Novel hybrid primers for sequencing of the mitochondrial cytochrome *b* gene in the rabbitfishes (Teleostei: Siganidae)

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Abstract   We describe novel hybrid primers for the successful amplification and sequencing of the mitochondrial cytochrome *b* (cyt*b*) gene in the family Siganidae. We aligned 403 cyt*b* sequences from 29 species and found multiple potential conserved hybrid primers (<2-fold degeneracy). The best set was selected, and successfully amplified and sequenced ~1100 bp of cyt*b* in the Siganidae.

Keywords   Acanthuridae · Cytochrome b · Hybrid primers · mtDNA · Siganidae · Zanclidae

The COI gene have been largely used for DNA barcoding of fish (Ivanova et al. 2007; Ward et al. 2009), but the cyt*b* gene is still preferred in some families like the rabbitfishes that represent an important artisanal fishery in the Indo-West Pacific region (Siganidae; Kuriwa et al. 2007; Lemer et al. 2007). Here, we designed versatile hybrid primers for cyt*b* in the family Siganidae. We used a design strategy based on a highly conserved 3' region (9–12 bp) and a 5' non-degenerate consensus region (Hoareau and Boissin 2010) to create versatile cyt*b* primers for the 30 species of Siganidae. The method helps detecting conserved genomic regions that minimize primers' degeneracy and optimize amplicons length (>500 bp).

We used a total of 403 sequences of cyt*b* assembled from Genbank and belonging to 24 species of Siganidae, four species of Acanthuridae and the Zanclidae *Zanclus cornutus* (Table S1). The sequences were aligned using the program MAFFT v7 online (https://mafft.cbrc.jp) applying the standard settings. The degenerate and non-degenerate consensus sequences (DCS and NCS, respectively) were obtained using the program BIOEDIT (http://www.mbio.ncsu.edu/bioedit/bioedit.html). We calculated a similarity index for all possible 11bp and 20bp regions along the alignment using the DCS (Hoareau and Boissin 2010) and we computed the proportion of sequences for each nucleotide site.

We tested the PCR amplification of the selected primers on 18 siganid species (Table S2). The population polymorphism was assessed by sequencing cyt*b* for both *Siganus sutor* (Valencienne 1835) and *S. laqueus* (von Bonde 1934) using specimens collected in a previous study (Kiper et al. 2018; Table S2). We extracted total genomic DNA using the DNeasy Blood and Tissue extraction kit (Qiagen, Valencia CA, USA). PCR amplifications were carried on in an Eppendorf Mastercycler with a final volume of 10 µl. The PCR mixture contained 1.5 mM of $MgCl_2$, 1x PCR buffer, 0.2 mM of dNTPs (Promega), 0.1 pM of each primer (Whitehead Scientifics, Johannesburg, South Africa), 0.04 U of Supertherm *Taq* polymerase (Southern Cross Biotechnologies, Cape Town, South Africa) and 20–60 ng of DNA template. PCR cycles were initiated with a denaturation step at 94°C for 3'00'', followed by 35 cycles of denaturation at 94°C for 0'30'', annealing temperature at 55°C for 0'30'', elongation step at 72°C for 1'00'', followed by a final elongation step at 72°C for 7'00''. The quality of the PCR product was confirmed on a 2% agarose gel. The PCR products were precipitated following the protocol from Kiper et al. (2018). The cycle sequencing reaction included 1 µl ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit v3.1 (Applied Biosystems, Foster City, USA), 5x sequencing buffer, 3.2 pM of forward primer and ~30 ng purified DNA product. After a precipitating

step of the cycle sequencing products, the sequencing was done at the University of Pretoria sequencing facility on an ABI3500x sequencer (Applied Biosystems).

