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# The Greenbug, *Schizaphis graminum* Rondani (Hemiptera: Aphididae), in Tunisia: Mitochondrial DNA Divergence and Haplotype Inference

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## ABSTRACT

The greenbug, *Schizaphis graminum* (Rondani), is a major pest of wheat worldwide. Based on host plant response to infestation, several biotypes of this pest have been identified and their phylogenetic relationships inferred using the mitochondrial COI gene. In this study, we have sequenced a fragment of 572 bp of COI gene from 10 *S. graminum* clones collected, over a three-year period, from 4 sites located in the north and south of Tunisia. Alignment of these sequences revealed the presence of 3 distinct haplotypes, that clustered with the agricultural biotypes I, J, E, K and C. Therefore, we suggest that resistance genes *Gb3*, *Gb4*, *Gb5* and *Gb6* in wheat should be the first choice for use in wheat improvement programs, for resistance against greenbug, in Tunisia.

Key words: *Schizaphis graminum*, haplotype, Cytochrome Oxidase subunit I (COI), gene sequencing, phylogenetics.

## INTRODUCTION

*Schizaphis graminum* Rondani, commonly known as the green aphid or greenbug, is a worldwide distributed pest of cereal crops. It causes severe injuries to host plants in all growth stages and often kills the entire cereal plant. It is prejudicial to the host, either due to the large quantity of sap it extracts, causing water and nutrients depletion (Cruz *et al.*, 1998) or due to vectoring Barley/Cereal Yellow Dwarf Virus (B/CYDV,

Gray *et al.*, 2007), Maize Mosaic Dwarf Virus (MMDV, Nault and Bradley, 1969) or Sugarcane Mosaic Virus (SCMV, Ingram and Summers, 1938).

*S. graminum* was first recorded on wheat in Argentina in 1889 (Arriaga, 1954). Up to date, over 20 greenbug biotypes have been recognized (Burd and Porter, 2006). All these biotypes (except biotype D, which has been identified on the basis of insecticide resistance) have been characterized, based on the preference for a host plant species and/or ability to damage specific cultivars of a defined species (Porter *et al.*, 1997; Nussley *et al.*, 2008). It was reported that there was no correlation between the emergence of *S. graminum* biotypes and dates of introduction of resistant varieties, suggesting that the biotypic divergence in greenbug populations does not result from selection exerted by resistance genes in the host plant. Shufran *et al*. (2000) compared mitochondrial DNA sequences from the available northern American biotypes and demonstrated that they cluster into 3 distinct clades; the first consisting of the so called “agricultural biotypes” (C, E, K, I and J), usually infesting wheat and sorghum; the second included biotypes F, G and NY; and the third comprised biotypes B, EUR and CWR. Biotypes of the two last clades are usually encountered on non cultivated hosts. In view of this, *S. graminum* biotypes could be characterized by mtDNA haplotypes and host nature (cultivated / not cultivated).

In Tunisia, *S. graminum* is a major pest of cereal crops (Boukhris-Bouhachem *et al.*, 2007). As the occurrence of greenbug biotypes in Tunisia is not well documented, we aimed in the present study to examine mtDNA nucleotide sequence divergence in Tunisian greenbug clones and estimate their relationship with reported biotypes.

## MATERIAL AND METHODS

### Insect Material

Ten greenbug clones were collected on wheat and barley from different sites in Tunisia, during spring 2009, 2010 and 2011. To avoid the effect of the previous host (Shufran *et al.*, 1992), these clones were reared on the susceptible barley cultivar “Custer’’ for three successive generations in a growth cabinet at 22°+/- 5°C, 50% relative humidity and 16h light: 8h dark photoperiod. Data on the sampling procedure and geographic location of the studied sites are given in table 1.

Table 1. Sampling data for *Schizaphis graminum*.

| Clone label | Host plant | Site | Geographic location in Tunisia | Date of collection |
|---|---|---|---|---|
| Sg1, Sg2 | Wheat | Jédeida (36° 51’ 13. 7’’ N, 9° 55’ 42.2’’ E) | North | March, 2009 |
| Sg3 | Wheat | Béja (36° 43’ 42.3’’ N, 9° 11’ 32.3’’ E) | North | March, 2009 |
| Sg4, Sg5 | Barley | Nefta (33° 52’ 42’’ N, 7° 53’ 1.4’’) | South | March, 2009 |
| Sg6, Sg7, Sg8, Sg9 | Barley | Ghannouch (33° 56’ 58.7’’ N, 10° 4’ 0.9’’ E) | South | March, 2010 |
| Sg10 | Barley | Ghannouch (33° 56’ 58.7’’ N, 10° 4’ 0.9’’ E) | South | March, 2011 |

**DNA Extraction, PCR and COI Gene Sequencing**

DNA was extracted from individual greenbugs belonging to each clone, using a CTAB ''hexadecyl-trimethylammonium bromide'' method (Doyle and Doyle, 1987). A 572 bp segment of the cytochrome oxidase subunit 1 (COI) gene was amplified from each insect individual, using a couple of original primers (SgF: 5'-ACCTGGATTTGGTT-TAATTTC-3'; SgR: 5'-GTTGAAATTATTGATCCAATTG-3') that we have designed, based on public nucleotide sequences (www.ncbi.nlm.nih.gov, accession numbers AF285916 and AF220511-AF220523) of cytochrome oxidase subunit I (COI) gene from different *S. graminum* clones and biotypes (Shufran *et al.*, 2000; Anstead *et al.*, 2002). The PCR reaction mix consisted of 50 ng of DNA, 0.1 unit of Taq polymerase (Promega), 200µM MgCl2, 0.1 mM of each primer and 0.2 mM dNTPs. Amplification was performed in a 2720 thermal cycler (Applied Biosystems), programmed as follows: an initial denaturation at 94°C for 5 minutes followed by 35 cycles each consisting of 3 steps: a denaturation (94°C, 1min), hybridization (48°C, 1 min) and elongation (72°C, 1 min); and a final extension at 72°C for 5 min. PCR products were visualized by electrophoresis on 1% low melting point agarose gel after staining with ethidium bromide. The appropriate bands were purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega) and sequenced by an ABI-373 automated DNA sequencing System using SgF and SgR primers.

