Fisheries Research 206 (2018) 287–291

Contents lists available at ScienceDirect

Fisheries Research

journal homepage: www.elsevier.com/locate/fishres

Short communication

Species delineation and hybrid identification using diagnostic nuclear markers for *Plectropomus leopardus* and *Plectropomus maculatus*

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ARTICLE INFO

Handled by J. Viñas

Keywords:
Coral trout
Hybridization
Diagnostic markers
Molecular identification

ABSTRACT

Diagnostic molecular markers are an essential tool in the study of species' ecology and evolution, particularly in closely related and sympatric species. Furthermore, the increasing awareness of wild-hybrids has led to a renewed interest in rapid diagnostic assays. Here, we test the ability of two mitochondrial (Cytb and COI) and two nuclear markers (ETS2 and TMO-4c4) to confidently discriminate purebred *P. leopardus* and *P. maculatus* and their first-generation hybrids. A sample of 48 purebred individuals and 91 interspecific hybrids were used in this study and their delineation confirmed using a set of microsatellite markers. Our results indicate mitochondrial markers could not distinguish even between species but both nuclear markers confidently identified species and first-generation hybrids. However, later-generation hybrids could not always be confidently identified due to ongoing introgression between species. Our findings provide a robust tool to distinguish purebred individuals and interspecific hybrids in a pair of species with an unexpectedly high incidence of hybridization. The quick species discrimination abilities provided by these diagnostic markers are important for stock assessment and recruitment studies of these important fishery species.

1. Introduction

Molecular markers are widely used in phylogenetic studies to reveal the history of species' evolution and are increasingly used in ecological or management frameworks for species identification. Coral trout (*Plectropomus* spp., family Serranidae) are commercially important and widely distributed coral reef fishes, and two species commonly occur on inshore and midshelf reefs of the Great Barrier Reef. The bar cheek coral trout, *P. maculatus* (Bloch, 1790), are generally more frequent on inner-shelf reefs, while the common coral trout, *P. leopardus* (Lacepède, 1802), are more abundant on mid- and outer-shelf reefs of the Great Barrier Reef (GBR), suggesting a difference in habitat preferences (Heemstra et al., 1993; Mapstone et al., 1998; Russ et al., 2008). The two species share many morphological and life history characteristics (Ferreira and Russ, 1994, 1992), and adult individuals are most easily identified by their distinctive spot patterns (Fig. 1). Newly settled juveniles of these two species however, can be very difficult to distinguish in the field. Where the two species co-occur in the GBR, they are also known to produce high proportions of viable hybrids (Harrison et al., 2017).

In the marine environment, fish hybridization is not a rare phenomenon, at least 111 natural fish hybrids have been reported, involving more than 173 species (Montanari et al., 2016). Hybridization may thus represent an important evolutionary mechanism in the marine environment, although the evolutionary significance of the process is somewhat debated (Marie et al., 2007; McMillan et al., 1999; Yaakub et al., 2007, 2006). Naturally occurring putative hybrids between *P. maculatus* and *P. leopardus* have been detected from field collections from reefs near Palm and Whitsunday Islands and the Capricorn Bunker Reefs in the GBR (Harrison et al., 2017; van Herwerden et al., 2006, 2002). Newly recruited juveniles of the two species are difficult to distinguish morphologically and adult hybrids may show some intermediate coloration and patterns such as half-elongated spots on the opercula, which may be subtle and are easily overlooked.

Previous molecular work on the genus has had mixed results. van Herwerden et al. (2002) sequenced individuals from four *Plectropomus* species but found that mitochondrial fragments (using primers L16007 and H00651 for the hypervariable region I of the control region) were not mutually exclusive and could not distinguish *P. leopardus* from *P. maculatus*. However, according to their study, the nuclear oncogene intron (ETS2) sequences provided some preliminary support to distinguish *P. leopardus*, *P. maculatus*, and interspecific hybrids. However,

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https://doi.org/10.1016/j.fishres.2018.05.022
Received 18 January 2018; Received in revised form 7 May 2018; Accepted 25 May 2018
Available online 01 June 2018
0165-7836/

