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Partial COI and 16S rRNA Genes Sequences of *Cephalopina titillator* Mitochondrial DNA: Evidence for Variation in Evolutionary Rates within Myiasis-Causing Species

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Abstract: *Cephalopina titillator* (Diptera: Oestridae) is an important nasal myiasis-producing parasite of camels. The aim of the current study is to assess the utility of the partial mitochondrial cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA) genes sequences to differentiate, identify and elucidate the phylogenesis of the *C. titillator* as a novel study for genomic investigation as well as diagnosis of such disease. Only the 2nd and 3rd instars larvae were collected from nasopharyngeal cavities of camels during post-mortem inspection in El-Bassatin and El-Warrak abattoir, Egypt. The *mt*DNA was isolated by different procedures. The universal primers sets (UEA1 and UEA10) and (UEA3 and UEA10) specifically amplify the sequence from tRNA-Tyr to tRNA-Leu of the COI yielded 1 amplicon of 580 bp and 2 amplicons of 1396 and 580 bp, respectively. On the other hand, the hyper variable part of the 16S rRNA gene sequence coding for the domain IV and V amplified 548 bp using the primers set (LR-N-13398 and LR-J-12887). Rooted phylogenetic trees were constructed for amplified fragments with the Maximum Parsimony method. Identical nucleotide sites, divergent sites and unique differences in sequences were calculated for each fragment. The evolutionary impression from presented calculations could not indicate sharp identity to specified genus in Oestrinae as well as other Oestridae subfamilies nor to closely related family Calliphoridae. Thus, it could be stated with reasonable confidence that the subfamily Oestrinae is not monophyletic. Therefore, the collection of additional molecular data (preferably nuclear genes) is obligatory before we can establish a reliable phylogenetic hypothesis or suggest new taxonomic classification of the genus *Cephalopina*.

Key words: Oestridae · *Cephalopina titillator* · COI gene · 16S rRNA gene · *mt*DNA · Phylogeny

INTRODUCTION

Substantial economic losses are recorded worldwide due to nasopharyngeal myiasis induced by *Cephalopina titillator* larvae that attack only camels [1, 2]. Infested camels lose their appetite, show difficulty in breathing, snort, sneeze, expel the larvae from their nostrils and may exhibit abnormal behavior resembling cranial coenuriasis leading to restlessness. In heavy infection, the breathing of the animal is greatly impaired due to blockage of the nasopharynx by larvae and/or mucofibrinous secretions simultaneously with stop feeding, infrequently, may finally die from meningitis caused by secondary bacterial or viral infections to the cerebrospinal canal [3]. Up to 80% of population have been reported to be infested since larvae are commonly found at post-mortem inspection [4-6]. Nevertheless, 85% efficacy of treatment with Ivermectin subcutaneously was recorded. In addition, diagnosis of infestation with this parasite is very difficult and rearing conditions of camels herds impair prophylaxis treatment [7]. Therefore, such myiasis is mostly underestimated in rearing flocks [8] justifying the little contributions concerning serological diagnosis in living animals [3, 7]. Moreover, despite the great insights into nucleotide and amino acid differences within and among species of insects in a phylogenetic context [9] which led to a broader understanding of the structure and function of insects genes and their relationship to

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distribution (phylogeography), physiology, development, evolution and ecology, however, molecular characterization of *C. titillator* genome yet needs to be better investigated [3].

*mt*DNA contains both conserved and variable regions within genes, reflecting the variation in evolutionary rate across the molecule [10]. Therefore, ability to be amplified using universal primers permits sequencing of the DNA of a species for which no previous sequence information exists. In addition to the protein coding, mitochondrial genes have relatively conserved nature across taxa, thus facilitate the collection of data on taxa not previously studied [9].

The mitochondrial cytochrome oxidase subunit I gene (*mt*COI) has been characterized in a comprehensive molecular data sets for seven orders which represent the main divisions of the class insecta with special concern to the most common myiasis-causing Oestridae spp. [11-13] in comparison to, Lepidoptera [14], Hymenoptera [15] and Diptera [2, 11, 12, 16-20], providing an essential database for their molecular identification, the assessment of phylogenetic relationships [2, 21], by means of PCR [17, 18, 20-23] and PCR-RFLP assay [19, 24-26]. Unfortunately, despite the abundance of data on myiasis producing larvae using COI gene marker approaches, there's no available data concerning *C. titillator* larvae.

