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Primers and polymerase chain reaction conditions for DNA barcoding teleost fish based on the mitochondrial cytochrome b and nuclear rhodopsin genes

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Abstract:

This report describes a set of 21 polymerase chain reaction primers and amplification conditions developed to barcode practically any teleost fish species according to their mitochondrial cytochrome b and nuclear rhodopsin gene sequences. The method was successfully tested in more than 200 marine fish species comprising the main Actinopterygii family groups. When used in phylogenetic analyses, its combination of two genes with different evolutionary rates serves to identify fish at the species level. We provide a flow diagram indicating our validated polymerase chain reaction amplification conditions for barcoding and species identification applications as well as population structure or haplotyping analyses, adaptable to high-throughput analyses.

ABSTRACT

This report describes a set of 21 PCR primers and amplification conditions developed to barcode practically any teleost fish species according to their mitochondrial cytochrome *b* and nuclear rhodopsin gene sequences. The method was successfully tested in more than 200 marine fish species comprising the main Actinopterygii family groups. When used in phylogenetic analyses, its combination of two genes with different evolutionary rates serves to identify fish at the species level. We provide a flow diagram indicating our validated PCR amplification conditions for barcoding and species identification applications as well as population structure or haplotyping analyses, adaptable to high-throughput analyses.

Teleosts account for > 95% of the estimated 30,000 fish species alive today (Miya et al. 2003; Nelson 2006). The unequivocal identification and classification of living organisms to the species level frequently relies on genetic evidence. Specific DNA sequences act as unrepeatable signatures and therefore constitute a unique DNA barcode for each species. Initiatives, such as The Barcode of Life Database (www.barcodinglife.org) including The Fish Barcode of Life (www.fishbol.org), use a DNA-based identification system based on a relatively small fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI). This short DNA sequence provides sufficient identification labels in terms of nucleotide positions (Hebert et al. 2003) to discriminate even between congeneric fish species, despite only 2% sequence divergence found in 98% of these species (Ward et al. 2005). It is nevertheless clear that longer length DNA barcodes will provide more efficient identification labels. Barcode efficiency can be further improved by the simultaneous use of two genes showing different evolutionary rates and genomic positions. The mitochondrial cytochrome *b* gene (cyt*b*) and the nuclear rhodopsin gene (rhod) fulfill these requirements. The cyt*b* gene, whose

(1) D, direct amplification; N, nested-PCR; 1st, first nested amplification reaction using outer primers; 2nd, second nested amplification reaction using inner primers; cp cyt*b*, amplification of the complete length cyt*b* gene (1141 bp); cyt*b*-5', amplification of the targeted 5' fragment of the cyt*b* gene (~750 bp); cyt*b*-3', amplification of the targeted 3' fragment of the cyt*b* gene (~700 bp).

(2) PCR cycles provided as "temperature in ºC – seconds" as follows:

Initial Denaturation / (Denaturation / Annealing / Extension) x Number of Cycles / Final Extension

(3) Remarks:

(*a*) Elongation step could be extended to 120 seconds.

(*b*) FishCytB-F or GluFish-F could be used as forward primer for sequencing.

(*c*) Polymerase used: Phusion High-Fidelity DNA polymerase (Finnzymes).

(*d*) Elongation step could be extended to 90 seconds.

(*e*) FishCytB-F and CytBI-5R are used for sequencing.

(*f*) THR-Fish-R and CytBI-1F could be used for sequencing.

(*g*) CytB-7F and TruccytB-R could be used for sequencing.

(*h*) Fish-seq is used for sequencing (instead of FishcytB-F).

(*i*) 7F-seq is used for sequencing (instead of CytB-7F).

(*j*) CytBI-1F and THR-Fish-R could be used for sequencing.

(*k*) Truccytb-R and THR-Fish2-R could be used for sequencing.

(*l*) GluFish-F or FishcytB-F and CytBI-2R or CytBI-3R could be used for sequencing.

(*m*) Hotstar Qiagen kit (Q-solutions).

phylogenetic performance is comparable to that of COI (Zardoya and Meyer 1996), has been widely used for identifying fish species and resolving fish phylogenies (Zardoya and Doadrio 1999; Farias et al. 2001; Chen et al. 2003; Dettai and Lecointre 2005). The intronless teleost fish rhod gene (Venkatesh et al. 1999) provides quantitatively-equal inter-species identification labels of targeted nuclear PCR amplification products throughout its coding sequence. This gene has also been used in fish phylogenetic studies (Chen et al. 2003). In addition, the nuclear and the mitochondrial genes serve mutually as a internal phylogenetic control to validate sequences obtained from a large number of samples.

