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Molecular diagnosis of native and quarantine pest thrips of southern European citrus orchards

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Abstract

The ITS-RFLP technique of the amplified internal transcribed spacer regions of ribosomal DNA has been used to establish the molecular identification of the most common pest thrips species of citrus orchards. Both native and quarantine thrips species have been tested and molecular keys are proposed through ITS-RFLP techniques. The restriction enzymes produced patterns that allowed unambiguous identification of seven thrips species examined (visual key). The sequences of species can also be verified using a molecular key that permits identification of a species through such parameters as primer pair used, restriction enzyme, length of PCR-product, fragments obtained. This study has shown that the use of genetic markers can be a valid alternative for quarantine workers and for epidemiological researchers, to whom the correct identification of pest species through classic morphological methods could be either very difficult and time-consuming or visually impossible.

Key words: molecular identification, ITS-RFLP, visual key, thrips, citrus pest.

Introduction

The insect order Thysanoptera includes in the family Thripidae about 50 species associated with economic crops and also quarantine species. Problems related to quarantine species are mainly due to identification: difficulties related to their small size, variability of body colour, morphological patterns, secondary sexual character states and the cryptic behaviour of some species which exhibit large differences in genetic compositions, habitat preference, *Tospovirus* transmission efficiency (Brunner *et al*., 2004; Hoddle *et al*., 2008; Brunner and Frey, 2010; Rugman-Jones *et al*., 2010; Jacobson *et al*., 2013). Classical morphological identification keys require experience and are difficult for non-specialists such as most quarantine workers. Moreover, these difficulties are greater when an identification of either larval stages or eggs that infest trade vegetables is required. Larval identification keys are rare (Kucharczyk, 2010; Vierbergen *et al*., 2010) and practically useless in terms of fast and accurate pest identification, but during the past 15 years identification systems which combine classic morphological knowledge with molecular identification methods have been produced (Moritz *et al*., 2000; 2004; 2009; 2010; 2013; Tyagi *et al*., 2015). Furthermore, improving molecular techniques have led to molecular keys that provide quick and correct identification ways for adults and all ontogenetic stages of the most common pest thrips species (Brunner *et al*., 2002; Toda *et al*., 2002; Kox *et al*., 2005; Rugman-Jones *et al*., 2006; Timm *et al*., 2008; Fekrat *et al*., 2015; Przybylska *et al*., 2015). The internal transcribed spacer (ITS), the non-coding fragment of the nuclear ribosomal region, has been one of the most widely used markers in thrips species identification (Rugman-Jones *et al*., 2006; Farris *et al*., 2010).

In the present article, a molecular technique is developed to identify the most common harmful thrips species on citrus plants in the southern areas of Italy and the Mediterranean Basin, together with a few exotic thrips species that are the most important quarantine pests for these crops. Results of molecular identification study of seven pest thrips species on citrus (Marullo and De Grazia, 2012) are presented and discussed; four of them are present in the Mediterranean Region, i.e. *Heliothrips haemorrhoidalis* (Bouche), *Frankliniella occidentalis* (Pergande), *Pezothrips kellyanus* (Bagnall), *Thrips tabaci* Lindeman, whereas three species, *Frankliniella bispinosa* (Morgan), *Scirtothrips aurantii* Faure, and *Scirtothrips citri* (Moulton) are considered quarantine species for EU territories.

Materials and methods

Collections of thrips species

Seventy individual thrips identified as seven different species were collected for this study (collecting details are summarized in table 1). The specimens of *F. bispinosa*, *S. citri* and *S. aurantii* from field populations, were provided by Prof. J Funderburk (University of Florida, USA) and Prof. L. Mound (CSIRO, Australia), respectively.

Molecular technique

Ten specimens of each species studied have been tested using a protocol which provides a few phases:

- DNA extraction: DNA has been extracted from single specimens using the Qiagen "DNeasy Tissue" kit (Germany) which allows the carcass of each specimen to be retrieved and mounted onto microscope slides. With the aid of a sterilized needle, we incised the specimen's abdomen and placed it in a 1.5 ml tube containing 20 µl proteinase K. After thoroughly mix by vortexing, the tube was incubated overnight at 55 °C. Then, we added 200 µl Buffer AL (provided with the kit) to the sample and vortexed it. After a short incubation (15 minutes at 70 °C) we added 200 µl ethanol (100%)

Table 1. Sampling details for the collected species.