Moreover, ribosomal genes have been used for phylogenetic analysis of a wide range of species and divergence levels including the deepest branches of the tree of life, since there're only two *mt*rRNA subunit genes (12S and 16S) [2]. With respect to myiasis-causing flies, the mitochondrial gene encoding for the 16S rRNA subunit has been studied for a few species belonging to the Calliphoridae, Sarcophagidae and Oestridae and it is demonstrated to be useful tool for molecular phylogenetic researches [2, 10, 17, 18, 27, 28].

In Egypt, this parasitic infestation needs more scientific attention on diagnosis of the disease in parallel to some insights on the genome of the parasite to overcome the national problem [29]. The aim of the current study was to assess the utility of the partial *mt*COI and 16S rRNA genes sequences to differentiate, identify and elucidate the phylogenesis of the *C. titillator* commonly retrieved in camels and its fruitful applicability as a novel study in genomic investigation as well as diagnosis of such disease.

MATERIALS AND METHODS

***C. titillator* Larval Instars:** Different instars of *C. titillator* larvae were collected from nasopharyngeal cavities of camels after being slaughtered at El-Bassatin and El-Warrak abattoir, Egypt. Characterization of different instars of *C. titillator* larvae was done according to Zumpt [30].

Molecular Characterization of Mitochondrial Target Genes of *C. titillator*:

Larval Mitochondrial DNA Extraction: *mt*DNA isolation procedures were performed using high salt concentration protocol [31] from 3rd instars of *C. titillator* larvae after internal organs had been retrieved. 20mg from the larval soft tissues were incubated with lysis buffer (10mM Tris-HCl, 10 mM EDTA, 3.4mM SDS and 20mM NaCl, pH 8.0, Sigma Aldrich), in addition to 1M DTT (Sigma Aldrich) and protinase K (20mg/ml) (Stratagin) at 56°C over night, then the supernatant was transferred to new collection tube (Coaster) to proceed in DNA isolation.

Oligonucleotides Primers Design: The universal primers used for COI amplification were UEA1, UEA3 and UEA10 designed according to Lunt *et al*. [32], Zhang and Hewitt [33] and Otranto *et al*. [21] (Table 1). While primers used for 16S rRNA amplification were 16Sbr and 16Sar designed according to Simon *et al*. [34] and Otranto *et al*. [17] (Table 1). Primers sets were synthesized by (Operon Technologies, Germany).

Amplification of COI and 16S rRNA *mt*DNA Genes Partial Sequences: PCR was performed in order to specifically amplify the sequence from tRNA-Tyr to tRNA-Leu of COI open reading frame from the *mt*DNA as well as the hyper variable part of the mitochondrial 16S rRNA gene sequence coding for the domain IV and V by the appropriate primer sets. For COI gene amplification, two separate PCR mixtures for (UEA1 and UEA10) and (UEA3 and UEA10) primers were separately performed in a total volume of 25µl under aseptic conditions. Each PCR mixture contained 100ng larval DNA, 50pM/µl of each primer, 20µl of Ready TaqMix complete master mix (AllianceBio, USA) and nuclease free water (Qiagen) to complete the total volume of the reactions. While 16S rRNA gene amplification was performed using one set of primers in 50µl total volume. All amplifications were performed in a PTC-100™ thermal cycler (MJ Research Inc., USA) utilizing one cycling profile [17, 21]. A reagent blank was run as control simultaneously with every PCR. Amplified products from the PCRs were electrophorised in 0.7% agarose gels in TBE buffer (45mM Tris-borate, 1mM EDTA, pH 8.3, Sigma Aldrich) and stained with ethidium bromide (Sigma Aldrich). A 100 bp ladder (Alliance Bio, USA) was used with each gel. Gel photo was analyzed by Lab Image soft ware.

Table 1: Primers used to amplify COI and 16S rRNA genes partial sequences.

