

Article

High Genetic Diversity in Geographically Remote Populations of Endemic and Widespread Coral Reef Angelfishes (genus: *Centropyge*)

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Abstract: In the terrestrial environment, endemic species and isolated populations of widespread species have the highest rates of extinction partly due to their low genetic diversity. To determine if this pattern holds in the marine environment, we examined genetic diversity in endemic coral reef angelfishes and isolated populations of widespread species. Specifically, this study tested the prediction that angelfish (genus: *Centropyge*) populations at Christmas and Cocos Islands have low genetic diversity. Analyses of a 436 base pair fragment of the mtDNA control region revealed that the endemic *C. jocularis* exhibited high haplotype ($h > 0.98$ at both locations) and nucleotide (Christmas $\pi\% = 3.63$, Cocos $\pi\% = 9.99$) diversity. Similarly, isolated populations of widespread angelfishes (*C. bispinosa* and *C. flavicauda*) had high haplotype ($h > 0.98$) and nucleotide ($\pi\% = 2.81$ and $\pi\% = 5.78\%$, respectively) diversity. Therefore, in contrast to terrestrial patterns, endemic and isolated populations of widespread angelfishes do not have low genetic diversity, rather their haplotype and nucleotide diversities were among the highest reported for marine fishes. High genetic diversity should reduce extinction risk in these species as it

could provide the evolutionary potential to adapt to the rapidly changing environmental conditions forecast for coral reefs.

Keywords: extinction risk; haplotype diversity; nucleotide diversity; Pomacanthidae; Christmas Island; Cocos (Keeling) Islands

1. Introduction

The highest rates of extinction have been recorded for endemic species and populations of widespread species inhabiting islands [1–3]. The high risk of extinction in this group has been attributed to a range of demographic, environmental and genetic factors [1,2,4]. The genetic characteristics that increase extinction risk in this group are low genetic diversity and inbreeding depression [1,2]. Low genetic diversity increases extinction risk because it reduces the potential for species to adapt to rapid environmental change. In endemics, and isolated populations of widespread species, inbreeding depression is thought to occur because of the low number of individuals forming the founder population and the small size of the extant population [5,6]. The importance of maintaining genetic diversity to reduce extinction risk is recognised by the IUCN and is considered to be a conservation priority [6,7].

Genetic diversity can be influenced by a range of factors including population size, natural selection, mutation rates, gene flow between populations, introgression from hybridisation and historical effects on these factors (e.g., population bottlenecks) [6]. In the terrestrial environment, island endemic species usually have small populations [5,8] and consequently, genetic diversity is predicted to be low as genetic variation is increasingly lost through genetic drift [9]. Isolated island populations of widespread species are also expected to have low genetic diversity because of limited gene flow with other populations and lower localised effective population sizes [6,10]. Therefore, low genetic diversity is expected to be a feature of terrestrial communities on remote islands because they contain a high proportion of endemic species and isolated populations of widespread species.

Coral reef fishes are the most diverse vertebrate communities in the world and are found throughout the tropics, including numerous remote islands. These isolated islands are hotspots of coral reef fish endemism [11–14] and also support isolated populations of species with broad geographic ranges. Determining whether these endemics and isolated populations conform to terrestrial patterns of low genetic diversity is crucial to assessing their risk of extinction. Of the known neo-extinctions of reef fishes, all have been from isolated islands or island groups [15,16]. An appreciation of the genetic diversity of endemic reef fishes is also of importance for ascertaining extinction risk of this group to changes in their environment caused by local and global anthropogenic disturbances [17,18].

Previous studies have revealed a range of genetic diversity values for populations of marine fishes, including those inhabiting coral reefs. Haplotype diversity (h) in populations of marine fishes has been found to vary vastly from 0 to 1, while nucleotide diversity ($\pi\%$) is known to vary from 0.07 to 31.8 [19–23]. For marine fishes, haplotype and nucleotide diversities are generally considered to be low where h and $\pi\%$ are less than 0.5 [19]. Therefore, if reef fishes conformed to predictions based on

terrestrial fauna, we would expect endemic species, and isolated populations of widespread fishes, to have low haplotype ($h < 0.5$) and nucleotide ($\pi < 0.5\%$) diversities.

