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Molecular and biologic characterization of a phytoplasma associated with *Brassica campestris* phyllody disease in Punjab province, Pakistan

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Abstract A phytoplasma-associated disease was identified in *Brassica campestris* (sarson) plants during a survey conducted in Punjab province of Pakistan in 2014–2016. The symptomatic plants showed characteristic symptoms of phyllody and witches' broom. Phytoplasma presence was detected by polymerase chain reaction on 16S ribosomal and tuf DNAs, followed by RFLP analysis and sequence comparison of the *16S rRNA* and *tuf* genes. The phytoplasma detected was classified in a new ribosomal subgroup designed 16SrIX-H. The phytoplasma presence in phloem tissues of symptomatic sarson samples was also confirmed through light microscopy and transmission studies to healthy plants through dodder and the leafhopper *Orosius albicinctus*. This is the first report of identification of 16SrIX-H subgroup phytoplasma associated with sarson and its natural vector in Pakistan.

Keywords Sarson phyllody · Phytoplasma · PCR-RFLP · 16SrIX-H subgroup · *Orosius albicinctus*

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Introduction

Sarson (*Brassica campestris* L., family Brassicaceae) is an important oilseed crop used as major cocking oil and fodder in Pakistan (Amjad 2014). Phytoplasmas colonize plants and insect species as obligate parasites: they inhabit sieve tubes and phloem feeding hemipteran insects that serve as efficient vectors belong to Cicadellidae: Deltocephalinae (Weintraub and Beanland 2006). Phytoplasma associated diseases can cause losses up to 70–100% in brassicacea and other species of cultivated plants (Bhowmik 2003; Bertaccini 2007) where they induce physiological disorders and interfering with plant defense pathways (Ahmad et al. 2013, 2014). More than 300 plant diseases in approximately 1000 plant species of 300 genera are reported worldwide (Bertaccini et al. 2014; Hoshi et al. 2007). The presence of phytoplasma diseases was reported in Pakistan since more than 40 years (Chapot 1970; Khan and Munir 1986; Khan et al. 1987) and phyllody diseases in chickpea, sesame, *Brassica napus*, parthenium, tomato, and onion were described (Akhtar et al. 2008, 2009a, b; Ahmad et al. 2015a, b, c).

In sarson cultivated fields in different districts of Punjab, Pakistan, a wide occurrence of potential insect vectors and symptoms such as malformed leaf with yellowish to purple discoloration, retarded growth, shoot proliferation, flower bud failure, virescence and phyllody are present. The aim of this study was to verify and confirm the association of these symptoms with phytoplasma presence by transmission trials and identification of the pathogen and its insect vector(s) to devise the best methods to manage the disease thereby reducing the economic losses caused by its presence.

Materials and methods

Plant material Eighty sarson (*B. campestris*) samples showing phyllody and witches' broom symptoms and 8 asymptomatic ones were collected from four districts (≈distance among districts: 150 Km to 370 Km) of Punjab (20 symptomatic and two asymptomatic plants per district collected randomly in the different areas surveyed): Khanewal (30°20″N, 71°55″E), Rahim Yar Khan (28°30″N, 70°25″E), Dera Ghazi Khan (30°05″N, 70°43″E) and Faisalabad (31°30″N, 73°05″E). Some symptomatic plants were uprooted in the field, transported and potted under insect proof glasshouse conditions for further transmission experiments.

DNA extraction and PCR detection Total DNA was extracted from symptomatic and asymptomatic sarson plants (leaf or full flower) and specimens from *O. albicinctus* were extracted as per protocol described by Angelini et al. (2001).

