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Important genetic diversity of '*Candidatus* Phytoplasma solani' related strains associated with bois noir grapevine yellows and planthoppers in Azerbaijan

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Abstract Bois noir (BN) is an important grapevine yellows endemic to the Euro-Mediterranean basin caused by '*Candidatus* Phytoplasma solani' ('*Ca.* P. solani'), a non culturable plant pathogenic *Mollicute*. Bois noir symptoms could be associated with '*Ca.* P. solani' in two Azerbaijanian vineyards where disease incidence and severity were recorded for five local *Vitis vinifera* cultivars. In order to gain insight into the epidemiology of Bois noir in Azerbaijan, '*Ca.* P. solani' isolates infecting plants were characterized by multi-locus sequence analysis and their *sec*Y and *stamp* gene sequences compared to that of the strains detected in other plants and in local *Cixiidae* planthoppers. Genotypes were determined for two non-ribosomal house-keeping genes, namely *tuf* and *sec*Y, as well as two variable markers namely *Stamp* and *mle*P1 genes, that respectively encode the antigenic membrane protein AMP and a 2-Hydroxycarboxylate transporter. The Azerbaijanian BN phytoplasma isolates corresponded to three *tuf*B and *sec*Y genotypes. A finer differentiation of Azerbaijanian '*Ca.* P. solani' isolates was obtained with *mle*P1 as five different *mle*P1 genetic variants were found. Finally, *Stamp* gene allowed differentiating four new genotypes in grapevine among the 10 new *Stamp* genotypes detected in various plants in Azerbaijan. The preliminary survey for infected insects conducted in northern Azerbaijan, led to the identification of *Hyalesthes obsoletus* and *Reptalus noahi* as potential vectors for two '*Ca.* P. solani' new genotypes phylogenetically distant from the known genetic clusters. Altogether these results indicate an important genetic diversity of BN phytoplasmas in Azerbaijan that certainly result from spread through local insect vectors.

Keywords Plant pathogen · *Vitis vinifera* · *Cixiidae* · MLSA · *Tuf* · *secY* · *mle*P1 · *Stamp*

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Introduction

Bois noir is an important grapevine yellows (GY) caused by the stolbur phytoplasma from the 16SrXII-A subgroup

which was recently described as '*Candidatus* Phytoplasma solani' (Laimer et al. 2009; Quaglino et al. 2013). This disease is a major limiting factor for viticulture and seriously affects quality and quantity of grape production in the Euro-Mediterranian basin (Laimer et al. 2009; Johannesen et al. 2012; Aryan et al. 2014; Cvrković et al. 2014; Ember et al. 2016). '*Ca.* P. solani' is mainly transmitted to grapevines by hemipteran polyphagous planthoppers that belong to the *Cixiidae* family namely *Hyalesthes obsoletus* (Maixner 1994) and *Reptalus panzeri* (Cvrković et al. 2014). Multilocus sequence analysis (MLSA) based on the amplification and sequencing of non-ribosomal gene is widely used in genotyping of phytoplasmas for taxonomy, evolution or epidemiology surveys (Lee et al. 2004; Arnaud et al. 2007; Danet et al. 2011; Johannesen et al. 2012). Molecular typing of '*Ca.* P. solani' isolates associated with grapevine yellows is also critical to investigate the different pathways of BN propagation (Langer and Maixner 2004; Cvrković et al. 2014; Aryan et al. 2014). Different molecular typing markers were developed for epidemiological studies on '*Ca.* P. solani' isolates, among which the most widely used are: gene *tuf* that encodes the translation elongation factor TU (Langer and Maixner 2004); gene secY, which encodes a component of the protein secretion machinery (Fialova et al. 2009); *Stamp* gene encoding the antigenic membrane protein AMP (Fabre et al. 2011a); and vmp1 gene encoding a variable membrane protein (Cimerman et al. 2009; Pacifico et al. 2009). In most cases these markers are used in combination to resolve regional epidemic cycles (Johannesen et al. 2012; Cvrković et al. 2014; Aryan et al. 2014; Kostadinovska et al. 2014; Atanasova et al. 2015; Kosovac et al. 2016; Delić et al. 2016). Upon restriction map of the gene *tuf*, '*Ca.* P. solani' isolates were classified into two main genotypes; tuf-a, and tuf-b. In Southwestern Germany, Langer and Maixner (2004) showed that the two tuf types were associated to different natural epidemic cycles of '*Ca.* P. solani'. While isolates of tuf-a type spread from the stinging nettle *Urtica dioica*, isolates of type tuf-b disseminated from the bindweed *Convolvulus arvensis*, due to their respective transmission by *U. dioica* or *C. arvensis*-specialized populations of *H. obsoletus*. A third minor genotype tuf-c was shown to propagate from the *Calystegia sepium* bindweed (Langer and Maixner 2004; Johannesen et al. 2012). Recently, a different tuf-b genotype, assigned to the new subtype tuf-b2, was associated to a sudden increase of BN propagating from *U. dioica* plant reservoir in Austria (Aryan et al. 2014).

The grapevine (*Vitis vinifera*) is one of the most cultivated plants and has a great economic importance for Azerbaijan Republic. Recent surveys conducted to the main grape-growing areas of Azerbaijan, revealed the presence of '*Ca.* P. solani' associated with grapevine yellows (Balakishiyeva et al. 2016). As a first step toward the deciphering of BN epidemiology in Azerbaijan, the genetic identity of BN phytoplasma isolates was determined in two main viticultural areas of the country, the Gabala region and Absheron peninsula. MLSA of '*Ca.* P. solani' BN isolates was therefore implemented by sequencing the two non-ribosomal house-keeping genes *tuf* and *sec*Y, the variable gene *Stamp* and *mle*P1, formerly described as *cit*S, that encodes a 2-Hydroxycarboxylate transporter (Balakishiyeva et al. 2012). '*Ca.* P. solani' strains detected in planthoppers of the family *Cixiidae* were also genetically characterized.

