Distribution of the Huanglongbing (Greening) Liberobacter Species in Fifteen African and Asian Countries

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ABSTRACT. The distribution of the two Huanglongbing (greening) liberobacter species, Liberobacter asiaticum and Liberobacter africanum, was studied by DNA-DNA hybridization with probe In 2.6 which is specific for L. asiaticum and AS 1.7 which is specific for L. africanum, as well as by PCR followed by *XbaI* restriction of the amplified DNA. Only L. asiaticum was found in isolates from the 11 Asian countries studied (i.e., India, Nepal, Sri Lanka, Vietnam, Cambodia, Malaysia, Indonesia, Thailand, the Philippines, Taiwan and China). Only L. africanum was found in samples from South Africa and Zimbabwe. Both L. asiaticum and L. africanum were present in Reunion and Mauritius, sometimes in separate trees or even co-existing in the same trees. Citrus leaves from New Caledonia and Western Samoa, showing zinc deficiency symptoms or yellows, but not blotchy mottle, were found to be free of liberobacters.

The microorganism responsible for citrus Huanglongbing (HLB) (Greening disease) was first observed by electron microscopy in 1970 in the phloem sieve tubes of infected Eloff sweet orange trees from Nelspruit, South Africa (12). The organism was soon found to be a walled bacterium rather than a wall-less mycoplasma (2, 17). It has a filamentous morphology but shows polymorphism with great variations in the length and diameter of the filaments as well as round forms of the bacterium in the sieve tubes (5). The HLB bacterium, because of its typical gram-negative bacterial cell wall, is easily distinguished from other phloem-restricted prokaryotes, such as phytoplasmas and spiroplasmas, which lack a cell wall and are only surrounded by a cytoplasmic membrane (2, 6, 17). As of today, the HLB bacterium has resisted *in vitro* cultivation, but has been taxonomically characterized and shown to belong to the alpha subdivision of the proteobacteria (9). Two "*Candidatus*" species have been recognized: "*Candidatus* Liberobacter asiaticum" and "*Candidatus* Liberobacter africanum" (9).

Electron microscopy was the only technique for the detection of the HLB liberobacters until 1992. However, with this technique, L. asiaticum and L. africanum could not be distinguished as they are morphologically identical.

In 1992, a DNA probe (In 2.6) was obtained for the detection of L. asiaticum (18), and in 1995, a similar probe (AS 1.7) was produced for the detection of L. africanum (16). More recently, PCR has been developed for the detection and identification of the two species (8, 10). This paper presents the results of a study of the liberobacter species present in 15 countries in Asia and Africa.

HLB liberobacters have been observed by electron microscopy in the phloem sieve tubes of leaf midribs or fruit axes of infected citrus trees collected in 13 Asian and eight African countries, as well as in Saudi Arabia and Yemen (3) and in Mauritius and Reunion Islands in the Indian Ocean (Tables 1 and 2). These numerous observations have shown that the liberobacters are restricted to the phloem sap in which they are introduced by their psyllid vectors; they are never observed within phloem parenchyma cells nor in the cytoplasm of phloem tissue.

HLB-infected leaf samples from trees sampled in 18 different countries were analyzed by DNA/DNA

TABLE 1
OCCURRENCE OF LIBEROBACTER ASIATICUM AND/OR LIBEROBACTER AFRICANUM IN VARIOUS COUNTRIES

Country	Presence of[a]		EM	No. of samples tested
	L. asiaticum	L. africanum		
India	+	-	+	150
Nepal	+	-	+	52
Sri Lanka	+	-	+	11
Vietnam	+	-	+	55
Cambodia	+	-	ND	1
Malaysia	+	-	+	20
Indonesia	+	-	+	1
Thailand	+	-	+	11
Philippines	+	-	+	3
Taiwan	+	-	+	1
China	+	-	+	3
South Africa	-	+	+	75
Zimbabwe	-	+	+	8
Reunion	+	+	+	15
Mauritius	+	+	+	50
New Caledonia	-	-	ND	20
Western Samoa	-	-	ND	15
Martinique	-	-	ND	1

[a]Determined by hybridization and/or PCR

hybridization with probes In 2.6 and AS 1.7 and/or by PCR to determine the Liberobacter species present (8, 10). Results are indicated in Table 1. In Asia, where more than 300 samples from 11 countries* were analyzed, only L. asiaticum was detected, and in Africa only L. africanum was present on the basis of 83 samples from South Africa and Zimbabwe. In Reunion and Mauritius, both liberobacter species were detected by DNA/DNA hybridization and PCR. The two species were generally present in different trees, but some trees were infected with both (7). Finally, the HLB liberobacter could not be detected in samples

TABLE 2
OCCURRENCE OF LIBEROBACTER IN ADDITIONAL COUNTRIES AS DETERMINED BY ELECTRON MICROSCOPY

COUNTRY	REFERENCE
Japan	Miyakawa and Tsuno (15)
Bangladesh	Catling et al. (4)
Pakistan	Garnier and Bové, unpublished
Saudi Arabia	Bové and Garnier (3)
Yemen	Bové and Garnier (3)
Burundi	Aubert et al. (1)
Cameroun	Aubert et al. (1)
Kenya	Garnier and Bové, unpublished
Malawi	Aubert et al. (1)
Rwanda	Aubert et al. (1)
Somalia	Garnier and Bové, unpublished

from New Caledonia, Western Samoa and Martinique, where the presence of the HLB liberobacter was questioned on the basis of some branch yellowing symptoms, even though the two psyllid vectors of HLB are known to be absent.

In conclusion, PCR or DNA/DNA hybridization techniques can be used to detect the two HLB liberobacter species. In Asia, only L. asiaticum, vectored by the psyllid *Diaphorina citri* Kuwayama, was present. In Africa, samples from the two countries examined (South Africa and Zimbabwe), were found to contain only L. africanum, vectored by the African psyllid, *Trioza erytreae* (Del Guercio). In Reunion and Mauritius, where both psyllids are present, both liberobacter species have been found, in separate as well as a few dually infected trees. Whether the two psyllids are able in nature, to transmit both liberobacter species, as was demonstrated experimentally (13, 14), remains to be determined.

Citrus is known to have originated from Asia and it is generally thought that most of their pathogens also have an Asian origin. The results described above suggest that L. africanum was not introduced from Asia into Africa with citrus. Thus, Liberobacter species are either insect-borne pathogens, or were acquired by the respective psyllids from indigenous rutaceous plants. Indeed, in Africa we have shown that *Toddalia lanceolata,* an indigenous rutaceous species, is a good host of both *T. erytreae* and L. africanum (11).

*Note: HLB-affected citrus trees have now also been found by us in Burma and Laos, and the agent is L. asiaticum.

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