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Molecular characterization of the camel nasal botfly, *Cephalopina titillator* (Diptera: Oestridae)

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Abstract

Background Larval stages of the camel nasal botfly, *Cephalopina titillator* (Diptera: Oestridae), rank among the top obligate endoparasites causing nasopharyngeal myiasis. Such infestations have adverse effects on camel health, reducing their economic value. The current study's objective is to assess the applicability of PCR using partial regions of the COI mtDNA and 28S rRNA gene sequences to identify, classify, and reveal the phylogenesis of *C. titillator.*

Results Larvae of the 2nd and 3rd instars of *C. titillator* were collected from the El-Bassatin abattoir in Egypt, extracted from the nasopharyngeal passage of the Arabian camel (*Camelus dromedarius*) during postmortem inspection. Genomic DNA was successfully isolated from 10 samples of 3rd instar larvae of *C. titillator* and amplified using primer pairs targeting partial mitochondrial COI (916 bp) and ribosomal 28S rRNA (830 bp) gene fragments. Nucleotide sequences from five samples have been sequenced and submitted to GenBank under accession numbers OP482168 to OP482172 for the COI gene and OP482160 to OP482164 for the 28S rRNA gene fragments. The COI gene exhibited 97.3% nucleotide identity across all specimens, while the 28S rRNA gene displayed 99.74% identical nucleotides. Maximum likelihood trees were constructed based on the generalized time-reversible (GTR) model. The resulting COI phylogenetic tree demonstrated that the subfamily Oestrinae does not exhibit monophyly. Additionally, it revealed that *C. titillator* is a sister group to the subfamily Gasterophilinae. Despite the scarcity of data available for the 28S rRNA gene, the phylogenetic analysis utilizing 28S rRNA revealed one distinct lineage for the Egyptian camel nasal bot fly.

Conclusions Molecular phylogenetic analysis was conducted using molecular markers of distinct origins (both mitochondrial and nuclear) to elucidate the evolutionary relationships within the family Oestridae. This analysis is particularly significant following the inclusion of *C. titillator*, a first-time discovery in Egypt.

Keywords Camel nasal botfly, *Cephalopina titillator*, Oestridae, Myiases, Phylogeny, Molecular identification, Cytochrome oxidase I, 28S rRNA

1 Background

The Arabian camel (*Camelus dromedarius*) is considered one of the crucial livestock animals that have a major impact on human life, especially economically. However, camels are often infected with various parasitic agents that cause diseases, thereby impairing their health and the quality of their products [1].

Cephalopina titillator (*C. titillator*) Clark, 1797 (Diptera: Oestridae), is a common parasitic problem that

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significantly impacts the camel industry in Egypt. The larvae of this species are the infesting stage in the insect lifecycle, causing obligate nasopharyngeal myiasis, while the adults are free-living [2]. This myiasis affects animal health, resulting in notable economic losses through animal abortion, reduced milk production, and weight gain retardation [2, 3]. Several methods have been proposed for the diagnosis of myiasis, including clinical investigation, morphological identification, and even the incidental detection of the adult fly [4].

In Egypt, the rate of infestation has been recorded in several studies from different abattoirs, mostly for the second and third larval instars of *C. titillator*. The prevalence of camel infestation was reported to be 53.23% (33 out of 62) at EL-Bassatin abattoir during the period from June 2019 to May 2020 [1]. It reached 41.67% (100 out of 240) during the period from September 2011 to March 2012 for the larvae collected from Tokh abattoir [5]. However, Cairo abattoir reported a higher rate of infestation, 71% (24 out of 30), during the period from January to May 2019 [6].

The development of molecular biology techniques has greatly advanced parasitological approaches for studying parasites of human and animal concern [7]. This molecular revolution has been facilitated not only by new techniques, such as Polymerase Chain reaction (PCR) and automated sequencing, but also by a better understanding of the many target genes, including mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA), and their role in evolutionary research. Despite the vast amount of widely available sequence data and their associated phylogenetic analysis of insects, there have been few studies investigating myiasis-producing larvae at the molecular biology level, especially within the family Oestridae [8, 9]. In contrast to Calliphoridae and Sarcophagidae, which have been the subjects of numerous molecular and phylogenetic studies due to their importance in the field of forensic entomology, the related sister group of the Oestridae family has received attention primarily in the context of control strategies and diagnostic techniques [10, 11].

Mitochondrial DNA has been regarded as the marker of choice for defining different taxonomic levels from phyla to species and has been widely used for resolving taxonomic controversies [12]. The reason for the adoption of mtDNA as a reliable and widely used genetic marker is that it possesses well-specified properties that make it preferable. Experimentally, mtDNA is relatively easy to isolate and amplify because it is present in high copy numbers within the cell, particularly in animal tissues, especially in the muscles of insects. Biologically, mitochondrial gene content exhibits high conservation across animals, featuring strict orthology of coding genes, the presence of genes/regions evolving at different rates, the advantage of having both conserved and variable segments, uniparental (maternal) inheritance, minimal or very low levels of recombination, very few duplications, and very short intergenic regions. These characteristics collectively enable the use of universal primers to isolate and amplify this gene fragment. These attributes together make this molecule a reliable and easy-to-use identification marker [13, 14]. Several studies have shown that the gene encoding the mitochondrial cytochrome oxidase subunit I (COI) is a particularly powerful target for molecular identification purposes, allowing phylogenetic questions for any insect to be defined [14–16].

One of the most frequently used markers is nuclear rDNA (28S rRNA), which is commonly employed to assess the evolutionary relationships between closely related taxa. The widespread usage of 28S rRNA in evolutionary analysis is attributed to its repetitive copies per genome [17]. The integrally diverse rates of genetic evolution of the 28S rRNA gene have enhanced its application across a broad phylogenetic range, making it suitable for investigating both higher (older) evolutionary relationships and relationships between more recently diverged species [18]. This is because it contains both conserved and highly variable regions (D expansions) in its conformation, making it a suitable marker for determining relationships at different hierarchical levels, and it has the capacity to distinguish between closely related species [19].

The aim of this study is to confirm the presence of camel nasal botfly larvae and to facilitate their identification through the use of PCR and automated sequencing molecular approaches, with the goal of characterizing the nucleotide sequences of partial gene fragments of mitochondrial COI and 28S rRNA among slaughtered camels in Egypt. Additionally, we aim to demonstrate the phylogenetic relationships within the family to establish the evolutionary profile of its members.