Name	Standard Affiliation	Position	Sequence
UEA1-F	TY-N-1438	tRNA-Tyr	5`-GAATAATGAATAATTCCCATAAATAGATTTACA-3`
UEA3-F	C1-J-1763	294	5`-TATAGCATTCCCACGACGAATAAATAA-3`
UEA10-R	TL2-N-3014	tRNA-Leu	5`-TCCAATGCACTAATCTGCCATATTA-3`
16Sbr-F	LR-J-12887	domain IV	5`-CCGGTCTGAACTCAGATCACGT-3`
16Sar-R	LR-N-13398	domain V	5`-CGCCTGTTTAACAAAAACAT-3`

Table 2: Accession numbers of used reference sequences during *C. titillator* genes analysis and phylogenetic trees construction.

Phylum	Subphylum	Class	Order	Suborder	Division	Section	Superfamily	Family	Subfamily	Genus	Accession Numbers	
											COI genes	*16S* genes
Arthropoda	Chelicerata	Arachnida	Acarina	Metastigmata			Ixodoidea	Ixodidae		*Amblyomma triguttatum*	NC005963	NC005963
	Myriapoda	Chilopoda	Lithobiomorpha					Lithobiidae		*Lithobius forficatus*	AJ270997	AJ270997
	Hexapoda	Insecta	Hymenoptera					Apidae		*A. mellifera*	NC001566	NC001566
			Lepidoptera					Crambidae		*O. furnacalis*	NC003368	NC003368
			Diptera	Nematocera			Culicoidae	Simuliidae		*S. damnosum*	-	AF081904
				Brachycera	cyclorrapha	Acalyptrate	Drosophiloidae	Drosophillidae		*D. subobscura*	-	AF126335
										D. Yakuba	NC001322	NC001322
										D. melanogaster	NC001709	NC001709
						Calyptrate	Hippoboscoidae	Glossinidae		*G. m morsitans*	-	AF072373
							Muscoidae	Muscidae		*Musca spp.*	-	AF322436
							Oestroidae	Oestridae	*Gasterophilinae*	*G .haemorrhoidalis*	AF497774	AF322439
										G. intestinalis	AF497773	-
										G. nasalis	AF497775	-
										G. pecorum	AF497776	-
									Hypodermatinae	*H. diana*	AF497763	AF322438
										H. bovis	AF497761	-
										H. lineatum	AF497762	-
										H. tarandi	AF497764	-
									Oestrinae	*C. stimulator*	AF497768	AF322441
										C. trompe	AF497769	-
										O. ovis	AF497767	-
										R. usbekistanicus	AF497771	-
									Cuterebrinae	*Cuterebra spp.*	-	AF322440
										C. baeri	AF497777	-
										C. jellisoni	AF497778	-
								Calliphoridae	*Lucillinae*	*L. cuprina*	AB112852	AF086858
									Calliphorinae	*C. quadrimaculata*	-	AF086857

Bioinformatic Analysis of Molecular Data:

Sequencing of PCR Products of COI and 16S rRNA *mt*DNA Genes: Each amplicon was purified for sequencing using the QIAquick spin PCR purification kit (Qiagen) according to the manifacturer's instructions. Sequencing reactions were performed with the Dye terminator DNA sequencing kit on an ABI 3100 DNA sequencer (Applied Biosystems, USA), as described by the manufacturer. Each sequencing reaction was repeated at least three times in both the forward and reverse directions before being accepted for analysis. The used primer sets for PCRs were used for sequencing of designated amplicons (Table 1).

Construction of Phylogenetic Tree: Sequences of COI and 16S rRNA amplified fragments were aligned using Nucleotide BLAST program of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) for sequence homology searches against genbank database. The obtained sequences were assembled using ChromasPro 1.49 beta (Technelysium PtyLtd, Tewantin, Australia). Multiple sequences were aligned using the ClustalW 1.8® program [35] and aligned sequences were edited by BioEdit sequence alignment editor (V. 7.0.9.0). Genbank records of related genuses belonging to phylum Arthropoda were used in construction of phylogenetic trees of COI and 16S rRNA genes (Table 2). Rooted phylogenetic trees were constructed with the Maximum Parsimony method [36]. The evolutionary distance was calculated by the Maximum Composite method [37]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was shown below the branches [38, 39]. Phylogenetic trees were constructed by MEGA4 [40]. For 16S rRNA gene, the tree was drawn to scale; with branch lengths calculated using the average pathway method [41] and were in the units of the number of changes over the whole sequence.

