Research Article

Morphological and molecular characterization of *Microplitis maculipennis* Szépligeti (Hymenoptera: Braconidae) from India with notes on its generic placement

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ABSTRACT: *Microplitis maculipennis* Szépligeti is an important parasitoid of castor semilooper *Acanthodelta janata* (L.) (Lepidoptera), a major pest of castor (*Ricinus communis* L.). *Microplitis* Förster shares remarkable morphological resemblance with moderately diverse genus *Snellenius* Westwood. In this study, molecular characterization of *M. maculipennis* was done using Cytochrome Oxidase I (COI) to confirm its generic placement in the respective genus. The Bayesian Inference (BI) and Maximum Likelihood (ML) phylogenetic analysis performed with a total of 354 published BOLD database sequences (after pre-processing of a total of 2257 COI sequences) of *Microplitis* and *Snellenius* species, representing 129 named species and 226 species determined only to genus raises doubts on the retention of both these genera separately. Our studies reveal that COI gene could not discriminate *Microplitis* and *Snellenius* species clearly.

**KEY WORDS:** *Acanthodelta janata,* barcode, COI, *Microplitis maculipennis***,** *Snellenius*

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INTRODUCTION

Microplitis (Förster, 1862), an apomorphic genus, is known with the type species *Microgaster sordipes* Nees. *Microplitis* Szepligeti contains almost 200 species worldwide (Fernández-Triana and Ward, 2017; Fernández-Triana *et al.,* 2015). The genus is diverse and well documented from the Holarctic region in comparison with the Neotropical, tropical and subtropical regions. It is also well known from the Australasian region (Austin and Dangerfield, 1992, 1993). We are exploring monophyly of *Microplitis* as this genus belongs to one of the group of wasps, the 'microgastroid complex,' which is a monophyletic assemblage of approximately 50,000 species within the family Braconidae that all employ viruses named Bracoviruses (BVs) during parasitism of lepidopteran hosts (Burke, 2016). The species used in our study *M. maculipennis* Szepligeti exclusively attacks lepidopteran hosts- *Acanthodelta janata* (L.), *Helicoverpa armigera* (Hübner), *H. zea* (Boddie), *Dysgonia algira* (L.) and *Elygea maternal* (Linn.) (Austin and Dangerfield, 1993; Gupta, 2013).

Microplitis maculipennis (Fig. 1) is an important parasitoid of castor semilooper *Acanthodelta janata* (L.) (Lepidoptera: Erebidae) (Fig. 2A) and causes 70-80% parasitization (Fig. 2B) (Singh *et al.,* 2008). *Acanthodelta janata* is a major pest of castor (*Ricinus communis* L.) and also attacks other host plants including *Vigna radiata*, *Bauhinia variegata*, *Rosa*, *Punica granatum*, *Ziziphus mauritiana*, *Mangifera indica*, *Citrus*, *Tridax*, *Cardiospermum*, *Ficus*, *Bauhinia*, etc. (Jairamaiah *et al.,* 1975; Somasekhar *et al.,* 1990).

