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‘*Candidatus* Phytoplasma cynodontis’, the phytoplasma associated with Bermuda grass white leaf disease

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Bermuda grass white leaf (BGWL) is a destructive, phytoplasmal disease of Bermuda grass (*Cynodon dactylon*). The causal pathogen, the BGWL agent, differs from other phytoplasmas that cluster in the same major branch of the phytoplasma phylogenetic clade in $<2{\cdot}5\,\%$ of 16S rDNA nucleotide positions, the threshold for assigning species rank to phytoplasmas under the provisional status ‘*Candidatus*’. Thus, the objective of this work was to examine homogeneity of BGWL isolates and to determine whether there are, in addition to 16S rDNA, other markers that support delineation of the BGWL agent at the putative species level. Phylogenetic analyses revealed that the 16S rDNA sequences of BGWL strains were identical or nearly identical. Clear differences that support separation of the BGWL agent from related phytoplasmas were observed within the 16S–23S rDNA spacer sequence, by serological comparisons, in vector transmission and in host-range specificity. From these results, it can be concluded that the BGWL phytoplasma is a discrete taxon at the putative species level, for which the name ‘*Candidatus* Phytoplasma cynodontis’ is proposed. Strain BGWL-C1 was selected as the reference strain. Phytoplasmas that are associated with brachiaria white leaf, carpet grass white leaf and diseases of date palms showed 16S rDNA and/or 16S–23S rDNA spacer sequences that were identical or nearly identical to those of the BGWL phytoplasmas. However, the data available do not seem to be sufficient for a proper taxonomic assignment of these phytoplasmas.

Bermuda grass white leaf (BGWL) is a destructive phytoplasmal disease of Bermuda grass (*Cynodon dactylon*), which was first reported in Taiwan (Chen *et al.*, 1972). The disease is known to occur in several Asian countries (Chen *et al.*, 1972; Zahoor *et al.*, 1995; Viswanathan, 1997; Sdoodee *et al.*, 1999), Sudan (Dafalla & Cousin, 1988) and Italy (Marcone *et al.*, 1997). The most characteristic symptoms are extensive chlorosis, proliferation of axillary shoots, bushy growing habit, small leaves, shortened stolons and rhizomes, stunting and death of the plants. A similar disease, termed cynodon white leaf (CWL), has been reported in Australia. The causal agent appeared to be related closely or identical to the BGWL phytoplasma (Padovan *et al.*, 1999; Tran-Nguyen *et al.*, 2000; Blanche *et al.*, 2003).

Phytoplasmas associated with Bermuda grass diseases form, together with the agents of white leaf diseases of other gramineous plants [including *Brachiaria distachya* (brachiaria grass), *Poa annua* (annual blue grass) and probably *Dactyloctenium aegyptum* (growfoot grass)], the BGWL phytoplasma group or 16SrXIV-A group (Lee *et al.*, 1998, 2000; Seemüller *et al.*, 1998; Sdoodee *et al.*, 1999). There is evidence that phytoplasmas associated with diseases of date palms (*Phoenix dactylifera*) (Cronjé *et al.*, 2000a, b) and carpet grass (*Axonopus compressus*) white leaf (CGWL) (Padovan *et al.*, 1999) are related closely to this group. More distantly related to the Bermuda grass pathogens are the phytoplasmas that are associated with sugarcane white leaf (SCWL), sugarcane grassy shoot (SCGS), rice yellow dwarf

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Abbreviations: BGWL, Bermuda grass white leaf; BraWL, brachiaria white leaf; BVK, leafhopper (*Psammotettix cephalotes*)-borne; CGWL, carpet grass white leaf; CirP, cirsium phyllody; CWL, cynodon white leaf; DacWL, dactyloctenium white leaf; DP-SD, date palm slow decline; DP-WTD, date palm white tip die-back; GaLL, galactia little leaf; RYD, rice yellow dwarf; SCGS, sugarcane grassy shoot; SCWL, sugarcane white leaf; SGS, sorghum grassy shoot.

