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'*Candidatus* Phytoplasma cynodontis' associated with white leaf disease of golden beard grass (*Chrysopogon acicalatus*)

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ABSTRACT

A phytoplasma was detected in golden beard grass (*Chrysopogon acicalatus*) displaying white leaf symptoms near vegetative fields at the Tatkone region in Myanmar, 2011. Based on restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes, including the 16S-23S spacer region and part of the 23S rRNA gene, the phytoplasma was identified as a member of the Bermuda grass white leaf phytoplasma (BGWL) group. The golden beard grass white leaf phytoplasma (GBGWL) 16S rRNA gene sequence exhibited over 98.7% similarity with all members of BGWL group phytoplasmas and 99.5% similarity with Thailand Bermuda grass white leaf phytoplasma (AF248961). In addition, the GBGWL phytoplasma was confirmed to be a member of '*Candidatus* Phytoplasma cynodontis' by phylogenetic analyses.

Key words: '*Candidatus* Phytoplasma cynodontis', golden beard grass, 16S rRNA gene.

Golden beard grass, *Chrysopogon acicalatus*, is a perennial plant of the Poaceae family, with extensively creeping stolons covered with imbricate scale-like sheaths, sending up numerous sterile leafy shoots. The grass is very resistant to grazing and drought due to its vigorous, deep root system. It is found in a variety of soil types, usually in locations favorable to moisture accumulation (Streeter, 2007). In Myanmar, most of uncultivated areas near or among cultivated fields are covered with these grasses. It is useful for pasturing and helpful for protection against soil erosion because of their deep root systems. In 2011, white patches of golden beard grass were observed sporadically around vegetative fields near a riverside in the Tatkone region. The conspicuous symptoms are whitening of leaves, bushy growing habit, little leaves and shortened internodes (Figure 1). These symptoms were similar to those described for white leaf diseases affecting other graminaeous plants caused by phytoplasmas (Nasare et al., 2007; Obura et al., 2010).

The Poaceae have the largest number of species associated with phytoplasma diseases worldwide. In terms of phytoplasma diseases on grasses, Napier grass stunt diseases (NGS) are associated with 16SrXI group phytoplasma in Kenya (Jone et al., 2004) and 16SrIII group phytoplasma in Ethiopia (Jone et al., 2007). White leaf diseases of other grasses are associated with '*Ca*. P. cynodontis' on Bermuda grass (*Cynodon dactylon*) and annual blue grass (*Poa annua*) in Italy (Lee et al., 1997; Marcone et al., 2004), Brachiaria grass (*Brachiaria distachya*), carpet grass (*Axonopus compressus*) and crowfoot grass (*Dactyloctenium aegyptum*) in Thailand (Wongkaew et al., 1997; Sdoodee et al., 1999; Jung et al., 2003a), and Delhi grass (*Dichanthium annulatum*) in India (Rao et al., 2009). Until now, no phytoplasma disease had been recorded on golden beard grass. Therefore, the white leaf symptoms on golden beard grass found in Myanmar were investigated to verify the causal agent, and if phytoplasma infection is confirmed, to identify the species of phytoplasma present and its phylogenetic relatedness with other known '*Ca.* Phytoplasma' species.

Five samples of symptomatic golden beard grass showing white leaf symptoms and also three asymptomatic plants were collected from the Tatkone region. Total DNA was extracted from 0.3 g of leaves samples using the CTAB-based method of Namba et al. (1993). The universal phytoplasma primer pair SN910601 (5'-GTT TGA TCC TGG CTC AGG ATT-3') and SN011119 (5'-TCG CCG TTA ATT GCG TCC TT-3') (Jung et al., 2003b) was used to PCR-amplify an approximately 1.8 kbp DNA fragment comprising the nearly full length 16S rRNA, the 16S-23S rRNA spacer region (SR), and part of the 23S rRNA gene. PCR assays were performed with an automated thermal cycler 2720 (Applied Biosystems, California, USA). Each PCR mixture (20 µL) contained 2 µL of total nucleic acid (100 ng), 2 µL of each primer (10 pmol), 0.4 µL of a 10 mM dNTP mix, 2 µL of 10x PCR buffer and 0.2 µL of *Taq* DNA polymerase (1 unit) (SolGent Co. Ltd, Daejeon, Korea). The PCR program consisted of 35 PCR cycles of the following program: 30 seconds (2 minutes for the first cycle) denaturation at 94°C, annealing for 30 seconds at 55°C, and primer extension for 90 seconds (7 minutes in the final cycle) at 72°C. The amplified PCR products were

