



Update of the *Xylella fastidiosa* outbreak in France: two new variants detected and a new region affected

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Accepted: 9 March 2022 / Published online: 19 March 2022
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Abstract *Xylella fastidiosa* is a xylem-limited bacterium native to America and classified as a priority pest for EU regulation. Since 2013, *X. fastidiosa* has been identified in European countries with a Mediterranean climate, such as Italy, France, Spain and Portugal, with different subspecies and sequence types (ST) detected. Since 2015 *X. fastidiosa* subsp. *multiplex* ST6 and/or ST7 has been detected in Corsica and the Provence-Alpes-Côte d'Azur in almost 70 plant species, whereas *X. fastidiosa* subsp. *pauca* ST53 has been found in only two host plants. In this study, we report two new variants, recently detected in two separated areas of the PACA region, genetically related to the subspecies *multiplex* and assigned to (i) ST88 detected on *Polygala myrtifolia*, *Hebe* sp., *Osteospermum ecklonis*, *Lavandula x intermedia*, *Coronilla glauca* and *Euryops chrysanthemoides* and (ii) ST89: detected on *Myoporum* sp. and *Viburnum tinus*. Both variant strains were isolated in vitro. Moreover, we report here the identification of *X. fastidiosa* subsp. *multiplex* ST6 in a new region of the South of France, Occitanie (Aude), in

plants from natural and urban settings and from a nursery.

Keywords *Xylella fastidiosa* subsp. *multiplex* · ST6 · ST88 · ST89 · MLST

X. fastidiosa (Wells et al., 1987) is a xylem-associated bacterium native to the Americas known to be the causal agent of Pierce's disease of grapevine and citrus variegated chlorosis (Baldi & La Porta, 2017; Chang et al., 1993; Hopkins & Purcell, 2002). Five subspecies have been described: *fastidiosa*, *multiplex*, *pauca*, *sandyi*, and *morus*, but studies have proposed that the subspecies *sandyi* and *morus* should be included within the subspecies *fastidiosa* (Denancé et al., 2019; Marcelletti & Scortichini, 2016).

X. fastidiosa can cause different types of symptoms, such as leaf scorching, wilting of the foliage, defoliation, chlorosis or bronzing along the leaf margin, and dwarfing (EPPO, 2019a). Up to now, 638 plant species belonging to 87 botanic families have been reported to be able to be either artificially or naturally infected (EFSA, 2021). At local scales the bacterium is disseminated by sap xylem feeding vectors, and at long distances by plant material movement. In America, different vectors have been identified, such as the glassy-winged sharpshooter *Homalodisca vitripennis* or the spittlebug *Philaenus spumarius* (Krugner et al., 2019). In Europe, the main known competent vectors are *P. spumarius* and *Neophilaenus campestris* (Cavalieri et al., 2019; Cornara et al., 2017).

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Even though the *X. fastidiosa* diseases have been described in America for over 100 years, *X. fastidiosa* has only recently been detected in Europe. Several subspecies were reported from the natural settings, agricultural and urban areas in Italy (*pauca* and *multiplex*) (EPPO, 2019b; Saponari et al., 2013), in France (*multiplex* and *pauca*) (Denancé et al., 2017), in Spain (*fastidiosa*, *pauca* and *multiplex*) (Landa, 2017) and in Portugal (*multiplex*) (EPPO, 2019b). In Italy, the subspecies *pauca* is responsible for Olive Quick Decline Syndrome (OQDS), and is causing dramatic economic losses in olive cultivation in Apulia (Olivicola Italia, 2019; Saponari et al., 2013). A recent study evaluated the impact of the strain causing OQDS on the major olive-cultivation European countries (Italy, Greece and Spain), using a spatially explicit bio-economic model (Schneider et al., 2020). They highlighted the importance of applying surveillance and adapting measures in infected areas to slow the spread of the disease.

