Genetic Diversity among *Rhynchophorus ferrugineus* Populations from Saudi Arabia and India

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ABSTRACT

The red palm weevil (*R. ferrugineus*), Rhynchophorus ferrugineus (Olivier) (Coleoptera, Curculionidae), is a great threat to the Saudi date palm *Phoenix dactylifera* L., which is the most important crop of Saudi Arabia. More than 400 date palm verities are grown with 25 of them classified as the most common. *R. ferrugineus* attacks coconut in India, therefore, the objective of this work was to assess possibility of classifinging indian *R. ferrugineus* as the source of infection in Saudi Arabia using PCR technique to determine the relationship between genetic variation of *R. ferrugineus* was determined for two populations: one from Saudi Arabia on date palm and the other from India on coconut. *R. ferrugineus* adults were collected from Alhasa (Saudi Arabia) and Goa (India) using insecticide free, food-baited pheromone (Ferrolure™) traps in both plantations. The weevils were subjected to genomic DNA extraction using molecular techniques to perform the Polymerase Chain Reaction (PCR), subsequent transformation, and extraction of recombinant plasmids and cloning for nucleotide sequencing. Results revealed that sequence identity were identical (99-100%), whereas the two populations were 96-97% identical. The sequence showed that *R. ferrugineus* populations from Saudi Arabia and India were likely to be from different geographical regions; suggesting that *R. ferrugineus* population in Saudi Arabia is not from India.

Key Words: CO1 gene, *Phoenix dactylifera*, Phylogenetic relationship, Polymerase Chain Reaction (PCR) *Rhynchophorus ferrugineus*.

INTRODUCTION

Date palm, *Phoenix dactylifera* L., is the most important crop of Saudi Arabia, which is one of the top date producing countries and growing over 400 date palm varieties of which 25 are the most common. Saudi Arabia produced about 766,800 tonnes of dates (based on imputation methodology) in 2014 while 1,095,158 tones in 2013 (FAO, 2014). The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera, Curculionidae), is attacking about 40 palm species (Save Algarve palms, 2014) including date palm in Saudi Arabia and Spreading rapidly in date palm plantations of the Arabian Peninsula in 1985 and coconut, *Cocos nucifera*, in India, where known to be existed over one hundred years (Abraham *et al*., 1998; Alhudaib, 1998; Faleiro, 2006; Maxwell-Lefroy, 1906; Wattanapongsiri, 1966; Zaid *et al*., 2002). Palm volatiles attract adult female weevils of *R. ferrugineus* to lay eggs, which hatch into damage inflicting grubs. The newly hatched grubs bore into the palm tissue resulting in extensive internal damage that is often difficult to detect at early stage of infestation.

FAO has identified *R. ferrugineus* as a category-1pest of date palm in the Middle East. Factors like high fecundity, difficulties of early detection due to its concealed nature, coupled with vast stretches of young plantations along with the lack of proper quarantine regimes to certify movement of pest-free planting materials have influenced the rapid increase and spread of *R. ferrugineus* in date palm plantations (Anonymous, 2004; Faleiro, 2006). To manage *R. ferrugineus* in both date palm and coconut plantations, a pheromone (ferrugineol) based on Integrated Pest Management (IPM) strategy is used to monitor and mass trap this pest in farms, where insecticides applied as major role in preventive and curative control methods (Abraham *et al.* 1998; El-Sabea *et al*., 2009; Faleiro, 2006).

A variation of spots color on pronotum of the genus *Rhynchophorus* has been existing with 24 color (Wattanapongsiri, 1966). El-Mergawy *et al*. (2011a) compared genetic relationship between *R. ferrugineus* and other four *Rhynchophorus* spp. in the mitochondrial gene region of Cytochrome c Oxidase subunit I (COI), *R. ferrugineus* was more similar to *R. bilineatus* than *R. palmarum*. Perez *et al*. (1996) suggested species synonym of *R. ferrugineus* and *R. vulneratus* while, Hallett *et al*. (2004) proposed that these two species considered as color morphs of the same species based on morphological, molecular-genetic and breeding data and given the same scientific name *R. ferrugineus*, which is called "Asian Palm Weevil".

