doi:10.14720/aas.2022.118.1.2132 Original research article / izvirni znanstveni članek

Morphological and molecular characterization and new distributional record of *Tetrastichus miser* (Nees, 1834) (Hymenoptera: Chalcidoidea: Eulophidae) from Kashmir

Ajaz RASOOL [1], Bashir A. GANAI [2], Shreevihar SANTHOSH [3] and Tariq AHMAD [1, 4]

Received March 12, 2021; accepted January 22, 2022.
Delo je prispelo 12. marca 2021, sprejeto 12. januarja 2022

Morphological and molecular characterization and new distributional record of *Tetrastichus miser* (Nees, 1834) (Hymenoptera: Chalcidoidea: Eulophidae) from Kashmir

Abstract: *Tetrastichus miser* (Nees, 1834) (Hymenoptera: Eulophidae: Tetrastichinae) is a parasitoid of Curculioninae and Scolytinae infesting various trees of economic importance. In the present study, it was collected from dried *Cedrus deodara* (Roxb.) G. Don (Pinaceae) infested with *Scolytus* beetles using sweep net and aspirator. The species is reported first time from Kashmir valley. Identification of a parasitoid is of paramount significance for studying its behavior, ecology, life cycle and usage in various biological control programmes. In addition to morphological description, molecular analysis using Cytochrome C Oxidase Subunit I was carried out to complement morphotaxonomy and to facilitate its easier identification for future studies. Phylogenetic analysis by Bayesian inference (BI) and Maximum Likelihood (ML) method showed Isolates of *Tetrastichusmiser* species clustering in same clade and separated from its closest match Tetrastichinae sp.Inter-specific divergence between *Tetrastichusmiser* and Tetrastichinae sp. was evident and ranged from 0.09 to 0.10 % (0.05 % mean). No overlap was observed between maximum distance within species and minimum distance between species.

Key words: *Tetrastichus miser*; *Cedrus deodara*; beetle; Cytochrome C Oxidase Subunit I; Clade; morphotaxonomy; molecular analysis

Morfološka in molekularna določitev ter novi podatki o razširjenosti vrste *Tetrastichus miser* (Nees, 1834) (Hymenoptera: Chalcidoidea: Eulophidae) v Kašmirju

Izvleček: Vrsta *Tetrastichus miser* (Nees) (Hymenoptera: Eulophidae: Tetrastichinae) je parazitoid hroščev iz podružin Curculioninae in Scolytinae, ki napadajo različne gospodarsko pomembne drevesne vrste. V raziskavi je bil parazitoid nabran s stresalnimi mrežami in aspiratorjem na posušenih himalajskih cipresah, *Cedrus deodara* (Roxb.) G.Don (Pinaceae), ki so bile napadene s hrošči iz rodu *Scolytus*. O vrsti poročajo prvič iz doline Kashmir. Določitev parazitoida je zelo pomembna za preučevanje njegovega obnašanja, ekologije, življenskega kroga in pri njegovi uporabi v različnih programih biotičnega zatiranja škodljivcev. Poleg morfološkega opisa je bila za lažjo določitev v bodočih raziskavah uporabljena molekularna analiza na osnovi podenote I citohrom C oksidaze. Filogenetska analiza z metodama Bayezinove inference (Bayesian inference, BI) in največje verjetnostni (Maximum Likelihood, ML) je pokazala, da so se izolati vrste *Tetrastichus miser* združevali v istem kladu, ločeno od najbližjih, ki se ujemajo s predstavniki poddružine *Tetrastichinae*. Ločitev vrste *Tetrastichus miser* in predstavnikov poddružine *Tetrastichinae* je bila očitna in je znašala od 0,09 do 0,10 % (v povprečju 0,05 %). Opaženega ni bilo nobenega prekrivanja med maksimalno razdaljo znotraj vrste in minimalno razdaljo med vrstami.

