Heat-tolerant Asian HLB meets heat-sensitive African HLB in the Arabian Peninsula! Why?

Josy Bové

This presentation is dedicated to the memory of Monique Garnier (1949-2003) who detected, by transmission electron microscopy, the HLB bacterium in the many countries reported in this presentation.

Abstract. It will be recalled how the notions of "heat-sensitive/heat-tolerant HLB" and "African HLB/Asian HLB" were developed. These notions benefited from the possibility to confirm, for the first time, the non-specific HLB symptoms by reliable laboratory techniques: detection of the HLB-associated bacterium by transmission electron microscopy from 1970 onwards to DNA-hybridization and PCR of the liberibacters in the 1990s. With these tools, the early history of citrus HLB in the many countries surveyed could be more precisely described. This presentation also shows or proposes why: (i) African HLB is heat-sensitive and Asian HLB, heat-tolerant; (ii) only one of the seven known liberibacters, namely *Candidatus* Liberibacter asiaticus (Las), is heat-tolerant, *Candidatus* Liberibacter africanus (Laf) being a heat-sensitive liberibacter; (iii) African HLB is native to Africa, and Asian HLB is native to Asia; (iv) "Continental Drift" supports astonishingly well the presence of Laf in Africa and that of Las in Asia; presence of Las in the Americas is the result of incursions; (v) HLB is not native to the Arabian Peninsula, but is the result of African and Asian HLB incursions into the peninsula; vi) recent presence of Las in Ethiopia is also the result of an incursion; vii) *Candidatus* Liberibacter americanus (Lam) in South America is the result of an incursion too; viii) while Laf and Las are of Gondwanan origin, *Candidatus* Liberibacter europaeus (Leu), Lam and *Candidatus* Liberibacter solanacearum (Lso) are of Laurasian origin.

On names!

Huanglongbing has been known under different names in different countries: greening in South Africa, citrus decline in India, likubin in Taiwan, leaf mottling in the Philippines, vein phloem degeneration in Indonesia, etc. In the Chaozhou district of southern China, the farmers were speaking their southern Chinese dialect and named the disease "huang long bing," "bing" standing for disease, "long" for shoot and "huang" for yellow, hence: "huang long bing" = yellow shoot disease. "Huang long" or yellow shoot was an early, characteristic symptom of the disease and referred to the yellow color of the new flush of growth on infected trees. Kung Hsiang Lin, from the South China Agricultural University in Guangzhou, Guangdong province, was the first, in the early 1950s, to show that huanglongbing was transmissible by graft inoculation, demonstrating in this way the infectious nature of the disease (Lin, 1956). Lin's work on huanglongbing was published in 1956, while similar work on graft transmission of South African greening (McClean and Oberholzer, 1965a) or Indonesian vein phloem degeneration (Tirtawidjaja et al.,

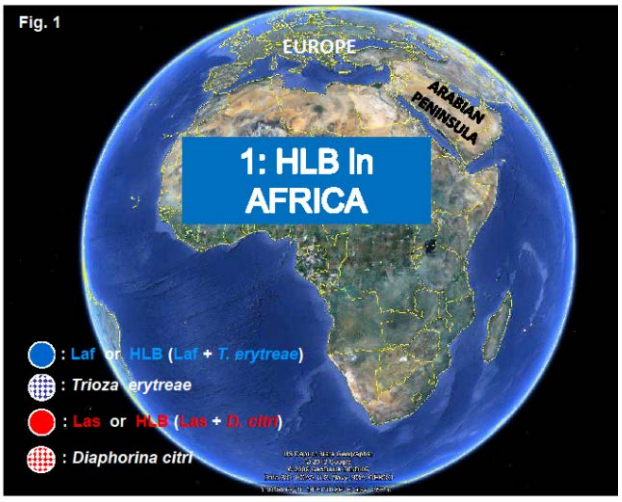
1965) was reported nine years later, in 1965. For these reasons, the name huanglongbing has precedence over greening, and the International Organization of Citrus Virologists (IOCV) proposed in 1995 at the 13th conference of the IOCV in Fuzhou (Fujian province, China) that the official name of the disease be huanglongbing (abbreviation: HLB), and this proposal was adopted (Moreno et al., 1996). Today, the term HLB is widely used for the African, American, and Asian forms of the disease.

On colors!

On the figures of this presentation, African HLB and/or *Candidatus* Liberibacter africanus (Laf) are represented by a circle whose inside is blue, while Asian HLB and/or *Candidatus* Liberibacter asiaticus (Las) have a circle with red inside. Similarly, the symbol of the African HLB psyllid is a circle with an inside of blue dots on a white background; for Las, the dots are red (see Fig. 1). So, blue relates to Africa and red, to Asia. Circles with white inside indicate absence of HLB.

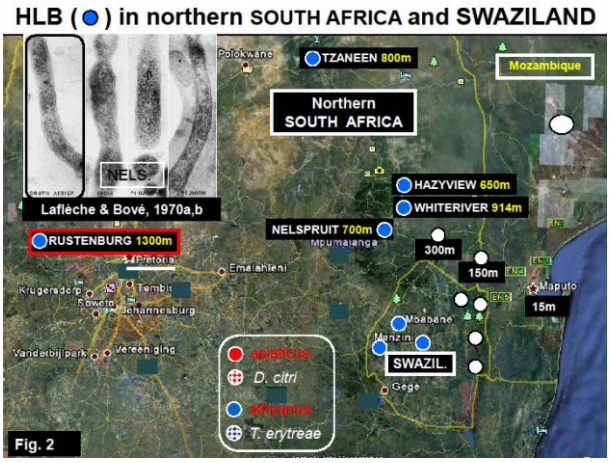
Part I:
Heat-sensitive, African HLB

1. Early research on HLB in South Africa: Temperature and the distribution of the disease and its African vector, *Trioza erytreae* (Fig. 1, 2, 3).

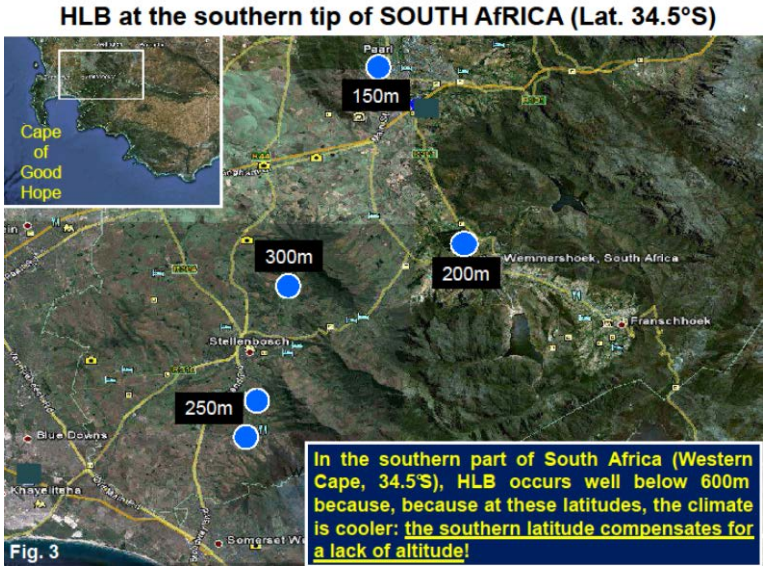


It is believed that symptoms of citrus HLB have been seen on the African Continent for the first time in South Africa in 1928/1929 (Oberholzer et al., 1965; Van der Merwe and Andersen, 1937) near the city of Rustenburg (altitude 1159m) at the foot of the Magaliesberg mountain range in western Transvaal (now: North West province). The disease became known as “yellow branch” in western Transvaal and “greening” in eastern Transvaal (now: Mpumalanga). The designation “greening” referred to poorly colored fruit, abnormally green especially at the stylar end, when it was already orange-colored at the peduncular end (symptom of “color” inversion). The term “greening” prevailed and became the most common name of the disease in Africa as well as in Asia, before it was replaced in 1995 by huanglongbing (Moreno et al., 1996).

Because HLB-affected citrus trees in South Africa often showed mineral deficiency symptoms (especially those of zinc), the disease was first thought to be a nutritional problem, until Schwarz in 1964 and McClean and Oberholzer in 1965 reported HLB to be transmissible by graft inoculation (McClean and Oberholzer,1965a). This result demonstrated that HLB was an infectious disease and probably a "virus" disease, since viruses were the only infectious, graft-transmissible agents of plants known in those days. In the same year 1965, McClean and Oberholzer also reported that the African citrus psyllid, *Trioza erytreae*, was an insect vector of the HLB agent (McClean and Oberholzer, 1965b). *T. erytreae* had been reported on citrus from South Africa in 1897 (Lounsbury, 1897). This was the first and earliest report on the African citrus psyllid on the African Continent. Del Guercio's report on the presence of the psyllid in Erytrea came "only" in 1918 (Del Guercio, 1918). The occurrence of *T. erytreae* on citrus can easily be detected by the presence, on the upper leaf face, of hemispherical "bumps", which result from the development, on the lower leaf face, of psyllid nymphs in concave "nests".



When, in 1965, it was realized that HLB was caused by an infectious agent transmitted by the African citrus psyllid, country-wide surveys were initiated in South Africa (Schwarz, 1967; Schwarz and Green, 1972; McClean et al., 1969) as well as in Swaziland (Catling and Atkinson, 1974) in order to better understand the somewhat unusual distribution of the disease. It was found that there was a strong correlation between the incidence of HLB and high psyllid populations. The psyllid populations were high in cool, moist upland citrus areas above ~600m altitude, and it was also in these areas that HLB was most widespread and severe. For instance, in Swaziland (Sw) and northern South Africa (SA), between latitudes 23°50'S (Tzaneen, SA) and 27°20'S (southern border of Sw), severe HLB occurred at Pretoria (SA,~1300m), Rustenburg (SA,~1159m), White River (SA,~914m), Tzaneen (SA,~800m), Nelspruit (SA,~700m), Mbabane (Sw,~1300m), Malkerns (Sw,~800m) and Manzini (Sw,~650m), but not at Malelane (SA,~300m), Tambankulu (Sw,~260m) and Big Bend (Sw,~100m). However, in the Western Cape Province, at the higher



southern latitudes, for instance between 33°43'S (Paarl) and 34°04'S (Sommerset West), HLB was present well below ~600m and was seen near Paarl (~150m), Wemmershoek (~200m) and to the North (~300m) and South (~250m) of Stellenbosch (Garnier et al;, 2000 a). In addition, it was found later that the agent involved in HLB in southern South Africa was the same as in northern South Africa, namely *Ca*. L africanus (Korsten et al., 1996; Garnier et al., 2000 a). Thus, South African HLB occurred well below ~600m at the high, southern latitudes because, at these latitudes, the climate was cool even at low altitudes, the high, southern latitudes compensating for the lower altitudes.

One of the reasons why psyllids were abundant in cool, moist areas was already well known (van der Merwe, 1941; Moran and Blowers, 1967; Catling, 1969): the African psyllid species, *T. erytreae*, was sensitive to high temperatures (~32°C and higher). Eggs and young instars of the insect were particularly sensitive to the combination of high temperatures and desiccation. A second reason for HLB to be restricted to cool surroundings was suggested by field experiments at Woodhouse (altitude 823m, ~10km Southwest of Nelspruit), where HLB was severe and psyllid populations were high (Schwarz and Green, 1972). In these 1968/1969 experiments, HLB-affected, 3-years-old sweet orange trees were grown in fiberglass cages and regularly sprayed with insecticides to prevent buildup of psyllids on the treated trees. The temperatures in the cages were found to be 8 to 10°C higher than the outside ambient temperatures. Under these high temperature conditions, fruit symptoms of HLB were drastically decreased, suggesting that, like the African psyllid vector, the HLB agent itself was also temperature-sensitive.

2. Stubborn, South African HLB, and two HLB-like diseases from Asia.

The influence of temperature on HLB symptoms was further studied under phytotron conditions at Gif sur Yvette, near Paris, France, in the frame of an international cooperation, which had been set up during the fifth conference of the International Organization of Citrus Virologists (IOCV) in November 1969 in Japan between the USA, South Africa, the Philippines, India and France including Reunion island. At this conference, HLB and stubborn diseases were still thought to be strains of the same "virus", HLB being in southern Africa a disease most severe in cool areas (McClean et al., 1969), while stubborn in California, Arizona, Iran and Morocco, being most severe in hot areas (30 to 35°C) (Olson and Rogers, 1969). These field results were the reason to study under controlled phytotron conditions not only HLB but also stubborn as well as two additional "HLB-like" diseases: Philippines leaf-mottling (Martinez and Wallace, 1968; Salibe and Cortez, 1968), and Indian citrus decline (Fraser and Singh, 1968). These Asian diseases had symptoms similar to those of African HLB. Symptoms of leaf mottling closely resembled those reported for South African HLB (Martinez and Wallace, 1968; Salibe and Cortez, 1968). The similarity between South African HLB and Indian citrus decline was stressed by Lilian Fraser who, after having surveyed all major citrus areas of India, concluded that citrus decline was caused by the "virus" responsible for South African HLB (Fraser and Singh, 1968). The Asian citrus psyllid, *Diaphorina citri*, had been reported in 1966 to

transmit Philippines leaf mottling (Celino Salibe and Cortez, 1966; Martinez and Wallace, 1967) and Indian citrus decline (Capoor et al., 1967). So, South African HLB, on the one hand, and Asian leaf mottling and citrus decline, on the other, had citrus psyllids as insect vectors.

3. Effect of controlled temperature conditions on California stubborn, South African HLB, Philippine leaf mottling and Indian citrus decline.

With the above information setting the stage, the effect of temperature on symptom expression of California stubborn, South Africa HLB, Philippine leaf mottling and India citrus decline were studied in two growth chambers of the French phytotron in 1969/1970 (Bové et al., 1974). In the cool chamber, the temperature was set at 22°C for 8-hr nights and 24°C for 16-hr days; in the warm chamber, the temperature consisted of 27°C for 8-hr nights and 32°C for 16-hr days. Artificial light was used to achieve a 16-hr light period. Relative humidity was 80%, except at night, when 60% was maintained in the warm chamber.

3.1. Stubborn.

In the case of stubborn, eight Madam Vinous sweet orange seedlings infected with California stubborn C189 (free of all other known graft-transmissible diseases) showed severe symptoms (small, cupped leaves with pale-green tips and mottling) within 5 weeks in the warm chamber, but only mild symptoms within 26 weeks in the cool chamber. Plants in the warm chamber were transferred to the cool chamber and *vice versa*. Plants now in the cool environment produced new growth with large leaves in contrast to the small cupped leaves from the previous growth under warm conditions. Conversely, small cupped, leaves were obtained under warm conditions on the plants held previously under cool conditions. When examined by transmission electron microscopy (TEM), the sieve-tubes of the small cupped leaves from the warm chamber contained many more of the helical mycoplasma organisms, characteristic of stubborn, than those from the cool chamber. It was from these small cupped leaves that the stubborn organism was cultured for the first time in 1970 (Saglio et al., 1971a), characterized as *Spiroplasma citri* in 1972 and shown to grow best at 32°C (Saglio et al., 1973), the latter result being in good agreement with the phytotron results. Therefore, the heat-tolerance of stubborn “disease” could be explained by the heat-tolerance of its “agent”, *S. citri*.

3.2. South African HLB.

Experimental plants. In the case of South African HLB, budwood sticks from a Hamlin sweet orange seedling infected with a Nelspruit strain of HLB transmitted through *T. erytreae* psyllids, were propagated on Orlando tangelo seedlings. Two batches of eight plants each were transferred immediately to the growth chambers. Uninoculated Hamlin sweet orange seedlings served as controls.

Results were as follows. Severe symptoms were obtained only under the cool conditions (22°C-24°C); at the warm conditions (27°C-32°C) no symptoms developed. After 30 weeks in the cool chamber, the affected plants measured only 35 cm in height as against 190 cm for those under the warm conditions. When, after 40 weeks in the cool chamber, severely affected plants were transferred to the warm

chamber, they quickly produced new, vigorous growth, recovered, and stayed symptomless during the remaining 10 months of the experiment.

3.3. Indian citrus decline, Philippine leaf mottling.

Experimental plants. A Mosambi sweet orange seedling, infected with the citrus decline agent (Poona strain) by *D. citri* psyllids, served as a source of inoculum to graft-inoculate Hamlin sweet orange seedlings by the leaf-patch technique of Calavan et al. (1972). Two batches of nine plants each were then cut back and transferred immediately to the growth chambers. In a second experiment, eight Madam Vinous and eight Hamlin sweet orange seedlings were inoculated with leaf patches from the Mosambi sweet orange seedling, cut back, and transferred to the growth chambers.

Ladu mandarin seedlings, infected with the Philippine leaf mottling agent (Lipa strain) by *D. citri* psyllids, were used to inoculate Hamlin and Madam Vinous sweet orange seedlings by the leaf-patch technique. Four inoculated seedlings of each variety were placed in the growth chambers.

Uninoculated Madam Vinous and Hamlin sweet orange seedlings were used as controls

Results. Indian citrus decline and Philippine leaf mottling reacted similarly, but differently from South African HLB. Indeed, with the two Asian diseases, severe symptoms were obtained, similarly to South African HLB, in the cool chamber, but, differently from South African HLB, also in the warm chamber. After 30 weeks under the warm conditions, the seedlings infected with citrus decline were 25 cm in height as against 180 cm for the healthy controls. After 17 weeks under the warm conditions, the seedlings infected with leaf mottling measured 40 cm and the healthy controls, 140 cm. These results indicated that South African HLB was heat-sensitive, as symptoms were only obtained under cool conditions; they confirmed the field observations of Schwarz and Green on reduction of fruit symptoms of HLB in fiberglass cages (see above) (Schwarz and Green, 1972). On the contrary, the two Asian HLB diseases were heat-tolerant, as the same symptoms develop not only under cool conditions (22°C-24°C), but also when the temperatures were much higher (27°C-32°C).

