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MULTILOCUS SEQUENCE ANALYSIS AS A POWERFUL TOOL TO MONITOR MOLECULAR EPIDEMIOLOGY OF '*CANDIDATUS* PHYTOPLASMA SOLANI' AT VINEYARD SCALE

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'*Candidatus* Phytoplasma solani' is a phytoplasma of the stolbur group (16SrXII-A subgroup), associated with grapevine Bois noir (BN), responsible for outbreaks in several European countries, and particularly in the Mediterranean area. It is transmitted by the polyphagous cixiid planthopper *Hyalesthes obsoletus* to a wide range of wild plants (inoculum sources) while grapevine is only occasionally infected. The multiple interactions with wild and cultivated annual and perennial host plants and insect vectors in different ecosystems might be responsible for generating genetic diversity of '*Ca.* P. solani'. Aim of this study was to estimate the '*Ca*. P. solani' molecular genotypes harbored in a single vineyard by multilocus sequence typing analysis for the *vmp*1, *stamp*, and *sec*Y genes. Several haplotypes per gene were detected, showing high genetic diversity even in a restricted area. Further analyses allowed to estimate the pressure of selection within a highly BN infected commercial vineyard by calculating the dN/dS ratio, which resulted particularly high (positive selection) for *stamp* and *vmp*1 genes. The high genetic variability, recorded in particular in genes encoding the membrane proteins, represents an adaptation strategy common to living microorganisms, particularly useful for a generalist pathogen that colonized different environments (host plant and vector tissues).

INTRODUCTION

Bois noir (BN) is a grapevine disease that is associated to '*Candidatus* Phytoplasma solani' ('Ca. P. solani'; 16SrXII-A subgroup) (QUAGLINO et al., 2013), and it is most common and widespread in Euro-Mediterranean regions (MAIXNER et al., 2011), becoming a real limiting factor for the productions. '*Ca*. P. solani' isolates are characterized by different degrees of genetic variability according to the genes involved (FOISSAC et al., 2013; QUAGLINO et al., 2013). The most variable genes are those that code for surface membrane proteins, which are directly exposed to host and vector interactions. In this study, we combined data coming from genotyping by multilocus sequence analysis with estimation of the dN/dS ratio. The latter is the ratio between the non-synonymous (dN) and the synonymous (dS) substitution rates in an alignment of amino-acid-coding sequences (NIELSEN, 2005), in order to estimate the richness of '*Ca*. P. solani' molecular genotypes and the pressure of selection within a highly BN infected commercial vineyard.

MATERIALS AND METHODS

The DNAs, extracted by CTAB protocol, were amplified in nested-PCR with specific primer pairs for *vmp*1, *stamp* and *sec*Y. *vpm*1 amplicons were digested in PCR-RFLP (FIALOVA et al., 2009; FABRE et al., 2011) in order to distinguish the molecular types. On the basis of the RFLP characterisation of *vmp*1 genes, representative samples within the vineyard, amplified with specific primer pairs for the *vmp*1, *stamp* and *sec*Y genes were purified and sequenced. The phylogenetic relationships were reconstructed, using the Mega v. 5.1 software, for the *vmp1, stamp*, and *sec*Y nucleotide sequences of '*Ca*. P. solani' that originated from the study vineyard, with respect to nucleotide sequences from other Italian regions and from Euro-Mediterranean countries that were available in Genebank. Moreover, the ratio between the proportion of non-synonymous and synonymous substitutions (dN/dS ratio), was determined for the nucleotide sequences at the study vineyard level in order to determine the type of selection interfere on *vmp*1 gene. Positive selection happens when dN/dS ratio >1, on the other hand a ratio <1 suggests a purifying selection process (NEI and KUMAR, 2000).

RESULTS AND DISCUSSION

In the study vineyard, the molecular characterization of *vmp*1, allowed to detect eight different *vmp*1 types (V3, V4, V9, V11, V12, V14, V15 and V18), most of them identified in *H. obsoletus* collected in Marche vineyard ecosystems (Landi et al., 2015). The wide genetic diversity of '*Ca*. P. solani' has been reported and generally related to complex interactions between the vector and the wide range of wild host plants (KESSLER et al., 2011). The dominant *vmp*1 genotypes were V14 and V12, while we sporadically detected V3.

The phylogenetic analysis was carried out on nucleotide sequences, which were representative of the RFLP types of the study vineyard, and on those available in GenBank. In the resulting dendrogram, the sequences generally clustered according to the PCR-RFLP patterns. Strains with the same RFLP pattern showed high nucleotide similarity (>99%) of sequences (MUROLO et al., 2010). The selective pressure in the *vmp*1, *stamp* and *sec*Y genes were estimated for the '*Ca*. P. solani' strains according to the abundance of non-synonymous mutations. For the *sec*Y gene, the overall dN/dS ratio was 1.02 (P =0.841), which suggested low neutral selection across this gene. The overall ratio between the non-synonymous to the synonymous mutations (dN/dS) was >1 for *vmp*1 (2.28; P = 0.001) and *stamp* (3.99; P = 0.019). These high values of dN/dS (i.e., >1) indicated detection of a high number of non-silent (dN) mutations. The higher genetic variability in the *vmp*1 and *stamp* genes with respect to the *sec*Y gene arose from the estimation of the rate of non-silent mutation (dN). According to this parameter, which is an indication of selective pressure, FABRE et al. (2011) defined the *sec*Y gene as a housekeeping gene, while the *vmp*1 and *stamp* genes were under positive selection, because they are involved in specific interactions as demonstrated for other phytoplasma (KAKIZAWA et al., 2006). The high genetic variability as well as the dN/dS ratio >1 of '*Ca*. P. solani' in *vmp*1 and *stamp* genes, within a restricted location (i.e. commercial vineyard) provide useful information to trace an inoculum source and the movement of pathogen strains over local and long distances (MUROLO and ROMANAZZI, 2015).

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