African Journal of Agricultural Research Vol. 7(7), pp. 1170-1176, 19 February, 2012
Available online at http://www.academicjournals.org/AJAR
DOI: 10.5897/AJAR11.1580
ISSN 1991-637X © 2012 Academic Journals

Full Length Research Paper

Genetic diversity of an invasive pest (*Oryctes agamemnon* Burmeister, *Coleoptera*: Scarabaeidae) of date palm in Tunisia, inferred from random amplified polymorphic DNA (RAPD) markers

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Accepted 13 December, 2011

The root borer (Rhinoceros beetle), *Oryctes agamemnon* (Burmeister, 1847) is an invasive coleopteran pest of date palm trees in southwestern Tunisia introduced accidentally in "Mrah Lahwar" (Department of Tozeur), spread out into "Rjim Maatoug" (Department of Kebili), then into most oases of Tozeur and Kebili departments, where it is now causing a serious damage. We used 101 samples of *O. agamemnon* collected from 7 oases and 2 date palm varieties, in order to assess several invasion parameters (e.g. level and distribution of genetic diversity, degree of gene flow between populations and number of introduction events). Analysis of molecular variance (AMOVA) of the random amplified polymorphic DNA (RAPD) markers exhibited no significant genetic differentiation in relation with departments (Tozeur vs. Kebili) or host plant varieties (Deglet Nour vs. Kenta), raising the hypothesis that a limited number of founder genotypes would have been at the origin of invasion. A substantial gene flow was revealed among populations, suggesting that the expansion of *O. agamemnon*, in Tunisian southwestern oases, has been most likely facilitated by human agency, through the propagation of date palm. These preliminary results would provide a framework for more detailed studies on introduced populations of *O. agamemnon*, in order to help the development of optimized management methods of this insect.

Key words: *Insecta*, *Coleoptera*, *Oryctes agamemnon*, introduced populations, gene flow, molecular markers.

INTRODUCTION

Biological invasions occur when organisms colonize and spread outside of their ancestral range, typically with negative consequences for areas where they have been introduced (Lockwood et al., 2007). In particular, invasive crop pests often result in substantial impacts on their host plants because of the disruption of natural food webs, following invasion (Bellows, 2001). Thus, the development of methods to manage those species is increasingly important in order to minimize economic losses. In most cases, biological invasions result from complex patterns of introduction, establishment and spread (Lozier et al., 2009). The path by which a species is introduced (including the geographical source and number of introductions) is the most important factor influencing its success in the new environment and conditions the demographic size and genetic diversity of a founding population (Lockwood et al., 2005). Nowadays, genetic methods offer the best tools to resolve such histories of non-native organisms and predict the adequate management approaches.

The insect order of *Coleoptera* contains the most destructive species of date palm tree, *Phoenix dactylifera* L., that cause damage to the stem and roots. In

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particular, three species of the genus *Oryctes* (*Oryctes agamemnon* Burmeister, *O. elegans* and *O. richteri*) infest date palm trees in North Africa and the Middle-East (Rochat et al., 2004). In Tunisia, *O. agamemnon* has been the most destructive pest of date palm trees in the southwestern oases. This species has been accidentally introduced in Tunisia from the United Arab Emirates around 1980 by means of imported offshoots of the date palm variety "Deglet Nour" (most cultivated variety in Tunisian oases) (Soltani, 2010). Later on, *O. agamemnon* has been disseminated into oases of "Rjim Maatoug" (Kebili department), 120 km to the south-west of "Tozeur", just to the south of the "Chott-El-Jerid" salt lake. *O. agamemnon* has been causing an increasing devastating damage to date palm, expressed by the sudden collapse of many productive palm trees.

Adults of *O. agamemnon* do not feed. The insect has one generation in a year, lasting about 336 ± 10 days when breeding in natural substrate (Soltani et al., 2008). Mating and oviposition usually occur in dark places inside the substrate. Mean fecundity is around 30 eggs per female (Soltani, 2010). After egg hatching, larvae remain in the substrate and develop until the pupal stage. Attacks of these insects were observed on different parts of the palm tree. The beetle burrows in the growing point of palms, feeds on unopened fronds, damaging inflorescences, reducing photosynthetic area, which decreases and/or delays fruit production. Young palms are mostly damaged by larvae, while large insect populations can affect mature palms. Heavy attacks of larvae invade the roots on the periphery of the crown, leading to fungal disease (Soltani et al., 2008).

