Chapter 8
Diagnostics and Identification of Diseases, Insects and Mites

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Abstract Rapid and reliable diagnostic methods for arthropod pests and pathogens allow for a rational and efficient use of plant protection products. Traditional detection methods based on visual assessment of plant symptoms, isolation, culturing in selective media, and direct microscopic observation of pathogens are frequently laborious, time-consuming and require extensive knowledge of classical taxonomy. Molecular techniques are faster, more specific, sensitive, and accurate than traditional techniques. Plant viral and bacterial diagnostics have been traditionally based on serological methods, such as ELISA or Lateral Flow Devices. New molecular techniques (qPCR, digital PCR, microarray) have been developed, optimized and validated in the last years with different applications to pest and pathogen detection and identification. HTS technologies are having an enormous impact on biological sciences, allowing the determination of genome variation within a species or a population. The use of field techniques, such as LAMP and portable platforms, is a

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promising tool to early and quickly detect pests. One of the critical points of on-site detection consists in the use of simple and user-friendly nucleic acid extraction procedure, involving a low number of steps. The choice of the diagnostic technique depends on the balance between the reliability and the cost of the analysis.

Keywords Digital PCR · ELISA · High throughput sequencing · In field diagnostics · LAMP · Lateral flow devices · Microarray · Molecular markers · Phylogeny · Real time PCR

8.1 Introduction

Arthropod pests and diseases negatively influence greenhouse production of vegetables and ornamentals. Preventive measures to avoid planting contaminated material are of crucial importance in the context of an integrated pest management. Rapid and reliable diagnostic methods allow a rational and efficient use of plant protection products and constitute an important requirement for the development of the horticultural sector. The trend in the European Union for detecting plant pathogens, outlined in the European and Mediterranean Plant Protection Organization (EPPO) protocols, integrates phenotypic, serological, and molecular techniques. The present chapter provides information on new methods for fast, accurate, reliable, and early detection of arthropod pests and pathogens.

8.2 Plant Pathogen Diagnostics

The easy spread of fungal spores, virus and bacteria combined with the intense globalization are key factors to allow the movement of pathogens around the world, which can become invasive in new areas and even cause the total destruction of the crop. The traditional detection methods based on visual assessment of plant symptoms, isolation, culturing in selective media, and direct microscopic observation of pathogens are frequently laborious, time-consuming and require extensive knowledge of classical taxonomy. The observation under microscope or stereoscopic microscope is used to determine the causal agent of the disease taking into consideration pathogenicity tests and morphological features, such as size and shape of the conidia and colony characteristics, such as colour. However, many microorganisms can produce the same symptoms in the plant making difficult the correct identification of the causal agent. As many plant pathogens remain latent in the planting material, and in very low numbers, methods of high sensitivity, specificity, and reliability are required. The difficulty of culturing some species in vitro and the inability for accurate quantification of the pathogen are other limitations. Early detection of pathogens in seeds and plant materials is of key importance to avoid further spreading and introduction of new pathogens into growing areas where they are not

present yet. These limitations have led to the development of molecular approaches with improved accuracy and reliability. Molecular techniques are faster, more specific, sensitive, and accurate than traditional techniques and they can identify non-cultivable microorganisms and facilitate early disease management decisions. The development of new instruments and platforms and the continuous increase of bioinformatics-data have been allowed the use of bioinformatics-based techniques as metagenomics, comparative genomics and genome sequencing as routine analysis. However, these techniques are associated with enormous quantity of information, which can only be managed by skilled personnel.

8.2.1 Immunological Methods

Advances in antibody production have boosted the development of new methods for the detection of plant pathogens. Polyclonal and monoclonal antisera are used to develop diagnostic systems to use in routine laboratories or for on-site detection. Plant viral and bacterial diagnostics have been traditionally based on serological methods, such as ELISA (Enzyme-linked immunosorbent assay) or LFD (Lateral Flow Devices) specific for the target organisms (Boonham et al. 2014). ELISA tests allow the diagnosis of the disease due to the use of specific antibodies against the target organisms. Commercial kits have been developed for the detection of phytopathogenic fungi, such as *Botrytis cinerea, Rhizoctonia solani, Pythium* spp. or *Septoria* spp., bacteria, such as *Acidovorax avenae* subsp. *citrulli, Clavibacter michiganensis*, *Pseudomonas syringae, Ralstonia solanacearum, Xanthomonas* spp., and a high number of virus.

On the other hand, the first LFD, designed by Danks and Barker (2000), based on the agglutination of only one band, simplify the interpretation of the results and the use on-site (Tomlinson et al. 2010a; Hodgetts et al. 2015). Despite the cost effectiveness of the serological methods, DNA-based methods have replaced antibody-based diagnosis analysis due to lower sensitivity of the serological methods, risk of false positives and negatives, and necessity of specific antibodies for each target.

8.2.2 DNA-Based Methods

DNA based methods are focused on the amplification of one or some regions of the DNA using specific primers and the comparison of the sequence with worldwide accessible databases, such as GenBank (https://www.ncbi.nlm.nih.gov/genbank/), databases of a specific genomic sequence, or databases of a genus, such as the Fusarium genome database hosted by the Broad Institute (https://www.broadinstitute.org/), to identify the causal agent of a disease. The main step includes an amplification of a target DNA or cDNA using primers or probes following a qualitative or quantitative polymerase chain reaction (PCR or qPCR). Both approaches require

the assessment of the sensitivity and specificity of the primers, by using taxonomically closely related genera/species, morphological 'look-a-likes' isolates, or other species commonly found in the target host.

Fusarium oxysporum is a worldwide-distributed soilborne and seedborne pathogen, which can cause high losses in favourable conditions. It is a good candidate to explain the difficulty present in developing some diagnostics assay, to obtain specific assays. The species includes both pathogenic and non-pathogenic strains. Over 100 *formae speciales* have been identified within the pathogenic strains based on the host species. No morphological features can distinguish the *formae speciales* or the races (identified in function of the virulence patterns on different host cultivars). Many studies have been performed to determine molecular markers able to distinguish the *formae speciales* and the elongation factor 1-alpha, the LSU, IGS or polygalacturonases genes could be useful target regions (Mbofung et al. 2007; Hirano and Arie 2009). PCR based assays were developed for the detection of a specific *forma specialis* (*F. oxysporum* f.sp. *lactucae* on lettuce seeds) (Mbofung and Pryor 2010) or even for its quantification by qPCR (*F. oxysporum* f.sp. *melonis* on cucurbits) (Haegi et al. 2013). RAPD (Random Amplification of Polymorphic DNA) and other fingerprint assays have been used to determine monomorphic bands specific for a *forma specialis,* such as *F. oxysporum* f.sp. *niveum,* causal agent of Fusarium wilt on watermelon (Lin et al. 2010), *F. oxysporum* f.sp. *radici-lycopersici* or *F. oxysporum* f.sp. *radicis-cucumerinum* (Validov et al. 2011).

In addition, the high number of transposable elements on the *Fusarium* genome has also exploited to design specific assays within the species: *Foxy* transposable elements have been used to discriminate *F. oxysporum* f.sp. *fragariae* (Suga et al. (2013), *Fot1* to determinate the presence of a new *F. oxysporum* pathogenic on Paris daisy (*Argyranthemum frutescens* L.; Pasquali et al. 2004), or even the to discriminate races among *F. oxysporum* f.sp. *lactucae* using the *Skippy* and *Han*-solo retrotransposons (Pasquali et al. 2007; Gilardi et al. 2016).

Specific-primers assays for the diagnosis of *Phytophthora,* an important oomycete genus, have been designed and tested for *P. nicotianae* and *P. cactorum* (Li et al. 2011), *P. cactorum, P. megasperma, P. plurivora, P. pseudosyringae* and *P. quercina* from soil samples (Nowakowska et al. 2017), or *P. infestans* in potato (Hussain et al. 2017). Specific primers have also been developed for phytopathogenic bacteria, such as *Pantoea ananatis, Burkholderia* spp., and *Enterobacter* in onion (Asselin et al. 2016).

8.2.3 Barcode Sequences

One of the most critical points in the DNA-based methods is the selection of a correct genetic marker or barcode, which is informative enough to obtain a species-level identification or even sub-species identification. A barcode is a short and standardized DNA sequence in a well-known gene, which is useful for the identification of species. The Internal Transcribed Spacer region (rDNA ITS) has been proposed by the Consortium for the Barcode of Life (CBOL) as the primary fungal

barcode (Begerow et al. 2010). The ITS region is part of the fungal ribosomal RNA genes and is present in a variable number of copies in the genome, ranging from 30 to 30,000 copies in eukaryotes (Prokopowich 2003), is composed by the conserved genes 18S (small ribosomal subunit), 5.8S, 28S (large ribosomal subunit) and 5S present only in some species. Two highly variable spacers flanked the 5.8S region, ITS1, and ITS2 are variable enough to determinate the species in the majority of the genera. The ITS region has been considered an optimal barcode due to the high number of copies, the easy amplification using universal primers such as ITS1 and ITS4 (White et al. 1990), the robust primers sites constructed in conserved regions of the 18S and 28S genes and the variability among species.

However, the ITS, due to its high variability among species, can cause problems in the identification of higher phylogeny ranks, such as families or orders, where other genetic regions should be used. On the other side, in many fungal genera the ITS is not decisive enough to differentiate the species and other molecular regions have been used. The elongation factor 1-alpha (EF1-α) gene, which codes for an elongation factor for protein translation, has been widely used to determine the species of *Fusarium.* In addition, the intergenic spacer region (IGS) of the nuclear ribosomal operon has been used as barcode for several *formae speciales* of *F. oxysporum* (Mbofung et al. 2007; Gherbawy et al. 2008; Srinivasan et al. 2010, 2012; Bertoldo et al. 2015). Other genes, such as beta tubulin, which codifies for the beta tubulin protein in the microtubules, have been demonstrated useful markers in other pathogenic and mycotoxigenic fungi such as *Penicillium* spp. (Frisvad 2014) and for some oomycetes.

In the case of oomycetes, the cytochrome oxidase subunit I and II were useful to identify species and sub-species with more resolution than the ITS within *Pythium* spp. pathogenic for leafy vegetables (Levesque and De Cock 2004). Other mitochondrial genes, such as *cox2, nad9, rps10* and *secY*, produced consistent results with the data from nuclear genes within *Phytophthora* genus (Martin et al. 2014).

A common approach is to combine different molecular markers for taxonomic purposes in a multilocus sequencing analysis (MLSA). In the MLSA analysis, the sequence of different gene sequences is concatenated and phylogenetic analysis could be carried out for species or sub-species identification obtaining a more accurate classification due to the higher amount of genetic information used.

In the case of bacteria, the 16S rRNA was used as universal barcode for bacteria identification. It is composed by 9 highly variable regions (V1–V9) ranging from 30 to 100 bp involved in the secondary structure of the small ribosomal subunit. The most common primers – 27F and 1492R – have been designed by Weisburg et al. in 1991.

