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The invasion of Tunisia by *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae): crossing an ocean or crossing a sea?

Paul F. Rugman-Jones[1,], Saida Kharrat[2], Mark S. Hoddle[1], and Richard Stouthamer[1]*

Abstract

The red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), is an invasive and injurious pest of palms that has extended its native range from Asia to Africa, the Middle East, and the Mediterranean Basin. In 2010, the morphologically indistinguishable *Rhynchophorus vulneratus* (Panzer) was detected and subsequently eradicated in California, USA. In 2011, a population of palm weevils was detected in ornamental palms in Tunis, Tunisia. Uncertainty over the identity of the weevil population in Tunisia led to conflicting speculation that the source populations emerged from infested palms illegally imported from either the USA or Italy. Mitochondrial haplotypes of specimens collected at multiple sites around Tunis were compared with haplotypes from global populations of *R. ferrugineus* and *R. vulneratus*, confirming that the Tunisian populations were *R. ferrugineus*. Moreover, the Tunisian populations had the same fixed mitochondrial haplotype ubiquitous in invasive populations throughout the Mediterranean Basin, and we conclude that the Tunisian populations most likely originated from imported infested palms from Europe, and not from palms imported from the USA.

Key Words: invasive pest; red palm weevil; *Rhynchophorus vulneratus*; phytosanitary regulation; mitochondrial DNA; *COI*

Resumen

El gorgojo rojo de la palma, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Dryophthoridae), es una plaga invasiva y nociva de palmas que ha extendido su área de distribución nativa en Asia a África, Medio Oriente y la Cuenca Mediterránea. En 2010, se detectó una especie, *R. vulneratus* (Panzer), morfológicamente indistinguible y luego erradicada de California, EE.UU. En 2011, se detectó una población de gorgojos de palma en palmas ornamentales en Tunis, Túnez. La incertidumbre sobre la identidad de la población de gorgojos en Túnez llevó a la especulación conflictiva de que la fuente de la población emergieron de palmas infestadas e importadas ilegalmente de los EEUU o de Italia. Se compararon los haplotipos mitocondriales de especímenes recolectados en múltiples sitios alrededor de Túnez con haplotipos de poblaciones globales de *R. ferrugineus* y *R. vulneratus*, lo que confirma que las poblaciones tunecinas eran de *R. ferrugineus*. Por otra parte, las poblaciones tunecinas tenían el mismo haplotipo mitocondrial fijo ubicuo en poblaciones invasoras en toda la Cuenca Mediterránea, y concluimos que las poblaciones tunecinas probablemente se originaron de palmas importadas de Europa y no de palmeras importadas de los Estados Unidos.

Palabras Clave: plaga invasiva; gorgojo rojo de la palma; *Rhynchophorus vulneratus;* regulación fitosanitaria; ADN mitocondrial; *COI*

The red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), is native to the Indian sub-continent and south-east Asia where it has been long recognized as a serious pest of several palm species, in particular the coconut palm (Murphy & Briscoe 1999; Faleiro 2006; Giblin-Davis et al. 2013). Voracious feeding by larvae within the trunk of infested palms typically leads to tree death. In the early 1980's, the red palm weevil was accidentally introduced into the Middle East, most likely in live coconut palms imported for landscaping. Following its introduction, this pest has had a serious impact on the date palm industries in these and neighboring countries (Ferry & Gomez 2002; El-Mergawy & Al-Ajlan 2011). Red palm weevil was first detected in the United Arab Emirates in 1985, and in the Kingdom of Saudi Arabia in 1987. Genetic differences between the populations in those countries suggested that they arose from independent invasion events (El-Mergawy et al. 2011; Rugman-Jones et al. 2013). These initial incursions propagated a large and rapid range expansion, most likely the result of accidental movement within live palms (Ferry & Gomez 2002), with the result that the red palm weevil is now established in most countries of the Persian Gulf, Mediterranean, and North Africa. Due to their ornamental and agricultural value, respectively, the Canary Island date palm, *Phoenix canariensis* Chabaud, **nom. coms. prop.** (Arecaceae), and the date palm, *Phoenix dactylifera* L. (Arecaceae), have been particularly impacted in invaded regions. Both palm species are preferred hosts of the weevil, and both have been extensively planted in invaded areas and neighboring countries.

