

**IDENTIFICATION OF *MEGASELIA SCALARIS* (DIPTERA : PHORIDAE)
BASED ON MORPHOLOGY AND MITOCHONDRIAL 16S rRNA
AND COI GENE SEQUENCES**

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Abstract

Megaselia scalaris (Diptera: Phoridae), commonly known as scuttle fly, is widely distributed all over the world. It is easily cultured in the laboratory condition making it a potential model organism. Besides, it has forensic importance. However, no report from Bangladesh could be retrieved about this fly. So, in the present study, identification of this species was attempted using both morphological and molecular approaches. Characteristics of male hypopygium and legs played key roles in morphological identification. To strengthen identification, mitochondrial COI and 16S rRNA gene fragments were amplified and sequenced. Blast search at NCBI provided highest hits to available COI and 16S rRNA sequences of *M. scalaris*. A neighbor joining phylogenetic tree was built using sequences of respective COI gene region to show its relationship among other closely related dipteran flies.

Introduction

Megaselia scalaris (Loew 1866) (Diptera : Phoridae) is a cosmopolitan, forensically important fly with a broad ecological habits⁽¹⁾. They are mainly found in warm climate regions with higher latitudes⁽²⁾. This fast moving, brownish fly shows a unique humpbacked structure on thorax, with hair-like processes, located between the facets of the compound eye⁽³⁾. The larvae of *M. scalaris* have been described as detritivore, parasite, parasitoid and facultative parasites (myiasis agents) of vertebrates, including human, and consume a wide range of organic materials⁽⁴⁾. Adult *M. scalaris* has been reported as a polyphagous organism, generally acting as saprophagous, sarcophagous or necrophagous⁽⁴⁻⁶⁾. Because of their voracious feeding habit, they can be easily maintained in laboratory condition⁽⁴⁻⁵⁾.

Several reports have been published around the world about occurrence of this human associated fly. *M. scalaris* is found in North America, Asia, Africa and Europe⁽⁶⁻⁹⁾, even on remote islands, like, Boatswainbird, Canary and Galápagos Islands⁽¹⁰⁻¹²⁾. But, no record is found from Bangladesh.

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For species identification, DNA sequencing is currently the most widely used method⁽¹³⁾. The process includes comparing genomic sequences of a target sample with a comprehensive reference database. For genomic sequence analysis, both mitochondrial and nuclear gene sequences are commonly used^(14,15). Saccone *et al.* 1999 has preferred mitochondrial genome than nuclear genome because of its lack of introns, limited exposure to recombination and haploid mode of inheritance⁽¹⁶⁾. Mitochondrial genes, such as, *Cytochrome oxidase 1 (COI)*, 16S and 12S rRNA are commonly used for identification purposes⁽¹⁷⁾. The objective of the present study has been identification of *Megaselia* species based on morphology and sequences of two mitochondrial genes, 16S ribosomal RNA (16S) and *cytochrome oxidase 1 (COI)* as the first attempt from Bangladesh.

Materials and Methods

The fly was collected from yeasted banana bait, generally used for collection of *Drosophila* flies. Collection site was Khilgaon, situated in the middle of Dhaka, Bangladesh and collection was made between January and February, 2015. The flies were reared in fly vials with semolina-agar-dextrose-yeast medium. To get isofemale population, male and female flies were separated and pairs were isolated and cultured. After two generations, flies were morphologically identified and some of them were used for molecular analysis.

A well-accepted key to this genus was provided by Disney, 1994⁽¹⁸⁾. The most recent keys to those of Europe, Galápagos Islands and Canary Islands were also provided by Disney^(4,6,10-12,18). All available keys were utilized for the identification of *Megaselia* flies.

