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Full Length Article

Molecular Identification and Phylogenetic Analysis of Distinct Geographical Populations of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) in Pakistan

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Abstract

The morphological identification using diagnostic morphological keys need tedious procedure of microscopic examination of male genital structures of adults and also require preparation of sample or specimens. Molecular identification and characterization for invasive insect pest based on mitochondrial cytochrome oxidase I (COI) and Inter transcribed spacer (ITS) is very important for correct identification. Red Palm Weevil (RPW) (*Rhynchophorus ferrugineus*) is an invasive palm pest and its mitochondrial cytochrome oxidase I (COI) and Inter transcribed spacer (ITS) based identification and genetic variation is very important because of its high polymorphism and existence of different *Rhynchophorus spp*. Here, for the first time, based on both COI and ITS primers, we have identified and characterized Red Palm Weevil (RPW) (*R. ferrugineus*) collected from four Provinces (KPK, Baluchistan, Sindh and Punjab) of Pakistan. The genetic analysis of various geographical population of Red Palm Weevil (RPW) (*R. ferrugineus*) was carried out through PCR. Phylogenetic analysis of nucleotides sequence done by framing maximum likelihood trees using MEGA6 indicated that there were no genetic variations among our *R. ferrugineus* strains showing 99–100% among themselves while in association with other *Rhynchophorus* strains reported from KSA, UAE, Greece and USA exposed 97 to 99% homology. Further, RPW population collected from 4 provinces of Pakistan shared 84, 85 and 87% homology for *R. palmarum, R. vulneratus* and *R. cruentatus* respectively. While, the evolutionary genetic divergence of our *R. ferrugineus* strains RPW1 (Acc. no: KY458178), RPW3 (Acc. no: KY458179) and RPW4 (Acc. no: KY458180) with other *R. ferrugineus* strains of other countries ranged 2–10 indicating maximum similarity index. We found value greater than 10 of evolutionary divergence for strains other than *R. ferrugineus* reported from other countries. This is the first evidence of *R. ferrugineus* widely occurrence, and its molecular identification and characterization from four provinces of Pakistan. This study will help us to develop species specific *R. ferrugineus* control measures. © 2018 Friends Science Publishers

Keywords: PCR; Red Palm weevil; COI; ITS; Phylogeny

Introduction

The Red Palm Weevil (*Rhynchophorus ferrugineus*; Order: Coleoptera and Family: Curculionidae), is documented to have a Southeast Asian origin. It was assumed that Red palm Weevil (hereafter read as RPW) dispersal occurred due to the shifting of ornamental palms across the Arabian Peninsula to Middle East including Pakistan (El-Mergawy *et al*., 2011a).

The invasion of *R. ferrugineus* was also reported in United States of America (USA), Japan, and Jordan (Kehat, 1999; Abe *et al.*, 2009; Suma *et al.*, 2014). The incidental presentation of invaded palm trees in the Middle East area, China and Japan made the red palm weevil to extend broadly (El-Mergawy and Al-Ajlan, 2011). Moreover, it is reflected as most disparaging pest of coconut trees (Abraham *et al*., 1975; Ferry and Gomez, 2002). Around 23 distinct genera, 3 families and more than 40 profitable date palm species are being affected by RPW (Faleiro *et al.*, 2012; Ministry of Agriculture, 2010) has claimed that 25 million palms in Saudi Arabia are at risk due to notorious and cryptic feeding nature of RPW. On the other hand, it was proclaimed that in case of India, RPW has caused approximate losses of 10–15% in coconut plantations (Murphy and Briscoe, 1999).

The taxonomic categorization is an important component to reduce the bewilderment between these

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weevils status (Wattanapongsiri, 1966). Sago palm weevil, Red strip weevil, Asiatic weevil and Coconut weevil are some names assigned to this pest according to different regions however, in South East Asia, proper identification of studied population was not accomplished. In the Middle East, there is a controversy that RPW is a Pakistani weevil (Rugman-Jones *et al*., 2013).

There are also controversies in the species status of *R. vulneratus* and *R. ferrugineus* after a similarity has been observed in their production and reaction to pheromones. A research is in progress to clear up this perplexity (Hallett *et al*., 1993; Perez *et al*., 1996). Orange form of *R. ferrugineus* was collected in the invaded territories of Egyptian and Middle East range (Hillis and Dixen, 1991; Salama and Sakar, 2002; Gadelhak and Enan, 2005; El-Mergawy *et al*., 2011a, b). The information about genetic variations can be verified by using the fundamental device which is the hereditary examination between the local and obtrusive species (Abulyazid *et al*., 2002). Fundamental knowledge is required for proper identification and environment friendly management techniques. Rugman-Jones *et al*. (2013) discussed that Random Amplified Polymorphic DNA (RAPD) markers and *Cytochrome oxidase I* (COI) sequence were utilized to dictate the synonymy between the two species *R. ferrugineus* and *R. vulneratus.* In fact, *R. ferrugineus* and *R. vulneratus* have been proved as two different species following ITS1 and ITS2 (Sadder *et al.*, 2015). The molecular scrutiny on genomic diversity among variant *R. ferrugineus* strains has been directed by several scientists from different countries including Egypt (Gadelhak and Evan, 2005), Middle East and Mediterranean Basin (El-Mergawy *et al*., 2011a), Phillipine (Abad *et al*., 2014) and Punjab, Pakistan (Yasin *et al.*, 2016).