The generic limits between *Microplitis* and *Snellenius* based on morphological studies have been controversial since long (Nixon, 1965; Mason, 1981; Austin and Dangerfield, 1992 1993; Fernandez-Triana *et al.,* 2015). The present study was undertaken to confirm the correct generic placement of the species *M. maculipennis*. As the majority of the BOLD sequences for these two genera were based on mitochondrial Cytochrome Oxidase I (COI) gene, the same was chosen for the present study. Recently
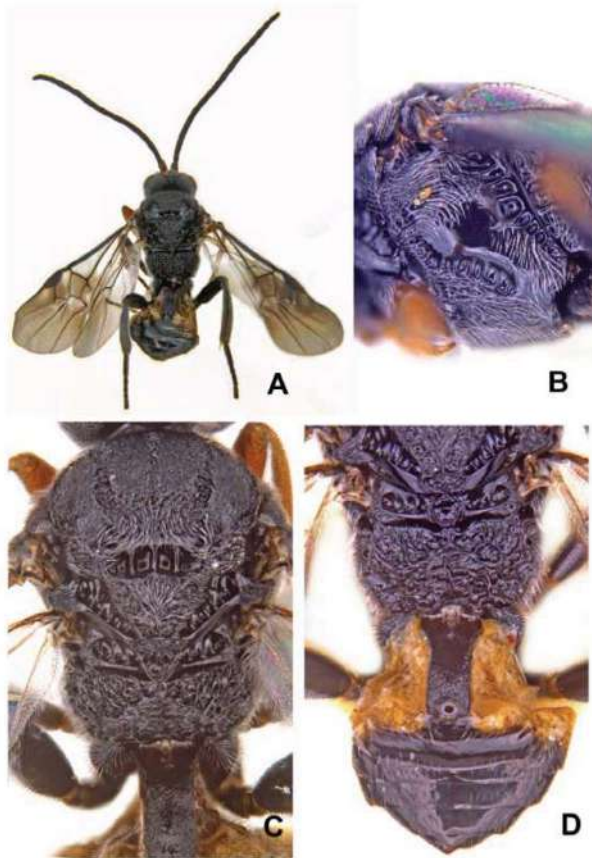
Fig. 1. ***Microplitis maculipennis*** **Szepligeti: A. Habitus in dorsal view; B. Mesopleuron in lateral view; C. Dorsal view of mesosoma with first tergite in part; D. Dorsal view of metasoma with mesosoma in part.**

Fig. 2A. **Unparasitized caterpillar *Acanthodelta janata* (Linnaeus).**

molecular based identification of insects using mitochondrial Cytochrome Oxidase I gene (COI) is gaining importance due to shortfalls in morphology-based identification (Erlandson *et al.,* 2017; Venkatesan *et al.,* 2016; Gupta *et al.,* 2016).

Fig. 2B. Parasitized *Acanthodelta janata* caterpillar with a cocoon of *Microplitis maculipennis.*

Cytochrome Oxidase I (COI) of the mitochondrial DNA has been widely used as markers for understanding the evolutionary relationships among different organisms (Õunap and Viidalepp, 2009). Furthermore, COI gene can be considered as universal bioidentification system for animals (Hebert *et al.,* 2003). Mardulyn and Whitefield (1999) used COI marker to study the phylogenetic relationships of hymenopteran parasitoids.

The genus *Microplitis* can be recognized by a large areolet, mesopleuron without prepectal carina, roughly sculptured propodeum often with a median longitudinal carina, propodeum evenly curved in the lateral view, shape and sculpture of first metasomal tergite, and with a weakly defined groove separating second and third tergum (Nixon, 1965; Mason, 1981; Austin and Dangerfield, 1992; 1993). Genus *Snellenius* Westwood was redescribed by Mason (1981). The species under *Snellenius* are characterized by highly exaggerated propodeum that comprises of two faces meeting transversely at a sharp angle of distinctly less than 90º; deep and strongly crenulate notauli; middle lobe of the mesoscutum raised above the level of lateral lobes and antenna with flagellar segments strongly compressed. Nixon (1965) mentioned that sharp line of division between the two genera is missing. *Microplitis* shares remarkable resemblance with closely allied genus *Snellenius* except for more sculptured notauli, coarsely reticulate propodeum with strong angulation and distinct prepectalcarina in the later (Nixon, 1965; Mason, 1981; Austin and Dangerfield, 1992). Many species are intermediate between *Snellenius* and *Microplitis* (especially in south-east Asia). Mason (1981) diagnosed *Snellenius* species to be with a partial or complete, usually irregular, prepectal carina. In Indo-Australian fauna this character appears to adequately separate these genera (Austin and Dangerfield, 1992). Fernández-Triana *et al.,* (2015) added that *Snellenius* can be separated by strongly excavated and sculptured notauli and scutellar disc, very wide and deep scutoscutellar sulcus,

and propodeum divided into two distinct faces clearly marked by a strong angulation (in the lateral view) and a transverse carina (in the dorsal view).