Genomic DNA was extracted from 5 - 6 whole flies by CTAB DNA extraction protocol⁽¹⁹⁾ with slight modification. The modifications include addition of 4 µl proteinase K to the sample before incubation for protein degradation and use of phenol-chloroform-isoamyl alcohol (25 : 24 : 1) instead of chloroform-isoamyl alcohol for purification. Precipitated DNA was dissolved in 50 µl sterilized distilled water and visualized in 1% agarose gel with a positive control of *Drosophila melanogaster* DNA sample.

DNA fragments of the mitochondrial 16 rRNA and *cytochrome oxidase 1 (COI)* genes were amplified using the oligonucleotide primers listed in Table 1. PCR amplifications were performed in 50 µl using 5 µl 10X PCR reaction buffer, 5 µl 25M MgCl₂, 1 µl 10 mM dNTP, 0.5 µl forward primers, 0.5 µl reverse primers, 0.5 µl *Taq* DNA polymerase (Thermo Scientific USA; Cat. EP0402) and 3.5 µl template DNA. Rest volume was filled using sterilized distilled water. The thermal cycling profiles that were programmed to amplify the gene by PCR for 30 cycles are as follows: 95°C, 2 min for initial denaturation; 95°C, 2 min for denaturation; 53°C, 1 min for annealing 16S rRNA primers or 57°C for annealing COI primers; 72°C, 1 min, for elongation and 72°C, 5 min for final elongation. Finally, PCR products were held at 22°C. PCR products were detected by agarose gel

electrophoresis. Amplified DNA fragments were then purified using Favor Prep PCR cleanup Mini kit (Favorgen Biotech Corp.).

Table 1. Oligonucleotide primers used in this study.

Primer name	Sequence (5 → 3)	Reference
16S rRNA – F	CGCCTGTTTAACAAAAACAT	Palumbi 1996
16S rRNA – R	CCGGTTTGAAGTCAGATCATG	Palumbi 1996
COI F	GGTCAACAAATCATAAAGATATTGG	Folmer <i>et al.</i> 1994
COI R	TAGACTTCTGGGTGGCCAAAGAATCA	Ward <i>et al.</i> 2005

PCR products were directly sequenced in both directions by Sanger Dideoxy Sequencing method in the Centre for Advanced Research in Sciences (CARS), University of Dhaka, Dhaka. Sequence chromatograms were read using FINCH TV software. Sequences of forward and reverse DNA strands were then edited and aligned manually using SEAVIEW software.

MEGA6⁽²⁰⁾ and SEAVIEW⁽²¹⁾ were used for sequence analysis. Blast searches at NCBI were performed for species identification. For multiple sequence alignment, complete COI gene sequences of some dipteran insects were collected from NCBI and their list is shown in Table 2. Sequences were aligned using CLUSTAL W⁽²²⁾. For phylogenetic analysis, MEGA6 and SEAVIEW were used. A neighbor-joining^(23,24) tree was constructed using MEGA6 to observe phylogenetic relationships of this fly with other dipteran genus. For protein coding nucleotide sequences, genetic code was selected for “invertebrate mitochondrial” and number of bootstrap replicons was set to 100.

Table 2. A list of COI gene sequences collected from NCBI database for present study.

Species	Accession number
<i>Calliphora vicina</i>	NC_019639
<i>C. vomitoria</i>	NC_028411
<i>Drosophila ananassae</i>	BK006336
<i>Haematobia irritans</i>	NC_007102
<i>Lucilia cuprina</i>	NC_019573
<i>Musca domestica</i>	KM200723
<i>Muscina stabulans</i>	NC_026292
<i>Tribolium castaneum</i>	KM009121
<i>Sarcophaga albiceps</i>	NC_028413
<i>Sarcophaga impatiens</i>	NC_017605

Results and Discussion

Before molecular analysis, the fly was morphologically identified as *M. scalaris*. It belongs to the family Phoridae and order Diptera of the phylum Arthropoda. Characteristic features of the fly are presented in Figs 1, 2.