Ključne besede: *Tetrastichus miser*; *Cedrus deodara*; hrošč; podenota I citohrom C oksidaze; klad; morfotaksonomija; molekularna analiza

1 Entomology Research Laboratory, Department of Zoology, University of Kashmir, Srinagar, India
2 Centre of Research for Development (CORD), University of Kashmir, Srinagar, India
3 Systematic Entomology Laboratory, Malabar Christian College – Calicut, Kozhikode, India
4 Corresponding author, e-mail: drtariqento@kashmiruniversity.ac.in

1 INTRODUCTION

Eulophidae (Hymenoptera: Chalcidoidea) is one of the largest chalcid families consisting of about 300 genera and 5000 speciesworldwide (Shree &Singh, 2015; Noyes, 2014). Eulophids have cosmopolitan distribution (Noyes, 2002) and are known to be entomophagous attacking insects and other arthropods like spiders and mites. Most eulophids are small in size (3 mm, average 1.5 mm). Family Eulophidae comprises of four subfamilies i.e. Eulophinae, Entedoninae, Euderinae and Tetrastichinae (Graham, 1975, Bouček & Graham, 1978). Eulophids are distinguished from other chalcidoid families by the presence of four segmented tarsi in both sexes and a short, straight protibial spur (as opposed to a generously proportioned, curved spur in most other chalcidoids) (Schauff, 1997). Species of eulophidae are mostly primary parasitoids or hyperparasitoids, parasitizing a wide range of hosts like cotton boll weevil, beetles, caterpillars of borers, midges, leaf miners, scale insects which are notorious pests of various horticultural, agricultural crops and forest plantations. Some eulophids are known to attack gall forming insects, mites (Bouček& Askew, 1968), eggs of spiders in silken egg sacs (LaSalle, 1990, 1994) and nematodes (van den Berg et al., 1990). Insect species belonging to more than 100 families and 10 orders are recorded as hosts for various eulophid chalcids (Talebi et al., 2010, 2011). Besides, most of the eulophids are parasitoids of insects hidden in plant tissue, such as wood borers, leaf miners, leaf rollers and gall makers.

The genus *Tetrastichus* Haliday (Hymenoptera: Eulophidae: Tetrastichinae) is far and wide distributed worldwide containing 518 species worldwide (Noyes, 2014). *Tetrastichus* species virtually occur in all terrestrial habitats in all geographic realms, and constitute a vital component of terrestrial ecosystems. Taxonomic work on Tetratsichinae was started by Burks (1943), who provided key to North American species of *Tetrastichus*. Tetrastichinae fauna of India includes 34 genera and 272 species (Hayat & Shah, 2004; Narendran, 2007). Still many species are yet to be explored and employed for various pest management programmes. The main diagnostic character include a submarginal vein with one seta (rarely 2-4), propodeum with inverted Y shaped paraspiracular carina and hind coxa with strong reticulations. *Tetrastichus miser* (Nees, 1834) was first reported from India by Narasimham (1984). The specimens of the present study were collected during surveys in Botanical garden of University of Kashmir from dried *Cedrus deodara* (Roxb.) G.Don tree infested with *Scolytus* beetles. Asthe species is new faunal record from Kashmir valley, the present study provides a brief diagnosis and photographic illustration to authenticate the new record. In addition, molecular identification via DNA barcoding of cytochrome C oxidase subunit I (COI) was also carried to complement morphotaxonomy and to facilitate its easier identification.

2 MATERIAL AND METHODS

Sampling on the prevalence of pest infestation was conducted in Botanical garden, University of Kashmir (34°08'09" N 74°49'14" E; 1590 m). Dead and dried *Cedrus deodara* was found infested with Scolytid beetles. 11 parasitoids were collected by hand picking, aspirator and using sweep net with ethyl acetate used as killing agent. Collection after proper isolation and separation was preserved in vials in 70 % alcohol for further taxonomic studies. After morphological identification, specimens which were needed for molecular analysis were preserved in 90 % alcohol and then frigid at -20 °C. For morphological studies, Card mounted specimens were examined under Leica M205A stereozoom microscope (Leica Microsystems, Germany).

2.1 DNA EXTRACTION AND SEQUENCING

2.1.1 DNA extraction

Before isolation, frigid samples were thoroughly washed with alcohol and formaldehyde to avoid contamination. The Genomic DNA was extracted from legs using DNA extraction kit (Nucleospin Insect DNA kit from Macheray Nagel, Germany) following manufacturer's protocol. PCR reaction mixture of 25 µl was prepared with following composition: 2.50 µl (10 x) of Taq assay buffer, 2.5 µl of dNTPs (each in 10 mM concentration), Forward primers and reverse primers each 0.2 µl (10 picomoles μl^{-1}), $MgCl_2$ buffer (1.5mM) 1.5 µl, Taq Polymerase 0.2 µl (1 U), DNA template 3 µl and Mili Q or sterilised water 14.9 µl. Universal primers (LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' (forward) and HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (reverse)) were used to amplify the COI region (Folmer et al., 1994). Amplification was done by using a Thermal Cycler (Biorad Laboratories, California) programmed to 98 °C for 5 minutes, followed by 30 cycles of 95 °C for 30 seconds, 45 °C for 45 seconds and 72 °C for 45 seconds and a final extension at 72 °C for 10 minutes. Amplified products were gel eluted in 0.8 % agarose stained with ethidium bromide and visualized using a UV trans-illuminator. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator

v 3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol at Rajiv Gandhi Centre for Biotechnology, Kerala, India.

