# MOLECULAR CHARACTERIZATION AND TRANSMISSION OF BERMUDA GRASS WHITE LEAF PHYTOPLASMA IN IRAN

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## SUMMARY

Symptoms of Bermuda grass white leaf (BGWL) were observed in several provinces of Iran. Among leafhoppers collected in BGWL-affected areas, *Exitianus capicola* tested positive for phytoplasma in PCR assays and transmitted the BGWL agent to healthy Bermuda grass plants. Using P1/P7 primer pair, target DNA fragments of approximately 1.8 kbp were amplified from diseased samples of Bermuda grass plants from 10 widely separated areas in six provinces. Restriction fragment length polymorphism (RFLP) analysis of P1/P7 PCR products identified two BGWL isolates from Firouzabad and Juyom (Fars province) denoted FBGWL and JBGWL as members of the Bermuda grass white leaf group, 16SrX-IV. Percent homology and phylogenetic analysis of 16S rDNA and 16S-23S spacer region (SR) sequences showed also that FBGWL and JBGWL are related to the 16SrX-IV group. Based on RFLP and phylogenetic analyses, FBGWL and JBGWL isolates were distinguishable from each other and were classified in subgroups 16SrXIV-A and 16SrXIV-B, respectively. This is the first report of BGWL disease and leafhopper transmission of the associated phytoplasma in Iran.

*Key words:* epidemiology, leafhopper transmission, *Exitianus capicola*, Bermuda grass white leaf, phytoplasma.

## INTRODUCTION

Bermuda grass white leaf (BGWL) disease, first reported from Taiwan (Chen *et al*., 1972), is now known to occur in other Asian countries (Zahoor *et al*., 1995; Lee *et al*., 2000; Rao *et al*., 2007; Jung *et al*., 2003), Africa (Daffala and Cousin, 1988), Australia (Padovan *et al*., 1999; Tran-Nguyen *et al*., 2000), Europe (Marcone *et al*., 1997) and Cuba (Arocha *et al*., 2005). Phytoplasmas associated with diseases of Bermuda grass, several other monocots and two dicots (*Circium arvensis* and *Galactia tenuifolia*) form the sugar cane white leaf (SCWL) branch in the monophyletic phytoplasma clade (Seemüller *et al*., 1994; Marcone *et al*., 2004). There are no reports on the identity of the vectors of these phytoplasmas. However, a phytoplasma found in the leafhopper *Psammotettix cephalotes* in Germany, was shown to belong to the same group (Seemüller *et al*., 1994).

BGWL-associated phytoplasmas together with phytoplasmal agents of white leaf diseases of brachiaria grass and annual blue grass are classified in the BGWL phytoplasma 16SrX1V-A subgroup (Lee *et al*., 1998, 2000). Taxonomic studies based on sequence analysis of PCR-amplified 16S rDNA, revealed that divergence of the BGWL strains from other members of the SCWL branch is above the recommended threshold of 97.5% sequence similarity for delineating novel phytoplasma species under the provisional status "*Candidatus*", according to the International Research Program on Comparative Mycoplasmology (IRPCM, 2004). However, on the basis of sequence analysis of the 16S-23S rDNA spacer region, serological comparisons, vector transmission specificity and plant host preference, phytoplasmas associated with BGWL are distinctly different from other members of the SCWL branch and are sufficiently homogenous to be included in a single species, *Candidatus* Phytoplasma cynodontis (Marcone *et al*., 2004).

In a 2002 survey of central and southern Iranian provinces, BGWL disease was found in fruit orchards, vegetable fields and uncultivated areas of Boushehr, Chaharmahal-Bakhtiari, Fars, Hormozgan, Kerman, Khuzistan and Sistan- Baluchistan provinces. BGWL is a lethal disease especially in warmer areas. The present study reports the etiology of BGWL disease, vector transmission and partial characterization of the BGWL phytoplasma from Iran. A preliminary report on molecular characterization of Iranian BGWL disease was published recently (Salehi *et al*., 2005).

