

'*Candidatus Phytoplasma oryzae*', a novel phytoplasma taxon associated with rice yellow dwarf disease

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In addition to rice yellow dwarf (RYD) phytoplasma, several phytoplasmas infect gramineous plants, including rice orange leaf, bermuda grass white leaf, brachiaria grass white leaf and sugarcane white leaf phytoplasmas. To investigate whether the RYD phytoplasma is a discrete, species-level taxon, several isolates of the aforementioned phytoplasmas were analysed using PCR-amplified 16S rDNA sequences. Two RYD isolates, RYD-J^T and RYD-Th, were almost identical (99.2%), but were distinct (similarities of 96.3–97.9%) from other phytoplasma isolates of the RYD 16S-group. The notion that the RYD phytoplasma constitutes a unique taxon is also supported by its unique insect vector (*Nephotettix* sp.), its unique host plant in nature (rice) and its limited geographical distribution (Asia). In Southern blot analysis, chromosomal and extrachromosomal DNA probes of the RYD phytoplasma reportedly did not hybridize with those of closely related phytoplasmas. These properties of the RYD phytoplasma clearly indicate that it represents a novel taxon, '*Candidatus Phytoplasma oryzae*'.

Rice yellow dwarf disease continues to be a problem for rice farmers in many regions of Asia. Infected rice (*Oryza sativa*) turns pale yellow and gradually starts to decay; it ultimately shows stunted growth and fails to produce grain. For many years after its discovery in 1919 (Anonymous, 1919), the agent that caused this disease was unknown and was believed to be a virus, until it was identified as a phytoplasma (then called a mycoplasma-like organism) (Nasu *et al.*, 1967). The causative agent, RYD phytoplasma, is transmitted by the leafhoppers *Nephotettix cincticeps*, *Nephotettix virescens* and *Nephotettix nigropictus*, and is present in most rice-growing countries in Asia (Ou, 1985; Nakashima & Murata, 1993). From its 16S rDNA sequences, geographical distribution and the specificity of its host and insect vector, RYD phytoplasma has been regarded as an independent taxon by Namba *et al.* (1993a) and Nakashima *et al.* (1993).

Abbreviations: BGWL, bermuda grass white leaf; BraWL, brachiaria grass white leaf; OY, onion yellows; Rhp, rape phyllody; ROL, rice orange leaf; RYD, rice yellow dwarf; SCWL, sugar cane white leaf.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences reported in this paper are AB052870 (ROL), AB052871 (BGWL-KK), AB052872 (BraWL-KK), AB052873 (RYD-Th) and AB052874 (SCWL-Ud).

RYD phytoplasma has been classified in the RYD 16S-group (Jung *et al.*, 2003). Its closest known relatives, based on 16S rDNA phylogenetic analyses, are the phytoplasmas associated with sugarcane white leaf (SCWL) and sugarcane grassy shoot (SCGS) found in sugarcane (*Saccharum officinarum*), annual bluegrass white leaf (ABGWL) found in annual bluegrass (*Poa annua*), bermuda grass white leaf (BGWL) in bermuda grass (*Cynodon dactylon*) and brachiaria grass white leaf (BraWL) in brachiaria grass (*Brachiaria* spp.) (Schneider *et al.*, 1995; Nakashima *et al.*, 1996; Lee *et al.*, 1997; Wongkaew *et al.*, 1997; Tran-Nguyen *et al.*, 2000). Although these phytoplasmas have many traits in common, such as symptoms (leaf chlorosis and proliferation of tillers in most cases) and host plants (gramineous plants), they have not been characterized in detail and little is known of the variability of the group. To determine whether RYD phytoplasma constitutes a discrete, coherent taxon, RYD isolates from two different areas, Japan and Thailand, were analysed and compared with other phytoplasmas studied to date.

