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Parentage Analysis of the Progenies of the Reciprocal Crosses of *Pangasianodon hypophthalmus* (Sauvage, 1878) and *Clarias gariepinus* (Burchell, 1822) using Cytochrome b Gene

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Abstract

In this study, parentage analysis of the progenies from reciprocal crosses of *Pangasianodon hypophthalmus* (PH) and *Clarias gariepinus* (CG) was accomplished by sequencing the cytochrome b gene and running phylogenetic analysis. The result obtained showed that the nucleotide composition of the cytochrome b gene of the reciprocal hybrid crosses exhibited high (99 %) similarity with those of the maternal parents notwithstanding the different morphology within the hybrid pool (i.e. Panga-like and Clarias-like). The analysis of 1076bp of the cytochrome b gene using three methods for inferring molecular relationships (Maximum Likelihood, Neighbour Joining, and Maximum Parsimony) revealed two main clusters. The upper cluster was a mix of *C. gariepinus* and the two distinct progenies of ♀CG × ♂PH (i.e. Panga-like and Clarias-like), while the lower cluster was a mix of the *P. hypophthalmus* and the progenies of ♀PH × ♂CG (i.e. Panga-like only). Hence, despite, phenotypic differences within the hybrid pool, progenies still inherit cytochrome b gene from the maternal parent alone. As a result, the direction of crosses of the reciprocal crosses could be accurately determined.

Keywords: Asian catfish, African catfish, Cytochrome b, Hybrid morphotype.

1. Introduction

The main aim of hybridization between different fish groups or species is to produce offsprings that perform better than the parental species (Bartley *et al.,* 2001; Okomoda *et al.,* 2017). However, unintended consequences have been experienced as a result of accidental backcrossing, hence, threatening the diversity of many freshwater fish species (Epifanio and Nielsen, 2000; Perry *et al.,* 2002; Senanan *et al.,* 2004; Na-Nakorn *et al.,* 2004). Aside from the fear of genetic homogenization of farmed and natural fish stocks (Hashimoto et al. 2010); hybrids may compete successfully with the native parental lineages in several ways (Ryman and Utter, 1987; Allendorf *et al.,* 2001; Rosenfield *et al.,* 2004), hence, the need for characterization.

The efficacy of molecular markers in the determination of the hybridization status, direction of crosses, and genetic introgression cannot be over emphasized (Forbes and Allendorf, 1991; Cianchi *et al.,* 2003; Hänfling *et al.,* 2005). This is because most hybrids inherit part of the genetic makeup of both species (Wilkins, *et al.,* 1994). As a result, the hybridization status of different crosses has been well investigated using nuclear markers since inheritance of this kind of DNA is from both parents (Rieseberg *et al.,* 1990; Sang *et al.,* 1995; Buckler *et al.,* 1997; Odorico and Miller, 1997). Similarly, morphological and cytogenetic analysis has been widely exploited for the same purpose. It is important to note that these methods command the same level of accuracy and at a very lower cost compared to the molecular characterization method (Hashimoto et al. 2010). However, they cannot be used to determine the direction of the hybridization of the crosses.

Although the potential of erythrocyte characterization for directional cross discrimination have been demonstrated in the earlier study by Okomoda *et al.,* (2018a), the outlined limitations of this approach largely reduce their usability in a wide array of species or studies. The most accurate method still remains the characterization of the mitochondrial DNA (mtDNA). This is because the mtDNA is primarily matrilineally inherited. As a result, their analysis can conveniently identify the maternal origin of the hybrids, and hence the direction of crosses (Pitts, 1995). The mitochondrial cytochrome b gene has particularly gained recognition as an important index in the comparisons of closely related taxa such as between populations or species (Degani 2004; Pfrender, *et*

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al., 2004; Perdices, *et al.*, 2005). However, it is conserved enough for clarifying deeper phylogenetic relationships (Allegrucci, *et al.*, 1999; Cunha, *et al.*, 2002; Sulaiman, *et al.*, 2006; Feulner, *et al.*, 2007).

While mtDNA has been exploited for the characterization of hybrids, the phylogenetic relationships between progenies were seldom analysed. This is particularly important in distant crosses which lead to the production of ploidy polymorphism of the hybrid progenies (i.e. different cytogenetic characteristics within the hybrid pool). A good example of such cross is the study on the hybridization between *Pangasianodon hypophthalmus* (Sauvage, 1878) and *Clarias gariepinus* (Burchell, 1822) where three distinct morphotypes were observed (Okomoda *et al.*, 2017, 2018a, 2018b) with different cytogenetic characteristics (Okomoda *et al.*, 2018c). Understanding the various levels of genetic relationships between hybrids and pure crosses could significantly aid in the development of management guidelines for commercial use and for conservation purposes (Birstein *et al.*, 2005; Freyhof *et al.*, 2005; Ludwig, 2008). Haven established the hybridization status of the reciprocal crosses between *P. hypophthalmus* and *C. gariepinus* in the study by Okomoda *et al.*, (2018b, 2018c), this study was designed for parentage determination and to establish phylogenetic relationship between pure and reciprocal progenies of the fishes using the cytochrome b gene.

2. Materials and Methods

Progenies of pure and reciprocal crosses between of *C. gariepinus*, *P. hypophthalmus* used in this study were obtained using the method previously described by Okomoda *et al.*, (2017, 2018 b, c). In brief, mature *P. hypophthalmus* and *C. gariepinus* were spawned by induced breeding using the Ovaprim® hormone (0.5mL/kg). Eggs and sperm were mixed to produce pure *C. gariepinus* (♀CH × ♂CH), pure *P. hypophthalmus* (♀PH × ♂PH), and the reciprocal crosses Clariothalmus (♀CG × ♂PH) and Pangapinus (♀PH × ♂CG). The progenies obtained from six different breeding trials were then cultured for one month in fibreglass tanks. At this point, two morphotypes were conspicuous in the Clariothalmus (Clarias-like and Panga-like), while all Pangapinus were of one morphotype (Panga-like) (See figure 1). All groups were continuously fed with commercial diet (35 % CP), and water quality was maintained at optimum (temperature = 33.0 ± 1.6^{0}C; pH = 7.0 ± 0.41; conductivity = 251 ± 0.31mg l^{-1}; total dissolved solids = 78.0 ± 0.89 mg l^{-1}; dissolved oxygen = 4.7 ± 0.39 mg l^{-1}).

Fifty fishes comprised of *P. hypophthalmus* (10), Clarias-like Clariothalmus (10), Panga-like Clariothalmus (10), Pangapinus (10), and *C. gariepinus* (10) were randomly selected and preserved in 95 % ethanol contained in appropriately labelled 1.5mL tubes. DNA was extracted from the fins of these fishes using specified protocol for Vivantis Nuclear Acid extraction kit (according to manufacturer's instruction). Amplification of the Cytochrome b, was carried out by polymerase chain reaction (PCR) using the universal primers Cyt b 3F (5'-CCACCGTTGTTATTCAACTATAGAAA-3') and Cyt b 3R (5'-AGAATRCTAGCTTTGGGAG-3') which was described by Bowen *et al.*, (2008).

