INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
10–609/SAE/2011/13–5–631–637
http://www.fspublishers.org

Full Length Article

Mitochondrial Genetic Variation and Invasion History of Red Palm Weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), in Middle-East and Mediterranean Basin

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ABSTRACT

The Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Olivier), (Coleoptera, Curculionidae, Rhynchophorinae), is an invasive pest of palm trees. RPW has invaded Middle East and several countries of the Mediterranean Basin during the last three decades. The mitochondrial genetic variation of RPW was investigated in the Middle-East and the Mediterranean basin areas using partial sequences of the C*ytochrome c oxidase* sub-unit 1 (CO1) gene. A 546-base pair portion of COI gene was sequenced for 310 individuals of RPW sampled from 14 different invaded countries resulting in eight different haplotypes. Eight haplotypes were subdivided into two phylogenetic groups according to their geographic positions. The obtained genetic diversity suggested that RPW population subdivided genetically into different sub-populations under the influence of genetic drift favored by founder events. RPW followed three different routes of invasion during the last 30 years. Likely, Middle-east populations and the Mediterranean ones are originating from different geographic populations of RPW. The data reported in this paper present an interesting and useful step toward the understanding of the genetic variation and invasion history of RPW. © 2011 Friends Science Publishers

Key Words: *Cytochrome c oxidase* sub-unit 1 (CO1); Invasive species; *Rhynchophorus ferrugineus*; Red palm weevil; Palm Tree; Middle-East, Mediterranean basin

INTRODUCTION

Invasive species are species that have been introduced by human activities behind their region of origin and that have experienced a demographic success in the colonized areas (Williamson, 1996; Sakai *et al.*, 2001; Armstrong, 2005). They have not only an economic impact but also have a negative effect on biodiversity (Sakai *et al.*, 2001).

The Red Palm Weevil (RPW) *Rhychophorus ferrugineus* (Olivier, 1790) (Coleoptera: Curculionidae) is considered as one of the most damaging invasive insect species (Wattanapongsiri, 1966; Faleiro, 2006). The geographic origin of RPW was claimed to be South East Asia and Melanesia (Abraham *et al*., 1975; Murphy & Briscoe, 1999; Vidyasagar, 2000; Ferry & Gomez, 2002). Multiple introductions of RPW to the Middle East, the Mediterranean Basin and US have occurred since mid 1980's through movement of infested offshoots (Faleiro, 2006).

Recently the RPW is being considered as a major pest of date palms (*Phoenix dactylifera*) and different ornamental palm species in the Middle East and the Mediterranean basin, Caribbean (Island of Curacao, Netherland Antilles), Lebanon and USA (Laguna Beach, Orange County, California) (Bokhari & Abuzuhari, 1992; Ferry & Gomez, 2002; Karut & Kazak, 2005; Faleiro, 2006; Kontodimas *et al*., 2007; EPPO, 2008; EPPO Reporting Service, 2008, 2009). The knowledge of genetic variation in RPW as an invasive species is a necessary step before investigating the genetic basis of its rapid adaptation and consequently its invasion success (Sakai *et al.*, 2001; Keane & Crawley, 2002; Wolfe, 2002). Furthermore, it is an essential topic before developing an effective management strategy (Grapputo *et al*., 2005; Monnerat *et al*., 2006; Marimuthu *et*

To cite this paper: El-mergawy, R.A.A.M., M.I. Nasr, N. Abdallah and J.F. Silvain, 2011. Mitochondrial Genetic Variation and Invasion History of Red Palm Weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), in Middle-East and Mediterranean Basin. *Int. J. Agric. Biol*., 13: 631–637

al., 2009; Sharma *et al*., 2009). Genetic variation of RPW was detected previously using random amplified polymorphic DNA-PCR (RAPD-PCR) technique (Abulyazid *et al*., 2002; Salama & Saker, 2002; Gadelhak & Enan, 2005; Al-Ayied *et al*., 2006).

The objective of this study was to trace the route of invasion of RPW through genetic analysis. Level of genetic similarity between different geographic populations is useful to determine from where a given population is coming. Here we used *Cytochrome c oxidase* I (COI) DNA sequences to investigate the genetic variation of RPW from 14 invaded countries. DNA-COI gene sequences were established as bio-identification tools and used in genetic variation, bare code studies, phylogeny and geographical distribution in various insect species (Hebert, 2003). This was due to their maternal inheritance, their rapid rate of evolution and their haploid nature (Avise *et al*., 1987; Behura, 2006; Singh, 2008).