RYD phytoplasma samples from rice plants showing typical yellow dwarf symptoms were collected at Tochigi, Japan, and designated RYD Japanese isolates (RYD-J). The

RYD-J phytoplasma was maintained in rice plants and green rice leafhoppers (*N. cincticeps*). Reference phytoplasma samples of the BGWL Thai isolate (BGWL-KK) and the BraWL Thai isolate (BraWL-KK), both collected in Khon Kaen, Thailand, and the SCWL Thai isolate (SCWL-Ud), collected in Udonthani, Thailand, have been described previously (Nakashima & Hayashi, 1995). In 1997, these referenced phytoplasma samples were re-collected. Samples from rice plants showing yellow dwarf symptoms in Thailand and those showing rice orange leaf (ROL) symptoms in the Philippines were collected and designated RYD Thai isolate (RYD-Th) and ROL Philippine isolate (ROL-Ph), respectively. Total nucleic acids were extracted from tissues, as described elsewhere (Namba *et al.*, 1993b), for use as PCR templates. A pair of previously designed primers (SN910610/SN011119; Jung *et al.*, 2003) was used to amplify 16S rDNA from each sample tested. The following thermal cycling program was used: 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 90 s at 72 °C, with a final elongation step of 7 min at 72 °C. Direct PCR using the primer pair SN910610/SN011119 amplified a fragment of the phytoplasma 16S rDNA of approximately 1.8 kbp in all the diseased plants examined (data not shown).

To investigate the phylogenetic relationships between phytoplasmas that infect gramineous plants, 16S rDNA of several gramineous plant-infecting phytoplasma isolates, including two RYD isolates, was sequenced. Primers used to sequence the 16S rDNA were reported previously (Namba *et al.*, 1993b; Jung *et al.*, 2003). Nearly complete 16S rDNA sequences were determined for the five novel isolates (RYD-Th, ROL-Ph, BGWL-KK, BraWL-KK and SCWL-Ud) and for one of the reported isolates (RYD-J). Almost complete 16S rDNA sequences were determined unambiguously (see Fig. 1 for accession numbers). These sequences were compared with each other and with those already reported in the database. 16S rDNA sequence analysis revealed that ROL-Ph is most closely related to onion yellows (OY) phytoplasma from Japan (Namba *et al.*, 1993a) and rape phyllody (Rhp) phytoplasma from the Czech Republic (Bertaccini *et al.*, 1998), both of which belong to the aster yellows subgroup of the AY 16S-group (Jung *et al.*, 2002). Judging from the 16S rDNA sequence similarity between ROL-Ph and OY (99.9%), Rhp (99.8%) and the other aster yellows subgroup members (98.9–99.8%), it is reasonable to classify the ROL phytoplasma in the AY 16S-group, thus distinguishing it from RYD, which belongs to the RYD 16S-group (Jung *et al.*, 2002). The ROL phytoplasma is transmitted by a leafhopper, *Recilia dorsalis* (Rivera *et al.*, 1963; Hibino *et al.*, 1987). The 16S rDNA sequences of the two RYD phytoplasma isolates, RYD-J and RYD-Th, were nearly identical (99.2%), confirming that these two isolates belong to the same species. In addition, the sequence of SCWL-Ud was almost identical (99.8%) to the reported sequence of SCWL-Th (accession no. X76432; Seemüller *et al.*, 1994). The sequence of BGWL-KK was 99.9% similar to that of another Thai isolate, BGWL-Th (accession no. AF248961; Davis & Dally, 2001), but only 97.9% similar to

that of an Italian isolate, BGWL-It (accession no. Y16388; Seemüller *et al.*, 1998), indicating heterogeneity among BGWL isolates. Unexpectedly, BraWL-KK was 99.9% similar to a phytoplasma that infects a different host, BGWL-Th, suggesting that these two phytoplasmas belong to the same species-level taxon. When the 16S rDNA sequences of the two RYD isolates were compared with those of other phytoplasma isolates in the RYD 16S-group, the sequence similarity ranged from 96.3% (RYD-J vs BGWL-It) to 97.9% (RYD-J vs BGWL-Th). The RYD, BGWL and SCWL phytoplasmas were previously shown to belong to the RYD 16S-group and data in this study suggest that BraWL should also be classified in this group.

