Anacanthotermes

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Mitochondrial *COXI* based molecular identification of harvester termite, *Anacanthotermes ochraceus* (Burmeister, 1839) in Riyadh Region, the Kingdom of Saudi Arabia

Abstract

Objective

Termites are well known for being the most destructive pests of both household commodities as well as agricultural crops on the globe. The termite fauna (Isoptera) has almost 2650 worldwide known termite species. Several species are the pest of crops and cause damage to wood structures.

Methods

In the present study, 29 specimens of termites collected from different localities of the Riyadh region were identified using mitochondrial gene *cytochrome peroxidase* subunit I (COX1) DNA sequence. COX1 gene was PCR amplified using universal primers (LCO 1490 and HCO 2198). MEGA7 software was used for phylogenetic tree construction which showed that all 29 isolates grouped together in a single clade indicated close relatedness of all the isolates.

Results

Pairwise nucleotide sequence divergence analysis showed that there was less divergence among all isolates ranging from 7.8% to 0%. Sequence analysis revealed the confirmed precise identification of 29 samples of *Anacanthotermes ochraceus* with COX1 barcode analysis.

Conclusions

Molecular data analysis has confirmed morphological identification of all 29 studied samples of *Anacanthotermes ochraceus*. However, this technology offers strong support for identification of cryptic species which are difficult to identify on the basis of morphological features. Further studies of complete mitogenome can be helpful for accurate identification of termites at species level.

Keywords: Isoptera, Blattodea, DNA barcoding, *Anacanthotermes ochraceus*, Riyadh

1. Introduction

Termites are members of the insect order Isoptera and are distinguished by their social behavioral traits. A termite colony typically includes a reproductive caste (a queen and a king) as well as workers and soldiers. Out of the 2650 identified termite species worldwide, around 300 species are serious pests that inflict significant damage to houses and timber structures (Edwards and Mill 1986, Abe et al., 2000). Although various termite species assist in ecological processes, the cycling of nutrients, they are largely known for their economic importance and as a significant pest of agricultural crops (Ahmed et al., 2006). Badawi has reported on the most of termite research in the Arabian Peninsula, particularly in Saudi Arabia (Badawi et al., 1986).

In fact, the Saudi termite fauna remains undiscovered, and there is no recent data in the literature. For any successful integrated pest management program, it is imperative to identify the pest. The accuracy of delimiting species is fundamental in the identification and discovery of insect species. As a result, we intend to review and identify the termite fauna of the Riyadh region using a molecular identification technique by COX1 gene sequence known as DNA-barcoding.

Over a decade ago, DNA barcoding was proposed as a fast, cost-effective, and simple taxonomic method based on the use of a unique, short and standardized gene region for the specimen identification and expediting discovery of putative new species (Hebert et al., 2003). DNA barcoding is elusive to many taxonomists. As barcoding numbers on commercial products, DNA barcoding tries to link a variety of biological sample with a specific portion of its DNA sequence, mostly mitochondrial gene 'Cytochrome Oxidase I' which is also known as COX1 gene (Ebach 2011). Fragment size of COX1 has been shown to provide high resolution to identify

cryptic species, thereby increasing taxonomy-based biodiversity estimates (Hebert et al., 2004) and its usefulness has been confirmed for several insect orders including Coleoptera (Löbl and Leschen 2005), Diptera (Scheffer et al., 2006), Ephemeroptera (Ball et al., 2005), Hemiptera (Lee et al., 2011), Hymenoptera (Smith et al., 2008) and Lepidoptera (Hajibabaei et al., 2006).

