Acta fytotechn zootechn, 28, 2025(2): 115–121
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Original Paper

DNA Barcoding and Phylogenetic Studies of *Pseudoleptonema quinquefasciatum* Martynov 1935 (Hydropsychidae: Trichoptera) from India

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Article Details: Received: 2024-11-25 | Accepted: 2025-04-04 | Available online: 2025-06-30

https://doi.org/10.15414/afz.2025.28.02.115-121

This study initiates DNA barcoding and phylogenetic investigations of *Pseudoleptonema quinquefasciatum* Martynov 1935, aiming to enhance species identification, assess genetic diversity, and clarify its evolutionary relationships. By analysing mitochondrial cytochrome c oxidase subunit I (COI) gene sequences from specimens collected in the Northwest Himalaya, we successfully generated the first DNA barcode for *P. quinquefasciatum* from India. Our findings establish a foundational framework for future taxonomic, ecological, and conservation studies to the genetic structure of *P. quinquefasciatum*. The generated DNA barcode data can facilitate precise species identification, aiding in resolving cryptic diversity and potential misidentifications in closely related taxa. Furthermore, our phylogenetic analyses shed light on the evolutionary affinities of *P. quinquefasciatum* within Hydropsychidae, reinforcing the significance of molecular approaches in taxonomy.

Keywords: DNA Barcoding, India, Phylogeny, Trichoptera

1 Introduction

The genus *Pseudoleptonema* Mosely, 1933, belongs to the subfamily Macronematinae within the family Hydropsychidae, a diverse group of caddisflies (order Trichoptera) found in freshwater ecosystems. Currently, *Pseudoleptonema* comprises fourteen species, five of which were recently transferred from the genus Trichomacronema to *Pseudoleptonema* based on morphological and molecular evidence (Oláh, 2013). These taxonomic revisions emphasizes the complexities in defining phylogenetic relationships within Hydropsychidae. In India, the genus *Pseudoleptonema* is represented by a single species, *Pseudoleptonema quinquefasciatum*, as documented by Parey et al. (2024). Despite its ecological significance, taxonomic information on this species remains scarce, necessitating further molecular investigations to clarify its phylogenetic placement.

The taxonomic classification of the subfamily Macronematinae has undergone multiple revisions due to the morphological similarities among its members and the limitations of traditional classification methods. Uy et al. (2018) highlighted the complexities associated with Macronematinae taxonomy, indicating the need for molecular approaches to supplement morphological studies.

1.1 DNA Barcoding and Its Importance

DNA barcoding, which utilizes a standardized fragment of the mitochondrial cytochrome *c* oxidase subunit I (COX I) gene, has proven to be an effective tool for species identification and phylogenetic studies (Hebert et al., 2004). The COX I gene exhibits a high degree of interspecific divergence while maintaining low intraspecific variation, making it a reliable molecular marker for distinguishing closely related species. DNA barcoding is an essential tool for biodiversity assessment, particularly in taxonomically challenging groups such as Trichoptera. The Barcode of Life Data System (BOLD) functions as a global repository for barcode records,

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integrating specimen information, genetic sequences, trace files, and images to facilitate species identification (Jalali et al., 2015). As of 2015, BOLD contained barcode data for 142,398 insect species worldwide, yet only 2,758 of these records were from Indian specimens. This highlights a significant gap in DNA barcoding efforts for Indian insect fauna
https://v3.boldsystems.org/index.php/TaxBrowser_Home

According to recent estimates by the Zoological Survey of India (ZSI, 2024), approximately 62,429 insect species across 595 families have been documented in India. Despite the rich biodiversity of India, only 2,330 species from 264 families have been barcoded, representing a mere 3.73% of the described insect species. The representation is even lower for Trichoptera, with less than 1% of Indian species having DNA barcode records (Shashank et al., 2022). This lack of molecular data poses challenges for accurate species identification, phylogenetic studies, and conservation planning.

1.2 Molecular Phylogeny of Trichoptera

The use of molecular data has significantly contributed to understanding the phylogenetic relationships within Trichoptera. Kjer et al. (2001, 2002) employed molecular markers, including COX I, ribosomal RNA, and elongation factor-1 alpha (EF-1α) gene sequences, to revise the higher phylogeny of Trichoptera. Their studies provided preliminary insights into the relationships among hydropsychid subfamilies, highlighting the potential of molecular approaches in resolving taxonomic ambiguities. The integration of molecular and morphological data is essential for refining the classification of Hydropsychidae, given the presence of cryptic species and convergent morphological traits.

