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Integrating Morphological and Molecular Approaches for Identifying *Pediculus humanus capitis* and Assessing the Resistance to Certain Pediculicides

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Abstract

Infestations of head lice continue to be a serious public health issue, notably in Egypt. For the implementation of efficient management measures, accurate species identification and researching their treatment approaches became crucial. So, the purpose of this research was to examine the morphological structure of *Pediculus humanus capitis* (*P. h. capitis*) using scanning electron microscopy (SEM) as a foundational study for further investigation into the function of sensory structures and their significance in the host choice. As well as it aimed to study the evolutionary relationships between *P. h. capitis* populations through the analysis of cytochrome c oxidase subunit I (COI) gene sequences, subsequently investigating the host's pediculicide resistance. Participants from Menoufia Governorate, Egypt provided head lice sample specimens. SEM analysis provided detailed insights into the external morphology of *P. h. capitis*, elucidating its features. The resulting COI sequences of Egyptian lice were investigated comparing them with those in different countries. In addition, molecular assessment of topical preparations of pediculicides (malathion, permethrin, phenothrin, and ivermectin) and garlic oil was performed after one and three weeks of treatment of the lice-infested individuals. Acetylcholine esterase (AChE), glutathione-s-transferase (GST), and cytochrome P450 (CYP450) gene expression were used to evaluate the efficacy of anti-lice preparations. The results of this study revealed that SEM of *P. h. capitis* indicated morphological features that could help in further studying the functions of sensory structures. In addition, phylogenetic analysis revealed that the Egyptian samples clustered together with head lice populations from Asia and Africa. Also, it was noticed that increased transcriptional levels of CYP450, AChE, and GST genes in malathion, permethrin, and phenothrin-treated groups suggested some level of resistance to these agents. While ivermectin and garlic oil were recommended at the same time as efficient substitutes. In conclusion, the COI gene sequences of *P. h. capitis* in Egypt are phylogenetically related to other countries. Additionally, molecular analysis revealed that several of the regularly used pediculicides result in resistance in the infested individuals.

KEYWORDS: *Pediculus humanus capitis*, SEM, COI gene, pediculicides, gene expression

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Introduction

Head lice, *P. h. capitis* are obligatory human hematophagous ectoparasites (1,2). This blood-feeding insect has coexisted with humans throughout history, and lives in the scalp area, causing significant discomfort and health concerns, particularly in children with a worldwide distribution regardless of hygienic conditions (3,4). The head louse is characterized by its narrow sucking mouthparts, which are concealed within the head, as well as its three pairs of clawed legs and small antennae have been designed particularly for grasping hair (3). It cannot live independently from its host (5). Head lice infestation, particularly among children, poses a significant public health concern all over the world as well as in Egypt (6). Studies have reported that approximately 22% of school students in Cairo, Egypt are affected by *P. h. capitis* infestations (7).This issue highlights the need for effective strategies to address and manage head lice infestations in the country.

Previous studies utilizing SEM have revealed valuable insights into the morphological characteristics of head lice, including variations in sensory structures, mouthpart adaptations, and other distinguishing features (8,9). However, Despite SEM's potential in the identification of head lice, there is still a need for comprehensive studies focusing on different populations, geographic regions, and variations within species.

Molecular techniques have been also used as a valuable tool for species identification among insects and utilized for ecological, biological, evolutionary, and phylogenetic investigations. Typically, mitochondrial genes such as cytochrome oxidase subunit I (COI) are employed for insect species identification due to high between (inter)-species variability and low with (intra)-species variation (10). Through the analysis of COI gene sequences, researchers can infer the genetic relatedness and evolutionary divergence between different populations of *P. h. capitis*, shedding light on the origin, dispersal patterns, and adaptation of these parasites.

Molecular studies on the mitochondrial DNA have revealed the presence of three noticeably diverse haplotypes of human head lice (11). Furthermore, understanding the evolutionary dynamics of *P. h. capitis* can provide insights into its potential for developing resistance to commonly used pediculicides, an ongoing challenge in public health.

The use of pediculicides has changed worldwide from DDT to carbaryl and malathion, and subsequently to pyrethroids (permethrin and phenothrin) (12). However, continuous usage of pyrethroids led to resistance (13) due to decreased nerve sensitivity (14,15) and higher cytochrome P450 detoxification capacity (16). However, treatment failure was reported in several case studies with carbaryl and malathion from Australia (17) and the United Kingdom (18). Pyrethroids share a common target site the voltage-sensitive sodium channel (VSSC), and act as neuroexcitants (19) in the nervous system. Malathion is an indirect nerve toxin organophosphorus insecticide, that acts as an irreversible inhibitor of acetylcholinesterase associated with the insect cholinergic nervous system. Resistance to commonly used pediculicides, including permethrin and malathion is increasing worldwide (20) and contributing to increased pediculosis incidence (21).

Ivermectin is produced by *Streptomyces avermitilis* and is globally used as an oral anthelmintic agent and used to treat and control head pediculosis (22). Ivermectin acts to paralyze pharyngeal pump muscles thus inhibiting feeding and attachment (23).