8.2.4 Quantitative PCR

The quantitative PCR or real time PCR (rt-PCR) (Heid et al. 1996; Raso and Biassoni 2014) consists in a PCR amplification with the real time measure of the accumulated product, by using intercalating dyes, such as SYBR GREEN or EVA

Green, or probes, such as TaqMan probes or Scorpion. The intercalating dyes allow the DNA quantification by fluorescent measurement after each replication cycle and comparing the results against a standard curve of known concentration of the target DNA. The specificity of the assay could be increased by a TaqMan qPCR, where the primers are combined with a TaqMan probe, a short oligonucleotide designed internally in the amplification product and labelled in the 5′ with a fluorophore and a quencher in the 3′. The annealing of the TaqMan probe to the DNA does not produce any fluorescence due to the proximity between the quencher and the reporter, whilst the detection of the fluorescence occurs during the extension step when the DNA polymerase excides the TaqMan nucleotides and therefore the quencher and fluorophore. The fluorescence is measured after each cycle and is later related with the accumulation of the product using absolute quantification methods (with standard curve) or relative quantification methods (comparing the target gene with an endogenous gene). One of the most important characteristics of this technique is the high sensitivity, which allows the determination of a plant pathogen even at femtogram level. The real time has been also miniaturized to obtain real time portable instruments (Koo et al. 2013).

This technique permits the determination of the presence and quantity of plant pathogens (Schaad and Frederick 2002; Sanzani et al. 2014; Mirmajlessi et al. 2016; Amaral Carneiro et al. 2017). Several qPCR assays have been recently developed for ascomycetes, such as *Alternaria solani* and *A. alternata,* causal agents of potato early blight and brown spot (Leiminger et al. 2014; Kordalewska et al. 2015), or for oomycetes, such as *Bremia lactucae* (Kunjeti et al. 2016), *Plectospherella cucumerina* (Gilardi et al. 2016), *Pythium irregulare* and *P. ultimum* directly from soil samples (Schroeder et al. 2006). The diagnosis of quarantine pathogens has to be accurate and rapid and qPCR assays have been developed in the last years for several quarantine pathogens, such as Potato Spindle Tuber Viroid (PSTVd) (Boonham et al. 2004).

8.2.5 Droplet Digital PCR

Digital PCR (dPCR) has been introduced in the last decade as a highly sensitive, precise and accurate acid nucleic quantification technique (Hindson et al. 2011). Initially described by Sykes et al. (1992), the dPCR combines the advantages of the end-point PCR, which consists in a semi-quantitative analysis measured by gel electrophoresis, and the qPCR, which uses fluorescence measurements of the accumulated products compared with a control (standards curve or reference gene). It is based on the detection of fluorescent probes without the necessity of the qPCR controls. The sample is diluted and partitioned into 20,000 droplets to obtain single template molecules, and, in each droplet, single amplifications occur. Subsequently, the droplets are quantified as positive or negative for the target sequence in function of the detection of fluorescence or not. Poisson statistics analysis of the positive and negative numbers allows the absolute quantification of the target sequence. This

technique overcomes the sensitivity issues and the difficulties in determining single nucleotide mutations and it is resilient to PCR inhibitors from plant, soil or water samples (Rački et al. 2014). dPCR assays have been developed for *Ralstonia solanacearum* causing potato brown rot (Dreo et al. 2014; Gutiérrez-Aguirre et al. 2015).

8.2.6 In Field Diagnostics Methods

The use of molecular techniques directly in the field on glass or plastic slides has been studied since the early 1980s. The first on-site test used in the diagnosis of plant pathogen was based on latex agglutination for the detection of plant viruses (Talley et al. 1980; Fribourg and Nakashima 1984). Since then, the objective has been to develop the fastest and more sensitive test, which produces results in short times without common laboratory tools and instruments.

One of the critical points of on-site detection consists in the use of simple and user-friendly nucleic acid extraction procedure, involving a low number of steps. The matrix rupture to extract the DNA usually combines enzymatic, chemical and mechanical methods to obtain the total cell disruption and a high extraction yield. However, the high yield with total disruption could be accompanied by the inhibition of downstream analyses. Post-extraction concentration and purification through a membrane or beads are widely performed. Many rapid, simple and easy protocols to extract the DNA in the field use membrane discs. However, the alkaline extraction is the most useful and easy technique for DNA extraction. Though it had been initially used for the extraction of plasmid from bacteria (Bimboim and Doly 1979), it has been recently optimized for a quick crude DNA extraction from plant material. Chomczynski and Rymaszewski (2006) used the polyethylene glycol-based method to lyse the cells and release the DNA to perform PCR. This type of nucleic acid extraction has been used to obtain a crude extract, which could be easily obtained by mechanical disruption of the cells directly in field.

Inexpensive but accurate tests should be used for routine programs including certification, breeding, plant quarantine or germplasm screening, or more diagnostics needs in the laboratory. The end-users are generally inexpert in molecular biology techniques, but the tests developed should maintain high specificity and sensitivity to avoid false positives and false negatives. The inspection in the fields requires easy techniques with a simple interpretation of the results. On-site testing is carried out in many cases with seeds without evident symptoms or plant material in pre-symptomatic infection stage where the pathogen is unnoticeable. The failure of an inspection test may allow the spread of the pathogen or the disease development until visible symptoms. A high sensitivity is a desirable characteristic in this type of tests.

Lateral Flow Devices (LFD) have been used for on-site testing, but the lower sensitivity compared to nucleic-acid methods as well as the difficult and long antibody production process is time-consuming and challenging, specially to detect

species or lower taxonomical levels. Moreover, the multiple detection of pathogens in the same assay could be a problem using this type of method.

8.2.7 Isothermal Amplification in the Field

Despite the advantages of the PCR and qPCR as diagnostic tools, their field application requires a complex equipment. These techniques are widely diffused among routine centralized laboratories where diary samples arrive from inspections and certifications. On-site diagnostic methods developed based on an antigen-antibody reaction, such as LFD, have been overcome by DNA-based methods. In field detection methods need to be specific and sensitive, easy to be interpreted and simple for the end-user.

Isothermal methods, which require a less complex equipment, present some advantages in contrast to PCR-based methods. The use of an isothermal water bath to perform the amplification, instead of a thermal cycler, has been considered an advantage to develop loop-mediated isothermal amplification (LAMP). A wide number of molecular techniques based on isothermal conditions have been taken into consideration, such as NASBA (acid-sequence-based amplification), 3SR (self-sustained sequence replication) and SDA (strand displacement amplification). However, these amplification methods have some drawbacks that LAMP overcame such as the use of a precise instrument to detect the product due to the low specificity becoming useless in the routine diagnosis. SDA overcomes some of these shortcomings by using four primers but it produces a high number of background products and the modified nucleotide increase the total cost of the technique making it unaffordable for phytopathological diagnostics.

The LAMP assay is able to amplify few copies of the target DNA in less than 1 h with high specificity and low susceptibility to inhibitors from the host matrix. The combination with the crude extraction method based on alkaline disruption of cells showed great potential for on-site detection (Franco-Ortega et al. 2018a).

The visualization of the results includes colour change reactions with HNB or calcein and $MnCl_2$, which vary from violet to blue and from orange to green after the reaction, respectively. Positive amplifications can be detected also due to an increase in turbidity caused by the precipitation of magnesium pyrophosphate observable only after centrifugation. The above-mentioned methods are quite subjective among the different users, particularly around the detection limit. Another visualization procedure includes the addition of intercalating dyes, such as SYBR Green and PicoGreen at high concentration, however the risk of cross-contamination increases with the amount of DNA produced using this reagent. A lower number of manipulations reduce possible cross-contaminations, which can cause false positive reactions.

The easiest procedure to detect positive LAMP results is the Real Time LAMP using instruments such as the OptiGene Genie II® and Genie III®. These small size

and battery-powered platforms have been designed as a suitable tool for on-site detection with closed-tubes to reduce post-amplification contaminations.

The conjunction of crude extraction procedures with the detection by real-time procedures to reduce the number of steps to analyse the samples and the risk of contamination is the most effective and suitable procedure to identify or confirm the causal agent of a disease in symptomatic material.

The high specificity of the LAMP assay has been exploited for the identification of the different *formae speciales* within *F. oxysporum,* such as *F. oxysporum* f.sp. *ciceris* using HNB (Ghosh et al. 2015), *Fusarium oxysporum* f.sp. *lactucae* (Franco Ortega et al. 2018b), or even races within a *forma specialis* as *F. oxysporum* f.sp. *lycopersici* race 1 (Ayukawa et al. 2016) using the portable Genie II®. LAMP assays for other Ascomycota as *Botrytis cinerea* (Tomlinson et al. 2010b; Duan et al. 2014), and Basidiomycota as *Rhizoctonia solani* (Patel et al. 2015) have been also developed and validated. Within the oomycetes, some LAMP assays have been designed in the last years for *Phytophthora nicotianae* (Li et al. 2015), *P. capsici* (Dong et al. 2015) and *Pythium aphanidermatum* in tomato (Li et al. 2011). For plant pathogenic bacteria, LAMP assays have been developed for '*Candidatus* Liberibacter solanacearum' (Ravindran et al. 2015) and *Ralstonia solanacearum* (Lenarčič et al. 2014) in potato.

The ease of using LAMP assays for the end users, has favoured the development of seed tests, overcoming the time consuming traditional approach of blotting, which is still recommended by the International Seed Testing Association (Abd-Elsalam et al. 2011; Franco Ortega et al. 2018b) or soil tests (Chen et al. 2013; Peng et al. 2014).

8.2.8 Microarray

DNA chips, DNA microarrays or macroarrays consist of a solid glass slide onto which dots of nucleic acid probes or primers have been printed. Each probe or primer is complementary to a target region in the genome of different genera/species, so the identification of the genus/species present in complex samples exploits the DNA hybridization, such as environmental samples, can be determined in a single assay (Zhou and Thompson 2002). Microarrays have been widely used in multiple gene expression studies, but they could be used to determine multiple bacterial or fungal species (Lievens et al. 2012), viruses (Boonham et al. 2007) or even mixtures of microorganisms, such as *Pantoea ananatis* and Maize Dwarf Mosaic Virus (MDMV) in maize (Krawczyk et al. 2017). This method allows a comprehensive vision of the population (Kristensen et al. 2007) or studying the population dynamics with high sensitivity, low reagent consumption, rapid and low cost approach. Miniaturized devices for DNA diagnostics, called 'lab-on-a-chip', have been applied to determine oomycetes species, such as *Phytopthora ramorum* and *Pythium* spp. (Julich et al. 2011).

8.2.9 High throughput Sequencing

In the last years, high throughput sequencing has become a feasible tool, which has been adopted across many biological fields, such as diagnosis of human, animal and plant diseases, population genetics and microbiology. Whole genome sequencing (WGS) approaches allow obtaining a comprehensive view of the genomics of a sample.

Since the first original Roche 454 platform, the innovation in the technologies has revolutionized the microbial ecology and the plant pathology diagnosis. High throughput Sequencing (HTS) platforms with long-read sequencing technologies are helping to resolve long repeated and complex sequences, which have been problematic to assemble with short-read sequencing platforms.