In this study, we sequenced a fragment of the COX I gene from a single individual of *P. quinquefasciatum* collected from India. This sequence represents the first molecular record of the species from the Northwest Himalaya, providing valuable data for future phylogenetic and taxonomic studies. The obtained sequence was deposited in NCBI GenBank and BOLD under accession number PP110970 and BIN: AAH8219 (Table 1). This DNA barcode will serve as a reference sequence for identifying *P. quinquefasciatum* and for comparing its genetic relationship with other hydropsychid species worldwide. By incorporating this sequence into global databases, we aim to contribute to the growing repository of DNA barcodes for Trichoptera, facilitating species identification and phylogenetic research. This study highlights the importance of molecular approaches in complementing traditional taxonomy, particularly in underrepresented insect groups such as *Pseudoleptonema*. Future studies should focus on sequencing additional individuals from different geographical regions to assess intraspecific variation and population structure.

2 Materials and Methods

Specimens were collected between April and October of 2019–2023, from the Northwest Himalaya (Uttarakhand) (Figure 1) using a 15-W ultraviolet fluorescent tube powered by a 12-volt rechargeable battery, and 160-W UV or mercury-vapor lamps. Each specimen was labelled with date, location, altitude, and collector names and preserved in 96% ethyl alcohol with a drop of glycerol. Morphological analysis focused on genital features, legs, wing patterns, venation, labial palps, antennae, and setal warts were carried out under Nikon SMZ25 stereo zoom microscope.

All specimens have been curated and stored in the Museum of the Department of Zoology at Baba Ghulam Shah Badshah University (BGSBU), Rajouri, India.

2.1 DNA isolation, Amplification and Sequencing

Genomic DNA was extracted by removing one leg and using DNeasy Kits (Qiagen) with a modified version of the manufacturer's protocol. The leg was initially immersed in a mixture of 20 µl Proteinase K and 200 µl ATL buffer. The mixture was incubated at 56 °C for 14 hours in the Proteinase K solution, with an extra 20 µl of Proteinase K introduced after 6 hours of incubation.

A PCR mixture was prepared containing 1 × Takara PCR buffer, 200 µM dNTPs, 1 µM of forward primer LepF1 (5'-ATTCAACCAATCATAAAGATATTGG-3') and reverse primer LepR1 (5'-TAAACTTCTGGATGTCCAAAAAATCA-3'), 0.2 U of Takara DNA polymerase (Kusatsu, Japan), grade water, and genomic DNA. PCR amplification was conducted as following: an initial step of denaturation at 94 °C for three minutes, followed by 30 cycles each consisting of denaturation at 94 °C of 30 seconds,

Table 1 GenBank and BOLD COI barcode data collected *Pseudoleptonema quinquefasciatum* from India

Species	Accession No.	BIN	Location	Coordinates
Pseudoleptonema quinquefasciatum Martynov, 1935	PP110970	BOLD:AAH8219	Naugaon Uttarkashi, UK	30° 79' 06.9" N 78° 13' 83.9" E