2 Results

2.1 Multiple sequence alignment analysis (MSA)

Throughout the MSA, a high degree of conservation among the sequenced specimens was confirmed. Regarding COI, it was revealed that out of an alignment length of 852 base pairs (bp), after sequence editing, there were 829 identical nucleotides, accounting for 97.3% of the total sequence. There were 23 different nucleotides, making up 2.69% in figure (Fig. 1). For 28S rRNA, out of an alignment length of 782 bp, there were 780 identical nucleotides, constituting 99.74%, while the number of different nucleotides was two, or 0.26% (Fig. 2).

Published and directly submitted sequences from the National Center for Biotechnology Information (NCBI)

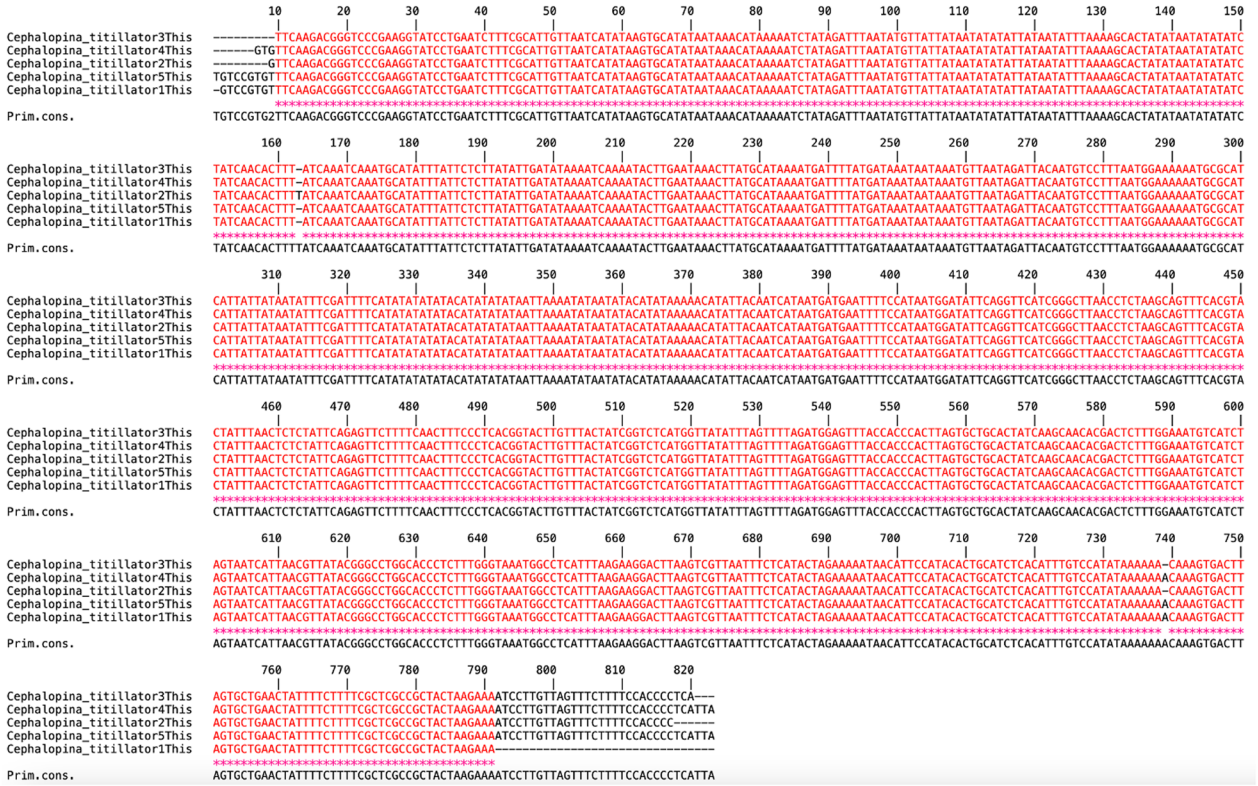

Fig. 1 CLUSTAL W alignment of the five sequences of COI gene fragment with the asterisk as an identity sign. The lower line of each alignment block represents the primary consensus (Prim. cons.) for this part

database for the four subfamilies of Oestridae were collected and tabulated for the two markers, in accordance with their accession numbers and references. For the COI marker, 24 sequences representing 20 different species from all the subfamilies were chosen to infer a representative historical evolution of the entire family (Table 1). For the 28S marker, only 10 sequences representing six species from three subfamilies were selected due to the limited data available for this gene (Table 2).

2.2 Phylogenetic tree construction and analysis

Phylogenetic trees were constructed for each marker using the maximum likelihood (ML) character-based method. These evolutionary trees illustrate the positions of different species and subfamilies within Oestridae after adding new sequences of *C. titillator* from Egypt. The phylogenetic tree of COI (Fig. 3) illustrates that the samples sequenced in this study clustered with the corresponding part of the gene length of the *C. titillator* reference sequence (NC_046479.1), confirming that they are the same species. The Egyptian *C. titillator* samples in this study showed a monophyletic relationship, as they clustered in the same clade with a most recent common ancestor displaying a branching pattern. This pattern grouped sample 2 (OP482169) and sample 3 (OP482170) in the same lineage, while samples 4 (OP482171) and 5 (OP482172) were in another lineage. Sample 1 (OP482168) was more closely related to the lineages of samples 4+5 than to the lineages of samples 2+3. This separation pattern is a result of nucleotide differences between the samples.

The tree topology indicates that the subfamily Oestrinae does not exhibit a monophyletic relationship, especially with the newly added *C. titillator*, which appears polyphyletic in relation to the rest of the subfamily. The *C. titillator* clade is positioned as a sister group to the subfamily Gasterophilinae. Additionally, it is evident that both Gastrophilinae and Cuterebrinae display monophyly. Notably, in the phylogenetic relationship of Hypodermatinae, while *Hypoderma* spp. exhibit monophyly, *Prezhevalskiana* spp. are separated in a polyphyletic relationship with the subfamily.