Evolutionary Rate Analysis Within Oestridae Species: Evolutionary rates of *C. titillator* COI and 16S rRNA genes were calculated by the Tajima test in comparison with other two genbank records. For 16S rRNA gene, the resolution of evolutionary rate between *C. titillator* and *Lucilia cuprina* (AF086858.1) was tested using *Drosophila melanogaster* (NC 001709.1) as an out-group, while for COI gene, the equality of evolutionary rate between *C. titillator* and *Oestrus ovis* (AF497767.1) was tested using *Lucilia cuprina* (AB112852.1) as an out-group in Tajima' relative rate test in MEGA4 [40, 42].

RESULTS

Amplified Partial COI and 16S rRNA Genes Sequences by PCR Assays: The results of PCRs for COI gene were varied according to the primer combination. The (UEA1 and UEA10) and (UEA3 and UEA10) primer sets reproducibly yielded 1 amplicon of 580 bp and 2 amplicons of 1396 and 580 bp, respectively (Figure 1). While the amplified hyper variable region of 16S rRNA gene fragment was reproducibly detected at 548 bp (Figure 2). The obtained mitochondrial sequences of both genes; the hyper variable part of the 16S rRNA coding for the domain IV and V as well as the part of the COI coding for the domain tRNA-Tyr to tRNA-Leu, of *C. titillator* larvae are shown in figures (3) and (4), respectively.

Phylogenetic Trees of Amplified Mitochondrial Partial COI and 16S rRNA Genes Sequences: Rooted phylogenetic trees constructed with the Maximum Parsimony method to infer the phylogenetic relationships between Egyptian *C. titillator* amplicons and other members of phylum Arthropoda published in genbank are shown in figures (5) and (6).

Evolutionary Rate Analysis within Oestridae Species with Regard to Mitochondrial Partial COI and 16S rRNA Genes Sequences: For COI gene sequence, there were a total of 512 positions in the final dataset, out of which 481 were parsimony informative. While in 16S rRNA gene sequence, there were a total of 456 positions in the final dataset, out of which 456 were parsimony informative and were in the units of the number of changes over the whole sequence aligned. The equality of evolutionary rate of *C. titillator* COI gene evaluated by the x^2 test statistic was 0.60 ($P = 0.44040$ with 1 degree of freedom) where *P*-value less than 0.05 was often used to reject the null hypothesis of equal rates between lineages. Identical nucleotide sites,

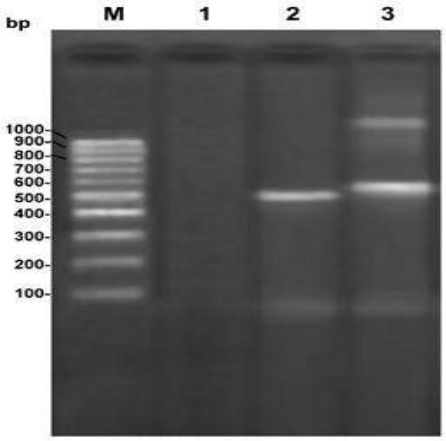

Fig. 1: 0.7% agarose gel of amplified PCR products of COI gene of *C. titillator* larvae stained with ethiduim bromide. Lane M: 100 bp DNA ladder, lane 1: Control negative, lane 2: 580 bp amplicon of primers set (UEA1-UEA10) and lane 3: 580 bp and 1396 bp amplicons of primers set (UEA3-UEA10).