Herein, we describe the use of 21 PCR primers capable of robustly and consistently amplifying targeted DNA sequences of practically any teleost fish species, and thus generating DNA sequence collections for species identification and phylogenetic purposes. The primers, 12 specific for cyt*b* (Table 1.A) and 9 for rhod (Table 1.C), target regions of low variability, flanking the PCR amplification areas of interest in the two genes (Fig. 1). The entire cyt*b* coding sequence (1141 bp) can be amplified in a single reaction. However, for improved amplification efficiencies two separate reactions, one for each of the 5' (~750 bp) and the 3' (~700 bp) fragments, with significant overlapping between the two regions (Fig 1A), are suggested. The rhod-specific primers are used for single step amplification of 460 bp in the gene coding sequence (Fig 1B).

DNA from tissue samples (mostly white muscle) obtained from fish specimens was extracted using standard phenol/chloroform procedures (Sambrook et al. 1989), a DNA isolation station (ABI PRISM™ 6100 Nucleic Acid PrepStation; Applied Biosystems, Inc.), or commercial column kits (Qiagen Dneasy Kit®, Qiagen Dneasy Tissue Kit® and QiAmp DNA mini kit®; QIAGEN GmbH, Hilden, Germany). DNA quality was checked on 0.8% agarose gels and DNA concentration was determined using the PicoGreen® DNA quantitation kit (Molecular

77 Probes) in a 96 multiwell microplate fluorometer reader, and a standard curve (0.2 ng/µl to
78 140 ng/µl).
79 Both genes were amplified in 25 µl reaction mixtures containing: 1 µl DNA template
80 (concentration range 20 ng/µl to 30 ng/µl); 1X PCR buffer; 0.4 mM dNTPs; 2.5 mM $MgCl_2$;
81 and 1.25 U Taq DNA polymerase. Forward and reverse primer concentrations were optimised
82 and adjusted to 0.25 ng/µl for cyt*b* and 0.5 ng/µl for rhod. For improved amplification
83 efficiencies and automated sequencing signal quality a nested or seminested PCR step was
84 found necessary in many cases. For nested and seminested PCR, 1-2 µl of the product from
85 the first reaction was used as template for the subsequent amplification of targeted fragments.
86 Table 2 provides alternative PCR protocols in case of amplification failure. Details of these
87 protocols including 33 alternative amplification conditions can be found in Supplementary
88 Table 1.
89 The final PCR products obtained were always of the expected length as determined by
90 agarose gel electrophoresis. After purification, products were processed for sequencing using
91 the same forward and reverse primers employed for amplification, except the the FishcytB-F
92 and CytBI-7F amplification products that were sequenced using Fish-seq and 7F-seq,
93 respectively (Table 1.B). The optimal sequencing DNA concentration was estimated at 20
94 ng/µl. PCR products were bidirectionally sequenced using an ABI 3730 capillary sequencer.
95 Table 2 provides a flow diagram of the protocol options available using the designed primers.
96 These protocols were tested on the complete list of species provided in FishTrace
97 (www.fishtrace.org) including 1028 teleost specimens comprising 220 species from 17
98 Actinopterigii orders (Anguilliformes, Clupeiformes, Osmeriformes, Salmoniformes,
99 Aulopiformes, Ophidiiformes, Gadiformes, Batrachoidiformes, Lophiiformes,
100 Atheriniformes, Beloniformes, Beryciformes, Zeiformes, Scorpaeniformes, Perciformes,
101 Pleuronectiformes and Tetraodontiformes), 75 families and 112 genera.

The first option (A1) in Table 2 was successful at amplifying either of the two cytb fragments or the rhod fragment in >60% of all barcoded FishTrace species. Approximately half of the remaining species were barcoded using option A2 and the rest using any of the other options. Overall, the above procedure yielded >99.9% successful amplifications. After validating the sequencing data, approximately 3% did not match the expected phylogeny, mainly due to sampling (misidentified specimens) or amplification-sequencing errors. In these cases, repeating the procedure using newly extracted DNA or new samples was sufficient to successfully amplify and sequence the target genes.

