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Ten real-time PCR assays for detection of fish predation at the community level in the San Francisco Estuary–Delta

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Abstract

The effect of predation on native fish by introduced species in the San Francisco Estuary–Delta (SFE) has not been thoroughly studied despite its potential to impact species abundances. Species-specific quantitative PCR (qPCR) is an accurate method for identifying species from exogenous DNA samples. Quantitative PCR assays can be used for detecting prey in gut contents or faeces, discriminating between cryptic species, or detecting rare aquatic species. We designed ten TaqMan qPCR assays for fish species from the SFE watershed most likely to be affected by non-native piscivores. The assays designed are highly specific, producing no signal from co-occurring or related species, and sensitive, with a limit of detection between 3.2 and 0.013 pg/μL of target DNA. These assays will be used in conjunction with a high-throughput qPCR platform to compare predation rates between native and non-native piscivores and assess the impacts of predation in the system.

Keywords: detection, diet analysis, environmental DNA, food webs, predator–prey interactions, TaqMan

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Introduction

DNA-based methods for diet analysis offer several advantages over the visual identification methods that are often used in predation studies (Sheppard & Harwood 2005; Carreon-Martinez & Heath 2010). They can detect prey composed of soft tissue, such as eggs and larvae (Symondson 2002; Albaina *et al*. 2010, 2012; Fox *et al*. 2012) as well as prey that have been digested and are no longer phenotypically identifiable. When thoroughly validated, TaqMan qPCR assays are specific enough to reliably identify species without additional confirmation such as sequencing or cloning, offering a faster turnover time than other DNA-based methods. Species-specific TaqMan assays have a number of uses, including distinguishing phenotypically similar species (Fox *et al*. 2005), detecting exogenous DNA from rare aquatic species (Amos & Whitehead 1992; Jerde *et al*. 2011), as well as detection of prey in faeces or gut contents (King *et al*. 2008; Baerwald *et al*. 2012; Shehzad *et al*. 2012).

In the San Francisco Estuary–Delta, predation by non-native piscivores has been hypothesized to contribute to the precipitous decline of a number of pelagic fishes (Sommer *et al*. 2007). Yet, predation remains relatively unstudied largely because direct estimates of predation are difficult to achieve via visual analyses. In particular, larval and early life stage fish have been shown to be visually unidentifiable 30–60 min postingestion (Schooley *et al*. 2008; Legler *et al*. 2010), whereas prey DNA may be detected in predator guts many hours, and sometimes days, after ingestion in controlled feeding experiments (Baerwald *et al*. 2012). As of 2013, three TaqMan qPCR assays have been designed for SFE species: Delta Smelt (*Hypomesus transpacificus*), Wakasagi Smelt (*Hypomesus nipponensis*) and Mississippi Silverside (*Menidia beryllina*) (Baerwald *et al*. 2011). We have designed a suite of complementary assays for other species hypothesized to be effected by predation, namely those comprising the SFE's Pelagic Organism Decline (Sommer *et al*. 2007): Longfin Smelt (*Spirinchus thaleichthys*), Threadfin Shad (*Dorosoma petenense*) and Striped Bass (*Morone saxatilis*). Predation has also been implicated in the decline of Chinook Salmon (*Oncorhynchus tshawytscha*) (Lindley & Mohr, 2003), so we have created an assay for Central Valley Chinook. To provide a more comprehensive view of the diets of SFE piscivores, we created assays for other species of conservation and management interest,

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including Sacramento Splittail (*Pogonichthys macrolepidotus*), Steelhead/Rainbow Trout (*Oncorhynchus mykiss*), Green Sturgeon (*Acipenser medirostris*) and White Sturgeon (*Acipenser transmontanus*). For control purposes, assays were designed for three putative predators: two non-native piscivores, Striped Bass and Smallmouth Bass (*Micropterus dolomieu*), as well as one native piscivore, the Sacramento Pikeminnow (*Ptychocheilus grandis*).

We developed a validation method to address the problem of pernicious low-level contamination, which is a concern for laboratories conducting forensics projects. Some laboratories have even been subject to audits (Jerde *et al.* 2013). The contamination issue is particularly acute when detecting mitochondrial genes, which occur in hundreds of copies per cell, and also when detecting low copy numbers of target sequence, such as with gut contents. In early validation experiments, we experienced what appeared to be nonspecific amplification with nontarget species. By cloning and sequencing the contaminants, we demonstrated that the target DNA was present in low levels in many of our samples. We hypothesized that the fin clips collected by our field sampling crews were contaminated with exogenous DNA from dirty sampling gear.