Species	Locality	Date of collection	Geographic coordinates	Host-plant
Frankliniella occidentalis	Reggio Calabria (South Italy)	23/05/2012	38°6'41.40"N 15°39'43.56"E 31 m asl	*Solanum lycopersicum*
Frankliniella bispinosa	Miami (Florida, USA)	15/04/2011	25°46'27"N 80°11'37"W 25 m asl	*Lagerstroemia speciosa*
Pezothrips kellyanus	Reggio Calabria (South Italy)	30/04/2012	38°6'41.40"N 15°39'43.56"E 31 m asl	*Citrus limon*
Heliothrips haemorrhoidalis	Lamezia Terme (South Italy)	20/09/2011	38°58'0.84"N 16°18'36.00"E 216 m asl	*Viburnum* spp.
Thrips tabaci	Lamezia Terme (South Italy)	22/05/2012	38°58'0.84"N 16°18'36.00"E 216 m asl	*Allium caepa*
Scirtothrips aurantii	Canberra (Australia)	12/10/2011	35°17'00"S 149°07'41"E 571 m asl	*Citrus* spp.
Scirtothrips citri	Davis (California, USA)	13/10/2011	38°32'41"N 121°44'25"W 17 m asl	*Citrus* spp.

and mix all again. We inserted the mixture into DNeasy Mini spin column (provided with the kit) placed in a 2 ml collection tube and centrifuged. We discarded flow-through and transferred the spin column to a new 2 ml tube where we added 500 µl Buffer AW1 (provided) and after centrifugation, we again discarded flow-through and transferred the spin column to a new 2 ml tube in which we added 500 µl Buffer AW2 (provided) and centrifuged to dry the DNeasy membrane. We placed the DNeasy Mini spin column in a clean 1.5 ml tube and we added 70 µl sterile, ultra pure water directly onto the DNeasy membrane. Finally we incubated at room temperature for 1 minute, and centrifuged to elute DNA;

- PCR conditions: the ITS regions have been amplified with 2 primer pairs (CS249/CS250: 5'-TCGTAACAAGGTTTCCG-3'; - 5'-GTTAGTTTCTTTTCCTC-3'; 18SMP/28SMP: 5'-TGAACCTGCGGAAGGAT-3'; 5'-TCTCACCTBAACTGAGG-3') and the polymerase chain reaction mixture contained: 10 µl of template; 5.0 µl of 10x PCR-buffer; 0.4 µl of 25 mM dNTPs; 4.0 µl of 25 mM MgCl2; 1.5 µl of each primer; 0.2 µl of Taq-polymerase and sterile distilled water to a final volume of 50 µl. The amplification is carried out in an Eppendorf Mastercycler gradient. The DNA has been denatured at 95 °C for 3 minutes, followed by 30 cycles of denaturation at 95 °C for 45 seconds. Annealing (the temperature at which the primer anneals or binds to the target site) at 47 °C (CS249-CS250) or 54 °C (18SMP-28SMP) for 45 seconds. and elongation at 72 °C for 2 minutes. The last cycle is followed by 4 minutes of incubation at 72 °C to complete any partially synthesized strands;
- Restriction: 7 µl of the PCR-products have been digested with 3 units of five enzymes (RsaI, HaeIII, MspI, HinfI and AluI) (Promega) and observed by electrophoresis in a 2% agarose gel with ethidium bromide to visualize the DNA restriction fragments (Moritz *et al*., 2000).

Results

The technique of RFLP of the amplified internal transcribed spacer region of ribosomal DNA (ITS1 and ITS2) enables the production of specific DNA fragment patterns for the identification of thrips species. The identification of a species has to be done through comparison of different patterns obtained with the same couple of primers (visual key) (figures 1-2).

Figure 1 shows the differences between *F. bispinosa*, *P. kellyanus*, *S. citri* and *T. tabaci* whose DNA was amplified using the primers 18SMP-28SMP. In all the species, patterns are represented by a sequence of bands for each restriction enzyme used (table 2). In particular, lane 3 corresponds to restriction fragments obtained by digestion of rDNA of *F. bispinosa* using RsaI enzyme; lane 4 coincides in restriction fragments by digestion of rDNA with HaeIII enzyme; lane 5 shows the bands obtained by MspI enzyme; lane 6 and 7 represent restriction fragments obtained with HinfI and AluI, respectively. Similarly, lanes 11-15, 19-23 and 27-31, represent the bands obtained through the digestion of rDNA of *P. kellyanus* , *T. tabaci*, *S. citri*, respectively.