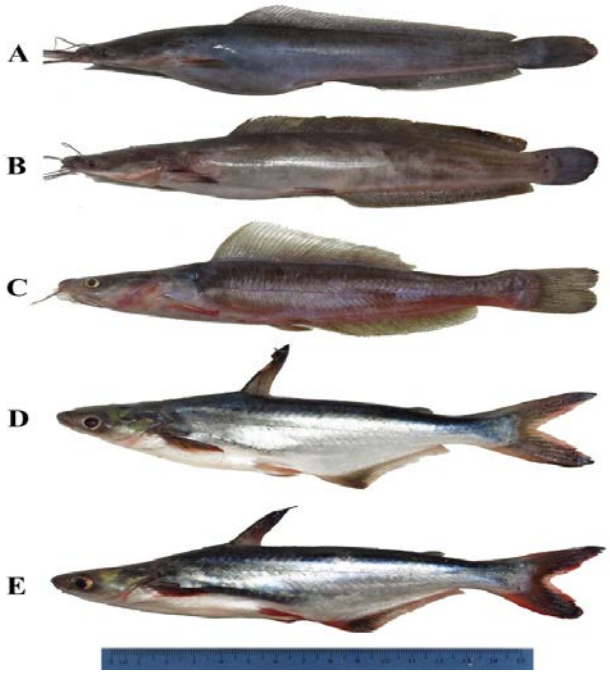

Figure 1. Morphology of (A) *Clarias gariepinus*; (B) Clarias-like Clariothalmus; (C) Panga-like Clariothalmus; (D) Pangapinus and (E) *Pangasianodon hypophthalmus*. (Source Okomoda *et al.*, 2018c)

A reaction volume of 25 µL was used containing 2.5 µL Easy *Taq* buffer, 2.0 µL dNTPs, 0.5 µL each of universal forward and reverse primer, 0.2µL *Taq* DNA polymerase (Easy *Taq*), 0.5µL template DNA, and 18. 8 µL sterile deionised water. Reactions were performed using an Eppendorf Mastercycler. The Cyt b PCR amplification was programed at 95^{0}C for two minutes, followed by thirty-five cycles of denaturation at 95^{0}C (thirty seconds), annealing temperature at 47^{0}C (forty-five seconds) and an extension of 72^{0}C (one minute), this was followed by a final single extension at 72^{0}C (seven minutes). An aliquot of the reaction was subjected to electrophoresis on a 1.0 % agarose gel. The gels were visualized using a gel documentation system (BIO RAD, USA).

PCR products were sent for sequencing at First Base Laboratories SDN BHD, Malaysia. All sequences were aligned and edited using ClustalW as implemented in MEGA version 6. Sequence ambiguities were resolved by comparing complementary strands of ten nucleotide sequences per group of fish. The identity of the sequences was confirmed using BLAST at the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastnandGE_TYPE=BlastSearchandLINK_LOC=blasthome). The percentages of similarities of all the progenies with the available sequences on the NCBI website were gotten.

The relationship between the different groups of fish was examined using neighbour joining (Saitou and Nei 1987), maximum parsimony and maximum likelihood (Felsenstein 1983) methods (using MEGA version 6). The confidence of the branching patterns was assessed by 1000 bootstrap replicates in the NJ analysis (Hillis and Bull 1993). The out group used was *Cyprinus carpio* (NCBI

Accession number: AB158806), while *C. macrocephalus* (NCBI Accession number: KJ533248) and *Pangasius nasutus* (NCBI Accession number: HM236395) were used as the sister groups in this study.

3. Results

Amplification of the universal primers in relation to the mitochondrial cytochrome b gene showed bands of approximately 1300bp for all the groups of fish and their different morphotypes (Appendix 1). The partial sequences obtained for the cytochrome b revealed very high similarity between the nucleotide composition of the reciprocal crosses and the maternal parents irrespective of their morphotypes. Blast also showed that the target sequence was gotten as the nucleotide compositions were 99 % identical with available cytochrome b sequences in the GenBank for *Clarias gariepinus* (accession number KJ533253.1) and *Pangasianodon hypophthalmus* (accession number KM434895.1) (respectively for pure and reciprocal progenies with Clarias and Panga maternal origins) (Appendix 2, 3, 4, 5 and 6). The results obtained by sequencing allowed the analysis of 1076bp of the Cyt b gene. Similar genetic pattern were obtained from the three methods used for inferring molecular relationships in this study (Maximum likelihood, Neighbour Joining, and Maximum Parsimony Respectively for Figure 2, Appendix 7 and 8). From the result obtained, two main groups were clearly defined. The upper group was a mix of *C. gariepinus*, Clarias-like Clariothalmus and Panga-like Clariothalmus with three haplotypes. The lower group, on the other hand, was a mix of the Pangapinus and the *P. hypophthalmus* with six observed haplotypes. The variations in the difference haplotypes occurred in three bases or less.

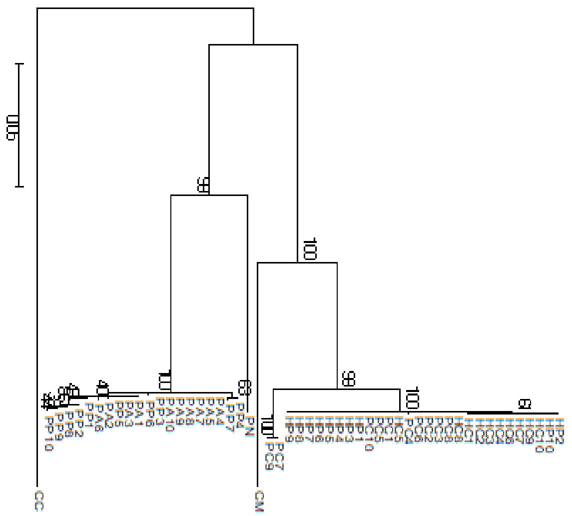

Figure 2. Phylogenetic relationships of the different progenies following Maximum likelihood analysis of 1076bp of the cytochrome b gene. PC1 - PC10 = *C. gariepinus*; HC1 – HC10 = Clarias-like Clariothalmus; HP1 – HP10 = Panga-like Clariothalmus; PA1 - PA10 = Pangapinus; PP1 - PP10 = *P. hypophthalmus*; CC = *Cyprinus carpio*; CM = *Clarias macrocephalus;* PN = *Pangasius nasutus.* (Support values are bootstrap values).