The Maghreb region of North Africa successfully avoided invasion by red palm weevil until 2008, when it was confirmed to be present in Morocco (Faleiro et al. 2012). Its presence was subsequently confirmed in Libya in 2009 (Al-Eryan et al. 2010), and in Dec 2011, despite laws established several decades earlier strictly forbidding the importation of live palms (EPPO 1999), red palm weevil was confirmed for the first time in Tunisia, in Carthage Township, Tunis (Chebbi 2011). Four

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years later, the red palm weevil has destroyed one third (~35,000) of the ornamental palms, mostly *P. canariensis*, in the greater Tunis area. In Tunisia, as in many other Arab and Mediterranean countries, palm trees represent an important element of cultural, historical, and architectural heritage (El-Juhany 2010; Manachini et al. 2013). Moreover, although red palm weevil is currently restricted to urban areas in Tunis, its establishment poses a major socio-economic threat to the date industry in the southern regions of the country. Tunisia is the biggest supplier of dates to the European Union (Ben-Amor et al. 2015), and it is estimated that ~12% of Tunisians derive income from date production (Faleiro et al. 2012). Furthermore, date production is the foundation of many oasian settlements in southern Tunisia (Benaoun et al. 2014). In 2011, an estimated ~51,000 Ha of dates were farmed in Tunisia, and this acreage produced ~180,000 tons of dates (Ben-Amor et al. 2015). Palms less than 20 years of age are most vulnerable to attack by red palm weevil and it is estimated that 50% of date palms in Tunisia (i.e., over 2 million trees) are in this age range (Faleiro et al. 2012). Date production in the Tunisian oases already has been impacted by another invasive coleopteran pest, the Arabian rhinoceros beetle, *Oryctes (Rykanoryctes) agamemnon arabicus* (Fairmaire) (Coleoptera: Scarabaeidae) (Abdallah et al. 2013). In response to the threat now posed by the red palm weevil, the Tunisian government has allocated a budget of 4 million Tunisian dinar (~US $2 million) for eradication.

The geographic source of the invasive red palm weevil population in Carthage is unknown, and the subject of much consternation among Tunisians. Most Tunisians blame the family of the ousted ex-president Zine El Abidine Ben Ali for the introduction of red palm weevil into Tunisia. In 2008 to 2009, Ben Ali's son-in-law, Sakher El Matri, built the opulent Palais de Sidi Dhrif, in the cliff-top town of Sidi Bou Said, adjacent to Carthage, which is within a few meters of the first infestation site. Newspaper reports claim that the palace gardens were planted with mature palms that were imported, in direct contravention of Tunisia's phytosanitary laws, from the USA, and that these imported palms were the source of the red palm weevil infestation in Tunisia (Ben Sassi 2015). However, contradictory claims from anonymous sources in the Tunisian Ministry of Agriculture, Water Resources and Fisheries, suggested that the palms (and other ornamental plants) used for landscaping Palais de Sidi Dhrif were imported from Italy.

There is good reason to doubt that the weevils originated from the USA. First, the red palm weevil has a checkered taxonomic history (Wattanapongsiri 1966; Hallet et al. 2004; reviewed by Rugman-Jones et al. 2013), and a recent study confirmed that the name has in fact been applied to at least 2 cryptic species, with highly variable and overlapping color-morphology (Rugman-Jones et al. 2013). *Rhynchophorus ferrugineus* sensu stricto, typified by its orange coloration and varying degrees of black maculation on the pronotum, has already successfully invaded most countries of the Persian Gulf and Mediterranean Basin, but has not been recorded from the USA (El-Mergawy et al 2011; Rugman-Jones et al. 2013). However, an isolated incursion of its cryptic twin, *Rhynchophorus vulneratus* (Panzer) (Coleoptera: Curculionidae), was detected in Laguna Beach, California, USA, in Aug 2010 (NAPPO 2010), and later eradicated (Hoddle et al. 2016). In California, all intercepted *R. vulneratus* specimens were predominantly black with a red stripe on the pronotum, but in its native range of Southeast Asia, the scope of color-variation in this species overlaps with *R. ferrugineus* and the 2 species can be reliably distinguished only with the aid of DNA sequencing (Rugman-Jones et al. 2013). The taxonomic status of red palm weevil in Tunisia has not been confirmed by DNA sequencing, but the population matches the color morphology of *R. ferrugineus* sensu stricto. Thus, although the timing of the detection in California was such that any movement of infested palms from there may have facilitated the introduction of *R. vulneratus* (not *R. ferrugineus*) to Tunisia, the discrepancy in color-morphology of the 2 populations suggests an alternative scenario. One caveat is that there may have been undetected color-variation within the *R. vulneratus* population in California, or perhaps there is a large environmental, as opposed to genetic, component to color-variation in these weevils. The uncertainty over species identity due to the lack of distinguishing morphological traits can be addressed by sequencing the DNA of the invasive weevil population in Tunis. The most parsimonious alternative hypothesis is that the invasive weevil population in Tunis is *R. ferrugineus*, and was accidentally introduced through infested palms imported from a neighboring country. All invasive red palm weevil populations in the countries neighboring Tunisia, studied to date, have been shown to be *R. ferrugineus*, and have been characterized as genetically poor, with a single, fixed mitochondrial haplotype, previously referred to as H8 (El-Mergawy et al. 2011; e.g., GenBank accession GU581319) or El-Mergawy H8 (Rugman-Jones et al. 2013; e.g., GenBank accession KF311438). Thus, the hypothesis that Tunisia's invasive red palm weevil population originated from a source closer to North Africa than the USA can be addressed by comparing DNA sequences of the Tunis population with sequences of other populations of invasive *Rhynchophorus* species.

