branches represent the relative genetic difference.

4. Discussion

4.1. Identification of the Samples

Pinctada fucata and *P. imbricata* are accepted in online marine taxonomic databases such as Molluscabase, GBIF and WoRMS. Cunha et al. [10] recognized the phylogenies of *Pinctada* species, and their analyses indicated that *P. fucata* and *P. martensii* were conspecific, with the species recovered as monophyletic compared to *P. imbricata*. Furthermore, Yu and Chu [9] observed low genetic distances among *P. fucata*, *P. fucata martensii* and *P. imbricata*, and indicated they may be conspecific. According to these earlier findings, *P. fucata* is the available specific name. In the present study, we identified the samples based on molecular markers and morphology, our results confirm that the species we collected from Mischief Reef is indeed *P. fucata*.

A series of detailed investigations (investigation time: July–August 1988, May 1989, May–June 1993 and March–April 1994) of the Nansha Islands by the China Nansha expedition showed the absence of *P. fucata* [13,40]. Therefore, our study represents the first record of *P. fucata* on the Nansha Islands. While *P. fucata* is of great economic value in pearl production, the emergence of non-native species may present a potential hazard to the indigenous ecosystem. The activities of introduced bivalve species, including shell construction, bioturbation, and filter feeding, could modify the processes and functions of ecosystems, thereby exerting detrimental effects on biodiversity and the environment [3,41]. Therefore, it is important to pay close attention to the new record of *P. fucata* in Mischief Reef.

4.2. Population Genetics Analysis

In the present study, we found that all *P. fucata* populations exhibited a significant level of genetic diversity. Additionally, the high haplotype diversity (0.93–1.00) with low nucleotide diversity (0.013–0.015) observed in our study is consistent with that reported in many other bivalve species [42,43].

In our study, we investigated the population genetic differentiation of six native *P. fucata* populations and one Mischief Reef population. The *F*st values and ML tree revealed that the differentiation among *P. fucata* populations was small. Previous studies have

suggested that the *P. fucata* populations in China could be considered a group [44]. We also found that many pairwise *F*st values were negative, indicating that differentiation between populations was very small [45]. For instance, the population sampled from Shenzhen showed negative *F*st values with four populations. This phenomenon may be explained by mixed germplasm resources resulting from mass hybridization and transport during the development of *P. fucata* aquaculture [25,46]. More importantly, it is noteworthy that among the six native populations, only the Lingshui population had a negative *F*st value (−0.0093) with the Mischief Reef population. Our results hinted that Lingshui had a very close relationship with *P. fucata* in Mischief Reef.

4.3. Possible Cause of Occurrence

Since we have not yet investigated the source of the *P. fucata* population in Mischief, we cannot directly address the invasion pathway and the exact origins. Thus, here we discuss the possible cause of the occurrence of *P. fucata*. Most marine invasive species are disseminated via ballast water or hull fouling, which are associated with maritime activities [4]. In North America, commercial shipping has been identified as the most significant introduction vector, responsible for 52–82% of nonindigenous species introductions over the past 30 years [47]. The pearl oyster exhibits a prolonged planktonic larval stage, which can last up to 17 days [48]. This characteristic may facilitate the entrainment of larvae in ballast tanks throughout the duration of a voyage. Other vectors, specifically hull fouling, floating ropes and aquaculture net cages, could also be introduction vectors. Previous studies have reported that aquaculture and other human activities have increased in frequency in the past decade, particularly in Lingshui and other cities in Hainan [49,50]. This raises the possibility that these activities may have facilitated the introduction of *P. fucata* from Hainan to Mischief Island, and could explain why *P. fucata* populations in Lingshui and Mischief Island exhibit a closer relationship. Marques and Breve [51] documented the presence of juvenile *P. imbricata* adhering to a floating rope found along the Uruguayan coast. Although there were no data indicating that the species was successfully and effectively settled, it could be a potentially invasive species in the Uruguayan coast. Furthermore, the expansion of native species into adjacent areas may also account for the presence of newly recorded species, as this process can produce effects similar to those generated by alien species [52]. As a result of human activities (reclamation, aquaculture, fishery), the habitat (including temperature, salinity, nutrients) in Mischief Reef may have been changed, which could benefit the migration of *P. fucata* from adjacent areas through ocean currents.

5. Conclusions

In the present study, we identified and described the occurrence of *P. fucata* in Mischief Reef. The results of the population genetics analysis hinted that the Lingshui population had a very close relationship with *P. fucata* in Mischief Reef. The presence of *P. fucata* should serve as a warning that anthropogenic disturbances have disrupted the local ecosystem in Mischief Reef. Investigating the invasion pathway of *P. fucata* is of great importance, as it highlights the necessity for management strategies to prevent the introduction of other invasive species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15040578/s1, Figure S1: The occurrence of *Pinctada fucata* in Mischief Reef; Table S1: Information of the downloaded ITS2 sequences from NCBI; Table S2: Haplotypes of the four *P. fucata* populations

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Institutional Review Board Statement: Not applicable.

Data Availability Statement: All sequences obtained in present study were deposited in NCBI. The accession numbers are OQ629249-OQ629288, MK748604 and MK748605.

Conflicts of Interest: The authors declare no conflict of interest.

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