The genetic distances within haplotypes of the same groups in this study (i.e. pure and hybrid crosses from the same maternal origin) ranged between 0.000 - 0.056 (Table 1). However, the genetic distance between the pure P. hypophthalmus and C. gariepinus ranges from 0.472 - 0.506.

Table 1: Genetic distance range between pure and reciprocal crosses of *Pangasianodon hypophthalmus* and pure *Clarias gariepinus*.

	PC	HC	HP	PA	PP
PC	0.000 – 0.056				
HC	0.000 – 0.056	0.000 – 0.056			
HP	0.000 – 0.056	0.000 – 0.054	0.000 – 0.056		
PA	0.472 – 0.502	0.475 – 0.504	0.472 – 0.506	0.010 – 0.051	
PP	0.472 – 0.506	0.470 – 0.491	0.472 – 0.494	0.000 – 0.052	0.000 – 0.050

Keys: PC = *C. gariepinus*; HC1 = Clarias-like Clariothalmus; HP1 = Panga-like Clariothalmus; PA1 = Pangapinus; PP1 = *P. hypophthalmus*.

4. Discussion

Different researchers had justified the need to discriminate reciprocal crosses of different species because hybrid progenies could display different biological and zootechnical characteristics (Tave, 1993; Toledo- Filho *et al.*1998; Porto-Foresti *et al.* 2008). More so, information on the directionality of mating can have significant effects on a number of factors including spatial, temporal, behavioural, physiological, and stock composition of the hybrid progenies (Pitts, 1995; Scribner *et al.*, 2001). This assumption is validated by the results of early studies which show differences in performance not only between the reciprocal crosses of *P. hypophthalmus* and *C. gariepinus,* but also between progenies of the hybrid Clariothalmus (i.e Clarias-like and Panga-like) (Okomoda *et al.*, 2018b, 2018c). Similarly, Dunham et al. (1982) had justified molecular discrimination of F1 progenies of the hybrids ♀*Ictalurus punctatus* × ♂*I. furcatus* and ♀*I. furcatus* × ♂*I. punctatus* on the bases of differential growth performances.

The cytochrome b gene is a highly conserved region (Moritz *et al.*, 1987; Olufeagba and Okomoda 2016); hence, the universal primer used in this study uniformly amplified 1300bp in all the progenies tested as expected. The observation of the very high similarity between the progenies of the reciprocal crosses and their maternal parent confirms the direction of crosses. Similarly, the phylogenetic analysis shows two separate groups of the test progenies in direction of the maternal origin. Observations of previous studies using different mtDNA genes are in line with the findings of this study. do-Prado *et al.*, (2011) had used 16S mitochondrial genes to discriminate reciprocal hybrids ‘pintachara’ and ‘cachapinta’ by the identification of the maternal parent of these crosses. The efficacy of differentiating reciprocal hybrids of *Leporinus macrocephalus* and *L. elongatus* using 16S mitochondrial DNA had also been reported by

Hashimoto *et al*., (2010). In addition, Olufeagba and Okomoda (2016) confirmed the maternal origin of the hybrid ♀ *C. gariepinus* × ♂ *C. batrachus* with the cytochrome b gene. Waldbrieser and Bosworth (2008) were able to discriminate reciprocal crosses of channel catfish (*I. punctatus*) and blue catfish (*I. furcatus*) hybrid using the mitochondrial cytochrome c oxidase 1 gene.

On the whole, mitochondrial DNA have proven to be a very effective molecular tool in the identification of the parental status of hybrids since it is cytoplasmically housed and only inherited from the mother to the offspring, and has no paternal contribution (Moritz *et al*., 1987; Wyatt *et al*., 2006). Hence, the pattern of genetic homogenization between progenies from the same maternal parent and distinct groups observed for the different maternal origins clearly shows the direction of crosses of the progenies. In line with this, Nazia *et al*., (2010) had earlier stated that high levels of genetic homogenization are large because of the common origin or the ongoing gene flow. Notwithstanding, different haplotype groups with close genetic distances were observed within the two main groups in this study. This observed variation within the progenies with similar maternal origins may have resulted from the different population of broodstocks used for the six different trials of breeding. Similar assumption has been advanced by several authors for the observation of haplotypes in fishes from different populations (e.g. Windsor and Hutchinson1995; Williams, *et al*., 1997; Doupe' and Lymbery 1999; Cross 2000; Englbrecht, *et al*., 2002; Frost et al. 2006; Norfatimah *et al*., 2009; Nazia *et al*., 2010).

Although *C. gariepinus* and *P. hypophthalmus* belong to different families, the phylogenetic analysis of the mitochondrial cytochrome b showed a considerably close genetic distance between these two species. Pardo *et al*., (2005) and Azevedo *et al*., (2008), have earlier hypothesized that close genetic distances between different fish families could explain the feasibility of hybridization between them. This assumption may be the reason for the successful hybridization of *C. gariepinus* and *P. hypophthalmus*. Although the hybridization status of the reciprocal crosses produced in this study were earlier confirmed using morphological (Okomoda *et al*., 2018b) and cytogenetic tools (Okomoda *et al*., 2018c), the direction of hybridization or cross combination could not be determined. The use of erythrocyte characterization had also been advanced in previous studies by the authors of this research, but with some limitations (Okomoda *et al*., 2018a). However, the characterization of the mitochondrial DNA still remains the most accurate and unambiguous tool for directional cross determination.

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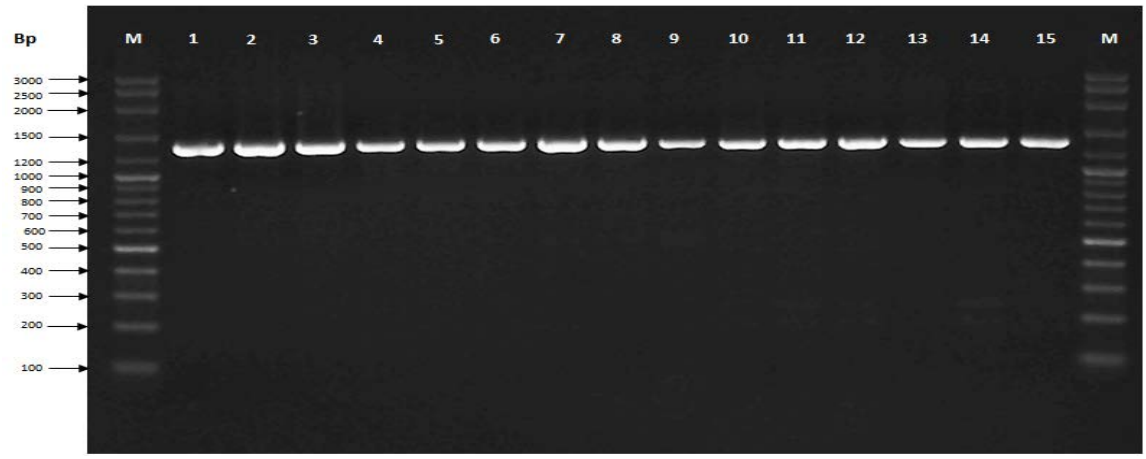
Appendix



Appendix 1. Agarose gel showing the amplified PCR products of the Cyt B of the progenies of pure and reciprocal crosses of *Pangasianodon hypophthalmus* and pure *Clarias gariepinus*. M: Marker; Lane 1-3: *C. gariepinus*; Lane 4-6: Clarias-like Clariothalmus; Lane 7-9: Panga-like Clariothalmus; Lane 10-12: Pangapinus: Lane 13-15: *P. hypophthalmus*.

