

Detection of 16S rDNA of Thai Isolates of Bacterium-Like Organisms Associated with Greening Disease of Citrus

Kazuo NAKASHIMA^{a)}, Maitree PROMMINTARA^{b)} Yoshihiro OHTSU^{c)},
Takeshi KANO^{c)}, Jun IMADA^{d)} and Meisaku KOIZUMI^{e)}

^{a)} *Biological Resources Division,
Japan International Research Center for Agricultural Sciences (JIRCAS)
(Tsukuba, Ibaraki, 305 Japan)*

^{b)} *Plant Pathology and Microbiology Division, Department of Agriculture (DOA)
(Bangkok, 10900 Thailand)*

^{c)} *Plant Protection Division, Fruit Tree Research Station (Tsukuba, Ibaraki, 305 Japan)*

^{d)} *Akitsu Branch, Fruit Tree Research Station (Akitsu, Hiroshima, 729-24 Japan)*

^{e)} *Okitsu Branch, Fruit Tree Research Station (Shimizu, Shizuoka, 424-02 Japan)*

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Abstract

Citrus greening disease caused by bacterium-like organisms (BLO; greening organisms: GOs) is one of the most destructive diseases of citrus in Thailand. An efficient and reliable detection method of the pathogen is urgently needed for the control of the disease. Recently, polymerase chain reaction (PCR) method for amplifying the 16S rDNA fragment of the GOs has been developed by Jagoueix *et al.* (1994; *Int. J. Syst. Bacteriol.* **44**: 379-386). We applied the method for the detection of Thai GO isolates, Nakorn-Pathum, Rangsit and Nan isolates, and succeeded in detecting the 16S rDNA fragments of the Thai isolates. Sequence analysis of the amplified 16S rDNA fragment of the Nakorn-Pathum isolate revealed that the isolate is very close to the Indian isolate of liberobacter phylogenetically. By simplifying the DNA extraction procedure, we were able to obtain DNA for PCR within 20 min, and detect the GO within 4.5 hr.

Additional key words : citrus greening, liberobacter, 16S rDNA, PCR, rapid diagnosis

Introduction

Citrus greening disease which occurs widely in citrus-growing areas of the African and Asian Tropics²⁾ is one of the most destructive diseases of citrus in Thailand. The prokaryote associated with greening disease was first observed in 1970 in the phloem of affected plants⁶⁾. It was initially considered that the causal agent was a mycoplasma-like organism (MLO, phytoplasma), but this organism was soon found to be enclosed by a 25 nm thick envelope, which was much thicker than the unit membrane envelope characteristic of MLOs (7-10 nm thickness)¹²⁾. By analogy with MLOs, these organisms have been called bacterium-like organisms (BLOs), or greening organisms (GOs). The organisms are transmitted by two insect vectors, the psyllid *Diaphorina citri* in Asia and the psyllid *Trioza erytreae* in Africa^{7,8)}. Because so far it had not been possible to culture GOs *in vitro*, microbiological/genetic information about the GOs has not been available. Recently, nucleotide sequences of the 16S rDNA of the Indian and African isolates of the GO have been determined⁴⁾. The results obtained indicated that the GOs are members of the α subdivision of the *Proteobacteria*. Jagoueix *et al.* proposed the name of "liberobacter" for the new phylogenetic group including GOs⁵⁾.

It has been difficult to detect the GOs. Detection of the GOs was based on the electron-microscopic examination of ultra-thin sections and transmission by grafting or insect vectors mainly. However, such methods are not practical. Ultra-thin sectioning is a tedious procedure and it requires expensive equipment. Moreover, it is usually difficult to detect the causal organisms in phloem elements because of the low titer of the pathogens. Transmission tests are of limited value due to the latency and the long incubation period in insects and plants. Progress has been made in the serological assay of GOs. Monoclonal antibodies (MAbs) were obtained against Indian, Chinese and African isolates of GOs^{3,4)}. It was found that the MAbs did not react to some GO

isolates because they are highly strain specific¹⁾. Many researchers have produced polyclonal antisera with partially purified GO antigens from diseased plants, but these antisera showed substantial cross-reactions with antigens from healthy plants. Therefore, an efficient and reliable method of detecting the pathogen is urgently needed for the control methods of the disease and basic research on the behavior of the pathogens. Recently hybridization procedure using DNA probes specific to the GO has been developed^{14,15)}. Moreover, it was shown that the polymerase chain reaction (PCR) procedure for amplifying 16S rDNA fragments using GO-specific primers enabled to detect several isolates of GO including the Nakorn Pathum (NP), Thailand isolate in periwinkle plants⁵⁾.