Other molecular marker like RAPD has also been used to study genetic diversity like for order Neuroptera (Yari et al., 2014). DNA barcodes could aid in the routine identification of insects in applied settings by enabling the recognition of morphologically cryptic species, by associating immature forms with adults (pest management), and by identifying eggs (phytosanitary applications) and fragmentary remains (food quality, ecological analyses) (Park et al., 2011). A crucial evidence of DNA barcoding in animals is that genetic variation within species is lower than genetic variation among species (Hebert et al., 2003, Hebert et al., 2003, Yari et al., 2014). In other words, there is an existence of 'barcoding gap' which allow unknown specimens to be identified as an existing species or flagged as a putative undescribed taxon. The presence of global barcoding gap in birds, fish, butterflies (Hebert et al., 2003, Ward et al., 2005, Dincă et al., 2011) sometimes disregarding the importance of local barcoding gaps (Meier et al., 2008). The accuracy of species delimitation also depends on the completeness of DNA reference library, the geographic extent of sampling, the intensity of intra-specific sampling and divergence time among closely related species. DNA barcoding has proved to be particularly expedient in the study of taxonomically thought-provoking taxa, where morphology-based identifications are maddened due to cryptic diversity (Witt et al., 2006) or phenotypic plasticity (Adamowicz et al., 2004). In present study cytochrome peroxidase subunit, I (COX1) based DNA barcode of *Anacanthotermes ochraceus* species of termites from different locations of Riyadh region have been analysed and significance of DNA barcode for accurate identification insect species has been discussed.

2. Materials and Methods

2.1. Sample Collection and Genomic DNA Extraction

Termite specimens were collected using aspirator, hand picking from a broad range of diverse habitats. The specimens were immediately preserved in absolute ethanol and placed in an ice box and shifted into a fridge at 4 °C in the laboratory. The geographic data of the surveyed sites (e.g. GPS coordinates) is given in the table 1. For genomic DNA isolation of insect samples, the modified protocol of CTAB method was used (Khan et al., 2017). Liquid nitrogen was used to crystallize the insects and followed by a process of grinding to a fine powder with pestle mortar. CTAB buffer containing 2% β-marceptoethanol (700μl) was used to make membrane porous and then incubated at 65°C for cellular disruption. Solution of chloroform and isoamyl alcohol (24:1) were used for separation of DNA and the reaction was precipitated by using 0.6 volume of isopropanol and centrifuge (x24000rpm) to pellet down DNA. The pelleted DNA was washed with 70% ethanol to remove the salts and the pellet was air dried. The purification DNA was analysed quantitatively by using nanodrop.

2.2. Amplification of Cytochrome Oxidase I gene

The isolated genomic DNA of the termite specimens was used as template in PCR reaction for amplification of COI gene. COI gene was amplified using universal primers (forward primer; LCO 1490: 5'- GGTCAACAAATCATAAAGATATTGG-3' and reverse primer; HCO 2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer O. 1994). For reaction, PCR protocol followed by initial denaturation temperature 95°C for 5 minutes, denaturation temperature 95°C for 1-minute, annealing temperature 45°C for 1 minute and elongation/extension temperature 72°C for 1 minutes. COI gene amplification was confirmed through Gel electrophoresis using 1% agarose gel.

2.3. Sequencing and Phylogenetic Analysis

Amplified PCR product of desired band size (680-700 bp) was purified using Illustra GFX PCR DNA and Gel Band Purification Kit (Healthcare Life Sciences, USA). Purified PCR products were sent to Macrogen Inc. Seoul, South Korea for DNA sequencing. The sequencing data was analyzed using the Lasergene package (DNASTAR, Madison, Wisconsin). Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search out closely related sequences from databases. Closely related sequences were obtained from databases in FASTA format; MEGA7.0 software also was utilised for multiple sequence alignment and phylogenetic analysis, while Tree View Software was used for tree display and manipulation. MegAlign application was benefited to pairwise distance analysis for evolutionary divergence between sequences (Tamura et al., 2004).

3. Results

Anacanthotermes ochraceus was sampled from agricultural farms covering different habitats of the Riyadh region in 2020. The sample collection sites and their detailed information regarding the coordinates are given in the Table 1. The samples were previously morphologically identified as *Anacanthotermes ochraceus* by our group (Sharaf et al., 2021). DNA extraction from the insect samples was carried out and subjected to PCR amplification of *COXI* gene using LCO 1490 and HCO 2198 primers (Folmer O. 1994). The amplified PCR products were analysed in 1% agarose gel electrophoresis and all samples PCR amplification showed bright bands of approximately 700 bp size (Figure 1). The purified PCR products were sent to Macrogen (Seol, South Korea) for DNA sequencing through its entirety in both orientations.