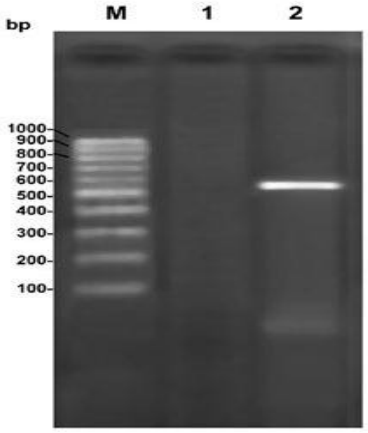

Fig. 2: 0.7% agarose gel of amplified PCR product of 16S rRNA gene of *C. titillator* larvae stained with ethidium bromide. Lane M: 100 bp DNA ladder lane 1: Control negative and lane 2: 548 bp amplicon of 16S rRNA gene.

divergent sites and unique differences in all three sequences calculated are represented in table (3). While for 16S rRNA gene sequence, the x^2 test statistic was 1.34 ($P = 0.24630$ with 1 degree of freedom) where *P*-value less than 0.05 was often used to reject the null hypothesis of equal rates between lineages. Identical nucleotide sites, divergent sites and unique differences in all three calculated sequences are represented in table (4).

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60
ccggtnnnnt tcagatcccg taagaattta aaagtcgaca GgCtTATTAT TTGaaCTACT
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         70         80         90        100        110        120
ACACCCAAAT ACATCTCTTA ATCCAACATC GAGGTCGCAA TCCTTTTTAT CGATAAGAAC
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        130        140        150        160        170        180
TCTCCAAAAA AATTACGCTG TTATCCCTAA AGTAACTTAT TTTTTTATTC ACCAATAATG
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        190        200        210        220        230        240
GATCAAACAC TCATAAATAA ATGAAAATTA TAATTAAAAG TTCATTAAAT TTTAATATCA
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        250        260        270        280        290        300
CCCCAATAAA ATATTATTAA AAAAAAAAAA TAAATACTAC CTAAAAATTC CTTTTTTACA
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        310        320        330        340        350        360
AAAATATAAA GATTTATAGG GTCTTCTCGT CTTTTAAATA AATTTTAGTT TTTTAACTAA
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        370        380        390        400        410        420
AAAATAAAAT TCTATAAAAA ATTAAAGGGA AACAGTCAAT ATTTCGTCCA ACCATTCATT
....|....| ....|....| ....|....| ....|....| ....|....| ....|....
        430        440        450        460        470
CCAGCCTCCA ATTAAAAAAC TAATGATTAT GCTACCTTTG CACAGTTAAA ATACTGCGG
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Fig. 3: The partial sequence of mitochondrial hyper variable part of the mitochondrial 16S rRNA gene coding for the domain IV and V of *C. titillator* larvae.

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....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60
acagttgtaa tagacattga tactcgagct tattttactt ctgcaactAt aaTgattGgt
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         70         80         90        100        110        120
attcGtacag gaattaaGAT TtGTaG--CA tTAtGt--ac --T-TT--A- TG---TCA--
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        130        140        150        160        170        180
TATT--ACTT GCATTGCAAG CCCAT----T GCTTA--CTA -TCCCTGCT- ---GT-TAAA
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        190        200        210        220        230        240
TGGGA--A-- -----T-AA- NTGC-ATTGA -AAAANNNTA NAT-ATGCTT TTTATG--GT
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        250        260        270        280        290        300
-NCTGNTGTC CCAAATAT-T NACCTATAAA T-CC--CTAT G---TGAGA- NTTCCTTTTT
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        310        320        330        340        350        360
TNATGNGCCG CCTTCA-TT- -AA----AAC ATTNNTTNNN CTGGTNTTNN GCATNGAA--
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        370        380        390        400        410        420
-CTNAGTNT- GTTNGCNCAA AANNNCTGAG A-TNACCNGT ATANNCTGAT AATATC-T--
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        430        440        450        460        470        480
--GTATNTC- AGNNGNTTTT CTTNTTNAAA NGTTCTCTCA T--NGGGGNA GCCCANNTTA
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        490        500        510        520        530        540
NCTNNCTANT C-TANTAT-- TTACT--TCN NTATCTGNNT ACAT-TTATA TCAACCCCAC
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        550        560        570        580        590        600
AATTTTTTTT TTACTCGT-A ATATTTTCTC TCGAATGGAT TAAGNCTTTC NCCCNNNCAN
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        610        620        630        640        650        660
CCNCTTCCTC ANNANTTCCC CCCCCC--CC NTTTTATTNG GGA--AAA-- TTTT--GGNT
....|.

