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25 **Abstract**

26 Faba bean samples with symptoms of yellowing, dwarfism, shoot proliferation,
27 internode shortening, leaf sprouts and lack of pod and seed production were
28 collected from Huancayo and Chupaca provinces, Junin-Peru, and analyzed to
29 verify phytoplasma presence and identity. After total nucleic acid extraction, the
30 amplification with universal phytoplasma primers, using nested polymerase chain
31 reactions, on the 16S ribosomal gene followed by restriction fragment length
32 polymorphism analysis and sequence analysis allowed the classification of the
33 detected phytoplasma in the subgroup 16SrIII-J. The alignment of 30 16S
34 ribosomal gene sequences from 15 faba bean symptomatic samples from single
35 plants allowed verifying the consistent presence of 5 single nucleotide
36 polymorphisms that are however not modifying the phytoplasma classification. The
37 phytoplasma identity was also corroborated by the amplification and the restriction
38 fragment length polymorphism analyses carried out on the ribosomal protein gene
39 amplicons obtained with primers specific for the phytoplasmas enclosed in the
40 16SrIII group. This is the first description in Peru of a disease associated with
41 phytoplasmas in faba beans.

42

43 **2 | INTRODUCTION**

44 *Vicia faba* L., known as faba bean, is a leguminous species originated in the
45 Mediterranean region and grown worldwide for its high content of carbohydrate,
46 protein, vitamins, minerals (Zn, Fe, Mg, Ca) and omega-3 (Etemadi et al. 2019). In
47 2017, Peru had a cropping area of 65,985 ha, with an average yield of 11,478
48 kg/ha and total seed production of 75,736 tons (FAOSTAT 2019). Faba bean is

49 worldwide infected by various fungi, bacteria, chromista, viruses, and nematodes.
50 The main diseases reported are chocolate spot (caused by *Botrytis fabae*), leaf
51 blight (*Xanthomonas campestris*), bacterial spot (*Pseudomonas syringae*), root rot
52 (*Fusarium solani*), powdery mildew (*Erysiphe cichoracearum*), and infections by
53 viral complexes (Etemadi et al. 2019). During the last decades *V. faba* was also
54 reported to be infected by phytoplasmas (Castro and Romero 2004; Al-Saleh and
55 Amer 2014; Salehi et al. 2016; Omar 2017), that are bacteria discovered in 1967
56 (Doi et al. 1967) lacking the cell wall and having a diameter of 200 to 800 nm. Their
57 genomes are of small size and possess variable numbers of extrachromosomal
58 fragments (Nishigawa et al. 2003). Phytoplasmas are hosted in the plant phloem
59 and in the hemolymph of their insect vectors that are mainly those having siphon-
60 like mouth systems, as leafhoppers and psyllids (Weintraub and Beanland 2006).
61 They infect hundreds of plant species in which different symptoms can be
62 observed; moreover, different phytoplasmas could be associated with
63 indistinguishable symptomatology in diverse plant host species (Bertaccini 2007,
64 Namba 2019). These phytoplasma-associated symptoms include leaf yellowing,
65 growth reduction, virescence, and witches‘ broom, shortening of internodes, small
66 leaves, phyllody, floral malformation, and plant death (Salehi et al. 2016; Kumari et
67 al. 2019).

68 Since their discovery it was difficult to detect and identify the phytoplasmas
69 in symptomatic plants since they are present in a reduced concentration in the
70 infected tissues where are unevenly distributed (Berges et al. 2000). Phytoplasmas
71 belong to the class *Mollicutes* provisionally enclosed in the ‘*Candidatus*
72 Phytoplasma’ genus (IRPCM 2004). PCR/RFLP analyses on their 16S ribosomal

gene allowed the phytoplasma classification in ribosomal groups and subgroups that is integrating the provisional classification for further differentiation among the detected strains (Bertaccini and Lee 2018).

In recent years in the faba bean fields located in the Junin region, symptoms related to phytoplasma presence have been observed with 8% incidence, however, to date the possible presence and identity of phytoplasmas was not studied. Therefore, the objective of this work was to identify the phytoplasma associated with the disease of the faba bean in the provinces of Huancayo and Chupaca in the Junin region of Peru.

