'*Candidatus Phytoplasma castaneae*', a novel phytoplasma taxon associated with chestnut witches' broom disease

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In Korea, Japanese chestnut trees (*Castanea crenata* Sieb. and Zucc.) showing symptoms indicative of witches' broom disease, including abnormally small leaves and yellowing of young leaves, were examined. Since the symptoms were suggestive of a phytoplasma infection, tissues were assayed for phytoplasmas by PCR analysis using a pair of universal primers that amplify a 1.4-kbp phytoplasma 16S rDNA fragment. The phytoplasma-specific fragment was amplified from diseased plants, but not from healthy plants, indicating that a phytoplasma was the causal agent of the chestnut witches' broom (CnWB) disease. The phylogenetic relationship of the CnWB phytoplasma to other phytoplasmas was examined by sequence analysis of the 16S rDNA. A phylogenetic analysis of 16S rDNA sequences of the phytoplasmas placed the CnWB phytoplasma within a distinct subgroup in the phytoplasma clade of the class *Mollicutes*. The phylogenetic tree indicated that the CnWB phytoplasma is related most closely to coconut phytoplasmas and suggested that they share a common ancestor. The unique properties of the CnWB phytoplasma sequences clearly establish that it represents a novel taxon, '*Candidatus Phytoplasma castaneae*'.

Keywords: '*Candidatus Phytoplasma castaneae*'

INTRODUCTION

Phytoplasmas are prokaryotes that lack a cell wall and are the causal agents of numerous plant diseases (McCoy *et al.*, 1989; Kirkpatrick, 1992). Phytoplasmas inhabit sieve elements in the phloem of plants and are transmitted between plants by phloem-feeding insects. Phytoplasmas are small enough to pass through bacteriological filters and, like mycoplasmas, are resistant to antibiotics that interfere with cell-wall formation. Chestnut trees (*Castanea* spp.) are grown widely in the temperate zone of the northern hemisphere as commercially important vegetable crops. The chestnut phytoplasma disease was first described tentatively as a graft-transmissible viral disease in

Japanese chestnut, *Castanea crenata* Sieb. and Zucc., in Japan, and was named chestnut yellows disease (Shimada & Kouda, 1954). However, it was later shown by transmission electron microscopy that a phytoplasma was associated with the disease (Okuda *et al.*, 1974). In Korea in 1993, chestnut trees showing the typical symptoms of small, yellow leaves were examined. Based on electron and fluorescence microscope analysis, a phytoplasma was implicated as the aetiological agent of the disease (Han *et al.*, 1997). It was named chestnut little leaf (CLL) disease, reflecting the most obvious symptom. In Italy, European chestnut trees (*Castanea sativa* Mill) showing small, yellowing leaves have also been found in chestnut orchards, but it is still not known whether the causal disease was associated with phytoplasmas (Mitterpergher & Sfalanga, 1998).

In Korea, Japanese chestnut trees showing the typical witches' broom symptoms, suggestive of phytoplasma

Abbreviations: CLL, chestnut little leaf; CnWB, chestnut witches' broom. The EMBL/DDBJ/GenBank accession number for the 16S rDNA sequence of '*Candidatus Phytoplasma castaneae*' reported in this paper is AB054986.

infection, have been observed. The disease, tentatively denoted as chestnut witches' broom (CnWB), also resulted in small, yellowing leaves, but differed from Korean CLL and the chestnut yellows of Japan in symptom severity; it has led to severe crop losses, particularly in Kyongnam and Chonbuk provinces.

RFLP analysis of PCR-amplified 16S rDNA provides a simple, rapid and reliable method to differentiate and classify unidentified phytoplasmas (Lee *et al.*, 1993; Prince *et al.*, 1993). The development of universal PCR primer pairs designed to amplify all known phytoplasma 16S rDNA has facilitated this approach significantly (Ahrens & Seemüller, 1992; Deng & Hiruki, 1991; Gundersen & Lee, 1996; Namba *et al.*, 1993a). Moreover, nucleotide sequence analyses of the amplified 16S rDNA fragments have made phylogenetic analysis of phytoplasma isolates possible, and the information obtained has been used as the basis for delineating phytoplasmas into several groups (Kuske & Kirkpatrick, 1992; Schneider *et al.*, 1995; Seemüller *et al.*, 1998; White *et al.*, 1998).

