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Identification of *Planococcus ficus* and *Planococcus citri* (Hemiptera: Pseudococcidae) by PCR-RFLP of COI gene

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The vine mealybug, *Planococcus ficus* (Signoret), and the citrus mealybug, *Planococcus citri* (Risso), are currently the most economically important pseudococcids in vineyards in Italy (Dalla Montà *et al.*, 2001). *Pl. ficus* is also considered to be a key pest in many other countries (Ben-Dov, 1994). The two species cause severe damage to the host plant, and significantly reduce the crop due to the production of honeydew, which allows the growth of sooty molds, and to their potential as virus vectors. The vine mealybug, in particular, is known to transmit grapevine leafroll and corky-bark diseases (Engelbrecht & Kasdorf, 1990; Tanne *et al*., 1989).

Populations of these two sibling species may coexist apparently without mutual interference (Rotundo & Tremblay, 1982), but some differences occur on their relationships with parasitoids. For instance, *Pl. citri* is successfully controlled by *Leptomastix dactylopii* Howard (Hymenoptera Encyrtidae) but this parasitic wasp is ineffective in controlling *Pl. ficus*, whereas *Anagyrus pseudococci* (Girault) (Hymenoptera Encyrtidae) can be employed in biological control programs for both mealybugs (Abdelkhalek *et al*., 1998; Mendel *et al*., 1999). Therefore, a well-timed identification of pests is very important for choosing the most suitable biocontrol agents for IPM.

The two scale insects are similar morphologically and their taxonomic separation is based on the distribution and presence of multilocular pores and tubular ducts on the adult females and these are difficult to detect (Cox & Ben-Dov, 1986; Williams & Granara de Willink, 1992). Therefore the discrimination between these two species needs a simpler and more reliable method, such as by molecular analyses.

In this study, restriction fragment length polymorphism (RFLP) analysis of regions of mitochondrial gene cytochrome oxidase subunit I (COI) was used to distinguish populations of these two mealybugs associated with grapevine.

Analysis methods. Twenty-six samples of *Planococcus* populations were collected between 2005-2007 in different Mediterranean localities (Table 1). From each sample, ten adult females were mounted on slides for morphological analysis and the identification was confirmed by comparison with slide-mounted vouchers deposited at the Di.S.Te.F. Scale Insects Collection. In addition, a total of 128 specimens, females and immature stages, were individually subjected to DNA extraction according to the protocol described by Walsh *et al.* (1991) and modified by De Barro and Driver (1997). Total DNA extracted by mealybug populations was amplified by PCR, following Bosco *et al*. (2006), with some changes. Samples of *Pseudococcus longispinus* Targioni Tozzetti were used as control species.

The PCR reaction was conducted using the primers C1-J-2195 (5'-TTGATTTTTTGGTCATCCAGAAGT-3') and TL2-N-3014 (5'-TCCAATGCACTAATCTGCCATATTA-3') (Simon *et al*., 1994). All PCR reactions were performed in 20 µl volumes with 0.85X of FailSafe™ PCR 2X premix F (Epicentre technologies), 0.5 µM of each primer 10 µM, 1.5 U of Taq polymerase 5U (Invitrogen) and 2 µl of DNA template. The cycling conditions were: 96°C for 5 min, then 35 cycles at 96°C for 45 s, 45°C for 1 min, 72°C for 1 min, followed by final cycle at 72°C for 10 min. Reactions and cycling conditions were conducted in an automated thermal cycler [GeneAmp® PCR System 2700 (Applied Biosystems)].