2 | MATERIALS AND METHODS

2.1| Sample collection and DNA extraction

Faba bean samples with symptoms of yellowing, dwarfism, shoot proliferation, internode shortening, leaf sprouts and lack of pod and seed production (Figure 1a-b) were collected from Huancayo (15 samples) and Chupaca provinces (16 samples), Junin-Peru, and analyzed to verify phytoplasma presence and identity. The 31 samples each collected from each from a separate plant, were processed and dehydrated with silica gel, then leaf tissues were sprayed with liquid nitrogen, and 25 mg were weighed for the extraction of DNA with innuPREP Plant DNA Kit (Analytik Jena, Germany) according to the instructions of the manufacturer. For the negative control, DNAs of four symptomless plants were also extracted following the same procedure. As positive control, the DNA of a strain from *Euscelidius variegatus* (16SrIII-B, API) maintained in the micropropagated collection (Bertaccini, 2014) was used. The quality of the obtained DNA was verified by

means of a 1% agarose gel and quantified with a spectrophotometer NanoDrop™ (ThermoScientific™).

2.2| PCR detection and RFLP analysis

Phytoplasma detection was achieved using PCR assays with P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995), R16mF2/mR1 primers followed in nested PCR reaction by R16F2n/R2 (Gundersen and Lee 1996) and fU5/rU3 (Lorenz et al. 1995) primers. The total nucleic acid used was about 20-40 ng for the PCR reactions and amplicons, diluted in sterile distilled water 1:30, were used as template for the nested PCR reactions. Cycling conditions reported (Schaff et al. 1992) were employed in all the PCR reactions. The amplicon visualization was obtained by electrophoresis in 1% agarose gel followed by ethidium bromide (10 ng/µl) staining. The R16F2n/R2 and fU5/rU3 amplicons were analyzed by RFLP using *Tru1*I and *Hpa*II (Fermentas, Vilnius, Lithuania) restriction enzymes and compared with the corresponding profile of the phytoplasma strain API in 6.7% polyacrylamide gels stained and visualized as for the agarose gels reported above. Further molecular characterization was achieved in nested PCR on the ribosomal protein (rp) operon with primer pairs rpL2F3/rp(I)R1A and rp(III)F1/rp(III)R1, specifically amplifying about 800 bp in the rp coding gene of the phytoplasmas enclosed in ribosomal group 16SrIII, under the reported conditions (Martini et al. 2007). The following phytoplasma strains enclosed in the ribosomal group 16SrIII and maintained in periwinkle (*Catharanthus roseus*) as a collection of micropropagated shoots (Bertaccini 2014) were used as controls: X disease phytoplasma (16SrIII-A, CX), a strain from *Euscelidius variegatus* (16SrIII-B, API), *Vaccinium* witches' broom (16SrIII-F, VAC), spirea stunt (16SrIII-E, SPI), and

poinsettia branching (16SrIII-H, JRI). The amplicons were analyzed by RFLP with the restriction enzyme *Tru1*I, observed after electrophoresis and visualized as for the agarose gels reported above.

2.3| Sequencing and phylogenetic analysis of 16S rDNA

The extraction from agar of R16mF2/R16mR1 and fU5/rU3 amplified fragments of approximately 1,400 and 876 bp respectively, was performed with the PCR Clean-Up System Kit (Promega, USA). Amplicons were then sent for both directions sequencing using the primer pairs employed in the amplification at Macrogen (Seoul, Korea). The sequence alignment and manual revision were done with the BLASTn program, followed by the comparison with phytoplasma sequences in the GenBank. The consensus sequence obtained was then subjected to virtual RFLP analysis with the *i*PhyClassifier using all the 17 available restriction enzymes with the 16SrIII subgroup strains (Zhao et al. 2009). Phylogenetic analysis with the Minimum Evolution system (Rzhetsky and Nei 1992) was also carried out using MEGA 6 (Tamura et al. 2013) with the 43 described '*Ca*. Phytoplasma' species and with a number of phytoplasma strains enclosing those classified in 16SrIII-J subgroup or reported from Peru and other South American agricultural areas.

