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Molecular Genetic Analysis of *Fruticicola Plectotropis Stschukini* Based on Its and COI Markers

Feruza Gaibnazarova[1,*], Khilola Khakberdiyeva[2], Ulugbek Musabekov[2]

[1]Associate Professor, Department of Biology, Gulistan State University, Syrdarya, Uzbekistan
[2]Gulistan State University, Syrdarya, Uzbekistan

Abstract In this study, the molecular genetic characteristics of the samples belonging to *Fruticicola plectotropis stchukini* were studied in association with ITS1–5.8S–ITS2 and mitochondrial COI markers. For analysis, genomic DNA was extracted from three samples, ITS and COI fragments were amplified by polymerase chain reaction and sequenced. The obtained nucleotide sequences were 630 bp long for ITS and 520 bp long for COI. Alignment analysis revealed the presence of a bitta CG'T polymorphism at the 57-bp position in the ITS marker, and five nucleotide polymorphisms at positions 156, 387, 414, 465 and 477 bp in the COI marker. These differences were interpreted as nucleotide substitutions. Phylogenetic trees were constructed using the Maximum Likelihood method in the association of ITS and COI accessions. The three samples in the ITS marker did not cluster into a monophyletic group, while the main samples in the COI marker M1, M2, and M3 revealed a monophyletic clade. The results showed that the COI marker is more informative than the ITS marker in molecular identification of *F. plectotropis stschukini* samples and in the search for phylogenetic relationships with related taxa.

Keywords Fruticicola plectotropis stschukini, Molecular genetics, ITS1–5.8S–ITS2, COI, Nucleotide polymorphism, Phylogenetics

1. Introduction

Molecular genetic methods are among the key tools for studying biodiversity, molecular identification of species, and determining phylogenetic relationships between them [1,2]. In particular, the use of DNA markers is an effective approach for reliably distinguishing morphologically close taxa [1,3]. Determining genetic variability based on comparison of nucleotide sequences allows assessing molecular differences within species and determining phylogenetic relationships of closely related taxa [2,4].

The internal transcribed spacer (ITS) region of nuclear ribosomal DNA and the mitochondrial cytochrome c oxidase I (COI) gene are widely used in molecular phylogenetic studies [1,3]. The ITS1–5.8S–ITS2 region is located in the nuclear genome and is considered an important molecular marker in studying phylogenetic relationships between species and higher taxonomic groups [1,2]. The COI gene is important in molecular species identification and DNA barcoding studies because it is located in the mitochondrial genome and relatively accurately reflects nucleotide changes [3,5].

* Corresponding author:
gaibnazarova@yahoo.com (Feruza Gaibnazarova)
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Land snails of the genus *Fruticicola* are morphologically conservative, and their taxonomic boundaries and phylogenetic relationships remain insufficiently resolved [4,5]. Molecular studies using multiple mitochondrial and nuclear markers have revealed the existence of cryptic and pseudocryptic species that are difficult to distinguish based on shell morphology alone [2,4]. These findings highlight the importance of employing multiple molecular markers to assess genetic differentiation and resolve taxonomic uncertainties within this genus [3,5].

The study of molecular genetic characteristics of the species *Fruticicola plectotropis stschukini* is important in terms of determining its phylogenetic relationships with close taxa, as well as assessing genetic variability within the species [1,6]. However, the ability of different molecular markers to reflect genetic differentiation and resolve phylogenetic relationships in samples of this species may not be the same [2,5]. Therefore, the parallel use of ITS and COI markers allows comparing their informativeness [3,6]. In this study, the molecular genetic characteristics of *Fruticicola plectotropis stschukini* samples were studied in association with ITS1–5.8S–ITS2 and mitochondrial COI markers, and the informativeness of these markers was comparatively assessed [1,4].

Purpose of the Research

The purpose of this study was to investigate the molecular

genetic characteristics of *Fruticicola plectotropis stschukini* samples using two independent marker systems — the nuclear ribosomal ITS1–5.8S–ITS2 region and the mitochondrial cytochrome c oxidase subunit I (COI) gene — and to comparatively assess the informativeness of these markers for molecular identification and phylogenetic analysis.

