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Genetic Variability of the Giant Black Aphid, *Pterochloroides persicae* (*Hemiptera: Aphididae*), Based on Sequences of the Mitochondrial Cytochrome *b* Gene

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ABSTRACT

The giant black aphid *Pterochloroides persicae* (Cholodkovsky) is one of the most damaging pests of Prunaceous trees. It has been reported in North Africa since 1987. In this study, we have used sequences of the mitochondrial cytochrome *b* gene (Cyt*b*), to elaborate the first phylogenetic analysis of this polyphagous pest. Sequences were obtained from 34 Tunisian clones covering different locations and different host plants. A total of 25 haplotypes were identified, among which 21 were unique. Haplotype H7 that was the most frequent would be the ancestral one. The maximum likelihood phylogenetic tree revealed that clones originating from distinct locations clustered into a single consistent group with little variations, reflecting the loss of genetic diversity that would have followed introduction of this pest. These results will have important implications for future studies of the genetic structure of this pest and other closely-related species.

Key words: Aphididae; *Pterochloroides persicae*; phylogeny; mtDNA; invasive pest.

INTRODUCTION

The giant black aphid, *Pterochloroides persicae* (Cholodkovsky), is a widely distributed aphid pest that was recorded in several parts of the world, such as Europe (Ciampolini and Martelli, 1980), the mediterranean area (Talhouk, 1972), Asia (Blackman and Eastop, 1994), the middle East (Abdullah and Eltayeb, 1996; Rakhshani *et al.,* 2005) and North Africa (El-Tariki and El-Sharif, 1987; Darwish *et al.,* 1989). This polypahgous pest has a high prevalence during most periods of the year, on several prunaceous trees (Blackman and Eastop, 1994). In Tunisia, outbreaks of *P. persicae* occur mostly on almond (*Prunus dulcis* (Mill.) D.A.Webb), peach (*Prunus persica* (L.), Batsch), plum (*Prunus domestica* (L.)) and apricot (*Prunus armeniaca* (L.)) (Ben Halima and Ben Hamouda, 2005). Damage and yield loss occur especially as a result

of the high insect density on tree trunk and branches (Abdullah and Eltayeb, 1996).

At species level, the genetic variation inferred from mitochondrial DNA (mtDNA) is a powerful tool in studies aiming to determine species historical and phylogenetic patterns and correlate the genetic diversity with the diversification mode, host association and biogeographic origins (Avise, 1994; Kim *et al.,* 2011). For these purposes, mtDNA variation has proven more efficient than nuclear DNA markers, owing to its haploid and monoparental (maternal) mode of inheritance and the absence of recombination (Brown *et al*., 1979; Reyes and Ochando, 2004). Mitochodrial DNA markers have been also widely used for infra-specific phylogeny at several taxonomic levels (Da Silva and Patton, 1993; Numm and Stanley, 1998; Eni *et al.,* 2001; Guryev *et al.,* 2001; Mezghani *et al.,* 2012), especially in several aphid genera, such as *Aphis* (Coeur D'acier *et al.,* 2007; Foottit, 2009), *Brachycaudus* (Coeur D'acier *et al.,* 2008), *Ropalosiphum* (Martinez-Torres *et al.,* 1997) and *Schizaphis* (Kharrat *et al.,* 2012).

In Tunisia, the major method of controlling the occurrence and outbreaks of *P. persicae* has been the application of pesticides, which adds environmental concerns to the already heavy financial impact caused by this pest. Although the biology and ecology of this aphid has been quite well studied, no information is available on the genetic diversity and population structure, which would facilitate its control. In the present study, we have used the mitochondrial cytochrome *b* gene (Cyt *b*), which has been already successfully applied to reveal sequence divergence in aphids (Coeur D'acier *et al.,* 2008; Kim *et al.,* 2011). The final aim was to provide a first report on the phylogeny and adaptation mode of this pest.