Download GenBank Graphics

Clarias gariepinus isolate BCG2 cytochrome b gene, partial cds; mitochondrial

Sequence ID: KJ533253.1 Length: 1114 Number of Matches: 1

Range 1: 39 to 1114 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1982 bits(1073)	0.0	1075/1076(99%)	0/1076(0%)	Plus/Plus

```
Query  1     CGACGCACTCATCGACCTTCCCGCCCCCTCTAATATCTCCGCATGATGAAACTTTGGCTC  60
Sbjct  39    CGACGCACTCATCGACCTTCCCGCCCCCTCTAATATCTCCGCATGATGAAACTTTGGCTC  98
Query  61    ACTACTATTACTATGTCTTGGAGTACAAATCCTCACAGGACTATTCCTAGCCATACACTA  120
Sbjct  99    ACTACTATTACTATGTCTTGGAGTACAAATCCTCACAGGACTATTCCTAGCCATACACTA  158
Query  121   CACTTCTGATATCTCAACCGCATTCTCATCAGTAGTACACATCTGCCGAGACGTCAACTA  180
Sbjct  159   CACTTCTGATATCTCAACCGCATTCTCATCAGTAGTACACATCTGCCGAGACGTCAACTA  218
Query  181   CGGATGAATCATCCGAAACCTTCACGCCAACGGAGCATCCTTCTTCTTCATCTGCATCTA  240
Sbjct  219   CGGATGAATCATCCGAAACCTTCACGCCAACGGAGCATCCTTCTTCTTCATCTGCATCTA  278
Query  241   CCTTCACATTGGCCGTGGTCTGTACTATGGCTCATACCTATACAAAGAGACCTGAAACAT  300
Sbjct  279   CCTTCACATTGGCCGTGGTCTGTACTATGGCTCATACCTATACAAAGAGACCTGAAACAT  338
Query  301   CGGCGTCGTACTACTCCTTTTAGTAATAATAACAGCCTTCGTAGGATACGTACTACCATG  360
Sbjct  339   CGGCGTCGTACTACTCCTTTTAGTAATAATAACAGCCTTCGTAGGATACGTACTACCATG  398
Query  361   AGGACAAATATCCTTCTGAGGTGCCACAGTAATCACAAACCTCTTATCAGCCGTACCCTA  420
Sbjct  399   AGGACAAATATCCTTCTGAGGTGCCACAGTAATCACAAACCTCTTATCAGCCGTACCCTA  458
Query  421   CATAGGAGATGCCCTAGTCCAATGAATCTGAGGAGGCTTCTCCGTAGACAATGCAACACT  480
Sbjct  459   CATAGGAGATGCCCTAGTCCAATGAATCTGAGGAGGCTTCTCCGTAGACAATGCAACACT  518
Query  481   TACACGATTCTTCGCATTCCACTTCCTCCTACCATTCACAATCATCGCAGCTACAATTCT  540
Sbjct  519   TACACGATTCTTCGCATTCCACTTCCTCCTACCATTCACAATCATCGCAGCTACAATTCT  578
Query  541   ACACGCACTATTCCTACACGAAACAGGATCAAACAACCCAATTGGACTAAACTCCGACGC  600
Sbjct  579   ACACGCACTATTCCTACACGAAACAGGATCAAACAACCCAATTGGACTAAGCTCCGACGC  638
Query  601   AGACAAAATCTCATTCCACCCATATTTCTCCTACAAAGACCTACTAGGATTTATCATTCT  660
Sbjct  639   AGACAAAATCTCATTCCACCCATATTTCTCCTACAAAGACCTACTAGGATTTATCATTCT  698
Query  661   ATTAACAGCCCTCGCATCTCTAAGCCTATTCTCCCCAAACCTTCTAGGCGACCCAGAAAA  720
Sbjct  699   ATTAACAGCCCTCGCATCTCTAAGCCTATTCTCCCCAAACCTTCTAGGCGACCCAGAAAA  758
Query  721   CTTCACCCCCGCCAACCCCCTAGTAACTCCACCTCACATCAAACCAGAATGATACTTCCT  780
Sbjct  759   CTTCACCCCCGCCAACCCCCTAGTAACTCCACCTCACATCAAACCAGAATGATACTTCCT  818
Query  781   ATTCGCATACGCCATCCTCCGATCCATCCCAAACAAACTAGGCGGAGTATTAGCACTATT  840
Sbjct  819   ATTCGCATACGCCATCCTCCGATCCATCCCAAACAAACTAGGCGGAGTATTAGCACTATT  878
Query  841   ATTCTCCATCCTAGTACTAATAGTAGTAGCACTACTACACCTCTCAAAACAACAGGGCCT  900
Sbjct  879   ATTCTCCATCCTAGTACTAATAGTAGTAGCACTACTACACCTCTCAAAACAACAGGGCCT  938
Query  901   AACCTTCCGACCTTTATCCCAAATCTTATTCTGAACCCTAGTAGCAGATGTAATAATCTT  960
Sbjct  939   AACCTTCCGACCTTTATCCCAAATCTTATTCTGAACCCTAGTAGCAGATGTAATAATCTT  998
Query  961   AACATGAATCGGCGGCATACCAGTAGAACATCCGTTCATCATTATCGGACAAATCGCCTC  1020
Sbjct  999   AACATGAATCGGCGGCATACCAGTAGAACATCCGTTCATCATTATCGGACAAATCGCCTC  1058
Query  1021  CATCCTCTACTTCTCCCTATTCCTCATCTTAAACCCACTAGCAGCCTGACTAGAAA  1076
Sbjct  1059  CATCCTCTACTTCTCCCTATTCCTCATCTTAAACCCACTAGCAGCCTGACTAGAAA  1114
```

Appendix 2. Aligned *Clarias gariepinus* cytochrome b sequence showing 99 % similarity with *Clarias gariepinus* isolate BCG2 cytochrome b gene, partial cds; mitochondrion (GenBank Accession Number KJ533253.1).

Appendix 3. Aligned Clarias-like Clariothalmus cytochrome b sequence showing 99 % similarity with *Clarias gariepinus* isolate BCG2 cytochrome b gene, partial cds; mitochondrion (GenBank Accession Number KJ533253.1).

