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Molecular Diagnosis and Genetic Variation of Termite Infest Palm Trees

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Abstract

A molecular diagnosis study was conducted on termite insect that invades palm trees. BLAST results showed that most of the samples were matched to the *Microserotermes* sp. with accession number KY224717.1, except one sample matched with *Amitermes desertorum* and *Amitermes vilis* with accession number KU523914.1, KU523912.1, respectively which is Garma-2 with query cover reaching 95.73% , 95.445 respectively. The results of the similarity and likelihood algorithm analysis showed the emergence of two main clusters, the first cluster includes taxa samples of the *Microcerotermes diversus* species with a taxa from the National Gene Bank (KY224717.1) for comparison, the second cluster includes the taxa from Gurma-2 region, which was morphologically classified as *Amitermes vilis*, with the comparison taxa from the National Gene Bank (KU523914.1) as out group (O.G) with a divergence branch length 0.06357. In general, the sequences of the cytochrome c oxidase (COXII) gene of the studied samples matched the sequences of the standard recorded samples in US GenBank with a matching rate of more than 97%, but some samples in phylogenetic tree showed a difference, as they gave a subcluster, which is an indicator of the development of a new subspecies or another species in the future perhaps.

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Introduction

Termite is one of the most important agricultural pests that invade palm orchards in central and southern Iraq(Abdulkader *et al.,* 2011; Al-Dosary, 2006) some areas of southwestern Iran (Latifian *et al.,* 2018) and northeastern Saudi Arabia (Faragalla and Al Qhtani, 2013), causing significant losses by reducing production and control costs (Shefik, 2010). The last taxonomic study conducted on termites in Iraq, more than four decades ago by Alalawi (1987), relied on morphological characteristics In the process of definition and classification.

In recent years, molecular genetic studies have expanded and branched out, but the most important is the determination of a genetic tree of geographically distributed species and higher taxonomic hierarchy, with regard to termites, it faces real challenges in the field of taxonomic diagnosis to differentiate between its species due to deficiency in cladistic analysis

(Kambhampati and Eggleton, 2000), a lack of taxonomic characteristics, a lack taxonomic characteristics, or insufficient taxon sampling and the characteristics that are utilized to classify species are frequently too diverse to make reliable decisions (Boake *et al*., 2002; Vargo and Husseneder, 2009). Recent developments in molecular methods, and more cooperation among researchers using different characters sets a promising solution to these issues (Kambhampati and Eggleton, 2000).

Molecular diagnostic techniques have several benefits that significantly reduce the degree of uncertainty associated with identification using morphological keys, they may be used on individuals of any caste or developmental stage and they can be carried out on a single individual at any time making it possible to conduct more thorough investigations of species distributions, identify species that have been introduced to places outside of their natural ranges (Austin *et al*., 2006; Szalanski *et al*., 2003).

Perhaps one of the most prominent modern techniques that have taken a large part in the field of genetically diagnosing of species is the Polymerase chain Reaction (PCR) technique, which aims to amplify a specific gene, so that the genetic fingerprint of this gene can be known later by knowing the sequence of its nitrogenous bases (Jenkins, 2006; Rock *et al.,* 2006).

Mitochondrial DNA (mtDNA) is also the most common in the field of diagnosing insects species, including termites, mtDNA is simple to use and lack recombination, and fast evolution compared with nuclear DNA (Austin *et al.,* 2007; Dong *et al.,* 2021) .

A number of researchers were able to benefit from genetic molecular analysis techniques to confirm the taxonomy of species Wang *et al*. (2009) confirmed the morphological classification of species belonging to the genus *Reticulitermes* in Indiana, by relying on the techniques of mitochondrial DNA amplification (COI,16S).

As the genus *Reticulitermes* is characterized by ambiguity in the process of classifying its species, it was possible to classify its species through molecular analysis techniques in the states of Georgia, Maryland, and North Carolina, and its geographical distribution (Austin *et al.,* 2007; Cameron and Whiting, 2007).

Also It was possible to distinguish the invasive species *Coptotermes formosanus* Shriaki (Isoptera: Rhinotermitidae) from the rest of the species in the United States of America by using multiplex genetic amplification techniques (multiplex-PCR) due to its economic importance in causing losses to buildings and forest trees (Janowiecki and Szalanski, 2015).