The aims of this study were, to confirm the species of invasive palm weevil populations in Tunisia (i.e., *Rhynchophorus ferrugineus* or *R. vulneratus*) and to determine the most likely area of origin (i.e., countries neighboring Tunisia or the USA). To achieve this we collected weevils at several sites around Tunis, the focal point of the red palm weevil invasion, and sequenced a section of the mitochondrial gene previously used to characterize global palm weevil populations (El-Mergawy et al. 2011; Rugman-Jones et al. 2013).

Materials and Methods

SPECIMEN COLLECTION

During Oct 2015, a total of 41 adult weevils were collected from 4 locations in Tunis. Date of collection, locality data, and geographical coordinates were recorded for each collection (Table 1). Adult weevils were extracted from infested *P. canariensis*, placed into labelled vials, and euthanized in 95% ethanol. Specimens were maintained in a −20 °C freezer in Tunis for several days, during which time the ethanol was changed after 24 and 48 h to ensure complete dehydration of each specimen and preservation of the DNA. Specimens were subsequently transported to University of California Riverside (UCR), California, USA, without temperature control, and then maintained at −20 °C. Weevils used in this study have been point-mounted and deposited in the Entomology Research Museum at UCR (Table 1).

DNA SEQUENCING AND ANALYSIS

A single tibia was removed and DNA was extracted from a small piece of leg muscle tissue of each individual specimen using the Chelex protocol detailed in Rugman-Jones et al. (2013). Polymerase chain reaction (PCR) was used to amplify a 528 bp section of the mitochondrial gene (mtDNA) *cytochrome oxidase subunit 1* (*COI*) using the primers C1-J-1718 and C1-N-2329 (Simon et al. 1994) according to Rugman-Jones et al. (2013). Amplification was verified by standard agarose gel electrophoresis. PCR products were purified using ExoSAP-IT® (Affymetrix, Santa Clara, California), and sequenced in both directions at the Institute for Integrative Genome Biology core instrumentation facility at UCR. Sequences were aligned manually using BioEdit version 7.0.9.0 (Hall 1999). Haplotypes were identified using DnaSP v5.10.01 (Librado & Rozas 2009), and compared with existing haplotypes (El-Mergawy et al. 2011; Rugman-Jones et al. 2013) held in GenBank (Benson et al. 2008).

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Table 1. Palm weevil populations sampled from dead or dying Canary Island date palms, *Phoenix canariensis*, around Tunis, during Oct 2015. Specimens were euthanized and preserved in ethanol, and, following the removal of a single tibia for DNA extraction, point-mounted and deposited in the Entomology Research Museum, University of California Riverside. *COI* sequences for all specimens were deposited in GenBank.