A lot of controlling strategies have been explored to manage this insect pest. For instance, manual extraction of adults and larvae from feeding galleries is being practiced in Tunisia, but is not useful for tall standing palms. Burning infected palm trees may cause environmental and health hazards. Frequent insecticide applications are also not very effective because adults and larvae of *O. agamemnon* spend most of their life concealed in galleries. Biological control is now approached in Tunisia, due to its effectiveness in some countries where Baculoviruses (Bedford, 1976; Zelazny, 1976; Rmale et al., 2005) or synthetic pheromones (Gries et al., 1994; Rochat et al., 2000, 2002; Hallet et al., 1995) were used to act against this pest.

A clear understanding of the mode of introduction of the invading insect, as well as the potential paths of gene flow, could provide useful insights for management. Several genetic markers can be used for analyzing relationships between populations of insect pests and investigate their dynamics from existing areas to new habitats (Darling and Blum, 2007). The Randomly-Amplified Polymorphic DNA-Polymerase Chain Reaction method (RAPD-PCR, Williams et al., 1990) has been frequently used because of its simplicity, economic cost, as well as the detailed amount of information about molecular polymorphisms it provides, without the necessity of previous knowledge of the DNA sequences to be studied. Although the use of this technique has been debated mainly because of the poor reproducibility of RAPD markers (Penner et al., 1993), several studies (Vanlerberghe-Masutti and Chavigny 1998; Raboudi et al., 2011) have succeeded to surmount this problem either by excluding the less intense bands, excluding the largest and smallest ones or conducting reactions twice, so that only reproducible bands serve to the subsequent analysis. In the present work, we applied RAPD-PCR to analyze genetic diversity of *O. agamemnon*, sampled from different sites of southwestern Tunisia.

MATERIALS AND METHODS

Insect sampling

Adults of *O. agamemnon* have been manually collected from most infected 7 oases of southwestern Tunisia ("Mrah Lahwar", "Chabbat", "Oudia" and "Dhafria" (in Tozeur department) and "Amal", "Ferdaoues" and "Nasr" (in Rjim Maatoug area, Kebili department) during June 2009 to July 2009 (Figure 1 and Table 1).

RAPD-PCR amplification and electrophoresis

DNA was extracted from the head of each sample of *O. agamemnon*, using the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1987). For RAPD-PCR amplifications, six single decamers (Operon Technologies, USA), OPA-13 (5'CAGCACCCAC3'), OPC-09 (5'CTCACCGTCC3'), OPG-03 (5'GAGCCCTCCA3'), OPH-01 (5'GGTCGGAGAA3'), OPH-02 (5'TCGGACGTGA3') and OPH-05 (5'AGTCGTCCCC3'), were used as primers. RAPD-PCR reactions were performed in 25 µl volume in a mixture containing 1X PCR buffer (Promega, USA); 0.1µM a single RAPD primer; 50 ng of insect DNA; 2.5 mM of $MgCl_2$; 0.1 mM of a dNTP equimolar mix (dATP, dGTP, dCTP and dTTP) and 1 U of *Taq* DNA polymerase (Promega, USA). Following a brief centrifugation, the samples were placed in a 2720 thermocycler (Applied Biosystems, USA) programmed for one step of initial denaturation (5 min at 94°C) then 35 cycles; each one consisting of one denaturation step (1 min at 94ºC), one annealing step (1 min at 36°C) and one extension step (1 min at 72°C). An extra extension step was also performed (7 min at 72°C). Amplification products were analyzed by electrophoresis in 1.5% agarose gel with 0.5X Tris Borate EDTA (TBE) buffer and detected by staining with Ethidium Bromide. Gels were observed under ultraviolet illumination and photographed. A molecular weight marker (100 bp Ladder, Invitrogen) was used as a standard. In order to avoid non reproducible markers, each experiment was conducted twice and only intense, reproducible fragments were considered for the statistical analysis.