Material and methods

Phytoplasma isolates and total nucleic acid extraction

Grapevine samples displaying leaf discoloration and rolling, shrivelling of grapes and incomplete lignifications of canes, were collected from three main grapevine-growing areas, in Ganja area, the Absheron peninsula in August 2014 and in Gabala region in September 2015 (Table 1). Symptomless grapevine plant materials were also sampled as negative control. Total nucleic acids were extracted from 1 g of fresh leaf vein or petioles of symptomatic and symptomless grapevine plants using the classical CTAB method described by Maixner et al. (1995). DNA extracts of '*Ca.* P. solani' isolates from vegetables and fruit crops previously collected in Absheron peninsula, Guba, Sheki regions of Azerbaijan (Balakishiyeva et al. 2010, 2012, 2013) and reference '*Ca.* P. solani' isolates maintained at INRA Bordeaux by grafting inoculation on *Catharanthus roseus* Madagascar periwinkle were also included to this study (Table 3).

Insect collection, identification and total nucleic acids extraction

In July 2013 insects were collected by sweep-netting on *Convolvulus arvensis* in Guba region. They were

Table 1 Grapevine samples collected in Azerbaijan and results of universal phytoplasma 16SrDNA PCR

V. vinifera sample name	Type of cultivar	Location	Number of positive 16S nested PCR / number tested
AZ. Gr01–14 to AZ.Gr.09–14	Red	Ganja	0/9
AZ. Gr10–14 to AZ.Gr.13–14	White	Ganja	0/4
AZ. Gr15–14 to AZ.Gr.20–14	White	Absheron	0/6
AZ. Gr21–14 to AZ.Gr.24–14	Red	Absheron	4/4
AZ. Gr05–15 to AZ.Gr.15–15	Red	Gabala	4/11

recovered with a mouth aspirator, separated according to family, morphologically examined under a binocular microscope to separate *Cixiidae* from *Cicadomorpha* or other planthoppers according to the Holzinger et al. (2003). *H. obsoletus* were tested immediately or kept at −25 °C in 70% ethanol for later testing. Total nucleic acids were extracted from 50 individual of the *H. obsoletus* species according to a previously published procedure (Foissac et al. 2000). DNA extract of '*Ca*. P. solani' isolate (AZ12-RN-15) from cixiid planthopper initially reported as *Setapius sp.* but finally as assigned to *Reptalus noahi* collected in June 2012 in Guba region (Balakishiyeva et al. 2013) was also included in this study.

Disease severity

Disease severity corresponded to the average percentage of canes exhibiting BN symptoms per diseased plant.

Phytoplasma detection

For phytoplasma detection, DNA extracts from 34 grapevine samples and 50 *Hyalesthes obsoletus* insects were tested by nested PCR using the 16S phytoplasma-specific primers R16mF2/R16mR1 followed by R16mF2n/R16mR2 (Gundersen and Lee 1996) and obtained amplicons sequenced. All PCR products were analysed by electrophoresis in 1% gel (MetaPhor® Agarose, USA), stained with ethidium bromide and visualized under UV light.

Typing on non-ribosomal genes

All of the grapevine BN phytoplasma isolates detected in Azerbaijan, '*Ca.* P. solani' isolates previously detected in annual and perennial plants in Guba, Sheki regions and Absheron peninsula (Balakishiyeva et al. 2010, 2012, 2013) and '*Ca.* P. solani' reference strains (Table 3) were subjected to genotyping on four non-ribosomal genes: *tuf*, *sec*Y, *Stamp* and *mle*P1. '*Ca.* P. solani' isolates extracted from *Cixiidae* planthoppers were also submitted to *sec*Y and *stamp* genotyping. The *tuf* gene was amplified using STOLTUF-F0 (5′-GCACGTTGATCACGGCAAAAC-3′) / STOLTUF-R0 (5'-CTGTTTTTCCACCTTCACGG-3′) primer pair for direct PCR and TufAYf/r primers for nested PCR, following the protocol described by Langer and Maixner (2004). Amplification of the *sec*Y gene was performed in nested PCR with primers PosecF1 and PosecR1 followed by PosecF3 and PosecR3 as previously reported (Fialova et al. 2009). The *Stamp* gene amplification was performed using the primer pair StampF/R0, and then by the nested primer pair StampF1/R1, according to the procedure described by Fabre et al. (2011a). Finally, a 1118 bp fragment of the *mle*P1 gene, deposited in European Nucleotide Archive study number PRJEB24172, was amplified using primer pair MleP1-F3 (5′- ATCCAACTAGTCGGATTACC -3′) / MleP1-R3 (5′- ACAACAATAAAAGCGCCTCC -3′) and nested primer pair MleP1-F5 (5′- TTATGCAT CATCGTCCCTTC -3′) / MleP1-R4 (5′- TGAGCAAA TGGCATTAACCC -3′). PCR conditions were 4 min at 94 °C as an initial denaturation step, followed by 35 cycles of 30s at 94 °C, 30 s at 55 °C and 1 min at 72 °C, and an additional elongation step of 7 min at 72 °C.

