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Original article

# Haplotype diversity of palm weevil in Saudi Arabia through ITS gene sequencing

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ABSTRACT

The red palm weevil, *Rhynchophorus ferrugineus* (Olivier 1790) caused a high economic loos in date palm in gulf region especially in Saudi Arabia. Previous estimations of palm weevil haplotype diversity in Saudi Arabia was not satisfactory because of choice of marker and small sampling area. Assessing haplotype diversity using COI gene was found ineffective. The present study was carried out to estimate the haplotypes of *R. ferrugineus* using internal transcribed spacer (ITS) sequences. Results showed 3 haplo-groups, among which, the haplotype UT9 was a singleton haplotype. The haplotypes UT16, UT2, UT6 UT10, UT14 and UT5 formed the second haplo-group whereas the remaining haplotypes were clustered as third group. K2P distance varied between 0 and 0.06 among the sequenced haplotypes and the maximum distance was recorded between two sets of haplotypes; *viz*; UT4 and UT3, UT4 and UT8. The overall distance of all the sequenced haplotypes were 0.02. Tajima's neutrality value was < 0 indicating the potentiality for further haplotype diversification. Median joining haplotype network diagram reveals the presence of 10 haplotypes and most mutations were at 244$^{th}$ and 381$^{st}$ nucleotide positions. Overall, total number of mutations leading to the evolution of the documented 10 haplotypes was 34. Through genetic data, we have provided a strong evidence that there were multiple haplotypes of *R. ferrugineus* which were bound to expand its diversity. Higher nucleotide diversity and Tajima's neutrality test values suggest that there were higher movement of infested palm trees, as desserts were natural barriers for movement of *H. ferrugineus*. The findings of this study will be useful for pest controlling agencies and to the workers involved in infested date palm quarantining.


## 1. Introduction

The red palm weevil, *Rhynchophorus ferrugineus* (Olivier 1790) (Family: Curculionidae) is native to Southeast Asia and Melanesia. Larvae of *R. ferrugineus* were known to destroy palm trees worldwide since its accidental introduction into USA, Caribbean, Mediterranean basin and Middle East (Rugman-Jones et al., 2013a; Rugman-Jones et al., 2013b; Rugman-Jones et al., 2013c). *R. ferrugineus* has pheromone receptors RferOR1 that are responsible for long-range orientation towards palm trees, according to a recent study (Antony et al., 2021). More than 16 genera and 26 various palm tree species were attacked by *R. ferrugineus* and hence been classified in A2 list as per European and Mediterranean Plant Protection Organization (EPPO, 2002). For example, Arabian gulf alone losses US$ 25.92 million during 2009 alone in data palm (*Phoenix dactylifera*) production, which is 30% of world's annual date palm production (El-Sabea et al., 2009). Over 25 million Saudi Arabian date palms were at risk during 2010 (Ministry of Agriculture, 2010). Usually, disease when detected is beyond remediation as infestation symptoms were visible only after severe damage of the palm tree. Hence early detection of larvae could be useful to stop the migration of the larvae to heart of the date palm vascular system to initiate infestation (Giblin-Davis, 2001). Many studies are focus-

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ing on using advanced technology to detect *R. ferrugineus* larvae sooner (Mao et al., 2021; Kurdi et al., 2021; Wang et al., 2021).

Identifying an organism based on a standardised gene sequences is called DNA barcoding and the gene used is commonly referred as a barcode gene (Hebert et al., 2004; Joly et al., 2014; Kress et al., 2015). DNA barcoding will allow easy and precise identification of *R. ferrugineus* and its early larval stages, as DNA barcodes from adult specimens have widely proven to be useful in identifying its early larval stages (Alcántar-Escalera et al., 2013; Khan et al., 2014; Steinke et al., 2016; Chu et al., 2019).

Previous estimations of palm weevil haplotype diversity in Saudi Arabia (Al-Ayied et al., 2006; Sadder et al., 2015a; Sadder et al., 2015b) revealed small diversity values reciprocating small sampling sizes. Recently, Sukirno et al. (2020a), Sukirno et al. (2020b) sequenced 297 palm weevil for COI genes sampling from 308 date palm plantations distributed in nine regions of Saudi Arabia and found 221 haplotypes grouped into 3 groups. When assessing haplotype diversity, COI were consider inferior as only few haplotypes were reported from previous studies (El-Mergawy et al., 2011a; El-Mergawy et al., 2011b; Rugman-Jones et al., 2013a; Rugman-Jones et al., 2013b; Rugman-Jones et al., 2013c). The present study aims to estimate the haplotypes of *R. ferrugineus* from different regions of Saudi Arabia using internal transcribed spacer (ITS) gene sequences.