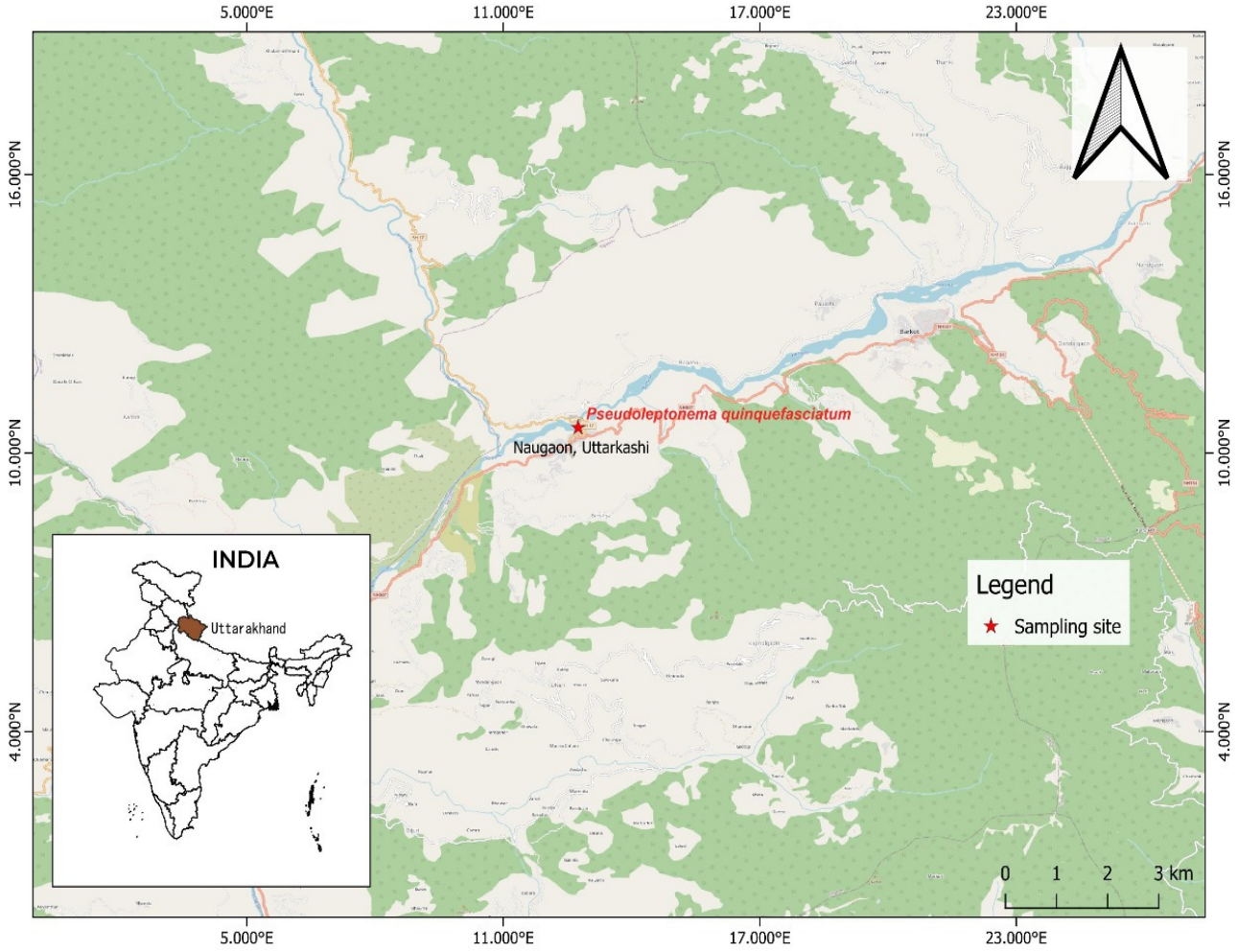

Figure 1 Map showing the collection site of *Pseudoleptonema quinquefasciatum* from Northwest Himalaya

annealing at 50 °C for 30 seconds, and one minute extension at 72 °C. Extension was repeated for ten minutes at 72 °C. Amplified template were then separated in 1% agarose gel stained with 1% ethidium bromide. After separation, the amplicons were excised from the gel and purified using the NucleoSpin Gel and PCR Cleanup Mini Kit (Düren, Germany) in accordance with the manufacturer's instructions. Purified The PCR products were sequenced using an ABI 3730XL or 3130XL sequencer (Biokart Private Limited, Bangalore, India) with BigDye Terminator v3.1 (Applied Biosystems). The sequencing reads obtained from both forward and reverse primers were assembled and trimmed, ensuring quality by evaluating each nucleotide based on the chromatograms.

2.2 Phylogenetic Analysis

The phylogenetic tree for *P. quinquefasciatum* cox-1 was constructed using Molecular Evolutionary Genetics Analysis version 11.0 (MEGA 11) (Kumar et al., 2018). Nucleotide sequences obtained from GenBank were also incorporated into the analysis, with their accession numbers and sequence origins provided in Table 2. The exact aligned matrix length was 658 bp. Among the 23 sequences analyzed, we used 10 sequences of *P. quinquefasciatum* Martynov 1935, one sequence of *Pseudoleptonema sinuatum*, two sequences of *Leptonema rafita*, three sequences of *Macrostemum midas*, three sequences of *Macrostemum radiatum*, two sequences of *Leptonema amazonense*, one sequence of *Leptonema woldianum* one sequence of *Macronema variipenne*, and one sequence of *Cheumatopsyche criseyde* (as outgroup).