In Egypt, specifically in Sinai, the morphological classification of some termite species was confirmed, its *Microcerotermes eugnathus* Silvestri and *Amitermes vilis* Hagen, It was also possible to save the mitochondrial gene sequences because they were not previously available, in addition to the two species *Microcerotermes palestinensis* Spaeth and *Amitermes desertorum* Desneux by amplifying the mitochondrial gene (COII,16S) (Ghesini and Marini, 2017). After the huge development of definition and classification based molecular studies depend on polymerase chain reaction (PCR) to genomic material (DNA), this study was conducted on species that infest palm trees in southern areas of Iraq to determine the molecular diagnosis and genetic variation between termite species.

Materials and Methods

Collecting samples of termite

Termite Samples were collected from eighteen areas in three provinces of southern Iraq as in Table 1. Samples were collected from palm trees infested with termites during the period of increased population density (out break) of termites from the beginning of October to December in 2022 and 2023. The bases of palm fronds infested with termites were cut and placed inside a plastic box (20 × 30 × 15 cm). These samples were brought to the laboratory under conditions of temperature and humidity (25±1C 0 and 75±3% RH).

Table 1 . Areas of southern Iraq which samples were collected from

Province	Region	Coordinates		No. samples
		N	E	
Basra	Garma	N30.987228	E47.401291	2
	Dier	N30.776181	E47.605675	2
	Zeraji	N30.746176	E47.698911	2
	Chibbasi	N30.590871	E47.800498	1
	Tanuma	N30.514968	E47.872646	2
	Gurna	N30.391694	E47.660827	1
Maysan	Mashtal	N31.794896	E47.195648	2
	Gsebah	N31.804068	E47.150542	2
	Maimona	N31.725455	E47,003573	2
	Kahla	N31.538037	E47.299224	2
	Al-Majar Al-Kabeer	N31.744047	E47.150648	1
	Qalaat Saleh	N31.711996	E47252821	1
Dhi Qar	Alnaser	E31.517569	E46.131368	2
	Alshtra	N31.460957	E46.151747	2
	Kalatsekar	N31.873683	E.46.064601	1
	Alrefaie	N31.765451	E46.104671	2
	Total			27

Morphological diagnosis of termites

In a 3 mL glass vial containing three soldiers, three workers, and three winged individuals with 70% ethyl alcohol inside, were sent to the Natural History Museum in Basra Governorate for the purpose of morphological diagnosis.

Preparing samples for extraction of genetic material (DNA)

The abdominal section of sixty workers of each sample was eliminated to reduce contamination of genetic material with microorganisms commensal in the hind guts of termites, the head and chest section was placed in a 2 mL apendorf tube with a little 70% alcohol.

Extraction of genetic material (DNA)

A special kit was used to extract DNA from Genaid Company in the Molecular Genetics Laboratory in the Department of Animal Production at the College of Agriculture, University of Basrah, and all the steps contained in the protocol attached to the kit were followed.

Mitochondrial DNA (mtDNA) amplification

The samples were sent to the South Korean company Macrogen to amplify the mitochondrial genetic regions, cytochrome oxidase 1 (COX1) and cytochrome oxidase 11 (COX11), using specialized primers, as shown in Table 2.

Table 2. Primers used to amplify mitochondrial DNA (mt DNA)

Primer name	Foreword Primer sequence	Primer name	Reverse primer sequence	Reference
(LCO1490)	5'-GGTCAACAAATCATAAAGATATTGG-3'	(HCO2198)	5'-TAACTTCAGGCTGACCAAAAAATCA-3'	(Murthy, 2020)
AK- F	5'- TACAGCCCACGCATTCGTTA-3'	AK- R	5'-TGCTAGGACTGGCAGGGATA-3'	Designed by Author
A-tLeu	5’-CAG ATA AGT GCA TTG GAT TT-3’	B-tLys	5’-GTT TAA GAG ACC AGT ACT TG-3	(Akoth *et al.,* 2022)

Design of primers and conditions

Temperature gradient (PCR efficiency of each PCR annealing temperature from 48°C ~ 68°C divided into 8 ranges) was conducted for primers LCO1490, HCO2198, AK-F, and AK-R to obtain the best temperature at which DNA fragments could bind to the original sequence as shown in Tabl 3.

Table 3. Temperature gradient for primers

Temperature	Time \ minute	Cycle
95	5	
95	30	35
48-68	30	
72	1	
72	10	
4	Storage	

For primers A-tLeu and B-tLys, a temperature 52 degrees Celsius was used for the binding stage (annealing) when the debonding temperature (denaturation) was 95 degrees Celsius and the rebonding temperature (extension) was 72 degrees Celsius.