To properly validate the assays, we needed a collection of samples that were free of contaminant DNA so we created a 'gold standard' panel. This type of panel is used to validate assays for medical purposes where the consequences of detecting a false positive are high – for example, when detecting a pathogen in food products (Malorny *et al.* 2003). The panel is a collection of DNA from organisms likely to co-occur with the target species which have been verified to be pure and are used to demonstrate the assays do not produce a false positive signal on nontarget DNA (OIE Manual 2010).

Materials and methods

Local barcode sequences

We sequenced common SFE fish species provided by colleagues at United States Bureau of Reclamation, United States Fish and Wildlife Service and California Department of Water Resources at cytochrome oxidase 1 (COI) and cytochrome b (CYTB) to ensure each TaqMan probe was unique amongst SFE fish – an *in silico* validation (Table 1). DNA was extracted with Qiagen DNeasy Blood and Tissue Kit (spin-column protocol) from fin clips and amplified using universal animal primers for CYTB (H15149 and L14724) (Kocher *et al.* 1989; Irwin *et al.* 1991) and universal fish primers for COI (FF2d and FR1d) (Ivanova *et al.* 2007). Sequences available in GenBank were used for SFE fish species when physical specimens were not available. Those sequences are listed in Table 1.

Assay design and optimization

For assay species, 6–10 individuals per species were sequenced at the mitochondrial genes COI and CYTB to assess sequence variation across SFE populations. Consensus sequences were created from individual sequences of each target species using SEQUENCHER version 4.8 (Gene Codes) and were entered into PRIMER EXPRESS Software version 3.0 (Life Technologies) to choose the most thermodynamically efficient combinations of primers and probe <150 base pairs in length. We selected 6-FAM MGB as the reporter dye with black hole quencher (BHQ) for all assays. Before empirical validation, specificity was confirmed in two ways. First, uniqueness of the combined primers and probe sequence was verified via BLASTn on NCBI. Assays sharing >98% identity with nontarget species occurring in western North America were redesigned. Second, prospective probe sequences were verified unique from analogous sequences of local fish by at least one polymorphism.

Probe concentrations were optimized in 5 μL qPCRs composed of 1× Applied Biosystems TaqMan Universal PCR Master Mix, No AmpErase UNG and 0.9 uM concentration of each primer. Thermocycling was performed using a Bio-Rad Chomo4 real-time detector with the following profile: 10 min at 95 °C, 40 cycles of 15 s denaturation at 95 °C and 1 min annealing–extension at 60 °C. Optimized concentrations of probe were 100 μM or 150 μM and are listed in Table 2.

Assay specificity

Before empirical validation, a set of 'gold standard' DNA samples was created. Whole fish were collected and preserved in 95% ethanol and processed in a laboratory that does not work with fish. We co-opted methods used in forensics laboratories such as not allowing personnel in the clean laboratory after being in a fish laboratory without first showering and changing clothes. Tissue samples were taken by removing the skin on a section of the fish and collecting the muscle tissue beneath with a tissue biopsy punch. All tools – surgical blades, forceps, biopsy punches and bench paper – were replaced with new or bleached and sterilized instruments between every sample. Extraction reagents were divided into single-use aliquots prior to sample preparation. Aliquots from the resulting extractions were divided into three vials and stored at −20 °C in the clean laboratory.

Assays were tested against the species in the 'gold standard' panel, those marked *** in Table 1. Thresholds were determined for each assay individually by setting each above the response curve for the no template controls (between 0.01 and 0.02).