Sequencing and phylogenetic analysis

All PCR products were directly sequenced on both strands by Beckman Coulter Genomics Company (Takeley, UK) on MegaBACE capillary sequencing instruments. The raw sequence chromatograms were

assembled and edited using the Phred-Phrap-Consed package version 29.0 (Gordon et al. 1998; Ewing et al. 1998). Reference for *tuf, secY* and *Stamp* sequences corresponding to previously published data were selected for phylogenetic analyses (Cimerman et al. 2006; Cimerman et al. 2009; Fabre et al. 2011a; Balakishiyeva et al. 2012; Johannesen et al. 2012; Cvrković et al. 2014; Cvrković et al. 2014; Delić et al. 2016). Multiple sequences alignments were performed using the CLUSTAL-W (Thompson et al. 1994). Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). The MP trees were obtained using the Subtree-Pruning-Regrafting (SPR) algorithm and the trees were drawn to scale, with branch lengths calculated using the average pathway method. The most parsimonious tree was shown. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. Statistical validity of phylogenetic branches was evaluated by 250 bootstrapping and the percentage of replicate trees in which the associated taxa clustered together in the 250 replicates bootstrap test was calculated. All sequences have been deposited in the European Nucleotide Archive under the study accession number PRJEB21652.

Results

Symptom surveys for BN were conducted in three main grape-growing areas in Azerbaijan, namely Ganja, Gabala region and Absheron peninsula (Table 1, Fig. 1a) in 2014 and 2015. During the surveys of different red and white *Vitis vinifera* cultivars, symptoms reminiscent of phytoplasma infection could been observed such as leaf reddening or yellowing depending on the cultivar, leaf rolling, drying of grapes and incomplete lignification of the canes. Phytoplasma universal 16SrDNA PCR amplification allowed to detect phytoplasmas in two locations namely Gabala and Absheron peninsula (Table 1). As indicated in a preliminary report, all 16SrDNA sequences amplified from Azerbaijanian grapes were identical to '*Ca.* P. solani' 16SrDNA (Balakishiyeva et al. 2016). In Absheron peninsula, BN symptoms associated with '*Ca.* P. solani' were observed in four different local red grapevine cultivars namely "Qara shani", "Ala shani", "Qara yay uzumu", and "Mahmudu", whereas in Gabala region BN symptoms were observed on the "Qara kishmish" red cultivars. The diseased cultivars appeared very sensitive to the disease as the severity, i.e. the proportion of canes displaying BN symptoms on a diseased plant, ranged from 50 to 100% (Table 2). '*Ca.* P. solani'-specific nested PCR test using the primers StampF/R0, followed by the primers StampF1/R1 revealed that 13 individuals were positive for '*Ca.* P. solani' among the 50 *H. obsoletus* planthoppers analysed. The eight grapevine samples as well as the 13 *H. obsoletus* individuals positive for '*Ca.* P. solani' were submitted to further genetic characterization of the associated phytoplasma isolates (Tables 1, 3).

All Azerbaijanian grapevine BN isolates yielded successful amplifications of the *tuf* gene. Phylogenetic analysis of *tuf* sequences using the maximum of parsimony method showed the presence of three '*Ca.* P. solani' *tuf* genotypes among Azerbaijanian grapevine BN isolates (Fig. 2a). BN isolate of grapevine cv. 'Qara yay uzumu' (AZ GR23–14) sampled from Absheron peninsula corresponded to the tuf-type 'b' common genotype in Western Europe known to be associated with bindweed (*C. arvensis*) and hereby referred as tuf-b1 (Langer and Maixner 2004). The other BN isolates from Absheron peninsula detected in cv. Qara shani' and 'Mahmudu' (AZ GR21–14 and AZ GR24–14) had a *tuf* sequence identical to that the isolate DEP and of the tuf-b2 genotype associated with *U. dioica* wild plant reservoir in Austria (Aryan et al. 2014). All the BN isolates detected in cv. 'Qara kishmish' collected from Gabala region (AZ GR05/06/08/15–15) gave the same genotype identical to the tuf-b3 genotype recently detected in a diseased potato plant collected in Romania (Ember et al. 2011; Foissac et al. 2013).

Sequencing of the *secY* gene also resulted in differentiating three distinct *secY* genotypes in Azerbaijanian grapevines (Fig. 2b). All of the '*Ca.* P. solani' isolates associated with BN detected in cv. 'Qara kishmish' collected from Gabala region had again the same *secY* genotype, a new genotype different to all reference '*Ca.* P. solani' isolates reported to date. This *secY* sequence differed by two single nucleotide polymorphisms (SNP) to the reference genotype S4 commonly found in Europe (Foissac et al. 2013). BN isolates detected in cv. 'Qara shani', cv. 'Ala shani' and cv. 'Qara yay uzumu' corresponded to the *secY* genotype S1 which is prevalent all over the Mediterranean area (Foissac et al. 2013). It was also detected in eggplant (AZ12AU6) and pepper (AZ12PV1) in Azerbaijan (Fig. 2b). Finally, BN isolates detected from cv.'Mahmudu' had a *secY* genotype distinct from all the other BN isolates collected in Absheron peninsula and was identical to the genotype S14, up to now only detected in French lavenders (Fabre et al. 2011a) and