## MATERIALS AND METHODS

**Disease sources.** Rhizomes of a Bermuda grass plant

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with typical symptoms of white leaf were collected in an affected patch in Firouzabad (Fars province), transferred to the greenhouse and used as a source of BGWL disease for vector studies. This isolate designated FBGWL and another isolate from Juyom (Fars province) designated JBGWL were used for phylogenetic analysis. Leaf samples from symptomatic and asymptomatic Bermuda grass plants were collected from various white leaf-affected areas and used for DNA extraction and PCR assays. The disease agent was maintained and propagated in the greenhouse using rooted rhizomes. Bermuda grass plants propagated from rhizomes of an asymptomatic Bermuda grass plant from a non-affected area were used as healthy controls.

**Search for vector.** Insects were collected from white leaf-affected patches in a citrus orchard at Firouzabad and identified to the species level. Samples of each species were subjected to nested PCR using P1/P7 (Schneider *et al.*, 1995) and R16F2n/R16R2 (Gundersen and Lee, 1996) primer pairs. Adults of PCR-positive species were separately transferred to wheat or barley seedlings for egg deposition and establishment of non-inoculative colonies, which were frequently monitored for freedom from phytoplasma contamination by nested PCR. Nymphs and young adults from non-inoculative colonies were caged on a white leaf-affected Bermuda grass plant for 4 weeks, then transferred in groups of 20 to 20 healthy Bermuda grass plants for a 4-week inoculation access period. Alternatively, insects collected in white leaf-affected patches of Bermuda grass in Firouzabad were caged (20 insects per plant) on 10 healthy Bermuda grass plants to test their transmission ability immediately after field collection.

**DNA extraction and PCR amplification.** Total nucleic acids (TNAs) were extracted from fresh leaves of diseased and healthy Bermuda grass using the small-scale procedure of Zhang *et al*. (1998) as modified by Abou-Jawdah *et al*. (2002), except that 170 mg of each sample were powdered in liquid nitrogen and transferred to a 1.5 ml Eppendorf tube for subsequent processing. DNA was extracted from the insects using a modified hot cetyltrimethylammonium bromide (CTAB) extraction method (Doyle and Doyle, 1990). Three insects of each species were homogenized in a 1.5 ml tube containing 600 µl of preheated (65°C) CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% PVP, 1% 2-mecaptoethanol) and incubated for 20 min at 65°C. Samples were extracted with chloroform-isoamyl alcohol (24:1). The aqueous nucleic acid layer was precipitated overnight at -20°C with 0.6 vol of isopropanol. The pellet obtained after centrifugation at 8000 rpm for 10 min was washed with 70% ethanol, vacuum-dried and suspended in 100 µl sterile water. The resultant suspension was used as DNA template for direct PCR amplification.

The universal phytoplasma primer pair P1/P7 (Schneider *et al.*, 1995) was used in direct PCR to amplify an 1.8 kbp fragment of ribosomal operon consisting of the 16S rRNA gene, 16S-23S intergenic spacer region (SR) and a portion of the 5' region of 23S rRNA gene. In nested PCR a 1:40 dilution of PCR products amplified by P1/P7 primer pair was used as template for a second round of PCR with primer pair R16F2n/R16R2 (Gundersen and Lee, 1996). PCR was performed in 50 µl reaction mixtures each containing 100 ng TNAs, 0.4 µM of each primer, 0.2 mM of each dNTP and lxPCR buffer (Cinagen, Iran). PCR conditions for the second round (nested) PCR were the same except that the annealing temperature was 58°C. The mixture was overlaid with 30 µl of mineral oil and subjected to 35 cycles of 1 min (2 min in the first round) at 94°C, 2 min at 55°C and 3 min (10 min for final cycle) at 72°C. Ten microliters of each PCR product were analyzed by electrophoresis in a 1% agarose gel in 1xTBE buffer (67 mM Tris-HCl, 22 mM boric acid, 10 mM EDTA, pH 0.8). DNA bands were stained with ethidiuim bromide and visualized with a UV transilluminator.