In conclusion, the protocol proposed is a powerful tool for barcoding practically all teleost fish species and was successfully used here to provide fully validated sequence data for the FishTrace genetic catalogue (www.fishtrace.org).

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Table 1.- Fish-versatile primers. **(A)** Primer pairs for the amplification of mitochondrial cytochrome *b* (1141 bp). **(B)** Primers for cyt*b* sequencing purposes. Fish-seq and 7F-seq were respectively used for sequencing FishcytB-F and CytBI-7F amplification products. **(C)** Primers pairs for amplification of the targeted fragment in the rhodopsin nuclear gene (460 bp).

(A)

(*a*)	Name (*b*)	Sequence (5'-3') (*b*)	Location (*c*)	Size (bp)	%GC	*Tm* (ºC) (*d*)
1	GluFish-F	AACCACCGTTGTTATTCAACTACAA	15329	25	36.0	57.7
2	FishcytB-F	ACCACCGTTGTTATTCAACTACAAGAAC	15330	28	39.3	60.7
3	CytBI-6F	TTCTCAGTAGACAACGCCACCCT	15862	23	52.2	61.0
4	CytBI-7F	CTAACCCGATTCTTTGCCTTCCACTTCCT	15883	29	48.3	68.3
5	CytBI-1F	CGATTCTTCGCATTCCACTTCCT	15889	23	47.8	62.5
6	*CytBI-5R*	*GGTCTTTGTAGGAGAAGTATGGGTGGAA*	16018	28	46.4	63.5
7	*CytBI-3R*	*GGGGTAAAGTTGTCTGGGTCTCC*	16111	23	56.5	60.9
8	*CytBI-2R*	*GCGGGGGTAAAGTTGTCTGGGTC*	16114	23	60.9	65.5
9	*CytBI-4R*	*AGGAAGTATCATTCGGGCTTAATATG*	16159	26	38.5	58.9
10	*TruccytB-R*	*CCGACTTCCGGATTACAAGACCG*	16528	23	56.5	64.6
11	*THR-Fish2-R*	*AACCTCCGACATCCGGCTTACAAGACCG*	16528	28	57.1	72.1
12	*THR-Fish-R*	*ACCTCCGATCTTCGGATTACAAGACC*	16529	26	50.0	64.4

(B)

(*a*)	Name	Sequence (5'-3')	Location (*c*)	Size (bp)	%GC	*Tm* (ºC) (*d*)
13	Fish-seq	CCACCGTTGTTATTCAACTACAAG	15331	24	41.7	56.6
14	7F-seq	CTAACCCGATTCTTTGCCTTC	15883	21	47.6	56.7

(C)

(*a*)	Name (*b*)	Sequence (5'-3') (*b*)	Location (*e*)	Size (bp)	%GC	*Tm* (ºC) (*d*)
15	RHO-30F:	CCNTAYGAYTAYCCNCARTAYTA	67	23	41.3	53.5
16	Rod-F2B:	GTCTGCAAGCCCATCAGCAACTTCCG	415	26	57.7	71.0
17	Rod-F2w:	AGCAACTTCCGCTTCGGTGAGAA	430	23	52.2	65.1
18	Rod-F2x:	AGCAACTTCCGCTTCGGCGAGAA	430	23	56.5	68.8
19	Rod-F2:	AGCAACTTCCGCTTCGGAGAGAA	430	23	52.2	64.4
20	*Rod-R4n:*	*GGAACTGCTTGTTCATGCAGATGTAGAT*	913	28	42.9	63.6
21	*Rod-4R:*	*CTGCTTGTTCATGCAGATGTAGAT*	913	24	41.7	57.2
22	*Rod-5R:*	*GGTGGTGATCATGCAGTGGCGGAA*	937	24	58.3	70.7
23	*RHO-319R:*	*TTNCCRCARCAYAANGTNGT*	955	20	45.0	66.6

(*a*): Numbers correspond to positions in Figure 1.

(*b*): Reverse primers in italics.

(*c*): Nucleotide position corresponding to the 5' position in the *Oncorhynchus mykiss* mitochondrial genome (GenBank accession number: NC_001717). Locations given for the reverse primers are based on the reverse-complementary primer sequence position.

