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Genetic Identification of Meriones spp. The Reservoir Host of Cutaneous Leishmaniasis in the North Western Region of Libya

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ABSTRACT

The identification and classification of reservoir hosts of zoonotic cutaneous leishmaniasis are very crucial towards the integrated management and control planning. Molecular techniques have been deployed and showed very sensitive and specific results to identify and differentiate rodents with great similarity in phenotypes including Meriones spp. Meriones species are very difficult to distinguish and separate according to their morphology and phenotype traits. This study was carried out in western region of Libya and three Meriones rodents were collected and their genetic identification was conducted by PCR technique using designed forward and reverse primers from Meriones spp. cytochrome b (cytb) gene sequence. This study has differentiated three haplotypes of M. shawi. with sequences similarities between samples of haplotypes were 99.13%, 99.42% and 99.71% between H1 and H2, between H1 and H3 and between H2 and H3 respectively. The current work is the first to use molecular techniques to study the genetic sequence of Meriones in Libya and has illustrated that PCR technique is powerful tool to discriminate Meriones species in western region of Libya.

التعريف الجيني للعائل الخازن لمرض اللشمانيا الجلدية Meriones spp. في منطقة الشمال الغربي بليبيا

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الكلمات المفتاحية:
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الملخص

يعتبر تعريف وتصنيف العوائل الخازنة لداء الليشمانيا الجلدي حيواني المنشأ أمرًا مهما من أجل تخطيط إدارة المكافحة المتكاملة. تم إستعمال التقنيات الجزيئية والتي أظهرت نتائج حساسة ومتخصصة جدا لتعريف وتمييز القوارض ذات التشابه الكبير في الأنماط الظاهرية بما في ذلك Meriones spp. يصعب التمييز والفضل بين أنواع Meriones وفقًا لسماتها المورفولوجية والنمط الظاهري. أجريت هذه الدراسة في المنطقة الغربية من ليبيا وتم تجميع ثلاثة قوارض من نوع Meriones والتي تم التعرف عليها جينيًا باستخدام تقنية PCR باستخدام بادئات أمامية وعكسية مصممة من تسلسل جينات (cytb) من Meriones spp . خلال هذه الدراسة تم تمييز ثلاثة

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أنماط فردية من M. shawi مع تسلسل تشابه بين عينات haplotypes والتي كانت 99.13٪ ، 99.42٪ و 99.71٪ بين H1 و H2 ، بين H1 و H3 وبين H2 و H3 على التوالي. تعتبر الدراسة الحالية اول دراسة في ليبيا تستعمل التقنيات الجزئيئة لدراسة التسلسل الجيني لقارض Meriones حيث اضهرت الدراسة إن تقنية (PCR) تعتبر أداة قوية للتمييز بين أنواع Meriones في الجزء الغربي من ليبيا.

Introduction

Zoonotic diseases are transmitted from animals to humans and cause major medical and economical loses worldwide. One of the most important diseases is leishmaniasis, which, is classified into three categories: cutaneous, mucocutaneous and visceral leishmaniasis [1]. Cutaneous leishmaniasis is caused by some species of protozoan genus leishmania named *leishmania major* and *leishmania tropica* that are transmitted by sand flies belong to *Phlebotomine sp.* in old world and to *Lutozomina sp*. in new world. The main reservoir host of cutaneous leishmania is some of rodent's species [2]. Leishmaniasis is an endemic infection in Libya with occurrence in many areas including Jabal, Nafousa, Sirt, Misurata, Bani-Walid and Tarhouna [3, 4, 5]. The first case was recorded in 1910 in Western region of Libya [6] and then hundreds of cases were reported every year. Rodents play a crucial role in the life cycle of cutaneous leishmaniasis [6,7].

About 25 species of rodents were identified to inhibit the country including *Psammomys sp* and *Meriones sp.* [8, 9]. *Meriones sp.* inhibit western cities in Libya and act as reservoir host of leishmania (10, 11] and there are three species were identified as *M. shawi, M. crassus and M. libycus* (9, 12, 13] in Libya including the Jabal Nafousa. Jabal Nafousa is extended to chain of Atlas Mountains between Libya and Tunisia. Ghawar *et al* [14] in his study has said that Meriones is the main reservoir for cutaneous leishmaniasis in Tunis.

