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A new variant of *Xylella fastidiosa* subspecies *multiplex* detected in different host plants in the recently emerged outbreak in the region of Tuscany, Italy

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Abstract The vector-borne bacterial pathogen *Xylella fastidiosa* is widely distributed in the Americas; in the last decade it has emerged as a serious threat for agricultural crops, natural environment and landscape in Europe. Following the first EU outbreak in 2013 in southern Italy, associated with a severe disease in olive trees, annual mandatory surveys are now in place in the Member States, leading to the discovery of bacterial outbreaks in different countries. Among the latest findings, an outbreak has been reported in the Italian region of Tuscany, with infections identified in seven different plant species. In this work, we report the isolation and the genetic characterization of isolates associated with this newly discovered outbreak. Multilocus sequence typing approach revealed the occurrence of isolates harbouring a new sequence type, denoted ST87, genetically related to strains of subsp. *multiplex*, but different from the genotypes of this subspecies previously characterized in Europe. Five cultured strains were successfully recovered from four of the seven host plants, an important achievement for advancing the studies on genomics and pathogenicity of these isolates and thus assess their potential threat for European agriculture.

Keywords *Xylella fastidiosa* · Tuscany · MLST · Sequence type · Host plants

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Xylella fastidiosa is a Gram-negative bacterium in the order Xanthomonadales, family Xanthomonadaceae (Wells et al. 1987) and is one of the most peculiar microorganisms among the "fastidious" vascular bacteria. It is vectored by xylem-feeding insects and infects a wide range of plant species. The pathogen has been intensely investigated because of its association with destructive diseases affecting important crops such as grapevine, olive, citrus, coffee and stone fruits, as well as numerous ornamental and forest species. It is the causal agent of the well-characterized grapevine Pierce's disease (Hopkins and Purcell 2002), citrus variegated chlorosis (Baldi and La Porta 2017; Chang et al. 1993) and Olive Quick Decline Syndrome (Saponari et al. 2017). However, several reports suggest that not all plant species allow symptomatic and long-term infections (Almeida and Nunney 2015), and the mechanisms of host plant-pathogen specificity remain largely unknown.

Although the number of available draft and complete genomes of *X. fastidiosa* are rapidly increasing, assessment of its genetic diversity is still principally based on Multilocus sequence typing (MLST) approach (Scally et al. 2005; Yuan et al. 2010), that allows bacterial

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genotypes clustering into four major subspecies: *fastidiosa*, *multiplex*, *pauca* and *sandyi*. Several analyses indicated that these subspecies evolved in geographical isolation with subsp. *pauca* native to South America (Nunney et al. 2012), subsp. *multiplex* native to temperate and subtropical North America (Nunney et al. 2012, 2014), subsp. *fastidiosa* presumably native to southern Central America (Nunney et al. 2010), and *sandyi* only detected in southern regions of the United States (Yuan et al. 2010).

International movements of infected plants for commercial or landscape planting are most likely the main pathway that contributed to the spread and establishment of *X. fastidiosa* outside of the Americas, where the pathogen was thought to be confined until the last twenty years, when the presence of the bacterium was reported in Taiwan, Iran and in several outbreaks detected in the European countries (Saponari et al. 2013; Denancé et al. 2017; Olmo et al. 2017; EFSA PLH Panel, 2018). The situation in Europe raised major concerns as: (i) the bacterium has been associated with the disease "Olive Quick Decline Syndrome", decimating olive trees in southern Italy; (ii) multiple subspecies have been detected, increasing risks for homologous recombination if multiple strains co-exist in the same environment/hosts; (iii) the extensive list of plant species found susceptible to the infection includes olive and almonds, both major affected crops in Europe; and (iv) the widespread presence of spittlebugs, the predominant European vector species so far found, in European and Mediterranean countries.