In this report, we attempted to detect several Thai GO isolates including the NP isolate maintained in citrus plants by the developed PCR procedure. Next, we determined the sequence of the amplified 16S rDNA fragment of GO-NP to define the phylogenetic position of the Thai GO isolate. Moreover, we tried to improve the detection method by simplifying the DNA extraction steps.

Materials and Methods

Materials. The Nakorn-Pathum isolate (NP), Rangsit isolate (R) and Nan (N) isolate of GO were collected in Nakorn Pathum, Rangsit and Nan in Thailand respectively. The GOs were transmitted from the infected citrus plants to sweet orange plants by psyllids *D. citri*. Thereafter, the GOs were transmitted to citrus plants (Mandarin and/or Ponkan) by grafting. Existence of the GOs was confirmed by electron microscopy.

DNA extraction. Crude vascular bundles (ca. 50mg) were collected from the midrib of a leaf and homogenized with 1 ml of 0.6 M mannitol, 20 mM Tris-HCl (pH 7.6), 5 mM magnesium acetate⁹⁾. After centrifugation of the suspension at 700 x g for 10min at 4°C, the supernatant was decanted in a new tube. The GO was concentrated in a pellet by centrifugation at 15,000 x g for 10 min at 4°C. DNA

was extracted from the pellet by the CTAB method¹⁰⁻¹¹⁾. The pellet was homogenized in 1 ml of CTAB solution (2 % [w/v] cetyltrimethylammoniumbromide, 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 1% [w/v] polyvinylpyrrolidone, 1% β -mercaptoethanol, and the homogenate was transferred to a microfuge tube. The homogenate was incubated at 65°C for 10 min, mixed gently with an equal volume of chloroform-isoamyl alcohol (24:1), and then centrifuged at 15,000 \times *g* for 5 min. The supernatant was decanted and saved. Nucleic acids were precipitated with 0.7 volume of ice-cold isopropanol. The pellet was washed with 70 % ethanol, dried, and resuspended in 100 μ l of TE buffer.

PCR. The 16S rDNA fragments of the GOs were amplified by the modified PCR method using a GO-specific primer set (OI1: 5' GCGCGTATGCA-ATACGAGCGGCA 3', OI2c: 5' GCCTCGCGACTTCGCAACCCAT 3', *cf.* Fig.2) described by Jagoueix *et al.*⁵⁾. The PCR reaction mixture contained the primers at a concentration of 1 μ M, each of the four deoxynucleoside triphosphates at a concentration of 200 μ M, 78 mM Tris-HCl (pH 8.8), 2mM MgCl₂, 17 mM (NH₄)₂SO₄, 10 mM β -mercaptoethanol, and 200 μ g of BSA per ml. The conditions used for amplification were as follows: 92°C for 30 sec; 40 PCR cycles, each consisting of 92°C for 1 min, 52°C for 30 sec, and 72°C for 90 sec; and 72°C for 10 min. Amplified DNA fragments were electrophoresed in a 1.0% agarose gel using TBE buffer containing ethidium bromide.

Cloning and Sequencing of 16S rDNA of GO. Cloning of the PCR-amplified 16S rDNA fragment of GO-NP was performed by a standard procedure¹³⁾ with some modification. The amplified DNA fragment was phosphorylated using T4 polynucleotide kinase, and ligated into *Sma* I cutting site of pBluescript SK II (-) using T4 DNA ligase. *Escherichia coli* XL-1 was transformed with the recombinant plasmid. *Eco* RI cutting fragments and *Taq* I cutting fragments of the cloned fragments were sub-cloned. Nucleotide sequencing of the insert DNA was performed by

the dideoxynucleotide chain termination method with fluorescent primers in an automatic DNA sequencer (model 373A; Perkin Elmer) using *Taq* Dye Primer Cycle Sequencing Kit (Perkin Elmer).

Results and Discussion

Detection of 16S rDNA of Thai GO isolates.