Trimming of DNA sequencing

Appropriate tremming was made to the nitrogenous base sequences using the Chromas Pro and BioEdit program.

Forward sequences of mitochondrial cytochrome oxidase II (COXII) 700bp genome fragments were used to compare with sequences of standard samples recorded at the National Center for Biotechnology Information (NCBI) with BLAST tool on web site: https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Drawing of phylogenetic tree

The phylogentic tree of termite samples was drawn using Miga software version 11, adopting the similarity and likelihood algorithm in the drawing.

Submission sample

All sequences are submitted to the National Center for Biotechnology Information NCBI to get a unique accsession no.

Results and Discussion

The results of electrophoresis of samples from the genetic material of termite samples extracted showed that there was no amplification in the polymerase reaction that targeted the mitochondrial gene cytochrome oxidase 1 (COX1) for primers LCO1490, HCO2198, AK-F, and AK-R, despite the grading process being performed as in Figure 1, 8 temperature grades were adopted: 48, 49.4, 51.8, 55.5, 60, 63.9, 66.4 and 68 degrees Celsius, even though Murthy (2020) stated that the amplification process in Primers LCO1490, HCO2198 was successful and effective.

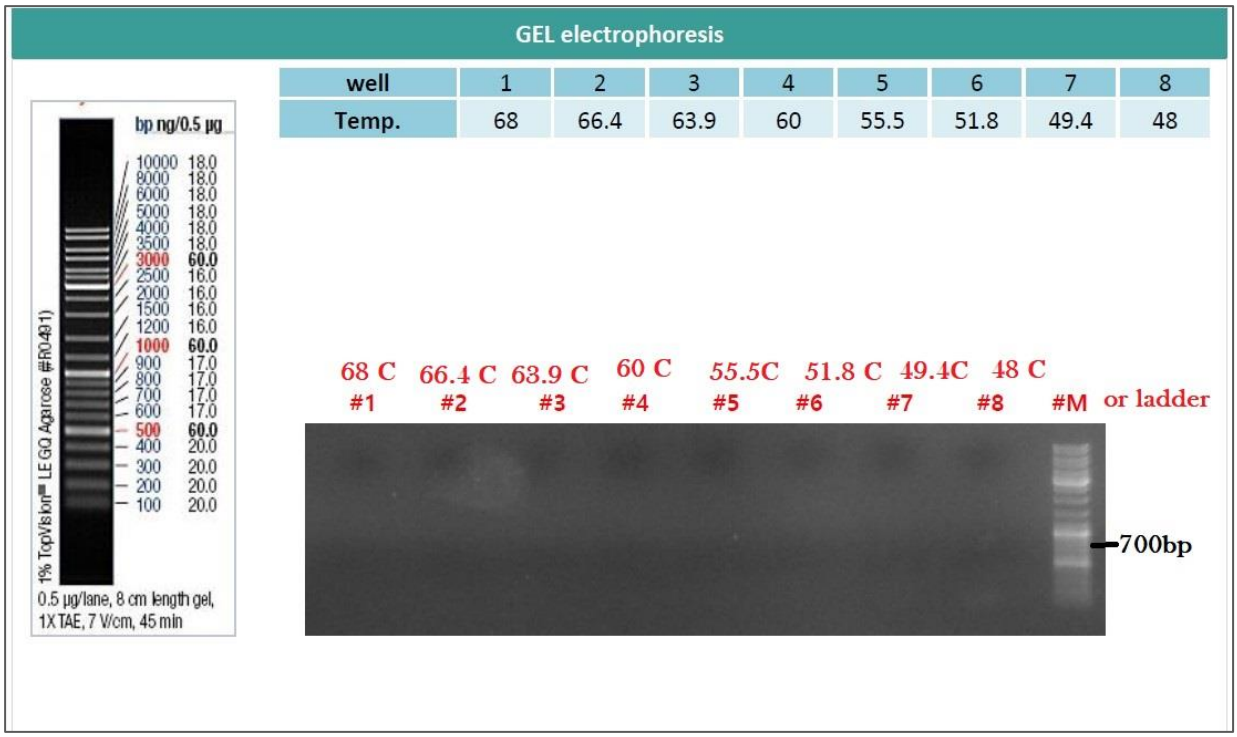


Figure 1. Electrophoresis bands do not appear for some samples amplified by primers LCO1490, HCO2198, AK-F, and AK-R