Males or females with wings (Fig. 1A); mid-tibia without paired bristles; hind tibia with a dorsal hair palisade (Fig. 1A); clearly forked vein 3 of wings.

Frons brownish yellow; halter knob yellowish; prothorax brown dorsally, scutellum pale brownish yellow (Fig. 1A); pleural regions mainly pale yellowish, but browner dorsally (Fig. 1D); abdominal tergites dark brown with yellow markings; legs long, dusky pale yellow in color; brownish veins and greyish brown membrane on wings.

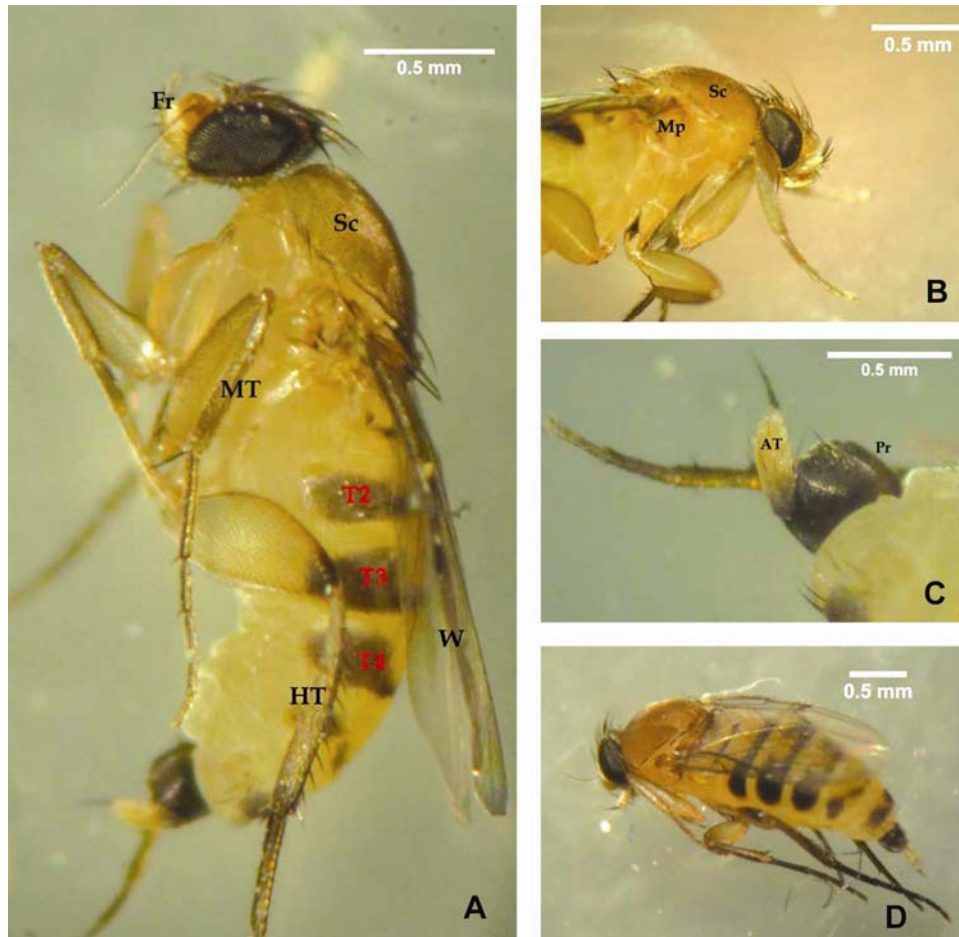


Fig. 1. *Megaselia scalaris* male. (A) Right face of whole body. (B) Left face of notopleuron. (C) Left wing. (D) Left face of hypogoeum. (E) Dorsal surface of the body. (Fr- Frons. Sc - Scutellum. MT - Mid Tibia. T2, T3 and T4 - Tergites 2, 3 and 4. HT - Hind Tibia. W - Wing. MP - Mesopleuron. AT - Anal Tube. Pr - Proctiger).