Coming to *M. maculipennis*, the species chosen in the present study, Gupta (2013) provided its detailed description (Fig. 1). Female body length 3.36–4.0 mm. Body black; scape and pedicel brown, flagellomeres dark brown to black; ocelli yellow brown, fore legs yellow-brown (more so on apical half of femur and tibia), mid legs dark brown (except yellowish brown apical tip of femur and basal tip of tibia), hind legs black; laterotergites of T1-T3 and first three sternites light yellow brown to off white; T1 median tergite black; median triangular field in T2, T4-T7 black; wings infuscate brown in apical 2/3rd, fore wing slightly darker than hind wing, with darker areas below stigma and through marginal cell, venation dark brown, stigma uniformly dark brown.

Head, densely pilose; eyes densely pilose; antennae as long as body or a little shorter in few specimens. Mesoscutum with medial lobe rugose punctuate and higher than lateral lobes; lateral lobes less rugose than median lode, with faint punctuations; notauli strongly indicated, meeting posteriorly into reticulate-punctate area, medial furrow impressed, crenulate-punctate; medial lobe slightly raised along longitudinal line; scutoscutellar sulcus very broad, deep, divided by wide costulae; dorsal scutellum very coarsely rugulose-punctate and pilose; propodeum with two faces that meet sharply at about 90°, very coarsely rugose-punctate; medial longitudinal carina not clear; mesopleuron with epicnemial area strongly raised, carinate, pilose, epicnemial furrow broad, coarsely crenulate; metapleuron coarsely reticulate-rugose and pilose. Fore wings infuscate in apical half, basal 1/3rd hyaline; pterostigma dark brown; 1-M very slightly curved; areolet of moderate size. Metasoma with T1 2.4–3.0 × as long as its apical width, slightly rugose except for shining apical patch, widest at extreme base, parallel sided, and widening very slightly in apical half; T2 smooth, pilose along posterior margin, triangular in shape, with median field indicated by median area; T2 as long as T3 medially, suture between T2 and T3 moderately distinct; T3-T7 with transverse rows of hairs, mostly in posterior 2/3rd, smooth and shining.

The members of this genus are koinobiont larval endoparasitoids of Lepidoptera more precisely, the noctuid genera *Helicoverpa* and *Spodoptera*. The majority of the hosts belong to the families Noctuidae, Erebidae, and to some extent Sphingidae and Lymantriidae (presently in Erebidae as Lymantriinae). Worldwide a huge anomaly regarding host association of *Microplitis* and *Snellenius* has been observed. In India, the majority of the *Microplitis* hosts are from families Noctuidae and Erebidae and exhibit solitary parasitism (Gupta, 2013; Ranjith *et al.,* 2015). Austin and Dangerfield (1993) recorded that *Microplitis* parasitizes the members of Noctuidae, Notodontidae and Erebidae, and the Oriental *Snellenius* parasitizes Noctuidae and Sphingidae. However, contradictory to the above host records, Fernández-Triana *et al.,* (2015) found that in ACG inventory of Costa Rica, *Microplitis* exclusively parasitizes sphingids, while *Snellenius* parasitizes members of Noctuidae and Erebidae.

In this study, *M. maculipennis* was identified morphologically and with molecular marker COI gene. Further, we analyzed the phylogeny of *Microplitis* and *Snellenius* by using the COI available in public database to ascertain whether species belonging to *Microplitis* and *Snellenius* form distinct clades or not.

MATERIALS AND METHODS

This paper is based on study of *Microplitis maculipennis* specimens reared from six different locations in southern India (housed at the ICAR-National Bureau of Agricultural Insect Resources (NBAIR), Bangalore, India).