The sequences were scored using CLCBio[R] Workbench 4.1.1 (CLCBio A/S, Aarhus, Denmark) and were deposited in Genbank (Table S2). We aligned the sequences using MAFFT and verified the quality of the sequences by checking for potential frameshifts (insertions and deletions), amino acid variants and for the presence of stop codon using MEGA6 (Tamura et al. 2013). We used the new sequences to reconstruct a Neighbour joining tree using the Kimura-two-parameters model in MEGA6 and edited it in FigTree v1.3.1 (http://tree.bio.ed.ac.uk).

We could identify multiple conserved regions to design cyt*b* primers from the set of sequences. Three regions showed a similarity index of 1.0 (no degeneracy) and a good sequence coverage (>40%; Fig. 1) when considering the standard 20 bp primers. Nine conserved regions were found when considering the 11 bp primers. We considered regions with good coverage, minimal degeneracy and that maximize the amplicon length. Using these criteria, we selected a forward 20 bp primer [CytbSig-F: ATGGCAAGCCTACGCAAAACC] and a reverse 11 bp primer (2-fold degenerate, in bold) to which we included a 10 bp of NCS [CytbSig-R: CGCTGAGCTA**CTARTGCATAC**]. The position of these forward and reverse primers in the mitochondrial genome of *Siganus fuscescens* (NCBI accession: NC_009572.1) are 14383–14402 and 15519–15539, respectively.

As expected by the highly conserved primers, the PCRs successfully amplified a fragment of ~1100 bp in all species of *Siganus* tested (18 out of 30 valid species), without non-target amplification. The sequences of *S. laqueus* and *S. sutor* provided good quality scores, aligned easily and were free of insertions, deletions, stop codons or changes in amino acids. Using the BLAST algorithm, the nearest 100 matches for these sequences were all from the genus *Siganus*. The phylogenetic tree built using representatives of Siganidae, Acanthuridae and Zanclidae confirmed that the sequences match the phylogeny by clustering all specimens of *S. sutor* and *S. laqueus* within the genus *Siganus* (Fig. 2). Moreover, the polymorphism within species is relatively high as cryptic diversity is detected within *S. sutor*.

In conclusion, these new cyt*b* primers provide a good genetic resolution at both inter- and intraspecific levels in Siganidae, and should help develop routine biodiversity survey in this taxon that is economically important in the Indo-Pacific region.

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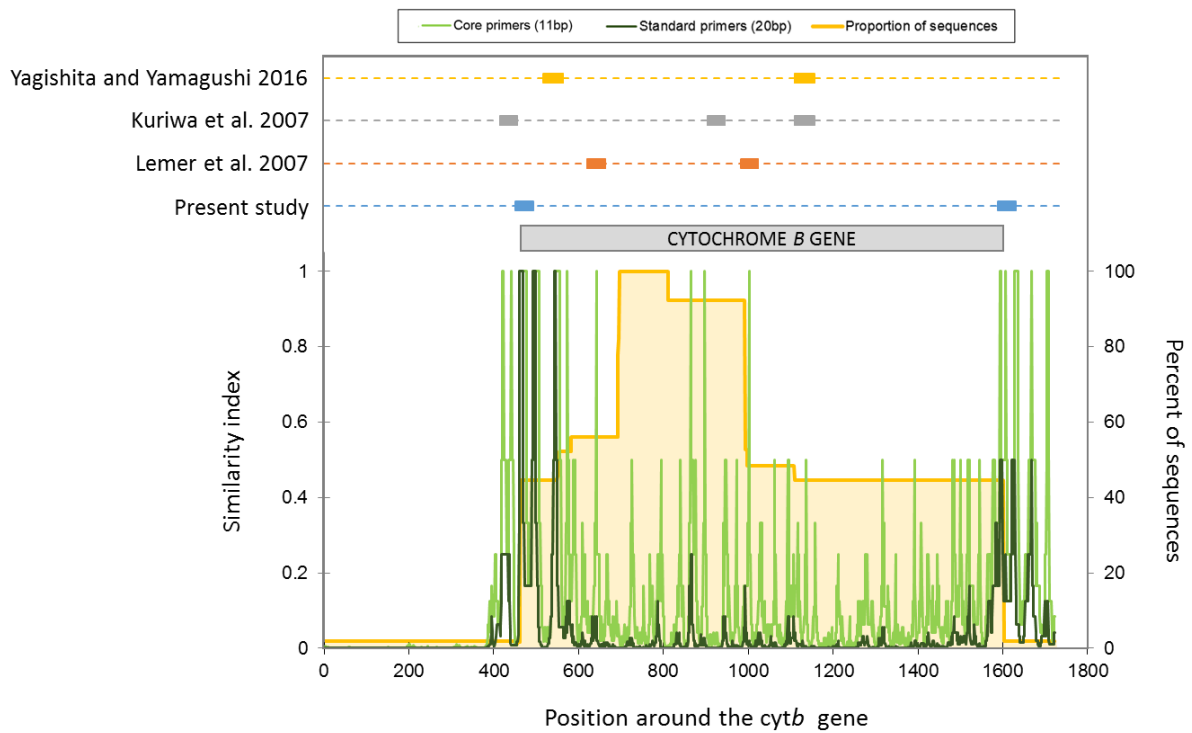
Fig. 1 Sequences similarity index, proportion of sequences (light yellow) and position of different primers from the present and previous studies along the cyt*b* gene obtained from the alignment of 403 sequences of Siganidae, Acanthuridae and Zanclidae. The 11bp core primers refer to the minimum size of the 3' part of the primer that is necessary for correct primer binding (Hoareau and Boissin 2010).

