J. Entomol. Res. Soc., 19(1): 81-90, 2017 *ISSN:1302-0250*

A Practical Molecular Diagnostic Tool of the Date Moth *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae) in Tunisia

Sawsen SEDGHIANI[1] Faten RABOUDI[2] Dhia BOUKTILA[1, 3*]
Hanem MAKNI[1,2] Mohamed MAKNI[1]

[1]Unité de Recherche Génomique des Insectes Ravageurs des Cultures d'intérêt agronomique (UR11ES10), Faculté des Sciences de Tunis, Université de Tunis El Manar, TUNISIA
[2]Institut Supérieur de l'Animation pour la Jeunesse et la Culture de Bir El Bey (ISAJC), Université de Tunis, TUNISIA
[3]Institut Supérieur de Biotechnologie de Béja (ISBB), Université de Jendouba, TUNISIA
e-mails: sedghianisawsen@yahoo.fr, faten.raboudi@isajc.rnu.tn,
*dhia_bouktila2000@yahoo.fr, hanem.makni@isajc.rnu.tn, md.makni@fst.rnu.tn

ABSTRACT

In Tunisia, date palms are infested by various insect pests, especially Pyralids, whose caterpillars feed inside dates, spoiling their quality. As a critical pre-requisite towards controlling these pests, their accurate taxonomic diagnosis must be applied at molecular level, independently from pest life stages. In the present study, we report the molecular diagnosis of the Pyralid moth *Ectomyelois ceratoniae* at the larvae stage, based on the Cytochrome Oxidase I (COI) gene sequence. We have sequenced a 710 base pairs fragment of the mitochondrial COI gene from 10 Tunisian *E. ceratoniae* specimens. The analysis of intra-specific diversity, using additional public sequences originating from Africa (GenBank: KP083440.1, KP083444.1, KP083442.1, KP083441.1 and JF748065.1) and Australia (GenBank: KF405701.1, KF40073.1 and KF397550.1) provided evidence of population differentiation over geographic scales, as the representatives of each continent (i.e. Africa or Oceania) formed a homogeneous cluster in phylogenetic analysis. Besides, we have used a quick method, based on Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP), to distinguish between *E. ceratoniae* and two other Pyralidae moths commonly found in dates following harvest, namely *Plodia interpunctella* and *Anagasta kuehniella*. This study describes a rapid and efficient molecular diagnostic tool for highly-damaging Pyralidae species. It represents a significant contribution that will impact future pest control strategies of date moths in Tunisia.

Key words: Date palm, Pyralidae, *Ectomyelois ceratoniae*, cytochrome oxidase I, DNA barcoding, PCR-RFLP.

INTRODUCTION

The date palm, *Phoenix dactylifera* L., is a strict dioecious evergreen tree capable of living over 100 productive years. It is one of the oldest domesticated trees, today playing important socio-economic roles in the Middle-East and North Africa (Mahmoudi *et al.*, 2008). It is thought that the earliest cultivation of *P. dactylifera* dates back to 3,700 BC in the area between the Euphrates and the Nile rivers, namely in what is now southern Iraq (Al-Mssallem *et al.*, 2013). Date palm was, historically, introduced by humans to northern India, North Africa, and southern Spain. As a part of North Africa,

Tunisia lends date palms a prominent place in its national economy, with a national production totaling 225,000 T, in 2014/2015 (Tunisian Ministry of Agriculture-Fruit inter professional group, 2015). In spite of their importance, date palms are susceptible to attacks by several pathogens and pests. Especially during harvest or storage, the fruit faces a high rate of infestation by moths belonging to the family of Pyralidae, such as the raisin moth *Cadra figulilella* (Gregson), The Indian mealmoth, *Plodia interpunctella* (Hübner), the Mediterranean flour moth, *Anagasta kuehniella* (Zeller), the almond moth, *Ephestia cautella* (Walker) and the carob moth *Ectomyelois ceratoniae* (Zeller). In Tunisia, *E. ceratoniae* has been considered as the most damaging pest of dates and the main constraint to dates export (Dhouibi, 1989; Jarraya, 2003).

