Sequencing Report of Cox1 Gene-Based Amplicons in Ten Isolates of Silver Carp

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Abstrast
One specific PCR fragment partially covering the coding regions of the cytochrome c oxidase subunit I encoding gene was selected in this study. The amplified fragments were directly exposed to Sanger sequencing experiments to assess the pattern of genetic polymorphism in the collected fish samples. Then, a specific comprehensive tree was generated to assess the accurate identification of the observed variants and their phylogenetic distribution Sequencing reactions showed the accurate identity of the investigated samples due to the presence of one species, as it was confirmed that S11-S20 belonged to silver carp (*Hypophthalmichthys molitrix*). Further details were also observed from the investigated sequencing reactions for the investigated S11-S20 samples. No nucleic acid variations were identified in the silver carp isolates. The generated phylogenetic tree was constructed to incorporate five phylogenetic clades with distinct phylogenetic distances. It was inferred from the tree that the silver carp group was suited in the vicinity to the clade of Prussian carp. This positioning referred to the fact that both species shared the highest homology than the other incorporated outgroup species.
Keywords: COX1(cytochrome oxidase), Polymorbhis , Sequence , Gene Diversity , Silver Carp.

1. Introduction

The silver carp (Hypophthalmichthys molitrix) has garnered interest due to its rising production in China, Bangladesh, India, the Russian Federation, Iraq, and Iran.
Feeds on phyto- and zooplankton, its natural habitat, it breeds upstream while its eggs and larvae flow downstream to floodplain zones. A species that leaps from the water when frightened. Swims near the surface. Juveniles and larvae eat zooplankton; from 15mm SL, phytoplankton. Sensitive to cold (below 5C°) and oxygen deficit. Dams affect reproductive success of organisms, (Ji et al., 2009; IUCN. 2011), (FAO 2009; Asgharzadeh et al., 2010; Hakimeh et al., 2010; Sass et al., 2014).
The present study was conducted to identify the pattern of the genetic variation of the investigated COX1 locus in ten isolates of silver carp. Based on the genetic variants of the investigated COX1, the pattern of the genetic diversity of these organisms was assessed in these samples (assigned S11 – S20) in the Middle Euphrates region in Iraq.

2. Materials and Method:

Nucleic acids sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced from both (forward and reverse) directions, following instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI (Applied Biosystem) sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed nucleic acid sequences of local samples with the retrieved nucleic acid sequences, the virtual positions, and other details of the retrieved PCR fragments were identified.

Interpretation of sequencing data

The sequencing results of the PCR products of the targeted samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in their corresponding position within the referring genome. The observed nucleic acids were numbered in PCR amplicons as well as in their corresponding positions within the referring genome. Each detected variant within the fish sequences was annotated by SnapGene Viewer ver. 4.0.4 (https://www.snapg ene.com).

Translation of nucleic acid variations into anima acid residues

The amino acid sequences of the targeted protein were retrieved online from the protein data bank (http://www.ncbi.nlm.nih.gov). The observed nucleic acid variants in the coding portions of the analyzed genetic loci were translated into a reading frame corresponding to the referring amino acid residues in the encoded protein using the Expasy online program (http://web.expasy.org/translate/). Multiple amino acid sequence alignment was conducted between the referring amino acid sequences and

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their observed mutated counterpart using the "align" script of the BioEdit server.

Comprehensive phylogenetic tree construction

A specific comprehensive tree was constructed in this study according to the neighbour-joining protocol described by Sarhan *et al.* (2019). The observed variants were compared with their neighbour homologous reference sequences using the NCBI-BLASTn server (Zhang *et al.* 2000). Then, a full inclusive tree, including the observed variant, was built by the neighbour-joining method and visualized as a circular cladogram using the iTOL suit (Letunic and Bork, 2019). The sequences of each incorporated group in the comprehensive tree were colored in an appropriate color.

3. Results and Discussion

Within this locus, ten samples were included in the present study (assigned as S11 to S20). These samples were screened to amplify the COX1 gene sequences of ten isolates of silver carp (Hypophthalmichthys molitrix) (S11-S20). Thus, the variation of the COX1 gene can be used for fish characterization due to its possible ability to adapt to variable genetic diversity as was seen in different fish organisms. The sequencing reactions indicated the exact identity after performing NCBI blastn for these PCR amplicons. Concerning the 655 bp amplicons, the NCBI BLASTn engine showed 100% sequence homology between the sequenced samples and the intended reference target sequences. By comparing the observed nucleic acid sequences of these investigated samples with the retrieved nucleic acid sequences (GenBank acc. MF180230.1), the accurate positions and other details of the retrieved PCR fragments were identified. The NCBI Blastn suit identified the accurate identity of the currently investigated S11-S10 samples. Silver carp was the identity of the S11-S20 isolates with an entire homology with the reference sequences (GenBank acc. MF180230.1). The total length of the targeted locus was localized in the NCBI server, and the positions of the start and end of the targeted locus were also confirmed within the most homologous target recognized (Fig. 1).