Statistical analyses

RAPD–PCR profiles for each aphid sample were identified visually by scoring the presence (1) or absence (0) of all reproducible bands. The finalized fragment data from all 6 primers were pooled to define a single binomial phenotype for each of the 101 samples. These binary data were used to calculate Fst genetic distances between pairs of populations. The resulting matrix was used to cluster the data using the "Multidimensional Scaling" (MDS) method

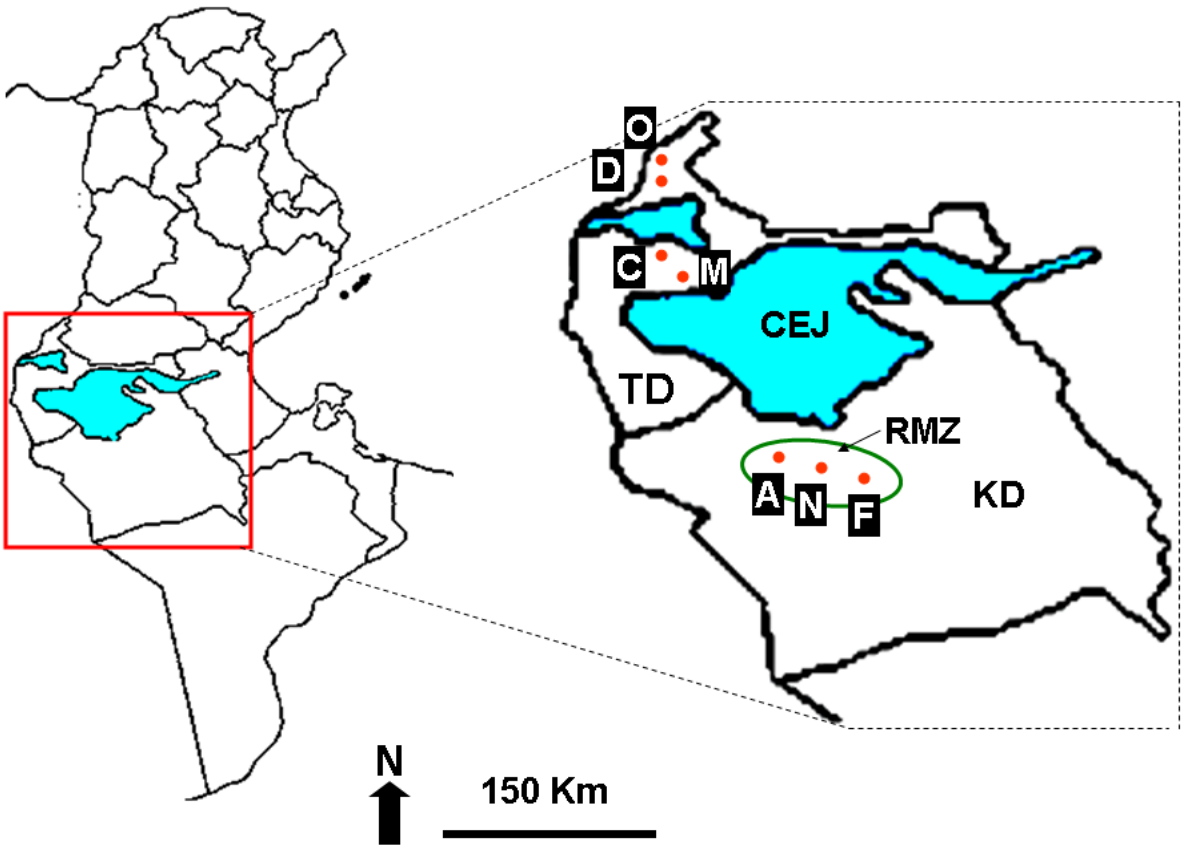

Figure 1. Map of Tunisia showing the geographic location of sampling sites for *O. agamemnon*. TD: "Tozeur" department; KD: "Kebili" department; RMZ: "Rjim Maatoug" zone; CEJ: "Chott-El-Jerid" salt lake; M: "Mrah Lahwar" oasis; C: "Chabbat" oasis; D: "Dhafria" oasis; O: "Oudia" oasis; F: "Ferdaous" oasis; A: "Amal" oasis; N: "Nasr" oasis.

Table 1. Sampling data for *Oryctes agamemnon.*

Department	Location	Host date palm variety	Sample size (adult insects)	Sampling date
Tozeur	"Chabbat"	"Deglet Nour"	8	23/06/2009
	"Dhafria"	"Deglet Nour"	11	24/06/2009
	"Mrah Lahwar"	"Deglet Nour"	13	23/06/2009
	"Oudia"	"Kenta"	18	25/06/2009
Kebili	"Amal"	"Deglet Nour"	15	14/07/2009
	"Ferdaoues"	"Deglet Nour"	18	18/07/2009
	"Nasr"	"Deglet Nour"	18	24/07/2009
Total	-	-	101	-

(Kruskal, 1964). A Mantel test was conducted to compare genetic and geographic distances matrixes. Hierarchical analysis of molecular variance (AMOVA) was used to quantify the diversity level and investigate genetic relationships among populations by partitioning the variation within and among defined groups. All statistical analyses were performed using the Arlequin software version 3.11 (Excoffier et al., 2005).