These sequences were compared with *P. quinquefasciatum* using nucleotide BLAST (Camacho et al., 2009) to search GenBank for similar sequences and downloaded all aligned and similar sequences. Bootstrapping analysis (1,000 repeats) was applied to assess node support. MEGA 11 (Tamura et al., 2021) was utilized to calculate uncorrected pairwise distances between barcode sequences of the selected specimens and those

Acta fytotechn zootechn, 28, 2025(2): 115–121
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Table 2 Uncorrected Pairwise distance (p distances) among *Pseudoleptonema* species

	PP110970	KX105405	KX104349	KY983386	FN179148	KY983384	KX291667	KY983385	KX296436	HM862458
PP110970 *Pseudoleptonema quinquefasciatum*		0.002	0.002	0.013	0.013	0.013	0.013	0.014	0.016	0.022
KX105405 *Pseudoleptonema quinquefasciatum*	0.002		0.000	0.013	0.013	0.013	0.013	0.014	0.016	0.022
KX104349 *Pseudoleptonema quinquefasciatum*	0.002	0.000		0.013	0.013	0.013	0.014	0.014	0.016	0.022
KY983386 *Pseudoleptonema quinquefasciatum*	0.096	0.098	0.008		0.004	0.004	0.012	0.012	0.014	0.021
FN179148 *Pseudoleptonema quinquefasciatum*	0.099	0.101	0.101	0.012		0.004	0.011	0.012	0.014	0.020
KY983384 *Pseudoleptonema quinquefasciatum*	0.103	0.105	0.105	0.011	0.008		0.012	0.013	0.014	0.021
KX291667 *Pseudoleptonema quinquefasciatum*	0.102	0.102	0.104	0.081	0.072	0.009		0.002	0.014	0.021
KY983385 *Pseudoleptonema quinquefasciatum*	0.103	0.103	0.103	0.081	0.072	0.079	0.002		0.014	0.022
KX296436 *Pseudoleptonema quinquefasciatum*	0.128	0.130	0.131	0.104	0.098	0.100	0.098	0.007		0.022
HM862458 *Pseudoleptonema sinuatum*	0.240	0.242	0.245	0.228	0.222	0.224	0.236	0.238	0.232	

of *P. quinquefasciatum* (Table 2). Nodes with bootstrap support below 70% were collapsed. The proportion of replicate trees in which the associated taxa clustered together during 1000 bootstrap replicates is indicated next to each branch. Substitution patterns and rates were calculated using the Kimura (1980) 2-parameter model (+G). A discrete Gamma distribution with five categories (+G) was applied to account for evolutionary rate variation among sites.

3 Results and Discussion

In this study, we successfully amplified a specific amplicon of cox-1 with the size of 683 base pairs (bp), from DNA samples of *P. quinquefasciatum*. In the phylogenetic tree, 24 sequences, including all available sequences from NCBI, were classified into their respective clades (Figure 2). Additionally, a sequence of *Cheumatopsyche criseyde* was correctly classified as an outgroup. Only 10 cox-1 sequences of *P. quinquefasciatum* isolates are available in the GenBank, including our 01 sequences from India. Among the 09 isolates, two were recorded from Nepal, and two from Thailand, one each from Malaysia, USA, Sweden, Laos and Myanmar. Due to lack of sequences and for good results, we compare our obtained DNA nucleotide sequence with other species of same subfamily Macronematinae in the Maximum Likelihood (ML) analysis (Figure 2). However, to estimate uncorrected pairwise distance we included only the sequences of genus *Pseudoleptonema* (Table 2).

The maximum intraspecific distance was found 1.6% between our collected species from India (PP110970) and Thailand (KX296436), while the minimum