115 **Table 1.** Detailed information of *Anacanthotermes ochraceus* specimens collected from different

116 places of Riyadh Region, Kingdom of Saudi Arabia

Sample No	Accession Number	Sample Code	Locality/Area	Coordinates
1	ON682943	Shaqra 37 isolate	Shaqraa	25°13.278'N, 45°16.760'E
2	ON529891	HBT 38 isolate	Hawtet bani Tamim (HBT)	23°36.809'N, 46°33.434'E
3	ON682942	Afif 35 isolate	Afif	23°51.837'N, 42°53.568'E
4	ON682941	Afif 34 isolate	Afif	23°51.859'N, 42°53.579'E
5	ON682940	Al-Bajadiah 32 isolate	Al-Bajadiah	24°17.782'N, 43°43.614'E
6	ON682939	Al-Bijadyah 31 isolate	Al-Bajadiah	24°17.804'N, 43°43.625'E
7	ON529890	AlBijadyah 30 isolate	Al-Bajadiah	24°17.783'N, 43°43.584'E
8	ON682937	Al-Bijadyah 26 isolate	Al-Bajadiah	24°18.334'N, 43°44.294'E
9	ON682936	Al-Bijadyah 25 isolate	Al-Bajadiah	24°18.335'N, 43°44.296'E
10	ON529889	AlBijadyah 23 isolate	Al-Bajadiah	24°17.937'N, 43°44.232'E
11	ON529887	Sajir 21 isolate	Sajir	24°09.878'N, 44°36.022'E
12	ON529886	Sajir 19 isolate	Sajir	25°13.332'N, 44°35.856'E
13	ON529885	Sajir 18 isolate	Sajir	25°13.306'N, 44°35.827'E
14	ON529884	Sajir 17 isolate	Sajir	25°12.645'N, 44°36.095'E
15	ON529883	AdDawadmi 16 isolate	Al Dawadmi	24°28.887'N, 44°21.555'E
16	ON529882	AdDawadmi 14 isolate	Al Dawadmi	24°28.701'N, 44°21.178'E
17	ON529881	AdDawadmi 13 isolate	Al Dawadmi	24°28.806'N, 44°20.881'E
18	ON529880	AdDawadmi 12 isolate	Al Dawadmi	24°28.805'N, 44°20.881'E
19	ON529879	AdDawadmi 10 isolate	Al Dawadmi	24°28.887'N, 44°21.540'E
20	ON529877	Dhurma 8 isolate	Dhurma	24°40.894'N, 46°00.364'E
21	ON529876	Dhurma 7 isolate	Dhurma	24°40.896'N, 46°00.367'E
22	ON529870	Dirab1 isolate	Dirab	24°25.094'N, 46°39.093'E
23	ON529892	AsSulayyil 43 isolate	As Sulayyil	20°27.903'N, 45°33.324'E
24	ON682946	As-Sulayyil 44 isolate	As Sulayyil	20°26.063'N, 45°31.302'E
25	ON682945	Al-Aflaj 40 isolate	Al Aflag	21°59.719'N, 46°32.657'E
26	ON682944	Howtat-Bani-Tamim 39 isolate	Hawtet bani Tamim (HBT)	23°36.752'N, 46°39.265'E
27	ON529872	Muzahmiyah 3 isolate	Al Muzahmiyah	24°29.502'N, 46°22.157'E
28	ON529871	Dirab 2 isolate	Dirab	24°25.322'N, 46°39.183'E
29	ON682935	Al-Bijadyah 24 isolate	Al-Bajadiah	24°17.787'N, 43°43.596'E