A roving survey was conducted in six locations *viz*., Attur, Malliyakkarai, Namagiripettai, Vazhapadi, Yethapur, and Thumbal of Tamil Nadu in southern India from October 2014 to December 2014 fortnightly (Table 1). Nearly 500 larvae of *Acanthodelta janata* were hand-collected from each location and observed for parasitization by *M. maculipennis* at room temperature and stored in vials for further studies at ICAR-National Bureau of Agricultural Insect Resources (ICAR-NBAIR). Parasitized larvae and adult wasps were characterized using COI. Also *A. janata* was also characterized using the above-mentioned marker for the host confirmation.

DNA Extraction and COI Amplification

Field collected *A. janata* were further reared on castor leaves in the laboratory and observed for parasitization. Larvae of *A. janata*, freshly formed cocoons and adults of *M. maculipennis* were stored in -20°C until further study. An individual sample of cocoon and adult of *M. maculipennis* and *A. janata* larvae were placed in 1.5 ml micro centrifuge tubes separately. Genomic DNA was isolated by using DNA extraction kit (QIAGEN DN easy blood and tissue kit Cat. 69504, Germany).

COI gene was amplified by PCR with the volume of 30μl reaction. It contained 2μl DNA template, 3μl PCR buffer, 1μl dNTPs 1.5μl forward LCO 1490 5'-GGTCAACAAA TCATAAAGATATTGG3' and reverse primers HCO 2198

Table 1: Per cent parasitism of *Microplitis maculipennis* on *Acanthodelta janata*

Location	GPS		Parasitism (%)			
	Longitude & Latitude	Elevation	Oct. 2014 (A month crop)	Nov. 2014 (2 months crop)	Dec.2014 (3 months crop)	Average
Attur	11.59629°N 78.59892°E	225.0 m	40	65	63	56
Malliyakkarai	11.56893°N 78.49935°E	274.5 m	73	72	69	71
Namagiripettai	11.46064°N 78.27415°E	259.8 m	63	69	75	69
Vazhapadi	11.65548°N 78.40126°E	311.9 m	62	60	60	61
Yethapur	11.66314°N 78.47662°E	277.3 m	88	89	81	86
Thumbal	11.77970°N 78.51923°E	393.4 m	67	60	79	67

5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.,* 1994), 1μl Taq Polymerase and 20μl of sterile distilled water. The cycling conditions for COI (initial denaturation at 95°C for 4 min followed by 34 cycles each of denaturation at 94°C for 30 sec, annealing at 50°C for 1.20 min and extension at 72°C for 2 min followed by a final extension step at 72°C for 7 min. The size of PCR product was determined with 1.2% agarose with a standard size. Then the PCR products were sent to automatic sequencing (Eurofins Genomics India Pvt. Ltd., Bangalore).

Sequence chromatograms of forward and reverse sequences were analyzed and trimmed for stop codons/nuclear copies in order to know the frame shift (https://www.ncbi.nlm.nih.gov/orffinder/). Further, the sequences were assembled using CLC Genomics Workbench 7. The similarity search of resulting consensus sequences was performed using Basic Local Alignment Search Tool (BLAST) against sequences in GenBank database to confirm that the sequence was indeed corresponding taxonomy. All COI generated consensus sequences have been deposited in NCBI GenBank database The accession numbers of *A. janata* and *M. maculipennis* are given in Table 3 and Table 4, respectively.

Sequence retrieval from BOLD database

In order to compare our isolated sequences with available database sequences, we referred to BOLD (http://v4.boldsystems.org/) to download COI sequences of *Microplitis* and *Snellenius*. The initial data set was comprised of 2257 COI sequences, but we pre-processed the data before further analysis. We removed poor quality sequences from the dataset which were having - symbol and N starches (>=5bp). The filtered dataset comprised of 1425 sequences which had > 600 bp length. Then, we included only one representative

sequence per *Microplitis* or *Snellenius* species. Thus, we ended retaining 354 sequences, representing 129 named species and 226 species determined only to genus level. Also, we included 12 COI sequences of *M. maculipennis* populations from our study, and one sequence of *Diolcogaster* sp. (KM996615) was included as an outgroup. After pre-processing, the final dataset consisted of 367 COI sequences.