Several investigations have proven genetic variation of *R. ferrugineus* from different regions using RAPD (Abulyazid *et al*., 2002; Salama and Saker, 2002; Gadelhak and Enan, 2005; Al-Ayied *et al*., 2006; El-Mergawy *et al*., 2011a,b,c).

Molecular technique, (*Cytochrome c oxidase* subunit I (COI)), was used to determine a relationship between genetic variation of *R. ferrugineus* populations from Saudi Arabia and India. Likewise, a relationship between genetic variation of the two populations was compared with available gene bank entries and COI locus was used to determine evolution rates between populations (Hebert *et al.*, 2003). The objective of this genetic variation study is to clarify that *R. ferrugineus* population of Saudi Arabia whether is from India or other region using PCR techniques.

MATERIALS AND METHODS

Collection of weevil adults:

Adults of R. *ferrugineus* for this study were attracted and collected using the standard bucket 5L four-window, insecticide free, food baited pheromone (Ferrolure^{TM}) traps in date palm and coconut plantations, respectively (Faleiro, 2006). In Saudi Arabia traps, dates were used as food bait, while traps in India were baited with sugarcane. Adults of *R. ferrugineus* were collected from 10 date palm locations in Alhasa, Eastern provence of Saudi Arabia and from 8 coconut locations in Goa (India) (Table 1). All adults were preserved in 95% ethanol at -80^{o}C to extract DNA. Molecular analyses were performed at Pests and Plant Diseases Unit, College of Agriculture and Food Science, King Faisal University.

Table 1. *R. ferrugineus* samples collected from Saudi Arabia and India

Sample locations	Village	GPS Coordinates	
Saudi Arabia		N	E
1	Aboshabal	25.26485	49.36139
2	Asfar	25.27619	49.44641
3	Batalyah	25.25693	49.37535
4	Fudul	25.22070	49.40532
5	Gowaybah	25.18115	49.36711
6	Hofuf	25.25472	49.39364
7	Kadood	25.22661	49.37178
8	Shobah	25.28166	49.37618
9	Shuquaiq	25.29446	49.33998
10	Suhemia	25.27746	49.36184
India			
1	Loutolim	03.90599	16.95817
2	Majorda	03.82876	16.92951
3	Mapusa	03.73027	17.25543
4	Raia	03.90123	16.92531
5	Raia	03.90702	16.93877
6	Sao Jose de Areal	03.93547	16.85757
7	Seraulim	03.85732	16.91283
8	Siolim	03.67742	17.26752

DNA extraction and sequencing:

Neck muscles of adult weevils were grounded with pestle in liquid nitrogen and suspended in 580 µL of SDS extraction buffer solution (Dellaporta *et al.,* 1983) (200mM Tris-HCl pH 7.5, 25mM EDTA pH 8.0, 50ml 10% SDS, 250mM NaCl, 650ml ddH2O) and 8 µL of RNase A solution (100mg/mL) was added. The ground neck muscles were vortexed for 10 seconds for proper mixing and than placed in water bath at 37°C for 1 hour. Ten µL of proteinase K solution (20mg/mL) was added and mixed gently than incubated at 50°C overnight. Equal volume of phenol:chloroform: Isoamyle alcohol (PCI) (25:24:1) was added. Mixtures were vortexed for 15 sec and centrifuged for 10 minutes at 10,000 rpm. Upper phase was transferred into new 1.5mL tube then 1/10 volume of 3 M Sodium acetate (pH 5.2) with 2 volume of chilled absolute ethanol were added and mixed by inverting tube and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant fluid was removed and pellet was washed with 800 µL of 70% ethanol and centrifuged for 3 minutes at 10,000 rpm. Ethanol was discarded completely without disturbing DNA pellet that was dried at room temperature. DNA was re-suspended in 100 µL TE buffer. Lastly, DNA samples were stored in a refrigerator at -20ºC.