3| RESULTS

From the 31 symptomatic samples collected from individual plants no or weak amplification was obtained with P1/P7 primers while two samples were amplified with primers R16mF2/R16mR1. By using primers amplifying shorter fragments in nested PCR the detection of phytoplasma expected length bands was obtained in

all the samples from symptomatic plants; in particular primers fU5/rU3 (amplicons of approximately 850 bp) provided 100% of amplification from all the samples from symptomatic plants and primers R16F2n/R2 (amplicons of approximately 1,200 bp) provided 80% of amplification (data not shown). The samples from asymptomatic plants and the sterile water used as negative controls did not produce bands in any PCR reaction. From the fU5/rU3 amplified fragments 10 samples were selected for direct sequencing, the remaining fragments were subjected to RFLP analysis with *Tru1*I and *Hpa*II (Figure 2a). The profiles obtained resulted identical to those of the strain from *Euscelidius variegatus* (16SrIII-B, API) (data not shown). The two 1,400 bp amplicons obtained with R16mF2/R16mR1 were sequenced in both directions and the consensus sequence of 1,320 bp (4 times coverage for each nucleotide in each position) was deposited in the GenBank under the accession number MT358307; this strain is referred here as faba bean yellows and witches' broom (FB-YWB) phytoplasma. The R16F2n/R2 sequence (1,240 bp) analyzed *in silico* RFLP by the *i*PhyClassifier (Figures 2b, c and d) was having a 1.00 as similarity coefficient to the phytoplasma strains enclosed in the subgroup 16SrIII-J. The phylogeny allowed clustering the FB-YWB sequence with phytoplasma strains enclosed in this ribosomal subgroup detected in other geographic areas of South America (Figures 3a and b). The alignment of 30 16Sr sequences amplified with fU5/rU3 primers (about 800 bp) from the faba bean phytoplasma strains detected in Peru showed the consistent presence of three SNPs (*i.e.* present in more than three sequences) at positions 384 (A/G), 442 (C/T), 831 (C/A) and of three insertions T at 492, C at 827, G at 1015 counted 3' to 5' in the deposited consensus sequence (data not shown). The specific amplification of the

phytoplasmas with rp 16SrIII group-specific primers followed by RFLP analyses with *Tru1*I (Figure 4), corroborated the close genetic relationship of this phytoplasma with those classified in the 16SrIII subgroup.

4| DISCUSSION

The detection of 16SrIII-J in symptomatic faba bean samples is the first identification of phytoplasmas enclosed in this ribosomal subgroup in Peru; they were reported first in Brazil (Montano et al. 2000) and later infecting several other crop species in South American states such as Argentina (Galdeano et al. 2013; Fernández et al. 2020), Brazil (Munhoz et al. 2019), and Chile (Quiroga et al., 2020). The identification of variability at the 16Sr level is not unusual in phytoplasmas classified in the 16SrIII group where the frequent presence of interoperon heterogeneity is reported in several strains (Davis et al. 2013).

The first studies of phytoplasmas in Peru date back to the late 1970s (Nault et al. 1979). Based on serological and microscopic techniques, the presence of phytoplasmas, spiroplasmas, and viruses in corn crops was verified. After that, a survey of phytoplasmas in different regions and crops allowed identifying phytoplasmas enclosed in ribosomal group 16SrI in diverse plants species, of group 16SrII in potato (*Solanum tuberosum*), while phytoplasmas in the 16SrIII group were detected in tomato (*Solanum lycopersicum*) and dandelion (*Taraxacum* sp.) (Hodgetts et al. 2009). More recently, a phytoplasma classified in the 16SrXV-A was detected in grapevine (Wei et al. 2017).

Phytoplasmas have not been identified in faba bean in Peru previously, however, a phytoplasma enclosed in the 16SrIII group was reported in Spain

(Castro and Romero 2004), while in Saudi Arabia (Al-Saleh and Amer 2014, Omar, 2017) and Iran (Salehi et al. 2016) 16SrII phytoplasma strains were identified in symptomatic plants. The identified phytoplasma is largely reported in South American countries it is therefore necessary to continue the studies to verify the presence of insect vectors following the recent demonstration of *Paratanus exitiousus* and *Bergallia valdiviana* in Chile (Quiroga et al. 2019). Furthermore, assays to verify a possible seed transmission, reported for other phytoplasmas in tomato, corn (*Zea mays*) and carrot (*Daucus carota*) (Calari et al. 2011; Satta et al. 2020) should be planned.

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353

354 **Figure 1** *Vicia faba* plants with symptoms of yellowing, dwarfism, leaf distortion,
355 and abnormal leaf sprouts near to healthy plants (a) and in a close up (b) in a
356 production field in the province of Chupaca, Peru.