Male fly is robust; feathered seta present at tip of proctiger, clearly longer and thicker than setae on circus; mesopleuron naked without any hairs or bristles (Fig. 1B). A short row of four or five spine-like bristles present, with bent over tips on hypogoeum, rather beneath base of hind femur. Hairs at tip of anal tube not differentiated (Fig. 1C).

Female is more distinctive than male; mesopleuron with hairs only, without bristles; palps straw yellow; no hair at base of vein 3; each labellum with fewer than 20 small spinules on lower face; tergites partly yellow; abdominal tergite 6 short and extremely broad; T3 and T4 tergites broader than T5 (Fig. 2A); T6 clearly broader than T5; scutellum with four bristles.

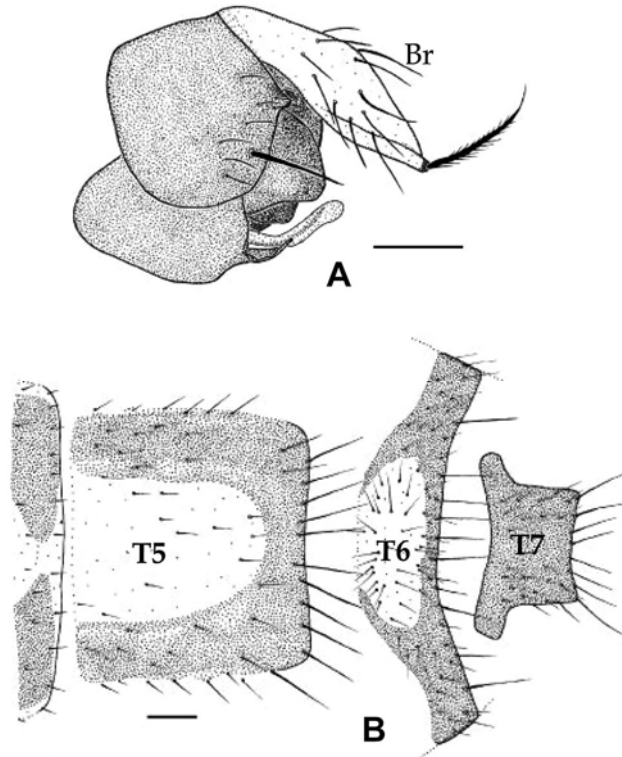


Fig. 2. *Megaselia scalaris*. (A) Left face of male hypopygium. (B) Female abdominal tergites 5 - 7. (Adapted from Disney *et al.* 2010) (Br- Bristles. T5, T6 and T7 - Tergites 5, 6 and 7).

Representative gel images of extracted DNA and amplified fragments of interests (mt 16S rRNA and COI) are presented in the Fig. 3.

Sequences are provided in Tables 3 and 4.

BLAST search of the 16S rRNA and COI gene sequences at NCBI returned highest query coverage (93% for 16S and 98% for COI gene) with *M. scalaris*.

The evolutionary history was inferred using the Neighbor-Joining method. The analysis involved 12 nucleotide sequences of COI gene of different dipteran flies. The evolutionary distances were computed using the Maximum Composite Likelihood method. All positions containing gaps and missing data were eliminated. There were a total of 560 positions in the final dataset.

Though molecular tools are being used in species identification nowadays, gene sequences of many species are still not available in the sequence databases. As a result, exact identification is not possible just by comparing sequences of an unknown organism with those available in the databases. So, identification using morphological keys plays important role before attempting a molecular approach. Genus *Megaselia* of phorid family