3.4. Similar bacteria are associated with South African HLB, Indian citrus decline and Philippine leaf mottling. One additional development strongly supported the similarities between South African HLB and the two Asian HLB-like diseases: the discovery, by TEM, of bacteria present not only in the phloem sieve-tubes from citrus affected with South African HLB, but also in the sieve tubes from citrus affected with Philippine leaf mottling and Indian citrus decline. These bacteria were initially thought to be mycoplasma-like organisms (MLOs) (Laflèche and Bové, 1970a, b), similar to those reported for the first time in 1967 in Japan (Doi et al., 1967). However, when they were compared to the true mycoplasmas seen in the sieve tubes of citrus affected by stubborn disease (Saglio et al., 1971a), they were clearly different from mycoplasmas, since their surrounding cell envelope was 200A° thick, while the cell envelope of the MLOs and the stubborn mycoplasma had a

thickness of only 100A°, characteristic of the wall-less, single unit-membrane envelope of mycoplasmas (Saglio et al., 1971b).

3.5. The nature of HLB. Several conclusions on the nature of HLB could be drawn from these results. **(i)** Stubborn and HLB were not virus diseases, as thought for many years, but were associated with bacteria. **(ii)** Stubborn and HLB were different diseases as the bacterium associated with stubborn was a mycoplasma, which could be cultured, was found to be helical, was vectored by a leafhopper and was characterized as *Spiroplasma citri* (Bové and Saglio, 1974; Saglio et al., 1973). On the contrary, the bacterium associated with HLB was not a mycoplasma, had a cell envelope twice as thick as the unit membrane of mycoplasmas, could not be cultured and was vectored by a psyllid. **(iii)** The bacterium having the 200A° thick cell envelope was present in the sieve tubes of citrus affected not only by South African HLB, but also Philippine leaf mottling, Indian citrus decline and Reunion island HLB (see below), strongly suggesting that all four of them belonged to the same group of diseases. **(iv)** Similarly to the situation with stubborn disease, the heat-sensitivity of South African HLB and the heat-tolerance of the two Asian diseases were properties of their respective bacteria, the bacterium associated with South African HLB being heat-sensitive and the bacterium associated with the Asian diseases being heat-tolerant. **(v)** The African heat-sensitive bacterium and the Asian heat-tolerant bacterium being morphologically indistinguishable by TEM, the fact that one was heat-sensitive and the others were heat-tolerant, revealed for the first time that they were not biologically identical. **(vi)** After the discovery of the MLOs in Japan in 1967 (Doi et al., 1967), the African and Asian bacteria turned out to be the first example of a non-MLO, bacterial plant-agent having a more complex envelope system than the single unit membrane of the MLOs. As shown in the next section, this more complex envelope system was that of a Gram-negative bacterium.

4. From the African bacterium and the Asian bacterium to respectively, "*Candidatus* Liberibacter africanus" (heat-sensitive) and "*Ca*. L. asiaticus" (heat-tolerant).

4.1. Characterization of the African and Asian bacteria. For twenty years, during the 1970s and 1980s, TEM detection of the bacterium in trees showing HLB symptoms remained the only technique capable of confirming that such trees were indeed affected by HLB (Garnier and Bové, 1996). While the stubborn agent could be characterized as soon as it had been cultured, the HLB bacterium could not be obtained in culture in spite of many attempts (Garnier and Bové, 1993), and it took a relatively long time for it to be characterized. Eventually, it was identified as a Gram-negative bacterium by cytology coupled to electron microscopy (Garnier et al, 1984a, 1984b). DNA-hybridization with specific probes made it possible for the first time to specifically detect and identify the African and the Asian HLB bacterium (Villechanoux et al., 1992). Monoclonal antibodies were developed against the African and Asian HLB bacteria and also revealed differences between the two types of bacteria (Garnier et al., 1991b; Gao et al., 1993). Finally, on the basis of their 16SrDNA sequences, the HLB bacteria were confirmed to be Gram-negative

organisms and found to belong to a new genus in the alpha subdivision of the *Proteobacteria*: the genus "Liberibacter", or more precisely, "*Candidatus* Liberibacter". (*Candidatus* indicates that the taxonomical identification of the bacterium could not be carried out with cultured organisms but involved DNA-based, molecular techniques). However, within the "*Candidatus* Liberibacter" genus, the South African bacterium and the bacterium from the Asian diseases represented two different species, which were named respectively *Candidatus* Liberibacter africanus (Laf) and *Candidatus* Liberibacter asiaticus (Las) (Jagoueix et al., 1994, 1997). The discovery of two different species was in agreement with the previously observed differences between the African and the Asian bacteria, as revealed by temperature sensitivity, DNA-hybridizations and serological reactions. Finally, from 1994 onwards, PCR techniques were developed to detect and identify specifically Laf and/or Las (Jagoueix et al., 1996; Hocquellet et al., 1999).

4.2. Liberibacters are endogenous bacteria. While many phytopathogenic bacteria, such as species of *Erwinia*, *Pseudomonas*, *Ralstonia* or *Xanthomonas*, are **exogenous** in that they are air/soil-borne, carried by air and rain, have no insect vectors, are not graft-transmissible, and colonize the intercellular space (apoplast) of their plant host, other plant bacteria are **endogenous** in that they are strictly phloem-restricted or xylem-restricted (Bové and Garnier, 2003). The liberibacters as well as the phytoplasmas (former MLOs), spiroplasmas and phlomobacters are phloem-restricted endogenous bacteria. *Xylella fastidiosa* is a xylem-restricted endogenous bacterium. The endogenous bacteria are graft-transmissible, and they are introduced directly into the phloem- or the xylem-cells by phloem sap- or xylem sap-feeding insect vectors, respectively. They do not need a special mechanism to gain access into their plant cell compartment. This is not so for exogenous bacteria, which need such a mechanism: the type III secretion system, for instance. This system is present in all the major groups of exogenous Gram-negative, plant pathogenic bacteria, except *Agrobacterium*. It has been speculated that the endogenous plant-associated bacteria in general would lack such systems (Bové and Garnier, 2003). Indeed, this has been found true for all endogenous bacteria whose genome has been sequenced, including liberibacters, phytoplasmas, Spiroplasma and *X. Fastidiosa*.

4.3. Contribution of periwinkle plants to the study of HLB. In 1981, transmission of Indonesian citrus vein-phloem degeneration to periwinkle plants (*Catharanthus roseus*) by dodder, was reported by Tirtawidjaja (1981). In France, in 1983, transmission of the HLB bacterium from citrus to periwinkle plants was achieved by dodder (*Cuscuta campestris*) (Garnier and Bové, 1983). The dodder transmission was performed with South African HLB, Indian citrus decline, Philippine leaf mottling, Chinese huanglongbing, and Thailand HLB. The titer of liberibacter cells in periwinkle plants was much higher than in citrus trees. Great numbers of infected periwinkle plants have been used as convenient sources for the phylogenetic and taxonomic characterization of the HLB bacterium, the production of monoclonal antibodies and the development of molecular detection techniques. Also, infected periwinkle plants reacted to temperature in the same way as citrus plants. Infected with Laf, they showed symptoms under cool conditions (27°C for 16-hr days and

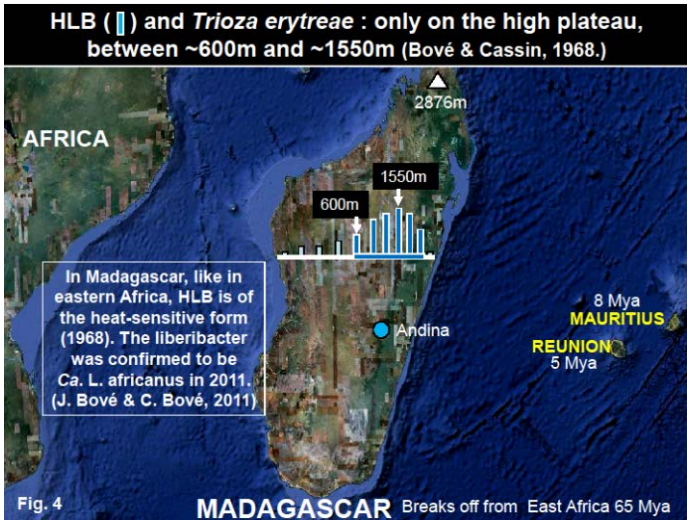
23°C for 8-hr nights) but not under warm conditions (32°C for 16-hr days and 27°C for 8-hr nights). Infected with Las, they showed symptoms under both temperature conditions. These results confirmed those with citrus, namely that the African HLB bacterium (Laf) was heat-sensitive and the Asian HLB bacterium (Las), heat-tolerant.

Citrus liberibacters have also been transmitted by dodder to solanaceous plants: tobacco (Ganier and Bové, 1993) and tomato (Duan et al., 2008), in which they induce symptoms and behave as pathogens.

4.4. African HLB and Asian HLB. So, by the early 1970s, it was well established that there were two forms of HLB: South African HLB and Asian HLB. South African HLB was heat-sensitive because (i) its psyllid vector, *T. erytreae*, and (ii) the HLB bacterium itself (later identified as *Ca*. L. africanus) were both heat-sensitive. As will be seen below, all forms of HLB in Africa and Madagascar Island are of the South African form, justifying the more general designation "African" HLB or "African form" of HLB. This form of the disease occurs in regions of cool, moist climate, often on highlands, with temperatures below ~30°C.

Philippine leaf mottling and Indian citrus decline were heat-tolerant because (i) their Asian psyllid vector, *D. citri*, and (ii) their HLB bacterium (later identified as *Ca*. L. asiaticus) were both heat-tolerant. As will be seen below (see Part II), additional Asian citrus diseases with leaf symptoms and fruit symptoms identical to those of HLB, such as Chinese huanglongbing, Taiwanese likubin, Indonesian vein-phloem degeneration, are also transmitted by the heat-tolerant *D. citri;* and their HLB bacterium (later identified as *Ca*. L. asiaticus) is heat-tolerant too. Hence, the designation "Asian" HLB or "Asian form" of HLB came into use. This form of HLB occurs even at temperatures of 32°C – 35°C.

5. In Madagascar, heat-sensitive *T. erytreae* and African HLB are present only on the central highlands (Fig. 4).



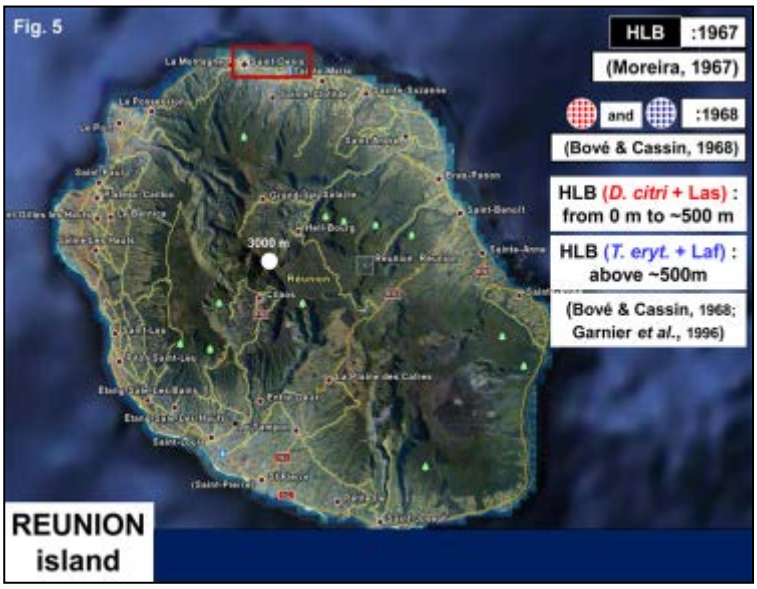
Madagascar, in the Indian Ocean, off the eastern coast of southern Africa, is the fourth largest island in the world. It is located between latitude 25°S (the latitude of Nelspruit in Mpumalanga) and 12°S (the latitude of northern Mozambique). The central highlands occupy more than 50% of the island's surface. They range from 800m to 1800m, the island culminating at 2876m. They slope gently to the West Coast, but have steep escarpments along the East Coast. The island was part of Gondwanaland and, conjoint to India, split away from eastern Africa at ~150 million years ago (Mya); it broke off from India and was on its own by ~88 Mya.

Citrus was introduced at the beginning of the 19th century. The African citrus psyllid, *Trioza erytreae*, was reported for the first time in 1961 (Caresche & Brenière, 1961; Brenière & Dubois, 1965). An island-wide survey for citrus diseases detected HLB in 1968 (Bové & Cassin, 1968a). Interestingly, the distribution of both the disease and the psyllid followed the patterns seen in northern South Africa: HLB and *T. erytreae* were only present on the cool central highlands, essentially between 600m and 1500m; they were totally absent from the hot coastal areas. When this survey was carried out, the liberibacters had not yet been discovered, and identification of the liberibacter species involved in Madagascar HLB was not feasible. In 2011, sweet orange leaves with blotchy mottle symptoms could, however, be collected; and PCR in Bordeaux, France, identified *Ca*. L. africanus, as expected (J.M. Bové, C. Bové, C. Saillard and M.P. Dubrana, unpublished).

6. In Reunion and Mauritius islands, heat-tolerant *D. citri* and Asian HLB were present from sea level to ~500m; heat-sensitive *T. erytreae* and African HLB were present from ~500m to ~1200m (Fig. 4, 5, 6, 7).

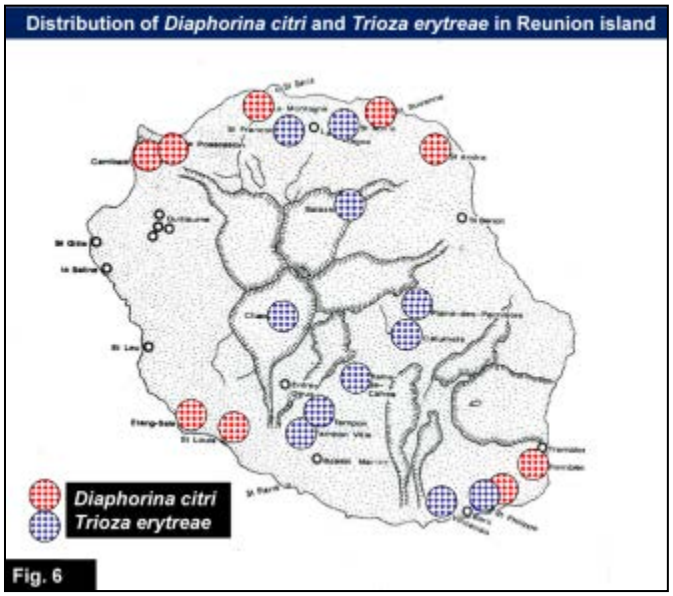
The above results were confirmed on two islands east of Madagascar (12°S-25°S), in the Indian Ocean; Reunion (20°50'S-21°23'S) and Mauritius (19°60'S-20°30'S). While Madagascar had Gondwanan origin and was detached from "India" by ~88 Mya, Reunion and Mauritius emerged from the Indian Ocean as volcanoes much later, respectively ~5 and ~8 Mya. While Reunion, the youngest island, culminates at 3070m, erosion has leveled the older Mauritius Island down to 828m. In both islands, HLB was first reported by Moreira in 1967 (Moreira, 1967).

6.1. Reunion (Fig. 5, 6). In Reunion in 1968, Bové and Cassin confirmed Moreira's identifications of HLB and *T. erytreae* (Moreira, 1967); and in addition, they reported the presence of the Asian citrus psyllid, *D. citri* (Bové and Cassin, 1968b). *D. citri* had been shown the previous year, in 1967, to be the psyllid vector of two Asian HLB-like diseases: citrus decline in India (Capoor et al., 1967) and citrus leaf mottling in the Philippines (Martinez and Wallace, 1967). Thus, Reunion turned out to be one of the first two regions, Mauritius being the second, where the African and Asian psyllid vectors were present concomitantly, even though in different zones (Bové and Cassin, 1968b). As in Madagascar and northern South Africa, the African citrus psyllid could only be seen at cool elevations, from ~500m up to ~1200m, indicating again that it was temperature-sensitive, while, interestingly enough, the Asian citrus psyllid was present only in the hot coastal areas and up to ~500m, strongly

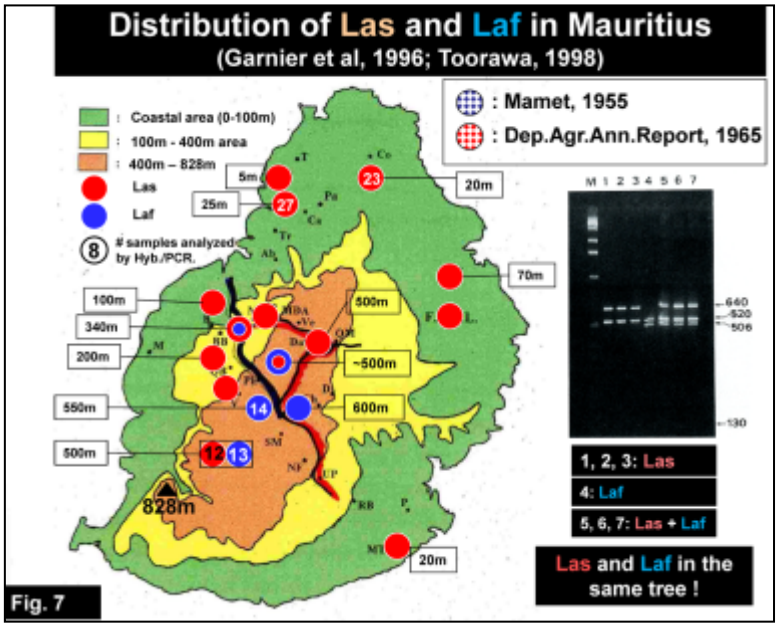


suggesting that it was temperature-tolerant (Bové and Cassin, 1968b; Catling, 1973). Indeed, later work showed that HLB in the zone inhabited by "*T. erytreae*" (~500m to 1200m) was associated with *Ca*. L. africanus while the disease in the "*D. citri* zone" (0 to ~500m) with *Ca*. L. asiaticus (Garnier et al., 1996; Garnier and Bové, 1996).