2.1.2 DNA sequence analysis

Trace files of sequences were subjected to quality check by recording Phred score with a minimum of 20Q assigned to qualify as actual and further edited and trimmed at ends using Chromas 2.2.2 and Sequence scanner V2.The Phred score logarithmically represents error probabilities in base calling, hence this algorithm is used by majority of sequence analysis softwares (Ewing et al., 1998). Sequences were also checked for indels and numts using BioEdit 7.2 (Hall et al., 2011). The homology search was performed for the resulting consensus sequences using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) and identification option of BOLD systems (Ratnasingham and Hebert 2013) against sequences in GenBank to confirm the corresponding sequence taxonomy. The generated COI consensus sequences were deposited in NCBI GenBank database and the corresponding accession numbers generated are MK419323, MT012501, MT017888, MT012523 and MT012515.

2.1.3 Phylogenetic analysis

Phylogenetic analysis was done for the homologous COI sequences obtained from Genbank database by performing similarity searches using BLASTn search algorithm (Altschulet al. 1990).A threshold of 3 % variation between individuals of Chalcid wasps for COI gene was used for differentiating putative species (Hebert et al., 2004; Santos et al., 2011; Smith et al., 2018).Only top hits (sequences) with high similarity score and E-values in BLASTn were considered and non-redundant species sequences were retained for further analysis. Our sequences did not reveal perfect matches, so a set of top 20 sequences were chosen for phylogenetic analysis and were aligned using Clustal W(Thompson et al., 1994) multiple alignment program inbuilt in MEGA X with the default alignment parameters (Kumar et al., 2018). Pair wise distance between each sequence was calculated using distance option of MEGA software. In addition, variable sites analyses from the alignment of the dataset were performed in MEGA X (Kumar et al., 2018).

2.1.4 Abbreviations and Acronyms

AIC: Akaike Information Criterion
BI: Bayesian Inference
BLAST: Basic Local Alignment Search Tool
BOLD: Barcode of Life Data System
CC: costal cell
COI: Cytochrome c Oxidase Subunit 1
DNA: Deoxyribonucleic acid
MCMC: Markov Chain Monte Carlo
MEGA: Molecular Evolutionary Genetic Analysis
ML: Maximum Likelihood
MV: marginal vein
NCBI: National Centre for Biotechnology Information
NJ: Neighbor Joining
SLG: sublateral groove
SMG: submarginal groove
SMV: submarginal vein
♀: Female

3 RESULTS

3.1 MORPHOLOGICAL IDENTIFICATION

3.1.1 Diagnosis

Body black with copper bronze metallic reflections; mesocutum with median line weaker towards pronotum, 5 long adnotaular setae on either side, in addition two rows of three setaeon either side of median line of mesonotum; eye with long pubescence; clava (0.72 x) distinctly shorter than scape; antennal formula 1 : 1 : 1 : 3 : 3; ratio of length of antennal segments 0.79: 0.32: 0.35 : 0.32 : 0.25 : 0.57. CC subequal to MV; SMV with one dorsal seta; malar space O.62 x length of eye; SMG of scutellum 1.7x from each other than from SLG, enclosed space 1.9 x as long as broad; callus with 4-7 setae arranged in two groups, one near the spiracle and the other near hind corner of propodeum; metasoma excluding ovipositor sheath a little shorter than mesosoma (2.5 : 2.6); hypopygium reaching 0.6 x length of gaster. The specimens slightly varies in the number and pattern of setae on mesonotum, the other characters are matching the redescription by Graham, 1991 (Fig. 1).