Site	Geographical Coordinates	Collect. Date	No. of Red Palm Weevil	UCR Entomology Museum Accession Numbers	GenBank Accession Numbers
El Kram	36.84565°N, 10.31868°E	12 Oct 2015	17	UCRC_ENT 00436529–37	KX228866–874
				UCRC_ENT 00436554–61	KX228891–898
La Souka	36.86294°N, 10.24004°E	15 Oct 2015	16	UCRC_ENT 00436538–53	KX228875–890
Carthage	36.85085°N, 10.32264°E	16 Oct 2015	6	UCRC_ENT 00436562–67	KX228899–904
La Marsa	36.88058°N, 10.32857°E	16 Oct 2015	2	UCRC_ENT 00436568–69	KX228905–906

Results

All 41 specimens harbored the same mitochondrial haplotype (GenBank accessions KX228866-906; Table 1). The haplotype confirms that the Tunisian population was *R. ferrugineus* sensu stricto, and matches the ubiquitous fixed haplotype that has previously been identified in all sampled red palm weevil populations collected from around the Mediterranean Basin, except those collected in Syria (Rugman-Jones et al. 2013). The haplotype has previously been referred to as H8 (El-Mergawy et al. 2011; e.g., GenBank accession GU581319) or El-Mergawy H8 (Rugman-Jones et al. 2013; e.g., GenBank accession KF311438).

Discussion

DNA sequences have become a fundamental tool for investigating taxonomic and phylogeographic questions, particularly in taxa where cryptic species occur. In the present study we sought to determine the specific status and potential geographic source of an invasive red palm weevil population first detected in Tunisia in 2011. We sequenced a 528 bp section of the *COI* gene from 41 specimens collected at multiple sites around Tunis and compared the sequences with existing sequences from a wide geographic sample of populations of *R. ferrugineus* and its cryptic congener *R. vulneratus* (El-Mergawy et al. 2011; Rugman-Jones et al. 2013). We conclusively showed that the Tunisian population is *R. ferrugineus*, thereby refuting the claim that the invasion occurred as a result of importing infested palms from the USA, as *R. ferrugineus* has never been recorded in the USA. In contrast, our finding of a single haplotype in Tunisia, that matches the ubiquitous El-Mergawy H8 haplotype present around the Mediterranean Basin (Rugman-Jones et al. 2013), provides strong support for the anonymous claims from the Tunisian Ministry of Agriculture, Water Resources and Fisheries, that the invasion of Tunisia by red palm weevil occurred as a result of the importation of infested palms from Italy. However, we do not have conclusive evidence for a specific country of origin, as the El-Mergawy H8 haplotype is ubiquitous in almost all red palm weevil populations around the Mediterranean Basin and is also present in populations in Malaysia, Thailand, Saudi Arabia, and Curaçao (Rugman-Jones et al. 2013). Regardless, we are confident that the red palm weevil did arrive in Carthage as a result of anthropogenic movement of palms, as the nearest potential source (i.e., infested area), Sicily (EPPO 2015), lies approximately 200 km away, across the Mediterranean Sea, a distance beyond the average dispersal capabilities of this insect (Hoddle et al. 2015).

The invasion of Tunisia by *R. ferrugineus* is an excellent example of the importance of phytosanitary regulations designed to prevent pest incursions. Importation of palm trees and palm products has been prohibited in Tunisia for decades to prevent the introduction of Bayoud disease (EPPO 1999), and strict enforcement of this prohibition doubtlessly contributed to Tunisia remaining free of red palm weevil for so long, relative to its neighbors. Currently, the red palm weevil invasion in Tunisia is restricted to the capital, Tunis, and isolated areas to the north. With timely intervention, and the determination of a government willing to commit approximately US $2 million to eradicating the red palm weevil, it may be possible to prevent rapid spread of this pest within Tunisia, raising hope that Tunisia's date industry, and the oasian culture that it supports, can still be spared. However, the loss of the ornamental palm trees from the urban landscape around Tunis is likely to be a lasting legacy of this invasion.

Finally, our study also highlights the importance of accurate species identification, and the key role that DNA-based analyses can play in this regard. Rugman-Jones et al. (2013) presented conclusive genetic evidence that the common name of red palm weevil was applied to 2 cryptic species, and that the species which invaded, and was eradicated from, California (USA) was not *R. ferrugineus,* but *R. vulneratus*. This critical distinction has been recognized by the European and Mediterranean Plant Protection Organization (EPPO 2016), but, despite acknowledging the genetic evidence, the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS) states that it will "…continue to refer to this detection as *Rhynchophorus ferrugineus* until additional information is available" (Molet et al. 2011 [revised 2014]). This stance ignores the definition of cryptic species (i.e., species that cannot be reliably distinguished based on their morphology), and in this instance, may have inadvertently supported the belief that the red palm weevil population arrived to Tunisia in palm trees imported from the USA.

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