Unlike other DNA-based methods, in this case, the HTS opens the door to multiple detection of even unknown species of bacteria, fungi or viruses, in a short time and with a low cost. As it is well known, only a small percentage of microorganisms can be cultured limiting the knowledge about the community present in a sample. However, during the analysis of the metagenomic-data it is essential to use high-quality database of the barcode, to avoid unclassified or misidentified *operational taxonomic unit* (*OTUs*). The most critical points are the not user-friendly bioinformatics pipelines, which require trained people with advanced informatics skills to facilitate the rapid and precise analysis and interpretation of the results. However, despite these drawbacks, HTS has been applied in the last years to multiple projects such as "The 1000 Fungal Genomes" project (http://1000.fungalgenomes.org/) managed by the Department of Energy of the USA to obtain the genome of 1000 fungal species from over 500 families. The interest of this project is to improve the knowledge of the genes involved in pathogenicity or virulence by using comparative genomics. The new available genomes could be used to obtain sequences specific for a genus/species/*forma specialis*/race. Specific primers for *F. oxysporum* f. sp. *conglutinans* on *Brassica oleracea* have been designed after comparative genomics among different *formae speciales* (Ling et al. 2016; van Dam et al. 2016) and arrays have been developed for *Alternaria longipes* and *A. alternata* after comparative genomics (Hou et al. 2016). Within the *Alternaria* genus, the species identification is controversial: often the results of traditional methods conflict with DNA-based taxonomy performed using informative nuclear and mitochondrial loci, such as ITS, BTUB, EF-1α, glyceraldehyde-3-phosphate dehydrogenase, actin, plasma membrane ATPase and calmodulin. The HTS approaches could overcome this taxonomic troubles, and substitute the MLSA approach (Woudenberg et al. 2015; Lawrence et al. 2016). Genomic data have been also useful to design real time LAMP assays for *Pseudoperonospora cubensis* (Rahman et al. 2017). Furthermore, HTS data have been used to design E-probes from different phytopathogens (bacteria, virus, fungi, and oomycetes) for E-probe Diagnostic Nuclei Acid Analysis (EDNA) (Stobbe et al. 2013; 2014).

Powerful advances in HTS technologies have been specially applied in virology (Adams et al. 2009; Boonham et al. 2014; Al Rwahnih et al. 2015; Roossinck et al.

2015). The most common methods for virus detection in plants are based on serological tests or DNA-based methods, such as PCR, however the low viral load in some samples makes difficult virus detection and identification. On the opposite, HTS technologies have improved the ability for WGS analysis and metagenomics removing the necessity of routine analysis, and therefore they have boosted the discovery of novel virus species (Adams et al. 2013a) or the complete genome sequencing of viruses (Adams et al. 2013b).

8.3 Detection and Identification of Insects and Mites

All arthropods have multiple genomes, mostly included in one of the following three categories: nuclear, mitochondrial and symbiont-associated DNA. Each genome has a different type of transmission, phylogenetic origin and variation rate that will determine which one should be selected as source for molecular markers.

Genetic information contained in the nuclear genome can be divided into different categories depending on their function and location in the chromosomes. These categories are non-repetitive (single-copy genes), middle-repetitive (ribosomal RNA, transfer RNA, histones, or transposable elements, among others) and highly repetitive DNA (satellite DNA). Moreover, DNA fragments between genes, the intergenic spacers (non-coding sequences), can be used as markers depending on the mutation rate and the purpose of the study.

Mitochondria and their haploid genome are inherited cytoplasmically and are transmitted primarily through maternal gametes. This genome is organized in a single circular chromosome where genes can be found in both DNA strands, sometimes with overlapping coding sequences. Because of its bacterial origin, the mitochondrial genome is made of a single continuous coding region being this a substantial difference compared with eukaryotic nuclear genes. The nature (bacterial origin) and transmission mode (maternal without recombination; evolution bottlenecks) of the mitochondrial genome affect its mutation rate. These characteristics make it especially valuable for phylogenetic studies. In addition, the actual gene composition and synteny (order of genes) are conserved enough to be used to compare between taxa. The main disadvantage of using mitochondrial DNA in arthropods is its maternal inheritance, which makes male dispersal patterns to be lost in those species where these patterns differ from females. Thus, for monitoring such populations, the use of nuclear DNA-based markers would be more convenient.

Arthropods keep intimate intra- and extracellular relationships with a diverse group of microorganisms (viruses, bacteria, rickettsias and yeasts), and often these relationships are obligate as none of the parts can live without the other one. These microorganisms have their own genome, which have been recently related to speciation and evolution of some insect Orders. This symbiont DNA is mainly transmitted by maternal lineage, as mitochondrial DNA, with the same restrictions (generational bottlenecks, single chromosome, gene structure and high mutation rate). However, as the relationship between arthropods and their symbiont is not as old as

that of mitochondria, the mutation rate of symbiont DNA is related to the evolution of the relationship. Therefore, it is possible to track symbiosis establishment and host speciation by studying the co-evolution of both genomes.

8.3.1 Arthropod Molecular Markers

In recent years, many molecular markers have been used not only to differentiate arthropod populations and species, but also to increase our understanding of their genetics (Behura 2006). These techniques have also shed light on the origin of invasive species and their distribution into new habitats (Xie et al. 2006). They have also facilitated the study of natural enemies commonly used in classical, inundative or conservation biological control (Symondson et al. 2002; Greenstone 2006). A large number of molecular markers have been developed and used in crop protection studies. Isozymes were the first molecular markers used, but they are now virtually obsolete. They are defined as variants of a single enzyme, performing the same or a similar biochemical function. The variants are due to differences in the enzyme amino acid sequence, which originates differences in their electrical charge and molecular weight. Isozymes have been used to analyse the diets of some predatory arthropods (Murray and Solomon 1978; Solomon et al. 1996).

The number of available molecular markers greatly increased after 1983, when KB Mullis conceived the Polymerase Chain Reaction (PCR) (Loxdale and Lushai 1998). These markers are differentiated according to the technique used, being the most common RFLPs (Restriction Fragment Length Polymorphism), RAPDs (Random Amplified Polymorphic DNA), AFLPs (Amplified Fragment Length Polymorphism), SCARs (Sequence-Characterized Amplified Region), SSRs (Simple Sequence Repeats) or microsatellites and SNPs (Single Nucleotide Polymorphisms). Microsatellites might have been the DNA molecular markers most used in ecology and population genetics (Guichoux et al. 2011). The availability of an increasing number of genomes in the databases has increased the generation of inexpensive microsatellite markers (Ge et al. 2013).

It has been traditionally considered that two individuals belong to the same species if they are able to mate and produce fully fertile offspring. However, nowadays this definition of species is being questioned. About 20 concepts based on genetic observations, biology, ecology, evolution and phylogenetics are considered to determine speciation, and only half of them recognize the processes of reproduction and competition as factors that contribute to the process of species evolution (Behura 2006). The speciation concept has benefited from the development of molecular techniques as isozymes and DNA markers, like RAPD and RFLP (Landry et al. 1993; Antolin et al. 1996; Silva et al. 1999; Unruh and Woolley 1999; Zhu and Greenstone 1999; Zhu et al. 2000). RAPD, RFLP and AFLP markers, as well as microsatellites, have also been proven to be effective for species differentiation and population genetic studies. The integrative taxonomy, which takes into account classical taxonomy and the amplification of DNA fragments, has proved to be very

useful in the differentiation of arthropod species (Ros and Breeuwer 2007; Matsuda et al. 2012; Castañé et al. 2013; Tyagi et al. 2015). These techniques are also becoming generalized in taxonomic studies of the entomofauna that lives in agricultural ecosystems (Gomez-Polo et al. 2013, 2014). However, in some cases, they are not informative enough to establish the real phylogenetic relationships among groups. Sequencing has arisen as the definitive technique for this purpose. The sequence analyses of nuclear ribosomal DNA (nrDNA) and mitochondrial DNA (mtDNA) have been used in these studies. The information they provide can contribute to answer relevant taxonomic questions for the biological control of pests, where cryptic and genetically close species are common (Hurtado et al. 2008a). There are no fixed rules to establish the amount of genetic variation associated with speciation. As in the case of taxonomic information, the boundaries between species should be the sum of the evidence obtained from several sources, including geographical, morphological, behavioural and genetic data.

8.3.2 Molecular Markers for Phylogeny and Phylogeography

Nuclear, ribosomal and mitochondrial DNA sequences are often used as molecular markers in phylogenetic studies of insects and mites (Yang et al. 2011). The nuclear ribosomal DNA and, in particular, the Internal Transcribed Spacer 2 (ITS2) is one of the most important markers in molecular systematics and evolution (Yli-Mattila et al. 2000; Ben-David et al. 2007; Hurtado et al. 2008a). For phylogenies at low taxonomic levels, the ITS2 region is usually recommended, as well as the 18S, 28S and the mitochondrial cytochrome oxidase I gene (COI). Compared with the ITS regions, the COI shows a low variability that limits the resolution of phylogenetic relationships to intraspecific and interspecific level (Yang et al. 2011). In general, the mitochondrial genome is highly conserved at the level of family and genus (Yuan et al. 2010). Mitochondrial sequences are greatly appreciated in taxon differentiation with a relatively recent divergence not exceeding several million years (Dabert 2006). These sequences are widely used as phylogenetic markers and their use to clarify the phylogeny in mites has increased during the last years (Gu et al. 2014). For example, several phylogenetic studies use the COI gene as a molecular marker in tetranyquids (Navajas and Boursot 2003; Ros and Breeuwer 2007) and other mite families, such as phytoseiids (Jeyaprakash and Hoy 2009; Tixier et al. 2011) or astigmatids (Yang et al. 2011). Pérez-Sayas et al. (2015) have established species boundaries, species barcodes and phylogenetic relationship among several clades of those groups. Sequence identity with a 10% of divergence has been established as species delimitation character, allowing to establish a barcode dataset for Acari identification (Pérez-Sayas 2016).

Phylogeographic studies within and among species are a very effective way to study the origins and impact of the colonization process. It is not easy to determine how colonization of invasive species has taken place. It is difficult to know if a pest has arrived due to human intervention or it has appeared as an explosion of an

already existing species, which was in a very low population density. If new habitats are colonized by individuals that come from an initial introduction event and their subsequent expansion, there will be a bottleneck. Over time, the settler population will continue to diverge genetically from the initial population, with a loss of alleles. This process corresponds to a genetic drift, which represents one of the engines that drives evolution. With a sufficient number of generations after isolation, the alleles found in the invading population will become monophyletic. That means that they share an ancestral allele, which can be used to trace back the population of origin. The degree of monophyly depends on two parameters: the effective population size and the number of generations since the invasive species was separated from the initial population (Roderick and Navajas 2003). Molecular markers can provide information about the origin and spread of a pest or a natural enemy. One of the reasons why mtDNA has been successfully used in phylogenetic studies is because it is very informative when it is used with restriction enzymes. The mtDNA is also much more sensitive than other markers for the detection of bottlenecks, since the effective size of mtDNA is one fourth of the chromosomal genes, thereby genetic structure changes of the population are better detected (Roehrdanz et al. 2002). The mitochondrial COI gene has been used in populations with founder effects and bottlenecks, such as those resulting from invasion processes (Gillespie and Roderick 2002). In the case of phytophagous pest mites, several studies have used molecular markers to determine genetic diversity, as well as population or species differentiation and invasion history (Navajas et al. 2002; Bailly et al. 2004; Carbonnelle et al. 2007; Hurtado et al. 2008b; Uesugi et al. 2009; Boubou et al. 2012). Microsatellites have become one of the most popular molecular markers used for population differentiation (Guichoux et al. 2011). They are common in eukaryotic organisms and have a very high polymorphism. Several microsatellite loci have been isolated for the two-spotted spider mite, *Tetranychus urticae* and other related mite species (Nishimura et al. 2003; Uesugi and Osakabe 2007; Sabater-Muñoz et al. 2012) and they have already been used for mite population genetic studies (Bailly et al. 2004; Li et al. 2009; Aguilar-Fenollosa et al. 2012, 2016; Pascual-Ruiz et al. 2014).