3.2 MATERIAL EXAMINED AND HOST ASSOCIATION

Card mounted ♀, INDIA: Jammu & Kashmir, Botanical garden, University of Kashmir (34°08'50.5"N, 74°52'00.9"E), elevation 1600 m), collected by Ajaz Rasool, May 2018, Graham (1991) reported it from *Rhynchaenus alni* (Linnaeus, 1758), *Rhynchaenus fagi*

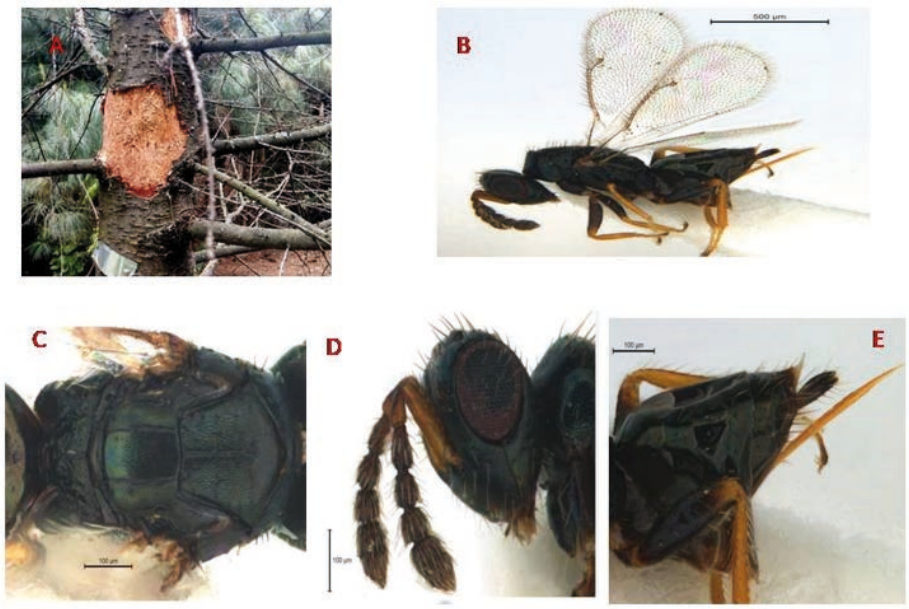

Figure 1: *Tetrastichus miser*, female: A infested *Cedrus deodara* tree, B Habitus, lateral view; C Mesosoma; D Head, Antennae; E Abdomen

(L., 1758), *Rhynchaenus pilosus* (J.C. Fabricius, 1781), *Rhynchaenus quercus* (L., 1758), *Rhynchaenus salicis* (L., 1758), and *Rhynchaenus oxyacantha* (Curculonidae). In the present study, it was reported from *Cedrus deodara* (Roxb.) G.Don trunk infested by Scolytidae (Coleoptera: Scolytidae).

3.3 DISTRIBUTION

Austria, Czech republic, Slovakia, Denmark, Finland, France, Germany, Hungary, India (Bangalore and J&K), Italy, Netherlands, Spain, Sweden, Ukraine, UK. Host records are in Universal Chalcidoidea Database (www.universalchalcidoideadatabase) (Noyes, 2014).

3.4 GENETIC DATA AND PHYLOGENETIC ANALYSIS

The accession numbers of sequences so generated areMK419323, MT012501, MT017888, MT012523 and MT012515. Barcoding of *Tetrastichus miser* from Kashmir has been carried for the very first time. Our sequences did not reveal perfect hits and hence were submitted to GenBank only after morphological identification. Nuclear copies of mitochondrial DNA (NUMT) contamination were fortified by performing amino acid translation by checking for the stop codons in the sequences. Translation of frame 3 of amino acid sequence containing 180 amino acids was performed using ExPASy bioinformatics resource portal. No haplotype diversity was seen in the *Tetrastichusmiser* isolates. All 5 isolates were collected from same host tree (*Cedrus deodara*) at same location. The final alignment of the data set resulted in 567 nucleotide sites having 488 conserved sites, 121 variable sites, 97 parsim-info sites and 24 singleton sites. The mean A+T content was revealed as 74.69 %.