Download GenBank Graphics

Clarias gariepinus isolate BCG2 cytochrome b gene, partial cds; mitochondrial

Sequence ID: KJ533253.1 **Length:** 1114 **Number of Matches:** 1

Range 1: 39 to 1114 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1982 bits(1073)	0.0	1075/1076(99%)	0/1076(0%)	Plus/Plus

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Query  1     CGACGCACTCATCGACCTTCCCGCCCCCTCTAATATCTCCGCATGATGAAACTTTGGCTC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  39    CGACGCACTCATCGACCTTCCCGCCCCCTCTAATATCTCCGCATGATGAAACTTTGGCTC  98

Query  61    ACTACTATTACTATGTCTTGGAGTACAAATCCTCACAGGACTATTCCTAGCCATACACTA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  99    ACTACTATTACTATGTCTTGGAGTACAAATCCTCACAGGACTATTCCTAGCCATACACTA  158

Query  121   CACTTCTGATATCTCAACCGCATTCTCATCAGTAGTACACATCTGCCGAGACGTCAACTA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  159   CACTTCTGATATCTCAACCGCATTCTCATCAGTAGTACACATCTGCCGAGACGTCAACTA  218

Query  181   CGGATGAATCATCCGAAACCTTCACGCCAACGGAGCATCCTTCTTCTTCATCTGCATCTA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  219   CGGATGAATCATCCGAAACCTTCACGCCAACGGAGCATCCTTCTTCTTCATCTGCATCTA  278

Query  241   CCTTCACATTGGCCGTGGTCTGTACTATGGCTCATACCTATACAAAGAGACCTGAAACAT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  279   CCTTCACATTGGCCGTGGTCTGTACTATGGCTCATACCTATACAAAGAGACCTGAAACAT  338

Query  301   CGGCGTCGTACTACTCCTTTTAGTAATAATAACAGCCTTCGTAGGATACGTACTACCATG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  339   CGGCGTCGTACTACTCCTTTTAGTAATAATAACAGCCTTCGTAGGATACGTACTACCATG  398

Query  361   AGGACAAATATCCTTCTGAGGTGCCACAGTAATCACAAACCTCTTATCAGCCGTACCCTA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  399   AGGACAAATATCCTTCTGAGGTGCCACAGTAATCACAAACCTCTTATCAGCCGTACCCTA  458

Query  421   CATAGGAGATGCCCTAGTCCAATGAATCTGAGGAGGCTTCTCCGTAGACAATGCAACACT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  459   CATAGGAGATGCCCTAGTCCAATGAATCTGAGGAGGCTTCTCCGTAGACAATGCAACACT  518

Query  481   TACACGATTCTTCGCATTCCACTTCCTCCTACCATTCACAATCATCGCAGCTACAATTCT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  519   TACACGATTCTTCGCATTCCACTTCCTCCTACCATTCACAATCATCGCAGCTACAATTCT  578

Query  541   ACACGCACTATTCCTACACGAAACAGGATCAAACAACCCAATTGGACTAAACTCCGACGC  600
             |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  579   ACACGCACTATTCCTACACGAAACAGGATCAAACAACCCAATTGGACTAAGCTCCGACGC  638

Query  601   AGACAAAATCTCATTCCACCCATATTTCTCCTACAAAGACCTACTAGGATTTATCATTCT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  639   AGACAAAATCTCATTCCACCCATATTTCTCCTACAAAGACCTACTAGGATTTATCATTCT  698

Query  661   ATTAACAGCCCTCGCATCTCTAAGCCTATTCTCCCCAAACCTTCTAGGCGACCCAGAAAA  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  699   ATTAACAGCCCTCGCATCTCTAAGCCTATTCTCCCCAAACCTTCTAGGCGACCCAGAAAA  758

Query  721   CTTCACCCCCGCCAACCCCCTAGTAACTCCACCTCACATCAAACCAGAATGATACTTCCT  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  759   CTTCACCCCCGCCAACCCCCTAGTAACTCCACCTCACATCAAACCAGAATGATACTTCCT  818

Query  781   ATTCGCATACGCCATCCTCCGATCCATCCCAAACAAACTAGGCGGAGTATTAGCACTATT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  819   ATTCGCATACGCCATCCTCCGATCCATCCCAAACAAACTAGGCGGAGTATTAGCACTATT  878

Query  841   ATTCTCCATCCTAGTACTAATAGTAGTACCACTACTACACCTCTCAAAACAACAGGGCCT  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  879   ATTCTCCATCCTAGTACTAATAGTAGTACCACTACTACACCTCTCAAAACAACAGGGCCT  938

Query  901   AACCTTCCGACCTTTATCCCAAATCTTATTCTGAACCCTAGTAGCAGATGTAATAATCTT  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  939   AACCTTCCGACCTTTATCCCAAATCTTATTCTGAACCCTAGTAGCAGATGTAATAATCTT  998

Query  961   AACATGAATCGGCGGCATACCAGTAGAACATCCGTTCATCATTATCGGACAAATCGCCTC  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  999   AACATGAATCGGCGGCATACCAGTAGAACATCCGTTCATCATTATCGGACAAATCGCCTC  1058