Among the latest reports in Europe, the bacterium was identified for the first time in December 2018 in the region of Tuscany (northern Italy) infecting different ornamentals plants (*Spartium junceum*, *Polygala myrtifolia*, *Cistus* spp., *Rhamnus alaternus* and *Lavandula* spp.) and an almond tree (*Prunus amygdalus*). Initial genetic characterization revealed the presence of bacterial sequences of the subsp. *multiplex* (Marchi et al. 2019). Notably, subsp. *multiplex* exhibits the greatest genetic diversity, comprising the highest number of genetically diverse strains, accounting for nearly 50% of the sequence types so far described (https://pubmlst.org/xfastidiosa/). Similarly, strains of subsp. *multiplex* have been associated with the highest number of susceptible plant species worldwide (EFSA 2018). In the EU, strains of subsp. *multiplex* are the most frequently detected and account for the majority of the susceptible EU host plants (European Commission 2018), being the predominant strains in France (Corsica and PACA Region) and mainland Spain (province of Alicante and Madrid), but also infecting different plant species in the Balearic Islands, and in the outbreak just recently reported in Portugal (Europhyt, Outbreak No.753). Characterization of the strains of subsp. *multiplex* in these EU outbreaks revealed the presence of at least three different, but closely related, variants (defined as Sequence Type, ST): ST6 (Corsica, PACA Region, province of Alicante and Madrid), ST7 (Corsica, PACA Region, Balearic Islands, Portugal) and ST81 (Balearic Islands).

In this study, we report the isolation of the bacterium from various infected plant species identified in the newly discovered outbreak in Tuscany, the complete identification of the genotype associated with the infections and its phylogenetic relationships, and the use of High Resolution Melting analysis (HRM) for rapid and large scale identification of the subspecies. Recent applications of HRM analysis in plant pathology proved to successfully differentiate genotypes of bacteria, viruses and viroids (Bester et al. 2012; Gori et al. 2012; Loconsole et al. 2013).

A total of 12 infected samples collected from *Rhamnus alaternus*, *Polygala myrtifolia*, *Rosmarinus officinalis*, *P. amygdalus*, *Cistus spp.*, *Lavandula spp.*, *Spartium junceum* were processed for DNA extraction, using the DNeasy mericon Food Kit (Qiagen, The Netherlands) and for isolation. These plants were from different sites, with the most distant being 3 km apart. Isolation and DNA extraction were performed within 3 days after sampling.

DNA was subjected to quantitative real time PCR (qPCR) (Harper et al. 2010) to confirm the presence of the bacterial infections prior to perform isolations, HRM and MLST assays.

Isolation was attempted from all infected species except *Cistus* spp. and *Lavandula* spp. using either petioles/midribs (*R. alaternus*, *P. myrtifolia*) or young shoots (*R. officinalis, S, junceum)* macerated in a phosphate-buffered saline (PBS) solution and plated onto periwinkle wilt-GelRite medium (PWG) (Hill and Purcell 1995), or using woody stems/cuttings (*P. myrtifolia*, *S, junceum*, *P. amygdalus*) squeezed and gently imprinted onto buffered charcoal yeast extract (BCYE) agar medium (Wells et al. 1981). *X. fastidiosa* colonies were readily recovered in approximately one week on PWG and 10 days on BCYE, from *S. junceum* (strain A014740), *P. myrtifolia* (strains A022187 and

A014725), *P. amygdalus* (strain A022183) and *R. alaternus* (strain A022177). Isolation failed from *R. officinalis*. Subcultures of these strains were made on PWG, BCYE and PD3 (Davis et al. 1980) media, clearly showing that the bacterium grew at a higher rate on PWG. Interestingly, values of the quantitation cycles (Cq) resulting from the qPCR assay indicated similar bacterial populations (Cq values close to 19) in *S. junceum* and *P. myrtifolia*, while the colony forming unit (CFU) estimated by plating serial dilutions of the sap indicated a concentration 10 times higher in *S. junceum* than in *P. myrtifolia,* 5.5×10^6 vs 6.5×10^5 CFU/ml, respectively. Moreover, only two colonies were recovered from *R. alaternus*, in agreement with qPCR assays yielding higher Cq values (~26.37). These comparative analyses, though limited to few samples, suggest that significant inhibition of bacterial growth may occur when plating the plant sap, affecting the estimation of absolute bacterial population size in the different plant matrices.

