Genetic Diversity of ND5 mitochondrial patterns in *Ceratitis capitata* (Diptera: Tephritidae) populations from Tunisia

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Abstract. The Mediterranean fruit fly (medfly) *Ceratitis capitata* (Diptera: Tephritidae) is known to be one of the most destructive and economically important agricultural pests worldwide. Several previous research projects have investigated the genetic makeup of regional populations of this pest and the relationships of populations from different areas of the world, including countries from the Mediterranean region. However, previously, little information has been reported on populations from Tunisia, despite the fact that this pest occurs in several agriculturally sensitive areas of this country. In order to study the genetic diversity of medfly populations within Tunisia, specimens were collected from the Coastal, Northern, Central and Southern regions of the country. Results using mitochondrial ND5 sequences show the presence of distinct haplotypes. This data used to analyze the levels of genetic variability within and between populations from Tunisia as well as from other countries in the Mediterranean region (Morocco and Israel) and in the world (Seychelles and Hawaii). This study also leads to a better understanding of the origin of new infestations and the colonization processes involving this pest.

Résumé. Diversité génétique des configurations mitochondrial ND5 chez les populations de *Ceratitis capitata* (Diptera : Tephritidae) de Tunisie. La mouche méditerranéenne des fruits *Ceratitis capitata* (Wiedemann) (Diptera : Tephritidae) est l'un des ravageurs les plus importants en agriculture dans le monde. Plusieurs travaux de recherche ont étudié les caractéristiques génétiques de la cératite ainsi que les relations entre populations de ce ravageur provenant de différentes régions du globe y compris les pays appartenant au bassin méditerranéen. Cependant, très peu d'informations sont disponibles concernant les populations de cératite en Tunisie, malgré l'importance économique de ce ravageur dans des zones clés de production fruitière tunisiennes. Dans le but d'étudier la diversité génétique des populations de cératite en Tunisie, des spécimens ont été collectés du Cap Bon, Nord, Centre et sud de la Tunisie. Les résultats basés sur l'étude des séquences du gène mitochondrial ND5 révèlent la présence d'haplotypes distincts. Ces haplotypes ont permis d'analyser les niveaux de variabilité génétique entre les populations tunisiennes et les autres populations de cératite du bassin méditerranéen (Maroc et Israël) ainsi que des populations prélevées dans d'autres régions du monde (Iles Seychelles et Hawaï). Cette étude a permis également de mieux caractériser l'origine de nouvelles infestations ainsi que les processus de colonisation de ce ravageur.

Keywords: Haplotype, medfly, mitochondrial DNA, molecular marker, sequencing.

Studies of the Mediterranean fruit fly (medfly) *Ceratitis capitata* (Wiedmann) (Diptera: *Tephritidae*) have revealed an extensive pattern of expansion of the geographic range of this highly destructive agricultural pest (Rössler 1989). Beginning from a presumed origin in Sub-Saharan Africa, in the past 200 years, this pest has expanded its geographic range to the Mediterranean basin as well as many other tropical and subtropical regions of the world (Sheppard *et al.* 1992; Meixner *et al.* 2002; Malacrida *et al.* 2007). In all of these areas the establishment of this pest causes considerable economic losses in fruit production i.e. citrus, apricots, peaches and figs. For the analysis of such invasive pest populations, DNA based genetic markers have showed a number of advantages in comparison to other types of genetic markers and to traditional methods based on morphological and/or behavioural characters. DNA markers used in previous studies of Mediterranean fruit fly populations include those derived from nuclear genes such as RAPDs (Haymer & McInnis 1994) and intron sequences (Gomulski *et al*. 1998; He & Haymer 1999), microsatellites (Bonizzoni *et al*. 2001), as well as those derived from mitochondrial DNA (mtDNA) (McPheron *et al.* 1994; Gasparich *et al.* 1995; Kourti 1997; Ochando *et al.* 2003). Mitochondrial DNA markers in particular have proved to be efficient for characterizing some aspects of the genetic diversity of medfly populations due in part to its haploid and uniparental (maternal) mode of inheritance and to the absence of recombination (Reyes & Ochando 2004). However, mitochondrial DNA studies have

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Table 1. Details of the different *C. capitata* specimens analyzed.