Query  1021  CATCCTCTACTTCTCCCTATTCCTCATCTTAAACCCACTAGCAGCCTGACTAGAAA  1076
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1059  CATCCTCTACTTCTCCCTATTCCTCATCTTAAACCCACTAGCAGCCTGACTAGAAA  1114
```

Appendix 4. Aligned Panga-like Clariothalmus cytochrome b sequence showing 99 % similarity with *Clarias gariepinus* isolate BCG2 cytochrome b gene, partial cds; mitochondrion (GenBank Accession Number KJ533253.1).

Download GenBank Graphics

Pangasianodon hypophthalmus isolate PSH02 cytochrome b (Cytb) gene, partial cds; mitochondrial

Sequence ID: KM434895.1 Length: 1139 Number of Matches: 1

Range 1: 42 to 1117 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1982 bits(1073)	0.0	1075/1076(99%)	0/1076(0%)	Plus/Plus

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Query  1     CGACGCACTAATTGACCTTCCTGCCCCATCCAATATTTCCGCATGATGAAACTTTGGTTC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  42    CGACGCACTAATTGACCTTCCTGCCCCATCCAATATTTCCGCATGATGAAACTTTGGTTC  101

Query  61    CCTACTATTATTATGCCTTATAGTACAGATCCTAACAGGACTTTTCCTAGCCATACATTA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  102   CCTACTATTATTATGCCTTATAGTACAGATCCTAACAGGACTTTTCCTAGCCATACATTA  161

Query  121   TACCTCAGACATCTCTACTGGCTTCTCATCCGTAGCCCACATCTGTCGAGATGTAAATTA  180
             |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  162   TACCTCAGACATCTCTACTGCCTTCTCATCCGTAGCCCACATCTGTCGAGATGTAAATTA  221

Query  181   CGGATGAGTCATCCGCAACTTACATGCCAACGGAGCTTCATTCTTTTTCATCTGTATTTA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  222   CGGATGAGTCATCCGCAACTTACATGCCAACGGAGCTTCATTCTTTTTCATCTGTATTTA  281

Query  241   CCTACACATCGGACGAGGATTATATTATGGCTCTTACTTATATAAAGAAACCTGAAATAT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  282   CCTACACATCGGACGAGGATTATATTATGGCTCTTACTTATATAAAGAAACCTGAAATAT  341

Query  301   TGGAGTAGTACTTCTCCTATTAGTTATAATAACCGCCTTCGTCGGATATGTTTTACCATG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  342   TGGAGTAGTACTTCTCCTATTAGTTATAATAACCGCCTTCGTCGGATATGTTTTACCATG  401

Query  361   AGGTCAAATATCATTTTGAGGCGCCACAGTAATCACAAATCTCCTATCAGCTGTCCCTTA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  402   AGGTCAAATATCATTTTGAGGCGCCACAGTAATCACAAATCTCCTATCAGCTGTCCCTTA  461

Query  421   CATAGGAGATATACTAGTACAATGAATTTGAGGTGGCTTCTCCGTAGACAATGCAACACT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  462   CATAGGAGATATACTAGTACAATGAATTTGAGGTGGCTTCTCCGTAGACAATGCAACACT  521

Query  481   AACACGATTCTTCGCATTTCACTTCCTACTTCCATTCGTAATTGTCGCAGCCACAGTATT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  522   AACACGATTCTTCGCATTTCACTTCCTACTTCCATTCGTAATTGTCGCAGCCACAGTATT  581

Query  541   ACATGCCTTATTCCTACACGAAACAGGCTCCAATAACCCAATTGGCCTAAACTCCGACGC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  582   ACATGCCTTATTCCTACACGAAACAGGCTCCAATAACCCAATTGGCCTAAACTCCGACGC  641

Query  601   AGACAAAATCTCCTTCCACCCATACTTCTCCTATAAAGATGTATTAGGATTCATAATCCT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  642   AGACAAAATCTCCTTCCACCCATACTTCTCCTATAAAGATGTATTAGGATTCATAATCCT  701

Query  661   CCTCACAGCCCTCGCATCTTTAGCCCTCTTCTCACCAAACCTTTTAGGAGATCCAGAAAA  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  702   CCTCACAGCCCTCGCATCTTTAGCCCTCTTCTCACCAAACCTTTTAGGAGATCCAGAAAA  761

Query  721   CTTCACCCCAGCCAACCCATTAGTAACACCGCCCCACATCAAACCAGAATGATACTTCCT  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  762   CTTCACCCCAGCCAACCCATTAGTAACACCGCCCCACATCAAACCAGAATGATACTTCCT  821

Query  781   ATTTGCATATGCCATCCTACGATCAATCCCAAATAAGCTAGGAGGGGTCCTGGCCCTACT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  822   ATTTGCATATGCCATCCTACGATCAATCCCAAATAAGCTAGGAGGGGTCCTGGCCCTACT  881

Query  841   ATTCTCCATCCTAGTATTAATAGTTGTTCCCCTATTACACACCTCTAAACAACAAGGCCT  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  882   ATTCTCCATCCTAGTATTAATAGTTGTTCCCCTATTACACACCTCTAAACAACAAGGCCT  941

Query  901   CACTTTCCGCCCCCTCTCCCAATTCCTATTCTGAGCCCTAGTAGCAGACGTAGCCATTCT  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  942   CACTTTCCGCCCCCTCTCCCAATTCCTATTCTGAGCCCTAGTAGCAGACGTAGCCATTCT  1001

Query  961   CACTTGAATTGGCGGTATACCAGTCGAACACCCATTCATCATTATCGGACAAATCGCCTC  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1002  CACTTGAATTGGCGGTATACCAGTCGAACACCCATTCATCATTATCGGACAAATCGCCTC  1061

Query  1021  CATTTTATATTTTCTCTTCTTCCTAGTCCTAAACCCCCTAGCAGGATGACTAGAAA  1076
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1062  CATTTTATATTTTCTCTTCTTCCTAGTCCTAAACCCCCTAGCAGGATGACTAGAAA  1117
