

## Survey of Mitochondrial DNA Markers in Populations of *Ceratitis capitata* (Wiedmann) from Tunisia

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**ABSTRACT:** The Mediterranean fruit fly, *Ceratitis capitata*, is considered to be one of the world's most destructive agricultural pests. Beginning from a presumed origin in Africa, in recent years this pest has considerably expanded its geographic range. In an effort to document the genetic relationships of worldwide populations of this pest species, mitochondrial DNA markers have been surveyed in several populations of *C. capitata*, but previously little or no information has been available from populations in Tunisia. To address this issue, specimens have been collected from several localities within the northern part of Tunisia (Cap Bon, Mornag, Bizerte and Kairouan). These are regions known for fruit production including oranges, peaches, apples and apricots. In addition, a sample population derived from the laboratory strain used for mass rearing at the INRA Institute in Tunis was also analyzed. We have used a PCR based approach to analyze mitochondrial DNA sequences and document the genetic variation present in these populations. Our analysis includes sequences of regions of the mitochondrial genome for analysis of mitochondrial haplotypes. Our results indicate that at least two distinct mitochondrial haplotypes are present in the Tunisian populations.

**Key Words:** genetics, haplotypes, Mediterranean fruit fly, polymorphic sequences

### INTRODUCTION

The Mediterranean fruit fly *Ceratitis capitata* (Wiedmann) (Diptera: *Tephritidae*) is highly polyphagous and has become established in many tropical and subtropical regions of the world (Sheppard *et al.*, 1992). Molecular markers derived from both mitochondrial and nuclear gene sequences have been surveyed in populations of this pest in several countries around the world including the Mediterranean region. These surveys have been used to analyze genetic relationships of populations and to examine the origin of biological invasions of this pest (Ochando *et al.*, 2003; He and Haymer 1999; Malacrida *et al.*, 1998; Kourti, 1997).

Mitochondrial markers have proved to be very useful for characterizing these populations. They have several advantages such as maternal inheritance, absence of recombination and relative ease of manipulation (Reyes and Ochando, 2004). Mitochondrial variation is often analyzed using the method RFLP-

PCR (restriction fragment length PCR –polymerase chain reaction).

In this method, a target DNA sequence is first amplified from individual specimens using PCR. The amplified target sequence is then digested with various restriction enzymes to detect DNA sequence polymorphisms based on the production of altered restriction fragment patterns. Using this type of approach, a system has been devised to encode mitochondrial haplotypes of *C. capitata* based on the presence or absence of restriction enzyme sites in three key regions of the mitochondrial DNA (Meixner *et al.*, 2002; Gasparich *et al.*, 1997, 1995; Sheppard *et al.*, 1992). This technique is very efficient for analyzing large scale samples and documenting the genetic variations between populations (Steck *et al.*, 1996).

Despite the extensive interest and utility of these mitochondrial markers, Tunisian populations, have remained largely uncharacterized to date (Gasparich *et al.*, 1997). The aim of this work is to initiate the investigation of the population's genetics from different areas within Tunisia and to compare their structure to other populations in the world.

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## MATERIAL AND METHODS

### Strains of *C. capitata*

Adults of *C. capitata* were collected from four geographic areas in Tunisia: Bizerte, Mor-nag, Cap Bon and Kairouan. Specimens from one laboratory reared strain were provided by the INRAT (Institut National de la Recherche Agronomique de Tunisie) and another was provided by the fruit fly rearing facility (USDA, Hawaii). Approximately 20 specimens were analysed from each locality.

### Genomic DNA extraction

Genomic DNA was extracted from the different samples using the Lifton rapid fly genomic DNA isolation protocol (Anleitner and Haymer, 1992). Briefly, each individual specimen is homogenized in 250 µl of grind buffer consisting of 0.2 M sucrose, 0.05 M ethylenediaminetetraacetic acid (EDTA), 1mM Tris, pH 9.0 and 0.5 % sodium dodecyl sulphate (SDS). Proteinase K is added at a concentration of 0.2 mg/ml and the mix is incubated at 65 °C for 1 h. Subsequently, 38 µl of potassium acetate (8 M) is added and the mixture is incubated at -20 °C overnight. After that, the mix is centrifuged at 10,000 rpm for 15 minutes at 4 °C to pellet proteins. The supernatant is retrieved and the DNA is precipitated with 600 µl of ethanol (95 %) and centrifuged as mentioned above. The pellet is then resuspended in 200 µl of TE followed by phenol extraction. After a final precipitation with sodium acetate (3 M), pH 6 at -20 °C for 1 hour to overnight, the pellet is spun down and washed with 500 µl of ethanol 70% and dried. Finally, the DNA pellet is resuspended in 20 µl of TE.