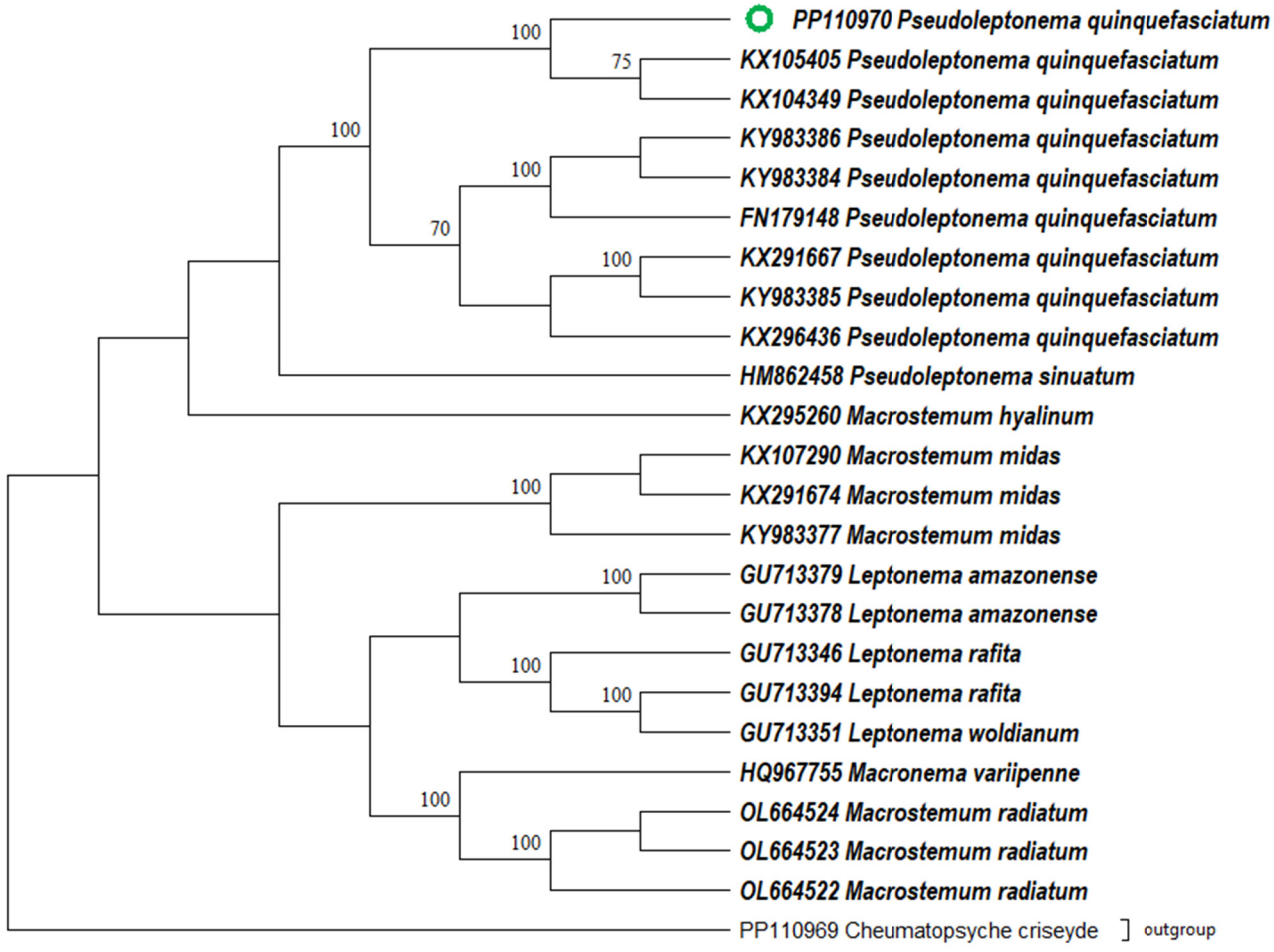

Figure 2 Maximum Likelihood (ML) analysis of 23 Macronematinae sequences based on COI data only bootstrap values ≥70% are shown
Specimen labels at branch tips include taxon and GenBank Sample ID. Green circle denotes the studied species

interspecific distance was found 24.2% (Table 2). The *p* distances were strongly supported by ML analysis where *P. quinquefasciatum* from India clustered with same species (2 sequences) from Nepal. Furthermore, the cladogram divided the operated species into 10 groups where the species from India grouped with the same species from other countries while *Pseudoleptonema sinuatum* forms a separate clade (Figure 2).