Rodents are very dependent on the existence of suitable environment for living and breeding. Therefore, species of *Meriones* cause a devastation to agricultural sector as it feeds on all types of plants and seeds. Their breeding behaviour made it very difficult to be controlled as they reproduce big numbers of offspring in short time [15]. Rodents interspecies in general and especially *Meriones* species can be distinguished from each other using several characteristics according to their morphology and phenotype traits such as colour of fur and tail (shapes and length) and their anatomy [9; 16]. Table 1: shows some differentiation characteristics of *Meriones spp.* by measurement in adult animal [8].

Table 1: shows differentiation of *Meriones spp*. by measurement [8].

Species	Total length	Tail	Hind foot	Ear
M. libycus	252-280 mm	130-143 mm	31-35 mm	16-19 mm
M. crassus	232-235 mm	114-121 mm	30-31 mm	17-18 mm
M. shawi	143 mm	140 mm	34 mm	19 mm

However, there is a disagreement over *Meriones* species classification in Libya between Setzer and Tochi division and Ranck classification, which, indicated the need for using accurate methods such as molecular techniques to classify *Meriones sp.* for establishing successful control program [17, 18].

Molecular techniques are very powerful and can be used to overcome any issues in the differentiation of rodents with great similarity in phenotypes, and also karyotyping techniques could be used for epidemiological studies, whereas identification of reservoir host is very important [19]. Many studies have found that Cytochrome b (Cyt b) gene is very good candidate for identifying small mammal species [20, 21].

Molecular techniques are very sensitive and specific and give precise results by using genetic material from different parts of body tissues and in severe circumstances do not require the presence of reservoir body tissues, as it can use rodents' faeces to extract DNA materials of intestinal (gut) cells to work on [22]. Few studies have been conducted to classify rodents in Libya. Therefore, this study was carried out using molecular tools to differentiate between *Meriones* species in Libya to establish an accurate classification. The current work is the first to use molecular techniques to study the genetic sequence of rodent in Libya

Material and Methods.

Study area and collection of samples:

This study was conducted within the geographical area of western region of Libya. One *Meriones shawi* rodent sample was collected from Arabra area, which, is located between (32º: 10': 59: 31") north and (12º: 50': 31: 67") east, and Two *Meriones shawi* samples were collected Wadilihai area, which is located (32º: 14' 16:31") north and (12º:47' 47:58") east. Three rodents were collected (one Meriones from Alrabta, two Meriones from Wadilihai). All of the rodents were collected by burrows flooding, whereas other techniques such as life traps and back breaker traps did not collect any samples.

Study primers

The designed primers used in the study for *Meriones spp*. were forward primer: Msh875F (5'-CAGCAGACA CAACAACAGCA-3') and Reverse primer: Msh875R (5'CCTCCAATTCA TGTAAGGATAAGT-3') with 875bp product size. The primers were designed from *Meriones spp*. cytochrome b (cytb) gene sequence from GenBank accession no (AJ851267.1) for *M. crassus* and GenBank accession no (KC480078.1) for *M. libycus* using the free online program primer 3 [23].

DNA extraction:

The work was carried out at the Laboratory of National Centre of Disease Control - Libya. The DNA was extracted from liver tissues of the rodents using QIAamp Tissue Kit (QIAGENE @, Hilden, Germany) according to manufacturer instructions.

DNA amplification, purification and sequencing

PCR protocol for DNA amplification composed of 25µl PCR reaction volume containing 25 -50 ng of DNA template, 2.5 µl 10X Buffer **(**QIAGENE**)**, 1.5 mM MgSO4, 0.2 mM of dNTPs, 2.5 µM of each primer and 1 unit of Hot start Taq Polymerase **(**QIAGENE**)**. PCR thermale profile cycling as follow : initial dénaturation at 94°C for 5 minute, follow by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 54°C and 1 min extension at 72°C followed by final elongation step for 10 min at 72 °C. Purification kit (PureLink, nitrogene by life technologies) was used to purify the amplified DAN according to manufacturer's instructions.