Taxonomic identification of pests is an essential issue in integrated pest management approaches. However, classical, morphology-based identification methods are sometimes unable to detect differences between specimens, especially those with deteriorate morphology or immature stages as eggs, pupae and larvae. The identification of these juvenile forms, which may have no distinguishable features, often needs to be delayed till maturity. Thus, the morphological approach is time-consuming, and requires a high expertise. Alternatively, molecular approaches, based on enzymatic amplification, digestion and/or sequencing of key genes could be of great help. The mitochondrial gene cytochrome oxidase subunit I (COI) has been nominated by Hebert *et al.* (2003a,b; 2004) as a standard gene for the identification of cryptic animal species. This gene has been used for resolving several insect complexes, such as *Bactrocera* sp. (Mezghani-Khemakhem *et al.*, 2012), *Mayetiola* sp. (Bouktila *et al.*, 2006), *Culex* sp. (Hemmerter *et al.*, 2009) and *Anopheles* sp. (Gutiérrez *et al.*, 2010). COI has several advantages; for example, it is devoid of introns and contains few insertions and deletions (Mardulyn and Whitfield, 1999). In addition, its evolution is rapid enough to discriminate not only related species but also phylogeographic groups within the same species (Cox and Hebert, 2001; Hebert *et al.*, 2003b). The aim of the DNA barcoding method is to assign unidentified specimens to a given taxon, at specific or infra-specific level, based on the COI gene sequence. Various studies have corroborated the success of this method (Hebert *et al.*, 2003a; Hebert *et al.,* 2004; Ward *et al.,* 2005; Hogg and Hebert, 2005; Tsao and Yeh, 2008; Wilson *et al.,* 2013). Furthermore, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was successfully applied to distinguish between several insects of cryptic species (Raboudi *et al.*, 2005; McKern and Szalanski, 2007; Garrick *et al.*, 2015). Taking this into consideration, the purpose of our present study was to identify and characterize larval specimens of *E. ceratoniae*, collected from date palms in different regions in Tunisia, based on COI gene sequencing and PCR-RFLP.

MATERIAL AND METHODS

Insect samples

Pyralidae larvae were collected from five oases located in southern Tunisia, in spring 2013. Geographic location and number of samples are given in Table 1. Specimens were stored at -20 °C until DNA extraction.

Table 1. Sampling and public sequence data.

Samples from Tunisian oases		
Insect species	***Samples labels***	***Sampling site***
Ectomyelois ceratoniae	K1, K2, K3, K4	Kebili (33° 42'N; 8°58'E)
	T1, T2	Tozeur (33° 55′ N; 8° 08′ E)
	N10	Nefta (33° 52′ N; 7° 53′ E)
	G1, G2	Gabes (33° 53′ N; 10° 07′ E)
	D1	Douz (33° 27′ N; 9° 01′ E)
Public sequences		
Insect species	**Accession numbers in Genbank or BOLDSYSTEMS databases**	**Geographical origin**
Ectomyelois ceratoniae	GenBank : KP083440.1	South Africa
	GenBank : KP083444.1	South Africa
	GenBank : KP083442.1	South Africa
	GenBank : KP083441.1	South Africa
	GenBank : JF748065.1	South Africa
	GenBank : KF405701.1	Western Australia
	GenBank : KF40073.1	Queensland (Australia)
	GenBank : KF397550.1	Queensland (Australia)
Plodia interpunctella	GenBank : KF492033.1	Maryland (USA)
	GenBank : KM572075.1	Austria
	GenBank : KF399544.1	Australia
	GenBank : GU096543.1	Ontario, Ottawa (Canada)
	GenBank : GU096544.1	Ontario, Ottawa (Canada)
Anagasta kuehniella	GenBank : GU828613.1	No data available
	BOLDSYSTEMS : MSWEB061-15	Ontario, Guelph (Canada)
	BOLDSYSTEMS : MSWEB064-15	Ontario, Guelph (Canada)
	BOLDSYSTEMS : MSWEB063-15	Ontario, Guelph (Canada)
	BOLDSYSTEMS : MSWEB062-15	Ontario, Guelph (Canada)