MATERIAL AND METHODS

Insect sampling

Thirty four (34) clones of *P. persicae* were used to investigate the impact of the geographical location and host association on the pest genotypes. These clones were sampled from four host plants and eight regions in Tunisia: Ariana (N 36.52.23, E 10.12.31), Béja (N 36.43.22, E 9.11.30), Grombalia (N 36.35.56, E 10.29.59) and Hammamet (N 36.24.0, E 10.36.51) in the northern area; Kasserine (N 35.10.8, E 8.49.43), Sousse (N 35.49.43, E 10.38.26), Monastir (N 35.46.27, E 10.49.30) and Sfax (N 34.43.4, E 10.44.9) in the central area. All samples were collected between June and July 2010 and preserved in 96% ethanol at -20°C (Table 1).

DNA isolation, amplification and sequencing

Total genomic DNA was isolated from each specimen according to the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1987). DNA samples were then used as templates for PCR amplification, using primers CP1 (5'-GAT GAT GAA ATT TTG GAT C-3') and CP2 (5'-CTA ATG CAA TAA CTC CTC C-3') (Harry *et al.,* 1998).

In order to amplify the Cytb gene from each insect individually, the PCR reaction mix consisted of 50 ng of DNA, 0.1 unit of Taq polymerase (Promega), 200µM $MgCl_2$,

0.1 mM of each primer and 0.2mM dNTPs. Amplification was performed in a 2720 thermal cycler (Applied Biosystems), programmed as follows: an initial denaturation at 94°C for 3 min, followed by 35 cycles of 30s at 94°C, 1min at 50°C and 1min at 72°C. A final extension step was run for 10min at 72°C. PCR products were visualized by electrophoresis on 1% agarose gel and stained with ethidium bromide. The appropriate bands were purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega), following the manufacturer's protocol; and sequenced by an ABI-373 automated DNA sequencing system. Sequences were manually edited using BioEdit version 7.0.5.3 software (Hall, 1999).

Table 1. *Pterochloroides pesrsicae* sampling data.

Clone label	Haplotype	Department	Host plant	GenBank accession	Date of collection
AbN1	H5	Ariana	*Prunus armeniaca*	KC462038	25/04/10
AbN2	H6	Ariana	*Prunus armeniaca*	KC462039	
AbN3	H7	Ariana	*Prunus armeniaca*	KC462040	
AbN4	H24	Ariana	*Prunus armeniaca*	JN996496	
AmN1	H20	Beja	*Prunus dulcis*	JN996490	10/05/2010
AmN2	H21	Grombalia	*Prunus dulcis*	JN996491	13/05/2010
AmN3	H22	Hammamet	*Prunus dulcis*	JN996492	14/05/2010
AmC1	H13	Kasserine	*Prunus dulcis*	KC462046	16/04/10
AmC2	H7	Kasserine	*Prunus dulcis*	KC462047	
AmC3	H7	Kasserine	*Prunus dulcis*	KC462048	
AmC4	H14	Sousse	*Prunus dulcis*	KC462049	
AmC5	H8	Sousse	*Prunus dulcis*	KC462050	
AmC6	H23	Monastir	*Prunus dulcis*	JN996493	
AmS1	H7	Sfax	*Prunus dulcis*	KC462056	13/05/10
AmS2	H17	Sfax	*Prunus dulcis*	KC462057	
AmS3	H18	Sfax	*Prunus dulcis*	KC462058	
AmS4	H22	Sfax	*Prunus dulcis*	JN996494	

Table 1. *Pterochloroides pesrsicae* sampling data. (Continued)