Five microliters of DNA (10–20 ng) were used as template for PCR amplifications with universal phytoplasma primers P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995); the PCR product was diluted 1: 200 with sterile distilled water and 1 μl was used in nested PCR with primers 16R758f/16R1232r (=M1/M2), F1/R0 (Lee et al. 1995) and M1/B6 (Duduk et al. 2013). Phytoplasma positive controls employed for the molecular analyses included DNA from phytoplasma reference strains maintained in periwinkle [*Catharanthus roseus* (L.) G. Don.] (Bertaccini 2015). In particular aster yellows (AY-1, 16SrI-B), clover phyllody (KVE, 16SrI-C), witches' broom disease of lime (WBDL, 16SrII-B), sesame phyllody from Thailand (SEPT, 16SrII-C), peach X disease (CX, 16SrIII-A), *Pichris echioides* yellows (PEP, 16SrIX-C)

were used. DNA of healthy sarson plants and samples devoid of DNA template were employed as negative controls. The 20 μl PCR mixture contained dNTPs (0.2 mM each), 0.5 μl of each primer pair (20 pmol), 1 U of Red*Taq* DNA polymerase (Sigma-Aldrich) and 10 X *Taq* polymerase buffer. The temperature conditions were as follows: denaturation at 94 °C for 1 min (1 min for first cycle), annealing at 55 °C for 2 min, and extension at 72 °C for 2 min. The last cycle was extended to 10 min at 72 °C. For the nested amplifications the thermal conditions were the same except that the annealing was at 50 °C for 2 min. Further amplification of the samples was carried out on *tuf* gene (Makarova et al. 2012) followed by RFLP analyses (Contaldo et al. 2011). All PCR amplifications were carried out in a thermal cycler Biometra (Germany). Six μl of amplified products were used for electrophoresis in 1.0% (*w/v*) agarose gel 1 X TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Staining was done with ethidium bromide and gels were observed under ultraviolet (UV) transilluminator to visualize DNA bands.

RFLP assays and phylogenetic analyses For RFLP analysis, 3 μl (about 300 ng of DNA) of M1/M2 and tuf cocktail PCR products were digested using fast enzymes *Tru1*I and *Tsp509*I at 65 °C as recommended by manufacturer (Fermentas, Vilnius, Lithuania). Restricted fragments were visualized through 6.7% polyacrylamide gel. The patterns obtained were visualized with staining in ethidium bromide and recorded in a gel documentation unit from Kodak (Rochester, NY, USA). Virtual RFLP analyses were also carried out on the obtained sequences in comparison with sequences of described 16SrIX subgroups (Lee et al. 2012) using the *i*PhyClassifier (Zhao et al. 2009).

Considering that all RFLP profiles were identical, a direct sequencing was carried out in both directions from two of the positive samples obtained with primers M1/B6 and F1/R0 amplifying the whole *16Sr RNA* gene after purification with a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA). The sequences were assembled by MEGA6 (Tamura et al. 2013) and the consensus aligned sequences were compared with nucleotide sequences in the GenBank database, using BLAST (version BLASTN 2.2.18) (National Center for Biotechnology Information, Bethesda, MD). Phylogenetic analyses were carried out on 16S rDNA sequences from sarson and from 46 '*Candidatus* Phytoplasma' strains representing the large majority of

officially described 'Candidatus' spp., using Acholeplasma laidlawii as the outgroup. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

Light microscopy A fresh 1–2 mm portion in length of stem or leaf midrib was cut with the help of fine razor blade from each of the 88 symptomatic and healthy sarson plant samples collected. They were then with Dienes' stained (stock solution: 0.5 g methylene blue, 10 g maltose, 1.25 g azure II and 0.25 g sodium carbonate dissolved in 100 ml distilled H_2O) 0.2% *v/v* in distilled water, for 10 min at 30 °C (Deenley et al. 1979). One drop of xylol was added before observation under a light microscope (MCX100 Daffodil Micros-Austria) at 40 X magnification.

Dodder transmission Dodder (*Cuscuta campestris* L.) strands germinated in sterile conditions and maintained on 20 symptomatic and 10 healthy sarson plants respectively, were used for transmission trials. Three dodder strands from phytoplasma symptomatic plants were allowed to establish on another group of nine healthy 4 week-old sarson plants for pathogen transmission. Similarly, phytoplasma free dodder strands from healthy plants were established on another group of nine healthy 4 week old sarson plants as control. After 3 weeks of stable dodder bridge establishment, plants were observed for symptoms development up to three months. The phytoplasma presence was verified by nested PCR assays as described below in all the plants before and after dodder transmission trials.