We amplified 16S rDNA fragments of the Thai GO isolates by using two primers OI1 and OI2c (Fig. 1). The DNA templates for PCR were as follows: healthy (lane 1), GO-NP-infected citrus plants maintained in a greenhouse in Japan (lane 2), healthy (lanes 3 and 4), GO-NP-infected (lanes 5 and 6), GO-R-infected (lanes 7 and 8), and GO-N-infected (lanes 9 and 10) citrus plants maintained in a greenhouse in Thailand. DNA samples of lanes 1, 3 and 4 were derived from different plants. DNA samples of the lanes 5, 7 and 9 were derived from leaves with severe symptoms, whereas those of lanes 6, 8 and 10 were derived from leaves with mild symptoms. We observed that DNA of the expected size (ca. 1,170 bp) was amplified in the GO-NP, -N, and -R-infected citrus plants, but no

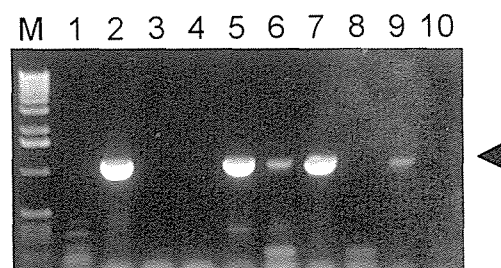


Fig. 1 Agarose gel electrophoresis of DNA fragments amplified from Thai GO isolates by the PCR by using primers OI1 and OI2c. The DNA templates for PCR were as follows: healthy (lane 1), GO-NP (Nakorn Pathum isolate)-infected citrus plants maintained in a greenhouse in Japan (lane 2), healthy (lanes 3 and 4), GO-NP-infected (lanes 5 and 6), GO-R (Rangsit isolate)-infected (lanes 7 and 8), and GO-N (Nan isolate)-infected (lanes 9 and 10) citrus plants maintained in a greenhouse in Thailand. DNA samples of lanes 5, 7 and 9 were derived from leaves with severe symptoms, whereas those of lanes 6, 8 and 10 were derived from leaves with mild symptoms. Lanes M contained 1 kb marker (BRL). Arrowheads indicate the position of the amplified 16S rDNA fragments of GOs.

amplification was obtained from healthy plants. It was often difficult to detect the 16S rDNA fragments of GO in leaves with mild symptoms and samples at an early infection stage (data not shown). These results indicated that the distribution of the GOs is uneven within plants, and suggested that DNA extracted from some leaves of one plant should be used as a template of PCR for the detection of the DNA of the pathogen.

Sequence Analysis of 16S rDNA of GO-NP.

Nucleotide sequence of the amplified 16S rDNA fragment of GO-NP was determined (Fig. 2). We

observed a 99.7% homology between the 16S rDNAs of GO-NP isolate and Poona isolate (India) of *Liberobacter* sp.⁵⁾ (determined nucleotides: 1,165 bp, primer sites: 45 bp, putative nucleotides: 5 bp, confirmed nucleotides: 1,115 bp, identical nucleotide: 1,113 bp), and 97.9% homology between the 16S rDNAs of GO-NP and Nelspruit isolate (Africa) of *Liberobacter* sp.⁵⁾ (determined nucleotides: 1,165 bp, primer sites: 45 bp, putative nucleotides: 5 bp, confirmed nucleotides: 1,115 bp, identical nucleotide: 1,093 bp). These results revealed that the Thai GO isolate was very close to