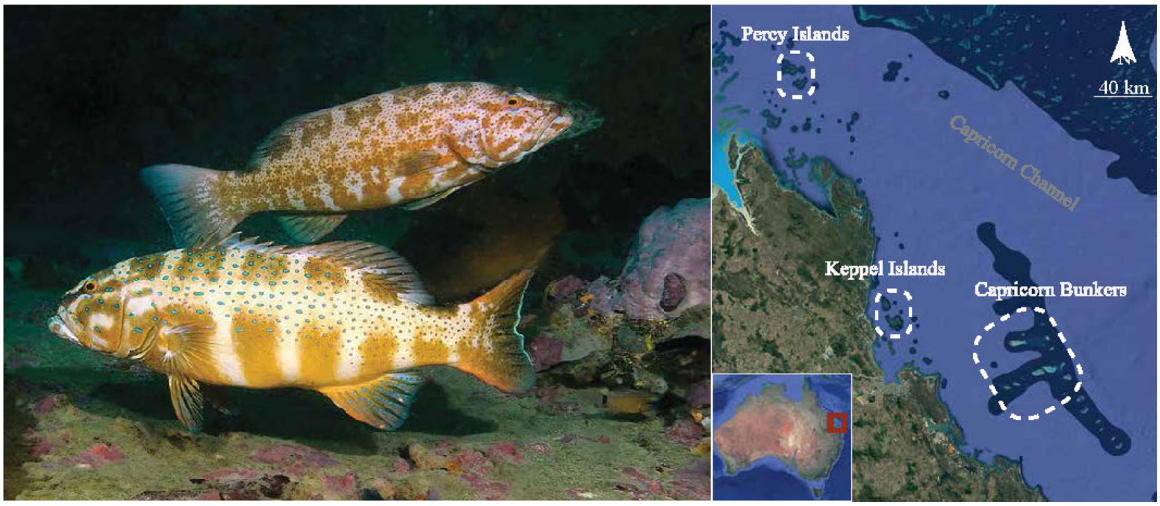

Fig. 1. *Plectropomus leopardus* (background) and *P. maculatus* (foreground) are easily distinguished by their characteristic blue spot patterns, which only become apparent after 2–3 months post-settlement. (Photo credit: Phil Woodhead). They were collected from reefs in the Percy Islands, Keppel Islands, and Capricorn Bunkers. The Capricorn Channel separates these inshore and mid-shelf reefs from the outer-shelf of the Great Barrier Reef. Sampling locations were indicated by dash line boxes. The sampling area is indicated by the red box in the inset. Map images from Google Earth. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

after analyzing additional individuals of *P. leopardus* and *P. maculatus* from the same area, the ETS2 region subsequently failed to delineate the two species (van Herwerden et al., 2006). Instead, a single nuclear microsatellite (locus 7–90 T) appears to have the ability to discriminate the two *Plectropomus* species, but putative hybrid samples were not tested using this marker (van Herwerden et al., 2006). Still, these markers have been a popular choice for phylogenetic studies for these species (e.g., Ding et al., 2006). Most recently, Ma et al. (2016) confirmed the very close phylogenetic relationship of *P. leopardus* and *P. maculatus* using the ETS2 marker. Subsequently, Harrison et al. (2017) used a panel of 25 microsatellite markers to show that *P. leopardus* and *P. maculatus* hybrids were common in wild populations on the GBR and that contemporary introgression had occurred.

A single diagnostic marker for *Plectropomus* spp. is important to extend further studies of interspecific hybrids, but also for stock assessment and recruitment studies of these important fishery species. The validation of a simple method to detect and confirm hybrid cases is the first step towards a better general understanding of the processes that underlie hybridization and would enable future studies exploring the associated evolutionary mechanisms involved. Furthermore, identification of juveniles may provide further insights into recruitment patterns and habitat association of newly recruiting individuals. Given the close relationship of *P. leopardus* and *P. maculatus*, the ability to quickly discriminate the two species using a single marker would also have many benefits.

The specific aim of this study was to identify diagnostic markers capable of distinguishing *P. leopardus*, *P. maculatus*, and their putative hybrids. Ideally, the diagnostic markers should be invariable within a species and have a fixed difference between species. In a previous study, 91 interspecific hybrids were identified from a collection of 2991 *P. leopardus* and *P. maculatus* sampled from reefs in the southern GBR (Fig. 1) (Harrison et al., 2017). These samples were identified using 25 microsatellite markers (Harrison et al., 2014) and were classified as purebred genotypes, first-generation hybrids (N = 20), or second-generation hybrids (N = 74). This previous work provides an ideal sample set containing individuals of known lineage (i.e., purebred of either species or a hybrid of the two) for further genetic investigations. For the present study, two mitochondrial and two nuclear markers were tested to identify potential diagnostic sites in a sample of 48 *P. leopardus*, 48 *P. maculatus* and 91 confirmed hybrids.

2. Material and methods

2.1. Sample collection

Samples were collected from the Keppel Islands (23° 10′ S, 150° 57′ E), the Percy Islands (21° 42′ S, 150° 18′ E), and the Capricorn Bunker reefs (23° 25′ S, 151° 46′ E) in the southern section of the GBR, Australia (Fig. 1). The Keppel and Percy Island groups are chains of high continental islands surrounded by fringing coral reefs, while the Capricorn-Bunker group is comprised of emergent platform reefs located on the outer margin of the continental shelf. Tissue samples for *Plectropomus* spp. were a subset of those used in Harrison et al. (2017). In brief, these samples were collected from the three previously mentioned locations. All samples were collected between August 2010 and August 2013 under Marine Parks Permit No. G11/3351, Queensland General Fisheries Permit No. 148534, and James Cook University Animal Ethics Permit A1625.

2.2. DNA analysis

The analyses presented in this study focused on 91 confirmed hybrids and 48 samples of each of the purebred parent species from Harrison et al. (2017). Ideally, diagnostic markers have no variations within a species, have fixed difference between parent species (i.e., they are mutually exclusive of each other), and have the potential to show hybrids as heterozygous (as an exception to the otherwise mutually exclusive nature of the marker). To investigate potential diagnostic markers, nuclear intron TMO-4c4 and ETS2 fragments were amplified by primers TMO-4C4F/TMO-4C4R and ETS2F/ETS2R (Lyons et al., 1997). To investigate maternal contributions to the hybrids, mitochondrial gene Cytb and COI fragments were amplified by primers Cytb9/Cytb7 (Song et al., 1998) and FishF2/FishR2 (Ward et al., 2005). It is important to note that many other potential diagnostic markers were assessed; including RAG1, RAG2, S7, and BMP4, none of which were suitable for diagnostic purposes (see Appendix 1 in the Supplementary material).

The QIAGEN Multiplex PCR Kit (Qiagen, Hilden, Germany) was used for the polymerized chain reaction. PCR cycling parameters were as follows: initial 95 °C denaturation for 15 min., followed by 35 cycles of 94 °C for 45 s., annealing for 60 s. (TMO-4C4F/TMO-4C4R: 60 °C; ETS2F/ ETS2R: 62 °C; Cytb9/Cytb7: 50 °C; FishF2/FishR2: 50 °C), and