Table 1 Assays were tested for cross-amplification with these species

Common name (*Latin name*)		Common name (*Latin name*)	
***	American Shad (*Alosa sapidissima*)	***	Red Shiner (*Cyprinella lutrensis*)
***	Bigscale Logperch (*Percina macrolepida*)	**	Redear Sunfish (*Lepomis microlophus*)
**	Black Crappie (*Pomoxis nigromaculatus*)	*	Redeye Bass (*Micropterus coosae*)
***	Bluegill Sunfish (*Lepomis macrochirus*)	*	Riffle Sculpin (*Cottus gulosus*)
***	Channel Catfish (*Ictalurus punctatus*)	*	Sacramento Perch (*Archoplites interruptus*)
***	Chinook (*Oncorhynchus tshawytscha*)	***	Sacramento Pikeminnow (*Ptychocheilus grandis*)
***	Common Carp (*Cyprinus carpio*)	***	Sacramento Splittail (*Pogonichthys macrolepidotus*)
***	Delta Smelt (*Hypomesus transpacificus*)	**	Sacramento Sucker (*Catostomus occidantalis*)
***	Fathead Minnow (*Pimephales promelas*)	***	Shimofuri Goby (*Tridentiger bifasciatus*)
***	Golden Shiner (*Notemigonus crysoleucas*)	*	Shiner Perch (*Cymatogaster aggregata*)
***	Green Sturgeon (*Acipenser medirostris*)	***	Smallmouth Bass (*Micropterus dolomieu*)
***	Hitch (*Lavinia exilicauda*)	*	Speckled Dace (*Rhinichthys osculus*)
**	Inland Silverside (*Menidia beryllina*)	*	Staghorn Sculpin (*Leptocottus armatus*)
***	Largemouth Bass (*Micropterus salmoides*)	***	Striped Bass (*Morone saxatilis*)
***	Longfin Smelt (*Spirinchus thaleichthys*)	***	Threadfin Shad (*Dorosoma petenense*)
*	Marbled Sculpin (*Cottus klamathensis*)	***	Three-spined Stickleback (*Gasterosteus aculeatus*)
***	Mississippi Silverside (*Menidia beryllina*)	***	Tule Perch (*Hysterocarpus traskii*)
*	Modoc Sucker (*Catostomus microps*)	**	Wakasagi Smelt (*Hypomesus nipponensis*)
***	Mosquitofish (*Gambusia affinis*)	***	Warmouth (*Lepomis gulosus*)
**	Pacific Herring (*Clupea pallasii*)	**	Western Mosquitofish (*Gambusia holbrooki*)
*	Pacific Lamprey (*Lampetra tridentata*)	*	White Bass (*Morone chrysops*)
*	Pink Salmon (*Oncorhynchus gorbuscha*)	***	White Catfish (*Ameiurus catus*)
*	Pit Sculpin (*Cottus pitensis*)	**	White Crappie (*Pomoxis annularis*)
***	Prickley Sculpin (*Cottus asper*)	***	White Sturgeon (*Acipenser transmontanus*)
**	Pumpkinseed (*Lepomis gibbosus*)	*	Yellow Perch (*Perca flavescens*)
***	Rainbow Trout (*Oncorhynchus mykiss*)	***	Yellowfin Goby (*Acanthogobius flavimanus*)
*	Rainwater Killfish (*Lucania parva*)		

*Validated *in silico* from nonlocal sequences.
**Validated *in silico* from local sequences.
***Validated empirically ('gold standard' panel).

Intraspecific validation

To confirm that the assays amplify DNA for the target species throughout our study region (i.e. avoiding false negatives), we tested the assay on target samples collected throughout each species' range in the watershed. When feasible, 40 individuals of each target species were tested. Collection locations are listed in Table 3. The selection of sampling locales reflects known subpopulation distributions and/or availability of samples.

Assay sensitivity

To determine the lowest concentration of DNA that is consistently detectable across replicates ($n = 8$), we performed a 5× serial dilution of target template (10000 pg/μL to 0.013 pg/μL) in the presence of 100 ng/μL nontarget template. The amplification plot threshold was set as detailed above. A reaction was considered positive if logarithmic amplification occurred before 40 cycles. The lowest concentration of target template that was detected in at least seven of eight reactions is included in Table 2.

Conformity of high-throughput platform

Functionality of the assays was verified on a second platform, the Fluidigm Biomark with a 5× serial dilution as performed above. The Biomark has several microfluidic chip formats and can perform up to 9216 qPCRs simultaneously by combining 96 species-specific assays with 96 predator gut DNA samples in nanolitre-scale reactions. The preferred format for detection is the gene expression chip; we used the 96.96 version. Fourteen assays were tested (ten from this study, two from a previous study (Baerwald *et al.* 2011) and two others) in replicates of six. No assay controls were included in two wells. Two extraction controls and four no template controls were also included on the chip. Sample DNA was prepared by performing a 14-cycle pre-amplification in a primer limited environment using standard PCR. Total volume for each pre-amplification reaction was 5 μL, composed of 0.18 μM forward and reverse primers from all assays, 1× Qiagen QuantiTect Multiplex PCR Master Mix (no ROX) and 1.25 μL template DNA. The pre-amplification was performed using an Applied Biosystems GeneAmp 9700