## 2. Materials and methods

### 2.1. Sample collection and preservation

Twenty samples of palm weevil, *R.ferrugineus* were collected across Saudi Arabia between June to October 2018. Sampling was done across multiple cities in 10 provinces of Saudi Arabia viz., Medina, Tabuk, Hail, Riyadh, Al-Qassim Region, Aljobail, Almoterfy in Al-Ahsa'a, Al-Shoiba, Al Aqiq and Bisha (Fig. 1**).** The exact geographical locations of individual samples with its corresponding geographical co-ordinates are given in Table 1. Adults of *R. ferrugineus* were hand picked from infested date palm plantations (*P. dactylifera* L.) and preserved directly into 70% ethanol (Scharlau, Barcelona) contained in 50 mL conical tubes. Coloured ethanol due to pigment diffusion was replaced with fresh 70% ethanol once reached the laboratory. Samples were kept at 4 °C until DNA extraction.

### 2.2. DNA isolation and PCR

For DNA isolation, approximately 3 $mm^2$ pronotal muscle was exercised from the preserved specimen using sterilized fine forceps and placed inside 200 μl PCR tubes. Ethanol in the tissues were evaporated either in room temperature or by placing the tubes in concentrator plus (Eppendrof, AG) at 45 °C for 10 min. DNA extraction and purification was carried out using the manufacture's protocol of DNeasy blood and tissue DNA isolation Kit (Qiagen, USA) excerpt only one by tenth of actual volume of the reagents given were used. The final elution was done in 50 μl elution buffer. The eluted DNA was rinsed trice and repeatedly eluted from the binding column to increase the DNA concentration. The isolation of DNA was confirmed by running 2 μl of elutant in 1.5% agarose gel along with Lamda DNA (Takara Bio) as standard.

During polymerase chain reaction (PCR), internal transcribed spacer (ITS) gene region was amplified using the primer pair; ITS-F: 5′-ATATGCTTAAATTCAGCGGG-3′ and ITS-R: 5′-GGGTCGATGAA GAACGCAGC-3′ (Navajas et al., 1998). 50 μl PCR mixture consisted of 1x PCR buffer (Promega), 15 mM $MgCl_2$, 0.2 mM dNTPs, 20 pM of each primers, 1u of Taq DNA polymerase (GoTaq, Promega), 40 ng DNA and ultra-pure water to a final volume of 50 μl. PCR condition was done with an initial denaturation for 5 min at 94 °C. The, 40 cycles consisted of a denaturation step at 94 °C for 30 sec., an annealing step at 50 °C for 30 sec. and an elongation step at 72 °C for 30 sec. The final extension step was for 7 min at 72 °C. The PCR amplicons were resolved by 1.5% agarose gel electrophoresis with ethidium bromide (0.5ug/ml) in 1X TBE buffer powered at 95 V for 30 min. A 100 bp DNA ladder (Thermo Fisher Scientific) was used as a standard to detect and assess the size of the amplicon. PCR products were visualized under UV light and photographed using a Gel Documentation System (BIO-RAD 2000). The PCR products were purified using QIAquick PCR purification kit (Qiagen, USA).

### 2.3. Sequencing and analysis

Sequencing was performed in ABI PRISM 3730XL Analyzer (Macrogen, South Korea) using Big Dye TM Terminator Cycle Sequencing kit following manufacture's instruction. Two way sequencing was performed and the chromatograms were compiled using BioEdit ver. 7.2 (Hall, 1999). Chromas Pro ver. 2.6.6 was used to double check the quality of the sequence chromatograms before compiling. Primer sequences were trimmed off from the final sequences. The sequences were aligned in Clustal X ver. 2.0.6 (Thompson et al., 1997) and the alignment was submitted to GenBank. Sequences could be accessed through GenBank accession numbers: MW575619-MW575638. Kimura-2 parametric distance analysis (Kimura, 1980) was conducted in Molecular Evolutionary Genetic analysis (MEGA) ver. X (Kumar et al., 2018).We calculated Tajima's Neutrality Test (Tajima, 1989) using segregation and nucleotide data using following equations; m = number of sequences, n = total number of sites, S = Number of segregating sites, ps = S/N, $\Theta$ = ps/a1, $\pi$ = nucleotide diversity, and D is the Tajima's test statistic. Tajima's test values reveals the difference between the number of segregation nucleotide sites and the average of pair-wise differences. The analysis was conducted in MEGA ver. X.