Fig. 2 Neighbour-joining tree based on the cyt*b* sequences of *Siganus sutor* and *S. laqueus* obtained using the new primers and additional sequences of Siganidae, Acanthuridae and Zanclidae. The bootstrap values >0.90 obtained after 1000 permutations are indicated for each nodes.

Supplementary Table 1. Genbank ID of the cytochrome *b* sequences of Acanthuridae, Siganidae and Zanclidae used to design the hybrid primers.

Family	Species	GenBank IDs
Acanthuridae	*Acanthurus lineatus*	EU273284.2;NC_010108.1
Acanthuridae	*Acanthurus leucosternon*	NC_009830.1
Acanthuridae	*Naso lopezi*	NC_009853.1
Acanthuridae	*Zebrasoma flavescens*	NC_009874.1
Siganidae	*Siganus woodlandi*	AB276792.1;AB276793.1;AB276794.1;AB276795.1;AB276796.1;AB276797.1;DQ898090.1;DQ898091.1
Siganidae	*Siganus argenteus*	AB276798.1;AB276799.1;AB276800.1;AB276801.1;AB276802.1;AB276803.1;AB276804.1;AB276805.1;AB276806.1;AB276807.1;AB276808.1;AB276809.1;AB276810.1;AB276811.1;AB276812.1;AB276813.1;AB276814.1;AY190542.1;DQ898028.1;DQ898029.1;DQ898030.1;DQ898031.1;DQ898032.1;DQ898033.1;DQ898034.1;DQ898035.1;DQ898036.1;EF210174.1
Siganidae	*Siganus spinus*	AB276815.1;AB276816.1;AB276817.1;AB276818.1;AB276819.1;AB276820.1;AB276821.1;AY190545.1;AY190554.1;DQ898079.1;DQ898080.1;DQ898081.1;DQ898082.1;EF210189.1;EF210190.1;EF210191.1;EF210192.1
Siganidae	*Siganus fuscescens*	AB276822.1;AB276823.1;AB276824.1;AB276825.1;AB276826.1;AB276827.1;AB276828.1;AB276829.1;AB276830.1;AB276831.1;AB276832.1;AB276833.1;AY190544.1;AY190551.1;AY190552.1;AY190553.1;DQ898040.1;DQ898041.1;DQ898042.1;DQ898043.1;DQ898044.1;DQ898093.1;DQ898094.1;EF210178.1;EF210179.1;EF210180.1;EF210181.1;EF210182.1;EF210183.1;EF210184.1;GU929680.1;GU929681.1;GU929682.1;GU929683.1;GU929684.1;GU929685.1;GU929686.1;GU929687.1;GU929688.1;GU929689.1;GU929690.1;GU929691.1;GU929692.1;GU929693.1;HQ843182.1;HQ843183.1;HQ843184.1;HQ843185.1;HQ843186.1;HQ843187.1;HQ843188.1;HQ843189.1;HQ843190.1;HQ843191.1;HQ843192.1