Country	Locality	coordinates	Host plant	Date	# of specimens
Tunisia	-Bizerte (North)	37°15'N 09°50'E	*Citrus sinensis* (orange)	II.2006	8
	-Kairouan (Centre)	35°45'N 10°05'E	*Prunus persica* (peach)	III.2006	6
	-Takelsa, Cap bon (coast)	37°01'N 11°02'E	*Citrus sinensis* (orange)	I.2006	6
	-Tozeur (south)	33°56'N 08°08'E	*Opuntia ficus-indica*	VII.2007	16
Morocco	-Sidi Slimane (Kenitra)	34° 15' N, 5°55'W	*Citrus sinensis* (orange)	II.2007	3
	-Sidi Abdelaziz (Kenitra)	32° 34' N 7° 40'W	*Citrus sinensis* (orange)	II.2007	2
Israel	-Rehovot	31°54'N 34°48'E	*Psidium guajava* (guava)	IX.2007	3
	-Maagan Michael	32°33'N 34°55'E	*Psidium guajava* .(guava)	IX.2007	12
Seychelles	-Mahé	09°22'S 46°28'E	*Citrus sinensis* (orange)	III.2007	2
Hawaii	-Kaui	22°03'N159°30'W	*Citrus sinensis* (orange)	III.2005	2

relied on the use of a PCR-RFLP method to identify diagnostic restriction site polymorphisms (McPheron *et al.* 1994) in genes such as the ND4 (NADH subunit 4) and ND5 (NADH subunit 5) genes (Sheppard *et al.* 1992; Gasparich *et al.* 1995; Meixner *et al.* 2002). This method was efficient to some extend but, it is used to detect only a very limited set of DNA sequence polymorphisms. This limitation may impact the ability to assess the true extent of diversity within and between populations, and in the case of the medfly, this may be especially significant for populations from some countries which show high levels of genetic variability compared to other regions of the world (Malacrida *et al.* 1998).

The main aim of this study is the characterization of the genetic diversity in Tunisian populations from different localities compared to other populations from 4 areas (Morocco, Israel, Seychelles and Hawaii Islands) using haplotype patterns identified through the direct analysis of DNA sequences from the mitochondrial ND5 gene.

Material and methods

Samples of *Ceratitis capitata*

Adults of *C. capitata* were collected from different geographical localities in Tunisia (Tab. 1). Infested fruits were collected from Citrus, Peaches, Guava and Figs and brought to the laboratory where pupation takes place. Collections from Israel, Morocco,

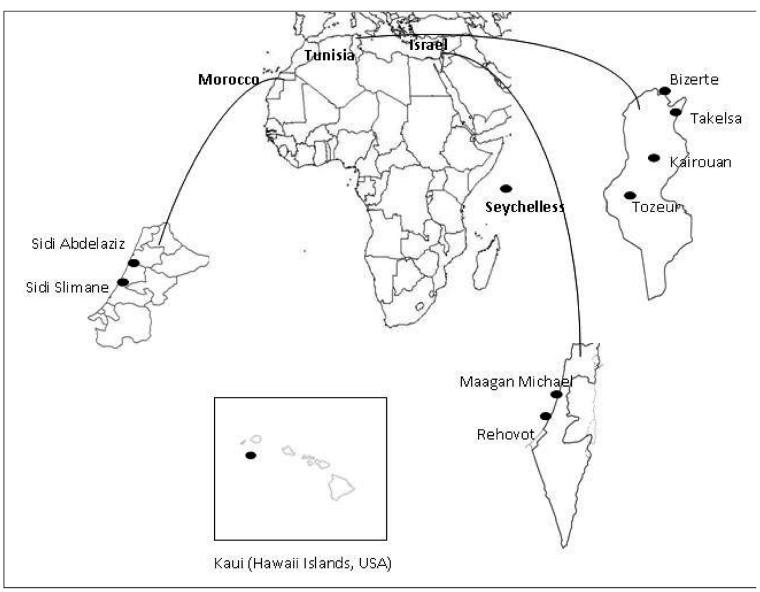

Figure 1
Map showing the geographical locations of the *Ceratitis capitata* populations studied.

