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Molecular identification of red palm weevil *Rhynchophorus ferrugineus* (Olivier) collected from three localities in Saudi Arabia

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The red palm weevil *Rhynchophorus ferrugineus* (Olivier) is one of the most destructive pests of date palms in several countries, including Saudi Arabia. Experiments were designed to explore if the *R. ferrugineus* spread in Saudi Arabia is one or more species. To achieve this objective, 124 larval and adult specimens were collected from Riyadh, Al-Kharj, and Al-Qassim cities and the mitochondrial cytochrome oxidase subunit 1 (*mtCO1*) gene was used as a barcoding region to investigate its specific status and genetic relatedness to other *Rhynchophorus* species distributed worldwide. Average uncorrected pairwise distances were obtained from 55 specimens (21 from Al-Kharj, 3 from Al-Qassim, and 31 from Riyadh). Results of partial direct sequencing of the mt*CO1* gene indicated genetic similarity between individuals of the three studied locations. In addition, maximum likelihood method for phylogenetic analysis confirmed the interference of mt*CO1* gene sequences of the collected samples. The present results are in accordance with those of the *R. ferrugineus* distributed in other countries.

Keywords: Rhynchophorus ferrugineus, Mitochondrial *CO1* gene, PCR, Sequencing, Phylogeny

INTRODUCTION

The red palm weevil (RPW) *Rhynchophorus ferrugineus* (*R. ferrugineus*) is a devastating palm pest that affects 40 palm species all over the world (Faleiro, 2006; Al-Dosary et al. 2016). It is native to southeast Asia, however, recently, its range has expanded vastly, as a result of multiple accidental anthropogenic introductions in the Middle East (Soroker et al. 2005; Al-Shawaf et al. 2013). *R. ferrugineus* is the most widespread and economically important pest of date palm in Saudi Arabia (Al-Dosary et al. 2016). *R. ferrugineus* completes its life cycle inside the trunk of palm trees (Avand, 1996; Abraham et al. 1998, 2001). This cryptic feeding habit of the RPW has rendered 25 million palms in Saudi Arabia at risk (Ministry of Agriculture, 2010). The invisible lifestyle of red palm weevil grubs in the trunk exhibits serious management challenges (Hussain et al. 2019).

Given the high distribution capacity of *R. ferrugineus* owing to its strong flight capability, its population is suspected to show some genetic diversity (Abulyazid et al. 2002; Rugman-Jones et al. 2013). Transporting infested trees and offshoots for burning and continued introduction of new date palm varieties into new regions makes it highly probable for the insect population to change genetically (El-Mergawy et al. 2011a, b; Al-Saad et al. 2018). Although traditional morphological methods of identification are successful in identifying taxonomic relationships at higher taxonomic levels, molecular methods of identification are important to elucidate the relationships among insect species and subspecies (Caterino et al. 2000). In this context, molecular examination of the genetic diversity of

different *R. ferrugineus* strains has been conducted by several researchers, in the Middle East and the Mediterranean Basin (Gadelhak & Enan, 2005; El-Mergawy et al. 2011a, b; Hashem, 2016). Furthermore, the study of genetic variations among invasive species (e.g., *R. ferrugineus*) is essential for developing suitable management strategies (Armstrong & Ball, 2005; Grapputo et al. 2005; Sharma et al. 2009).

The mitochondrial cytochrome oxidase subunit 1 (mt*CO1*) gene sequence has been used as a bio-identification tool for detecting genetic variations, phylogeny, and geographical distribution and in barcode studies of different insect species (Hebert et al. 2003). The use of mitochondrial DNA markers in insects has several advantages, such as presence of maternal inheritance, high rate of evolution, haploid status, availability of universal primers, and the capability to be used for species in which their sequences are not known (Roehrdanz, 1993; Zhang & Hewitt, 2003).

Molecular identification and characterization of invasive insect pests, such as *R. ferrugineus*, based on mt*CO1* is important for their proper identification, because of high gene polymorphism and the existence of different *Rhynchophorus* spp. (Manzoor et al. 2018). Furthermore, the mt*CO1* marker has also been used to investigate the invasion history and origins of *R. ferrugineus* (Rugman-Jones et al. 2013).

In the present study, I aimed to identify *R. ferrugineus* collected from three localities in Saudi Arabia (Riyadh, Al-Kharj, and Al-Qassim) by direct sequencing of the *mtCO1* gene. Phylogenetic analysis of the nucleotide sequence was carried out by framing maximum likelihood trees, based on the Tamura–Nei model (Tamura & Nei, 1993). Proper identification of RPW can potentially aid in pest management by eco-friendly control measures.