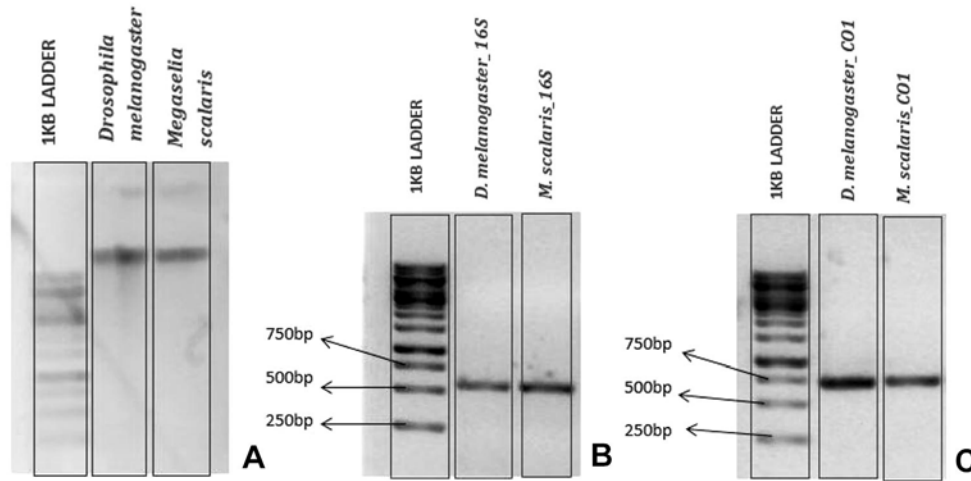


Fig. 3. Extracted DNA of *Drosophila melanogaster* and *Megaselia scalaris* (A), PCR products of 16S rRNA (B) and COI gene (C) regions of both species, all visualized in 1% agarose gel. *D. melanogaster* sample was used as a positive control.

Table 3. Partial sequences of 16S rRNA gene determined in the present study.

>16S rRNA_Partial_ *M. scalaris*
TTTTATTTATGGCTGCAATATCTTAATGTGCAAAGGTAGCATAATCATTAACTCTGAAATTGAGGG
CTGGTATGAATGGTTGGATGAAATATTAAGTGTTTTAAATAAATTTATAATAGAAATTTATTTTTT
AGTTATAAAGCTAAAATAATTTAAAAGACGAGAAGACCCTATAGAAAAATCTTTATATATAAGTT
ATTTTAATTATATAGATAAAAATTAATTTATTATAAATTTATATATTTTATTGGGGTGATATTAAT
TTGTTAAATTTTAAATCTATAATAAAACATAAATTAATGAATTATTGATCCATTAATTAATGATTA
AAAAATCAAGTTACTTTAGGGATAACAGCGTAATTTTTTTAGAGAGTTCTTTTTTGATAAAAAA
GACTGCCCCCTTGATGTTTGGATTAAGATACAGTTTTTTAGGTGCAGCAGCTTAAACTTAAAGT
CTGTTCCGACTTTTTAAATCTTACATGATCTGAGTTCCAAACC = 503bp

includes around 1,400 species of flies distributed in tropical and subtropical areas^(6,25-26). Among the species of this genus, only a few has been represented in the sequence databases. So, the existing morphological keys to *Megaselia* species have been enormously valuable prior to molecular identification.

Features of male hypopygium are mainly used in morphological identification of *Megaselia* species^(4,25). Male *M. scalaris* are closely related with *M. longiseta* and *M. rufipes* in the keys of *Megaselia* species of canary island⁽¹¹⁾. A short row of four or five spine-like bristles generally occur beneath the base of hind femur of *M. longiseta* with different

hypopygium structure, which are not observed in *M. scalaris*. Moreover, *M. scalaris* possess feathered bristles at the tip of anal tube, which *M. rufipes* lacks. Female *M. scalaris* are differentiated from closely related *M. bistruncata* and *M. oxybelorum* by broader T3 and T4 tergites compared to T5. Besides, yellowish abdominal tergites with short T6 tergite of *M. scalaris* differ from *M. intermedia*. In addition, *M. scalaris* possess variable sizes, and anterior scutellar bristles are often shorter than other related species. The Neotropical and Nearctic *M. imitatrix* is apparently the most closely related species to *M. scalaris*^(12,26). In Borgmeier's (1962) primary keys to Neotropical species, the male of *M. pruinosifrons* has a short anal tube and the anterior scutellars are minute hairs, instead of bristles. The female abdominal tergite 6 is not broader than T5, as is the case in *M. scalaris* (and *M. imitatrix*)^(1,11).