While samples amplified with the primers A-tLeu and B-tLys, which targeted the mitochondrial gene cytochrome oxidase 11 (COX11) were successful to give an amplicon, This primer also succeeded in amplifying COX11 of termite samples in Akoth *et al.* (2022) showed the possibility of identifying of three genera of termites, *Macrotermes, Amitermes* and *Odontotermes*, using this primer, and the possibility of drawing a phylogenetic tree with the species registered in the National Center for Biotechnology Information (NCBI), that samples showed the presence of bands during the polymerase chain reaction process as shown in the Figure 2, after work will be done on sequencing the nitrogenous bases to be approved and compared with the species registered in the National Center for Biotechnology Information to possibility of drawing a phylogenetic tree of convergence and divergence for termite species that invade palm trees in southern Iraq for the first time and recording their sequences of mtDNA in the National Gene Bank.

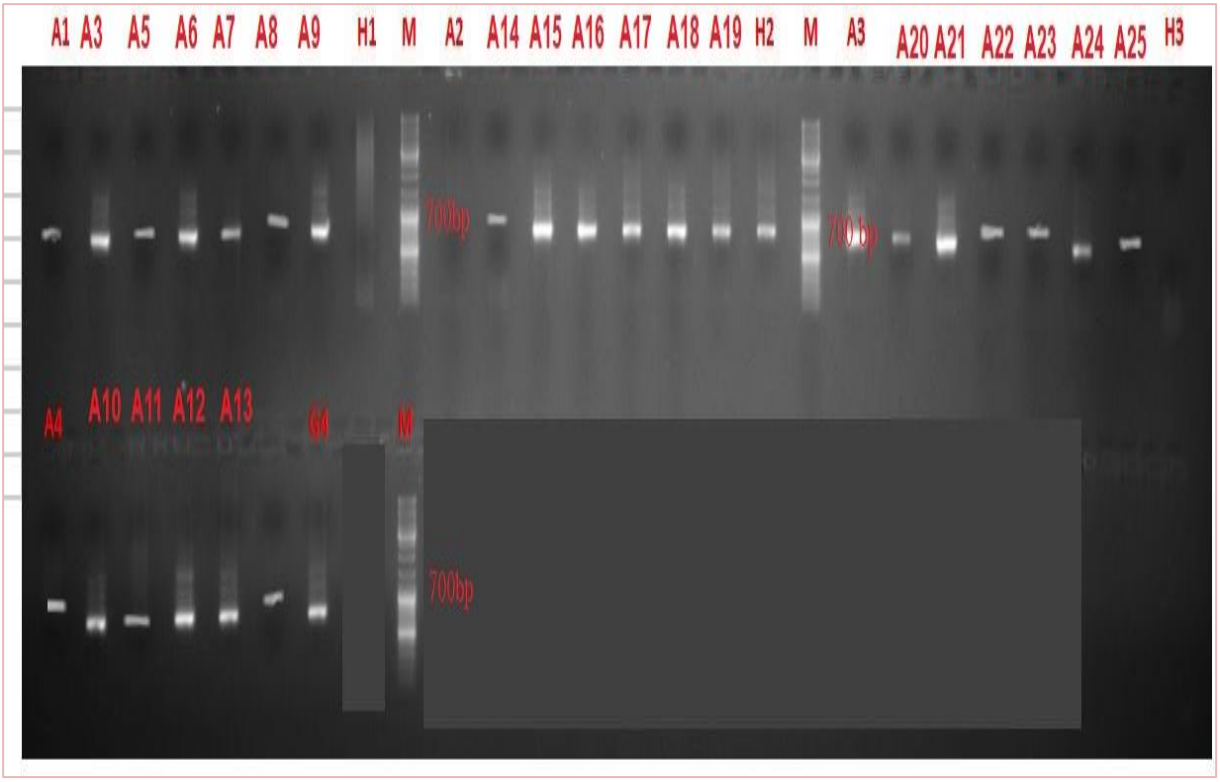


Figure 2. Success of the primers A-tLeu and B-tLys in forming the amplicon and the appearance of DNA bands

Diagnostic results at the Natural History Museum in Basra confirmed the presence of two species of termites in the samples sent after they were collected from the areas shown in Table 1. The first is the species *Microcerotermes diversus* Silvestri and the second species is *Amitermes* vilis . To confirm these results, a molecular comparison of the DNA extracted from the samples was conducted with the species recorded in the gene bank at the National Center for Biotechnology Information to determine the extent of their compatibility and the percentage of query cover between them as in Table 4.