(*d*): *Tm* calculated using PrimerExpress™ 2.0 (Applied Biosystems).

(*e*): Nucleotide position corresponding to the 5' position in the *Astyanax mexicanus* genomic rhodopsin gene (GenBank accession number: U12328). Locations given for the reverse primers are based on the reverse-complementary primer sequence position.

Table 2.- Flow diagram providing alternative PCR protocols for amplification of the target fragments of the cyt*b* and rhodopsin genes. Nested or seminested PCRs comprise two reactions. The first reaction uses a pair of *outer* primers (designated A to D in both genes), which in cyt*b* flank the whole gene. The second reaction has specific *inner* primers (numbered) for each gene fragment. Preferential reactions are designated with a letter followed by a number e.g., for cyt*b*-5' **A1** is the first choice and **B1** the third choice. **(A)** Preferential PCR conditions (primer pair and thermocycling program) for the amplification of cyt*b* as two PCR fragments: cyt*b*-5' and cyt*b*-3'. **(B)** Preferential PCR conditions for the amplification of the targeted fragment of the rhod gene.

(A)

Cyt*b*: consecutive options 1st reaction(1)			
A	B	C	D
FishcytB-F + TruccytB-R	GluFish-F + TruccytB-R	FishcytB-F + THR-Fish-R	GluFish-F + THR-Fish-R

Cyt*b*-5': consecutive opt. 2nd reaction(2)	
1	2
FishcytB-F + CytBI-5R	FishcytB-F + CytBI-4R

Cyt*b*-3': consecutive options 2nd reaction(2)			
1	2	3	4
CytbI-7F + THR-Fish-R	CytbI-7F + TruccytB-R	CytbI-6F + THR-Fish-R	CytbI-6F + TruccytB-R

(B)

Rhod: consecutive options 1st reaction(3)			
A	B	C	D
Rod-F2B + Rod-5R	Rod-F2B + Rod-5R	RHO-30F + RHO-319R	Rod-F2B + Rod-5R

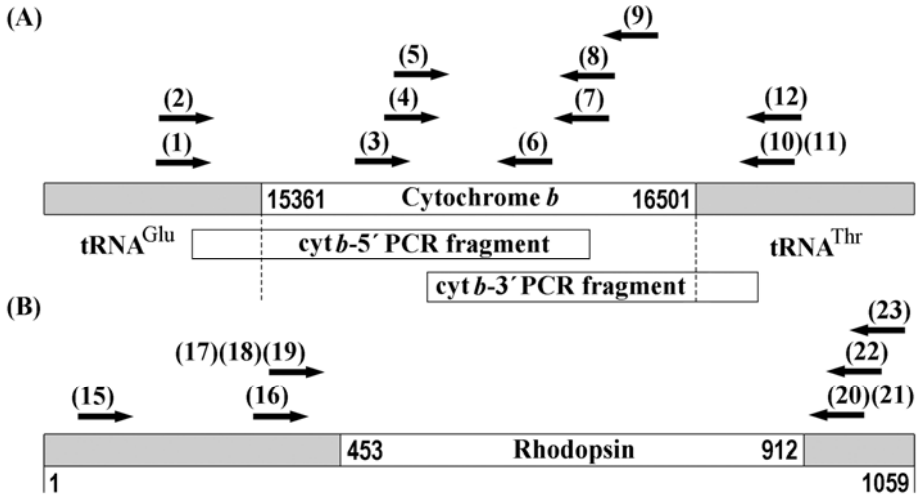
Rhod: consecutive opt. 2nd reaction(4)		
1	2	3
Rod-F2W + Rod-R4n	Rod-F2X + Rod-R4n	Rod-F2 + Rod-R4n

PCR programs given as "temperature in ºC – seconds" as follows: Initial Denaturation / (Denaturation / Annealing / Extension) x Number of Cycles / Final Extension: (1) 95-420 / (94-30 / 55-35 / 72-120) x35 / 72-420; (2) 95-420 / (94-30 / 55-35 / 72-45) x38 / 72-420; (3) 95-420 / (94-30 / 62-30 / 72-30) x40 / 72-420; (4) 95-420 / (94-30 / 56-30 / 72-30) x40 / 72-420.

