

MOLECULAR DIAGNOSTICS AND DNA TAXONOMY

Multiplex PCR and RFLP approaches for identification of rabbitfish (*Siganus*) species using mitochondrial gene regions

R. G. RAVAGO-GOTANCO, M. T. MANGLICMOT and M. J. R. PANTE

Marine Science Institute, College of Science, University of the Philippines, Diliman, Quezon City 1101, Philippines

Abstract

Molecular assays are described for the identification of six rabbitfish (*Siganus*) species. A multiplex PCR assay using primers targeting the mitochondrial cytochrome b region simultaneously identifies four species: *Siganus canaliculatus*, *S. fuscescens*, *S. javus*, and *S. spinus*. Subsequent RFLP assays of multiplex amplicons differentiate between *S. virgatus* and *S. corallinus* based on diagnostic fragments from the mitochondrial cytochrome oxidase I region. Assays were validated with known specimens demonstrating accuracy of the molecular identification. Applied to morphologically indistinguishable early developmental stages, these assays can facilitate studies on species-specific spatio-temporal patterns of larval dispersal and population connectivity to aid fishery management.

Keywords: multiplex PCR, rabbitfish, RFLP, *Siganus*, species identification

Received 13 November 2009; revision accepted 30 November 2009

Rabbitfishes (Siganidae) are widely distributed in shallow coastal habitats throughout the Indo-Pacific, representing important herbivores and commercially valuable fishery, aquaculture and ornamental species (Woodland 1990). In the Philippines, rabbitfish species inhabiting reef flats and seagrass meadows are particularly exploited. Harvesting begins at the presettlement stage as fish arrive in dense schools to settle in seagrass meadows, while juveniles and adults are fished year-round (Campos *et al.* 1994; Soliman *et al.* 2009).

In patchily distributed reef habitats, the extent of dispersal between populations (connectivity) has important implications to the dynamics of populations, their resilience to disturbance, and how they can be managed and protected (Jones *et al.* 2009). For rabbitfishes, the planktonic larval phase represents potential for extensive dispersal. Species identification of early developmental stages is essential towards inferring species-specific larval dispersal pathways and demographic connectivity. Although late-stage siganid larvae can be distinguished from other families using morphological characters (Leis & Carson-Ewart 2000), species identification is difficult as

the fish have not yet developed distinct colour patterns used for taxonomic identification (Woodland 1990). Presettlement siganids on seagrass meadows have been reported to consist of three species: *Siganus fuscescens*, *S. spinus*, and *S. virgatus* based on nucleotide sequence analysis of the mitochondrial cytochrome b (cytb) region (Ochavillo 2002). We have developed a multiplex PCR and RFLP assay for rapid identification of these three target species as well as three additional species: *S. canaliculatus*, *S. corallinus* and *S. javus*.

Reference specimens for optimization and testing of assays consisted of adult samples of 14 *Siganus* species known to occur throughout the Philippine archipelago. Specimens were morphologically identified based on colour pattern and body shape, with the exception of *S. canaliculatus* which were identified based on mitochondrial DNA control region and cytb sequences (R. Ravago-Gotanco, unpublished). DNA was extracted from ethanol-preserved muscle tissue using a Chelex-proteinase K method (Yue & Orban 2005).

The mitochondrial DNA (mtDNA) cytb region was used as a marker for species identification (Lemer *et al.* 2007). Sequence data for 22 *Siganus* species (GenBank Accession Nos. AB276792–AB276961, EF210174–EF210192, DQ898028–DQ898094) were aligned using Geneious software (Drummond *et al.* 2007). Primer 3

Correspondence: R. G. Ravago-Gotanco, Fax: +63-2-924-7678; E-mail: rachelr@upmsi.ph

(Rozen & Skaletsky 2000) was used to design species-specific forward primers for *S. canaliculatus*, *S. fuscescens*, and *S. spinus* to amplify unique-sized fragments in combination with a universal reverse primer (Lemer *et al.* 2007; Table 1). For *S. virgatus*, species-specific forward and reverse primers were designed. As a positive control of the reaction, primers were designed for the mtDNA cytochrome oxidase subunit I (CoI) gene from *Siganus* sequences (GenBank Accession Nos. EU620476 to EU620494).