While the 28S rRNA marker holds substantial importance for inferring evolutionary relationships between taxa, the phylogenetic tree for 28S rRNA lacks various biological sequences for the family. Despite the limited number of sequences, this marker provides an overview of the evolutionary events in the

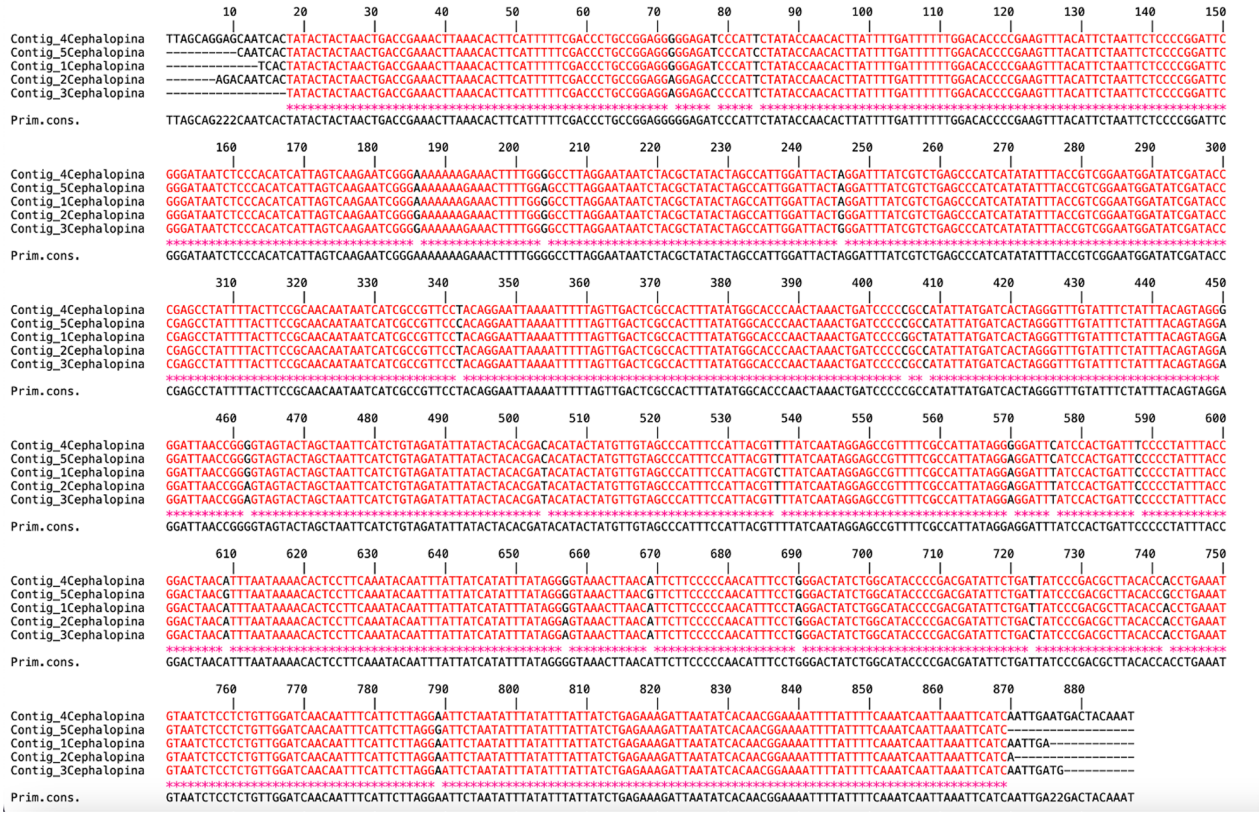

Fig. 2 CLUSTAL W alignment of the five sequences for 28S rRNA gene with the asterisk as an identity sign. The lower line of each alignment block represents the primary consensus (Prim. cons.) for this part

family. The ML phylogenetic tree (Fig. 4) shows that the *Egyptian C. titillator* samples in this study exhibit monophyly by sharing a common ancestor. Due to the very low number of nucleotide differences between samples, there are no branching patterns between *C. titillator* samples, as if they were of the same sequence. The Cuterebrinae subfamily displays a monophyletic relationship by sharing the same recent ancestor. Additionally, it positions the samples of *C. titillator* as a paraphyletic group of Gasterophilinae (only represented by *Gasterophilus intestinalis*) and Cuterebrinae. The absence of the subfamily Hypodermatinae is due to the lack of sequencing records for 28S rRNA in GenBank.

2.3 Discussion

In the last few decades, molecular taxonomy has become an essential approach to support conventional taxonomy for the accurate identification of insect species [25]. This is particularly important for the endoparasitic larval stages of myiasis-causing dipteran insects that inhabit internal tissues and organs, making them difficult to collect in sufficient numbers from different life stages for comprehensive taxonomic studies [25]. Furthermore, several studies have recommended molecular approaches for the diagnosis and identification of the Oestridae family to enable reliable and effective fly control decisions [4, 26].

Although the camel nasal bot fly, *C. titillator*, is currently represented by only one species, the molecular identification carried out in this study holds great value in explaining the evolutionary relationships within the Oestridae family and providing a comprehensive overview of the taxonomic status of this species. This is particularly important as it is one of the highly specialized oestrid pests infesting camels.

In this study, partial regions of both COI (mitochondrial marker) and 28S rRNA (nuclear marker) genes were utilized to amplify fragments of the expected sizes, 916 and 830 bp, respectively. These markers have been employed for the accurate identification of many species (e.g., *Oestrus ovis* (*O. ovis*), *Rhinoestrus purpureus*, *Gasterophilus* spp.) within the Oestridae family [21, 25], as well as for subsequent molecular phylogenetic studies. The primers used in this study, whether custom-designed "COI" or published "28S rRNA" [21], efficiently amplified the target regions of interest.

