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Development of a molecular tool to identify brown bullhead catfish (*Ameiurus nebulosus*) from environmental DNA in water

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Cover picture: Brown bullhead catfish, *Ameiurus nebulosus*. Fork length 415 mm, 1035 g. Photo: Brendan Hicks.

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Executive summary

The aim of this work was to develop a molecular tool to positively identify brown bullhead catfish using environmental DNA (eDNA) that is suspended within the water column. The project had five main objectives: **1)** to develop a protocol for the collection of eDNA, **2)** to select and test primer sets that are specific to brown bullhead catfish, **3)** to optimise DNA extraction and amplification, **4)** to validate the methodology through sequencing the amplified DNA, and **5)** to evaluate its sensitivity by sampling at various sites that had a range of catfish abundances.

Water was sampled from Lake Rotoiti from a boat with a 1 litre labelled plastic container attached to a telescopic pole, retrieving water samples about 30 cm above the lake bottom in about 2 m of water depth and from deeper water. Each container was deployed container-opening facing down, and on reaching the desired collection depth, the opening was rotated uppermost to allow the entry of water. The full container was then pulled back to the surface, a plastic screw cap firmly screwed onto the container, and then subsequently placed in a chilly bin on a 20-cm layer of crushed ice. The collection was recorded on a GPS instrument.

The biggest challenge with the environmental samples are the inhibitors they contain that will affect the efficiency of the polymerase chain reaction. Much of the projects time involved developing methods to remove inhibitors to the Taq polymerase that is essential to the PCR process. A methodology has been devised that has a repeatable and stable protocol to remove inhibitors from water samples collected for eDNA analysis which involves three steps: **1)** phenol-chloroform extraction of eDNA, **2)** electrophoresis of the eDNA and removing part of the gel containing the band of amplified DNA, and **3)** centrifugation of the gel piece at 16,000 G to release the eDNA into a supernatant for amplification and final quantification by real-time qPCR.

Sequenced eDNA from Lake Rotoiti was specific to the genus *Ameiurus* with a 98.5 to 99.0% probability of it originating from brown bullhead catfish (*Ameiurus nebulosus*). This confirmed that a repeatable method for determining the presence of brown bullhead catfish in the Te Arawa (Rotorua) lakes, had been devised. Primer analysis shows they are not completely specific for brown bullhead catfish and are also able to pick up two other *Ameiurus* catfish species (*A. natalis*, yellow bullhead catfish and *A. melas*, black bullhead catfish). Fortunately, it is known that New Zealand only has *Ameiurus nebulosus*, so cross matching with other *Ameiurus* species does not affect the reliability of these primers for detecting brown bullhead catfish in the Te Arawa lakes and the techniques developed are fit for purpose.

Comparison of amplified and sequenced eDNA from sites with a range of catfish abundances suggests that a C_t (cycle threshold) value of about 38 reflects the presence of brown bullhead catfish using qPCR and the lowest amount of eDNA present for reliable detection. The positive signal from the eastern shoreline site suggest that catfish are now there at low abundance. The lack of an eDNA signal from Okawa Bay in April 2019, where catfish had a mean catch rate of 2.1 fish net^{-1} night^{-1} in 2018, may be attributable to seasonal movements of catfish to deeper water in cool weather as catfish appear to use deeper water in winter.

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1. Introduction

The North American brown bullhead catfish (*Ameiurus nebulosus*) was introduced to NZ in 1877 and is recognised internationally as an invasive species. This catfish species in New Zealand is commonly 200–300 mm in length but can grow to 480 mm and more than 2 kg in weight in New Zealand (McDowall 1990). Catfish are primarily nocturnal bottom feeders that eat a diverse range of food. They prefer shallow, weedy habitat in lakes and rivers, and are usually found in the littoral zone at depths of <6 m (Dedual 2002). In the Waikato, catfish have been recorded at 455 mm and 1630 g at 7 years of age (Patchell 1977). Catfish in NZ present potential adverse ecological impacts on lakes due to their benthic feeding, which can add to nutrients and sediment to the water column. They also prey on and compete with native crayfish (kōura) and other fish. Catfish are tolerant of turbid and eutrophic water quality, low dissolved oxygen concentrations, and a wide range of water temperatures. They are sexually mature at 2 years of age (about 180-220 mm long) and produce a few hundred to 6,000 eggs per female (McDowall 1990). Brown bullhead catfish have a wide thermal tolerance. Optimal growth occurs at 29 to 30°C with preferred temperatures of 26 to 31°C. The critical thermal maximum is 38°C, and though the minimum spawning temperature is 14°C, the optimum spawning temperature is 21°C, with an optimum of 23°C for egg development (**Fig. 1**). Peak gonad development usually occurs in September, followed by spawning in spring and early summer. The prolific breeding that was seen as a pulse of juveniles in Lake Rotoiti in summer in January-May 2018 was almost certainly a result of a 4-5°C anomaly of warmer water temperatures in Lake Rotoiti in November and December 2017 (Hicks and Allan 2018). Catfish are a significant threat to kōura (freshwater crayfish) in NZ (Barnes and Hicks 2003) and are found throughout the Waikato River system including Lake Taupō. They are tolerant of high and low water temperatures and low dissolved oxygen, and are very tolerant of pollution.

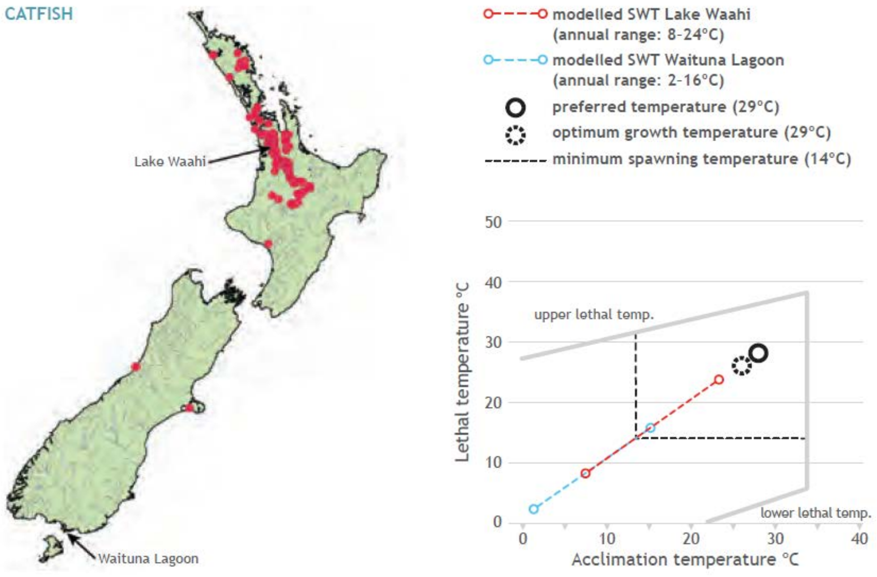


Figure 1. Thermal tolerance polygon, minimum spawning temperature, and New Zealand distribution for catfish, *Ameiurus nebulosus*. SWT = surface water temperature. Source: Collier et al. (2015).

Catfish have most likely been in Lake Rotoiti for more than 20 years, due to juvenile catfish observed to fall out of a hollow-framed boat trailer after a boat launching in 1995. This boat had been parked on its trailer overnight in Lake Taupo at Motuapa, where catfish are abundant, some hours before the boat was driven to and launched in Lake Rotoiti. There were no further confirmed sightings of catfish in Lake Rotoiti until January 2009 when a dead adult catfish about 450 mm long was found on the shore (Blair and Hicks 2009). However, in 2015, the owners of Lake Rotoiti hot pools reported seeing live catfish, and in March 2016, catfish were caught first by a weed harvester and then by fyke netting in Te Weta Bay, Lake Rotoiti. Since then, catfish have been found more widely in Lake Rotoiti, in the Ohau Channel (January-May 2018) and in Lake Rotorua in December 2018 (Francis 2019).

Currently, the principal method of determining the spread of catfish in the Rotorua lakes has been overnight sets of fyke nets. As effective as this method has been, with over 41,802 catfish caught in about 6,931 net nights between March 2016 and September 2018 (Francis 2019), it is very laborious and is dependent on the number of nets available, weather, and staff time to set and retrieve nets. It is critical that the Bay of Plenty Regional Council develops a technique that can identify catfish rapidly from a water sample that will allow determination of the presence or absence of catfish from a single site visit. Overseas, investigators have used eDNA techniques to determine the presence of brown bullhead catfish from water samples (e.g., Béres et al. 2017). Due to this, the Bay of Plenty Regional Council (BOPRC) contracted the University of Waikato to develop a molecular method to positively identify brown bullhead catfish from eDNA in water.

2. Development of a protocol for identification of catfish by eDNA

The aim of this work is to develop a molecular method to positively identify brown bullhead catfish using environmental DNA (eDNA) that is suspended in the water column. The project had five main objectives: **1)** to develop a protocol for the collection of eDNA, **2)** to select and test primer sets that are specific to brown bullhead catfish, **3)** to optimise DNA extraction and amplification, **4)** to validate the methodology through sequencing the amplified DNA, and **5)** to evaluate its sensitivity by sampling at various sites that had a range of catfish abundances.

Selection of control A. nebulosus DNA and primers

For initial tests of the the eDNA extraction protocol, 50 mL of water from a 5,000 litre tank that held 4 catfish (about 2 kg in total weight) for about 3 months. Adipose fin tissue was also taken from one of these catfish to extract tissue DNA to act as the positive control, to test the general technique, and to compare amplicon length. We initially used the universal primers of Ivanova et al. (2007) (FF2d 5’-TTCTCCACCAACCACAARGAYATYGG-3’ and FR1d 5’-CACCTCAGGGTGTCCGAARAAYCARAA-3’), which amplify a 708-bp region of the cytochrome c oxidase 1 (COI) gene from the brown bullhead catfish. We also used the primers of Béres et al. (2017) (Neb_F 5’−CTGCTACATGCTAAGGCTAACG−3’ and Neb_R 5’−GGATTATTGTGGCGATTGGT−3’) to amplify a 457-bp region of the cytochrome-B (Cyt-B) gene.

Because these amplified products are too long for qPCR, which needs a <300 bp product, we also used the primers of Clusa and García-Vázquez (2018) (Am-16S-F 5’−CGTCAAGAACYCAGTTRAACT−3’ and Am-16S-R 5’−GWTTCTGYGACTTAGAGTTGTCA−3’), amplify a 134-bp region of the 16S ribosomal DNA (16S rDNA) gene, to amplify extracted eDNA from the tank water and DNA extracted from catfish fin tissue samples. The amplified DNA was electrophoresed in a 2% agarose TAE gel at 110 V for 30 min (TAE agarose gel buffer comprised 100 mM Tris base, 50 mM sodium acetate, and 10 mM EDTA at pH 8.8). After electrophoresis, the amplified DNA in the gel was visualised by ethidium bromide staining using UV excitation (312 nm wavelength).

Each gel that was used to confirm the amplicon size also included a GenScript PCR DNA ladder that allowed the size of the amplified DNA to be estimated as it comprises nucleic acid fragments of specific base-pair sizes incrementing in steps of 100 bp from 100 to 1,500 bp. The 500 bp band of the ladder is more intense so that it is easily distinguishable as a reference point.

Isolation of eDNA from Lake Oranga

Initially, three 500-mL water samples were taken from the hypertrophic and highly turbid Lake Oranga on the University of Waikato campus, which has about 51 kg/ha of brown bullhead catfish (Hicks et al. 2015). Approximately 300 mL of water was filtered through through a Whatman glass-fibre filter with a 0.8-µm pore size until the filter blocked. From this filter, 30 µL of eDNA with ~20 ng/µL of DNA (~600 ng in total) was extracted. Initial PCR amplification by both sets of primers was negative, due to the probable presence of inhibitors that were co-extracted with eDNA. This PCR inhibition was removed using a combination of phenol-chloroform extraction followed by agarose gel purification of the eDNA. This then allowed the specific amplification of catfish DNA.

Filtration of eDNA

eDNA extraction began with water being filtered through a Whatman glass-fibre filter (60-mm diameter, 0.8-µm pore size) under vacuum. The filter equipment, including a 2-L side-arm Büchner flask and attached glass filter funnel, is shown in **Fig. 2**. Depending on the amount of suspended material in the water, 300-1000 mL of the water sample could be filtered before the glass-fibre filter blocked. This was a successful collection method because all of the eDNA binds or adsorbs to the silica in the glass-fibre filter.

Figure 2. Vacuum system used to filter water sample through a glass-fibre filter.

Extraction of eDNA from the filter

Following eDNA filtration, the filter was placed in a sterile 50-mL centrifuge tube containing 1.5 mL of cetyl trimethyl ammonium bromide extraction (CTAB) solution (10% W/V stock solution, diluted to a final CTAB concentration of 1%) and the filter was washed at 65ºC for 1 h. This solution was removed and saved. The washing step was repeated with an additional 1 mL of warm (65ºC) CTAB extraction solution. Both collected solutions combined and mixed with an equal volume of 50:50 phenol:chloroform. This solution was shaken vigorously for 20 s and placed on a rotating wheel for 10 min. The tubes were then centrifuged at 16,000 x g for 10 min and the supernatant containing the DNA was removed. To 0.7 mL of this solution, 70 µL of 10% SDS and 77 µL of 5-M NaCl was added; the final solution was incubated at 65ºC for 10 min. Once the solution had cooled, an equal volume of chloroform was added to it and the solution shaken vigorously for 10 mins and centrifuged at 16,000 x g for 10 min. Eight hundred µL of the supernatant was removed and placed into a new tube and the DNA was precipitated with an equal volume of 10% polyethylene glycol containing 1.2 M NaCl overnight at 4ºC. The precipitated DNA was recovered using centrifugation at 16,000 x g for 10 min and the DNA pellet was washed twice with 1 mL of 80% ethanol by centrifugation using 16,000 x g for 2 min. Samples were left to air dry to remove any remaining ethanol and the DNA was then dissolved in 40 µL of TE buffer (10-mM tris and 1 mM EDTA at pH 8.0), and the DNA concentration and the 260/280 and 260/230 ratios were measured using a Nanodrop spectrophotometer.

