

# A morphological and molecular characterization of vine mealybug populations (Hemiptera, Pseudococcidae) from Tunisia

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## Abstract

Some vine mealybug, *Planococcus ficus* (Signoret) populations in Tunisian vineyards have been morphologically and genetically characterized. The morphological examination was based on the main distinctive characteristics of species of *Planococcus*, namely the number and distribution of the multilocular disc pores and tubular ducts on the adult female. This showed the existence of two different vine mealybug populations in Tunisia. Likewise, in the molecular analyses, two separate clades were revealed in the neighbour-joining phylogenetic tree, supporting the morphological studies and suggesting that there are two distinct populations of *P. ficus* on grapevine in Tunisia.

## Introduction

The Coccoidea (Hemiptera: Sternorrhyncha) contains nearly 8000 species of plant-feeding scale insects in up to 32 families (Gullan and

Cook, 2007). The most common families are those with the most species, namely the *Diaspididae*, *Pseudococcidae* and *Coccidae* (Kondo *et al.*, 2008). *Pseudococcidae* (mealybugs) is the second most species-rich family with 2291 species described (Ben-Dov *et al.*, 2011). It is found worldwide and is considered the most economically important scale insect group infesting grapevine (*Vitis vinifera* L.) (Dalla Montà *et al.*, 2001; Daane *et al.*, 2006; Walton *et al.*, 2009; Charles *et al.*, 2010). More specifically, *Planococcus ficus* (Signoret) is considered to be the most serious mealybug threat to grapevine in many grape-growing areas around the world, *e.g.* Italy, Iran, California (USA), Argentina and South Africa (Trjapitzin and Trjapitzin, 1999; Dalla Montà *et al.*, 2001; Daane *et al.*, 2006; Moghaddam, 2006; Walton *et al.*, 2009). Due to its phloem-feeding habit, *P. ficus* eliminates honeydew, which supports the growth of sooty mold fungi on leaf and fruit surfaces, thus inhibiting photosynthesis. Furthermore, *P. ficus* has been shown to transmit grapevine viruses (Mahfoudhi *et al.*, 2009; Tsai *et al.*, 2010).

Mealybugs are often impossible to identify in the field and have, therefore, been preserved in alcohol and then mounted on microscope slides for identification (Williams and Watson, 1988). Some species are morphologically very similar (*e.g.* *P. ficus* and *P. citri*, Risso) and identification using morphological characteristics can be difficult and/or inaccurate. For these reasons, molecular studies have recently been introduced to better discriminate and characterize some closely related mealybug species (Cavalieri *et al.*, 2008; Hardy *et al.*, 2008; Malausa *et al.*, 2011). Such new molecular tools can complement and confirm the morphological studies.

In Tunisia, *P. ficus* and *P. citri* have been shown to coexist on grapevines within the same vineyard (Mansour *et al.*, 2009) and *P. ficus* is now considered to be the most common mealybug species in vineyards in Tunisia (Mansour *et al.*, 2011). However, there is still no published information on combined morphological and molecular studies of vine mealybug populations from Tunisia. In this context, the present study was undertaken to characterize some vine mealybug populations to be found in Tunisia to verify whether morphologically and genetically different populations of this insect occurring in some Tunisian grape-growing areas.

## Materials and methods

### Sampling of mealybugs

Sampling was carried out during the summer 2009 in 11 table-grape vineyards belonging to 8 grape growing sites (Table 1). Most of the vineyards investigated were planted with the variety *Muscat of Italy*, a variety that is pergola trained and drip irrigated. Adult females were collected mainly from vine trunks and then preserved

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in small vials containing 70% ethanol until morphological or molecular characterization was carried out.