Table 1 Oestridae species COI sequences used to study the phylogenetics of *C. titillator* with their accession number on NCBI-GenBank

Subfamily	Species	Accession number	References
Oestrinae (Nasopharyngeal Myiasis)	*Cephalopina titillator*	OP482168**:** OP482172 (5 samples)	This study
	Cephalopina titillator	NC_046479.1	[27]
	Cephenemyia trompe	NC_045881.1	[27]
	Rhinoestrus usbekistanicus	NC_045882.1	[27]
	Cephenemyia stimulator	MW145178.1	Unpublished sequence
	Oestrus ovis	MW145179.1	Unpublished sequence
Gasterophilinae (Gastrointestinal Myiasis)	*Gasterophilus intestinalis*	KR230407.1	Unpublished sequence
	Gasterophilus nasalis	NC_042781.1	[35]
	Gyrostigma rhinocerontis	NC_042379.1	[35]
	Gasterophilus inermis	NC_042780.1	[35]
	Gasterophilus haemorrhoidalis	NC_042779.1	[35]
	Gasterophilus nigricornis	MG920506.1	[35]
	Gasterophilus pecorum	KU578262.1	[37]
	Gasterophilus flavipes	MK412089.1	[25]
Hypodermatinae (Cutaneous Myiasis)	*Hypoderma sinense*	EU181169.1	[38]
	Hypoderma lineatum	GU584123.1	[39]
	Hypoderma bovis	EU181164.1	[38]
	Przhevalskiana silenus	AF257119.1	Unpublished sequence
Cuterebrinae (Cutaneous Myiasis)	*Dermatobia hominis*	MK593540.1	[42]
	Cuterebra fontinella	JF439549.1	[40]

Table 2 Oestridae species 28s rRNA sequences used to study the phylogenetics of *C. titillator* with their accession number on NCBI-GenBank

Subfamily	Species	Accession number	References
Oestrinae (Nasopharyngeal Myiasis)	*Cephalopina titillator*	OP482160: OP482164 (5 samples)	This study
Cuterebrinae (Cutaneous Myiasis)	*Cuterebra fontinella*	JF439574.1	[40]
	Cuterebra austeni	KP954361.1	[41]
	Cuterebra spp.	JQ246649.1	[43]
	Dermatobia hominis	JQ246650.1	[43]
Gasterophilinae (Gastrointestinal Myiasis)	*Gasterophilus intestinalis*	AJ551429.1	[45]

Throughout this study, specific primers (custom-designed for COI or published for 28S rRNA) successfully amplified the targeted regions with their expected lengths.

For COI, the reference sequence of *C. titillator* (NC_046479.1) [27] exhibited identical mapping to the forward and reverse primers designed to amplify the region of interest. The Basic Local Alignment Search Tool (BLAST) homology search revealed that the amplified region, confined between the forward and reverse primers on the reference sequence, spans from the external loop 4 (E4) base number (2210) to the carboxyl terminal (–COOH) base number (3125), which significantly contributes to the identification of myiasis-causing Oestridae. Several studies have employed this region to analyze intraspecific variation between the species, even using different sets of primers, for example, *Oestrus* spp., *Rhinoestrus* spp., *Hypoderma* spp., *Gasterophilus* spp., and *Cuterebra* spp. [21, 25, 28, 29].

For 28S rRNA, the used primers successfully amplified the D1–D2 expansion region of interest. Since there were no records of such a gene available in the NCBI-GenBank repository for *C. titillator*, the conserved primer pair was retrieved from some related genera [18, 21]. Many studies have relied on the properties of such a region to assess the molecular taxonomic position of various dipterous

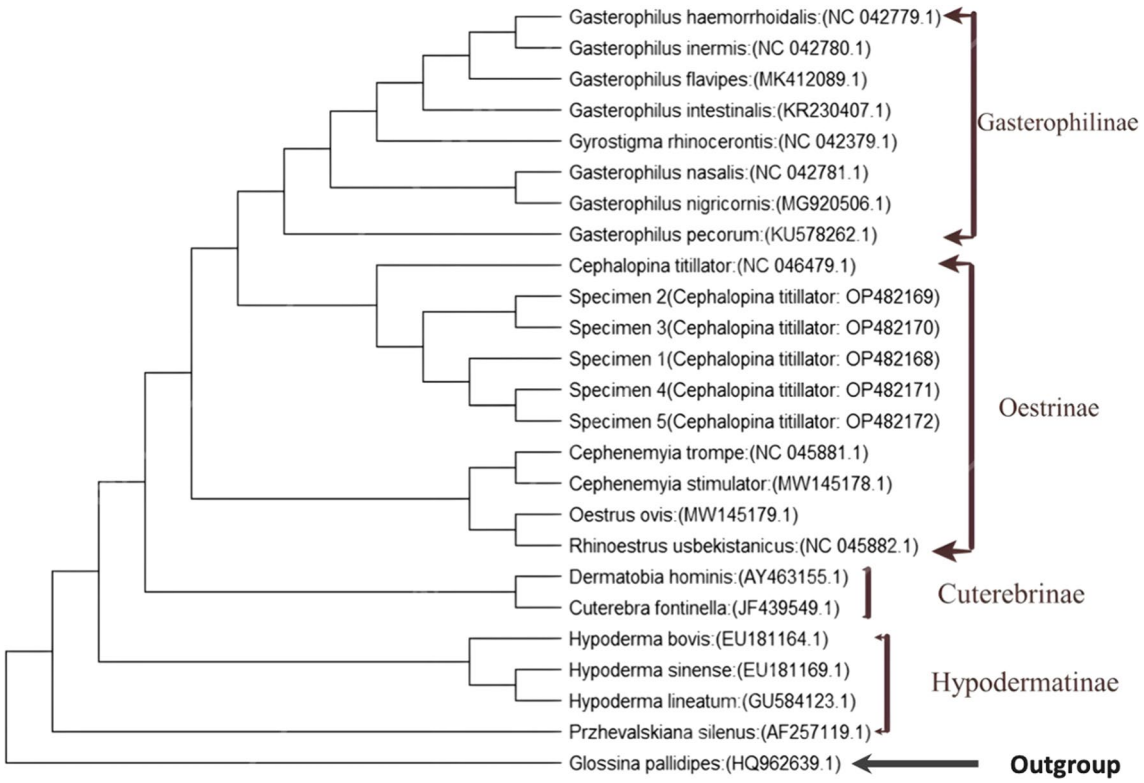

Fig. 3 Phylogenetic tree of Oestridae constructed by maximum likelihood (ML) analysis using generalized time-reversible model. The analysis inferred from the sequence region of *mt*COI gene describing the relationships of *Cephalopina titillator* to homologous sequences retrieved from the GenBank. This analysis involved 25 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). *Glossina pallidipes* is used as an outgroup to the rest of the tree

species at both the inter- and intraspecific levels. For example, *Oestrus* spp. and *Rhinoestrus* spp. [16, 21].