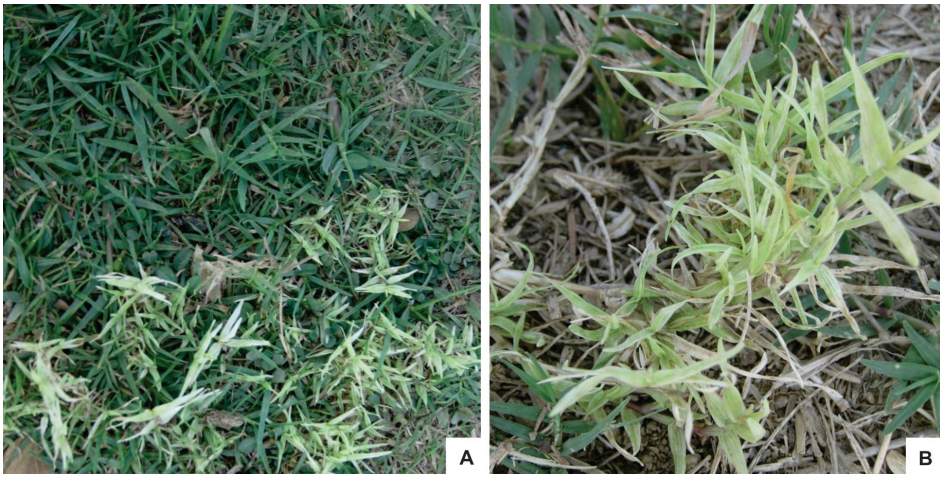

FIGURE 1 - White leaf symptoms of golden beard grasses (*Chrysopogon acicalatus*). **A**. white leaf diseased grasses; **B**. Little leaves and shorten internodes of a phytoplasma affected grass in the field.

analyzed by electrophoresis and visualization of DNA bands using a UV transilluminator.

PCR products were digested separately with nine restriction endonuclease enzymes (*Alu* I, *Eco*R I, *Hae* III, *Hha* I, *Hinf* I, *Mse* I, *Msp* I, *Sau*3A I and *Taq* I). The restriction products were resolved in an 8% polyacrylamide gel followed by staining in ethidium bromide and visualization of DNA band using a UV transilluminator. Direct sequencing of the PCR products was performed after purification with ExoSAP-IT (USB Co., USA) using six primers as described (Jung et al., 2003b). The obtained sequences were then assembled and edited using DNASTAR's laserGene software and consensus sequences were generated. The sequences were compared with those present in GenBank using the BLAST algorithm. Sequence alignments were performed using CLUSTAL W. A phylogenetic tree was constructed using the neighbor-joining method and the data with 1,000 bootstrap replications. The phylogenetic relatedness of the 16S rRNA gene sequence of the phytoplasma detected in golden beard grass in Myanmar was compared with those of 26 previously described phytoplasmas obtained from GenBank. The acholeplasma, *Acholeplasma laidlawii*, was used as an outgroup.

All five symptomatic plants examined resulted phytoplasma-positive in PCR assays with the amplified DNA fragments of 1.8 kbp, whereas no PCR products were obtained from the three asymptomatic plants tested. Phytoplasma infection in golden beard grass displaying white leaf symptoms was confirmed and is heretofore referred as GBGWL phytoplasma. RFLP analyses of PCR products digested by nine restriction enzymes is shown in Figure 2. *Hha* I, *Hinf* I, *Hae* III, and *Mse* I RFLP profiles were similar to those of Italian Bermuda grass white leaf (BGWL), 16SrXIV group (Marcone et al., 1997; Salehi et al., 2009). For *Taq* I, two additional bands were observed between ~750 bp and ~350 bp in the GBGWL phytoplasma. These bands indicate the presence of sequence heterogeneity of the rRNA operons, as observed in other phytoplasma-infected plants (Jomantiene et al., 2002). The RFLP analyses shows that the GBGWL agent is closely related to the BGWL phytoplasma.