357

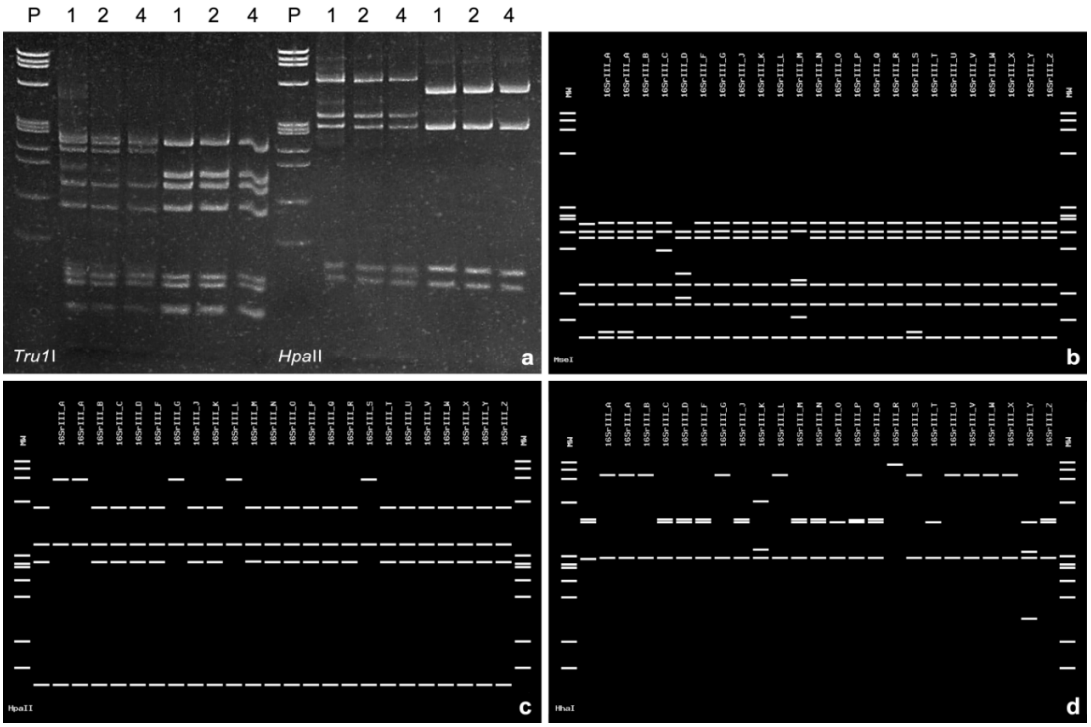

Figure 2 a) Polyacrylamide 6.7% gels showing the restriction profiles of 16S ribosomal amplicons of faba bean phytoplasma strains from Peru: from left digestion with *Tru1*I of R16F2n/R16R2 amplicons followed by digestion of the same samples with the same enzyme of fU5/rU3 amplicons; after the second marker (P) the same amplicons digested with *Hpa*II. Samples 1, 2, and 4, broad bean samples from the diverse localities. b), c) and d) *In silico* RFLP patterns of the R16F2n/R2 sequence of the FB-YWB strain (GenBank accession number MT358307) in comparison with the 16SrIII available phytoplasma subgroups using the *i*PhyClassifier. Ribosomal subgroups are listed at the top; lane with no name is strain FB-YWB. *Tru1*I and *Mse*I are isoschizomer enzymes. P and MW, marker ΦX174 digested with *Hae*III and having the fragment sizes from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72 base pairs, respectively.