Previous reports investigated the implantation of the cytochrome P450 gene in the assessment of the efficacy of insecticides (24). Insecticide resistance is based on an increase in detoxification enzyme levels, or related to the reduction in target-site sensitivity. Such enzymes belong to large enzyme families, including cytochrome P450s, glutathione S-transferase, and esterase genes (25,26).

Herein, the study aimed to describe *Pediculus h. capitis* morphological structure by SEM to serve as a pilot study for additional investigation into the pediculus sensory structure involvement in selecting the right host. In addition, it was intended to explore the evolutionary relationships of *P. h. capitis* populations through the analysis of COI gene sequences. Subsequently, the study was to assess different anti-lice preparations at a molecular level for safe alternatives to chemical ones.

Materials and Methods

Ethical approval:

The study was conducted according to the ethical approval of the Research Ethics Committee, Faculty of Medicine, Menoufia University (6/2023/PARA).

Collection of *P. h. capitis*

A total of 515 randomly selected adult and young student participants from primary, preparatory, and secondary schools, nurseries, and patients attending dermatology outpatient clinics at Menoufia University Hospitals, Egypt were examined and selected for acquiring head lice samples. Using a fine-toothed comb, each student was checked for the presence of head lice. Students identified with active *P. h. capitis* infestations were included in the study. Lice were collected from student heads by hand using sterile gloves and with a small narrow teeth comb morphological structure of *Pediculus humanus capitis* (*P. h. capitis*) using scanning electron microscopy (SEM) comb hairbrush as described by Rain *et al* (27). Live lice were immediately transferred to separate sterile vials containing 70% ethanol for temporary storage until examined with the scanning electron microscope and DNA extraction.

Morphological identification of lice by scanning electron microscopy (SEM)

In this work, the external morphology of male and female *P. h. capitis* adults were described. At the Electron Microscope Unit (EMU), Faculty of Science, Alexandria University, Egypt, collected lice were processed and scanned with a scanning microscope JEOL (JSM-5300). Before SEM analysis, the lice were carefully rinsed with distilled water to remove any residue or debris. Subsequently, they were dehydrated using a series of ethanol solutions with increasing concentrations (e.g., 50%, 70%, 90%, and 100%). The dehydrated lice were then subjected to critical point drying that helps to prevent structural damage to the lice during the drying process. The dried lice were mounted onto SEM stubs. The samples were covered with a thin coating of conductive substance and they were then ready for SEM imaging. The acquired SEM images were analyzed.

Extraction of genomic DNA and quantification

Individually, whole genomic DNA was extracted from each sample following the manufacturer's instructions using a commercial DNA extraction kit (Gene JET Genomic DNA Extraction Kit, Thermo Fisher Scientific, USA) following guidelines particular to louse. Primers targeting the COI gene of *P. h. capitis* were selected based on previously published studies as described in (28,29). According to Folmer *et al* (30), the forward primer sequences were (GGTCAACAAATCATAAAGATAT) and the reverse primer sequences were (TAAACTTCAGGGTGACCAAAAA), in T100 thermocycler (Bio-Rad, CA, USA). The quantity and quality of DNA were assessed using a Nanodrop Qubit 3.0 Fluorometer.

COI amplification and sequencing

The volume of the PCR reaction mixture was increased to 25 µl and comprised 12.5 µl of AmpliTaq Gold Master Mix (Thermo Fisher Scientific, USA), 1µl of forwarded primer, 1 µl of reverse primer, 8 µl of genomic DNA and completed to 25 µl nuclease-free water. The amplification procedure includes a preliminary denaturation stage at 92° C for 4 min, followed by a predetermined number of cycles (40) of denaturation at 92° C for 1, annealing at 48° C for 30s, extension at 72° C for 1 min, and a concluding extension step at 72° C for 10 min. Agarose gel electrophoresis was used to examine PCR results to verify effective amplification. The typical marker was a 100 bp DNA ladder. According to the manufacturer's instructions,

positive PCR products were sequenced using the BigDye Terminator v3. and cycle sequencing kit (Applied Biosystems, USA) and purified using the QIAquick Gel Extraction Kit (QIAGEN, USA). To verify their identification, the acquired DNA sequences were examined using BLAST techniques and compared to known COI gene sequences in databases.

Phylogenetic analysis

Using MEGA11 (28) and the COI gene of head lice from representative populations from various geographical locations, a neighbor-joining phylogenetic tree was created. The collection locality for each specimen was provided alongside its corresponding GenBank accession number on the tree topology with 1000 replicates For the Bootstrap value.

Ex-vivo assessment of topical pediculicides at a molecular level

For bystanders of various ages, WHO (2016) (31) reported that the exposure to malathion should not exceed the tolerable systemic dose (TSD) (8%, 10%, and 20% of the TSD, in adults, children, and toddlers, respectively). Therefore, local solutions were employed in the current investigation in low quantities as directed and permitted. Samples were collected from lice-infested school children, nurseries, and patients attending dermatology outpatient clinics. Three hundred lice-infested individuals who attended to Dermatology Clinic were divided into six groups, each with 50 persons; group 1 included infested persons with no treatment, groups from 2 to 5 included infested persons treated with anti-lice therapeutic preparations containing malathion (0.5%, lotion twice/week, Western Union Ph), permethrin (5%, cream once/week, Misr), ivermectin (5%, lotion once/week, Unipharma) and phenothrin (2%, shampoo once/week, Mash), respectively. Group 6 included infested persons treated with pure garlic oil (garlic essential oil – pure natural therapeutic grade, once/week, NineLife - Egypt). Lice were collected after one and three weeks of treatment.