Some amplified COI fragments of *Pl. ficus*, *Pl. citri* and *Ps. longispinus* were sent to a sequencing service (BMR Genomics). The obtained sequences showed high similarity when compared with those of *Pl. ficus*, *Pl. citri* and *Ps. longispinus* deposited at GenBank. The sequences were then analyzed for restriction reception sites using the program NEBcutter V2.0 (Vincze *et al*., 2003). The chosen restriction enzymes were: *Bsp*PI, *Hinf*I, *Hph*I, *Mbo*II, *Ssp*I. Amplified DNA of all specimens was then cut with these five enzymes to produce multiple fragments which were used as

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markers to identify species. Digest reaction was performed in 15 µl volumes with 1X buffer, 2 units of restriction enzyme and 5 µl of PCR product. Samples were digested for 4 h at 37°C for *Hinf*I, *Hph*I, *Mbo*II (Fermentas), *Ssp*I (Promega) and at 55°C for *BspP*I (Fermentas). The restriction enzyme profiles were resolved on a 2% agarose gel, stained with ethidium bromide and visualized with UV light.

PCR-RFLP performance.The PCR amplification of the COI gene from both *Planococcus* species produced a fragment of about 900 bp. Analysis of predicted restriction sites in the COI gene revealed four enzymes (*BspP*I, *Hinf*I, *Mbo*II, *Ssp*I) with restriction sites for *Pl. ficus* and three (*Hinf*I, *Hph*I, *Ssp*I) for *Pl. citri*.

Digestion of the products with five restriction endonucleases showed that *BspP*I, *Hinf*I and *Ssp*I produced polymorphic patterns that distinguished *Pl. ficus* from *Pl. citri* and separated both from *Ps. longispinus* (Fig. 1), while *Mbo*II and *Hph*I patterns produced ambiguous identification of these species. *Hinf*I gave a clearer distinction between the three species than *BspP*I and *Ssp*I. *Hinf*I patterns produced two discrimination bands at about 300 bp and about 400 bp for *Pl. ficus* and single bands at about 700 bp for *Pl. citri* (Fig. 2) and at about 350 bp for *Ps. longispinus*. For this reason, *Hinf*I was used to identify the mealybugs collections. This identification method was able to amplify all the specimens regardless of instar (females and immature stages) and geographic origin. The results showed that each population was composed of individuals belonging to one species (Table 1). All the analyzed samplings gave the same outcomes with molecular and morphological methods.

More recently, other methods were developed to distinguish between the two mealybugs using RAPD-PCR and multiplex-PCR (Demontis *et al*., 2007; *Ulubaş Serçe et al*., 2007; Saccaggi *et al.*, 2008). RAPD-PCR is a useful tool to discriminate species for which no genetic data are available but it isn't easily reproducible because it is sensitive to small changes in reaction conditions (McEwan *et al*. 1998; Pérez *et al*., 1998). Multiplex-PCR, based on more than one pair of primers to amplify multiple PCR products, is difficult to develop because all the primers require the same melting temperature, they should not interact with one another and the amplified products have to be of different sizes (Hoy, 2003). The PCR-RFLP used in the present study is more reproducible than RAPD-PCR and it is easier to develop than Multiplex-PCR. The technique provides researchers and technicians with a powerful tool for the rapid, accurate and unequivocal identification of these mealybugs in different agricultural fields, especially for the correct choice of parasitoids in integrated pest management.