A BLAST tool showed that Most of the samples were matched to the *Microserotermes* sp. with accession number KY224717.1 except one sample matched with *Amitermes desertorum* and *Amitermes vilis* with accession number KU523914.1, KU523912.1 respectively which is Garma-2 with query cover reach to 95.73% , 95.445 respectively. The E. values for each sample was equal to 0.00 that mean all sequences are far away from the accidence arrangement and are a real match with NCBI data base.

The highest percentage of matching with the species registered with accession number KY224717.1 above was in the areas of Qalaat Saleh reaching 97.46% in 100% Query cover. And then comes Garma-1 and Tanuma-1 with matching similarity reach to 97.43% in 100% Query cover.

The lowest percentage of matching was with Gurnna area sample it was 96.58% with Query cover 99%.

It is noteworthy that the sample from the garma-2 was classified as *Amitermes vilis* but

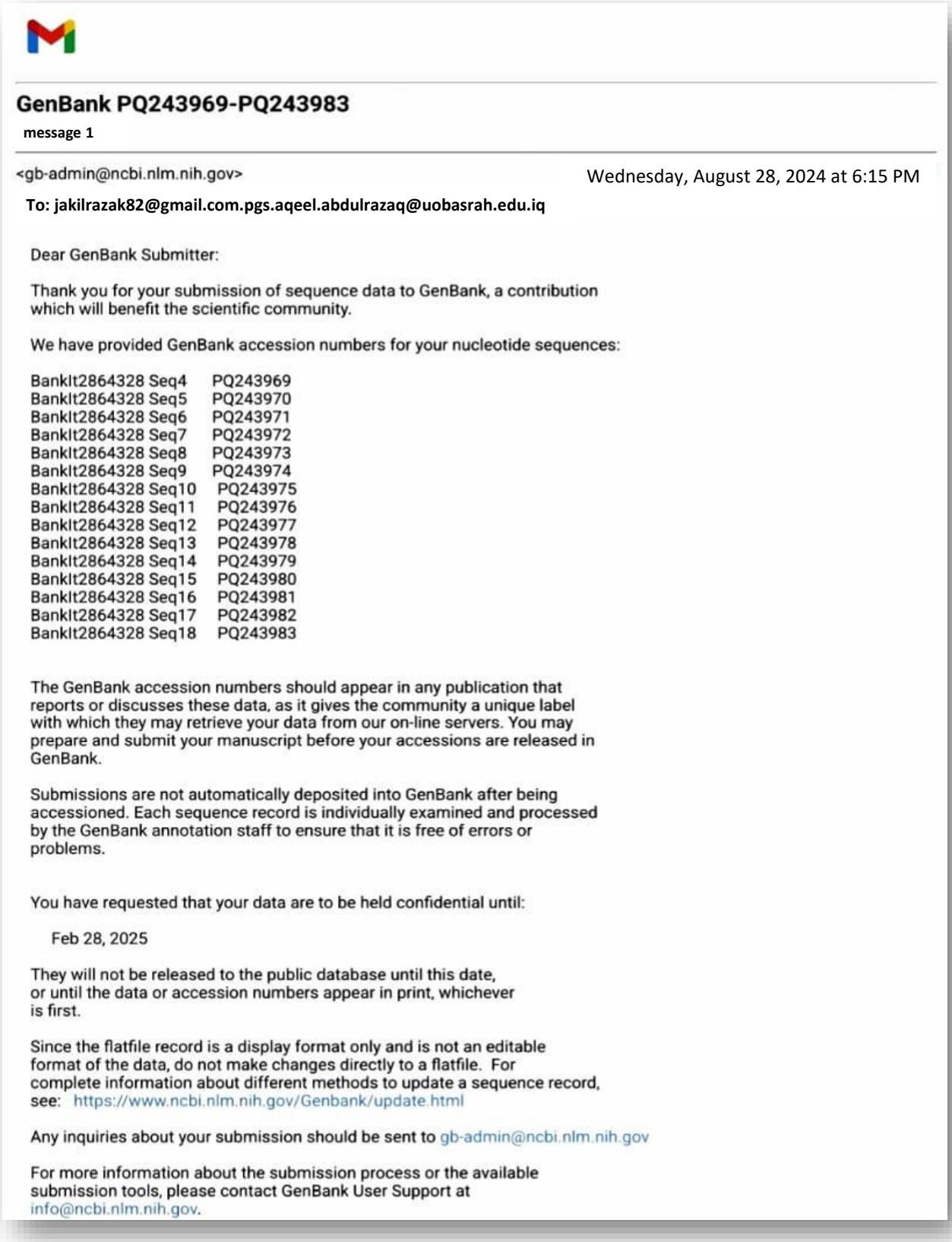
when matched with National Gene Bank specimens it showed great similarity to the species *Amitermes disortiorum* rather than to *Amitermes vilis* this may support the hypothesis of (Akoth *et al.,* 2022; Dong *et al.,* 2021) that confirm this region of the mitochondrial DNA (mtDNA) that Which performs the genetic expression of the enzyme cytochrome C oxidase, which is important in the process of oxidizing respiratory compounds necessary for the living organism sub unit 2 (COXII) generally exhibits high interspecies (Between species) but low intraspecies(same species) separation, indicating that species frequently form clearly defined groupings also Akoth *et al.* (2022) pointed out that some samples may belong to a new taxonomic group due to low sequence similarity with Gene bank samples however, it needs more description based on morphological and chemical features is still required.