Gene expression of resistance and detoxification-related genes

Acetylcholine esterase (AChE), glutathione-s-transferase (GST), and cytochrome P450 (CYP450) gene expression were used to evaluate the efficacy of anti-lice preparations. RNA was extracted using the Gene Jet RNA purification Kit from Thermo Scientific (USA). Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used to create cDNA following the manufacturer's instructions. Utilizing the Maxima SYBR Green/ROX qPCR Master Mix Kit from Thermo Scientific (USA), quantitative real-time PCR (qRT-PCR) was carried out in a Biosystem step one plus instrument. After 10 minutes at 95 °C, reaction mixtures underwent 40 cycles of 15 s at 95 °C, 58–60 s at 58 °C, and finally 60 s at 72 °C. The entire analysis was performed using actin as an internal standard. Table (1) includes a list of the primers utilized in the investigation. Three replicates were used for every test group (32).

Statistical analysis:

Data were tabulated and analyzed using one-way analysis of variance (ANOVA), using IBM SPSS software version 25 (New York, NY, USA). The values with $P < 0.05$ were considered significant. Data was presented as means and standard deviation and qRT-PCR data was analyzed through relative quantification (33).

Table 1: List of primers and their sequences used in the current study

Primer name	Forward sequence	Reverse sequence	Accession number
AChE	ACAAGGCGGTGGAAATAGCA	TTAGTGCCACGTTCAGCCAT	AB266614
GST	TTTCCTGTCACCGCATTGGG	TGCCGGACGTTTTTCTATCCA	XM_002426844
CyP450	CCATTTTGGGTACGCGTTGG	ACTTCGTCGTCCAGGAAACC	XM_002425597
Actin	TGCCACATGCTATTCTCCGT	CGGCAGTGGTAGTGAATGAA	(Kwon et *al*, 2014)

RESULTS

Morphological characterization results

In the current study, the findings of the evaluation of the external characteristics of the male and female adults of *P. h. capitis* were presented. Morphological characterization with SEM provides detailed descriptions of species and allows precise examination of subtle morphological variations. Besides mitochondrial DNA identification, it assists the integration of knowledge to develop effective control methods and explore the evolutionary relationships of *P. h. capitis* populations.

The head louse body is dorsoventrally compressed, elongated, and distinct into three body parts (head, segmented abdomen, and an unsegmented thorax with three pairs of clinging legs) (Fig. 1A-1C). The anteriorly located mouthpart is highly sclerotized. (Fig. 1D, 1E). The ventral surface of the head has a deep longitudinal invagination called the buccal slit that interrupts the ventral part of the haustellum, (Fig. 1D). At the distal end of the clypeus near the haustellum, there are two distinct types of sensory hairs indicated as short and long clypeus bristles (Fig. 1F). The cylindrical head has a pair of segmented antennae (five segments) (Fig. 1G) with different antennal setae (Fig. 1H) and scattered antennal bristles (Fig. 1I).