The positive control containing catfish fin DNA showed an amplified band whereas the the eDNA samples known to contain catfish DNA failed to amplify, most likely because it was inhibited. Isolation of eDNA is often associated with inhibition of the PCR amplification (Hunter et al. 2019, Sanches and Schrier 2020). This necessitated the development of an additional cleaning step before amplification by PCR. The actual PCR inhibitors involved were not identified, but the combined phenol-chloroform extraction followed by gel electrophoresis were sufficient to overcome inhibition of the PCR in all water samples. The gel-electrophoresis protocol for clean-up of extracted eDNA involved agarose gel electrophoresis of the eDNA as described above with the DNA band being excised from the gel and centrifuged at 16,000 x g for 5 min to collect the eDNA. Two volumes of 100% ethanol was added to the eDNA to precipitate the DNA by incubating at -20ºC for 1 h and the DNA recovered by centrifugation at 16,000 x g for 15 min. The DNA pellet was then washed with 1 mL of 70% ethanol and finally resuspended in 10 µL of tris-EDTA (TE) buffer. Two µL of this DNA was then used for amplification with both conventional PCR and qPCR.

Select and test primer sets that are specific to brown bullhead catfish by qPCR

Once it was clear that inhibition was removed and that the eDNA was able to be amplified, work began developing the qPCR protocol. The primers known to amplify a 134 bp region of the *Ameiurus* spp. mitochondrial 16S ribosomal DNA, Am-16S-F and Am-16S-R, (Clusa and García-Vázquez 2018), were tested.

The sensitivity of detection of the catfish DNA within qPCR was improved by using SYTO™ 82 nucleic acid stain for fluorescent detection of amplified DNA. SYBR® Green was avoided as the fluorescent dye due to its tendency to inhibit qPCR. Solis Biodyne Hot FirePol Taq polymerase was used to make up the qPCR master mix (**Tables 1** and **2**), where 20 µL of the 200 µL qPCR stock (Table 2) was added to 2 µL of extracted eDNA (equivalent to 40-100 ng eDNA). Further confirmation of specific amplification within qPCR was achieved by amplicon melting temperature, checking the length of the amplicon by gel electrophoresis, and DNA sequencing of the amplificon. To test for contamination of our qPCR reagents, a negative control that contained everything but the template was included within each qPCR run in addition to a positive control to control for amplicon inhibition.

Table 1. Initial qPCR master mix.

Components	Amounts (mL)	Final concentrations
10x concentrated buffer B2	1	1x
25 mM $MgCl_2$	0.8	4 mM
10 mM dNTPs	0.25	250 mM
DEPC-treated water	7.95	

Table 2. qPCR mix to make up 200 μL of working concentration. Each qPCR reaction contains 20 μL of this mix.

Components	Amounts (μL)	Final concentrations
qPCR master mix	200	
10 μM Forward primer	2	~0.1 μM
10 μM Reverse primer	2	~0.1 μM
Taq polymerase (5 U/μL)	1.5	~0.4 U
5 mM SYTO^{TM} 82	0.4	~10 μM

Conventional PCR

Before commencing qPCR, we conducted conventional PCRwith two positive controls (firstly, with known catfish DNA only, and secondly, the extracted eDNA together with the known catfish DNA to check for inhibition of amplification), tubes with the extracted eDNA samples, and a negative control with all the reagents but no DNA. To further prevent amplification of non-specific products, a touchdown programme from 65^oC to 55^oC was used to enable specific annealing of primers. The entire conventional PCR programme consisted of: 95^oC for 15 min, followed by 10 cycles of 95^oC for 15 s, 65^oC for 15 s with a decrease of 1^oC/cycle, and 72^oC for 20 s, followed by 35 cycles of 95^oC for 15 s, 55^oC for 15 s, and 72^oC for 20 s. The amplicons were visualised by TAE agarose electrophoresis containing ethidium bromide at 1 µg/mL as the nucleic acid stain. The TAE buffer was composed of 100 mM Tris base, 50 mM sodium acetate, and 10 mM EDTA at pH 8.8). If the positive control with known catfish DNA and eDNA was inhibited (i.e., failed to amplify), we proceeded to remove the inhibition before the qPCR.

qPCR method

A Corbett Rotogene 6000 was used for qPCR amplification of the eDNA with the specified primers. a The same touchdown programme as for conventional PCR was used to enable specific annealing of primers. The qPCR programme consisted of: 95^oC for 15 min, followed by 10 cycles of 95^oC for 15 s, 65^oC for 15 s (with a decrease of 1^oC/cycle), 72^oC for 20 s, followed by 35 cycles of 95^oC for 15 s, 55^oC for 15 s, 72^oC for 20 s, and 80^oC for 10 s. Signal acquisition occurred during the 72°C and at 80°C steps. A melt curve analysis was programmed to occur within a range of 80°C to 90°C to check the specificity of amplification. Each qPCR run contained a positive control with DNA extracted from fin tissue to check for inhibition and a negative control with all the reagents but no DNA to check for contamination.

Purifying eDNA for sequencing

Gel electrophoresis of the amplified eDNA from Lake Oranga field samples indicated the presence of minor nonspecific DNA in addition to the 134 bp amplicon, which prevented direct sequencing of this amplicon to confirm its identity. To remove the nonspecific amplified DNA for sequencing, which would confound downstream Sanger sequencing, we developed a nested PCR approach with custom-designed 16S-rDNA outer primers (CFOF 5'-GCA GAA GCG GAC ATA CAC CT-3', CFOR 5'-GGC AGG TAG CCG GAT CTT TT-3') to initially amplify a larger outer 227 bp fragment that incorporated the 134-bp target within its sequence. The outer primers were used to pre-amplify the purified eDNA for 20 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 20 s. 0.5 µL of this pre-amplified DNA was then used as the template for the second round of qPCR amplification using primers Am-16S-F and Am-16S-R using the above programme for 30 cycles. This amplified the 134-bp fragment was purified for sequencing by treating with exonuclease I and alkaline phosphatase. The digested amplicon was then sent to the Massey Genome Service, Massey University for sequencing. BLAST (Basic Local Alignment Search Tool) analysis of the sequences received from Massey University confirmed the identity of *A. nebulosus*.

3. Application of the eDNA methodology to identify catfish in Lake Rotoiti

Initial sequence amplification of eDNA from Lake Rotoiti

Water was sampled from Lake Rotoiti from a boat with a 1-L labelled plastic container attached to a telescopic pole (Mighty Gripper KG H3 three-stage telescopic handle, 1100 to 2800-mm long, from Envco Global Ltd, NZ). During sample collection, sediment introduction was avoided to limit the blocking of the filter and fresh nitrile gloves were worn for each sample to avoid cross contamination between samples. Water was retrieved from about 30 cm above the lake bottom in about 2 m of water depth and from deeper water (**Fig. 2**). Each container was deployed with container opening facing down, which was rotated to be upper-most to allow the entry of water on reaching the desired collection depth. The full container was then pulled back to the surface, a plastic screw cap firmly screwed onto the container, which was subsequently placed in a chilly bin on a 20-cm layer of crushed ice. The position where the sample was taken from was recorded on a CX-60 Garmin GPS instrument (**Fig. 3**).

Samples were transported back to the laboratory on ice for processing within six hours of collection and immediately underwent filtration and eDNA extraction as outlined on p9 in the section *Extraction of eDNA from the filter*.

Figure 3. Water sample collection system, showing a 1-L plastic screw-top container mounted on a 3-m telescopic pole before rotation for filling.

Figure 4. Global positioning system locations of water sample collection sites for eDNA in Te Weta Bay, Lake Rotoiti, showing approximate water column depth in metres on 13 September 2018.

To confirm the identity of the amplified eDNA, the sequenced qPCR product amplicon was aligned to referenced sequences from GENBANK (NC_036387.1, KR476911.1, KR476830.1) using Geneious 11.14. A consensus sequence alignment was created from the eDNA samples amplified by the second-round primers (Am-16S-F and Am-16S-R) from the three sites in Te Weta Bay, Lake Rotoiti. The water samples were from sites about 2 m deep where large (>200 mm fork length) catfish had been captured (CF1L) and where small (<150 mm fork length) catfish had been captured (CF2S). eDNA extracted from a third water sample from water too deep for conventional fyke netting (about 4 m deep) close to the containment net (CF3N).

The consensus sequences were then entered into a BLAST search through the NCBI web-based procedure (https://blast.ncbi.nlm.nih.gov/Blast.cgi), in order to identify if they were related to known brown bullhead catfish sequences, already within the nucleotide database.

Test field sensitivity of the qPCR technique along a gradient of catfish abundances

To further evaluate the sensitivity of our eDNA collection and extraction techniques sites in Lake Rotoiti that were known to have mean catfish catch rates that ranged from 0 to 64 catfish net^{-1} night^{-1} in 2018 (**Fig. 5 and 6**, GPS points 431-439), were sampled. Sites in Lake Rotorua on the eastern shore and near Mokoia Island **(Fig. 6**, GPS points 440-442) were also sampled for analysis.

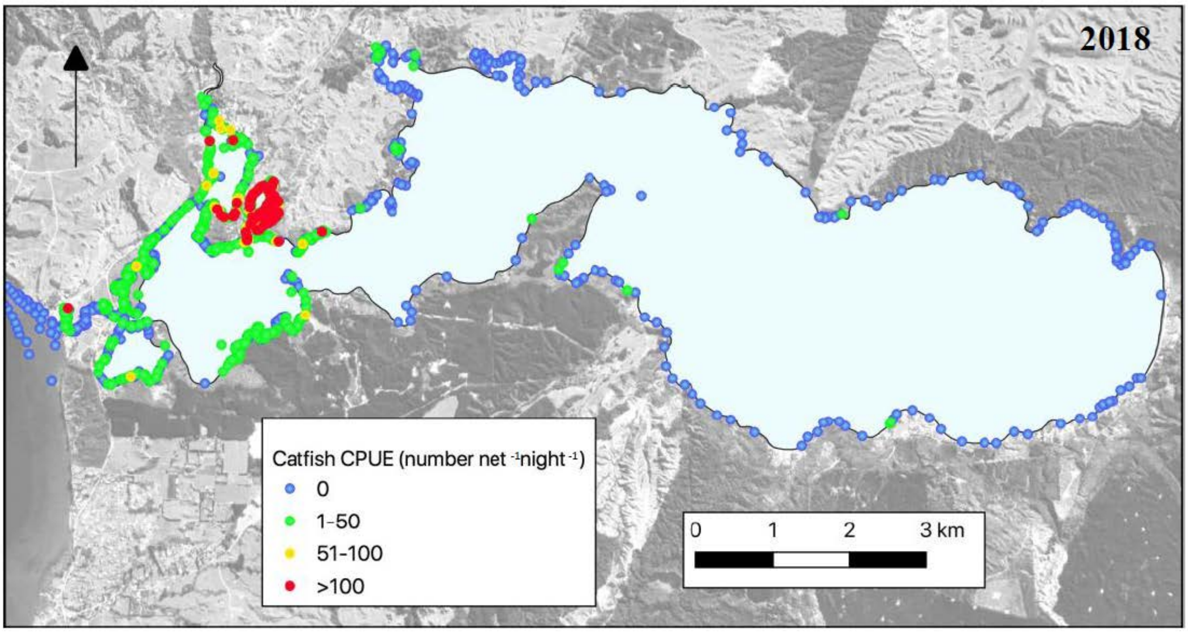


Figure 5. Catfish catch rates from fyke nets set in Lake Rotoiti in 2018 during routine monitoring for catfish. Source: Francis (2019).

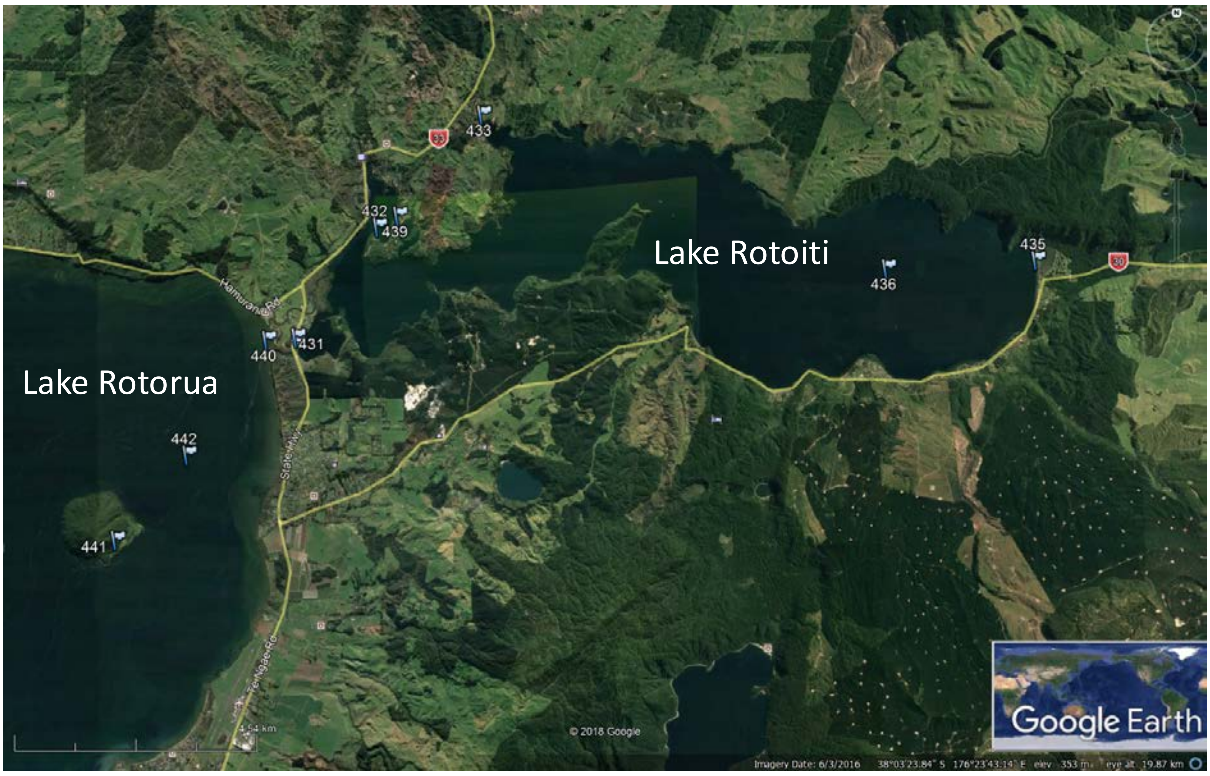


Figure 6. GPS locations of sites at which 1-L water samples were collected for catfish eDNA sampling on 16 April 2019.