**Nucleotide Sequence Computer Processing**

Nucleotide sequence alignments were performed using the software package ClustalX version 2.00 (Larkin *et al.*, 2007). Fourteen public sequences (www.ncbi.nlm.nih.gov, accession numbers AF285916 and AF220511-AF220523), corresponding to partial sequences of cytochrome oxidase subunit I (COI) gene from different *S. graminum* clones and biotypes (Shufran *et al.*, 2000; Anstead *et al.*, 2002), were used for comparison. Genetic distances were estimated by the maximum composite likelihood model (Tamura *et al.*, 2004). Phylogenetic analyses were conducted using maximum likelihood method (Tamura and Nei, 1993), neighbor joining method (NJ) (Saitou and Nei, 1987) and maximum parsimony method (Nei and Kumar, 2000). A bootstrap resampling was applied to assess the support for individual nodes using 1000 pseudo-replicates. All genetic distances and phylogenetic analyses were conducted using MEGA 4.0 software (Tamura *et al.,* 2007). Newly determined sequences were deposited in GenBank database (www.ncbi.nlm.nih.gov) under accession numbers: HQ848297-HQ848306.

## RESULTS AND DISCUSSION

Following sequence checking and editing, the total length of DNA sequence used from each individual was 572 bp for all samples. Three distinct forms were identified and given haplotype designations. These haplotypes were identified based on two nucleotide polymorphisms in positions 471 and 528. The most common haplotype found in clones from all sites in the north and the south of Tunisia was designated

TUN3. In addition, two rare haplotypes were detected: TUN1 found in clone Sg2 from Jédeida, and TUN2 found in clone Sg9 from Ghannouch (Table 2). Genetic distances between haplotypes were 0.0018 (between TUN1 and TUN2), 0.018 (between TUN1 and TUN3) and 0.0035 (between TUN2 and TUN3). Aphid clones belonging to the same site did not always share the same mitochondrial haplotype. Indeed, two haplotypes (TUN1 and TUN2) were identified in clones from Ghannouch and two (TUN1 and TUN3) in clones from Jédeida.

Table 2. Haplotype distribution among 10 clones of *S. graminum* analyzed.

| Haplotype | Polymorphism (bp) | | Haplotype distribution | | | |
|---|---|---|---|---|---|---|
| | 471 | 528 | North | | South | |
| | | | Jédeida | Béja | Ghannouch | Nefta |
| TUN1 | T | T | 1 | - | - | - |
| TUN2 | C | C | - | - | 1 | - |
| TUN3 | C | T | 1 | 1 | 4 | 2 |

Sequence alignment produced the same phylogenetic topology reported in Shufran *et al.* (2000), although the aligned sequences were shorter here (572 bp). Maximum likelihood, maximum parsimony and neighbor-joining phylogenies generated congruent cladograms, differing only in the branching order of biotypes. Tunisian greenbug clones clustered with agricultural biotypes, I, J, E, K and C (Fig.1).

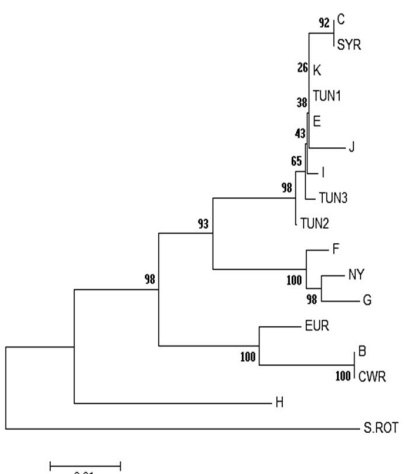


Fig. 1. Neighbor-Joining homology tree produced from nucleotide sequences from a 572 bp fragment of the COI gene of *S. graminum*, showing clustering of Tunisian haplotypes.

*SYR: S. graminum isolate from Syria (Anstead et al., 2002); C, E, K, I, J: Agricultural biotypes (Shufran et al., 2000); F, G and NY, B, EUR, CWR: Biotypes of non cultivated hosts (Shufran et al., 2000); TUN1, TUN2, TUN3: Tunisian COI haplotypes; S.ROT: Subclytia rotundiventris (Diptera: Tachinidae) used as root; The percentages of replications supporting each branch are shown.*

Genotype characterization and/or biotype identification of pests is the first step for any cereal-breeding program to obtain resistant cultivars. Based on the COI

sequences, our study has shown that Tunisian greenbug haplotypes belonged to clade 1 which includes biotypes avirulent towards wheat genes *Gb3*, *Gb4*, *Gb5* and *Gb6*, conferring resistance to the greenbug (Porter *et al.*, 1997; Nuessly *et al.*, 2008). In spite of the limited sampling in this study, results might be already of interest in foreseeing the possibility to implement a Tunisian cereal breeding program against *S. graminum*, taking into account the identity of local biotypes of this pest, in order to deploy efficient resistance genes. Ideally, a more extensive survey, incorporating additional molecular markers, should be carried out to better explore the relationships between insect genotypes. In this context, mitochondrial genes such as the cytochrome b (cytb) and NADH dehydrogenase (ND) ones have already been used successfully to detect DNA polymorphisms in greenbug (Aikhionbare and Mayo, 2000).

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