### Morphological characterization

The mealybugs were prepared on microscope slides according to the method described by Williams and Watson (1988). For each sample vineyard, 20 slide mounted specimens (20 replicates) were selected for morphological characterization. The identification of mealybug species was performed using the keys of Tranfaglia and Tremblay (1982), Cox (1989) and Williams and Granara de Willink (1992). The main characteristics studied were those for discriminating between species in the genus *Planococcus* Ferris (Tranfaglia and Tremblay, 1982; Cox, 1989; Williams and Granara de Willink, 1992): the total number of multilocular disc pores on pro-, meso- and metathorax, and the number of tubular ducts on the head and near cerarii VIII (counting from the head). Thus, for each site, the population was characterized by the mean (n=20) of each of the five characteristics mentioned above in order to highlight any morphological differences between mealybug populations belonging to different grape-growing sites. To compare the same characteristic between the different sites, data were analyzed using one-way ANOVA and the means separated using the Least Significant Difference (LSD)-test at P=0.05. All statistical analyses were performed using Statistica 7.0 (StatSoft Inc., 2004).

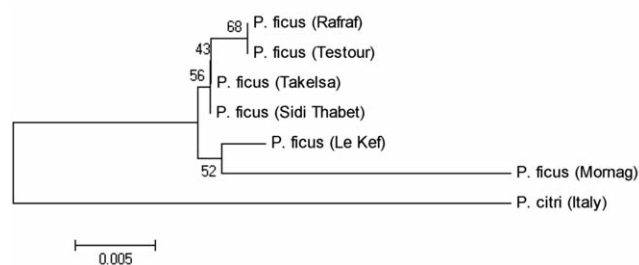
### Molecular characterization

Single mealybug individuals were subjected to total DNA extraction using Chelex 100 resin (BioRad), following the protocol described by Walsh *et al.* (1991) and modified by De Barro and Driver (1997). Each sample was homogenized in 40  $\mu$ L Chelex 5% and then incubated at 54°C for 15 min and at 98.8°C for 7 min, before being cooled at -20°C for 5 min and then centrifuged at 13,200 rpm for 10 min. The supernatant containing DNA was recovered and was stored at -20°C to be used for polymerase chain reaction (PCR) assay.

The relationship between different populations of *P. ficus* was investigated by amplification and sequencing of Ribosomal ITS1 region (Internal Transcribed Spacer 1). DNA amplification was conducted using TW81 forward primer: 5'-GTTTCCGTAGGTGAACCTGC-3' (Brust *et al.*, 1998) and reverse primer 5.8R 5'-ATCCGCGAGCCGAGT-GATCC-3' (De Barro *et al.*, 2000). PCR reactions were conducted in 21  $\mu$ L volumes with 8.5  $\mu$ L buffer premix 2x F (FailSafe™ PCR Premix Selection Kit, Epicentre Technologies), 1  $\mu$ L of each primer (10  $\mu$ M), 0.5  $\mu$ L Taq polymerase (Invitrogen) and 2  $\mu$ L DNA template. The cycle parameters were: 94°C for 5 min, then 30 cycles at 94°C for 1 min, 52°C for 1 min, 72°C for 1.5 min. PCR products were run in 1.6% agarose gel and highlighted with SYBR® Safe DNA gel stain (Invitrogen). PCR products of approximately 500 bp were sequenced by BMR Genomics sequencing service (Padua, Italy) and analyzed through neighbor-joining (Saitou and Nei, 1987) and Tamura-Nei distances as implemented in MEGA5 (Tamura *et al.*, 2011). The citrus mealybug *P. citri* was used as the outgroup.