DNA sequences were compared with GenBank reference sequences through Basic local alignment search tool (BLAST) (Altschul et al., 1990) using default parameters for searching the entire nucleotide collection using the conditions optimized for highly similar sequences. First five reference sequences of closest match to the query sequences of the present study were extracted and the repeated sequences were removed before being used for the phylogenetic tree construction. Neighbor-Joining tree (Saitou and Nei, 1987) was drawn using Molecular Evolutionary Phylogenetic Analysis software version 10.1.7 (Kumar et al., 2018) to infer the phylogenetic relationship among the sequenced haplotypes.The evolutionary distances were computed using the Kimura 2-parameter method (Kimura et al., 1980). The percentage of replicate trees in which the associated taxa clustered together was calculated using the bootstrap test (100 replicates) (Felsenstein, 1985).Interactive Tree Of Life (iTOL) (Letunic and Bork, 2019) (https://itol.embl.de/) was used for the better representation of constructed phylogenetic tree.

Median joining haplotype network diagram was drawn using Network ver 10.2. (https://www.fluxus-engineering.com/sharenet.htm), developed by Fluxus Technology Ltd. Aligned DNA sequences in FASTA format was converted into RDF (Resource Description Framework) format using FAS to RDF convertor tool (http://fas2rdf.herokuapp.com/) and output file is used as input data to draw the network diagram.

## 3. Results

### 3.1. Sequence identification

DNA was recovered from an ethanol-preserved specimen at a concentration of 5.34 ± 2.43 μg/μl. About 700 bp PCR amplicon


**Fig. 1.** Map reveals sample collection sites across Saudi Arabia. The color integers represents sample number and the position of the integer indicate the approximate area of the respective collected samples. Total of 20 samples were collected from 20 different study area.

**Table 1**
The details of sampled location with geographical coordinates were given below.

| S. No | Collection area | Latitude (North) | Longitude (East) |
|---|---|---|---|
| 1 | Medina | 24.60 | 39.46 |
| 2 | Medina | 24.57 | 39.60 |
| 3 | Medina (Alula) | 26.62 | 37.92 |
| 4 | Tabuk (Al Amir Fahd Ibn Sultan Rd ) (Madinah Rd) | 28.38 | 36.55 |
| 5 | Tabuk (King Khalid Rd) (Amman Road) | 28.39 | 36.56 |
| 6 | Hail (Eastern Alkhitah) | 27.98 | 41.80 |
| 7 | Hail (al- Qaid) | 27.88 | 41.70 |
| 8 | Hail (Western Alkhitah) | 27.95 | 41.66 |
| 9 | Riyadh (Alhaeer) | 24.41 | 46.85 |
| 10 | Riyadh (Aloiayenah) | 24.47 | 46.85 |
| 11 | Al-Qassim Region (Riyadh Al Khabra) | 26.06 | 43.56 |
| 12 | Al-Qassim Region(Slaam) | 25.66 | 43.80 |
| 13 | Al-Qassim Region (Al Bukayrīyah) | 26.17 | 41.13 |
| 14 | Aljobail (Eastern Region) | 27.08 | 49.54 |
| 15 | Almoterfy in Al-Ahsa'a (Eastern Region) | 25.29 | 49.48 |
| 16 | Al-Shoiba (Makkah Region) | 21.40 | 39.78 |
| 17 | Al Aqiq (Makkah Region) in Taif city | 21.53 | 39.79 |
| 18 | Saqefah in Khobar (Eastern Region) | 24.47 | 39.60 |
| 19 | Bisha (Asir Region) | 20.03 | 42.62 |
| 20 | Bishaaleubla (Asir Region) | 19.96 | 42.59 |

was produced from all specimens. All sequences shared on average of 98.7% identity (96.06 to 100%) with the ITS sequences of *R. ferrugineus* in the GenBank (Table 1). While few sequences closely resembles the other ITS sequences previously sequenced from *R. ferrugineus* captured in Saudi Arabia (n = 4), many (n = 16) shared close identity with *R. ferrugineus* collected from Greece, India and Israel (Table 1). For better interpretation all haplotypes were named as UT (University of Tabuk) 1–20 and correlated with the corresponding Genbank accession numbers in the Table 2.