2. Materials and Methods

DNA isolation. For molecular genetic studies, tissue samples were obtained from the soft body of *Fruticicola plectotropis stschukini* specimens. Total genomic DNA was isolated using a modified version of the CTAB method [Doyle J.J., Doyle J.L., 1987]. The tissue was frozen using liquid nitrogen and thoroughly homogenized. 600 µl of 2×CTAB solution was added to the resulting homogenate and incubated at 60 ℃ for 20 minutes. During incubation, the samples were mixed carefully every 5 minutes.

In the next step, 600 µl of chloroform:isoamyl alcohol (24:1) was added to the sample, mixed thoroughly, and centrifuged at 10,000 rpm for 5 minutes. The resulting 600 µl of the upper phase was transferred to a new microtube, and 60 µl of 10×CTAB solution was added. After mixing, another 600 µl of chloroform:isoamyl alcohol (24:1) was added, and the sample was centrifuged at 10,000 rpm for 5 minutes.

The upper phase was transferred to a new 600 µl tube and an equal volume (1:1) of CTAB Precipitation solution was added. To ensure DNA precipitation, the sample was incubated at 60 ℃ for 30 minutes. Then, it was centrifuged at 14,000 rpm for 3 minutes and the supernatant was removed.

300 µl of RNase-containing High Salt solution was added to the resulting precipitate, vortexed for 10 seconds and incubated at 37°C for 15 minutes. Then, 250 µl of isopropanol stored at –20 ℃ was added and the sample was gently mixed for 2 minutes. The sample was stored at –20 ℃ for 20 minutes and centrifuged at 10,000 rpm for 15 minutes. After removing the supernatant, the DNA precipitate was dried at room temperature for 15–25 minutes. The pellet was dissolved in 100 µl TE buffer, vortexed, and stored at –20 ℃ after brief centrifugation.

The presence and quality of the isolated genomic DNA was assessed by 1% agarose gel electrophoresis.

PCR amplification. The ITS1 and ITS4 universal primers were used to amplify the ITS1–5.8S–ITS2 fragment in the ribosomal DNA of *Fruticicola plectotropis stschukini* samples [White T.J., Bruns T., Lee S., Taylor J., 1990]:

ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′

ITS4: 5′-TCCTCCGCTTATTGATATGC-3′

PCR was performed using the GenPak® PCR MasterMix kit. The reaction was prepared in a total volume of 20 µl and consisted of 10 µl 2× MasterMix, 8.2 µl double distilled water, 0.4 µl ITS1 primer, 0.4 µl ITS4 primer, and 1 µl genomic DNA.

Amplification was initiated with an initial denaturation at 94 ℃ for 5 min. Subsequently, 45 seconds of denaturation at 94 ℃, 45 seconds of primer annealing at 58 ℃, and 130 seconds of elongation at 72 ℃ were repeated for 35 cycles. The final elongation was performed at 72 ℃ for 7 minutes. After PCR, the samples were stored at +4 ℃.

In addition, the mitochondrial cytochrome c oxidase I (COI) gene fragment of Fruticicola plectotropis stschukini samples was amplified using the primers HCO2198-L and LCO1490-L:

HCO2198-L:

5′-TAAACTTCWGGRTGWCCAAARAATCA-3′

LCO1490-L:

5′-GGTCWACWAATCATAAAGATATTGG-3′

PCR amplification of the COI fragment was also performed using the GenPak® PCR MasterMix kit in a total reaction volume of 20 µl. The reaction mixture consisted of 10 µl of 2× MasterMix, 8.2 µl of double-distilled water, 0.4 µl of HCO2198-L primer, 0.4 µl of LCO1490-L primer, and 1 µl of genomic DNA.

The amplification products of the ITS and COI fragments were visualized using ethidium bromide-stained 1% agarose gel electrophoresis. Samples that gave positive amplification were selected for further sequencing.

Sequencing. The PCR products that gave positive amplification of the ITS1–5.8S–ITS2 and COI gene fragments were sent to the Russian company “Syntol” for nucleotide sequence determination. The nucleotide sequences obtained as a result of sequencing were prepared for further bioinformatic analyses.

The three nucleotide sequences obtained during the study were deposited in the NCBI GenBank database.

Comparison of nucleotide sequences and identification of genetic differences. The initial analysis and mutual comparison of the obtained ITS and COI nucleotide sequences were carried out in the SnapGene program. The sequences were entered into the program and aligned to each other using alignment. Based on the alignment results, the corresponding and different positions between the nucleotide sequences of the study samples were determined, and nucleotide substitutions were visually assessed.