	O11 primer site	
GO(Th)	<u>GCGCGTATGC AATACGAGCG GCAGACGGGT GAGTAACGCG TAGGAATCTA CCTTTTCTA</u> -CGGGATAAC GCATGGAAC GTGTGCTAAT ACCGTATACG	99
GO(In)		100
GO(Af)	TT T	100
GO(Th)	CCCTATTGGG GAAAAGATT TATTGGAGAG AGATGAGCCT GCCTGGATT AGCTAGTTGG TAGGGTAAGA GCCTACCAAG GCTACGATCT ATAGCTGGTC	199
GO(In)		200
GO(Af)	AG	200
GO(Th)	TGAGAGGACG ATCAGCCACA CTGGGACTGA GACACGGCCC AGACTCCTAC GGGAGGCAGC AGTGGGAAT ATTGACAAT GGGGGCAACC CTGATCCAGC	299
GO(In)		300
GO(Af)	CA	300
GO(Th)	CATGCCCGGT GAGTGAAGAA GGCCTTAGGG TTGTAAAGCT CTTTCGCCGG AGAAGATAAT GACGGTATTC GGAGAAGAAG CCCCGCTAA CTCCTGCCA	399
GO(In)		400
GO(Af)	NNN	400
GO(Th)	GCAGCCGGG TAATACGAAG GGGCGGAGG TTGTTGGAA TAACTGGCCG TAAAGGGCCG GTAGCCGGCG ATTAAGTTAG AGGTGAAATC CCAGGCTCAA	499
GO(In)		500
GO(Af)		500
GO(Th)	CCTTGGAACT GCCTTAATA CTGGTTGTCT AGAGCTTAG GAGAGGTGAG TGGAAATCCG AGTGTAGAGG TGAAATTCGT AGATATTCGG AGGAACACCG	599
GO(In)		600
GO(Af)	A C	599
GO(Th)	GTGGCGAAGG CCGCTCACTG GCCTGATACT GACGCTGAGG CGCGAAAGCG TGGGGAGCAA ACAGGATTAG ATACCCCTGGT AGTCCACGCC GTAACCATG	699
GO(In)		700
GO(Af)	T	699
GO(Th)	AGTGCTAGCT GTTGGTGGT TTACCATTCA GTGGCCGACG TAACGCATTA AGCACTCCGC CTGGGGAGTA CGGTCCGAAG ATTAATAACT AAAGGAATTG	799
GO(In)	N N	800
GO(Af)		799
GO(Th)	ACGGGGGCC GCACAAGCGG TGGAGCATGT GGTTAATTC GATGCAACGC GCAGAACCTT ACCAGCCCTT GACATGTATA GGACGATATC AGAGATGGTA	900
GO(In)		900
GO(Af)	N A G T A	899
GO(Th)	TTTTCTTTTC GGAGACCTTT ACACAGGTGC TGCATGGCTG TCGTCAGCTC GTGTCTGAG ATGTGGGTT AAGTCCCGCA ACGAGCGCAA CCCCTGCCCTC	1000
GO(In)		1000
GO(Af)	T C T A	999
GO(Th)	TAGTTGCCAT CAAGTTTAGG TTTTACCTA GATGTTGGGN ACTTTATAGG GACTGCCGGT GATAAGCCGG AGGAAGGTGG GGATGACGTC AAGTCTCAT	1100
GO(In)		1100
GO(Af)	A - T T	998
	O12c primer site	
GO(Th)	GGCCCTTATG GCCTGGGCTA CACACGTGCT ACAATGGTGG TTACA <u>TGGG TTGCCAAGTC GCGAGGC</u>	1166
GO(In)	CG	1166
GO(Af)	CG	1164

Fig. 2 Sequence alignment of 16S rDNA fragments amplified from Nakorn Pathum (Thailand), Poona (India), and Nelspruit (Africa) isolates of GO. A blank indicates that the nucleotide is the same as that in the GO-NP sequence. Gaps, included to maximize the alignment, are indicated by dashes. The numbers refer to the positions of the nucleotides from 5' terminus of the sequences shown. GO(Th), Thai GO isolate Nakorn Pathum; GO(In), Indian GO isolate Poona; GO(Af), African GO isolate Nelspruit.

the Indian isolate of *Liberobacter* sp. phylogenetically.

Improvement of detection method of 16S rDNA of GO. We tried to improve the DNA extraction procedure to simplify the detection. We used GO-NP-infected citrus plants as templates of PCR. We used 1 μ l of several fractions obtained from experiment A, B, and C for 10 μ l of PCR solution. PCR and electrophoresis were performed as described above.

Experiment A. We determined whether the "homogenization" of leaf tissues was necessary. Fraction A-1 corresponded to the CTAB solution mixed with cut pieces (ca. 1 mm) of midrib of GO-NP-infected leaves (step CTAB, Fig. 3 A, lane 1), fraction A-2 corresponded to the solution obtained after treatment at 65°C for 5 min (step 65C, Fig. 3 A, lane 2), fraction A-3 to the supernatant after centrifugation (15,000 x g, 5 min) following extraction with chloroform/isoamyl alcohol (step CHL, Fig. 3 A, lane 3), and fraction A-4

corresponded to the DNA solution (10 μ l, TE) of the pellet after centrifugation (15,000 x g, 5min) following incubation (5min) with 0.7 vol. of isopropanol (step ISO, Fig. 3 A, lane 4). Only the fraction A-4 was positive after the PCR reaction. These results indicated that we can omit the most tedious step, homogenization of the leaf tissues.