Technology transfer

The future plan is to involve BOPRC staff in the collection process to begin the technology transfer for sample collection if required.

4. Results and discussion

Recovery and amplification of catfish eDNA from tank water and Oranga Lake

The primers of Ivanova et al. (2007) amplified a 708 bp amplicon from COI primers with conventional PCR (**Fig. 7**). The ladder refers to a 1500-base DNA ladder, which is a sequence of base-pair size markers from 100 to 1500 bases in 100-base increments. These show as discrete bands, with the lightest (smallest) sizes migrating the furthest from the loading wells at the top of the gel. In contrast, primers Am-16S-F and Am-16S-R amplified a product of 134 bp of the mitochondrial 16S-rDNA sequence of the brown bullhead catfish (*Ameiurus nebulosus*).

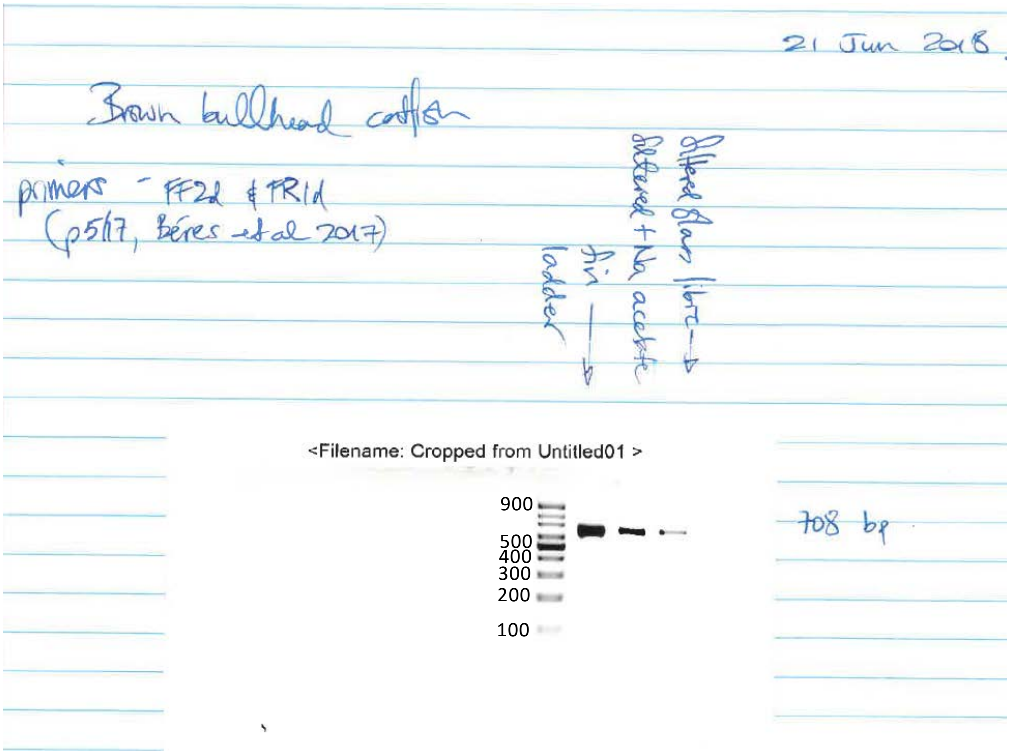


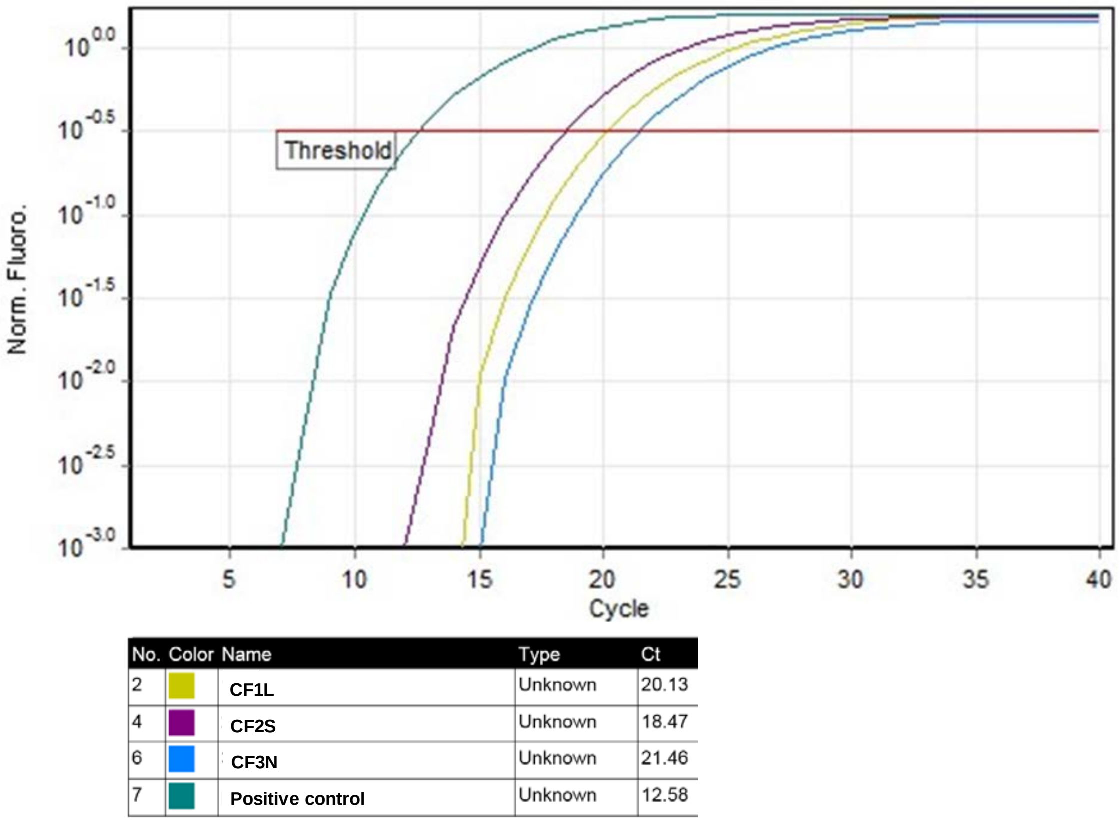
Figure 7. Electrophoresis gel showing amplicons for brown bullhead catfish generated using primers FF2d and FR1d (Ivanova et al. 2007; Béres et al. (2017) that confirmed successful extraction of eDNA from tank water samples. The sizes of the bands on the ladder are the number of bases for each band.

Amplification of catfish eDNA from Lake Oranga

Oranga Lake on the University of Waikato campus, which has about 51 kg/ha of catfish, also has several other fish species (goldfish, koi carp, shortfin eels, gambusia, and common bullies), abundant zooplankton, phytoplankton, bacteria, and suspended sediment was used as the proof of concept. These other biological species provide a background of competing DNA that can potentially confound species-specific eDNA identification. The first hurdle was the recovery of the eDNA from the lake water, which led to the successful use of the glass fibre filters with a 0.8-µm pore size. In addition, components from the water and suspended sediment contained inhibitory molecules, preventing direct amplification of eDNA using a qPCR approach. To remove this inhibitory effect, 20 µL of extracted eDNA underwent electrophoresis on a TAE gel. The DNA band was removed in a gel fragment and then underwent centrifugation. The liquid that was centrifuged out of the gel fragment containing the eDNA was then precipitated in ethanol, washed and resuspended in 10 µL of TE buffer. Two µL of this eDNA was used as the template for qPCR. The eDNA from Oranga Lake was amplified by qPCR and the primers Am-16S-F and Am-16S-R. Successful amplification at a modest C_t (cycle threshold) detection of 19-22. A C_t of 22 is well within acceptable limits of qPCR sensitivity.

Initial field test of the methodology with water samples from Lake Rotoiti

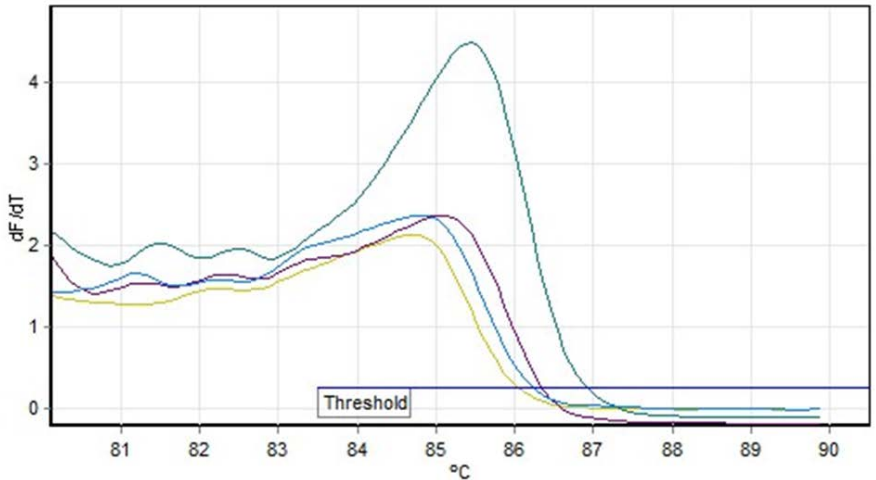
Duplicate water samples were collected from three sites in Te Weta Bay, Lake Rotoiti (**Fig. 3**). These sites were selected because it has the greatest catch rates of catfish in Lake Rotoiti. C_t values for the eDNA samples from Te Weta Bay were between 18.5 to 21.5 compared to the positive control, which had a C_t of 12.6 (**Fig. 8**).



No.	Color	Name	Type	Ct
2	🟨	**CF1L**	Unknown	20.13
4	🟪	**CF2S**	Unknown	18.47
6	🟦	**CF3N**	Unknown	21.46
7	🟩	**Positive control**	Unknown	12.58

Figure 8. qPCR amplification of bullhead catfish DNA, using eDNA from water samples from Te Weta Bay, collected on 13 Sept 2018. The cycle threshold (C_t) was read at the fluorescence of $10^{-0.5}$. Positive control was from fin tissue.

Melt curves of the amplified products were collected between 80 and 90ºC and compared, further confirming the successful amplification of the brown bullhead catfish eDNA (**Fig. 9**).



No.	Color	Name	Genotype	Peak 1
2	🟨	**CF1L**		84.700
4	🟪	**CF2S**		85.075
6	🟦	**CF3N**		84.800
7	🟩	**Positive control**		85.425

Figure 9. Melt curves obtained between 80-90ºC for the PCR products obtained from the eDNA samples. Results show a similar profile for the positive control (fin tissue) and eDNA samples, indicating successful amplification of the correct product.

Nesting amplified eDNA for identification by DNA sequencing

Nested PCR was necessary for field DNA samples because of the minor smearing seen above the 227 band (**Fig. 10**). An initial band of 227 bp was amplified using primers CFOF and CFOR (**Fig. 10**). 0.5 µL from this first round of amplification was then used as the template to amplify a band of 134 bp, using second-round primers Am-16S-F and Am-16S-R, which was then quantified using nanodrop (**Table 3**) and sent to Massey University for sequencing.

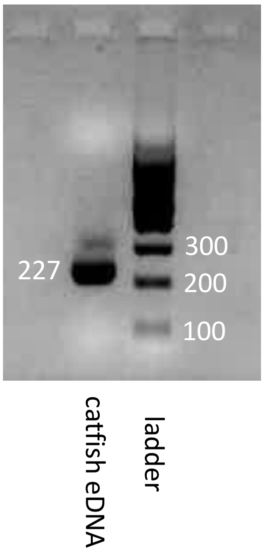


Figure 10. Electrophoresis gel showing the 227 bp amplicon produced using CFOF and CFOR with a DNA ladder for reference. Numbers refer to bases in the the amplicon (catfish eDNA) and nucleic acid fragments in the ladder.

Table 3. Nanodrop DNA quantitation showing 280/260 and 260/230 ratios.

		Absorbance		Ratios	
Sample	Nucleic acid (ng/µL)	A260	A280	260/280	260/230
CF1L	117.3	2.347	1.236	1.90	1.98
CF2S	29.8	0.596	0.303	1.97	2.39
CF3N	90.1	1.802	0.935	1.93	2.09

BLAST analysis of the sequenced eDNA

Consensus sequences were obtained through the alignment of multiple sequences obtained from each site (**Table 3**). These consensus sequences were then aligned to each other (**Fig. 11**) and also were used in BLAST analysis, showing the DNA had identities of between 98.5-99.0% to the 16S rDNA gene of *Ameiurus nebulosus* with GenBank sequence numbers of NC_036387.1, KR476911.1, and KR476830.1). There were some cross matches with two other catfish species, showing that the amplified eDNA was entirely consistent with the genus *Ameiurus* but could not be distinguished from *A. natalis* (yellow bullhead catfish) and *A. melas* (black bullhead catfish).