### 3.2. Haplo-groups and distance analysis

We identified three haplogroups based on the NJ tree analysis which were highlighted through 3 different colors (red, orange and blue) (Fig. 2). The haplotype UT9 was a singleton haplotype in the analysis. The haplotypes UT16, UT2, UT6 UT10, UT14 and UT5 formed the second haplo-group whereas the remaining haplotypes were clustered as third group at the lower end of the phylogenetic tree. Interestingly all reference sequences extracted from GenBank was classified within the third cluster of the Haplogroup. Lack of reference ITS sequences for first and second haplogroups may be due their new origin and may be specific to Saudi Arabia. Also the members of third haplo-group such as UT13, UT14, UT15, UT17, UT18, UT19, and UT20 did not had even single nucleotide variation and were exactly identical.

During K2P distance analysis, all nucleotide positions containing gaps and missing data in the alignment were eliminated and there were a total of 468 nucleotide positions in the final aligned dataset.K2P distance varied between 0 and 0.06 among the sequenced haplotypes (Table 3). Maximum distance was recorded between two sets of haplotypes; *viz*; UT4 and UT3, UT4 and UT8. The overall distance of all the sequenced haplotypes were 0.02.

The optimal tree with the sum of branch length = 0.12658351 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using

**Table 2**
All ITS sequences were compared with reference sequences in GenBank through BLAST analysis and their highest percentage of similar sequences were presented as reference sequence. All sequences uniformly shared various percentage of similarity with ITS sequences of *Rhynchophorus ferrugineus* in the GenBank.

| N | Seq. ID | GenBank acc. number | Sim. (%) | Ref. seq. acc. No. | Country | Reference |
|---|---|---|---|---|---|---|
| 1 | UT1 | MW575619 | 98.77 | JX292126 | India | El-Mergawy et al., 2011a; El-Mergawy et al., 2011b |
| 2 | UT2 | MW575620 | 99.38 | KC954631 | Saudi Arabia | Unpublished |
| 3 | UT3 | MW575621 | 96.22 | KM503122 | Greece | Unpublished |
| 4 | UT4 | MW575622 | 96.32 | JX292126 | India | El-Mergawy et al., 2011a; El-Mergawy et al., 2011b |
| 5 | UT5 | MW575623 | 97.96 | HM043673 | Egypt | El-Mergawy et al., 2011a; El-Mergawy et al., 2011b |
| 6 | UT6 | MW575624 | 98.99 | HM043674 | Egypt | El-Mergawy et al., 2011a; El-Mergawy et al., 2011b |
| 7 | UT7 | MW575625 | 100 | KF311695 | Israel | Rugman-Jones et al., 2013a; Rugman-Jones et al., 2013b; Rugman-Jones et al., 2013c |
| 8 | UT8 | MW575626 | 96.22 | KC954638 | Saudi Arabia | Unpublished |
| 9 | UT9 | MW575627 | 99.13 | KC954633 | Saudi Arabia | Unpublished |
| 10 | UT10 | MW575628 | 98.75 | KC954632 | Saudi Arabia | Unpublished |
| 11 | UT11 | MW575629 | 96.06 | KM503122 | Greece | Unpublished |
| 12 | UT12 | MW575630 | 97.82 | KC954638 | Saudi Arabia | Unpublished |
| 13 | UT13 | MW575631 | 100 | KF311695 | Israel | Rugman-Jones et al., 2013a; Rugman-Jones et al., 2013b; Rugman-Jones et al., 2013c |
| 14 | UT14 | MW575632 | 99.38 | KC954638 | Saudi Arabia | Unpublished |
| 15 | UT15 | MW575633 | 100 | KF311695 | Israel | Rugman-Jones et al., 2013a; Rugman-Jones et al., 2013b; Rugman-Jones et al., 2013c |
| 16 | UT16 | MW575634 | 98.90 | KM503122 | Greece | Unpublished |
| 17 | UT17 | MW575635 | 100 | KF311695 | Israel | Rugman-Jones et al., 2013a; Rugman-Jones et al., 2013b; Rugman-Jones et al., 2013c |
| 18 | UT18 | MW575636 | 100 | KF311695 | Israel | Rugman-Jones et al., 2013a; Rugman-Jones et al., 2013b; Rugman-Jones et al., 2013c |
| 19 | UT19 | MW575637 | 100 | KF311695 | Israel | Rugman-Jones et al., 2013a; Rugman-Jones et al., 2013b; Rugman-Jones et al., 2013c |
| 20 | UT20 | MW575638 | 100 | KF311695 | Israel | Rugman-Jones et al., 2013a; Rugman-Jones et al., 2013b; Rugman-Jones et al., 2013c |