Seychelles and Hawaii Islands were also considered for this study (Fig. 1). All the specimens were preserved in Ethanol 95%.

Genomic DNA Extraction

Genomic DNA was extracted from individuals belonging to the various collections using the Lifton rapid fly genomic DNA isolation protocol (Anleitner & Haymer 1992). Each individual specimen is homogenized in 250 µl of grind buffer consisting of 0.2 M sucrose, 0.05 M EDTA (ethylenediaminetetraacetic acid), 1mM Tris pH 9.0 and 0.5 % SDS (Sodium Dodecyl Sulfate). Proteinase K was added at a concentration of 0.2 mg /ml and the mix is incubated at 65 °C for 1 h. Subsequently, 38 µl of 8 M Potassium acetate is added and the mixture is incubated at -20 °C overnight. The mix was then 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 again. The pellet is then resuspended in 200 µl of TE (10mM Tris-1mM EDTA, pH = 8) followed by equal volume of phenol extraction. After a final precipitation with 3 M Sodium acetate, pH 6 at -20 °C overnight, the DNA 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 and sequencing

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

N-5-J-7991: TAATAAACTCATTCAATCAA

N-4-N-8916: ATAGAAGCTCCTGTATCTGG

This primer pair amplifies a portion of the NADH – *dehydrogenase subunit 5* (ND5). DNA was amplified in 50µl polymerase chain reaction containing 3µl $MgCl_2$ (3mM), 0.5 µl primers (20pmol/µl), 45 µl Invitrogen Platinum Supermix and 1µl of genomic DNA corresponding to 50ng. The amplification program has an initial denaturation step of 2 minutes at 94 °C, followed by 40 cycles of 30 seconds denaturation at 94 °C, 30 seconds annealing at 46 °C and 2 minutes extension at 66 °C, and a final extension of two minutes at 72 °C. In order to check the PCR reaction success, the amplicons were run on 1% agarose gels in TBE 0.5 X buffer (45mM Tris, 45mM Borate, 1mM EDTA) with the 2-Log ladder (New England Biolabs, Beverly, MA) as a molecular weight marker. PCR products from individual specimens were isolated using the "Gene clean" method (Qbiogene, Solon, OH, USA) as described by the manufacturer and resuspended in a total volume of 10 µl (Vogelstein & Gillespie 1979). One to 2 µl of template DNA from each individual were used for sequencing reactions performed with BigDye terminator chemistry (Applied Biosystems, Inc., Carlsbad, CA) on an ABI 3730XL capillary-based automated DNA sequencer.

Data Analysis

Sequences obtained from the PCR products were aligned using the software program CLUSTALW as implemented in DS GENE 2.0 (ACCELERYS Inc., San Diego, CA). The analysis of molecular variance (AMOVA) was computed using ARLEQUIN version 3.1 (Weir & Cockerham 1984; Excoffier *et al.* 1992), to determine the population genetic variability. Genetic diversity was analyzed for 5 population groupings based on countries. The TCS software version 1.21 (Templeton *et al.* 1992), was used to draw the parsimonious haplotype network. The program NEB cutter 2.0 (Vincze *et al.* 2003) was used to generate virtual enzyme restriction sites maps from the ND5 sequences.

Results

Mitochondrial ND5 sequences from the Mediterranean fruit fly were generated from a total of 60 individual specimens representing populations from 5 countries (Tunisia, Morocco, Israel, Seychelles and USA (Hawaii)). The total length of the ND5 products was 900 bp (Fig. 2). In order to avoid any accidental