The 16S rDNA sequences of the RYD phytoplasmas and related phytoplasmas in the RYD 16S-group (RYD-J, RYD-Th, BGWL-Th, BGWL-KK, BraWL-KK and SCWL-Ud) were compared with sequences from phytoplasmas of other groups. The base positions were numbered as described previously (Namba *et al.*, 1993a). Sequence analysis of the phytoplasmas of the RYD 16S-group indicated that there are several signature sequences unique to this group: 5'-AACACTG-3' (positions 604–610) and 5'-GCAA-3' (999–1002) are not found in any other phytoplasmas belonging to other 16S-groups. In the RYD 16S-group, 5'-TATCAGACTA-3' (626–635) is also conserved, with just two exceptions: SCWL and BVK phytoplasmas have T instead of C (629). Additionally, 5'-AAATCTTCGGA-TTTT-3' (61–75) is conserved, except in the RYD isolates; both RYD-J and RYD-Th have T instead of C at position 65. Interestingly, this sequence alteration clearly distinguishes the RYD phytoplasmas from other RYD 16S-group isolates. The RYD phytoplasmas also possessed other unique 'signature' nucleotide alterations not found in any other phytoplasmas, including RYD 16S-group isolates. For example, single bases of RYD phytoplasma 16S rDNA differed from the corresponding sequences in all other phytoplasmas at positions 149 (G to A), 181 (C to T), 263 (A/C to T), 1148 (G to A), 1409 (A to G) and 1447 (G to A). The presence of these RYD-specific signature sequences in the 16S rDNA provides evidence for the genetic divergence of this pathogen from other phytoplasmas.

To clarify the phylogenetic relationships between RYD phytoplasma and the other RYD 16S-group phytoplasma isolates, almost complete 16S rDNA sequences (~1.4 kbp) from 48 phytoplasmas and a related mollicute, *Acholeplasma laidlawii*, as an outgroup, were aligned using the program CLUSTAL W (version 1.6) (Thompson *et al.*, 1994). A distance matrix and phylogenetic tree were constructed with CLUSTAL W using the neighbour-joining method (Saitou & Nei, 1987). Genetic distances between the sequences were estimated using the K_{nuc} value (Kimura, 1980). Confidence values (%) were estimated by the bootstrap sampling method (100 replicates). Sequences of the other organisms used in this study were obtained from DDBJ (<http://www.ddbj.nig.ac.jp/>). The sources of the 16S rDNA sequences used in this study are listed in Table 1. The RYD 16S-group

Table 1. Strains of phytoplasmas and *Acholeplasma* used in this study and associated diseases

Strain	Associated plant disease
AAV	American aster yellows
AlmWB-A4 ^T	Almond witches'-broom
AP	Apple proliferation
AshY1 ^T	Ash yellows
AUSGY	Australian grapevine yellows
BWB	Buckthorn witches'-broom
BGWL-It	Bermuda grass white leaf
BGWL-KK	Bermuda grass white leaf
BraWL-KK	Brachiaria grass white leaf
BVGY	Buckland valley grapevine yellows
BVK	—*
CbY	Chinaberry yellows
CirP	<i>Cirsium arvense</i> phyllody
CnWB	Chestnut witches'-broom
CP	Clover proliferation
PPWB	Caribbean pigeon pea witches'-broom
ESFY	Erigeron witches'-broom
EY	Elm yellows
FD	Flavescence dorée of grapevine
GaLL	Galactia little leaf
GLL-Eth	Ethiopian <i>Gliricidia</i> little leaf
HibWB	<i>Hibiscus</i> witches'-broom
IBS	Italian bindweed stolbur
JHP	Japanese <i>Hydrangea</i> phyllody
JWB-G1 ^T	Jujube witches'-broom
LDG	Coconut lethal yellowing
LDT	Coconut lethal yellowing
LfWB	Loofah witches'-broom
LY	Coconut lethal yellowing
OY	Onion yellows
PinP	<i>Pinus sylvestris</i> yellows
PpYC	Papaya yellow crinkle
ROL	Rice orange leaf
RYD-J ^T	Rice yellow dwarf
SbS	Sorghum bunchy shoot
SCWL-Ud	Sugarcane white leaf
SpaWB	<i>Spartinum</i> witches'-broom
StLL	Stylosanthes little leaf
STOL	<i>Capsicum annuum</i> stolbur
ViLL	<i>Vigna</i> little leaf
WBDL	Lime witches'-broom
WTWB	Weeping tea tree witches'-broom
WX	Western X-disease
<i>Acholeplasma laidlawii</i>	—