The genetic difference between the sequences was assessed based on the nucleotide substitutions identified in the alignment results. The different nucleotide positions were recorded, and the total nucleotide difference between the sequences was expressed in percentages. The alignment results were saved in a graphical form and used to visually represent the research results.

Phylogenetic tree construction. For phylogenetic analysis, the ITS and COI sequences obtained during the study and the homologous sequences selected from the NCBI GenBank database were entered into the MEGA 12 program. The sequences were aligned using the ClustalW algorithm.

The phylogenetic tree was constructed using the Maximum Likelihood (ML) method. To assess the statistical reliability of the tree topology, 1000 bootstrap resamplings were performed. The bootstrap values generated in the phylogenetic tree represented the level of statistical support for the corresponding nodes. Using the phylogenetic trees constructed based on ITS and COI markers, the phylogenetic

relationships of *Fruticicola plectotropis stschukini* specimens with the studied Bradybaena representatives and other close taxa were assessed.

3. Results and Discussion

Results of genomic DNA isolation. Genomic DNA was isolated from soft body tissues of Fruticicola plectotropis stschukini specimens using a modified version of the CTAB method. The isolated DNA samples were examined using 1% agarose gel electrophoresis. As a result of electrophoresis, high molecular weight DNA bands characteristic of genomic DNA were observed in the samples (Figure 1).

A total of 3 *Fruticicola plectotropis stschukini* samples were analyzed in the study. All samples had sufficient quantity and quality of DNA and were used for further PCR amplification.

PCR amplification results. ITS1–5.8S–ITS2 fragment.

The ITS1–5.8S–ITS2 fragment of *Fruticicola plectotropis stschukini* samples was amplified using ITS1 and ITS4 primers. As a result of 1% agarose gel electrophoresis, clear DNA bands were observed in the samples that gave positive amplification.

The estimated length of the amplified ITS fragments was 600-630 base pairs (bp). Successful amplification of the ITS fragment was observed in all 3 samples.

Positive PCR products were selected for further sequencing.

COI gene fragment. The mitochondrial COI gene fragment of *Fruticicola plectotropis stschukini* samples was amplified using primers HCO2198-L and LCO1490-L. As a result of agarose gel electrophoresis, DNA bands corresponding to the COI fragment were observed in the samples that gave positive amplification.

The length of the COI fragments was approximately 500-530 bp. Positive amplification was recorded in all 3 samples. Successfully amplified products were selected for sequencing.

Sequencing results. Positive PCR products of ITS1–5.8S–ITS2 and COI gene fragments were sent to Syntol for sequencing. As a result of sequencing, ITS and COI nucleotide sequences of 3 samples were obtained.

The three sequences obtained during the study on the ITS fragment were deposited in the NCBI GenBank database and assigned accession numbers PX339959.1, PX339960.1 and PX339961.1. These sequences were recorded as Fruticicola plectotropis stschukini M1, *Fruticicola plectotropis stschukini* M2 and *Fruticicola plectotropis stschukini* M3, respectively.

The length of the obtained sequences was 630 bp for ITS and 520 bp for COI.

Comparison of nucleotide sequences and genetic differences. The nucleotide sequences of the ITS1–5.8S–ITS2 fragments of samples M1, M2 and M3 of the species *Fruticicola plectotropis schukini* were compared using the SnapGene program based on alignment. As a result, it was found that the sequences of the studied samples generally corresponded to a high degree of similarity.

The alignment results showed a nucleotide difference at position 57 bp. At this position, the nucleotide C was detected in samples M1 and M2, and the nucleotide T in sample M3. Thus, a C→T nucleotide exchange was observed at this position. Since two of the three samples contained a C nucleotide, the consensus nucleotide at this position was formed as C.