TTGNAA
```

Fig. 4: The partial sequence of the mitochondrial COI gene coding for the domain tRNA-Tyr to tRNA-Leu of *C. titillator* larvae.

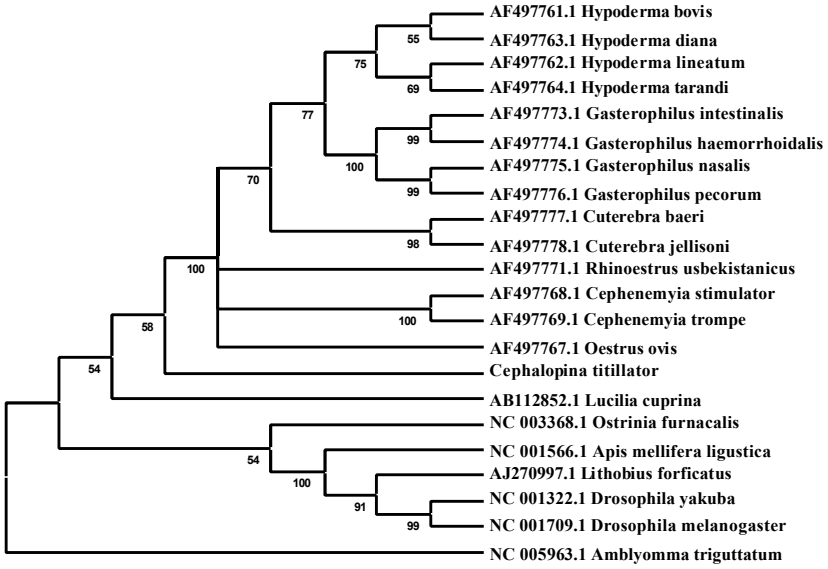

Fig. 5: Rooted phylogenetic tree constructed with the Maximum Parsimony method to infer the phylogenetic relationships between aligned sequences of partial COI gene of *C. titillator* in comparison to other genbank sequences records. The evolutionary distance was calculated by the Maximum Composite method.

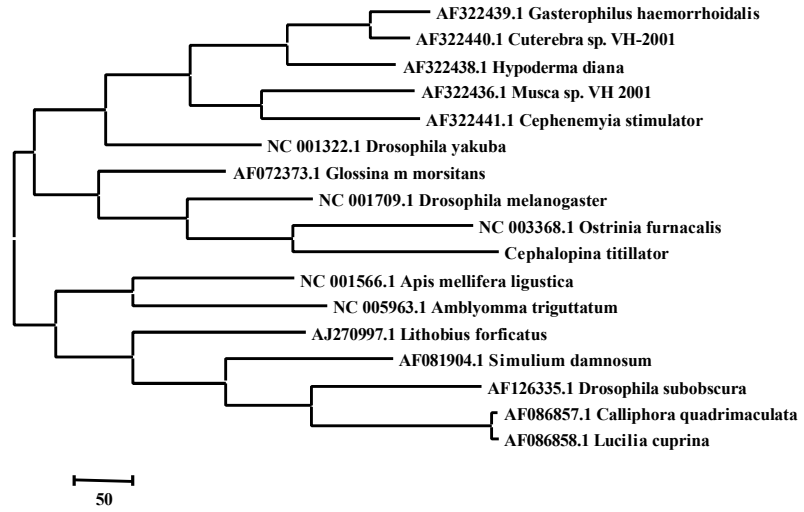

Fig. 6: Rooted phylogenetic tree constructed with the Maximum Parsimony method to infer the phylogenetic relationships between aligned partial 16S rRNA gene of *C. titillator* in comparison to other genbank sequences records. The evolutionary distance was calculated by the Maximum Composite method.

Table 3: Evolutionary rate of amplified partial *C. titillator* larvae COI gene sequence calculated by the Tajima test in comparison to identical fragments of genbank records of *O. ovis* and *L.cuprina*

Configuration	Count
Identical sites in all three sequences (*C. titillator, O. ovis* and *L. cuprina*) (m_{iii})	70
Divergent sites in all three sequences (*C. titillator, O. ovis* and *L. cuprina*) (m_{ijk})	133
Unique differences in sequence (*C. titillator*) (m_{ijj})	79
Unique differences in sequence (*O. ovis*) (m_{iji})	89
Unique differences in sequence (*L. cuprina*) (m_{iij})	141

Table 4: Evolutionary rate of amplified partial *C. titillator* larvae 16S rRNA gene sequence calculated by the Tajima test in comparison to identical fragments of genbank records of *L. cuprina* and *D. melanogaster*

Configuration	Count
Identical sites in all three sequences (*C. titillator, L. cuprina, D. melanogaster*) (m_{iii})	61
Divergent sites in all three sequences (*C. titillator, L. cuprina, D. melanogaster*) (m_{ijk})	118
Unique differences in sequence (*C. titillator*) (m_{ijj})	116
Unique differences in sequence (*L. cuprina*) (m_{iji})	99
Unique differences in sequence (*D. melanogaster*) (m_{iij})	81

DISCUSSION

It is difficult or even impossible to identify larvae to species level by morphology alone, so an increasing amount of DNA sequence data has been obtained with the goal of using molecular phylogenetic methods for species identification [10]. Several studies have been addressed using DNA sequences to identify insects, most choosing to use *mt*DNA as the basis for sequencing [20]. Since *mt*DNA is providing more accurate identifications of the species to which larvae belong, even closely related species can have different developmental characteristics [2].

Unfortunately, there's no available literature concerning molecular data of *C. titillator*. Nonetheless, the available literatures on other closely related Oestridae were in disagreement with the present data [11, 17, 18, 20, 21]. All previous studies that had succeeded to amplify the COI gene in most common Oestridae larvae causing myiasis; including *Hypoderma bovis, Hypoderma lineatum, O. ovis, P. silenus* and *Gasterophilus* spp., using the primers sets (UEA1 and UEA10) and (UEA3 and UEA10) yielded different sized amplicons equals 1630 and 1300 bp, respectively [21]. Other amplifications of the most variable part of COI gene encoding the region spanning from E4 to COOH were utilizing primers sets (UEA7 and UEA8) and (UEA9 and UEA10) which had yielded amplicons of 412 bp and 307 bp, respectively, for *H. bovis, H. lineatum, Hypoderma diana, Hypoderma tarandi, Hypoderma actaeon, P. silenus, O. ovis, Cephenemyia stimulator, C. trompe, C. ulrichi, Rhinoestrus usbekistanicus, R. phacochoeri, Contarinia baeri, Cephenemyia jellisoni* [11] and *Rhinoestrus purpureus* [17]. While, *Gasterophilus intestinalis, Gasterophilus haemorrhoidalis, Gasterophilus nasalis and Gasterophilus pecorum* yielded the same amplicons sizes using the primers sets (UEA7 and GaRev) and (GaFor and UEA10), respectively [11, 17]. On the other hand, Pawlas-Opiela and coworkers [20] amplified 649 bp fragment of COI gene of *G. intestinalis* and *G. nasalis* using primers set (Gco1s and Gco1an). However the present study recorded that *C. titillator* mitochondrial 16S rRNA gene amplicon was reproducibly detected at 548 bp using the primers set (LR-N-13398 and LR-J-12887). This result was in agreement with previous studies on closely related Oestridae, where 16S rRNA gene was specifically amplified using the same primers set yielding amplicons sized 550 bp and 549-551 bp for *Rhinoestrus* spp. and *Gasterophilus* spp., respectively [17, 18], since slight variations in size were accounted for deletion/insertion of blocks of contiguous nucleotides [34].

When the equality of evolutionary rate of the sequenced *C. titillator* COI partial gene was calculated in comparison to most related genbank records of *O. ovis* using *L. cuprina* as an out-group, the results could not indicate sharp identity to the specified genus within Oestrinae. However, the records had illustrated higher similarity with *O. ovis* sequence (family Oestridae: subfamily Oestrinae: genus *O. ovis*) as well as *L. cuprina* sequence (family Calliphoridae: subfamily Lucillinae: genus *L. cuprina*) than to other Oestrinae haplotypes (family Oestridae: subfamily Oestrinae: genus; *C. stimulator, C. trompe* and *R. usbekistanicus*). On contrary, in spite that 16S rRNA gene sequence had shown to be phylogenetically more informative for relationships among closely related species when annotating the hyper variable region encoding for domains IV and V [17, 18], the evolutionary calculations could not indicate sharp identity to specified genus in Oestrinae (family Oestridae: subfamily Oestrinae: genus; *C. stimulator, C. trompe, O. ovis* and *R. usbekistanicus*) nor to other Oestridae subfamilies (Gasterophilinae, Hypodermatinae and Cuterebrinae) or to closely related Calliphoridae (subfdamily Lucillinae: genus *L. cuprina*). In continence, the calculated evolutionary records illustrated higher similarity with *D. melanogaster* (section Acalyptrate: superfamily Drosophiloidae: family Drosophillidae: genus *D. melanogaster*). It worth to be noted that chooses of counter parts in the corresponding phylogenetic tree as well as the taxonomical arrangement within the super family Oestroidae depended on morphological parameters of classification [30] as well as the highly conserved genetic structure and arrangement of *mt*DNA [10, 20].

