MOLECULAR DIAGNOSIS OF THE MITOCHONDRIAL DIVERSE GROUP, CLADE A, HAPLOTYPE A5 OF THE HEAD LICE *Pediculus humanus capitis* IN IRAQ WITH THE INVESTIGATION OF ITS PHYLOGENETIC AND SECONDARY STRUCTURE ANALYSIS

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ABSTRACT

This study was aimed to investigate molecular diagnosis of the mitochondrial diverse group (haplogroup and haplotype) of the head lice, *Pediculus humanus capitis* in Iraq, using CYTB gene sequence analysis. For this purpose, louse sampling was performed over a period ranging from 1st April 2021 to the 31th May 2021 among primary school children in the center of Erbil city, Kurdistan region , Iraq. Genomic DNA from isolated sample were achieved followed by amplify a partial sequence of CYTB and sequencing it. All sequences were recorded in International Center for Biotechnology Information (NCBI) under the accession number, OL684637. OL684638 OL684639 OL684640 and OL684641. BLAST results showed that the query sequence was 97.72% similar to *Pediculus humanus capitis*, Clade A, Haplotype A5. (identity percentage is 97.44%) which is consider as a first molecular investigation for the determination of the type of genetic diverse group in Iraq. Phylogenetic tree was constructed and the results appear that there is a phylogenetic close relationship with African mitochondrial genetic diverse group.

Keywords: pediculosis; rna folding; diversity; thermodynamic energy; taxa; erbil.

تشخيص الجزيئي لمجموعة المايتوكوندريا المتنوعة Clade A ، Haplotype A5 لقمل الراس، *Pediculus humanus capitis* في العراق مع استكشاف موقعه الفايلوجيني وتحليل هيكل الحمض النووي الريبي RNA الثانوي

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المستخلص

الهدف من هذة الدراسة هو تشخيص الجزيئي لتحديد فئة تنوع الوراثي المايتوكوندرية (Haplogroup و Haplotype) لقمل الرأس في العراق، باستخدام تحليلات الجيننية لي CYTB سيكوينس. ولهذا الغرض، تم أخذ عينات من القمل الراس على مدى فترة تتراوح بين الاول من نيسان 2021 الى 31 مايس 2021 بين أطفال المدارس الابتدائية في وسط مدينة أربيل، إقليم كردستان العراق، ثم عزل الحمض النووي الجينومي DNA من عينات عشوائيا ثم العمل على PCR لسيكوينس CYTB واجراء عملية سيكوينسينك (Sequencing). تم تسجيل جميع سيكونس في المركز الوطني لمعلومات التكنولوجيا الحيوية(NCBI) تحت رقم الانضمام،OL684637 ;OL684638 ;OL684639 ;OL684640 وةOL684641. أظهرت نتائج BLAST أن السيكوينس المعزولة كان 97.72٪ مشابها ل *Pediculus humanus capit*is انحيازيها (alignment) بنجاح مع clade A، haplotype A5 (نسبة تشابه 97.44 ٪) التي تعتبر أول تشخيص جزيئي لتحديد فئة تنوع الوراثي المايتوكوندرية لقمل الرأس في العراق. تم رسم الفايلوجيني لايجاد موقع الفايلوجيني ، واظهرت النتائج بان هناك علاقة الوثيقة بين سكوينس المعزولة .

الكلمات المفتاحية: ثبات RNA، التنوع، الطاقة الديناميكية الحرارية، Taxa، اربيل.

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INTRODUCTION

The association between man and the head lice is one of the oldest relationship and its history return to 10000 years ago (1). Blood sucking head lice belong to Kingdom Animalia, Class: Insecta, Order: Phiroptera, Family: Pediculidae, Genus: *Pediculus*, Species: *humanus* and Subspecies: *capitis* that's obligate parasite on human this highly specialized to suck blood and close association with their host where completed its entire life cycle (12). In spite of the fact that invasion by this parasite does not cause a serious wellbeing issue, couldbean individua l and open wellbeing burdenphysically, mental ly, and socially (30) and the most common health related in early 6-12 aged group children with the intensive of the scalp is the main clinical symptoms of this parasite in addition initiate of secondary infection by various other microorganism that caused due to head lice biting as described by Madke and Khopkar (26). Strong phylogenetic considers of human lice based on mitochondrial DNA, mainly cytochrome b (CYTB) and cytochrome oxidase subunit 1 (COX1) qualities, have deduced *P. humanus capitis* into six unique mitochondrial clades (haplogroups): A, D, C, E, B and F, each with distinct geographical conveyance and haplotypes (24, 11, 9 and 3). Head lice clade determination can have achieved either by utilizing clade-specific quantitative real-time PCR measures that focused on a part of cytochrome b (CYTB) gene particular to each of the six clades, as already used by Koyo *et al*. (20) and Amanzougaghene *et al*. (7) or through utilizing mitochondrial gene sequencing as used by Al-Shahrani *et al.* (3). The former is unsuccessful method for the clade determination while the latter is the method of choice as mentioned by Koyo *et al*. (21).