Insect transmission The leafhopper *Orosius albicinctus* Distant was investigated to verify its role as insect vector after its collection from naturally infected symptomatic and asymptomatic plants and rearing under cages. For disease acquisition, adults (250) were caged with severely symptomatic sarson plants for 3 days. After acquisition access period (5 days), the insects (25 per plant) were transferred to 12 single caged healthy sarson plants 4 week-old. After 7 days of inoculation period the insects were killed and groups of five individuals were employed for phytoplasma PCR detection after nucleic acid extraction as reported above. The same was also done with insects fed on healthy plants and transferred to 12 caged healthy sarson plants. Then, the plants were observed daily for symptom development for up to three months. All the experiments were carried out under glasshouse condition at 30 ± 5 °C.

Results

Symptoms observation Sarson plants exhibited mainly virescence, phyllody, and witches' broom symptoms (Fig. 1) that varied according to the different stage of
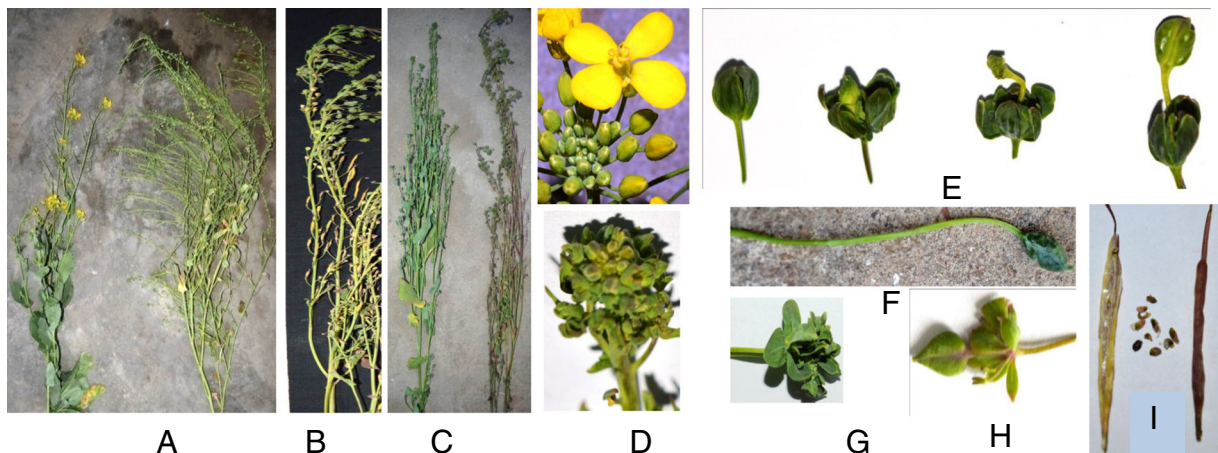
Fig. 1 *B. campestris* (sarson): **a**. Left healthy and right infected shoots (phyllody, virescence and witches' broom symptoms). **b**. Infected *Brassica* showing abnormal pods. **c**. Left mild infection (symptoms with greenish coloration) and right severe symptoms with brownish coloration. **d**. Malformed infected buds. **e**. Abnormal development from budding to pod formation in infected plant. **f**. Abnormal capsule with elongated pedicel. **g**. Phyllody and virescence. **h**. Infected floral parts. **i**. Infected pods producing abnormal seeds

growth. The incidence of the symptomatology in the fields examined was between 10 and 30%. Infection at early growth stages caused reduced leaf sizes, elongated internodes and overall growth retardation. Some of the pods produced wrinkled seeds (Fig. 1i). The flower calices were budded and the top of the short pedicels was distorted (Fig. 1f). Virescence, phyllody, and witches' broom, excessive proliferation, and severe malformation along with no development of seeds in capsules was observed during late infections (Figs. 1g-h). The most common symptom was phyllody; the carpels showed leaf fusions at the margins and elongated shoot-like structures were developed instead of ovaries; the false ovary was flat, short and showed wrinkled surface. The stamens were flat, leaf-like and anthers, if produced, had abnormal pollen grains mostly green with pods having a leaf-like structure. Plants infected during the flowering stage had severe symptoms on the upper part, whereas infection before flowering induced severe symptoms on the entire plant, occasionally producing some flowers and very small capsules with little abnormal seeds.

