Both Huanglongbing (Greening) Liberobacter Species Are Present in Mauritius and Reunion

M. Garnier, S. Jagoueix, P. Toorawa, M. Grisoni, R. Mallessard, A. Dookun, S. Saumtally, J. C. Autrey, and J. M. Bové

ABSTRACT. Thirteen leaf samples were collected on citrus trees showing Huanglongbing (HLB) (Greening) or HLB-like symptoms in Reunion Island in December 1993 and analyzed by DNA-DNA hybridization with probes In 2.6 and AS 1.7 specific for Liberobacter asiaticum and L. africanum, respectively. Nine samples reacted with AS 1.7 only, 1 with In 2.6 only, 2 with both AS 1.7 and In 2.6; 1 was negative with both probes. Thirty-eight samples were also collected in Mauritius on trees showing HLB or HLB-like symptoms in December 1993 and analyzed as above. Sixteen were found infected with L. africanum, six with L. asiaticum, and two with both liberobacter species. Fourteen samples which came from symptomless trees or trees showing zinc deficiency symptoms without mottle, did not react with the probes. In 1995, 37 additional samples were collected in Mauritius and tested by DNA-DNA hybridization and PCR. Four samples came from healthy trees and gave negative results with both techniques. Three samples consisted of symptomless leaves collected on HLB affected shoots were negative by hybridization, while one was positive by PCR. Twenty-one of the 30 samples with mottled or yellow leaves gave a positive hybridization signal with probe In 2.6 specific for L. asiaticum, and one sample reacted with probe AS 1.7 specific for L. africanum. Twenty-eight of the 30 samples gave positive PCRs, and the *Xba*I profiles showed 27 corresponded to L. asiaticum and one to L. africanum. These data show that both L. africanum and L. asiaticum occur in the two neighboring islands either in the same or in different trees.

We have shown recently that the Huanglongbing (HLB) (Greening) bacterium exists as two species; Liberobacter africanum in Africa and Liberobacter asiaticum in Asia (2). Reunion (France) and Mauritius, as well as the border region between Saudi Arabia and Yemen, are the only areas in the world where the two insect vectors of HLB, the Asian psyllid, *Diaphorina citri* Kuwayama, and the African psyllid, *Trioza erytreae* (Del Guercio), occur together. In nature, *D. citri* is known to transmit the heat-tolerant L. asiaticum (1) and *T. erytreae* the heat-sensitive L. africanum (6). However, experimentally, each psyllid can transmit either one of the two liberobacter species (4, 5). Until recently, there were no techniques to detect and distinguish the two species and, therefore, it was not known if both species occurred in Reunion and Mauritus, even though the two psyllid vectors were known to be present. This paper shows for the first time that the two liberobacter species are present in both Reunion and Mauritius. This result is based on two recently developed techniques: (i) DNA/DNA hybridization with probes In 2.6, specific for L. asiaticum and probe AS 1.7, specific for L. africanum (7, 8); and (ii) PCR followed by *Xba*I digestion, the two liberobacters having differential restriction profiles (3).

Hybridization results are summarized in Table 1. They show that 33 samples reacted only with probe In 2.6 and 20 samples only with probe AS 1.7, indicating that the two liberobacter species are present in Mauritius and Reunion islands. In four samples, the two liberobacter species were present simultaneously, in similar amounts, as strong reactions (+) were obtained with the two probes. However, in nine samples, a strong hybridization signal was obtained with one probe, but only a faint signal (±) with the other probe. In these cases, it is difficult to draw clear-cut conclusions because probe In 2.6 gives faint hybridization signals when large amounts of L. africanum DNA occur, and probe AS 1.7 gives similar cross-hybridization with large amounts of L. asiaticum DNA.

TABLE 1
SPECIES OF LIBEROBACTER PRESENT IN LEAF SAMPLES TAKEN FROM HUANGLONGBING-AFFECTED TREES IN MAURITIUS AND REUNION. DETECTION BASED ON HYBRIDIZATION WITH LIBEROBACTER-SPECIFIC PROBES

Hybridization		No. of samples			Liberobacter present	
		Reunion[z]	Mauritius[y]			
In 2.6	AS 1.7	Dec. 1993	Dec. 1993	Apr. 1995	L. asiaticum	L. africanum
+	-	1[x]	8[t]	24[o]	+	-
+	±	0	3[s]	0	+	?
-	+	7[w]	12[r]	1[n]	-	+
±	+	2[v]	4[q]	0	?	+
+	+	2[u]	2[p]	0	+	+

[z]Samples were collected in December 1993
[y]Samples were collected in December 1993 and April 1995.
[x]Sample 46
[w]Samples 35, 38, 39, 40, 41, 42, 44
[v]Samples 36, 37
[u]Samples 43. 45
[t]Samples 72 to 75, 78,80, 82, 84
[s]Samples 62, 63, 65
[r]Samples 49 to 55, 60, 61, 64, 67, 70
[q]Samples 57, 68,71, 83
[p]Samples 58, 59
[o]Samples 1 to 4, 6, 7, 9 to 22, 26, 27, 35, 36
[n]Sample 23

In these doubtful cases, PCR was found useful. This is illustrated in Fig. 1 for samples 27, 35, 36, 54, 68, 70, and 71 and summarized in Table 2 where the PCR results have been compared with those of hybridization. Samples reacting with probe In 2.6 only (Fig. 1, lanes 1, 2, 3 and Table 2) gave, upon digestion of the PCR amplified DNA, the characteristic profile of L. asiaticum. Similarly, samples reacting with probe AS 1.7 (Fig. 1, lane 4 and Table 2) gave, upon digestion of the PCR amplified DNA, the characteristic profile of L. africanum (3), except sample 70 (lane 6) which showed, upon PCR, the mixed profiles of both L. asiaticum and L. africanum. In addition, the mixed PCR profiles were also obtained with two samples hybridizing strongly with one probe and only weakly with the other (Fig. 1, lanes 5, 7 and Table 2, samples 68 and 71).