It is obvious that the genealogical relationships between Oestridae were sufficiently complex suggesting that *mt*DNA data alone may be insufficient for full species identification as was reported elsewhere [13]. The warning was based on two factors. First, the subfamilies do not exhibit a pattern of reciprocal monophyly in *mt*DNA phylogenetic trees based on COI nor 16S rRNA genes sequences. In continence, some Oestrinae haplotypes (*C. stimulator, C. trompe, O. ovis* and *R. usbekistanicus*) are much more closely related to other Oestridae subfamilies; Gasterophilinae, Hypodermatinae and Cuterebrinae than to *C. titillator* which is until now classified as member of subfamily Oestrinae [30]. Second, the revealed topology from the constructed tree in the present study indicated that *C. titillator* found more related to family Calliphoridae (subfamily Lucillinae: genus *L. cuprina*) than it is to other Oestrinae haplotypes (*C. stimulator, C. trompe, O. ovis* and *R. usbekistanicus*) as well as other Oestridae subfamilies (Gasterophilinae, Hypodermatinae and Cuterebrinae). This may strongly propose gene intermixture or recombination done along successive evolutionary developmental changes which deviate *C. titillator* genes dramatically from the ancestral arthropod genes order inferring an evolutionary genetic divergence [44, 45] that were presented in the *mt*DNA coding sequences but did not affect the functions and/or arrangements of the open reading frames of the genes of these genuses [11]. In addition, mitochondrial protein coding genes display a high rate of substitution in the third codon position; often synonymous changes, resulting in no change [43]. This made nucleotide sequence data a useful subject for study of lower taxonomic levels, while the amino acids sequences is of greater use in higher level analyses [44, 45]. Moreover, family Oestridae had an enormous diversity in terms of life style, geographic distribution and parasite behavior within the host [9]. Until now, relatively few studies have been investigated the phylogenies of Oestridae [9, 46]. Despite that identification of strains and/or species needs to be supported by a detailed analysis of the genetic material via DNA sequencing [46, 47] to obtain species specific markers [10, 28, 48], hence, the nuclear DNA of flies evolved at the same rate as *mt*DNA [49, 50].

In conclusion, *mt*DNA contains both conserved and variable regions within genes, reflecting the variation in evolutionary rate across the molecule in phylum Arthropoda. The complex issues surrounding DNA-based discrimination between *C. titillator* and Oestrinae haplotypes into distinct clades may have a deeper biological origin, supported by the fact that there is a long-recognized division of Oestrinae into morphologically different genuses which highlighted the effectiveness of parasite-host interaction as well as the geographical and socio-ecological patterns on the genetic constitution of the parasite and the pressure they exhibit even on highly conserved *mt*DNA. Thus DNA-based species identification must be based on a database comprising replicate samples from the same genus collected from wide range which was not possible in the present study.

Finally, the present work is motivating future phylogenetic studies on the genus *Cephalopina* and a possible taxonomic revision, which appears to be necessary since we can now state with reasonable confidence that the subfamily Oestrinae is not monophyletic, hence, both genes analyzed in this study are mitochondrial genes that are not independently inherited. Therefore, the collection of additional molecular data (preferably nuclear genes) is obligatory before we can establish a reliable phylogenetic hypothesis or suggest new taxonomic classification of the genus *Cephalopina*.

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