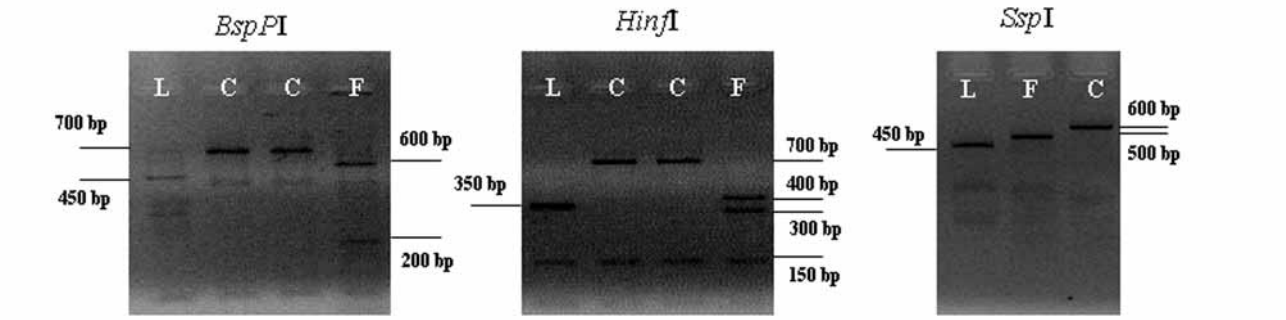

FIGURE 1. COI gene restriction patterns of *Ps. longispinus* (L), *Pl. citri* (C) and *Pl. ficus* (F) generated by *BspP*I, *Hinf*I and *Ssp*I.

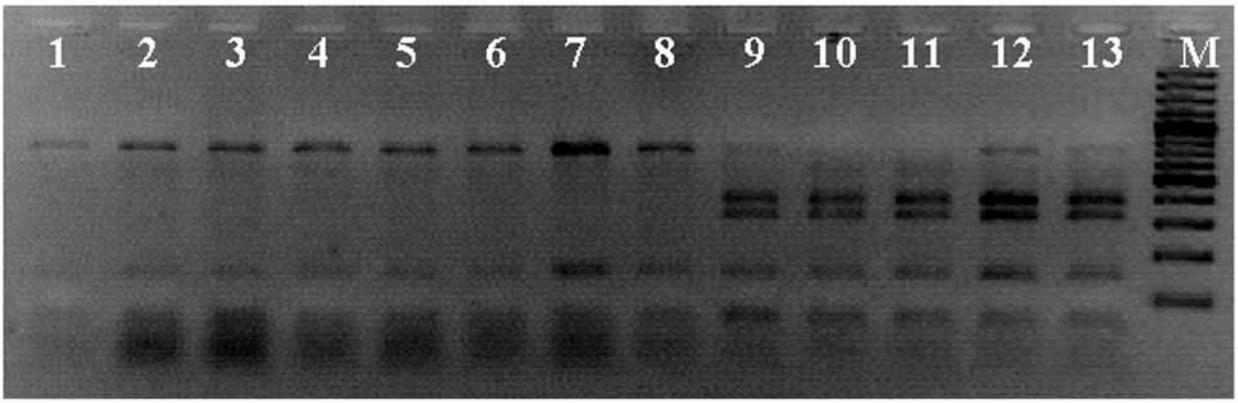

FIGURE 2. RFLP analysis with *Hinf*I restriction enzyme of COI gene. Digested products were separated on 2% agarose gels stained with ethidium bromide. Lanes: 1-8, *Pl citri*; 9-13, *Pl ficus*; M, 100 bp ladder.

TABLE 1. Results of analysis of PCR-RFLP of cytochrome oxidase subunit I using *Hinf*I on *Planococcus* collections.

Provenance		Total samplings	Hostplant	Species obtained by RFLP analysis
Italy	Acate	4	*Vitis vinifera* L.	*Pl. ficus*
	Caltagirone	3	*Vitis vinifera* L.	*Pl. ficus*
	Catania	1	*Citrus sinensis* (L.) Osbeck	*Pl. citri*
	Chiaramonte Gulfi	3	*Vitis vinifera* L.	*Pl. ficus*
	Partinico	1	*Vitis vinifera* L.	*Pl. citri*
	S. Croce Camerina	1	*Vitis vinifera* L.	*Pl. ficus*
	Siracusa	1	*Ficus carica* L.	*Pl. ficus*
	Vittoria	5	*Vitis vinifera* L.	*Pl. ficus*
Morocco	Rabat	1	*Citrus* sp.	*Pl. citri*
Portugal	Lisbon	2	*Solanum tuberosum* L.*	*Pl. citri*
Tunisia	Takelsa	1	*Hibiscus* sp.	*Pl. ficus*
		1	*Solanum tuberosum* L.*	*Pl. citri*
	Tunis	2	*Solanum tuberosum* L.*	*Pl. citri*

* potato sprouts in laboratory rearings

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