COI gene amplification and sequencing

DNA was extracted from larvae, using Cetyl Trimethylammonium Bromide (CTAB) method (Doyle and Doyle, 1987). Extracted DNA was resuspended in 30 µL of sterile Milli-Q water. Polymerase Chain reactions were conducted with primers LCO1490: 5'-GGT-CAA-CAA-ATC-ATA-AAG-ATA-TTG-G-3' and HCO2198: 5'-TAA-ACT-TCA-GGG-TGA-CCA-AAA-AAT-CA-3' (Folmer et *al.*, 1994). These primers amplified a 710 base pairs fragment in the 5' end of the mtDNA cytochrome oxidase subunit I (COI). Each reaction, performed in 25µL volume, used 50ng of DNA template, 0.1 unit of Taq polymerase (Promega), 200µM $MgCl_2$, 0.1 mM of each primer and 0.2 mM dNTPs. PCR conditions consisted of an initial denaturation at 94°C for 5

minutes, followed by 35 cycles each consisting of 3 steps: denaturation (94°C, 1 min), annealing (54 °C, 1 min) and extension (72°C, 1 min); and a final extension at 72°C for 5 min. PCR products were separated by 1% agarose gel and stained with ethidium bromide. The appropriate bands were purified using Quik Gel Extraction (Invitrogen) before being sequenced by an automated DNA sequencing system.

PCR-RFLP assay

Fifteen (15) µL of the COI gene PCR product of each larva were incubated in a total reaction volume of 20 µL with 5 U of the endonuclease *Dra*I and 10X digestion buffer (Promega) at 37 ºC for 4 hours. The digested products were visualized on 2% agarose gel, stained with ethidium bromide and photographed under ultraviolet light.

Bioinformatic analyses of sequences

In order to characterize *E. ceratoniae* specimens, at intra-species level, based on COI gene sequence, we have enriched the genetic COI gene pool, by 8 additional public sequences, corresponding to partial sequences of cytochrome oxidase subunit I (COI) gene of *E. ceratoniae*. Five of these sequences were from South Africa, while three originated from Australia (Table 1). The COI gene sequences obtained through DNA amplification and those retrieved from databases were aligned using ClustalW algorithm implanted in BioEdit 7.2.5 (Hall, 1999). The method reported by Elsasser *et al.* (2009) and Shokralla *et al.* (2011), based on Kimura 2-parameter (K2P) model of base substitution (Kimura, 1980), was used for phylogeny reconstruction and for generating a Neighbor Joining (NJ) phylogenetic tree by MEGA software version 6 (Tamura *et al.*, 2013), with a bootstrap analysis of 1000 replications. Besides, in an attempt to identify discriminatory restriction sites between *E. ceratoniae* and other Pyralidae species, we have analyzed restriction polymorphisms, using NEBcutter v.2.0 software (Vincze *et al.*, 2003), and three consensus sequences: (a) a consensus of *E. ceratoniae* COI gene sequences that was developed based on the combined 10 local sequences and the eight public accessions of this species, shown in Table 1, and (b) two consensus sequences of each *P. interpunctella* and *A. kuehniella* COI gene that were generated from public accessions of these species, shown in Table 1.

RESULTS

Correlation between genetic diversity and geographical distribution

The 710 bp fragment of the COI mitochondrial gene was amplified and sequenced for each sample (n=10). After checking and manual editing, the total length of DNA sequence used from each individual was adjusted to 604 bp. A Blast search in Genbank (Benson *et al.*, 2013) and BOLDSYSTEMS (Ratnasingham and Hebert, 2013) databases showed that all specimens belonged to *E. ceratoniae* species, with BOLDSYSTEMS compatibilities ranging between 99.83% and 100%, indicating a high “DNA barcode” sequence similarity between the studied Tunisian specimens and their most similar conspecific *E. ceratoniae* ones. As expected in insect mitochondrial DNA,

these sequences contained a high AT percentage (Crozier and Crozier, 1993). No insertions, deletions, or stop codons were observed in the sequences, indicating the absence of nuclear copies of mitochondrial origin (NUMTs) (Bensasson *et al.*, 2001; Song *et al.*, 2008). The alignment at the nucleotide level showed that COI sequences were either identical or highly similar. Moreover, the translation of these sequences revealed identical amino acid sequences (data not shown).