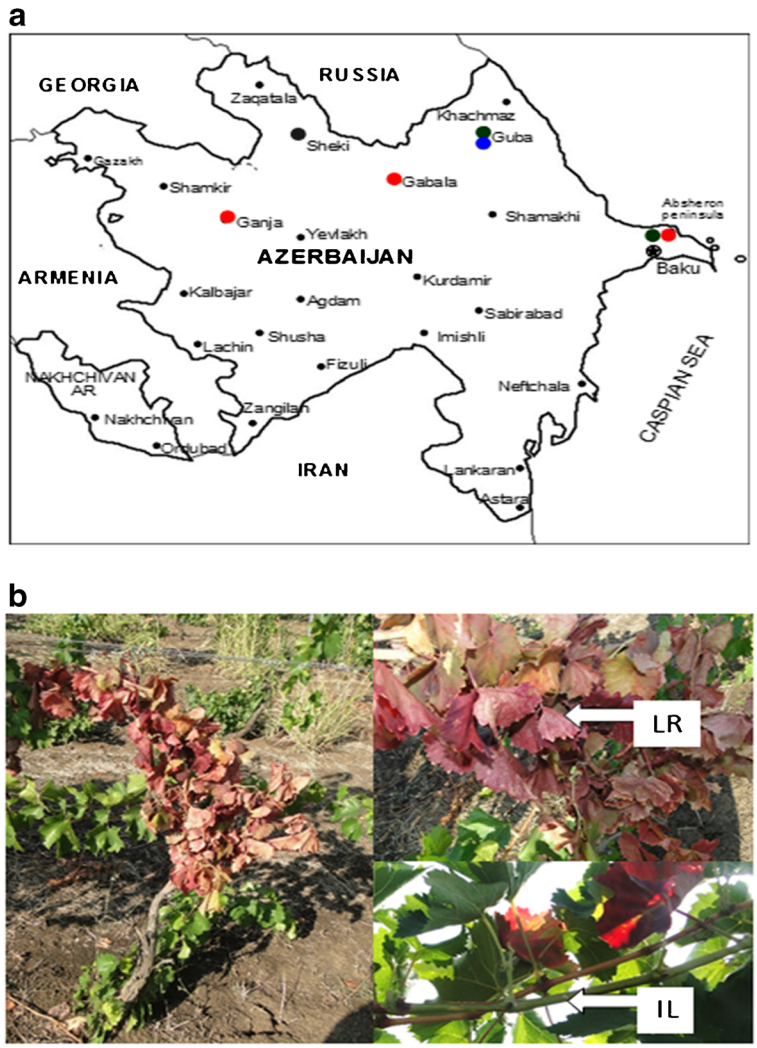

Fig. 1 Sampling location and typical symptoms of grapevine yellows in Azerbaijan. Location of grapevine, vegetable, fruit tree and insect sampling in Azerbaijan (**a**). Locations of grapevine sampling are indicated in red circles. Location of vegetable and fruit tree samples collected previously are indicated in green circle. Location of insect sampling is indicated by blue circle. Typical symptoms of grapevine yellows on young red cultivar "Qara kishmish" in Gabala region of Azerbaijan (**b**), including leaf reddening (LR) and incomplete lignification of the cane (IL)

in the reference isolate DEP from *Lavandula intermedia* maintained in Madagascar periwinkle. A fourth *secY* sequence not detected in grapevine, was detected in the Guba region in a declining *Prunus myrobolan* tree

Table 2 Azerbaijanian BN grapevine isolates and disease severity in the corresponding plot

Isolate	Grapevine cultivar	Cultivar type	Collected location	Disease severity in corresponding field
AZ-GR-21-14	"Qara shani"	Red	Absheron peninsula	60%
AZ-GR-22-14	"Ala shani"	Red	Absheron peninsula	100%
AZ-GR-23-14	"Qara yay uzumu"	Red	Absheron peninsula	50%
AZ-GR-24-14	"Mahmudu"	Red	Absheron peninsula	80%
AZ-GR-05-15	"Qara kishmish"	Red	Gabala	100%
AZ-GR-06-15	"Qara kishmish"	Red	Gabala	50%
AZ-GR-08-15	"Qara kishmish"	Red	Gabala	70%
AZ-GR-15-15	"Qara kishmish"	Red	Gabala	100%

Table 3 Azerbaijanian (previously collected) and reference '*Ca*. P. solani' isolates used in this study

Code	Original plant host / experimental host	Country of origin
AZ3-CE	*Prunus avium*	Azerbaijan/Guba region
AZ7-PV1	*Capsicum annuum*	Azerbaijan/Guba region
AZ7-AUB3	*Solanum melanogera*	Azerbaijan/Guba region
AZ8-NEF40	*Mespilus germanica*	Azerbaijan/Sheki region
AZ8-TO41	*Solanum lycopersicum*	Azerbaijan/Sheki region
AZ8-TO42	*S. lycopersicum*	Azerbaijan/Absheron peninsula
AZ12-PV1	*Capsicum annuum*	Azerbaijan/Absheron peninsula
AZ12-PV3	*Capsicum annuum*	Azerbaijan/Absheron peninsula
AZ12-AU6	*Solanum melanogera*	Azerbaijan/Absheron peninsula
AZ12PRM13	*Prunus myrobolan*	Azerbaijan/Guba region
AZ12-RN-15	*Reptalus noahi*	Azerbaijan/Guba region
AZ13-H05 to AZ13-H44	*Hyalesthes obsoletus*	Azerbaijan/Guba region
P7	*Catharanthus roseus*	Lebanon
Moliere	*Prunus avium* / *C. roseus*	France
PO	*Hyalesthes obsoletus* /*C. roseus*	France
LG	*Solanum lycopersicum*/ *C. roseus*	France
Char1	*H. obsoletus*/ *C. roseus*	France
STOL	*Capsicum annuum* / *C. roseus*	Republic of Serbia
L973	*Lavendula intermedia*	France
19–25	*Vitis vinifera*/ *C. roseus*	Germany
GGY	*V. vinifera*/ *C. roseus*	Germany
CH1	*V. vinifera*/ *C. roseus*	Italy
T292	*S. lycopersicum*/ *C. roseus*	Italy
SB1	*V. vinifera*	Croatia
LOZA2	*V. vinifera*	Serbia
BG4560	*V. vinifera*	Bulgaria
GR13	*V. vinifera*	Greece
GR328	*C. annuum*	Greece
CZ3148	*Convolvulus arvensis*	Czech republic
CZ3169	*Apium graveolense*	Czech republic
RO_R597	*Solanum tuberosum* L.	Romania
DE 30003-T3	*V. vinifera*	Germany
DEP	*Lavandula intermedia* / *C. roseus*	France

sampled in 2012. This genotype was also detected in six infected *H. obsoletus* insects collected on bindweed in 2013 in the vicinity of the same Prunus orchard.