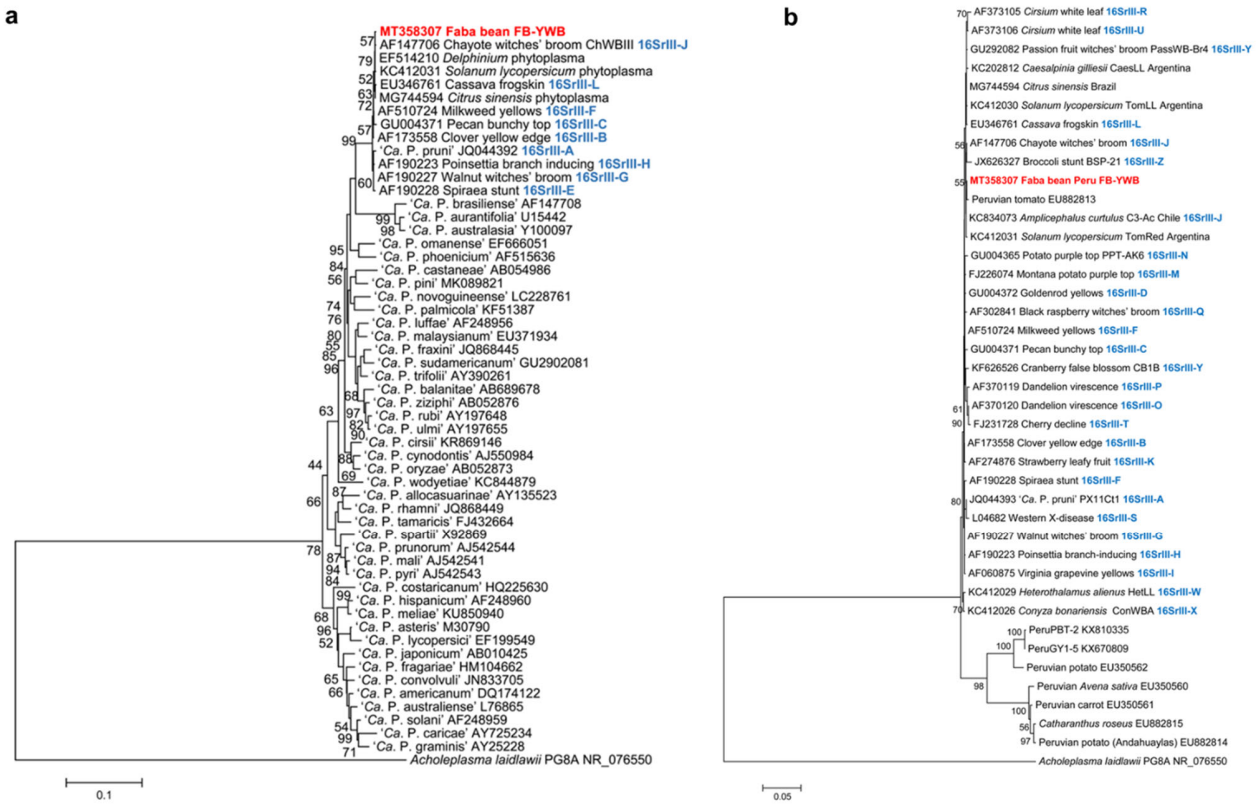

Figure 3 a) and b) Phylogenetic trees showing relationships at the 16S ribosomal RNA gene level of the faba bean yellows and witches' broom phytoplasma strain from Peru (FB-YWB) (in red) compared with the classified '*Ca*. Phytoplasma' species and with some phytoplasmas classified in the group 16SrIII (reported ribosomal subgroups are listed on the right in blue) using the Minimum Evolution method. The replicate tree percentage indicating the clustering of the associated taxa are next to the branches (Felsenstein 1985). The Tamura-Nei method (1993) was used to calculate the evolutionary distances that are expressed in base substitutions per site, only values above 50 are reported. The out group employed is the *Acholeplasma laidlawii*. The numbers of the GenBank of the sequences employed are on the right for the '*Ca*. Phytoplasma' species and on the left for the strains in the 16SrIII group.

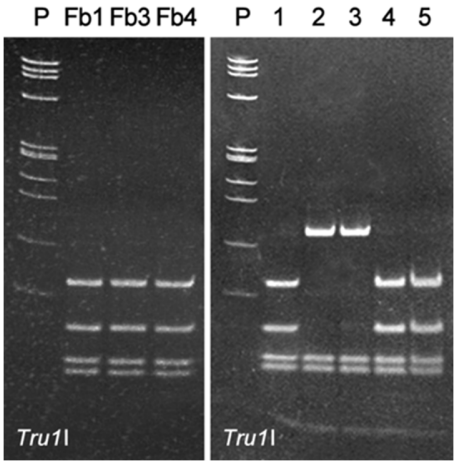

Figure 4 Restriction fragment length polymorphism patterns of ribosomal protein amplicons from faba bean yellows and witches' broom phytoplasma from Peru (Fb1, Fb2, and Fb4) and other phytoplasma strains classified in the ribosomal group 16SrIII after polyacrylamide 6.7% gel electrophoresis. The amplification was carried out in nested PCR with rpIIIF1/rpIIIR1 primers; the enzyme used is listed at the bottom. Lanes 1, X disease (16SrIII-A, CX), 2, strain from *Euscelidius variegatus* (16SrIII-B, API), 3, *Vaccinium* witches' broom (16SrIII-F, VAC), 4, spirea stunt (16SrIII-E, SPI), 5, poinsettia branching (16SrIII-H, JRI). P, marker ΦX174 digested with *Hae*III and having the fragment sizes from top to bottom of 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118 and 72 base pairs.