Up to now, 87 sequence types (ST) of *X. fastidiosa* have been reported all around the world, and the corresponding partial sequences of seven housekeeping genes related to *X. fastidiosa* were registered in the PubMLST database (Jolley et al., 2018) dedicated to *X. fastidiosa* (<https://pubmlst.org/organisms/xylella-fastidiosa>). Among the 39 STs belonging to subspecies *multiplex*, two new variants were reported in Europe during the last four years: (i) ST81 in Balearic Islands, Spain, (Landa, 2017), and later in California, USA (O'Leary et al., 2020); (ii) ST87 in Tuscany, Italy, (Saponari et al., 2019).

In 2015, the subspecies *multiplex* ST6 and ST7 genotypes were detected in France in Corsica and in PACA regions (Alpes-Maritimes and Var), and annual official surveys with plant and vector samplings confirmed the presence and dissemination of these STs in Corsica and PACA regions (Cunty et al., 2020; Denancé et al., 2017). The presence of the subspecies *pauca* ST53 genotype was reported in only one location, in Menton in PACA region, in 2015 (Denancé et al., 2017) and four years later at the same location on one olive tree (Cunty et al., 2020). In 2020, a new region, Occitanie (Aude), was found to be impacted by the presence of *X. fastidiosa* subspecies *multiplex* (EPPO, 2020). The methods applied to detect the bacterium and identify the ST in plants are in line with the EPPO protocol PM7/24 (EPPO, 2019a). Briefly, the detection is performed using the real-time PCR Harper and the identification of the ST by applying an MLST scheme of seven

housekeeping genes and using the PubMLST database to identify allele numbers and ST (EPPO, 2019a; Harper et al., 2010; Yuan et al., 2010).

The aim of this study was to provide an overview of the current *X. fastidiosa* situation in France. We report the identification of *X. fastidiosa* subsp. *multiplex* (i) two new variants in the PACA region, ST88 in Var and ST89 in Alpes-Maritimes, and (ii) ST6 in a new French area, Aude in Occitanie region, in different plant species, including the new host plant *Perovskia abrotanoides*.

Since 2015 more than 24,000 plants were sampled and analysed in the PACA region within the framework of the surveillance. A sample was considered positive when the Cq value was lower or equal to 38, as described by Harper et al. (2010). Among the Harper-positive *X. fastidiosa* samples, two new MLST profiles, from two distinct locations, were identified. MLST performed on samples of *Polygala myrtifolia* and *Hebe* sp., both collected in 2020 in the same area in Saint-Raphaël (Var), revealed a new allele *malF*_19, similar to *malF*_13 (T at position 301 and A at position 486) and the sequence was registered on Genbank (accession number OL353884). The MLST profile, such as: *leuA*_3, *petC*_3, *malF*_19, *cysG*_7, *holC*_3, *nuoL*_3 and *gltT*_3, was assigned to ST88 (Fig. 1). This profile varied in only one gene from the ST7 previously found in this region. Strain isolation was performed on these positive plant samples, using petioles, midribs or young shoots dilacerated and macerated in sterile water, plated on modified PWG (PWGm) medium and incubated at 28 °C according to EPPO standard PM 7/24 (EPPO, 2019a). After 10–15 days, typical colonies were recovered (strain named LSV52.37) from *P. myrtifolia* (presenting leaf scorch) with the same MLST profile as the one obtained directly from plant macerate. In some plant samples received in 2021 from Saint-Raphaël, ST88 was found, including *Osteospermum ecklonis*, *Lavandula x intermedia*, *Coronilla glauca* and *Euryops chrysanthemoides*. This indicated the potential maintenance of this variant over time at this location. Moreover, a sample of *P. myrtifolia* was found to be co-infected by ST7 and ST88. Additionally, another variant was detected in Villeneuve-Loubet (Alpes-Maritimes) in 2020. A new allele *nuoL*_23, similar to *nuoL*_3 (T at position 185), was identified and the sequence was registered on Genbank (accession number OL353885). The MLST profile, such as: *leuA*_6, *petC*_3, *malF*_5, *cysG*_18, *holC*_4, *nuoL*_23 and *gltT*_7, was assigned to ST89 (Fig. 1). This profile was very different from ST6