Biological control of *T. erytreae* and *D. citri* with the ectoparasites *Tamarixia dryi* and *Tamarixia radiata,* respectively, has been initiated successfully in Reunion island in the 1970s (Etienne and Aubert, 1980) and has changed the level of the psyllid populations. By 1979-1980, *T. erytreae* was totally eradicated and the levels of *D. citri* were greatly reduced (Aubert et al., 1996).



6.2. Mauritius (Fig. 7). In Mauritius, the genus *Trioza* (most likely the species *T. eytreae*) was reported as early as 1923 (D'Emmerez et Gebert, 1923) and was mentioned again under the synonymous name *Trioza merwei* Pettey in 1955 (Mamet, 1955). *D. citri* was mentioned in the 1965 annual report of the department of agriculture, Port Louis, Mauritius, and cited by Moreira in 1967 (Moreira, 1967). Thus both islands, Reunion and Mauritius, harbored the two citrus psyllids species: *T. erytreaea* and *D. citri*. (Garnier et al., 1996). The distribution of the two psyllid species followed patterns similar to those in Reunion Island, D. citri occupying the zone from sea level up to ~ 500m and T. erytreae, preferring the central highlands between ~ 500m and ~ 828m, the highest point on the island. In the frame of a collaboration between Mauritius and France, Parveen Toorawa (Toorawa, 1998) exhaustively studied the distribution of the two HLB liberibacters in Mauritius at a time when DNA-DNA hybridization and PCR, respectively, with liberibacter-specific probes and primers, had been developed and made it possible to identify and detect *Ca*. L. africanus (Laf) and/or *Ca*. L. asiaticus (Las) (Jagoueix et al., 1996). She subdivided the island into three zones: (i) a coastal zone comprised between sea level and ~100m, (ii) an intermediary zone between



~100m and ~400m, and (iii) the central plateau, above ~400m. She selected citrus trees with blotchy mottle symptoms in several orchards of the three zones and identified the liberibacters present in these trees. In seven orchards of the coastal zone, Las was detected in 76 trees; none of the analyzed trees carried Laf. Within four orchards in the intermediary zone (~100m to ~400m), Las was detected 9 times and Laf, twice. On the central plateau (above ~400m), nine orchards had 18 trees infected with Las and 28 trees infected with Laf. Noteworthy, all 18 Las-infected trees and 12 of the 28 Laf-infected trees were between ~400m and ~500m; the remaining 16 Laf-infected trees were above ~500m. Thus, only Las was found in the coastal zone (0m-~100m) and only Laf above ~500m; the majority of Las infected trees (82%) were between sea level and ~400m, and most of the Laf-infected trees (94%) were above ~400m. One orchard in the 400m to 500m zone had three types of HLB-infected trees: infection with Laf, infection with Las, and infection with both Las and Laf (Fig. 7). These data showed again that Laf-associated African HLB was heat-sensitive and Las-associated Asian HLB, heat-tolerant.

Biological control of *T. erytreae* and *D. citri*, respectively, with *T. dryi and T. radiata* has also been initiated in Mauritius.

7. In Ethiopia/Erytrea, between latitudes 18°N and 4° N, regions cool enough to support heat-sensitive *T. erytreae* and African HLB have to be above an altitude of 1000m-1200m, while in South Africa the suitable altitudes were lower: only ~600m at latitude 25° S or ~100m at latitude 35° S (Fig. 8, 9).

All experiments summarized in the above paragraphs were carried out in the southern hemisphere, between relatively high southern latitudes: 34°50'S (southern tip of Africa) and latitude 12°S (northern Madagascar). Similar work was also carried out in the northern hemisphere of Africa, in Ethiopia/Erytrea, between latitude 18°N (northern Erytrea) and latitude 4°N (southern Ethiopia).

The political situation of Erytrea has changed over the years. Previously part of Ethiopia ("greater" Ethiopia), Erytrea became in 1890 an Italian colony. Occupied by the British in 1941, it came back to Ethiopia from 1952 to 1993; it became independent in 1993.

Commercial citrus production started in the 1920s when a large number of trees on rough lemon were introduced from South Africa into Erytrea at Elaboret, near Asmara. In 1967 as well as some years later, indexed trees were imported from California (cited by Van Bruggen and Almaz Yilma, 1985).

Del Guercio reported the presence of *T. erytreae* in Erytrea in 1918 during the Italian occupation (Del Guercio, 1918). *T. erytreae* was mentioned again in "greater" Ethiopia in 1971 (Schmutterer, 1971), a time when Ethiopia and Erytrea were reunited. Considering that the psyllid was present in Erytrea in 1918, it is very likely that it occurred also, in those early years, in Ethiopia, South of Erytrea.

Symptoms of HLB in "greater" Ethiopia were reported by Chapot (1970), Schwarz (1976), Dereje *et al*. (1977), and van Bruggen and Almaz Yilma (1985); the last two authors have also confirmed the disease by biological indexing on citrus seedlings. Symptoms were found on all kinds of citrus and in most citrus-growing areas: area 1 (elevation: ~1,720m), area 2 (1,180-1,750m), area 3 (~1,200m), area 4 (1,400-2,100m), area 5 (1,710-1,800m), area 6 (~1,600m), area 7 (1,050-1,880m), area 8 (Awara Melka, Melka Sadie, ~1,550m), area 9 (Shoa Robi, ~1,230m), area 10 (Mek'ele, ~2,200m), area 11 (Elaboret, ~1,600) and area 12 (Erer Gota/Urso, ~1200m) (Fig. 8). All these areas were at elevations higher than ~1,000m. HLB symptoms were not seen in the following areas (Fig. 8): area 8 (Melka Werer, ~730m; Metahara, 950m), area 13 (Gode, ~200m), area 14 (Dubti, ~300m) and area 15 (Dilla/Yirga Alem, 1600-1800m). Most of these areas without HLB symptoms were below ~1000m.

Thus, already widely distributed when reported for the first time in the 1970s, the disease was probably present in "greater" Ethiopia much earlier, but remained undetected and/or unreported for many years.

In Ethiopia, a survey for HLB was carried out in the late 1980s along latitude ~9°N in the lower Awash River valley (Melka Werer, ~730m), the middle Awash valley (Nura Era, ~1200m), the upper Awash valley (Awasa/Koka, ~1600m) and in backyard citrus trees at higher elevations (Debre Zeit: ~1850m; Addis Ababa: ~2400m) (Aubert et al., 1988). Based on (i) symptoms, (ii) TEM detection of the sieve tube-restricted HLB bacterium and (iii) *T. erytreae* adults as well as nymph bumps on citrus leaves, HLB occurred at Addis Ababa {~2400m, mean annual maximum Temperature (mamT): 22.8°C}, Debre Zeit (~1850m, 26.1°C), Awasa/Koka (~1600m, 24.3°C) and Tibila (~1400m). HLB and *T. erytreae* were not seen at Nura Era (~1200m, 30.8°C), Metahara (~950m, 32.3°C), Awash (~919m, 33.4°C) and Melka Werer (~730m, 34.3°C) (Fig. 9).

Finally, at latitude ~18°N, HLB and *T. erytreae* were present in Erytrea, at Elaboret (~1600m, 23°C), but not at Kassala (~529m, 35,9°) 150km farther west in

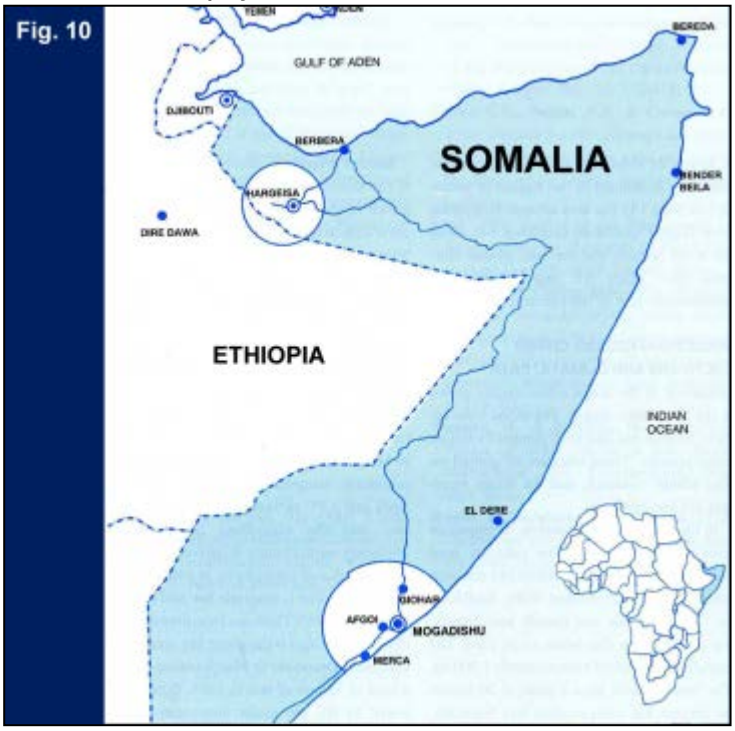
neighboring Sudan (Fig. 9). *T. erytreae* was mentioned in the Sudan in 1965 (Gentry, 1965).

So, in Ethiopia and Erytrea, taking into account all the data, regions cool enough to support the African citrus psyllid and HLB had to be above elevations of 1000-1200m, with mamT below ~26°C. In northern South Africa, along latitude 25°S, and in Western Cape at latitude 34°.50S, the critical altitudes were respectively, ~600m and ~100m.

8. Somalia (Fig. 9, 10)

The Food and Agriculture Organization of the United Nations (FAO), supported a survey for virus and virus-like diseases of citrus was conducted in two citrus regions of Somalia in October-November, 1986: the area around Mogadishu, from Goluin in the South to Giohar in the North, at elevations below 50m, and the area around Hargeisa at ~1300m altitude in the northwestern region of the country (Bové, 1995d).

No evidence for HLB was seen in the Mogadishu region. The African psyllid vector of the disease, *Trioza erytreae,* has never been reported, and no signs of its presence were found during the survey. The climate of the Mogadishu region is most likely too hot for both the African disease and its psyllid vector.



In contrast to Mogadishu, symptoms of HLB were observed in the Hargeisa region at Arapsiyo (~1350m altitude). Electron microscopy revealed the presence of the HLB bacterium in the sieve tubes of symptomatic trees. In some trees, both the HLB bacterium and citrus tristeza virus (CTV) were detected in the sieve tubes and CTV was confirmed by ELISA.

Citrus was introduced into the Hargeisa region in 1930 from Cyprus and Australia. Two citrus nurseries functioned from 1951 to 1962, one at Arapsiyo and one at Geed Deeble. New citrus introductions were made in 1959 from various countries and, in particular, from Kenya and Ethiopia. In Kenya, citrus tristeza and HLB were widely distributed by 1981 (Bové, 1981 survey, unpublished). CTV was present in both the highlands and the coastal lowlands, while HLB was present only in the highlands above ~700m. Hence, it is possible that in the Hargeisa region, the trees infected with both CTV and the HLB bacterium were imported from Kenya. Ethiopia is another possibility. Indeed, HLB and citrus tristeza disease as well as their insect vectors, *Toxoptera citricida, Aphis*

gossypii for CTV and *T. erytreae* for HLB, have all been reported from the citrus region at Dire Dawa, the second largest city in Ethiopia, at a distance of only 100km from Hargeisa (Schwarz, 1976; Dereje et al., 1977; Crowe and Kamal, 1979; van Bruggen and Almaz Yilma, 1985).

HLB and *T. erytreae* being present at Dire Dawa (elevation: ~1200m) and HLB being confirmed at Hargeisa (~1300m), one would expect *T. erytreae* to be present at Hargeisa also. Unexpectedly enough, the African psyllid was not detected during the October-November survey in the Hargeisa region. No adults and no nymphs of *T. erytreae* were seen on citrus leaves. Nymphs develop on the lower leaf-side and each nymph produces a highly characteristic, concave depression which, on the upper leaf-side, looks like a bump. These leaf-bumps never disappear. No bumps could be found, neither on young leaves nor on adult leaves of any age. In spite of these negative results, and knowing that in nearby Ethiopia the African psyllid has been reported from all areas where typical symptoms of HLB have been observed, it is most probable that *T. erytreae* is present in the Hargeisa citrus areas. However, in view of the presence of *D. citri* in southwestern Saudi Arabia, South of Mecca, (see below), it might be worthwhile to look for the presence of the Asian citrus psyllid.

9. Additional countries with *T. erytreae and* African HLB (Fig. 11).

9.1. East Africa. Besides **Erytrea**, **Ethiopia**, **Somalia**, **South Africa** and **Swaziland** (see above), additional countries in East Sub-Saharan Africa carry *T. erytreae* and HLB: **Kenya**, **Malawi, Rwanda/Burundi**, **Tanzania**, **and Zimbabwe**. HLB symptoms in several of these countries were confirmed by detection of the HLB bacterium by TEM, DNA-hybridization and/or PCR: Kenya, Malawi, Rwanda/Burundi, Somalia, and Zimbabwe (Aubert et al., 1988; Garnier and Bové, 1996; Magomere et al., 2009). In all the countries, the distribution of HLB and *T. erytreae* followed the same general patterns as those in South Africa, Madagascar or Ethiopia: presence of heat-sensitive, African HLB and *T. erytreae* only in highland regions at altitudes high enough to offer cool and moist environmental conditions. Most of **Malawi** (10°S-17°S) is located above 1000m elevation, and HLB is widespread all over the country (Aubert et al., 1988); *T. erytreae* was reported in 1967 (Commonwealth I of E, 1967). In **Tanzania** (1°S-11°S), *T. erytreae* was recorded in 1967 (Commonwealth I of E 1967) and 1988 (Grisoni, 1988; Evers and Grisoni, 1991). Above an elevation of 1200m, HLB is most severe and *T. erytreae* populations, most dense. Between 1200m and 800m, psyllid populations are fairly

high, but HLB symptoms are moderate. Between 800m and 500m, HLB infection is light with relatively small vector populations. Below 400m, HLB and psyllids are absent (Evers and Grisoni, 1991).

9.2. West Africa. Only two HLB-affected countries belong to West Africa: Nigeria (4°N-13°N) and Cameroon (2°N–13°N). HLB symptoms were reported from **Nigeria** in 1984, but no mention of *T. erytreae* was made (Varma and Atiri, 1993). In **Cameroon**, the presence of *T. erytreae* was mentioned in 1967 (Commonwealth I of E, 1967). HLB and the HLB bacterium were reported in 1988 (Aubert et al., 1988). The distribution of *T. erytreae* and HLB has been extensively studied from 1992 to 1996 (Tamesse et al., 1999) Again, a strong correlation between severe HLB and important psyllid populations was observed. High maximum temperatures were the main constraint on psyllid outbreaks. No psyllids were present in regions where the number of days, with temperatures equal to or higher than 32°C, exceeded 130. Numerous psyllids were found between ~500m and ~1670m and all regions at altitudes between ~500m and ~700m had psyllids. On the basis of these results, HLB in West African Cameroon is of the African form, similar to HLB in East Africa.

9.3. Indian Ocean Islands. The cases of Madagascar, Reunion and Mauritius have been covered above (see 5. and 6.). Madagascar has characteristic heat-sensitive African HLB on the central plateau. Reunion and Mauritius are the only two regions with heat sensitive African HLB as well as heat-tolerant Asian HLB. In Reunion, Asian HLB was present from sea level to ~500m and African HLB, at elevations between ~500m and ~1200m. In Mauritius, the majority of Las-infected trees (82%) were between sea level and ~400m, and most of the Laf-infected trees (94%) were above ~400m.

10. Countries and islands with the African citrus psyllid, but no reports of HLB.

In the following countries, *T. erytreae* has been reported, but not HLB: Angola, Congo (Dem. R. of), Gabon, Sudan, Uganda and Zambia.

Some islands off the Atlantic coast of Africa carry *T. erytreae* but not HLB: Madeira (Portugal), Canarias islands (Spain): Tenerife, La Gomera, La Palma and El Hierro (Fig. 40), São Tomé and Principe, and St. Helena.

11. The case of Candidatus Liberibacter africanus, subspecies capensis (Lafc).

The “capensis” subspecies of *Candidatus* Liberibacter africanus was detected by PCR in an ornamental rutaceous species, *Calodendrum capense*, the Cape chesnut tree in the Stellenbosch area of Western Cape province, South Africa in 1998 (Garnier et al., 2000a and b). Leaves of the three affected trees showed characteristic blotchy mottle even though it has been reported more recently that symptoms may be mild or absent, suggesting an endophytic relationship between *C. Capense* and LafC. The new liberibacter was characterized by serology and the sequences of its 16S rDNA and the intergenic 16S/23S rDNA region, and ribosomal protein genes of the bacterial beta-operon. Phylogenetic analysis showed the new liberibacter to be more closely related to *Ca*. L. africanus than to *Ca*. L. asiaticus and received a subspecies assignation, *Ca*. L. Africanus subsp. Capensis (Lafc).

So far, Lafc has never been detected in commercial varieties of citrus (Garnier et al., 2000b; Pietersen et al., 2010a; Phahladira et al., 2012). Its host, the Cape chesnut, has a wide distribution (Pietersen et al., 2010b). It occurs along the south and eastern coast of southern Africa and is present in the Western Cape, the Eastern Cape, KwaZulu-Natal, Mpumalanga, Gauteng, North West and Northern Provinces, Swaziland, Tanzania and Ethiopia. Lafc has also a wide distribution as it was detected on *C. Capense* trees in most of the South African localities tested (Western and Eastern Cape, KwaZulu-Natal, Mpumalanga, Gauteng, and Limpopo). The subspecies was even found In the Eastern Cape, a province free of *Ca*. L. africanus and HLB. Infection of *Calodendron capense* with Lafc in countries North of South Africa has not yet been studied, but probably exists.