After receiving accession numbers, final alignment of top 20 hits was done each roughly 567bp long of which 5 sequences represent the current study, 6 represent *Baryscapus* sp., 2 represent *Aprostocetus* sp., 4 *Tetrastichinae* sp. and 3 eulophid species. The nucleotide composition revealed high A-T content (74.69 %) which is common for arthropods. The nucleotide frequencies include 34.25 % (A), 40.44 % (T/U), 12.61 % (C), and 12.70 % (G). Phylogenetic trees for *Tetrastichus miser* species were constructed by Bayesian inference (BI) and Maximum Likelihood (ML) methods. Both methods were used to confirm the evolutionary history of *Tetrastichus* species. For BI method, model selection was based on the Akaike information criterion (AIC) computed by Partitionfinder version 2.1.1 software (Lanfear, 2012). The subset partitions with positions 1, 2 and 3 were done and the best fit substitution models were predicted. The BI analyses was performed using MrBayes version 3.2.2 (Ronquist et al., 2012), a stop rule convergence value of 0.01 was set, which occurred on the 1140000 Markov chain Monte Carlo (MCMC) generation and two incrementally heated chains. MCMC started from a random tree, sampling

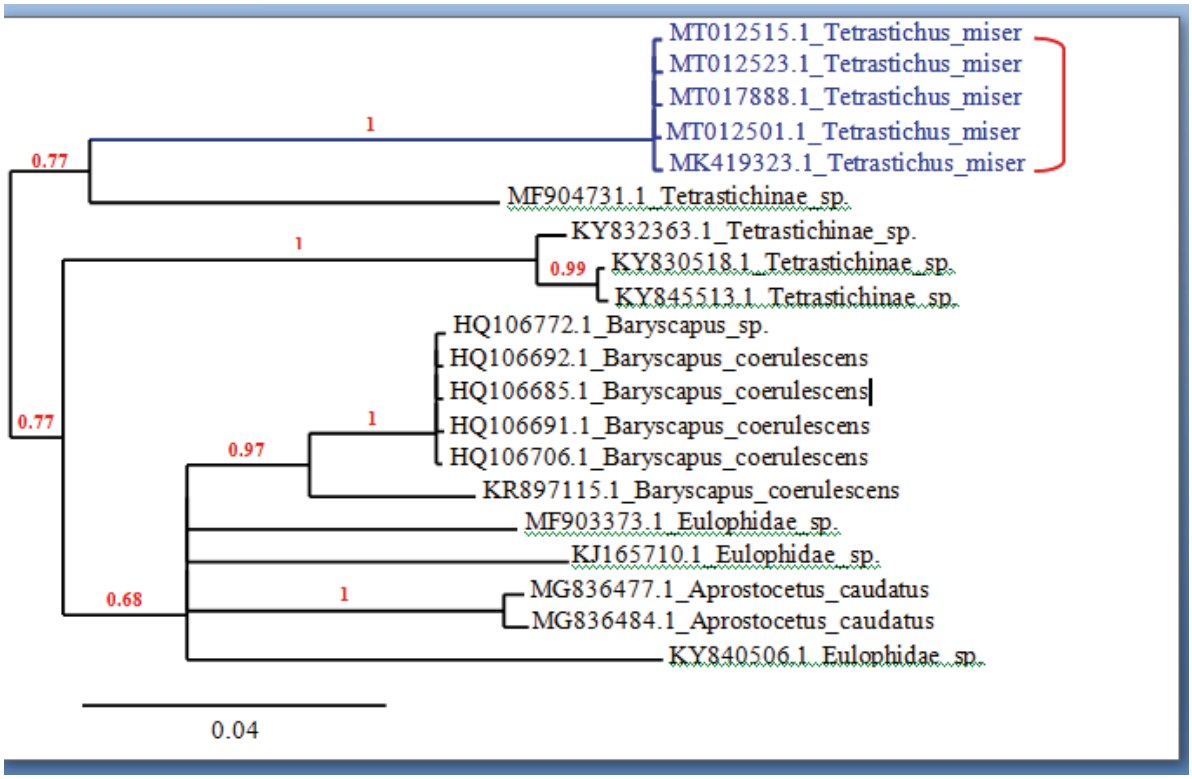

Figure 2: Bayesian inference phylogenetic tree of COI gene sequences of *Tetrastichus* spp. The scale bar indicates the number of substitutions per nucleotide position

one of every 500 generations, with the first 550 (25 %) of the trees discarded as burn-in out of 2200. The resulting tree was imported, edited and visualized using TreeDyne (Chevenet et al., 2006) inbuilt in Phylogeny.fr (Dereeper et al., 2008) (Fig. 2).For ML method, evolutionary history was inferred based on the Kimura 2-parameter model (Kimura 1980) in MEGA X (Kumar et al., 2018). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985) (Supplementary file). In both trees, similar typology was found and *Tetrastichus miser* isolates clustered in a single clade with strong bootstrap value (100 %) and separated from other species.