Table 4. Sequences of eDNA samples extracted from three sites in Te Weta Bay, Lake Rotoiti where large (CF1L) and small (CF2S) catfish were caught or in deeper water (about 4 m) by the containment net (CF3N).

Sequence Name	**Sequence**
CF1L (130bp)	TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCTAAGTCRCAGA
CF2S (133bp)	TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCTAAGTCRCAGAAWC
CF3N (136bp)	TCGTCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCTAAGTCRCAGAAWC

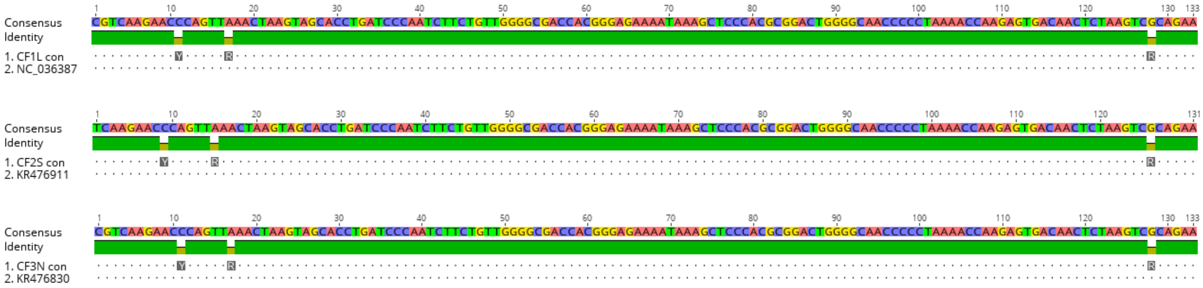


Figure 11. Consensus sequence alignments for eDNA samples extracted from three sites in Te Weta Bay, Lake Rotoiti where large (CF1L con) and small (CF2S con) catfish were caught and in deeper water (about 4 m) by the containment net (CF3N con).

Comaprison of field sensitivity of the qPCR technique with a gradient of catfish abundances in Lake Rotoiti

Low DNA yields from sampling on 16 April 2019 meant that sites with low catfish abundance in 2018 (LROI03 and LROI04) failed to produce a DNA signal by qPCR (**Table 5**). From the four sites that had both DNA products and an estimate of catfish abundance (**Figure 12**) we calculated a log-linear equation of $C_t = -0.817\ X + 38.1$ ($r^2 = 0.947$), where C_t = cycle threshold from qPCR and X = natural log of catfish catch rate in fish net^{-1} night^{-1} +1.

Table 5. qPCR results for catfish eDNA in water samples collected from Lake Rotoiti on 16 April 2019 compared to site-specific catfish catch rates in 2018, including sites with no eDNA signal (–).

Location	Sample code	Ray's code	Way point	Description	Catfish catch rate in 2018 (fish net^{-1} night^{-1})	Cycle threshold (C_t)	Melt temperature (°C)
Lake Rotoiti	LROI01A & B	1B	431	Okawa Bay	2.1	–	–
Lake Rotoiti	LROI02A & B	2B	432	Okere Arm	17.5	35.4	85.8
Lake Rotoiti	LROI03A & B	3B	433	Te Karaka Bay - Otaramarae	0.2	38.4	85.9
Lake Rotoiti	LROI04A & B	4B	435	Eastern shoreline	0	–	–
Lake Rotoiti	LROI04A & B	4Bresin	435	Eastern shoreline	0	37.8	85.2
Lake Rotoiti	LROI05A & B	5B	436	Middle of eastern basin		–	–
Lake Rotoiti	LROI06A & B	6B	439	Te Weta Bay jetty - same as TEW2 13 Sep 2018	63.7	34.9	85.3
Lake Rotoiti	LROI06A & B	6Bresin	439	Te Weta Bay jetty - same as TEW2 13 Sep 2018	63.7	–	–
Lake Rotorua	LROI07A & B	rot1	440	Eastern shore		–	–
Lake Rotorua	LROT02A & B	rot2	441	Mokoia Island		35.9	85.8
Lake Rotorua	LROT03A & B	rot3	442	Deep water between Mokoia Island and Ohau Channel		37.4	85.6

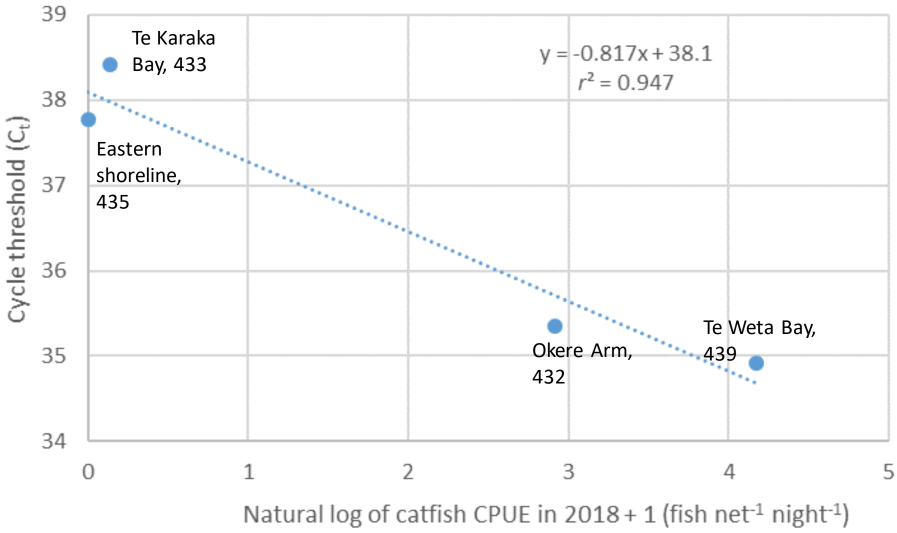


Figure 12. qPCR results for catfish eDNA in water samples collected from Lake Rotoiti on 16 April 2019 compared to site-specific catfish catch rates in 2018. Numbers refer to GPS locations in Fog. 6.

5. Conclusions

The successful amplification of catfish sequences from Lake Rotoiti with 98.5 to 99.0% identity to the 16S rDNA mitochondrial gene from the genus *Ameiurus* confirms a repeatable method for determining the presence of brown bullhead catfish in the Te Arawa (Rotorua) lakes using eDNA. Although the primers used in this study (Am-16S-F and Am-16S-R, Clusa and García-Vázquez 2018) were found to share identity with two other *Ameiurus* catfish species (*A. natalis*, yellow bullhead catfish and *A. melas*, black bullhead catfish), *A. nebulosus* is the only member of this genus within NZ, so this molecular detection method is specific for brown bullhead catfish here.

It has been well reported that most environmental water samples collected will contain inhibitors of some kind to the polymerase chain reaction (e.g., Clusa and García-Vázquez 2018; Hunter et al. 2019; Sanches and Schrier 2020). Much of the time in this project was consumed in developing a technique to remove amplifcation inhibitors from the eDNA samples to allow the Taq polymerase to carry out its activity, which is essential to the PCR process. We have devised a repeatable and stable protocol to remove inhibitors from water samples collected for eDNA analysis. This involves three steps: **1)** phenol-chloroform extraction of eDNA, **2)** electrophoresis of the eDNA and removing part of the gel containing the band of amplified DNA, and **3)** centrifugation of the gel piece at 16,000 G to release the eDNA contained in the gel into a supernatant for amplification and final quantification by real-time qPCR.

Comparison of eDNA from sites with a range of catfish abundances suggests that a C_t value of about 38 reflects the lowest amount of eDNA showing detection of brown bullhead catfish by the qPCR method. The positive signal from the eastern shoreline site suggest that catfish are now there at low abundance, despite the zero catch rates up to 2018. The lack of an eDNA signal from Okawa Bay in April 2019, where catfish had a mean catch rate of 2.1 fish net^{-1} night^{-1} in 2018, may be attributable to seasonal movements of catfish to deeper water in cool weather as catfish appear to use deeper water in winter (Dedual 2002). Additionally, eDNA techniques, like all sampling techniques, are vulnerable to false negatives for a variety of reasons, including fish movement and eDNA degradation.

We are now in a position to transfer the water sample collection protocols to Bay of Plenty Regional Council staff.

6. Acknowledgements

This research was funded by Bay of Plenty Regional Council. We thank Rob Donald of Bay of Plenty Regional Council for his review. Olivia Patty performed the consensus alignments and BLAST analyses. Geoff Ewart’s assistance was essential for aligning collection sites to known catfish abundance. We are deeply indebted to Steve Bird for his review, which improved the final version considerably.

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Appendix 1. How to read the BLAST information in Appendices 2-4.

```
>NC_036387 Ameiurus nebulosus strain NEFC_F16-028 mitochondrion, complete genom
e

Length = 16512

E-value = 1.05e-56, Score = 124, Bitscore = 230.105, Identities = 127/130 (97%),
Positives = 130/130 (100%), Gaps = 0/130 (0%)
Frame = +1
CF1L consensus      1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG   60

                      TCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG

NC_036387        2278 TCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 2337

CF1L consensus     61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT  120

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NC 036387        2338 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 2397

CF1L consensus    121 AAGTCRCAGA  130
                      AAGTC+CAGA
NC_036387        2398 AAGTCGCAGA 2407
```

Matched reference

Expect value (E) describes the number of hits one can "expect" to see by chance when searching a database of a particular size. The lower the E-value, or the closer it is to zero, the more "significant" the match is.

Identities is an actual count of the number of matching bases between the CF1L consensus and the reference it matched to.

Alignment: first line is the CF1L consensus, second line shows the differences between the 2 sequences, third line the reference sequence.

The 3 '+' in the alignment show why identity is 97%, the nucleotides at these positions in the CF1L consensus were unable to be discerned due to poor sequencing quality at that spot or heterogeneity.

Appendix 2. BLAST (basic local alignment search tool) results from CF1L consensus sequence (top 10 results)

```
1) >NC_036387 Ameiurus nebulosus strain NEFC_F16-028 mitochondrion, complete genome

Length = 16512

E-value = 1.05e-56, Score = 124, Bitscore = 230.105, Identities = 127/130 (97%),
Positives = 130/130 (100%), Gaps = 0/130 (0%)
Frame = +1

CF1L consensus      1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG   60
                      TCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG
NC_036387        2278 TCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 2337

CF1L consensus     61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT  120
                      GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT
NC_036387        2338 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 2397

CF1L consensus    121 AAGTCRCAGA  130
                      AAGTC+CAGA
NC_036387        2398 AAGTCGCAGA 2407
```

View GenBank record on NCBI website (opens browser)

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LOCUS       NC_036387              16512 bp ds-DNA     linear   VRT 24-AUG-2018
DEFINITION  Ameiurus nebulosus strain NEFC_F16-028 mitochondrion, complete
            genome.
ACCESSION   NC_036387
VERSION     NC_036387.1  GI:1314948533
DBLINK      BioProject: PRJNA420704
KEYWORDS    RefSeq.
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 16512)
  AUTHORS   Maloy,A.P., Schroeter,J.C., Rees,C.B. and Bartron,M.L.
  TITLE     Family specific long-range PCR primers for the amplification of
            fish mitochondrial DNA and whole genome sequencing
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 16512)
  CONSRTM   NCBI Genome Project
  TITLE     Direct Submission
  JOURNAL   Submitted (24-AUG-2018) National Center for Biotechnology
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REFERENCE   3  (bases 1 to 16512)
  AUTHORS   Maloy,A.P., Schroeter,J.C., Rees,C.B. and Bartron,M.L.
  TITLE     Direct Submission
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            length.
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Positives = 130/130 (100%), Gaps = 0/130 (0%)
Frame = +1

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                      TCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG
MF621734         2278 TCAAGAACTCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 2337

CF1L consensus     61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT  120
                      GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT
MF621734         2338 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 2397