Polymerase Chain Reaction (PCR):

PCR was done in a 25 µL reaction containing 1 µL of the total DNA extract (40 ng of total DNA), 2 mM MgCl2, 2.5 of 10x PCR buffer, 1.5 µL of 10 µM of CO1 primer (forward, 5'-TATAGCATTCCCCGTTTA-3' and reverse,5'-TCCTAATAAACCAATTGC-3'), 2.5 µL of 10 mM dNTPs, 0.3 µL of 5U Taq DNA Polymerase and reaction was completed to 25 µL with Nuclease-free water. PCR was conducted in the ESCO Swift Maxi Thermal Cycler with primary denaturation at 94°C for 5 min, then by 40 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min, final cycle was a polymerization cycle performed at 72°C for 5 min. Obtained PCR products were loaded on 1.5% agarose gel, 100 bp ladder was used as a reference for sizing of amplified PCR fragments. Five EasyStain II µL was added to 100 ml agarose solution and DNA fragments were visualized by UV illumination using Gel Documentation System (Syngene UK).

Cloning and Sequencing:

Cloning of PCR products were conducted using pGEM®T-Easy vector (Promega, USA). Ligation of amplified PCR into the vector was done in the mixture containing 1 µL of pGEM®T-Easy vector (Promega, USA), 2 µL of PCR product, 5 µL of 2X Rapid buffer, 1 µL of T4 DNA ligase and ddH_2O were added to make the final volume of 10 µL. The mixture was pipetting gently and incubated overnight at 4°C. Recombinant vector was transformed into competent cells which were thawed on ice for 30 min. Hundred µL of DH5α Competent Cells (Invitrogen) (ThermoFisher Scientific, USA) was transferred to cooled 5 ml tube then 5 µL of ligated DNA was added, the mixture was incubated on ice for 30 min. Cells were exposed to heat for 2 min by immersing tubes into water bath at 42°C then transferred on ice for 2 min. after transformation 1 ml LB medium was added and tubes were incubated for 1 h at 37°C in shaking water bath. Hundred µL of transformed cells were grown on LB agar medium amended with 100 ppm ampicillin over night at 37°C. bacterial white colonies in which plasmid was successfully transformed were selected and inoculated on LB amended with 100 ppm ampicillin in a shaking water bath at 37°C overnight.

Wizard plus SV Minipreps DNA Purification kit was used to extract Recombinant plasmids as described by manufacturer's instructions (Promega, USA). The extracted minipreps was digested by restriction endonuclease *Eco*RI to ensure that the amplified PCR was inserted. One µl of 10X buffer H and 6µl of extracted DNA mini-prep. 0.5µl of *Eco*RI and the mixture was completed to 10 µl by adding ddH_2O, and then placed in water bath at 37°C for 4 hours. DNA fragments were separated on 1.5% agarose gel stained

with EasyStain II. Clones of *R. ferrugineus* containing 545bp were sequenced in both directions. Sequencing was conducted at Macrogen Company, Seoul, South Korea using 3730XL automated DNA capillary sequencer. Sequence alignments and phylogenetic relationship were done by MEGA6 (Tamura *et al*., 2013).

RESULTS AND DISCUSSION

Figure (1) showed the results of amplified fragments obtained using the primer pairs. All test samples produced a 546 bp fragment.

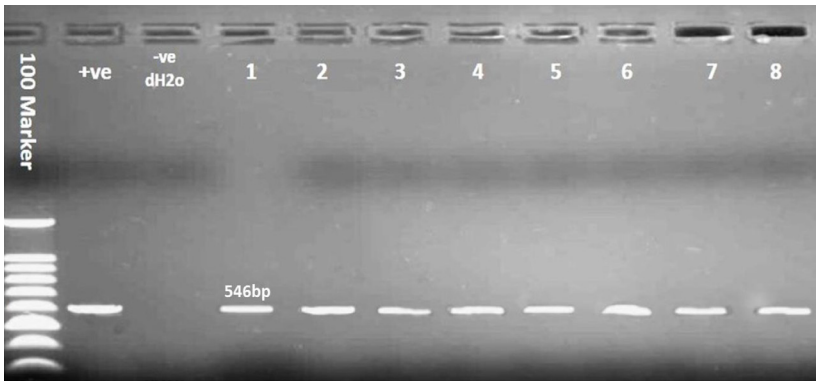

Fig. 1. Results of PCR amplification products. M:100 bp DNA ladder, +ve: positive control, -ve: negative control (*R. ferrugineus* band samples using CO1f/r primers).