FIGURE LEGENDS:

Figure 1.- Amplification schemes: targeted gene regions and primer positions. **(A)** Typical vertebrate cytochrome *b* organization indicating flanking genes ($tRNA^{Glu}$, $tRNA^{Thr}$) and their nucleotides positions (15361 to 16501) within the *Oncorhynchus mykiss* mitochondrial genome (GenBank accession number: NC_001717). The relative lengths of targeted cyt*b*-5' and cyt*b*-3' PCR fragments are represented. Detailed information on represented primer pairs is given in Table 1.A. **(B)** Rhodopsin amplification scheme. Targeted 460bp-length fragment and primer location given as that corresponding to the 5' position in the *Astyanax mexicanus* rhodopsin gene (GenBank accession number: U12328). Detailed information on the represented primer pairs is given in Table 1.B.

Figure 1


(A)
(9)
(5)
(8)
(2)
(4)
(7)
(12)
(1)
(3)
(6)
(10)(11)
15361
Cytochrome b
16501
tRNAGlu
cyt b-5´ PCR fragment
tRNAThr
cyt b-3´ PCR fragment
(B)
(23)
(17)(18)(19)
(22)
(15)
(16)
(20)(21)
453
Rhodopsin
912
1
1059

Supplementary Table 1.- PCR conditions detailing direct and nested amplifications, and alternative strategies for fish DNA barcoding. **(A)** Cytochrome *b*. **(B)** Rhodopsin 460bp-length fragment.

(A)