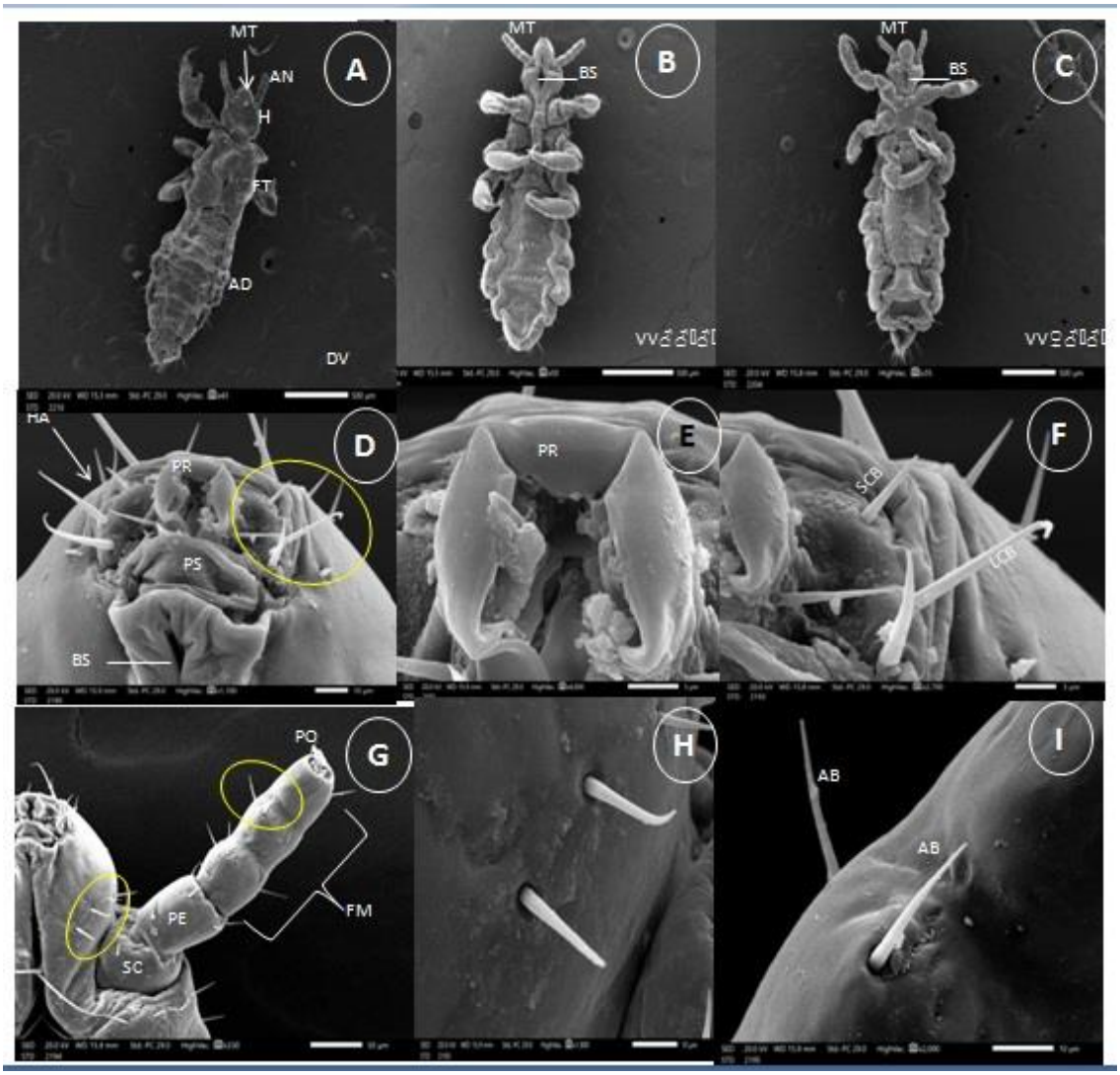

Fig.1: SEM image of *P. h. capitis*, revealing the detailed external morphology of the head louse. The photo highlights (A) the dorsal view of the general structure of the elongated body, (B) the ventral view of the female head louse, (C) the ventral view of the male head louse, (D-F) the ventral view of the apical part of the mouthparts (haustellum) with its surrounding bristles, (G) the segmented antennae, (H) antennal setae, (I) details of the antennal bristles. **MT**: Mouthparts, **AN**: Antenna, **H**: Head, **FT:** Fused Thorax, **AD:** Abdomen, **DV:** Dorsal view, **BS:** Buccal slit, **VV:** Ventral view, **HA**: Haustellum, PR: Proboscis, **SCB:** Short clypeus bristles, **LCB**: Long clypeus bristles, **SC:** Scapus, **PE:** Pedicellum, **FM**: Flagellum, **PO**: Peg organ, **AB:** Antennal bristles

On the apical region of the 5th antennal segment, there is a peg organ (sensilla basiconica), in which 10 basiconica sensilla were seen at its distal end, including four finger-like ones, four with sharp tips, and two with rounded ends (Fig. 2A). Three fused thoracic segments make up the thorax, each of which is equipped with two pairs of legs (Fig. 2B). On each leg, there is a terminal tarsus that possesses a noticeable, articulated projected hooked nonsmooth claw (Fig. 2C). The inner surface of the tarsal claw has three projections (Fig. 2D, 2E). There is only one pair of spiracles on the mesothorax which is located dorso-laterally on both sides.

The abdomen consists of nine segments (Fig. 3C♂, 3D♀), and the posterior dorsal side of each segment has a transverse row of setae (Fig. 3A, 3B). The posterior end of the abdomen differentiates between females and males. In males, it is roundly protruded (Fig. 3C), while in females, this end is branched (Fig. 3D). A detailed morphological structure of the external genital reproductive part of male *p. h. capitis* (Fig. 3E, 3F) showing genital opening with long setae located medial to its edge and short setae covering its anterior border. The abdomen has six pairs of spiracles which are located dorso-laterally on the segments, each spiracle has a plate (Fig. 3G).