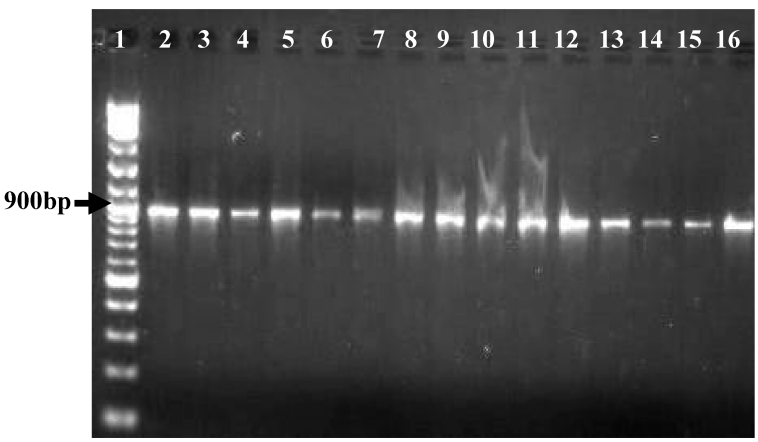

Figure 2
Gel picture (Agarose 1%) of the ND5 amplified fragment of *Ceratitis capitata* specimens from Bizerte (Tunisia).

Table 2. Haplotype frequency distribution among the populations of *Ceratitis capitata* analyzed.

Code	Base pair positions of nucleotide changes																																					Haplotype distribution									
																																						Tunisia				Israel		Morocco		Seychelles	Hawaii
	3	50	51	52	53	54	64	72	91	97	275	303	315	354	372	560	591	648	842	843	844	845	850	851	852	853	854	855	857	858	Bizerte	Takelsa	Kairouan	Tozeur	M. Michael	Rehovot	Sidi Slimane	Sidi Abdelaziz		
I	A	A	A	A	A	T	G	G	T	C	A	T	A	G	A	A	G	G	A	T	C	T	A	C	T	C	G	C	A	T	4	2	1	9								
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VI	.	.	.	.	.	C	.	.	C	.	.	C	G	A	.	.	A	A	.	.	.	.	.	.	.	.	.	T	.	.						1						
VII	.	.	.	.	C	C	.	.	C	.	.	C	G	A	.	.	A	A	.	.	.	.	.	.	.	.	.	T	.	.					3							
VIII	.	.	.	.	C	.	.	.	C	.	.	C	G	A	.	.	A	A	.	.	.	.	.	.	.	.	.	T	.	.					1							
IX	.	.	.	.	.	.	.	.	C	.	.	C	G	A	.	.	A	.	.	.	.	.	.	.	.	.	.	T	.	.			1									
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XIV	T	.	.	.	.	.	.	.	C	.	.	C	G	A	.	.	A	A	.	.	.	.	.	.	.	.	.	T	.	.		1										
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XVI	.	C	C	T	T	.	.	.	C	.	.	C	.	A	.	.	A	A	.	.	.	.	.	.	.	.	.	T	.	.								1				
XVII	.	C	C	C	T	.	.	.	C	.	.	C	.	A	.	.	A	A	.	.	.	.	.	.	.	.	.	T	.	.								1				
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XIX	.	C	C	.	.	.	.	.	C	.	.	C	.	A	.	.	A	A	.	.	.	.	.	.	.	.	.	T	.	.							1					
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XXI	.	C	C	C	T	.	.	A	C	.	.	T	.	A	.	.	A	A	.	.	.	.	.	.	.	.	.	T	.	.											1	
XXII	T	C	C	T	T	.	.	.	.	T	G	T	.	A	G	G	A	A	T	C	T	A	C	T	C	G	T	A	T	A									1			
XXIII	A	C	C	T	T	.	.	.		T	G	T	.	A	G	G	A	A	.	.	.	.	.	.	.	.	.	T	.	.									1			