Clone label	Haplotype	Department	Host plant	GenBank accession	Date of collection
PeN1	H8	Ariana	*Prunus persica*	KC462041	01/05/10
PeN2	H9	Ariana	*Prunus persica*	KC462042	
PeN3	H10	Ariana	*Prunus persica*	KC462043	
PeN4	H11	Ariana	*Prunus persica*	KC462044	
PeN5	H12	Ariana	*Prunus persica*	KC462045	
PeN6	H19	Ariana	*Prunus persica*	JN996497	
PeC1	H15	Kasserine	*Prunus persica*	KC462051	16/04/2010
PeC2	H16	Kasserine	*Prunus persica*	KC462052	
PeC3	H4	Kasserine	*Prunus persica*	KC462053	
PeC4	H7	Kasserine	*Prunus persica*	KC462054	
PeC5	H7	Kasserine	*Prunus persica*	KC462055	
PeC6	H7	Kasserine	*Prunus persica*	JN996495	
PrN1	H1	Ariana	*Prunus domestica*	KC462034	01/05/2010
PrN2	H2	Ariana	*Prunus domestica*	KC462035	
PrN3	H3	Ariana	*Prunus domestica*	KC462036	
PrN4	H4	Ariana	*Prunus domestica*	KC462037	
PrN5	H25	Ariana	*Prunus domestica*	JN996497	

Computer processing of nucleotide sequences

The Blastn algorithm (Altschul *et al.,* 1997) was used to search for homologous *P. persicae* sequences in the NCBI database (http://ncbi.nlm.nih.gov/). Multiple nucleotide sequence alignments were performed using ClustalW (Thompson *et al.,* 1994; http://www.ebi.ac.uk/clustalw/) with default settings. Phylogenetic analyses were conducted using MEGA v.5.1 Beta3 (Tamura *et al.,* 2011). The genetic distance among accessions was calculated using the Kimura-Two-Parameter (K2P) distance model (Kimura 1980). Phylogenetic analyses were conducted using the maximum likelihood method (Sneath and Sokal, 1973). The consensus tree was evaluated from 500 bootstrap replications. Newly determined sequences, listed in Table 1, were deposited in NCBI Genbank database (http://ncbi.nlm.nih.gov/). Sequences from NCBI database corresponding to partial sequences of Cyt*b* gene from different

aphid species and clones were used for comparison. Using the program TCS 1.21 (Clement *et al.,* 2000), a network of the *Cyt*b haplotypes was generated with respect to their frequencies (number of individuals / haplotype). A Kolmogorov-Smirnov plot showing the percentage of identity between samples was constructed, using GeneDoc (Nicholas and Nicholas, 1997; http://www.nrbsc.org/gfx/genedoc/).

RESULTS

Assessment of the intra-clonal sequence variation

A preliminary analysis was carried out, in order to determine the level of sequence variation in the mitochondrial Cytb gene fragment from *P. persicae.* In this analysis, 4 to 6 individuals representing each population/clone were analyzed. This preliminary analysis revealed a high within-population conservation of the Cytb sequences. Consequently, it was decided to study only a limited number (4 to 6) individuals from each population.

Sequence alignment, blast searches and phylogenetic analysis

PCR amplification, using CP1 and CP2 primers with 34 aphid DNA samples, revealed a PCR fragment of about 810 bp, which was sequenced. A partial 690 bp sequence was uniformly cropped from all sequences and used for the alignment with MEGA v.5.1 Beta3. The highest pairwise distance of all sequences using the K2P method was 0.022 between clones AbN4 and AmC4. Sequences had 20 variable and 17 phylogenetically informative sites; and the average nucleotide composition was T: 42.65%, C: 12.02%, A: 36.67%, G: 8.66%. Such a high A+T content (79.32%) was expected, as it represents a general feature of the Cytb region in arthropods as it was reported in similar studies on insect taxa (Simmons and Weller, 2001). Our Cytb sequences showed 92% identity with *Nippolachnus piri* Matsumura (Gb accession JX035597.1), 90% identity with *Eulachnus nigricola* Pašek (JX035544.1), 91% with *Cinara tujafilina* Del Guercio (Gb accession JX035715.1), 89% with *Aphis spiraecola* Patch (Gb accession GU205364.1), 88% with *Aphis gossypii* Glover (Gb accession AM085356.1), and 89% with *Hyalopterus pruni* Geoffroy (Gb accession GU457815.1) in the NCBI databases.