CF1L consensus    121 AAGTCRCAGA  130
                      AAGTC+CAGA
MF621734         2398 AAGTCGCAGA 2407
```

View GenBank record on NCBI website (opens browser)

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VERSION     MF621734.1  GI:1276391806
KEYWORDS    .
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 16513)
  AUTHORS   Maloy,A.P., Schroeter,J.C., Rees,C.B. and Bartron,M.L.
  TITLE     Family specific long-range PCR primers for the amplification of
            fish mitochondrial DNA and whole genome sequencing
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 16513)
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  TITLE     Direct Submission
  JOURNAL   Submitted (08-AUG-2017) Northeast Fishery Center, US Fish and
            Wildlife Service, PO Box 75, Lamar, PA 16848, USA
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flat format, export it.
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Positives = 130/130 (100%), Gaps = 0/130 (0%)
Frame = +1

CF1L consensus      1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG   60
                      TCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG
MF621733         2278 TCAAGAACTCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 2337

CF1L consensus     61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT  120
                      GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT
MF621733         2338 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 2397

CF1L consensus    121 AAGTCRCAGA  130
                      AAGTC+CAGA
MF621733         2398 AAGTCGCAGA 2407
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View GenBank record on NCBI website (opens browser)

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            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 16513)
  AUTHORS   Maloy,A.P., Schroeter,J.C., Rees,C.B. and Bartron,M.L.
  TITLE     Family specific long-range PCR primers for the amplification of
            fish mitochondrial DNA and whole genome sequencing
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 16513)
  AUTHORS   Maloy,A.P., Schroeter,J.C., Rees,C.B. and Bartron,M.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-AUG-2017) Northeast Fishery Center, US Fish and
            Wildlife Service, PO Box 75, Lamar, PA 16848, USA
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flat format, export it.
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                     AAGTC+CAGA
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View GenBank record on NCBI website (opens browser)

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REFERENCE   1  (bases 1 to 546)
  AUTHORS   Clusa,L. and Garcia-Vazquez,E.
  TITLE     An easy and fast method to detect the seven commonest invasive fish
            species in Europe from environmental DNA
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 546)
  AUTHORS   Clusa,L. and Garcia-Vazquez,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-NOV-2016) Department of Functional Biology,
            University of Oviedo, Avda Julian Claveria s/n, Oviedo, Asturias
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            sequencing ; ##Assembly-Data-END##
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      121 tctccccttt caagtcaatg aaattgatct gcccgtgcag aagcggacat acacctacaa
      181 gacgagaaga ccctttggag cttaagatac aagatcaact acgtcaagaa cccagttaaa
      241 ctaagtagca cctgatccca atcttctgtt ggggcgacca cgggagaaaa taaagctccc
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      361 accaaaagat ccggctacct gccgaccaac gaaccaagtt accctaggga taacagcgca
      421 atcccctttc agagtccata tcgacaaggg ggtttacgac ctcgatgttg gatcaggaca
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5. >KR476911 Ameiurus nebulosus isolate Ameneb1 16S ribosomal RNA gene, partial se
quence; mitochondrial

Length = 496

E-value = 1.05e-56, Score = 124, Bitscore = 230.105, Identities = 127/130 (97%),
Positives = 130/130 (100%), Gaps = 0/130 (0%)
Frame = +1

CF1L consensus     1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG  60
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CF1L consensus    61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 120
                     GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT
KR476911         279 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 338

CF1L consensus   121 AAGTCRCAGA 130
                     AAGTC+CAGA
KR476911         339 AAGTCGCAGA 348
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View GenBank record on NCBI website (opens browser)

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LOCUS       KR476911                 496 bp ds-DNA     linear   VRT 03-MAR-2016
DEFINITION  Ameiurus nebulosus isolate Ameneb1 16S ribosomal RNA gene, partial
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ACCESSION   KR476911
VERSION     KR476911.1  GI:868611071
KEYWORDS    .
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 496)
  AUTHORS   Thalinger,B., Oehm,J., Mayr,H., Obwexer,A., Zeisler,C. and
            Traugott,M.
  TITLE     Molecular prey identification in Central European piscivores
  JOURNAL   Mol Ecol Resour 16 (1), 123-137 (2016)
  PUBMED    26053612
REFERENCE   2  (bases 1 to 496)
  AUTHORS   Thalinger,B., Oehm,J., Mayr,H., Obwexer,A., Zeisler,C. and
            Traugott,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-MAY-2015) Institute of Ecology, University of
            Innsbruck, Technikerstr. 25, Innsbruck, Tyrol 6020, Austria
COMMENT     ##Assembly-Data-START## ; Sequencing Technology :: Sanger dideoxy
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      121 cctttcaagt caatgaaatt gatctgcccg tgcagaagcg gacatacacc tacaagacga
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6. >KR476830 Ameiurus nebulosus isolate Ameneb2 16S ribosomal RNA gene, partial se
quence; mitochondrial

Length = 498

E-value = 1.05e-56, Score = 124, Bitscore = 230.105, Identities = 127/130 (97%),
Positives = 130/130 (100%), Gaps = 0/130 (0%)
Frame = +1

CF1L consensus     1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG  60
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CF1L consensus    61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 120
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KR476830         281 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 340

CF1L consensus   121 AAGTCRCAGA 130
                     AAGTC+CAGA
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View GenBank record on NCBI website (opens browser)

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LOCUS       KR476830                 498 bp ds-DNA     linear   VRT 03-MAR-2016
DEFINITION  Ameiurus nebulosus isolate Ameneb2 16S ribosomal RNA gene, partial
            sequence; mitochondrial.
ACCESSION   KR476830
VERSION     KR476830.1  GI:868610848
KEYWORDS    .
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 498)
  AUTHORS   Thalinger,B., Oehm,J., Mayr,H., Obwexer,A., Zeisler,C. and
            Traugott,M.
  TITLE     Molecular prey identification in Central European piscivores
  JOURNAL   Mol Ecol Resour 16 (1), 123-137 (2016)
  PUBMED    26053612
REFERENCE   2  (bases 1 to 498)
  AUTHORS   Thalinger,B., Oehm,J., Mayr,H., Obwexer,A., Zeisler,C. and
            Traugott,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-MAY-2015) Institute of Ecology, University of
            Innsbruck, Technikerstr. 25, Innsbruck, Tyrol 6020, Austria
COMMENT     ##Assembly-Data-START## ; Sequencing Technology :: Sanger dideoxy
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      121 cccctttcaa gtcaatgaaa ttgatctgcc cgtgcagaag cggacataca cctacaagac
      181 gagaagaccc tttggagctt aagatacaag atcaactacg tcaagaaccc agttaaacta
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      301 cggactgggg caacccccta aaaccaagag tgacaactct aagtcgcaga atctctgacc
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7. >JX899750 Ameiurus nebulosus voucher ANSP 182780 12S ribosomal RNA gene, partia
l sequence; tRNA-Val gene, complete sequence; and 16S ribosomal RNA gene, partia
l sequence; mitochondrial

Length = 2657

E-value = 1.05e-56, Score = 124, Bitscore = 230.105, Identities = 127/130 (97%),
Positives = 130/130 (100%), Gaps = 0/130 (0%)
Frame = +1

CF1L consensus      1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG   60
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JX899750         2191 TCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 2250

CF1L consensus     61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT  120
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JX899750         2251 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 2310

CF1L consensus    121 AAGTCRCAGA  130
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JX899750         2311 AAGTCGCAGA 2320
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View GenBank record on NCBI website (opens browser)

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LOCUS       JX899750                2657 bp ds-DNA     linear   VRT 16-JAN-2013
DEFINITION  Ameiurus nebulosus voucher ANSP 182780 12S ribosomal RNA gene,
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            ribosomal RNA gene, partial sequence; mitochondrial.
ACCESSION   JX899750
VERSION     JX899750.1  GI:441420517
KEYWORDS    .
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 2657)
  AUTHORS   Sullivan,J.P., Muriel-Cunha,J. and Lundberg,J.G.
  TITLE     Phylogenetic relationships and molecular dating of the major groups
            of catfishes of the neotropical superfamily Pimelodoidea
            (Teleostei, Siluriformes)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2657)
  AUTHORS   Sullivan,J.P., Muriel-Cunha,J. and Lundberg,J.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-OCT-2012) Ichthyology, Academy of Natural Sciences of
            Philadelphia, 1900 Benjamin Franklin Parkway, Philadelphia, PA
            19103, USA
COMMENT     ##Assembly-Data-START## ; Assembly Method :: Sequencher v. 4.1 ;
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     1981 cccagtgaca tgttaaacgg ccgcggtatt ttgaccgtgc gaaggtagcg caatcacttg
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7. >DQ421891 Ameiurus nebulosus voucher FLEBr1 16S ribosomal RNA gene, partial seq
uence; mitochondrial

Length = 501

E-value = 1.05e-56, Score = 124, Bitscore = 230.105, Identities = 127/130 (97%),
Positives = 130/130 (100%), Gaps = 0/130 (0%)
Frame = +1

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DQ421891         319 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 378

CF1L consensus   121 AAGTCRCAGA 130
                     AAGTC+CAGA
DQ421891         379 AAGTCGCAGA 388
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View GenBank record on NCBI website (opens browser)

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DEFINITION  Ameiurus nebulosus voucher FLEBr1 16S ribosomal RNA gene, partial
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ACCESSION   DQ421891
VERSION     DQ421891.1  GI:90995081
KEYWORDS    .
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 501)
  AUTHORS   Padhi,A.
  TITLE     Mitochondrial DNA sequence revealed contrasting demographic history
            between the black bullhead (Ameiurus melas) and its cryptic lineage
            in North America
  JOURNAL   Aquat. Living Resour. 23, 325-333 (2010)
REFERENCE   2  (bases 1 to 501)
  AUTHORS   Padhi,A. and Collier,G.E.
  TITLE     Cryptic speciation in North American catfish of the genus Ameiurus
            (Siluriformes: Ictaluridae)
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 501)
  AUTHORS   Padhi,A. and Collier,G.E.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-MAR-2006) Department of Biological Sciences,
            University of Tulsa, 600 S. College Ave., Tulsa, OK 74104, USA
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                     /organelle="mitochondrion"
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      121 agacctgtat gaatggtgga acgagggctt agctgtctcc cctttcaagt caatgaaatt
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8. >DQ421876 Ameiurus melas voucher MTMBL12 16S ribosomal RNA gene, partial sequen
ce; mitochondrial

Length = 501

E-value = 1.05e-56, Score = 124, Bitscore = 230.105, Identities = 127/130 (97%),
Positives = 130/130 (100%), Gaps = 0/130 (0%)
Frame = +1

CF1L consensus     1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG  60
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DQ421876         259 TCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 318

CF1L consensus    61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 120
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DQ421876         319 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 378

CF1L consensus   121 AAGTCRCAGA 130
                     AAGTC+CAGA
DQ421876         379 AAGTCGCAGA 388
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View GenBank record on NCBI website (opens browser)

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LOCUS       DQ421876                 501 bp ds-DNA     linear   VRT 04-JAN-2013
DEFINITION  Ameiurus melas voucher MTMBL12 16S ribosomal RNA gene, partial
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ACCESSION   DQ421876
VERSION     DQ421876.1  GI:90995066
KEYWORDS    .
SOURCE      mitochondrion Ameiurus melas (black bullhead)
  ORGANISM  Ameiurus melas
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 501)
  AUTHORS   Padhi,A.
  TITLE     Mitochondrial DNA sequence revealed contrasting demographic history
            between the black bullhead (Ameiurus melas) and its cryptic lineage
            in North America
  JOURNAL   Aquat. Living Resour. 23, 325-333 (2010)
REFERENCE   2  (bases 1 to 501)
  AUTHORS   Padhi,A. and Collier,G.E.
  TITLE     Cryptic speciation in North American catfish of the genus Ameiurus
            (Siluriformes: Ictaluridae)
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 501)
  AUTHORS   Padhi,A. and Collier,G.E.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-MAR-2006) Department of Biological Sciences,
            University of Tulsa, 600 S. College Ave., Tulsa, OK 74104, USA
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                     /haplotype="16sBlk1"
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                     /collected_by="Mike Ruggles, Ryan Lott and Ben Davis,
                     Fisheries Biologists, Montana Fish Wildlife & Parks"
     rRNA            1..>501
                     /product="16S ribosomal RNA"
     BLAST_Hit       259..388
ORIGIN
        1 caaaaacatc gcctcctgca aaaatcaacg tataggaggt cttgcctgcc cagtgacatg
       61 ttaaacggcc gcggtatttt gaccgtgcga aggtagcgca atcacttgtc tcttaaatgg
      121 agacctgtat gaatggtgga acgagggctt agctgtctcc cctttcaagt caatgaaatt
      181 gatctgcccg tgcagaagcg gacatacacc tacaagacga gaagaccctt tggagcttaa
      241 gatacaagat caactacgtc aagaacccag ttaaactaag tagcacctga tcccaatctt
      301 ctgttggggc gaccacggga gaaaataaag ctcccacgcg gactggggca accccctaaa
      361 accaagagtg acaactctaa gtcgcagaat ctctgaccaa aagatccggc tacctgccga
      421 ccaacgaacc aagttaccct agggataaca gcgcaatccc ctttcagagt ccatatcgac
      481 aagggggttt acgacctcga t
//
```

```
9. >AY458873 Ameiurus nebulosus voucher TNHC 47496 12S ribosomal RNA gene, partial
 sequence; tRNA-Val gene, complete sequence; and 16S ribosomal RNA gene, partial
 sequence; mitochondrial

Length = 2042

E-value = 1.05e-56, Score = 124, Bitscore = 230.105, Identities = 127/130 (97%),
Positives = 130/130 (100%), Gaps = 0/130 (0%)
Frame = +1

CF1L consensus      1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG   60
                      TCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG
AY458873         1745 TCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 1804

CF1L consensus     61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT  120
                      GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT
AY458873         1805 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 1864

CF1L consensus    121 AAGTCRCAGA  130
                      AAGTC+CAGA
AY458873         1865 AAGTCGCAGA 1874
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View GenBank record on NCBI website (opens browser)

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LOCUS       AY458873                2042 bp ds-DNA     linear   VRT 29-APR-2004
DEFINITION  Ameiurus nebulosus voucher TNHC 47496 12S ribosomal RNA gene,
            partial sequence; tRNA-Val gene, complete sequence; and 16S
            ribosomal RNA gene, partial sequence; mitochondrial.
ACCESSION   AY458873
VERSION     AY458873.1  GI:41353717
KEYWORDS    .
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 2042)
  AUTHORS   Wilcox,T.P., Garcia de Leon,F.J., Hendrickson,D.A. and Hillis,D.M.
  TITLE     Convergence among cave catfishes: long-branch attraction and a
            Bayesian relative rates test
  JOURNAL   Mol. Phylogenet. Evol. 31 (3), 1101-1113 (2004)
  PUBMED    15120403
REFERENCE   2  (bases 1 to 2042)
  AUTHORS   Wilcox,T.P., Garcia de Leon,F.J., Hendrickson,D.A. and Hillis,D.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (04-NOV-2003) Section of Integrative Biology, University
            of Texas, 1 University Station C0930, Austin, TX 78712, USA
FEATURES             Location/Qualifiers
     source          1..2042
                     /organism="Ameiurus nebulosus"
                     /organelle="mitochondrion"
                     /mol_type="genomic DNA"
                     /specimen_voucher="TNHC 47496"
                     /db_xref="taxon:27778"
     misc_feature    1..>2042
                     /note="contains 12S ribosomal RNA, tRNA-Val and 16S
                     ribosomal RNA genes"
     BLAST_Hit       1745..1874
ORIGIN
        1 cccagatgta ttcttacaca cacatccgcc cgggtactac gagcatagct taaaacccaa
       61 aggacttggc ggtgtctcag acccacctag aggagcctgt tctataaccg ataaccccg
      121 ttaaacctca ccacttcttg ttaataccgc ctatataccg ccgtcgtcag cttaccctgt
      181 gaaggtctaa cagtaagcaa aatgggcccg cccaaaaacg tcaggtcgag gtgtagcgta
      241 cgaagtggga agaaatgggc tacattttct acaactagaa tattaaacga atggcattat
      301 gaaaattatt gcctgaaggt ggatttagta gtaaaaagca aacagagtgt ccttttgaat
      361 taggctctga gacgcgcaca caccgcccgt cactctcccc tacacataac tattccacat
      421 atataataca ctccacatca atacggggag gcaagtcgta acatggtaag tgtaccggaa
      481 ggtgcacttg gaacaatcag gacgtggctg agatagccaa gcatctccct tacaccgaga
      541 agacatccat gcaaattgga tcgccctgag ccaaaaagct agcttaaccc ccagaatacc
      601 taaaacaaca ttaaaatact ccaaaaaacc acaatatccc aaattaaaac attctaccgc
      661 cctagtacgt gtgacagaaa aggcaataat aaagcaataa ataaagtacc gcaagggaac
      721 gctgaaagag aaatgaaata gatcattaaa gcacaataaa gcagagatta aacctcgtac
      781 cttttgcatc atgatttagc cagtcctccg agcaaagcgc actttagttc tagaccccga
      841 aactaagtga gctaccccga gacagcctat aaattagggc caacccgtct ctgtggcaaa
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      901 agagtgggaa gatctccggg tagaggtgac aagcctaccg aacttagtta tagctggttg
      961 cctaggaagt gaatagaagt tcagcctcgt acccctcacc tcaccaacac ctttatacta
     1021 cacgagcccg agagacctac gagagttagt caaagggggt acagcccctt tgatgcagga
     1081 cacaacctca tcaggaggtt aaagattata ctaaacaaga tatgccgctc cagtgggcct
     1141 aaaagcagcc acctgaacag aaagcgttaa agctctggcg gattctaatc tattatttaa
     1201 atattatatc ttaaacccct aattatacta ggccaatcca tgcccgcatg gaagagacac
     1261 tgctaaaatg agtaataaga aggaaccccc ttctcctagc ctacgtgtat gctaaattgg
     1321 accccccact agcaattaac gaacccaatt aaagagggca ttgtggacat attaaatatc
     1381 aagaaaaccc cacacacccc catcgttaac cccacaccgg aaggcactat aggaaagact
     1441 aaaagaaaag gaaggaactc ggcaaacata agcctcgcct gtttaccaaa aacatcgcct
     1501 cctgcaaaaa tcaaagtata ggaggtcttg cctgcccagt gacatgttaa acggccgcgg
     1561 tattttgacc gtgcgaaggt agcgcaatca cttgtctctt aaatggagac ctgtatgaat
     1621 ggtggaacga gggcttagct gtctcccctt tcaagtcaat gaaattgatc tgcccgtgca
     1681 gaagcggaca tacgcctaca agacgagaag accctttgga gcttaagata caagatcaac
     1741 tacgtcaaga acccagttaa actaagtagc acctgatccc aatcttctgt tggggcgacc
     1801 acgggagaaa ataaagctcc cacgcggact ggggcaaccc cctaaaacca agagtgacaa
     1861 ctctaagtcg cagaatctct gaccaaaaga tccggctacc tgccgaccaa cgaaccaagt
     1921 taccctaggg ataacagcgc aatccccttt cagagtccat atcgacaagg gggtttacga
     1981 cctcgatgtt ggatcaggac atcctaatgg tgcagccgct attaagggtt cgtttgttca
     2041 ac
//
```

```
10.>AY430234 Ameiurus nebulosus 16S ribosomal RNA gene, partial sequence; mitochon
drial

Length = 375

E-value = 1.05e-56, Score = 124, Bitscore = 230.105, Identities = 127/130 (97%),
Positives = 130/130 (100%), Gaps = 0/130 (0%)
Frame = +1

CF1L consensus     1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG  60
                     TCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG
AY430234         161 TCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 220

CF1L consensus    61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 120
                     GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT
AY430234         221 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 280

CF1L consensus   121 AAGTCRCAGA 130
                     AAGTC+CAGA
AY430234         281 AAGTCGCAGA 290



View GenBank record on NCBI website (opens browser)

LOCUS       AY430234                 375 bp ds-DNA     linear   VRT 26-AUG-2004
DEFINITION  Ameiurus nebulosus 16S ribosomal RNA gene, partial sequence;
            mitochondrial.
ACCESSION   AY430234
VERSION     AY430234.1  GI:40841837
KEYWORDS    .
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 375)
  AUTHORS   Lopez,J.A., Chen,W.-J. and Orti,G.
  TITLE     Esociform phylogeny
  JOURNAL   Copeia 2004 (3), 449-464 (2004)
REFERENCE   2  (bases 1 to 375)
  AUTHORS   Chen,W.-J. and Orti,G.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-OCT-2003) School of Biological Sciences, University
            of Nebraska-Lincoln, 314 Manter Hall, Lincoln, NE 68588, USA
FEATURES             Location/Qualifiers
     source          1..375
                     /organism="Ameiurus nebulosus"
                     /organelle="mitochondrion"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:27778"
     rRNA            1..>375
                     /product="16S ribosomal RNA"
     BLAST_Hit       161..290
ORIGIN
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       61 cccctttcaa gtcaatgaaa ttgatctgcc cgtgcagaag cggacatacg cctacaagac
      121 gagaagaccc tttggagctt aagatacaag atcaactacg tcaagaaccc agttaaacta
      181 agtagcacct gatcccaatc ttctgttggg gcgaccacgg gagaaaataa agctcccacg
      241 cggactgggg caaccccccta aaaccaagag tgacaactct aagtcgcaga atctctgacc
      301 aaaagatccg gctacctgcc gaccaacgaa ccaagttacc ctagggataa cagcgcaatc
      361 ccctttcaga gtcca
//
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Appendix 3. BLAST results from CF2S consensus sequence (top 10 results).