Genetic diversity for each group take place continuously as mentioned by Hammoud *et al.* (16) so it's important to observe all isolated sequences in the present study in order to determine type of its clade (Haplogroup and haplotypes). Cytochrome b is broadly utilized in orderly considers to resolve divergences at numerous ordered levels and the nucleotide arrangement of the CYTB gene contains species-specific data and has been utilized in the diagnosis of macro/microorganism species (13). Since this fragment has by distant the largest ordered representation in nucleotide databases and now, there are a large number of CYTB gene within the GenBank/ NCBI/EMBL/DDJB and this information set is continually developing as mentioned by Parson *et al* (28). Cytochrome b gene is a profitable particle for the developmental connections among individuals, population and species (18). The development of evolutionary trees on the premise of distance methods such as neighbor-joining depends significantly on the exact estimation of the evolutionary separations from the watched grouping dissimilarities (32). Several study on the genetic diversity on other organisms (rather than head lice) were conducted in Iraq during the last years including those of Hussein and Jubrael (17); Al-Khalaf *et al.* (2) and Hadi *et al*. (15). The aim of this study is to molecular diagnosis of the head lice, *P. humanus capitis* in Iraq, using CYTB gene sequences. This goes more specifically to find phylogenetic trees of Iraqi isolated head lice sequences with the investigation of its haplogroup and haplotype in addition to its secondary structure prediction analysis.

MATERIALS AND METHODS

Sampling: Louse sampling was performed over a period ranging from 1st April 2021 to the 31th May 2021 among primary school children in the center of Erbil city, Kurdistan region , Iraq. 144 head lice were collected manually from the hair of infested children (student) using lice combe. Each louse was examined with a microscope and identify according to Mullen and Durden (27) as a morphological confirmation step. Photos were taken with HUAWEI NOVA 7i, model JNY-LX1, 2019 and each photo saved electronically.

Prepare and preserve of samples for genomic DNA extraction: Some of collected lice were preserved in 95% alcohol and other in sterile injected water then stored frozen under 0 Cº at home and laboratory refrigerator till use to ovoid its genomic DNA degradation in order to get to good extraction quality. Before DNA extraction ethanol was removed

from parasites. specimens were air-dried to remove ethanol as performed by Amanzougaghene *et al*. (7).

DNA extraction

Genomic DNA from more than random five (5) collected sample were achieved using extraction kit from tissue, Jena Bioscience Animal DNA preparation Kit (Jena Bioscience GmbH .07749 Jena) conferring to the production's instruction with little alterations.

Quantification and qualification of genomic DNA: DNA concentration quantity and quality were achieved using NanoDrop (ND- 1000, USA). The output of genomic DNA samples with more than 0.5µg amount and (A260–A320) / (A280– A320) ratio with greater than 1.7 qualities were achieved.

DNA amplification

Universal primer were designed to amplify a partial sequence of *cytochrome b* (5'-G A G C G A C T G T A A T T A C T A A T C 3'), 20 pmol reverse primer (5'-C A A C A A A A T T A T C C G G G T C C-3'),) as utilized by Li *et. al.* (23). PCR amplification for CYTB partial gene was done in 20 µl of reaction mixture containing; 2x Taq DNA Polymerase Master Mix (AMPLIQON A/S Stenhuggervej 22), 20 Picomol (pmol) of forward promer (5'-G A G C G A C T G T A A T T A C T A A T C-3'),20Picomole (pmol) reverse primer (5'-C A A C A A A ATT A T C C G G G T C C-3'), DNase free water and template DNA (Table 1) by Bioresearch PTC-200 Gradient thermocycler. Temperature profile included step one is an initial denaturation at 95 C for 5 min, step two followed by 35 cycles of a denaturation at 95C for 40 second, a primer annealing at 55C for 45 second, an extension at 72C for 1 min and final step is an extra extension at 72C for 5 min.

Table 1. Cytochrome b PCR Amplification Reagents

PCR components	Concentration	Volume (µl)
Master Mix	2x	25
Forward Primer	20 Pmol	2
Reverse Primer	20 Pmol	2
DNase free Water	-	18
Template DNA	50ng/µl	3
Total		50

Agarose gel electrophoresis separation

According to Lee *et al.* (22) a total of 0.5- 2% of agarose powder was melted in 1X TBE buffer and heated by microwave until clear solution was obtained. The solution was allowed to cool to about 45-37C°, by swirling the flask. 5-10µl of ethidium bromide solution was added to the melted agarose gel and mixed well. The melted agarose solution was poured into the casting tray and was allowed to solidify. The combs were pulled out carefully. The gel was placed in the electrophoresis chamber. Enough TBE Buffer was added so that there is about 2-3 mm of buffer over the gel. 2-3µl of 6X loading buffer staining was mixed to 5 µl of DNA extracted each samples and pipetted into separate wells in gel . DNA ladder (100- 1500 base pair) was carefully pipetted into separate well in the gel. For loading of PCR products were same procedure but samples have not need to mixing with 6X loading buffer staining because AMPLIQON master mix was contained red loading buffer staining. The electrode wires were connected to the power supply, making sure the positive (red) and negative (black) were connected correctly. The power supply was set to about 50-100 volts. The power supply was turned off after the samples had run sufficiently, the gel was removed using gloves and visualized under U.V. light. photographed and documented using UV trans-illuminator.

Sequencing of DNA

Five samples of PCR product cytochrome b partial gene have sequenced by ABI Prism Terminator Sequencing Kit (Applied Biosystem) at Macrogen Molecular Company in Korea. Chromatograms of cytochrome b gene were checked for editing using Finch TV program software.

Sequence analysis

The isolated sequences had been submitted To NCBI (National Center for Biotechnology Information) in order to obtain on accession number. Sequences were aligned at Basic Local Alignment Search Tool (BLAST) usinghttps: // blast. ncbi. nlm. nih. Gov / Blast. cgi to comparing and alignment laboratory or query sequence with the same sequencing fragment marker (CYTB) to find out more similarity with *Pediculus humanus capitis.* In order to find phylogenetic position of the

isolated sequences, multiple alignment had been done among several related lice sequences (20 highly query sequence identity >96.96 with the E-value of 1e-25). For this purpose, related sequences were selected and downloaded from NCBI then aligned with each other (adding *Pthirus pubis* as an out group) using Muscle model within MEGA software v.11 (Program Files\ MEGA11 \ MEGA_64.exe). in order to determine type of isolated head lice clade (Haplogroup and haplotypes), a second phylogenetic position of the isolated head lice among several recorded head lice haplotypes of the haplogoupe A, B, C, D, E and F were described as used by Hammoud *et al.* (16). The minimum free energy prediction had been drawn for the secondary structure of the isolated sample sequences using RNAfold web server (RNAfold web serverhttp://rna.tbi.univie.ac.at › cgi-bin).

