INSECT-TRANSMITTED PROCARYOTES

Improved Sensitivity in the Detection and Differentiation of Citrus Huanglongbing Bacteria from South Africa and the Philippines

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ABSTRACT. Desiccated leaves infected with the citrus huanglongbing bacterium (HLB) were obtained from South Africa, the Philippines and the Collection of Exotic Citrus Pathogens, Beltsville, MD. Total DNA was isolated and a ca.1,160 bp amplicon of the HLB 16S ribosomal DNA was amplified using published (4) HLB-specific primers. Yields of the amplicon varied widely and often were not visible on gels. Higher yields were obtained using the KlenTaq (Sigma) mixture of DNA polymerases. Two new PCR primers (CN265/CN266) were designed to yield a 448 bp amplicon near the 3' end of the 1,160 bp amplicon. These primers enhanced the detection of the HLB DNA both by "nested" PCR from low yield samples and directly from desiccated tissue. They also enabled discrimination between the African and Asian species of HLB by encompassing the *Xba*I restriction site located at base 1,029 of the 1,160 bp amplicon, which is characteristic of the African species (5). The 1,160 bp amplicon was sequenced, and consistent characteristic differences were observed between the samples from South Africa and the Philippines, including the presence of one and two restriction sites for *Xba*I in the Asian and African species of HLB, respectively. Only the African species was detected in samples from South Africa, and only the Asian species was detected in the samples from the Philippines.

Huanglongbing (HLB), commonly called greening, is an important disease of citrus which limits production in much of Asia and Africa and part of the Arabian Peninsula (1, 3). In South Africa, HLB was first detected in 1928 in the Western Transvaal, where it was known as yellow branch. It caused crop losses of 30-100%, and citrus production was eliminated in three major production areas (1). Control measures in South Africa and many countries are based on reducing the inoculum by control of the psyllid vectors, removal of infected branches and the use of certified disease-free nursery stock. The non-specific nature of the foliar symptoms make it easy to confuse HLB with nutrient deficiencies, root diseases and other stress related factors. Therefore, it is essential that a reliable assay procedure be available to distinguish between the latter and HLB. Several indirect approaches with symptomatic tissue were used to prepare monoclonal antibodies and DNA for producing DNA probes (2, 3, 5). The use of monoclonal antibodies for field diagnosis has proven unsatisfactory, with results being inconsistent (2). The use of specific DNA probes has proven to be more reliable and as sensitive as electron microscopy (5), but it has the drawback of being very time consuming. The recent discovery of HLB in the Western Cape Region of South Africa, an area where this disease was previously absent, as well as its increasing spread in the Philippines prompted our need for a diagnostic procedure that could be used to identify diseased trees and eliminate them before the disease became endemic in new areas.

Recently, two related species of bacteria, "*Candidatus* Liberibacter asiaticus" and "*Candidatus* L. africanus" were identified as the causal agents of the citrus greening disease

in Asia and Africa, respectively (5). The species were reliably differentiated by molecular methods, which included amplification of a ca. 1,160 bp amplicon of the 16S ribosomal DNA by polymerase chain reaction (PCR) and subsequent digestion by the restriction enzyme *Xba*I. The amplicon from "*Candidatus* L. asiaticus" was hydrolyzed into two fragments of ca. 640 and 520 bp, whereas that of "*Candidatus* L. africanus" was hydrolyzed into three fragments of ca. 520, 506 and 130 bp. The exact sizes of the amplicons and fragments vary by a few bases, depending on which sequence is used for comparison (4).

We tested the published procedure and a modified procedure on field samples collected from South Africa and the Philippines to increase the sensitivity of the procedure and to gain additional information on the causal agents of HLB in these two countries.