**RFLP analysis.** For RFLP analysis, 8 µl of the P1/P7 primed PCR products were digested with restriction enzymes *Alu*I, *hae*III, *Hha*I, *Hin*fI, *Hpa*II, *Mse*I, *Taq*I and *Tas*I according to instructions of the manufacturer (Roche) at 37°C (65°C for *Taq*I) overnight. The products of digestions were analyzed by electrophoresis through a 2.5% agarose gel followed by staining with ethidium bromide and visualization of DNA bands with UV transilluminator.

**Cloning and sequencing.** P1/P7 primed PCR products were ligated onto pTZ57R/T vector and cloned into *Escherichia coil* DH5α cells using InsT/A clone™ PCR Product Cloning Kit (Fermentas, Lithuania) according to manufacturer's instructions. Presence of the correct insert was confirmed by restriction endonuclease analysis using *Eco*RI and *Pst*I enzymes. Plasmid DNA from cultures of recombinant colonies was purified using High Pure Isolation Kit (Roche, USA). Sequencing was performed by SEQLAB (Göttingen, Germany). M13 forward and reverse primers were used as sequencing primers. Internal primers, when required, were designed and used by the sequencing company. The 16S-23S rDNA SR was further analyzed using P3 and P7 (Smart *et al*., 1996) as markers. BLAST search was performed to determine the closest phytoplasma relatives of sequenced isolates.

**Sequence homology, phylogenetic and putative restriction site analyses.** 16S rDNA and SR sequences of JBGWL and FBGWL isolates with similar reference phytoplasmas were separately aligned using Clustal X

1.81 (Thompson *et al.*, 1997). Phylogenetic tree was constructed using the NJ plot option of Clustal X program. *Acholeplasma laidlawii* was used as an outgroup to root the tree. Putative restriction site maps of 16S rRNA gene plus SR sequences of Iranian BGWL isolates (JBGWL and FBGWL) and *Ca*. Phytoplasma cynodontis were generated using MapDraw option of DNASTAR program (DNASTAR Inc., USA) and were manually aligned to compare restriction sites for the endonucleases *Hin*fI, *Mse*I and *Taq*I. The SR sequence homology between strains was evaluated after alignments were generated using homology matrix option of DNAMAN program version 4.02 (Lynnon Corporation, Canada).

## RESULTS

**Symptomatology and disease incidence.** The major natural symptoms of the disease were chlorosis and whitening of above ground plant parts, reduction of leaf size, shortening of internodes, stolons and rhizomes, proliferation of axillary buds, stunting and plant death (Fig. 1A). These symptoms are similar to those of Italian BGWL (Marcone *et al.*, 1997). Similar symptoms were obtained under greenhouse conditions by vector inoculation of Bermuda grass plant (Fig. 1B). Disease incidence rate in certain patches of Bermuda grass in Firouzabad and Juyom approached 100%. Variable amounts of white leaf-affected Bermuda grass were also found in Boushehr, Chaharmahal-Bakhtiari, Hormozgan, Kerman, Khuzistan and Sistan-Baluchistan provinces.

**Vector transmission.** Four leafhopper species, *Aconurella prolixa*, *Recilia schmidgeni*, *Exitianus capicola* and *Psammotettix striatus* (Cicadellidae) and the planthopper *Toya propinqua* (Delphacidae) were collected on white leaf-affected Bermuda grass at Firouzabad. Only 3 out of 8 samples of *E. capicola* were phytoplasma positive when tested by nested PCR. Four of 10 plants exposed to *E. capicola* naturally colonizing symptomatic Bermuda grass from the field became infected. When non-inoculative leafhoppers of the above species fed first on white leaf-affected Bermuda grass plants were transferred to healthy Bermuda grass, only *E. capicola* was capable of transmitting the BGWL agent to 7 of 20 inoculated plants. The disease incubation period in Bermuda grass was at least 4 months. Infection of Bermuda grass plants was verified by PCR. Other leafhoppers and the planthopper were neither PCR-positive nor capable of transmitting the disease agent.