This study determines whether endemic and isolated populations of widespread angelfishes, at Christmas and Cocos Islands have low genetic diversity ($h < 0.5$ and $\pi < 0.5\%$). These islands are an ideal location to examine genetic diversities of isolated marine species because they are among the most-isolated islands in the tropical Indian Ocean [24]. Three congeneric pygmy angelfish (genus: *Centropyge*) species were examined. *Centropyge jocularis* is endemic to these two locations and is not found anywhere else in the world [25]. *C. bispinosa* and *C. flavicauda* are among the most widely distributed angelfishes, ranging from east Africa to the central Pacific Ocean [25], with Christmas Island supporting geographically-isolated populations of these species. Christmas and Cocos Islands are separated by approximately 1000 km, therefore, we also predicted that the two populations of the endemic species (*C. jocularis*) might be genetically subdivided between these locations due to low genetic exchange [26].

2. Experimental Section

2.1. Field Collections

Genetic material for this study was obtained by collecting angelfishes from both Christmas (10°29'S, 107°37'E) and Cocos (Keeling) Islands (12°04'S, 96°48'E). Individuals of *C. jocularis* were collected from both locations (Christmas $n = 44$ and Cocos $n = 41$). Samples of the widespread study species were only collected from Christmas Island ($n = 24$ for *C. bispinosa* and $n = 28$ for *C. flavicauda*), as they are absent from the Cocos Islands. All angelfishes were collected by spearfishing whilst SCUBA diving in May to August 2005. Shortly after capture, a small fin clip ($\sim 1 \text{ cm}^2$) was taken from each fish and preserved separately in 80% ethanol.

2.2. Laboratory Procedures

The mitochondrial (mtDNA) control region (D-loop) was used to determine genetic diversity of endemic and isolated populations of widespread angelfishes. Samples were washed in TE buffer and DNA was obtained by first digesting with Proteinase K followed by standard salt (NaCl) extraction [27]. Amplification of a 436 base pair section of the mtDNA control region was performed using universal primers LI5995 (5'-AATTCTCACCCCTAGCTCCCAAAG-3') and HI6498 (5'-CCTGAAGTAGGAACCAGATG-3') [28]. Amplification involving polymerase chain reaction (PCR) was conducted using a 20 μl volume containing the following reagents: 2.5 mM Tris pH 8.7, 5 mM KCl, 5 mM $(\text{NH}_4)_2\text{SO}_4$, 200 μM each dNTP, 2 mM MgCl_2 , 0.5 μM forward primer LI595, 0.5 μM reverse primer HI6498, 1 U *Taq* polymerase (Qiagen), and at least 10 ng of DNA template. The PCR was conducted as follows: a denaturing step of 94 °C for 30 sec, followed by an annealing temperature of 51 °C for 30 sec, then 72 °C for 90 sec and a final extension of 72 °C for 10 min. This cycle was repeated 34 times. The success of PCR reactions was verified after running 2 μL of PCR product through a 2% agarose gel. The gel also revealed a single band of PCR product and a ladder verified that the amplified DNA fragment was the correct size. The remaining PCR product was cleaned using isopropanol, dried and sent to Macrogen Inc. (Seoul, Korea) for standard Sanger

sequencing. Both the forward and reverse sequences were obtained using primers LI5995 and HI6498 (respectively).

2.3. Data Analyses

To determine genetic diversity, sequences were first visually aligned using Sequencher 4.5 (Gene Code Corporation, MI, USA), and then manually adjusted in BioEdit (Version 7.0.9) [29] in preparation for importing into molecular statistical programs.