The GenBank/EMBL/DDBJ accession numbers for the P1/P7 amplimer (16S rRNA gene and 16S–23S rDNA spacer region) of phytoplasma strains BGWL-C1, BGWL-C2 and BGWL-CA are AJ550984, AJ550985 and AJ550986, respectively.

(RYD) and sorghum (*Sorghum stipoideum*) grassy shoot (SGS). Aside from these monocot diseases, BGWL-related phytoplasmas have been identified in two dicotyledons, *Cirsium arvensis* (Canada thistle) and *Galactia tenuifolia*, and in the leafhopper *Psammotettix cephalothes* (Seemüller *et al.*, 1994; Schneider *et al.*, 1997).

The above-mentioned phytoplasmas form a major branch, thereafter referred to as the SCWL branch, in the monophyletic phytoplasma clade. Previous taxonomic studies on these phytoplasmas, based on sequence analysis of PCR-amplified 16S rDNA, showed that most of them share $>97{\cdot}5\,\%$ sequence similarity, the standard threshold for defining putative phytoplasma species under the provisional status '*Candidatus*' for incompletely described prokaryotes according to Murray & Stackebrandt (1995). However, the phytoplasmas that cluster in this branch appeared to be too diverse to be included in a single species. Thus, the objectives of this work were to examine (i) whether the phytoplasmas identified in Bermuda grass were sufficiently homogeneous to be included in a single species, and (ii) whether the Bermuda grass agents are distinctly different from other phytoplasmas of the SCWL branch to justify separation at the putative species level. It is recommended that phytoplasmas differing in $<2{\cdot}5\,\%$ of 16S rDNA nucleotide positions should only be regarded as putative species when the separation is supported by data based on molecular markers other than 16S rDNA, such as plant host range, insect vector transmission and serological comparisons (IRPCM, 2000). Based on data obtained in previous work and additional sequence analysis carried out in this study, our comparisons indicate that the phytoplasmas associated with BGWL and CWL are largely identical and represent a distinct taxonomic entity that we propose should be distinguished at the putative species level.

Phylogenetic analysis of rDNA

Phytoplasmas examined and GenBank accession numbers of retrieved and newly submitted rDNA sequences are listed in Table 1. Strain BGWL-C1, examined previously by RFLP and sequence analysis under the designations Cy.da. and

Table 1. Phytoplasmas related to the Bermuda grass white leaf agent examined in this study

Phytoplasma (strain)	Geographical origin	Sequence*	GenBank accession no.	Reference/collector(s)
Bermuda grass white leaf:				
BGWL-C1	Italy	16S, SR	AJ550984	C. Marcone
BGWL-C2	Italy	16S, SR	AJ550985	C. Marcone
BGWL-CA	Italy	16S, SR	AJ550986	C. Marcone
BGWL-T	Thailand	16S, SR	AF248961	Davis & Dally (2001)
BGWL-S	Sudan	SR	AF100412	Cronjé *et al.* (2000a)
BGWL-C	China	SR	AF025423	N. A. Harrison, J. A. Richardson & J. H. Tsai
BGWL-I	Indonesia	SR	Y14645	Padovan *et al.* (1999)
Cynodon white leaf:				
CWL	Australia	16S	AF509321	Blanche *et al.* (2003)
CWL	Australia	SR	Y15868	Padovan *et al.* (1999)
Brachiaria white leaf (BraWL)	China	16S, SR		N. A. Harrison
Sugarcane white leaf:				
SCWL	Thailand	16S	X76432	Seemüller *et al.* (1994)
SCWL	Taiwan	SR	AY139874	S. M. Hsyu & C. P. Lin
Sugarcane grassy shoot (SCGS)	Thailand	SR	Y15861	Sdoodee *et al.* (1999)
Sorghum grassy shoot:				
SGS-1	Australia	16S	AF509324	Blanche *et al.* (2003)
SGS-2	Australia	16S	AF509325	Blanche *et al.* (2003)
SGS	Australia	SR	Y15867	Padovan *et al.* (1999)
Rice yellow dwarf:				
RYD	Japan	16S	D12581	Namba *et al.* (1993)
RYD	Taiwan	SR	AY139873	S. M. Hsyu & C. P. Lin
Carpet grass white leaf (CGWL)	Thailand	SR	Y15858	Sdoodee *et al.* (1999)
Date palm white tip die-back (DP-WTD)	Sudan	SR	AF100411	Cronjé *et al.* (2000a)
Date palm slow decline (DP-SD)	Sudan	SR	AF268000	Cronjé *et al.* (2000b)
Cirsium phyllody (CirP)	Germany	16S, SR	X83438	Schneider *et al.* (1997)
Galactia little leaf (GaLL)	Australia	16S, SR	Y15865	Padovan *et al.* (1999)
Leafhopper-borne (BVK)	Germany	16S	X76429	Seemüller *et al.* (1994)