N = numbers, seq. = sequence, sim. = similarity, acc. = accession, No. = number.

the Kimura 2-parameter method and are in the units of the number of base substitutions per site. There were a total of 468 positions in the final dataset.

There were a total of 44 segregation sites with overall nucleotide diversity was 0.019 (Table 4). Tajima's neutrality value was < 0 indicating population expansion in terms of haplotype diversity.

### 3.3. Haplo-type network

Median joining haplotype network diagram reveals the presence of 10 haplotypes (Fig. 3). The haplotypes UT16, UT12, UT10, UT5, UT4 UT1, UT16 and UT11 are unique haplotypes represented by single members in this study. UT2 and UT3 bubbles in the network diagram also contains UT6 and UT8 haplotypes respectively. All remaining haplotypes were aggregated in UT15 bubble of the network diagram and forms the largest haplotype group (n = 9). There were a total of 7 median vectors connecting triplet of the different haplotypes. In the ITS sequences aligned, 244th position was found to be most frequently mutated (n = 6) position followed by 381$^{st}$ position (n = 4). Overall, total number of mutations leading to the evolution of these 10 haplotypes was 34.

## 4. Discussion

Agriculture ministry of Saudi Arabia has tried various measures to control *R. ferrugineus* and the economic losses incurred on behave.Though multiple pest managing procedures and pheromone-centric trapping protocols were used, *R. ferrugineus* continue to spread in Saudi Arabia and its surrounding countries. Studying genetic diversity based on the morphology of *R. ferrugineus* does not yield reliable estimates as environmental factors have profound influence on the morphology. Earlier studies using RAPD markers have shown the presence of 3 haplogroups of *R. ferrugineus* in Egypt (Salama and Saker, 2002), which is similar to the observation we made in this study using ITS gene sequences. Similarly another study estimating the haplotype diversity of *H. ferrugineus* acknowledges the presence of three haplogroups in UAE.

Even though mitochondrial cytochrome *C* oxidase subunit I gene (COI) is used to identify *R. ferrugineus* (Sukirno et al., 2020a; Sukirno et al., 2020b), the nuclear copies could lead to over estimation of taxonomic or haplotype diversity (Song et al., 2008). However earlier studies with COI have also concluded with the presence of 3 haplogroups (Sukirno et al., 2020a; Sukirno et al., 2020b). Also very few haplotypes (n = 1) were reported from previous studies using COI (El-Mergawy et al., 2011a; El-Mergawy et al., 2011b; Rugman-Jones et al., 2013a; Rugman-Jones et al., 2013b; Rugman-Jones et al., 2013c). However when sampling sizes were increased, increase in COI haplotypes were evidenced (Sukirno et al., 2020a; Sukirno et al., 2020b). A recent study that used a whole genome approach to decode *R. ferrugineus* haplotype variation identified new chemosensory and neuropeptide genes that could be targeted for pest control (Dias et al., 2021). We used ITS sequences which were proven for precise estimation of molecular diversity of *R. ferrugineus* (Sadder et al., 2015a; Sadder et al., 2015b). Like the previous studies (Sadder et al., 2015a; Sadder et al., 2015b), the present study also reveals rapid diversification of the pest and Tajima's test support rapid expansion of territory. Though the present study suffers by not having duplicate samples, 10 haplotypes with potential of further expansion was detected. In general, ITS gene sequences are widely used for identification and