RESULTS AND DISCUSSION

In this study, DNA sequence of the head lice was a CYTB value of 350bp, the amplified fragment was 350 base pair and the remaining of 321-333bp after editing (Figure 1). Results of the sequencing procedure were re-redo in Korea country several time and the best sequences graph had been recorded then sequenced nucleotide had been submitted to the Gene bank of NCBI (accession number OL684637, OL684638, OL684639, OL684640, OL684641were obtained and recorded). Head lice species are genetically identical to other recorded head lice species present with the same sequencing fragment marker (CYTB) from National Center for Biotechnology Information (NCBI) and the BLAST results showed that the query sequence was 97.72% similar to *Pediculus humanus capitis* with the E. value of 5e-129 which is locate at the significant levels of similarity (<-50) and the query sequence cover of 100% (Figure 2). In order to find phylogenetic position of the isolated sequences, multiple alignment had been done among several related lice sequences (20 highly query sequences identity >96.96 with the E-value >1e-125. For this purpose, related sequences were selected and downloaded from NCBI then aligned with each other (adding *Pthirus pubis* as an out group) using Muscle model within MEGA software v.11 software program (Program Files\ MEGA11\ MEGA_ 64.exe).

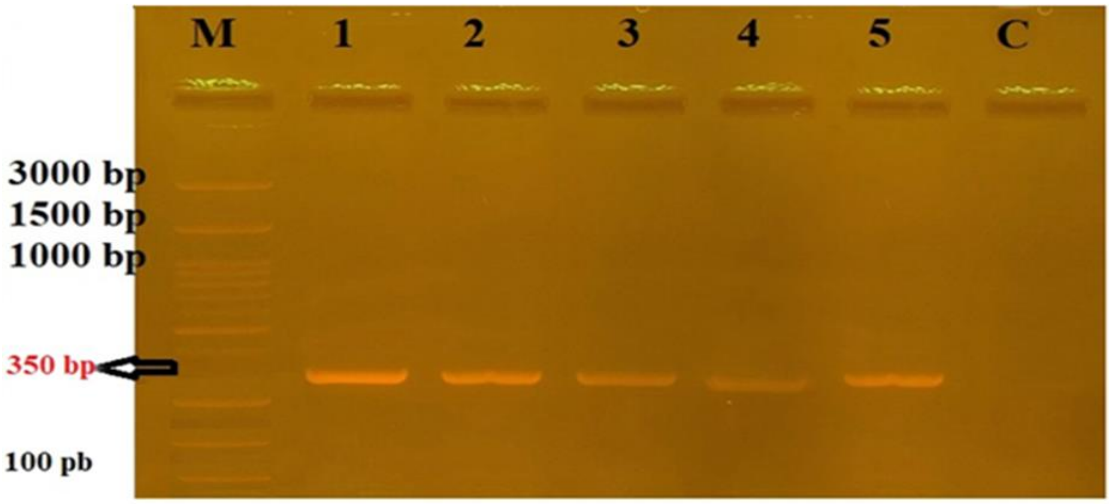

Figure 1. Agarose gel electrophoresis for CYTB gene sequence of the head lice, shows sequence amplification with 350 bp. (Ladder line =100 bp).

Results of multiple alignment shows significant alignment among several genetically related groups. After sequence editing (using jalveiw software program, https: // www. jalview. org / getdown /release/), the phylogenetic tree build (neighbor joining with P-Distance model and 1000 bootstrap replication). Result appear that all isolated sequences make assister groups with each other with bootstrap value of 100% and having a same (common) node ancestor with the other previous recorded sequences of head lice under the accession number, KC685773, KC685791, KC685779 and KC685777 with the bootstrap value of 41% (Figure 3). Head lice clade determination can have achieved