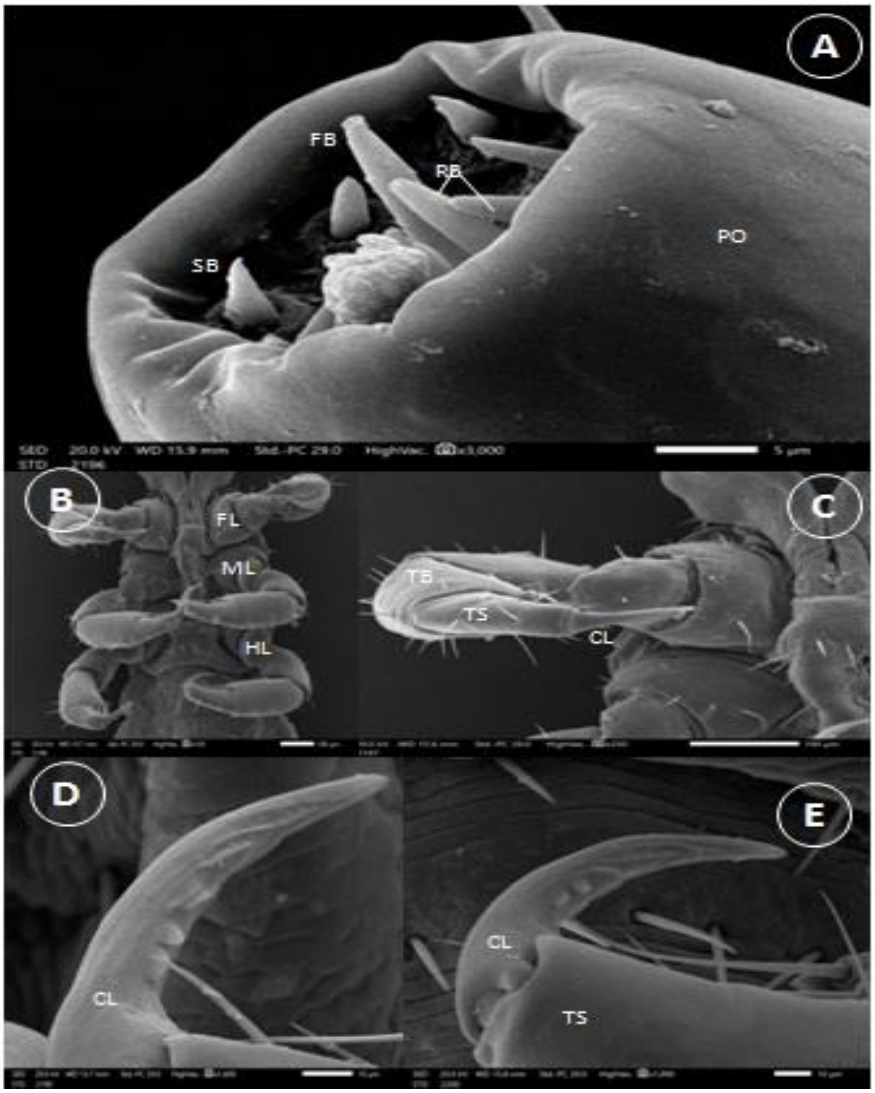

Fig. 2: Scanning electron micrograph depicting (A) Magnification of the distal antennal end (peg organ) with its different types of basiconica sensilla, (B) three clinging legs, (C) detailed structure of one leg, (D, E) the protrusions on the inter surface of the claws. **SB**: Sharp end basiconica sensilla, **FB**: Finger like basiconica sensilla, **RB:** Round end basiconica sensilla, **FL**: Foreleg, **ML:** Middle leg, **HL:** Hind leg, **TB:** Tibia, **TS**: Tarsus