The PCR amplification results observed on the agarose gel exhibited approximate molecular sizes as expected for the ten investigated samples for each marker used. The same length was reported for the relatively close species, *O. ovis*, using the same 28S rRNA primers, which amplified a region of 830 bp [16]. This may be due to the absence of large mutational events that reflect differences in fragment size length polymorphisms. Additionally, the amplified region from the COI gene displayed the same fragment size length among the different individuals used. These molecular results indicated no apparent intraspecific variation in *C. titillator*, as no size polymorphisms were observed.

The alignment process using CLUSTAL W for the five sequenced samples for COI revealed that out of the 852 bp alignment length, 829 nucleotides were identical, accounting for 97.3%, while the different nucleotides numbered only 23 (2.69%), including nine parsimony informative sites and 14 singletons. The nucleotide frequency composition of our *C. titillator* COI sequences was 30.00% (A), 32.65% (T), 22.72% (C), and 14.62% (G), with the AT content (62.65%) being higher than the GC content (37.34%). These results not only align with the nucleotide composition of family Oestridae members, with their higher AT%, but also coincide with the nature of mtDNA [25, 30, 31].

For 28S rRNA, the CLUSTAL W alignment for five sequenced samples revealed that 780 out of 782 nucleotides were identical, accounting for 99.74%, while only 2 nucleotides differed (0.26%). The nucleotide frequency composition for these 28S rRNA sequences was 35.64% (A), 35.90% (T), 16.67% (C), and 11.79% (G), resulting in a higher AT content (71.54%) than GC content (28.46%). Otranto [16] employed the same primer set (D1-D2) to amplify the 28S rRNA gene in four *Rhinoestrus* morphotypes. They found no nucleotide differences insertion/deletion (inDels) detected, indicating that their specimens belonged to the same species. Monero [21] reported a high degree of identity (98.9–99.7%) among *Oestrus* spp. collected from domestic sheep, domestic goats, and European mouflon using the same primer sets. This might be attributed to the nature of the 28S rRNA

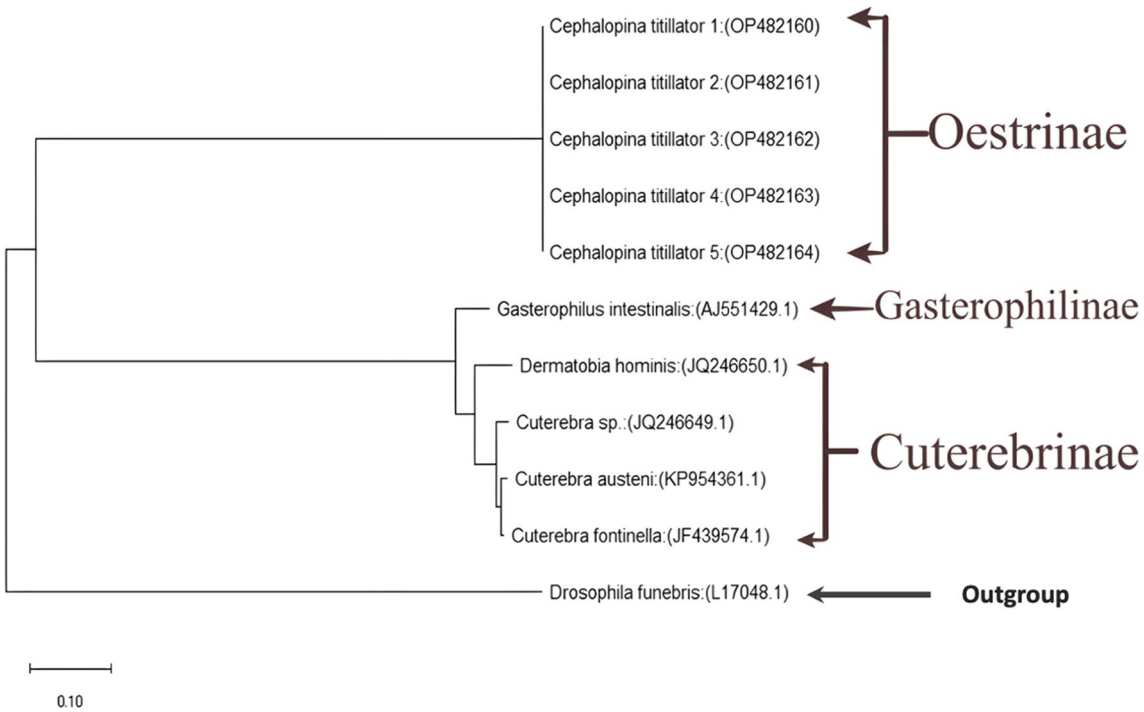

Fig. 4 Phylogenetic tree of Oestridae constructed by maximum likelihood (ML) analysis using Kimura 2-parameter model. The analysis inferred from the sequences of 28S rRNA gene describing the relationships of *Cephalopina titillator* against homologous sequences retrieved from the GenBank. This analysis involved 11 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). Subfamily Hypodermatinae is not represented in this tree due to the lack of representing sequence record. *Drosophila funebris* is used as an outgroup to the tree

gene, which is typically used to elucidate relationships among closely related species.

Phylogenetic relationships within the family Oestridae are still largely unresolved; only a few recent attempts have been made to use molecular data to improve its traditional taxonomy and define evolutionary relationships [30, 32]. The lack of unambiguous identification criteria creates an essential need not only for producing intensive molecular studies on the family but also for utilizing the accumulated molecular data in an attempt to resolve the enigma and decipher the broader evolutionary route.

This study is a first-time report on the camel bot fly, *C. titillator*, of Egypt, investigating its relationship between different species and genera in the family, and illustrating the evolutionary relationship among the four Oestridae subfamilies. This is especially important for subfamily Oestrinae, as the branching pattern of Oestrinae is moderately supported, and new sequence data are needed to clarify the ambiguous relationships within the whole family [32].