either by utilizing clade-specific quantitative real-time PCR measures that focused on a part of cytochrome b (CYTB) gene particular to each of the six clades, as already used by Koyo *et al*. (20) and Amanzougaghene *et al*. (7) or through utilizing mitochondrial gene sequencing as used by Al-Shahrani *et al.* (3). In order to determine type of its clade (Haplogroup and haplotypes), phylogenetic position of the isolated head lice among several recorded head lice haplotypes of the haplogoupe A, B, C, D, E and F were described as used by Hammoud *et al.* (16). From a total of eighteen different haplotype sequences of the head lice (clade A, B, C, D, E and F) which was selected from NCBI, only 4 sequence were blasted and aligned successfully with all isolated sequences (OL684637, OL684638, OL684639, OL684640 and OL684641). Results appear that all isolated sequences were successfully aligned with the Clade A haplogroup, haplotype A5 (identity percentage is 97.44%, Figure 4). Present study measure only the isolated sequence, OL684637 in all calculation because depending on the blast result, this sequence having high percentage of sequence identity (97.44%) than other isolated sequences with the expected value of 4e-115 (Table 2). The neighbour joining tree was drawn, using P. distance model with 1000 bootstrap replication. Tree was constructed with a significant bootstrap value between of 40-99% and the isolated were located at position clade A, Haplotype A5 (KM579542.1) with a bootstrap value of 72% and make a sister group with previously isolated haplotype A57 (KX444540.1) and A61 (MF672002.1) with a bootstrap value of 97%. It is important, to mention that the isolated sample have a common node with clade D, haplotype D62(X249768.1), D67 (KX249773.1) and D75 (H230923.1) with a bootstrap value of 82%as represented in Figure (5). Clade A is most prevalent haplogroup that's present in most countries throughout the world (16) while clade D is recorded from Democratic Republic of Congo for the first time by Drail *et al*. (11) then it was recorded in South Africa, Egypt and Pakistan (10) followed by Ethiopia (6). In Iraq previously, head lice haplogroup and its haplotypes were not reported, so the present study confirmed the occurrences of haplogroup A and its haplotype A5 through using of sequencing technique for the first time in Iraq and investigation of its phylogenetic close relationship with African clade D haplotypes, D62, D67 and D75 consider the first attempt in Iraq and second one in the middle East. It's very important to point out that the analysis of the secondary structure prediction is important which will be compared with the reference structure to measure prediction accuracy as mentioned by Zhu *et al.* (33). For this purpose the minimum free energy prediction had been drawn for the secondary structure of the isolated sample sequences, OL684637, OL684638, OL684639, OL684640 and OL684641(-68.8 kcal/mol, -75.00 kcal/mol, -69.00 kcal/mol, -70.60 kcal /mol and -69.60 kcal / mol respectively) using RNA fold web server (RNAfold) web serverhttp://rna.tbi.univie.ac.at › cgi-bin). For sequences compression with other closely related sequences, several other previous recorded clade A haplotypes under the accession number MF672002, KX444540, KM579542 and clade D haplotypes sequence were used under the accession number, KX249773, KX249768 and MH230923 (which are reported by Hammoud *et al.* (16) with the minimum free energy of -57.60 kcal /mol, -56.30 kcal /mol, -57.20 kcal/mol, -51.78 kcal/mol, -46.20 kcal/ mol and 52.90 kcal/ mol respectively (Figure 6 and 7). All isolated sequences were compared in type and number of loops which are appeared in the secondary structure of the isolated sequences map. Types of loop which are observed in all isolated sequences include, External, Internal, Bulge, Hairpin, Helices and Multi-branch loops closely a similar total number of loops, 36, 35, 33, 36, 42 for each sequence, OL684637, OL684638, OL684639, OL684640 and OL684641 (mean loops number = 36.4) were recorded respectively (Table 3). Relatively similar loop types were observed in all sequences. An External loop only was found in OL684640 while the other sequences without External loops. High number of Internal loops (12 loops) were found in OL684641 while the lowest one (3 loop) was found in OL684640. Both OL684637 and

OL684641 shows minimum Bulge loops while OL684640 with maximum Bulge loops (4 loops). Number of Harpin loops in OL684638 and OL684639 same and equal to 3 loops in each of them. Also similar number of Hairpin loops were observed in all three other sequences (OL684637, OL684640, OL684641). Relatively a similar number of Helices loops were noticed in all isolated sequences, OL684637, OL684638, OL684639, OL684640 and OL684641 (18, 20,18,19,20 loops respectively). Low number of Multi-branched loops were recorded among all study sequences (Figure 8). The present result is closely similar to the results which had been mapped from previous isolated sequences which was recorded by Hammoud *et al.* (16) as mentioned in Figure (9). The value of minimum free energy of all isolated sequences (-68.8 kcal/mol, -75.00 kcal/mol, -69.00 kcal/mol, -70.60 kcal/mol and - 69.60 kcal/ mol respectively) relatively similar (57.20 kcal/mol) to the reference sequences of the haolotype A5 under the accession number KM579542 this differences is due to sequence length , because the minimum free energy (MFE) of ribonucleic acids (RNAs) decrease with the length of gene sequences (Table 4) as mentioned by Trotto (31), so if the length of isolated sequences to downsize, the minimum free energy of the study sample is closely similar (-52.1 kcal/mol) to the MFE of referenced haplotype A5 sequence (-57.20Kcal/mol) as represented in Figure (10). In addition to the similarity in the simple indices of the RNA folding stability which can obtained by divided the MFE by the number of sequence nucleotides, so depending on what mentioned by the later author's hypothesis, both isolated sequences under the accession number OL684637 and haplotype A5 having nearly RNA folding stability index value (-52.1/272bp = -0.19 and -57.20/272bp = -0.21 respectively) as mentioned in Table (4). Previously several other studies had been done on the head lice diversity and clade determination through using mitochondrial cytochrome b gene sequence includes those of Kittler *et al*. (19); Light *et al*. (24); Raoult *et al.* (29); Boutellis *et al*. (10); Drali *et al*. (12); Amanzougaghene *et al*. (6); Amanzougaghene *et al*. (5); Al-Shahrani *et al*. (3); Ascunce *et al*. (8); Louni *et al*. (25); Amanzougaghene *et al*. (4); Koyo *et al*. (20); Haama *et al*. (14); Amanzougaghene *et al.* (7) and Hammoud *et al.* (16).