Arlequin (version 2.0), [30] was used to calculate haplotype diversity index (h), nucleotide diversity index ($\pi\%$) and the number of nucleotide changes as input for constructing a minimum spanning tree of the haplotype network [31] for each of the three study species. Haplotype diversity index followed Nei [32] where $h = n(1 - \sum x_i^2)/(n-1)$, and n is the total number of individuals and x_i is the frequency of a given haplotype in the population. For the endemic *C. jocularis*, haplotype and nucleotide diversity were calculated for each of the two populations (Christmas and Cocos), as well as for the total (*i.e.*, both populations combined). Genetic population structure was examined in *C. jocularis* using analysis of molecular variance (AMOVA) conducted in Arlequin. For the widespread species (*C. bispinosa* and *C. flavicauda*), haplotype and nucleotide diversity values were calculated for Christmas Island populations.

Phylogenetic analyses were also undertaken to determine how evolutionary history affects genetic diversity in *C. jocularis*. Neighbour joining (NJ, Tamura-Nei model, 1000 bootstrap replicates) and Maximum Parsimony (MP) approaches were used to construct a phylogram in MEGA (Molecular Evolutionary Genetics Analysis, Version 4.0), [33]. Maximum Likelihood (ML) analyses were also performed in GARLI (Genetic Algorithm for Rapid Likelihood Inference), [34]. Ten independent ML analyses were run, each comprised of 10,000 generations, with the best tree determined by the lowest log-likelihood score. Information from the best tree was imported into PAUP (Phylogenetic Analysis Using Parsimony, version 4.0), [35] to produce a 50% consensus tree with NJ and MP support values added to the tree nodes. The tree was rooted using *C. bispinosa* and *C. flavicauda* as the outgroups.

3. Results

3.1. Endemic *C. jocularis*

Examination of the 436 bp control region mtDNA sequenced from 85 *C. jocularis* individuals (Christmas $n = 44$ and Cocos $n = 41$) revealed 161 polymorphic sites, 143 transitions, 42 transversions and 8 indels. The AT:CG ratio was biased 31:19. There were 54 unique haplotypes and 12 shared haplotypes (shared between 2 to 6 individuals) (Figure 1). Haplotype diversity was high ($h > 0.98$) in both the Christmas and Cocos populations (Table 1). Nucleotide diversity was also high for both populations ($\pi\% = 3.63$ and 9.99 for Christmas and Cocos respectively) due to the high number of pairwise differences between individuals arising from the large number of base pair substitutions (Table 1).

Phylogenetic analyses revealed two distinct groups within the endemic species (Figure 1), with an ancestral group comprised of 10 individuals found only at the Cocos Islands. The second group (the more recently diverged group) contained fish from both Christmas and Cocos, and included the

majority of the sampled individuals (75 of 85). The 10 individuals in the ancestral group all had unique haplotypes, and haplotype diversity was also high in the more recently derived group ($h > 0.98$), although there were 12 shared haplotypes. Nucleotide diversity was high for both groups ($\pi\% = 3.34$ to 3.66, Table 1).

There was a significant difference in the haplotype frequency of the Christmas and Cocos populations of *C. jocularis* ($\Phi_{ST} = 0.011$, $p = 0.0007$). This difference may have been driven by the presence of the localised ancestral group at the Cocos Islands. However, re-analysing the data without the ancestral group (10 Cocos individuals removed) showed that the Christmas and Cocos populations remain genetically differentiated, indicating restricted gene flow between the two populations ($\Phi_{ST} = 0.013$, $p = 0.001$).