MATERIALS AND METHODS

Sample preparation and PCR amplification. Desiccated leaves infected with HLB were obtained from the Nelspruit region of South Africa, the Baguio City region of the Philippines and the Collection of Exotic Citrus Pathogens (CECP) in Beltsville, MD. Petioles and midribs were excised with a razor blade and extracts were prepared by protocol 2 using the Wizard (Promega) DNA purification column as previously described (5). PCR amplification was performed in 50 µl reaction mixtures essentially as described previously (5), except that the leaf samples had been desiccated, and two different heat stable DNA polymerases [*Taq* polymerase (various sources) and KlenTaq LA polymerase (Sigma)] were tested. The 1,160 bp amplicon was amplified using a mixture of the OA1 and OI1 5' end (forward) primers (5'GCGCGTATTTTATACGAGCGGCA3' and 5'GCGCGTATCCAATACGAGCGGCA3' for the African and Asian species, respectively) and the 3' end (reverse) primer OI2c (5'GCCTCGCGACTTCGCAACCCAT 3') from Jagouiex et al (4). Two additional primers were tested, CN265 (5' TGGGTGGTTTACCATTCAGT3' and CN266 (5'CGCGACTTCGCAACCCAT3'), which amplify the 448 bp near the 3' end of the 1,160 bp amplicon and encompass the single *Xba*I restriction site characteristic of the African HLB species at base 1,029. The 1,160 bp amplicons were cloned in the pGEM-T vector (Promega) and sequenced in the DNA Sequencing Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida. Sequence comparisons were made with published sequences for a South African (Nelspruit) isolate and an Indian isolate (Poona) (5) (GenBank accessions L22533 and L22532, respectively) and an unpublished sequence of a Japanese (Okinawa) isolate (GenBank accession AB008366).

Restriction enzyme analysis. Amplified DNA was digested with the *Xba*I restriction enzyme (Biolabs or Promega) in a final volume of 20 µl at 37°C overnight according to the manufacturer's instructions. Digested DNA was analysed by electrophoresis on 1.0% and 1.5% agarose or 8% polyacrylamide gels.

RESULTS AND DISCUSSION

The 1,160 bp amplicon was amplified to visible quantities from four of 12 samples from the CECP in Beltsville using a mixture of the OA1, OI1 and OI2c primers and the KlenTaq DNA polymerase, but the yields varied considerably (Fig. 1, lanes 1, 8, 12, and 13). With the *Taq* polymerase alone, visible amplification was obtained with only one of the same12 samples (Fig. 2, lane 1). Thus KlenTaq, a mixture of two DNA polymerases and with proof reading ability, was more effective than ordinary *Taq* polymerase in

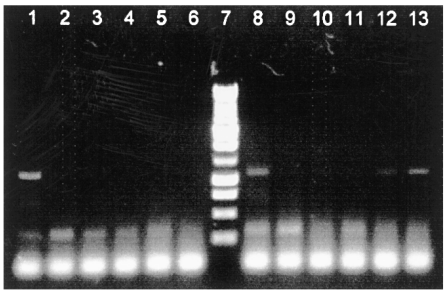

Fig. 1. Electrophoresis on 1.0% agarose gel of DNA amplified using the KlenTaq DNA polymerase and the OA1/OI1/I2c primer combination and samples from Reunion (lane 1), Taiwan (lane 2), India (lane 3), Thailand (lane 4), Taiwan (lane 5), China (lane 6), 1 kb ladder (Promega) (lane 7), India (lane 8), China (lane 9), and Reunion (lanes 10-13).

amplifying the HLB amplicon. The research which developed the PCR method for HLB detection was performed using fresh tissue (4, 5). Our method utilized desiccated tissue, which may, in part, explain our low and erratic yields. In samples with less than visible yields of the 1,160 bp replicon, we were uncertain as to whether any amplification had occurred. Therefore, we designed primers CN265 and CN266 for use in "nested" PCR to re-amplify the 448 bp portion at the 3' end (Fig. 5) of those 1,160 bp replicons which had been replicated to less than visible levels. Using 1.0 µl of the PCR products from Fig. 1 as template, we were then successful in obtaining

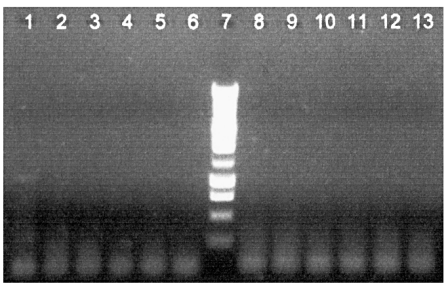

Fig. 2. Electrophoresis on 1.0% agarose gel of DNA amplified using *Taq* DNA polymerase, the OA1/OI1/I2c primer combination and samples as in Fig. 1.