Nucleic acids extracts recovered from the 12 infected plant samples were also subjected to HRM analysis (Montes-Borrego et al. 2017). To this end, SYBR Green-based qPCR reactions were set using the primers HL5/HL6 (Francis et al. 2006) targeting the gene encoding for the hypothetical protein HL. Following the amplification cycles, melt curve analyses were performed over a temperature range of 65–95 °C in 0.5 °C increments, and the resultant melting profiles evaluated using the Precision Melt Analysis™ software (Bio-Rad, USA). Reference strains included in the HRM test were: strain De Donno (CFBP 8402, subsp. *pauca*), strain Temecula-1 (ATCC 700964, subsp. *fastidiosa*), strain ESVL (subsp. *multiplex*) and strain CFBP 8416 (subsp. *multiplex*). All 12 infected samples displayed a Tm of 83 °C and based on the HRM analysis all clustered together and with the reference strains ESVL and CFBP 8416 of subsp. *multiplex* (Fig. 1), with a confidence interval greater than 99% (data not shown). Thus, under our experimental conditions, HRM analysis proved to be a useful tool for rapid and preliminary identification of the genetic relationships of the bacterial strain(s) and consequently the subspecies causing the infection(s).

Genetic typing of the strains isolated and cultured from *S. junceum*, *P. myrtifolia* and *P. amygdalus* was performed using the MLST approach. Briefly, the bacterial DNA was subjected to end-point PCR with seven pairs of primers, according to the scheme described in