8.3.3 Molecular Markers for Biological Control

Biotechnology and genomics have become an indispensable tool also in studies related to crop protection and biological control. New insights into the ecology, population structure and biological control of pest species have benefited from the application of these molecular techniques, which have increased the speed, sensitivity and accuracy of pest detection, diagnosis and management.

In classical biological control, natural enemies are usually searched where the pest is indigenous. Species that have a worldwide distribution are expected to have high levels of genetic variability, however, when considering introduced control agents, its genetic diversity can drop by means of character fixation and heterozygotes loss. With the use of DNA-based markers, natural enemies can be studied like

the pest, to determine the population structure, which allows selecting the appropriate races or biotypes of the control agent adapted to the local race of the target pest. The phylogenetic relationships can determine changes or adaptations of the biological control agent to the host. It can also identify patterns of establishment and expansion of the introduced biological control agent. Moreover, to maximize the success of introduction it is necessary to mass-produce and release natural enemy populations with a high genetic variability. In addition, a population introduced into a new habitat has to compete with a wide range of organisms that may not be present at their place of origin. In general, an appropriate estimate of genetic variability can help in determining the survival potential, adaptation to mass-breeding process, and release in the field. Thus, predators, parasitoids or entomopathogens may have a greater chance to adapt to new habitat, respond to new environmental conditions and control the pest properly. However, mass breeding is a limiting process that could induce an increase of consanguinity and a decrease of genetic variability by genetic drift. In such process, some of the natural enemy traits (as insecticide resistance, host-detection ability, etc.) can be lost and the biological control program could be compromised. Molecular markers can provide information on whether these traits are going to remain unchanged during rearing (quality control system) and also become powerful tools for monitoring releases (Roderick and Navajas 2003).

Sequence determination is the ultimate polymorphism detection system, as it allows to clearly identify a single individual. In the last decade, HTS is having an enormous impact on biological sciences allowing the determination of genome variation within a species or a population. Comparative genome analysis of the forthcoming genome sequences will allow the identification of highly conserved gene families, conserved regulatory elements, repeated elements, ingested prey, symbionts, etc., on which new markers will be designed (Kaufman et al. 2002; Belosludtsev et al. 2004). Furthermore, new targets for pest control based on interference RNA (RNAi) on species-specific genes, symbionts suppression through plant biotechnology or by new generation pesticides will become available in the near future (Wang et al. 2011).

8.3.4 Arthropod Trophic Interactions

Monoculture systems, where a plant species is grown in a wide area, could be perceived as a simplification of a natural ecosystem. In these systems, the concept of trophic chain has been traditionally assumed as the relationship between a phytophagous pest and a single biological control agent. However, the concept of trophic relationships in biological control had been changing towards the existence of multiple ecological interactions that form complex networks (González-Chang et al. 2016). The methodology traditionally used for establishing relationships between plants, pests and natural enemies were tedious and provided limited information. In some cases, it also depended on the nature of the natural enemy (predator or parasitoid) and feeding regime (chewing or sucking). In the past, the effect caused

by predators was usually determined by direct observation in the field, being sometimes replaced, in the case of chewing predators, by dissection and the subsequent morphological identification of the solid residues present in their gut (Sunderland et al. 1987; Breene et al. 1990). In sucking predators, which suck the liquid content of the prey, as many polyphagous predators do (i.e. bugs, spiders, etc.), predation can be evaluated by the presence of the remaining exoskeletons of the predated preys in the field, although in some cases it is difficult or impossible. Therefore, the analysis, identification and quantification of these multitrophic relationships using traditional methods based on direct observation or dissection followed by visual identification of stomach contents was complicated. However, studies that use molecular methods to analyse prey DNA within predators, allow shedding light on the understanding of these multitrophic dynamics and their application to improve biological control (Furlong 2015; González-Chang et al. 2016; Gurr and You 2016). The molecular techniques used can be classified into two main groups, those used for protein detection and those for DNA detection. The first are based either on detection of isozymes, as mentioned above or on the development of specific polyclonal or monoclonal antibodies followed by a subsequent analysis by serological techniques based on antigen-antibody reaction, like ELISA (*Enzyme-Linked Immunosorbent Assay*) (Greenstone 1996; Agustí et al. 1999a). These serological techniques allow a fast analysis of a large number of predators, although the development of the antibodies is very expensive and laborious (Symondson et al. 1999). They are also specific to only one developmental stage (egg, larva or adult) of the pest, underestimating the total predatory activity on a particular prey. In general, the most common antibodies have been developed for the detection of the eggs vitelline (main egg protein), allowing the detection of only eggs and gravid females, and excluding nymphs and adult males. For this reasons, the use DNA-based methodologies to analyse predator gut contents began to gain ground. Although serial analysis of PCR-based markers can be a bit more laborious, the development of molecular markers is much faster, cheaper and simpler than monoclonal antibodies. These techniques allow knowing the real prey spectrum of a polyphagous predator and/or to determine the range of predators that can feed on a particular pest species.

Once a target gene sequence of each agent involved in the food chain is identified, it is necessary to design species-specific primers for the detection of prey within the predator. It is important that the primers are specific to each prey in order to avoid the amplification of other non-target species. Prey DNA detection is possible if the amplified fragment is present in multiple copies and if the amplified sequences are short, as they are degraded during digestion and can be detected for a longer time after ingestion (Agustí et al. 1999b; Zaidi et al. 1999). Therefore, multicopy regions, such as the COI and COII mitochondrial genes, have been used (Agustí et al. 2003a, b; Chen et al. 2000).

The first studies using DNA-based techniques related with biological control studies in agroecosystems were those of Agustí et al. (1999b, 2000), in which two specific molecular markers were developed to study predation by Miridae bugs on two pest species of tomato crops in the Mediterranean area, the lepidopteran *Helicoverpa armigera* (Hübner) and the whitefly *Trialeurodes vaporariorum*

Westwood. After that, these techniques have been used in numerous studies to evaluate predation of a large number of pest species and to identify potential biological control agents (King et al. 2008; Moreno-Ripoll et al. 2012; Romeu-Dalmau et al. 2012). For example, the generalist predators *Pardosa cribata* Simon (Araneae: Lycosidae) and *Pseudophonus rufipes* (DeGeer) (Coleoptera: Carabidae) present in citrus orchards were identified as main predators of the soil stages (L3, pupae and newly emerged adult) of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Monzó et al. 2010, 2011).

Commonly, in the study of trophic relationships related to biological control, a great variety of predatory and prey species are involved, which requires the design of several species-specific pairs of primers to detect the potential ingested prey. This may increase the number of necessary PCR reactions, the time and the material resources needed. For this reason, the use multiplex PCR to simultaneously detect multiple preys within a predator, started to be used (Harper et al. 2005). This technique is capable of using several pairs of primers in a single amplification reaction for the simultaneous detection of several prey species (King et al. 2011; Pompanon et al. 2012; Sint et al. 2012; Kamenova et al. 2017). It has been recently used to study the trophic relationships between *T. urticae* and *P. citri* and their natural enemies, particularly phytoseid mites (Pérez-Sayas et al. 2015), as well as to study predation by the five most common phytoseid species (*Euseius stipulatus* (Athias-Henriot), *Phytoseiulus persimilis* Athias-Henriot, *Neoseiulus californicus* (McGregor), *Neoseiulus barkeri* Hughes, *Typhlodromus phialatus* Athias-Henriot) on tetranychids and trips (Gómez-Martínez et al. 2019).

Nevertheless, PCR multiplex may be limited to detect other food sources not considered in the multiplex PCR design. In this case, the use of HTS technologies, which is based on the massive amplification of DNA fragments using universal primers, can be very useful, because of being able to amplify all prey DNA and provide a much more detailed information about the trophic networks. This methodology may show a higher complexity of the ecosystems, particularly about the importance of alternative food sources or intragremial predation (Wirta et al. 2014; Gómez-Polo et al. 2015). This method is particularly suited to agricultural studies where the focus is often on the predation of one pest species by several potential predator species (Boyer et al. 2016). For example, a food web comprising seven species over two trophic levels in a Mediterranean lettuce crop has been reconstructed using HTS (Gomez-Polo et al. 2016). However, HTS could also have some limitations. Although these methods allow detecting the DNA of all ingested prey species, it is necessary to have a complete sequence database with the DNA sequences of the whole spectrum of potential prey species in order to detect and identify them. When a DNA sequence of a particular species is not available, this prey cannot be detected and identified. Fortunately, sequence databases, such as GenBank, contain a large number of arthropod sequences and increase day by day. In addition, it has been described that these techniques can produce biases when quantifying the number of prey that the predator has actually consumed (Deagle et al. 2013). Therefore, most of these studies provide only qualitative results, as it happens with conventional PCR. Nevertheless, these new molecular methods are

very powerful tools in order to evaluate existing trophic networks in agricultural ecosystems and their impact on biological control.

8.4 Conclusions

New molecular techniques have been developed, optimized and validated in the last years with different applications to pest and pathogen detection and identification. The combination of traditional and molecular techniques permits to characterize, detect, identify and quantify different pests and pathogens. The limit of detection of pathogens, by comparing the molecular techniques, can reach nanograms of DNA for PCR, picograms of DNA for biosensors, and femtograms of DNA for qPCR and digital PCR. HTS technologies are having an enormous impact on biological sciences, allowing the determination of genome variation within a species or a population. Comparative analysis of the genome sequences allows the identification of highly conserved gene families, conserved regulatory elements, repeated elements, uncultured pathogens, new species, symbionts, etc., on which new markers could be designed. On the other side, the use of field techniques, such as LAMP and portable platforms, is a promising tool to early and quickly detect pests and a useful decision support system for appropriate pest and disease management.