Sequencing Reaction

Samples were loaded into Sequencer (Genetic analyses 3500XL). Samples were sequenced using forward primer Msh875F (5'-CAGCAGACA CAACAACAGCA-3') and Reverse primer: Msh875R (5'CCTCCAATTCA TGTAAGGATAAGT-3').

The protocol of sequencing reaction composed of 20µl volume containing: 4µl BDT v3.1 reaction mix, 4µl 5X sequencing buffer, 1µl 3.2pmol/µl primer and 1µl purified PCR product, 10µl deionsied H_2O. it was mixed by flicking and put in the thermocycler using the following profile: initial dénaturation at 96°C for 1 min, follow by 25 cycles of 10 second denaturation at 96°C, 1 min annealing at 53°C and 1 min extension at 72°C followed by final elongation step for 4 min at 60 °C. Purification kit (PureLink, nitrogene by life technologies) was used to purify the amplified DAN according to manufacturer's instructions [24].

Purification protocol.

To precipitate 20 µl sequencing reaction the following protocol was done as follows: remove the PCR reaction tubes from the thermal cycler and briefly spin, add 80 µl deionized water to the reaction, transfer the contents to a 1.5 ml tube. add 20 µl of 125 mM EDTA pH 8.0, mix by inverting 4-5 times, Add 259 µl of 100% ethanol to each tube. Cap the tubes and mix by inverting 4-5 times. Incubate at

room temperature for 20 min. spin the tubes at 14000 rpm for 15 min. Carefully discard the supernatant. Add 300 µl 0f 70% ethanol to each tube, and invert 2-3 times. Spin the tubes at 14000 rpm for 5 min. carefully discard the supernatant. Invert the tubes while open on tissue, to drain the remaining ethanol and dry the pellet at 45°C to 50°C till all alcohol evaporated and dissolve the pellet in 20 µl of Hi-Di formamide. Vortex and spin briefly. Denature the samples at 95°C for 2-3 min and chill at 4°C and finally load the samples in 96-well plate for sequencing.

Data analysis:

Sequencing products were aligned by naked eye using GenBank. Data from MtDNA region (Cytb) were analysed to identify nucleotide composition using MEGA 6. The molecular phylogenetics tree was created using distance-based method MEGA 6. DNAsp program was used for statistical analysis.

Ethical Approval:

All animal experimentations comply with institutional, national and international guidelines. The study and rodents handling protocols were approved by the ethical committee at NCDC of Libya.

Results

The DNA sequencing results showed that the three used samples represent three different haplotypes of *Meriones shawi*. The analyses of the amplified fragment of MtDNA cytochrome b gene sequences for *Meriones spp.* showed 697 bp. with no gaps in between. Three haplotypes were found among the samples due to the diversity in six polymorphic locations as illustrated in table 2.

Table 2. Positions of variable nucleotides *Meriones shawi* haplotypes.

Variable Nucleotides Haplotypes	222	255	262	294	364	495	594	661
Haplotype1	A	C	G	C	A	C	T	T
Haplotype2	G	T	A	T	G	C	C	T
Haplotype3	G	C	A	C	G	C	C	T
Reference *Meriones sp* AJ815264	G	C	A	C	G	A	C	G

The value of haplotype diversity (Pi) was 0.074 and (SD) was 0.272. The similarity between samples haplotypes H1 and H2 is 99.13% and H1 and H3 is 99.42% whereas between H2 and H3 is 99.71% as shown in the table 3.

Table 3. Sequences similarities between sample of *Meriones shawi* haplotypes.

	H1	H2	H3	Ref AJ851264
H1	100 %	–	–	–
H2	99.13%	100%	–	–
H3	99.42%	99.71%	100%	–
Ref AJ851264	99.1%	99.4%	99.7%	100%

Pairwise genetic distances between the haplotypes are given in table 4. The samples showed small genetic distances among the haplotypes and the reference sequence for *Meriones shawi*. (AJ851264). Joining tree for *cyto* b between samples, references and outgroup is illustrated in figure 1.