**PCR amplification.** DNA fragments of approximately 1.8 kbp were amplified with the universal primer pair P1/P7 by one step PCR from 20 diseased samples of Bermuda grass (2 samples per region) from Firouzabad, Khafr, Juyom, and Jahrom (Fars province), Dezful and Ahwaz (Khusistan province). Cheshme Dimeh (Chaharmahal–Bakhtiari province), Nikshahr (Sistan-Baluchistan province), Minab (Hormozgan province) and Jiroft (Kerman province) (Fig. 2). No amplification was obtained from symptomless plants and other leafhopper samples examined. With nested PCR using primer pair P1/P7 followed by R16F2n/R16R2, fragments of 1.2 k bp were amplified from all symptomatic Bermuda grass plants and 3 of 8 individuals of *E. capicola* collected on white leaf-affected Bermuda grass plants. No PCR products were obtained from asymptomatic Bermuda grass plants collected in the field, *E. capicola* from non-inoculative colonies and other leafhopper samples.

**RFLP analysis.** P1/P7 PCR products (1.8 kbp) were analyzed by digestion with *Alu*I, *hae*III, *Hba*I, *Hin*fI, HpaII, *Mse*l, *Taq*I and *Tas*I (Fig. 3). Collectively, RFLP

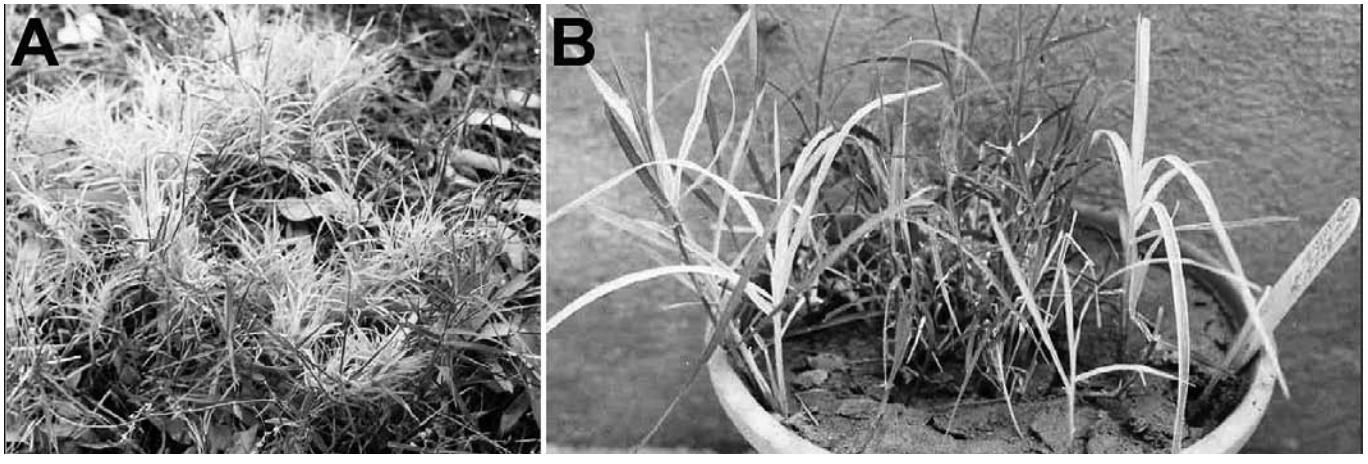


**Fig. 1.** Symptoms of Bermuda grass white leaf: (A) Chlorosis and whitening of above ground plant parts, small leaves, bushy growth and stunting in a patch of Bermuda grass in Firouzabad, Iran. (B) Whitening of leaves and stems in Bermuda grass experimentally inoculated with Firouzabad Bermuda grass white leaf agent by *Exitianus capicola*.