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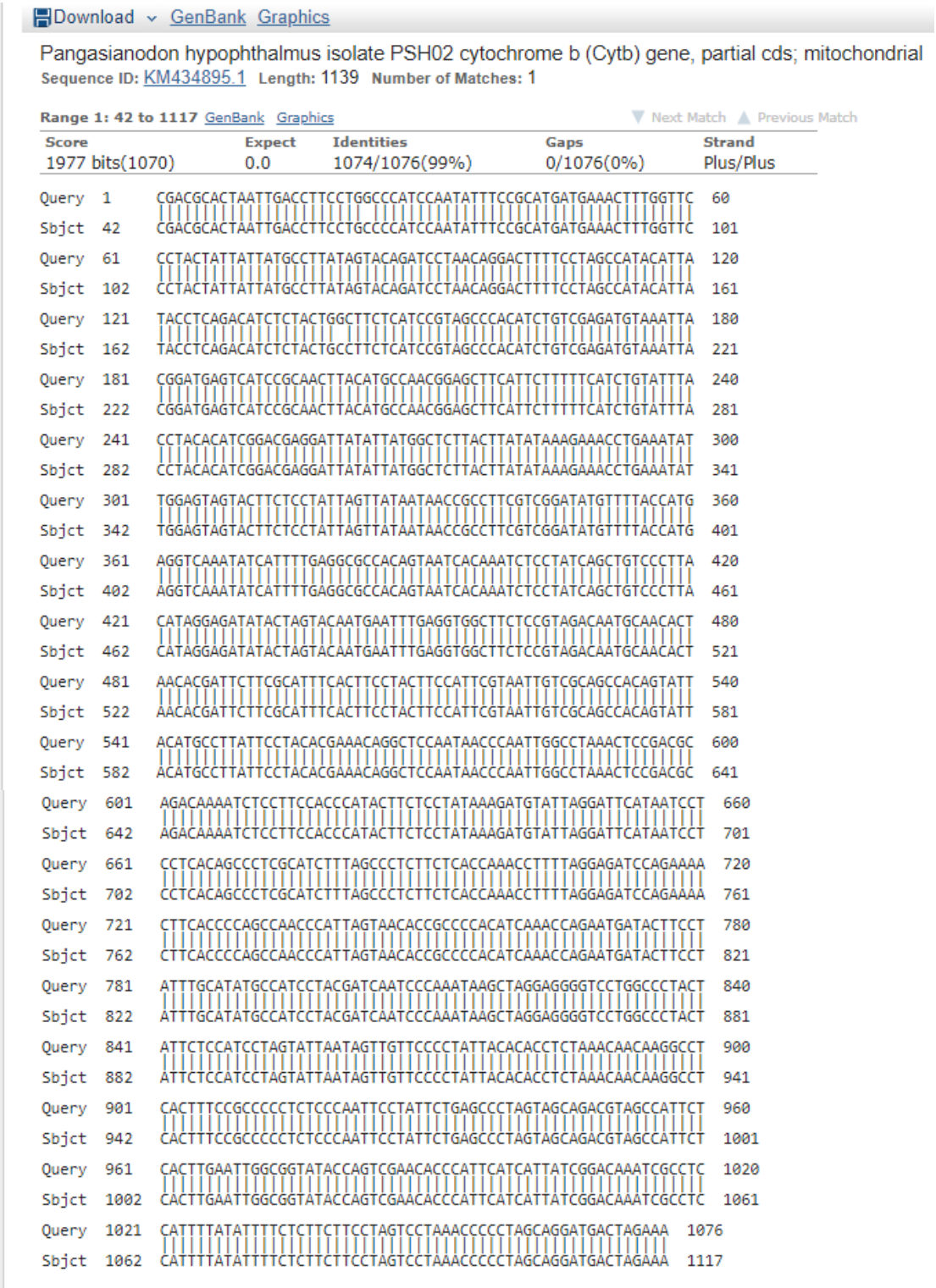
Appendix 5. Aligned Pangapinus cytochrome b sequence showing 99 % similarity with *Pangasianodon hypophthalmus* isolate PSH02 cytochrome b (Cytb) gene, partial cds; mitochondrial sequence (GenBank Accession Number KM434895.1).



Download GenBank Graphics

Pangasianodon hypophthalmus isolate PSH02 cytochrome b (Cytb) gene, partial cds; mitochondrial

Sequence ID: KM434895.1 Length: 1139 Number of Matches: 1

Range 1: 42 to 1117 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1977 bits(1070)	0.0	1074/1076(99%)	0/1076(0%)	Plus/Plus

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Query  1     CGACGCACTAATTGACCTTCCTGGCCCATCCAATATTTCCGCATGATGAAACTTTGGTTC  60
             ||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  42    CGACGCACTAATTGACCTTCCTGCCCCATCCAATATTTCCGCATGATGAAACTTTGGTTC  101

Query  61    CCTACTATTATTATGCCTTATAGTACAGATCCTAACAGGACTTTTCCTAGCCATACATTA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  102   CCTACTATTATTATGCCTTATAGTACAGATCCTAACAGGACTTTTCCTAGCCATACATTA  161

Query  121   TACCTCAGACATCTCTACTGGCTTCTCATCCGTAGCCCACATCTGTCGAGATGTAAATTA  180
             |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  162   TACCTCAGACATCTCTACTGCCTTCTCATCCGTAGCCCACATCTGTCGAGATGTAAATTA  221

Query  181   CGGATGAGTCATCCGCAACTTACATGCCAACGGAGCTTCATTCTTTTTCATCTGTATTTA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  222   CGGATGAGTCATCCGCAACTTACATGCCAACGGAGCTTCATTCTTTTTCATCTGTATTTA  281

Query  241   CCTACACATCGGACGAGGATTATATTATGGCTCTTACTTATATAAAGAAACCTGAAATAT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  282   CCTACACATCGGACGAGGATTATATTATGGCTCTTACTTATATAAAGAAACCTGAAATAT  341

Query  301   TGGAGTAGTACTTCTCCTATTAGTTATAATAACCGCCTTCGTCGGATATGTTTTACCATG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  342   TGGAGTAGTACTTCTCCTATTAGTTATAATAACCGCCTTCGTCGGATATGTTTTACCATG  401

Query  361   AGGTCAAATATCATTTTGAGGCGCCACAGTAATCACAAATCTCCTATCAGCTGTCCCTTA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  402   AGGTCAAATATCATTTTGAGGCGCCACAGTAATCACAAATCTCCTATCAGCTGTCCCTTA  461

Query  421   CATAGGAGATATACTAGTACAATGAATTTGAGGTGGCTTCTCCGTAGACAATGCAACACT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  462   CATAGGAGATATACTAGTACAATGAATTTGAGGTGGCTTCTCCGTAGACAATGCAACACT  521

Query  481   AACACGATTCTTCGCATTTCACTTCCTACTTCCATTCGTAATTGTCGCAGCCACAGTATT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  522   AACACGATTCTTCGCATTTCACTTCCTACTTCCATTCGTAATTGTCGCAGCCACAGTATT  581

Query  541   ACATGCCTTATTCCTACACGAAACAGGCTCCAATAACCCAATTGGCCTAAACTCCGACGC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  582   ACATGCCTTATTCCTACACGAAACAGGCTCCAATAACCCAATTGGCCTAAACTCCGACGC  641

Query  601   AGACAAAATCTCCTTCCACCCATACTTCTCCTATAAAGATGTATTAGGATTCATAATCCT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  642   AGACAAAATCTCCTTCCACCCATACTTCTCCTATAAAGATGTATTAGGATTCATAATCCT  701

Query  661   CCTCACAGCCCTCGCATCTTTAGCCCTCTTCTCACCAAACCTTTTAGGAGATCCAGAAAA  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  702   CCTCACAGCCCTCGCATCTTTAGCCCTCTTCTCACCAAACCTTTTAGGAGATCCAGAAAA  761

Query  721   CTTCACCCCAGCCAACCCATTAGTAACACCGCCCCACATCAAACCAGAATGATACTTCCT  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  762   CTTCACCCCAGCCAACCCATTAGTAACACCGCCCCACATCAAACCAGAATGATACTTCCT  821

Query  781   ATTTGCATATGCCATCCTACGATCAATCCCAAATAAGCTAGGAGGGGTCCTGGCCCTACT  840
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Sbjct  822   ATTTGCATATGCCATCCTACGATCAATCCCAAATAAGCTAGGAGGGGTCCTGGCCCTACT  881

Query  841   ATTCTCCATCCTAGTATTAATAGTTGTTCCCCTATTACACACCTCTAAACAACAAGGCCT  900
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Sbjct  882   ATTCTCCATCCTAGTATTAATAGTTGTTCCCCTATTACACACCTCTAAACAACAAGGCCT  941

Query  901   CACTTTCCGCCCCCCTCTCCCAATTCCTATTCTGAGCCCTAGTAGCAGACGTAGCCATTCT  960
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Sbjct  942   CACTTTCCGCCCCCCTCTCCCAATTCCTATTCTGAGCCCTAGTAGCAGACGTAGCCATTCT  1001

Query  961   CACTTGAATTGGCGGTATACCAGTCGAACACCCATTCATCATTATCGGACAAATCGCCTC  1020
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Sbjct  1002  CACTTGAATTGGCGGTATACCAGTCGAACACCCATTCATCATTATCGGACAAATCGCCTC  1061

Query  1021  CATTTTATATTTTCTCTTCTTCCTAGTCCTAAACCCCCTAGCAGGATGACTAGAAA  1076
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1062  CATTTTATATTTTCTCTTCTTCCTAGTCCTAAACCCCCTAGCAGGATGACTAGAAA  1117
```
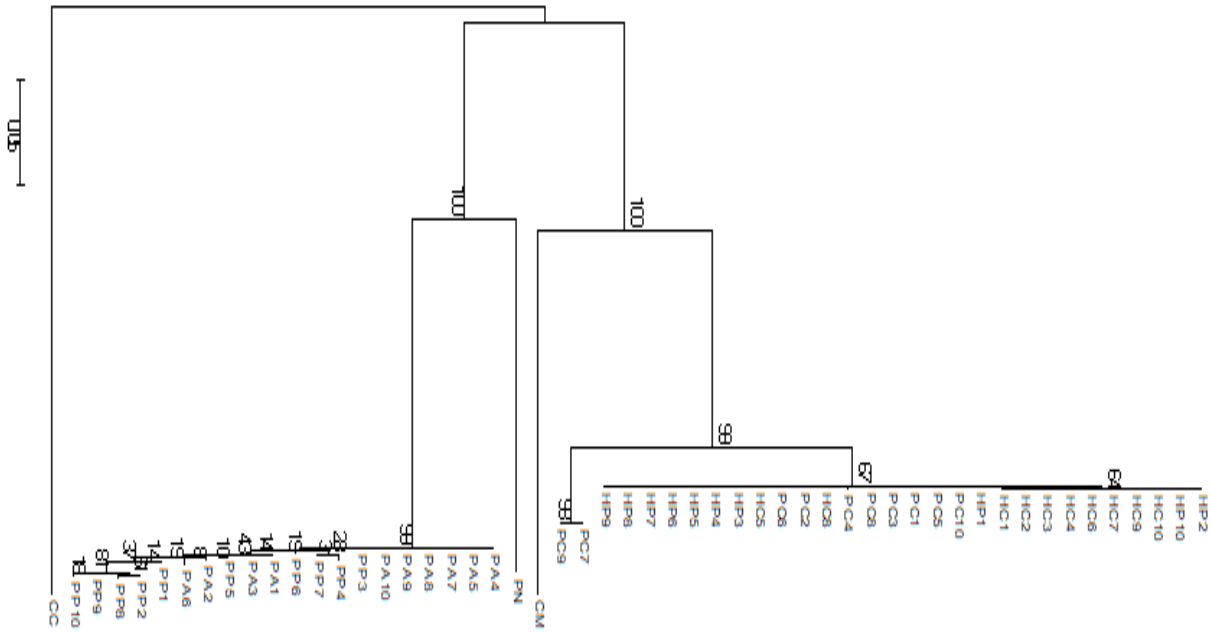
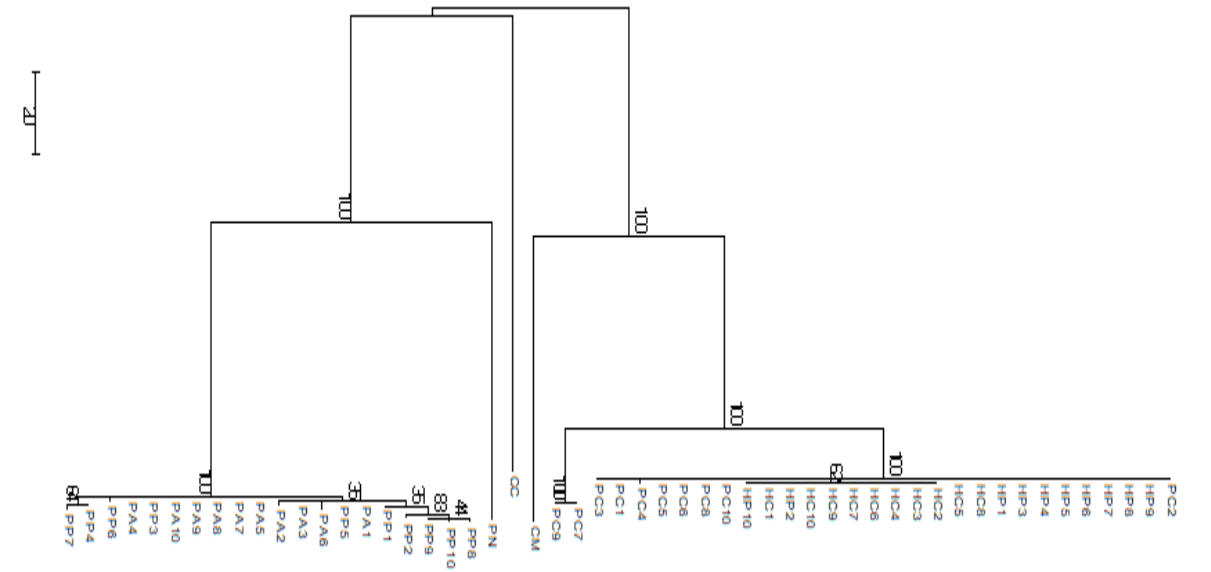