*BVK phytoplasma originates from a leafhopper, *Psammodettix cephalotes*, and has no associated disease.

forms a stable phylogenetic cluster in the tree, as judged by branch lengths and bootstrap values (100%). However, the RYD 16S-group cluster contains several internal nodes with weak bootstrap values, which also reflect an internal

branching order that is slightly different from previous results (Seemüller *et al.*, 1998; Jung *et al.*, 2002). This highlights the difficulty in depicting subgroups within the RYD 16S-group using the internal branching order (Fig. 1). The bootstrap value (100%) at the actual node indicates statistical support for BGWL and BraWL phytoplasmas sharing a common ancestral node. However, 16S rDNA sequence identity alone may not be enough to classify RYD phytoplasma as an independent species-level taxon.

It has been proposed previously that two phytoplasma isolates should be considered to belong to different species-level taxa if their 16S rDNA similarity is less than 97% or if their biological features, such as host plant or vector insect, are distinct (Jung *et al.*, 2002). Recently, the Phytoplasma and Spiroplasma Working Team of the International Research Program on Comparative Mycoplasmaology (IRPCM) announced their recommendation that two phytoplasmas that have less than 97.5% 16S rDNA sequence similarity may be designated two separate 'Candidatus Phytoplasma' species, and that those that have more than 97.5% 16S rDNA similarity may be designated separate species only if they can satisfy the following three criteria: (i) they are transmitted by different vectors; (ii) they have different natural plant hosts; and (iii) there is evidence of molecular diversity between the two phytoplasmas (Anonymous, 2002). All of these criteria are clearly met by RYD phytoplasma as follows. Only three species of leafhopper (*Nephotettix* spp.) transmit RYD phytoplasma, and no other insect vectors have been reported. These insect species are distributed only in Asia (Ou, 1985) and the diseases are naturally restricted to Asia (Nakashima *et al.*, 1993). It is also believed that RYD phytoplasma infects only rice (*Oryza sativa*) under natural conditions. Evidence for the spread of RYD phytoplasma from rice to other gramineous plants is limited, although it is believed to occur through root grafts and occasionally by leafhopper transmission. The plant-host specificity of RYD phytoplasma in nature may be associated with insect-vector feeding preferences, which are controlled by biophysical and biochemical mechanisms (Viswanathan & Kalode, 1986) and ultimately by genetic factors. The plant-host specificity may also be due to resistance of a particular plant, since RYD phytoplasma has not been transmitted by a dodder into periwinkle plants. Southern blot analyses showed that one chromosomal and six extrachromosomal DNA fragments of RYD phytoplasma hybridized only with DNA from plants infected by RYD, and not with those infected with other phytoplasmas, including the closely related SCWL phytoplasma (Nakashima *et al.*, 1993). All these lines of evidence and our findings in this study support the recognition of RYD phytoplasma as a unique, novel species-level taxon. The results of base-by-base comparisons of the RYD sequence are consistent with the hypothesis that the RYD subgroup should be regarded as a separate taxon. The geographical distribution of RYD phytoplasma in Asia may have provided ecological isolation, favouring the evolution of a distinct RYD phytoplasma. The presence of