The detected difference is a 1 bp single nucleotide substitution (SNP). In this case, no additional base was introduced into the nucleotide sequence or the existing base was dropped, that is, no insertion or deletion was observed. The difference was represented only by the replacement of one nucleotide with another nucleotide at the 57-bp position. Thus, as a result of the alignment of the ITS1–5.8S–ITS2 fragments, along with a high level of nucleotide conservation in the samples of F. plectotropis schukini, a C/T polymorphism was detected at the 57-bp position. At this position, the sequences of samples M1 and M2 were identical, and sample M3 differed from them by one nucleotide substitution. This C/T polymorphism was noted as one of the signs expressing molecular-genetic variability between the studied samples. (Figure 1)

The nucleotide sequences of the mitochondrial cytochrome c oxidase I (COI) gene fragment of samples M1, M2 and M3 of the species *Fruticicola plectotropis schukini* were compared based on the sequences obtained using primers HCO2198-L and LCO1490-L. The obtained sequences were aligned using the SnapGene program, and the corresponding and different nucleotide positions between the samples were determined.

The alignment results showed a high degree of similarity in the sequences of the COI gene fragments of samples M1, M2 and M3. At the same time, differences represented by nucleotide exchanges were detected at positions 156, 387, 414, 465 and 477 bp. These positions are indicated by red arrows in the figure. (Figure 2)

At position 156 bp, nucleotide A was recorded in sample M1, while in samples M2 and M3, this position was represented by nucleotide G. Therefore, an A/G type polymorphism was observed at this position.

On the contrary, at the 387-bp position, the G nucleotide was detected in the M1 sample, and the A nucleotide in the M2 and M3 samples. This situation is characterized by a G/A nucleotide exchange.

At the 414-bp position, the C nucleotide was preserved in the M1 and M2 samples, while the T nucleotide was recorded in the M3 sample. As a result, a C/T polymorphism was observed at this position. This difference is one of the nucleotide characteristics that distinguish the M3 sample from the M1 and M2 samples.

At the 465-bp position, the A nucleotide was present in the M1 and M3 samples, and the G nucleotide was detected in the M2 sample. This situation was recorded as an A/G type nucleotide substitution.