In this study, we have demonstrated that the pathogen causing CnWB is indeed a phytoplasma (CnWB phytoplasma). We then determined the phylogenetic placement of the CnWB phytoplasma by amplifying and sequencing its 16S rDNA. Our results indicate clearly that this phytoplasma is unique among all previously described phytoplasmas. We also identified nucleotide sequences unique to the CnWB phytoplasma 16S rDNA, which will allow specific detection of this phytoplasma.

METHODS

Source of phytoplasma strains. Samples from Japanese chestnut trees (*Castanea crenata* Sieb. and Zucc.) displaying disease symptoms of CnWB and from trees without symptoms were collected from several independent diseased areas in Kyongnam and Chonbuk provinces in Korea. Healthy tissues were collected from greenhouse-grown chestnut seedlings.

Primer pairs and PCR conditions. Total nucleic acids were extracted from tissues as described previously (Namba *et al.*, 1993a) for use as PCR templates. The extracted nucleic acids were quantified by agarose gel electrophoresis. A pair of previously designed universal primers (SN910610 and SN910502; Namba *et al.*, 1993a) was used to amplify 16S rDNA in each sample tested. PCR conditions were described previously (Sawayanagi *et al.*, 1999). PCR products were electrophoresed in 0.7% agarose gels in TAE buffer and visualized with a UV transilluminator following ethidium bromide staining.

Nucleotide sequencing and PCR specific for CnWB phytoplasma. Primers used to sequence the 16S rDNA were reported previously (Namba *et al.*, 1993a, b). The PCR-amplified 16S rDNA products of at least ten independent CnWB-infected plant samples were sequenced using Dye Terminator cycle sequencing kits (Applied Biosystems). A pair of oligonucleotides was designed for specific amplification of CnWB phytoplasma DNA according to the 16S rDNA sequence. PCR conditions were as described by

Sawayanagi *et al.* (1999) except that the annealing temperature was 54 °C and 30 cycles were run.

Cladogram construction. Nearly complete 16S rDNA sequences from almost all phytoplasmas reported and from *Acholeplasma palmae* (Table 1) were aligned with that from the CnWB phytoplasma using the program CLUSTAL W (Thompson *et al.*, 1994) and base positions were numbered using a previously described system (Namba *et al.*, 1993b). Sequences of other organisms used in this study were obtained from the DDBJ. Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura, 1980) and a phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987), with *A. palmae* as the outgroup. The topology of trees was evaluated by bootstrap analysis of the sequence data with CLUSTAL W, based on 100 random samplings.

Nucleotide sequence accession numbers. GenBank accession numbers of the 16S rDNA sequences of the CnWB phytoplasma and other phytoplasmas used in this study and of *A. palmae* are listed in Table 1.

RESULTS AND DISCUSSION

Detection of phytoplasma in diseased chestnut trees

PCR using universal primers SN910610 and SN910502 was used to amplify the phytoplasma 16S rDNA and detected one fragment, approximately 1.4 kbp in size, representing the 16S rDNA of the phytoplasmas in all diseased chestnut trees examined (data not shown). Under the same conditions, no amplification products were obtained from asymptomatic plants collected from the same area or from healthy plants grown in a greenhouse.

Nucleotide sequencing of amplified 16S rDNA from the CnWB phytoplasma

Many phytoplasmas have been classified by analysing 16S rDNA sequences and RFLP profiles (Tymon *et al.*, 1997; Montano *et al.*, 2000). Sequence analysis of 16S rDNA has also played an important role in the description of novel phytoplasma species (Zreik *et al.*, 1995; Davis *et al.*, 1997; White *et al.*, 1998; Griffiths *et al.*, 1999; Sawayanagi *et al.*, 1999; Montano *et al.*, 2001). This study used similar approaches to determine the phylogenetic relationships between the CnWB phytoplasma and other phytoplasmas.

All of the 16S rDNA sequences of the CnWB phytoplasma isolated from several independent areas were identical (data not shown).

Sequence similarity and phylogenetic relationships

Sequence comparisons revealed that similarity between the CnWB phytoplasma and other phytoplasmas ranged from 86.8 to 94.9% and that between the CnWB phytoplasma and other mollicutes ranged from 70.1 to 86.4%. Stackebrandt & Goebel (1994) proposed that any two bacteria having 16S rRNA similarity of 97% or higher needed additional data before they could be classified into different species.