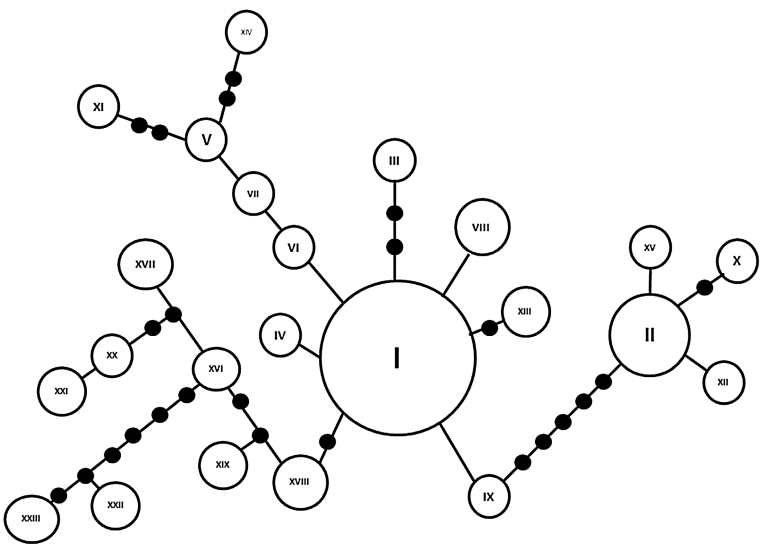

Figure 3
Parsimonious haplotype network generated from ND5 sequences of *Ceratitis capitata* populations. The ellipse size corresponds to the haplotype frequency. (●): indicates mutation steps representing hypothetical haplotypes which have not been detected among specimens analyzed.

base changes due to artifacts during experimental procedures, we considered only a fragment of 860 bases for all sequences. After deleting primer sequences, observed sequence polymorphisms were verified. Unique variants were re-amplified and re-sequenced to eliminate alterations representing PCR or sequencing artifacts. Then, using the ORF finder function from the NCBI website (set for invertebrate mitochondrial code), the sequences were checked to verify that there was no disruption of open reading frames. The complete sequence of one representative sequence is available through Genbank (accession number: FJ474404).

Haplotype analysis

The ND5 sequences alignment revealed 23 haplotypes which are encoded according to one single change per base pair position. Haplotypes as well as their distribution per country and locality are shown in Tab. 2. There are 20 unique haplotypes, 16 of them are identified in populations from the southern Mediterranean group. In fact, in Tunisia, there are 1, 2, 4, 1 unique haplotypes respectively in Bizerte, Takelsa, Kairouan and Tozeur localities. In Israel, there are two unique variants in Maagen Michael and one in Rehovot and in Morocco there are two unique variants in each locality (Sidi Abdelaziz and Sidi Slimane). Among all the specimens studied, the most variable localities are Kairouan (6 haplotypes) and Takelsa (4 haplotypes) in Tunisia, and Maagen Michael (4 haplotypes) in Israel. Among all the variants identified, haplotype II as encoded in Tab. 2 is commonly found in the southern Mediterranean basin. It is a dominant haplotype, detected in 6 out of 10 populations sampled from the Mediterranean countries (Tunisia, Morocco and Israel).

In addition to identification of a diverse array of haplotypes, the sequence based approach used here is conducive to network based analyses. As described by Posada and Crandall (2001), the analysis of networks effectively depicts a wide range of phylogenetic phenomena operating at the species and population levels. Consistent with this, the network produced here shows that within these populations essentially all of the variation present in this segment of DNA has been captured using this approach (Fig. 3). It confirms the presence of the dominant haplotype II and shows the absence of 21 mutation steps that hypothetically connect the variants. The populations from Seychelles and Hawaii Islands show only unique haplotypes.

Analysis of Molecular Variance (AMOVA)

The analysis of molecular variance AMOVA (Tab. 3) showed that the fixation index among groups (representing the 5 countries) is significant (FSC = -0.00349, $P < 0.0001$). This indicates that there is a genetic differentiation between the 5 countries considered in this study. The fixation index among populations is also significant (FST = 0.55892, $P < 0.0001$), this result highlights the genetic divergence between the 10 populations collected from different geographical localities. At the population level, the fixation index is also highly significant (FCT = 0.56045, $P < 0.0001$) and almost the same as the percentage of variation among groups.

Discussion

The analysis of mitochondrial DNA variability among medfly population specimens collected from the southern Mediterranean basin and other areas in the world is conducted to investigate the genetic structure of *C. capitata*. Given its maternal inheritance, small size, lack of recombination and presence of polymorphisms, the mtDNA proved to be a valuable tool to study the various patterns existing in many insect species such as *Drosophila* (Lewis *et al*. 1995), *Phlebotomus* (Esseghir *et al*. 1997) and *Ceratitis capitata* populations (Spanos *et al*. 2000). Initial studies on the medfly genetic diversity were based on a RFLP-PCR-based approach using the ND5-tRNA-ND4 mitochondrial locus to detect the haplotype variants.