```
1. >LC198795 Ameiurus melas mitochondrial gene for 16S ribosomal RNA, partial sequ
ence, isolation_source: laboratory tissue sample A_melas_002

Length = 99

E-value = 1.43e-40, Score = 95, Bitscore = 176.552, Identities = 96/97 (98%),
Positives = 97/97 (100%), Gaps = 0/97 (0%)
Frame = +1

CF2S con    35 CCAATCTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAAC  94
               CCAATCTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAAC
LC198795     1 CCAATCTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAAC  60

CF2S con    95 CCCCTAAAACCAAGAGTGACAACTCTAAGTCRCAGAA 131
               CCCCTAAAACCAAGAGTGACAACTCTAAGTC+CAGAA
LC198795    61 CCCCTAAAACCAAGAGTGACAACTCTAAGTCGCAGAA  97
```

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```
LOCUS       LC198795                  99 bp ds-DNA     linear   VRT 05-DEC-2017
DEFINITION  Ameiurus melas mitochondrial gene for 16S ribosomal RNA, partial
            sequence, isolation_source: laboratory tissue sample A_melas_002.
ACCESSION   LC198795
VERSION     LC198795.1  GI:1287461964
KEYWORDS    .
SOURCE      mitochondrion Ameiurus melas (black bullhead)
  ORGANISM  Ameiurus melas
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 99)
  AUTHORS   Clusa,L. and Garcia,E.
  TITLE     An easy and fast method to detect the seven commonest invasive fish
            species in Europe from environmental DNA
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 99)
  AUTHORS   Clusa,L. and Garcia,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (25-NOV-2016) Contact:Laura Clusa University of Oviedo,
            Functional Biology; Avenida Julian Claveria, Oviedo, Asturias
            33006, Spain
FEATURES             Location/Qualifiers
     source          1..99
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                     /organelle="mitochondrion"
                     /mol_type="genomic DNA"
                     /isolation_source="laboratory tissue sample A_melas_002"
                     /db_xref="taxon:219545"
                     /tissue_type="muscle"
                     /PCR_primers="fwd_name: Am-16S-F, fwd_seq:
                     cgtcaagaacycagttraact, rev_name: Am-16S-R, rev_seq:
                     gwttctgygacttagagttgtca"
     rRNA            1..>99
                     /product="16S ribosomal RNA"
     BLAST_Hit       1..97
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       61 cccctaaaac caagagtgac aactctaagt cgcagaatc
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```
2. >LC198796 Ameiurus melas mitochondrial gene for 16S ribosomal RNA, partial sequ
ence, isolation_source: aquarium water sample 3

Length = 94

E-value = 8.63e-38, Score = 90, Bitscore = 167.319, Identities = 91/92 (98%),
Positives = 92/92 (100%), Gaps = 0/92 (0%)
Frame = +1

CF2S con    40 CTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCT  99
               CTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCT
LC198796     1 CTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCT  60

CF2S con   100 AAAACCAAGAGTGACAACTCTAAGTCRCAGAA 131
               AAAACCAAGAGTGACAACTCTAAGTC+CAGAA
LC198796    61 AAAACCAAGAGTGACAACTCTAAGTCGCAGAA  92
```

View GenBank record on NCBI website (opens browser)

```
LOCUS       LC198796                  94 bp ds-DNA     linear   VRT 05-DEC-2017
DEFINITION  Ameiurus melas mitochondrial gene for 16S ribosomal RNA, partial
            sequence, isolation_source: aquarium water sample 3.
ACCESSION   LC198796
VERSION     LC198796.1  GI:1287461965
KEYWORDS    .
SOURCE      mitochondrion Ameiurus melas (black bullhead)
  ORGANISM  Ameiurus melas
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 94)
  AUTHORS   Clusa,L. and Garcia,E.
  TITLE     An easy and fast method to detect the seven commonest invasive fish
            species in Europe from environmental DNA
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 94)
  AUTHORS   Clusa,L. and Garcia,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (25-NOV-2016) Contact:Laura Clusa University of Oviedo,
            Functional Biology; Avenida Julian Claveria, Oviedo, Asturias
            33006, Spain
FEATURES             Location/Qualifiers
     source          1..94
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                     /organelle="mitochondrion"
                     /mol_type="genomic DNA"
                     /isolation_source="aquarium water sample 3"
                     /db_xref="taxon:219545"
                     /country="Spain:Aragon, Zaragoza"
                     /PCR_primers="fwd_name: Am-16S-F, fwd_seq:
                     cgtcaagaacycagttraact, rev_name: Am-16S-R, rev_seq:
                     gwttctgygacttagagttgtca"
     rRNA            1..>94
                     /product="16S ribosomal RNA"
     BLAST_Hit       1..92
ORIGIN
        1 cttctgttgg ggcgaccacg ggagaaaata aagctcccac gcggactggg gcaaccccct
       61 aaaaccaaga gtgacaactc taagtcgcag aatc
//
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```
3. >NC_036387 Ameiurus nebulosus strain NEFC_F16-028 mitochondrion, complete genom
e

Length = 16512

E-value = 3.02e-57, Score = 125, Bitscore = 231.952, Identities = 128/131 (97%),
Positives = 131/131 (100%), Gaps = 0/131 (0%)
Frame = +1

CF2S con       1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG   60
                 TCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG
NC_036387   2278 TCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 2337

CF2S con      61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT  120
                 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT
NC_036387   2338 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 2397

CF2S con     121 AAGTCRCAGAA  131
                 AAGTC+CAGAA
NC_036387   2398 AAGTCGCAGAA 2408
```

View GenBank record on NCBI website (opens browser)

```
LOCUS       NC_036387              16512 bp ds-DNA     linear   VRT 24-AUG-2018
DEFINITION  Ameiurus nebulosus strain NEFC_F16-028 mitochondrion, complete
            genome.
ACCESSION   NC_036387
VERSION     NC_036387.1  GI:1314948533
DBLINK      BioProject: PRJNA420704
KEYWORDS    RefSeq.
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 16512)
  AUTHORS   Maloy,A.P., Schroeter,J.C., Rees,C.B. and Bartron,M.L.
  TITLE     Family specific long-range PCR primers for the amplification of
            fish mitochondrial DNA and whole genome sequencing
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 16512)
  CONSRTM   NCBI Genome Project
  TITLE     Direct Submission
  JOURNAL   Submitted (24-AUG-2018) National Center for Biotechnology
            Information, NIH, Bethesda, MD 20894, USA
REFERENCE   3  (bases 1 to 16512)
  AUTHORS   Maloy,A.P., Schroeter,J.C., Rees,C.B. and Bartron,M.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-AUG-2017) Northeast Fishery Center, US Fish and
            Wildlife Service, PO Box 75, Lamar, PA 16848, USA
COMMENT     PROVISIONAL REFSEQ: This record has not yet been subject to final
            NCBI review. The reference sequence is identical to MF621731.
            COMPLETENESS: full
            length.
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This sequence is too large to show in the text view.  To see the entire sequence in GenBank
flat format, export it.
```

```
4. >MF621734 Ameiurus nebulosus voucher NEFC_F16-115 mitochondrion, complete genom
e

Length = 16513

E-value = 3.02e-57, Score = 125, Bitscore = 231.952, Identities = 128/131 (97%),
Positives = 131/131 (100%), Gaps = 0/131 (0%)
Frame = +1

CF2S con      1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG   60
                TCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG
MF621734   2278 TCAAGAACTCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 2337

CF2S con     61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT  120
                GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT
MF621734   2338 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 2397

CF2S con    121 AAGTCRCAGAA  131
                AAGTC+CAGAA
MF621734   2398 AAGTCGCAGAA 2408
```