or ST7 usually found in PACA, such as only *petC_3* was in common. The ST89 was detected directly on DNA extracted from samples of *Myoporum* sp. and *Viburnum tinus*, both collected in 2020 in the same area. One strain (strain named LSV52.52) was isolated from *Myoporum* sp. (presenting leaf scorch) with same MLST profile. Interestingly, this MLST profile was previously found at the same location in 2018 only on one sample of *Veronica elliptica*, but the isolation of the strain was unsuccessful. However, this confirmed the maintenance of this variant over time at this location. In order to evaluate the phylogenetic relationships of the 87 STs available on PubMLST database and the two new variants ST88 and ST89 identified here, the sequences of the seven housekeeping genes were concatenated, aligned, and the phylogenetic networks were retrieved using Splits Tree4 (Huson & Bryant, 2005) (Fig. 2). Both new STs grouped with strains belonging to the subspecies *multiplex*. The ST88 was closely related to STs already found in Europe, such as ST6 (France, mainland Spain), ST7 (France, Balearic Islands, Portugal), ST81 (Balearic Islands) and ST87 (Italy) (Landa, 2017; Saponari et al., 2019), while ST89 was closer related to STs found in USA such as ST41 and ST43 (Nunney et al., 2013).

After Corsica and PACA, Occitanie is the third region of France most impacted by the presence of *X. fastidiosa*. In Occitanie region (south-west of France), approximately 5000 plant samples, (representing more than 200 plant species) symptomatic or asymptomatic (nursery, natural settings, agricultural or urban areas), were analysed for

the presence of *X. fastidiosa* from August 2020 to August 2021. Among these samples, 132 were found to be *X. fastidiosa* positive in the real-time PCR Harper et al. (2010) with Cq values ranging from 19.66 to 38. Among the 132 Harper-positive samples, 120 were typed as *multiplex*, and for 98 the ST identification succeeded, all being ST6. Based on EU list (<https://eur-lex.europa.eu/legal-content/fr/TXT/?uri=CELEX%3A32020R1201>), one new host plant was reported: *Perovskia abrotanoides*, which was asymptomatic. Moreover, the bacterium was first detected on *Lavandula x intermedia* (variété ‘Grosso’) in a nursery, and then in the natural settings on: *Coronilla valentina* (4), *Fraxinus* sp. (1), *Laurus nobilis* (2), *Lavandula x allardii* (5), *Lavandula x intermedia* (12), *Lavandula* sp. (19), old cultivated *Medicago sativa* (5), *Phlomis fruticosa* (1), *Platanus* sp. (1), *Prunus dulcis* (Mill.) D.A Webb (12), *Rhamnus* (2), *Rosa canina* (3), *Salvia rosmarinus* (2), *Spartium junceum* (38), *Spartium* sp. (29). It was interesting to notice that no *P. myrtifolia* was found to be infected with *X. fastidiosa* in this new infected region, in contrast with the Corsica and PACA regions. It is linked to the fact that *P. myrtifolia* is very scarce as an ornamental in Occitanie (Aude) due to lower winter temperatures at this area. Strain isolation was performed, as described above, and three strains presenting typical colonies were recovered from three different plant genera: *Lavandula x intermedia* (variété ‘Grosso’), *L. nobilis* and *Spartium* sp., for which Ct value on plant extract were 28.83; 23.42 and 19.87, respectively. The strains identified were confirmed applying real-time PCR