MATERIALS AND METHODS

Sampling: A total of 310 RPW individuals were collected over a four year period (2003 to 2007) from 52 geographic localities representing fourteen countries (Fig. 1 & 2). Also, individuals of *R. palmarum* from French Guiana were obtained to be used as an out-group in genealogical analysis.

DNA extraction and PCR amplification: Genomic DNA was extracted from RPW samples using DNeasy Tissue Kit (QIAGEN) according to the manufacturer's protocol. The PCR was performed in a total volume of 25 µL containing 1 x reaction buffer, 3 mM $MgCl_2$, 0.24 mM dNTPs, 1.4 µM of each primer [Bron (5'-TATAGCATTCCCCGTTTA-3' and Simon (5'-TCCTAATAAACCAATTGC-3')] modified from Simon *et al*. (1994) (Eurogentec), 1U Go Taq Flexi DNA polymerase (Promega Corp.) and 2.5 µL of DNA (a 100 time dilution of the original DNA). The PCR program was as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 min. PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega Corp). Purified PCR products were analyzed by electrophoresis in 1% agarose gel. The molecular size of the amplified products was estimated using 100 bp DNA ladder (Invitrogen).

DNA sequencing: Purified PCR products were sequenced using AB1 PRISM Dye Terminator Cycle Sequencing Ready Reaction System, version 1.1. (Applied Biosystems) Sequencing reactions were carried out in 20 µL volumes containing 1 µL of 3.2 µM primer, 2 µL of Big Dye Terminator mix, 3 µL of sequencing buffer and a template/sterile water mixture, containing 30 to 90 ng templates. The program used was as follows: 96°C for 1 min followed by 24 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min and a final extension at 60°C for 5 min.

Data analyses: Obtained sequences were aligned manually using MacClade 4.05 software (Maddison & Maddison, 2003). After manual alignment, invalid end sequences were trimmed, this resulted in a final alignment of 546 bp in length. Sequences were subjected to Basic Local Alignment Search Tool (BLAST) in order to perform sequence similarity searches, according to Zhang *et al*. (2000). All CO1 haplotypes identified in this study have been deposited in GenBank (accession numbers GU581629-GU581319).

Nucleotide frequencies were calculated using MEGA4 software (Tamura *et al*., 2004, 2007; Yang, 2006), where missing data were eliminated from the dataset (Complete-deletion option). InDels (Insertion-Deletion polymorphism**)** were estimated using DnaSP software (Librado & Rozas, 2009), the same software was used to detect variable and conserved sites.

The genetic distances between haplotypes were calculated based on the Tamura-Nei model of base substitution (Tamura & Nei, 1993) using MEGA4 software (Tamura *et al*., 2007). In order to estimate genetic variation among and within different geographic populations of RPW, different genetic diversity indices were calculated: (a) number of segregating (S)/polymorphic sites (the number of sites which are occupied by at least two different nucleotides) (Tajima, 1993), (b) total number of mutations, (c) number of fixed differences (sites at which all of the sequences in one population are different from all of the sequences in a second population), (d) haplotype diversity (h) (a measure of the uniqueness of a particular haplotype in a given population) and (e) nucleotide diversity (Pi) (the average number of nucleotide differences per site between two sequences) (Nei, 1987) using DnaSP software (Librado & Rozas, 2009).

Haplotype network, phylogenetic and demographic analyses: A network of the mitochondrial RPW haplotypes was inferred using statistical parsimony, as implemented in TCS v. 1.21 (Clement *et al*., 2000). The method links haplotypes with the smallest number of differences as defined by a 95% confidence criterion.

Phylogenetic trees were reconstructed using two different reconstruction methods: (a) Neighbour joining (NJ; Saitou & Nei, 1987) and (b) maximum parsimony (MP; Eck & Dayhoff, 1966). The NJ tree was reconstructed using the Tamura-Nei model (Tamura & Nei, 1993), while MP tree was searched using the Close-Neighbour-Interchange (CNI) algorithm (Felsenstein, 1985; Nei & Kumar, 2000) at a search level of 3. The initial trees for MP were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Genealogical analyses were conducted in MEGA4 software (Tamura *et al*., 2007). Bootstrap support values were obtained from 1000 replications using both methods. *R. palmarum* was used as an out-group taxon. The values of F_{ST} (genetic differentiation), *Nm* (gene flow) and the Tajima's *D*

neutrality tests (Tajima, 1989) were calculated using DnaSP software (Librado & Rozas, 2009).