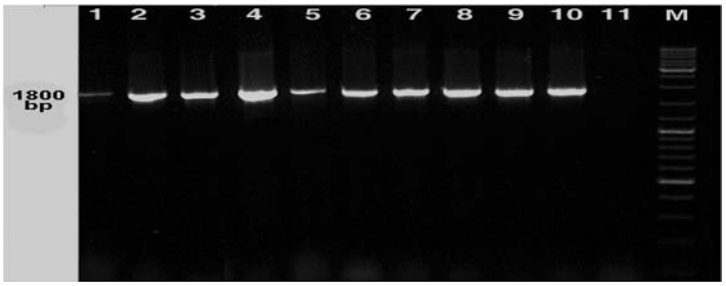


**Fig. 2.** Amplification of phytoplasmal DNA from white leaf – affected Bermuda grass from Firouzabad, Khafr, Juyom and Jahrom in Fars province, Dezful and Ahwaz in Khuzistan province, Cheshme Dimeh in Chaharmahal-Bakhtiari province, Nikshahr in Sistan-Baluchistan province, Minab in Hormozgan province and Jiroft in Kerman province (lanes 1-10, respectively) by PCR using P1/P7 primer pair. M, DNA Ladder. Lane 11: healthy Bermuda grass sample.

patterns analyzed with these enzymes were similar to those of Bermuda grass white leaf, 16SrXIV group (Lee *et al.*, 1998; Marcone *et al.*, 1997; Tran-Nguyen *et al.*, 2000; Blanche *et al.*, 2003). FBGWL isolate was indistinguishable from Indonesian and Thailandese BGWL (IBGWL and TBGWL, respectively) (Marcone *et al.*, 1997), but JBGWL differed from other isolates because of the absence of a *Hin*fI site which was present in FBGWL, IBGWL and TBGWL, and the presence of a *Taq*I site which was absent in the other isolates.

Putative restriction site analysis of 16S rRNA gene plus SR sequences (almost 1750 bp) revealed also that as BGWL group members, FBGWL and JBGWL isolates are distinguishable because of the presence of a *Taq*I site at 3' end of the 16S rRNA gene of JBGWL (absent in FBGWL) and the presence of *Hin*fI and *Mse*I sites (in the SR region and at 5' end of 16S rRNA gene, respectively) in FBGWL, which are absent in JBGWL. Based on this analysis, FBGWL and BGWL-CL (*Ca*. Phytoplasma cynodontis ) appeared to be indistinguishable (Fig. 4).

**Sequence homology and phylogenetic analysis.** Blast search showed that the 16S rRNA gene and SR sequences of JBGWL (GenBank accession Nos. EF444486 and DQ195216, respectively) and of FBGWL (GenBank accession Nos. EF444485 and DQ195215, respectively) isolates shared closest homology with the 16S rRNA gene and SR sequences of members of BGWL, 16SrXIV, phytoplasma group.

When the FBGWL and JBGWL 16S rDNA and SR sequences were aligned and compared with similar reference phytoplasmas, trees were obtained (Figs. 5 and 6, respectively) that were in good agreement with previous reports (Marcone *et al.*, 2004). In these analyses, FBGWL and JBGWL isolates clustered close to other known members of the 16SrXIV group. Phylogenetic tree constructed with SR sequences revealed that members of 16SrXIV group phytoplasmas can be divided into two subgroups. FBGWL isolate together with Italian BGWL isolates C1, C2 and CA (GenBank accession Nos. AJ550984, AJ550985, AJ550986), Thailandese and Indonesian BGWL phytoplasmas (AF248961 and Y14645), Sudan date palm slow decline (AF268000) and Sudan date palm white tip die-back (AF100411) form subgroup A whereas JBGWL, together with Chinese BGWL (AF025423) and Thailand carpet grass white leaf (Y15858), form subgroup B. The percent homology between SR sequences was determined and the result is presented as a matrix (Table 1) which shows