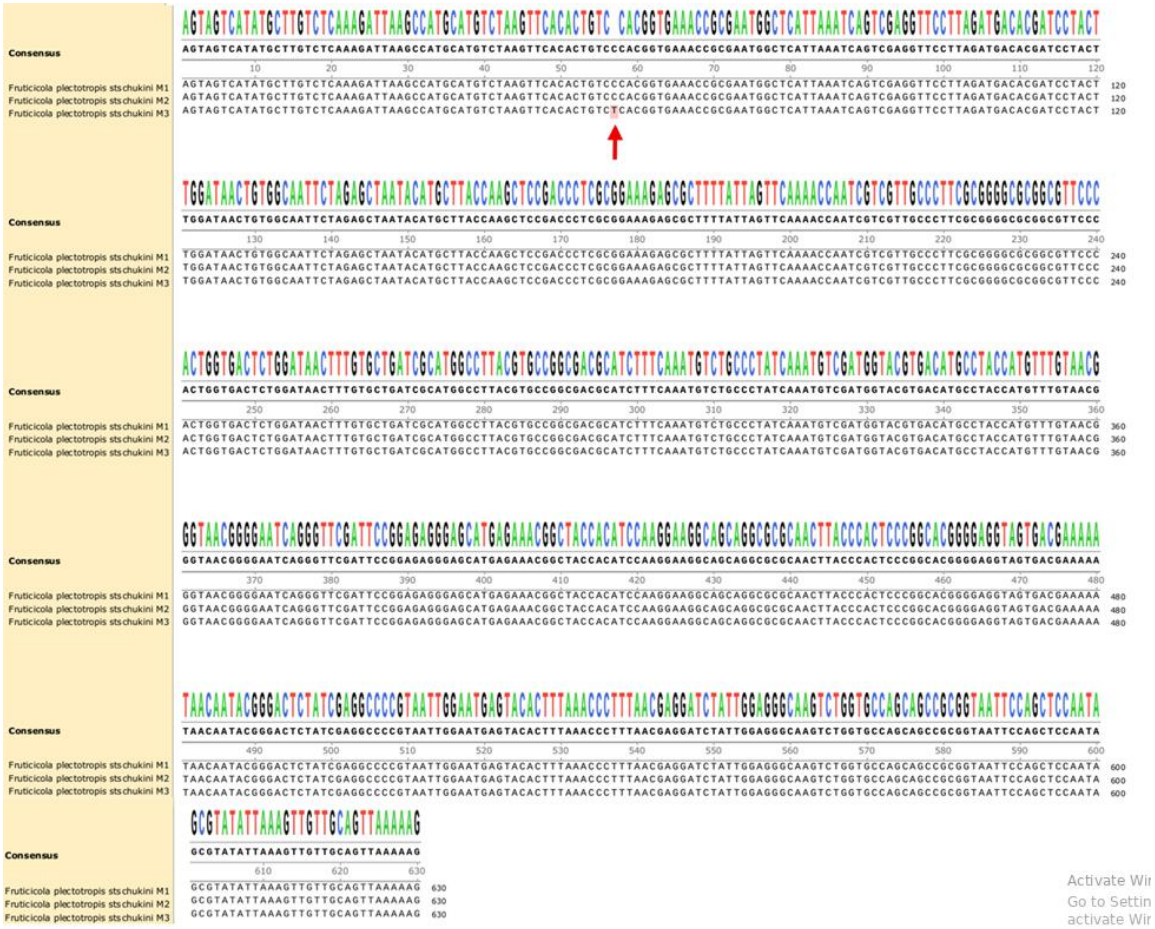

Figure 1. Results of SnapGene alignment of ITS nucleotide sequences of *Fruticicola plectotropis stschukini* samples. Different nucleotide positions are indicated by red arrows

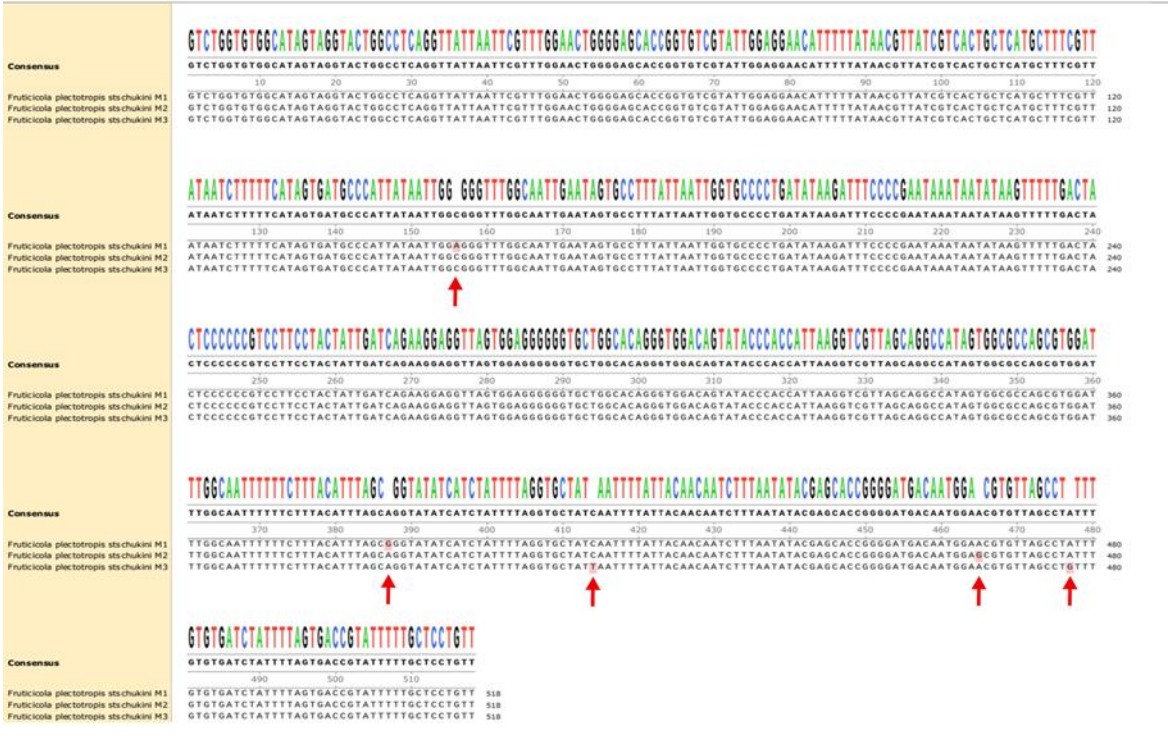

Figure 2. Results of SnapGene alignment of COI nucleotide sequences of *Fruticicola plectotropis stschukini* samples. Different nucleotide positions are indicated by red arrows

At the 477-bp position, the T nucleotide was observed in the M1 and M2 samples, and the G nucleotide in the M3 sample. Thus, a T/G polymorphism was detected at this position.