*16S, 16S rRNA gene; SR, 16S–23S rRNA spacer region.

BGWL (accession no. Y16388; Marcone *et al.*, 1997; Seemüller *et al.*, 1998), was resequenced in order to obtain unambiguous data. Sequencing of both strands of P1/P7 PCR products (Schneider *et al.*, 1995) was performed by a commercial service (Bio Molecular Research and Biotechnology Centre, University of Padua, Padua, Italy) using internal primers, as described previously (Seemüller *et al.*, 1994; Marcone *et al.*, 1997). rDNA sequences were analysed by using the software package HUSAR (Biocomputing Service Group, German Cancer Research Center, Heidelberg). Gaps and ambiguities were removed from the final dataset. Phylogenetic and molecular evolutionary analyses were conducted by using the neighbour-joining program of the genetic analysis software MEGA, version 2.1 (Kumar *et al.*, 2001). The data were resampled 500 times; bootstrap percentage values are given at nodes of the trees. Maximum-likelihood and parsimony methods yielded similar results in tree construction. Phylogenetic distances were calculated by pairwise comparison. Numbering of nucleotide positions corresponds to that of the 16S rDNA molecule of aster yellows phytoplasma strain OAY (Lim & Sears, 1989).

Phylogenetic relatedness of the Bermuda grass strains to each other and to other phytoplasmas is shown in Fig. 1. Italian strains BGWL-C1 (proposed as the reference strain), BGWL-C2 and BGWL-CA were identical in 16S rDNA sequence, whereas Thai strain BGWL-T differed from them in four nucleotide positions. Australian strain CWL showed one polymorphism in comparison to strain BGWL-T and three polymorphisms in comparison to the Italian strains. From this data, it can be concluded that the Bermuda grass phytoplasma strains examined are highly uniform and that strain CWL can be included with the BGWL phytoplasma. The BraWL agent is related very closely to the BGWL phytoplasma. It differs in five positions from strain BGWL-T and in nine positions from the Italian strain, corresponding to 99·7 and 99·4 % 16S rDNA sequence similarity, respectively. Of the other phytoplasmas that cluster in the SCWL branch, those related most closely to the BGWL phytoplasma are the SGS and SCWL agents, which share 98·3–98·5 and 98·2–98·4 % 16S rDNA sequence similarity with them. By showing divergences of 3·3–3·4 and 2·6 %, respectively, strains CirP and GaLL from dicotyledonous hosts are related most distantly to the BGWL agent in the SCWL branch. Phytoplasmas from other phylogenetic groups differ from the BGWL agent in >5 % of 16S rDNA positions (Fig. 1). Comparisons of 16S rRNA gene sequences from the BGWL strains with those of phytoplasmas representing the major phylogenetic groups revealed that the BGWL phytoplasma has the following unique signature sequence: 5′-AATTAGAAGGCATCTTTTAAT-3′ (positions 169–189), which is not present in any other organism.