All these insect species belonged to the *Hemiptera* order and, thus, were used for the construction of the phylogenetic tree (Fig. 1). The phylogenetic tree inferred from Cytb sequences was in agreement with the sequence analysis, as all *P. persicae* populations clustered together in a single group, with no clustering associated with the geographic origin or habitat.

Haplotype analysis

The network of the Cytb haplotypes, generated by TCS 1.2 program, revealed 25 distinct haplotypes, which were encoded according to a single change per nucleotide position. There were 21 unique haplotypes ; among which only four (H4, H7, H8 and H22) were detected in more than a single location (Table 2). Three groups of haplotypes

(H4, H9), (H7, H22, H23) and (H8, H12, H13, H15) were directly connected within the network; which can be explained by a single base substitution between haplotypes of each group. Moreover, Haplotype H7 was characterized as the most frequent one (Fig. 2). Kolmogorov-Smirnov plotting revealed a high homogeneity within the studied 34 accessions of *P. persicae* collected over an extensive area (Fig. 3).

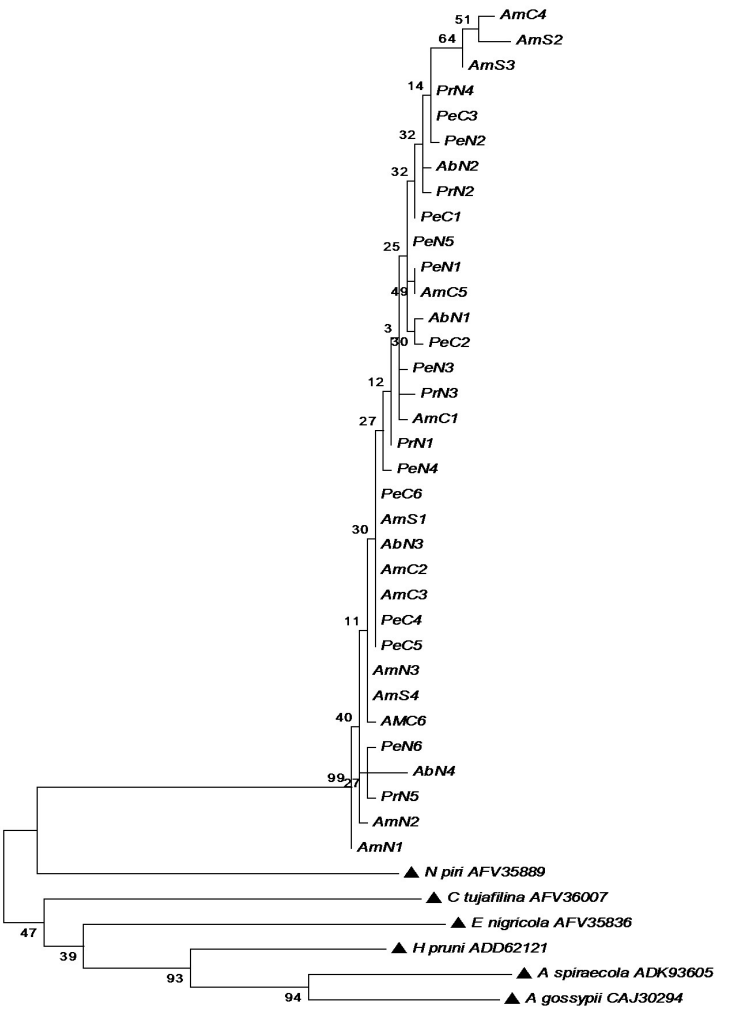

Fig. 1. Maximum likelihood phylogenetic inference, from a 690 bp fragment of *Pteochloroides persicae* Cytb gene, showing the clustering of Tunisian haplotypes in a single group. ▲: Cytb sequences from *Hemiptera* taxa, used as reference: *N. piri* (Gb accession AFV35889), *E. nigricola* (AFV35836), *C. tujafilina* (AFV36007), *A. spiraecola* (ADK93605), *A. gossypii* (CAJ30294); *H. pruni* (ADD62121).