Figure 2 reproduces the patterns of *F. bispinosa* and *F. occidentalis* by using the primers CS249-CS250. Each of the seven species constitutes a distinct pattern. Figure 3 shows the patterns of the second instar larvae of *F. occidentalis* and *P. kellyanus* obtained by digestion of rDNA with 5 restriction enzymes (RsaI, HaeIII, MspI, HinfI and AluI).

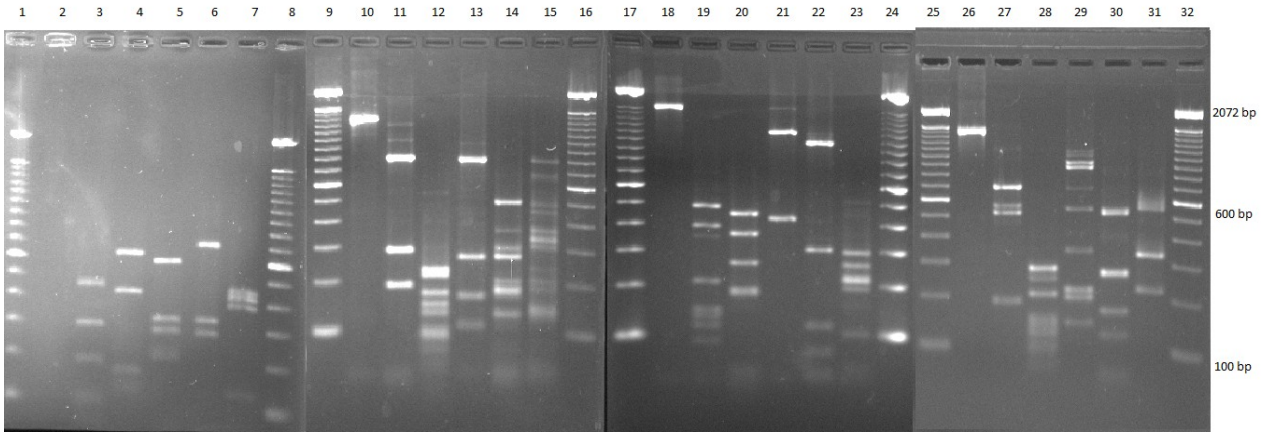

Figure 1. Agarose gel showing the ITS-RFLP patterns of *F. bispinosa* (lanes 3-7), *P. kellyanus* (lanes 11-15), *T. tabaci* (lanes 19-23) and *S. citri* (lanes 27-31) obtained by digestion of amplified ITS regions of rDNA (18SMP-28SMP) with RsaI, HaeIII, MspI, HinfI and AluI. Lanes 1, 8, 9, 16, 17, 24, 25 and 32, 100 bp DNA ladder. Lanes 2, 10, 18 and 26, PCR products of *F. bispinosa*, *P. kellyanus*, *T. tabaci* and *S. citri* respectively.

Table 2. Size (in base pairs) of the ITS-RFLP restriction fragments of each species tested.

Species	Primer Pair	PCR-product	RsaI	HaeIII	MspI	HinfI	AluI
S. aurantii	18SMP-28SMP	1252	693, 176, 141	555, 506, 432, 278, 167, 144	663, 352, 225	647, 244, 145	628, 495, 167
S. citri	18SMP-28SMP	1420	692, 569, 523, 192	292, 256, 213, 154, 131	942, 901, 555, 349, 229, 156	526, 274, 175, 127	583, 345, 216
F. occidentalis	18SMP-28SMP	1360	358, 305, 254	562, 408	553, 369, 223, 141	460, 310, 253, 191	485, 280
F. bispinosa	18SMP-28SMP	1380	455, 300, 202	640, 428, 177	596, 330, 290	708, 332, 285	431, 394
P. kellyanus	18SMP-28SMP	1299	800, 288, 187	224, 214, 170, 146, 127	771, 257, 162, 104	485, 290, 264, 198, 176, 127	782, 654, 485, 446, 360, 328
T. tabaci	18SMP-28SMP	1619	481, 398, 211, 146	451, 365, 263, 193	1141, 435	1010, 408, 305	300, 255, 217, 198
H. haemorrhoidalis	CS249-CS250	865	690	315, 249, 173	700	374	390
F. occidentalis	CS249-CS250	1400	464, 390, 276	595, 521, 204	591, 457, 260, 182	476, 320, 294	543, 281
F. bispinosa	CS249-CS250	1349	478, 239, 130, 112, 102	688, 473, 114, 106	608, 277, 249, 148	732, 320, 279	465, 400, 180
P. kellyanus	CS249-CS250	1370	850, 314, 195	235, 193, 165, 144	810, 271, 164	495, 279, 223, 187, 154	819, 368, 192

