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Intercepted isolates of *Xylella fastidiosa* in Europe reveal novel genetic diversity

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Abstract After the first confirmed outbreak of *Xylella fastidiosa* in the European Union (EU), associated with an olive disease denoted olive quick decline syndrome, mandatory surveys are now carried out in the member States and inspections increased at EU entry points such as ports. Such activities led to the interception of *X. fastidiosa*-infected coffee plants in consignments originating from Central America. Similarly, the geographic expansion of the olive decline epidemic area of the Apulia region (southern Italy) prompted investigations to identify new host plants. Here we report the interception of three novel bacterial sequence types in Italy, based on multi-locus sequence typing, that cluster with different *X. fastidiosa* subspecies, illustrating the risk of the introduction of additional pathogen genetic diversity into Europe. In the epidemic area of Apulia, new foci as well as host plant species positive with *X. fastidiosa*, including cherry, myrtleleaf and rosemary, were found to be all infected with the same sequence type of this bacterium (ST53, or CoDiRO strain). This work highlights the limited knowledge of *X. fastidiosa* phylogenetic and phenotypic diversity, the risk of novel *X. fastidiosa* introductions via contaminated plant material, and corroborates other studies indicating that the Apulia epidemic emerged from a single introduction of this pathogen into the region.

Keywords Xylella fastidiosa · Olive disease · Pierce's disease, vector-borne

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Introduction

The recent emergence of *Xylella fastidiosa* into Europe, as evidenced by its widespread distribution in the southern region of Apulia in Italy (Martelli et al. 2015), as well as its presence throughout Corsica (France) (EPPO 2015) has greatly increased the awareness of the threat this pathogen poses to European agriculture and environment (Purcell 1997). Thus, interest has emerged about the possibility of limiting the number of potential introductions into Europe. A recent analysis of introduction pathways suggested that the importation of contaminated plant material presents high risk (EFSA 2015a). Similar conclusions have been reached in previous analyses (e.g. Rathé et al. 2012). However, most *X. fastidiosa*-colonized plants do not express symptoms (Purcell and Saunders 1999), many symptomatic hosts take several months to show symptoms after infection (Almeida and Nunney 2015),

and symptoms are often difficult to use as a diagnostic tool for large scale surveillance programs because they are often similar or associated to those induced by water stress (McElrone et al. 2003; Daugherty et al. 2010). Thus, detection of *X. fastidiosa* at ports of entries at the European Union (EU) level represents a logistical challenge, in addition to associated high costs.

Surveillance efforts established after the reporting of *X. fastidiosa* in Italy in October 2013 have led to interceptions of infected plants at a market south of Paris, as well as 11 positive plants intercepted at European ports during a two-week survey in late 2014 (EFSA 2015b). In addition, plants were also reported to be positive after introduction and maintenance in greenhouse conditions in France (Legendre et al. 2014). More recently, detection of *X. fastidiosa* throughout Corsica highlighted some of the challenges involved in detection and surveillance of a pathogen previously considered absent from the EU. Accordingly, many *X. fastidiosa* diseases are the result of long-distance introductions, most likely via contaminated plant material (Almeida and Nunney 2015). Examples include a North American genotype causing plum leaf scald in South America (Nunes et al. 2003), a Pierce's disease of grapevines genotype from the United States in Taiwan (Su et al. 2013), with the United States Pierce's disease clade itself having been introduced from Central America (Nunney et al. 2010). The recent introduction of a genotype of *X. fastidiosa* subspecies *pauca*, previously limited to South America, to Central America (Nunney et al. 2014a) and Italy (Loconsole et al. 2014; Elbeaino et al. 2014), represents another example. Therefore, long-distance dispersal followed by establishment has occurred multiple times, occasionally having devastating consequences.