RESULTS

RAPD-PCR amplification overview

All amplification products used in the statistical analysis were reproducible ones. A total of 89 different markers

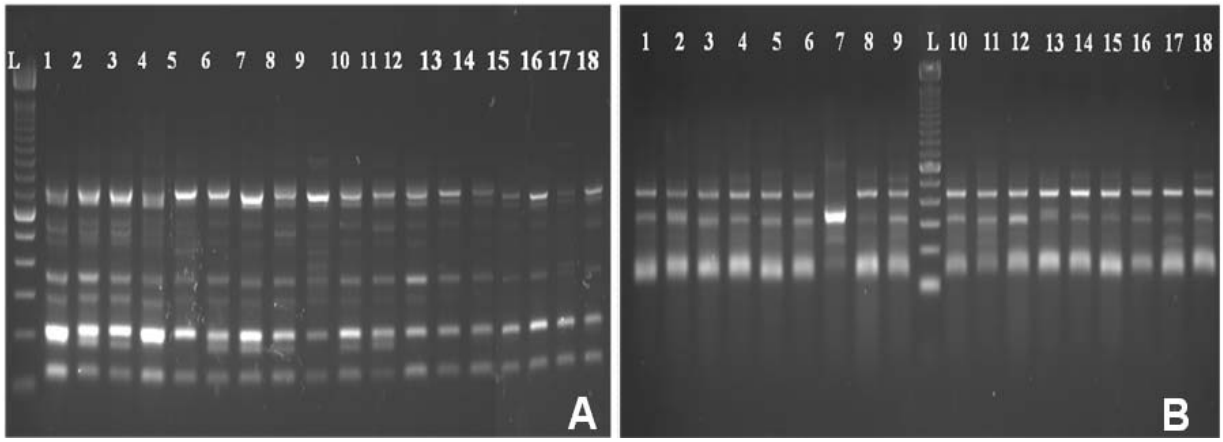

Figure 2. RAPD-PCR patterns, obtained using primers OPH-01 and OPG-03 and DNA from 2 natural populations of *O. agamemnon*. A: Primer OPH-01 and DNA from *O. agamemnon* collected in "Oudia" oases; B: primer OPG-03 and DNA from *O. agamemnon* collected in "Nasr" oases; L: molecular weight standard (100 bp ladder, Invitrogen).

Table 2. Pair-wise genetic distances (Fst) between seven studied populations of *Oryctes agamemnon*.

	Mrah Lahwar	Chabbat	Oudia	Dhafria	Ferdaoues	Amal	Nasr
Mrah Lahwar	0.0000						
Chabbat	**0.2925***	0.0000					
Oudia	0.4875	0.5064	0.0000				
Dhafria	0.3874	0.4982	0.5780	0.0000			
Ferdaoues	0.7228	0.7485	0.5850	0.7048	0.0000		
Amal	0.6843	0.6773	0.5192	0.6049	**0.7553***	0.0000	
Nasr	0.4762	0.5231	0.3773	0.5785	0.6582	0.5181	0.0000

*The highest and lowest genetic distances are indicated in bold.

were scored with the six primers used, ranging from 130 to 1000 pb in size. Figure 2 shows examples of RAPD patterns obtained with primers OPH-01 and OPG-03.

Within-population variability

The percentage of polymorphic markers using the 6 RAPD primers varied between 27.77%, in "Mrah Lahwar", and 55.44%, in "Nasr", with an average of 40.22% across all the studied populations.

Among-population diversity

Sixty-seven (67) different phenotypes were recorded in the 101 *O. agamemnon* studied samples, among which 51 (76.12%) were redundant in two or more locations. This fact clearly indicates that there are overlapping genotypes between the studied populations.

Genetic distance values ranged from 0.2925 between "Chabbat" and "Mrah Lahwar" to 0.7553 "Ferdaoues" and "Amal" (Table 2). No correlation between genetic and geographic space was found by Mantel test. Indeed, populations Nasr and Oudia were comparatively close in genetic space (0.3773), although they are distant in geographic space (Figure 1). Conversely, populations "Ferdaoues" and "Amal" which are close in geographic space (Figure 1) were separated by a comparatively high genetic distance (0.7553).