MATERIALS AND METHODS

Ethics statement

Red palm weevils were collected from a private date palm farm, with the owner's permission. To the best of my knowledge, the collections included herein were not from National Parks or wild protected areas. Furthermore, these weevils are most certainly not an endangered species.

RPW sampling

Insect specimens, both adult males and females, averaging 3.62 ± 0.08 cm and 3.26 ± 0.06 cm in length, respectively, and full-grown larvae (≥3.6 cm in length) were collected during 2016, from infested date palms at three different localities in Saudi Arabia: Riyadh, Al-Kharj and Al-Qassim. A total of 124 specimens were collected, of which 48, 16 and 60 were from Al-Kharj, Al-Qassim and Riyadh, respectively. Each collected specimen was carefully washed in a physiological saline, labeled with the date and time of collection, transferred to firmly closed vials containing 95% ethanol and stored at 4 ℃ until processed.

DNA extraction and polymerase chain reaction (PCR)

Before isolation, a small piece of each ethanol-preserved specimen was cut, rinsed with distilled water, placed in a sterile 2 mL microcentrifuge tube, and crushed properly, prior to DNA extraction. QIAGEN DNeasy® Blood & Tissue Kit (Cat No. /ID: 51304, QIAGEN, Germany) was used to extract genomic DNA from individual specimens as per the manufacturer's instructions.

Amplification of the barcoding region of the mt*CO1* gene from each DNA sample was carried out by PCR, using the primer with forward sequence (COI- LCO1490 F) (5′-GGTCAACAAATCCATAAAGATATTGG-3′) and reverse sequence (COI- HCO2198 R) (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994). PCR was performed in 20 µL reaction volume, comprising of 4.0 µL of Master Mix (Solis BioDyne 5 × FIREPol®, Estonia), 12.5 mM MgCl2 (1 × PCR solution, 2.5 mM MgCl2) and 2 mM dNTPs of each (1 × PCR solution, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, and 200 µM dTTP)], 0.6 µL of each primer, 2.0 µL of the DNA template and 12.8 µL of nuclease-free water. Thermal Cycler (Thermal cycler, Applied Biosystems by Thermo Fisher Scientific, USA) was used for PCR and the conditions for thermal cycling were as follows: 1st stage of initial denaturation at 95 ℃ for 15 min, followed by 2nd stage of 35 cycles at 95 ℃, an annealing step at 51 ℃ and extension at 72 ℃, for 45 s each. This was followed by the 3rd stage of final extension at 72 ℃ for 10 min. PCR amplicons were run on 1.5 % agarose gel at 10 V/cm for 60 min, stained with ethidium bromide visualized under U.V light transilluminator (BDA compact-gel imager, Biometra Analytic jena, Germany).

DNA sequence and bioinformatics analyses

The PCR products were direct-sequenced using the aforementioned primers (COI- LCO1490 F and COI- HCO2198 R), with the Big Dye terminator V3.1 sequencing kit (Applera, Foster

City, CA, USA). They were then analyzed with the ABI 3700 DNA Analyzer (Applied Biosystem, Foster City, CA, USA), at the Central Laboratory of King Saud University.

BioEdit Sequence Alignment Editor version 7.2.5 (Hall, 1999) was used and the sequences were trimmed to approximately 645 bp, followed by multiple alignment with the reference mt*CO1* gene of *R. ferrugineus* using ClustalW adjusted to a maximum number of 1000 iterations (Thompson et al. 1994). The evolutionary history was inferred using the maximum likelihood method based on the Tamura–Nei model (Tamura & Nei, 1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log-likelihood value. The analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 418 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). Number of bootstrap replications is 100.

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RESULTS

All collected specimens were processed for DNA extraction, however, successful results were achieved for only 55 specimens (21 from Al- Kharj, 3 from Al-Qassim, and 31 from Riyadh). Analysis of PCR products indicated that RPW specimens had a band size of approximately 700 bp (Fig. 1).

These were then identified to the lowest possible taxonomic level, using sequence alignments of the mt*CO1* gene, in order to make comparative analysis of the species with respect to their environment.

In the present study, all sequences were identified using the NCBI BLAST tool, and then aligned with the sequences of other RPW from GenBank database, as shown in Fig. (2). Direct nucleotide sequences of the mt*CO1* gene showed that all RPW individuals were 99.98% identical. The least genetic distance was recorded as intraspecific variations among the RPW individuals, ranging from 0.0 to 3.0%. One sequence from samples collected from each location was selected randomly and deposited in the Genebank as the followings: Riyadh (Acc. #: MH016278), Al-Kharj (Acc. #: MH016277) and Al-Qassim (Acc. #: MH016279).