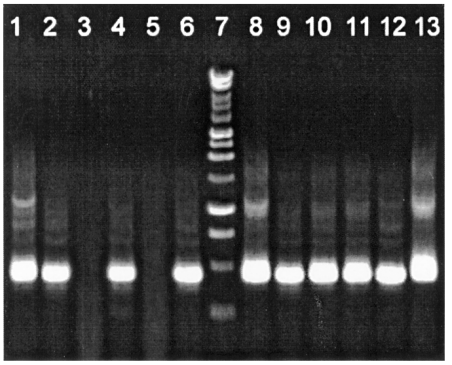

Fig. 3. Electrophoresis on 1.5% agarose gel of DNA re-amplified from 1 µl of the respective PCR products in Fig. 1 using KlenTaq DNA polymerase and the CN265/CN266 primer combination. The sample numbers and sources are as in Fig. 1.

amplification of the 448 bp amplicon from 10 of the 12 samples (Fig. 3), indicating that a low level of amplification of the 1,160 bp had occurred from the majority of the samples. We then tested the ability of primers CN265 and CN266 to amplify the 448 bp amplicon directly from tissue extracts and were successful with all 10 samples tested (Fig. 4). Digestion of these amplicons with *Xba*I yielded hydrolysis products of 312 and 136 bp from those samples from South African (Fig. 4, lanes 2 to 5), whereas the 448 bp amplicon was undigested for those samples from

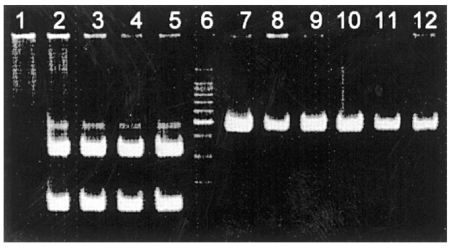

Fig. 4. Electrophoresis on 8% polyacrylamide gel of DNA amplified using KlenTaq DNA polymerase and the CN265/CN266 primer combination and then digested with *Xba*I. The sample sources were: uninoculated sweet orange (lane 1), South Africa (lanes 2-5), 1 kb ladder (Promega) (lane 6), Philippines (lanes 7-11) and China (lane 12).

the Philippines (Fig. 4, lanes 7-11) and one sample from China (Fig. 4, lane 12). Thus primers CN265 and CN266 are useful both to enhance the sensitivity of detection of the HLB and also to discriminate between the two species.

The 1,160 bp amplicon was cloned and sequenced from four samples from South Africa and three samples from the Philippines. The complete sequences were aligned and compared to the published and GenBank accession sequences cited