The putative insect vector required to transmit Lafc to *C. capense* is not known. Adults of *T. erytreae*, the psyllid vector of Laf, feed on the Cape chesnut, but this rutaceous tree was totally unsuitable as a host for the nymphal development of the citrus psyllid (Moran, 1968a and b).

It has been suggested that subspecies “capensis” may represent the “parent lineage” from which the species *Ca.* L. Africanus on citrus emerged through host species jumping and selection (Pietersen et al., 2010b). Preliminary data seem to suggest haplotype relationships between Laf and Lafc (W. R. Nelson, personal communication.

12. African HLB: Conclusions.

12.1 African HLB: a heat-sensitive pathosystem

On the African continent, in all the cases where HLB has been detected, the only citrus liberibacter identified was *Candidatus* Liberibacter africanus (Laf) (Garnier and Bové, 1996; Korsten et al., 1996; Pietersen et al., 2010a). In several African, HLB-affected regions, Laf has not been directly identified by adequate techniques (DNA-hybridization, PCR). However, the distribution of HLB in these regions has always been that of heat-sensitive, African HLB, thus identifying Laf indirectly. Also, when, in such an HLB-affected region (Madagascar for instance, or Kenya), it became possible to identify the liberibacter several years later, it always turned out to be Laf.

Laf is heat-sensitive (Bové et al., 1974; Garnier et al., 1983). Five other liberibacter species are also heat-sensitive: (i) *Ca*. L. africanus subsp. capensis, (ii) *Ca*. L. americanus (Lam) (Bové and Bonnet, 2007, unpublished; Lopes et al., 2009), (iii) *Ca*. L. solanacearum (Lso) (Munyaneza et al., 2012), (iv) *Ca*. L. europaeus (Leu) (Camerota et al., 2012), and (v) Liberibacter crescens (Lcr) (Leonard et al., 2012). Only *Ca*. L. asiaticus (Las) is heat-tolerant (Bové et al., 1974 ; Garnier et al., 1983; Lopes et al., 2009). Heat-sensitivity seems to be the rule with the liberibacters; Las is an exception (see 33 and fig. 53, 54, 55).

The citrus psyllid, *T. erytreae*, is the only known insect-vector of Laf in Africa, but it is most probably not a vector of Laf subsp. capensis (see above). Like Laf, *T. erytreae* is also heat-sensitive (van der Merwe, 1941; Moran and Blowers, 1967; Catling, 1969).

Laf and *T. erytreae* being heat-sensitive, African HLB is also heat-sensitive: it is a heat-sensitive pathosystem. It occurs only in cool environments at more or less high elevations according to latitudes.

12.2. *Trioza erytreae*: native to Africa.

T. erytreae is widely distributed throughout eastern sub-Saharan Africa with probable incursions into West Africa. It is also present on islands off the African coast. It is the only HLB psyllid in Madagascar, in agreement with its proposed Gondwanan origin, in contrast to Reunion and Mauritius islands where both *T. erytreae* and *D. citri* occur as the result of incursions (see 35).

T. erytreae was reported in South Africa as early as 1897 (Lounsbury, 1897) and in Erytrea and Kenya in 1918 (*Del Guercio*, 1918).

T. erytreae is the only *Trioza* species to feed and develop on *Rutaceae* plants, not only citrus, but also citrus relatives, including (i) *Vepris lanceolata* (Lam.) { = *Vepris undulata* (Thumb.), = *Toddalia lanceolata* (Lam.)} and (ii) *Clausena anisata* (Willd.), two preferred, native hosts of the African citrus psyllid. *Fagara capensis* (Thumb.) {= *Zanthoxylum capense* (Thumb.)} is also an adequate indigenous host for *T. erytreae* development (Moran, 1968 a and b).

Thus, before citrus and its hybrids (which are not indigenous to Africa) were introduced into Africa, *T. erytreae* had at least two native rutaceous hosts to complete its development: *Vepris lanceolata* and *Clausena anisata.* These two plants are among the original host plants of *T. erytreae* (Moran, 1968 a and b).*Vepris lanceolata* is not only a preferred host of the citrus psyllid, it has also been reported as a host the HLB bacterium, Laf (Korsten et al., 1996).

T erytreae also develops very adequately on *Citrus limon* and must have been well pre-adapted to this host at the time of the introduction of citrus to Africa. For this pre-adaptation purpose, *Fagara capensis* was almost as suitable as *C. limon* for the development of *T. erytreae* (Moran, 1968 a and b).

12.3. *Ca*. L. africanus: ancient species, native to Africa.

Worldwide, the distribution of Laf concerns only one very precise and homogenous zone, the African zone, comprising: (i) the southern and eastern coast of Sub-Saharan Africa and only two countries in West Africa, Cameroon and Nigeria; (ii) islands off the East Coast of Africa: Madagascar, Reunion and Mauritius. While Madagascar carries only typical African HLB with Laf *and T. erytreae*, Reunion and Mauritius, before biological control was effective, were affected by Asian HLB with Las and *D. citri* along the coastal areas, and African HLB with Laf and *T. erytreae* at higher elevations.

Out of the African zone, Laf has only been detected on the southwestern part of the Arabian Peninsula (**see Part III**) but never eastwards, beyond the Arabian Peninsula, in Asia and South-East Asia. Similarly, America is free of Laf, but carries Las and Lam. Europe, Australia, New Zealand and New Caledonia are free of citrus liberibacters.

Phylogenetically, *Ca*. L. africanus and *Ca*. L. asiaticus are closely related species. It has been calculated that speciation of the liberibacter ancestor into laf and Las started 147 Mya, much before the introduction of citrus into the African zone: Laf

and Las have a long life history (Teixeira et al., 2008a). However, in the absence of citrus, non-citrus, native, African rutaceous plants were available as plant hosts for early Laf forerunners. Even at present times, *Vepris lanceolata* is a host of Laf. In addition, in the African zone, *T. erytreae* was available for coevolution and a life-style alternating between the ancestral *T. erytreae* species as an insect host and a plant host, for instance *V. lanceolata*.

Ca. L. europaeus is a plant endophyte in certain plants (fruit tree *Rosaceae*) (Raddadi et al., 2010; Camerota et al., 2012) and a pathogen in other hosts (Scotch broom) (Thompson et al., 2013). Laf in its native African host might also have been plant endophytic, but turned out to be pathogenic in the citrus hosts when these were introduced into Africa, very recently regarding geological times.

Finally, very early, the ancestral Laf as an endosymbiont might have been of insect origin rather than plant origin.

For all these reasons, Laf is seen as a species of ancient origin with a long history, indigenous to Africa. This conclusion, as far as Laf is concerned, was also reached by Beattie et al. (2005, 2008).

The origin of Laf in Africa will be examined below (Part III).

13. Heat-tolerant Asian Citrus liberibacter in Africa: ETHIOPIA 2010 (Fig. 9)!

In 2010, Las was reported for the first time from Africa and, more precisely, from northern **Ethiopia** (Saponari et al., 2010). Citrus trees in orchards as well as trees for budwood sources in nurseries were inspected in the warmer citrus growing areas of Tigray and North Wollo. This survey revealed nearly 100 trees with symptoms of leaf yellowing with a blotchy mottle pattern, dead branches and poor fruit. Eight sweet orange trees (three symptomatic orchard trees, two symptomatic budwood-source trees and three symptomless trees) were sampled in April 2009. PCR amplification of (i) the ribosomal protein gene of *rplKAJL-rpoBC* with primers A2/J5 according to Hocquellet et al. (1999) and (ii) the 16S/23S intergenic region with primers OI2/23S1 according to Jagoueix et al. (1997), as well as amplicon sequencing, revealed Las in the samples from the symptomatic trees but not in those from the symptomless ones.

In areas where Las was confirmed, eradication of symptomatic trees and additional surveys to determine the spread of the disease were recommended. It is also urgent to identify the psyllid vector involved. Efforts to encourage the Ethiopian authorities to do so have failed in 2012 and 2013. The psyllid could be either *T. erytreae* or *D. citri*, as the two psyllid vectors can each transmit, experimentally at least, the African liberibacter as well as the Asian liberibacter (Massonié et al., 1976; Lallemand et al., 1986). *T. erytreae* was reported from Erytrea as early as 1918 (Del Guercio, 1918). If present in Ethiopia, *D. citri* could have been introduced accidentally, across the Red Sea, from south-western Saudi Arabia where the Asian citrus psyllid is abundant and was reported in 1972/1973 (FAO, 1972; Wooler et al., 1974). In addition to the Asian citrus psyllid, Asian HLB has also been discovered from the same southwestern region of Saudi Arabia in 1981-1983 (Bové and Garnier,

1984; Bové, 1995c) (See below Part III). It is very possible that Las might have been introduced into Ethiopia from south-western Saudi Arabia.

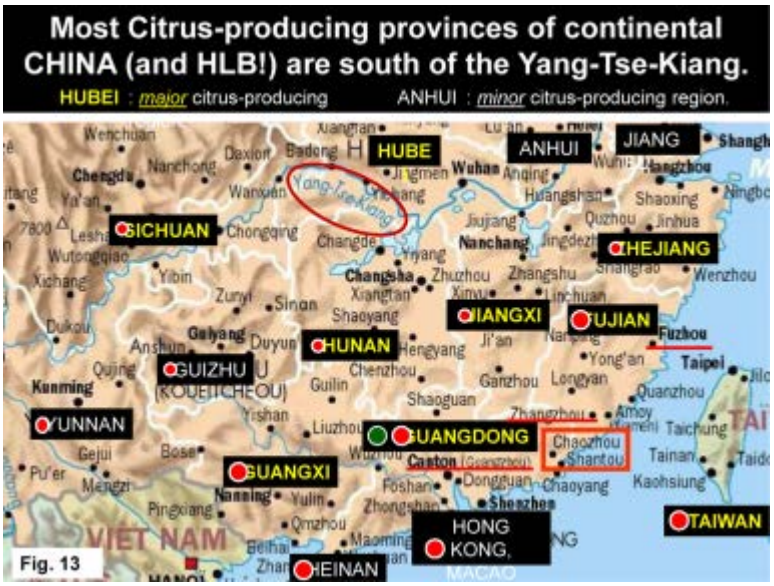
The presence of Las is a threat not only for Ethiopia but also for warm citrus growing areas of sub-Saharan Africa where heat-sensitive African HLB and *T. Erytreae* are absent or not well-established. This is the case for many sub-Saharan African countries.

Part II:
Heat-tolerant, Asian HLB

In the case of Africa, it is well accepted that symptoms of greening/HLB have been mentioned first in 1928/1929 in the Rustenburg area of South Africa, North West province. The situation in Asia is not as clear. Three countries have been involved in the debate on where and when HLB showed up first in Asia: China, India and the Philippines, and we shall start "Part II, Asian HLB", with these three countries. However, none of these countries claimed to be the first in Asia, they only described the HLB situation within their own boundaries.

14. Continental China (Fig. 12, 13).

It is assumed that the first publication, in which the name "yellow shoot" appeared, is the report by Otto August Reinking in 1919 on his survey of diseases of economic plants in sSouthern China (Reinking, 1919). For this reason it has been assumed that the first official record of HLB in China was made by Reinking in 1919. The report appeared in the "Philippine Agriculturist" as Reinking was a mycologist from the College of Agriculture at Los Baños, Philippines. According to Reinking's survey, citrus yellow shoot was of little importance. At that time the nature of the disease was not known. Water logging, high water table in lowlands, poor drainage in uplands,



nematode soil-infestations, fusarium infections of roots or micronutrient deficiencies were evoked as possible causes of HLB. Reinking himself thought that the disease was the result of water logging (Lin, 1956). As pointed out by Lin (Lin, 1956), both HLB and water logging are characterized by leaf yellowing. The two types of decolourations are quite different, but can be mistaken, and the possibility exists, as was pointed out by Beattie et al. (2008) that Reinking was not looking at HLB symptoms, but probably at those of water logging and should not get credit for the first report of HLB in China, if not Asia (Beattie et al., 2008). Apparently, the first accurate observation and description of the disease in China was given by Chen Q. Bao in 1943, when the disease had already become a serious problem (Chen, 1943). He observed the disease in the Chaozhou/Shantou district of North Eastern Guangdong province. It was precisely in this citrus district that, according to Lin (Lin, 1956), the disease originated and was gradually spread with planting material from there to other citrus places. It was also in the Chaozhou/Shantou district that the disease was called Huang Long Bing (=Yellow shoot disease) by the farmers in their southern Chinese dialect. In other districts, the disease had other names, but finally "huanglongbing" prevailed and was used by Lin in his 1956 publication, the one that demonstrated transmission of HLB by graft-inoculation (Lin, 1956). Before Lin, Chen Q. Bao (1943) had also used grafting experiments to document the transmissibility of HLB, but, instead of using graft-inoculation, he used graft-propagation, which demonstrated only "carry over" of the disease (Lin, 1956). Between 1949 and 1954, Lin made observations suggesting natural transmission and spread of HLB, but he did not mention *Diaphorina citri* (Lin, 1956) {*Identification of the citrus psyllids as vectors of HLB came only in 1965 in South Africa (*T. erytreae*) (McClean and Oberholzer, 1965b), and shortly thereafter in India and the Philippines (*D. Citri*) (Capoor et al., 1967; Salibe and Cortez, 1968; Martinez and Wallace, 1967)*}.

Following the dodder transmission, from citrus to periwinkle plants, of the South African greening bacterium and the Indian citrus decline bacterium in 1983 (Garnier and Bové, 1983), similar transmissions were also reported in 1988 for the Chinese huanglongbing bacterium and confirmed the 1983 results (Ke et al., 1988). The Chinese HLB bacterium was identified as Las by PCR (Jagoueix et al., 1996; Garnier and Bové, 1996; Tian et al., 1996).

Diaphorina citri was recorded as present in southern China in the late 1910s by Crawford (Crawford, 1917, 1919) and in 1933 by Clausen (Clausen, 1933). For Hoffmann, who started his work on citrus pests in Guangdong in 1926 (Jiang et al., 1935), the first record of the psyllid in Guangdong was in 1934, and these psyllids carried no parasitoids (Hoffmann, 1936). Luh, who worked on citrus insect pests in Huangyen in 1935 did not mention the psyllid. Hence, according to Beattie et al. (2008), these data suggest that *D. citri* was either not present in China before the 1930s or, if present, it was not widely spread.

In summary, in continental China, HLB probably originated in the Chaozhou/Shantou area and has begun to spread, first with planting material, next with *D. citri*, in the 1930s-1940s, to other citrus growing regions of China.

Taiwan and the Japanese Ryukyu islands are not involved in the debate on the origin of HLB in Asia; they will however be mentioned, immediately below, because of their proximity with Continental China.

15. Taiwan (Fig. 14).

D. citri was mentioned in Taiwan by Kuwayama in 1908 (Kuwayama, 1908), Crawford in 1919 (Crawford, 1919), and Clausen in 1933 (Clausen, 1933). The first record of HLB (named Likubin in Taiwan) is not known with precision, but the disease "has seriously affected citrus trees...since 1951" (Su and Huang, 1990) and "has been prevalent ...since 1958" (Su and Matsumoto, 1972).

The disease agent was first thought to be related to citrus tristeza virus (Matsumoto and Su, 1966; Su and Matsumoto, 1972). Its bacterial nature was eventually recognized (Chen et al., 1971; Su and Leu, 1972; Su and Chang, 1976), once the HLB bacterium had been first described in France (Laflèche and Bové, 1970a; Saglio et al., 1971a). The bacterium was identified as Las by PCR in 1995 (Jagoueix et al., 1996, Garnier and Bové, 1996).

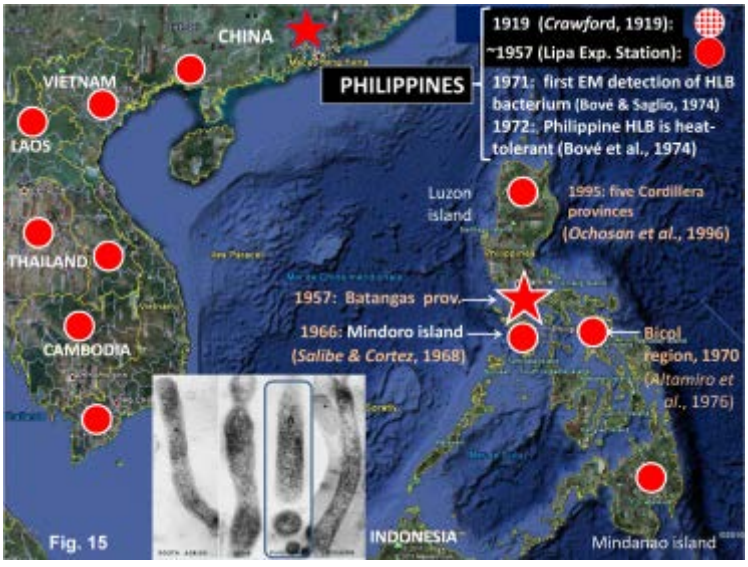
16. Ryukyu islands of Japan (Fig. 14).

The Ryukyu Islands are located between Taiwan and Japan's Kyushu island. One of the southern Ryukyu Islands is only 70 miles from Taiwan. *D. citri* has been reported from the Ryukyu Islands in 1965 (Miyatake, 1965) and the psyllid populations were higher in the southern islands than the northern ones (Miyakawa, 1972). In the early 1970s, extensive field observations gave no evidence of HLB and TEM did not reveal the HLB bacterium (Miyakawa, 1972). The disease was first found in 1988, close to Taiwan, on Iriomote Island, Okinawa prefecture (Miyakawa and Tsuno, 1989) and, in spite of removal of affected trees, the disease was back in 1993. The disease seemed to be moving northward, as in 1994, HLB was found on Okinawa Island (Kawano et al., 1997) and by 1999, nearly all the Okinawa region was contaminated with the exception of the Daito islands (Naito et al., 2001). Surveys carried out North of Okinawa between 2002 and 2006 showed that four of the five Amami islands were found to be affected, with no HLB on Amami, the most northern island (Shinohara et al., 2006). Since 1997, HLB symptoms have been confirmed by PCR and Las was the only Liberibacter detected (Subandiyah et al., 2000; Naito et al., 2001; Shinohara et al., 2006).