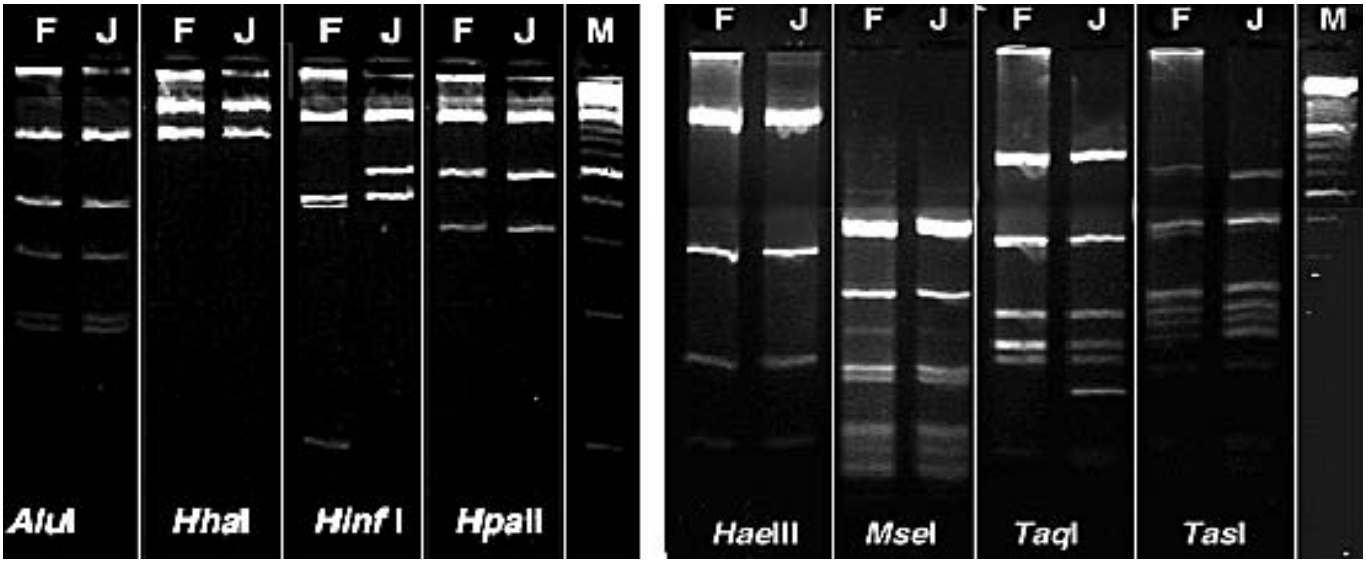


**Fig. 3.** Restriction fragment length polymorphism (RFLP) profiles of rDNA fragment (1.8 kbp) amplified by P1/P7 primer pair from Firouzabad (F) and Juyom (J) Bermuda grass white leaf phytoplasmas. Lane M, DNA ladder. DNA products were digested with *Alu*I, *Hha*I, *Hin*fI, *Hp*aII (A), *HaeIII*, *Mse*I, *Taq*I and *Tas*I (B) enzymes.