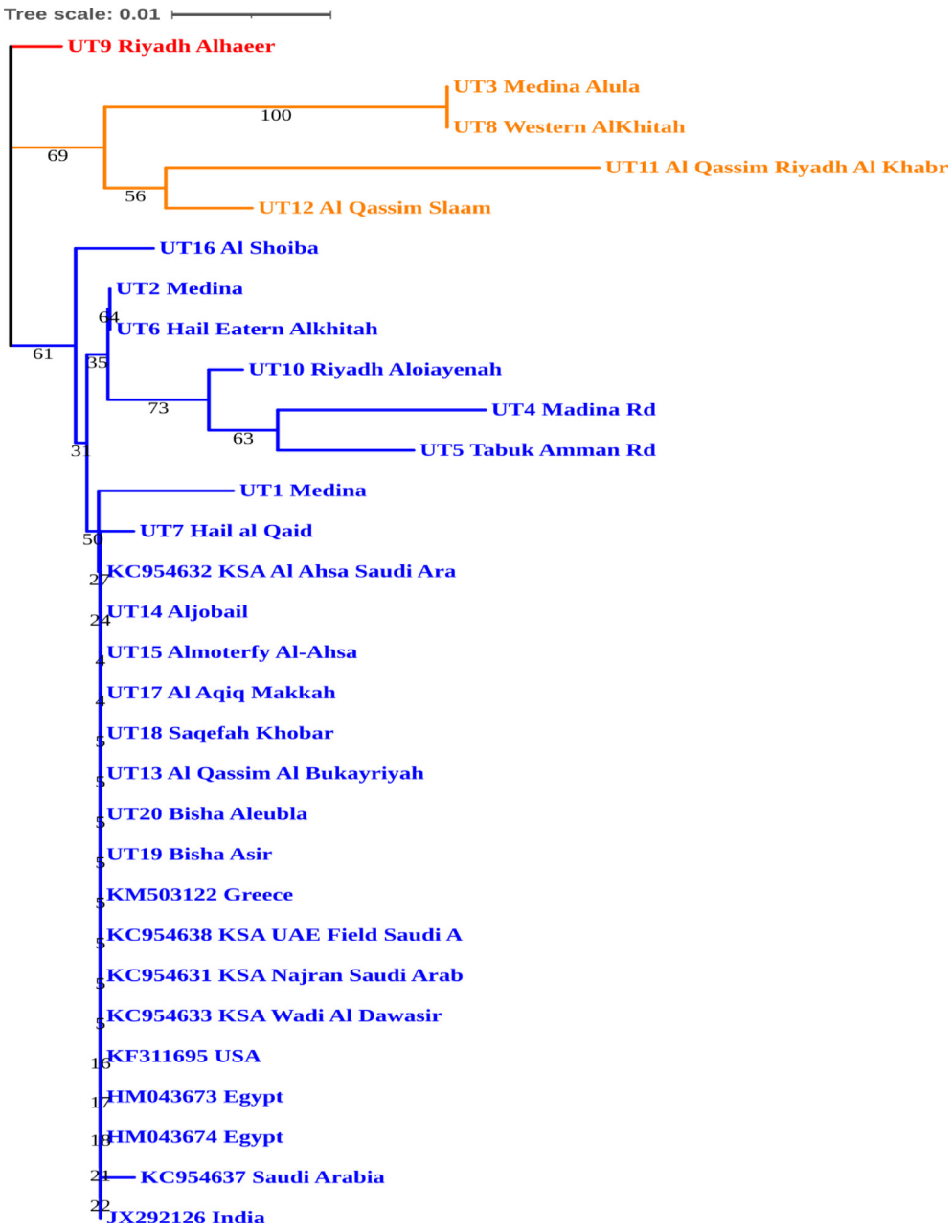


**Fig. 2.** The phylogenetic tree constructed using Neighbor-Joining method for specimens under investigation.

assessing the molecular diversity of fungi, plants and animals. Recently this nuclear region of DNA is found efficient for the estimation of diversity and identification of insects such as Coleopterans (Sayed, 2016).