3.5 INTRA- AND INTER- SPECIFIC EVOLUTIONARY DIVERGENCE

Pairwise Kimura-2-parameter (K2P) distance was calculated to comprehend the evolutionary divergence rate among the species based on COI gene (Kimura 1980). Datasets which were used for phylogenetic analysis were used for estimation of Pairwise intra and inter species K2P distances among the species using option implemented in MEGA X (Kumar et al., 2018). Among *Tetrastichus miser* isolates, no divergence was reported as such with intraspecific nucleotide difference of 0.00 %. Tetrastichinae sp. showed intraspecific divergence of 0.01 to 0.09 % whereas among *Baryscapus coerulescens* (Ashmead, 1898) species it ranged between 0.00 to 0.03 %. Interspecific divergence between *Tetrastichus miser* and Tetrastichinae sp. ranged from 0.09 to 0.10 % (0.05 % mean). Between *Baryscapus coerulescens* and *Tetrastichus miser* species interspecfic divergence of 0.08 to 0.09 % (0.05 % mean) was reported.

4 DISCUSSION

Identification of a parasitoid is of paramount significance for studying its behavior, ecology, life cycle and usage in various biological programmes. *Tetrastichus miser* parasitizes a wide range of Curculionid beetles infesting various deciduous trees (Graham, 1991). Management of various bark beetles therefore requires information of natural enemies associated with them for future biological control programs. Morphologically, it has been described in India but owing to new record from Kashmir, current study was complemented by molecular analysis

for future taxonomic purposes. There have been only few molecular studies on chalcid wasps from India (Rasool et al., 2018). Employing both morphological and molecular analysis for identification and characterization of *T.miser* species has been carried out for the first time. COI gene was used as marker gene for barcoding purposes for this species. Card pointed specimens were observed under LeicaM205A stereozoom microscopefor morphological studies. Key morphological characters like single SMV dorsal setae, black body with metallic reflections, were consonance with studies of Narasimham (1984).The specimens slightly varies in the number and pattern of setae on mesonotum, the other characters are matching the re-description by Graham,(1991).

The final alignment of the data set resulted in 567 nucleotide sites having 488 conserved sites, 121 variable sites, 97 parsim-info sites and 24 singleton sites. The mean A+T content was revealed as 74.69 %. The data was analyzed for sequence divergence at different taxonomic levels in MEGA X software. Pairwise distance using K2P parameter in MEGA X was used to calculate the distance matrix (Kimura, 1980). Interspecific divergence between *Tetrastichus miser* and Tetrastichinae sp. was noticeable and ranged from 0.09 to 0.10 % (0.05 % mean). No overlap was observed between maximum K2P distance within species and minimum distance between species. High nucleotide variations indicate geographical isolation and hence limited gene flow between species (Santos et al., 2011; Powell et al., 2019).

For phylogenetic analysis, Maximum likelihood method and Bayesian Inference methods were used to infer evolutionary history so as to look for clustering of clades in different trees. Both methods resulted in somewhat similar typology. In both the methods congeneric species cohesively clustered together with closely related genera. Besides, taxa belonging to a particular species more often than not formed a coherent cluster indicating that CO1 gene sequences are useful in identification of species. The bootstrap consensus tree inferred from 1000 replicates is taken to correspond to the evolutionary history of the taxa analyzed (Felsenstein, 1985). The taxa belonging to the same species, genus or family clustered together with healthy bootstrap support. It was also found that sequences from same country and genus or species clubbing in the same clade in both trees. Along with low support values at nodes, some high values were also reported when nodes include sister or conspecific sequences. The reason for this is that the COI gene fragment has been reported to best resolve shallow species-level relationships in arthropod fauna but showed poor results when family level and beyond relationships were taken into consideration (Waugh, 2007) and same was the case with our sequence data. Nevertheless, molecular analysis supported morphological results, besides adding its barcode to the databases for further exploration. Considering the role of *T. miser* in biological control programmes, molecular data of this study will serve as an elite DNA barcode for species identification, future molecular studies, better understanding of bio-control services and other related taxonomic studies in future.