(S11-S20) silver carp (Hypophthalmichthys molitrix)

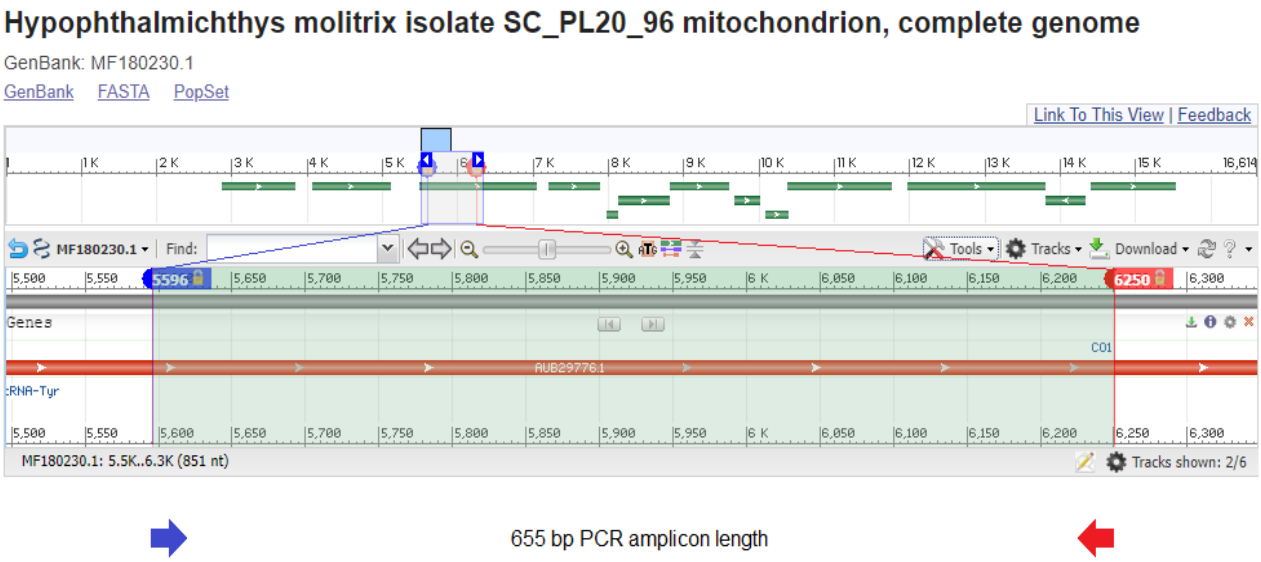

Fig. 1. The exact position of the retrieved 655 bp amplicon partially covered a portion of the COX1 gene within ten isolates of silver carp genomic sequences (GenBank acc. no. MF180230.1). The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

After positioning the 655 bp amplicons' sequences within the genomic sequences of silver carp samples, the details of its sequences were highlighted, and the total length of the amplified amplicons was also determined (Table 1).

Table 1. The position and length of the 655 bp PCR amplicons that used to amplify a portion of the *COX1* gene within the genomic sequences of silver carp (GenBank acc. no. MF180230.1).

Amplicon	Reference locus sequences (5′ - 3′)	length
COX1 gene nucleic acid sequences of silver carp(S11-S20)	TCTCATTCGAGCCGAACTAAGCCAACCCGGATCACTTCTGGGTGATGACCAAATTTATAATG TTATTGTTACTGCCCATGCCTTCGTAATAATTTTCTTTATAGTAATACCAATCCTTATTGGAGG GTTTGGAAACTGACTCGTGCCCCTAATGATTGGAGCACCTGATATAGCATTCCCACGAATAA ATAATATAAGCTTTTGACTCCTACCCCCATCTTTCCTTCTACTACTAGCCTCTTCTGGTGTTGA GGCCGGGGCCGGAACAGGATGAACAGTTTATCCACCACTCGCGGGCAATCTTGCCCACGC AGGAGCATCCGTAGACCTAACAATTTTCTCTCTTCACCTAGCAGGTGTGTCATCAATTTTAGG AGCAATTAACTTCATCACCACAACTATTAATATAAAACCACCAGCCATCTCTCAATATCAAAC ACCTCTCTTTGTTTGAGCTGTGCTCGTAACAGCCGTACTTCTTCTCTTATCCTTACCAGTTTTA GCTGCTGGAATTACAATACTCCTTACAGACCGTAATCTTAATACCACATTCTTTGACCCAGCA GGGGGAGGAGACCCAATTCTATATCAACACCTATTCTGATTCTTTGGTCACCCAGAAGTTTA CATTCTTATTTTACCTGGATTTGGAATC	655 bp