No.	PCR[1]	Forward + Reverse	PCR cycles[2]	Remarks[3]
1	D cp cyt*b*	FishcytB-F + THR-Fish-R	95-420 / (94-30/50-30/72-30)x40 / 72-420	
2	D cp cyt*b*	FishcytB-F + THR-Fish-R	95-300 / (95-30/50-30/72/45)x40 / 72-420	
3	D cp cyt*b*	FishcytB-F + THR-Fish2-R	95-420 / (94-30/50-35/72-90)x40 / 72-420	***(a)***
4	D cp cyt*b*	FishcytB-F + TruccytB-R	95-420 / (94-30/50-35/72-90)x40 / 72-420	***(a)***
5	D cp cyt*b*	GluFish-F + TruccytB-R	95-420 / (94-30/50-35/72-90)x40 / 72-420	***(a)***
6	D cyt*b*-5'	FishcytB-F + CytBI-5R	95-420 / (94-30/50-35/72-60)x40 / 72-420	***(b)***
7	D cyt*b*-5'	FishcytB-F + CytBI-5R	95-300 / (95-30/50-30/72-45)x40 / 72-420	
8	D cyt*b*-5'	FishcytB-F + CytBI-5R	98-40 / (98-15/60-30/72-30)x45 / 72-420	***(c)***
9	D cyt*b*-5'	FishcytB-F + CytBI-4R	95-420 / (94-30/50-35/72-60)x40 / 72-420	***(d)***
10	D cyt*b*-5'	FishcytB-F + CytBI-3R	95-420 / (94-30/50-35/72-60)x40 / 72-420	***(d)***
11	D cyt*b*-5'	FishcytB-F + TruccytB-R	95-420 / (94-30/50-35/72-90)x40 / 72-420	***(e)***
12	D cyt*b*-3'	CytbI-7F + THR-Fish-R	95-300 / (95-30/52-30/72-30)x40 / 72-420	
13	D cyt*b*-3'	CytbI-7F + THR-Fish2-R	95-420 / (94-30/50-35/72-60)x40 / 72-420	***(d)***
14	D cyt*b*-3'	CytbI-7F + THR-Fish2-R	98-40 / (98-15/60-30/72-30)x45 / 72-420	***(c)***
15	D cyt*b*-3'	CytbI-7F + TruccytB-R	95-420 / (94-30/50-35/72-60)x40 / 72-420	***(d) (f)***
16	D cyt*b*-3'	CytbI-7F + TruccytB-R	95-300 / (95-30/50-30/72-45)x40 / 72-420	
17	D cyt*b*-3'	CytbI-6F + THR-Fish-R	95-360 / (95-55/52-55/72-55)x40 / 72-600	
18	D cyt*b*-3'	CytbI-6F + THR-Fish2-R	95-420 / (94-30/50-35/72-60)x40 / 72-420	***(d)***
19	D cyt*b*-3'	CytbI-6F + TruccytB-R	95-300 / (95-30/52-30/72-45)x40 / 72-400	
20	D cyt*b*-3'	FishcytB-F + TruccytB-R	95-420 / (94-30/50-35/72-90)x40 / 72-420	***(g)***
21	N 1st	FishcytB-F + TruccytB-R	95-420 / (94-30/55-35/72-120)x35 / 72-420	
	N 2nd cyt*b*-5'	FishcytB-F + CytBI-5R	95-420 / (94-30/55-35/72-45)x38 / 72-420	***(h)***
	N 2nd cyt*b*-5'	FishcytB-F + CytBI-5R	95-420 / (94-30/50-35/72-60)x38 / 72-420	
	N 2nd cyt*b*-5'	FishcytB-F + CytBI-4R	95-420 / (94-30/55-35/72-45)x38 / 72-420	***(h)***
	N 2nd cyt*b*-3'	CytbI-7F + THR-Fish-R	95-420 / (94-30/55-35/72-45)x38 / 72-420	***(i)***
	N 2nd cyt*b*-3'	CytbI-7F + TruccytB-R	95-420 / (94-30/55-35/72-45)x38 / 72-420	***(i)***
	N 2nd cyt*b*-3'	CytbI-6F + THR-Fish-R	95-420 / (94-30/55-35/72-45)x38 / 72-420	***(i)***
	N 2nd cyt*b*-3'	CytbI-6F + TruccytB-R	95-420 / (94-30/55-35/72-45)x38 / 72-420	***(i)***
22	N 1st	FishcytB-F + THR-Fish-R	95-420 / (94-30/50-35/72-90)x40 / 72-420	
	N 2nd cyt*b*-5'	FishcytB-F + CytBI-2R	95-420 / (94-30/50-35/72-60)x40 / 72-420	***(d)***
	N 2nd cyt*b*-3'	CytbI-7F + THR-Fish2-R	95-420 / (94-30/50-35/72-60)x40 / 72-420	***(d)***
	N 2nd cyt*b*-3'	CytbI-7F + TruccytB-R	95-420 / (94-30/50-35/72-60)x40 / 72-420	***(j)***
	N 2nd cyt*b*-3'	CytbI-7F + THR-Fish-R	95-420 / (94-30/50-35/72-60)x40 / 72-420	***(d)***
	N 2nd cyt*b*-3'	CytbI-6F + THR-Fish-R	95-420 / (94-30/50-35/72-60)x40 / 72-420	***(k)***
23	N 1st	FishcytB-F + THR-Fish-R	94-240 / (94-30/55-35/72-120)x35 / 72-420	
	N 2nd cyt*b*-5'	FishcytB-F + CytBI-5R	94-240 / (94-30/55-35/72-45)x38 / 72-420	
	N 2nd cyt*b*-3'	CytbI-7F + THR-Fish-R	94-240 / (94-30/55-35/72-45)x38 / 72-420	
	N 2nd cyt*b*-3'	CytbI-7F + TruccytB-R	94-240 / (94-30/55-35/72-45)x38 / 72-420	
	N 2nd cyt*b*-3'	CytbI-6F + TruccytB-R	94-240 / (94-30/55-35/72-45)x38 / 72-420	
22	N 1st	FishcytB-F + TruccytB-R	95-240 / (94-30/55-30/72-30)x40 / 72-420	
	N 2nd cyt*b*-5'	FishcytB-F + CytBI-3R	95-240 / (94-30/55-30/72-30)x35 / 72-420	
	N 2nd cyt*b*-3'	CytbI-7F + TruccytB-R	95-240 / (94-30/55-30/72-30)x35 / 72-420	
26	N 1st	GluFish-F + TruccytB-R	95-420 / (94-30/55-35/72-120)x35 / 72-420	
	N 2nd cyt*b*-5'	FishcytB-F + CytBI-5R	95-420 / (94-30/55-35/72-45)x38 / 72-420	***(h)***
27	N 1st	FishcytB-F + TruccytB-R	95-420 / (94-30/50-35/72-90)x40 / 72-420	
	N 2nd cyt*b*-3'	CytbI-7F + TruccytB-R	95-420 / (94-30/50-35/72-60)x40 / 72-420	
24	N 1st	FishcytB-F + THR-Fish2-R	95-420 / (94-30/50-35/72-90)x40 / 72-420	
	N 2nd cyt*b*-3'	CytbI-7F + THR-Fish2-R	95-420 / (94-30/50-35/72-60)x40 / 72-420	***(l)***