To examine the extent of intra-specific divergence, 8 additional sequences of *E. ceratoniae* COI were downloaded from Genbank and BOLDSYSTEMS database. The pair-wise sequence comparison across *E. ceratoniae* individuals showed that genetic distances ranged from 0.0% to 4.1%, with an average of 1.2%. These values of intra-specific divergence, were influenced by the presence of two groups showing 3.9% divergence: The first, included specimens from Tunisia and South Africa (African continent), while the second was made of specimens originating from Queensland and Western Australia (Oceania). Each group was homogeneous, with low mean divergence rates of 0.4% and 0.2% for the first and second groups, respectively. Indeed, when specimens from Oceania were excluded, the overall intra-specific divergence dropped significantly from 1.2% to 0.4%. Because of their deep divergence, sequences from Queensland and Western Australia formed a distinct cluster in Neighbor-joining tree (Fig. 1).

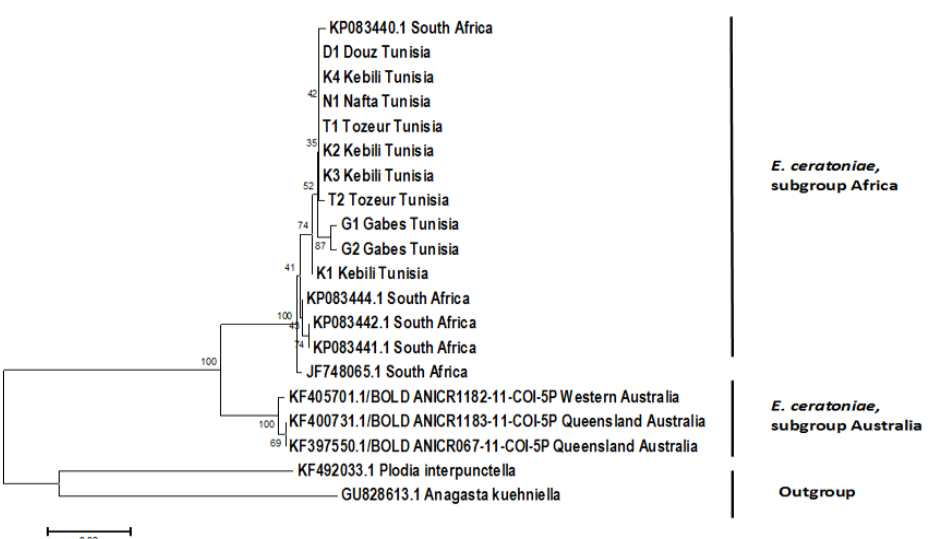

Fig. 1. Neighbor-joining phylogenetic tree, based on a 604 bp COI gene fragment, showing genetic relationships among *Ectomyelois ceratoniae* specimens from Tunisia, South Africa and Australia. Lepidoptera species *Plodia interpunctella* and *Anagasta kuehniella* were used as outgroup. The numbers above branches refer to the bootstrap values based on 1000 replications.

COI-PCR-RFLP: an efficient tool for distinguishing between *E. ceratoniae* and the complex *P. interpunctella* and *A. kuehniella*

The COI sequences of *E. ceratoniae, P. interpunctella* and *A. kuehniella* were

examined for their recognition site of restriction enzyme using the virtual sequence digestion program NEBcutter. *Dra*I was chosen because of its discriminatory potential. It yielded, *in silico*, diagnostic RFLPs that discriminated easily between *E. ceratoniae*, on one hand, and *P. interpunctella* and *A. kuehniella*, on the other hand. Indeed, *Dra*I endonuclease acted specifically at a diagnostic restriction site at position 570 in *E. ceratoniae*, but not in *P. interpunctella* and *A. kuehniella* (Fig. 2). The visualized PCR-RFLP patterns, obtained in wet lab experimentation for 20 Tunisian specimens of *E. ceratoniae*, were similar to those predicted *in silico*, showing a standard profile with a 570 bp and a 140 bp DNA fragments on agarose gel (Fig. 3), confirming that all specimens belong to *E. ceratoniae* species.