Sequencing of the *mle*P1 gene allowed the highest differentiation of Azerbaijanian BN isolates with five different *mle*P1 genotypes detected in grapevine (Fig. 2c). All the four '*Ca.* P. solani' isolates collected in 2014 in Absheron peninsula gave different *mle*P1 sequences, whereas all the four isolates collected in 2015 in Gabala had the same *mle*P1 gene. The *mle*P1 sequence of the BN isolate AZ GR22–14 from Absheron peninsula was also detected in tomato, pepper, cherry and common medlar previously collected in Guba, Sheki regions and Absheron peninsula of Azerbaijan and in seven French, Italian, German and Lebanese '*Ca*. P. solani' reference isolates maintained in periwinkle. Similarly the mleP1 genotype detected in AZ GR23–14 was also detected in an eggplant collected in 2007 in Northern Azerbaijan as well as in

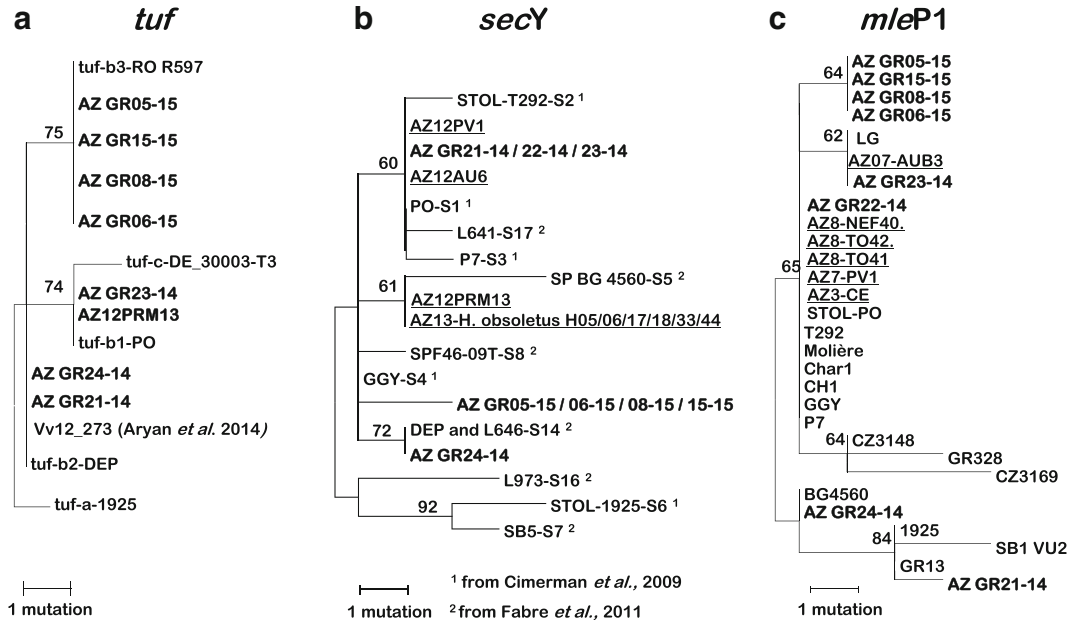

Fig. 2 Phylogenetic analyses of *tuf, sec*Y and *mle*P1 sequences using maximum parsimony. **a** gene *tuf*. **b** gene *sec*Y. **c** gene *mle*P1. Azerbaijanian BN isolates are indicated in bold letters and other 'Ca. *P. solani*' isolates from Azerbaijan are underlined. The most parsimonious trees are shown with boostrap values indicated along the branches

the French isolate LG originally transmitted from tomato to *C. roseus* using *Cuscuta campestris.* The most distant variant was detected in AZ GR21–14 as it differed by 3 SNP to AZ GR24–14, 4 SNP to AZ GR22–14 and 5 SNP to the others Azerbaijanian BN isolates. This mleP1 distant sequence was clustering with *mle*P1 sequences of reference isolates corresponding to type tuf-a.

The phylogenetic analysis of *Stamp* genes allowed to distinguish four different *Stamp* genotypes among eight Azerbaijanian grapevine BN isolates (Fig. 3). The two BN isolates detected in cv. 'Qara kishmish' from Gabala region, corresponded to the same new *Stamp* genotype, whilst the other Azerbaijanian grapevine BN isolates showed three different sequences. *Stamp* gene amplified from the BN isolate AZ GR22–14, AZ GR06–15 and AZ GR15–15 yielded ambiguous sequences that could not be analysed. Maximum parsimony phylogenetic analysis including already published *Stamp* sequences showed four genetic clusters, i.e. I, II, III, IV (Fabre et al. 2011a, b) in addition of two phylogenetic clusters exclusively composed of Azerbaijanian isolates. The three *Stamp* genotypes detected in grapevine from Absheron peninsula (AZ GR21–14, 23–14 and 24–14) clustered within the cluster III with four other *Stamp* genotypes detected in eggplants, peppers, tomato, common medlar and cherry collected in Azerbaijan from 2003 to 2012 and with '*Ca*. P. solani' isolates detected in Balkans and in Lebanon. No Azerbaijanian isolates was clustering with the already described clusters I, II and IV. The two *Stamp* sequences detected in BN from Gabala, AZ GR05–2015 and GR08–2015, clustered with two genotypes detected in *H. obsoletus.* These genotypes constituted a distinct branch supported by a bootstrap of 100, characterized by a unique GCT deletion at position 94, as well as possessed a 12 nt deletion in common with genotypes from clusters I, II and III. This sequence was also deleted in the unique *Stamp* genotype AZ12PRM13 detected in *P. myrobolan* and in eleven *H. obsoletus* and one *Reptalus noahi* (Emeljanov 1995) collected in Guba region, seventy kilometres away from Gabala (Fig. 1a). This unique sequence was distant from other *Stamp* clusters and was characterized by a unique GTT deletion signature. This *Stamp* genotype was not evidenced in any of the Azerbaijanian BN isolates.