Table 3. Analysis of Molecular Variance (AMOVA) of the studied *Ceratitis capitata* populations. Level of significance at P = 0.0001. FSC: fixation index among groups. FST: fixation index among populations within groups. FCT: fixation index within populations.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation indexes
Among groups	4	81.256	2.18440	56.04	FSC = –0.00349*
Among populations	5	8.403	–0.00597	–0.15	FST = 0.55892*
Within populations	50	85.958	1.71917	44.11	FCT = 0.56045*
Total	59	175.617	3.89759		

This method uses a set of three enzymes (EcoRV/XbaI/MnlI). The haplotype code is based on the presence (A) or absence (B) of the restriction site of these enzymes (Sheppard *et al.* 1992). For Instance, a study conducted by Gasparich *et al.* (1997), revealed the presence of one haplotype in Hawaii (BBB), seven haplotypes in Subsaharan Africa (AAA, AAB, BAB, ABA, BBA, BAA and BBB) and two haplotypes in the Mediterranean area (AAA and AAB). Recently, Barr (2009) examined the medfly pathway using a revised protocol of the previous above mentioned study and reported two more haplotypes in the Mediterranean basin (BBB and BAA). However, in our study, we relied on the use of direct sequencing to detect the polymorphic patterns in the populations considered. We detected a total of twenty three haplotypes, nineteen out of them are identified in Mediterranean populations. All the detected variants correspond to only one RFLP form (BBA) predicted with NEB cutter 2.0 program (Vincze *et al*. 2003). This form was reported in West Africa (Gaparich *et al.* 1997). It was considered controversial by Barr (2009), who suspected high failure rate for the XbaI enzyme. Overall, the number of haplotypes uncovered here confirms the high level of variability in the populations of this pest within Tunisia. This consistent with the idea that the Tunisian populations, which are "ancient" by medfly standards (Malacrida et al. 1998), are expected to be more diverse than populations recently established (Reyes and Ochando 2004). Previous studies had shown that the first major expansion of this pest into non native areas included countries of the Mediterranean basin (Malacrida et al. 1998), but quantification of the level of variability in these populations had remained elusive when populations, such as those from Tunisia, proved to be difficult to analysis using the PCR-RFLP approach (Gasparich et al. 1997). As described previously, one of the limitations of any PCR-FRLP approach is the inability to detect novel variants. Certainly, it is clear from our results that the populations from Tunisia contain a wide range of such novel forms. It can be inferred from our study that RFLP forms detected in previous studies can be considered as haplogroups that comprise multiple polymorphic variants. It is the case of the study on medfly populations in Argentina which used both approaches (RFLP and direct sequencing) and uncovered a large array of sequence haplotypes corresponding to only one RFLP form (AAB) (Lanzavecchia *et al.* 2008). Despite the small number of sequences from Morocco, 4 unique haplotypes were detected, which suggests high levels of polymorphism in this area. This is in agreement with the medfly genetic diversity study based on allozymes, which classified Morocco in the Iberian African group and considered Moroccan populations as high polymorphic and ancestral populations (Malacrida *et al.* 1998).

In conclusion, the direct analysis of mitochondrial DNA sequences from the ND5 region has uncovered a rich array of distinct haplotypes within populations of the Mediterranean fruit fly. This approach makes it possible to use sophisticated tools for the analysis of networks of intraspecific gene genealogies (Posada & Crandall 2001) without sacrificing the traditional advantages of using mitochondrial DNA (Steck et al. 1996; Spanos et al. 2000) to analyze populations of this and other pest species. Furthermore, we have shown that our approach allows for the detailed analysis of highly variable populations, such as those from countries like Tunisia, which had proven to be refractive to analysis using the PCR-RFLP method. The enhanced variability identified by the direct analysis of sequence based haplotypes will allow a range of questions relevant to pest population dynamics such as the origin of new infestations and the relationships of existing populations to be examined in greater detail than had been possible previously.

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