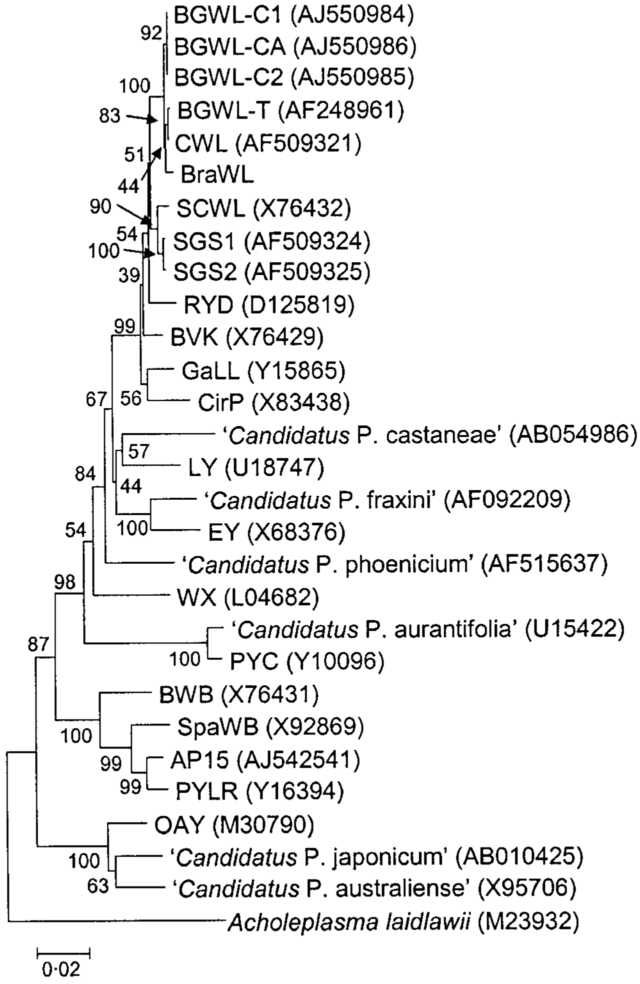

Fig. 1. Phylogenetic tree constructed by using the neighbour-joining method with 16S rDNA sequences from strains of the Bermuda grass white leaf phytoplasma (BGWL-C1, BGWL-CA, BGWL-C2, BGWL-T and CWL), BGWL-related agents (BraWL, SCWL, SGS1, SGS2, RYD, BVK, GaLL and CirP) and 15 reference phytoplasmas. *Acholeplasma laidlawii* was used as the outgroup. Other abbreviations are: AP15, apple proliferation; BWB, buckthorn witches'-broom; EY, elm yellows; LY, coconut lethal yellowing; OAY, oenothera aster yellows; PYC, papaya yellow crinkle; PYLR, peach yellow leaf roll; SpaWB, spartium witches'-broom; WX, western X-disease. Bar, 2 substitutions in 100 nt.

The relationships of the SCWL-branch phytoplasmas based on the sequence of the 16S–23S rDNA spacer region (approx. 250 residues in length) are depicted in Fig. 2. The tRNA^{Ile} gene was present in all strains and the sequence of this gene was identical in all BGWL strains and most other SCWL-branch phytoplasmas. Also, the regions flanking the tRNA^{Ile} gene were highly conserved. Strains BGWL-CA, BGWL-C1, BGWL-C2, BGWL-S, BGWL-T, BGWL-I and CWL and the two date palm strains, DP-WTD and DP-SD, were identical. Strains BGWL-C, BraWL and CGWL differed from them in two positions, upstream and downstream of the tRNA^{Ile} gene, where G residues were replaced by A residues. These data confirm the results of the 16S rDNA analysis, i.e. that strains from Bermuda grass are