In addition to the inherent challenges of reducing the number of likely introductions of *X. fastidiosa* into new areas, as well as of new genotypes into areas already with endemic or established *X. fastidiosa*, there are problems associated with the limited knowledge of *X. fastidiosa* genetic and phenotypic diversity. Very few genotypes/clades have had their host range studied (Almeida and Purcell 2003; Almeida et al. 2008), thus limiting inferences on potential threats. Furthermore, *X. fastidiosa* taxonomy has had a convoluted story, which now is understood to be a consequence of homologous recombination as this bacterium is naturally competent (Kung and Almeida 2011). The emergence of a previously unknown genotype in Italy (Loconsole et al. 2014; Elbeaino et al. 2014), coupled with its first report from Costa Rica during the same year (Nunney et al. 2014a), highlights the risk as well as threat of such knowledge gap, as no information was previously available about the host plant range or biology of this specific genotype (known as ST53). The introduction of multilocus sequence typing (MLST, Yuan et al. 2010) revolutionized *X. fastidiosa* taxonomy and organized phylogenetic clades in a meaningful manner in the face of frequent homologous recombination.

MLST uses data from seven housekeeping genes that are not under positive selection and are distributed throughout the chromosome. Individual sets of alleles are used to assign isolates to a Sequence Type (ST). We report on the detection and phylogenetic placement of *X. fastidiosa* isolates obtained from various plant species in the epidemic area of Apulia (southern Italy), as well as from coffee plants intercepted in three nurseries in northern Italy. Our results indicate that three of these isolates represent novel STs, with loci originating from different *X. fastidiosa* subspecies; these isolates were all intercepted in northern Italy. A fourth ST, ST53, has already been described in Italy and Costa Rica (Nunney et al. 2014a; Loconsole et al. 2014). These findings corroborate previous analyses that *X. fastidiosa*-colonized plant material represents a major pathway for the long-distance dispersal of this bacterium.

Material and methods

Bacterial isolates and host plants

Xylella fastidiosa isolates were recovered from different host plants in Italy. Specifically, four isolates (CO33026, CO33, CO15 and COBZ) were from ornamental coffee plants (*Coffee arabica*) intercepted in three nurseries in northern Italy, where they were recently imported from Costa Rica through the Netherlands (the origin of COBZ remains unknown). Most of these infected plants (CO33026, CO33, CO15) belong to the same contaminated lot of coffee plants identified in October 2014 in Netherlands (Bergsma-Vlami et al. 2015; EFSA 2015b). Fifteen other isolates were obtained from the outbreak area of Apulia (southern Italy) from symptomatic and asymptomatic naturally infected host plants (Table 1, Fig. 1). Isolates recovered from olive trees were collected from seven geographically distant foci in Apulia, including the one recently identified beyond the area demarcated as contaminated area (as of May 2015), in

Table 1 List of isolates used in this study, with available information as well as multi-locus sequence typing-assigned sequence type (ST). Site numbers correspond to locations in Fig. 1

Isolate	Host plants	Site	Symptoms	Origin	ST
CO33026	*Coffea arabica*	-	Mild leaf scorching	Costa Rica	ST76
CO33	*Coffea arabica*		Mild leaf scorching	Costa Rica	ST72
CO15	*Coffea arabica*		Symptomless	Costa Rica	ST72
COBZ	*Coffea arabica*		Symptomless	Unknown	ST73
ALM-1	*Prunus amygdalus*	1	Leaf scorching	Apulia, Italy	ST53
OLDR-1	*Nerium oleander*		Leaf scorching and decline	Apulia, Italy	ST53
OLG-2	*Olea europaea*		Quick decline	Apulia, Italy	ST53
Tr1	*Olea europaea*		Quick decline	Apulia, Italy	ST53
PW-1	*Catharanthus roseus*		Symptomless	Apulia, Italy	ST53
Ch-1	*Prunus avium*		Leaf scorching	Apulia, Italy	ST53
PM-1	*Polygala myrtifolia*		Leaf scorching and decline	Apulia, Italy	ST53
WF-1	*Westringia fruticosa*		Leaf scorching and decline	Apulia, Italy	ST53
KM13	*Olea europaea*	2	Quick decline	Apulia, Italy	ST53
H123	*Olea europaea*	3	Quick decline	Apulia, Italy	ST53
I31	*Olea europaea*	4	Quick decline	Apulia, Italy	ST53
I18	*Olea europaea*		Quick decline	Apulia, Italy	ST53
C24	*Olea europaea*	5	Quick decline	Apulia, Italy	ST53
C5	*Olea europaea*	6	Quick decline	Apulia, Italy	ST53
Br-1	*Olea europaea*	7	Quick decline	Apulia, Italy	ST53

the province of Brindisi. The remaining isolates were from plant species in the same area where *X. fastidiosa* was first detected in October 2013 (site 1, Fig. 1).