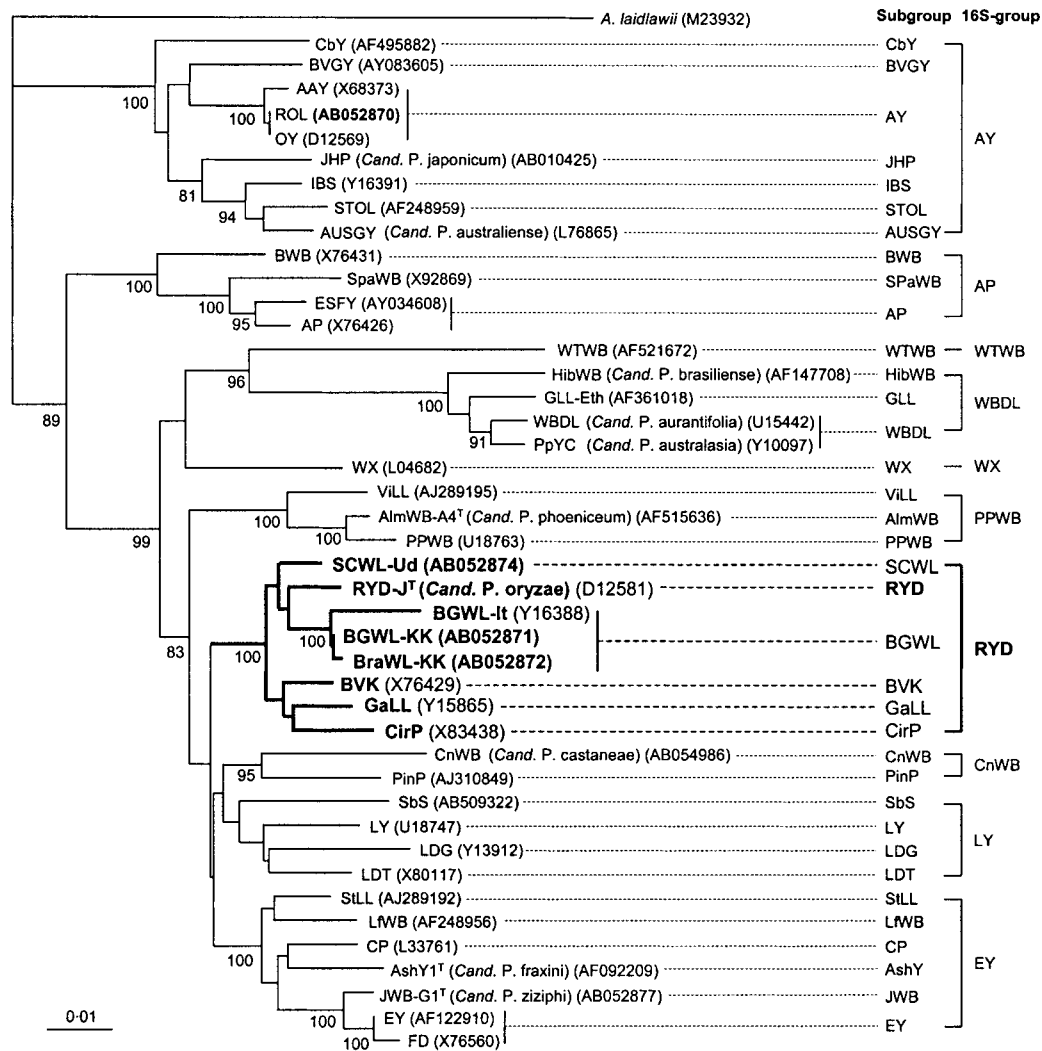


Fig. 1. Phylogenetic distance tree constructed by the neighbour-joining method, comparing the 16S rDNA sequences of RYD phytoplasma with those of other representative phytoplasmas from GenBank. *Acholeplasma laidlawii* was used as the outgroup. Accession numbers are shown in parentheses; those determined in this study are in bold. Numbers on branches are confidence percentages obtained from 100 bootstrap replicates (only values above 80% are shown). The corresponding phylogenetic group and subgroup names reported previously (Jung *et al.*, 2003) are shown on the right. Bar, phylogenetic distance of 1%. Phytoplasma abbreviations are given in Table 1; *Cand.*, *Candidatus*.

RYD-specific signature sequences and sequences unique to RYD phytoplasma in the 16S rDNA sequence also support this proposition and provide evidence for the genetic divergence of this pathogen from other phytoplasmas. It has been demonstrated that RYD phytoplasma isolates are phylogenetically distinct from all previously described strains belonging to the '*Candidatus* Phytoplasma' genus and it is proposed that they should be designated a novel *Candidatus* species with the following description: '*Candidatus* Phytoplasma oryzae' [(Mollicutes) NC; NA; O; NAS (GenBank numbers D12581 and AB052873), oligonucleotide sequences of unique regions of the 16S rDNA 5'-AACTGGATAGGAAATTTAAAAGGT-3' and 5'-ATGAGACTGCCAATA-3', P (rice, phloem); M].

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