In Pakistan, previously, RPW was collected only from limited date palm growing regions of Punjab and KPK and its genetic study was carried out using COI based primers (Yasin *et al*., 2016). The major date palm growing regions of Sindh and Baluchistan were still ignored. First time, we did complete survey in all date palm growing areas of Pakistan and collected RPW for their molecular characterization.

It is always better to use more than one gene for proper DNA barcoding and phylogenetic study to get accurate and trustable results. DNA sequences of both genes of RPW was deposited at NCBI. Molecular biologist and taxonomist will use DNA barcoding information of both genes for population study and RNAi based control strategy. Therefore, the principle goal of the present research was to collect, identify and distinguish the local population of RPW from 4 distinct provinces (Punjab, Sindh, Baluchistan and KPK) on the basis of both COI and ITS genes based primers in Pakistan. We used sequences of two genes (ITS and CO1) and made comparison with already described species at NCBI in the Genebank for identification and the evolutionary divergence of palm weevil.

Materials and Methods

Samples Collection

In 2015, weevils were captured during February to April from Punjab (Layyah, Muzaffargarh, Bahawalpur), Sindh (Ghotki, Sukhar, Larkana), Baluchistan (Panjgur, Khezdar) and Khyber Pakhtunkhawa (Dera Ismail Khan) Provinces from deceased as well as fallen trees in orchard of date palms as shown in Fig. 1. Many of the weevils were collected for the purpose of rearing but province wise collection was also carried out to undertake their molecular identification. RPW samples to be used for sequence analysis were stored in 95% ethanol and then preserved in a freezing condition of -20°C at IGCDB Laboratory, PARS Campus, University of Agriculture Faisalabad, Pakistan.

DNA Extraction

DNA was isolated from the collected samples by using a ISOHAIR kit according to the manufacturer protocol (Tanaka *et al.*, 2012). The incubation of samples was done at condition of 60^{o}C temperature for 20 min in each case. A ratio of 1:10 of dilutions for initial extraction of DNA lead to resilient amplification in contrast to full strength and dilutions ratio of 1:100 (results not shown). Another ratio (1:10) for dilutions was employed to template DNA or tDNA for performing polymerase chain reaction. Formation of dilution was done through diluting fully strength products of DNA along with buffer (TE buffer) having pH value of 8.0.Quantification of isolated deoxyribonucleic acid was carried out by performing electrophoresis of gel.

Amplification of PCR Products and Electrophoresis

A Thermal Cycler (Applied biosystems brand, Veriti Thermal Cycler) was used for PCR. Overall size for every PCR reaction was 25 μL. Every single reaction comprised of master mix (12.5 microliter) of Apex brand, deionized water, individual primer (1microliter) at working conc. of 100 micro mole while DNA with 1 μL quantity. Conditions for polymerase chain reaction comprised of initial denaturation temperature (95°C) for 3 min followed by 35 cycles with conditions (temp: 94°C for 30 Sec; temp: 50°C for 30 sec; temp: 72°C for 1 min) denaturation, annealing and extension respectively. The final extension was performed for 10 min of time interval at 72°C temperature. For RPW samples, two genes including mtCO1 and ITS-1 and ITS-2 with ribosomal subunit 5.8S were used for the sequence analysis of Pakistani weevils. Polymerase chain reaction products were run on 1% agarose gel electrophoresis (pre-stained via ethidium bromide) for time period of 90 min at voltage (100 volt) in a buffer 1x TAE containing chemicals like Tris acetate (40 m*M*)-, EDTA (1 m*M*) with pH value of 8.0. DNA ladder used in molecular analysis was Hyperladder VI (Bioline, USA). Blue light

based edition of bands led gels staining through the use of Syber Gold ordered by Life technologies, USA. Wizard gel was helped out from the gel splinter for recovering the DNA by the implementation of PCR cleanup kit for cleaning PCR product (Promega, United State of America). After that gels were detected underneath the UV illumination (U.V. Trans-illuminator, "TFX 35 Life Technologies"-, CA-, U.S.A.) as well as snapshots were developed through working with Gel-Doc 1000 (Bio-Rad Lab.-, C.A-, U.S.A). Furthermore, molecular size of amplified products was assessed via "Phi X-174 DNA/Hae-III marker" (Invitrogen, Life Tech.-, C.A-, USA).

Sequencing and Phylogenetic Analysis

Sequencing was carried out by the ICBR (*Interdisciplinary Center for Biotechnology Research*), situated at Mowry Road, Sanger lab., Room 178, UF, USA. Investigations of sequencing reads were done by employing the Geneious version software (Kearse *et al.*, 2012). Sequences were analyzed, and the obtained sequences were uploaded at NCBI with accession numbers: Seq6-KY458180, Seq5-KY458179, Seq4-KY458178, Seq3-KY458177, Seq2-KY458176 and Seq1-KY458175. Data recovered from the NCBI Genebank for ITS2 sequencings consisting of 728 base pairs for *Rhynchophorus* species. Alignments were made in the Bioedit with the help of multiple ClustalW alignment function described by Hall (1999). Alignments were exposed according to maximum likelihood methodologies by using DNAML protocols and they were 250 times bootstrapped all the way through SEQBOOT function according to the Felsenstein's protocol (1989).