RESULTS AND DISCUSSION

Genetic variation and invasion history of RPW in 14 invaded countries of the South-Asia, Middle-East and the Mediterranean Basin were investigated using *Cytochrome c oxidase* subunit I (COI).

PCR products, nucleotide composition and frequencies: The electrophoretic analysis of PCR products based on partially amplified CO1 gene resulted in a single amplified DNA band of about 600 bp in length from each individual. The nucleotide composition was biased towards the adenine and thymine (A-T), as the A-T content ranged from 337 to 341 with frequencies of 61.7% to 62.4%, respectively. The guanine and cytosine (G-C) content ranged from 205 to 209 with frequencies of 37.6% to 38.3%, respectively. Similarly, Smith (2005) and Li *et al.* (2009) found also that the base composition of the mitochondrial CO1 gene sequence of other insect species was biased towards adenine and thymine.

Variable and conserved sites: The obtained CO1 sequences showed no insertion or deletion (InDels). Similarly, no InDels was detected in CO1 sequences in studies of other insect species such as: *Homalodisca coagulata* (Homoptera: Cicadellidae) (Smith, 2005), *Helicoverpa* spp. (Lepidoptera: Noctuidae) (Behere *et al*., 2007) and *Caligula japonica* (Lepidoptera: Saturniidae) (Li *et al*., 2009). The variation among CO1 sequences due to nucleotide substitutions: Out of the total 546 bp, 523 were constant sites and 23 (4.2%) were variable. Out of the 23 variable sites 22 were transitional substitutions, while one was transversional. From the total of the 23 variable sites there were seven parsimony informative sites (substitution shared by at least two sequences). The conserved sequences were subdivided into two regions: (1) a region started at nucleotide 234 in our data and ended at nucleotide 299; (2) a region started at nucleotide 406 and ended at nucleotide 546.

Number of haplotypes and introduction events: Genetic variation was detected among the fourteen geographic populations of RPW as merging homologous sequences revealed eight different haplotypes designated as H1 to H8. Those haploypes were differentially distributed among the Middle-East and the Mediterranean area (Fig. 1).

Variation was detected only within populations of RPW from UAE and Oman (Fig. 1). In comparison, the analyses of mitochondrial DNA in other insect species demonstrated 14 haplotypes in *H. coagulata* (Homoptera: Cicadellidae) (Smith, 2005), 33 haplotypes in *Helicoverpa* spp. (Lepidoptera: Noctuidae) (Behere *et al*., 2007) and 6 haplotypes in *C. japonica* (Lepidoptera: Saturniidae) (Li *et al*., 2009). A low number of haplotypes clearly reflects a reduction in genetic variation as expected for invasive species (Puillandre *et al*. 2007). The presence of more than one haplotype in the population of RPW from UAE and Oman may be explained as follows: either the introduced RPW came from different source populations; or it came from only one source, either through different introduction events or from a single one containing more than one haplotype (Fig. 2).

The local populations of RPW in Egypt were fixed for a single mitochondrial haplotype (H8). This may be explained by a unique introduction event, a single successful one or multiple introductions of the same haplotype. Rapid

Fig. 1: RPW sampled geographic areas and distribution of CO1 haplotypes

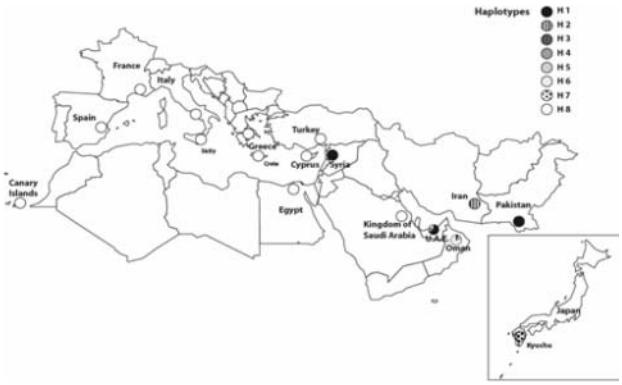
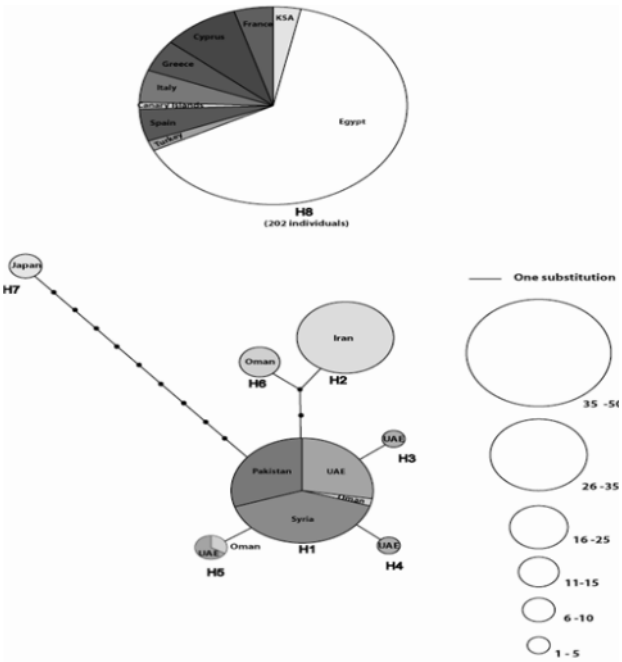