Interestingly, the alignment results of the 655 bp samples revealed the presence of no nucleic acid variants in the investigated samples of the silver carp group in comparison with the most similar referring reference nucleic acid sequences (Fig. 2).

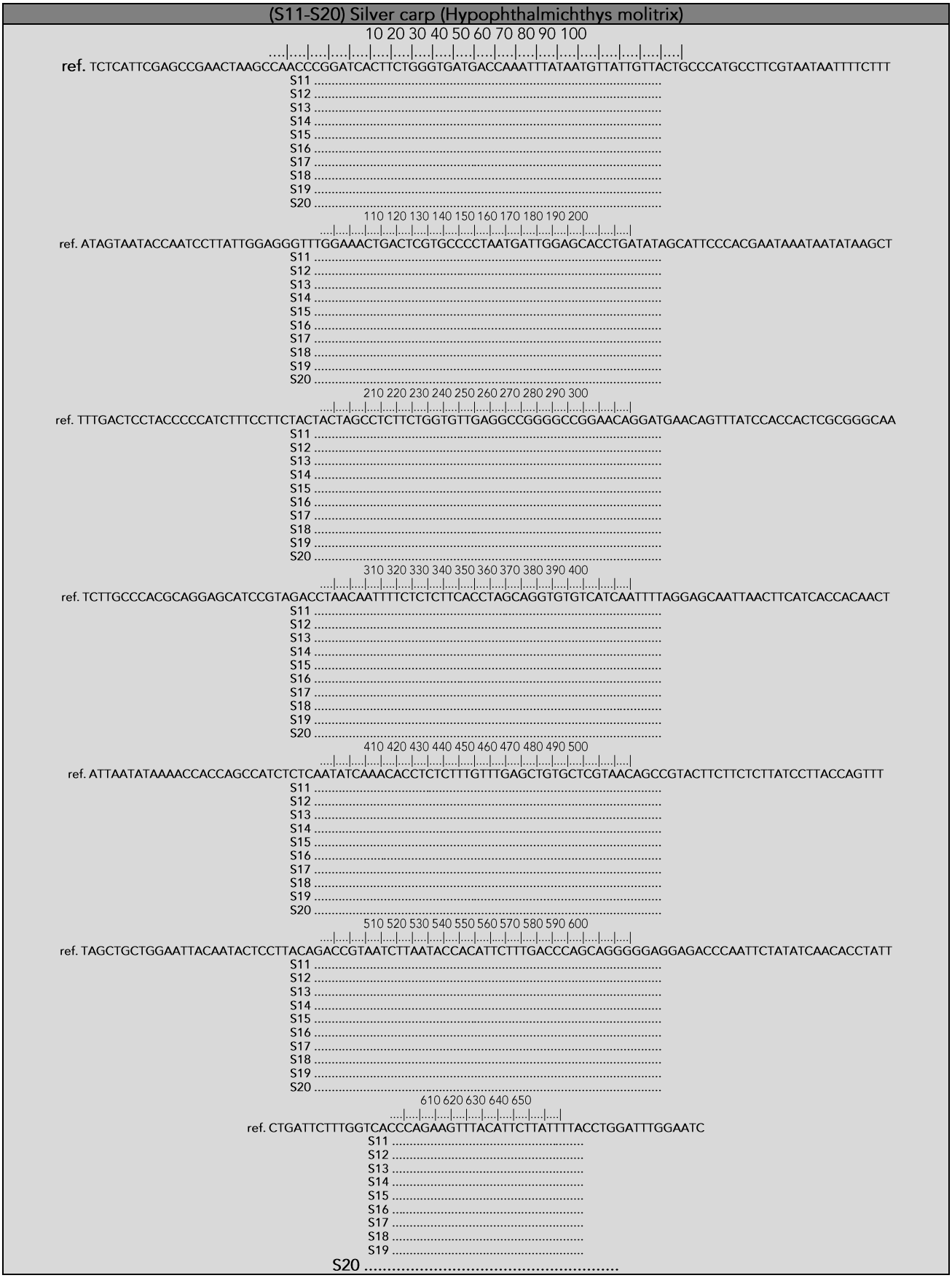

Fig. 2. Nucleic acid sequences alignment of ten samples of silver carp with their corresponding reference sequences of the 655 bp amplicons of the *COX1* genetic sequences. The symbol "ref" refers to the NCBI referring sequence, letter "S", followed by a number refers to the sample number.