Global re-organization (tranversion/transition) for each source to a plausible group added and deleted for the last specie was recommended for the tree. Phylogeny tree buildup constituting protracted dominance decree of homology was made through a source of condenses sequence software (Tree View Software) according to Page (1996). With the intention of putting distinction from Pakistan keen on worldwide perception, we combined our sequencing results in the research previously described (El-Mergawy and Al-Ajlan, 2011; Rugman-Jones *et al*., 2013; Wang *et al*., 2015) with accessions at NCBI: KF413063-KF413073, KF311358-KF311740, GU581319-GU581628. All the sequences were trimmed to homogenous length of 728 sequences with 728 bp each. Haplotypes were built by the distortion of sequences through DnaSP v5.10.01 and statistical parsimony procedure was aided for the buildup of haplotypes globally.

Sequences were distorted again into haplotypes by utilizing the DnaSP v5.10.01 and a worldwide haplotype network was generated with the assistance of statistical parsimony method depicted by Templeton *et al.* (1992) by using TCS programming software (Version 1.21) mentioned by Clement *et al*. (2000). Phylogenetic analysis and evolutionary divergence were measured according to Tamura *et al.* (2013), Nei and Kumar (2000) respectively following Neighbour joining methodology of Saitou and Nei (1987).

Results

PCR and Gel Electrophoresis

After sample collection from various provinces of Pakistan (Fig. 1 and Table 1), PCR was performed using CO1 and ITS based primer pairs and then Gel casting was practiced according to prescribed procedure. I kb Molecular marker was used during electrophoresis for comparison with our PCR amplified fragments. Clear bands at 710 bp length were obtained for our RPW specimens collected from different provinces (KPK, Punjab, Sindh and Baluchistan) (1-2, L3-L4, L5-6, L7- L8) as shown (Fig. 2). The control samples showed no amplification of DNA fragment (L11).

Phylogenetic and Blast Analysis

PCR amplified products were cleaned and Sequenced by using forward and reverse primers (*Cytochrome Oxidase Subunit-1* and *Internal Transcribed Spacer*) from the weevils collected from the Punjab, KPK, Sindh and Baluchistan Provinces of Pakistan (Fig. 1). Sequences were obtained by PCR products from the weevils collected from Pakistan. Unnecessary and null sequences were trimmed manually till final alignment of 728 bp was achieved. There was no any deletion or insertion. Weevils from above mentioned districts showed resemblance.

Phylogenetic tree (Fig. 3) and evolutionary divergence (Table 2) built by utilization of COI gene indicated a single clade in which our *R. ferrugineus*-isolates (RPW1-RPW4) exhibited 99–100% genetic homology among themselves. Similarly, our isolates displayed 99% homology with other *R. ferrugineus* isolates from Pakistan (Acc. nos: KU696507 and KU696492) and Egypt (Acc. no: GU581539). On the other hand, the percentage matching of our isolates was 97% in association with other *R. ferrugineus* isolates from Egypt (Acc. nos: KU366272 and KU366273), China (Acc. nos: KF413067; KF413064 and KF413073), USA (Acc. nos: KF311362 and KF311474) and Greece (Acc. no: KM503130). Moreover, percent homology of our isolates was noted to be 87% when matched with other *R. vulneratus* strains (Acc. nos: KF311568; KF311633 and KF311587). In addition, 85% and 84% homology of our Pakistani *R. ferrugineus* isolates was experienced in connection with *R. cruentatus* (Acc. no: AY131113) from USA and *R. Palmarum* (AY131121) respectively.

Table 1: Sampling sites in the palm orchards in Provinces (Sindh, KPK, Balochistan and Punjab)

Population	Collection date	No. of specimens	Province	Geographical characteristics		
				Alt. (m)	Lat.	Long.
D.I.Khan	8 March, 2015	29	KPK	166	31.490° N	70.520 °E
Layyah	15 Feb, 2015	38	Punjab	143	30.580° N	70.560° E
Muzaffar Garh	16 Feb, 2015	20	Punjab	119	30°05'N	71°14'E
Bahawalpur	16 Feb, 2015	27	Punjab	115	29°24'N	71°40'E
Punjgur	27 March, 2015	14	Baluchistan	980	26.7303° N	64.1478° E
Khezdar	28 March, 2015	10	Baluchistan	1237	27.81°0'N	66.61°0'E
Sukhar	22 Feb, 2015	34	Sindh	62	50.13° N	12.83° E
Larkana	23 Feb, 2015	13	Sindh	147	27.5570° N	68.2028° E
Ghotki	23 Feb, 2015	30	Sindh	72	28°05'N	69°21'E

Table 2: Estimates of Evolutionary Divergence between Sequences of different *Rhynchophorus* species according to Nei and Kumar, (2000). The number of base differences per sequence from between sequences are shown. Standard error estimate(s) are shown above the diagonal. $1^{st}+2^{nd}+3^{rd}$+non-coding are codon positions. 26 nucleotides were analyzed and positions with missing date were removed with a final data set of 337 positions. MEGA-6 software was used for