The cytb primer pairs were evaluated in separate PCR amplifications against 14 *Siganus* species to test for selective amplification of fragments of the expected size. Primers were species-specific for *S. canaliculatus* and *S. spinus*. The *S. fuscescens* forward and universal reverse primer pair cross-amplified *S. javus*. Addition of an *S. fuscescens*-specific reverse primer to the reaction selectively amplified an additional fragment diagnostic for *S. fuscescens* whereas *S. javus* had a single amplicon. Primer pairs designed for *S. virgatus* also cross-amplified *S. corallinus*. The low sequence divergence ($d = 0.018$ net uncorrected p-distance) and the small number of positions with fixed nucleotide differences between *S. virgatus* and *S. corallinus* (8 sites out of 1141 bases) limited the design of *S. virgatus*-specific primers. No cross-amplification was observed for eight species: *S. argenteus*, *S. guttatus*, *S. puellus*, *S. punctatissimus*, *S. punctatus*, *S. unimaculatus*, *S. vermiculatus*, and *S. vulpinus*. CoI primers amplified an ~780 bp fragment for all 14 species.

Nine primers were subsequently combined in a multiplex PCR assay: four cytb species-specific forward oligos, two cytb species-specific reverse oligos, one cytb universal reverse oligo, and forward and reverse oligos for CoI (Table 1). The multiplex PCR assay was evaluated for efficiency in distinguishing between *S. canaliculatus*, *S. fuscescens*, *S. javus*, *S. spinus* and *S. virgatus/S. corallinus* in a single reaction. The optimized assay consists of 10 μ L reactions containing 1 \times PCR Buffer, 2 mM MgCl₂, 200 μ M of each dNTP, 0.25 units of Taq DNA polymerase

(Invitrogen), 0.3 μ M each primer, and 1 μ L of DNA template. Cycling conditions were 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 2 min, with a final extension at 72 °C for 5 min. Fragments were resolved on 2% TBE-buffered agarose gels, and detected following ethidium bromide staining. Multiplex PCR yielded multiple amplicons identifying target species: a ~780-bp positive control CoI fragment and smaller fragments diagnostic for *S. spinus* (150 bp), *S. canaliculatus* (200 bp), *S. virgatus/S. corallinus* (230 bp), *S. fuscescens* (340 and 430 bp), or *S. javus* (430 bp) (Fig. 1A). Molecular identification was consistent with morphological identifi-

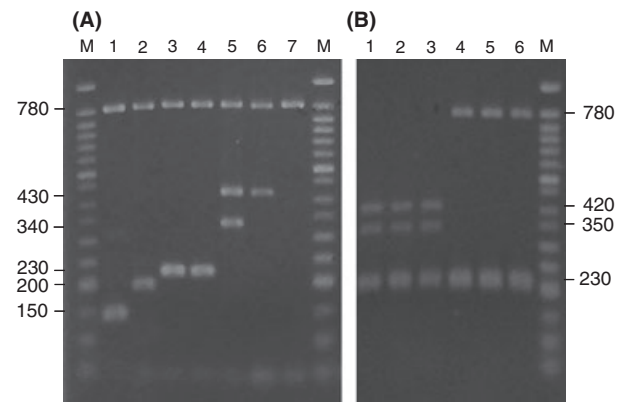


Fig. 1 Multiplex PCR fragment profiles for *Siganus* species consisting of species-specific cytochrome b (cytb) amplicons and a positive control cytochrome oxidase I (CoI) amplicon (A). The CoI amplicon (~780 bp) and cytb species-diagnostic amplicons from *S. spinus* (lane 1, ~150 bp), *S. canaliculatus* (lane 2, ~200 bp), *S. virgatus* and *S. corallinus* (lanes 3 and 4, ~230 bp), *S. fuscescens* (lane 5, ~340 bp and 430 bp) and *S. javus* (lane 6, ~430 bp) are shown. Only the positive control amplicon is observed for other *Siganus* species (lane 7). Species-diagnostic multiplex PCR-RFLP profile of *S. virgatus* and *S. corallinus* following digestion with *HhaI* is shown in (B). Lanes 1 - 3, *S. corallinus*; lanes 4 - 6, *S. virgatus*. Diagnostic fragments were generated from the CoI amplicon while the 230-bp cytb fragment was undigested. M, 50 bp DNA ladder. Fragment sizes are indicated in basepairs (bp).

Gene region and target species	Primer name	Sequence (5' to 3')
Cytochrome b		
<i>Siganus canaliculatus</i>	Scan-350F	TCGTTCTCCTTCTCCTGGTT
<i>Siganus fuscescens</i>	Sfus-122F	TGATCATCCAAATCCTCACG
	Sfus-465R	GTAGGGGACGGCAGAGAGT
<i>Siganus spinus</i>	Sspi-225F	TGGCTGACTAATTCGGAACC
<i>Siganus virgatus/</i>	Svir-663F	CCCATACTTTTCATACAAAGACCTGT
<i>Siganus corallinus</i>	Svir-892R	TTGATGCCAGAAGTGCTAGG
Siganidae, universal reverse (Lemer <i>et al.</i> 2007)		AGGAAGTGGAAKGC GAAGAA
Cytochrome oxidase I		
	CoI-510F	CATGAAACCTCCYGTATYTCC
	CoI-1286R	TGTTGTGGGAAGAAAGTYAGRIT

Table 1 Oligonucleotide primers used in multiplex PCR to identify *Siganus* species

cation for a total of 125 specimens assayed (40 individuals each for *S. fuscescens*, *S. javus* and *S. spinus*; five individuals of *S. canaliculatus*). Only the positive control amplicon was observed for eight non-target *Siganus* species (20 individuals per species).