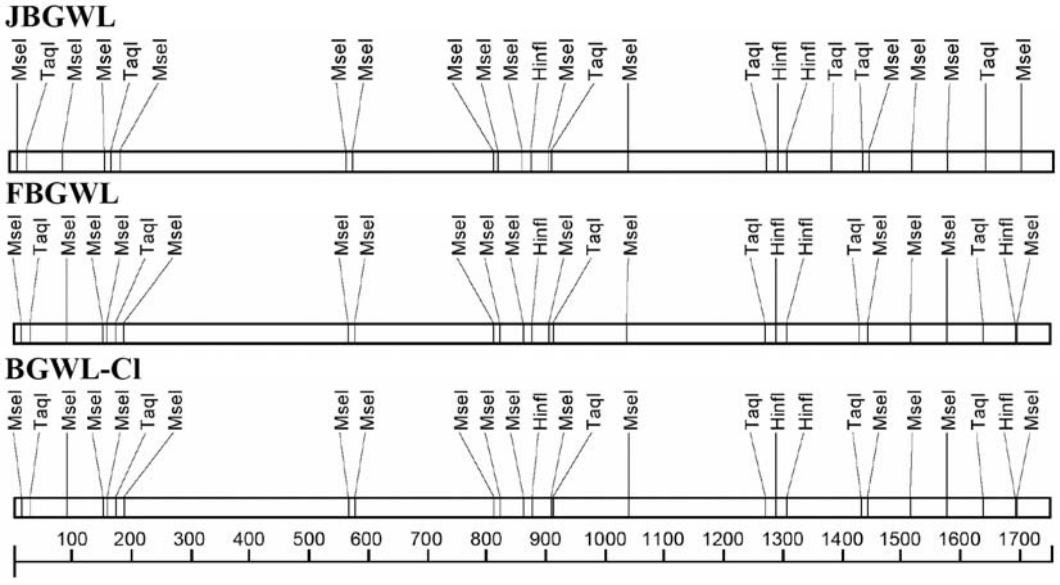


**Fig. 4.** Putative restriction site maps of 16S rRNA plus SR sequences of Juyom and Firouzabad Bermuda grass white leaf phytoplasmas and *Ca*. Phytoplasma cynodontis (BGWL-Cl). Maps were generated using the MapDraw option of DNASTAR program (DNASTAR Inc.). Phytoplasma abbreviations and accession numbers (in parantheses): BGWL-Cl, Bemuda grass white leaf, Italy (AJ550984); FBGWL, Firouzabad Bemuda grass white leaf (EF444485, DQ195215); JBGWL, Juyom Bemuda grass white leaf (EF444486, DQ195216).

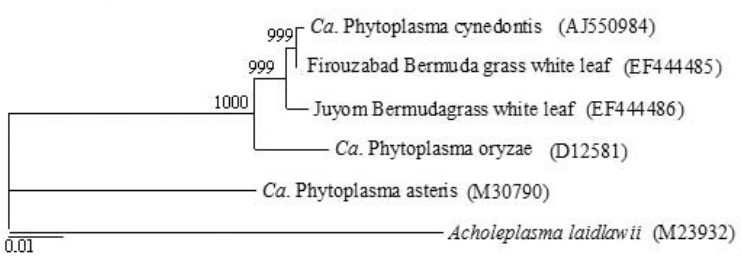


**Fig. 5.** Phylogram of full length 16S rDNA sequences of 5 phytoplasmas and *Acholeplasma laidlawii* as outgroup and position of Juyom and Firouzabad Bermuda grass white leaf phytoplasmas in the phylogram. The tree was constructed using NJ plot option of Clustal X program. GenBank accession Nos are in parentheses to the right of phytoplasma names.

that JBGWL and FBGWL isolates share 99.2 % homology. FBGWL was most similar to subgroup A and JBGWL to subgroup B members.

## DISCUSSION

RFLP and phylogenetic analyses and percent homology revealed that as members of 16SrXIV group, Iranian phytoplasma isolates FBGWL and JBGWL are distinguishable from one other and fall into two subgroups. Marcone *et al.* (2004) also showed that on the basis of SR sequence analysis, BGWL strains are not completely homogenous and BGWL-C is distinguishable from other BGWL strains. Putative restriction site