We found that a haplotype from Riyadh region (UT9) was unique and the earlier studies have also declared many unique haplotypes from Riyadh and concluded this site as hot spot of infestation (Sukirno et al., 2020a; Sukirno et al., 2020b). Our observations coincides with the previous study that Riyadh and its surrounding regions would have witnessed multiple introductions of *R. ferrugineus* from different sources or single introduction of multiple haplotypes (Sukirno et al., 2020a; Sukirno et al., 2020b). Sukirno et al. (2020a), Sukirno et al. (2020b) recognise a third haplotype to be more prevalent in Saudi Arabia, however cross verification of sequences of this study (ITS) with Sukirno et al. (2020a), Sukirno et al. (2020b) (COI) was not possible. We recognise through phylogenetic grouping that, third haplogroup (bottom of the tree) were ubiquitous while first two group were more localised in Saudi Arabia. Since in past few decades, date palms are more rapidly transported across Saudi Arabia (especially from Riyadh regions) (MOA, 2014), rapid infestations and expansions of new territory is possible.

## 5. Conclusion

Through genetic data, we have provided a strong evidence that there were multiple haplotypes of *R. ferrugineus* which were bound to expand its diversity. Higher nucleotide diversity and Tajima's neutrality test values suggest that there were higher movement of infested palm trees, as desserts were natural barriers for movement of *H. ferrugineus*. The findings of this study will be useful for

**Table 3**
Kimura-2 distance data of ITS sequences of 20 haplotypes of *H. ferrugineus*. Greener the shades, lower the scores. Redder the shades higher the scores.

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | UT2_Medina | | | | | | | | | | | | | | | | | | | | |
| 2 | UT6_Hail_Eastern Alkhitah | 0.000 | | | | | | | | | | | | | | | | | | | |
| 3 | UT14_Aljobail | 0.002 | 0.002 | | | | | | | | | | | | | | | | | | |
| 4 | UT1_Medina | 0.011 | 0.011 | 0.009 | | | | | | | | | | | | | | | | | |
| 5 | UT15_ Al-Ahsa_Almoterfy_ | 0.002 | 0.002 | 0.000 | 0.009 | | | | | | | | | | | | | | | | |
| 6 | UT7_Hail_al_Qaid | 0.004 | 0.004 | 0.002 | 0.011 | 0.002 | | | | | | | | | | | | | | | |
| 7 | UT9_Riyadh_Alhaeer | 0.011 | 0.011 | 0.009 | 0.017 | 0.009 | 0.011 | | | | | | | | | | | | | | |
| 8 | UT3_Medina_Alula | 0.035 | 0.035 | 0.033 | 0.040 | 0.033 | 0.035 | 0.031 | | | | | | | | | | | | | |
| 9 | UT8_ Hail_Western_AlKhitah | 0.035 | 0.035 | 0.033 | 0.040 | 0.033 | 0.035 | 0.031 | 0.000 | | | | | | | | | | | | |
| 10 | UT11_Al_Qassim_Riyadh_Al_Khabr | 0.046 | 0.046 | 0.044 | 0.051 | 0.044 | 0.042 | 0.042 | 0.051 | 0.051 | | | | | | | | | | | |
| 11 | UT12_Al_Qassim_Slaam | 0.019 | 0.019 | 0.022 | 0.031 | 0.022 | 0.024 | 0.017 | 0.033 | 0.033 | 0.033 | | | | | | | | | | |
| 12 | UT17_Taif_Al_Aqiq | 0.002 | 0.002 | 0.000 | 0.009 | 0.000 | 0.002 | 0.009 | 0.033 | 0.033 | 0.044 | 0.022 | | | | | | | | | |
| 13 | UT18_ Al Khobar_Saqefah | 0.002 | 0.002 | 0.000 | 0.009 | 0.000 | 0.002 | 0.009 | 0.033 | 0.033 | 0.044 | 0.022 | 0.000 | | | | | | | | |
| 14 | UT13_Al_Qassim_Al_Bukayriyah | 0.002 | 0.002 | 0.000 | 0.009 | 0.000 | 0.002 | 0.009 | 0.033 | 0.033 | 0.044 | 0.022 | 0.000 | 0.000 | | | | | | | |
| 15 | UT20_Bisha_ Asir | 0.002 | 0.002 | 0.000 | 0.009 | 0.000 | 0.002 | 0.009 | 0.033 | 0.033 | 0.044 | 0.022 | 0.000 | 0.000 | 0.000 | | | | | | |
| 16 | UT19_Bisha_Asir | 0.002 | 0.002 | 0.000 | 0.009 | 0.000 | 0.002 | 0.009 | 0.033 | 0.033 | 0.044 | 0.022 | 0.000 | 0.000 | 0.000 | 0.000 | | | | | |
| 17 | UT16_Al_Shoiba_Makkah Region | 0.009 | 0.009 | 0.006 | 0.015 | 0.006 | 0.009 | 0.013 | 0.037 | 0.037 | 0.044 | 0.022 | 0.006 | 0.006 | 0.006 | 0.006 | 0.006 | | | | |
| 18 | UT4_Tabuk_Madina_Rd | 0.024 | 0.024 | 0.026 | 0.035 | 0.026 | 0.028 | 0.035 | 0.060 | 0.060 | 0.053 | 0.035 | 0.026 | 0.026 | 0.026 | 0.026 | 0.026 | 0.028 | | | |
| 19 | UT10_Riyadh_Aloiayenah | 0.009 | 0.009 | 0.011 | 0.019 | 0.011 | 0.013 | 0.019 | 0.044 | 0.044 | 0.046 | 0.020 | 0.011 | 0.011 | 0.011 | 0.011 | 0.011 | 0.013 | 0.019 | | |
| 20 | UT5_Tabuk_Amman_rd | 0.019 | 0.019 | 0.022 | 0.028 | 0.022 | 0.024 | 0.031 | 0.051 | 0.051 | 0.044 | 0.035 | 0.022 | 0.022 | 0.022 | 0.022 | 0.022 | 0.028 | 0.022 | 0.015 | |