Accessions	Species/Isolates	Divergence Values (Black)																	standard errors (Blue)								
KX228866.1_R. ferrugineus_isolate_RED15		1.0	1.0	1.0	1.4	0.0	1.0	2.1	2.9	3.0	3.2	3.0	3.0	3.1	3.1	1.0	3.8	1.0	5.3	5.3	6.0	6.2	6.3	6.0	6.1	6.6
KF413073.1_R. ferrugineus_isolate_NP1	1.0		0.0	0.0	1.0	1.0	0.0	1.9	2.7	2.7	2.9	2.7	2.7	2.8	2.9	1.0	3.6	1.0	5.3	5.3	5.9	6.1	6.2	5.9	6.1	6.6
GU581319.1_R. ferrugineus_isolate_M310	1.0	0.0		0.0	1.0	1.0	0.0	1.9	2.7	2.7	2.9	2.7	2.7	2.8	2.9	1.0	3.6	1.0	5.3	5.3	5.9	6.1	6.2	5.9	6.1	6.6
KF311362.1_R. ferrugineus_isolate_RED009	1.0	0.0	0.0		1.0	1.0	0.0	1.9	2.7	2.7	2.9	2.7	2.7	2.8	2.9	1.0	3.6	1.0	5.3	5.3	5.9	6.1	6.2	5.9	6.1	6.6
KF413064.1_R. ferrugineus_isolate_FZ3	2.0	1.0	1.0	1.0		1.4	1.0	2.2	2.9	2.9	3.1	2.9	2.9	3.0	3.1	1.4	3.8	1.4	5.2	5.2	5.9	6.1	6.2	5.9	6.1	6.6
KM503130.1_R. ferrugineus_isolate_TEIC	0.0	1.0	1.0	1.0	2.0		1.0	2.1	2.9	3.0	3.2	3.0	3.0	3.1	3.1	1.0	3.8	1.0	5.3	5.3	6.0	6.2	6.3	6.0	6.1	6.6
KF311474.1_R. ferrugineus_isolate_RED888	1.0	0.0	0.0	0.0	1.0	1.0		1.9	2.7	2.7	2.9	2.7	2.7	2.8	2.9	1.0	3.6	1.0	5.3	5.3	5.9	6.1	6.2	5.9	6.1	6.6
KF413070.1_R. ferrugineus_isolate_XM2	5.0	4.0	4.0	4.0	5.0	5.0	4.0		2.9	3.1	3.3	3.1	3.1	3.2	3.2	2.1	3.8	2.1	5.4	5.4	5.9	6.3	6.3	5.9	6.1	6.7
KF413067.1_R. ferrugineus_isolate_FQ4	9.0	8.0	8.0	8.0	9.0	9.0	8.0	10.0		2.3	2.4	2.3	2.3	2.1	2.5	2.9	3.1	2.9	5.3	5.3	5.8	5.9	5.9	5.8	5.9	6.7
GU581539.1_R. ferrugineus_isolate_NF49	9.0	8.0	8.0	8.0	9.0	9.0	8.0	10.0	6.0		1.0	0.0	0.0	0.9	1.3	3.0	2.7	3.0	5.4	5.4	6.0	5.9	6.1	6.0	6.2	6.8
KY458177.1_R. ferrugineus_isolate_RPW4	10.0	9.0	9.0	9.0	10.0	10.0	9.0	11.0	7.0	1.0		1.0	1.0	1.3	1.0	3.2	2.5	3.2	5.6	5.6	5.8	6.1	6.0	5.8	6.2	6.8
KY458176.1_R. ferrugineus_isolate_RPW3	9.0	8.0	8.0	8.0	9.0	9.0	8.0	10.0	6.0	0.0	1.0		0.0	0.9	1.3	3.0	2.7	3.0	5.4	5.4	6.0	5.9	6.1	6.0	6.2	6.8
KY458175.1_R. ferrugineus_isolate_RPW1	9.0	8.0	8.0	8.0	9.0	9.0	8.0	10.0	6.0	0.0	1.0	0.0		0.9	1.3	3.0	2.7	3.0	5.4	5.4	6.0	5.9	6.1	6.0	6.2	6.8
KU696507.1_R. ferrugineus_isolate_LY-1	10.0	9.0	9.0	9.0	10.0	10.0	9.0	11.0	5.0	1.0	2.0	1.0	1.0		1.5	3.1	2.7	3.1	5.4	5.4	5.9	5.9	6.1	5.9	6.1	6.7
KU696492.1_R. ferrugineus_isolate_B-4	11.0	10.0	10.0	10.0	11.0	11.0	10.0	12.0	8.0	2.0	1.0	2.0	2.0	3.0		3.1	2.8	3.1	5.6	5.6	5.8	6.1	6.0	5.8	6.3	6.8
KU366273.1_R. ferrugineus_isolate_Ismailia	1.0	1.0	1.0	1.0	2.0	1.0	1.0	5.0	9.0	9.0	10.0	9.0	9.0	10.0	11.0		3.8	0.0	5.3	5.3	6.0	6.2	6.3	6.0	6.1	6.6
KF413066.1_R. ferrugineus_isolate_PT3	15.0	14.0	14.0	14.0	15.0	15.0	14.0	16.0	12.0	8.0	7.0	8.0	8.0	9.0	8.0	15.0		3.8	5.8	5.8	5.8	6.3	6.1	5.8	6.1	6.6
KU366272.1_R. ferrugineus_isolate_Qina-Eg	1.0	1.0	1.0	1.0	2.0	1.0	1.0	5.0	9.0	9.0	10.0	9.0	9.0	10.0	11.0	0.0	15.0		5.3	5.3	6.0	6.2	6.3	6.0	6.1	6.6
KF311636.1_R. bilineatus_isolate_RED1151	39.0	38.0	38.0	38.0	37.0	39.0	38.0	39.0	42.0	40.0	41.0	40.0	40.0	39.0	42.0	39.0	45.0	39.0		0.0	4.9	5.1	5.3	4.9	6.6	6.6
KF311637.1_R. bilineatus_isolate_RED1152	39.0	38.0	38.0	38.0	37.0	39.0	38.0	39.0	42.0	40.0	41.0	40.0	40.0	39.0	42.0	39.0	45.0	39.0	0.0		4.9	5.1	5.3	4.9	6.6	6.6
KF311587.1_R. vulneratus_isolate_RED498	43.0	42.0	42.0	42.0	41.0	43.0	42.0	42.0	44.0	43.0	42.0	43.0	43.0	42.0	43.0	43.0	46.0	43.0	28.0	28.0		3.5	2.8	0.0	6.5	7.1
KF311633.1_R. vulneratus_isolate_RED1144	44.0	43.0	43.0	43.0	42.0	44.0	43.0	44.0	43.0	42.0	43.0	42.0	42.0	43.0	44.0	44.0	45.0	44.0	31.0	31.0	12.0		3.4	3.5	6.7	7.0
KF311568.1_R. vulneratus_isolate_RED254	48.0	47.0	47.0	47.0	46.0	48.0	47.0	48.0	45.0	46.0	45.0	46.0	46.0	45.0	46.0	48.0	48.0	48.0	30.0	30.0	9.0	13.0		2.8	6.8	7.1
KF311587.1_R. vulneratus_isolate_RED498	43.0	42.0	42.0	42.0	41.0	43.0	42.0	42.0	44.0	43.0	42.0	43.0	43.0	42.0	43.0	43.0	46.0	43.0	28.0	28.0	0.0	12.0	9.0		6.5	7.1
AY131113.1_R. cruentatus	50.0	50.0	50.0	50.0	49.0	50.0	50.0	50.0	48.0	48.0	48.0	48.0	48.0	47.0	49.0	50.0	48.0	50.0	50.0	50.0	51.0	53.0	54.0	51.0		7.0
AY131121.1_R. palmarum	55.0	55.0	55.0	55.0	56.0	55.0	55.0	57.0	57.0	57.0	58.0	57.0	57.0	56.0	59.0	55.0	57.0	55.0	56.0	56.0	61.0	60.0	63.0	61.0	54.0	