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Query: Pediculus humanus capitis isolate KARWAN-1 cytochrome b (CYTB) gene, partial cds Query ID:
OL684637.1 Length: 263

>Pediculus humanus capitis voucher B2517H cytochrome b (CYTB) gene, complete cds; mitochondrial
Sequence ID: KC685777.1 Length: 1074
Range 1: 471 to 733

Score:453 bits(245), Expect:5e-129,
Identities:257/263(98%),  Gaps:0/263(0%), Strand: Plus/Plus

Query  1    AGGAGGGTTTTCAGTTAGACATTCTACTTTAGAGCGGCTGTTTACTCTTCACTTTCTTTT  60
            ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  471  AGGAGGGTTTTCAGTTAGACATCCTACTTTAGAGCGGCTGTTTACTCTTCACTTTCTTTT  530

Query  61   ACCGTTTGTCTTATTGGGGCGTCTTGTACCTCACATTATTCTCCTCCACCAACACGGTTC  120
            |||||||||||||||||  | || || ||||||||||||||||||||||||||||||||
Sbjct  531  ACCGTTTGTCTTATTGGGGTTTGTTATAGCTCACATTATTCTCCTCCACCAACACGGTTC  590

Query  121  TAGAAATCCTTTAGGATTGGATTTGGATAGTGATAAAGtttatttttatccttactttta  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  591  TAGAAATCCTTTAGGATTGGATTTGGATAGTGATAAAGTTTATTTTTATCCTTACTTTTA  650

Query  181  tctaaaagatattttaggaggttttgtgtgtttatttttatttgttttgatttgcattta  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  651  TCTAAAAGATATTTTAGGAGGTTTTGTGTGTTTATTTTTATTTGTTTTGATTTGCATTTA  710

Query  241  ttCGCCGGACTTCTTCATGGACC  263
            |||||||||||||||||||||||
Sbjct  711  TTCGCCGGACTTCTTCATGGACC  733
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Figure 2. Pairwise alignment of cytochrome b sequence of the isolated head lice (OL684637). Query is the study sequence and Subject is the GenBank sequenc

CONCLUSION

Several cytochrome b gene sequences were isolated from the head lice, *P. humanus capitis*, clade A, haplotype A5 and recorded under the accession number OL684637. OL684638 OL684639 OL684640 and OL684641 in NCBI which are consider as the first molecular investigation of the head lice in Iraq. Cytochrome b is considering as a best genetic marker for the head lice mitochondrial

clade determination to resolve divergences at numerous ordered levels and determination of the phylogenetic position in addition to compression among types, number, thermodynamic energy and RNA folding stability.

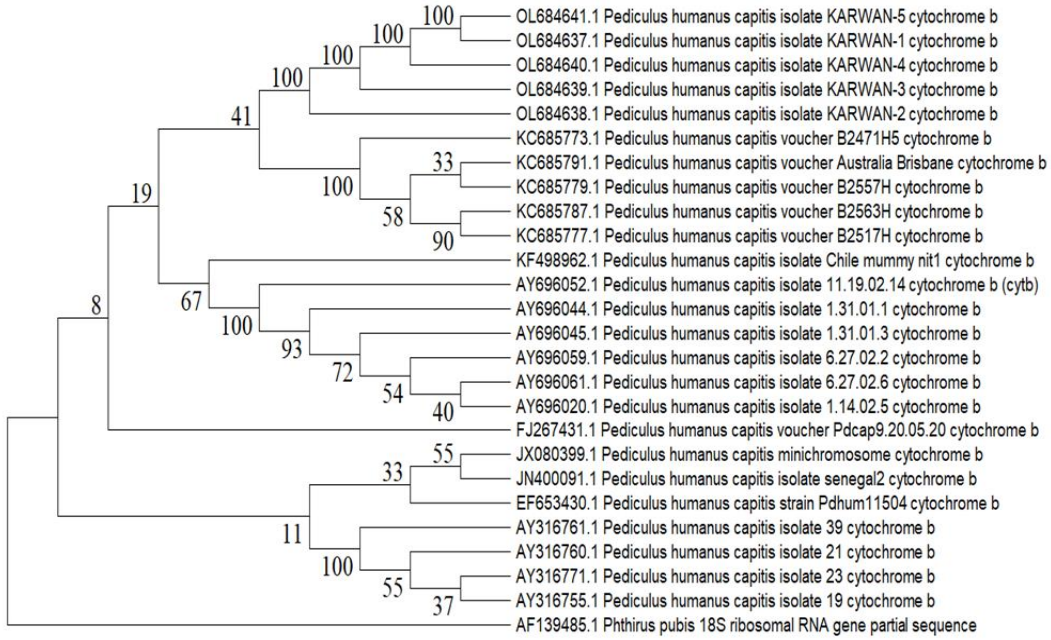

Figure 3. Phylogenetic position of the isolated head lice according to cytochrome b gene sequence represented in neighbour joining tree with bootstrap value

Table 2. Results of sequence blasting with several different haplotypes sequences of the head lice from NCBI

