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Multi-gene barcoding to discriminate sibling species within a morphologically difficult fish genus (*Sillago*)

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ABSTRACT

Fisheries species that cannot be reliably identified based on unique morphological features pose a challenge for research, monitoring and management. DNA barcoding, which refers to a standardized procedure for genetic species identification based on the mitochondrial cytochrome oxidase 1 (CO1) gene, is an important tool to confront this problem. Here, we present a multi-gene barcoding approach to discriminate two fisheries species flagged as sibling species within the morphologically difficult fish genus *Sillago*: *S analis* and *S. ciliata*. CO1 revealed low interspecific variation that was insufficient for genetic distance-based species identification. Despite this, one diagnostic site did permit genetic character-based identification. However, four cases of mismatch among morphological identifications (mIDs) and CO1 barcodes required analysing additional genes and loci. A spot test approach was used to screen five other genes in the mitochondrial genome (mtDNA) and four genes in the nuclear genome (nuDNA). The nuDNA recombination activating gene 2 (RAG2) revealed most diagnostic sites, correctly clustering mIDs of all individuals using both genetic distance- and character-based analysis. Three of four cases of mID/CO1 mismatches were found to be simultaneous CO1/RAG2 (mtDNA/nuDNA) mismatches indicative of past hybridization. The fourth mID/CO1 mismatch was found to represent an initial morphological misidentification. Five spot-tested genes, containing either fully or partially diagnostic sites (mtDNA: NADH dehydrogenase 2 [ND2], ATP synthase [ATPase] and cytochrome b; nuDNA: histone protein 3a and ring finger protein 213), confirmed these findings. Based on our preliminary data, we recommend (1) that *S. analis* and *S. ciliata* retain their taxonomic status as sibling species with full corresponding recognition as separate management and conservation units, and (2) precautionary management of *S. ciliata* fisheries until further research into hybridization is carried out. The multi-gene spot test approach and efficient markers (RAG2 in combination with ND2, ATPase or CO1) may help identifying other problematic fish species.

1. Introduction

Most animal species have been classified and can be identified based on unique morphological features. If morphological species identification is not possible, or if it is restricted to certain life history stages, monitoring, scientific study and harvest management may be complicated, perhaps even unfeasible. Hebert et al. (2003b) proposed a standardized protocol to tackle such problems based on a global catalogue of animal DNA covering approximately 650 base pairs (bp) of the mitochondrial cytochrome oxidase subunit 1 (CO1) gene. This was the beginning of the so-called DNA barcoding campaign, which has since been further developed (Frezal and Leblois, 2008; Hebert et al., 2003a, 2004a,b; Ratnasingham and Hebert, 2007; Savolainen et al., 2005; Ward et al., 2009).

Considering the wide spectrum of differentiation among recognized species, DNA barcoding has been criticized for its simplicity. Criticism has focused on the use of a single and mitochondrial gene sequence, distance-based methods to assess sequence divergence, and the use of sequence divergence thresholds for species delimitation (DeSalle et al., 2005; Rubinoff, 2006; Rubinoff et al., 2006). However, controversies largely centre on the utility of DNA barcoding for species discovery, while its efficiency for identifying recognized species is widely acknowledged (DeSalle, 2006). Initial results of the Fish Barcode of Life campaign (FISH-BOL,

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www.fishbol.org), for example, indicated that CO1 barcodes separate about 98% of marine fish throughout their life history (Ward et al., 2009). This estimate appears to hold true for those fish species that have so far been barcoded, currently about 25% of all known species, facilitating science, monitoring and management (Becker et al., 2011; Ward, 2012). However, CO1 barcodes have been reported to give inadequate or misleading results when complex (e.g. recently radiated, closely related, or hybridizing species) or understudied taxa are involved (Conflitti et al., 2012; Kaila and Stahls, 2006; Meyer and Paulay, 2005; Morgan et al., 2012; Ward, 2009).

In this study, we use DNA barcoding to discriminate amongst two members of the family of whiting (Sillaginidae), which comprises 31 bottom dwelling species of teleost fish. Most sillaginids are important for fisheries and/or aquaculture in many countries of the Indo-Pacific region, but remain poorly researched (McKay, 1992). The most diverse genus, *Sillago*, is characterized by considerable interspecific similarity, which has caused much confusion with species recognition within this group (McKay, 1992). For some *Sillago*, such as the sibling species *S. analis* (golden-lined whiting) and *S. ciliata* (sand whiting), this situation complicates biological studies and fisheries monitoring that must rely on robust species identification throughout life history stages (Krück et al., 2009; Weng, 1983). Here, we report the results of research on these two sibling species aimed at (i) investigating their taxonomic status based on gene sequence data and (ii) establishing genetic protocols for robust species identification.