The COI-based phylogenetic tree for the Oestrinae subfamily exhibited a polyphyletic relationship, with no recent common ancestor included, especially after adding *C. titillator* to the phylogenetic tree throughout this study. The *C. titillator* branch was positioned as a sister group with Gasterophilinae, while the rest of Oestrinae (*O. ovis*, *Rhinoestrus usbekistanicus*, *Cephenemyia trompe*, and *Cephenemyia stimulator*) were separated. There was also a consensus split between *Oestrus* spp. and *Rhinoestrus* spp. from *Cephenemyia* spp. Otranto and Steven [7] indicated the monophyly of the Oestrinae subfamily using a partial COI gene without including *C. titillator*. However, when they used the entire COI sequence in their study, they obtained a conflicting result with the grouping of *O. ovis* (Subfamily: Oestrinae) with *Hypoderm bovis* (*H. bovis*) and *Hypoderma lineatum* (*H. lineatum*) (Subfamily: Hypodermatinae). Dong [33] and Shamsi [34] assessed the relation between the Oestrinae subfamily to be polyphyletic using the same represented species.

For the subfamily Gasterophilinae, this study revealed a monophyletic relationship, which corroborates the result of Otranto [19], who also confirmed its monophyly. The results of Li [25] and Yan [35], using the third instars of horse stomach bot fly larvae, were consistent with the findings of this study. *Gasterophilus haemorrhoidalis* and *Gasterophilus inermis* appeared as sister taxa within the same clade, sharing a most recent common ancestor, as

did *Gasterophilus nasalis* and *Gasterophilus nigricornis*. In addition, *Gasterophilus intestinalis*, *Gasterophilus flavipes*, and *Gasterophilus pecorum* formed a separate branch. However, a recent study revealed new findings after adding *Cobbolida* (elephant bot fly) species (Gasterophilinae), resulting in a polyphyletic relationship within this subfamily. The *Cobbolida* spp. clade separated as an outgroup in the entire family tree [36].

The Cuterebrinae subfamily formed a distinct clade with its representative genera of *Cuterebra* spp. and *Dermatobia* spp. This result aligns with many studies that support their monophyletic relationship [21, 30, 33, 36]. However, the position of Cuterebrinae relative to the other subfamilies may differ. In this study, it showed a paraphyletic relationship with Gasterophilinae + Oestrinae and a polyphyletic relationship with Hypodermatinae. Several studies that included only *Cuterebra* species as representatives of the subfamily found that it exhibited a polyphyletic relationship with the other three subfamilies [30, 33]. Moreno [21] supported its paraphyletic status with Hypodermatinae and Gasterophilinae, while indicating polyphyly with Oestrinae. Karademir [36] suggested that Cuterebrinae is a sister group to Hypodermatinae, as they share a recent common ancestor.

The relationship between Hypodermatinae and the other subfamilies was illustrated to be paraphyletic. Hypodermatinae's position is relatively ambiguous in different studies relative to the other subfamilies. Some studies assigned it as a sister group to the subfamily Gasterophilinae [21, 30, 33], while others related it to the subfamily Cuterebrinae [36]. Otranto and Stevens [7] showed a monophyletic relationship with *O. ovis* (Oestrinae) when using the entire COI. This subfamily is mainly represented by the two genera: *Hypoderma* sp. and *Prezhevalskiana* sp. In this study, the different species corresponding to *Hypoderma* (*H. bovis*, *H. sinense*, and *H. lineatum*) showed a monophyletic relationship. This aligns with all the other studies that included this genus in a phylogenetic relationship. However, when using *Przhevalskiana silenus*, this species exhibited a polyphyletic relationship with *Hypoderma* sp. and all the other subfamilies. Regarding *Prezhevalskiana* species, several studies reported them to have a monophyletic relationship with the *Hypoderma* sp. clade [21, 30].

2.4 Conclusions

This work illuminates the role of molecular approaches in identifying and assessing the evolutionary profile of the obligate endoparasitic larvae within the family Oestridae. Additionally, it paves the way for future phylogenetic studies on the camel nasal bot fly, *C. titillator*, given the ongoing need for greater taxonomic confidence and the clarification of its position within the subfamily Oestrinae, which is not yet established as monophyletic. Further molecular investigations encompassing the entire mitochondrial genome can provide a comprehensive overview of the entire family Oestridae.

3 Methods

3.1 Larvae sample collection and morphological identification

Between 2019 and 2020, the 2nd and 3rd larval samples were obtained from slaughtered camels at the El-Bassatin abattoir in Cairo Governorate, Egypt (29° 59′ 45" N, 31° 16′ 34" E). Most of the camels tested had been brought from Sudan and were over three years old. A total of 62 butchered camel heads were separated from the body, and the skulls were incised through the throat to reach the nasopharynx, where the presence of the larvae was checked in the nasal and pharyngeal cavities.

The obtained larvae were collected in sterilized plastic cups labeled with the date of collection and then transferred to the Department of Entomology laboratory. In the laboratory, the live larvae were washed with tap water to eliminate any remaining camel tissue mucus, identified based on the features indicated by Zumpt [20], and subsequently differentiated into 2nd and 3rd larval instars. These were then preserved in 70% ethyl alcohol for molecular studies.

3.2 DNA extraction and molecular analysis

Ten mature 3rd instar larvae were used to extract total genomic DNA following the manufacturer's manual for the commercial kit (Wizard® Genomic DNA Purification Kit, Promega, USA). Aliquots of 5 µl were taken from each DNA sample and quantified using a NanoDrop spectrophotometer (Thermo Scientific NANODROP 2000). The final DNA concentration in each stored sample was diluted to reach 100 ng/µl.

The amplification of COI gene fragments was carried out using custom-designed COI primers generated with the Primer-BLAST program (Primer designing tool available at nih.gov), based on the published *C. titillator* whole mitochondrial genome [GenBank identification number (ID): NC_046479.1] [11]. The full length of the COI gene in the mitochondrial genome ranges from base number 1625 to 3158, with a total length of 1534 nucleotides. To ensure comprehensive coverage of the most representative COI region for the family Oestridae, which contains variable sequences, we selected a partial region for amplification, spanning from base number 2210 to 3125 (as shown in Fig. 5). The forward and reverse primer lengths were 22 and 20 bases, respectively, designed to amplify a fragment with the expected length of 916 bp (as indicated in Table 3).