Fig. 2: CO1 haplotype 95% statistical parsimony network. Each haplotype is labelled by its number. The size of each sector indicates the number of individuals. Different shadings represent different populations. Haplotypes not sampled or extinct are designated by small black circles. Each line portion represents single nucleotide mutation



expansion of the H8 haplotype in the Egyptians' governorates could have resulted from a series of secondary invasion events through transportation of infested young or adult date palm trees and offshoots from contaminated to uninfected areas.

According to Ferry and Gomez (2002), RPW was introduced to Egypt through an infected offshoot offered by the UAE, but our results showed that the Egyptian haplotype was not similar to any of the four haplotypes detected to date in the UAE. There are two possible hypotheses: (1) the offered offshoot of the UAE was not infected by RPW and in the same time infected offshoots have been introduced from another country; (2) RPW may have been introduced to Egypt through the offshoot offered by the UAE but we have not been able until now to find H8 haplotype among UAE samples due to limited sampling effort in that country. One can underline the fact that during the five years period of RPW sampling in Egypt only haplotype H8 has been found in that country. That observation supports the hypothesis of a single accidental RPW introduction event and suggests a low level of date and ornamental palm exchange rate between Gulf countries and Egypt.

The H8 haplotype was also fixed in RPW from KSA, Spain (mainland & Canary Islands), Italy, Greece, Cyprus, Turkey and France populations. This may suggest, at least for the Mediterranean countries, multiple introductions from a single source or successive introductions from one country to the other. A mixed scenario is also possible including Spain, the first European Community invaded country, as a relay for the invasion of some European Community countries. The wide geographic distribution pattern of H8 haplotype may indicate a very high invasive potential when introduced by human.

Genetic distances among haplotypes: The genetic distances among the eight haplotypes ranged from 0.002 to 0.034 (Table I). The mean genetic distance among all populations (0.014) was higher than the one that was found previously in populations of other insect species such as *Antheraea pernyi* (0.002-0.006) and *C. japonica* (0.006) (Zhu *et al*., 2008; Li *et al*., 2009).

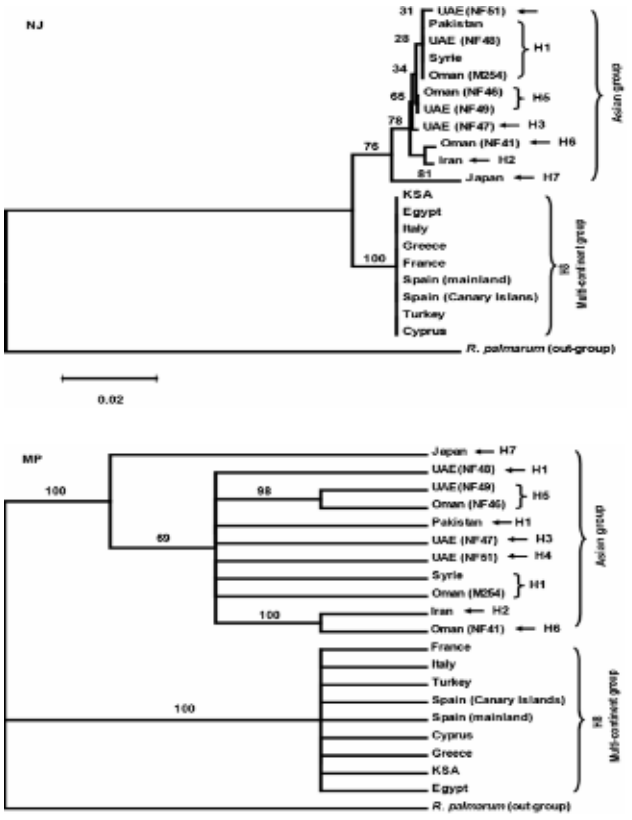
The highest value of pairwise genetic distance 0.034 was observed between H7 and H8 haplotypes, while the lowest value 0.002 was observed three times between haplotypes: (1) (H1 & H3), (2) (H1 & H4) and (3) (H1 & H5). The relatively low genetic distances (0.002-0.007) among the H1 to H6 haplotypes signify close genetic relationships among them. This close relationship may suggest a recent divergence from their common ancestor. On the other hand, the relatively high genetic distance among the above mentioned six haplotypes and both the H7 (0.021-0.026) and H8 (0.023-0.027) haplotypes indicate that they are fairly distantly related.