Table 2 Assay details: primer and probe sequences for each species including Accession no., optimized probe concentration, PCR efficiencies and lowest concentration reliably detected

Common name (*Latin name*)	Gene	Primer and probe (5′ to 3′)	Accession no.	Probe concentration	PCR efficiency	Limit of detection
Chinook Salmon (*Oncorhynchus tshawytscha*)	CYTB	Fwd-CCTAAAAATCGCTAATGACGCACTA Rev-GGAGTGAGCCAAAGTTTCATCAG Probe-AGCACCCTCTAACATTTCAG	KF013235	100 μM	93.1	0.64 pg/μL
Green Sturgeon (Acipenser medirostris)	COI	Fwd-AGGGAAAAAATGGTTAGGTCTACAGA Rev-CCCCACTGGCGGGAAA Probe-CTCCCGCATGGGCTA	KF558288	150 μM	94.0	0.013 pg/μL
Longfin Smelt (Spirinchus thaleichthys)	CYTB	Fwd-CTCTGCCGGGACGTCAAT Rev-CCCGTTAGCGTGCATATTCC Probe-ACGGCTGACTAATC	KF013249	100 μM	94.2	0.64 pg/μL
Rainbow Trout/ Steelhead (Oncorhynchus mykiss)	COI	Fwd-AACATAAAACCTCCAGCCATCTCT Rev-AGCACGGCTCAAACGAAAA Probe-AGTACCAAACCCCC	KF558313	150 μM	92.4	3.2 pg/μL
Sacramento Pikeminnow (Ptychocheilus grandis)	COI	Fwd-TCTTCGTATGGGCCGTACTTG Rev-GCCAGGACTGGTAGTGATAACAGA Probe-AACAGCCGTTCTTC	KF558277	150 μM	103	0.64 pg/μL
Smallmouth Bass (Micropterus dolomieu)	COI	Fwd-ATCCTAGGGGCCATCAATTT Rev-ACCAAACAAACAGGGGTGTCT Probe-AAACCCCCAGCTATTTCCC	KF558298	100 μM	94.5	0.64 pg/μL
Sacramento Splittail (Pogonichthys macrolepidotus)	COI	Fwd-CCAGGACTGGCAGTGATAGG Rev-CTCCCAATATCAAACACCTCTCTT Probe-AGGAGAACAGCAGTTACA	KF558276	100 μM	101	0.64 pg/μL
Striped Bass (Morone saxatilis)	COI	Fwd-TCCCCGAATGAACAACATAAGTT Rev-GAAGCTAGAAGGAGGAGGAAGGA Probe-TTGACTGCTTCCCCC	KF558274	100 μM	100	0.64 pg/μL
Threadfin Shad (Dorosoma petenense)	CYTB	Fwd-AAGTCCTCGGCCGATGTG Rev-CATGCAAACGGAGCATCCT Probe-CGTAGATACAAATGAAAAAG	KF013218	150 μM	104	0.64 pg/μL
White Sturgeon (Acipenser transmontanus)	CYTB	Fwd-CCCCGTTTGCATGAATGTTT Rev-CGCCCACATCTGCCGAGAT Probe-ATTAGTCATCCGTAATTCA	KF013247	100 μM	101	0.026 pg/μL

thermocycler with the following profile: 95 °C for 10 min, 14 cycles of 95 °C for 15 s and 60 °C for 4 min and a final hold temperature of 10 °C. The pre-amplified product was diluted 1:5 with low concentration (0.1 mM) EDTA buffer.

The assays and template DNA are loaded separately and mixed on the chip. The assays are loaded as a 10× concentration mix composed of 3 μL 2× Assay Loading Reagent (Fluidigm, PN 85000736) and 3 μL of 20× TaqMan Gene Expression Assay. The 20× TaqMan Gene Expression Assay is 18 uM each primer and 4 uM each probe in TRIS. The sample mix is 2.5 μL Applied Biosystems TaqMan Universal PCR Master Mix, No AmpErase UNG, 0.25 μL GE Sample Loading Reagent (Fluidigm, PN 85000746) and 2.25 μL diluted pre-amplified DNA. The temperature profile on the Biomark includes a 'thermal mix' and UNG-hot start period before the PCR cycles begin. The thermal mix was 2 min at 50 °C, 30 min at 70 °C and 10 min at 25 °C, which was followed by the UNG-hot start segment composed of 2 min at 50 °C and 10 min at 95 °C. Finally, 40 cycles of 95 °C for 15 s and 60 °C for 60 s was performed to amplify the DNA.