Experiment B. We determined whether "cutting" of the leaf midrib was necessary. We tried to detect the DNA of the pathogen by using a solution in which a GO-NP-infected leaf was dipped as first fraction B-1 (Fig. 3 B, lane 1). The fractions B-2, B-3, and B-4 were obtained by steps similar to those in experiment A, step 65C, CHL, and ISO (Fig. 3 B, lanes 2, 3, and 4). No DNA was amplified from any fractions. These results indicated that we need to use the cut pieces of the leaf midrib for indexing materials.

Experiment C. We determined whether all the steps in experiment A were necessary. Each fraction after step CTAB corresponded to the

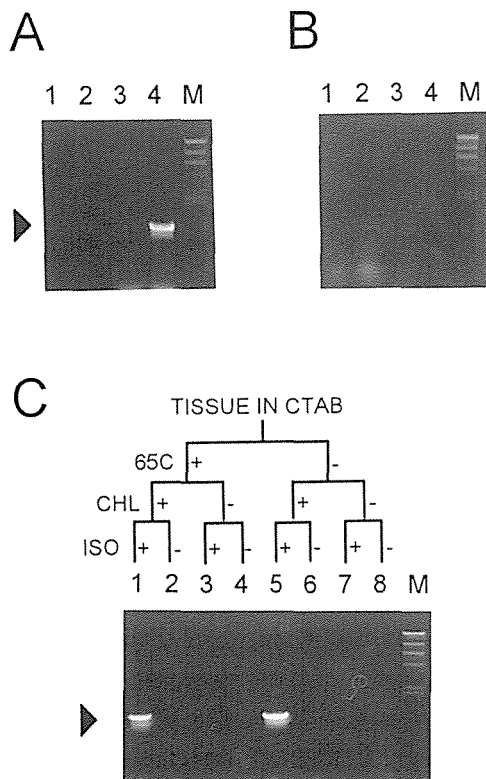


Fig. 3 Agarose gel electrophoresis of DNA fragments amplified from several fractions by the PCR by using primers O11 and O12c. Templates of PCR were obtained from the following fractions. (A) CTAB solution with ca. 1 mm pieces of midribs of GO-infected leaves (step CTAB, lane 1), the solution after treatment for 5 min at 65 C (step 65C, lane 2), supernatant after centrifugation (15,000 x g, 5 min) following extraction with chloroform/isoamyl alcohol (step CHL, lane 3), and DNA solution of the pellet after the centrifugation (15,000 x g, 5 min) following incubation (5 min) with 0.7 vol. of isopropanol (step ISO, lane 4). (B) CTAB solution in which a GO-infected leaf is dipped as the first fraction (lane 1). The next steps were the same as steps, 65C, CHL, and ISO (lanes 2, 3, and 4). (C) The reference was as follows : CTAB (first step), step 65C, +; step CHL, +; and step ISO, + (lane 1); step 65C, +; step CHL, +; and step ISO, - (lane 2); step 65C, +; step CHL, -; and step ISO, + (lane 3); step 65C, +; step CHL, -; and step ISO, - (lane 4); step 65C, -; step CHL, +; and step ISO, + (lane 5); step 65C, -; step CHL, +; and step ISO, - (lane 6); step 65C, -; step CHL, -; and step ISO, + (lane 7); step 65C, -; step CHL, -; and step ISO, - (lane 8). Lanes M contained lambda/HindIII digests. Arrowheads indicate the position of the amplified 16S rDNA fragments of GOs.

following reference (Fig. 3 C): fraction C-1 : step 65C, +; step CHL, +; and step ISO, + (Fig. 3 C, lane 1); fraction C-2 : step 65C, +; step CHL, +; and step ISO, - (Fig. 3 C, lane 2); fraction C-3 : step 65C, +; step CHL, -; and step ISO, + (Fig. 3 C, lane 3); fraction C-4 : step 65C, +; step CHL, -; and step ISO, - (Fig. 3 C, lane 4); fraction C-5 : step 65C, -; step CHL, +; and step ISO, + (Fig. 3 C, lane 5); fraction C-6 : step 65C, -; step CHL, +; and step ISO, - (Fig. 3 C, lane 6); fraction C-7 : step 65C, -; step CHL, -; and step ISO, + (Fig. 3 C, lane 7); and fraction C-8 : step 65C, -; step CHL, -; and step ISO, - (Fig. 3 C, lane 8). 16S rDNA fragments were amplified from the fractions C-1 and C-5. These results showed that step 65C was not necessary, unlike steps CHL and ISO.