Table 1. Strains of phytoplasma and *Acholeplasma* used in this study, associated diseases and 16S rDNA sequence accession numbers

Strain	Associated plant disease	16S rDNA accession no.	Strain	Associated plant disease	16S rDNA accession no.
AAY	American aster yellows	X68373	LDT	Tanzanian coconut lethal disease	X80117
ACLR	Apricot chlorotic leaf roll	X68338	LDY	Yucatan coconut lethal decline	U18753
AGY	Australian grapevine yellows	X95706	LfWB	Loofah witches' broom	L33764
AlfS	Alfalfa stunt	AF177384	LWB3	Lilac witches' broom	AF105317
AIY	Alder yellows	Y16387	LY	Coconut lethal yellowing	U18747
APS	Apple proliferation	X76426	MIAY	Michigan aster yellows	M30970
AshY	Ash yellows	X68339	OAY	<i>Oenothera</i> aster yellows	M30790
AshY1	Ash yellows	AF092209	OY	Onion yellows	D12569
AshY2	Ash yellows	AF189215	PaWB	<i>Paulownia</i> witches' broom	AF279271
AshY3	Ash yellows	AF105315	PD	Pear decline	X76425
AshY4	Ash yellows	L33759	PD-I	Pear decline	Y16392
AshY5	Ash yellows	AF105316	PEP	<i>Picris echinoides</i> phyllody	Y16393
AT	Apple proliferation	X68375	PEY	<i>Picris echinoides</i> yellows	Y16389
AUSGY	Australian grapevine yellows	L76865	PnWB	Peanut witches' broom	L33765
AY-H	Ash yellows	AF245439	PpDB	Papaya dieback	Y10095
AY1	Maryland aster yellows	L33767	PPER	European stone fruit yellows of peach	X68374
BAWB	Black alder witches' broom	X76431	PLL	Pigeon pea little leaf	AJ289191
BB	Tomato big bud	L33760	PpM	Papaya mosaic	Y10096
BGWL	Bermudagrass white leaf	Y16388	PPT	Potato purple top	AF217247
BLL	Brinjal little leaf	X83431	PPWB	Pigeon pea witches' broom	L33735
BLWB	Black locust witches' broom	AF244363	PPWB-Ch	Chinese pigeon pea witches' broom	AF028813
BoLL	nk	Y15863	PpYC	Papaya yellow crinkle	Y10097
BVK	Blutenverkleinerung	X76429	PYL1	<i>Phormium</i> yellow leaf	U43569
CaC	Cactus phytoplasma	AF200718	PYL2	<i>Phormium</i> yellow leaf	U43570
ChWB-C	Chayote witches' broom	AF147706	PYLRL	Peach yellow leaf roll	Y16394
ChWB-M	Chayote witches' broom	AF147707	RpPh	Rape phyllody	U89378
CIRP	<i>Cirsium arvense</i> phyllody	X83438	RuS	<i>Rubus</i> stunt	Y16395
CnWB	Chestnut witches' broom	AB054986	RYD	Rice yellow dwarf	D12581
CP	Clover proliferation	L33761	SAY	Western severe aster yellows	M86340
CPh	Clover phyllody	L33762	SCWL	Sugarcane white leaf	X76432
CPhI	Italian clover phyllody	X77482	ScYPI-Ba	Sugarcane yellows	AF056094
CPPWB	Caribbean pigeon pea witches' broom	U18763	SLL	<i>Stylosanthes</i> little leaf	AJ289192
CX	Canadian peach X-disease	L33733	SLY	Strawberry lethal yellows	AJ243045
CYE	Clover yellow edge	L33766	SPAR	<i>Spartium</i> witches' broom	X92869
CYE-C	Clover yellow edge	AF175304	SPLL	Sweet potato little leaf	X90591
CYE-L	Clover yellow edge	AF173558	SPWB	Sweet potato witches' broom	L33770
CYE-Or	Clover yellow edge	AF189288	StGP	Strawberry green petal	AJ243044
Elm-AH	Elm phytoplasma	AF268895	StLL	<i>Stylosanthes</i> little leaf	Y17055
ESFY	European stone fruit yellows of apricot	Y11933	STOL	Stolbur of <i>Capsicum annuum</i>	X76427
EY	Elm yellows	L33763	STRAWB1	Strawberry witches' broom	U96614
EY1	Elm yellows	AF122910	STRAWB2	Strawberry witches' broom	U96616
EY2	Elm yellows	AF189214	SunHP	Sun hemp witches' broom	X76433
FBP	Faba bean phyllody	X83432	SVN	Soybean veinal necrosis	AF177383
FD	Flavescence dorée of grapevine	X76560	TBB	Australian tomato big bud	Y08173
FM	<i>Fragaria multicapita</i> phytoplasma	AF036354	TBB-A	Australian tomato big bud	AJ289194
GaLL	<i>Galactia</i> little leaf	Y15865	TWB	Tsuwabuki witches' broom	D12580
GPh	<i>Gerbera</i> phyllody	AB026155	ULW	<i>Ulmus</i> witches' broom	X68376
HD1	Hemp dogbane	AF122912	VAC	<i>Vaccinium</i> witches' broom	X76430
HibWB	<i>Hibiscus</i> witches' broom	AF147708	VGR	Virginia grapevine yellows	AF060875
IABW	Italian alfalfa witches' broom	Y16390	VILL	<i>Vigna</i> little leaf	Y15866
IBS	Italian bindweed stolbur	Y16391	VK	Grapevine yellows	X76428
JHP	Japanese <i>Hydrangea</i> phyllody	AB010425	VLL	<i>Vigna</i> little leaf	AJ289195
KAP	<i>Knautia arvensis</i> phyllody	Y18052	WBDL	Witches' broom disease of lime	U15442
KV	Clover phyllody	X83870	WVEY	Elm yellows	AF122911
LDG	Ghanaian Cape St Paul's wilt disease	Y13912	WX	Western X-disease	L04682
LDN	Nigerian Awka disease	Y14175	<i>A. palmar</i>	–	L33734