Description	Query Cover	E value	Per. Ident
KM579542.1 *Pediculus humanus* haplotype A5 cytochrome b (cytb) gene, partial cds; mitochondrial	**88%**	**4e-115**	**97.44%**
KX444540.1 *Pediculus humanus capitis* haplotype A57 cytochrome b (Cytb) gene, partial cds; mitochondrial	**88%**	**8e-112**	**96.58%**
MF672002.1 *Pediculus humanus capitis* haplotype A61 cytochrome b (cytb) gene, partial cds; mitochondrial	**87%**	**4e-110**	**96.54%**
MH230923.1 *Pediculus humanus capitis* haplotype D75 cytochrome b (CYTB) gene, partial cds; mitochondrial	**88%**	**4e-95**	**92.31%**

```
Query: OL684637.1 Pediculus humanus capitis isolate KARWAN-1 cytochrome b (CYTB) gene,
partial cds Query ID: lcl|Query_53606 Length: 263

>KM579542.1 Pediculus humanus haplotype A5 cytochrome b (cytb) gene, partial cds;
mitochondrial
Sequence ID: Query_53610 Length: 272
Range 1: 39 to 272

Score:399 bits(216), Expect:4e-115,
Identities:228/234(97%), Gaps:0/234(0%), Strand: Plus/Plus

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            ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  39   AGGAGGGTTTTCAGTTAGACATCCTACTTTAGAGCGGCTGTTTACTCTTCACTTTCTTTT  98

Query  61   ACCGTTTGTCTTATTGGGGCGTCTTGTACCTCACATTATTCTCCTCCACCAACACGGTTC  120
            |||||||||||||||||  | || || ||||||||||||||||||||||||||||||||
Sbjct  99   ACCGTTTGTCTTATTGGGGTTTGTTATAGCTCACATTATTCTCCTCCACCAACACGGTTC  158

Query  121  TAGAAATCCTTTAGGATTGGATTTGGATAGTGATAAAGtttatttttatccttactttta  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  159  TAGAAATCCTTTAGGATTGGATTTGGATAGTGATAAAGTTTATTTTTATCCTTACTTTTA  218