Phytoplasma molecular identification All 80 samples from symptomatic plants resulted positive by nested PCR amplification with M1/B6, M1/M2 and tuf cocktail primers producing the expected length amplicons of about 1100, 500, and 450 bp respectively (data not shown). No amplification was obtained from asymptomatic samples and from negative control without DNA template. The RFLP analyses on amplicons obtai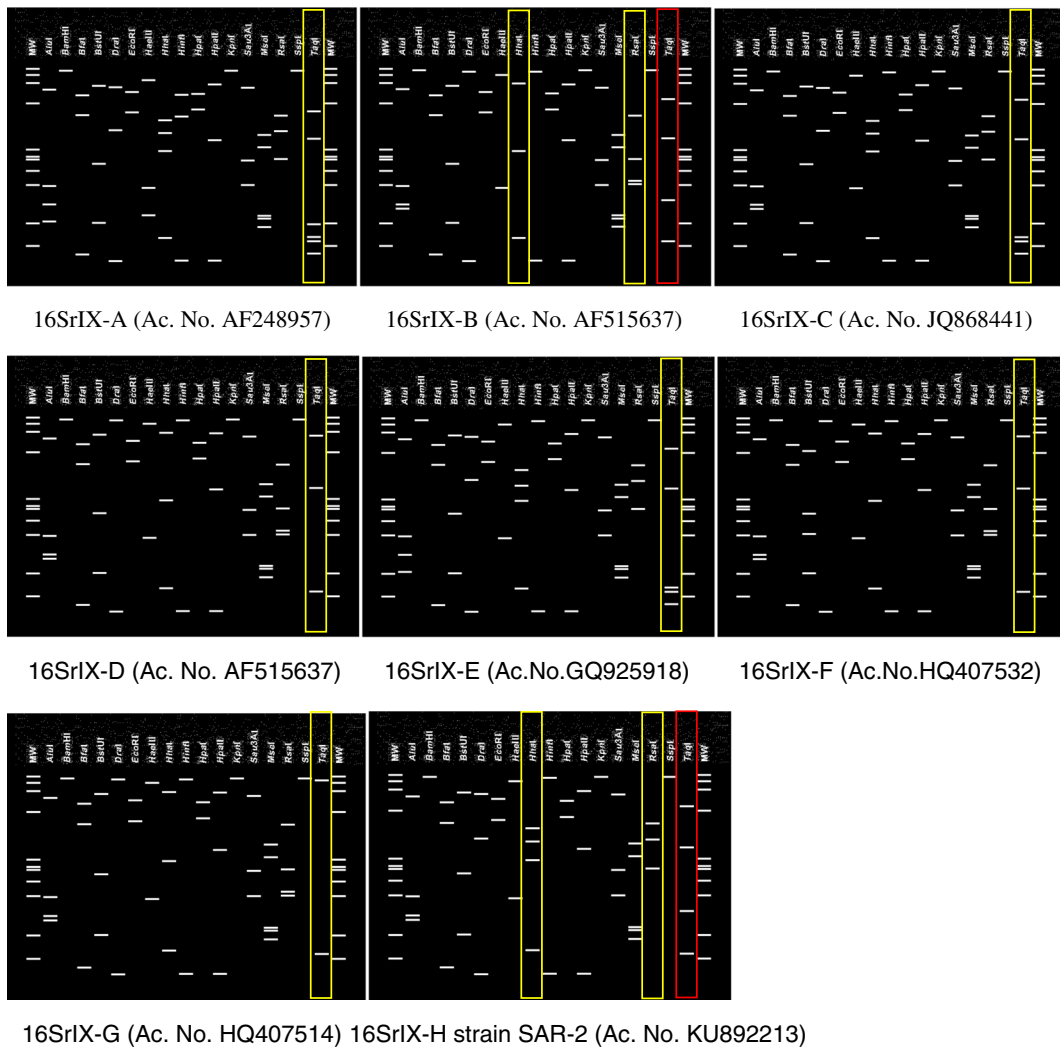ned with primers M1/M2 and tuf cocktail of the sarson samples showed that the phytoplasma detected in all samples were identical to each other and to those of 16SrIX-C phytoplasma group employed as reference (Fig. 2). M1/B6 and F1/R0 amplicons from sarson strains SAR-1 (Br5) and SAR-2 (Br6), selected as representative of the disease studied, were directly sequenced on both strands and 908 bp and 1320 bp sequences were obtained that resulted 100% identical to each other and were submitted to GenBank under accession numbers KT253604 and KU892213 respectively. These sequences showed 100% and 99% homology, respectively with those of phytoplasma strains affiliated with subgroups in 16SrIX group. The sequence of strain SAR-2 was employed for virtual RFLP in *i*PhyClassifier and resulted differentiable from all reported phytoplasma subgroups by the combination of profiles obtained with *Taq*I, *Hha*I and *Rsa*I restriction enzymes (Fig. 3). This was suggesting the assignment of this sarson phytoplasma strain to a new ribosomal subgroup designed 16SrIX-H. Phylogenetic analysis using 46 additional strains confirmed the phytoplasma placement in the clade containing phytoplasmas assigned to the 16SrIX group, where it is however clearly differentiable from all reported subgroups with a robust branch support (Fig. 4).