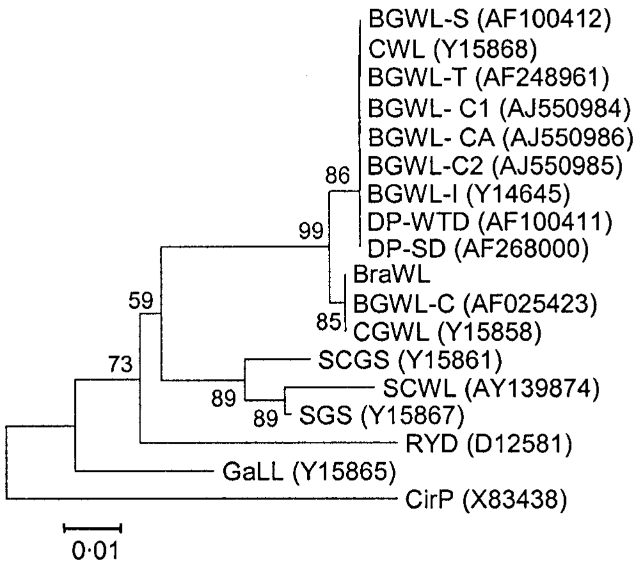

Fig. 2. Phylogenetic tree constructed by using the neighbour-joining method with 16S–23S rDNA spacer region sequences from strains of the Bermuda grass white leaf agent and related phytoplasmas. See Table 1 for strain abbreviations. Bar, 1 substitution in 100 nt.

identical or nearly identical and that the BraWL agent is related to them very closely. It also appears that the phytoplasmas associated with CGWL and date palm diseases are very similar to the BGWL agent. These findings largely confirm the results of previous work (Wongkaew *et al.*, 1997; Padovan *et al.*, 1999; Sdoodee *et al.*, 1999; Cronjé *et al.*, 2000a, b; Tran-Nguyen *et al.*, 2000). 16S–23S rDNA spacer sequence similarity of the other SCWL-branch phytoplasmas to the BGWL agent is considerably lower than on the 16S rDNA level, ranging from 93·3 % for the RYD phytoplasma to 86·3 % for the GaLL agent.

RFLP analysis, primer specificity and genome size

In previous work, numerous BGWL strains from various geographical areas were examined by RFLP analysis of PCR-amplified rDNA by using several restriction enzymes. No polymorphisms were identified (Marcone *et al.*, 1997; Wongkaew *et al.*, 1997; Lee *et al.*, 1998; Tran-Nguyen *et al.*, 2000). Also, no differences were found between a white leaf agent from annual blue grass and a BGWL phytoplasma strain from Thailand (Lee *et al.*, 1997). Although there is no firm evidence from previous work that phytoplasmas associated with BraWL, CGWL and date palm diseases can be differentiated from the closely related BGWL agent by RFLP analysis of rDNA, putative restriction site mapping carried out in this work revealed a *Taq*I site at position 1424 in the BraWL agent that was not present in the BGWL phytoplasma strains. In contrast, a *Mae*III site at position 1067 was identified in the BGWL phytoplasma that does not occur in the BraWL agent. In all previous comparisons, the BGWL phytoplasma could be distinguished clearly by RFLP analysis from several more distantly related SCWL-branch phytoplasmas, including the SCWL, SCGS and SGS agents (Marcone *et al.*, 1997, 2001; Padovan *et al.*, 1999; Sdoodee *et al.*, 1999; Tran-Nguyen *et al.*, 2000; Blanche *et al.*, 2003).

Specific amplification of BGWL phytoplasma rDNA is possible by using reverse primer rCWL, derived from the 16S–23S rDNA spacer sequence of strain CWL in combination with primer fSCWL, deduced from the 16S rDNA sequence of the SCWL phytoplasma (Tran-Nguyen *et al.*, 2000). No amplification products were obtained from the SCWL, SCGS and SGS phytoplasmas. On the other hand, the SGS-specific primer rSGS in combination with primer fSCWL did not amplify the target DNA from the BGWL phytoplasma (Tran-Nguyen *et al.*, 2000).