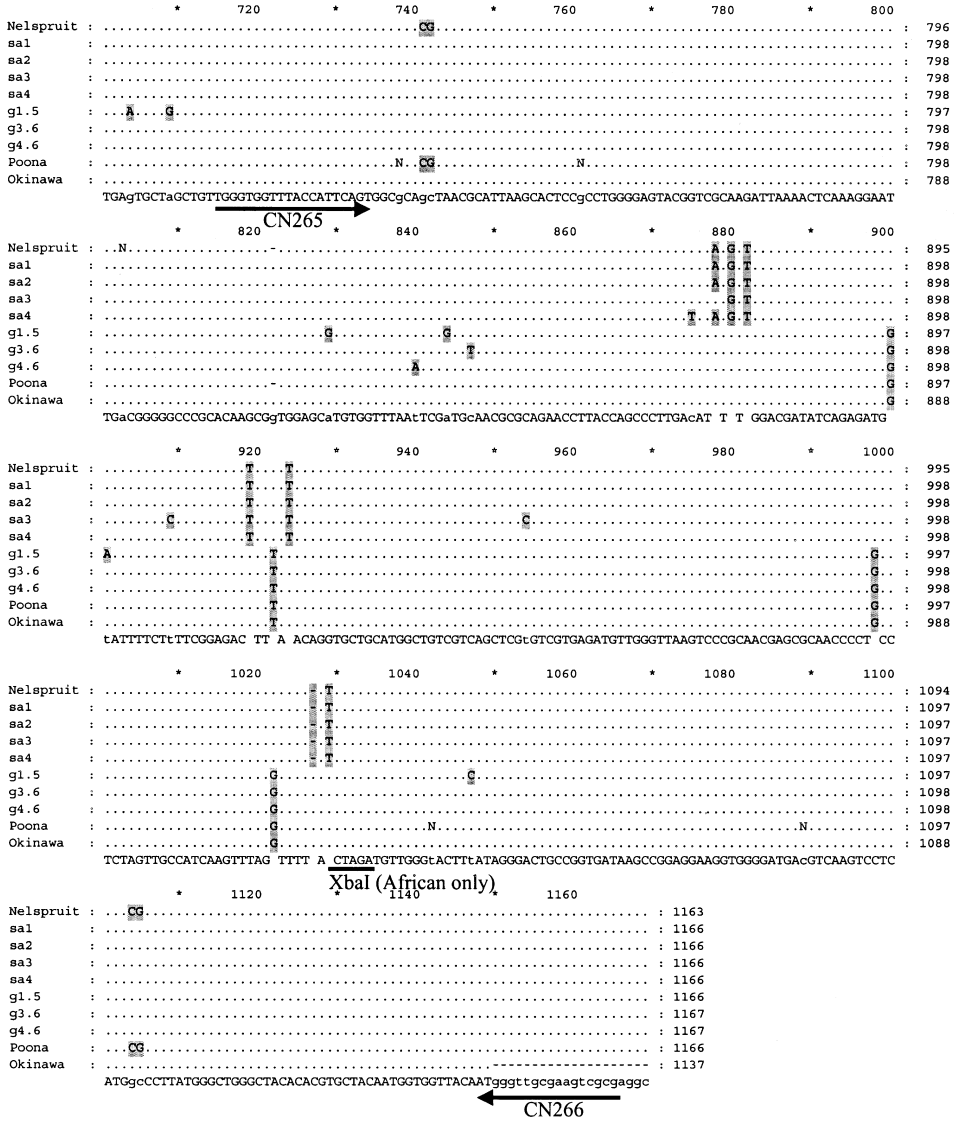

Fig. 5. Sequence alignment of a portion of the bacterial 16S ribosomal DNA from citrus Huanglongbing infected samples from the Nelspruit area of South Africa (sa1-sa4) and the Baguio City region of the Philippines (g1.5-g4.6) with published sequences from isolates from South Africa (Nelspruit), India (Poona) and Japan (Okinawa) (4, 5). Dots and the capital letters at the bottom indicate the identical nucleotides for all sequences; small letters and gaps indicate one or more nucleotide differences, and dashes indicate deletions. The locations of primers CN265 and CN266 and the *Xba*I site for the African species are indicated beneath their sequences.

above. Our sequences are being submitted to GenBank, and for economy of space, only 3' partial sequences are shown in Fig. 5. With only minor variations, the sequences from the South African samples were essentially as reported for the Nelspruit isolate from South Africa (4), and those from the Philippines were essentially as reported for the Poona isolate from India (5) and the Okinawa isolate from Japan. Of particular importance was the presence of the characteristic two *Xba*I restriction sites in the amplicons from all four samples from South Africa, and the presence of the single site in all three amplicons from the Philippine samples. The second *Xba*I site (TCTAGA) common to the African species is shown at base 1029 in Fig. 5, and it occurs only in the sequences from the South African samples. Other characteristic differences between the sequences of the two HLB species in this region of the ribosomal DNA can be seen around bases 880, 920 and 1020.

We were unable to find references on the molecular characteristics of the HLB from the Philippines except for (4). These investigators demonstrated the amplification of the 1,160 bp replicon with the primer combination of OA1, OI1 and OI2c and its hydrolysis by *Xba*I into fragments of 640 and 520 bp, indicating that their sample from the Philippines contained the Asian species of the HLB. We confirmed these results by sequencing three different replicons from samples from the Baguio City region of the Philippines and demonstrated the single *Xba*I site at base 520 and its absence at base 1,026 of the 1,160 bp replicon.

Our results are consistent with those reported previously on the molecular characteristics of the 16S ribosomal DNA regions of "*Candidatus* L. asiaticus" and "*Candidatus* L. africanus" and their usefulness in differentiating the two species of bacteria. In addition, we have developed more sensitive procedures to amplify the DNA from desiccated or low yield samples and have confirmed the presence of "*Candidatus* L. asiaticus" as the causal agent of citrus greening disease in the Philippines by its DNA sequence.

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