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References

Abd-Elsalam K, Bahkali A, Moslem M, Amin OE, Niessen L (2011) An optimized protocol for DNA extraction from wheat seeds and Loop-Mediated Isothermal Amplification (LAMP) to detect *Fusarium graminearum* contamination of wheat grain. Int J Mol Sci 12(6):3459–3472. https://doi.org/10.3390/ijms12063459

Adams IP, Glover RH, Monger WA, Mumford R, Jackeviciene E, Navalinskiene M, Samuitiene M, Boonham N (2009) Next-generation sequencing and metagenomic analysis: a universal diagnostic tool in plant virology. Mol Plant Pathol 10(4):537–545. https://doi.org/10.1111/j.1364-3703.2009.00545.x

Adams IP, Glover R, Souza-Richards R, Bennett S, Hany U, Boonham N (2013a) Complete genome sequence of arracacha virus B: a novel cheravirus. Arch Virol 158(4):909–913. https://doi.org/10.1007/s00705-012-1546-x

Adams IP, Miano DW, Kinyua ZM, Wangai A, Kimani E, Phiri N, Reeder R, Harju V, Glover R, Hany U, Souza-Richard R, Deb Nath P, Nixon T, Fox A, Barnes A, Smith J, Skelton A, Thwaites R, Mumford R, Boonham N (2013b) Use of next-generation sequencing for the identification and characterization of *Maize chlorotic mottle virus* and *Sugarcane mosaic*

virus causing maize lethal necrosis in Kenya. Plant Pathol 62(4):741–749. https://doi.org/10.1111/j.1365-3059.2012.02690.x

Aguilar-Fenollosa E, Pina T, Gómez-Martínez MA, Hurtado MA, Jacas JA (2012) Does host adaptation of *Tetranychus urticae* populations in clementine orchards with a *Festuca arundinacea* cover contribute to a better natural regulation of this pest mite? Entomol Exp Appl 144(2):181–190. https://doi.org/10.1111/j.1570-7458.2012.01276.x

Aguilar-Fenollosa E, Rey-Caballero J, Blasco JM, Segarra-Moragues JG, Hurtado Ruiz MA, Jaques Miret JA (2016) Patterns of ambulatory dispersal in *Tetranychus urticae* can be associated with jost plant specialization. Exp Appl Acarol 68(1):1–20. https://doi.org/10.1007/s10493-015-9969-1

Agustí N, Aramburu J, Gabarra R (1999a) Immunological detection of *Helicoverpa armigera* (Lepidoptera: Noctuidae) ingested by heteropteran predators: time related decay and effect of meal size on detection period. Ann Entomol Soc Am 92(1):56–62. https://doi.org/10.1093/aesa/92.1.56

Agustí N, de Vicente MC, Gabarra R (1999b) Development of sequence amplified characterized region (SCAR) markers of *Helicoverpa armigera*: a new polymerase chain reaction-based technique for predator gut analysis. Mol Ecol 8(9):1467–1474. https://doi.org/10.1046/j.1365-294x.1999.00717.x

Agustí N, de Vicente C, Gabarra R (2000) Developing SCAR markers to study predation on *Trialeurodes vaporariorum*. Insect Mol Biol 9(3):263–268. https://doi.org/10.1046/j.1365-2583.2000.00185.x

Agustí N, Unruh TR, Welter SC (2003a) Detecting *Cacopsylla pyricola* (Hemiptera: Psyllidae) in predator guts by using COI mitochondrial markers. B Entomol Res 93(3):179–185. https://doi.org/10.1079/BER2003236

Agustí N, Shayler SP, Harwood JD, Vaughan IP, Sunderland KD, Symondson WOC (2003b) Collembola as alternative prey sustaining spiders in arable ecosystems: prey detection within predators using molecular markers. Mol Ecol 12(12):3467–3475. https://doi.org/10.1046/j.1365-294X.2003.02014.x

Al Rwahnih M, Daubert S, Golino D, Islas C, Rowhani A (2015) Comparison of next-generation sequencing versus biological indexing for the optimal detection of viral pathogens in grapevine. Phytopathology 105(6):758–763. https://doi.org/10.1094/PHYTO-06-14-0165-R

Amaral Carneiro G, Matic S, Ortu G, Garibaldi A, Spadaro D, Gullino ML (2017) Development and validation of a TaqMan real time PCR assay for the specific detection and quantification of *Fusarium fujikuroi* in rice plants and seeds. Phytopathology 107(7):885–892. https://doi.org/10.1094/PHYTO-10-16-0371-R

Antolin MF, Guertin DS, Petersen JJ (1996) The origin of gregarious *Muscidifurax* (Hymenoptera: Pteromalidae) in North America: an analysis using molecular markers. Biol Control 6(1):76–82. https://doi.org/10.1006/bcon.1996.0010

Asselin JAE, Bonasera JM, Beer SV (2016) PCR primers for detection of *Pantoea ananatis*, *Burkholderia* spp., and *Enterobacter* sp. from onion. Plant Dis 100(4):836–846. https://doi.org/10.1094/PDIS-08-15-0941-RE

Ayukawa Y, Komatsu K, Kashiwa T, Akai K, Yamada M, Teraoka T, Arie T (2016) Detection and differentiation of *Fusarium* oxysporum f. sp. *lycopersici* race 1 using loop-mediated isothermal amplification with three primer sets. Lett Appl Microbiol 63(3):202–209. https://doi.org/10.1111/lam.12597

Bailly X, Migeon A, Navajas M (2004) Analysis of microsatellite variation in the spider mite pest *Tetranychus turkestani* (Acari: Tetranychidae) reveals population genetic structure and raises questions about ecological factors. Biol J Linn Soc 82(1):69–78. https://doi.org/10.1111/j.1095-8312.2004.00316.x

Begerow D, Nilsson H, Unterseher M, Maier W (2010) Current state and perspectives of fungal DNA barcoding and rapid identification procedures. Appl Microbiol Biot 87(1):99–108. https://doi.org/10.1007/s00253-010-2585-4

Behura SK (2006) Molecular marker systems in insects: current trends and future avenues. Mol Ecol 15(11):3087–3113. https://doi.org/10.1111/j.1365-294X.2006.03014.x

Belosludtsev YY, Bowerman D, Weil R, Marthandan N, Balog R, Luebke K, Lawson J, Johnston SA, Lyons CR, Obrien K, Garner HR, Powdrill TF (2004) Organism identification using a genome sequence-independent microarray probe set. BioTechniques 37(4):654–660

Ben-David T, Melamed S, Gerson U, Morin S (2007) ITS2 sequences as barcodes for identifying and analyzing spider mites (Acari: Tetranychidae). Exp Appl Acarol 41(3):169–181. https://doi.org/10.1007/s10493-007-9058-1

Bertoldo C, Gilardi G, Spadaro D, Gullino ML, Garibaldi A (2015) Genetic diversity and virulence of Italian strains of *Fusarium oxysporum* isolated from *Eustoma grandiflorum*. Eur J Plant Pathol 141(1):83–97. https://doi.org/10.1007/s10658-014-0526-2

Bimboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7(6):1513–1523. https://doi.org/10.1093/nar/7.6.1513

Boonham N, González Pérez L, Mendez MS, Lilia Peralta E, Blockley A, Walsh K, Barker I, Mumford RA (2004) Development of a real-time RT-PCR assay for the detection of *Potato spindle tuber viroid*. J Virol Methods 116(2):139–146. https://doi.org/10.1016/j.jviromet.2003.11.005

Boonham N, Tomlinson J, Mumford R (2007) Microarrays for rapid identification of plant viruses. Annu Rev Phytopathol 45:307–328. https://doi.org/10.1146/annurev.phyto.45.062806.094349

Boonham N, Kreuze J, Winter S, van der Vlug R, Bergervoet J, Tomlinson J, Mumford R (2014) Methods in virus diagnostics: from ELISA to next generation sequencing. Virus Res 186:20–31. https://doi.org/10.1016/j.virusres.2013.12.007

Boubou A, Migeon A, Roderick GK, Auger P, Cornuet JM, Magalhães S, Navajas M (2012) Test of colonisation scenarios reveals complex invasion history of the red tomato spider mite *Tetranychus evansi*. PLoS One 7(4):e35601. https://doi.org/10.1371/journal.pone.0035601

Boyer S, Snyder WE, Wratten SD (2016) Molecular and isotopic approaches to food webs in agroecosystems. Food Webs 9:1–3. https://doi.org/10.1016/j.fooweb.2016.07.004

Breene RG, Sweet MH, Olson JK (1990) Analysis of the gut contents of Naiads of *Enallagma civile* (Odonata, Coenagrionidae) from a Texas pond. J Am Mosq Control 6(3):547–548

Carbonnelle S, Hance T, Migeon A, Baret P, Cros-Arteil S, Navajas M (2007) Microsatellite markers reveal spatial genetic structure of *Tetranychus urticae* (Acari: Tetranychidae) populations along a latitudinal gradient in Europe. Exp Appl Acarol 41(4):225–241. https://doi.org/10.1007/s10493-007-9068-z

Castañé C, Agustí N, Arnó J, Gabarra R, Riudavets J, Comas-Angelet J, Alomar O (2013) Taxonomic identification of *Macrolophus pygmaeus* and *Macrolophus melanotoma* based on morphometry and molecular markers. Bull Entomol Res 103(2):204–215. https://doi.org/10.1017/S0007485312000545

Chen Y, Giles KL, Payton ME, Greenstone MH (2000) Identifying key cereal aphid predators by molecular gut analysis. Mol Ecol 9(11):1887–1898. https://doi.org/10.1046/j.1365-294x.2000.01100.x

Chen Q, Li B, Liu P, Lan C, Zhan Z, Weng Q (2013) Development and evaluation of specific PCR and LAMP assays for the rapid detection of *Phytophthora melonis*. Eur J Plant Pathol 137(3):597–607. https://doi.org/10.1007/s10658-013-0273-9

Chomczynski P, Rymaszewski M (2006) Alkaline polyethylene glycol-based method for direct PCR from bacteria, eukaryotic tissue samples, and whole blood. BioTechniques 40(4):454–458. https://doi.org/10.2144/000112149

Dabert M (2006) DNA markers in the phylogenetics of Acari. Biol Lett 43:97–107

van Dam P, Fokkens L, Schmidt SM, Linmans JH, Kistler HC, Ma JL, Rep M (2016) Effector profiles distinguish *formae speciales* of *Fusarium oxysporum*. Environ Microbiol 18(11):4087–4102. https://doi.org/10.1111/1462-2920.13445

Danks C, Barker I (2000) On-site detection of plant pathogens using lateral-flow devices. EPPO Bull 30(3–4):421–426. https://doi.org/10.1111/j.1365-2338.2000.tb00922.x

Deagle BE, Thomas AC, Shaffer AK, Trites AW, Jarman SN (2013) Quantifying sequence proportions in a DNA-based diet study using ion torrent amplicon sequencing: which counts count? Mol Ecol Resour 13(4):620–633. https://doi.org/10.1111/1755-0998.12103

Dong Z, Liu P, Li B, Chen G, Weng Q, Chen Q (2015) Loop-mediated isothermal amplification assay for sensitive and rapid detection of *Phytophthora capsici*. Can J Plant Pathol 37(4):485–494. https://doi.org/10.1080/07060661.2015.1119733

Dreo T, Pirc M, Ramšak Ž, Pavšic J, Milavec M, Zel J, Gruden K (2014) Optimising droplet digital PCR analysis approaches for detection and quantification of bacteria: a case study of fire blight and potato brown rot. Anal Bioanal Chem 406(26):6513–6528. https://doi.org/10.1007/s00216-014-8084-1

Duan YB, Ge CY, Zhang XK, Wang JX, Zhou MG (2014) Development and evaluation of a novel and rapid detection assay for *Botrytis cinerea* based on Loop-Mediated Isothermal Amplification. PLoS One 9:e111094. https://doi.org/10.1371/journal.pone.0111094

Franco Ortega S, Tomlinson J, Hodgetts J, Spadaro D, Gullino ML, Boonham N (2018a) Development of loop-mediated isothermal amplification assays for the detection of seed-borne fungal pathogens, *Fusarium fujikuroi* and *Magnaporthe oryzae*, in rice seeds. Plant Dis 102:1549–1558. https://doi.org/10.1094/PDIS-08-17-1307-RE