PFGE analysis revealed a chromosome size of 530 kbp for two BGWL isolates from southern Italy (Marcone *et al.*, 1999). This value was confirmed by examining five other BGWL isolates that were collected at different locations in the same area (C. Marcone, unpublished results). The estimated genome size represents not only the smallest mollicute chromosome reported to date, but also the smallest genome known for any self-replicating organism.

Serological differentiation and plant host and insect vector specificity

Polyclonal antisera raised to the BGWL and SCWL phytoplasmas revealed no cross-reactivity in serological comparisons (Sarindu & Clark, 1993; Viswanathan, 1997, 2001). Also, an antiserum raised to BGWL phytoplasma antigens did not react with BraWL and DacWL phytoplasma antigens (Sarindu & Clark, 1993). This indicates that the BraWL and DacWL agents are not identical to the BGWL phytoplasma.

By serological methods, PCR amplification using various primers and RFLP analysis of amplified DNA, the BGWL phytoplasma *sensu stricto* has only been identified in white leaf-diseased Bermuda grass and not in other plants. In reciprocal transmission experiments with the leafhopper *Matsumuratettix hiroglyphicus*, the natural vector of the SCWL phytoplasma, there was no evidence for transmission of the BGWL phytoplasma from Bermuda grass to sugarcane or vice versa (Anonymous, 1979).

Conclusion and taxonomic description

This taxonomic study showed that, based on 16S rDNA and 16S–23S rDNA spacer sequence analysis, phytoplasma isolates from Bermuda grass are identical or nearly identical. At the 16S rDNA level, they differ from most other phytoplasmas in the SCWL branch, including the SCWL, SGS and RYD agents, in 1·5–2·3 % of nucleotide positions. Thus, divergence of the BGWL agent from these phytoplasmas is below the recommended threshold of 97·5 % sequence similarity for defining a novel phytoplasma species under the provisional status '*Candidatus*', according to the International Research Program on Comparative Mycoplasmology (IRPCM, 2000). However, from sequence analysis of the 16S–23S rDNA spacer region, serological comparisons, vector transmission specificity and plant host preferences, there is supporting evidence that the BGWL agent is sufficiently different from these phytoplasmas to be described as a novel putative species.

At the 16S rDNA and/or 16S–23S rDNA spacer sequence level, several phytoplasmas, including the BraWL and CGWL agents and isolates associated with diseases of date palms, proved to be identical or nearly identical to the BGWL phytoplasma. However, their classification does not presently seem advisable, as only 16S–23S rDNA sequence data are available from the most closely related phytoplasmas (from date palms); this is a less significant taxonomic tool than 16S rDNA sequence. Also, there is little information on other taxonomic markers of these phytoplasmas. It is only known that antibodies raised to the BGWL agent did not react with BraWL phytoplasma antigens. Thus, taxonomic assignment of these phytoplasmas must await results of additional investigations, particularly on biological properties, such as vector and plant host specificity. It is conceivable that they are pathovars or other subtypes of the BGWL phytoplasma.

Due to its unique properties, we propose to designate the BGWL phytoplasma as a novel '*Candidatus*' species according to guidelines proposed by Murray & Schleifer (1994) for uncultivated prokaryotes whose uniqueness is defined only by very limited characteristics. The BGWL phytoplasma is designated as a novel distinct species with the following description: '*Candidatus* Phytoplasma cynodontis' (*cynodontis* epithet referring to the plant host) [(Mollicutes) NC; NA; O, wall-less; NAS (GenBank accession no. AJ550984; oligonucleotide sequence complementary to unique region of the 16S RNA gene is 5′-AATTAGAAGGCATCTTTTAAT-3′; P (*Cynodon dactylon*); M]. The reference strain is BGWL-C1. DNA samples from this strain are available from C. M.

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