nk, Not known.

Thus, the CnWB phytoplasma can safely be considered to constitute a novel taxon or species. For phylogenetic analysis, CnWB phytoplasma 16S rDNA sequences were aligned with those of most reported phytoplasmas (Table 1). Phylogenetic analysis of 16S rDNA sequences from 110 diverse phytoplasmas, plus CnWB phytoplasma and *A. palmar*, produced one neighbour-joining tree (Fig. 1). Bootstrap analysis revealed that the phylogenetic tree was reliable and it was also in good agreement with a tree constructed previously

with 57 phytoplasma accessions (Seemüller *et al.*, 1998). As a result of using a larger number of accessions, the current tree has several additional branches. The tree indicated that the CnWB phytoplasma constitutes a distinct branch, with a long phylogenetic distance from any other phytoplasma. The tree also indicated that the CnWB phytoplasma branch is included in a larger group that includes three other branches: a branch composed of LDG plus LDN, the branch containing LDT and a branch

Fig. 1. Phylogenetic distance tree constructed by the neighbour-joining method, comparing the nearly complete 16S rDNA sequence of the CnWB phytoplasma with those of other phytoplasmas from GenBank. *Acholeplasma palmarum* was used as the outgroup. Numbers on the branches are confidence values obtained for 100 replicates (only values above 80% are shown). The bar represents a phylogenetic distance of 1%.

composed of LDY plus LY. Except for the CnWB phytoplasma, all of the phytoplasmas included in this larger group infect the coconut. Judging from the branch lengths between CnWB and these other phytoplasmas, the CnWB phytoplasma may have diverged from LDG/LDN and related phytoplasmas early in the evolution of this phytoplasma group.

RFLP and heteroduplex mobility analyses of amplified phytoplasma 16S rDNA revealed that the pathogen that causes CLL disease, which has symptoms similar to CnWB, was a phytoplasma closely related to aster yellows (AY) phytoplasma (Hiruki *et al.*, 1998), but is distinct from the CnWB phytoplasma (Fig. 1). It has been reported that different phytoplasmas may cause

similar symptoms in the same host plant. Examples include grapevine infected by *flavescence dorée* or *bois noir* phytoplasmas (Daire *et al.*, 1993) and Spanish broom (*Spartium junceum*) infected by apple proliferation or elm yellows phytoplasmas (Marcone *et al.*, 1996). Thus, it is possible that the CLL and CnWB diseases, which sometimes show similar symptoms, are caused by distinct phytoplasma species, the CLL and CnWB phytoplasmas. Nucleotide sequence analyses of PCR-amplified 16S rDNA and phylogenetic analyses of 16S rDNA sequences revealed that the CnWB phytoplasma is not related to AY, unlike CLL, but is more closely related to isolates of coconut phytoplasmas. These data suggest strongly that the CnWB phytoplasma is a novel phytoplasma that infects chestnut trees.