View GenBank record on NCBI website (opens browser)

```
LOCUS       MF621734               16513 bp ds-DNA     linear   VRT 18-NOV-2017
DEFINITION  Ameiurus nebulosus voucher NEFC_F16-115 mitochondrion, complete
            genome.
ACCESSION   MF621734
VERSION     MF621734.1  GI:1276391806
KEYWORDS    .
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 16513)
  AUTHORS   Maloy,A.P., Schroeter,J.C., Rees,C.B. and Bartron,M.L.
  TITLE     Family specific long-range PCR primers for the amplification of
            fish mitochondrial DNA and whole genome sequencing
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 16513)
  AUTHORS   Maloy,A.P., Schroeter,J.C., Rees,C.B. and Bartron,M.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-AUG-2017) Northeast Fishery Center, US Fish and
            Wildlife Service, PO Box 75, Lamar, PA 16848, USA
//
This sequence is too large to show in the text view.  To see the entire sequence in GenBank
flat format, export it.
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5. >MF621733 Ameiurus nebulosus voucher NEFC_F16-113 mitochondrion, complete genom
e

Length = 16513

E-value = 3.02e-57, Score = 125, Bitscore = 231.952, Identities = 128/131 (97%),
Positives = 131/131 (100%), Gaps = 0/131 (0%)
Frame = +1

CF2S con      1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG   60
                TCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG
MF621733   2278 TCAAGAACTCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 2337

CF2S con     61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT  120
                GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT
MF621733   2338 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 2397

CF2S con    121 AAGTCRCAGAA  131
                AAGTC+CAGAA
MF621733   2398 AAGTCGCAGAA 2408
```

View GenBank record on NCBI website (opens browser)

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LOCUS       MF621733               16513 bp ds-DNA     linear   VRT 18-NOV-2017
DEFINITION  Ameiurus nebulosus voucher NEFC_F16-113 mitochondrion, complete
            genome.
ACCESSION   MF621733
VERSION     MF621733.1  GI:1276391792
KEYWORDS    .
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 16513)
  AUTHORS   Maloy,A.P., Schroeter,J.C., Rees,C.B. and Bartron,M.L.
  TITLE     Family specific long-range PCR primers for the amplification of
            fish mitochondrial DNA and whole genome sequencing
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 16513)
  AUTHORS   Maloy,A.P., Schroeter,J.C., Rees,C.B. and Bartron,M.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-AUG-2017) Northeast Fishery Center, US Fish and
            Wildlife Service, PO Box 75, Lamar, PA 16848, USA
//
This sequence is too large to show in the text view.  To see the entire sequence in GenBank
flat format, export it.
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```
6. >KY231825 Ameiurus melas isolate A_melas_002 16S ribosomal RNA gene, partial se
quence; mitochondrial

Length = 546

E-value = 3.02e-57, Score = 125, Bitscore = 231.952, Identities = 128/131 (97%),
Positives = 131/131 (100%), Gaps = 0/131 (0%)
Frame = +1

CF2S con     1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG  60
               TCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG
KY231825   224 TCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 283

CF2S con    61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 120
               GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT
KY231825   284 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 343

CF2S con   121 AAGTCRCAGAA 131
               AAGTC+CAGAA
KY231825   344 AAGTCGCAGAA 354
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View GenBank record on NCBI website (opens browser)

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LOCUS       KY231825                 546 bp ds-DNA     linear   VRT 30-NOV-2017
DEFINITION  Ameiurus melas isolate A_melas_002 16S ribosomal RNA gene, partial
            sequence; mitochondrial.
ACCESSION   KY231825
VERSION     KY231825.1  GI:1270025115
KEYWORDS    .
SOURCE      mitochondrion Ameiurus melas (black bullhead)
  ORGANISM  Ameiurus melas
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 546)
  AUTHORS   Clusa,L. and Garcia-Vazquez,E.
  TITLE     An easy and fast method to detect the seven commonest invasive fish
            species in Europe from environmental DNA
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 546)
  AUTHORS   Clusa,L. and Garcia-Vazquez,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (24-NOV-2016) Department of Functional Biology,
            University of Oviedo, Avda Julian Claveria s/n, Oviedo, Asturias
            33006, Spain
COMMENT     ##Assembly-Data-START## ; Sequencing Technology :: Sanger dideoxy
            sequencing ; ##Assembly-Data-END##
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                     /isolate="A_melas_002"
                     /db_xref="taxon:219545"
                     /country="Spain"
                     /PCR_primers="fwd_name: 16S-Ar, fwd_seq:
                     cgcctgtttatcaaaaacat, rev_name: 16S-Br, rev_seq:
                     ccggtctgaactcagatcacgt"
     rRNA            1..>546
                     /product="16S ribosomal RNA"
     BLAST_Hit       224..354
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      121 tctccccttt caagtcaatg aaattgatct gcccgtgcag aagcggacat acacctacaa
      181 gacgagaaga ccctttggag cttaagatac aagatcaact acgtcaagaa cccagttaaa
      241 ctaagtagca cctgatccca atcttctgtt ggggcgacca cgggagaaaa taaagctccc
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      421 atcccctttc agagtccata tcgacaaggg ggtttacgac ctcgatgttg gatcaggaca
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7. >KR476911 Ameiurus nebulosus isolate Ameneb1 16S ribosomal RNA gene, partial se
quence; mitochondrial

Length = 496

E-value = 3.02e-57, Score = 125, Bitscore = 231.952, Identities = 128/131 (97%),
Positives = 131/131 (100%), Gaps = 0/131 (0%)
Frame = +1

CF2S con     1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG  60
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KR476911   219 TCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 278

CF2S con    61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 120
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KR476911   279 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 338

CF2S con   121 AAGTCRCAGAA 131
               AAGTC+CAGAA
KR476911   339 AAGTCGCAGAA 349
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View GenBank record on NCBI website (opens browser)

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LOCUS       KR476911                 496 bp ds-DNA     linear   VRT 03-MAR-2016
DEFINITION  Ameiurus nebulosus isolate Ameneb1 16S ribosomal RNA gene, partial
            sequence; mitochondrial.
ACCESSION   KR476911
VERSION     KR476911.1  GI:868611071
KEYWORDS    .
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 496)
  AUTHORS   Thalinger,B., Oehm,J., Mayr,H., Obwexer,A., Zeisler,C. and
            Traugott,M.
  TITLE     Molecular prey identification in Central European piscivores
  JOURNAL   Mol Ecol Resour 16 (1), 123-137 (2016)
  PUBMED    26053612
REFERENCE   2  (bases 1 to 496)
  AUTHORS   Thalinger,B., Oehm,J., Mayr,H., Obwexer,A., Zeisler,C. and
            Traugott,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-MAY-2015) Institute of Ecology, University of
            Innsbruck, Technikerstr. 25, Innsbruck, Tyrol 6020, Austria
COMMENT     ##Assembly-Data-START## ; Sequencing Technology :: Sanger dideoxy
            sequencing ; ##Assembly-Data-END##
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                     /organelle="mitochondrion"
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                     /db_xref="taxon:27778"
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                     /product="16S ribosomal RNA"
     BLAST_Hit       219..349
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       61 atcacttgtc tcttaaatgg agacctgtat gaatggtgga acgagggctt agctgtctcc
      121 cctttcaagt caatgaaatt gatctgcccg tgcagaagcg gacatacacc tacaagacga
      181 gaagaccctt tggagcttaa gatacaagat caactacgtc aagaacccag ttaaactaag
      241 tagcacctga tcccaatctt ctgttggggc gaccacggga gaaaataaag ctcccacgcg
      301 gactggggca accccctaaa accaagagtg acaactctaa gtcgcagaat ctctgaccaa
      361 aagatccggc tacctgccga ccaacgaacc aagttaccct agggataaca gcgcaatccc
      421 ctttcagagt ccatatcgac aagggggttt acgacctcga tgttggatca ggacatccta
      481 atggtgcagc cgctat
//
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```
8. >KR476830 Ameiurus nebulosus isolate Ameneb2 16S ribosomal RNA gene, partial se
quence; mitochondrial

Length = 498

E-value = 3.02e-57, Score = 125, Bitscore = 231.952, Identities = 128/131 (97%),
Positives = 131/131 (100%), Gaps = 0/131 (0%)
Frame = +1

CF2S con     1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG  60
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KR476830   221 TCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 280

CF2S con    61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 120
               GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT
KR476830   281 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 340

CF2S con   121 AAGTCRCAGAA 131
               AAGTC+CAGAA
KR476830   341 AAGTCGCAGAA 351
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View GenBank record on NCBI website (opens browser)

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LOCUS       KR476830                 498 bp ds-DNA     linear   VRT 03-MAR-2016
DEFINITION  Ameiurus nebulosus isolate Ameneb2 16S ribosomal RNA gene, partial
            sequence; mitochondrial.
ACCESSION   KR476830
VERSION     KR476830.1  GI:868610848
KEYWORDS    .
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 498)
  AUTHORS   Thalinger,B., Oehm,J., Mayr,H., Obwexer,A., Zeisler,C. and
            Traugott,M.
  TITLE     Molecular prey identification in Central European piscivores
  JOURNAL   Mol Ecol Resour 16 (1), 123-137 (2016)
  PUBMED    26053612
REFERENCE   2  (bases 1 to 498)
  AUTHORS   Thalinger,B., Oehm,J., Mayr,H., Obwexer,A., Zeisler,C. and
            Traugott,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-MAY-2015) Institute of Ecology, University of
            Innsbruck, Technikerstr. 25, Innsbruck, Tyrol 6020, Austria
COMMENT     ##Assembly-Data-START## ; Sequencing Technology :: Sanger dideoxy
            sequencing ; ##Assembly-Data-END##
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                     /organelle="mitochondrion"
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                     /db_xref="taxon:27778"
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                     /product="16S ribosomal RNA"
     BLAST_Hit       221..351
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      121 cccctttcaa gtcaatgaaa ttgatctgcc cgtgcagaag cggacataca cctacaagac
      181 gagaagaccc tttggagctt aagatacaag atcaactacg tcaagaaccc agttaaacta
      241 agtagcacct gatcccaatc ttctgttggg gcgaccacgg gagaaaataa agctcccacg
      301 cggactgggg caacccccta aaaccaagag tgacaactct aagtcgcaga atctctgacc
      361 aaaagatccg gctacctgcc gaccaacgaa ccaagttacc ctagggataa cagcgcaatc
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9. >JX899750 Ameiurus nebulosus voucher ANSP 182780 12S ribosomal RNA gene, partia
l sequence; tRNA-Val gene, complete sequence; and 16S ribosomal RNA gene, partia
l sequence; mitochondrial

Length = 2657

E-value = 3.02e-57, Score = 125, Bitscore = 231.952, Identities = 128/131 (97%),
Positives = 131/131 (100%), Gaps = 0/131 (0%)
Frame = +1

CF2S con      1 TCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG   60
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JX899750   2191 TCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCACGG 2250

CF2S con     61 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT  120
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JX899750   2251 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 2310

CF2S con    121 AAGTCRCAGAA  131
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JX899750   2311 AAGTCGCAGAA 2321
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View GenBank record on NCBI website (opens browser)

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LOCUS       JX899750                2657 bp ds-DNA     linear   VRT 16-JAN-2013
DEFINITION  Ameiurus nebulosus voucher ANSP 182780 12S ribosomal RNA gene,
            partial sequence; tRNA-Val gene, complete sequence; and 16S
            ribosomal RNA gene, partial sequence; mitochondrial.
ACCESSION   JX899750
VERSION     JX899750.1  GI:441420517
KEYWORDS    .
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 2657)
  AUTHORS   Sullivan,J.P., Muriel-Cunha,J. and Lundberg,J.G.
  TITLE     Phylogenetic relationships and molecular dating of the major groups
            of catfishes of the neotropical superfamily Pimelodoidea
            (Teleostei, Siluriformes)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2657)
  AUTHORS   Sullivan,J.P., Muriel-Cunha,J. and Lundberg,J.G.
  TITLE     Direct Submission
  JOURNAL   Submitted (03-OCT-2012) Ichthyology, Academy of Natural Sciences of
            Philadelphia, 1900 Benjamin Franklin Parkway, Philadelphia, PA
            19103, USA
COMMENT     ##Assembly-Data-START## ; Assembly Method :: Sequencher v. 4.1 ;
            Sequencing Technology :: Sanger dideoxy sequencing ;
            ##Assembly-Data-END##
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                     /country="USA: Pennsylvania, Delaware River"
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     tRNA            944..1015
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                     AAGACATCCATGCAAATTGGATCGCCCTGA"
     rRNA            1016..>2657
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      121 ccaagacgcc ttgctacgcc actcccccaa gggaactcag cagtaataga cattaagcca
      181 taagtgtaaa cttgacttag ttagggttat tagggccggt aaaattcgtg ccagccaccg
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      841 ctcccctaca cataactatt ccacatatat aatacactcc acatcaatac ggggaggcaa
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     1381 ctaccgaact tagttatagc tggttgccta ggaagtgaat agaagttcag cctcgtaccc
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     1621 ctggcggatt ctaatctatt atttaaatat tatatcttaa acccctaatt atactaggcc
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     1741 cctagcctac gtgtatgcta aattggaccc cccactagca attaacgaac ccaattaaag
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     1861 caccggaagg cactatagga aagactaaaa gaaaaggaag gaactcggca aacataagcc
     1921 tcgcctgttt accaaaaaca tcgcctcctg caaaaatcaa agtataggag gtcttgcctg
     1981 cccagtgaca tgttaaacgg ccgcggtatt ttgaccgtgc gaaggtagcg caatcacttg
     2041 tctcttaaat ggagacctgt atgaatggtg gaacgagggc ttagctgtct cccctttcaa
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10. >DQ421891 Ameiurus nebulosus voucher FLEBr1 16S ribosomal RNA gene, partial seq
uence; mitochondrial