Phylogenetic analysis

Since, there are no other sequences available in any public database for this species, all analyses were carried out using COI gene nucleotide sequences. All the sequences were aligned using the program MUSCLE with the default alignment parameters (Edgar, 2004). To refine and correct the alignment, we used trim AI software to concatenate trimmed alignment with the automated1 option before substitution model prediction (Capella-Gutiérrez *et al.,* 2009). Phylogenetic relationships were estimated by Bayesian-Inference (BI), with support (clade posterior probabilities values) showed on the respective tree. Best-fit substitution models were selected by the Akaike information criterion as implemented in PartitionFinder version 1.1.1 software (Lanfear *et al.,* 2012). Bayesian-Inference analysis was performed in MrBAYES v3.1.2 (Ronquist *et al.,* 2012), using the (MC)3 algorithm, with three heated and one cold simultaneous Markov chains per run and two independent runs per analysis as executed by default. The stop-rule was set and we increased the number of generations until the average deviation of split frequencies reached a value below 0.01. Markov Chain Monte Carlo (MCMC) started from a random tree, sampling one of every 500 generations. The analyses consisted of 14,830,000 MCMC generation, with the first 7415 (25%) of the trees discarded as burn-in out of 29,660 trees. The remaining trees were used to build a majority-rule

consensus tree on which the Posterior Probabilities (PP) was shown. The resulting nexus formatted BI phylogenetic tree was imported, edited and visualised in the Fig Tree software version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). In addition to Baysian tree, we generated Maximum Likelihood tree using RAxML (v7.0.4) tool with parameters 'ML+ Rapid Bootstrap' and kept 1000 searches bootstrap support values for each node (Stamatakis, 2006) (Fig. 4). Tree file edited in the FigTree software.

RESULTS AND DISCUSSION

Sequence and phylogenetic analysis

We obtained a total of 354 published BOLD database sequences (after pre-processing of a total of 2257 COI sequences) of *Microplitis* or *Snellenius* species, representing 129 named species and 226 species determined only to

Table 2. Summary statistics for COI loci from *Microplitis* species

Characteristics	COI
No. of sequences analyzed	12
Total no. of sites	675
Conserved sites	452
Variable sites	221
Parsimony informative sites	150

genus. This dataset was in the range of 600 to 658 bp in length. Twelve populations of *M. maculipennis* reared from southern India were sequenced from mt DNA COI with 614 to 624 bp. The data were aligned using MUSCLE (Thompson *et al.,* 1994). The alignment of the COI dataset resulted in a total of 675 nucleotide sites, of which 221 (47.78 %) were variable sites and 150 (40.82%) were parsimony-informative (Table 2). For the Bayesian inference analysis, the model was applied to the subset partitions positions 1, 2, and 3. For all the three positions, the General Time Reversible (GTR) with gamma distribution (G)+Invariant (I) substitution model was predicted with best-fit partitioning schemes (lnL: -16713.73231 and AIC: 34953.46462) using Partition Finder version 1.1.1 and further it was used to generate a phylogenetic tree.

The Bayesian Inference (BI) and Maximum Likelihood (ML) phylogenetic trees of the *Microplitis* sp. and *Snellenius* sp. is shown in Fig. 3 and Fig. 4, respectively. The clades Posterior Probability (PP) values are colored based on the auto-scale range between minimum and maximum values in Fig. 3. In case of ML tree (Fig. 4), Bootstrap Values (BV) is given at each node. In BI tree, we have obtained two distinct clades, A (PP=0.99) as a single separate cluster, while B clade (PP=0.88) is further divided into 11 major subclades B_1 -B_{11}. On similar line, we have obtained two major Clade A and B in ML tree, Clade contains seven sequences of *Snellenius*