Query  181  tctaaaagatattttaggaggttttgtgtgtttatttttatttgttttgatttg  234
            |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  219  TCTAAAAGATATTTTAGGAGGTTTTGTGTGTTTATTTTTATTTGTTTTGATTTG  272
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Figure 4. Pairwise alignment of cytochrome b sequence of the isolated head lice (OL684637). Query is the study sequence and Subject is the GenBank sequence

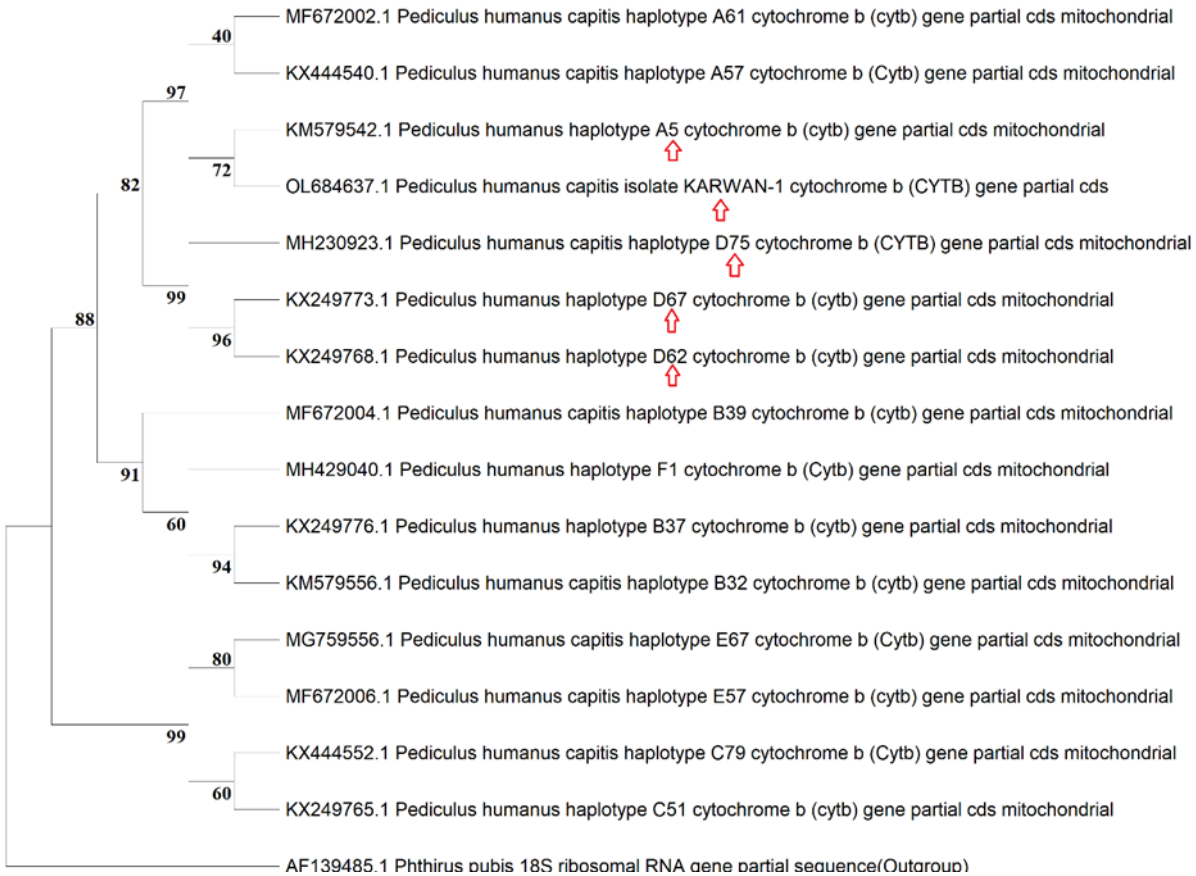

Figure 5. Represent a phylogenetic position of the isolated head lice among several recorded head lice haplotypes represented in neighbour joining.

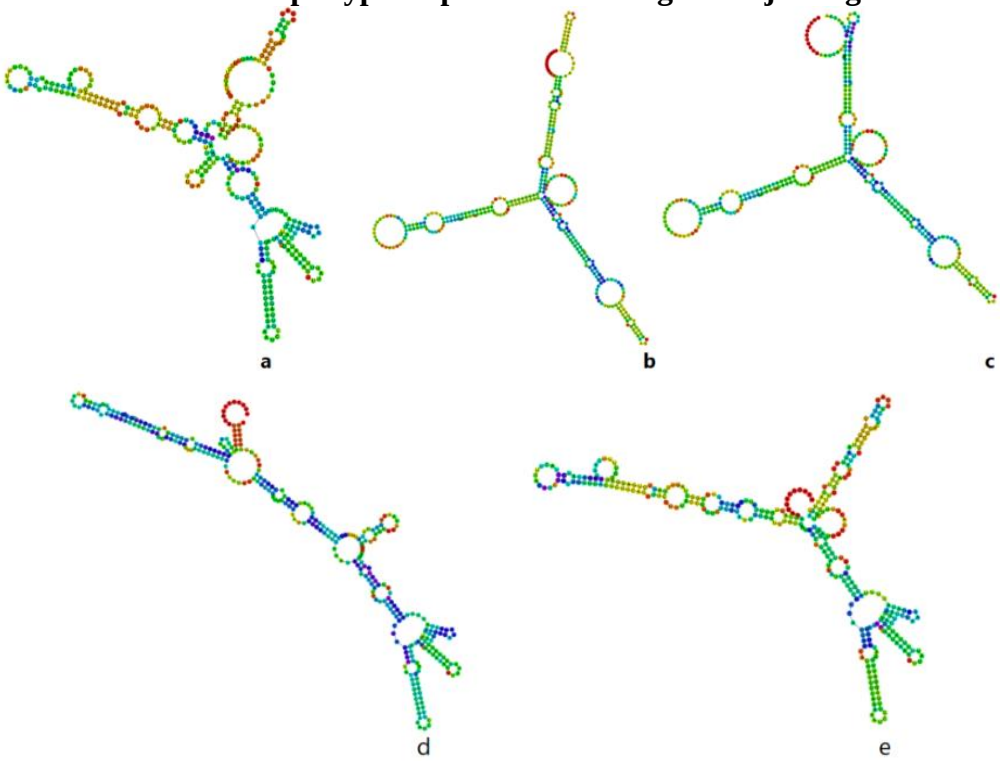

Figure 6. Schematic representation of the Cytochrome b gene expected secondary sequence of the isolated head lice sequences (a, b, c, d and e for OL684637, OL684638, OL684639, OL684640 and OL684641sequnces respectively).

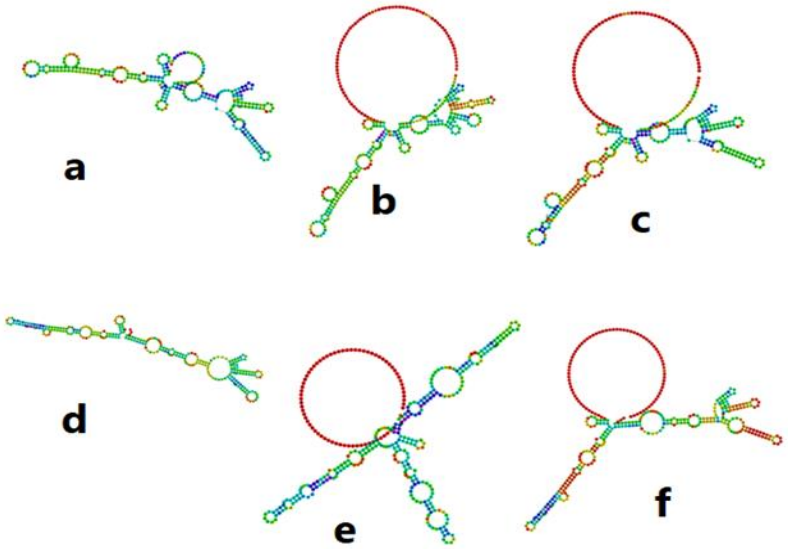

Figure 7. Schematic representation of the Cytochrome b gene expected secondary structure sequence of the reference sequences of the clade A (a, b, c, MF672002, KX444540, KM579542 respectively) and Clade D (d, e, f, KX249773, KX249768 and MH230923 sequences respectively

Table 3. Types and number of loops calculated from secondary structure analysis of cytochrome b sequences of the isolated sample and previous recorded sequences of clade D head lice by Hammoud *et al.* (2021).