analysis of dataset (Tamura *et al.*, 2013)

Phylogenetic tree (Fig. 5) and evolutionary divergence (Table 3) construction through ITS gene indicated that our *R. ferrugineus*-isolates (RPW1 and RPW4) exhibited 100% genetic matching with each other and 99% with *R. ferrugineus* isolate-RPW3 isolate. Likewise, our isolates displayed 99% homology with other *R. ferrugineus* isolates

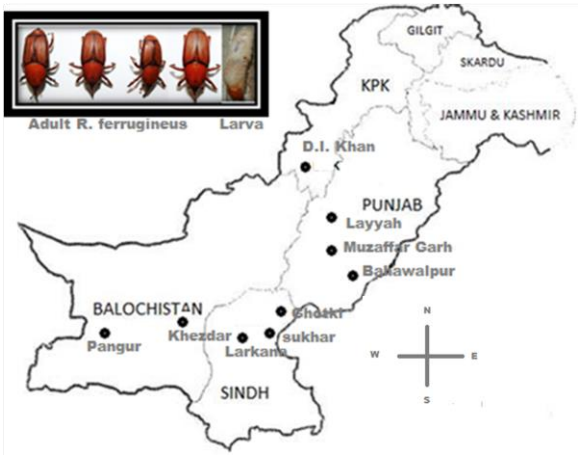

Fig. 1: A map showing various collection sites for *R. ferrugineus* in different Provinces of Pakistan

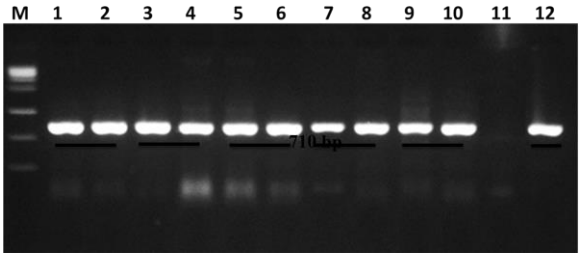

Fig. 2: Agarose Gel Electrophoresis of COI Polymerase Chain (PCR) RPW from 10 *Rhynchophorus ferrugineus* specimens collected from different provinces, Punjab (Sample 1, Lane 1-2), KPK (Sample 2, Lane 3-4), Sindh (Sample 3, Lane 5-6, Lane 7-8) and Baluchistan (Sample 4, Lane 9-10) provinces of Pakistan. Lane 11 (-) and Lane 12 (+) control. *M.* molecular weight marker (1 kb ladder)