Figure 1. (a) Outgroup rooted phylogram based on 436 bp control region mtDNA sequences from 85 *Centropyge jocularis* individuals (44 from Christmas Island and 41 from the Cocos Islands). The NJ, MP and ML majority rule bootstrap consensus support values are displayed at each node. (b) Minimum spanning tree of *C. jocularis* haplotypes. Black filled circles are Christmas Island individuals and unfilled circles are individuals from the Cocos Islands. The size of the circle indicates the relative frequency of each haplotype (smallest circle = 1 individual, largest circle = 6 individuals). Bars indicate the number of substitutions between haplotypes, with thin bars = 1 substitution, medium bars = 5 substitutions, and thick bars = 10 substitutions. (c) Mismatch distribution of pairwise sequence differences for *C. jocularis* and the expected distribution of pairwise sequence differences under an expansion model.

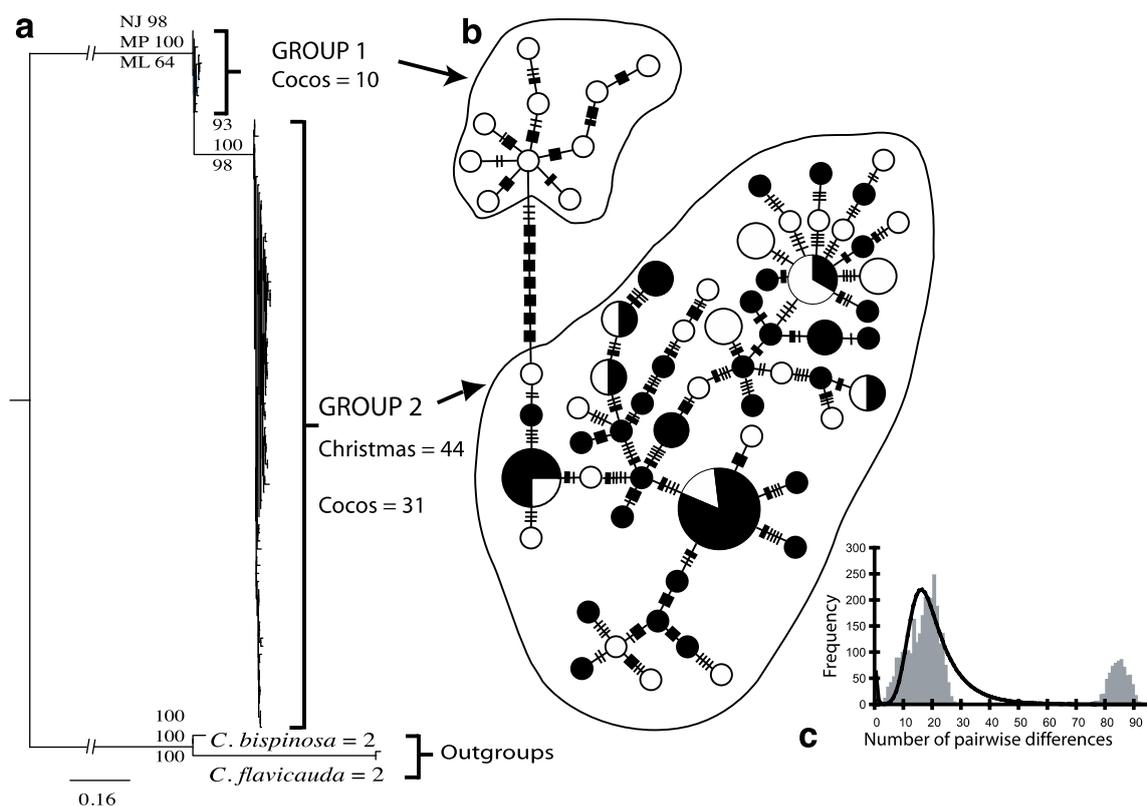


Table 1. Genetic diversity measures for the endemic *Centropyge jocularis* from Christmas and Cocos Islands, and for the widespread species *C. bispinosa* and *C. flavicauda* from Christmas Island.