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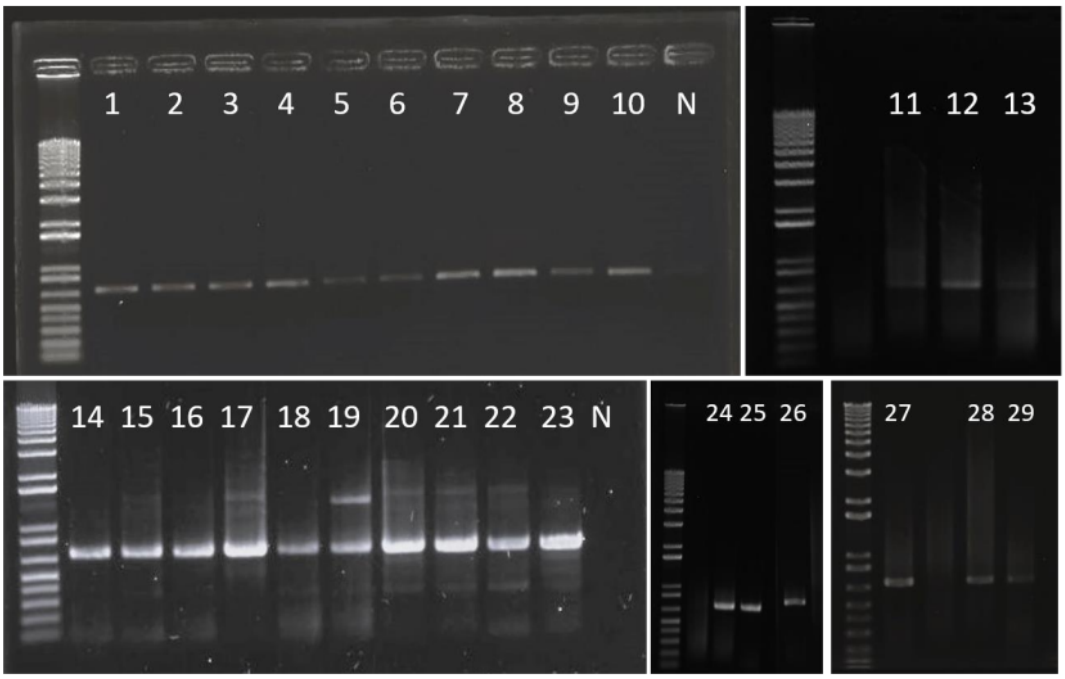


Figure 1. PCR amplification of COX1 gene from sampled specimens of *Anacanthotermes ochraceus* resolved on 1% agarose gel electrophoresis.

3.1. Phylogenetic Analysis of Anacanthotermes ochraceus

COXI gene sequences of 29 samples of *Anacanthotermes ochraceus* species were analyzed and barcode sequences were submitted into Genbank database with accession numbers ON529870-ON529872, ON529876-ON529877, ON529879- ON529887, ON529889- ON529892, ON682935-ON682937, ON682939-ON682946. Using basic local alignment search tool (BLAST), it was found that our samples belong to Hodotermitidae family of termites and there was no COX1 sequence of *Anacanthotermes ochraceus* species in the databases but those of two other member of the family Hodotermitidae i.e. *Microhodotermes viator* (EU253837) and *Hodotermes mossambicus* (EU253836) were retrieved from the NCBI database. COX1 gene sequence of a fruit fly *Bactrocera zonata* (MG770092) was used as an outgroup sequence. The sequences of closely species of termites were retrieved in fasta format. Those downloaded sequences along with our

sample sequences were subjected to multiple sequence alignment and phylogenetic tree was constructed using maximum likelihood algorithm. Phylogenetic analysis showed that all the isolates of our samples grouped together into a single monophyletic clade and confirmed to be highly closely related with one another and being the members of same species. Second closely related clade was the group of different members of genera Microhodotermes and Hodotermes which belong to the family Hodotermitidae. Species of genera *Amitermes* Silvestri, 1901 and *Macrotermes* Holmgren, 1910 form separate clades (Figure 2). Pairwise sequence identity percentage analysis showed that our samples barcode sequences show 88.5-100% nucleotide sequence identity with one another. This is showing highly conserved nucleotide sequence homology within the collected samples of the *A. ochraceus* species. Among the sequences retrieved from database *Microhodotermes viator* (Latreille, 1804) (EU253837) showed highest nucleotide sequence identity of 88.9% with one of our samples *A. ochraceus* because *M. viator* is the member of same family Hodotermitidae. Nucleotide sequence identity with members of other families of termites ranged 82.6-87.3% (Table 2).