Franco Ortega S, Tomlinson J, Gilardi G, Spadaro D, Gullino ML, Garibaldi A, Boonham N (2018b) Rapid detection of *Fusarium oxysporum* f.sp. *lactucae* on soil, lettuce seeds and plants using loop-mediated isothermal amplification. Plant Pathol 67:1462–1473. https://doi.org/10.1111/ppa.12855

Fribourg CE, Nakashima J (1984) An improved latex agglutination test for routine detection of potato viruses. Potato Res 27(2):237–249

Frisvad JC (2014) Taxonomy, chemodiversity, and chemoconsistency of *Aspergillus*, *Penicillium*, and *Talaromyces* species. Front Microbiol 5:773. https://doi.org/10.3389/fmicb.2014.00773

Furlong MJ (2015) Knowing your enemies: integrating molecular and ecological methods to assess the impact of arthropod predators on crop pests. Insect Sci 22(1):6–19. https://doi.org/10.1111/1744-7917.12157

Ge C, Sun JT, Cui YN, Hong XY (2013) Rapid development of 36 polymorphic microsatellite markers for *Tetranychus truncatus* by transferring from *Tetranychus urticae*. Exp Appl Acarol 61(2):195–212. https://doi.org/10.1007/s10493-013-9684-8

Gherbawy Y, El-Deeb B, Hassan S, Altalhi A, Rai M (2008) Molecular markers for *Fusarium oxysporum formae speciales* characterization. In: Gherbawy Y, Mach R, Rai M (eds) Current advances in molecular mycology. Novapublishers, NewYork. Chapter 6

Ghosh R, Nagavardhini A, Sengupta A, Sharma M (2015) Development of Loop-Mediated Isothermal Amplification (LAMP) assay for rapid detection of *Fusarium oxysporum* f. sp. *ciceris* – wilt pathogen of chickpea. BMC Res Notes 8:997. https://doi.org/10.1186/s13104-015-0997-z

Gilardi G, Webb K, Ortu G, Gullino ML, Garibaldi A (2016) Real-time PCR for rapid in planta detection of *Plectosphaerella cucumerina* on wild rocket (*Diplotaxis tenuifolia*). Phytopathol Mediterr 55:285–292

Gillespie RG, Roderick GK (2002) Arthropods on islands: colonization, speciation, and conservation. Annu Rev Entomol 47:595–632. https://doi.org/10.1146/annurev.ento.47.091201.145244

Gómez-Martinez MA, Pina T, Aguilar-Fenollosa E, Jaques JA, Hurtado MA. (2019) Tracking mite trophic interactions by multiplex PCR. Pest Manag Sci. https://doi.org/10.1002/ps.5555

Gómez-Polo P, Alomar O, Castañé C, Riudavets J, Agustí N (2013) Identification of *Orius* spp. (Hemiptera: Anthocoridae) in vegetable crops using molecular techniques. Biol Control 67(3):440–445. https://doi.org/10.1016/j.biocontrol.2013.09.017

Gómez-Polo P, Traugott M, Alomar O, Castañé C, Rojo S, Agustí N (2014) Molecular identification of six common syrphid species of Mediterranean vegetable crops and their parasitism using multiplex PCR. J Pest Sci 87:371–378

Gómez-Polo P, Alomar O, Castañé C, Lundgren JG, Piñol J, Agustí N (2015) Molecular assessment of predation by hoverflies (Diptera: Syrphidae) in Mediterranean lettuce crops. Pest Manag Sci 71(9):1219–1227. https://doi.org/10.1002/ps.3910

Gomez-Polo P, Alomar O, Castañé C, Agustí N (2016) Molecular tracking of arthropod predator–prey interactions in Mediterranean lettuce crops. Food Webs 9:18–24. https://doi.org/10.1016/j.fooweb.2016.01.001

González-Chang M, Wratten SD, Lefort MC, Boyer S (2016) Food webs and biological control. A review of molecular tools used to reveal trophic interactions in agricultural systems. Food Webs 9:4–11. https://doi.org/10.1016/j.fooweb.2016.04.003

Greenstone MH (1996) Serological analysis of arthropod predation: past, present and future. In: Symondson WOC, Liddell JE (eds) The ecology of agricultural pests. Chapman & Hall, Londres, pp 267–321

Greenstone MH (2006) Molecular methods for assessing insect parasitism. B Entomol Res 96(1):1–13. https://doi.org/10.1079/BER2005402

Gu XB, Liu GH, Song HQ, Liu TY, Yang GY, Zhu XQ (2014) The complete mitochondrial genome of the scab mite *Psoroptes cuniculi* (Arthropoda: Arachnida) provides insights into Acari phylogeny. Parasites Vector 7:340. https://doi.org/10.1186/1756-3305-7-340

Guichoux E, Lagache L, Wagner S, Chaumeil P, Léger P, Lepais O, Lepoittevin C, Malausa T, Revardel E, Salin F, Petit RJ (2011) Current trends in microsatellite genotyping. Mol Ecol Resour 11(4):591–611. https://doi.org/10.1111/j.1755-0998.2011.03014.x

Gurr GM, You M (2016) Conservation biological control of pests in the molecular era: new opportunities to adress old constraints. Front Plant Sci 6:1256. https://doi.org/10.3389/fpls.2015.01255

Gutiérrez-Aguirre I, Rački N, Dreo T, Ravnikar M (2015) Droplet digital PCR for absolute quantification of pathogens. Methods Mol Biol 1302:331–347. https://doi.org/10.1007/978-1-4939-2620-6_24

Haegi A, Catalano V, Luongo L, Vitale S, Scotton M, Ficcadenti N, Belisario A (2013) A newly developed real-time PCR assay for detection and quantification of *Fusarium* oxysporum and its use in compatible and incompatible interactions with grafted melon genotypes. Phytopathology 103(8):802–810. https://doi.org/10.1094/PHYTO-11-12-0293-R

Harper GL, King RA, Dodd CS, Harwood JD, Glen DM, Bruford MW, Symondson WOC (2005) Rapid screening of invertebrate predators for multiple prey DNA targets. Mol Ecol 14:819–827. https://doi.org/10.1111/j.1365-294X.2005.02442.x

Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6(10):986–994. https://doi.org/10.1101/gr.6.10.986

Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, Bright IJ, Lucero MY, Hiddessen AL, Legler TC, Kitano TK, Hodel MR, Petersen JF, Wyatt PW, Steenblock ER, Shah PH, Bousse LJ, Troup CB, Mellen JC, Wittmann DK, Erndt NG, Cauley TH, Koehler RT, So AP, Dube S, Rose KA, Montesclaros L, Wang S, Stumbo DP, Hodges SP, Romine S, Milanovich FP, White HE, Regan JF, Karlin-Neumann GA, Hindson CM, Saxonov S, Colston BW (2011) High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem 83(22):8604–8610. https://doi.org/10.1021/ac202028g

Hirano Y, Arie T (2009) Variation and phylogeny of *Fusarium oxysporum* isolates based on nucleotide sequences of polygalacturonase genes. Microbes Environ 24(2):113–120. https://doi.org/10.1264/jsme2.ME08554

Hodgetts J, Karamura G, Johnson G, Hall J, Perkins K, Beed F, Nakato V, Grant M, Studholme DJ, Boonham N, Smith J (2015) Development of a lateral flow device for in-field detection and evaluation of PCR-based diagnostic methods for *Xanthomonas campestris* pv. m*usacearum*, the causal agent of banana Xanthomonas wilt. Plant Pathol 64(3):559–567. https://doi.org/10.1111/ppa.12289

Hou Y, Ma X, Wan W, Long N, Zhang J, Tan Y, Duan S, Zeng Y, Dong Y (2016) Comparative genomics of pathogens causing brown spot disease of tobacco: *Alternaria longipes* and *Alternaria alternata*. PLoS One 11:e0155258. https://doi.org/10.1371/journal.pone.0155258

Hurtado M, Agustí N, Sabater-Muñoz B (2008a) Aplicación de técnicas moleculares al control biológico de plagas. In: Jacas JA, Urbaneja A (eds) Control biológico de plagas agrícolas. Futuro del control biológico. Phytoma-España, S.L, Phytoma-España, pp 453–468

Hurtado MA, Ansaloni T, Cros-Arteil S, Jacas JA, Navajas M (2008b) Sequence analysis of the ribosomal internal transcribed spacers region in spider mites (Prostigmata: Tetranychidae) occurring in citrus orchards in Eastern Spain: use for species discrimination. Ann Appl Biol 153(2):167–174. https://doi.org/10.1111/j.1744-7348.2008.00250.x

Hussain T, Singh BP, Anwar F (2017) Development of specific marker for PCR diagnostic of late blight of potato caused by *Phytophthora infestans* using RAPD based SCAR methodology. J Saudi Soc Agric Sci 16(4):299–305. https://doi.org/10.1016/j.jssas.2015.10.001

Jeyaprakash A, Hoy MA (2009) First divergence time estimate of spiders, scorpions, mites and ticks (subphylum: Chelicerata) inferred from mitochondrial phylogeny. Exp Appl Acarol 47(1):1–18. https://doi.org/10.1007/s10493-008-9203-5

Julich S, Riedel M, Kielpinski M, Urban M, Kretschmer R, Wagner S, Fritzsche W, Henkel T, Möller R, Werres S (2011) Development of a lab-on-a-chip device for diagnosis of plant pathogens. Biosens Bioelectron 26(10):4070–4075. https://doi.org/10.1016/j.bios.2011.03.035

Kamenova S, Bartley TJ, Bohan D, Boutain JR, Colautti RI, Domaizon I, Fontaine C, Lemainque A, Le Viol I, Mollot G, Perga ME, Ravigné V, Massol F (2017) Invasions toolkit: current methods for tracking the spread and impact of invasive species. Adv Ecol Res 56:85–182. https://doi.org/10.1016/bs.aecr.2016.10.009

Kaufman TC, Severson DW, Robinson GE (2002) The Anopheles genome and comparative insect genomics. Science 298(5591):97–98. https://doi.org/10.1126/science.1077901

King RA, Read DS, Traugott M, Symondson WOC (2008) Molecular analysis of predation: a review of best practice for DNA-based approaches. Mol Ecol 17(4):947–963. https://doi.org/10.1111/j.1365-294X.2007.03613.x

King RA, Moreno-Ripoll R, Agustí N, Shayler SP, Bell JR, Bohan DA, Symondson WOC (2011) Multiplex reactions for the molecular detection of predation on pest and non-pest invertebrates in agroecosystems. Mol Ecol Resour 11(2):370–373. https://doi.org/10.1111/j.1755-0998.2010.02913.x

Koo C, Malapi-Wight M, Kim HS, Cifci OS, Vaughn-Diaz VL, Ma B, Kim S, Abdel-Raziq H, Ong K, Jo YK, Gross DC, Shim WB (2013) Development of a real-time microchip PCR system for portable plant disease diagnosis. PLoS One 8:e82704. https://doi.org/10.1371/journal.pone.0082704

Kordalewska M, Brillowska-Dąbrowska A, Jagielski T, Dworecka-Kaszak B (2015) PCR and real-time PCR assays to detect fungi of *Alternaria alternata* species. Acta Biochim Pol 62:707–712

Krawczyk K, Uszczyńska-Ratajczak B, Majewska A, Borodynko-Filas N (2017) DNA microarray-based detection and identification of bacterial and viral pathogens of maize. J Plant Dis Protect 124(6):577–583. https://doi.org/10.1073/pnas.242579699