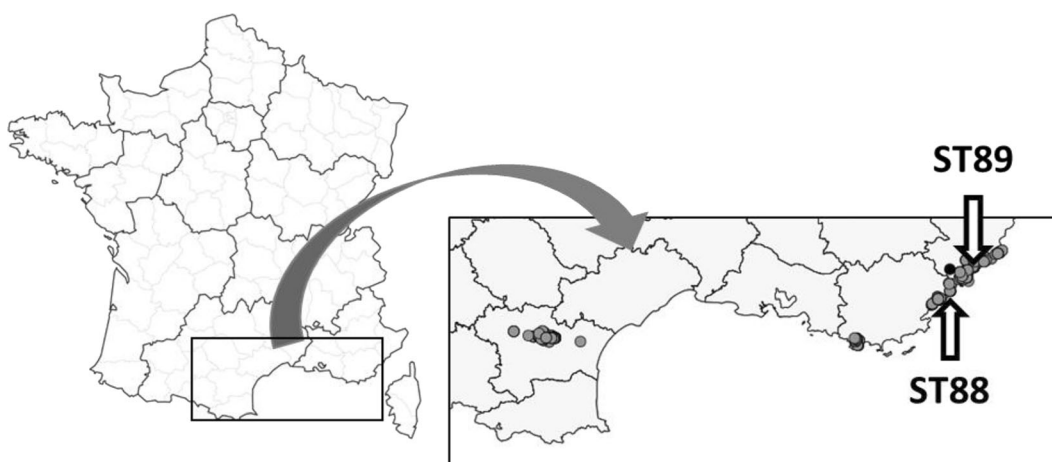


Fig. 1 Map representing the location of positive plants to *Xylella fastidiosa* detection (south-west: Occitanie; south-east: PACA), and the location of two new variants ST88 and ST89. The map was adapted from R Shiny dedicated for *X. fastidiosa* (EAS unit, Anses)

Harper and typed by MLST as ST6 (allele number 3 for the seven housekeeping genes), named LSV52.20; LSV52.39 and LSV52.40, respectively and stored at -80°C . Same MLST profiles were obtained on strains and on plant macerates from which these strains were isolated. Up to now in Occitanie, only ST6 has been identified in different plants, mainly in *S. junceum*, *Spartium* sp. and *P. dulcis* coming from the wild environment and *Lavandula* sp. coming from a nursery and urban areas. Moreover, *M. sativa* had been found positive. In North America, cultivated alfalfa was already

identified as a plant reservoir of *X. fastidiosa* subspecies *fastidiosa*, and the epidemiological role of alfalfa in the spread of the bacterium in the case of Pierce's disease was confirmed when alfalfa were located surrounding vineyards (Hewitt & Houston, 1941). Assays performed in the framework of the H2020 POnTE project (artificial inoculations of alfalfa with different strains of *X. fastidiosa*), confirmed the capability of a strain ST6, isolated from Corsica, to maintain and multiply in alfalfa (data not shown). It is therefore relevant to pursue surveillance of this crop in France.