PCR products were sequenced and analyzed to confirm COI gene (Fig 2). Phylogenetic analyses were conducted using MEGA6 (Tamura *et al.,* 2013). Our data showed that there is no different between female and male in term of CO1 gene.

TGACTCTTACCCCCCTCTCTCACTCTTCTCTTAA
TAAGAAGAGTCGTAGAAAAGGGGGCAGGAACA
GGTTGAACAGTATATCCTCCTTTAGCAGGAAATG
TAGCCCACAGAGGAGCATCTGTAGATTTAGCTAT
TTTTAGTCTTCATATAGCAGGGATCTCCTCTATT
CTAGGGGCTATTAACTTTATCTCTACAGCTATTA
ATATACGACCAACGGGCATACTTTCTGATCGCCT
CTCTTTATTTGTTTGAGCTGTAAGAATTACTGCC
CTTCTTCTTCTTCTCTCCCTTCCTGTCCTAGCGG
GAGCAATTACTATGCTATTAACTGACCGAAATAT
CAATACATCATTTTTCGATCCTGCGGGAGGCGG
AGACCCTATTCTTTACCAACATTTATTTTGATTT
TTTGGACACCCAGAAGTTTATATTCTTATCCTTC
CAGGATTTGGAATAATTTCCCATATTATTACTAA
TGAAAGAGGAAAAAAAGAAACCTTTGGAATTCT
TGGTATAATTTATGCCATAATAGCAATTGGTTTAT
TAGGA

Fig. 2. Nucleotide sequence of CO1 gene 546 bp of *R. ferrugineus* sample.

Al-Ayied *et al.*, 2006 evaluated phylogenetic relationship between three phenotypically different forms of *R. ferrugineus* populations collected from date palm plantations of Alhasa, Saudi Arabia, using a PCR based RAPD technique finding that black and brown color morphs were genetically closer. Intra color variation remained minimum in black but brown spotted morphs exhibited more variation indicating a generation of new mutants or a different race. This could have major implications for *R. ferrugineus* control strategies; regarding to response of adult weevils to semiochemicals (Faleiro, 2006).

Phylogenetic analysis of CO1 gene of *R. ferrugineus*, representative of Saudi Arabia and India (Fig3).

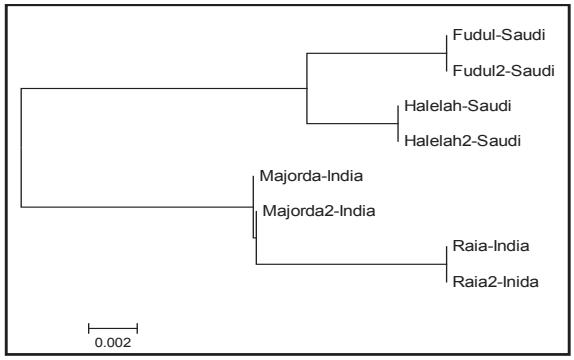

Fig. 3. Phylogenetic tree constructed based on analysis of CO1 gene sequences of *R. ferrugineus*. The 0.002 bar indicates one nucleotide change per 5000 nucleotides.

equence identity showed that Fudul and Halelah (Saudi Arabia) were identical 99-

100% as same 99-100% with Majorda and Raia (India). However 96-97% identical between Saudi Arabia and India populations, this support that *R. ferrugineus* is different origins (Rugman-Jones *et al*., 2013) (Table 2).