Bacterial culturing and sequencing of MLST loci

Xylella fastidiosa was cultured from all host plants during the Spring and Summer of 2015, except for samples CO15 and COBZ, using standard protocols (Almeida et al. 2001; Cariddi et al. 2014) and triply cloned prior to recovering the DNA for the amplification and sequencing of the set of genes part of the MLST scheme (*leuA*, *petC*, *malF*, *cysG*, *holC*, *nuoL*, and *gltT*; Yuan et al. 2010). MLST data for CO15 and COBZ were obtained via direct PCR of total DNA extracted from infected plant material; DNA was extracted using CTAB buffer following the procedure reported by Loconsole et al. (2014). GenBank accession numbers for deposited sequence data are: for allele *leuA*, 7 = KJ406216, 12 = KT722773; for *petC*, 6 = KJ406222, 12 = KT722779, 13 = KT722774; for *malF*, 16 = KJ406228, 15 = KT722775; for *cysG*, 24 = KJ406252, 26 = KT722776, 27 = KT722780; for *holC*, 10 = KJ406234, 24 = KT722777; for *nuoL*, 10 = KJ406240, 18 = KT722778; for *gltT*, 14 = KJ406246.

MLST and phylogenetic analyses

START2 (Jolley et al. 2004) was used to obtain a summary of the MLST data and the allelic profile for all samples. For the subsequent phylogenetic analyses, the data set included all STs available at the pubmlst *X. fastidiosa* database (http://pubmlst.org/fastidiosa) (Yuan et al. 2010), as well as the three new STs described in this study. Maximum likelihood and parsimony searches were performed for individual loci and concatenated sequences, neighbor-joining trees were also generated; all tree topology and bootstrap (1000 bootstraps for neighbor-joining and maximum parsimony, 100 for maximum likelihood) analyzes were performed with PAUP*4 (Swofford 2002). jModeltest (Posada 2008) was used to determine the best evolutionary models for each maximum likelihood search. Because *X. fastidiosa* is naturally competent (Kung and Almeida 2011) and the MLST data