3.2. Intraspecific and Interspecific Sequence Divergence

Degree of sequence divergence at intraspecific as well as interspecific level was studied using MegAlign software application of DNA Star (DNA Star Inc., Madison, WI, USA). *Anacanthotermes ochraceus* species, 29 samples were analyzed. Intraspecific sequence divergence among studied 29 isolates ranged from 0% to 7.8% indicating low divergence and highly conserved barcode sequence indicating all isolates belong to the same species. Interspecific sequence divergence between studies isolates and other species sequences retrieved from databases showed a high degree of divergence with a minimum interspecific value of 12% with *M. viator* (EU253837) (Table 3).

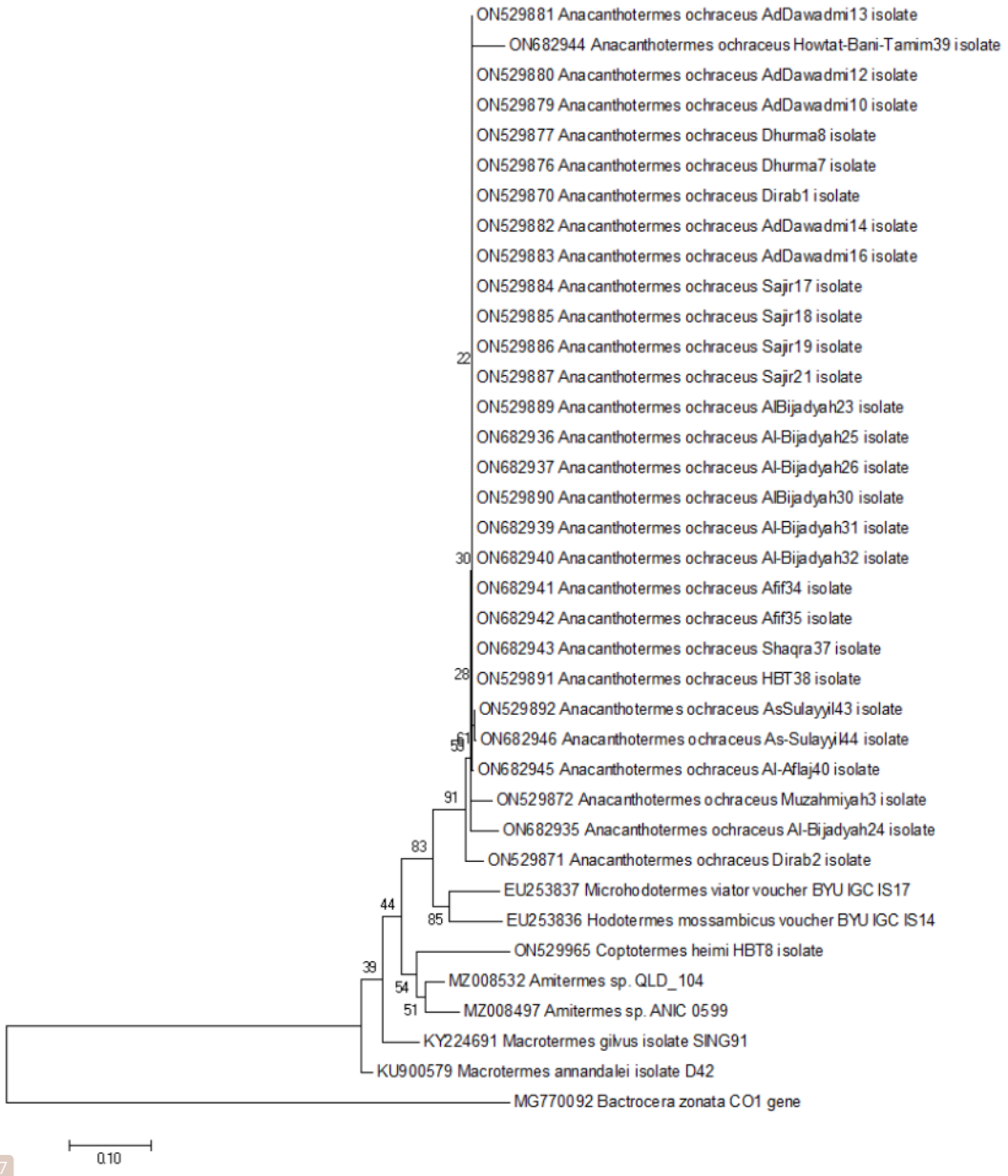