Table 2. Genetic similarity index among the eight CO1 of *R. ferrugineus* based on the pair wise analysis of 564 bp of CO1 gene sequences using the Neighbor-Joining method substitution model (Tamura *et al*., 2013)

	Fudul Saudi	Fudul2 Saudi	Halelah Saudi	Halelah2 Saudi	Majorda India	Majorda2 India	Raia1 India	Raia2 India
Fudul Saudi	100%							
Fudul2Saudi	100%	100%						
HalelahSaudi	99%	99%	100%					
Halelah2Saudi	99%	99%	100%	100%				
Majorda India	97%	97%	97%	97%	100%			
Majorda2India	97%	97%	97%	97%	97%	100%		
Raia1 India	96%	96%	96%	96%	99%	99%	100%	
Raia2 India	96%	96%	96%	96%	99%	99%	100%	100%

Several investigations have been done by El-Mergawy *et al*. (2011b) using RAPD-PCR technique to explain genetic relationships between *R. ferrugineus* from 30 countries. The analysis results discriminated by tested populations into twelve clusters. Several genetic variation studies conducted to clarify *R. ferrugineus* origin supposing that were two main sources, first was related to all discovered populations in Mediterranean countries and Egypt, while second was detected populations in Pakistan, Iran and Arabian Peninsula. Furthermore, El-Mergawy *et al.* (2011c) divided *R. ferrugineus* populations from 12 countries into 3 haplotypes depending on the differences of Cytochrome b and ITS2 partial sequence. In contrast, Rugman-Jones *et al*. (2013) reported H9, H1, H14, H15, and H16, haplotypes were typically found in India, and (H1, H5) haplotypes were in Pakistan, whereas H8 and H17 haplotypes were in Saudi Arabia (Alhasa) (El-Mergawy *et al.*, 2011c; Yasin *et al*., 2016). Similarly, previous studies showed that Malaysia and Thailand were same H8 haplotype (El-Mergawy *et al.*, 2011c; Wang *et al*., 2015). Based on the results given in figures 2, 3, and table 2, it is clear that the relationship of the examined *R. ferrugineus* was not so close to support the claim that the Saudi collected samples were diven genetically from the same gene pool of the Inidian group since the values of genetic similarity was as low as 96%. Figure 3 also indicated that the Indian *R. ferrugineus* tree was not closely attached to the Saudi one based on Co1 gene.

Therefore, it could be concluded from obtained sequence and data that *R. ferrugineus* populations in Saudi Arabia were from different origins rather than India.

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التنوع الجيني لعشائر سوسة النخيل الحمراء (*Rhynchophorus ferrugineus*) من المملكة العربية السعودية والهند

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الملخص

نخيل التمر (*Phoenix dactylifera* L.) أهم محصول في المملكة العربية السعودية، ويزرع أكثر من 400 نوع من النخيل؛ 25 هي الشائعة. وتعتبر سوسة النخيل الحمراء، *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) الآفة الرئيسية لنخيل التمر في المملكة العربية السعودية ولنخيل جوز الهند في الهند.

تم تحديد العلاقة بين التنوع الوراثي لعشيرتين من سوسة النخيل الحمراء (*R. ferrugineus*) واحدة من المملكة العربية السعودية والأخرى من الهند. تم جمع الحشرات الكاملة من سوسة النخيل الحمراء من الأحساء (المملكة العربية السعودية) ومن غوا (الهند) بواسطة مصائد فرمونية (Ferrolure™) غذائية خالية من المبيدات في مزارع نخيل التمر ونخيل جوز الهند. تم استخراج الحمض النووي باستخدام التقنيات الجزيئية وتفاعل البلمرة المتسلسل، ومنه تم استنساخ الجين المطلوب، ومن ثم تعرف تسلسل النوكليوتيدات الخاص به.

كشفت النتائج تطابق تسلسل النوكليوتيدات (99-100%) بينما كان تطابق العشيرتين (96-97%). وأظهرت نتائج التسلسل أنه من المحتمل أن تكون عشيرتا المملكة العربية السعودية والهند من مناطق جغرافية مختلفة؛ مما يوحي بأن عشيرة سوسة النخيل الحمراء (*R. ferrugineus*) في المملكة لم يكن مصدرها الهند.

الكلمات المفتاحية: جين CO1، سلسلة تفاعل البلمرة (PCR)، العلاقة الجينية، نخيل التمر (*Phoenix dactylifera*).