All 5 identified differences were single nucleotide substitutions. The alignment results did not show any insertions or deletions associated with the addition or deletion of nucleotides at these positions. In general, in the studied fragment of the COI gene, along with a high level of nucleotide similarity in samples M1, M2 and M3, a total of 5 polymorphic nucleotide positions were identified at positions 156, 387, 414, 465 and 477 bp. These differences were noted as signs of molecular genetic variability based on the COI marker of Fruticicola plectotropis schukini samples.

Phylogenetic tree results. Phylogenetic analysis was performed based on ITS and COI gene fragments. The nucleotide sequences of the study samples were aligned with homologous sequences selected from the GenBank database, and a phylogenetic tree was constructed based on the Maximum Likelihood method (Figure 3).

For the ITS gene, the phylogenetic tree included the M1, M2, and M3 samples of *F.plectotropis stschukini* obtained during the study, as well as the ITS1–5.8S–ITS2 sequences of *Bradybaena similaris*, *Fruticola fruticum*, *Fruticola schrenckii*, and Bradybaena transbaicalia selected from the GenBank database. The Acharax sp. sample was used as an outgroup.

In the phylogenetic tree, it was observed that the M1, M2, and M3 samples of *F. plectotropis stschukini* were located on different branches. The M2 and M3 samples were located closest to each other, forming a close group. This group also includes GenBank sequences belonging to *Bradybaena transbaicalia* and *Fruticola schrenckii.* Sample M1, however, was located separately from samples M2 and M3, and was included in the same group as sequences from *Bradybaena similaris*, *Fruticola fruticum*, and *Fruticola schrenckii*.

The resulting phylogenetic topology showed that the genetic uniformity of F. plectotropis stschukini samples in the ITS1–5.8S–ITS2 region is incomplete. In particular, the close location of samples M2 and M3 indicates a high degree of similarity of ITS sequences between these samples.

Acharax sp., located at the root of the phylogenetic tree, was isolated from other taxa as an outgroup on a separate branch.

Therefore, according to the results obtained, the ITS1–5.8S–ITS2 region did not serve as a sufficiently informative marker for resolving the phylogenetic relationships of *F. plectotropis stschukini* with related taxa at high resolution in this study. Although the phylogenetic topology obtained from the ITS marker showed the closeness between some specimens, it did not unite the *F. plectotropis stschukini* specimens into a single clade.