DISCUSSION AND CONCLUSIONS

Our results are in agreement with previous studies reporting that the levels of intra-specific variation rarely exceed 2% in Lepidoptera (Hebert *et al.*, 2003b; 2009; Hajibabaei *et al.*, 2006; Hausmann *et al.*, 2011). The K2P distances calculated between samples from the two continents were higher (1.2% mean intra-specific divergence) compared with those calculated from the representatives of a single continent (0.4% and 0.2% for Africa and Oceania, respectively). This fact pleads in favor of a population differentiation over geographic scales. Likewise, several entomological studies reported that geography and genetic distances were co-dependent (Bergsten *et al.*, 2012; Bouktila *et al.*, 2012). Similarly, Čandek and Kuntner (2014) found that increased geographical sampling affects slightly the intra-specific distance variation but did not hamper the performance of the DNA barcodes as a tool for species identification. This variation may be the result of isolation by distance (Wright, 1943) or distance decay (Nekola and White, 1999).

Although DNA sequencing based on cytochrome oxidase I (COI) gene is an effective and reliable tool for insect identification, the use of this method could be time-consuming and/or expensive. For this reason, we suggest that a combination of COI genotyping and PCR-RFLP will be a rapid diagnostic tool between Pyralid moths, as this method was previously applied to several insect pests (Raboudi *et al.*, 2005; McKern and Szalanski, 2007; Valenzuela *et al.,* 2007; Mezghani-Khemakhem *et al.*, 2012). We have shown that the endonuclease *Dra*I has a specific cleavage site in *E. ceratoniae* COI gene sequences, facilitating the distinction between this species and other Pyralids, namely *P. interpunctella* and *A. kuehniella.* Results reported, in this paper, will facilitate species identification, not only by specialized entomologists, but also non-specialists who are not familiarized with the morphology of Pyralidae.

ACKNOWLEDGEMENTS

This work was supported by the Tunisian Ministry of Higher Education and Scientific Research. The authors greatly acknowledge all the industrial staff of ‘Boudjebel SA VACPA’ Company for specimen supply.