from variant regions of Kingdom of Saudi Arabia including UAE (Acc. no: KC954638), Al Ahsa (Acc. no: KC954638), Qatif (Acc. no: KC954634), Najran (Acc. no: KC954631) and Mecca (Acc. no: KC954637), likewise the percentage (99%) association was noted when matched with *R. ferrugineus* isolate TEIC-RPW1 (Acc. no: KM503122). On another hand, the percentage matching of our isolates was 97% in association with other *R. Vulneratus* isolate from Indonesia (Acc. nos: KC954642). Moreover, %age homology of our isolates was confirmed as 96% when matched their association with *R. vulneratus* RED034 strain (Acc. no: KF311715). In addition, %age homology of our isolates was 92% in connection with *R. bilineatus* strains (Acc. nos: KF311739 and HM043701).

Furthermore, the blast analysis with pair base alignment and employing COI gene revealed that our all *R. ferrugineus* isolates are same strains and their similarity percentage ranged between 97–100% sharing same clade for both COI and ITS based constructed tree as shown in Fig. 4.

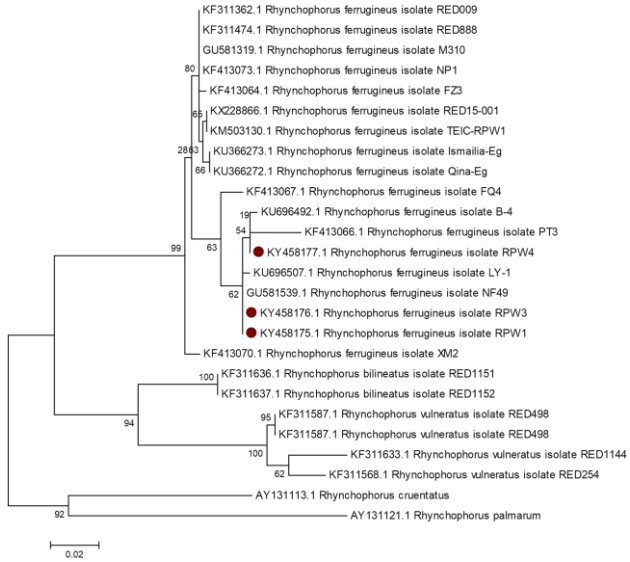

Fig. 3: Phylogeny tree based on COI produced by Maximum Likelihood Method using MEGA6 according to according to Tamura *et al.* (2013)

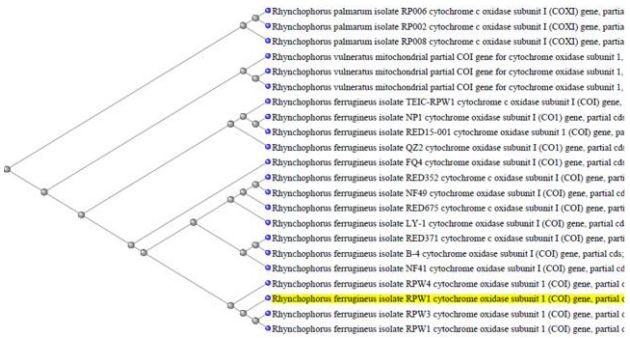

Fig. 4: Maximum parsimony tree constructed through BLAST pair base alignment

Evolutionary Genetic Divergence among Population

Calculated genetic evolutionary divergence (GD) based on COI sequences among all the 26 haplotypes ranged from 1.0 to 63.0 as described in Table 3. The GD among verified strains were assessed. The GD among our strains (RPW1, RPW3 and RPW4) ranged from 1 to 2 showing minimum genomic divergence. Similarly, the lowest divergences of our isolates among other *R. ferrugineus* strains were established that fluctuate from between 1 to10 with NCBI submitted accession numbers: (KF413073, GU581319, KF311362, KF413064, KM503130, KF311474, KF413070, KF413067, GU581539, KY458177, KY458176, KY458175, KU696507, KU696492 and KU366273). On the other hand, maximum GD in the range (40–41) was noted when compared with other *R. billineatus* strain (Acc. no: KF311636), 43–46 in case of *R. vulneratus* strains (Acc. nos: KF311587, KF311633 and KF311568),

Table 3: Genetic-divergence-data-table conducted according to Nei and Kumar (2000)