Figure 2. Phylogenetic tree was created in MEGA 7.0 software using maximum-likelihood algorithm. The examination shows relationship of COX1 gene sequences of *Anacanthotermes ochraceus* with that of other closely related termite species from same family and other families of termites. All termite species used in the phylogenetic tree were labelled by their scientific name along with specific accession number. Numeric values at the base of each branch indicated the percentage of bootstrap value reiterated 1000.

Table 2. Matrix of pairwise nucleotide sequence identity percentage of COX1 gene of 29 samples of *Anacanthotermes ochraceus* with other related sequences retrieved from NCBI database.

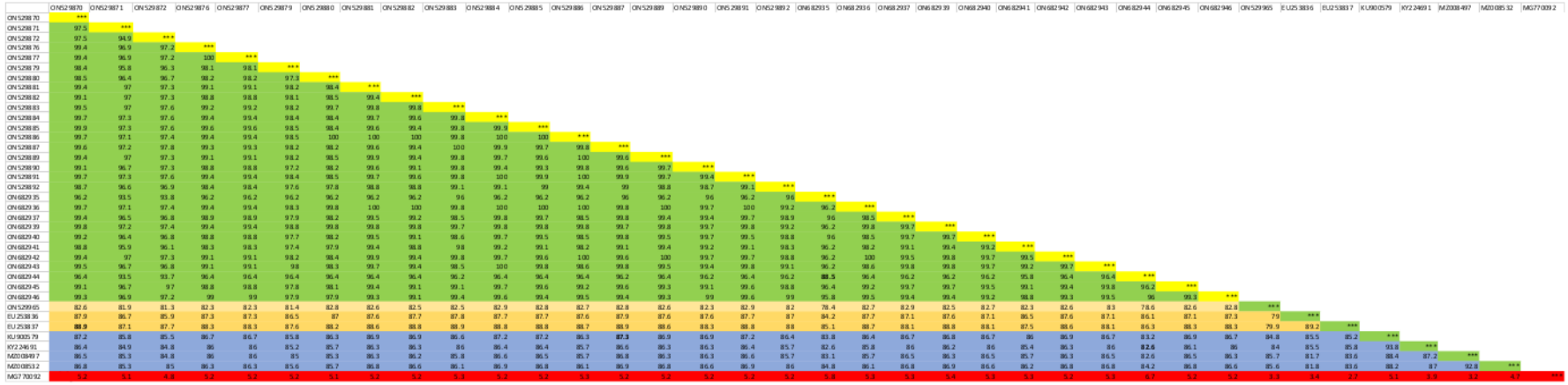
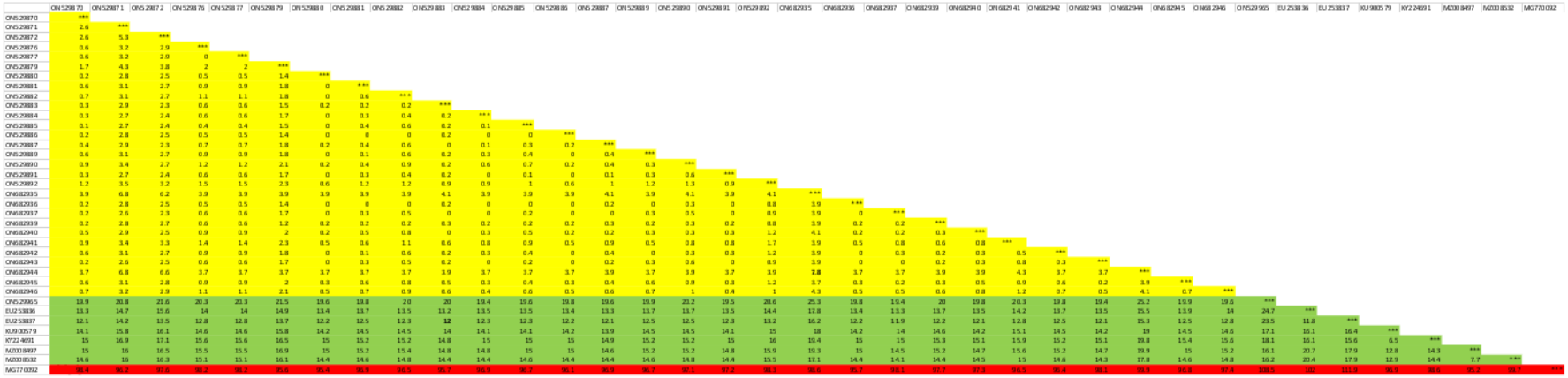