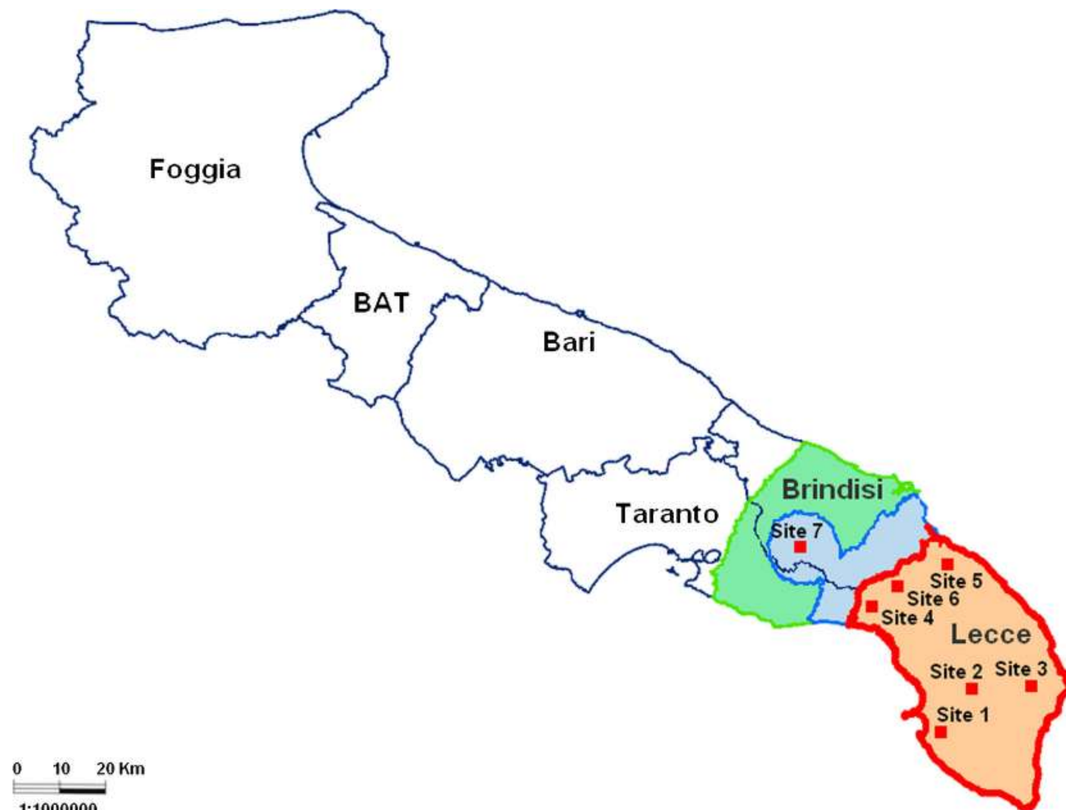

Fig. 1 Map of the Apulia region, with its different provinces, showing the demarcated areas (*orange* is the contaminated area, *blue* is the buffer zone, and *green* the surveillance zone), and the sites were *Xylella fastidiosa* isolates were collected

set includes recombinant STs (Almeida et al. 2008; Nunney et al. 2013, 2014b) as well as loci with intragenic evidence of recombination (Nunney et al. 2013), underlying assumptions of tree-building algorithms are often violated. Therefore we also used SplitsTree v.4. (Hudson and Bryant 2006) to build a NeighborNet phylogenetic network representing the evolutionary relationships among STs, where poorly supported branches are shown as a network. RDP4 version 4.14 (Martin and Rybicki 2000) was used to identify intragenic recombination in the three newly described STs; no intragenic recombination was detected for any of the new alleles reported here.

Results

Infected host plants

Leaf scorching on mature leaves were present on the intercepted coffee plants CO33026 (ST76), CO33 and CO15 (ST72); whereas the isolate COBZ (ST73) was associated with a symptomless coffee plant. All olive-tree *X. fastidiosa* isolates from Apulia were from symptomatic plants showing severe and persistent symptoms of branch dieback, desiccation and decline. Infected myrtleleaf (*Polygala myrtifolia*) and coastal rosemary (*Westringia fruticosa*) showed a similar disorder with desiccation of the foliage and branch dieback persisting over the seasons (Fig. 2a-b). Almond and cherry trees displayed leaf scorching affecting the mature leaves of some branches (Fig. 2c-d); such symptoms appeared and became evident during the summer season, which was also the period when the bacterium was detected from leaves of both of these hosts with PCR-based tools (Saponari et al. 2014).

MLST allelic profile and phylogenetic analyses

All isolates collected from the Apulia region belonged to ST53; details of the allelic profile of ST53 have been analyzed in detail elsewhere (Nunney et al. 2014a). Four

Fig. 2 Symptoms on host plants infected by *Xylella fastidiosa* strain CoDiRO. Infected hosts were identified during surveys made in the infected area during summer 2014. **a** *Polygala myrtifolia*; **b** *Westringia fruticosa*; **c** *Prunus amygdalus*; **d** *Prunus avium*

of the seven MLST loci were shared between ST53 and ST73 (Table 2); however, the alleles for the other three loci were phylogenetically clustered together (Fig. 3), suggesting that these STs have a shared evolutionary history. All loci from ST53 and ST73 belonged to well supported monophyletic groups with other alleles originated from isolates that cluster with *X. fastidiosa* subspecies *pauca*. The phylogenetic network derived from all MLST data publicly available indicated, as expected based on the allele relationships among the seven loci, that ST53 and ST73 are genetically closely related.

A similar relationship was observed for ST72 and ST76; all but one of the seven alleles was shared between these genotypes. The only locus not shared was *petC*, but the alleles were still clustered together. Most other loci of ST72 and ST76 have alleles that were loosely, but not exclusively, associated with *X. fastidiosa* subsp. *fastidiosa* (Fig. 3). In the case

Table 2 Allelic profiles associated with isolates of *Xylella fastidiosa* obtained from Italy. The CoDiRO isolate represents ST53, which is associated with the epidemic in Apulia

Isolate	ST	MLST loci						
		leuA	*petC*	*malF*	*cysG*	*holC*	*nuoL*	*gltT*
CO33026	76	12	13	15	26	24	18	1
CO33	72	12	12	15	26	24	18	1
CO15	72	12	12	15	26	24	18	1
COBZ	73	7	6	8	27	10	16	8
CoDiRO	53	7	6	16	24	10	16	14