The phylogenetic tree constructed using Maximum-Likelihood method based on multiple sequence alignment of the mt*CO1* gene showed that the samples of RPW obtained from different geographic localities were of the same species. In addition, they were 99-100% identical to other *R. ferrugineus* deposited in the Genebank under the accession numbers: KT428893.1 and KF311360.1 as they were grouped the phylogenetic tree in one clade while the outgroup represented by *Dermestes maculatus* was placed in other clade. The genetic relatedness revealed the least genetic divergence observed for this species from different infested governorates of Saudi Arabia (Fig. 3).

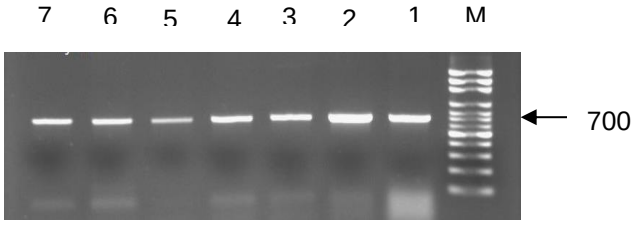

Figure1: Gel electrophoresis for PCR products of randomly selected *R. ferrugineus* specimens (1–3 from Riyadh; 4, 5 from Al-Kharj; 6, 7 from Al-Qassim). M: DNA marker (100bp).

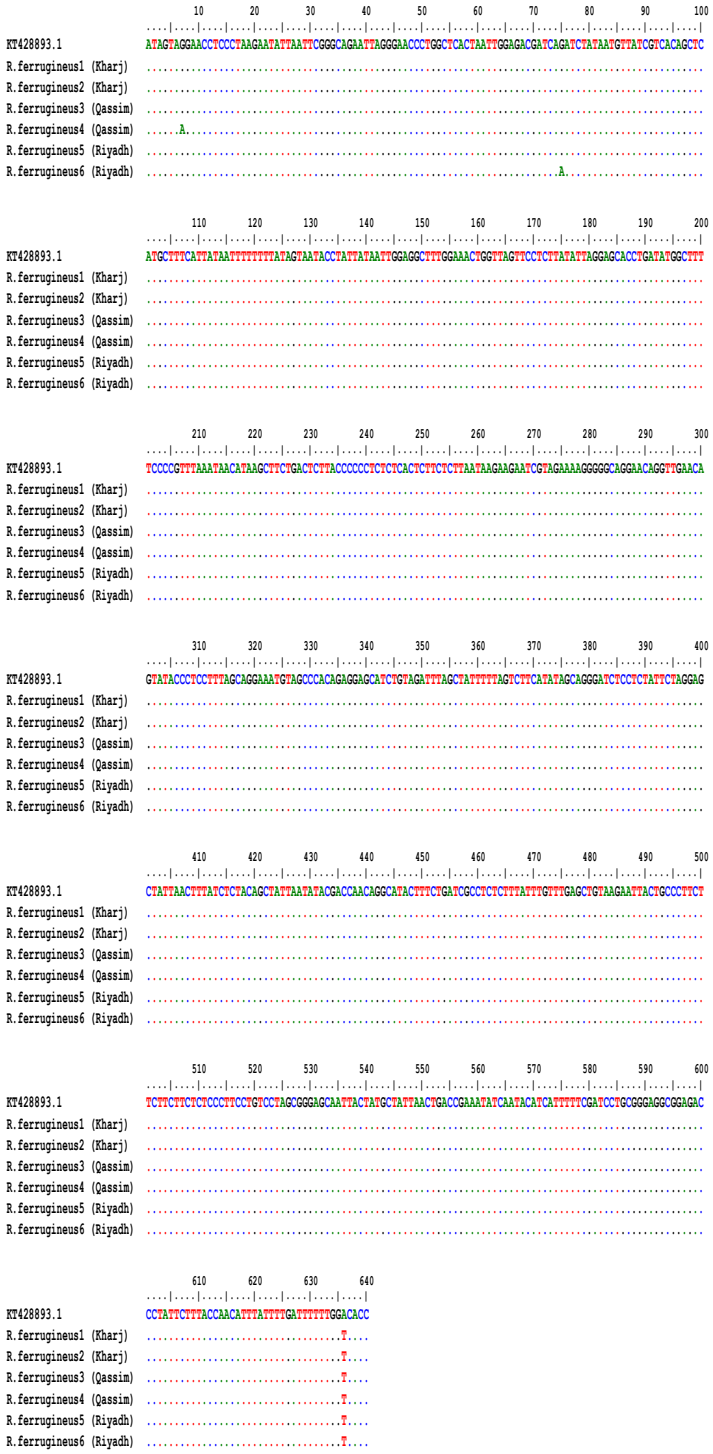

Figure2: Alignment of the partial sequence of the mitochondrial *CO1* gene of *R. ferrugineus* specimens (1–6), from various regions with the reference species, *R. ferrugineus* mitochondrion, complete genome (KT428893.1).