**Table 4**
Results from Tajima's Neutrality Test.

| | *S* | *Ps* | *Θ* | *π* | *D* |
|---|---|---|---|---|---|
| 20 | 44 | 0.094 | 0.026 | 0.019 | −1.111 |

n = total number of sites, S = Number of segregating sites, ps = S/N, Θ = ps/a1, π = nucleotide diversity, and D is the Tajima test statistic.

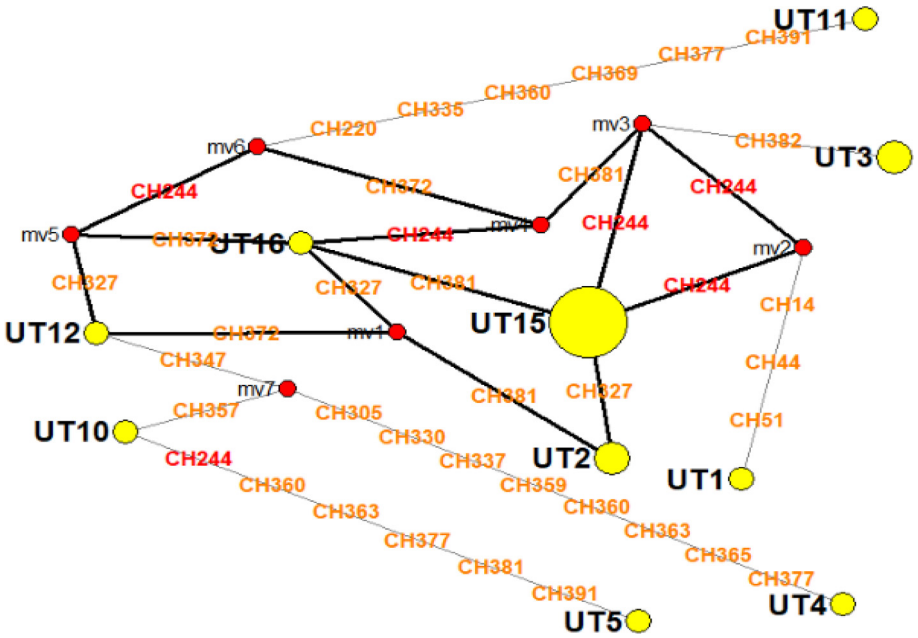


**Fig. 3.** Median joining network diagram showing 10 haplotypes (yellow circles) of *R. ferrugineus* in this study. Majority consensus generated sequences was indicated by red points (median vectors; mv) which connects triplet of sequences. Mutations in the position of the characters were indicated CH and most frequent mutation (n = 6) was at CH244 position (indicated by red text). Size of the circle corresponds to the number of the haplotypes present.

pest controlling agencies and to the workers involved in infested date palm quarantining.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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