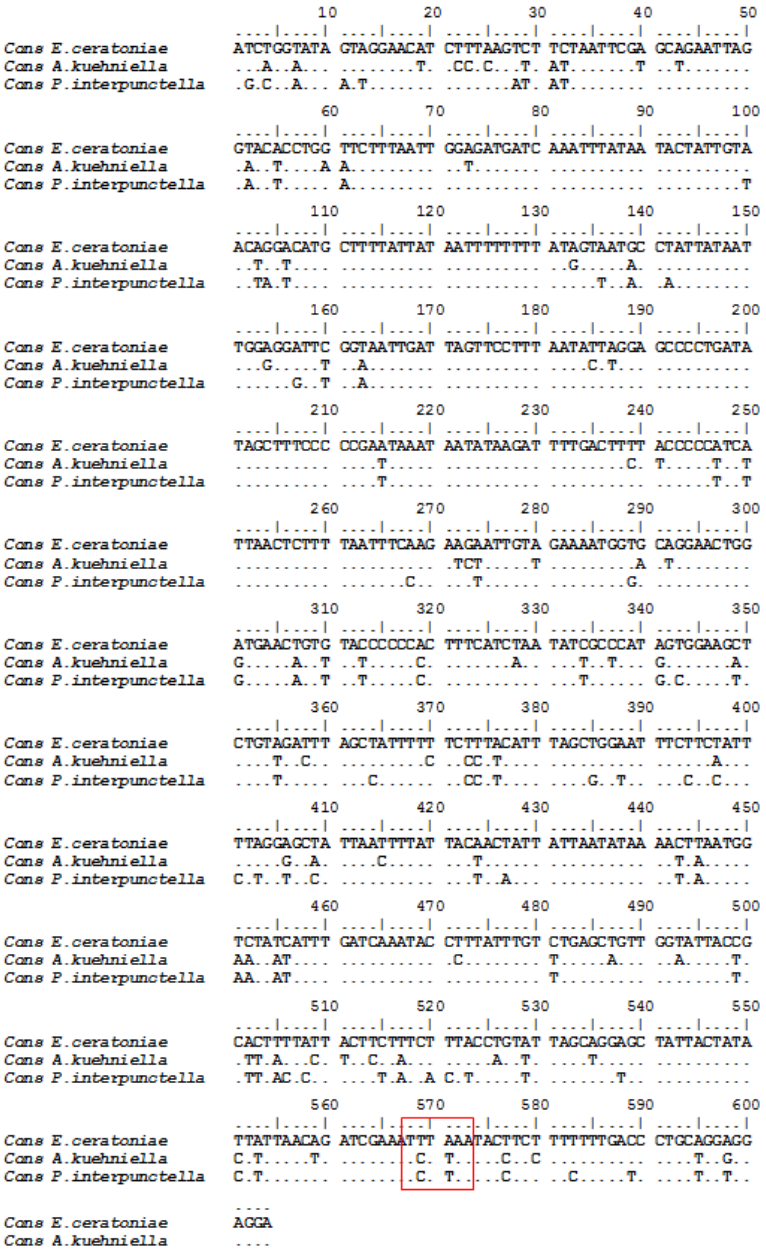

Fig. 2. Comparison of a 604 bp COI gene portion between *Ectomyelois ceratoniae*, *Anagasta kuehniella* and *Plodia interpunctella*. The red frame refers to the differential *Dra*I restriction site.

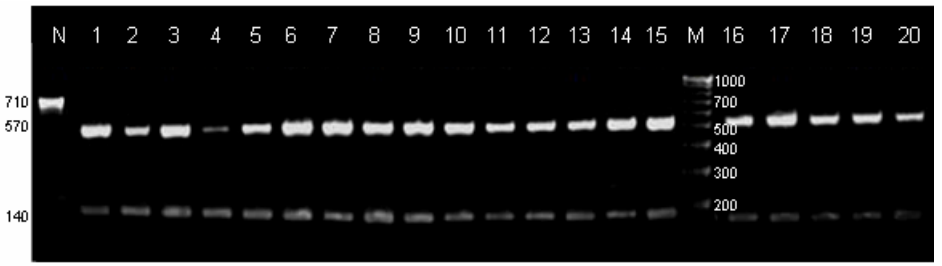

Fig. 3. Banding profiles of the COI gene obtained from 20 *E. ceratoniae* individuals, after digestion with *Dra*I. N: native (undigested) 710 bp fragment of the COI gene; M: Molecular weight marker (1000 bp).

REFERENCES

Al-Mssallem, I. S., Hu, S., Zhang, X., Lin, Q., Liu, W., Tan, J., Yu, X., Liu, J., Pan, L. *et al.*, 2013, Genome sequence of the date palm *Phoenix dactylifera* L. *Nature Communications,* 4 (2274): 1-9.

Bensasson, D., Zhang, D. X., Hartl, D. L., Hewitt, G. M., 2001, Mitochondrial pseudogenes: evolution's misplaced witnesses. *Trends in Ecology and Evolution,* 16: 314-321.

Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., Sayers, E. W., 2013, GenBank. *Nucleic Acids Research*, 41(Database issue): 36-42.

Bergsten, J., Bilton, D. T., Fujisawa, T., Elliott, M., Monaghan, M. T., Balke, M., Hendrich, L., Geijer, J., Herrmann, J., Foster, G. N., Ribera, I., Nilsson, A. N., Barraclough, T. G., Vogler, A. P., 2012, The effect of geographical scale of sampling on DNA barcoding. *Systematic Biology,* 61: 851-869.

Bouktila, D., Mezghani, M., Marrakchi, M., Makni, H., 2006, Genetic variation and relatedness in Tunisian wheat midges of the genus *Mayetiola* (Diptera: Cecidomyiidae), inferred from biological and molecular data. *Acta Entomologica Sinica,* 49: 822-828.