Based on Fst distances, factorial analysis using "Multidimensional Scaling" (MDS) method (Figure 3) showed two major clusters: Cluster 1 containing "Chabbat", "Mrah Lahwar" and "Dhafria" populations while cluster 2 having populations of "Oudia" and "Nasr". "Ferdaoues" and "Amel" populations were clearly separated from both clusters. Therefore, no differentiation between the studied populations could be evidenced on the basis of department (Tozeur / Kebili). Also, populations were not distinguished according to the host date palm variety, since population "Oudia" (collected on "Kenta" variety) was not genetically distant from the remaining populations that were collected on "Deglet Nour" variety. Finally, molecular variance analysis (AMOVA) showed that the genetic structure was not associated with department ($P = 0.916$) or date palm

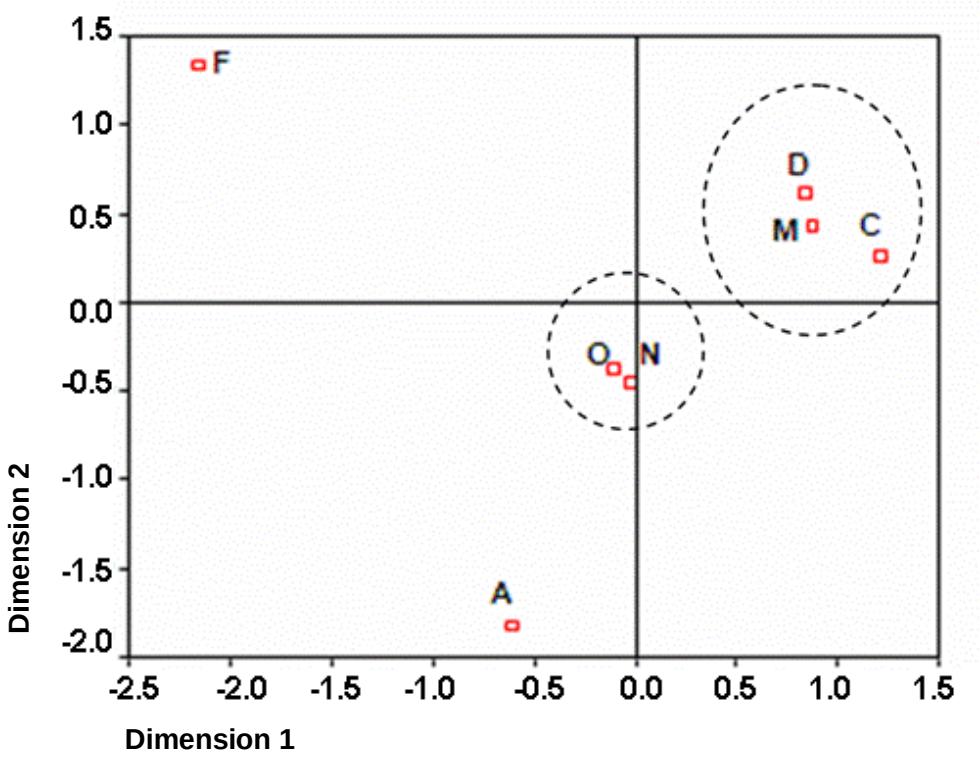

Figure 3. Multidimensional scaling scatter plot showing patterns of diversity among 7 *Oryctes agamemnon* populations, on the basis of Fst genetic distances. *F: Ferdaoues; M : Mrah Lahwar; C: Chabbat; O : Oudia; D: Dhafria; A : Amal; N: Nasr.*

Table 3. Genetic variation among defined groups of populations, revealed by AMOVA, based on RAPD data sets.

Source of variation	d. f.	Sum of squares	Total variation (%)	P-value
Among departments (Kebili vs. Tozeur)	1	113.619	-4.539	0.916 (NS)
Among date palm varieties	1	81.142	-1.838	1.000 (NS)

variety (P = 1.000) (Table 3).