On the basis of the bacteriophage-type DNA polymerase gene (DNA-pol) present in the las genome, two distinct Las genotypes can be distinguished: Las genotypes with the DNA-pol sequence (DNA-pol$^+$ genotype) and those without (DNA-pol$^-$ genotype) (Tomimura et al., 2010). Taiwanese Las isolates were of the DNA-pol$^+$ genotype (Taiwan genotype). The Las isolates from nine of the eleven Ryukyu Islands between Taiwan and Okinawa had also the Taiwan genotype. However, all Las isolates from Okinawa and Amami islands were of the DNA-pol$^-$ genotype (Japan genotype) (Tomimura et al., 2010).

17. Philippines (Fig. 15).

In 1921, H. A. Lee described mandarin and Valencia sweet orange trees grafted on two different rootstocks (Lee, 1921). On mandarin rootstock, the trees grew well, without symptoms, but on pummelo rootstock, the trees were severely diseased and showed mottled leaf, hence the title of Lee's 1921 paper: "the relation of stocks to mottled leaf of citrus trees". The words "mottled leaf" were reminiscent of a major leaf symptom of huanglongbing (HLB): blotchy mottle. However, Martinez and Wallace (1968) have discussed the possibility that, in fact, the above trees were infected with citrus tristeza virus (CTV). If so, from what we know today, only the trees on pummelo rootstock represented a CTV-sensitive scion/rootstock combination and they showed, as expected, the "mottled leaf" symptom, most likely zinc deficiency symptoms, whereas the trees on mandarin rootstock were CTV-tolerant and, as expected, showed no symptoms. The likelihood of CTV being present in the Philippines has been documented by Wallace et al. (1956). Records of the Philippine Experiment Station show that, in the early part of the 20th century, many introductions of citrus came from countries infected with CTV. Also, budwood of Batangas mandarin, introduced to the United States from the Philippines prior to 1930, carried CTV. Therefore, as pointed out by Beattie et al., (2008), Lee, in his 1921 publication, was not describing HLB symptoms, but probably those of CTV. The situation is similar to that in China in 1919 when Reinking was probably looking at trees affected by water logging, rather than not HLB.



There is now a general consensus that HLB was first noticed by technicians of the Lipa Experiment Station in the Batangas province of Luzon island in 1957 (Salibe and Cortez, 1968; Martinez and Wallace, 1968; Cortez and Celino, 1972; Altamirano et al., 1976). Citrus trees in Batangas province (South Luzon Island) and surrounding provinces became severely affected and were practically wiped out. The origin of the

disease was seen in the introduction, prior to 1957, of infected citrus plants from China and India (Altamirano et al., 1976; Benemerito, 1956). The spread of the disease occurred from Batangas to other parts of the country with planting material shipped to areas like the Bicol Region, where citrus was expanding. In 1966, the disease was seen in Calapan, northeastern Mindoro Island (Salibe and Cortez, 1968) By 1993, five provinces of the northern Cordillera region of Luzon Island were contaminated (Ochosan et al., 1996). Affected trees were mainly found in the southern Benguet province, most probably due to its proximity to the lowland areas where the disease was most prevalent. It was also the major entry point of planting material from contaminated nurseries in the lowlands.

The great similarity of Philippine leaf mottling with vein-phloem degeneration in Java, Indonesia (Tirtawidjaja et al., 1965), huanglongbing in southern China (Lin, 1956), likubin in Taiwan (Su and Matsumoto, 1972) and greening in South Africa was stressed by Salibe and Cortez (1968) and Martinez and Wallace (1968). Salibe and Cortez (1968) were also among the very few ones who, in 1966, drew attention on K. H. Lin's work on huanglongbing in China (Lin, 1956) as well as on A. Ciccarone's article on Lin's work in an Italian citrus journal in 1957 (Ciccarone, 1957). Published when times were difficult in China, this article was important because it revealed Lin's work to the western world.

As mentioned in Part I.3, Philippine leaf mottling and Indian citrus decline were the first two Asian HLB diseases shown to be heat-tolerant, while South African greening was found heat-sensitive (Bové et al., 1974). Also, historically, the HLB bacterium was detected for the first time by TEM in citrus affected by each one of the three above diseases (Saglio et al., 1971a). The HLB bacterium from the Philippine leaf mottling disease was identified as Las by DNA hybridization and PCR (Villechanoux et al., 1992; Garnier and Bové, 1996; Jagoueix et al, 1996) and these results were confirmed later (Harakava et al., 2000). Cruz et al. (2010) have also detected "*Candidatus* Liberibacter sp" by PCR, but they did not determine whether the involved species was Laf or Las.

D. citri was recorded as present in the Philippines in 1919 (Crawford, 1919). The psyllid was widely distributed in the country, but its population density varied considerably (Cortez and Celino, 1972). Batangas province in South Luzon, where leaf mottling was severe, had also a large psyllid population. Higher North, in Laguna and Cavite provinces, disease incidence was intermediate and psyllid populations ranged from low to high. Shortly after 1965, when *T. erytreae*, the African citrus psyllid, was reported to transmit greening in South Africa (McClean and Oberholzer, 1965b), *D. citri,* the Asian citrus psyllid, was found to transmit leaf mottling in the Philippines (Salibe and Cortez, 1968; Martinez and Wallace, 1967) and citrus decline in India (Capoor et al., 1967).

18. Indian Subcontinent

In recent times, countries on the Indian Subcontinent underwent historical changes. In 1947, India became independent, but lost newly founded Pakistan (East and West), and the Punjab was split between India and West Pakistan. In 1971, East

Pakistan became independent as Bangladesh, and West Pakistan became just Pakistan.

18.1. India/Pakistan (Fig. 16, 17, 18).

Citrus in India/Pakistan has been known to suffer seriously from certain disorders resulting in low production, twig dieback, slow death, and even sudden wilting. These symptoms were attributed to "decline", a disease supposed to have been first observed by Roghoji Bhonsale (cited by Capoor, 1963) in the 18th century, soon after the introduction of citrus into India, as well as by Bonavia in 1888 in Assam (Bonavia, 1988). However, the problem with Indian decline comes from the fact that it is not a disease with specific symptoms (Asana, 1958) and, over the years, many factors, including soil disorders, nutritional deficiencies, twig fungi, and viruses such as citrus tristeza virus (CTV), were evoked to account for it (Capoor, 1963). Eventually, support for the involvement of HLB in decline came in 1958 in the Coorg region of southern India when characteristic leaf mottling symptoms of HLB were observed (Asana, 1958) and especially in 1966 when a survey led by Lilian Fraser in all major citrus areas of India, concluded that decline was caused by the "virus" responsible for greening in South Africa (Fraser & Singh, 1968). Unquestionable proof for the presence of HLB in India came in 1967 when successful transmission of the decline agent, free of CTV, was obtained with the Asian citrus psyllid, *D. citri,* by Capoor in Poona, Maharashtra (Capoor *et al*., 1967). Finally, in 1971, Bové and co-workers detected the HLB bacterium in a Musambi sweet orange seedling, made available to them by Prof. Capoor and experimentally infected, through *D. citri* transmission, with Poona decline (Poona HLB isolate) (Laflèche and Bové, 1970a; Saglio et al., 1971a). Furthermore, together with Philippine leaf mottling, the Poona citrus decline was found to be heat-tolerant in 1972 (Bové et al., 1974).

However, the above account does not give full credit to the early studies of HLB and its vector, *D. citri*, in India/Pakistan. Indeed, the history of HLB in the 1920s, had to be amended when Bindra (1970) and in particular Beattie et al. (2008, 2010) drew attention on a 1927 publication of Husain and Nath (Husein and Nath, 1927). In their memoir, the latter authors described, as early as 1927, symptoms characteristic of HLB on citrus trees at Sargodha, Faisalabad and Gujranwala in the Punjab of today's Pakistan (Fig. 16). Ironically enough, Husein and Nath didn't realize that they were looking at HLB! They thought that the symptoms they observed were due to damage caused by *D. citri* psyllids feeding on the trees. The Asian citrus

psyllid, *D. citri,* was known in India at least since 1922, when Waterston (1922) reported the presence of the parasitoid, *Tamarixia radiata,* on the psyllid in the Punjab.

Therefore, it is accepted today that the description of HLB symptoms by Husein and Nath in India/Pakistan in 1927 (Husein and Nath, 1927), took place before such symptoms were reported from China and the Philippines, as the 1919 report of Reinking in China (Reinking, 1919) and the 1921 report of Lee in the Philippines (Lee, 1921) did not describe HLB but, respectively water logging and CTV symptoms, as seen above. Thus, it is India/Pakistan, but not China, the Philippines or any other zone, which probably represents the region where, in Asia, HLB originated. It will be seen below that other arguments, in particular those based on the Gondwanan origin of Laf and Las, support this conclusion (Nelson et al., 2013).

In 1987 in today's **Pakistan**, the HLB bacterium has been detected by TEM by Garnier and Bové in mottled leaves of sour orange seedlings in the Peshawar district (Bové, 1995b), and by Catara and coworkers (Catara et al., 1988) in sweet orange in the Sahiwal-Okara district and in rough lemon leaves at the Faisalabad Research Station. In 2007, PCR and sequencing of the amplified DNA identified Las in citrus samples collected at Rabaat, Temurgrah and Peshawar, North-West Frontier Province (Chohan et al., 2007).

The distribution of HLB in today's **India** was studied via an Indo-French project from 1990 to 1993. Leaves with and without blotchy mottle (BM) symptoms were collected in various regions of India. Detection of the HLB bacterium in midribs of these leaves was carried out by TEM, dot blot DNA hybridization (dbH) and ELISA with monoclonal antibodies (MAs). The DNA probe, In 2.6, and the MAs, 2D12 and 10A6, were produced from the Asian, heat-tolerant Poona isolate of HLB (Bové et al.,1993a). In the case of leaf samples showing BM, 41 of 48 samples were positive by TEM, 39 of 42 by dbH, and only 4 of 14 by ELISA, the 4 positives being in only 1 of 4 orchards. None of the 11 samples without BM was positive by either one of the techniques. This showed the advantage of using BM leaves for testing. Most samples found positive by TEM were also positive by dbH. Elisa with MAs directed against the Poona isolate of HLB detected the HLB bacterium in only 1 of 4 orchards tested, showing that these MAs were unsuitable (too specific) for diagnostic purposes. The presence of HLB as based on the detection of the HLB bacterium was confirmed in the following regions: Andhra Pradesh (Hindupur, Tirupati), Delhi

(Agricultural Research Center), Karnataka (Bangalore, Coorg area), Maharashtra (Poona), Orissa (Angul, Subalda) and Rajasthan (Jhalawar) (Varma et al., 1993).

In 1991, the HLB bacterium was also detected by TEM in **Uttar Pradesh** at Dakrani where HLB incidence and natural transmission were low, and especially at Jeolikote where HLB incidence and natural transmission were high (Azzaro et al., 1993). In the Jeolikote area, at an elevation of 1220m and an average maximum monthly temperature (a.m.m.T) of 35°C, the conditions were more favourable for *D. citri* than at Dakrani with an elevation of 500m and an a.m.m.T as high as 39°C.

The presence of HLB in the North-Eastern region of India (**Sikkim, West Bengal, Meghalaya, Assam**) has been confirmed by PCR analyses, which identified Las not only in suspicious plant samples but also in psyllids from affected orchards in the regions visited (Das et al., 2007; Miyata et al., 2011). Las has also been reported from Vidarbha in Maharashtra (Das, 2009).

In Karnataka State (southern India), 13 mandarin leaf samples from HLB-affected mandarin trees and 5 samples from asymptomatic trees were collected in different localities and analyzed for liberibacters by (i) PCR amplification of 16SrDNA (Jagoueix et al., 1996) and (ii) beta-operon ribosomal protein genes (Hocquellet et al., 1999). Las was detected in all symptomatic leaf samples, while all symptomless samples tested negative. The 16SrDNA amplicons from two representative Karnataka HLB-affected mandarin trees were sequenced. The two sequences were identical (100% identity) and shared 97-99% identity with 50 previously reported Las sequences from India (Poona, Nagpur), Japan, China, Taiwan, Malaysia, Brazil (São Paulo) and USA (Florida) (Adkar-Purushothama et al., 2009).

18.2. Bangladesh (Fig. 17, 18).

Citrus occurs in the hilly areas to the Northwest of the country, near the Assam border. *D citri* was first recorded in East Pakistan, today's Bangladesh, in 1962 and 1965 (Alam, 1962, 1965). Typical symptoms of HLB blotchy mottle were observed in 1974/1975 on sweet orange and lemon trees. The HLB bacterium was detected by TEM in symptomatic leaves from these trees (Catling et al., 1978) This was the first report on HLB in Bangladesh.

18.3. Afghanistan (Fig. 16).

D. citri has been reported from Afghanistan in 1971 (Faizyar, 1971) but it is not known whether HLB is present or not.

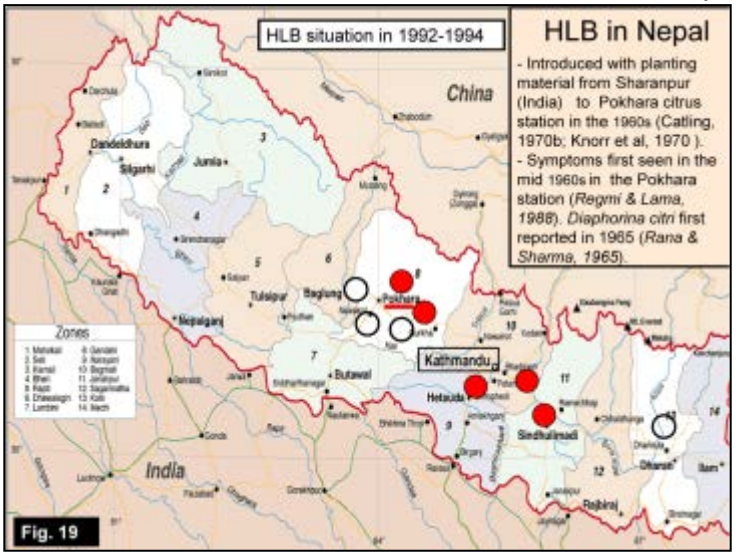
18.4. Sri Lanka (Fig. 17).

In 1993, 11 leaf samples were collected on trees showing blotchy mottle (Garnier and Bové, 1996). Ten were positive by TEM detection of the HLB bacterium

and by DNA hybridization with probe In 2.6, specific of Asian HLB (Villechanoux et al, 1992). This was the first evidence for the presence of Las-associated HLB in Sri Lanka. Occurrence of *D. citri* was mentioned in 1961(Ahmad, 1961).

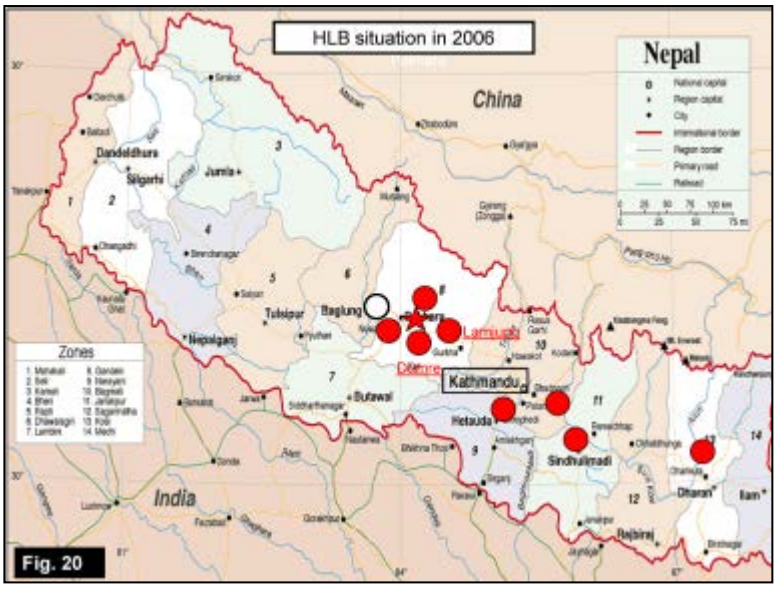
18.5. Nepal (Fig. 18, 19, 20, 21).

In Nepal, most commercial citrus trees are seedling mandarin trees, grown essentially in the low- and mid-hills, between 400 and 1600m. The presence of *D. citri* was reported in 1965 (Rana and Sharma, 1965). HLB was first observed in the mid 1960s in citrus orchards of the Horticulture Research Station at Pokhara (altitude 935m), half way between the western and eastern limits of the country (Regmi and Lama, 1988). The Pokhara station was opened in 1960 to produce planting material to increase the area under mandarin cultivation (Budathoki and Pradhanang, 1992).



During that period large numbers of citrus trees were imported from Sharanpur in Uttar Pradesh, India, and distributed to citrus growers in the Pokhara valley. This is how, unknowingly, HLB was imported from India and why Pokhara became the initial focus of the disease (Knorr et al., 1970), a disease which has wiped out the mandarin industry in the Pokhara valley. As the trees were seedlings, spread of the HLB agent was not through infected budwood but by *D. citri*, the psyllid vector of HLB, reaching high populations in May, June and July (Regmi and Lama, 1988), in the absence of any control measures and in spite of attempts to initiate biological control (Lama et al., 1988).