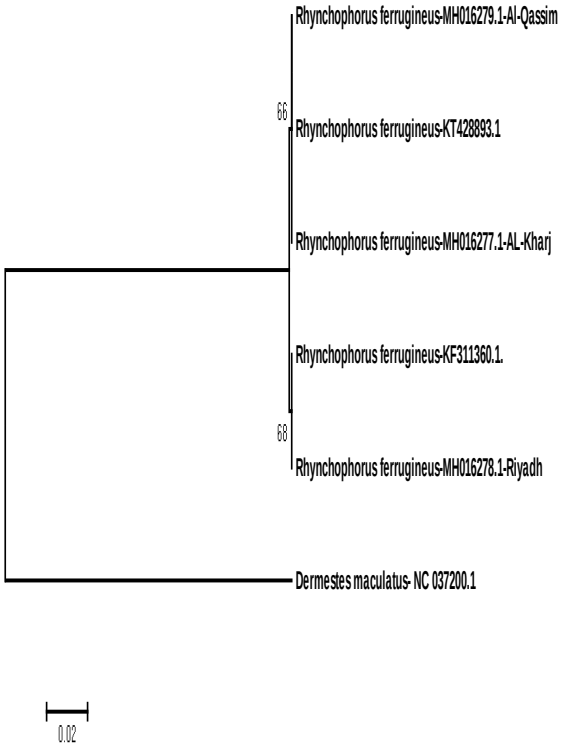

Figure 3:Molecular phylogenetic analysis of *R. ferrugineus* by the maximum likelihood method.

DISCUSSION

In the present study, PCR products of mt*CO1* resulted in a single band of 700 bp, for the three strains of R. ferrugineus collected from Al-Kharj, Al-Qassim, and Riyadh regions. Similarly, Manzoor et al. (2018) obtained clear bands of 710 bp for RPW specimens collected from different provinces in Pakistan (Khyber Pakhtunkhwa (KPK), Punjab, Sindh, and Baluchistan).

Phylogenetic analysis of *R. ferrugineus* strains from Riyadh (Acc. #: MH016278), Al-Kharj (Acc. #: MH016277) and Al-Qassim (Acc. #: MH016279) revealed that they were genetically similar (99.98%) to each other and to other *R. ferrugineus* strains identified worldwide (Acc. #: KT428893.1 and KF311360.1). Previous studies have shown a maximum range of 97–99% identity among individuals of *R. ferrugineus* from different regions, including Egypt (Gadelhak and Enan, 2005), the Middle East and the Mediterranean Basin (El-Mergawy et al. 2011a, c), and Philippines (Abad et al. 2014). In addition, previous mt*CO1*-based analyses revealed that geographically different populations from various localities of Saudi Arabia are genetically similar to each other and to *R. ferrugineus* from other countries, for example, Pakistan (Manzoor et al. 2018) and Egypt (Gadelhak and Enan, 2005; Hashem, 2016).

In the present investigation, we could not find major genetic variations among the samples collected from different geographical localities in Saudi Arabia. This suggests that a single species of *R. ferruginous* exists in the studied areas of Saudi Arabia. This finding ensures the previous suggestion that little genetic variations over the geographic populations do not cause inherent changes (Marimuthu et al. 2009).

CONCLUSION

In conclusion, molecular identification of RPW samples collected from three different geographical regions of Saudi Arabia revealed that RPW populations are genetically similar, not only to each other but also to *R. ferrugineus* identified from other countries. These results might help in drafting species-specific policies (e.g., RNAi technique) and allow for better management of *R.*

ferrugineus in Saudi Arabia, thus preventing RPW from spreading to other date palm-growing areas. Furthermore, such findings may be helpful for drafting quarantine protocols at Saudi Arabia's air and sea ports. Additional studies are recommended in the RPW-invaded regions of Saudi Arabia to certainly show if one or more species of RPW exists.

CONFLICT OF INTEREST

The author declared that she has no conflict of interest regarding the content of this article.

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AUTHOR CONTRIBUTIONS

Reem Alajmi has designed and done all the works and all analyses of the present work.

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