Appendix 6. Aligned *Pangasianodon hypophthalmus* cytochrome b sequence showing 99 % similarity with *Pangasianodon hypophthalmus* isolate PSH02 cytochrome b (Cytb) gene, partial cds; mitochondrial sequence (GenBank Accession Number KM434895.1).



Appendix 7. Phylogenetic relationships of the different progenies following Neighbour joining analysis of 1076bp of the cytochrome b gene. PC1 - PC10 = *C. gariepinus*; HC1 – HC10 = Clarias-like Clariothalmus; HP1 – HP10 = Panga-like Clariothalmus; PA1 - PA10 = Pangapinus; PP1 - PP10 = *P. hypophthalmus*; CC = *Cyprinus carpio*; CM = *Clarias macrocephalus;* PN = *Pangasius nasutus.* (Support values are bootstrap values).



Appendix 8. Phylogenetic relationships of the different progenies following Maximum Parsimony analysis of 1076bp of the cytochrome b gene. PC1 - PC10 = *C. gariepinus*; HC1 – HC10 = Clarias-like Clariothalmus; HP1 – HP10 = Panga-like Clariothalmus; PA1 - PA10 = Pangapinus; PP1 - PP10 = *P. hypophthalmus*; CC = *Cyprinus carpio*; CM = *Clarias macrocephalus;* PN = *Pangasius nasutus.* (Support values are bootstrap values).