LY and related strains

The grouping of the coconut phytoplasmas is controversial. For instance, Liefting *et al.* (1996) included isolates LY, LDY, LD and PPWB in a phylogenetic analysis and recognized that these strains formed a cluster. They proposed naming this group as a seventh ‘strain cluster’, after the classification system of Seemüller *et al.* (1994) and Schneider *et al.* (1995). However, Tymon *et al.* (1998) showed that PPWB and coconut phytoplasmas should be separated into different clusters in a phylogenetic analysis and recognized that the coconut phytoplasma cluster formed three distinct ‘subclades’: (i) LY and LDY, (ii) LDT and (iii) LDG and LDN. According to the classification system of Gundersen *et al.* (1994a), these were respectively named subclades vii, xii and xiv. Conversely, Lee *et al.* (1998) determined that these strains formed two subclades, VI (PPWB) and VII (LY, LDY and LD), using a phylogenetic analysis of nearly full-length 16S rDNA sequences from 42 diverse phytoplasmas. Thus, the coconut phytoplasma group is now considered to represent a unique and distinct primary group. In our study, we found that PPWB should be separated from five coconut phytoplasmas plus CnWB phytoplasma and, thus, it forms a different cluster in a phylogenetic analysis, in agreement with previous work (Tymon *et al.*, 1998; Lee *et al.*, 1998).

PCR specific for CnWB phytoplasma

A primer set was designed using the CnWB phytoplasma 16S rDNA sequence. The primers were forward primer CnWBF1 (5′-CTAGTTTAAAAACAA-TGCTC-3′, corresponding to nucleotides 587–606 in the CnWB 16S rDNA sequence) and reverse primer CnWBR1 (5′-CTCATCTTCCTCCAATTC-3′, complementary to nucleotides 1145–1162). In PCR samples containing CnWBF1 and CnWBR1, a 575-bp DNA fragment was amplified when the template DNA was derived from plants naturally affected by CnWB disease. No amplifications were observed when the template was derived from healthy plants (data not shown). In the future, this primer pair will facilitate the screening of putative insect vectors for the diseases

found in Korea, although transmission of the phytoplasma has not yet been identified in the field.

Phytoplasma signature sequences and unique 16S rDNA sequences

The 16S rDNA sequence of the CnWB phytoplasma was compared with sequences from 110 other phytoplasmas. Of three sequences reported previously to be unique to phytoplasmas (Gundersen *et al.*, 1994b; Namba *et al.*, 1993b), TTTTAAAG (at positions 187–195) and GCTT (224–227) were found in the 16S rDNA sequence of the CnWB phytoplasma. However, the sequence ACTGGA (155–160) has a G at position 155 in the 16S rDNA sequence of CnWB and coconut phytoplasmas (LDG, LDN, LDT, LDY and LY). Additionally, in the CnWB sequence, the nucleotide at position 156 is T. Furthermore, GTGT (276–279) and CCC (487–489) sequences unique to phytoplasmas have a T at positions 278 and 487 in the 16S rDNA of the CnWB phytoplasma.

In addition to the above unique sequences, the 16S rDNA of the CnWB phytoplasma contains other unique sequences compared with all other phytoplasmas studied. For example, single bases of the CnWB phytoplasma 16S rDNA differed from the corresponding sequences in coconut phytoplasmas as well as all other phytoplasmas at positions 128 (G to A), 232 (C to A), 262 (A to C), 463 (C/G/T to A), 597 (C/G/T to A), 650 (A to G), 884 (G to A), 909 (C to T), 990 (A/G/T to C), 999 (T/C to G), 1124 (T to C), 1145 (A to G) and 1158 (G to A).

In comparisons of the CnWB phytoplasma with the coconut phytoplasmas, there are base differences between CnWB and LY, LDT and LDG/LDN at 44, 37 and 44 positions, respectively. Single bases at positions 248 (T to A), 497 (C to T), 526 (G to A), 598 (T to A), 602 (C to T), 606 (T to C), 638 (A to T), 657 (C to T), 684 (T to C), 709 (G to A), 822 (G to T), 1120 (G to C), 1127 (G to A), 1166 (T to C), 1221 (A to G), 1223 (T to G), 1268 (A to G), 1269 (T to C) and 1331 (C to T) differ between the CnWB phytoplasma and the coconut phytoplasmas. The presence of CnWB-specific signature sequences and sequences unique to the CnWB phytoplasma in the 16S rDNA also supported this proposition and provided evidence for the genetic divergence of this pathogen from other phytoplasmas. Therefore, we conclude that CnWB is a phylogenetically distinct taxon.