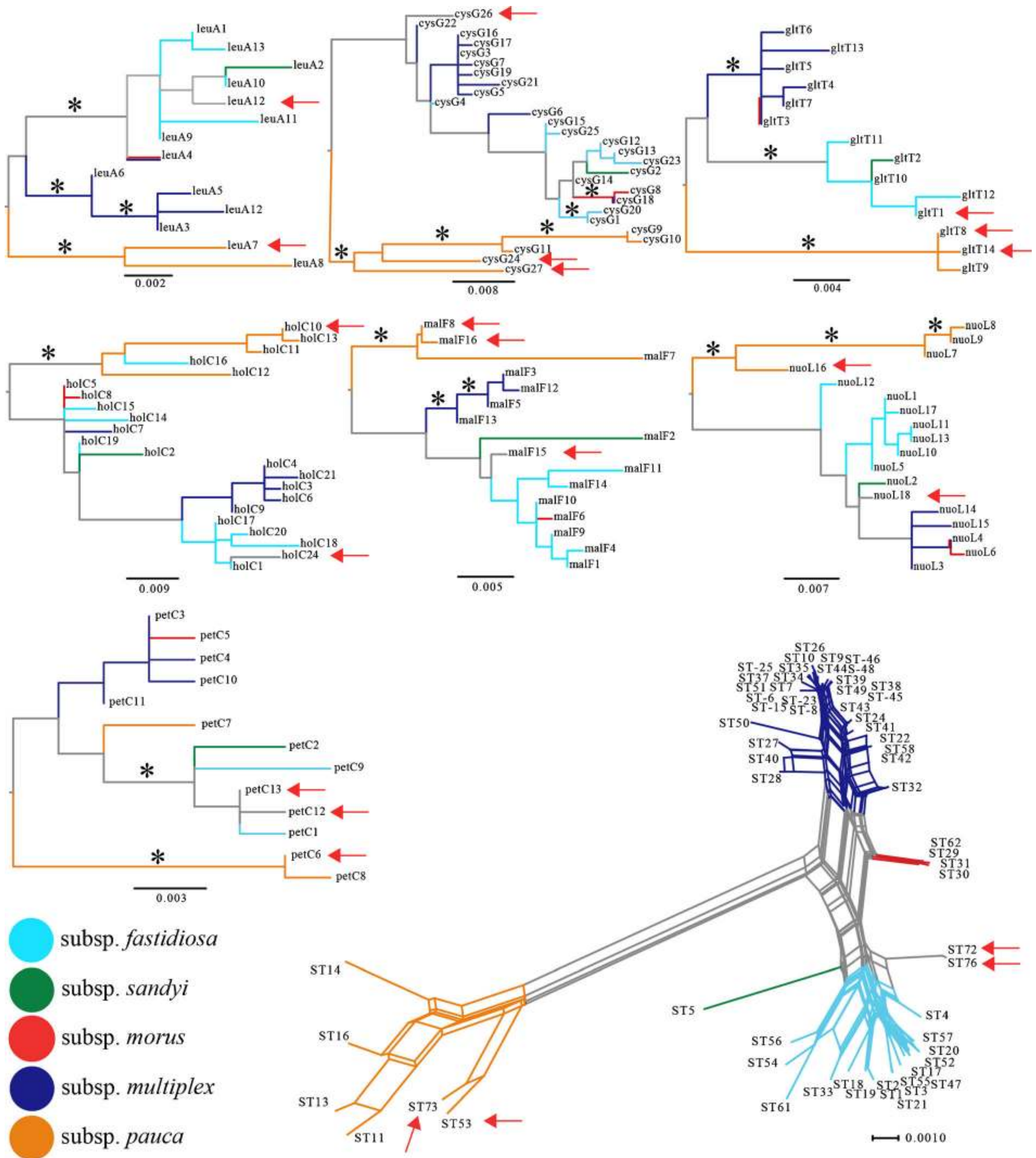

Fig. 3 Phylogenetic network of *Xylella fastidiosa* sequence types (STs, neighbor-net analyses of concatenated sequences) and single-locus maximum likelihood trees of *X. fastidiosa* loci used for multi-locus sequence typing analysis. *Asterisks* in loci trees represent branches supported by 80 % bootstrap support with maximum parsimony, maximum likelihood, and neighbor-joining tree searching methods. *Red arrows* indicate alleles and STs present (ST53) or intercepted (others) in Italy. Branches were color-coded based on phylogenetic placement of the concatenated STs, following current taxonomy of the species; branches in single-locus trees are color coded based on respective placement of the concatenated ST