Table 3. Characterization of *Acanthodelta janata* using CO1

Sl.No.	Location	Stage	Strain	GenBank Acc. No. (COI)
1.	Attur	*A. janata* larva	AJ-1-AT	KP765518
3	Vazhapadi	*A. janata* larva	AJ-4-VA	KP765521
5	Thumbal	*A. janata* larva	AJ-5-TH	KP765522
7	NamagiriPettai	*A. janata* larva	AJ-7-NA	KP765524

Table 4. Characterization of *Microplitis maculipennis* using COI gene

Sl.No.	Location	Stage	Strain	GenBank Acc. No.
1	Malliyakari	Cocoon	MM-8-MA	KP759295
2	Malliyakari	Adult	MM-1-MA	KP759288
3	NamagiriPettai	Cocoon	MM-11-NA	KP759298
4	NamagiriPettai	Adult	MM-2-NA	KP759289
5	Attur	Cocoon	MM-7-AT	KP759294
6	Attur	Adult	MM-6-AT	KP759293
7	Thumbal	Cocoon	MM-10-TH	KP759297
8	Thumbal	Adult	MM-4-TH	KP759291
9.	Vazhapadi	Adult	MM-5-VA	KP759292
10.	Vazhapadi	Cocoon	MM-9-VA	KP759296
11.	Yethapur	Adult	MM-3-YE	KP759290
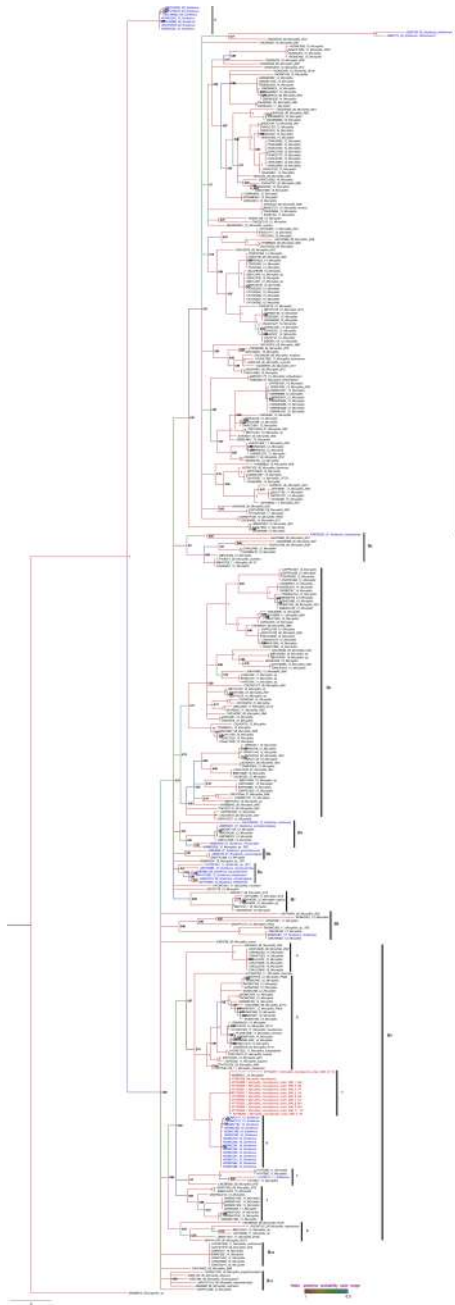
Fig. 3. The Bayesian inference phylogenetic tree of the *Microplitis* sp. and *Snellenius* sp.

species with confidence BV=99. Further, B_1 (PP=0.61) is split into many subclades in BI tree, we found that the majority of the species belongs to *Microplitis* in this clade except for two sequences of *Snellenius phildevriesi* and *S. isidrochaconi*. These two species form a separate clade with *Microplitis* jft81 with PP=0.71. In ML tree, same species sequence are grouped in clade B2C10. Similar scenario is observed in subclade B2, *Snellenius irenebakerae* which is clustered with other species of *Microplitis* (PP=0.63) and the rest of sequences are dominated with *Microplitis* species. In ML tree, same species sequence are grouped in clade B2C6

with BV=59. In BI tree, species belonging to *Microplitis* formed several strongly supported an independent subclades, B3 (PP=0.73), B7 (0.82), B10 (PP=1) and B11 (0.58) the latter with lower support. In ML tree, we observed same clades species in B2C8 (BV=91), B2C7 (BV=99), and B2C1 (BV=100).