KY458180.1		0.9	0.9	2.3	2.1	2.1	2.3	2.1	3.7	4.0	3.9	3.8	3.9	4.0	2.1
KY458179.1	1.0		0.0	2.3	2.1	2.1	2.3	2.1	3.7	4.0	3.9	3.6	3.9	4.0	2.1
KY458178.1	1.0	0.0		2.3	2.1	2.1	2.3	2.1	3.7	4.0	3.9	3.6	3.9	4.0	2.1
KC954634.1	6.0	6.0	6.0		1.0	1.0	1.3	1.0	2.8	3.3	3.2	3.2	3.1	3.3	1.3
KC954638.1	5.0	5.0	5.0	1.0		0.0	0.9	0.0	2.8	3.3	3.3	3.3	3.1	3.3	0.9
KC954632.1	5.0	5.0	5.0	1.0	0.0		0.9	0.0	2.8	3.3	3.3	3.3	3.1	3.3	0.9
KC954637.1	6.0	6.0	6.0	2.0	1.0	1.0		0.9	3.0	3.5	3.5	3.4	3.3	3.5	1.3
KC954631.1	5.0	5.0	5.0	1.0	0.0	0.0	1.0		2.8	3.3	3.3	3.3	3.1	3.3	0.9
KC954642.1	15.0	15.0	15.0	9.0	10.0	10.0	11.0	10.0		1.9	2.7	2.9	1.3	1.9	2.9
KF311715.1	18.0	18.0	18.0	13.0	14.0	14.0	15.0	14.0	4.0		3.3	3.5	1.4	0.0	3.2
KF311739.1	17.0	17.0	17.0	12.0	13.0	13.0	14.0	13.0	9.0	11.0		1.7	2.9	3.3	3.1
HM043701.1	17.0	16.0	16.0	12.0	13.0	13.0	14.0	13.0	11.0	14.0	3.0		3.1	3.5	3.3
KF311717.1	16.0	16.0	16.0	11.0	12.0	12.0	13.0	12.0	2.0	2.0	9.0	12.0		1.4	3.0
KF311715.1	18.0	18.0	18.0	13.0	14.0	14.0	15.0	14.0	4.0	0.0	11.0	14.0	2.0		3.2
KM503122.1	5.0	5.0	5.0	2.0	1.0	1.0	2.0	1.0	11.0	13.0	12.0	13.0	11.0	13.0	

[1]KY458180.1_*Rhynchophorus_ferrugineus*_isolate_RPW4[2]KY458179.1_*Rhynchophorus_ferrugineus*_isolate_RPW3[3]KY458178.1_*Rhynchophorus_ferrugineus*_isolate_RPW1[4]KC954634.1_*Rhynchophorus_ferrugineus*_isolate_KSA_Qatif[5]KC954638.1_*Rhynchophorus_ferrugineus*_iolate_KSA_UAE_field[6]KC954632.1_*Rhynchophorus_ferrugineus*_isolate_KSA_Al_Ahsa[7]KC954637.1_*Rhynchophorus_ferrugineus*_isolate_KSA_Mecca[8]KC954631.1_*Rhynchophorus_ferrugineus*_isolate_KSA_Najran[9]KC954642.1_*Rhynchophorus_vulneratus*_isolate_Indonesia[10]KF311715.1_*Rhynchophorus_vulneratus*_isolate_RED034[11]KF311739.1_*Rhynchophorus_bilineatus*_isolate_RED1149[12]HM043701.1_*Rhyncophorus_bilineatus*_isolate_NF22[13]KF311717.1_*Rhynchophorus_vulneratus*_isolate_RED06[14]KF311715.1_*Rhynchophorus_vulneratus*_isolate_RED034[15]KM503122.1_*Rhynchophorus_ferrugineus*_isolate_TEIC-RPW

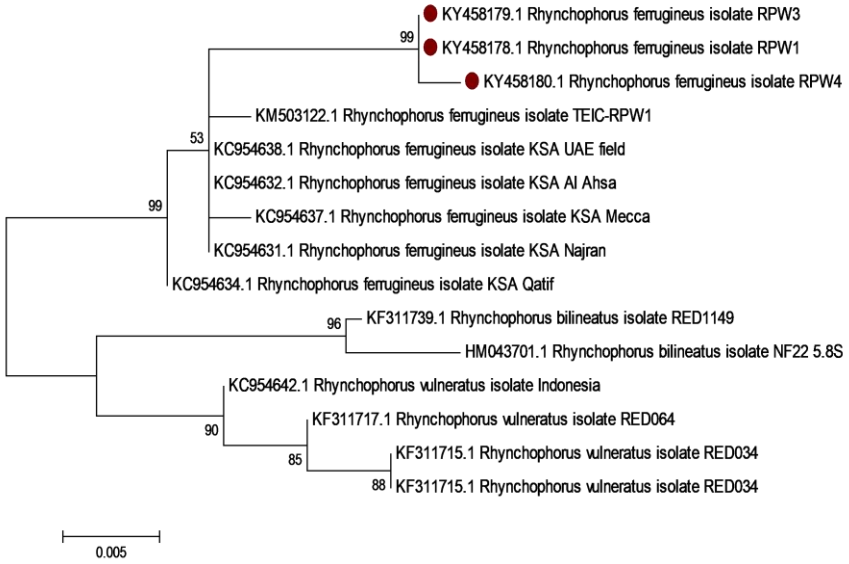

Fig. 5: ITS based Molecular Phylogenetic analysis through Maximum Likelihood using MEGA6 according to Tamura *et al.* (2013).

while the GD for *R. cruentatus* (AY131113) and *R. palmarum* (AY131121) was ranged from 48 and 57 respectively. Same pattern was also found regarding evolutionary divergence based on ITS amplified sequences as indicated in Table. Tamura-Nei Model based Maximum likelyhood method was implemented for the history analysis of RPW. Highest log likelihood was -856.91 and percentages are shown in front of the each brach of each taxon. Heauristic tree was initially made through NJM (Neighbour joining method) and pairwise matrix distances were shown by MCL technique. Irrelevant data were removed and final data constituted of 465 positions in a nucleotide sequence of 15 bases analyzed by MEGA-6.