It is noted in Table 4 that all sample sequences were submitted to the US National Center for Biotechnology Information (NCBI) for the purpose of registering them and provided them a unique access number for the possibility of accessing and using in subsequent scientific studies, access numbers were indeed granted and are now being processed until their release is completed in February 2025, as in email in which some of those numbers were provided as in Figure 3.

Table 4. Match percentages of termite samples with species registered in the National Center for Biotechnology Information (NCBI)

Sample source	scientific name of the sample after morphological identification	Access number (in processing)	Query coverage	E. Value	Percent identity	Scientific name	Access number
Garma-1	*Microcerotermes diversus*	PQ227059	100%	0.0	97.43%	*Microcerotermes* sp.	KY224717.1
Garma-2	*Amitermes vilis*	PQ227060	97% 97%	0.0 0.0	95.73% 95.44%	*Amitermes desertorum* *Amitermes vilis*	KU523914.1 KU523912.1
Dier-1	*Microcerotermes diversus*	PQ227061	100%	0.0	97.11%	*Microcerotermes* sp.	KY224717.1
Dier-2	*Microcerotermes diversus*	PQ218454	100%	0.0	97.01%	*Microcerotermes* sp.	KY224717.1
Zeraji-1	*Microcerotermes diversus*	PQ218455	100%	0.0	97.42%	*Microcerotermes* sp.	KY224717.1
Zeraji-2	*Microcerotermes diversus*	PQ218456	100%	0.0	97.12%	*Microcerotermes* sp.	KY224717.1
Chibasi	*Microcerotermes diversus*	PQ218457	100%	0.0	97.11%	*Microcerotermes* sp.	KY224717.1
Tanuma-1	*Microcerotermes diversus*	PQ218458	100%	0.0	97.43%	*Microcerotermes* sp.	KY224717.1
Tanuma-2	*Microcerotermes diversus*	PQ218459	100%	0.0	97.23%	*Microcerotermes* sp.	KY224717.1
Gurna	*Microcerotermes diversus*	PQ218460	99%	0.0	96.58%	*Microcerotermes* sp.	KY224717.1
Mashtal-1	*Microcerotermes diversus*	PQ243972	100%	0.0	97.10%	*Microcerotermes* sp.	KY224717.1
Mashtal-2	*Microcerotermes diversus*	PQ243973	100%	0.0	97.41%	*Microcerotermes* sp.	KY224717.1
Gsebah-1	*Microcerotermes diversus*	PQ243974	100%	0.0	97.28%	*Microcerotermes* sp.	KY224717.1
Gsebah-2	*Microcerotermes diversus*	PQ243975	100%	0.0	97.28%	*Microcerotermes* sp.	KY224717.1
Maimona-1	*Microcerotermes diversus*	PQ243976	100%	0.0	97.28%	*Microcerotermes* sp.	KY224717.1
Maimona-2	*Microcerotermes diversus*	PQ243977	100%	0.0	97.28%	*Microcerotermes* sp.	KY224717.1
Kahla-1	*Microcerotermes diversus*	PQ243978	100%	0.0	97.18%	*Microcerotermes* sp.	KY224717.1
Kahla-2	*Microcerotermes diversus*	PQ243979	100%	0.0	97.21%	*Microcerotermes* sp.	KY224717.1
Al-Majar Al-Kabeer	*Microcerotermes diversus*	PQ243980	100%	0.0	97.28%	*Microcerotermes* sp.	KY224717.1