3.1 Maximum Likelihood Estimation of the Gamma Parameter for Site Rate Variation

The mean evolutionary rates for these categories were 0.00, 0.06, 0.28, 0.93, and 3.72 substitutions per site. The nucleotide frequencies were as follows: A = 25.00%, T/U = 25.00%, C = 25.00% and G = 25.00%. To estimate the maximum likelihood (ML) values, the tree topology was automatically determined. The highest Log likelihood for this analysis was -3,793.627. The analysis included 23 nucleotide sequences, and all codon positions (1st, 2nd and 3rd) were considered. Positions with less than 95% site coverage were excluded, meaning fewer than 5% alignment gaps, missing data, and ambiguous bases were permitted at any position.

4 Conclusions

This study represents a valuable contribution to the understanding of genetic diversity and phylogenetic relationships within *Pseudoleptonema quinquefasciatum*, particularly from an Indian perspective. By successfully amplifying and analyzing a 658 bp fragment of the mitochondrial cytochrome *c* oxidase subunit I (cox-1) gene, we were able to position the Indian isolate within a broader phylogenetic framework alongside global sequences. The phylogenetic reconstruction, performed using the Maximum Likelihood (ML) approach, along with pairwise genetic distance estimations, revealed a maximum intraspecific divergence of 1.6% and a minimum interspecific divergence of 24.2%. These findings confirm the genetic clustering of the Indian isolate with Nepalese sequences, indicating a closer phylogenetic affinity within this biogeographical region. Additionally, the clear separation of *Pseudoleptonema sinuatum* into a distinct clade further supports its phylogenetic distinctiveness within the subfamily Macronematinae. This study emphasizes the importance of molecular data in resolving taxonomic uncertainties and highlights the necessity of further genetic investigations to enhance our understanding of regional biodiversity and evolutionary relationships among Hydropsychidae members. The clustering of the Indian isolate with Nepalese sequences is expected due to their shared biogeographical distribution within the Himalayan mountain range. However, our study provides molecular evidence supporting this relationship, quantifying the genetic divergence and reinforcing the phylogeographic connectivity of *Pseudoleptonema quinquefasciatum* within the region. The observed variations, when interpreted in a phylogeographic context, further highlight the role of geographic and ecological factors in shaping genetic differentiation.

In the present study, the mean evolutionary substitution rates varied across different site categories, with observed values of 0.00, 0.06, 0.28, 0.93, and 3.72 substitutions per site. These rates reveal the differential evolutionary pressures acting on various codon positions, which is a crucial aspect of molecular evolution studies. Typically, synonymous substitutions at third codon positions tend to evolve at a faster rate compared to non-synonymous substitutions at first and second positions due to the relaxed selective constraints on synonymous changes.

The nucleotide composition analysis revealed an equal distribution of base frequencies, with adenine (A), thymine/uracil (T/U), cytosine (C), and guanine (G) each comprising 25.00% of the total nucleotide content. This uniformity suggests a balanced representation of nucleotide bases across the dataset, which may influence the genetic stability and codon usage bias within the studied sequences. To infer the phylogenetic relationships, the Maximum Likelihood (ML) approach was employed, wherein the tree topology was determined automatically to optimize the likelihood estimation. The ML analysis resulted in a highest log-likelihood value of -3,793.627, indicating the best-supported phylogenetic hypothesis given the dataset. Log-likelihood values serve as a measure of model fit, with higher (i.e., less negative) values suggesting a better representation of evolutionary relationships.

The analysis incorporated total 23 nucleotide sequences, encompassing all codon positions (1st, 2nd, and 3rd) to ensure comprehensive phylogenetic reconstruction. To maintain data integrity and minimize alignment biases, positions with less than 95% site coverage were excluded. This exclusion criterion allowed for the retention of high-confidence sites while filtering out those with excessive alignment gaps, missing data, or ambiguous bases (i.e., positions with uncertainties in nucleotide identity). By enforcing this threshold, the analysis ensured that the reconstructed phylogeny was based on well-supported sequence data, minimizing potential errors arising from incomplete or ambiguous regions.

Acknowledgements

The authors express their sincere gratitude to the Science and Engineering Research Board (SERB), Government of India, for their financial support of this research under the Core Research Grant (CRG) scheme (Sanction Order No. CRG/2020/001494, dated December 1, 2020). We thank Dr. John C. Morse of Clemson University for his valuable suggestions and Prof. Hans Malicky for providing relevant literature.

Authors' Contributions

AM: Conceptualization, Methodology, Investigation, Writing – original draft; SHP: Investigation, Validation, Writing – review & editing; MSP: Investigation, Validation, Writing – review & editing

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