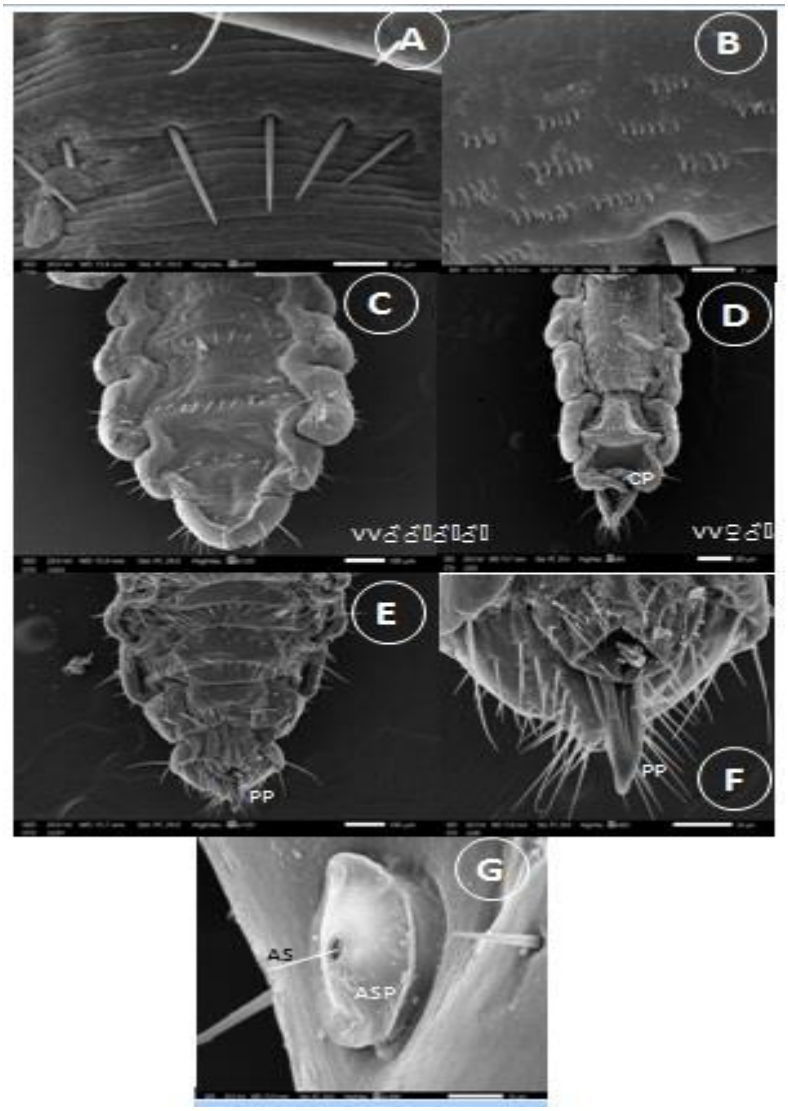

Fig. 3: SEM micrograph displaying (A, B) A high magnification of the ventral side of abdominal segments showing the tiny hairs, (C) the ventral view of male head louse abdomen, (D) the ventral view of female head louse abdomen, (E, F) a detailed structure of the external genital/reproductive part of male head louse, (G) A high resolution magnified abdominal spiracle plate with the abdominal spiracle opening. **CL**: Claw, **CP:** Clasper, **PP**: Pseudopenis, **AS**: Abdominal spiracle, **ASP**: Abdominal spiracle plate.

The overall SEM findings provided information on the distribution of several sensilla types on *P. h. capitis*, facilitating further research into their roles in host preference. Additionally, the inside side of the leg claw had a rough texture; this important discovery may have medical implications in the forceful grabbing of the *Pediculus* in the hair and may be the source of certain alterations to the afflicted scalp.

Sequencing and phylogenetic results

The partial COI gene (650 bp) was successfully amplified in almost all collected head lice from different geographical regions of Egypt. The COI gene from *P. h. capitis*, incomplete cds; mitochondrial (MK913649), shared 99.85–100% similarity with all of these sequences. With the accession codes OR041586–OR041588 and OR050968–OR050973, representative samples were entered into the GenBank. The current research advances our understanding of the taxonomy, population dynamics, and evolutionary adaptations of head lice, helping the improvement of control methods and public health measures. In addition, this study emphasizes the value of performing thorough research that includes many populations,

geographies, and species differences to better our understanding of head lice.

Phylogenetic analysis

The phylogenetic analysis of the COI gene in the current study was compared with samples from different geographical regions worldwide and exclusively revealed the presence of our studied sequences in human head lice from Asia and Africa that belong to the same phylotype lineage (Fig. 4). Also, it revealed that the current amplified sequences from Egypt shared the same haplotype with the head lice of France, Australia, New Zealand, and USA. Such results match the migration route of human head lice based on molecular data of the COI gene from previous studies.

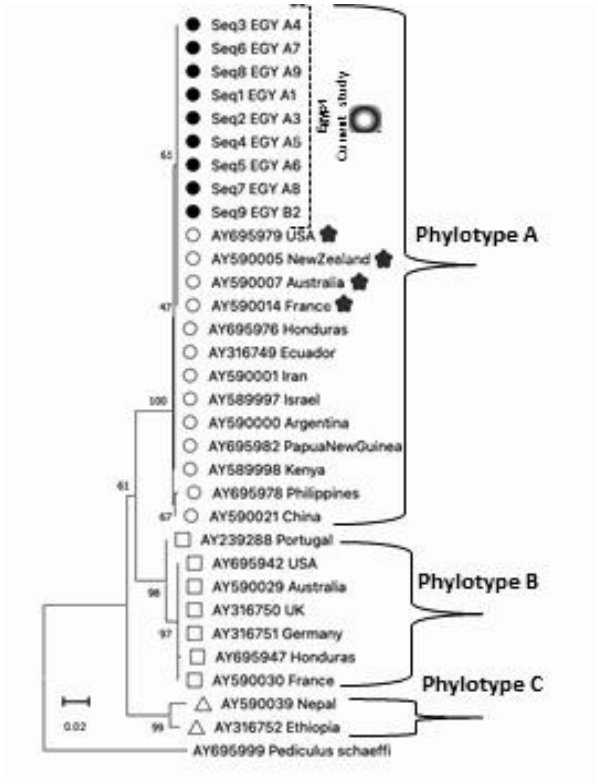

Fig. 4: A neighbor-joining phylogenetic tree was constructed based on the COI mitochondrial DNA gene, revealing the presence of three distinct internationally approved clades exclusively consisting of human head lice. AY695999 *P. schaeffi*, was used as out group. The tree utilized a bootstrap value of 1000, ensuring the robustness and reliability of the inferred relationships.

Expression of detoxification and resistance-related genes in *P. h. capitis*

Based on the follow-up of patients seen at the outpatient clinic, it was found that the efficiency of the lice treatments employed had decreased (unpublished data), suggesting that there may have been an emergence of resistance to these treatments. So, to evaluate and assess the most frequently utilized preparations, a molecular-based evaluation was carried out. The current results demonstrated that the highest up-regulation of acetylcholine esterase, glutathione-s-transferase, and cytochrome P450 genes was observed in malathion and permethrin-treated groups followed by phenothrin-treated groups after one week of treatment compared to control group (Fig. 5). Similar pattern of up-regulation was also observed after three weeks of treatment. However, no significant alterations in the transcriptional level of the above-mentioned genes were observed in ivermectin and garlic oil treated after one and three weeks of treatment groups reflecting its effectiveness and less possibility of raising resistance against them.