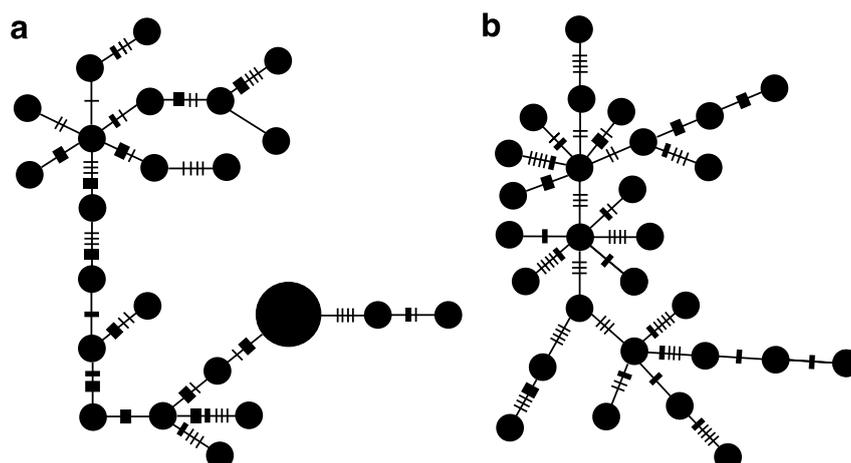
Species	Location	<i>n</i>	<i>n_h</i>	<i>h</i> (±SD)	π%(±SD)	Mean pairwise differences (±SD)
<i>C. flavicauda</i>	Christmas Island	28	28	1 ± 0.010	2.81 ± 0.39	12.08 ± 5.63
<i>C. bispinosa</i>	Christmas Island	24	23	0.989 ± 0.012	5.78 ± 0.73	24.04 ± 10.95
<i>C. jocularis</i>	Christmas Island	44	35	0.983 ± 0.011	3.63 ± 1.83	15.70 ± 7.14
<i>C. jocularis</i>	Cocos Islands	41	37	0.995 ± 0.007	9.99 ± 4.91	41.65 ± 18.45
<i>C. jocularis</i>	Christmas and Cocos Islands	85	66	0.991 ± 0.004	6.92 ± 0.34	30.16 ± 13.30
<i>C. jocularis</i>	Group 1 (Cocos Islands)	10	10	1 ± 0.045	3.34 ± 1.85	14.49 ± 7.10
<i>C. jocularis</i>	Group 2 (Christmas and Cocos Islands)	75	56	0.988 ± 0.005	3.66 ± 1.83	15.83 ± 7.14

n = number of individuals, *n_h* = number of haplotypes, *h* = haplotype diversity index. Nucleotide diversity is given as a percentage (π%) and standard deviations (SD) are provided.

3.2. Widespread Species *C. bispinosa* and *C. flavicauda*

A 436 bp section of the mtDNA control was successfully sequenced from 24 *C. bispinosa* individuals collected from Christmas Island. The sequences revealed 81 polymorphic sites, with 78 transitions, 2 transversions, 4 indels and an AT:CG ratio of 7:3. Haplotype diversity was very high (*h* = 0.99) as 23 of the 24 sequenced individuals had unique haplotypes (Figure 2, Table 1). Nucleotide diversity in this isolated population was also very high (π% = 5.78) due to the large number of base pair differences between individuals (mean = 24, Table 1, Figure 2a).

Figure 2. Minimum spanning tree for haplotypes of widespread species a) *Centropyge bispinosa* (*n* = 24), and b) *C. flavicauda* (*n* = 28) collected from Christmas Island. The size of the circle indicates the relative abundance of each haplotype (smallest circle = 1 individual, largest circle = 2 individuals). Bars indicate the number of substitutions between haplotypes, with thin bars = 1 substitution, medium bars = 5 substitutions, and thick bars = 10 substitutions.



The same section of mtDNA was sequenced in 28 *C. flavicauda* individuals from Christmas Island. These sequences comprised 74 polymorphic sites, with 65 transitions, 8 transversions, 7 indels, and an AT:CG ratio of 31:14. All 28 individuals had a unique haplotype ($h = 1$), and nucleotide diversity was also high ($\pi\% = 2.81$) (Table 1, Figure 2b).