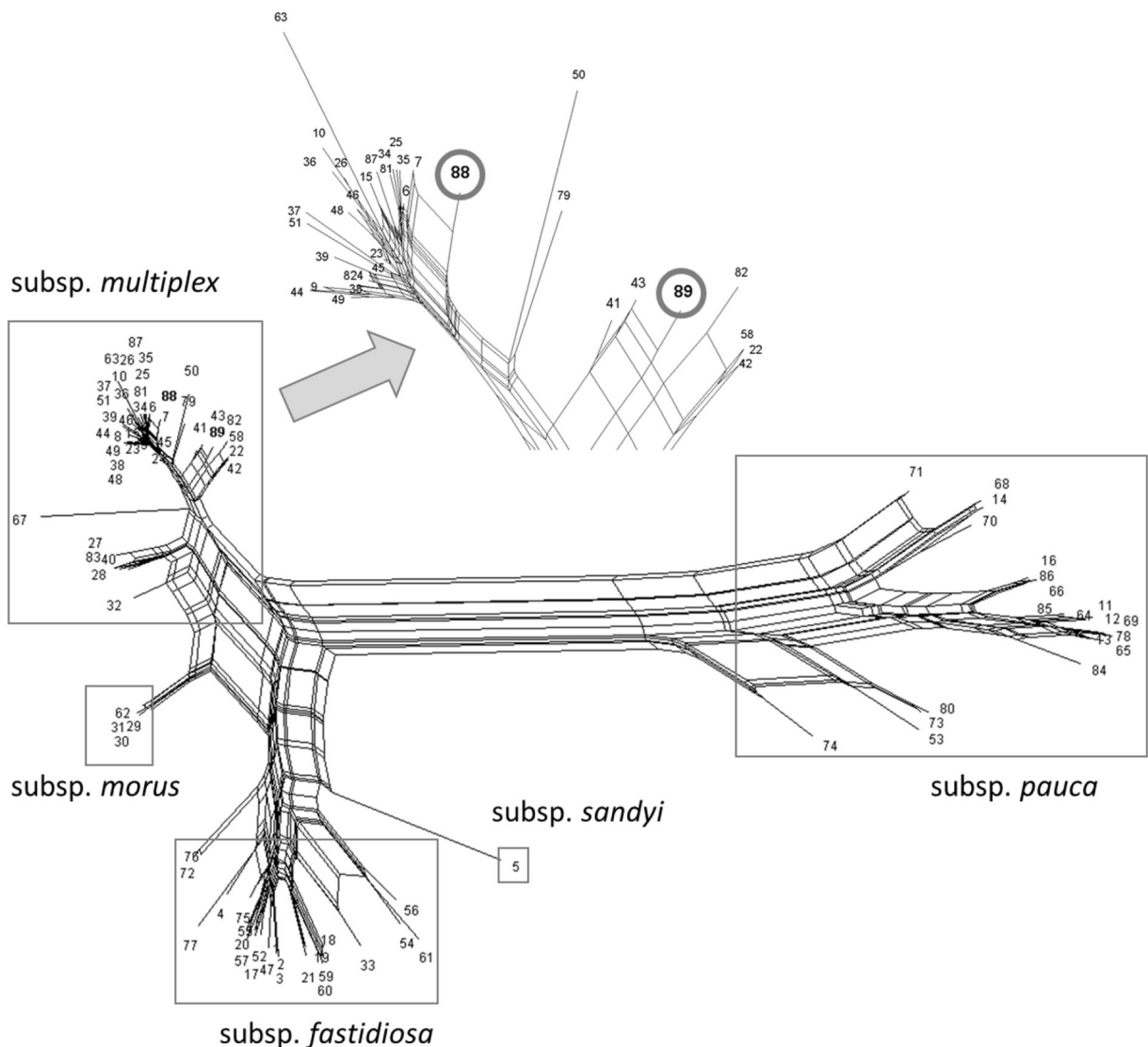


Fig. 2 Phylogenetic relationships of the 87 sequence types (STs) of *Xylella fastidiosa* available on PubMLST database and the two new variants ST88 and ST89 identified in these study, revealed

using Splits Tree4 (Huson & Bryant, 2005). The subspecies were highlighted by squares, and focus was done on the top of the figure for the ST88 and ST89, which were surrounded by grey circles

In summary, we revealed the presence of ST88 and ST89, two new STs of the subspecies *multiplex*, in the PACA region, on different host plants and at two different geographical locations, which are maintained over time. Based on the MLST results, we can hypothesize that in France ST88 and ST89 result from two distinct events. ST88 and ST7 belong to the same clonal complex as there are only two different nucleotides on only one gene out of the seven analysed by MLST. Both STs were found in the same location, and also in co-infection, suggesting that they share the same ecological niche. As the subspecies *multiplex* was found to exhibit a high number of recombination events (Vanhove et al., 2019) it is possible that other variants may be found in the PACA region. The strain ST89 is very different from the STs already present in the PACA region, and in Europe, as it belongs to a clonal complex including ST41 and ST43, reported only in the USA (Nunney et al., 2013). The presence of ST89 probably results from a new introduction, possibly from America. For the strains of ST6 identified in the Occitanie region, it will be interesting to go further and identify whether these strains are closely related to the French ST6 or the Spanish ST6, which is more closely related to French ST7 than to French ST6 (Landa et al., 2020). So, work is in progress on the genomic analyses of ST88 and ST89 strains, and also on the ST6 strains isolated in the Occitanie, to improve the knowledge of the subspecies diversity, and to give new insights into the origins and dates of introduction of *X. fastidiosa* subsp. *multiplex* strains present in France, and in Europe.

Acknowledgments We thank Fredon PACA, Fredon Occitanie, SRAL PACA and SRAL Occitanie for the collection of plants, and more particularly Denis Ferrieu (SRAL PACA) and Christine Colas (SRAL Occitanie).

Author's contributions BL performed the diagnostic tests; BL and AF performed strain isolation; CD, AF and SP performed the MLST-PCR and sequence analyses; AC performed the MLST analysis; AC prepared the manuscript; AC, BL, PdJ and VO revised the manuscript.

Declarations This article does not contain any studies with human or animal subjects performed by any of the authors.

Conflict of interest The authors declare that they have no conflicts of interest.

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