Bouktila, D., Kharrat, S., Mezghani-Khemakhem, M., Jerraya, A., Makni, M., 2012, Genetic diversity in different populations of citrus leafminer, *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) in Tunisia, assessed by RAPD-PCR. *Journal of Crop Protection,* 1: 189-199.

Čandek, K., Kuntner, M., 2014, DNA barcoding gap: reliable species identification over morphological and geographical scales. *Molecular Ecology Resources,* 15: 268-277.

Cox, A. J., Hebert, P. D. N., 2001, Colonization, extinction and phylogeographic pattering in a freshwater crustacean. *Molecular Ecology,* 10: 371-386.

Crozier, R. H., Crozier, Y. C., 1993, The mitochondrial genome of the honeybee *Apis melhfera:* complete sequence and genome organization. *Genetics*,, 133: 97-117.

Dhouibi, M. H., 1989, *Biologie et Écologie d'Ectomyelois ceratoniae Zeller (Lepidoptera- Pyralidae) Dans Deux Biotopes Différents au sud de la Tunisie et Recherche de Méthodes Alternatives de Lutte*. PhD Thesis, University of Paris VI, France, 176.

Doyle, J. J., Doyle, J. L., 1987, A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Photochemical Bulletin,* 19: 11-15.

Elsasser, S. C., Floyd, R., Hebert, P. D. N., Schulte-Hostedde, A. I., 2009, Species identification of North American guinea worms (Nematoda: Dracunculus) with DNA barcoding. *Molecular Ecology Resources*, 9: 707-712.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek R., 1994, DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology,* 3: 294-299.

Garrick, R. C., Collins, B. D., Yi, R. N., Dyer, R. J., Hyseni, C., 2015, Identification of Eastern United States *Reticulitermes* termite species via PCR-RFLP, assessed using training and test data. *Insects*, 6: 524-537.

Gutiérrez, L. A., Orrego, L. M., Gómez, G. F., López, A., Luckhart, S., Conn, J. E., Correa, M. M., 2010, A new mtDNA COI gene lineage closely related to *Anopheles janconnae* of the Albitarsis complex in the Caribbean region of Colombia. *Memórias do Instituto Oswaldo Cruz*, 105:1019-1025.

Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., Hebert, P. D. N., 2006, DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of the National Academy of Science of the United States of America,* 103: 968-971.

Hall, T. A., 1999, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series,* 41: 95-98.

Hausmann, A., Haszprunar, G., Hebert P. D. N., 2011, DNA Barcoding the Geometrid fauna of Bavaria (Lepidoptera): Successes, surprises, and questions. *Plos One,* 6: e17134.

Hebert, P. D. N., Cywinska A., Ball, S. L., De Waard, J. R., 2003a, Biological identification through DNA barcodes. *Proceedings of the Royal Society, Biological Sciences,* 270: 313-321.

Hebert, P. D. N., Ratnasingham, S., De Waard, J. R., 2003b, Barcoding animal life: Cytochrome C oxidase subunit I divergences among closely related species. *Proceeding of the Royal Society, Biological Sciences,* 270: 896-899.

Hebert, P. D., Penton, E. H., Burns, J. M., Janzen, D. H., Hallwachs W., 2004, Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America,* 101: 14812-14817.

Hebert, P. D. N., Dewaard, J. R., Landry, J. F., 2009, DNA barcodes for 1/1000 of the animal kingdom. *Biology letters,* 6: 359-362.

Hemmerter, S., Slapeta, J., Beebe, N. W., 2009, Resolving genetic diversity in Australasian *Culex* mosquitoes: incongruence between the mitochondrial cytochrome c oxidase I and nuclear acetylcholine esterase 2. *Molecular and Phylogenetic Evolution*, 50: 317-25.

Hogg, I. D., Hebert, P. D. N., 2005, Biological identifications of springtails (Hexapoda: Collembola) from the Canadian arctic, using mitochondrial barcodes. *Canadian Journal of Zoology,* 82: 749-754.