Sequences of species determined by ITS-RFLP analysis were used to develop two molecular identification keys to confirm the identification of a species through a series of parameters as: primer pair used, restriction enzyme, length of PCR-product, fragments obtained. In a first key (table 3) six species studied were included, considering differences in size of the PCR products and restriction fragment lengths using the primer pair 18SMP-28SMP. These primers did not work on *H. haemorrhoidalis*. Therefore, a second key for the identification of four species (table 4) was obtained using the primer pair CS249-CS250. Keys require the user to make a choice between only two data at a time. The choices are numbered and refer to the size of the ITS band and the number of fragments obtained by a precise restriction enzyme. In addition, for a further confirmation of the correct identification of a species it is possible to connect to the web-data base: http://moritz.zoologie.uni-halle.de/ (Moritz, 2010).

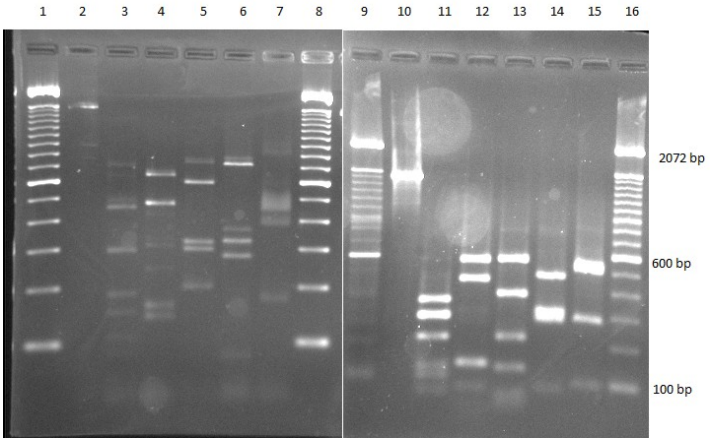

Figure 2. Agarose gel showing the ITS-RFLP patterns of *F. bispinosa* (lanes 3-7) and *F. occidentalis* (lanes 11-15), obtained by digestion of amplified ITS regions of rDNA (CS249-CS250) with RsaI, HaeIII, MspI, HinfI and AluI. Lanes 1, 8, 9 and 16, 100 bp DNA ladder. Lanes 2 and 10, PCR products of analysed species.

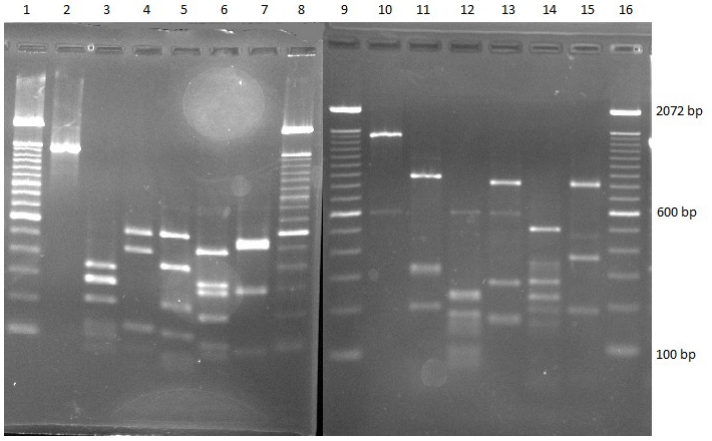

Figure 3. Agarose gel showing the ITS-RFLP patterns of larval stages of *F. occidentalis* (lanes 3-7) and *P. kellyanus* (lanes 11-15) obtained by digestion of amplified ITS regions of rDNA (CS249-CS250) with RsaI, HaeIII, MspI, HinfI and AluI. Lanes 2 and 10 represent PCR products. Lanes 1 and 16, 100 bp DNA ladder.

Table 3. Molecular key to thrips species based on ITS-RFLP products obtained with 18SMP-28SMP.