no nucleic acid variations were identified in silver carp samples as complete homology was found with their corresponding reference sequences. To confirm the investigated sequences, the sequencing chromatograms of the investigated samples, as well as their detailed annotations, were verified and documented, and the chromatograms of their sequences were shown according to their positions in the PCR amplicons. However, the observed nucleic acid sequences were further analyzed to identify the corresponding positions of these isolates in the cytochrome c oxidase subunit I. All nucleic acid sequences of the amplified S11 to S20 PCR products were translated to their corresponding amino acid sequences using the Expasy translate suite (Fig. 3).

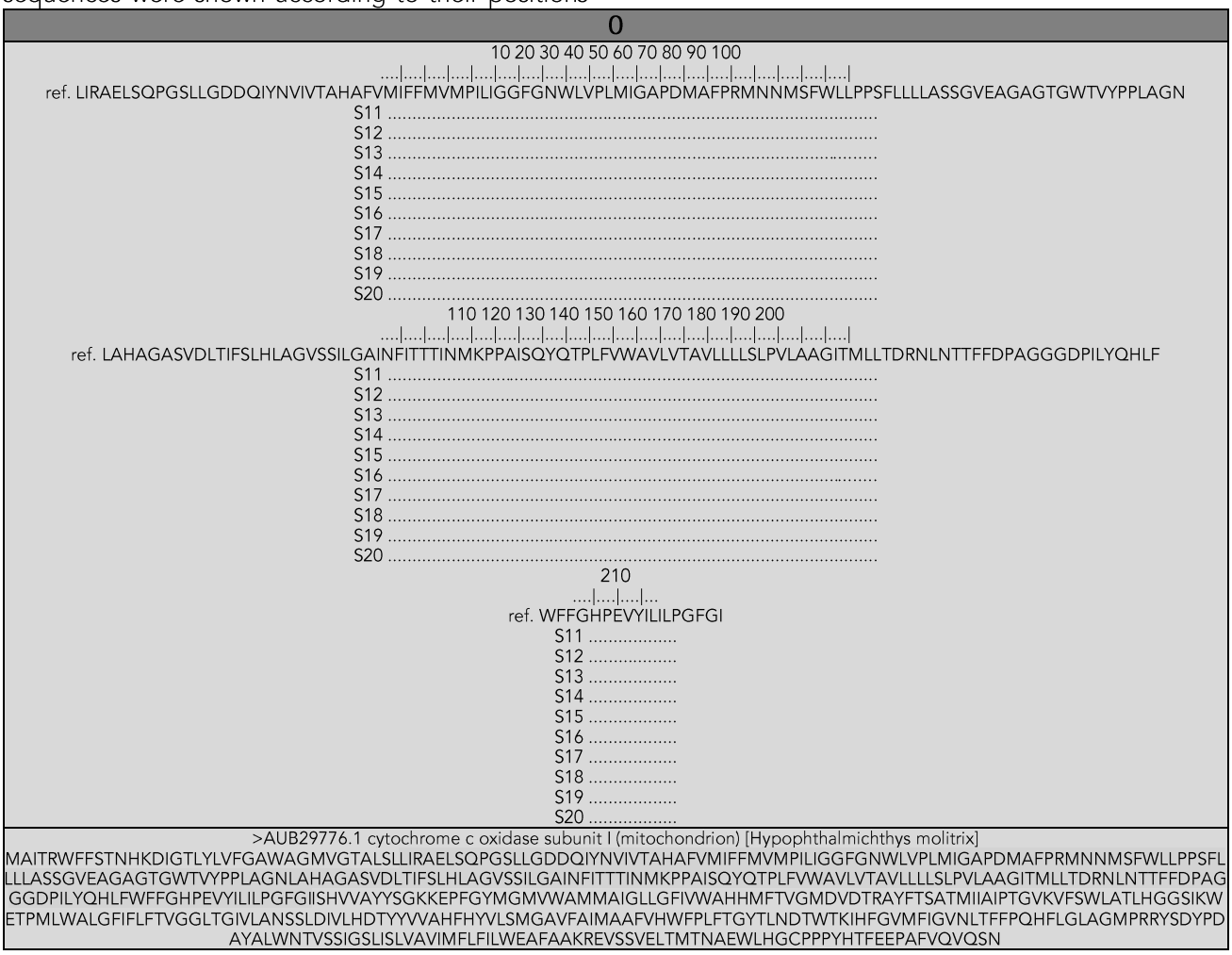

Fig. 3. Amino acid residues alignment of the detected variations of the cytochrome c oxidase subunit I within the investigated silver carp samples as they are highlighted according to their corresponding positions within the amplified 655 bp fragment and according to their corresponding positions within the entire protein. The grey highlights refer to the amplified region of the *COX1*-encoded cytochrome c oxidase subunit I.

All the investigated *COX1* sequences were deposited in the NCBI web server, and unique accession numbers were obtained for all analyzed sequences, starting from GenBank OP456562 which was deposited to represent the S11 sample, to the GenBank OP456571 which was deposited to represent the S20 sample.

A comprehensive phylogenetic tree was generated in the present study according to nucleic acid sequences observed in the amplified 655 bp of the *COX1* gene amplicons. This phylogenetic tree contained S11 to S20 samples alongside other relative nucleic acid sequences of grass carp (*Ctenopharyngodon idella*), Eurasian carp (*Cyprinus carpio*), Prussian carp (*Carassius gibelio*), and Binni (*Mesopotamichthys sharpeyi*) sequences.

Within this tree, our investigated samples were incorporated alongside other relative sequences to constitute five major clades of incorporated sequences within the cladogram. These major clades were represented by the grass carp, Eurasian carp, Prussian carp, and Binni group sequences. These four clades were used as outgroups to compare the ratio of phylogenetic homology between silver carp and the other related organisms in the same area of study. Phylogenetic results showed that all five clades were suited in distinct phylogenetic clades away from each other's sequences. This observation indicated an intermediate homology between the five incorporated organisms within the same tree. Other than this observation, these data indicated the ability of *COX1* gene-based amplicons to detect these fish species without including any noticeable homology with other sequences of other species whether being in the same genus or other outgroup