4. Discussion

In the terrestrial environment, genetic diversity of endemics and isolated populations of widespread species tends to be low and this may be an important factor contributing to their extinction risk [1,9]. Based on this, and the known range of genetic diversity for marine fishes [19], we predicted that island endemic reef fishes and isolated populations of species with broad geographic ranges would have low haplotype ($h < 0.5$) and nucleotide ($\pi < 0.5\%$) diversity. Instead, we found that genetic diversity values of the endemic and isolated angelfish populations ($h > 0.98$, $\pi > 2.8\%$) were among the highest recorded for marine fishes [19,21–23,36–39]. Hence, contrary to expectations based on terrestrial species, it appears that there are processes operating that are promoting, rather than reducing, genetic diversity in these endemic and isolated populations.

There are a number of factors that influence genetic diversity. Endemic species usually have low abundance [5,8] and therefore are predicted to have low genetic diversity because of the increasingly deleterious effects of inbreeding and genetic drift on small populations [9,40]. However, marine endemics often have high abundance [41] and thus the effect of drift on genetic diversity may be minimal. Indeed, the abundance of the endemic study species *C. jocularis* is more than 30 times greater than that of the 16 widespread species of angelfishes that are present at Christmas and Cocos Islands [42]. High genetic and nucleotide diversity is expected in fishes that maintain large populations through their evolutionary history [19]. If the current high abundance of *C. jocularis* has been a feature of its evolutionary history then this will have contributed to its high haplotype and nucleotide diversity.

Irrespective of population size, genetic diversity can also be high if endemics occur in refugia (e.g., Pleistocene refugia: [43]) where environmental conditions have been relatively stable over evolutionary time scales allowing for the accumulation of genetic variation (see [44] and references therein). Christmas and Cocos Islands are oceanic islands that have the Indonesian Archipelago as their nearest neighbour. Indonesia is an area where Pleistocene sea level fluctuations caused significant disruption to marine habitats and fauna [45]. If Christmas and Cocos Islands were environmentally stable then they may have served as Pleistocene refugia. Also, *C. jocularis* may have had high genetic diversity if it was once more widespread and as its range contracted this diversity may have been condensed and maintained in the relict populations at Christmas and Cocos Islands. These islands also support endemic relict populations of other reef fishes (e.g., *C. flavissima* and *Stegastes insularis*, [25,46]) whose disjunct geographic ranges are separated by the historically tumultuous Indonesian Archipelago [45]. Therefore, the high genetic diversity of *C. jocularis* at Christmas and Cocos may also be because it is endemic to refugia and/or because it is a relict of a once widespread species.

Endemics can also have high genetic diversity if they represent a fusion of clades or have interbred with other species (i.e., introgression from hybridisation) [47–50]. Christmas and Cocos Islands represents a suture zone where different clades or species come into secondary contact and interbreed [51,52]. *C. jocularis* has both high haplotype and nucleotide diversity, which is a signature

of secondary contact between different clades or lineages [19]. Indeed, two genetically distinct groups were detected in *C. jocularis* and while the historical cause(s) for the origin of these groups is not known, it has clearly increased the genetic diversity of this species.

The restricted gene flow between Christmas and Cocos populations of *C. jocularis* supports the notion that endemic reef fishes have limited dispersal [26]. Restricted gene flow may have also influenced genetic diversity in *C. jocularis*. If local adaptation occurs at each island location then this will lead to differences in the gene pools of the two populations. Rare dispersal events may allow for enough gene flow to increase genetic diversity in each population, but gene flow may be insufficient to homogenise the genetic composition or to counteract adaptive changes of both populations.