TABLE 2
HUANGLONGBING IN MAURITIUS (1993, 1995). ANALYSIS OF HYBRIDIZATION-POSITIVE SAMPLES BY LIBEROBACTER-SPECIFIC PCR

Hybridization results		Liberobacter profile by PCR		
In 2.6	AS 1.7	L. asiaticum	L. africanum	No. samples
+	-	+	-	24[z]
-	+	-	+	2[y]
-	+	+	+	1[x]
±	+	+	+	2[w]

[z]Sample Mauritius 1995: 1 to 4, 6, 7, 9 to 22, 26, 27, 35, 36
[y]Sample Mauritius 1993: 54; 1995: 23
[x]Sample Mauritius 1993: 70
[w]Sample Mauritius 1993: 68, 71

The use of DNA/DNA hybridization and PCR has allowed us to demonstrate for the first time that the two liberobacter species, L. asiaticum and L. africanum, are present in both Reunion and Mauritius, two islands in which the two psyllid vectors occur concomitantly. The results of hybridization and PCR are usually congruous, but PCR is easier to use. It is also slightly more sensitive than hybridization and it is able, after *Xba* I digestion of the amplified DNA, to detect the two liberobacter species in samples where hybridization detects only the most predominant one or gives a strong signal with one probe and a faint one with the other probe. Even though the two liberobacter species are present in Reunion and Mauritius, only a few trees have been shown to be infected simultaneously with the two species. Whether the two psyllids can also be infected simultaneously by the two liberobacter species is under investigation.

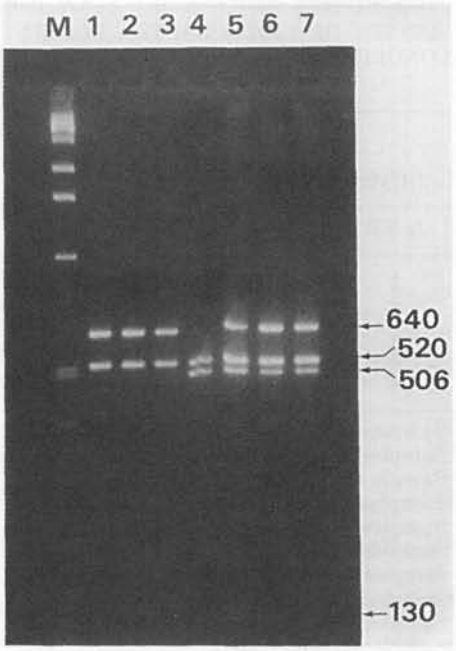

Fig. 1. *Xba*1 digestions of the PCR amplified DNA from citrus leaf midribs of samples collected in Mauritius. Lane 1: sample 27, lane 2: sample 35; lane 3: sample 36; lane 4: sample 54; lane 5: sample 68; lane 6: sample 70; lane 7: sample 71; M: 1 Kb ladder.

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LITERATURE CITED

1. Capoor, S. P., D. G. Rao, and S. M. Viswanath
   1967. *Diaphorina citri* Kuway., a vector of greening disease of citrus in India. India J. Agr. Sci. 37: 572-576.
2. Garnier, M. and J. M. Bové
   1996. Distribution of the Huanglongbing (greening) liberobacter species in fifteen African and Asian countries, p. 388-391. *In*: Proc. 13th Conf. IOCV. IOCV, Riverside.
3. Jagoueix, S., J. M. Bové, and M. Garnier
   1996. PCR detection of the two liberobacter species associated with greening disease of citrus. Mol. Cell. Probes 10: 43-50.
4. Lallemand, J., A. Fos, and J. M. Bové
   1986. Transmission de la bactérie associée à la forme africaine de la maladie du "greening" par le psylle asiatique *Diaphorina citri* Kuwayama. Fruits 41: 341-343.
5. Massonié, G., M. Garnier, and J. M. Bové
   1976. Transmission of Indian citrus decline by *Trioza erytreae* (Del G.), the vector of South African greening, p. 18-20. *In*: Proc. 7th Conf. IOCV., IOCV, Riverside.
6. McClean, A. P. D. and P. C. J. Oberholzer
   1965. Citrus psylla, a vector of the greening disease of sweet orange. S. Afr. J. Agr. Sci. 8: 297-298
7. Planet, P., S. Jagoueix, J. M. Bové, and M. Garnier
   1995. Detection and characterization of the African citrus greening liberobacter by amplification, cloning and sequencing of the *rpl*KAJL-*rpo*BC operon. Curr. Microbiol. 30: 137-141.
8. Villechanoux, S., M. Garnier, J. Renaudin, and J. M. Bové
   1992. Detection of several strains of the bacterium-like organism of citrus greening disease by DNA probes. Curr. Microbiol. 24: 89-95.