Light microscopy Under light microscopy dark blue areas were observed in the phloem region of sections from infected plants after Dienes' staining and no similar stained areas were observed in sections from asymptomatic tissues; intense color in infected samples but not in asymptomatic stem cross sections was observed (data not
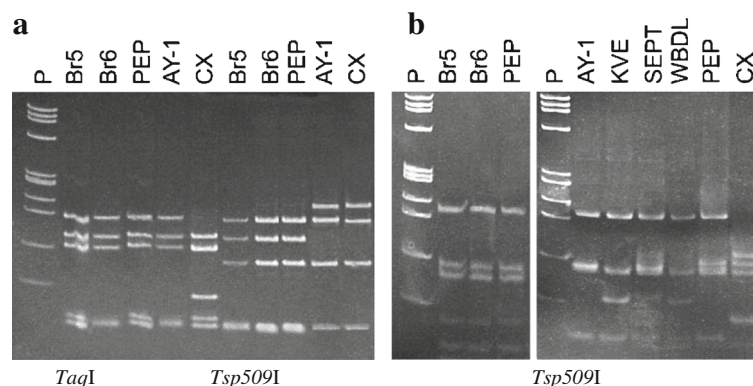
Fig. 2 Restriction fragment length polymorphism patterns of phytoplasmas from sarson (Br5 and Br6 = SAR1 and SAR2) compared with reference strains from periwinkle: PEP, *Pichris echioides* yellows (16SrIX-C); AY-1, aster yellows (16SrI-B); CX, peach X disease (16SrIII-A); KVE, clover phyllody (16SrI-C); SEPT, sesame phyllody from Thailand (16SrII-C); WBDL, witches' broom disease of lime (16SrII-B). The amplification was obtained in nested PCR with primers M1/M2 in **a**) and in **b**) with cocktail primers amplifying the *tuf* gene, the restriction enzymes used are at the bottom of the figures. P, marker phiX174 *Hae*III digested

16SrIX-A (Ac. No. AF248957)   16SrIX-B (Ac. No. AF515637)   16SrIX-C (Ac. No. JQ868441)

16SrIX-D (Ac. No. AF515637)   16SrIX-E (Ac.No.GQ925918)   16SrIX-F (Ac.No.HQ407532)

16SrIX-G (Ac. No. HQ407514) 16SrIX-H strain SAR-2 (Ac. No. KU892213)

Fig. 3 Virtual restriction fragment length polymorphism patterns with 17 restriction enzymes of phytoplasmas from sarson strain SAR-2 (Br6) compared with profiles from described 16SrIX subgroups (Ac. No., Genbank accession number) produced using the *i*PhyClassifier (Zhao et al. 2009). Coloured squares show the profiles of differential restriction enzymes that allow 16SrIX-H subgroup discrimination from all other subgroups in group 16SrIX

shown). In all other tissues observed, there were no major alterations, and no color differentiation was observed between healthy and infected tissues.