1	Size of the ITS band > 1260 bp	2
	Size of the ITS band ≤ 1260 bp	*S. aurantii*
2	Size of the ITS band > 1400 bp	3
	Size of the ITS band ≤ 1400 bp	4
3	PCR product restricted with MspI gives > 4 bands	*S. citri*
	PCR product restricted with MspI gives ≤ 3 bands	*T. tabaci*
4	PCR product restricted with HaeIII gives 2 bands	*F. occidentalis*
	PCR product restricted with HaeIII gives ≥ 3 bands	5
5	PCR product restricted with MspI gives 3 bands	*F. bispinosa*
	PCR product restricted with MspI gives ≥ 4 bands	*P. kellyanus*

Table 4. Molecular key to thrips species based on ITS-RFLP products obtained with CS249-CS250.

1	Size of the ITS band > 1000 bp	2
	Size of the ITS band < 1000 bp	*H. haemorrhoidalis*
2	Size of the ITS band > 1350 bp	3
	Size of the ITS band < 1350 bp	4
3	PCR product restricted with RsaI gives 3 bands	4
	PCR product restricted with RsaI gives > 3 bands	*F. bispinosa*
4	PCR product restricted with MspI gives 3 bands	*P. kellyanus*
	PCR product restricted with HaeIII gives > 3 bands	*F. occidentalis*

Discussion

DNA patterns generated by ITS-RFLP have potential as useful tool in the identification of pest thrips species. Furthermore, we have successfully tested this method on instar stages, for which morphological keys are scanty or difficult to utilize, and a similar system has been applied to identify single eggs (Moritz *et al*., 2004). The technique might improve the efficacy of pest quarantine diagnoses. The identification to species level of larval stages is usually avoided by quarantine workers. Larval thrips are often mistaken for Collembola, or adults are confused with Staphylinid beetles (Vierbergen, 1995). Such misidentifications might be important in the case of thrips species that have a predatory behaviour, such as *Karnyothrips* or *Scolothrips* species (Marullo and De Grazia, 2013).Therefore, a need exists for a system of identifying adult and larval stages of pest thrips species. Recently, the molecular technique known as loop-mediated isothermal amplification (LAMP) was employed to discriminate the presence of DNA of *T. tabaci* from other species, using material from different stages of development (Fekrat *et al*., 2015) and to detect *Thrips palmi* Karny, a pest species under quarantine regulation in the European Union. These studies highlighted the sensitivity and the usefulness of LAMP to identify all the stages of the life cycle, including the preimaginal stage, which otherwise could be only differentiated by expert taxonomists (Przybylska *et al*., 2015).

In this study, two molecular keys have been presented, one with six of the seven species investigated and the other was developed to include *H. haemorrhoidalis* that was not represented in the first key. Production of a molecular key that involves members of different genera can be difficult, and certain constraints have to be considered. In the present work analysis, a few technical problems were encountered, such as the absence of results for *H. haemorrhoidalis* using the primer pair 18SMP-28SMP in order to amplify the ITS regions. Also, the primer pair CS249-CS250 was ineffective for *T. tabaci*, *S. citri* and *S. aurantii* . A further weakness of the ITS-RFLP method is that it still requires morphological identification of specimens, as similarly asserted by Rugman-Jones *et al*. (2006) for the most important pest species of the genus *Scirtothrips*. This aspect might be an hindrance for non-specialist taxonomists not familiar with thrips' dichotomous and electronic keys (Hoddle *et al*., 2012; ThripsWiki, 2014).

Another critical consideration is that studies based on PCR are able to detect only a few species at a time. It is essential to develop a more efficient method for simultaneous screening of mass samples. Possibly, DNA barcoding methods in combination with the mitochondrial COI gene could be a future way to molecular studies (Mehle and Trdan, 2012). An integrated approach of morphology and DNA barcoding for the invasive pest thrips, *Thrips parvispinus* (Karni) is proposed from Tyagi *et al*., 2015, in order to support how DNA barcoding data may be immense use for accurate species identification and phylogenetic analysis (Mound *et al*, 2010).

Moroever, recent studies (Chung *et al*., 2011; Lee *et al*., 2013; Yeh *et al*., 2015) report the microarray assay as a valid method for insect pest identification. Particularly, Yeh *et al*. (2015), using DNA microarray based on species-specific primers referring to ITS1 sequences of 15 agriculturally important thrips, provided an efficient tool for the simultaneous identification and monitoring of a number of thrips species. In conclusion, the availability of a molecular key could be extremely useful in the identification of species. The advantage of such an identification system over morphology-based taxonomic methods and other molecular techniques is that it is quick, specific, requires only basic laboratory skills, and can be performed with DNA extracted from single individuals, which can be preserved, mounted on slides and used for future reference. On balance, molecular methods for identification of thrips species represent an important possibility when classical morphological methods are difficult, time consuming or visually impossible.

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