of *nuoL* and *malF* these novel STs were placed more closely to subsp. *sandyi*, while in the case of *cysG* the allele (*cysG*26) was more closely associated with alleles derived from subsp. *multiplex*. It should be noted that many of the branches for the seven MLST loci were not supported with bootstrap analyses, which is not surprising as intragenic recombination has been previously reported for much of this dataset (Nunney et al. 2014c). Furthermore, the presence of intergenic recombination in *X. fastidiosa* is illustrated with the same dataset, where one *holC* allele originated from a subsp. *fastidiosa* ST (*holC*16) is nested within subsp. *pauca* alleles. Only three alleles (*leuA*4, *cysG*18, and *nuoL*4) were shared among subspecies, always between *multiplex* and *morus*. Despite being informative, relationship inferences among alleles should be performed with these caveats in mind.

The lack of strong association of the alleles of ST72 and ST76 with any subspecies, as observed with ST73 and subsp. *pauca*, is highlighted by the placement of these STs in the phylogenetic network. When the placement of ST5 (the sole ST associated with subsp. *sandyi*) is considered, ST72 and ST76 are relatively basal to the subsp. *fastidiosa* cluster. The boundaries of *X. fastidiosa* subspecies are largely based on geography and host plant species in which STs cause disease, rather than a quantitative approach. There is no information available about the host range of the isolates belonging to ST72 and ST76, other than their isolation from coffee plants introduced from Central America. Thus, we have refrained from assigning ST72 and ST76 to a subspecies, as it appears that it could indirectly impact the status of subsp. *sandyi* (ST5). It should be noted that the placement of subsp. *sandyi* alleles (ST5) is often closely related or nested within subsp. *fastidiosa* (Fig. 3). Furthermore, alleles for the seven loci of ST72 and 76 were also associated with subsp. *sandyi* alleles for *malF* and *nuoL*.

Discussion

The bacterium *X. fastidiosa* has been largely considered a pathogen of the Americas, so much so that review articles have addressed possible reasons why it was not present in Europe, for example, as long as twenty years ago (Purcell 1997). However, *X. fastidiosa* had been detected in Taiwan as early as 1992 (Leu and Su 1993), as well as in the Balkans in the mid-1990s (Berisha et al. 1998). During the last couple of years, however, it has been reported in Italy (Saponari et al. 2013), France (EPPO 2015), and Iran (Amanifar et al. 2014). The threat of *X. fastidiosa* to Europe and other regions, therefore, has led to additional work attempting to better understand introduction pathways as well as increased surveillance efforts. Here we report on the identification of three novel *X. fastidiosa* STs (i.e. genotypes) that were intercepted in northern Italy; in addition to a broader survey of infected plants in southern Italy, which we found to be all associated with one single ST (ST53).

We sampled symptomatic olive trees in the provinces of Lecce and Brindisi, all sampled trees were infected with the same genotype, ST53. The association of ST53 with the olive quick decline has been previously reported (Elbeaino et al. 2014; Loconsole et al. 2014,), but this survey over a larger geographical area, including the outbreak in the Brindisi province (site 7) at the northern edge of the epidemic indicates that only one pathogen introduction is associated with this emerging sanitary problem. Furthermore, five other plant species sampled from the infected zone were also infected with ST53, supporting the single introduction hypothesis. ST53 is also found in Costa Rica (Nunney et al. 2014a), in addition, shipments of plant material from Central America have also been found to be frequently infected with *X. fastidiosa* (EFSA 2015b). Altogether, this work provides additional support to the current hypothesis that infected plant material introduced from Central America was responsible for the current epidemic in southern Italy, which is driven by a single genotype. The five other plant species sampled here were previously reported to also be associated with *X. fastidiosa* (Saponari et al. 2014), except that here we also show they were infected with ST53.