Isolated sequences		External loops	Internal loops	Bulge loops	Hairpin loops	Helices loops	Multi-branched	Total loop
OL684637		0	9	1	6	18	2	36
OL684638		0	9	2	3	20	1	35
OL684639		0	8	3	3	18	1	33
OL684640		1	3	4	6	19	3	36
OL684641		0	12	1	6	21	2	42
Reference sequences (clade A)	Haplotypes							
MF672002	A61	0	6	1	7	18	2	34
KX444540	A57	0	5	1	7	17	2	32
KM579542	A5	0	5	1	7	17	2	32
Reference sequences (clade D)	Haplotypes							
KX249773	D67	0	9	3	4	18	1	35
KX249768	D62	0	10	3	5	18	1	37
MH230923	D75	0	6	1	5	18	2	32

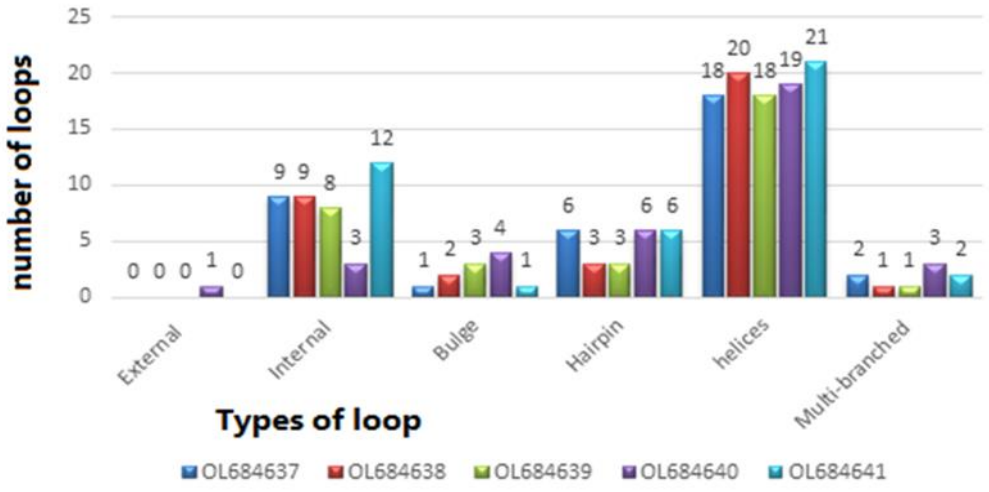

Figure 8. Histogram shows variation in the number and types of cytochrome b sequence loops of the isolated head lice.

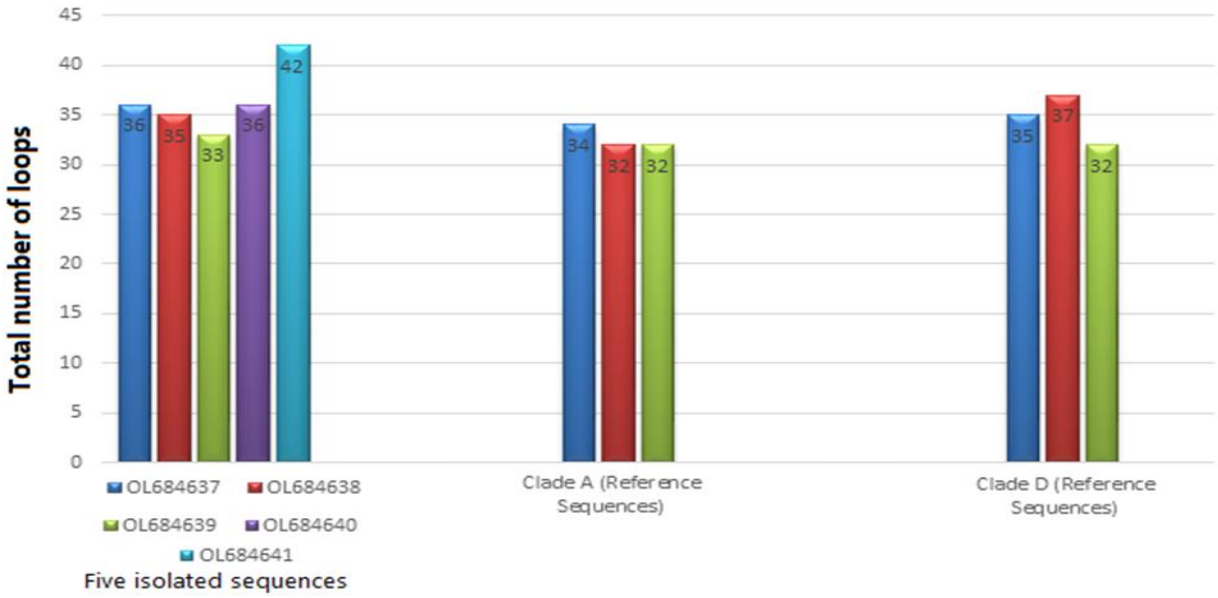

Figure 9. Histogram shows similarity in the number and of cytochrome b sequence loops of the isolated head lice sequences and referenced (Hammoud *et al.*, 2021) sequences of the clad A and D

Table 4. Shows that isolated sequences length versus Minimum free energy (MFE) and RNA folding stability

Isolated sequences	Sequences length (bp)	MFE (Kcal/mol)	Index of RNA folding stability (Kcal/mol)/bp
OL684637	320	-68.8	-0.22
OL684638	333	-75	-0.23
OL684639	322	-69	-0.21
OL684640	322	-70.6	-0.22
OL684641	321	-69.6	-0.22
OL684637	272	-52.2	-0.19
OL684638	272	-52.1	-0.19
OL684639	272	-52.1	-0.19
OL684640	272	-52.1	-0.19
OL684641	272	-52.1	-0.19

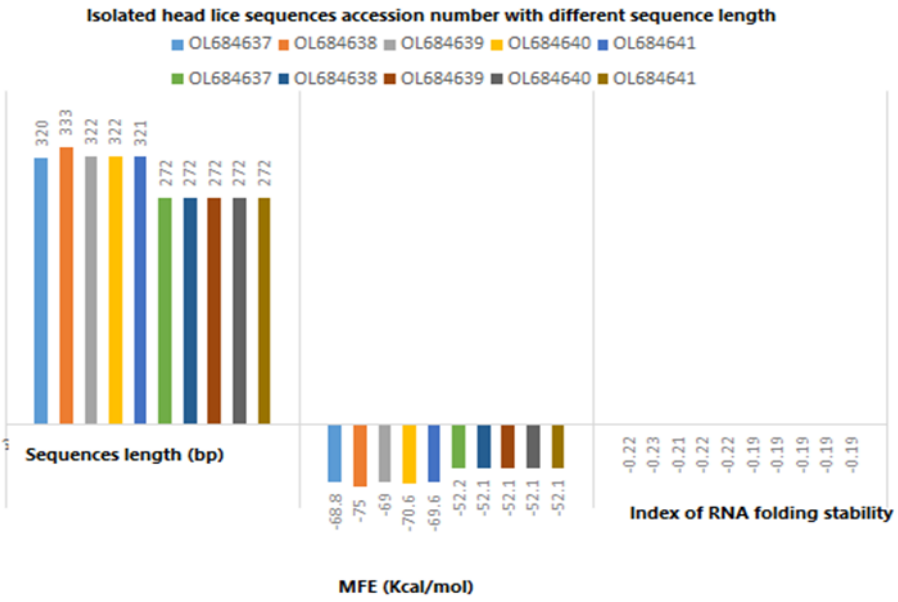

Figure 10. Shows that isolated sequences length versus Minimum free energy (MFE) and RNA folding stability index

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