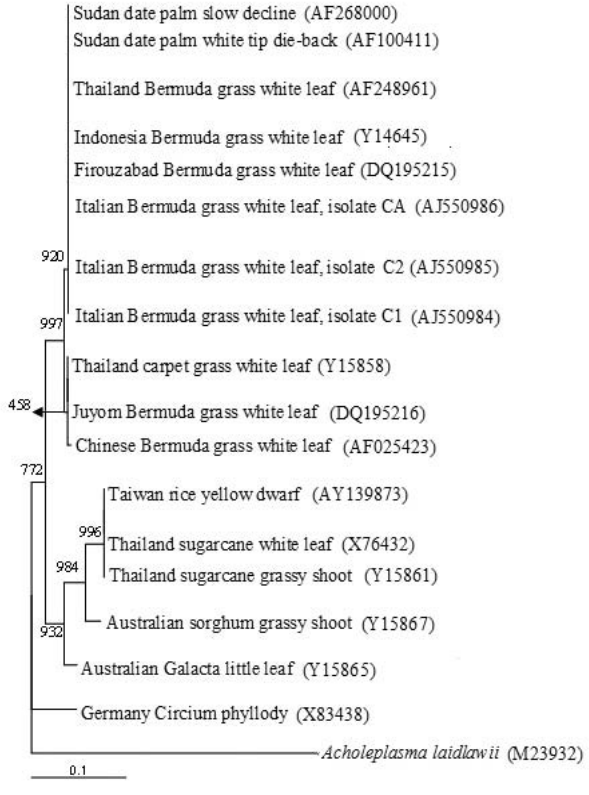


**Fig. 6.** Phylogram of 16S-23S rDNA spacer region (SR) sequences of 17 phytoplasmas and *Acholeplasma laidlawii* as outgroup and position of Firouzabad and Juyom Bermuda grass white leaf phytoplasmas in the phylogram. The tree was constructed using NJ plot option of Clustal X program. GenBank accession Nos are in parentheses to the right of phytoplasma names.

**Table 1.** Percent similarity among Juyom and Firouzabad white leaf isolates and other reference phytoplasmas as determined by analysis of 16S-23S rDNA spacer sequences.

| | JBGWL | FBGWL | BGWL-C1 | BGWL-C2 | DPWTD | BGWL-C | SCWL | RYD | CirP |
|---|---|---|---|---|---|---|---|---|---|
| JBGW | | 99.2 | 99.2 | 99.2 | 99.2 | 100 | 91.2 | 94 | 88.1 |
| FBGWL | | | 100 | 100 | 100 | 98.9 | 90.8 | 93.6 | 88.1 |

JBGWL and FBGWL: Bermuda grass white leaf isolates from Juyom (DQ195216), and Firouzabad (DQ195215); BGWL-C1 (AJ550984) and BGWL-C2 (AJ550985): Bermuda grass white leaf isolates from Italy; BGWL-Ch: Bermuda grass white leaf from China (AF025423); RYD: Rice yellow dwarf, Taiwan (Ay139873); SCWL: Sugarcane white leaf, Taiwan (AY139874); DPWTD: date palm white tip die back, Sudan (AF100411); Cirp: Circium phyllody, Germany (X83438).

analysis confirmed RFLP analysis and showed that FBGWL isolate is distinct from JBGWL on the basis of both 16S rRNA gene and SR sequences. Based on Marcone *et al*. (2004) and the present study, JBGWL together with BGWL-C and BraWL phytoplasmas can be designated as members of the 16SrXIV-B subgroup.

In previous works many BGWL strains from different geographical areas were examined by RFLP analysis of PCR amplified rDNA by various restriction enzymes (Marcone *et al*., 1997; Wongkaew *et al*., 1997; Lee *et al*., 1998; Tran-Nguyen *et al*., 2000) including those used in the present study, but no polymorphisms were identified. These studies were not based on SR sequences and did not include BGWL- C or JBGWL strains. PCR-positive samples of white leaf-affected Bermuda grass plants of areas other than Firouzabad and Juyom were not molecularly analyzed.

To our knowledge, *E. capicola* is reported for the first time as the natural and experimental vector of the Bermuda grass white leaf agent. It can now be added to list of five cicadellid species, i.e. *Criculifer haematoceps*, *Orocius albicinctus*, *Neoaliturus fenestratus*, *Psammotettix striatus* and *Macrosteles laevis* reported as vectors of phytoplasmal agents in Iran (Salehi and Izadpanah, 1992; Salehi *et al*., 1995, 2005). *E. capicola* is one of the main insects of Bermuda grass fauna. Breeding on Bermuda grass under both greenhouse and natural conditions indicated that *E. capicola* is well adapted to this species. *E. capicola* has been previously reported as vector of a phytoplasma associated with Limonium hybrid crop (Weintraub *et al.*, 2004).

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