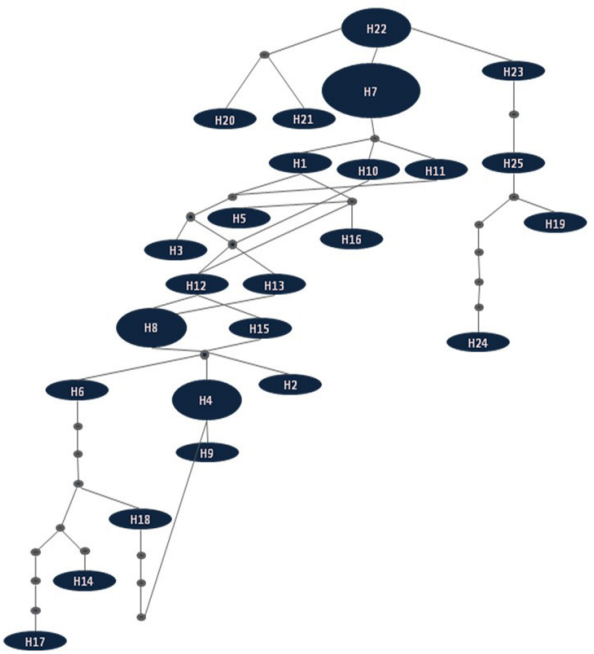

Fig. 2. Haplotype network generated from the analysis of a partial Cytb region of the mitochondrial DNA. The size of each ellipse shows the relative frequency of each haplotype among all the individuals sampled. The filled circles represent forms inferred but not detected among the haplotype samples.

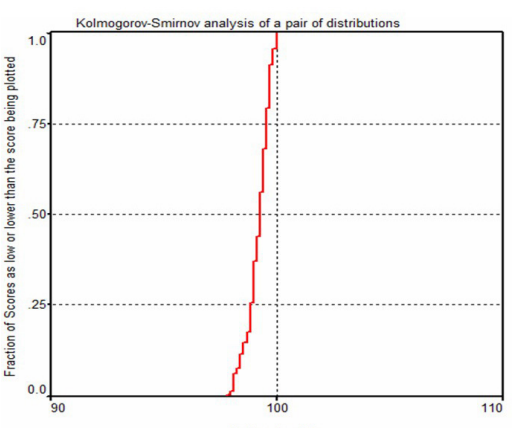

Fig. 3. Kolmogorov-Smirnov plot showing the percentage of identity between samples inferred from 34 DNA sequences of mitochondrial Cytochrome b gene.

Table 2. Haplotype distribution among clones of *Pterochloroides pesrsicae* analyzed.