sequences. The total number of the aligned nucleic acid sequences in this comprehensive tree was fifty-one.

As indicated above, the investigated samples were clustered into five phylogenetic clades of highly recognized phylogenetic distances within the incorporated sequences of silver carp, in addition to grass carb, Eurasian carp, Prussian carp, and Binni. The most interesting fact observed in our investigated fish isolates is correlated with the ability of the utilized *COX1* gene-based amplicons to categorize the silver carp, grass carb, Eurasian carp, Prussian carp, and Binni sequences into these observed phylogenetic distributions.

The most interesting observation of the currently generated tree was represented by the positioning of the silver carp group beside the sequences of the clade of grass carp. After the silver carp, the rectangular cladogram had clearly shown that the grass carp exerted another phylogenetic positioning, which was followed by the Eurasian carp (**Fig. 4A**). On the contrary, the most recent descendent of the incorporated five groups were represented by the Prussian carp since it was positioned at the utmost distance away from the roots of the current tree. However, the Binni clade followed the Prussian carp by its descendant phylogenetic positioning.

Concerning the clade of silver carp (S11-S20), twenty sequences of the same species were incorporated. Our investigated sequences of S11-S20 have exerted even distribution within the same major clade. This is due to the absence of any nucleic acid substitutions in these samples with respect to their corresponding reference sequences of the silver carp group (**Fig. 4B**). The incorporated samples within this clade showed the presence of various strains of the silver carp sequences that have been deposited from variable American origins (GenBank acc. no. MF180230.1 and KP013119.1, and KJ746961.1).

It was inferred from the constructed tree that the detected nucleic acid substitutions showed a slight evolutionary effect of the variations observed in the fish samples in comparison with the other investigated wild-type fish samples. This style of sample positioning indicated the presence of a slight evolutionary effect of the observed genetic variant in inducing a possible deviation in the evolutionary positioning of these fish samples.

The presence of remarkable evolutionary distances among silver carp sequences from one side, and grass carp, Eurasian carp, Prussian carp, and Binni sequences from the other side indicated the high resolution of the currently utilized PCR products of the *COX1* sequences in the efficient detection and discrimination with the related organisms.