### PCR Reaction

The DNA isolated from Mediterranean fruit fly specimens was amplified using two primer pairs (N-4-J-8883/N-4-N-9243) and (N-5-J-7991/N-4-N-8916) with the following sequences as described in Gasparich *et al.* (1995) :

N-4-J-8883: TAATAATCCATATCCTCCTA

N-4-N-9243: TTAGTTTTAACATTTAGAAG

N-5-J-7991: TAATAAACTCATTCAATCAA

N-4-N-8916: ATAGAAGCTCCTGTATCTGG

The first primer pair (N-4-J-8883/N-4-N-9243) is used to amplify a portion of the NADH subunit 4 (ND4) gene and the second one (N-5-J-7991/N-4-N-8916) amplifies a portion of the NADH subunit 5 (ND5) gene.

The PCR mix contains 17.3 µl dH<sub>2</sub>O, 2.5 µl PCR buffer, 3µl MgCl<sub>2</sub>, 0.5 µl dNTPs, 0.5 µl primers, 1µl DNA and 0.2µl Taq Polymerase. The amplification program has an initial denaturation step of 2 minutes at 94°C, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 46°C and 2 minutes at 66°C, and a final extension of two minutes at 72°C. The amplification products were analyzed by electrophoresis in 1% agarose gels in TBE 0.5 X buffer with the 2-Log ladder (Biolabs) as a molecular weight marker.

### DNA sequencing

PCR products from individual specimens representing different populations were isolated using the "Gene clean" method. In this method, 20 µl of each PCR product is transferred to a microcentrifuge tube and 5 volumes of the GENECLEAN TURBO for PCR Salt Solution are added. The solution is transferred to a cartridge tube containing a membrane that will trap the DNA. The mixture is then centrifuged at less than 14,000 rpm for 5 seconds and the liquid is transferred to a catch tube. Then, 500 µl of prepared GENECLEAN Turbo for PCR Wash is added to the membrane, the mix is centrifuged as mentioned above for 5 seconds and then for an additional 4 minutes to drive the last of the wash Solution from the Turbo cartridge. After that, 30 µl of GENECLEAN Turbo for PCR Elution Solution is added and the mixture is incubated for 5 minutes at room temperature, followed by a centrifugation at less than 14000 rpm for 30 seconds to elute the DNA in to a new tube.

The gene cleaned PCR products were used as templates for sequencing reactions car-

ried out using BigDye terminator chemistry (Applied Biosystems, Inc.) and ABI 3730XL capillary-based automated DNA sequencers. Sequencing was performed at ASGPB (Advanced studies in genomics, proteomics and bioinformatics) center at the University of Hawaii at Manoa.

## RESULTS AND DISCUSSION

Previous investigations have analyzed the genetic diversity of worldwide medfly populations using a wide range of molecular markers including microsatellites, allozymes, and RFLPs (Restriction fragment length polymorphism) (Ochando *et al.*, 2003; He and Haymer 1999; Malacrida *et al.*, 1998; Kourti, 1997). Among these genetic tools, markers based on mtDNA sequences seem to have distinct advantages for studying polymorphisms (Reyes and Ochando, 2004). Although mtDNA markers have been studied in many worldwide populations of the medfly (Gasparich *et al.*, 1997), to date there have been no detailed studies of the genetic makeup of populations of this pest species from Tunisia.

Figure 1 shows the localities of the populations from Tunisia that were surveyed in this study. Approximately 20 specimens from each of these localities, as well as specimens from a lab strain maintained at INRAT, were analyzed. Using PCR, DNA fragments approximately 600 base pairs in length containing portions of ND4 gene 4 and ND5 gene, were amplified from each of these specimens. These PCR products were sequenced from representative individuals for analysis.

Figure 2 shows representative sequences from Tunisian individuals aligned with sequences obtained from a GenBank entry (U12924) containing the ND4 and ND5 regions of the medfly genome. Sequence polymorphisms are highlighted with red boxes.