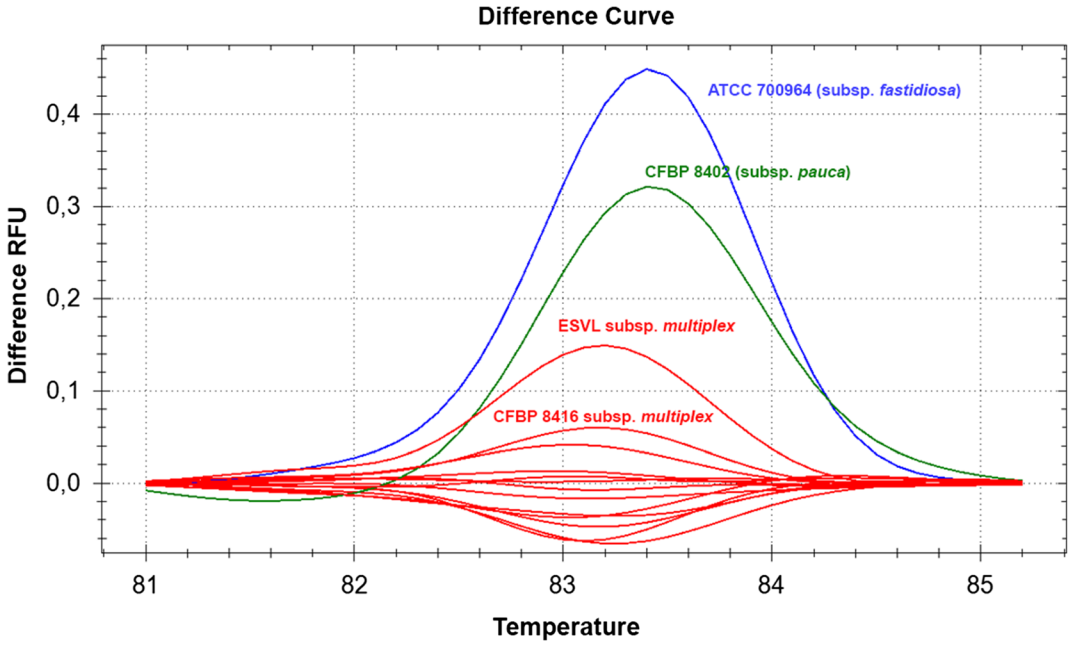

Fig. 1 High Resolution Melting (HRM) analysis. The resolution of the melting curves of the HL amplicons (Francis et al. 2006) results in the clustering of the samples as a function of the melting temperature. The reference strains belonging to subsp. *fastidiosa* (ATCC 700964), *pauca* (CFBP 8402) and *multiplex* (ESVL and CFBP 8416) generate three clusters indicated respectively by the colours blue, green and red. The samples recovered in the outbreak in Tuscany clustered with the reference strains of subsp. *multiplex* (red curves)

the reference database (https://pubmlst.org/xfastidiosa/), that amplify fragments belonging to the core genome of the bacterium, that are not under positive selection. Those genes encode 2-isopropylmalate synthase (*leuA*), ubiquinol-cytochrome C oxidoreductase (*petC*), ABC transporter sugar permease (*malF*), siroheme synthase (*cysG*), DNA polymerase III holoenzyme, chi subunit (*holC*), NADH-ubiquinone oxidoreductase (*nuoL*), glutamate symport protein (*gltT*).

The sequences recovered for each amplicon were compared with alleles registered in the reference database, to define the allele at each gene locus. Sequence similarity analysis revealed the presence of a new allele variant for the gene *nuoL*, identified as *nuoL*_21 and made available in GenBank under the accession number MK353403; which differs for a single nucleotide (G to A at position 276 of the nucleotide sequence) from allele *nuoL*_3, already characterized in genotypes belonging to subsp. *multiplex*. Allele sequences at *leuA*, *petC*, *malF*, *cysG*, *holC*, *gltT* loci showed 100% identity with allele variants already described in the database and associated to subsp. *multiplex*. The combination of the 7 alleles at each locus was deposited in the dedicated PubMLST database, and a new Sequence Type, identified as ST87 was assigned. This ST was conserved among the three cultured isolates and it is characterized by the following profile: *leuA*_5, *petC*_3, *malF*_5, *cysG*_3, *holC*_3, *nuoL*_21, *gltT*_3.

The concatenated sequence was used to generate the Neighbor-Net phylogenetic network (Fig. 2), by using Splits Tree4 (Huson and Bryant 2005), that showed a high resolution of the phylogenetic relationships among the