Discussion

Bois noir epidemiology is peculiar by comparison to Flavescence dorée, another grapevine yellows that

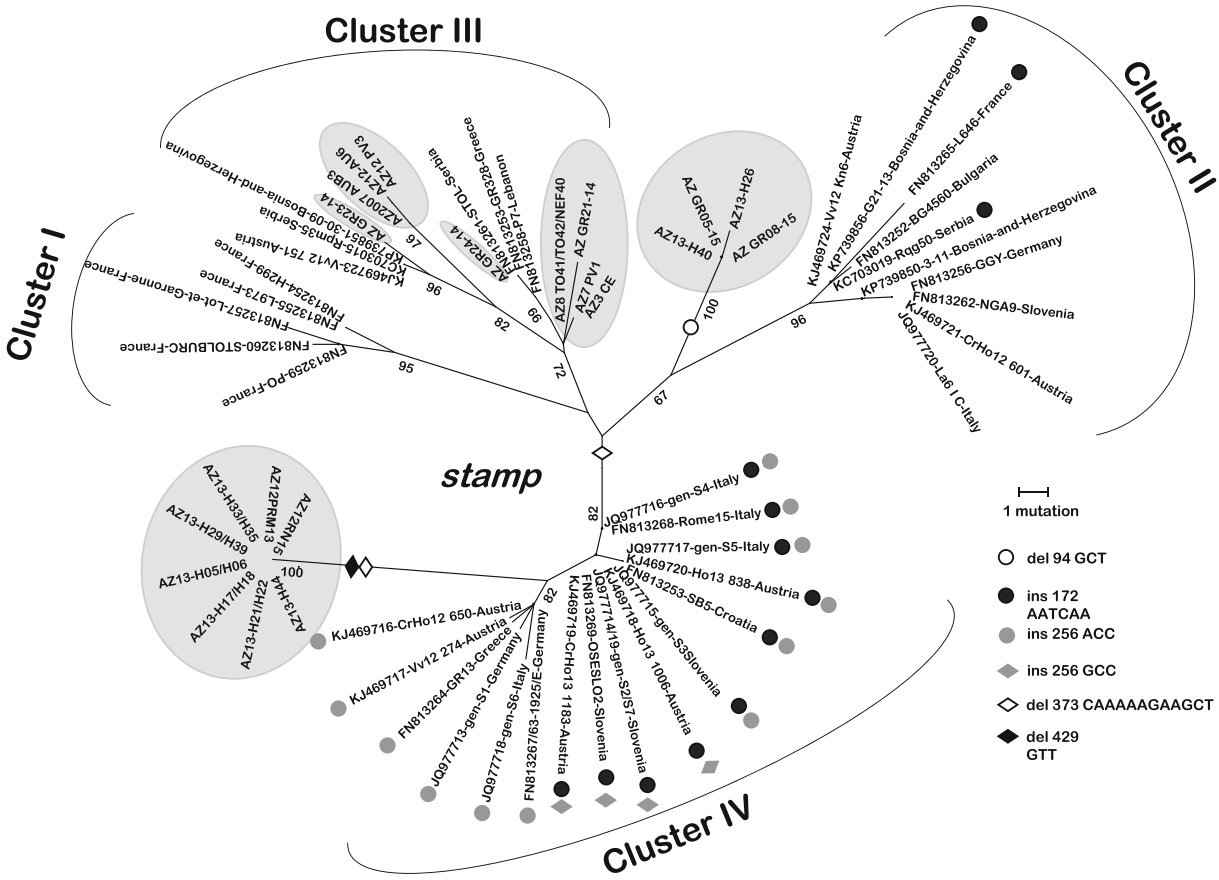

Fig. 3 Phylogenetic analysis of *Stamp* sequences using Maximum parsimony. Azerbaijanian '*Ca.* P. solani' isolates are is grey background. The most parsimonious tree is shown with boostrap values indicated along the branches. Insertion and deletion specific to isolates or phylogenetic branches are indicated at the top of isolate names or along branches. *Stamp* genetic clusters are those described in Fabre et al. 2011b