The improved and simple method of detection of the DNA of the pathogen is illustrated in Fig. 4. Pieces of midrib of leaves were dipped in 200 μ l of CTAB solution and 200 μ l of chloroform-isoamyl alcohol (24:1), mixed completely, and centrifuged at 15,000 \times g for 3 min. A 100 μ l aliquot of the supernatant was decanted and saved in a new tube containing 70 μ l of isopropanol. After allowing the preparation to stand for 3 min, nucleic acids were precipitated by centrifugation for 3 min. The pellet was suspended in 100 μ l of TE buffer. A 1 μ l aliquot of the DNA solution should be used for 10 μ l of PCR solution. By applying the improved DNA extraction method, we were able to obtain DNA for PCR within 20 min and GO would be detected within 4.5 hr in total. Moreover we were able to detect the pathogen from the CTAB solution mixed with the diseased leaf tissues at room temperature (ca. 25°C) for one month (data not shown). This finding indicated that the diagnosis can be made later in the laboratory after cutting samples with disposable razors and preparing the CTAB solution in the field. We consider that the method of diagnosis using PCR is suitable for detecting the GOs rapidly, easily, and reliably. Although we must determine whether we can detect all the isolates of GOs with this method, epidemiological studies of the GOs may be facilitated, studies of the GOs may be promoted,

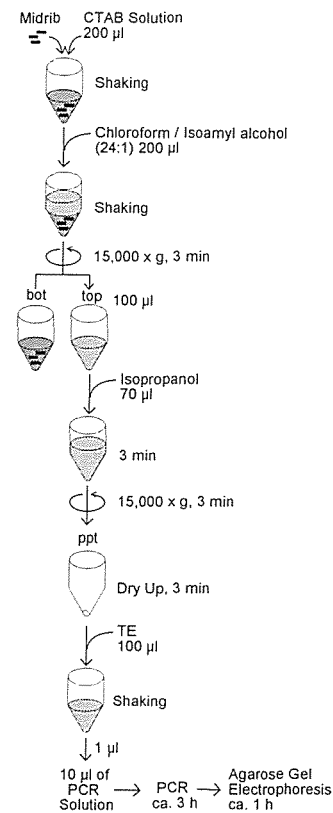


Fig. 4 Illustrated scheme of the improved DNA extraction and detection method of 16S rDNA of GOs.

and eventually destructive citrus greening disease may be controlled.

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カンキツグリーニング病の病原バクテリア様微生物（タイ株）の 16S rDNAの検出

中島一雄^{a)}、マイトレー プロミンターラ^{b)}、大津善弘^{c)}
加納 健^{c)}、今田 準^{d)}、小泉銘册^{e)}

a) 国際農林水産業研究センター生物資源部（〒305 茨城県つくば市大わし1-2）

b) タイ農業局植物病理・微生物部（タイ王国バンコク市チャトチャック区10900）

c) 果樹試験場保護部（〒305 茨城県つくば市藤本2-1）

d) 果樹試験場安芸津支場（〒729-24 広島県豊田郡安芸津町三津338-1）

e) 果樹試験場興津支場（〒424-02 静岡県清水市興津中町）

摘 要

バクテリア様微生物（BLO; グリーニング病病原体: GO）により引き起こされるカンキツグリーニング病は、熱帯地域のカンキツ類において、最も被害が大きい病気である。本病を防除するためには、感染樹の早期検定・除去が必要であり、迅速で信頼性の高い病原体検出法の開発が必要とされていた。最近、Jagoueixら（1994; *Int. J. Syst. Bacteriol.* 44: 379-386）によって、PCR法によりGOの16S rDNAを増幅する方法が開発された。我々は、

このPCR法の適用により、GOのタイ株であるNakorn Pathum株、Rangsit株、Nan株の16S rDNA断片を検出することに成功した。Nakorn Pathum株の16S rDNA断片の塩基配列を解析した結果、本株はliberobacter インド株と、系統進化的に極めて近いことが明らかになった。PCR用のDNAの抽出法を簡易化することにより、4.5時間以内にGOを検出することが可能になった。

キーワード：カンキツグリーニング病, liberobacter, 16S rDNA, PCR, 迅速診断