Table 4. Partial sequences of COI gene determined in the present study.

>COI_Partial _*M. scalaris*
 GGGGCCTGAGCTGGAATAGTAGGAACATCTTTAAGTATTATAATTGAGCTGAATTAGGGCACC
 CTGGTGCTTTAATTGGTGATGATCAAATTTATAAAGAAATTGTTACTGCCCATGCATTTATTATAA
 TTTTTTTATAGTAATACCTATTATAATAGGAGGATTTGGAAATTGATTAGTTCGCCTAATATTAG
 GGGCACCTGATATGGCCTTTCCACGAATAAATAATATAAGTTTTTGAATACTTCCCCCTTCTCTA
 ACTCTTTTATTAGCAAGAAGTATAGTAGAAAATGGAGCTGGAACCTGGTTGAACAGTTTATCCAC
 CCCTATCTTCTAGAATTGCCCATAGAGGAGCTTCAGTCGATTAGCAATTTTTTTCATTACATCTT
 GCCGGAATTTCTTCTATTCTTGGAGCAGTAAATTTTACTACTACAATTATTAATATACGATCCACA
 GGAATTACTTTTGATCGAATACCTTTATTTGTATGATCAGTAGGTATTACTGCTCTTTTATTATACT
 TTCCTACTGTTCTAGCAGGTGCTATTACTATCTATTAACAGATCGAAATTTTAATACATCATTC
 TTTGACCCTGCAGGAGGGGGAGACCCATTCTATATCAACATCTATT = 636bp

Mitochondrial COI gene sequence is used as a genetic marker for identification of species^(27,28). The region of COI amplified in the present study has about 98% sequence similarity with *M. scalaris* of previous literatures^(29,30). The use of 16S rRNA has also been preferred in many cases as a marker in DNA taxonomy⁽³¹⁻³³⁾. About 93% sequence similarity has been found in case of 16S rRNA gene fragment of the present specimen when aligned with *M. scalaris* of previous study⁽³⁰⁾, though only *M. scalaris* sequences are the topmost BLAST hits (NCBI nucleotide BLAST). Since 16S rRNA sequence is more conserved than that of COI⁽³⁴⁾, this discrepancy in the result might be due to sequencing artifacts. Nevertheless, it is not impossible that there are more variations in the 16S rRNA gene sequence among *M. scalaris* flies distributed in different regions of the world. So, further sequencing of the 16S rRNA gene region of a sizable population of *M. scalaris* can be valuable in this circumstance.

The phylogenetic tree constructed using COI gene sequences of flies of different dipteran families indicates that the specimen is *M. scalaris* in conformity with morphological identification. *M. scalaris* is differentiated from other species belonging to dipteran families as found in previous studies^(35,36). Aschiza (Phoridae) and Schizophora