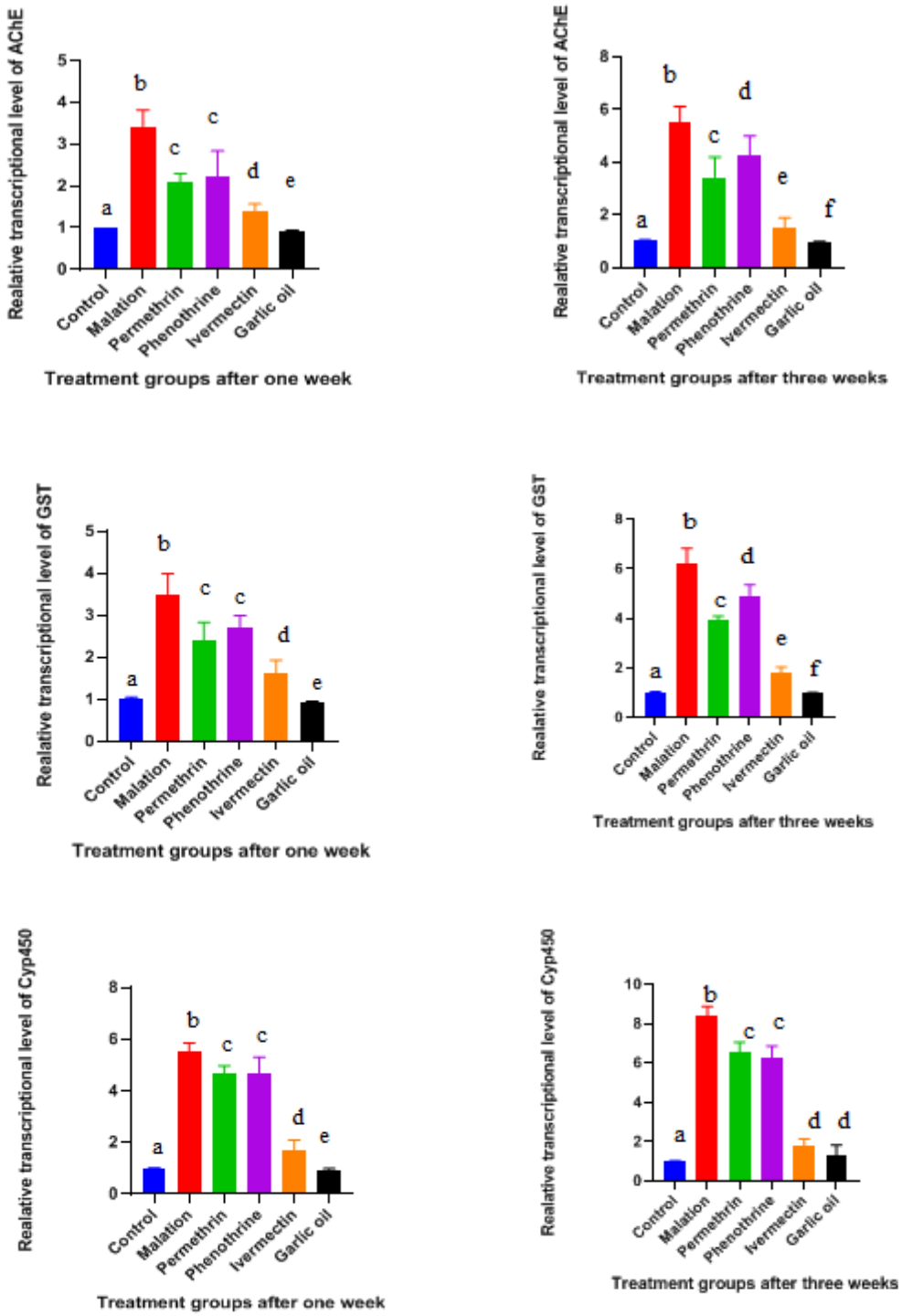

Fig. 5: Representative figure of relative expression patterns of acetylcholine esterase, glutathione-s-transferase, and cytochrome P450 genes in malathion, permethrin, phenothrin, Ivermectin, and garlic-treated groups after one and three weeks of treatment. Results are expressed as means ± SD. Different letters on error bars indicate significant differences between different treatments according to ANOVA (Tukey's) test ($p < 0.05$).

Discussion

Head lice infestation remains a persistent global health concern. The use of scanning electron microscopy offers a valuable approach to investigate the morphological features of *P. h. capitis*. Through a detailed examination of the external structures, SEM can shed important light on the population dynamics, adaptability, and taxonomy of head lice. This study aims to contribute to the existing knowledge base, advancing our understanding of head lice morphology and its implications for effective control measures.

The detection of a live louse, nymph, or viable nit on the head is the most accurate way to determine whether someone has head lice. However, visual inspection without combing poses challenges due to head lice's aversion to light and their rapid crawling behavior (3,34).