Results

Assay specificity

Specificity was demonstrated by testing for amplification with the DNA of 30 co-occurring fish species from the 'gold standard' panel. No signal was detected for any of these empirically validated species (those listed with *** in Table 1). They were validated *in silico* to have at least one polymorphism in the probe sequence from all SFE fish species. Some of these *in silico* tests were with locally sampled fish (those listed with ** in Table 1), and for

Table 3 Sampling locations for intraspecies validation

Common name (*Latin name*)	Sampling locations
Chinook Salmon (*Oncorhynchus tshawytscha*)	6 BC, 10 MRH, 10 MR, 7 DC, 4 SR, 3 SJR
Green Sturgeon (*Acipenser medirostris*)	27 SPB, 7 TW, 6 FW
Longfin Smelt (Spirinchus thaleichthys)	5 LW, 6 HR, 5 SM, 5 SSFB, 10 CI, 10 SFB
Largemouth Bass (*Micropterus salmoides*)	9 OR, 13 YB, 2 LNS
Rainbow Trout/ Steelhead (*Oncorhynchus mykiss*)	3 AR, 5 CHS, 3 EL, 4 PRHS, 5 HC, 7 MC, 8 KR, 3 CAS
Sacramento Pikeminnow (*Ptychocheilus grandis*)	4 DSC, 8 YB
Smallmouth Bass (*Micropterus dolomieu*)	9 MS, 2 SM, 1 LB
Sacramento Splittail (*Pogonichthys macrolepidotus*)	8 NR, 13 CV, 7 SJR, 9 YB
Striped Bass (*Morone saxatilis*)	6 OR, 10 YB, 10 DSC, 4 SB
Threadfin Shad (*Dorosoma petenense*)	23 YB, 3 LSR, 3 OR,2 SJRM, 1 CI, 1 LBS
White Sturgeon (*Acipenser transmontanus*)	40 SPB

Location guide: American River (AR), Butte Creek (BC), California Steelhead (CAS), Coleman Hatchery Strain (CHS), Chipps island (CI), Central Valley (CV), Deer Creek (DC), Deepwater Shipping Channel (DSC), Eagle Lake (EL), Fremont Weir (FW), Hot Creek (HC), Humboldt River (HR), Kern River (KR), Lake Berryessa (LB),Liberty Slough (LBS), Lindsey Slough (LNS), Lower Sacramento River (LSR), Lake Washington, WA (LW), Milestone Creek (MC), Merced River (MR), Merced River Hatchery (MRH), Miner's Slough (MS), Napa River (NR), Old River (OR), Pit River Hatchery Strain (PRHS), Suisun Bay (SB), San Francisco Bay (SFB), San Joaquin River (SJR), San Joaquin River at Mossdale (SJRM), Suisun Marsh (SM), San Pablo Bay (SPB), Sacramento River (SR), South San Francisco Bay (SSFB), Tisdale Weir (TW), Yolo Bypass (YB).

those SFE species we were unable to sequence, the *in silico* validation was performed with nonlocal sequences of SFE fish (those marked as * in Table 1). Lastly, the entire assay sequence diverged by at least 2% from any species with a remote possibility of occurring in the SFE.

Intraspecific validation

The assays were also verified to work on target species throughout the SFE watershed. Locations of the within-species validation samples are in Table 3. Some reactions failed as single technical replicates (<5%), although these samples amplified when technical replicates were made. Before using these assays in a different watershed, it would be prudent to verify the assays amplify the target species for the new locality.

Assay sensitivity

Serial dilutions were performed in the presence of 100 ng/μL nontarget template DNA. The lowest concentration of target template that was detected in at least seven of eight reactions was between 3.2 and 0.013 pg/μL (Table 2).