Table 5. Blast search result

Description	Max. score	Total score	Query cover (%)	E value	Identical (%)	Accession
Blast Hits for <i>Megaseliascalaris</i> mitochondrion, complete genome	699	699	97	0.0	93	KF974742.1
<i>Megaselia</i> sp. A 16S ribosomal RNA gene, partial sequence	682	682	97	0.0	92	AF154806.1
16S rRNA gene	680	680	96	0.0	92	AF154805.1
<i>Megaselia</i> sp. A 16S ribosomal RNA gene, partial sequence	676	676	96	0.0	92	AF154804.1
<i>Megaseliascalaris</i> 16S ribosomal RNA gene, partial sequence	634	634	88	6e-178	93	AF126340.1
<i>Megaselia</i> sp. A 16S ribosomal RNA gene, partial sequence	606	606	86	1e-169	92	AF154807.1
Blast Hits for <i>Megaseliascalaris</i> mitochondrion, complete genome	1110	1110	100	0.0	98	KF974742.1
<i>M. scalaris</i> COI gene	1109	1109	99	0.0	98	KT879896.1
<i>Megaseliascalaris</i> voucher RAMegsca-01 cytochrome oxidase subunit I (COXI) gene, partial	1072	1072	100	0.0	97	JQ941745.1
<i>Megaseliascalaris</i> isolate 01 cytochrome oxidase subunit I (COI) gene, partial	1061	1061	99	0.0	97	AB907181.1
<i>Megaseliascalaris</i> mitochondrial COI gene for cytochrome oxidase I, complete cds	1050	1050	100	0.0	96	KC407773.1
<i>Megaseliascalaris</i> voucher KOR-D17-001 cytochrome oxidase I (COI) gene, partial cds	981	981	93	0.0	96	KU949579.1
<i>Megaseliascalaris</i> isolate HCORPTEH10 cytochrome oxidase subunit I (COI) gene, partial cds						

groups (Calliphoridae, Muscidae and Drosophilidae) are clearly distinguished. Members of the Schizophora occur together showing monophyletic origin and fit taxonomic classification of these dipteran species.

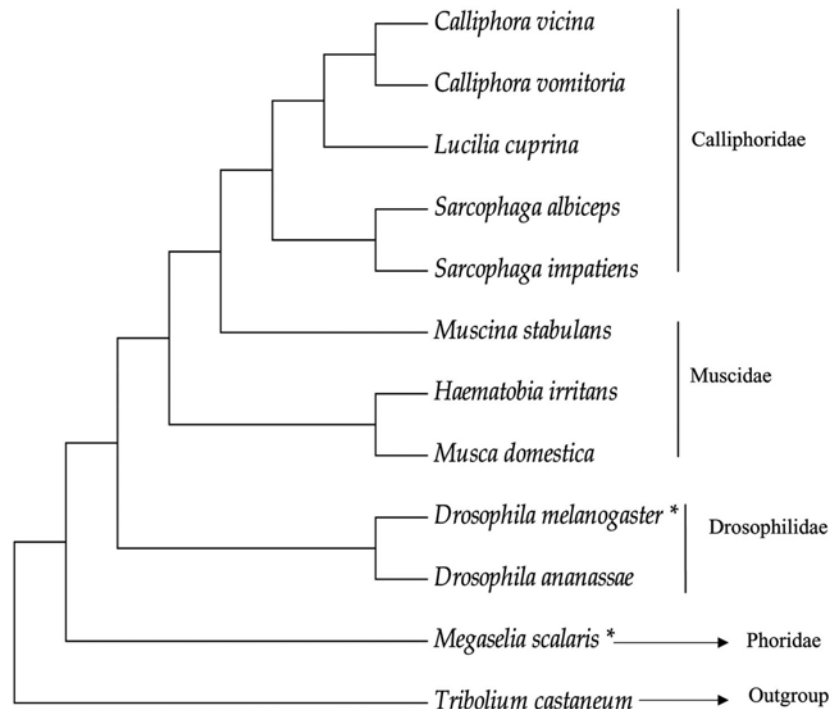


Fig. 4. Phylogenetic relationship among COI genes from different dipteran species. (NCBI accession numbers are given in Table 2, *Marked sequences were generated during this work).

In conclusion, this is the first report of *Megaselia scalaris* from Bangladesh. Morphological key has provided a clear identification of the species. Since, respective regions of COI and 16S rRNA genes of other species under genus *Megaselia* are not available in the sequence databases, molecular analysis alone could not be decisive about the species though it is a valuable addition for future such studies.

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