epidemically propagates through a grapevine-specific leafhopper vector. Indeed, BN does not naturally propagate from infected grapevine but spreads from wild plant host reservoirs that act as reproduction hosts for planthopper vectors of the family *Cixiidae* (Langer and Maixner 2004; Cvrković et al. 2014). The genetic characterization of '*Ca.* P. solani' strains associated to Bois noir led to the identification of different epidemiological cycle involving different weeds and insect vector species or ecotypes (Langer and Maixner 2004; Cvrković et al. 2014; Aryan et al. 2014). To control BN, tracing the presence of the '*Ca.* P. solani' genotypes in wild plant hosts surrounding the infected vineyards requires a precise genetic characterization of the phytoplasma strains associated with BN cases. The development of *mle*P1 as an additional variable genotyping marker will improve the resolution of the MLSA typing tools. In Azerbaijan, the high genetic diversity of '*Ca.* P. solani' isolates, found in grapevine and in annual vegetable crops, supports an active spreading through insect vectors rather than the vegetative propagation of '*Ca.* P. solani' –infected grapevine planting material. In addition, the *Stamp* genotypes detected in BN affected grapes in Azerbaijan are unique and are not likely to result from the importation of infected planting material from European countries. The presence of the three tuf-b genotypes tuf-b1, b2 and b3 in analyzed Azerbaijanian BN isolates, certainly reflects a complex epidemiology that may involve more than one plant reservoir or insect vector species. These wild plant reservoirs remain to be identified in Azerbaijan. Up to now, the bindweed species *C. arvensis* and the stinging nettle *U. dioica* have been reported to play important roles in the epidemiology of tuf-b1 and tuf-b2 genotypes respectively and should therefore focus attention. Their role as reservoir hosts for '*Ca.* P. solani' in Azerbaijan needs to be

demonstrated. Nevertheless, as new genotypes, especially for gene *Stamp*, were found in Azerbaijan, the involvement of other plant reservoirs such as *Vitex agnus castus* that was recently highlighted as a possible origin of several genotypes occurring in central Europe cannot be ruled out (Kosovac et al. 2016). In addition, the reservoir host of the new tuf-b3 genotype is not yet established. The preliminary survey for infected *Cixiidae* planthoppers in Azerbaijan shows that not only *H. obsoletus* was found infected but also species like *Reptalus noahi* can be infected by '*Ca.* P. solani'. However, the vectoring ability and plant hosts of Azerbaijanian populations of *H. obsoletus* and populations of other species such as *R. noahi* must be further investigated to sustain practical solutions to reduce the incidence of the disease.

Author contribution All authors have been personally and actively involved in substantive work leading to the manuscript. Conceived and designed the experiments: GB, XF, IH; Performed the survey: GB, AM, EV, JB, XF. Identified insects: J-LD; Performed the molecular experiments: GB, JB, AM, PS, IE; Analyzed the data: GB, IH, XF; Contributed reagents/materials/analysis tools: IH, XF. Wrote the paper: GB, XF.FundingThis study was funded by Science Development Foundation under the President of Azerbaijan Republic, Grant № EIF-2013-9(15)-46/28/3) and the INRA meta-programme Sustainable Management of Crop Health SMACH LYCOVITIS.

Compliance with ethical standards

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

Human and animal rights This research does not include any animal and/or human trials.
All sequences have been deposited in the European Nucleotide Archive under the study accession number PRJEB21652.

References

Arnaud, G., Malembic-Maher, S., Salar, P., et al. (2007). Multilocus sequence typing confirms the close genetic interrelatedness of three distinct flavescence doree phytoplasma strain clusters and group 16SrV phytoplasmas infecting grapevine and alder in Europe. *Applied and Environmental Microbiology, 73*, 4001–4010.

Aryan, A., Brader, G., Mörtel, J., Pastar, M., & Riedle-Bauer, M. (2014). An abundant '*Candidatus* Phytoplasma solani' tuf b strain is associated with grapevine, stinging nettle and *Hyalesthes obsoletus*. *European Journal of Plant Pathology, 140*, 213–227.

Atanasova, B., Jakovljević, M., Spasov, D., et al. (2015). The molecular epidemiology of bois noir grapevine yellows caused by '*Candidatus* Phytoplasma solani' in the republic of Macedonia. *European Journal of Plant Pathology, 142*, 759–770.

Balakishiyeva, G., Danet, J.-L., Qurbanov, M., Mamedov, A., Kheyr-Pour, A., & Foissac, X. (2010). First report of phytoplasma infections in several temperate fruit trees and vegetable crops in Azerbaijan. *Journal of Plant Pathology, 92*, 115.

Balakishiyeva, G., Danet, J.-L., Mamedov, A., Huseynova, I. M., & Foissac, X. (2012). Genetic variability of stolbur phytoplasmas in Azerbaijan. *Reports of Azerbaijan National Academy of Sciences LXVIII, 2*, 66–73.

Balakishiyeva, G., Danet, J.-L., Mammadov, A., Huseynova, I., & Foissac, X. (2013). Survey of 'Candidatus Phytoplasma solani' and its potential vectors in Northern regions of Azerbaijan. In E. Torres, A. Lavina, A. Batlle (Eds.), Third European Bois Noir Workshop (pp. 56–58). Barcelona.

Balakishiyeva, G., Mamedov, A., Foissac, X., Huseynova, I., & Aliyev, J. (2016). First report of grapevine 'bois noir' in Azerbaijan. *Plant Disease, 100*, 2522.

Cimerman, A., Pacifico, D., Salar, P., Marzachi, C., & Foissac, X. (2009). Striking diversity of *vmp*1, a variable gene encoding a putative membrane protein of the stolbur phytoplasma. *Applied and Environmental Microbiology, 75*, 2951–2957.

Cimerman, A., Arnaud, G., & Foissac, X. (2006). Stolbur phytoplasma genome survey achieved using a suppression subtractive hybridization approach with high specificity. *Applied and Environmental Microbiology, 72*, 3274–3283.

Cvrković, T., Jović, J., Mitrović, M., Krstić, O., & Toševski, I. (2014). Experimental and molecular evidence of Reptalus panzeri as a natural vector of bois noir. *Plant Pathology, 63*, 42–53.