DNA divergence and polymorphism at inter-population level: The total number of mutations among all haplotypes was 24, the overall haplotype diversity (hd) was 0.556 and

Table I: Genetic distances among the eight CO1 haplotypes based on the pairwise analysis of 564 bp of CO1 gene sequences using Tamura-Nei substitution model

CO1 haplotypes	H1	H2	H3	H4	H5	H6	H7
H1							
H2	0.006						
H3	0.002	0.007					
H4	0.002	0.007	0.004				
H5	0.002	0.007	0.004	0.004			
H6	0.006	0.004	0.007	0.007	0.007		
H7	0.021	0.024	0.023	0.023	0.023	0.026	
H8	0.025	0.026	0.023	0.027	0.023	0.026	0.034

Fig. 3: Phylogenetic hypotheses. Phylogenetic trees were reconstructed using: neighbour joining (NJ) and maximum parsimony (MP) methods, Bootstrap support values (1000 replicates) are indicated above the lines



the total nucleotide sequence diversity (Pi) was 0.01238. Number of fixed differences among the different populations of RPW varied from one to seventeen. The nucleotide diversity (Pi) among different geographical populations of RPW ranged from 0.00069 to 0.01193 (Table II).

DNA polymorphism at intra-population level: Number of segregating (S) sites was 3 and 4, the haplotypic diversity (hd) was 0.58571 and 0.34167 and the nucleotide diversity

Table II: Number of fixed differences and nucleotide diversity (Pi) among different geographical populations of *R. ferrugineus*

Populations	H8 populations		UAE		Oman		Syria		Iran		Pakistan		Japan	
	FD	Pi	FD	Pi	FD	Pi	FD	Pi	FD	Pi	FD	Pi	FD	Pi
H8 populations	0	0	11	0.0064	11	0.006	13	0.006	14	0.009	13	0.00481	17	0.00439
UAE	11	0.006	0	0	0	0.003	0	0.0007	3	0.003	0	0.00078	0	0.00886
Oman	11	0.006	0	0.0035	0	0	0	0.0028	1	0.002	0	0.00303	10	0.01193
Syria	13	0.006	0	0.0007	0	0.003	0	0	3	0.003	0	0	10	0.00829
Iran	14	0.009	3	0.0032	1	0.002	3	0.0026	0	0	3	0.00235	12	0.00758
Pakistan	13	0.005	0	0.0008	0	0.003	0	0	3	0.002	0	0	10	0.00912
Japan	17	0.004	0	0.0089	10	0.012	10	0.0083	12	0.008	10	0.00912	0	0

H8 populations: Egypt, KSA, Turkey, Spain, Italy, France, Greece and Cyprus
FD: Number of fixed differences
Pi: Nucleotide diversity

(Pi) was 0.001240 and 0.00221 within UAE and Oman populations, respectively.

Haplotype network and Phylogenetic analyses: The obtained 95% parsimony haplotype network (Fig. 3) separated the most common haplotype (H8), represented in nine countries from the other seven haplotypes: H1 to H7. Genealogical analysis was investigated using two different phylogenetic methods: neighbor-joining (NJ) and maximum parsimony (MP) (Fig. 4). For maximum parsimony (MP), a tree out of 55 most parsimonious trees (length= 111). The consistency index was 0.653846, the retention index was 0.526316 and the composite index was 0.483642 (0.344130) for all sites and parsimony-informative sites (in parentheses).