The obtained phytoplasma sequences comprising the nearly full-length 16S rRNA gene, the spacer region and part of the 23S rRNA gene shared the same levels of nucleotide identity. Therefore, only one sequence has been deposited in Genbank database under the accession number of AB642601. A BLAST search showed that the sequences of the GBGWL phytoplasma share over 98.7% similarity with all members of the BGWL phytoplasma group (16SrXIV); AF248961, EU294011, GQ403689, AB052872, EU032485 and EF444485. The 16S rRNA gene sequence similarity is 99.5% with Thailand Bermuda grass white leaf (AF248961) and 99.3% with the type strain, '*Ca*. P. cynodontis' (AJ550984). The phylogenetic relatedness of 16S rRNA gene sequences of the GBGWL phytoplasma to other phytoplasmas is shown in Figure 3. The phylogenetic analysis indicated that the GBGWL phytoplasma clusters together with the phytoplasmas from the BGWL group and '*Ca*. P. cynodontis', while the phytoplasma isolates in other groups clustered separately forming distinct groups.

Based on the combined results of RFLP analyses and sequence analysis of the 16S rRNA gene sequences, we propose that the GBGWL phytoplasma in white leaf-affected golden beard grass in Myanmar is a BGWL phytoplasma-related agent. On the other hand, the GBGWL phytoplasma is clearly distinguished from the sugarcane white leaf agent, a member of the rice yellow dwarf group ('*Ca*. P. oryzae') by RFLP patterns of some enzymes (Marcone et al., 1997). As the 16S rRNA gene sequence similarity is greater than 97.5%, the GBGWL phytoplasma could be considered as a member of the *Candidatus* species '*Ca.* P. cynodontis'. Based on the previous reports, white leaf diseases in grasses occurring from various geographical areas are largely identical and represent members of '*Ca*. P. cynodontis'. This study highlights the diversity of phytoplasmas in Myanmar.

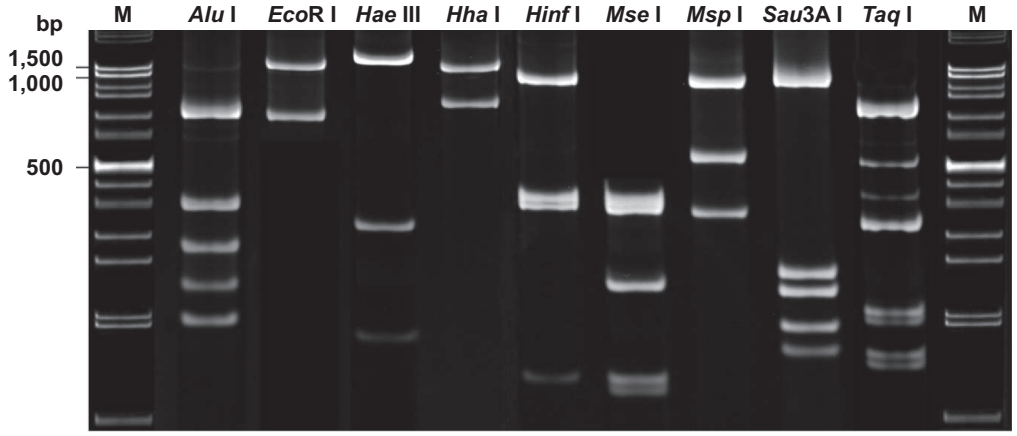

FIGURE 2 - *Alu* I, *Eco*R I, *Hae* III, *Hha* I, *Hinf* I, *Mse* I, *Msp* I, *Sau*3A I and *Taq* I RFLP profiles of PCR products (1.8 kbp) obtained with primers SN910601/ SN011119, comprising the 16S-23S rRNA gene region of the golden beard grass white leaf phytoplasma. Lane M: 1 kbp plus 100 bp Marker.