Danet, J.-L., Balakishiyeva, G., Cimerman, A., et al. (2011). Multilocus sequence analysis reveals the genetic diversity of European fruit tree phytoplasmas and the existence of inter species recombination. *Microbiology, 157*, 438–450.

Delić, D., Balech, B., Radulović, M., et al. (2016). Vmp1 and *Stamp* genes variability of '*Candidatus* phytoplasma solani' in Bosnian and Herzegovinian grapevine. *European Journal of Plant Pathology, 145*, 221–225.

Ember, I., Acs, Z., Munyaneza, J. E., et al. (2011). Survey and molecular detection of phytoplasmas associated with potato in Romania and southern Russia. *European Journal of Plant Pathology, 130*, 367–377.

Ember, I., Bodor, P., Zsófi, Z., et al. (2016). Impact of bois noir disease on grapevine performance and wine quality. *Mitteilungen Klosterneuburg, 66*(1), 79–83.

Emeljanov, A. F. (1995). The new taxa of the tribe *Pentastirini* (*Homoptera, Cixiidae*) from Palaearctic. *Zoologicheskii Zhurnal, 74*(9), 73–89.

Ewing, B., Hillier, L., Wendl, M., & Green, P. (1998). Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Research, 8*, 175–185.

Fabre, A., Danet, J.-L., & Foissac, X. (2011a). The stolbur phytoplasma antigenic membrane protein gene *Stamp* is submitted to diversifying positive selection. *Genes, 472*, 37–41.

Fabre, A., Balakishiyeva, G., Ember, I., et al. (2011b). *Stamp* encoding the antigenic membrane protein of stolbur phytoplasma is useful for molecular epidemiology. *Bulletin of Insectology, 64*, 21–22.

Fialova, R., Valova, P., Balakishiyeva, G., et al. (2009). Genetic variability of stolbur phytoplasma in annual crop and wild plant species in south Moravia (Czech Republic). *Journal of Plant Pathology, 91*, 411–416.

Foissac, X., Carle, P., Fabre, A., Salar, P., Danet, J. L., & STOLBUR-EUROMED consortium. (2013). 'Candidatus Phytoplasma solani' genome project and genetic diversity in the Euro-Mediterranean basin. In E. Torres, A. Lavina, A. Batlle (Eds.), Invited conference, Third European Bois Noir Workshop (pp. 11–13). Barcelona.

Foissac, X., Danet, J. L., Zreik, L., et al. (2000). Cloning of the *spo*T gene of '*Candidatus* Phlomobacter fragariae' and development of a PCR-restriction fragment length polymorphism assay for detection of the bacterium in insects. *Applied and Environmental Microbiology, 66*, 3474–3480.

Gordon, D. C., Abajian, C., & Green, P. (1998). Consed: A graphical tool for sequence finishing. *Genome Research, 8*, 195–202.

Gundersen, D. E., & Lee, I. M. (1996). Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea, 35*, 114–151.

Johannesen, J., Foissac, X., Kehrli, P., & Maixner, M. (2012). Impact of vector dispersal and host-plant fidelity on the dissemination of an emerging plant pathogen. *PLoS One, 7*, e51809.

Holzinger, W. E., Kammerlander, I., & Nickel, H. (2003). *The Auchenorrhyncha of Central Europe. Die Zikaden Mitteleuropas: 1. Fulgoromorpha, Cicadomorpha excl. Cicadellidae*. Leiden: Brill.

Kosovac, A., Radonjić, S., Hrnić, S., et al. (2016). Molecular tracing of the transmission routes of bois noir in Mediterranean vineyards of Montenegro and experimental evidence for the epidemiological role of *Vitex agnus-castus* (Lamiaceae) and associated *Hyalesthes obsoletus* (Cixiidae). *Plant Pathology, 65*, 285–298.

Kostadinovska, E., Quaglino, F., Mitrev, S., et al. (2014). Multiple gene analyses identify distinct 'Bois noir' phytoplasma genotypes in the republic of Macedonia. *Phytopathologia Mediterranea, 53*, 300–310.

Laimer, M., Lemaire, O., Herrbach, E., Goldschmidt, V., Minafra, A., Bianco, P., & Wetzel, T. (2009). Resistance to viruses, phytoplasmas and their vectors in the grapevine in europe: a review. *Journal of Plant Pathology, 91*, 7–23.

Langer, M., & Maixner, M. (2004). Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur group based on RFLP-analysis of non-ribosomal DNA. *Vitis, 43*, 191–199.

Lee, I. M., Martini, M., Marcone, C., & Zhu, S. F. (2004). Classification of phytoplasma strains in the elm yellows group (16SrV) and proposal of 'Candidatus Phytoplasma ulmi' for the phytoplasma associated with elm yellows. *International Journal of Systematic and Evolutionary Microbiology, 54*, 337–347.

Maixner, M. (1994). Transmission of German grapevine yellows (Vergilbungskrankheit) by the planthopper *Hyalesthes obsoletus* (Auchenorrhyncha: Cixiidae). *Vitis, 33*, 103–104.

Maixner, M., Ahrens, U., & Seemuller, E. (1995). Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. *European Journal of Plant Pathology, 101*, 241–250.

Pacifico, D., Alma, A., Bagnoli, B., et al. (2009). Characterization of bois noir isolates by restriction fragment length polymorphism of a Stolbur-specific putative membrane protein gene. *Phytopathology, 99*, 711–715.

Quaglino, F., Zhao, Y., Casati, P., et al. (2013). 'Candidatus Phytoplasma solani', a novel taxon associated with stolbur- and bois noir-related diseases of plants. *International Journal of Systematic and Evolutionary Microbiology, 63*, 2879–2894.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution, 30*, 2725–2729.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research, 22*, 4673–4680.