Phylogenetic analyses of the geographical populations of RPW revealed a sub-structure in RPW as tested populations of RPW were subdivided into two distinct groups according to their geographic positions. All phylogenetic trees agreed that the eight CO1 haplotypes subdivided into two major phylogenetic groups designated as the "Asian group" and the "Multi-Continent group" according to their geographic position. The Asian group that formed a monophyletic clade included six different populations of RPW collected from five Asian countries such as: Iran, Japan, Oman, Pakistan, Syria and UAE. The Asian group contained seven haplotypes: H1 detected in Pakistan, UAE, Oman and Syria; H2 detected in Iran; H3 and H4 detected in UAE; H5 detected in UAE and Sultanate of Oman; H6 detected in Oman; H7 detected in Japan. The Multi-continent group included eight populations belonging to three different continents: (1) Africa - Egypt; (2) Asia - KSA, Turkey; (3) Europe - Spain (mainland & Canary Islands), Italy, Greece, Cyprus & France). The Multi-continent group contained only a single haplotype H8.

This phylogeny also revealed that the ''Multi-continent group'' was sister group of the Asians. The Japanese population (harboring H7 haplotype) was the sister population of the Asian group, accordingly, the Asian group was subdivided into two subgroups, one contained the RPW population from Japan and the other contained the other populations of the Asian group.

Evolutionary and demographic inference of the genetic variation: Tajima's D neutral test, F_{ST} (genetic differentiation) and Nm (gene flow) values calculated among the different geographical populations of RPW were 2.05, 0.98 and 0, respectively. These values showed that the 14 populations of RPW diverged genetically under the influence of genetic drift likely through multiple founder events. Positive Tajima's parameter D value was found among the different geographic populations of RPW ''i.e.,'' 2.05448 suggests a recent founder event; a phenomenon that was observed for many alien insect species (Puillandre *et al*., 2007). This means that only a fraction of the genetic structure of the original population was introduced to the invaded countries.

The value of F_{ST} calculated among the fourteen populations of RPW was 0.98 indicating a major genetic differentiation among the fourteen populations (Wright, 1978), with each fixed for one or a limited number of haplotypes. This differentiation can be due to a bottleneck event (populations rest in small size for a long time) that enhanced the founder effect (Maruyama & Fuerst, 1985; Campbell & Reece, 2008). This bottleneck event was supported by the null value of Nm that signified the absence of gene flow among the tested RPW populations. However, we have only access here to mitochondrial diversity (maternal lineage); consecutively gene flow at nuclear level cannot be excluded. In contrast, a low value of F_{ST} (0.07) was estimated among populations of *Helicoverpa spp.* (Lepidoptera: Noctuidae) (Behere *et al*., 2007), indicating the absence of genetic structure likely due to gene flow.

The paradoxes: Results showed three paradoxes: first, the haplotype that was found in KSA (H8) was the same as in the ''Multi-continent group'' but was different from those observed in the KSA neighboring countries; Second, although Syria is situated in the Mediterranean area where the H8 haplotype dominates, Syrian RPW populations were harboring H1 haplotype, the most abundant haplotype in the Asian area; Third, although no natural barriers can prevent the distribution of RPW between Pakistan and India (the possible area of origin of RPW), only one haplotype H1, was found in RPW samples obtained from Pakistan.

CONCLUSION

The phylogenetic relationships of all 14 RPW populations based on COI region were sufficiently resolved.

According to sequence divergence between single haplotype found in the ‘’Multi-continent group’’ and the different haplotypes of the ‘’Asian group’’ we suggest that RPW followed three different routes of invasion during the last 30 years; one towards the East of the area of origin that gave rise to the H7 haplotype in Japan and two routes towards the West. The invaded area is divided in the West between Middle East, where 6 haplotypes were found and the Mediterranean basin where the haplotype H8 was detected in nine countries. These three invasion roads are corresponded to three different genetic lineages of RPW populations, which had independent evolutionary histories.

Acknowledgement: The authors are grateful to all the colleagues who provided us with *R. ferrugineus* samples: Vassilis Vasiliou, Didier Rochat, Eric Chapin, Laurence Olivier, Luc Olivier Brun, Brigitte Fréro, Dr. J.P. Morin, Dimitris C. Kontodimas, Raffaele Griffo, Santi Longo, Tomas Cabello Garcia and Cengiz Kazak.

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(Received 20 November 2010; Accepted 17 December 2010)