2. Materials and methods

2.1. Fish collections

Fish were collected in September 2009 from two different sampling areas chosen to represent species-specific habitat types (Fig. 1). Hays Inlet (HI) within northern Moreton Bay, Queensland, Australia is a mangrove-fringed estuary with muddy sediments considered a habitat typical for *Sillago analis*. Point Lookout (PL) on North Stradbroke Island, where all remaining fish for this study were caught, is an ocean beach habitat considered typical for *S. ciliata*. All fish at HI were caught using rod and line. All fish from PL were caught by commercial fishers. Live-caught fish were killed using an overdose of clove oil. Tissue samples were taken from the caudal fins of fish and preserved in 100% ethanol for DNA extraction. All fish were preserved at −20 °C. Animal treatment in this study was carried out in accordance with all legal and welfare matters pertaining to the use of animals for research and teaching within the scope of the Animal Care and Protection Act Qld (2002) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition 2004). Study protocols were approved by the Animal Ethics Committee of the University of Queensland (permit number: CMS/816/08).

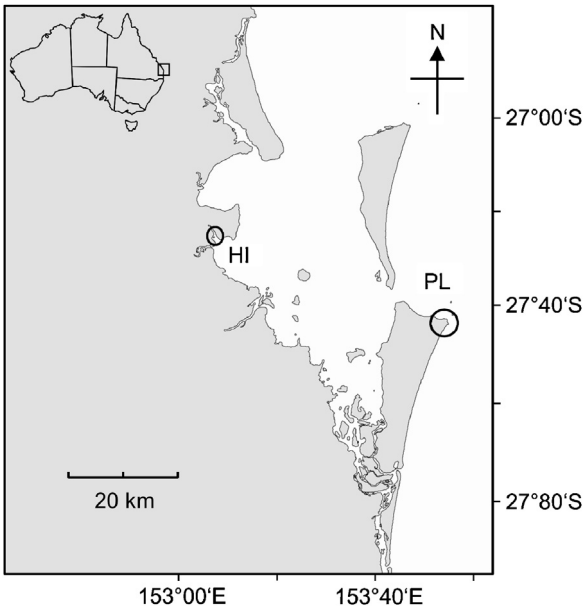

Fig. 1. Map of Moreton Bay in Queensland, Australia showing sampling locations at Hays Inlet (HI) and Point Lookout (PL). HI is a muddy estuarine habitat considered typical for *Sillago analis*, while PL is a sandy ocean beach habitat considered typical for *S. ciliata*.

2.2. Morphological analysis

Initial identification of the two study species was based on visual examinations. Species-specific features in external morphology given by McKay (1992) were used. A primary feature (distinct numbers of lateral line scales [LLS, 54–61 in *S. analis* and 60–69 in *S. ciliata*]) and a secondary feature (a dusky or blue-black blotch at the base of the pectoral fin in *S. ciliata*, which is absent or very faint in *S. analis*) were used in combination. No other characteristics in external morphology were found to reliably help discriminate between the two species. In common with the findings of McKay (1985, 1992), for example, the range and distribution of all fin ray counts (dorsal, anal and pectoral fins) between *S. ciliata* and *S. analis* were found to be almost identical. Vertebral counts and morphometrics of a large number of specimens of the two species were presented by McKay (1985), but these were not stated to have value in distinguishing them. McKay (1985) also indicated some variation in the range of several proportional measurements (e.g. snout length, eye diameter and interorbital width in head length) between the two species. However, when specimens of similar standard lengths were compared in this study the results were so strongly overlapping that these measurements were considered of negligible diagnostic value. All morphological examinations were carried out under a stereo microscope at Queensland Museum.

2.3. Genetic analysis

DNeasy blood and tissue kits were used to extract DNA from fin tissue by following the manufacturer's instructions (Qiagen). Ten gene regions in both the mitochondrial genome (mtDNA) and nuclear genome (nuDNA) were targeted for DNA barcoding. MtDNA gene regions included CO1, the control region (CR), cytochrome b (Cytb), ATP synthase (ATPase), and NADH dehydrogenase 2 (ND2) and 4 (ND4). NuDNA gene regions included recombination activating genes 1 (RAG1) and 2 (RAG2), histone protein 3a (H3a), and ring finger protein 213 (RNF213). The choice of these markers was based on a combination of previous experience with the genetic identification of inshore fisheries species in Queensland, the availability of universal primers, and prior success of PCR amplifications in other *Sillago* species.

Primers Fish-F1, Fish-F2, Fish-R1 and Fish-R2 (primarily the combination Fish-F1 and Fish-R2) were used to amplify a 655 base pair (bp) fragment of mtDNA from the 5′ region of the CO1 gene (Ward et al., 2005). A 810 bp region of nuDNA RAG2 was recovered using the primer pair RAG2-f1 and RAG2-r3 (Westneat and Alfaro, 2005). Both CO1 and RAG2 sequences were analysed using all DNA samples. The following gene fragments were analysed for a subset of at least eight DNA samples. A 278 bp fragment of mtDNA from the Cytb region was amplified using primer CB2H-15175 (Martin and Palumbi, 1993) and a new light strand (L) primer for *Sillago* (CBL-Sillago: 5′-TTCTGGGGCTATGCTTGG-3′). A 900 bp fragment of mtDNA in the ATPase gene region was recovered using primers