(B)

No.	PCR[1]	Forward + Reverse	PCR cycles[2]	Remarks[3]
25	D	Rod-F2W + Rod-R4n	95-240 / (94-30/60-30/72-45)x40 / 72-490	
26	D	Rod-F2W + Rod-R4n	96-60 / (96-30/50-30/60-240)x25 / 4-∞	
27	N 1st	Rod-F2B + Rod-5R	95-420 / (94-30/62-30/72-30)x40 / 72-420	
	N 2nd	Rod-F2W + Rod-R4n	95-420 / (94-30/56-30/72-30)x40 / 72-420	
	N 2nd	Rod-F2W + Rod-R4n	95-420 / (94-30/54-30/72-30)x40 / 72-420	
	N 2nd	Rod-F2X + Rod-R4n	95-420 / (94-30/56-30/72-30)x40 / 72-420	
28	N 1st	Rod-F2B + Rod-5R	95-420 / (94-30/60-30/72-30)x40 / 72-420	
	N 2nd	Rod-F2W + Rod-R4n	95-420 / (94-30/56-30/72-30)x40 / 72-420	
	N 2nd	Rod-F2X + Rod-R4n	95-420 / (94-30/56-30/72-30)x40 / 72-420	
29	N 1st	RHO-30F + RHO-319R	95-420 / (94-30/62-30/72-30)x40 / 72-420	
	N 2nd	Rod-F2W + Rod-R4n	95-420 / (94-30/56-30/72-30)x40 / 72-420	
30	N 1st	Rod-F2B + Rod-5R	94-240 / (94-30/60-35/72-120)x35 / 72-420	
	N 2nd	Rod-F2 + Rod-4R	94-240 / (94-30/60-35/72-40)x35 / 72-420	
31	N 1st	Rod-F2B + Rod-5R	95-600 / (95-55/54-55/72-55)x40 / 72-600	
	N 2nd	Rod-F2 + Rod-4R	95-600 / (95-55/72-55/72-55)x35 / 72-600	
32	N 1st	Rod-F2B + Rod-5R	95-240 / (94-30/55-30/72-30)x40 / 72-420	
	N 2nd	Rod-F2 + Rod-4R	95-240 / (94-30/55-30/72-30)x40 / 72-420	
33	N 1st	Rod-F2B + Rod-5R	95-600 / (94-55/56-55/72-55)x40 / 72-600	***(m)***
	N 2nd	Rod-F2 + Rod-4R	95-600 / (94-55/56-55/72-55)x40 / 72-600	***(m)***

(1) D, direct amplification; N, nested-PCR; 1st, first nested amplification reaction using outer primers; 2nd, second nested amplification reaction using inner primers; cp cyt*b*, amplification of the complete length cyt*b* gene (1141 bp); cyt*b*-5', amplification of the targeted 5' fragment of the cyt*b* gene (~750 bp); cyt*b*-3', amplification of the targeted 3' fragment of the cyt*b* gene (~700 bp).

(2) PCR cycles provided as "temperature in ºC – seconds" as follows:

Initial Denaturation / (Denaturation / Annealing / Extension) x Number of Cycles / Final Extension

(3) Remarks:

(*a*) Elongation step could be extended to 120 seconds.

(*b*) FishCytB-F or GluFish-F could be used as forward primer for sequencing.

(*c*) Polymerase used: Phusion High-Fidelity DNA polymerase (Finnzymes).

(*d*) Elongation step could be extended to 90 seconds.

(*e*) FishCytB-F and CytBI-5R are used for sequencing.

(*f*) THR-Fish-R and CytBI-1F could be used for sequencing.

(*g*) CytB-7F and TruccytB-R could be used for sequencing.

(*h*) Fish-seq is used for sequencing (instead of FishcytB-F).

(*i*) 7F-seq is used for sequencing (instead of CytB-7F).

(*j*) CytBI-1F and THR-Fish-R could be used for sequencing.

(*k*) Truccytb-R and THR-Fish2-R could be used for sequencing.

(*l*) GluFish-F or FishcytB-F and CytBI-2R or CytBI-3R could be used for sequencing.

(*m*) Hotstar Qiagen kit (Q-solutions).