To distinguish between *S. virgatus* and *S. corallinus*, we utilized nucleotide sequence variability of the Col amplicon in a restriction fragment length polymorphism (RFLP) assay. Restriction site analysis of Col sequences (GenBank Accession Nos. EU620482–620483, EU620486–620487) using Geneious software identified two putatively diagnostic enzymes, *Hha*I and *Rsa*I. Separate digestions were performed in 10 µL reactions containing one unit of enzyme, buffer, water, and 3 µL multiplex PCR product, incubated for 3 h at 37 °C. Each enzyme yielded species-diagnostic RFLPs from the Col amplicon to differentiate *S. virgatus* and *S. corallinus*. The 230-bp *cytb* amplicon was uncut (Fig. 1B). The RFLP assay correctly identified 80 reference specimens ($N = 40$ individuals each species), with no observed intraspecific variability in RFLP profiles.

These protocols provide a rapid method for identification of six *Siganus* species. Multiplex PCR enables a single-step identification of four species, *S. canaliculatus*, *S. fuscescens*, *S. javus* and *S. spinus*. Although an additional RFLP assay is needed to diagnose *S. virgatus* and *S. corallinus*, the combined methods are a low-cost alternative to DNA sequencing-based methods, unambiguously identifying 205 reference specimens. Applied to early developmental stages, the method is valuable for studies on the early life history of *Siganus* species inhabiting reef flats, particularly for species-specific spatio-temporal patterns of larval dispersal, settlement, and recruitment. The assays are expected to facilitate genetic and demographic connectivity studies for management of these highly exploited species. The method is also applicable for differentiating between morphologically similar *S. fuscescens* and *S. canaliculatus*, to facilitate studies on the geographical distribution and ecology of these sibling species.

Acknowledgements

This article results from research funded by the Center of Excellence for Southeast Asia of the Coral Reef Targeted Research (CRTR) Program, a GEF-World Bank-University of Queensland international program. This is contribution number 384 from the University of the Philippines Marine Science Institute (UP-MSI).

References

- Campos WL, Del Norte-Campos AGC, McManus JW (1994) Yield estimates, catch, effort and fishery potential of the reef flat in Cape Bolinao, Philippines. *Journal of Applied Ichthyology*, **10**, 82–95.
- Drummond AJ, Ashton B, Cheung M *et al.* (2007) Geneious v4.7. Available from <http://www.geneious.com> (Last accessed 15 November 2009).
- Jones GP, Almany GR, Russ GR *et al.* (2009) Larval retention and connectivity among populations of corals and reef fishes: history, advances and challenges. *Coral Reefs*, **28**, 307–325.
- Leis JM, Carson-Ewart BM (2000) *The Larvae of Indo-Pacific Coastal Fishes: An Identification Guide to Marine Fish Larvae*. Brill, Leiden, The Netherlands. 850 pp.
- Lemer S, Aurelle D, Vigliola L, Durand J, Borsa P (2007) Cytochrome b barcoding, molecular systematics and geographic differentiation in rabbitfishes (Siganidae). *Comptes Rendus Biologies*, **330**, 86–94.
- Ochavillo DG (2002) *The dispersal of coral reef fish larvae: Genetic markers, planktonic duration and behavior*. PhD Dissertation, University of Southern California, Los Angeles, 231 pp.
- Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, NJ. Source code available at <http://fokker.wi.mit.edu/primer3> (Last accessed 15 October 2009).
- Soliman V, Bobiles RU, Yamaoka K (2009) Overfishing in three siganid species (Family: Siganidae) in Lagonoy Gulf, Philippines. *Kuroshio Science*, **2**, 145–150.
- Woodland DJ (1990) Revision of the fish family Siganidae with descriptions of two new species and comments on distribution and biology. *Indo-Pacific Fishes*, **19**, 136
- Yue GH, Orban L (2005) A simple and affordable method for high-throughput DNA extraction from animal tissues for polymerase chain reaction. *Electrophoresis*, **26**, 3081–3083.