Length = 501

E-value = 3.02e-57, Score = 125, Bitscore = 231.952, Identities = 128/131 (97%),
Positives = 131/131 (100%), Gaps = 0/131 (0%)
Frame = +1

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DQ421891   319 GAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCT 378

CF2S con   121 AAGTCRCAGAA 131
               AAGTC+CAGAA
DQ421891   379 AAGTCGCAGAA 389
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View GenBank record on NCBI website (opens browser)

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LOCUS       DQ421891                 501 bp ds-DNA     linear   VRT 04-JAN-2013
DEFINITION  Ameiurus nebulosus voucher FLEBr1 16S ribosomal RNA gene, partial
            sequence; mitochondrial.
ACCESSION   DQ421891
VERSION     DQ421891.1  GI:90995081
KEYWORDS    .
SOURCE      mitochondrion Ameiurus nebulosus (brown bullhead)
  ORGANISM  Ameiurus nebulosus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Actinopterygii; Neopterygii; Teleostei; Ostariophysi; Siluriformes;
            Ictaluridae; Ameiurus.
REFERENCE   1  (bases 1 to 501)
  AUTHORS   Padhi,A.
  TITLE     Mitochondrial DNA sequence revealed contrasting demographic history
            between the black bullhead (Ameiurus melas) and its cryptic lineage
            in North America
  JOURNAL   Aquat. Living Resour. 23, 325-333 (2010)
REFERENCE   2  (bases 1 to 501)
  AUTHORS   Padhi,A. and Collier,G.E.
  TITLE     Cryptic speciation in North American catfish of the genus Ameiurus
            (Siluriformes: Ictaluridae)
  JOURNAL   Unpublished
REFERENCE   3  (bases 1 to 501)
  AUTHORS   Padhi,A. and Collier,G.E.
  TITLE     Direct Submission
  JOURNAL   Submitted (01-MAR-2006) Department of Biological Sciences,
            University of Tulsa, 600 S. College Ave., Tulsa, OK 74104, USA
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                     /organelle="mitochondrion"
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                     /specimen_voucher="FLEBr1"
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                     /collected_by="Rich Caleteux, Fisheries Biologist, Florida
                     Fish & Wildlife Conservation Commission"
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      121 agacctgtat gaatggtgga acgagggctt agctgtctcc cctttcaagt caatgaaatt
      181 gatctgcccg tgcagaagcg gacatacgcc tacaagacga gaagaccctt tggagcttaa
      241 gatacaagat caactacgtc aagaacccag ttaaactaag tagcacctga tcccaatctt
      301 ctgttggggc gaccacggga gaaaataaag ctcccacgcg gactggggca accccctaaa
      361 accaagagtg acaactctaa gtcgcagaat ctctgaccaa aagatccggc tacctgccga
      421 ccaacgaacc aagttaccct agggataaca gcgcaatccc ctttcagagt ccatatcgac
      481 aagggggttt acgacctcga t
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Appendix 4. BLAST results from CF3N consensus sequence (top 10 results).

```
1. >LC198795 Ameiurus melas mitochondrial gene for 16S ribosomal RNA, partial sequ
ence, isolation_source: laboratory tissue sample A_melas_002

Length = 99

E-value = 1.46e-40, Score = 95, Bitscore = 176.552, Identities = 96/97 (98%),
Positives = 97/97 (100%), Gaps = 0/97 (0%)
Frame = +1

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               CCAATCTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAAC
LC198795     1 CCAATCTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAAC  60

CF3N con    98 CCCCTAAAACCAAGAGTGACAACTCTAAGTCRCAGAA 134
               CCCCTAAAACCAAGAGTGACAACTCTAAGTC+CAGAA
LC198795    61 CCCCTAAAACCAAGAGTGACAACTCTAAGTCGCAGAA  97
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Ameiurus melas mitochondrial gene for 16S ribosomal RNA, partial sequence, isolation_source: laboratory tissue sample A_melas_002

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  |        |         |         |         |         |
  CCAATCTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGA
  CTGGGGCAACCCCCTAAAACCAAGAGTGACAACTCTAAGTCGCAGAA
```

```
2. >LC198796 Ameiurus melas mitochondrial gene for 16S ribosomal RNA, partial sequ
ence, isolation_source: aquarium water sample 3

Length = 94

E-value = 8.80e-38, Score = 90, Bitscore = 167.319, Identities = 91/92 (98%),
Positives = 92/92 (100%), Gaps = 0/92 (0%)
Frame = +1

CF3N con    43 CTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCT 102
               CTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCT
LC198796     1 CTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCT  60

CF3N con   103 AAAACCAAGAGTGACAACTCTAAGTCRCAGAA 134
               AAAACCAAGAGTGACAACTCTAAGTC+CAGAA
LC198796    61 AAAACCAAGAGTGACAACTCTAAGTCGCAGAA  92
```

Ameiurus melas mitochondrial gene for 16S ribosomal RNA, partial sequence, isolation_source: aquarium water sample 3

```
  1        10        20        30        40        50
  |        |         |         |         |         |
  CTTCTGTTGGGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGG
  GCAACCCCCTAAAACCAAGAGTGACAACTCTAAGTCGCAGAA
```

```
3. >NC_036387 Ameiurus nebulosus strain NEFC_F16-028 mitochondrion, complete genom
e

Length = 16512

E-value = 2.38e-58, Score = 127, Bitscore = 235.645, Identities = 130/133 (97%),
Positives = 133/133 (100%), Gaps = 0/133 (0%)
Frame = +1

CF3N con       2 CGTCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC   61
                 CGTCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC
NC_036387   2276 CGTCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC 2335

CF3N con      62 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT  121
                 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT
NC_036387   2336 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT 2395

CF3N con     122 CTAAGTCRCAGAA  134
                 CTAAGTC+CAGAA
NC_036387   2396 CTAAGTCGCAGAA 2408
```

Ameiurus nebulosus strain NEFC_F16-028 mitochondrion, complete genome

```
  1        10        20        30        40        50
  |        |         |         |         |         |
  CGTCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTG
  GGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCC
  TAAAACCAAGAGTGACAACTCTAAGTCGCAGAA
```

```
4. >MF621734 Ameiurus nebulosus voucher NEFC_F16-115 mitochondrion, complete genom
e

Length = 16513

E-value = 2.38e-58, Score = 127, Bitscore = 235.645, Identities = 130/133 (97%),
Positives = 133/133 (100%), Gaps = 0/133 (0%)
Frame = +1

CF3N con      2 CGTCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC   61
                CGTCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC
MF621734   2276 CGTCAAGAACTCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC 2335

CF3N con     62 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT  121
                GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT
MF621734   2336 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT 2395

CF3N con    122 CTAAGTCRCAGAA  134
                CTAAGTC+CAGAA
MF621734   2396 CTAAGTCGCAGAA 2408
```

Ameiurus nebulosus voucher NEFC_F16-115 mitochondrion, complete genome

```
  1        10        20        30        40        50
  |        |         |         |         |         |
  CGTCAAGAACTCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTG
  GGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCC
  TAAAACCAAGAGTGACAACTCTAAGTCGCAGAA
```

```
5. >MF621733 Ameiurus nebulosus voucher NEFC_F16-113 mitochondrion, complete genom
e

Length = 16513

E-value = 2.38e-58, Score = 127, Bitscore = 235.645, Identities = 130/133 (97%),
Positives = 133/133 (100%), Gaps = 0/133 (0%)
Frame = +1

CF3N con      2 CGTCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC   61
                CGTCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC
MF621733   2276 CGTCAAGAACTCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC 2335

CF3N con     62 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT  121
                GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT
MF621733   2336 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT 2395

CF3N con    122 CTAAGTCRCAGAA  134
                CTAAGTC+CAGAA
MF621733   2396 CTAAGTCGCAGAA 2408
```

Ameiurus nebulosus voucher NEFC_F16-113 mitochondrion, complete genome

```
  1        10        20        30        40        50
  |        |         |         |         |         |
  CGTCAAGAACTCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTG
  GGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCC
  TAAAACCAAGAGTGACAACTCTAAGTCGCAGAA
```

```
6. >KY231825 Ameiurus melas isolate A_melas_002 16S ribosomal RNA gene, partial se
quence; mitochondrial

Length = 546

E-value = 2.38e-58, Score = 127, Bitscore = 235.645, Identities = 130/133 (97%),
Positives = 133/133 (100%), Gaps = 0/133 (0%)
Frame = +1

CF3N con     2 CGTCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC  61
               CGTCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC
KY231825   222 CGTCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC 281

CF3N con    62 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT 121
               GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT
KY231825   282 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT 341

CF3N con   122 CTAAGTCRCAGAA 134
               CTAAGTC+CAGAA
KY231825   342 CTAAGTCGCAGAA 354
```

Ameiurus melas isolate A_melas_002 16S ribosomal RNA gene, partial sequence; mitochondrial

```
  1        10        20        30        40        50
  |        |         |         |         |         |
  CGTCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTG
  GGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCC
  TAAAACCAAGAGTGACAACTCTAAGTCGCAGAA
```

```
7. >KR476911 Ameiurus nebulosus isolate Ameneb1 16S ribosomal RNA gene, partial se
quence; mitochondrial

Length = 496

E-value = 2.38e-58, Score = 127, Bitscore = 235.645, Identities = 130/133 (97%),
Positives = 133/133 (100%), Gaps = 0/133 (0%)
Frame = +1

CF3N con     2 CGTCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC  61
               CGTCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC
KR476911   217 CGTCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC 276

CF3N con    62 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT 121
               GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT
KR476911   277 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT 336

CF3N con   122 CTAAGTCRCAGAA 134
               CTAAGTC+CAGAA
KR476911   337 CTAAGTCGCAGAA 349
```

Ameiurus nebulosus isolate Ameneb1 16S ribosomal RNA gene, partial sequence; mitochondrial

```
  1        10        20        30        40        50
  |        |         |         |         |         |
  CGTCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTG
  GGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCC
  TAAAACCAAGAGTGACAACTCTAAGTCGCAGAA
```

```
8. >KR476830 Ameiurus nebulosus isolate Ameneb2 16S ribosomal RNA gene, partial se
quence; mitochondrial

Length = 498

E-value = 2.38e-58, Score = 127, Bitscore = 235.645, Identities = 130/133 (97%),
Positives = 133/133 (100%), Gaps = 0/133 (0%)
Frame = +1

CF3N con     2 CGTCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC  61
               CGTCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC
KR476830   219 CGTCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC 278

CF3N con    62 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT 121
               GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT
KR476830   279 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT 338

CF3N con   122 CTAAGTCRCAGAA 134
               CTAAGTC+CAGAA
KR476830   339 CTAAGTCGCAGAA 351
```

Ameiurus nebulosus isolate Ameneb2 16S ribosomal RNA gene, partial sequence; mitochondrial

```
  1        10        20        30        40        50
  |        |         |         |         |         |
  CGTCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTG
  GGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCC
  TAAAACCAAGAGTGACAACTCTAAGTCGCAGAA
```

```
9. >JX899750 Ameiurus nebulosus voucher ANSP 182780 12S ribosomal RNA gene, partia
l sequence; tRNA-Val gene, complete sequence; and 16S ribosomal RNA gene, partia
l sequence; mitochondrial

Length = 2657

E-value = 2.38e-58, Score = 127, Bitscore = 235.645, Identities = 130/133 (97%),
Positives = 133/133 (100%), Gaps = 0/133 (0%)
Frame = +1

CF3N con      2 CGTCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC   61
                CGTCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC
JX899750   2189 CGTCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC 2248

CF3N con     62 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT  121
                GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT
JX899750   2249 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT 2308

CF3N con    122 CTAAGTCRCAGAA  134
                CTAAGTC+CAGAA
JX899750   2309 CTAAGTCGCAGAA 2321
```

Ameiurus nebulosus voucher ANSP 182780 12S ribosomal RNA gene, partial sequence; tRNA-Val gene, complete sequence; and 16S ribosomal RNA gene, partial sequence; mitochondrial

```
  1        10        20        30        40        50
  |        |         |         |         |         |
  CGTCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTG
  GGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCC
  TAAAACCAAGAGTGACAACTCTAAGTCGCAGAA
```

```
10. >DQ421891 Ameiurus nebulosus voucher FLEBr1 16S ribosomal RNA gene, partial seq
uence; mitochondrial

Length = 501

E-value = 2.38e-58, Score = 127, Bitscore = 235.645, Identities = 130/133 (97%),
Positives = 133/133 (100%), Gaps = 0/133 (0%)
Frame = +1

CF3N con     2 CGTCAAGAACYCAGTTRAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC  61
               CGTCAAGAAC+CAGTT+AACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC
DQ421891   257 CGTCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTGGGGCGACCAC 316

CF3N con    62 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT 121
               GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT
DQ421891   317 GGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCCTAAAACCAAGAGTGACAACT 376

CF3N con   122 CTAAGTCRCAGAA 134
               CTAAGTC+CAGAA
DQ421891   377 CTAAGTCGCAGAA 389
```

Ameiurus nebulosus voucher FLEBr1 16S ribosomal RNA gene, partial sequence; mitochondrial

```
  1        10        20        30        40        50
  |        |         |         |         |         |
  CGTCAAGAACCCAGTTAAACTAAGTAGCACCTGATCCCAATCTTCTGTTG
  GGGCGACCACGGGAGAAAATAAAGCTCCCACGCGGACTGGGGCAACCCCC
  TAAAACCAAGAGTGACAACTCTAAGTCGCAGAA
```