5 CONCLUSION

Tetrastichus miser is well known parasitoid of scolytid beetles infesting various tree species of economic importance. Morphologically, species has been defined and described in India, but molecular taxonomy was missing. There was rarely an entry in the GenBank database for this species. This study was carried to add to knowledge of chalcid wasps from Kashmir valley and also complement its morphologically defined taxonomy. COI gene of mitochondrial genome was used as DNA barcode for the molecular analysis. The sequenced segment was found to be 567 bp long and was found to be AT rich in content. The sequence showed low percentage of match in NCBI and BOLD database systems with the closest match being Tetrastichinae sp. (91 %).Phylogenetic analysis inferred close match between *Tetrastichus* isolates, clustering into same clade with good bootstrap support. No overlap was observed between maximum K2P distance within species and minimum distance between species. Considering the obscurity in identification of diverse insect fauna, this exercise will complement taxonomic analysis and sequence data will serve as an elite DNA barcode for species identification and other related taxonomic studies in future.

6 CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

7 ACKNOWLEDGEMENT

Authors are highly grateful to Head of Department of Zoology and Director of the Centre of Research for Development (CORD) for providing working facilities. Authors, SS and TA acknowledge the financial assistance from SERB, Department of Science and Technology, Government of India, New Delhi for the financial assistance (File No. EMR/2017/005528 & EMR/2017/000215 respectively).

8 REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, *25*(17), 3389-3402. https://doi.org/10.1093/nar/25.17.3389

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2008). GenBank. *Nucleic Acids Research*, *37*(suppl_1), D26-D31. https://doi.org/10.1093/nar/gkn723

Boucek, Z., & Askew, R. R. (1968). *Palearctic Eulophidae (excl. Tetrastichinae), Hym. Chalcidoidea*. Le Francois.

Boucek, Z., & Graham, M. D. V. (1978). British check-list of Chalcidoidea (Hymenoptera): taxonomic notes and additions. *Entomologist's Gazette*, *29*(4), 225-235.

Burks, B. D. (1943). The North American parasitic wasps of the genus *Tetrastichus* a contribution to biological control of insect pests. *Proceedings of the United States National Museum*. https://doi.org/10.5479/si.00963801.93-3170.505

Chevenet, F., Brun, C., Bañuls, A. L., Jacq, B., & Christen, R. (2006). TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics*, *7*(1), 1-9. https://doi.org/10.1186/1471-2105-7-439

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F.& Gascuel, O. (2008). Phylogeny. fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research*, *36*(suppl_2), W465-W469. https://doi.org/10.1093/nar/gkn180

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792-1797. https://doi.org/10.1093/nar/gkh340

Ewing, B., Hillier, L., Wendl, M. C., & Green, P. (1998). Basecalling of automated sequencer traces usingPhred. I. Accuracy assessment. *Genome Research*, *8*(3), 175-185. https://doi.org/10.1101/gr.8.3.175

Felsenstein, J. (1985), Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, *39*(4), 783-791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

Folmer, O., Black, M., Hoeh, W., Lutz, R and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology, 3,* 294-299.

Graham, M. D. V. (1976). Relationships and synonymy of *Winnemana* Crawford (Hymenoptera Eulophidae). *Journal of Entomology Series B, Taxonomy*, *44*(3), 281-282. https://doi.org/10.1111/j.1365-3113.1976.tb00018.x

Graham, M. D. V. (1991). A reclassification of the European Tetrastichinae (Hymenoptera: Eulophidae): revision of the remaiming genera. *Memoirs of the American Entomological Instinstitute*, *49*, 1-322.

Hall, T., Biosciences, I., & Carlsbad, C. (2011). BioEdit: an important software for molecular biology. *GERF Bull Biosci*, *2*(1), 60-61.

Hayat, M., & Shahi, M. H. (2004). Taxonomic notes on Indian Eulophidae (Hymenoptera: Chalcidoidea)—1. On the types of some Tetrastichinae. *Oriental Insects*, *38*(1), 303-314. https://doi.org/10.1080/00305316.2004.10417396

Hebert, P. D., Penton, E. H., Burns, J. M., Janzen, D. H., & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator. Proceedings of the National Academy of Sciences*, *101*(41), 14812-14817. https://doi.org/10.1073/pnas.0406166101

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*(2), 111-120. https://doi.org/10.1007/BF01731581

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547-1549. https://doi.org/10.1093/molbev/msy096

Lanfear, R., Calcott, B., Ho, S. Y., & Guindon, S. (2012). PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, *29*(6), 1695-1701. https://doi.org/10.1093/molbev/mss020