Qalaat Saleh	*Microcerotermes diversus*	PQ243981	100%	0.0	97.46%	*Microcerotermes* sp.	KY224717.1
Alnaser-1	*Microcerotermes diversus*	PQ243982	100%	0.0	97.27%	*Microcerotermes* sp.	KY224717.1
Alnaser-2	*Microcerotermes diversus*	PQ243983	100%	0.0	97.14%	*Microcerotermes* sp.	KY224717.1
Alshtra-1	*Microcerotermes diversus*	PQ243984	100%	0.0	97.10%	*Microcerotermes* sp.	KY224717.1
Alshtra-2	*Microcerotermes diversus*	PQ243985	100%	0.0	97.10%	*Microcerotermes* sp.	KY224717.1
Qalatsekar	*Microcerotermes diversus*	PQ243986	100%	0.0	97.10%	*Microcerotermes* sp.	KY224717.1
Alrefaie-1	*Microcerotermes diversus*	PQ243987	100%	0.0	97.28%	*Microcerotermes* sp.	KY224717.1
Alrefaie-2	*Microcerotermes diversus*	PQ243988	100%	0.0	97.10%	*Microcerotermes* sp.	KY224717.1



GenBank PQ243969-PQ243983

message 1

<gb-admin@ncbi.nlm.nih.gov> Wednesday, August 28, 2024 at 6:15 PM

To: jakilrazak82@gmail.com.pgs.aqeel.abdulrazaq@uobasrah.edu.iq

Dear GenBank Submitter:

Thank you for your submission of sequence data to GenBank, a contribution which will benefit the scientific community.

We have provided GenBank accession numbers for your nucleotide sequences:

BankIt2864328 Seq4	PQ243969
BankIt2864328 Seq5	PQ243970
BankIt2864328 Seq6	PQ243971
BankIt2864328 Seq7	PQ243972
BankIt2864328 Seq8	PQ243973
BankIt2864328 Seq9	PQ243974
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BankIt2864328 Seq11	PQ243976
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BankIt2864328 Seq15	PQ243980
BankIt2864328 Seq16	PQ243981
BankIt2864328 Seq17	PQ243982
BankIt2864328 Seq18	PQ243983

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Feb 28, 2025

They will not be released to the public database until this date, or until the data or accession numbers appear in print, whichever is first.

Since the flatfile record is a display format only and is not an editable format of the data, do not make changes directly to a flatfile. For complete information about different methods to update a sequence record, see: https://www.ncbi.nlm.nih.gov/Genbank/update.html

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For more information about the submission process or the available submission tools, please contact GenBank User Support at info@ncbi.nlm.nih.gov.