Kristensen R, Gauthier G, Berdal KG, Hamels S, Remacle J, Holst-Jensen A (2007) DNA microarray to detect and identify trichothecene- and moniliformin-producing *Fusarium* species. J Appl Microbiol 102(4):1060–1070. https://doi.org/10.1111/j.1365-2672.2006.03165.x

Kunjeti SG, Anchieta A, Martin FN, Choi YJ, Thines M, Michemore RW, Koike ST, Tsuchida C, Mahaffee W, Subbarao KV, Klosterman SJ (2016) Detection and quantification of *Bremia lactucae* by spore trapping and quantitative PCR. Phytopathology 106(11):1426–1437. https://doi.org/10.1094/PHYTO-03-16-0143-R

Landry BS, Dextraze L, Boivin G (1993) Random amplified polymorphic DNA markers for DNA fingerprinting and genetic variability assessment of minute parasitic wasp species (Hymenoptera, Mymaridae and Trichogrammatidae) used in biological control programs of phytophagous insects. Genome 36(3):580–587

Lawrence DP, Rotondo F, Gannibal PB (2016) Biodiversity and taxonomy of the pleomorphic genus *Alternaria*. Mycol Prog 15(1):3. https://doi.org/10.1007/s11557-015-1144-x

Leiminger J, Bäßler E, Knappe C, Bahnweg G, Hausladen H (2014) Quantification of disease progression of *Alternaria* spp. on potato using real-time PCR. Eur J Plant Pathol 141(2):295–309. https://doi.org/10.1016/S0378-1097(03)00759-6

Lenarčič R, Morisset D, Pirc M, Llop P, Ravnikar M, Dreo T (2014) Loop-mediated isothermal amplification of specific endoglucanase gene sequence for detection of the bacterial wilt

pathogen *Ralstonia solanacearum*. PLoS One 9(4):e96027. https://doi.org/10.1371/journal.pone.0096027

Levesque CA, De Cock A (2004) Molecular phylogeny and taxonomy of the genus *Pythium*. Mycol Res 108(12):1363–1383. https://doi.org/10.1017/S0953756204001431

Li T, Chen XL, Hong XY (2009) Population genetic structure of *Tetranychus urticae* and its sibling species *Tetranychus cinnabaribus* (Acari: Tetranychidae) in China as inferred from microsatellite data. Ann Entomol Soc Am 102(4):674–683. https://doi.org/10.1603/008.102.0412

Li M, Asano T, Suga H, Kageyama K (2011) A multiplex PCR for the detection of *Phytophthora nicotianae* and *P. cactorum* , and a survey of their occurrence in strawberry production areas of Japan. Plant Dis 95(10):1270–1278. https://doi.org/10.1094/PDIS-01-11-0076

Li B, Liu P, Xie S, Yin R, Weng Q, Chen Q (2015) Specific and sensitive detection of *Phytophthora nicotianae* by nested PCR and loop-mediated isothermal amplification assays. J Phytopathol 163(3):185–193. https://doi.org/10.1111/jph.12305

Lievens B, Justé A, Willems KA (2012) Fungal plant pathogen detection in plant and soil samples using DNA macroarrays. Methods Mol Biol 835:491–507. https://doi.org/10.1007/978-1-61779-501-5_30

Lin YH, Chen KS, Chang JY, Wan YL, Hsu CC, Huang JW, Chang PF (2010) Development of the molecular methods for rapid detection and differentiation of *Fusarium oxysporum* and *F. oxysporum* f. sp. *niveum* in Taiwan. New Biotechnol 27(4):409–418. https://doi.org/10.1016/j.nbt.2010.05.005

Ling J, Zhang J, Zeng F, Cao Y, Xie B, Yang Y (2016) Comparative genomics provide a rapid detection of *Fusarium oxysporum* f. sp. *conglutinans*. J Integr Agric 15(4):822–831. https://doi.org/10.1016/S2095-3119(15)61237-0

Loxdale HD, Lushai G (1998) Molecular markers in entomology. B Entomol Res 88(6):577–600. https://doi.org/10.1017/S0007485300054250

Martin FN, Blair JE, Coffey MD (2014) A combined mitochondrial and nuclear multilocus phylogeny of the genus *Phytophthora*. Fungal Genet Biol 66:19–32. https://doi.org/10.1016/j.fgb.2014.02.006

Matsuda T, Hinomoto N, Singh RN, Gotoh T (2012) Molecular-based identification and phylogeny of Oligonychus species (Acari: Tetranychidae). J Econ Entomol 105(3):1043–1050. https://doi.org/10.1603/EC11404

Mbofung GCY, Pryor BM (2010) A PCR-based assay for detection of *Fusarium oxysporum* f. sp. *lactucae* in lettuce seed. Plant Dis 94(7):860–866. https://doi.org/10.1094/PDIS-94-7-0860

Mbofung GY, Hong SG, Pryor BM (2007) Phylogeny of *Fusarium oxysporum* f. sp. *lactucae* inferred from mitochondrial small subunit, elongation factor 1-alpha, and nuclear ribosomal intergenic spacer sequence data. Phytopathology 97:87–98. https://doi.org/10.1094/PHYTO-97-0087

Mirmajlessi SM, Loit E, Mand M, Mansouripour SM (2016) Real-time PCR applied to study on plant pathogens: potential applications in diagnosis – a review. Plant Protect Sci 51(4):177–190. https://doi.org/10.17221/104/2014-PPS

Monzó C, Sabater-Muñoz B, Urbaneja A, Castañera P (2010) Tracking medfly predation by the wolf spider, *Pardosa cribata* Simon, in citrus orchards using PCR-based gut-content analysis. B Entomol Res 100(2):145–152. https://doi.org/10.1017/S0007485309006920

Monzó C, Sabater-Muñoz B, Urbaneja A, Castañera P (2011) The ground beetle *Pseudophonus rufipes* revealed as predator of *Ceratitis capitata* in citrus orchards. Biol Control 56(1):17–21. https://doi.org/10.1016/j.biocontrol.2010.09.004

Moreno-Ripoll R, Gabarra R, Symondson WOC, King RA, Agustí N (2012) Trophic relationships between predators, whiteflies and their parasitoids in tomato greenhouses: a molecular approach. B Entomol Res 102(4):415–423. https://doi.org/10.1017/S0007485311000836

Murray RA, Solomon MG (1978) A rapid technique for analysing diets of invertebrate predators by electrophoresis. Ann Appl Biol 90:7–10. https://doi.org/10.1111/j.1744-7348.1978.tb02603.x

Navajas M, Boursot P (2003) Nuclear ribosomal DNA monophyly versus mitochondrial DNA polyphyly in two closely related mite species: the influence of life history and molecular drive. P Roy Soc Lond B Bio 270(Suppl 1):S124–S127. https://doi.org/10.1098/rsbl.2003.0034

Navajas M, Perrot-Minnot JM, Lagnel J, Migeon A, Bourse T, Cornuet JM (2002) Genetic structure of a greenhouse population of the spider mite *Tetranychus urticae*: spatio-temporal analysis with microsatellite markers. Insect Mol Biol 11(2):157–165. https://doi.org/10.1046/j.1365-2583.2002.00320.x

Nishimura S, Hinomoto N, Takafuji A (2003) Isolation, characterization, inheritance and linkage of microsatellite markers in *Tetranychus kanzawai* (Acari: Tetranychidae). Exp Appl Acarol 31:93–103

Nowakowska JA, Malewski T, Tereba A, Oszako T (2017) Rapid diagnosis of pathogenic *Phytophthora* species in soil by real-time PCR. Forest Pathol 47:e12303. https://doi.org/10.1111/efp.12303

Pascual-Ruiz S, Gómez-Martínez MA, Ansaloni T, Segarra-Moragues JG, Sabater-Muñoz B, Jacas JA, Hurtado-Ruiz MA (2014) Genetic structure of a phytophagous mite species affected by crop practices: the case of *Tetranychus urticae* in clementine mandarins. Exp Appl Acarol 62(4):477–498. https://doi.org/10.1007/s10493-013-9755-x

Pasquali M, Acquadro A, Balmas V, Migheli Q, Gullino ML, Garibaldi A (2004) Development of PCR primers for a new *Fusarium oxysporum* pathogenic on Paris daisy (*Argyranthemum frutescens* L.). Eur J Plant Pathol 110(1):7–11

Pasquali M, Dematheis F, Gullino ML, Garibaldi A (2007) Identification of race 1 of *Fusarium oxysporum* f. sp. *lactucae* on lettuce by inter-retrotransposon sequence-characterized amplified region technique. Phytopathology 97(8):987–996. https://doi.org/10.1094/PHYTO-97-8-0987

Patel JS, Brennan MS, Khan A, Ali GS (2015) Implementation of loop-mediated isothermal amplification methods in lateral flow devices for the detection of *Rhizoctonia solani*. Can J Plant Pathol 37:118–129. https://doi.org/10.1080/07060661.2014.996610

Peng J, Zhang H, Chen F, Zhang X, Xie Y, Hou X, Pu J (2014) Rapid and quantitative detection of *Fusarium oxysporum* f. sp. *cubense* race 4 in soil by real-time fluorescence loop-mediated isothermal amplification. J Appl Microbiol 117(6):1740–1749. https://doi.org/10.1111/jam.12645

Pérez-Sayas C. 2016. Determinación de las relaciones filogenéticas y tróficas de la acarofauna en cítricos. PhD Thesis, Universitat Jaume I de Castelló, Castellón, Spain

Pérez-Sayas C, Pina T, Gómez-Martínez MA, Camañes G, Ibáñez-Gual MV, Jaques JA, Hurtado MA (2015) Disentangling mite predator-prey relationships by multiplex PCR. Mol Ecol Resour 15(6):1330–1345. https://doi.org/10.1111/1755-0998.12409

Pompanon F, Deagle BE, Symondson WO, Brown DS, Jarman SN, Taberlet P (2012) Who is eating what: diet assessment using next generation sequencing. Mol Ecol 21(8):1931–1950. https://doi.org/10.1111/j.1365-294X.2011.05403.x

Prokopowich CD, Gregory TR, Crease TJ (2003) The correlation between rDNA copy number and genome size in eukaryotes. Genome 46(1):48–50. https://doi.org/10.1139/g02-103

Rački N, Dreo T, Gutierrez-Aguirre I, Blejec A, Ravnikar M (2014) Reverse transcriptase droplet digital PCR shows high resilience to PCR inhibitors from plant, soil and water samples. Plant Methods 10(1):42. https://doi.org/10.1186/s13007-014-0042-6

Rahman A, Miles TD, Martin FN, Quesada-Ocampo LM (2017) Molecular approaches for biosurveillance of the cucurbit downy mildew pathogen, *Pseudoperonospora cubensis*. Can J Plant Pathol 39:282–296. https://doi.org/10.1080/07060661.2017.1357661

Raso A, Biassoni R (2014) Twenty years of qPCR: a mature technology? In: Methods in molecular biology, Clifton, pp 1–3

Ravindran A, Lévy J, Pierson E, Gross DC (2015) Loop-mediated isothermal amplification procedure (LAMP) for detection of the potato zebra chip pathogen "Candidatus Liberibacter solanacearum". Methods Mol Biol 1302:85–97. https://doi.org/10.1007/978-1-4939-2620-6_7