LaSalle, J. (1990). Tetrastichinae (Hymenoptera: Eulophidae) associated with spider egg sacs. *Journal of Natural History*, *24*(6), 1377-1389. https://doi.org/10.1080/00222939000770831

LaSalle, J. (1994). North american genera of Tetrastichinae (Hymenoptera: Eulophidae). *Journal of Natural History*, *28*(1), 109-236. https://doi.org/10.1080/00222939400770091

Narasimham, A. U. (1984). Comparative studies on *Tetrastichus hagenowii* (Ratzeburg) and *T. asthenogmus* (Waterston), two primary parasites of cockroach oothecae, and on their hyperparasite *Tetrastichus* sp. (*T. miser* (Nees) group)(Hymenoptera: Eulophidae). *Bulletin of Entomological Research*, *74*(2), 175-189. https://doi.org/10.1017/S0007485300011329

Narendran, T. C. (2007). Indian chalcidoid parasitoids of the Tetrastichinae (Hymenoptera: Eulophidae). *Occasional Paper-Records of the Zoological Survey of India*, (272).

Noyes, J. S. (2002). *Universal Chalcidoidea database*. http://www.nhm.ac. Uk/entomology / chalcidoids.

Noyes, J.S. (2014). *Universal Chalcidoidea Database*. World Wide Web electronic publication.http://www.nhm.ac.uk/chalcidoids. (accessed, 3rd Feberuary, 2021).

Powell, C., Caleca, V., Sinno, M., van Staden, M., van Noort, S., Rhode, C., Allsopp, E&Van Asch, B.(2019). Barcoding of parasitoid wasps (Braconidae and Chalcidoidea) associated with wildand cultivated olives in the Western Cape of South Africa. *Genome*, *62*(3), 183-199. https://doi.org/10.1139/gen-2018-0068

Rasool, A., Ahmad, T., & Ganai, B. A. (2018). An overview of molecular identification of insect fauna with special emphasis on chalcid wasps (Hymenoptera: Chalcidoidea) of India. *Acta agriculturae Slovenica*, *111*(1), 229-239. https://doi.org/10.14720/aas.2018.111.1.22

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, *19*(12), 1572-1574. https://doi.org/10.1093/bioinformatics/btg180

Santos, A. M., Besnard, G., & Quicke, D. L. (2011). Applying DNA barcoding for the study of geographical variation in host–parasitoid interactions. *Molecular Ecology Resources*, *11*(1), 46-59. https://doi.org/10.1111/j.1755-0998.2010.02889.x

Schauff, M. E. (1997). Eulophidae. *Annotated Keys of the Genera of Neartic Chalcidoidea (Hymenoptera)*, 325-429.

Shree, A., & Singh, S. (2015). Diversity of family Eulophidae (Hymenoptera: Chalcidoidea) in forest, horticulture, and habitats of Doon valley of Uttarakhand, India. *Indian Forester*, *141*(8), 873-876.

Smith, M. A., Rodriguez, J. J., Whitfield, J. B., Deans, A. R., Janzen, D. H., Hallwachs, W., & Hebert, P. D. (2008). Extreme diversity of tropical parasitoid wasps exposed by iterative integration of natural history, DNA barcoding, morphology, and collections. *Proceedings of the National Academy of Sciences*, *105*(34), 12359-12364. https://doi.org/10.1073/pnas.0805319105

Sujeevan, R., & Hebert, P. (2007). BOLD: the Barcode of life data system. *Molecular Ecology Notes*, *7*, 355-364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Talebi, A. A., Asadi, R., Fathipour, Y., Kamali, K., & Moharramipour, S. (2010). Eulophid parasitoids of agromyzid leafminers genus *Liriomyza* (Dipt.: Agromyzidae) in Tehran, Iran. *IOBC WPRS Bulletin*, *28,* 263-266.

Talebi, A. A., Khoramabadi, A. M., & Rakhshani, E. (2011). Checklist of eulophid wasps (Insecta: Hymenoptera: Eulophidae) of Iran. *Check List*, *7*(6), 708-719. https://doi.org/10.15560/11008

Van Den Berg, E., Prinsloo, G. L. & Neser, S. 1990. An unusual host association: *Aprostocetus* species, (Eulophidae), a hymenopterous predator of the nematode, *Subhanguina mobilis* (Chit & Fisher, 1975) Brezski, 1981 (Anguinidae). *Phytophylactica*, *22*, 125-127