Discussion

The genomic variation among distinct geographical population of RPW from each Province of Pakistan (Punjab, KPK, Sindh and Baluchistan) were studied implying *Cytochrome oxidase I* (COI)/Inter Transcribed SpacerI/II (ITS sequences as observed to differentiate the synonymy between the species *R. ferrugineus* and *R. vulneratusfrom* Rugman-Jones *et al.*, 2013; Sadder *et al.*, 2015.

Our Phylogenetic analysis of *R. ferrugineus* strains including RPW1 (Acc. no: KY458178), RPW3 (Acc. no: KY458179) and RPW4 (Acc. no: KY458180) are genetically similar (99–100%) with each other and with other *R. ferrugineus* strains from the world with maximum range of 97–99% homology as described by several scientist from different countries including Egypt (Gadelhak and Evan, 2005), Middle East and Mediterranean Basin (El-Mergawy *et al*., 2011), Phillipine (Abad *et al*., 2014). While minimum percentage similarity (85%) was noticed in association with another strain of palm weevil, *R. cruentatus* (Acc. no: AY131113) from Unites State of America observed by Perring *et al.* (1993). Gadelhak and Enan (2005) identified 61 red palm weevil populations exhibiting highest genetic distance values. Previously, RPW was collected from limited regions of Pakistan (Punjab, KPK) and identified by using only COI primers (Yasin *et al*., 2016). Sindh and Baluchistan are major date palm growing areas of Pakistan and severely affected by RPW. These areas particularly Baluchistan have been previously ignored regarding RPW infestation and identification on molecular level. But recently in our study, RPW was collected from all major date palm cultivated regions of Pakistan including Baluchistan and Sindh and characterized on both COI and ITS based primers.

In our case of genetic divergence, minimum GD (1 to 2) was noticed in comparison with our isolates while with other *R. ferrugineus* strains, the GD in the range of 1 to 10 was marked. Moreover, maximum GD of 57.0 of our aforementioned strains in comparison with *palmarum* strain (AY131121) was noticed. We suggest that *R. ferrugineus* is intrusive to Pakistan given by the similarity between the weevils found in Pakistan and Arab areas of Saudi Arabia and has very close association with *R. ferrugineus* identified from gulf countries. According to Marimuthu *et al.* (2009) least continuous genetic variations over the geographic populations does not cause inherent changes but Sharma *et al*. (1998) was unable to found inherited homogeneity in the studied insect samples because 100% polymorphism has also been found among *C. quincuefaciatus*. Contrarily, dissimilarity in insect populationshas been also observed (Srivastava *et al.,* 2005) explaining various genetic distance values related to different insects (*B. mori*) as diverse regions introduce more diverse populations (Vieira *et al.,* 2007; Gunderina *et al*., 2009). We could not fiund major genetic variation in our samples and suggested that single species of RPW (*R. ferruginous)* exist in all date palm growing areas of Pakistan.

According to COI and ITS based analysis, geographically different populations of various provinces of Pakistan are genetically similar among themselves as well as from other *R. ferruginous* reported from other countries. The phylogenetic and genetic evolutionary divergence has led us to conclude that the Pakistani RPW have close association with the RPW population of Gulf countries as indicated by the clades formed by the phylogenetic dendrograms. This is the first report of RPW widely presence in Pakistan and molecular characterization showing that only one species of RPW (*R. ferruginous)* exist in all provinces of Pakistan and there is not any significant genetic variation among themselves

R. ferrugineus is very dangerous invasive pest causing billions of dollars loss annually. In Pakistan, it is widely spreading in almost all date palm growing areas. It is recommended that during date palm suckers transportation from one region to another for plantation, control measures should be strictly followed.

This study will be very helpful for species specific policy making and better management of *R. ferruginous* in Pakistan. For example, RNAi is latest technique used control species specific insect pest and management of insecticides resistance. Currently, RPW (*R. ferruginous*) species specific targeted genes used for RNAi methodology have been identified which i will be applied in field and management of insecticide resistance in Pakistan. Because, only one species of RPW (*R. ferruginous*) exist in Pakistan, so, making of one species specific management policy will stop RPW infestation into other date palm growing areas of Pakistan. Further, This would be useful tool in quarantine work at Pakistani air and sea ports and this molecular techniques presented here will be applicable to individuals from all over country geographical area as great similarity between the present alignment exercise of COI and ITS sequences from overall date palm growing districts of Pakistan and those from other countries has been found, it is likely that this method might also be useful for individuals of the same species from other countries.

Conclusion

In conclusion, molecular identification and characterization of RPW samples collected from different geographical regions of Pakistan clearly showed that RPW population are genetically similar not only from each other but also from other *R. ferruginous* Reported from other countries. RPW from Pakistan have very closed genetic and evolutionary link from RPW prevailed in Gulf countries. This study will be very helpful for species specific policy making against *R. ferruginous* and in quarantine work at Pakistani air and sea ports for its better management.

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