Disease transmission All the dodder and the healthy plants of sarson used for the transmission experiments were PCR tested before the transmission trials and resulted to be phytoplasma free. The agent of the phyllody disease was successfully transmitted from infected to healthy plants via both dodder and insects. Dodder transmission occurred by symptoms appearance with in 28–45 days in six out of the nine plants employed and all these symptomatic plants were PCR-positive to

phytoplasma DNA, while the non-symptomatic were negative. *O. albicinctus* also transmitted the disease and 10 out of 12 sarson plants used become symptomatic within 35–55 days after the end of the transmission period. Only the symptomatic plants resulted positive to phytoplasma DNA after nested PCR assays.

Discussion

The work carried out on sarson phyllody allowed to achieve the molecular and biological characterization
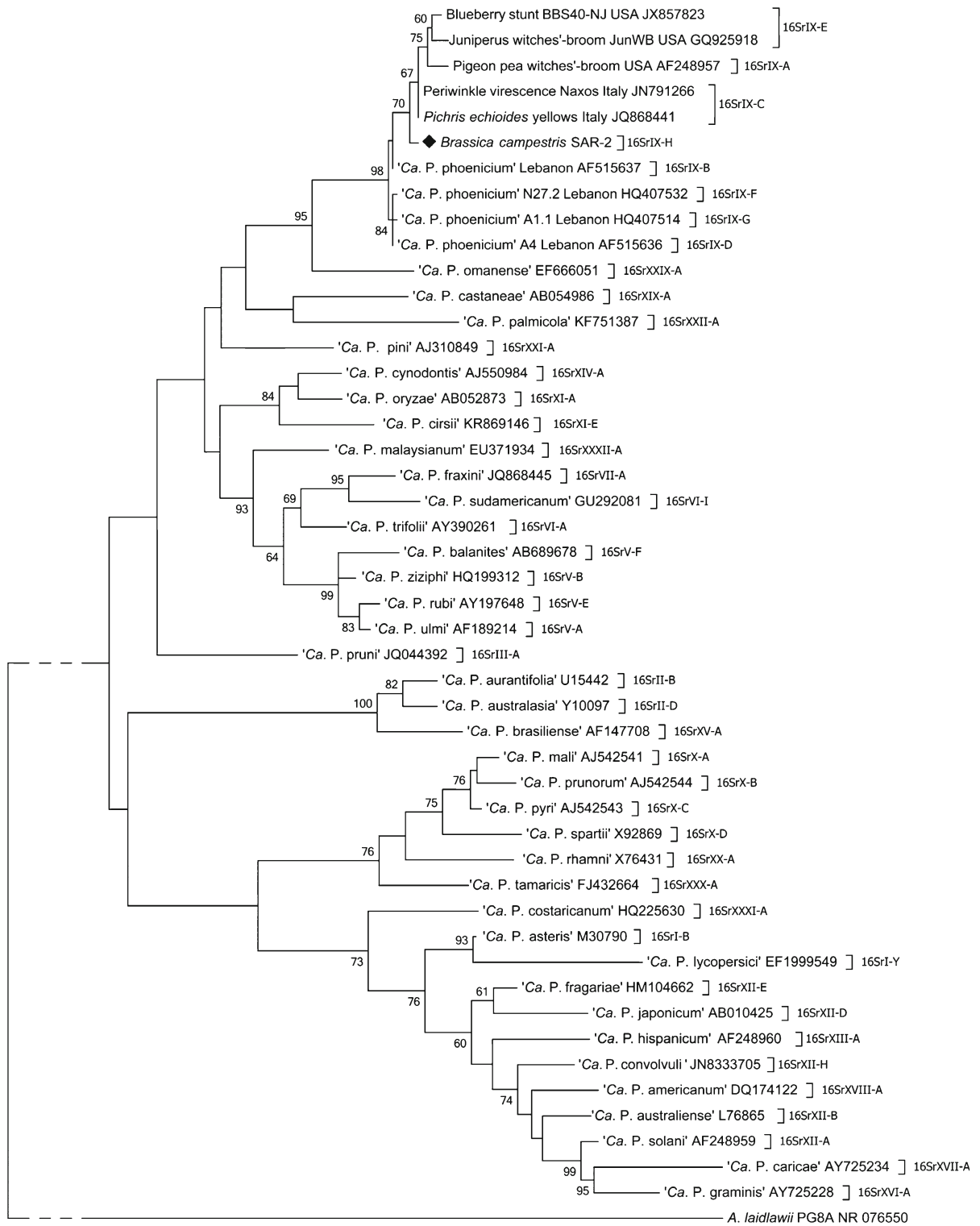
Fig. 4 Phylogenetic tree showing the evolutionary relationships of 46 phytoplasmas plus the sarson strain SAR-2 (Genbank Ac. No.: KU892213) from *B. campestris* from Pakistan on *16Sr RNA* gene. *A. laidlawii* was used as outroot. Phytoplasma '*Candidatus*' (Ca. P.) name or strain name or acronym together with the country are indicated on the branches followed by the Genbank Ac. No. of the sequence employed and the ribosomal subgroup classification. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach based on the Tamura-Nei model (Tamura and Nei 1993). The analysis involved 48 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1168 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013)