Few members of *Snellenius* (*S. johnkresssi, S. lucindamcdadeae, S. velvaruddae, S. gerardoherreai, S. warrenwageri, S. mariakuzminae, S. kerrydressleare, S. bobdressleri, S. sandyknappae,* and *Snellinus* Whitfield 19) formed clades B4, B5, and B6 but with low support (PP 0.5-0.57). In case of ML tree, same species are grouped in subclade B2C9. In subclade B8 (PP=0.98) in BI tree and B1C2 (BV=100) in ML tree, *S. vickifunkae* species showed close relationship with *Microplits* sp. sff3 and other members with high support (PP=1). B9 (PP=0.66) clade in BI tree and B2C4 clade in ML tree was split into 7 subclades: a-g. Our study sequences of *M. maculipennis* are closely allied to *Snellenius* species with high support value (PP=1; BV=98), while Clade a(PP=1), b(PP=1), f(PP=0.85), g(PP=0.93) consists exclusively of *Microplitis* members. In clade e (PP=1), only one *Snellenius* is embedded within *Microplitis* species. In case of Maximum likelihood phylogenetic tree, The COI gene tree displayed a very close topology with BI tree and both methods gave similar branching, which slightly differed at the internal nodes (Fig. 4).

Microplitis maculipennis is an important parasitoid of castor semilooper *A. janata* and this was much evident from our field collected populations. This species is distributed in Australia, India (widespread), Malaysia, Papua New Guinea and Thailand. Austin and Dangerfield (1993) stated that *Microplitis* shares remarkable resemblance with *Snellenius* except for more impressed and sculptured notauli and propodeum, and distinct prepectalcarina in the later. Since many of the Indian specimens of *M. maculipennis* do not posses distinct prepectal carina, hence the species was placed in the genus *Microplitis* (Gupta, 2013).

In this study, an effort was made to recognize and discriminate *Microplitis* species and understand their evolutionary relationships based on COI gene, through publically available COI sequences and phylogentic analysis. The clear-cut generic limits between *Microplitis* and *Snellenius* and the generic placement of *M. maculipennis* based on morphological studies have always been controversial. Our studies show that all the new sequences of *M. maculipennis* have close similarity with the *Snellenius* species from the Oriental region. This is in congruence with the fact that the intergeneric delimitation of *Microplitis* and *Snellenius* is more difficult in the Oriental region.

Fig. 4. The Maximum Likelihood Phylogenetic tree of the *Microplitis* **sp. and** *Snellenius* **sp.**

In our previous molecular-based studies, it was confirmed that the DNA barcoding of trichogrammatids (Hymenoptera: Trichogrammatidae) by using the mitochondrial cytochrome oxidase-I marker sequences was a practical approach for shaping molecular diversity (Venketesan *et al.,* 2016). In addition to that, the phylogenic analysis was performed to identify and classify various species-groups of the genus *Glyptapanteles* Ashmead, 1904 (Insecta: Hymenoptera: Braconidae: Microgastrinae) (Gupta *et al.,* 2016).

CONCLUSION

The present analysis suggests that these two genera demands comprehensive study of the world fauna as it raises doubts about the identity of many of the sequences which exist in the Gen Bank for which many of the voucher specimens need to be re-examined in order to confirm which species should strictly fall into *Microplitis* or otherwise, if they are two discrete genera. There is also a strong possibility that *Microplitis* and *Snellenius* might not retain their separate generic status in future based on combined morphological and molecular analysis and considering the huge anomaly in their respective host associations. Furthermore, multigene phylogeny is required to differentiate the species under both genera substantially.

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