;HQ843193.1;HQ843194.1;HQ843195.1;HQ843196.1;HQ843197.1;HQ843198.1;HQ843199.1;HQ843200.1;HQ843201.1;HQ843202.1;HQ843203.1;HQ843204.1;HQ843205.1;HQ843206.1;HQ843207.1;HQ843208.1;HQ843209.1;HQ843210.1;HQ843211.1;HQ843212.1;HQ843213.1;HQ843214.1;HQ843215.1;HQ843216.1;HQ843217.1;HQ843218.1;HQ843219.1;HQ843220.1;HQ843221.1;HQ843222.1;HQ843223.1;HQ843224.1;HQ843225.1;HQ843226.1;HQ843227.1;HQ843228.1;HQ843229.1;HQ843230.1;HQ843231.1;HQ843232.1;HQ843233.1;HQ843234.1;HQ843235.1;HQ843236.1;HQ843237.1;HQ843238.1;HQ843239.1;HQ843240.1;HQ843241.1;HQ843242.1;HQ843243.1;HQ843244.1;HQ843245.1;HQ843246.1;HQ843247.1;NC_009572.1
Siganidae	*Siganus canaliculatus*	AB276834.1;AB276835.1;AB276836.1;AB276837.1;AB276838.1;AB276839.1;AB276840.1;AB276841.1;AB276842.1;AB276843.1;AB276844.1;AB276845.1;AB276846.1;AB276847.1;AB276848.1;AB276849.1;AB276850.1;AB276851.1;DQ274055.1;EF210175.1;EF210176.1;GU929677.1;GU929678.1;GU929679.1
Siganidae	*Siganus javus*	AB276852.1;AB276853.1;AB276854.1;AY190555.1;DQ898052.1;DQ898053.1;DQ898054.1
Siganidae	*Siganus puellus*	AB276855.1;AB276856.1;AB276857.1;AB276858.1;AB276859.1;AB276860.1;AB276861.1;AB276862.1;AB276863.1;AB276864.1;AB276865.1;AB276866.1;AB276867.1;DQ898063.1;DQ898064.1;DQ898065.1;DQ898066.1;DQ898067.1
Siganidae	*Siganus punctatissimus*	AB276868.1;AB276869.1;AB276870.1;AB276871.1;AB276872.1;AY190543.1
Siganidae	*Siganus punctatus*	AB276873.1;AB276874.1;AB276875.1;AB276876.1;AB276877.1;AB276878.1;AB276879.1;AB276880.1;DQ898068.1;DQ898069.1;DQ898070.1;DQ898071.1;DQ898072.1;DQ898073.1;DQ898074.1;DQ898092.1;EF210187.1
Siganidae	*Siganus uspi*	AB276881.1
Siganidae	*Siganus magnificus*	AB276882.1
Siganidae	*Siganus unimaculatus*	AB276883.1;AB276884.1;AB276885.1;AB276886.1;AB276887.1;AB276888.1;AB276889.1;AB276890.1;AB276891.1;AB276892.1;NC_013148.1
Siganidae	*Siganus vulpinus*	AB276893.1;AB276894.1;AB276895.1;AB276896.1;AB276897.1;AB276898.1;AB276899.1;AB276900.1;AB276901.1;AB276902.1;AB276903.1;AY19054