of the associated phytoplasmas. However due to the limitations of detection by nested PCR additional possibility that other graft and dodder transmissible organisms may be involved cannot be ruled out. On the other hand the majority of the symptoms observed was clearly related to the presence of phytoplasmas as reported in the worldwide literature. The detected phytoplasma belonged to a new 16SrIX subgroup and its presence was confirmed through light microscopy, insect and dodder transmission. Phytoplasma diseases are spreading in Pakistan also due to the wide presence of insect vectors and phyllody diseases associated with 16SrII-D subgroup phytoplasma presence were reported in chickpea, sesame (Akhtar et al. 2008, 2009b; Ahmad et al. 2015a), *Parthenium,* radish and tomato (Ahmad et al. 2015a, 2015b, 2015c). Brassica plants showing phytoplasma disease symptoms i.e. rape phyllody with green petals were reported in western Canada and Hungary since decades (Sackston 1953; Horváth 1969; Lehmann and Skadow 1971). In Pakistan, phytoplasmas were found in *Brassica napus* plants showing stunted growth, phyllody and virescence as well as misshapen seed and swollen inflorescences; 5–10% of brassica cultivated fields in Punjab show the presence of symptomatic plants (Ahmad et al. 2015a). Symptoms similar to those reported in Pakistan were associated with phytoplasmas belonging to two subgroups (16SrI-A and 16SrI-B) in Alberta, Canada (Wang and Hiruki 2001; Olivier et al. 2010), while subgroup 16SrVI-A, '*Candidatus* Phytoplasma trifolii', was associated with an Iranian cabbage disease (Salehi et al. 2007). The 16SrIX group phytoplasma was associated with Indian toria phyllody

(Azadvar et al. 2009, 2011; Azadvar and Baranwal 2010) and winter oilseed rape symptomatic plants infected by 16SrI-B phytoplasmas were described in Italy, Czech Republic and Poland (Bertaccini et al. 1998; Kamińska et al. 2012). The rape seed phyllody disease was reported as transmitted by *Eucelis plebejus* Fall., and *Macrosteles* spp. (Kolte 1985). *Macrosteles quadrilineatus* Forbes can transmit phytoplasmas in different subgroup to 200 plants species in about 42 families (Weintraub and Beanland 2006). The leafhopper *Circulifer haematoceps* was confirmed able to transmit rape seed phyllody to various species of plants (Salehi et al. 2010). However insect vectors of phyllody diseases in Pakistan are still unknown, however the widely spread presence of *O. orientalis* in parthenium fields showing phyllody symptoms was reported (Ahmad et al. 2015b, 2015c). The ability of *O. albicinctus* to transmit the sarson-phyllody associated phytoplasma (16SrIX-H) from infected to healthy sarson in Pakistan represents a new finding, since it was previously reported to transmit 16SrII-D subgroup phytoplasmas associated with chickpeas and sesame phyllody (Akhtar et al. 2009a, 2009b).

Additional work is in progress to verify the distribution and the economic importance of this phytoplasma in the cultivated areas of Pakistan and by further verifying the range of its insect vectors and alternate host plants in order to devise effective containment measures.

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