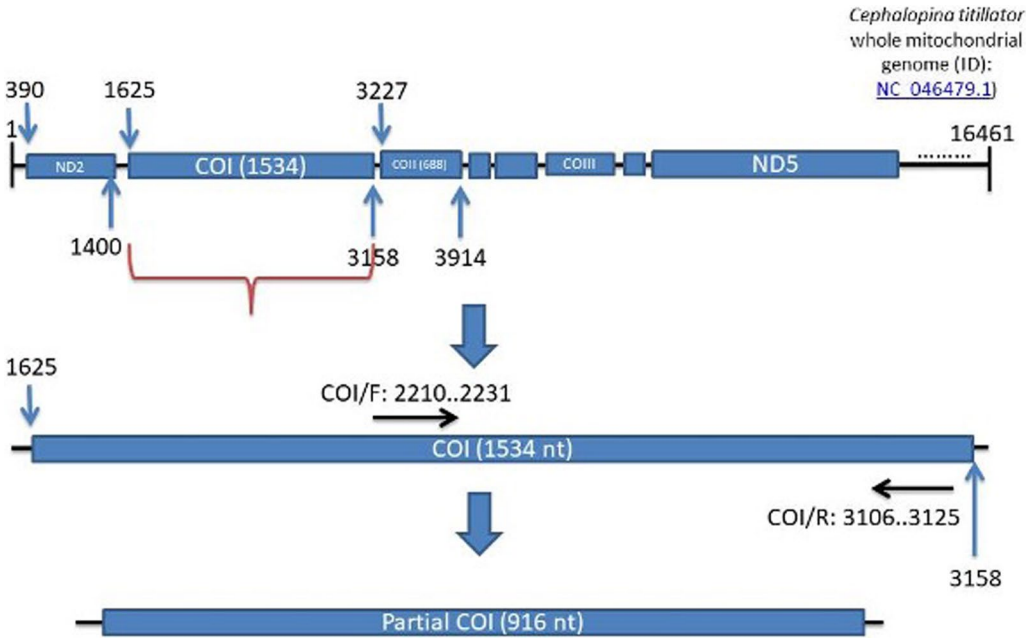

Fig. 5 Diagrammatic representation showing the COI primer design of *Cephalopina titillator* used to amplify the partial COI (916 bp), including the position of forward (COI/F) and reverse (COI/R) primers. The reference sequence was retrieved from the NC_046479.1 whole mitochondrial genome

Table 3 The primer sequences of COI and 28S rRNA markers in both the forward and reverse directions

Primer	Direction	Primer sequence	Expected gene fragment length	References
COI	"Forward" COI/F	5′- TCTCTACCAGTTTTAGCAGGAG -3′	916 bp	Custom-designed in this study
	"Reverse" COI/R	5′- GTTCAGCAGGTGGTGTATTT -3′		
28S rRNA	D1 "Forward"	5′- CCCCCTGAATTTAAGCATAT -3′	830 bp	[18, 21]
	D2 "Reverse"	5′-GTTAGACTCCTTGGTCCGTG -3′		

The second targeted gene fragment, 28S rRNA, was amplified using forward (D1) and reverse (D2) primers, each 20 bases in length. These primers were designed to amplify a gene fragment with the expected length of 830 bp (as shown in Fig. 6 and Table 3) [18, 21]. All COI and 28S rRNA primers were synthesized and obtained from Macrogen Company, Korea.

3.3 PCR procedures and amplicons sequencing

The PCR reaction was carried out in a total volume of 20 µl. This included 2 µl of larval-extracted genomic DNA (DNA template) at a concentration of 100 ng/µl, except for the blank control where it was substituted with nuclease-free water (NF-H_2O). Additionally, 1 µl of each forward and reverse primer for each marker was used at a working dilution of 0.01 nmoles/µl. To complete the reaction mix, 10 µl of ready-to-use amaROnePCR master mix (1X, 250 reactions, Catalogue Number (Cat. No.): SM213-0250) was added. The remaining 6 µl was made up of nuclease-free water.

PCR amplification followed the protocol outlined by Stevens and Wall [18], with minor modifications in the cycle times. The amplification was conducted using a Wee32 HIMEDIA thermocycler, India.

The PCR reaction conditions were as follows: one cycle of initial DNA template denaturation at 94 °C for 4 min, followed by a cycling loop of 35 cycles, each consisting of a denaturation step at 94 °C for 45s, a primer annealing step at 55 °C (for COI) and 60 °C for 45s (for 28S rRNA), an extension step at 72 °C for 45s, and a final extension step at 72 °C for 7 min. Simultaneously with each reaction, a blank reagent was run as a control.

Upon completion, the PCR reactions were held at a final step and stored at 4 °C for immediate analysis or frozen in the refrigerator for further processing.

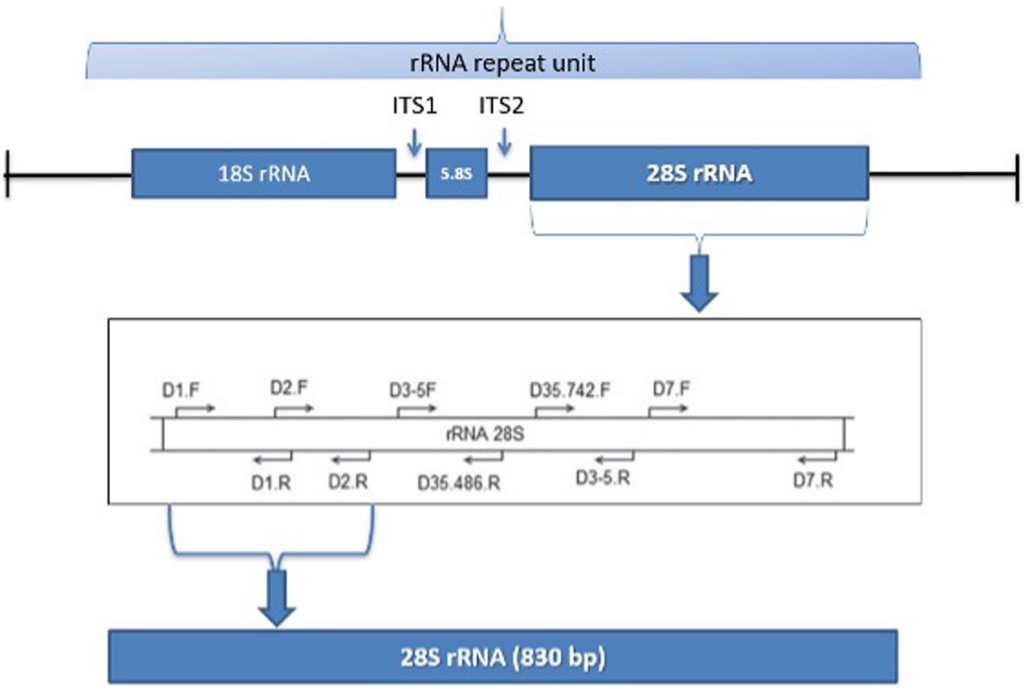

Fig. 6 Diagrammatic representation showing the 28S rRNA D1-D2 primers used to amplify a gene fragment from *Cephalopina titillator* of expected length, 830 bp. Reference of figure [44]