		8.1;DQ898084.1;DQ898085.1;DQ898086.1;DQ898087.1;DQ898088.1;DQ898089.1
Siganidae	*Siganus vermiculatus*	AB276904.1;AY190556.1;DQ898083.1
Siganidae	*Siganus guttatus*	AB276905.1;AB276906.1;AB276907.1;AB276908.1;AB276909.1;AB276910.1;AB276911.1;AB276912.1;AB276913.1;AY190547.1;DQ898046.1;DQ898047.1;DQ898048.1;DQ898049.1;DQ898050.1;HQ843248.1;HQ843249.1;HQ843250.1;HQ843251.1;HQ843252.1;HQ843253.1
Siganidae	*Siganus lineatus*	AB276914.1;AB276915.1;AB276916.1;AB276917.1;AB276918.1;AB276919.1;AB276920.1;AB276921.1;AB276922.1;AB276923.1;DQ898051.1;DQ898055.1;DQ898059.1;DQ898060.1;DQ898061.1;DQ898062.1
Siganidae	*Siganus corallinus*	AB276924.1;AB276925.1;AB276926.1;AB276927.1;AB276928.1;AB276929.1;AB276930.1;AB276931.1;AB276932.1;AB276933.1;AB276934.1;AB276935.1;AB276936.1;AB276937.1;AB276962.1;DQ898037.1;EF210177.1
Siganidae	*Siganus virgatus*	AB276938.1;AB276939.1;AB276940.1;AB276941.1;AB276942.1;AB276943.1;AB276944.1;AB276945.1;AB276946.1;AB276947.1;AB276948.1;AB276949.1;AB276950.1;AY190546.1;AY190549.1;AY190550.1
Siganidae	*Siganus doliatus*	AB276951.1;AB276952.1;AB276953.1;AB276954.1;AB276955.1;AB276956.1;AB276957.1;AB276958.1;AB276959.1;AB276960.1;AB276961.1;DQ898038.1;DQ898039.1
Siganidae	*Siganus luridus*	AM949022.1;AM949023.1;AM949024.1;AM949025.1;AM949026.1;AM949027.1;AY249548.1;AY249549.1;AY249550.1;AY249551.1;AY249552.1;AY249553.1;AY249554.1;AY249555.1;DQ898056.1;DQ898057.1;DQ898058.1;EF210185.1;EF210186.1
Siganidae	*Siganus rivulatus*	AM949028.1;AM949029.1;AM949030.1;AM949031.1;AY167612.1;AY167613.1;AY167614.1;AY167615.1;AY167616.1;AY249540.1;AY249541.1;AY249542.1;AY249543.1;AY249544.1;AY249545.1;AY249546.1;AY249547.1;DQ898075.1;DQ898076.1;DQ898077.1;DQ898078.1
Siganidae	*Siganus sp.*	DQ898045.1
Siganidae	*Siganus randalli*	EF210188.1
Siganidae	*Siganus sutor*	EF210193.1;EF210194.1
Zanclidae	*Zanclus cornutus*	NC_009852.1

Supplementary Table 2. Details of the specimens of *Siganus* used in the present study for the amplification and the sequencing tests, including specimen ID, collection date, sampling location and GenBank accession numbers.