Although only four coffee plants were intercepted and tested as part of this study, three novel STs were obtained. A series of relevant inferences may be made from this finding. First, there is not only a large amount of potentially contaminated plant material entering the EU, as already reported (EFSA 2015b; Bergsma-Vlami et al. 2015), but there is also a significant amount of genetic diversity being introduced. One of the three new STs (ST73), is related to ST53, clustering with subsp. *pauca*. These STs differ at three loci (*malF*, *cysG* and *gltT*); however, the alleles for all of these loci were clustered together and associated with subspecies *pauca* alleles, indicating a shared evolutionary history. ST72

and ST76 differ at only one locus, *petC*, but the placement of alleles also suggests a close evolutionary relationship between these STs. Intergenic recombination was not addressed in this study, although it is common in *X. fastidiosa* (Nunney et al. 2012), but it is clear that it has occurred with ST72 and ST76 due to the phylogenetic placement of allele *cysG*26 (no evidence of intragenic recombination was observed in the dataset). However, ST72 and ST76 form a divergent clade of *X. fastidiosa*, which is loosely associated with subsp. *fastidiosa* and subsp. *sandyi.* Therefore, in addition to the interception of an isolate with novel alleles (ST73) associated with a clade already present in Italy, the coffee plants surveyed also included a new and divergent clade of *X. fastidiosa* and a large set of previously undescribed alleles. Virtually nothing is known about ST72 and ST76. In addition, these data raise important questions about our knowledge of *X. fastidiosa* diversity, an important but ignored issue that has been previously discussed (Almeida and Nunney 2015).

The phylogenetic placement of ST72 and ST76 raises questions about *X. fastidiosa* taxonomy at large. The current scheme, which is generally accepted by the community, is based on MLST data (Almeida and Nunney 2015). The MLST approach provided a necessary framework for *X. fastidiosa* diversity to be studied in the presence of high rates of homologous recombination, but formal boundaries among subspecies were never proposed. Subspecies *sandyi* is represented by a sole ST (ST5), which is a sister clade to subsp. *fastidiosa*. ST72 and ST76 have alleles that are associated with alleles from STs belonging to subsp. *fastidiosa* and subsp. *sandyi*, and the concatenated sequence does not place these new genotypes within either subspecies. For these reasons, we refrained from assigning these STs to any subspecies due to their inconclusive placement. We hope that these STs serve as an opportunity for the *X. fastidiosa* community to consider how to integrate biological and genetic diversities into a well-supported, robust and useful taxonomic framework. It should be noted that information about *X. fastidiosa* biological diversity, especially in the context of host plant range, lags significantly behind knowledge about its genetic diversity.

Xylella fastidiosa is naturally competent (Kung and Almeida 2011) and natural populations have been demonstrated to frequently recombine (Almeida et al. 2008; Nunney et al. 2012, 2014b, 2014c). Furthermore, as recently reviewed, there is evidence supporting the hypothesis that gene flow via homologous recombination may be one of the drivers leading to the emergence of *X. fastidiosa* diseases (Almeida and Nunney 2015). The perceived threat of novel introductions of *X. fastidiosa* to the EU should be considered in relation to the fact that the pathogen already occurs in the region. In addition to the intrinsic risks associated with a new introduction, there is substantial risk of gene flow among genotypes if they occur sympatrically. For example, Nunes et al. (2003) clearly demonstrated how genetic material from subsp. *pauca* introgressed into the genome of a subsp. *multiplex* genotype introduced into South America. Therefore, the interception of *X. fastidiosa* via contaminated plant material does not only bring additional strain-specific risks, but also unforeseeable outcomes if in contact with ST53 in Italy, for example. The European flora has, to our knowledge, not been previously exposed to *X. fastidiosa*, so the potential host range of *X. fastidiosa* in the region remains to be estimated. In addition, there is no *X. fastidiosa* genotype-insect vector species specificity (Almeida et al. 2005) and there are potential *X. fastidiosa* vectors throughout the region (EFSA 2015a). The implementation of strategies aimed at reducing the probability of novel introductions into the EU is highly desirable in face of the potential risks.

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Compliance with ethical standards Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the authors.

Conflict of interest The authors declare no conflict of interest.

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