Infestations of head lice persist despite insecticidal therapy being available. To highlight certain anatomical features that could be useful in the treatment of head lice infestations, a scanning electron microscopy examination may be beneficial (8). The external morphology of different types of basiconica in the chemoreceptors peg organ (35) was previously well demonstrated (8). In our examination for the present study, we observed and confirmed the findings of the same morphological description. There weren't any considerable variations of physical characteristics, such as body shape, mouthparts composition, antennal shape and composition claw size, and structure spiracle composition (36,8,37) the differentiation between males and females (9).

The main noticeable difference observed compared to previous studies was in the texture of the internal side of the leg claw which wasn't smooth as mentioned before. This may be crucial in the clasping of the host's hairs during oviposition and the lice's adaptability to being attached to the hair for a prolonged period. This agrees with Nunez *et al* (38) whose work provides a secure attachment to host hairs. It has been noted that *P. h. capitis* claws are connected to the substrate to which they are attached, reflecting adaptation to human hair thickness, preventing male louse from losing firmness to attach themselves to females, increasing chances for sex, and preventing female louse from losing firmness to attach themselves to males. The function of legs as taxonomic characteristics that are used to separate species and identify potential metric changes within the same species is illustrated by morphological description using SEM.

Through analysis of the cytochrome c oxidase subunit I (COI) gene, the present study sought to investigate the evolutionary connections of *P. h. capitis* populations. The COI gene, a popular genetic marker, has been successful in shedding light on *P. h. capitis* evolutionary connections (39, 40, 41, 42). The conserved nature of the COI gene across individuals allowed for reliable comparisons, while the presence of distinct sequence variations provided insights into the genetic differentiation among populations. The genetic characterization of head lice from Egypt was successfully achieved through this technique.

Molecular studies on the mitochondrial DNA revealed the presence of three noticeably diverse phylotypes of human head lice (43,44). The most shared phylotype is found among both head and body lice commonly known as (phylotype A) which is widely distributed worldwide. The second type (phylotype B) contains only head lice and has been found in Europe, and Australia. The third type (phylotype C) exclusively has head lice from Nepal and Ethiopia.

By examining the genetic variation and phylogenetic relationships across different geographical regions, we sought to uncover patterns of migration, genetic differentiation, and potential factors influencing the evolutionary trajectory of these parasites. Our phylogenetic analysis of the COI data obtained in the current study with those retrieved from the GenBank confirmed earlier findings of (40,41,43,44) in showing the three distinct clades of the head lice (Fig. IV). Moreover, the genetic sequences of Egyptian head lice exhibited an intriguing discovery, as they demonstrated a shared lineage with head lice from France, Australia, New Zealand, and the USA. This observation aligns with the evolutionary patterns of

head lice's geographical distribution worldwide as previously studied in detail by (40,43,44).

Resistance due to insecticide treatments is considered a major factor in increasing infestation numbers by head lice especially topical pediculicides (13). Suggested resistance mechanisms included acceleration in detoxification of insecticides by reduction enzymes, esterification, and altered acetylcholinesterase (13) which is consistent with the results of the current study. Elevated transcriptional levels of Cyp450 and acetylcholine esterase reported in malathion, permethrin, and phenothrin-treated groups in the current study are confirmed by previous reports stating that mechanisms of malathion resistance involved increased metabolism by Cyp P450, and glutathione-S-transferases genes (45).

Results regarding an increase in AChE and Cyp450 expression in permethrin-treated groups agree with those from Subahar *et al* (46) who reported that the increased activity was associated with resistance of lice to permethrin. Moreover, elevated detoxifying enzyme levels were associated with insecticide resistance in other insect species, i.e. *Anopheles* (47). Karunaratne *et al*, (48) reported that increased AChE activity resulted in increasing its resistance to insecticides. So, it can be deduced that the lice resistance mechanism is probably associated with increased detoxifying enzyme activity. Consequently, these genes are suggested as molecular markers of insecticide resistance assessment.

In this work, mt DNA is used for the first time in Egypt to identify head lice and to evaluate pediculicides at the molecular level. The results of this study will advance our understanding of head lice population dynamics and evolutionary adaptations, which will help us create more effective prevention methods and public health initiatives.

Conclusion

The COI gene sequences used in this study to investigate the evolutionary relationships of *P. h. capitis* populations revealed that the Egyptian samples clustered with head lice populations from Asia and Africa. This study used SEM to report complete morphological details of *P. h. capitis.* Ivermectin and garlic oil are suggested as efficient anti-louse remedies in place of chemical ones by reassessment studies of anti-louse formulations and raised transcriptional levels of the examined genes.

Author Contributions: SHM: Designed the experiments, SHM, SFO, and WAS: Conducted field research and sample Collection, SHM and SFO: conducted laboratory work, analyzed data, and wrote the original manuscript. SFO and WAS: revised and edited the manuscript. All authors read and approved the final manuscript.

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Institutional Review Board Statement:

The study was conducted according to the ethical approval of the Research Ethics Committee, Faculty of Medicine, Menoufia University (6/2023/PARA).

Informed Consent Statement: Written informed consent has been obtained from the patient (s) (or one of his parents if child) to publish this paper.

Data Availability Statement: Data are available within the article.

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Conflicts of Interest:

The authors affirm that there are no conflicts of interest.

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