Conformity of high-throughput platform

Serial dilutions performed on the Fluidigm Biomark were made in the presence of 100 ng/μL nontarget template DNA, and the lowest concentration of target template that was detected in at least four of six reactions was between 3.2 and 0.64 pg/μL. Chinook Salmon, Green Sturgeon, Sacramento Pikeminnow, Sacramento Splittail, Smallmouth Bass, Striped Bass and Threadfin Shad were detectable at 0.64 ng/uL and Longfin Smelt, Rainbow Trout and White Sturgeon were detectable at 3.2 ng/uL.

Discussion

Using local sequences

Assays can rely on a single polymorphism to distinguish between related species, so using sequences from online databases (e.g. NCBI or the Barcode Of Life Database) may result in a nonspecific signal, particularly in a multi-species sample. To avoid false positives, we sequenced as many local samples as possible to verify the probe for each species is unique amongst local fish. This database provided confidence in the specificity of the assays for species we were unable to test empirically for cross-amplification.

'Gold standard' panel

Bringing a laboratory accustomed to genotyping up to forensics standards is not trivial. We spent a considerable amount of time tracking down sources of contamination and checking for cross-amplification. Ultimately, the 'gold standard' panel was required for expedient valida-

tion of these assays. We recommend this technique to any group using assays in a similar context.

High-throughput platform

The assays were marginally less sensitive on the Biomark system than with the 96-well format. Inconsistency of the Biomark platform to detect low levels of target DNA may be the result of stochastic amplification of samples at the lower limits of detection. The Biomark uses 6.7 nL volume reactions, which may be small enough to have uneven numbers of template in each reaction. However, it is worth noting the Biomark was at least as sensitive as the 96-well format if the criterion for a positive reaction is changed to amplification in one of six technical replicates.

Conclusion

Together, these assays represent many of the fish species of conservation and management interest in the SFE. Using these assays in combination with the high-throughput Biomark platform, we will be able to study fish diets on a broader scale than previously possible. Comparisons between native and non-native piscivores, as well as spatial and temporal relationships for specific predator–prey interactions, will provide important information to those interested in top-down trophic effects in the watershed.

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S.B. acquired, analysed and interpreted the data and drafted the manuscript. G.S. contributed ideas that were key to the study concept and design and provided support in the acquisition of data and in the analysis and interpretation of data. B.M.S. and J.L.C. provided critical revision and input for the study concept and design. B.M. contributed critical revisions and analysis and interpretation of data. M.R.B. provided the study concept and

design and assisted in drafting the manuscript, and analysis and interpretation of data.

Data Accessibility

Provided below are GenBank Accession nos. for the sequences the assays are based on as well as individuals used for *in silico* validation. See supporting information for sequence alignments and sample information.

Assays: KF013235, KF558288, KF013249, KF558313, KF558277, KF558298, KF558276, KF558274, KF013218, KF013247.

COI validation: KF558279.1, KF558287.1, KF558304.1, KF558302.1, KF558303.1, HQ557524.1, KF558297.1, HQ557218.1, KF558280.1, KF558296.1, KF558314.1, KF558315.1, EU523994.1, HQ971432.1, JN025112.1, JN025122.1, FJ164543.1, KF558283.1, KF558284.1, KF558307.1, KF558308.1, KF558305.1, KF558289.1, GU440367.1, KF558316.1, EU524714.1, KF558292.1, KF558286.1, KF558285.1, HQ579042.1, KF558298.1, KF558299.1, KF558300.1, EU524140.1, KF558274.1, KF558310.1, KF558311.1, KF558312.1, KF558313.1, KF558293.1, EU524238.1, KF558295.1, KF558294.1, KF558275.1, KF558276.1, KF558281.1, KF558282.1, KF558277.1, EU524337.1, KF558278.1

CYTB validation: KF013221.1, KF013239.1, KF013247.1, KF013228.1, KF013232.1, AY225665.1, AF454872.1, KF013233.1, KF013229.1, KF013230.1, KF013219.1, JX484684.1, JX484677.1, JX484657.1, AF370623.1, KF013225.1, KF013227.1, DQ010200.1, KF013248.1, KF013231.1, AF257125.1, KF013242.1, KF013234.1, KF013245.1, AY225708.1, KF013246.1, KF013213.1, KF013214.1, AY374295.1, KF013217.1, KF013240.1, KF013241.1, GU251088.1, KF013243.1, KF013244.1, KF013235.1, AF546115.1, KF013237.1, GQ275159.1, KF013216.1, KC686831.1, KF013223.1, KF013224.1, AY366273.1, KF013220.1