Genetic diversity in isolated populations of widespread species is predicted to be low because population size is usually small and gene flow to other populations is low [1]. However, genetic diversity was high despite the very small population sizes observed for the two widespread study species at Christmas Island (0.05 to 0.18 individuals per 300 m²: [42]). It is possible these isolated populations still receive sufficient gene flow from other populations because these species are among the most widely distributed angelfishes in the world [25] and gene flow would be required to establish and maintain this broad distributional range. Isolated populations of other widespread *Centropyge* species also have high haplotype diversity and high genetic connectivity [37,53]. Furthermore, the recent arrival of *C. acanthops* at the Cocos Islands (Hobbs personal observation) demonstrates that larvae of widespread *Centropyge* species are capable of dispersing over 2500 km. Broad dispersal in widespread *Centropyge* may be facilitated by broadcast spawning, a pelagic larval duration of 30 to 35 days and competent swimming abilities of late stage larvae [54,55], which may be further aided by favorable currents at the time of spawning. Moderate to high genetic diversity (haplotype and/or nucleotide) has also been reported in several other widespread reef fishes present at Christmas and Cocos Islands [21–23,39]. The gene flow received by populations of these widespread species at Christmas and Cocos Islands may be sufficient to counteract the loss of genetic diversity associated with genetic drift. For the widespread species examined in this study, additional sampling from other populations would be required to confirm that gene flow was the reason for their high genetic diversity at Christmas Island.

Genetic diversity can also be related to taxonomy with some groups having higher diversity due to faster mutation rates [9]. High haplotype diversity in an Atlantic *Centropyge* angelfish was partly attributed to the high mutation rate of the control region of mtDNA [37]. Similarly, a very high rate of mutation rate has been detected in the mtDNA control region of butterflyfishes [56], which are the sister group to angelfishes [25,57]. Therefore, the high genetic diversity observed in all three angelfishes in this study is probably influenced (at least partly) by the fast mutation rate of the mtDNA control region in this taxonomic group.

In this study we used mtDNA to examine genetic diversity. A more detailed assessment of genetic diversity would require sequencing regions of the nuclear genome and developing microsatellites to estimate genotypic diversity from multiple independent loci. However, mtDNA diversity provides a reasonable proxy for nuclear diversity in marine fishes [58] and therefore we expect that the angelfishes in this study do not have low nuclear DNA diversity. Research on other coral reef fishes has used mtDNA control region and nuclear DNA and both markers produced comparable results: endemics have higher genetic diversity than widespread congeners [50]. The hypotheses of this study

did not test if endemics and isolated populations of angelfishes had high genetic diversity, but rather tested if they had low genetic diversity (as per expectations based on terrestrial patterns). Despite the limitations of this study, we are confident that these hypotheses can be rejected (that is, endemics and isolated populations of angelfishes do not have low genetic diversity). Even though we did not sample central populations of the widespread species, it is evident just from sampling the isolated populations that isolated populations do not have low genetic diversity. Both nucleotide and haplotype diversity in the isolated populations are among the highest seen for reef fishes and approach the maximum possible.

5. Conclusions

This study found that geographically remote populations of endemic and widespread angelfishes had high genetic diversity and thus did not conform to terrestrial patterns of low genetic diversity. Endemic terrestrial species usually have low genetic diversity because they often have low abundance [1,9]. However, endemic reef fishes frequently have high abundance (reviewed in [41]) and therefore they may not have low genetic diversity. In the terrestrial environment, isolated populations of widespread species usually have low genetic diversity because they have small populations and low gene flow. However, the dispersive larval phase of reef fishes increases the potential for genetic exchange over large areas and this gene flow may be sufficient to maintain high genetic diversity in isolated populations [21–23]. Further research on the genetic diversity of endemic marine species and populations at remote locations is required to determine if there are differences in geographic patterns of genetic diversity between marine and terrestrial species. Determining patterns of genetic diversity is important because if geographically remote populations of endemic and widespread marine species have high genetic diversity than this should increase their evolutionary potential to adapt to escalating impacts [17,18].

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