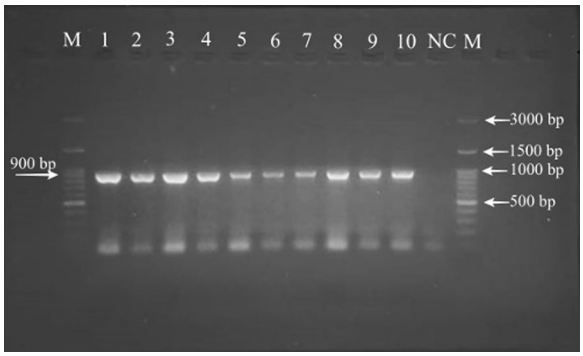

Fig. 7 PCR products of COI gene of *Cephalopina titillator* larvae on 1.5% agarose gel stained with ethidium bromide. Lane M 100:3000 bp DNA ladder, lane 1–10: 916bp amplicon using primers set (Forward & Reverse COI) and lane (NC) blank control

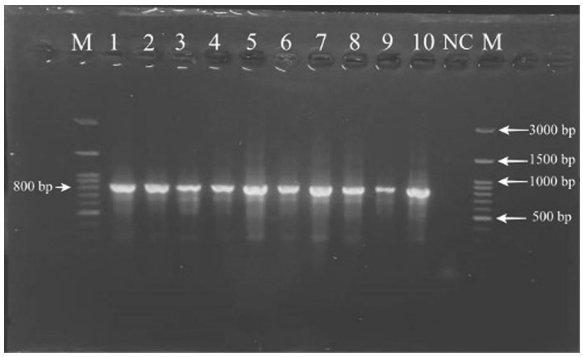

Fig. 8 PCR products of 28S rRNA gene of *Cephalopina titillator* larvae on 1.5% agarose gel stained with ethidium bromide. Lane M 100:3000 bp DNA ladder, lane 1–10: 830 bp amplicon using primers set (D1-D2) and lane (NC) blank control

The PCR products were detected by electrophoresis in a 1.5% agarose-Tris–acetate-EDTA (TAE) gel containing 5 µl of ethidium bromide dye (10 mg/ml stock, Vivantis). The visual assessment was carried out under ultraviolet light using a transilluminator (model: CSLUVTS365L, company: Cleaver Scientific Ltd.). The sizes of the amplified DNA bands for both genes were determined by referencing them to DNA ladder band sizes (100–3000 bp ladder, GeneDirex, Cat. No. DM003-R500) (as depicted in Figs. 7 and 8).

The amplicons from five specimens were purified from the agarose gel using a silica-based spin column PCR Purification Kit (Favorgen, Cat. No.: FAGCK 001) and subsequently sequenced on an Applied BioSystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) at Macrogen, Inc., Seoul, Korea. The sequencing was performed using the Big

Dye terminator cycle sequencing kit v.3.1. Notably, all fragments were sequenced in both directions.

3.4 Data editing and Phylogenetic approaches

The obtained sequences for both gene markers underwent a quality control and base editing process using DNASTAR's Lasergene SeqMan software (V.8) [22]. During the trimming step, low-quality bases from both the 5′ and 3′ ends were removed. In the assembly step, the forward and reverse sequences of each gene marker were aligned, overlapping sequences were excised, and they were combined into a single consensus DNA sequence with no gaps or false joins [22].

The consensus sequences for each sample were employed for comparison on the NCBI-GenBank website using BLAST to retrieve homologous sequences through similarity searches across all available sequence data. Multiple sequence alignment between the generated and collected homologous DNA sequences was conducted using CLUSTAL W [23] within the Molecular Evolutionary Genetics Analysis (MEGA, version 11.0) [24].

The phylogenetic trees were constructed using MEGA-11 with a character-based method represented by the ML method. The best evolutionary model was determined to be the generalized time-reversible model (GTR) with a discrete Gamma distribution, selected based on the lowest Bayesian information criterion (BIC) scores.

Abbreviations

28S rRNA	28S ribosomal RNA
BIC	Bayesian information criterion
BLAST	Basic local alignment search tool
bp	Base pair
C. titillator	*Cephalopina titillator*
Cat. No.	Catalogue number
COI	Mitochondrial cytochrome oxidase subunit I
Fig	Figure
GTR	Generalized time-reversible model
H. lineatum	Hypoderma lineatum
H. bovis	Hypoderma bovis
H. sinense	Hypoderma sinense
inDels	Insertion/deletion
MEGA	Molecular evolutionary genetics analysis
ML	Maximum likelihood
MSA	Sequence alignment analysis
mtDNA	Mitochondrial DNA
O. ovis	*Oestrus ovis*
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
TAE	Tris-acetate-EDTA

Authors contributions

MGS: Assisted in study design and methodology, Provided expertise in entomology and phylogenetics, Reviewed and revised the manuscript, Assisted in the interpretation of the molecular data. SHF: Personally collected and processed the larval specimens, Assisted in data collection and molecular analyses, Conducted all the molecular analyses and sequencing (in case of questions please contact: sherifhamed@sci.asu.edu.eg). EIK: Conceived and designed the study, Provided guidance on study design, Reviewed and revised the manuscript. MAK: Conceived and designed the study. EHG: Provided expertise in entomology and phylogenetics, Assisted in the interpretation of the molecular data, Assisted in writing the first draft of the paper.

Declarations

Ethical approval

This study was ethically approved by the Research Ethics Committee of Faculty of Science, Ain Shams University, Cairo, Egypt (ASU-SCI/ ENTO/ 2023/6/2).

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