A citrus rehabilitation project involving Nepal, France, Spain and Brazil, supported surveys for graft-transmissible diseases of citrus were conducted in various parts of Nepal. Symptomatic leaf samples from trees suspicious of HLB were collected for liberibacter detection by (i) DNA hybridization (Regmi et al., 1996) in December 1992 (23 samples) and April-May 1994 (29 samples) or (ii) PCR according to Teixeira et al., (2005b) in October 2004 (8 samples), October 2005 (14 samples) and December 2006 (38 samples). The



altitudes of the sampled orchards were recorded with an altimeter. Presence of *D. citri* was also noted.

On the basis of the 2004-2006 surveys and PCR analyses (Regmi et al., 2010), Asian HLB with Las and *D. citri* was confirmed in the following regions: (i) Pokhara region (Pokhara ~900m, Hamta ~900m, Lamachaur ~900m, Batulechaur ~900m, Pokhere ~950m, Syangja ~850m, Bandipur/Dumre ~450m, and Lamjung ~1000m), (ii) Kathmandu region (Shankuthree ~1300m, Kirtipur ~1350m)), (iii) Sindhulimadi region (~650m), (iv) Dhankuta/Karmitar region (Dhankuta ~1120m, Paripatle Horticulture Station ~1200m / ~1350m).

Some of the above locations, positive for HLB in 2004/2006, were still negative in 1992/1994: (i) Pokhara region (Syangja, even though *D. citri* was already present, and Bandipur/Dumre, *D. citri* being still absent) and (ii) Dhankuta/Karmitar region (Dhankuta and Paripatle Horticulture station, *D. citri* being still absent). The Bandipur/Dumre orchard, without HLB in 1994, was by 2006 totally destroyed by the disease. Except for the Kathmandu area, liberibacter-infected trees were not found at altitudes higher than ~1300m, and similarly, the psyllid vector, *D. citri*, was not seen above this altitude. Areas at or above 1300m where HLB and *D. citri* were not seen, even in 2006, were: Lumle (~1750m, West of Pokhara), and Bijayachap (~1300m) above Sindhulimadi (which itself is at ~600m and has both HLB and *D. citri*). The Paripatle horticulture station is on a steep hill side between ~1200m and ~1350m; only trees in the lowest location were affected by HLB.

Fig. 21

HLB in NEPAL

Location	Altitude	1992-1994		2006	
		D. citri	HLB	*D. citri*	HLB
Lumle (West of Pokhara)	1750m	–	–	–	–
Pokhara region					
Pokhara	~900m	+	+	+	+
Hamta	~900m	+	+	+	+
Batulechaur	~900m	+	+	+	+
Pokhere	~950m	+	+	+	+
Syangja (South of Pokhara)	~850m	+	–	+	+
Dumre (East of Pokhara)	~450m	–	–	+	+
Lamjung (Northeast of Pokh.)	~1000m	– in 1986	– in 1986	+	+
Kathmandu region					
Shankuthree	~1300m	+	+	+	+
Kirtipur	~1350m	+	+	+	+
Sindhulimadi	~650m	+	+	+	+
Dhankuta/Karmitar region					
Dhankuta	~1120m	–	–	+	+
Paripatle Hort. Stat.	1200m-1350m	–	–	+	+

These data as a whole suggest that HLB in Nepal is the result of incursions from India and further spread by *D. citri*. Both the disease and the psyllid vector occur below ~1400m.

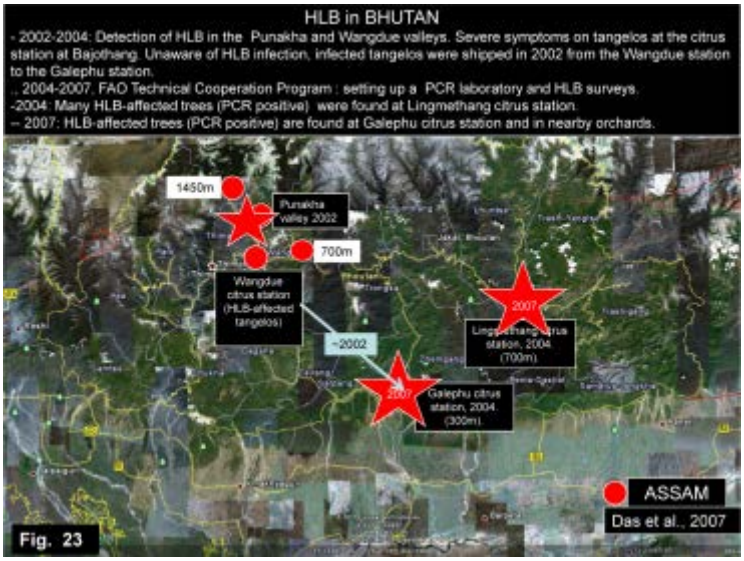
18.6. Bhutan (18, 22, 23).

Mandarin seedling trees represent the most important horticultural crop in Bhutan. They are grown on an estimated area of 5 500 hectares, for the most part at an altitude of 200 meters, 1 800 meters being the upper limit. The annual mandarin production in Bhutan is over 29 000 tonnes. However, the mandarin industry has been plagued with decline problems. The possibility arose that the decline could be caused by HLB. A survey was carried out by Yuichi Tomiyasu (JICA) from June to December 2000. Symptoms of huanglongbing (HLB) were observed on trees located

below 1000 m of altitude, but not on those above 1400 m, *i.e.* at altitudes where the insect *Diaphorina citri*, the Asian psyllid-vector of HLB, cannot survive. However, in the absence of a diagnostic laboratory in Bhutan, the putative HLB symptoms could not be confirmed by PCR detection of the HLB-bacterium and no precise conclusions could be drawn from the survey.

To further investigate the problem, two surveys, one in August 2002 (Garnier and Bové, 2002) and one in March 2004 (Duran-Vila and Bové, 2004), were carried out. In addition, a three-years-long Technical Cooperation Program (TCP/BHU/3001 A) was set up by FAO. Three surveys were conducted in the frame of the TCP: in October 2004 (Bové, 2004), April/May 2006 (Bové, 2006), and September 2007 (Duran-Vila, 2007; Yamamoto, 2007). The presence of HLB in Bhutan was established on the basis of (i) characteristic symptoms, and in particular leaf blotchy mottle, (ii) detection of the associated liberibacter in symptomatic leaves by specific PCR reactions, and (iii) presence of the insect-vector, *Diaphorina citri*. Leaf samples from the first three surveys (08/2002, 03/2004 and10/2004) had to be analyzed by PCR in the Bordeaux laboratory; those from the April/May 2006 and September 2007 surveys were worked up in the new PCR laboratory, established in the frame of the FAO-supported TCP at the National Plant Protection Center (NPPC), Timphu,. Results of the surveys were as follows (Bové, 2008).

18.6.1. HLB in Punakha and Wangdue valleys (~1450m to ~700m). The "mother" river Mo Chu (coming from North West) and the "father" river Pho Chu (coming from North East) meet at Punakha to form the Puma Tsang Chu, which flows down South through Wangdue city and Kamechu orchard, crosses the Bhutan–India border at Kalikhola and eventually joins the Brahmaputra river. The surveys in the valley have shown that HLB extends from the most northern orchard visited, the Rimchu orchard at ~1450 m, North of Punakha,



all the way down to the most southern orchard inspected, the Kamechu orchard, at ~700 m, South of Wangdue. The most severely affected area was that comprising the Phuntshopelri and Sonagasa orchards at ~1350m near Punakha. Orchards at the northern and southern limits of the survey were much less affected.

The initial trees of the **Phuntshopelri orchard** (~1350m) were imported from India and were 25 years-old in 2002. The mandarin trees grew well until the 1990s, when decline became noticeable. By 2002, large numbers of trees were affected and many trees had already been replaced. Blotchy mottled leaves characteristic of HLB were present and PCR detected Las in these leaves. The presence of HLB in Bhutan was thus confirmed (Garnier and Bové, 2002; Doe Doe et al., 2003). Similar results were obtained by others (Ahlawat et al., 2003).

The **"Lower Sonagasa" orchard** (~1350m), separated from the Phuntshopelri orchard by the Mo Chu river, was planted in 1994 on a flat piece of land. In 2002, it was the perfect example of an orchard in the process of being invaded by HLB. Some of the mandarin trees were still nice and green. Others were partly green, partly yellow. Still others were totally yellow. Mottled leaves could be seen within the yellow parts of the trees. Such mottled leaves were collected on affected trees, and gave strong positive PCR reactions for Las. Most likely, infection of the Lower Sonagasa orchard came from the nearby Phuntshopelri orchard.

The **Rimchu orchard** (~1450m) was the most northern and the most elevated one of those surveyed in 2002, when the orchard had only a few trees with HLB symptoms and testing positive for Las by PCR. In September 2007, many trees were still in good shape, even though some trees had HLB symptoms and were PCR positive. The **Kamechu orchard** (~700m) was at the lowest altitude of those surveyed in 2002, when only a few trees in the orchard tested positive for HLB. However, in October 2004 many more trees were affected by HLB, and in September 2007, most of the trees had conspicuous symptoms and were declining. These results show that in mandarin orchards at the relatively high altitude of 1450m, HLB evolution and HLB incidence is not as severe as in orchards at lower altitudes, such as the Kamechu orchard, at 700m. This observation was already made by Yuichi Tomiyasu (JICA) after his surveys of June to December, 2000. Obviously, at 700m, the climatic conditions are much more favorable for *D. citri* than at 1450m. As a matter of fact, the Bhutanese entomologists have not seen the psyllid vector at Rimchu, but did find it at Kamechu.

Bajothang research center (~1250m). The Renewable Natural Resources Research (RNRR) Center at Bajothang, North of Wangdue, had a mother tree collection as well as collections of mandarins, satsumas and tangelos. Budwood from certified citrus species and varieties had been imported from the Citrus Experiment Station in the French island of Corsica (free of HLB), through Mr. John Goelet and Yop Carlier, and grafted on various rootstocks. Because of unawareness of HLB, all trees were planted in the open, and became an easy prey for the HLB psyllid vector. HLB was detected at the Bajothang center during the 2002 survey. Trees with HLB symptoms were again seen in October 2004 as well as in May 2006, in spite of the fact that some HLB-affected trees had been removed after the October 2004 visit.

Severe HLB symptoms were still present in September 2007 and confirmed positive for Las by PCR. Particularly strong symptoms of leaf blotchy mottle were observed on Minneola, Seminola and Iyo tangelos, as well as Bears lime, and Las was detected by PCR.

In **conclusion**, the origin of HLB in the Punakha/Wangdue valleys is to be found in the Phuntshopelri orchard in which some of the initial trees imported from India in 1977 were most probably infected with the HLB agent. The most southern orchard affected by HLB was the Kamechu orchard (~700m). Further down South, the **Damphu/Tashipang** region, where many trees had HLB-like branches and shoots with yellow leaves, was surveyed in March 2004. The symptoms were not due to HLB, but caused by Phytophthora foot-rot and/or gummosis, all PCR tests for liberibacters being negative (Duran-Vila & Bové, 2004).

18.6.2.. **HLB in the Galephu Region** (Sarpang district). In May 2004, two samples from the **Bhur RNRR substation** (~260m) at Gelephu, close to the Bhutan/India border, were examined by PCR for liberibacters by Dr. C. Regmi, National Academy of Science and Technolgy, in Kathmandu, Nepal, and found positive for Las.

On September 20, 2007, in the frame of the FAO-TCP, we have surveyed the citrus trees at the Bhur substation, and have observed severe HLB symptoms on many cultivars. PCR confirmed the presence of Las in trees of Seminole tangelo, Mineola tangelo, Oroblanco lemon, Kinnow mandarin, Lanelate sweet orange, and Satsuma. This situation resembled very much the HLB situation at the Bajothang RNRR Center, as described above. Indeed, the citrus varieties affected by HLB at Bhur were introduced from the Bajothang center in the early 2000s, a time when HLB was already spreading at Bajothang, but nobody being aware of it. This is a perfect example of how a disease has been introduced unwillingly from one region into another region by importation of infected plant material.

Thus, in September 2007 at the time of the survey, HLB infected trees have been present at the Bhur station since several years. This length of time has probably been enough for the disease to get out of the station and invade private, commercial orchards. Indeed, at **Tatopani**, near the hot spring shrine, HLB symptoms, confirmed by PCR for Las, have been observed during the September 2007 survey on several mandarin trees in a large commercial orchard.

18.6.3. HLB in the Lingmethang Region (Mongar district). Mr. Yuichi Tomiyasu (JICA) is said to have suspected HLB in the Lingmethang substation around 2000.

HLB symptoms were detected at the **Lingmethang RNRR substation** for the first time in October 2004, at the occasion of the first TCP mission, and positive PCR tests confirmed the presence of Las (Bové, 2004). Surveys for HLB in nearby commercial mandarin orchards were negative. Therefore, it was recommended in the first TCP report *"that all citrus trees be removed from the substation in order to avoid propagation of the disease throughout the whole region"*. On April 29, 2006, during the second TCP mission, it was found that only two trees had been removed, and it was recommended again that **all** trees be pulled out (Bové, 2006). Eventually, the

trees were removed on August 14, 2006, but an orchard of Thai mandarin and pummelo trees from Wengkhar RNRR Center (Mongar district) was planted immediately thereafter, on August 20, 2006. Fortunately, at the **Wengkhar center**, citrus is located at **~**1450m, an altitude where the HLB psyllid vector is thought to be absent and has not been detected. No HLB symptoms have been observed at the Wengkhar center in October 2004 and September 2007, and no HLB symptoms were observed on the trees from Wengkhar at the Lingmethang substation during the visit of September 18, 2007. Unfortunately, HLB symptoms were observed the same day, on September 18, 2007, on a mandarin tree in an orchard at **Thridangbi village,** within the Lingmethang region, and PCR has confirmed the presence of Las. In other words, the disease has escaped the substation and has entered private orchards, a situation similar to that witnessed in the Galephu region (see above).

18.6.4. *Diaphorina citri* and *Diaphorina communis* in Bhutan. During our surveys from 2002 to 2007, ***Diaphorina citri*** has been seen in orchards at altitudes between ~1350m and ~260m. However, there are no records as to when the Asian citrus psyllid has been detected for the first time in Bhutan. During the 2007 survey, the psyllid has also been seen on *Murraya paniculata*, its favoured host, in particular at Galephu (Yamamoto, 2007).

On his arrival in Bhutan for the 2007 TCP survey, Dr. Pedro Takao Yamamoto, the TCP entomologist, was told of a dark-colored psyllid. He collected several specimens of this "black" psyllid and sent them to Dr Daniel Burckhardt (Naturhistorisches Museum Augustinergasse 2 CH-4001 Basel, Switzerland) for identification. The answer was: "***Diaphorina communis* Mathur, 1975",** and this was the first identification of this psyllid species in Bhutan (Yamamoto, 2007; Bové, 2008). In his TCP report to the Bhutan government, Yamamoto also suggested investigating the possibility for *D. communis* to transmit Las, the Asian HLB liberibacter, and he provided protocols to do so. The presence of *D. communis* in Bhutan has been recently confirmed, and, in addition, the presence of Las in the psyllid has been reported (Donovan et al., 2012). *D. communis* has been described from Uttar Pradesh, India (Mathur, 1975). Common on *Murraya koenigii*, it occurs occasionally on citrus.

18.6.5. Conclusion: HLB detection in Bhutan. In conclusion, HLB and Las, the associated, Asian liberibacter, have been identified in the following districts from 2002 to 2007 in the frame of the FAO-supported TCP: Punakha, Wangdue, Samtse, Sarpang, Zhemgang and Mongar. Additional HLB-affected districts have been identified after 2007: Dagana, Tsirang, Tashi Yangtse, Pema Gastel and Samdrup-Jongkhar. Some of these districts reach the border between Bhutan and the North East regions of India, where Las-associated HLB and Las-infected *D. citri* psyllids have been detected (Das et al., 2007). Like in Nepal, the origin of HLB in Bhutan is to be seen in the importation of infected planting material from India and redistribution of unknowingly infected plants within the country, as well as by *D. citri*, in the southern districts in particular.

19. Indochina (Fig. 24).

Indochina, between the Indian Subcontinent and Continental China, comprises Myanmar (*ex* Burma) to the West, Vietnam to the East, and Thailand, Cambodia, and Laos in between. Some of these countries have had wars and revolutions during the 20th century and these periods of unrest did not favor observations in plant pathology in general and HLB in particular.

19.1. Myanmar.

D. citri was reported from Myanmar in 1933 (Clausen, 1933), 1959 (Ebeling, 1959) and 1993 (Waterhouse, 1993). Samples with blotchy mottle or yellow leaves were collected in April 1996 and taken to Bordeaux, France, for 16SrDNA-based PCR detection of the involved liberibacters (Jagoueix et al., 1996, 1996b) The liberibacter detected in all positive samples was Las. Samples (sa.) positive for Las were from Pegu/Sarlay (1 pummelo sa., 1 lime sa.), Mandalay (2 lime sa.), Kalaw (1 mandarin seedling sa.), Pindaya (4 mandarin sa., 1 lime sa., 1 rough lemon sa.), Aungban (2 navel sa.), and Inle lake (1 rough lemon sa., 1 mandarin sa., 1 lime sa.), all together 16 positive samples out of 18 (Garnier and Bové, 2000). This was apparently the first report of HLB in Myanmar.