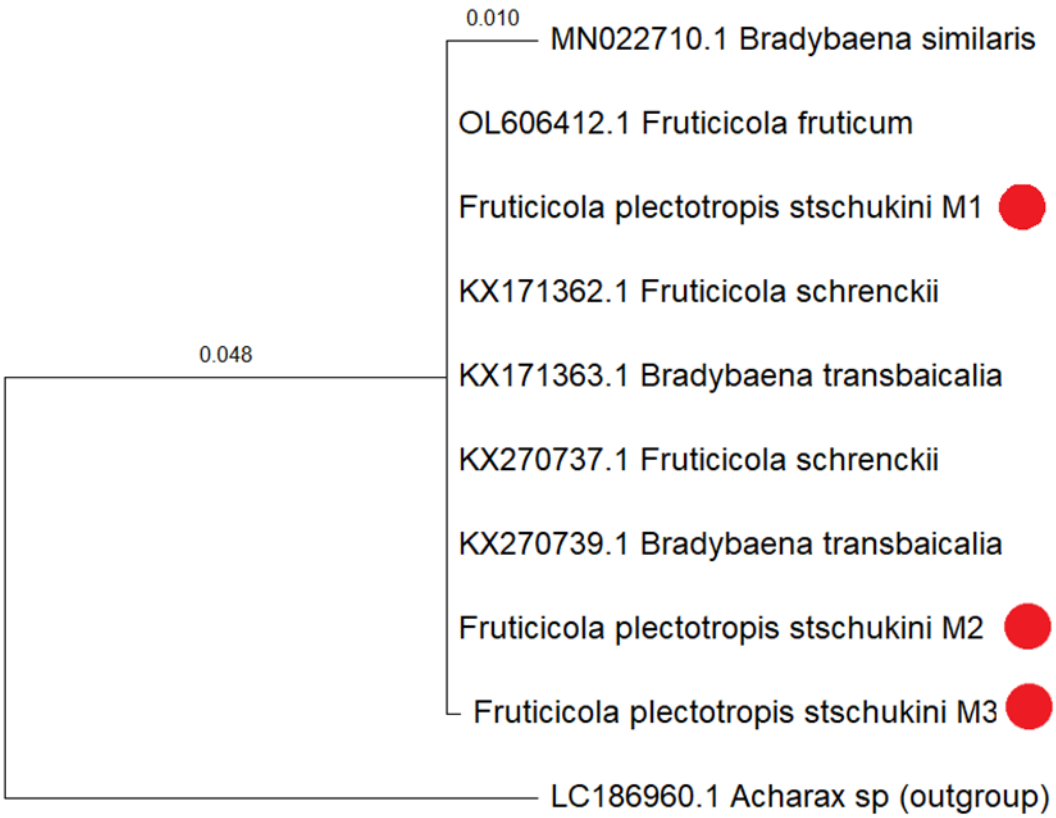

Figure 3. Phylogenetic tree of *Fruticicola plectotropis stschukini* and related taxa constructed using the Maximum Likelihood method based on ITS1–5.8S–ITS2 nucleotide sequences