Phytoplasma phylogenetic classification

In this study, by including almost all available phytoplasma 16S rDNA sequences (110 sequences) in a single phylogenetic tree, the relationships among reported strains were made clearer (Fig. 1). Evidently, the phylogenetic tree we generated can be divided into several major clusters, which are supported by high bootstrap values. Thus, a total of eight distinct phytoplasma monophyletic groups based on 16S rDNA sequences can now be recognized. These groups

are as follows: 16S-group I, AY and related strains; 16S-group II, apple proliferation (AP) and related strains; 16S-group III, peanut witches' broom (PnWB) and related strains; 16S-group IV, Canadian peach X-disease (CX) and related strains; 16S-group V, pigeon pea witches' broom (PPWB) and related strains; 16S-group VI, LY and related strains; 16S-group VII, rice yellow dwarf (RYD) and related strains; and 16S-group VIII, EY and related strains. Our classification of phytoplasmas into '16S-groups' is generally in good accord with previously reported classifications into 'groups' or 'subclades' (Seemüller *et al.*, 1998). However, the '16S-groups' have two notable features: (i) they use almost all reported sequences (110 sequences) as opposed to 'groups' or 'subclades' that used limited numbers of sequences and (ii) the numbering of the 16S-groups concurs with the branching order in the whole tree.

Any pair of phytoplasmas belonging to different 16S groups is so phylogenetically different that the similarity of 16S rDNA sequences between the two is always lower than 96%. If 97% similarity is regarded as the threshold for defining species, as discussed above (Stackebrandt & Goebel, 1994), this indicates that each 16S-group contains multiple taxa comparable to 'species'. Therefore, in an attempt to clarify relationships between phytoplasma isolates further within an individual 16S-group, we divided each 16S-group further into smaller taxa when the similarity of the 16S rDNA sequences of two isolates was less than 97% or when two isolates showed clear biological isolation, such as striking heterogeneity in host plants and insects. Since the phytoplasma is an endocellular microbe and is totally dependent on its hosts, the second criterion should be important for phytoplasma classification. Using such criteria, we identified 29 distinct smaller taxa or 'subgroups'. In the phytoplasma phylogeny, six *Candidatus* species have so far been registered (Fig. 1) and each *Candidatus* species corresponds well to a 'subgroup' proposed here. When one looks at Fig. 1, one may think that other 16S-groups are possible. For example, the largest 16S-group, 16S-group I, could be divided into two 16S-groups, 16S-groups Ia and Ib, respectively containing the AY subgroup and the STRWB1/JHP/IBS/AUSGY/STOL subgroups. However, the highest 16S rDNA sequence similarities between members of the AY subgroup and those of the STRWB1/JHP/IBS/AUSGY/STOL subgroups were 96.5% (between RaPh and VK), 96.4% (between RaPh and STOL) and 96.2% (between KV and STOL), which are higher than the allowed similarity between any of the proposed 16S-groups (always less than 96%). Thus, we conclude that the eight 16S-groups proposed in this paper are appropriate at present.

Conclusions

We have identified the pathogen that causes CnWB disease in Japanese chestnut trees in Korea as a novel species of phytoplasma, CnWB phytoplasma. So far,

the phylogenetic analysis of phytoplasmas found in chestnut trees from Korea and elsewhere has been limited. Phylogenetic analyses indicated that the CnWB phytoplasma constitutes a clearly defined subgroup and belongs to 16S-group VI, together with the LY, LDG and LDT subgroups. Furthermore, its 16S rDNA sequence dissimilarity, signature sequences and phylogenetic position distinguish it as a novel species. In addition, in a PCR approach using a primer pair specific to CnWB (CnWBF1/CnWBR1), specific amplifications provided evidence for a novel phytoplasma species.

We propose that the CnWB phytoplasma be designated as a novel, distinct *Candidatus* species, '*Candidatus* Phytoplasma castaneae', with the following description: '*Candidatus* Phytoplasma castaneae' [(*Mollicutes*): NC; NA; O; NAS (GenBank acc. no. AB054986), oligonucleotide sequences of unique regions of the 16S rDNA 5'-CTAGTTTAA-AAACAATGCTC-3' (587–606) and 5'-CTCATCTT-CCTCCAATTC-3' (1145–1162) (*Castanea*, phloem); MJ].

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