Roderick GK, Navajas M (2003) Genes in new environments: genetics and evolution in biological control. Nat Rev Genet 4(11):889–899. https://doi.org/10.1038/nrg1201

Roehrdanz RL, Degrugillier ME, Black WC (2002) Novel rearrangements of arthropod mitochondrial DNA detected with Long-PCR: applications to arthropod phylogeny and evolution. Mol Biol Evol 19(6):841–849. https://doi.org/10.1093/oxfordjournals.molbev.a004141

Romeu-Dalmau C, Piñol J, Agustí N (2012) Detecting aphid predation by earwigs in organic citrus orchards using molecular markers. B Entomol Res 102(5):566–572. https://doi.org/10.1017/S0007485312000132

Roossinck MJ, Martin DP, Roumagnac P (2015) Plant virus metagenomics: advances in virus discovery. Phytopathology 105(6):716–727. https://doi.org/10.1094/PHYTO-12-14-0356-RVW

Ros VID, Breeuwer JAJ (2007) Spider mite (Acari: Tetranychidae) mitochondrial COI phylogeny reviewed: host plant relationships, phylogeography, reproductive parasites and barcoding. Exp Appl Acarol 42(4):239–262. https://doi.org/10.1007/s10493-007-9092-z

Sabater-Muñoz B, Pascual-Ruiz S, Gómez-Martínez MA, Jacas JA, Hurtado MA (2012) Isolation and characterization of polymorphic microsatellite markers in *Tetranychus urticae* and cross amplification in other Tetranychidae and Phytoseiidae species of economic importance. Exp Appl Acarol 57(1):37–51. https://doi.org/10.1007/s10493-012-9529-x

Sanzani SM, Li Destri Nicosia MG, Faedda R, Cacciola SO, Schena L (2014) Use of quantitative PCR detection methods to study biocontrol agents and phytopathogenic fungi and oomycetes in environmental samples. J Phytopathol 162(1):1–13. https://doi.org/10.1111/jph.12147

Schaad NW, Frederick RD (2002) Real-time PCR and its application for rapid plant disease diagnostics. Can J Plant Pathol 24(3):250–258. https://doi.org/10.1080/07060660209507006

Schroeder KL, Okubara PA, Tambong JT, Lévesque CA, Paulitz TC (2006) Identification and quantification of pathogenic *Pythium* spp. from soils in Eastern Washington using real-time polymerase chain reaction. Phytopathology 96(6):637–647. https://doi.org/10.1094/PHYTO-96-0637

Silva IMMS, Honda J, van Kan F, Hu JG, Neto L, Pintureau B, Stouthamer R (1999) Molecular differentiation of five *Trichogramma* species occurring in Portugal. Biol Control 16(2):177–184. https://doi.org/10.1006/bcon.1999.0755

Sint D, Raso L, Traugott M (2012) Advances in multiplex PCR: balancing primer efficiencies and improving detection success. Methods Ecol Evol 3(5):898–905. https://doi.org/10.1111/j.2041-210X.2012.00215.x

Solomon MG, Fitzgerald JD, Murray RA (1996) Electrophoretic approaches to predator-prey interactions. In: Symondson WOC, Liddell JE (eds) The ecology of agricultural pests- biochemical approaches. Chapman and Hall, London, pp 457–468

Srinivasan K, Gilardi G, Spadaro D, Garibaldi A, Gullino ML (2010) Molecular characterization through IGS sequencing of *formae speciales* of *Fusarium oxysporum* pathogenic on lamb's lettuce. Phytopathol Mediterr 49:309–320. https://doi.org/10.14601/Phytopathol_Mediterr-5460

Srinivasan K, Spadaro D, Poli A, Gilardi G, Gullino ML, Garibaldi A (2012) Genetic diversity and pathogenicity of *Fusarium oxysporum* isolated from wilted rocket plants in Italy. Phytoparasitica 40(2):157–170. https://doi.org/10.1007/s12600-011-0207-z

Stobbe AH, Daniels J, Espindola AS, Verma R, Melcher U, Ochoa-Corona F, Garzon C, Fletcher J, Schneider W (2013) E-probe Diagnostic Nucleic acid Analysis (EDNA): a theoretical approach for handling of next generation sequencing data for diagnostics. J Microbiol Methods 94(3):356–366. https://doi.org/10.1016/j.mimet.2013.07.002

Stobbe AH, Schneider WL, Hoyt PR, Melcher U (2014) Screening metagenomic data for viruses using the E-Probe diagnostic nucleic acid assay. Phytopathology 104(10):1125–1129. https://doi.org/10.1094/PHYTO-11-13-0310-R

Suga H, Hirayama Y, Morishima M, Suzuki T, Kageyama K, Hyakumachi M (2013) Development of PCR primers to identify *Fusarium oxysporum* f. sp. *fragariae*. Plant Dis 97(5):619–625. https://doi.org/10.1094/PDIS-07-12-0663-RE

Sunderland KD, Crook NE, Stacey D, Fuller BJ (1987) A study of feeding by polyphagous predators on cereal aphids using ELISA and gut dissection. J Appl Ecol 24(3):907–933. https://doi.org/10.2307/2403989

Sykes PJ, Neoh SH, Brisco MJ, Hughes E, Condon J, Morley AA (1992) Quantitation of targets for PCR by use of limiting dilution. BioTechniques 13(13):444–449

Symondson WOC, Erickson ML, Liddell JE, Jayawardena KGI (1999) Amplified detection, using a monoclonal antibody, of an aphid-specific epitope exposed during digestion in the gut of a predator. Insect Biochem Mol 29(10):873–882

Symondson WOC, Sunderland KD, Greenstone MH (2002) Can generalist predators be effective biocontrol agents? Annu Rev Entomol 47:561–594. https://doi.org/10.1146/annurev.ento.47.091201.145240

Talley J, Warren FHJB, Torrance L, Jones RAC (1980) A simple kit for detection of plant viruses by the latex serological test. Plant Pathol 29:77–79. https://doi.org/10.1111/j.1365-3059.1980.tb01184.x

Tixier MS, Hernandes FA, Guichou S, Kreiter S (2011) The puzzle of DNA sequences of Phytoseiidae (Acari: Mesostigmata) in the public GenBank database. Invertebr Syst 25(5):389–406. https://doi.org/10.1071/IS11013

Tomlinson J, Dickinson MJ, Boonham N (2010a) Rapid detection of *Phytophthora ramorum* and *P. kernoviae* by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. Phytopathology 100(2):143–149. https://doi.org/10.1094/PHYTO-100-2-0143

Tomlinson JA, Dickinson MJ, Boonham N (2010b) Detection of *Botrytis cinerea* by loop-mediated isothermal amplification. Lett Appl Microbiol 51(6):650–657. https://doi.org/10.1111/j.1472-765X.2010.02949.x

Tyagi K, Kumar V, Singha D, Chakraborty R (2015) Morphological and DNA barcoding evidence for invasive pest thrips, *Thrips parvispinus* (Thripidae: Thysanoptera), newly recorded from India. J Insect Sci 15(1):105. https://doi.org/10.1093/jisesa/iev087

Uesugi R, Osakabe M (2007) Isolation and characterization of microsatellite loci in the two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae). Mol Ecol Notes 7:290–292. https://doi.org/10.1111/j.1471-8286.2006.01583.x

Uesugi R, Kunimoto Y, Osakabe M (2009) The fine-scale genetic structure of the two-spotted spider mite in a commercial greenhouse. Exp Appl Acarol 47(2):99–109. https://doi.org/10.1007/s10493-008-9201-7

Unruh TR, Woolley JB (1999) Molecular methods in classical biological control. In: Bellows TS, Fisher TW (eds) Handbook of biological control. Academic, San Diego, pp 57–85

Validov SZ, Kamilova FD, Lugtenberg BJJ (2011) Monitoring of pathogenic and non-pathogenic *Fusarium oxysporum* strains during tomato plant infection. Microb Biotechnol 4(1):82–88. https://doi.org/10.1111/j.1751-7915.2010.00214.x

Wang Y, Zhang H, Li H, Miao X (2011) Second-generation sequencing supply an effective way to screen RNAi targets in large scale for potential application in pest insect control. PLoS One 6(4):e18644. https://doi.org/10.1371/journal.pone.0018644.t003

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173(1):697–703

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, New York, pp 315–322

Wirta HK, Hebert PD, Kaartinen R, Prosser SW, Várkonyi G, Roslin T (2014) Complementary molecular information changes our perception of food web structure. Proc Natl Acad Sci 111(5):1885–1890. https://doi.org/10.1073/pnas.1316990111

Woudenberg JHC, Seidl MF, Groenewald JZ, de Vries M, Stielow JB, Thomma BPHJ, Crous PW (2015) *Alternaria* section *Alternaria*: species, *formae speciales* or pathotypes? Stud Mycol 82:1–21. https://doi.org/10.1016/j.simyco.2015.07.001

Xie L, Hong XY, Xue XF (2006) Population genetic structure of the two-spotted spider mite (Acari: Tetranychidae) from China. Ann Entomol Soc Am 99(5):959–965. https://doi.org/10.1603/0013-8746(2006)99[959:PGSOTT]2.0.CO;2

Yang B, Cai J, Cheng X (2011) Identification of astigmatid mites using ITS2 and COI regions. Parasitol Res 108(2):497–503. https://doi.org/10.1007/s00436-010-2153-y

Yli-Mattila T, Paavanen-Huhtala S, Fenton B, Tuovinen T (2000) Species and strain identification of the predatory mite *Euseius finlandicus* by RAPD-PCR and ITS sequences. Exp Appl Acarol 24(10–11):863–880

Yuan ML, Wei DD, Wang BJ, Dou W, Wang JJ (2010) The complete mitochondrial genome of the citrus red mite *Panonychus citri* (Acari: Tetranychidae): high genome rearrangement and extremely truncated tRNAs. BMC Genomics 11:597. https://doi.org/10.1186/1471-2164-11-597

Zaidi RH, Jaal Z, Hawkes NJ, Hemingway J, Symondson WOC (1999) Can multiple-copy sequences of prey DNA be detected amongst the gut contents of invertebrate predators? Mol Ecol 8:2081–2087

Zhou J, Thompson DK (2002) Challenges in applying microarrays to environmental studies. Curr Opin Biotech 13:204–207. https://doi.org/10.1016/S0958-1669(02)00319-1

Zhu YC, Greenstone MH (1999) Polymerase chain reaction techniques for distinguishing three species and two strains of *Aphelinus* (Hymenoptera: Aphelinidae) from *Diuraphis noxia* and *Schizaphis graminum* (Homoptera: Aphididae). Ann Entomol Soc Am 92(1):71–79. https://doi.org/10.1093/aesa/92.1.71

Zhu YC, Burd JD, Elliott NC, Greenstone MH (2000) Specific ribosomal DNA marker for early polymerase chain reaction detection of *Aphelinus hordei* (Hymenoptera: Aphelinidae) and *Aphidius colemani* (Hymenoptera: Aphidiidae) from Diuraphis noxia (Homoptera: Aphididae). Ann Entomol Soc Am 93(3):486–491. https://doi.org/10.1603/0013-8746(2000)093[0486:SRDMFE]2.0.CO;2