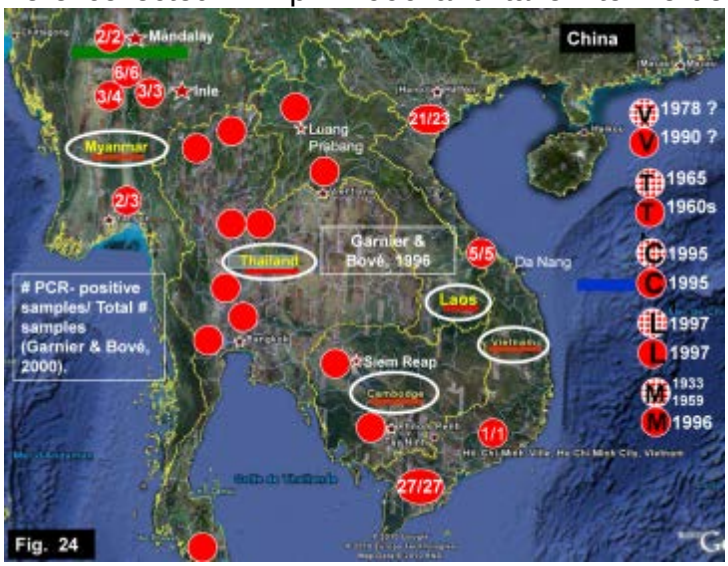


Fig. 24

19.2. Thailand. *D. citri* was reported in 1965 (Anonymous, 1965), 1968 (Thrower, 1968), 1970 (Catling, 1970) and 1973 (Schwarz et al., 1973). According to Schwarz et al. (1973), HLB first appeared in the 1960s. The devastation of citrus in Thailand by HLB was described by Roistacher (Roistacher, 1996).

The HLB bacterium was detected by Koizumi *et al*. in symptomatic leaves during their 1992-1993 surveys, which showed the disease and its vector to be present in all the citrus orchards surveyed throughout the country (Koizumi et al., 1997).

Eleven blotchy mottle leaf samples from northern, central and southern Thailand were shown by TEM to carry the HLB bacterium; DNA hybridization and/or PCR identified the bacterium as Las (Garnier and Bové, 1996).

Las was also detected by PCR in symptomatic citrus leaves and/or *D. citri* psyllids by Nakashima et al. (1998) and Tomimura et al. (2009).

19.3. Cambodia.

Blotchy mottle leaf samples were collected in March 1995 on pummelo trees with strongly lopsided fruits near Pnom Penh and Siem Reap. They were taken to Bordeaux, France, for DNA hybridizations with Probe In 2.6, specific for Las (Villechanoux et al., 1992). Hybridizations identified Las in all symptomatic samples. *D. citri* was observed during the survey (Garnier and Bové, 2000). This is apparently the first report of HLB and *D. citri* in Cambodia.

19.4. Laos.

Blotchy mottle leaf samples (sa.) were collected in Aprll 1997 near Vientiane (1 pummelo sa.), Mong Buathong (2 pummelo sa., 1 lime sa.), Louangprabang (1 pummelo sa., 1 *Murraya paniculata* sa.), and Ban Xiengleck (1 lemon sa., 1pummelo sa.). In Bordeaux, PCR detected Las in 6 samples out of 8, the negative samples being the *M. paniculata* sample and the pummelo sample from Ban Xiengleck (Garnier, and Bové, 2000). This is apparently the first report of HLB and *D. citri* in Laos.

19.5. Vietnam. *D. citri* was indirectly mentioned in 1978 as a host from which the psyllid parasite *Aphidencyrtus diaphorinae* was reared in Vietnam (Myartseva and Trjapitzin., 1978). It is unclear when HLB was first reported in Vietnam. According to Ha Minh Trung (Ha, 1991), the disease became serious in the 1970s. Symptoms of the disease were described in 1990 after several surveys by Whittle, Van Velsen, or Grisoni (Ha, 1991).

The first symptomatic leaf samples (sa.) for symptom confirmation by laboratory methods [TEM, DNA-hybridization (Villechanoux et al., 1992) and PCR (Jagoueix et al., 1996)] were collected in 1995 in North- (23 sa.), Central- (5 sa.) and South- (27 sa.) Vietnam (Bové et al., 1996). In Ninh Binh and Hoa Binh provinces of North Vietnam, 18 of 23 samples gave positive hybridizations with probe In 2.6, specific of Asian HLB, while 16SrDNA PCR detected Las in 14 of 16 samples. In Hue province of Central Vietnam, 4 of 5 samples were positive by hybridization and 5 of 5 samples, by PCR. In Dong Nai, Tien Giang, Vinh Long, Ben Tre, and Can Tho provinces of South Vietnam, 26 of 27 samples gave positive hybridizations and 27 of 27 samples were PCR positive for Las. The HLB bacterium was detected by TEM in samples from North, Central and South Vietnam.

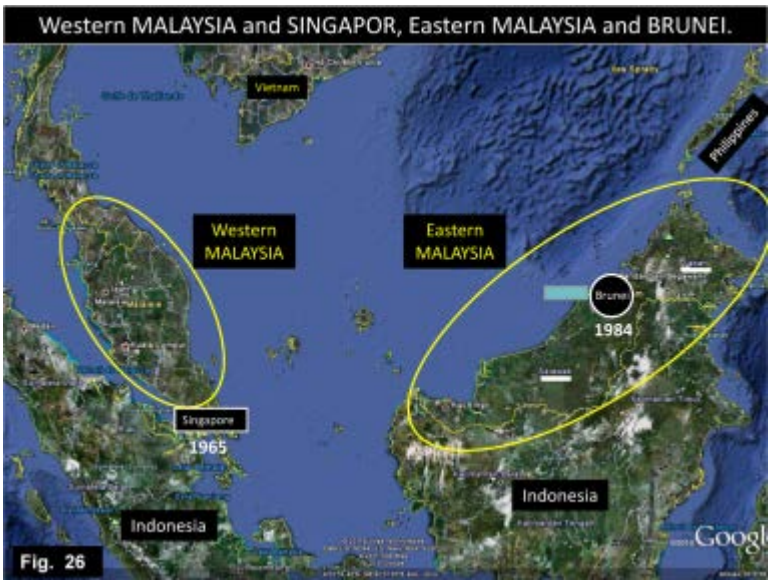
Las was also detected by PCR amplification of 16SrDNA (Jagoueix et al., 1996) and 16/23S intergenic region (Jagoueix et al., 1997) in five Vietnamese field sources by Tomimura et al. (2009).

These results show a wide distribution of HLB in Vietnam, probably due to two major reasons: use of infected nursery plants and transmission by *D. citri.*

20. Malaysia (Fig. 25, 26, 27, 28).

The country is composed of two parts, (i) a western or peninsular part, also named **Malaya**, with the capital, Kuala Lumpur, and (ii) an eastern part comprising two regions of Kalimantan (Borneo), **Sabah** and **Sarawak**, located North of the Indonesian part of Kalimantan.

D. citri was reported from the Malay Archipelago in

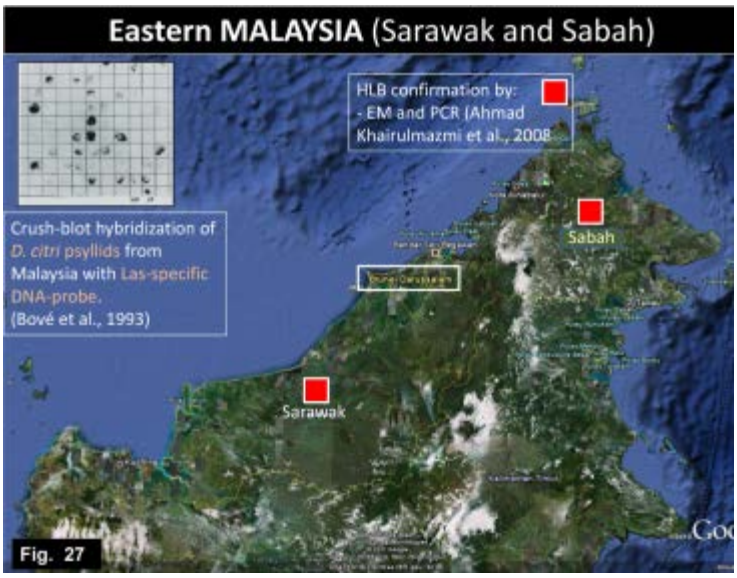


1919 (Crawford, 1919), Malaya in 1933 (Clausen, 1933), and Malaysia in 1959 (Ebeling, 1959) and 1980 (Yunus and Ho, 1980). In the late 1980s, large psyllid populations were seen by Aubert (1989) and Shamsudin and Lim (1989) in many parts of the country.

Until 1989, HLB was reported only on the basis of symptoms and the presence of *D. citri* (Catling, 1985; Ko and Shamsudin, 1987; Shamsudin and Lim, 1989; Aubert, 1989).

20.1. Eastern Malaysia (Fig. 27).

In Sarawak, citrus production involved 2000ha and was mainly for local consumption. Farmers planted essentially the mandarin variety Langkat. In the mid-1980s, HLB started to appear in some citrus farms. By early 1990s, a majority of farms were infected. Farmers abandoned citrus and planted other crops (Teo Chan Hock, 1998). In 1996, the Department of Agriculture started to produce disease-free planting material through shoot-tip grafting. However, only less than 50% of the farmers followed the recommendations (removal of affected trees instead of pruning affected branches, use citrus material from shoot-tip grafting, but discard citrus propagation by air-layering, *D. citri* control). By 2001-2002, HLB became widespread again. By 2006, in one orchard, 11.7% symptomatic trees (55 of 470 trees) were found HLB-positive by PCR at the Plant Pathology laboratory, Semongok (Eng, 2007).

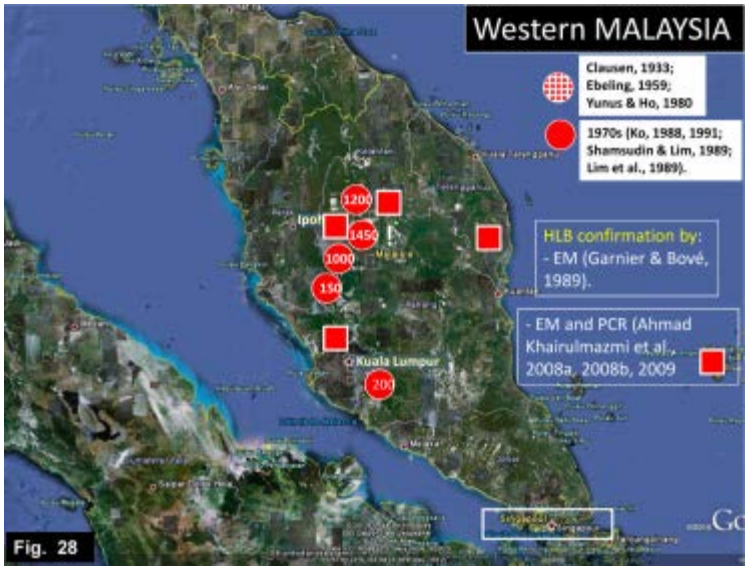


The HLB bacterium was detected for the first time in psyllids collected in September 1991 and May 1992, each time on the same HLB-affected trees within a single orchard, by Dr. Teo Chan Hock, Agricultural Research Center, Sarawak Department of Agriculture. The psyllids were sent to Bordeaux by 48-hour-delivery mail. The psyllids were crushed individually on nylon membranes and analyzed by crush blot hybridization with probe In 2.6, specific of Las (Bové et al., 1993a). Forty-five of the 115 psyllids (39%) from the September 1991crush-blot hybridized with the

probe. On the May 1992 crush-blot, only 5% of the psyllids showed hybridization. Uninfected psyllids raised in Bordeaux and used as negative controls have never given positive reactions.

20.2. Western Malaysia (Fig. 28).

In July 1989, samples of (i) leaf-midribs from symptomatic leaves with blotchy mottle or zinc deficiency pattern and (ii) pedoncular ends of fruit axes from small, lopsided fruits with brownish seeds, were collected in citrus orchards of Kuala Terla (altitude 1200m), Tanah Rata (1450m), Bertam Valley (1000m), Kampong (150m) and Titi (200m). The samples were fixed in glutaraldehyde and treated for TEM detection of the HLB bacterium in Bordeaux (Garnier and Bové, 1989). The HLB bacterium was found in 11 of 12 leaf samples analyzed and in 3 of 5 fruit samples; the 2 negative fruit samples came from 2 trees whose leaf samples were positive and, therefore, no further efforts were made to also detect the HLB bacterium in additional ultrathin sections from these two fruit samples. Detection of the HLB bacterium as reported in this work was the first laboratory confirmation of HLB in Malaysia.



Khairulmazmi et al. (2008a) have used PCR amplification of 16SrDNA with primers OI2c/OI1 (Jagoueix et al., 1996) to detect citrus liberibacters. In this technique, not only Las, but also Laf, yield an 1167bp amplicon. Therefore, when an 1167bp amplicon is obtained, it means that a given "*Candidatus* Liberibacter species" is present, either Las or Laf; it does not mean that Las is present, as was assumed by Khairulmazmi et al. To know whether the 1167 ampicon corresponds to Las or Laf, the 1167bp amplicon can be restricted with restriction enzyme *Xba1*; when three restriction fragments (520bp, 506bp, 130bp) are obtained, the *Candidatus* Liberibacter species involved is Laf; with two restriction fragments (640bp, 520bp), it is Las (Jagoueix et al., 1996). This restriction-enzyme identification of Las or Laf was missing in the work of Khairulmazmi et al. Nevertheless, their PCR work, as well as their TEM detection of the HLB bacterium, showed the presence of an HLB liberibacter, Las or Laf, in the major citrus growing areas of Peninsular Malaysia at both low altitudes (less than 700m) or high altitudes (more than 700m): Selangor (low), Terengganu (low), Pahang (high) and Kelantan (high).

In further work, Khairulmazmi et al. (2008b) did however identify the Malaysian HLB liberibacter as Las from the nucleotide sequence of the 16SrDNA PCR-amplicon of a Honey Mandarin HLB isolate from Terengganu (HLB-T) and Selangor (HLB-S). Two additional HLB isolates, one from pummelo (HLB-Pummelo) and one from mandarin

(HLB-Mandarin) were also identified as Las (Khairulmazmi et al., 2009) from (i) the 16SrDNA nucleotide sequence and (ii) the sequence of the OMP gene (Bastianel et al., 2005), coding for an outer membrane protein. The similarities of the **16SrDNA** sequences among the four Malaysian HLB isolates were between 96 and 99%; the highest similarity (99%) was between HLB-Pummelo and HLB-Mandarin. The latter two isolates had also high 16SrDNa sequence similarities with Indian Las-Poona (99%, 98%), Japanese Las-Okinawa (100%, 99%), Chinese Las-Guangdong (99%, 99%) and Chinese Las-Fujian (99%, 99%). The Malaysian Las 16SrDNA sequences had 84% to 85% similarities with the Laf sequence and 81% to 82% similarities with the Lam sequence. Comparison of the nucleotide sequence of the **OMP** gene showed that there was a high nucleotide similarity (99%) between the two Malaysian isolates, HLB-Pummelo and HLB-Mandarin. Comparisons between the OMP sequences of the latter two Malaysian isolates with sequences of isolates from Thailand, Philippines and China also showed high nucleotide similarity (99%). The similarity of the OMP amino acid sequences of HLB- Mandarin and HLB-Pummelo was equally high (98%). Finally, high amino acid similarities (98-99%) were observed between the Malaysian isolates and isolates from China, Thailand and Philippines.

21. Indonesia (Fig. 29).

21.1. Java and Sumatra.

In 1959, attention was drawn on a type of chlorosis resembling mineral deficiency symptoms affecting citrus in West Java. From 1960 onwards, not less than three million trees were destroyed in West Java and the disease affected also Sumatra (Thrower 1959; Tirtawidjaja, 1980). The fact that Java and Sumatra suffered most was related to the introduction and distribution of citrus nursery stock by the "Pasar Minggu" center located in one of the southern districts of Jakarta, Java. Pasar Minggu has still today a famous fruit-market. The center was blamed for having spread the disease with contaminated citrus material. By 1985, eastern Kalimantan (Borneo), southern Sulawesi (Celebes) and Bali were affected (Aubert et al., 1985).

Fig. 29

Pioneering work on HLB in Indonesia has been carried out by Tirtawidjaja (1964) in the frame of his PhD and Tirtawidjaja et al., (1965). It was shown that the chlorotic decline affecting citrus trees in Indonesia was accompanied by vein-phloem collapse and accumulation of starch, and the disease received the name "citrus vein phloem degeneration" (CVPD). CVPD was found to have similarities to HLB in

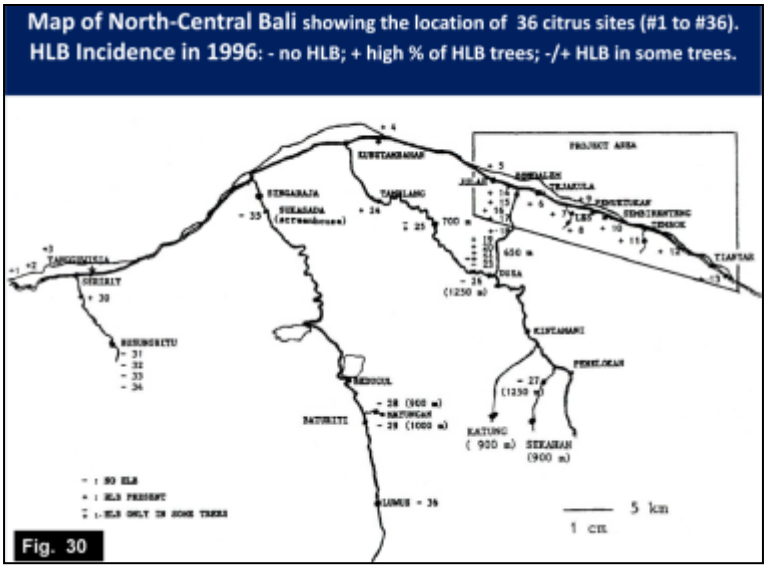
Taiwan (Likubin). Salibe and Cortez (1968) also stressed the similarities of CVPD and HLB in southeastern Asia. In addition, Tirtawidjaja and colleagues transmitted CVPD by graft-inoculation from citrus to citrus, thus establishing its infectious nature, and eliminated not only citrus tristeza virus as a possible cause, but also *Toxoptera citricida* as a vector. Transmission from citrus to periwinkle plants (*Vinca rosea*) by dodder (*Cuscuta* sp., probably *C. australis*) and back transmission from periwinkle to citrus were also obtained (Tirtawidjaja, 1981). This is apparently the first transmission of HLB by dodder, as African and Asian HLB were transmitted from citrus to periwinkle plants only in 1983 (Garnier and Bové, 1983).