Table 4: Genetic distance between reference sample and *Meriones shawi* haplotypes.

Species	*Meriones* sp(R)	H1	H2	H3	*M .libycus china*	*M libycus tunis*	*M libycus saudia*
Meriones sp (AJ851264)	.						
H1	0.0088	.					
H2	0.0058	0.0088	.				
H3	0.0029	0.0058	0.0029	.			
Meriones libycus AB381902	0.1460	0.1504	0.1478	0.1460	-		
Meriones libycus JQ927404	0.1628	0.1674	0.1647	0.1628	0.0208	-	
Meriones libycus KC480078	0.1478	0.1522	0.1497	0.1478	0.0014	0.0193	-
Meriones libycus AJ851267	0.1350	0.1428	0.1386	0.1350	0.1113	0.1319	0.1130

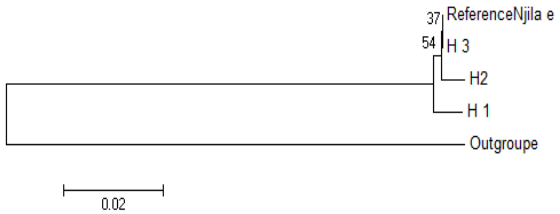

Figure 1. Neighbour joining tree for *cyto* b between samples, references and outgroup.

Discussion

Leishmaniasis is an endemic disease in Libya with occurrence in many parts of the country including Jabal Nafousa, Sirt, Misurata, Zliten, BaniWalid and Tarhouna [3, 4, 5]. The disease requires the presence of rodents as reservoir hosts, which play a key role in the life cycle of cutaneous leishmaniasis. This study was conducted in western north of Libya in Alrabta and Wadi-Alhai areas. These areas are rich with plants that make it suitable habitat for rodents such as *Meriones spp and Psammomys spp*. (6). *Meriones spp*. is distributed in the area of western region of Libya [11]. Three species of Meriones were identified in Libya as *M. shawi, M. crassus and M. libycus* [9]. The identification of reservoir hosts (rodents) is very crucial step in the integrated control management programs [19]. Cytochrome b gene (Cyt b) is one of the most useful tools and very powerful to distinguish between species of rodents in different approaches [20]. The identification was done using molecular techniques (mitochondrial cytochrome b) as this method showed to be very reliable tool [17 and 25]. Three haplotypes of *Meriones spp.* were identified in this study by comparison of their sequences and references samples that were deposited in the gene bank [25]. Few samples were included in this study due to the fact that *Meriones spp.* are very difficult to be collected despite using different techniques for samples collection. Samples of this study were collected from an area of Psammomys, which, was similar to the finding reported by Osborn and Helmy [26]. The molecular study of *Meriones spp.* was done using the designed initiation codes (KC480078) for *M. libycus* and (AJ851267) for *M. cerssus*. The biggest genetic distance between rodents of this study and reference sequence for *M. libycus* was

0.1674, and for *M. crassus was 0.135*. The results confirm that rodents' samples do not belong to both species. The current finding shows that the three haplotypes belong to the species of *M. shawi*. Sequences similarities between samples of *Meriones shawi* haplotypes were 99.13%, 99.42% and 99.71% between H1 and H2, between H1 and H3 and between H2 and H3 respectively (table: 2). Results illustrate that the used technique is powerful tool to discriminate *Meriones* species that found in western region of Libya from each other, as the genetic distance for cyt b gene showed high values and indicates that samples are isolated, and represent true biological species [27]. The current work is the first study used molecular technique on rodents in Libya and has successfully identified different haplotypes of *Meriones shawi*, and indicated that used primers in this study are high-quality for further and similar studies to identify more reservoir hosts of cutaneous leishmaniasis.

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Conflict of interests
The authors declare that they have no conflicting interests.

Author Contributions
All authors contribute in carrying out the work and preparing and reviewing the manuscript.

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