Jarraya, A., 2003, *Principaux nuisibles des Plantes Cultivées et des Denrées Stockées en Afrique du Nord: Leur Biologie, Leurs Ennemis Naturels, Leurs Dégâts et Leur Contrôle*. Maghreb Editions, Tunis, Tunisia.

Kimura, M., 1980, A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16: 111-120.

Mahmoudi, H., Hosseininia, G., Azadi, H., Fatemi, M., 2008, Enhancing date palm processing, marketing and pest control through organic culture. *Journal of Organic Systems,* 3: 29-39.

Mardulyn, P., Whitfield, J. B., 1999, Phylogenetic signal in the COI, 16S, and 28S genes for inferring relationships among genera of *Microgastrinae* (Hymenoptera; Braconidae): Evidence of a high diversification rate in this group of parasitoids. *Molecular Phylogenetics and Evolution,* 12: 282-294.

McKern, J. A., Szalanski, A. L., 2007, Molecular diagnostics of economically important clearwing moths (Lepidoptera: Sesiidae). *Florida Entomologist,* 90: 475-479.

Mezghani-Khemakhem, M., Ben Lazahr, W., Bouktila, D., Ben Slimen, H., Makni, H., Makni, M., 2012, A rapid diagnostic technique of *Bactrocera cucurbitae* and *Bactrocera zonata* (Diptera: Tephritidae) for quarantine application. *Pest Management Science,* 69: 744-746.

Nekola, J. C., White, P. S., 1999, The distance decay of similarity in biogeography and ecology. *Journal of Biogeography,* 26: 867-878

Raboudi, F., Mezghani, M., Makni, H., Marrakchi, M., Rouault, J. D., Makni, M., 2005, Aphid species identification using cuticular hydrocarbons and cytochrome b gene sequences. *Journal of Applied Entomology,* 129: 75-80.

Ratnasingham, S., Hebert, P. D. N., 2013, A DNA-based registry for all animal species: The barcode index number (BIN) system. *Plos One*, 8: e66213.

Shokralla, S., Zhou, X., Janzen, D. H., Hallwachs, W., Landry, J. F., Jacobus, L. M., Hajibabaei, M., 2011, Pyrosequencing for mini-barcoding of fresh and old museum specimens. *Plos One*, 6: e21252. doi:10.1371/journal.pone.0021252.

Song, H., Buhay, J. E., Whiting, M. F., Crandall, K. A., 2008, Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified, *Proceedings of the National Academy of Sciences of the United State of America,* 105: 13486-13491.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013, MEGA 6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution,* 30: 2725-2729.

Tsao, W. C., Yeh, W. B., 2008, DNA-based discrimination of subspecies of swallowtail butterflies (Lepidoptera: Papilioninae) from Taiwan. *Zoological Studies*, 47: 633-643.

Tunisian Ministry of Agriculture-Fruit inter professional group, 2015, http://gifruits.com/?page_id=2311andlang=fr (12.02.2015).

Valenzuela, I., Hoffmann, A. A., Malipatil, B. B., Ridland, P. M., Weeks, A. R., 2007, Identification of aphid species (Hemiptera: Aphididae: Aphidinae) using a rapid polymerase chain reaction restriction fragment length polymorphism method based on the cytochrome oxidase subunit I gene. *Australian Journal of Entomology,* 46: 305-312.

Vincze, T., Posfai, J., Roberts, R. J., 2003, NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Research,* 31: 3688-3691.

Ward, R. D., Zemlak, T. S., Iness, B. H., Last, P. R., Hebert, P. D. N., 2005, DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society*, *Biological Sciences*, 360: 1847-1857.

Wilson, J. J., Sing, K. W., Sofian-Azirun, M., 2013, Building a DNA Barcode Reference Library for the True Butterflies (Lepidoptera) of Peninsula Malaysia: What about the Subspecies? *Plos One,* 8: e79969.

Wright, S., 1943, Isolation by distance. *Genetics*, 31: 114-138.

Received: March 09, 2016 *Accepted: November 02, 2016*