## Results and discussion

Statistical analyses did not reveal any differences between vine mealybug populations in either the number of tubular ducts on head (F5, 114=1.22; P=0.3) or near cerarii VIII (F5, 114=1.95; P=0.08) (Table 2). By contrast, significant differences were found in the total number of multilocular disc pores on the prothorax (F5, 114=4.26; P=0.01), on the mesothorax (F5, 114 = 5.41; P=0.0001) and on the metathorax (F5, 114=10.74; P<0.0001). Overall, the number of multilocular disc pores on *P. ficus* was significantly lower in populations



**Figure 1. Molecular characterization. Neighbour-joining phylogenetic tree of vine mealybug *Planococcus ficus* populations from six vine areas in Tunisia. *P. citri*, was used as the outgroup.**

**Table 1. Tunisian table-grape growing areas sampled (summer 2009).**

Grape-growing site	Geographic coordinates	N vineyards	Sampling date
Mornag	36°41'7.04"N, 10°17'17.01"E	3	16/6; 2/7; 28/8
Takelsa	36°47'24"N, 10°37'48"E	1	27/6
Le Kef	36°10'45.38"N, 8°42'50.9"E	1	30/6
Sidi Thabet	36°54'50"N, 10°2'10"E	1	22/7
Rafrat	37°11'50.53"N, 10°10'43.43"E	2	7/7
Testour	36°33'17.38"N, 9°26'41.49"E	1	11/6
Bousalem	36°36'0"N, 8°41'60"E	1	11/6

**Table 2. Mean number of tubular ducts on adult female vine mealybugs (n = 20) from six vine growing areas in Tunisia.**

Grape-growing site	Mean n. ( $\pm$ SE) of tubular ducts on the head	Mean n. ( $\pm$ SE) of tubular ducts near cerarii VIII
Le Kef (NW)	0.15 $\pm$ 0.08	1.5 $\pm$ 0.32
Mornag (NE)	0.2 $\pm$ 0.09	0.75 $\pm$ 0.26
Takelsa (NE)	0.15 $\pm$ 0.08	0.8 $\pm$ 0.22
Testour (NW)	0.3 $\pm$ 0.10	1.7 $\pm$ 0.40
Rafrat (EN)	0.45 $\pm$ 0.20	1.45 $\pm$ 0.28
Bousalem (NW)	0.1 $\pm$ 0.07	0.85 $\pm$ 0.27

Within columns, there is no significant difference in the means at 5% level of significance (P>0.05). NW, north-west Tunisia; NE, north-east Tunisia; EN, extreme north of Tunisia. SE, standard error.

**Table 3. Mean number of multilocular disc pores on adult female vine mealybugs (n=20) from six vine growing areas in Tunisia.**

Grape-growing site	Mean n. ( $\pm$ SE) of multilocular disc pores on prothorax	Mean n. ( $\pm$ SE) of multilocular disc pores on mesothorax	Mean n. ( $\pm$ SE) of multilocular disc pores on metathorax
Le Kef (NW)	4.5 $\pm$ 0.55 <sup>a</sup>	0.9 $\pm$ 0.23 <sup>a</sup>	1.95 $\pm$ 0.37 <sup>a</sup>
Mornag (NE)	5.05 $\pm$ 0.89 <sup>ab</sup>	1.8 $\pm$ 0.37 <sup>ab</sup>	3.7 $\pm$ 0.67 <sup>a</sup>
Takelsa (NE)	6.75 $\pm$ 0.59 <sup>bc</sup>	4.5 $\pm$ 0.83 <sup>d</sup>	6.7 $\pm$ 0.7 <sup>b</sup>
Testour (NW)	7 $\pm$ 0.68 <sup>c</sup>	3.4 $\pm$ 0.61 <sup>cd</sup>	8.15 $\pm$ 0.94 <sup>b</sup>
Rafrat (EN)	7.85 $\pm$ 0.65 <sup>c</sup>	2.9 $\pm$ 0.49 <sup>bc</sup>	7 $\pm$ 0.59 <sup>b</sup>
Bousalem (NW)	7.9 $\pm$ 0.71 <sup>c</sup>	2.7 $\pm$ 0.45 <sup>bc</sup>	6.35 $\pm$ 0.83 <sup>b</sup>

Within columns, there is no significant difference in means followed by the same letter at 5% level of significance (LSD test). NW, north-west Tunisia; NE, north-east Tunisia; EN, extreme north of Tunisia. SE, standard error.