Four of these five sequence polymorphisms are shared by individuals from collections (D) and (E), INRA laboratory strain and Bizerte wild collection, respectively yet are absent in all other individuals. The fifth is a single nucleotide polymorphism (SNP) found only in this individual (#16) from collection (C) from Kairouan (the central coastal part of the country). These results suggest that there are at least two distinct mitochondrial haplotypes present in Tunisia. Furthermore, the shared polymorphisms between the (D) and (E) collections are also consistent with the fact that the INRAT strain (D) was originally derived from a collection in the Bizerte (E) region (Cheikh *et al.* 1975).



**Figure 1.** A map of Tunisia showing sample collection sites

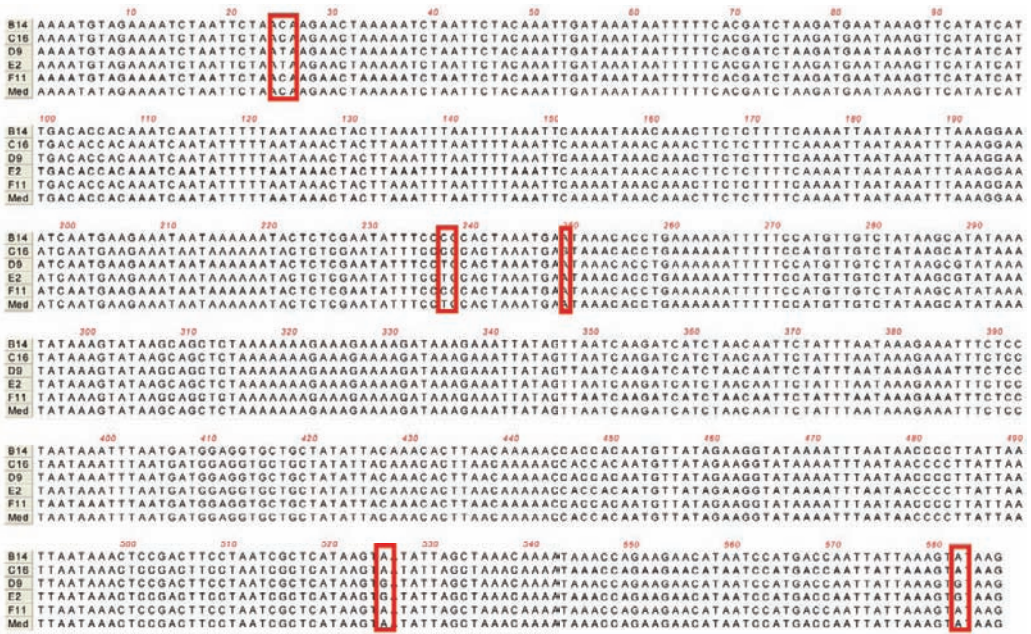
The findings of the present study confirm the existence of at least two haplotypes in Tunisia. The presence of a limited number of

haplotypes has been found in other Mediterranean countries. It remains to be seen whether any of the Tunisian haplotypes reported by Gasparich *et al.* (1997) are the same as those identified here.

Recently, up to nine mitochondrial haplotypes were identified from Mediterranean fruit fly populations in Spain (Reyes and Ochando, 2004). Based on this, these authors suggest that Spain has the highest level of mitochondrial diversity followed by Italy and Greece in the Mediterranean area. Further investigations will analyze additional specimens from the Tunisian and Mediterranean collections to further clarify these relationships.

### CONCLUSION

Collections of medfly specimens from various regions of Tunisia have been used to successfully initiate molecular genetic investigations of these populations. Sequence polymorphisms found in at least one wild collection (Area E – Bizerte) appear to be absent in other areas, suggesting that at least two distinct mitochondrial haplotypes exist in Tunisia, one of these is from the extreme north (Bizerte) and another haplotype is from the central coastal area (Kairouan). This preliminary evidence suggests that there is genetic differentiation between populations from different areas in Tunisia.



**Figure 2.** Alignment of DNA sequence from the ND4 and ND5 regions of the mitochondrial DNA (Tunisia samples (B14: individual 14 from Takelsa, C16: individual 16 from Kairouan, D9: individual 9 from INRAT, E2: individual 2 from Bizerte, F11: individual 11 from Soliman) and Med: Medfly Gen Bank entry (U 12924, containing sequences from the ND4 and ND5 regions). Sequence changes are highlighted.

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