Table 3. Matrix of intraspecific and interspecific nucleotide sequence divergence of COX1 gene of 29 samples of *Anacanthotermes ochraceus* with other related sequences retrieved from NCBI database.



4. Discussion

Termites are abundantly present everywhere both in tropical and subtropical regions of the world and exhibit wide genetic diversity (Roy et al., 2006; Cheng et al., 2011). In the past, insect taxonomists were exclusively dependent on the morphological and histological features for the identification of the insect specimens which sometimes might be incorrect and which could lead to revisions of the taxonomic identification and classification of the same organisms. It is also important that specimen can be difficult to identify in different developmental stages (egg, larva, pupa and adult) of sample under study (Hussain et al., 2020). But this present era of the genomics and molecular biology has strengthened the field of taxonomy and systematic identification of species on the basis of DNA sequences (Seifert 2009). The DNA barcoding has successfully delineated the species borders in insects proving itself a supportive technique for the accurate identification of specimen and discovering new species (Rasool et al., 2020, Sukirno et al., 2020, Wikantyoso et al., 2021, Zaman et al., 2022).

The present study includes 29 COX1 gene sequences which were amplified with LCO 1490 and HCO 2198 universal primers. These primers have already proved 100% success for insect DNA barcode amplification (Hebert et al., 2003). All the 29 specimens from the given study represented the *A. ochraceus* species. The sequence analysis of several termite specimens collected from different localities in the Riyadh region confirmed the morphological based identification of the species *A. ochraceus*, which were previously identified by (Sharaf et al., 2021). DNA based identification of soldier caste of genus *Coptotermes* spp. has been done from Indonesia. The other mitochondrial genes 12S and 16S sequences were used to study phylogenetic relationships and genetic divergence among different species of genus *Coptotermes* (Wikantyoso et al., 2021). In line with these findings the 29 specimens of *A. ochraceus* analysis using maximum-likelihood algorithm for phylogenetic study showed highly conserved single clade indicating all specimens

belong to same species confirming the morphological studies. Maximum likelihood algorithm has been previously used for phylogenetic relationships of termites by (Zaman et al., 2022), (Bourguignon et al., 2013). Similar results have been reported by (Sharma et al., 2013) where several morphological and molecular studies of termites were performed and molecular data confirmed the morphological data. Moreover, the present pairwise nucleotide sequence identity as well as divergence percentage data also confirmed both phylogenetic and morphological information. All specimens identified as same species (intraspecific) showing higher nucleotide sequence identity percentage (88.5-100%) and lower divergence of (0-7.8%). On the other hand, interspecific nucleotide identity was as low as 82.6-87.3% and divergence was as high as 12%. Similar studies also confirmed the genetic divergence of termites at intraspecific as well as interspecific level (Cheng et al., 2011).

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e.g. role of the present work in facilitating others work, future programs, plans, etc.

5. Conclusions

The specimens of termite species *Anacanthotermes ochraceus* already identified using morphological traits were revalidated deploying molecular analysis of a mitochondrial gene COX1 sequence. Molecular data analysis has confirmed morphological identification of all 29 studied samples of *Anacanthotermes ochraceus*. However, due to limited availability of data about COX1 barcode sequences about different species of termites in public databases may become hurdle in discovery of new species without the support of morphological data. However, this technology offers strong support for identification of cryptic species which are difficult to identify on the basis of morphological features. Further studies of complete mitogenome can be helpful for accurate identification of termites at species level.

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