Species name	Specimen no.	Collection date	Collection site	Genbank ID
Siganus argenteus	IFA0001	11 Dec. 2013	Ifaty, Mangily, Madagascar	NA
	S006	4 Feb. 2005	Nouméa, New Caledonia	NA
Siganus canaliculatus	Scan02	20 Nov. 2006	Singapore	NA
	Scan07	20 Nov. 2006	Singapore	NA
Siganus corallinus	S002	4 Feb. 2005	Nouméa, New Caledonia	NA
Siganus doliatus	S003	4 Feb. 2005	Nouméa, New Caledonia	NA
	S007	4 Feb. 2005	Nouméa, New Caledonia	NA
Siganus fuscescens	Scff25	20 Nov. 2006	Tekka mall, Singapore	NA
	Sfus18	Aug. 2006	Honiara market, Solomon Islands	NA
Siganus guttatus	Sgut01	24 Nov. 2007	Sorong, West Papua	NA
	Sgut02	24 Nov. 2007	Sorong, West Papua	NA
Siganus javus	Sjav04	1 Nov. 2007	PulauUntungJawa, Java Sea, Indonesia	NA
	Sjav05	1 Nov. 2007	PulauUntungJawa, Java Sea, Indonesia	NA
S. laqueus				NA
	RB09-241	1 Dec. 2009	Nampula, Mangrove channel, Mozambique	MF326182
	T-66	16 Apr. 2005	Victoria Market, Seychelles	MF326187
	KEN0001	18 Jan. 2014	Kilifi, Kenya	MF326163
	KEN0012	14 Apr. 2014	Msambweni, Kenya	MF326164
	KEN0013	14 Apr. 2014	Msambweni, Kenya	MF326165
	KEN0030	29 Dec. 2013	Shimoni, Kenya	MF326166
	TZW067	26 Oct. 2009	Southwestern part of Chwaka Bay, Zanzibar, Tanzania	MF326188
Siganus lineatus	Slin02	24 Nov. 2007	Sorong, West Papua	NA
	Slin13	24 Nov. 2007	Sorong, West Papua	NA
Siganus luridus	IFA0162	3 Jan. 2014	Ifaty, Madagascar	NA
	Slur01	2002	Lattaqieh, Syria	NA
Siganus puellus	S004	4 Feb. 2005	Nouméa, New Caledonia	NA
Siganus punctatus	Spun01	24 Nov. 2007	Sorong, West Papua	NA
	S001	4 Feb. 2005	Nouméa, New Caledonia	NA
Siganus rivulatus	Sriv01	2002	Lattaqieh, Syria	NA
	Sriv06	2002	Lattaqieh, Syria	NA
Siganus spinus	Sspi02	13 Jun. 2006	Ngela, Solomon Islands	NA
	Sspi12	27 Aug. 2006	Manus Island, Papua New Guinea	NA
Siganus sutor				NA
	INS0001	31 Mar. 2014	Inhassoro, Mozambique	NA
	R0301-211	23 Dec. 2014	La Réunion	MF326167
	R0301-212	23 Dec. 2014	La Réunion	MF326168
	R0301-213	23 Dec. 2014	La Réunion	MF326169
	R0301-214	23 Dec. 2014	La Réunion	MF326170
	R0301-215	23 Dec. 2014	La Réunion	MF326171
	R0301-216	23 Dec. 2014	La Réunion	MF326172

	R0301-217	23 Dec. 2014	La Réunion	MF326173
	R0301-218	23 Dec. 2014	La Réunion	MF326174
	R0301-219	23 Dec. 2014	La Réunion	MF326175
	R0301-220	23 Dec. 2014	La Réunion	MF326176
	R0301-221	23 Dec. 2014	La Réunion	MF326177
	R0301-222	23 Dec. 2014	La Réunion	MF326178
	R0301-223	23 Dec. 2014	La Réunion	MF326179
	R0301-224	23 Dec. 2014	La Réunion	MF326180
	R0301-225	23 Dec. 2014	La Réunion	MF326181
	Ssut10	1 Aug. 2006	Rodrigues Island, Mauritius	MF326183
	Ssut14	1 Aug. 2006	Rodrigues Island, Mauritius	MF326184
	Ssut17	1 Aug. 2006	Rodrigues Island, Mauritius	MF326185
	Ssut21	1 Aug. 2006	Rodrigues Island, Mauritius	MF326186
Siganus vermiculatus	Srev01	24 May. 2015	Padang, Indonesia	NA
Siganus vulpinus	S005	4 Feb. 2005	Nouméa, New Caledonia	NA
Siganus woodlandi	Swo05	8 Jul. 2005	Nouméa, New Caledonia	NA
	Swo06	8 Jul. 2005	Nouméa, New Caledonia	NA