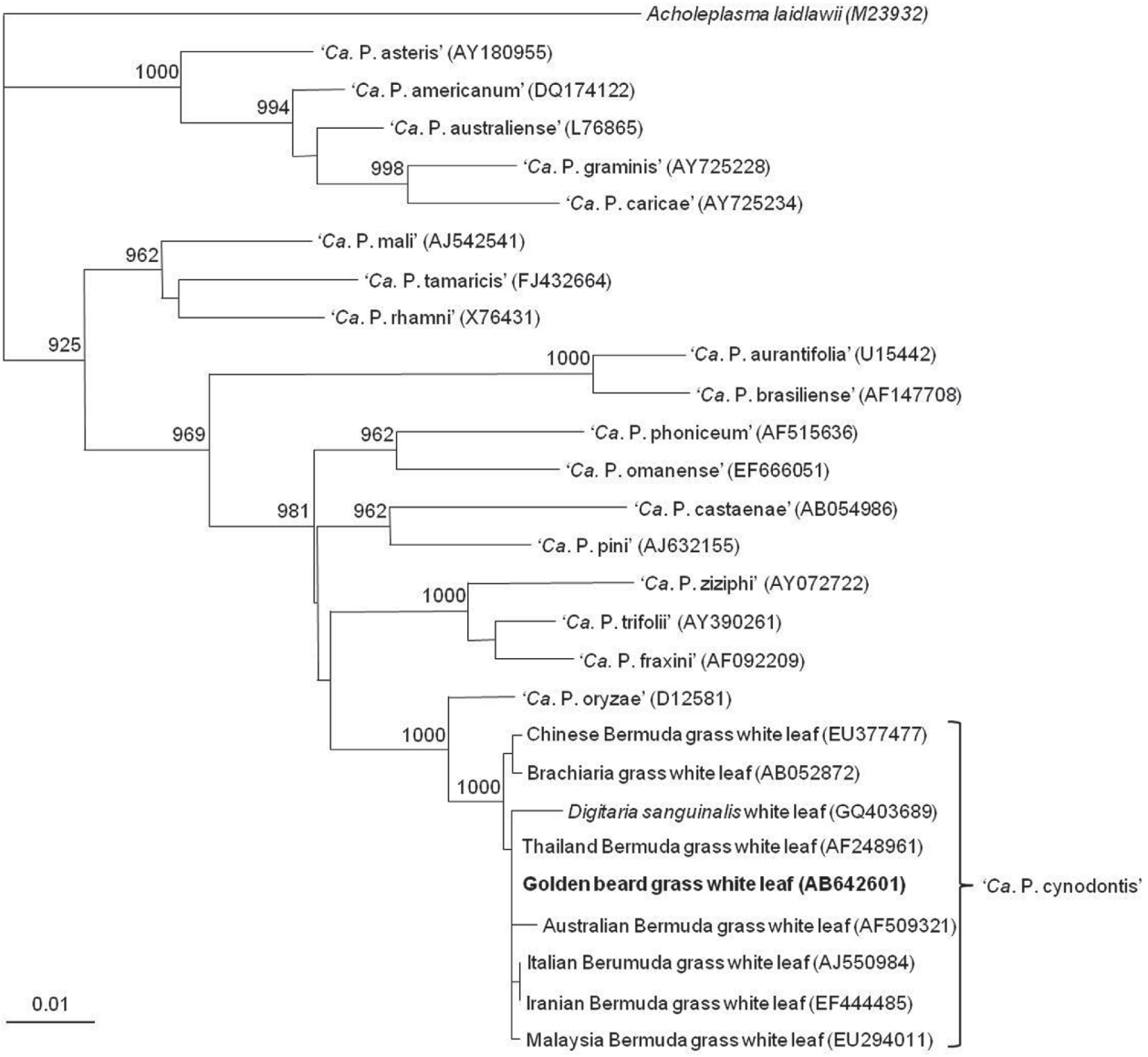

FIGURE 3 - Phylogenetic tree, constructed by the neighbor-joining method, based on the nearly full length 16S rRNA gene sequences from GBGWL phytoplasma and previously described phytoplasmas retrieved from GenBank. *Acholeplasma laidlawii* was used as an outgroup. Numbers on branches are confidence values obtained from 1,000 bootstrap replications (only values above 80% are shown).

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