Figure 3. A provided accession number for sequences are submitted to NCBI

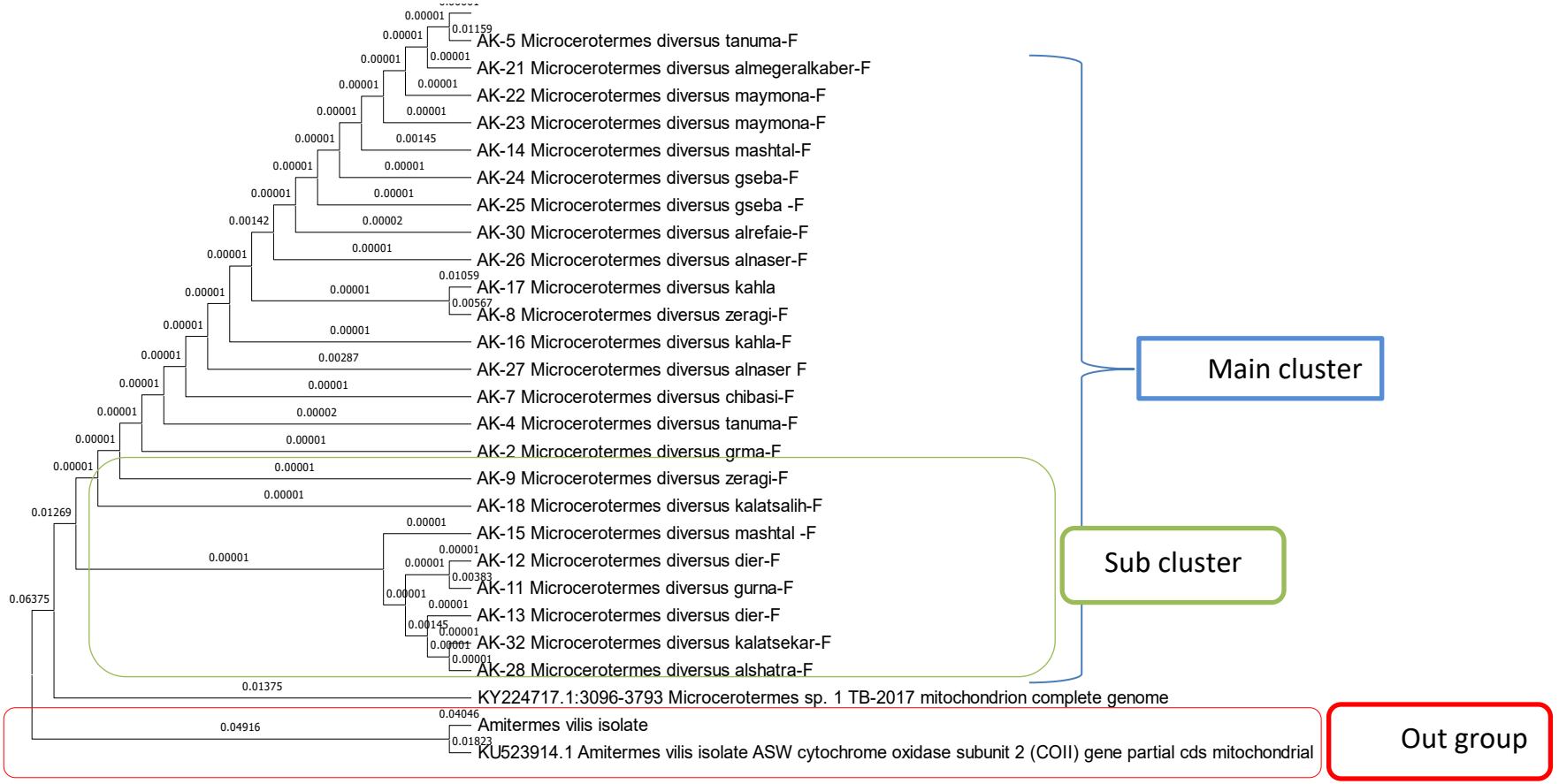

Figure 4. Phylogenic tree of termite samples in the study areas

The results of the similarity and likelihood algorithm analysis of the phylogenetic tree in MEGA program V. 11 showed the emergence of two main clusters, the first cluster includes taxa samples of the *Microcerotermes diversus* species with a taxa from the National Gene Bank (KY224717.1) for comparison purposes, as shown in Figure 4, and the second cluster includes the taxa from Gurma-2 region, which was morphologically classified as *Amitermes vilis*, with the comparison taxa from the National Gene Bank (KU523914.1) as out group (O.G) with a divergence branch length 0.06357 ,it's consistent with (Akoth *et al.,* 2022) pointed out that the minimum branch length among species should be more than 0.03.

According to a phylogram topology of the molecular characterization, COII sequences ensured accurately that tips divided into distinct clusters and high degree of consistency compared to their assigned morphospecies nodal support was shown by the common ancestors that directly defined the clusters because region of the mtDNA COXII generally exhibits high interspecies but low intraspecies separation, indicating that species frequently form clearly defined groupings on phylogenetic trees (Akoth *et al.,* 2022).

It is worth noting that there is a sub-cluster in the main cluster belonging to the *M. diversus*, there is a sub-cluster that may indicate the potential development of a new species or subspecies that can adapt to the changing climatic conditions or by genetic drift (Aizatul *et al.,* 2021) this genetic variation required further studies and comparisons with sequences from other regions to support this hypothesis.

Conclusions

The molecular diagnosis of termites infested palm trees using Polymerase chain reaction technique (PCR) of cytochrome c oxidase gene (COX II) showed that there is a match with samples registered in the American GenBank for the two species *Microcerotermes diversus* and *Amitermes vilis* at high rates reached to 97.46% and 95.73% respectively. also, the genetic variation of the evolutionary tree showed the presence of a secondary subcluster, which is an important indicator to the evolution of termite *Microcerotermes diversus* in the environment to maybe form a new species or subspecies in the future.

Conflicts of Interest

Regarding the publication of this manuscript, the authors declare that there are no conflicts of interest**.**

Acknowledgments

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