The current observation of this tree has confirmed sequencing reactions because it explained the actual neighbour-joining-based positioning in such observed variations. Interestingly, the American origins of our investigated samples could not be ignored.

Interestingly, the utilization of the *COX1* gene sequences in this study has given further proof for the presence of the accurate identification of the actual positoining of these types of fish sequences. This *COX1* gene-based comprehensive tree has provided comprehensive evidence about the high competency of such genetic fragments to efficiently identify this sort of phylogenetic distribution. This, in turn, gives a further indication of the ability of the currently utilized *COX1* gene-specific primers to describe the investigated silver carp and to discriminate this type from its related sequences by generating accurate phylogenetic positions.

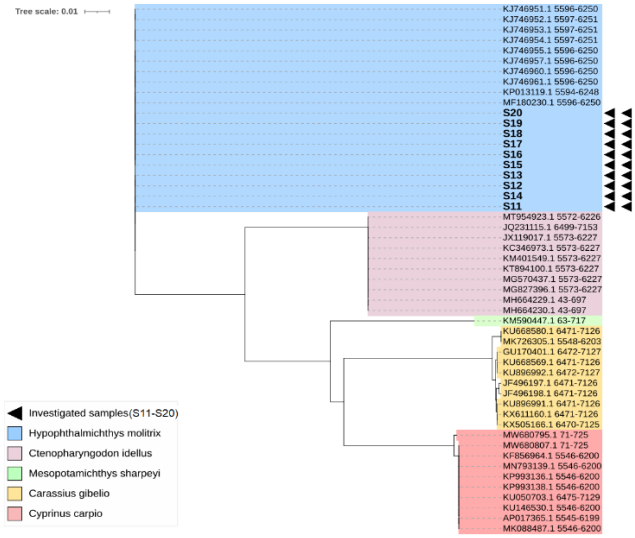

Fig. 4A. A comprehensive rectangular cladogram phylogenetic tree of genetic variants of the *COX1* gene fragment of ten samples belonging to silver carp. The black-colored triangle refers to the analyzed fish variants. All the mentioned numbers referred to GenBank accession number of each referring species. The number "0.01" at the top portion of the tree refers to the degree of scale range among the comprehensive tree-categorized organisms. The letter "S#" refers to the code of the investigated samples.

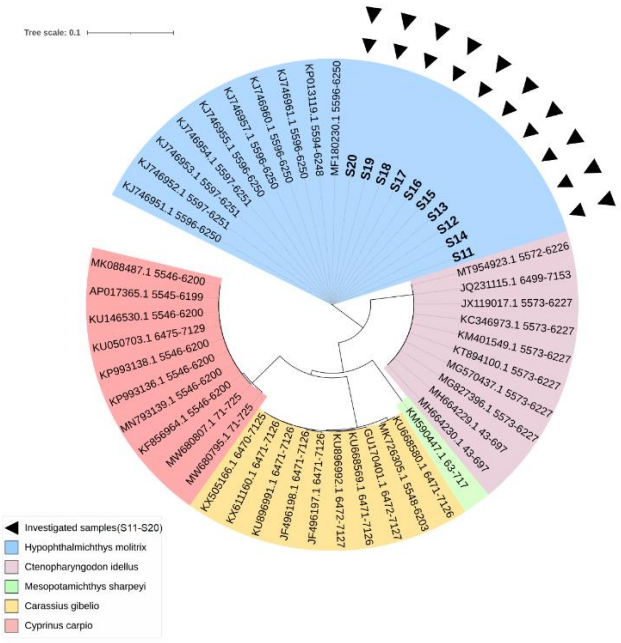

Fig. 4B. A comprehensive circular cladogram

phylogenetic tree of genetic variants of the COX1 gene fragment of ten samples belonging to silver carp sequences. The black-colored triangle refers to the analyzed fish variants. All the mentioned numbers referred to GenBank accession number of each referring species. The number "0.1" at the top portion of the tree refers to the degree of scale range among the comprehensive tree-categorized organisms. The letter "S#" refers to the code of the investigated samples.

4. Conclusion

This study found the ability of all the utilized genetic locus COX1 to identify the precise identity of the investigated samples and to discriminate among them. Moreover, this study suggests possible employment for the amplicons of cytochrome c oxidase subunit I of having the most specific power to discriminate between the phylogenetic diversity among the other implemented tools. These PCR fragments can efficiently be used to detect the biological diversity of a wider spectrum of fish sequences and can therefore be explored to discover further details within these identified groups.

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