**Table 4. Pairwise genetic distances between *Planococcus ficus* populations belonging to different sites. The citrus mealybug *Planococcus citri* was used as the outgroup.**

Specimens	<i>P. citri</i> (Italy)	<i>P. ficus</i> (Le Kef)	<i>P. ficus</i> (Mornag)	<i>P. ficus</i> (Sidi Thabet)	<i>P. ficus</i> (Takelsa)	<i>P. ficus</i> (Rafraf)	<i>P. ficus</i> (Testour)
<i>P. citri</i> (Italy)	0.000	0.011	0.012	0.010	0.010	0.010	0.010
<i>P. ficus</i> (Le Kef)	0.048	0.000	0.007	0.003	0.003	0.004	0.004
<i>P. ficus</i> (Mornag)	0.061	0.021	0.000	0.007	0.007	0.007	0.007
<i>P. ficus</i> (Sidi Thabet)	0.043	0.005	0.021	0.000	0.000	0.002	0.002
<i>P. ficus</i> (Takelsa)	0.043	0.005	0.021	0.000	0.000	0.002	0.002
<i>P. ficus</i> (Rafraf)	0.046	0.007	0.023	0.002	0.002	0.000	0.000
<i>P. ficus</i> (Testour)	0.046	0.007	0.023	0.002	0.002	0.000	0.000

from the Le Kef and Mornag sites compared to populations from the sites at Takelsa, Testour, Rafraf and Bousalem (Table 3). More specifically, the most significant difference between these populations were found in the number of multilocular disc pores on the metathorax (Table 3).

These morphological results were supported by our molecular analysis. The neighbour-joining phylogenetic tree (Figure 1) shows that the *P. ficus* populations are clearly separated into two clades, suggesting that *P. ficus* is comprised of two genetically different populations on grapevine in Tunisia. Indeed, the mealybugs from the sites of Le Kef and Mornag (the pairwise genetic distances between these populations and the other four populations ranged from 0.005 to 0.023) were genetically different from populations collected from the sites at Rafraf, Takelsa, Sidi Thabet and Testour (pairwise genetic distances between these populations ranged from 0 to 0.002) (Table 4). Into this clade the genetic distances of the four population are very low. Using the Internal Transcribed Spacer 1 (ITS1) region, no genetic differences were found between the Rafraf and Testour populations or between the Takelsa and Sidi Thabet populations, respectively. Further studies are needed to confirm these results and facilitate recognition of the vine mealybug populations in Tunisia. These results suggest two possible hypotheses. First, that the populations from the Mornag and Le Kef sites are F1 hybrids from either the cross *P. ficus* females x *P. citri* males or the cross *P. citri* females x *P. ficus* males. In fact, to support this possible hypothesis, both male and female *P. ficus* and *P. citri* have been recorded from the same vineyard in Tunisia (Mansour, 2008; Mansour *et al.*, 2009). If such hybrids were present on grapevines, then identification based solely on morphological characters would probably be difficult and might inaccurate identification, making it necessary to carry out molecular analyses for more accurate discrimination (characterization). Consequently, if further study does confirm the presence of two distinct morphs, then combining morphological and molecular analyses could be important. The second hypothesis might be that the two populations of *P. ficus* represent two different biotypes. In this context, performing biological crosses between males and females from each population would be useful to determine whether or not they are reproductively incompatible. Understanding how these morphological and molecular differences in *P. ficus* populations could influence biological traits is clearly important with regard to integrated pest management. Furthermore, differences in their ability to transmit grapevine viruses need to be evaluated. Further studies should be focused on carefully investigating other vine mealybug populations from vineyards throughout the Mediterranean basin, as this is the area of origin of this pseudococcid species. These would widen our understanding of the phylogenetic (evolutionary) relationships among vine mealybug populations from different Mediterranean countries.

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