DISCUSSION

The major aim of this study was to document the level and distribution of genetic diversity in populations of the root borer, *O. agamemnon*, the most destructive pest of date palm trees in the southwestern oases of Tunisia that was introduced from the United Arab Emirates around 1980. As an introduced species, *O. agamemnon* is supposed to be present in biogeographic region where it did not evolve and to which it might be poorly adapted, encountering a suite of novel stresses and selection pressures, such as abiotic conditions and/or availability of the host plant (Prentis et al., 2008). Bulman et al. (2005) explained that the genetic adaptative potential of an invasive species; and therefore, its genetic diversity in the new habitat, is linked to three key factors: period of sexuality, abiotic conditions and/or availability of the host plant. The length of generation time is considered as a major factor in determining the amount of genetic diversity in insect populations, because it is directly related to the risk of death before the reproductive stage (Nylin and Gottard, 1998). In the present study; the level of intra-population genetic diversity in root borer in Tunisia was found moderate, as it could be inferred from the percentage of polymorphic markers (40.22%). We hypothesize that since the introduction of *O. agamemnon* into the south of Tunisia, its slow rhythm of sexuality (one generation per year) was insufficient to generate a great amount of variability to overcome the founder effect. In contrast, selection pressure, in relation with abiotic conditions, seems to have less or no effect on the genetic diversity, most probably because *O. agamemnon* adults are hidden inside the stem of date palm tree. Also, no

effect of host plant could be concluded, as population "Oudia" was collected from the variety "Kenta" although it displayed the same diversity level as other populations that were collected on "Deglet Nour" variety.

The analysis of the genetic structure, by MDS, Mantel test and AMOVA revealed no significant genetic differentiation in relation with departments (Tozeur vs. Kebili) or host plant varieties (Deglet Nour vs. Kenta). Even not being in strict correlation with departments or date palm varieties, the MDS analysis has revealed the existence of at least two clusters: cluster 1 constituted by "Dhafria", "Mrah Lahwar" and "Chabat" populations; and cluster 2 including "Oudia" and "Nasr" ones. Cluster 1 reflected a comparatively more homogenous genetic structure for populations from the department of Tozeur, while those from Kebili were more dispersed, without constituting a unique genetic pool contrasted to Tozeur populations.

The absence of clear genetic differentiation in relation with departments or host plant varieties is further supported by the redundancy in RAPD phenotypes. Indeed, 76.12% of 67 phenotypes generated in total in this study were present in 2 or more populations, suggesting a high level of nuclear gene flow over long geographic distances. As pointed out by several authors (Hartl et al., 1993; Suchentrunk et al., 2000), such a genetic overlapping between populations of an invasive insect pest could be explained by a "genetic bottleneck" effect, meaning that the invasive population was initially founded by a small number of individuals that were isolated from a larger gene pool in the initial habitat (Arab United Emirates). These genetic bottlenecks are thought to decrease the quantitative variation within the invasive population (Dlugosh and Parker, 2008). Therefore, we think that a unique invasion of Tunisian palm date trees by root borer would have occurred, that was followed by dispersal through a long distance. Based on the big size of belly that characterize adults of *O. agamemnon* (which implies a limited flight), along with the standing of Chott-El-Jerid as a natural barrier separating the different oases studied in this research; we suggest that between-oases dispersal of the species would have resulted mainly from human activity through the transportation of biological material from infested oases. It was reported that herbivorous insects attacking agricultural crop systems are among the most transported organisms (Lozier et al., 2009). Human activities such as travel and trade (Di Castri, 1989; Mack et al., 2000) could lie behind the transportation of such infected plant material.

In conclusion, the limited genetic intra-population diversity along with the substantial gene flow revealed among populations and the absence of genetic structure in relation with geography or host variety, plead in favor of non-differentiation of pest strains, making the management efforts be homogenous for all the palm grove of South-West Tunisia. The results obtained in the present study provide baseline information for monitoring the diversity level and spread mode of *O. agamemnon* in Tunisia. However, the conclusions herein presented will need to be more fully analyzed, through the use of additional molecular markers and populations and larger sample sizes within each population.

ACKNOWLEDGEMENTS

Funding support of the Tunisian Ministry of "Higher Education and Scientific Research" is gratefully acknowledged. The authors address special thanks to Ali ZOUBA (Centre de Recherches Phénicicoles de Dégache), Ali BELHAJ (Office de Développement de Réjim Maatoug), M'hamed HCINE (Institut des Régions Arides de Kebili) and Mohamed-Sadok BELKADHI (Institut des Régions Arides de Kebili), for their valuable help.

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