	Nucleotide position																					North				Centre			
	54	59	107	237	365	379	420	421	445	487	495	512	531	583	602	632	660	663	671	686	Beja	Grombalia	Hammamet	Ariana	Sousse	Kasserine	Monastir	Sfax
H1	C	T	T	A	T	G	G	C	T	G	A	A	G	T	T	T	G	T	T	T				1				
H2	.	.	A	.	.	A	.	.	.	A	.	.	A	.	.	.	A	.	.	.				1				
H3	.	.	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	G	.	A				1				
H4	.	.	T	.	.	A	T	.	.	A	.	.	A	.	.	.	A	.	.	.				1		1		
H5	.	.	T	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A				1				
H6	.	.	T	.	.	A	.	.	.	A	.	.	A	A	.	.	A	.	.	.				1				
H7	.	.	T	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.				1	2	2	1	1
H8	.	.	T	.	.	A	.	.	.	A	.	.	.	.	.	.	A	.	.	.				1	1			
H9	.	.	A	.	.	A	T	.	.	A	.	.	A	.	.	.	A	.	.	.				1				
H10	.	.	T	.	.	.	.	.	.	A	G	.	.	.	.	.	.	.	.	.				1				
H11	.	.	T	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A				1				
H12	.	.	T	.	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.				1				
H13	.	.	T	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	.						1		
H14	.	.	A	.	G	A	T	A	.	A	.	.	A	.	.	A	A	G	.	A					1			
H15	.	.	T	.	.	A	.	.	.	A	.	.	A	.	.	.	.	.	.	.						1		
H16	.	.	T	.	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.						1		
H17	.	.	A	G	.	A	.	A	G	A	.	.	A	A	.	A	A	G	C	A								1
H18	.	.	T	.	.	A	T	.	.	A	.	.	A	A	.	A	A	G	.	A								1
H19	A	C	T	G	.	.	.	.	.	A	G	.	.	.	.	.	.	G	.	.				1				
H20	A	.	A	G	.	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.	1							
H21	A	.	T	G	G	.	.	.	.	.	G	.	.	.	.	.	.	G	.	.		1						
H22	A	.	T	G	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.			1					1
H23	A	.	T	G	.	.	.	.	.	.	G	.	.	.	.	.	A	.	.	.							1	
H24	A	.	T	G	.	.	.	.	G	A	G	C	.	.	G	.	.	G	C	A				1				
H25	A	.	T	G	.	.	.	.	.	A	G	.	.	.	.	.	A	G	.	.				1				
Total																					1	1	1	14	2	9	1	4

Conclusion and Discussion

The giant black aphid has been reported in Tunisia since 1987 and considered as one of the most damaging pests of Prunaceous trees (El Tariki and El Sharif, 1987). Since the arrival of this invasive pest in Tunisia, it has expanded dramatically through geographical areas, where Prunaceous trees are cultivated, causing important economic losses to the hosts. Herein, the genetic variability of this pest was analyzed, based on the sequencing of the Cytb gene, using 34 clones from different populations of *P. persicae,* collected over an extensive area. Twenty five (25) haplotypes of Cytb including one predominant haplotype (H7) were identified. The existence of such a rich array of distinct haplotypes of *P. persicae* was not accompanied by a phylogenetic distinction between clones. This fact was expected, as haplotypes differ only by punctual substitutions. Indeed, the maximum likelihood phylogenetic tree indicated that populations from distinct geographic origins clustered into a single consistent group with little variations. This result is in agreement with those of Cifuentes *et al.* (2011) and Flores *et al*. (2003), who observed a high genetic homogeneity when analyzing the genetic structure of another invasive pest, *T. absoluta*, based on molecular polymorphisms. Similar results have also been reported on *Oryctes agamemnon*, an invasive pest of Palm trees in Tunisia (Abdallah *et al.,* 2012). The lack of genetic heterogeneity in invasive pests could be attributed to the fact that introduced species frequently undergo selection pressures that abolish most, if not all, of their genetic variability. Introduced populations often are also genetically less variable than the original population from which they derive, because they frequently contain only a subset of the genetic diversity present in the native population (Chakraborty and Nei, 1977). Although such reductions of the genetic diversity are generally believed to be harmful, they occasionally contribute to the success of some invasive species, as it was reported in the aphid *S. avenae* (Figueroa *et al.,* 2005).

In our study, the Cytochrome b marker was useful for monitoring the differentiation of *P. persicae* new haplotypes in Tunisia. Nevertheless, a more complete knowledge of the dispersal mode, genetic phylo-geographical structure and insecticide resistance mechanisms of *P. persicae* are still required for developing effective management strategies. For this purpose, additional genetic markers, such as microsatellites, could be used in future studies that would reveal higher amounts of genetic heterogeneity.

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