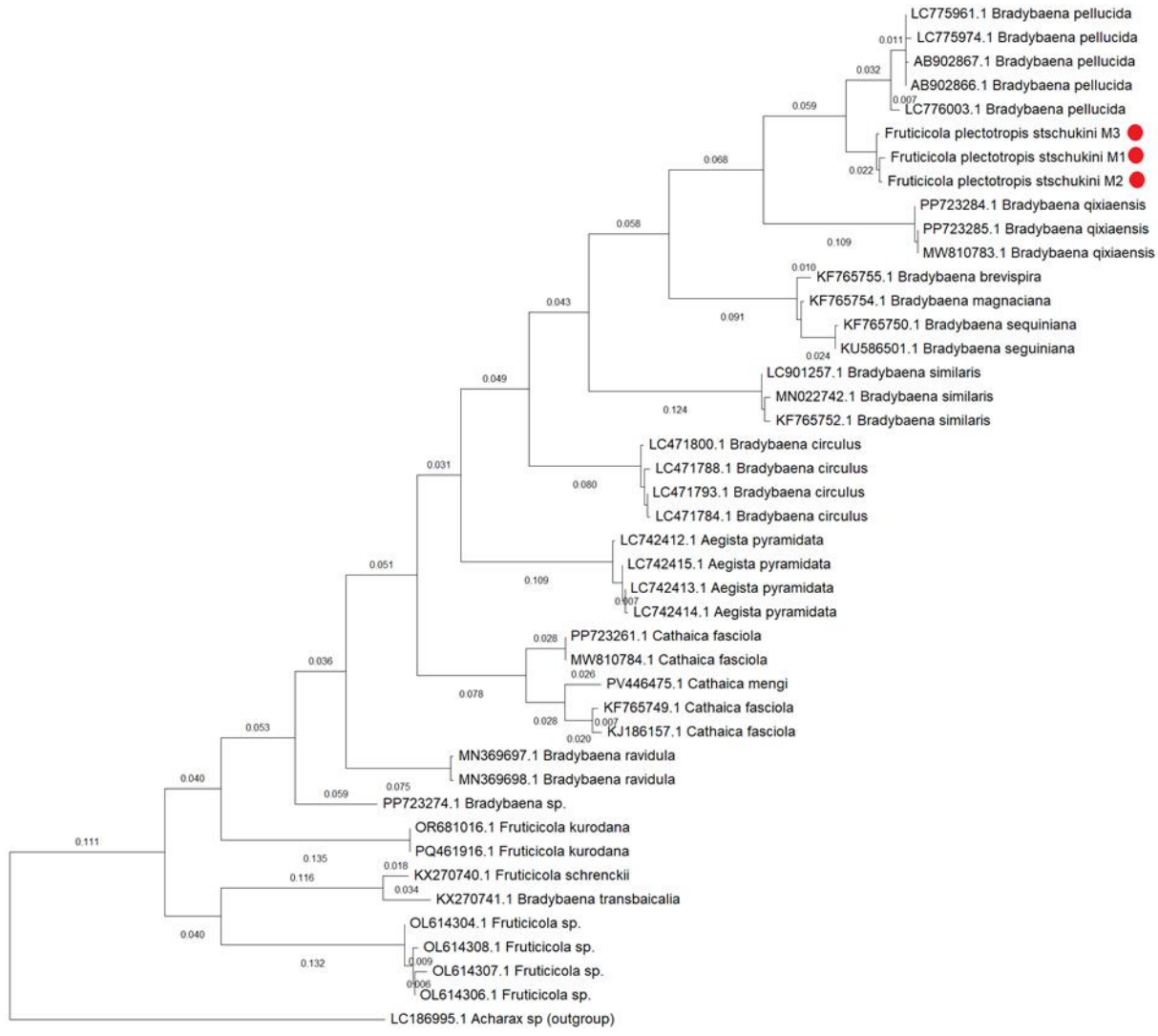

Figure 4. Phylogenetic tree of *Fruticicola plectotropis stschukini* and related taxa constructed using the Maximum Likelihood method based on COI nucleotide sequences

The phylogenetic relationships of *Fruticola plectotropis stschukini* and related taxa were analyzed using the Maximum Likelihood (ML) method based on the COI nucleotide sequences. The phylogenetic tree included the M1, M2, and M3 samples of *F. plectotropis stschukini* obtained during the study, as well as the COI sequences of *Bradybaena pellucida, Bradybaena qiaxiensis, Bradybaena brevispira, Bradybaena magnaciana, Bradybaena sequiniana, Bradybaena similaris, Bradybaena circulus, Aegista pyramidata, Cathaica fasciola, Bradybaena ravida, Fruticola kurdona, Fruticola schrenckii, Bradybaena transbaicalia,* and *Fruticola sp. selected* from the GenBank database. The *Acharax sp*. sample was used as an outgroup. (Figure 4)

It was observed that the M1, M2, and M3 samples of *F. plectotropis stschukini* were placed together in the phylogenetic tree, forming a single monophyletic group. Samples M1 and M2 are located closest to each other, joined at a common node, while sample M3 is located as the next sister group to this pair. This indicates that there are common COI genetic features that unite all three samples within a single species.

The *F. plectotropis stschukini* M1–M3 group is closely related to the GenBank sequences of *Bradybaena pellucida.* Five sequences belonging to B. pellucida also formed a separate group. However, the *F. plectotropis stschukini* samples are not included in this group, but are located on a separate branch. This indicates that *F. plectotropis stschukini* can be distinguished from B. pellucida based on COI sequences.

Elsewhere in the tree, taxa belonging to the genera *Bradybaena* and *Fruticola* are also grouped together in separate groups. The separate placement of *Fruticola kurdona*, *F. schrenckii*, and *Bradybaena transbaicalia* from the *F. plectotropis stschukini* M1–M3 group indicates genetic differentiation between these taxa based on COI sequences.

In general, in the phylogenetic tree constructed based on the COI marker, it was observed that the M1, M2 and M3 specimens of *F. plectotropis stschukini* were united into a single monophyletic clade. This result allows us to group these three specimens within the same species based on the COI marker. The closest arrangement of specimens M1 and

M2, and the placement of specimen M3 in the same clade with them, indicates the genetic affinity between the studied specimens.

These results, compared with the previous phylogenetic topology obtained based on the ITS1–5.8S–ITS2 markers, indicate that the COI marker provides a relatively higher phylogenetic resolution in grouping *F. plectotropis stschukini* specimens and separating them from related taxa. While the M1, M2, and M3 specimens are located on different branches in the ITS tree, the three specimens are united into a single monophyletic group in the COI tree. Therefore, in the framework of this study, the COI marker can be used as a more effective molecular marker for assessing the phylogenetic relationships of *F. plectotropis stschukini* and related taxa.

4. Conclusions

Three Fruticicola plectotropis stschukini specimens (M1–M3) were analysed using nuclear ITS1–5.8S–ITS2 and mitochondrial COI markers. The reported alignments identified one C/T substitution at ITS position 57 and five variable COI positions (156, 387, 414, 465 and 477 bp). The resulting phylogenetic trees placed the three specimens on separate branches in the ITS analysis but together in a monophyletic group in the COI analysis. Within this small dataset, COI therefore provided greater resolution for grouping the analysed specimens than ITS.

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