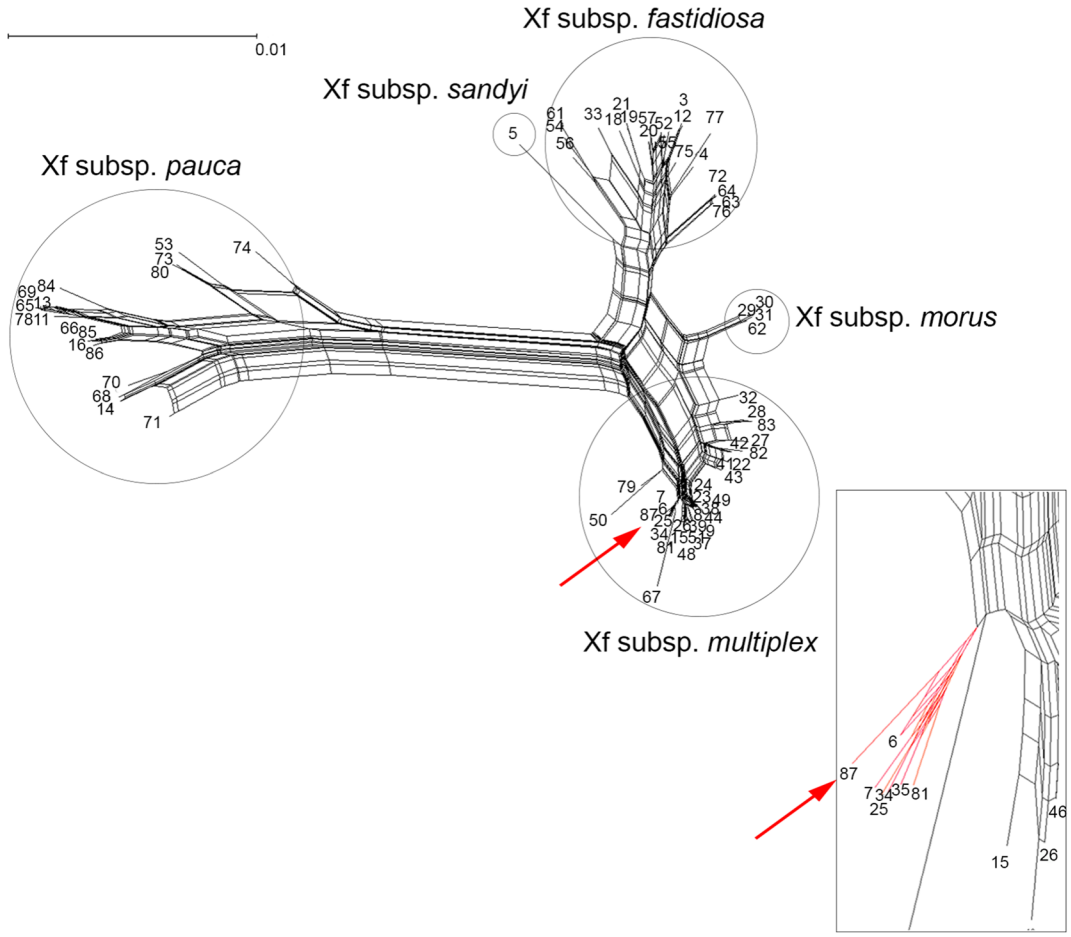

Fig. 2 Phylogenetic network of *Xylella fastidiosa* sequence types (STs, Neighbor-Net analyses of concatenated sequences). Taxonomic clusters corresponding to subsp. *fastidiosa, sandyi, morus, multiplex* and *pauca* are indicated with circles. Red arrows indicate the taxonomic allocation of the newly characterizes genotype ST87. A close up of the ramification harboring ST87 is shown in the right corner at the bottom of the figure

STs. The phylogenetic tree showed that ST87 grouped with STs belonging to *X. fastidiosa* subsp. *multiplex*, on the same branch comprising all the STs so far identified in Europe, ST6 and ST7 found in Corsica, France, Balearic Islands and mainland Spain, and ST81 in the Balearic Islands (EFSA PLH Panel, 2018). However, more insights into the genetic relatedness among the isolates grouping in this cluster will be gathered through the ongoing whole-genome sequencing.

This work further extends the characterization of the isolates recovered in the recently emerged outbreak in Tuscany, supporting the finding of a unique and hitherto undescribed genotype, herein identified as ST87, in all the infected samples so far characterized. The noteworthy finding of ST87 in almond confirms the susceptibility of this species to strains of subsp. *multiplex*, responsible for the majority of the infections reported in almond in North America and associated with almond leaf scorch disease.

The emergence of this new outbreak in the EU territory raises several epidemiological questions that still need to be addressed. However, the preliminary data herein collected, along with those reported by Marchi et al. (2019), suggest that this outbreak most probably is linked to a single introduction of this new genotype in the area and, as such, no relations exist with the previous reports of strains of the subsp. *multiplex* in Europe.

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Authors contributions MS conceived the experiments and performed isolations; MS and DB revised the manuscript; GD prepared the manuscript and contributed to culture the strains; GA and SZ performed the diagnostic tests; RAK performed MLST-PCR and sequencing; GL performed the MLST analysis; DR provided the infected materials.

Compliance with ethical standards The authors declare that ethical standards have been followed and that no human participants or animals were involved in this research.

Competing interests The authors declare that they have no competing interests.

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