Diaphorina citri was recorded as present in Java in 1919 (Crawford, 1919) and 1974 (Ashari; Eveleens, 1974), in Indonesia in 1927 (Husein and Nath, 1927), 1959 (Ebeling, 1959, cited by Catling, 1970) and Aubert, 1987) and 1993 (Waterhouse, 1993). Aubert (1989 b) noticed the psyllid in Flores (or Ende) island.Transmission of CVPD by *D. citri* to citrus (*C. reticulata*, *C. jambhiri*, *C. amblycarpa*, *C. aurantifolia*, *C. limonia*) was reported in 1981(Tirtawidjaja, 1981). From this result, and knowing that Philippine HLB (Leaf motlling) and Indian HLB (citrus decline) had been transmitted by *D. citri* in 1966/1967 (Salibe and Cortez, 1968; Martinez and Wallace, 1967; Capoor et al., 1967), it appeared that CPVD was "*a very severe strain of [HLB], which has wiped out whole areas of citrus in some areas of Java and Sumatra*". Finally, in 1985 the HLB bacterium was detected in mandarin trees by TEM for the first time in Indonesia in the Purworejo coastal plain, near Cangkrep, Central Java (altitude 100m), where HLB was well established since several years, and in the highlands near Majetan, East Java (altitude 1000m), where suspicious symptoms had appeared in recent years (Aubert et al., 1985). These results confirmed the presence of HLB in Indonesia.

21.2. Bali (Fig. 29, 30).

In the early 1970s, when it was learned that HLB was not a virus disease, but a bacterial one, thus theoretically sensitive to antibiotic treatments, Indonesia started a large nation-wide program to control HLB by injecting tetracycline into trees. The program met with total failure. In the mid 1980s, a citrus rehabilitation program based on (i) eradication of infected trees and (ii) production of disease-free budwood by shoot-tip grafting, was initiated (Supriyanto and Whittle, 1991). In Bali alone, this resulted in the removal of 3.6 million trees or 70% of all citrus in the island. With the belief that HLB had been eradicated, more than one million Tejakula mandarin trees on rough lemon rootstock were planted between 1991 and 1993 on the North Bali coastal area, centered on Tejakula city. An irrigation project, financed by the European Union, was started in August 1993 to support the Tejakula mandarin project. Unfortunately, by July 1994, 39% of the newly planted mandarin trees showed HLB symptoms, and by May 1995, the percentage had reached 76%, showing that HLB had not been eradicated. A new rehabilitation program was initiated in 1996 in the frame of the irrigation project (Bové et al., 2000b). A diagnosis laboratory with molecular detection facilities was established at the Punten extension of the Tlekung Agricultural Research Station, near Batu, East Java. PCR amplification of 16SrDNA with primers OI1 and OI2c and/or DNA hybridization with

liberibacter specific probes (Jagoueix et al., 1996b) were used to detect HLB liberibacters in leaf samples from over 200 trees. HLB was confirmed to be present throughout the coastal zones surveyed, from Tegallingah in the West to Tianyar in the East. Almost 100% of the Tejakula mandarin trees were affected and showed severe chlorosis (yellow leaves) and stunting. Many trees had mottled leaves and fruits with color inversion. The liberibacter detected was always Las. Abundant populations of *D. citri* were present and in certain orchards up to 15% of psyllids were positive, the liberibacter being Las (Bové et al., 2000b).



A major finding in North Bali relates to the occurrence of HLB as a function of altitude (Bové et al., 2000b). The North Bali coast, including the irrigation project area, is bordered to the South by steep slopes of two volcanoes: Mount Batur (1,717 m) and Mount Agung (3,142 m). Surveys for HLB were conducted towards the South along the road which climbs over a distance of 16 km from Bondalem on the coast at an altitude of ~10 m to Dusa at ~1,250 m. In **April 1996**, as shown by symptomatology and PCR, HLB was present at a very high incidence (close to 100% infected trees) all along the coast and up to altitudes of about ~500 m. The incidence declined sharply between ~500 and ~700 m. At ~850 m and higher, no HLB-affected trees could be found. As a result of this survey, HLB-affected trees were eradicated in the coastal area and up to an altitude of ~500 m in late 1996 to early 1997, and 90,000 Tejakula mandarin trees, grown from healthy buds, were planted in January 1997 in the zones where trees had been eradicated. A second eradication campaign was conducted from October to December 1997. The young trees planted in January 1997 received insecticide treatments every two weeks for psyllid control. Following these eradication and replanting operations, and as shown by the **August 1998** survey, the areas between ~10 and ~500 m had, as expected, a very low HLB incidence, the ~500 to ~700 m zone had 50% affected trees and between ~700 and ~1,300 m, there were many more trees than in the 1996/1997 surveys.

The Sekahan / Katung area of North-Central Bali, the major Siam mandarin production zone, was also found to be affected by HLB during the 1998 survey. Between ~700 and 1,000 m, HLB incidence was high, but still low above 1,000 m.

21.3. Comparing Las from Indonesia with Las from other Asian countries.

Subandiyah et al. (2000) used leaf midribs of 9 HLB isolates (is.) from several Asian countries (Indonesia: 2 is.; Japan: 4 is. from Okinawa island and 1 is. from Iriomote island; Philippines: 1 is.; Thailand: 1 is.) to amplify liberibacter 16SrDNA with

PCR primers OI1 and OI2c according to Jagoueix et al. (1996 b) and the 16/23S ribosomal intergenic region with PCR primers OI2 and 23S1 according to Jagoueix et al. (1997). Identification of Las was based (i) on restriction of the 16SrDNA amplicons with *Xba1* (Jagoueix et al., 1996 b) as well as (ii) on the nucleotide sequence of the 16SrDNA amplicons and the amplicons from the16/23S intergenic region (Jagoueix et al., 1997).

For all 9 isolates studied, the HLB liberibacter identified was Las. The Las 16SrDNA sequences were identical among the nine isolates studied and they were very similar to published sequences of Las from Thailand (99.4 to 100% identity), Nepal (100% identity), and India (98.8% identity). The sequences of the Las 16/23S intergenic region were identical among the nine isolates studied as well as published isolates from Nepal and Thailand; they were close to published isolates of India and China (99.2% identity). Thus, the 16SrDNA region and the 16S/23S intergenic region are highly conserved among the nine Las isolates from Southeast Asia (Subandiyah et al., 2000). Similarly, Tomimura et al. (2009) have sequenced the same two regions of as many as 31 Las isolates from Southeast Asia and have observed no nucleotide differences among the 31 isolates, thus confirming the results of Subandiyah et al. (2000), namely that the 16SrDNA and 16S/23S regions of many Las isolates from Southeast Asia are highly conserved.

22. Timor island

Timor island, one of the easternmost Lesser Sunda islands, is located North of Australia. It is divided between **East Timor,** an independent, sovereign state since 2002, and **West Timor,** which is part of Indonesia.

22. 1. East Timor (Fig. 29, 31).

Surveys for HLB and *D. citri* were conducted in July 2000, May 2002 and April 2003 (Weinert et al., 2004). HLB symptoms were confirmed by 16SrDNA PCR amplification, followed by *Xba1* restriction of the amplicon to identify Las (Jagoueix et al., 1996 b). A selection of positive and negative samples was checked in the laboratories of Dr. D. Hailstones, NSW, Australia, and Dr. M. Garnier, Bordeaux, France. HLB was found to be widespread in the western half of East Timor. Twenty positive samples were collected from the towns of Dili, Ermera, Liquica, Ailieu, Maubisse, Suai, Hera and Maliana. *D. citri* was present in each of these locations. None of the sixteen samples collected in five locations from the eastern part of the country was positive, and the psyllid vector was not

detected in these locations.

Las was again identified in three leaf samples from East Timor by Miyata et al. (2011).

The detection, in East Timor, of HLB with Las and *D. citri* is a significant threat to citriculture not only in East Timor and nearby West Timor, but also in Australia.

22.2. West Timor (Indonesia)

D. citri has been recorded in West Timor (Weinert et al., 2004). PCR tests have confirmed that HLB was among the diseases causing decline of citrus in West Timor (Mudita, 2009) but the local government has refused to acknowledge this finding, in spite of the fact that HLB is widespread in the western part of East Timor.

23. New- Guinea island (Fig. 29, 32)

Like Tmor island, New-Guinea island is also split in two, with, in the East, **Papoua New-Guinea** (PNG), independent since 1975, and in the West, **Papoua**, former Dutch New-Guinea, today a part of Indonesia, renamed “Irian Jaya” in 1973.

23.1. Papoua (Irian Jaya, Indonesia)

In 1994, an HLB outbreak took place near Sorong in the western part of Papoua, where *D. citri* was present. Eradication of the Sorong HLB was attempted. However, surveys in 1998 and 1999, and PCR tests according to Jagoueix et al., (1996), detected HLB again near **Sorong** as well as **Jayapura**, a coastal town near the border with Papoua-New Guinea (PNG), more than 1,000 km to the East of Sorong., The results showed that HLB eradication in Sorong in 1994 had not been successful and confirmed the presence of *D. citri* at Sorong and Jayapura. However, HLB was not found across the border in parts of PNG adjacent to Jayapura (Davis et al., 2000).

23.2. Papoua New-Guinea

Even though the above 1998 / 1999 surveys did not identify HLB in PNG, the disease as well as *D. citri* were eventually detected in PNG in September 2002 in one citrus tree in the border town of **Vanimo** on the northeastern coast of PNG, at a distance of only ~100 km from Jayapura in Irian Jaya, where HLB had been discovered in 1998 / 1999. After the first HLB-affected tree in Vanino, a delimiting survey discovered a second and a third infected tree close to the first one, a fourth one at some distance away in Vanimo and a fifth one in a neighboring village, several km away. The five infected trees represented 7% of the 72 trees tested. In 2003, 48 trees were examined and 12 were found infected (25%).The PCR tests for these

surveys were according to Jagoueix et al. (1996). They were carried out in Australia and some samples were confirmed in France. In all cases, the HLB liberibacter detected was Las. *D. citri* was found throughout Vanimo and in nearby villages in a strip along ~50 km of the coast.

The large number of healthy trees, the widespread distribution of the vector, *D. citri*, and the spatial separation of the disease foci suggested that introduction of HLB was recent and may have occurred on more than one occasion.

The above results are from Pest Alert, 2003, Weinert et al., 2004, and Davis et al., 2005.

24. Australia

Australia, New Zealand and New Caledonia are free of HLB and *Diaphorina. citri*. However, interestingly enough, *D. citri*, the Asian citrus psyllid, was found in 1915 near Stapleton (13°.10S, 131°E) in the Northern Territory (N.T.) of Australia ! Indeed, the British Museum of Natural History, London, holds 20 pinned specimens of *D. citri* bearing the labels “Australia, Stapleton, N.T. 16.vii.1915.

Surveys were conducted in 2002 on suitable hosts around Stapleton to investigate if the *D. citri* infestation of 1915 had persisted. These inspections failed to detect either *D. citri* or Las (Bellis et al., 2005). It is presumed that *D. citri* was eradicated fortuitously between 1919 and 1922, when all introduced species and hybrids of the genus Citrus in the Northern Territory, North of latitude 19°S, were destroyed, mostly by burning, during a successful campaign to eradicate citrus canker (*Xanthomonas citri* subsp. citri).

The presence of *D. citri* and Las-associated HLB in Timor island [East Timor as well as West Timor (Indonesia)], and in New Guinea island [both Papoua-New Guinea and Papoua (Indonésia)] is obviously a threat to Australia.

25. Iran (Fig. 33, 35).

In Asia, New-Guinea Island is the most **eastern** region to carry HLB, while Iran is the most **western** region. Iran is also one of the countries in Asia where HLB has been reported most recently: in 2009. The HLB-associated liberibacter was Las. (Faghihi et al., 2009).

In Iran, *D. citri* was detected in late 1997 on the coast at Chah Bahar and inland at Gasre Gand (southern Sistan/Baluchistan), close to the border with Pakistan, at the occasion of a survey for *Hishimonus phycitis*, the presumed insect vector of witches’ broom disease of lime (Bové et al., 2000a). By 2011 (Salehi et al;, 2012), the Asian

citrus psyllid was found in all surveyed citrus growing areas of southern Iran provinces [Sistan/Baluchistan, Hormozgan, Kerman (Jiroft and Kahnooj), and Fars (Darab and Lar)]. This was the first time that the psyllid was detected in Fars province. Direct PCR and nested PCR showed psyllids from Ghasre Gand (Sistan/Baluchistan), and Minab and Roodan (Hormozgan) to be positive for citrus liberibacter. The same PCR methods detected also liberibacters in 23 sweet orange trees and one mandarin tree from Nikshar and Sarbaz (Sistan/Baluchistan) and 16 sweet orange trees from Roodan and Senderk (Hormozgan). PCR-amplified *omp* gene segments from two HLB isolates from Sistan/Baluchistan and Hormozgan were cloned and sequenced. The two sequences were 100% identical and were those of Las and not those of Laf. Finally, graft-inoculation of grapefruit and sweet orange seedlings with scions from a symptomatic sweet orange in Sarbaz caused blotchy mottle symptoms, characteristic of HLB.

In conclusion, *D. citri* and Las-associated HLB are widely distributed in citrus growing regions of Sistan/Baluchistan and Hormozgan provinces. The Asian citrus psyllid was detected in Fars province, but not (yet) HLB. It is most likely that both the Asian psyllid vector of Las and Las itself were introduced into Iran from neighbouring Pakistan/India where evidence was already present since the 1920s for *D. citri* and HLB (Waterston, 1922, Husain and Nath, 1927; see also above: 18.1.); the HLB-associated liberibacter is Las, in agreement with the fact that only Las has been identified in the Indian subcontinent (see above: 18). *D. citri* has been reported from Afghanistan in 1971 (Faizyar, 1971).

26. Conclusion : Asian HLB zone and African HLB zone

In all the many Asian citrus-growing regions studied above (Part II), extending from Iran to New Guinea island, HLB is characterized by two remarkable facts: (i) the liberibacter is always Las and (ii) the psyllid vector of Las is always *D. citri*. The African liberibacter, Laf, and the African psyllid vector, *T. erytreae*, widely distributed in southern and eastern Africa, have never been seen in the above Asian countries. The pathosystem “Citrus + Las + *D. citri* “ can be called “Asian HLB”, occupying the “Asian HLB zone”, in the same way that “Citrus + Laf + *T. erytreae*” is called African HLB, occupying the “African HLB zone”. These two zones do not overlap and the major difference between the two pathosystems comes from the fact that Asian HLB is heat tolerant because both Las and *D. citri* are heat-tolerant, while African HLB is heat-sensitive because both Laf and *T. erytreae* are heat-sensitive.

The following part concerns a third zone, the Arabian Peninsula, where Asian HLB meets African HLB (Fig. 34).

Part III:
Arabian Peninsula,
where
Heat-tolerant, Asian HLB meets Heat-Sensitive, African HLB.
Fig. 34, 35, 36, 37, 38, 39, 40.

In November 1981, while conducting a survey on behalf of FAO on virus and virus-like diseases of citrus in the Arabian Peninsula, J. M. Bové discovered Asian HLB in the southwestern part of Saudi Arabia: in the Mecca and Asir provinces. During the April 1982 survey, distribution of Asian HLB was further examined in these provinces as well as in Jizan province; in addition, African HLB, including *T. erytreae,* was observed for the first time in North Yemen (Yemen Arab Republic). In December 1983, African HLB was further studied in North Yemen and was also found in Saudi Arabia, close to the border with North Yemen; finally, both *D. citri* and *T. erytreae* were seen to be present together in HLB-affected orchards in the Brehim Khaibar oasis of the Abha/Khamis Mushait area of Asir province. A survey in 1987 showed *T. erytreae* to be present across the border between North and South Yemen (People's Democratic Republic of Yemen). *T. erytrae* was reported in Najran in 1984; by 1993, the African citrus psyllid had disappeared and was replaced by *D. citri*, introduced on plant material from northern Asir.

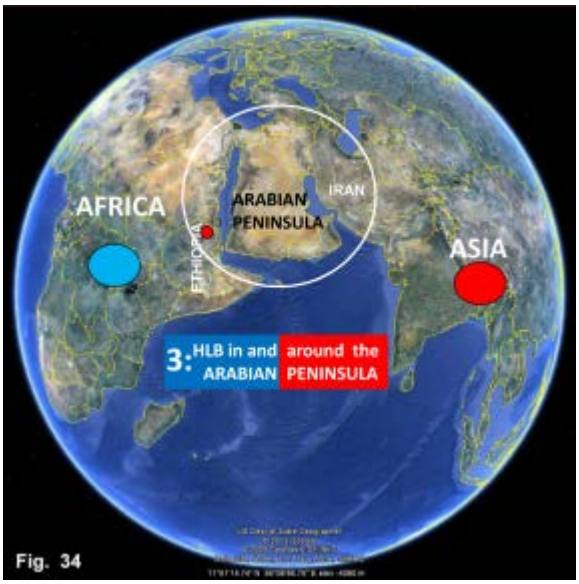


Fig. 34

In the 1980s, identification of the HLB bacterium as Laf or Las by DNA-hybridization or PCR was not yet available and diagnosis of HLB was based on symptom expression and transmission electron microscopy (TEM) detection of the HLB-associated bacterium in the sieve tubes of symptomatic leaves and fruits; TEM was carried out in Bordeaux by M. Garnier on samples kept in 2% glutaraldehyde.

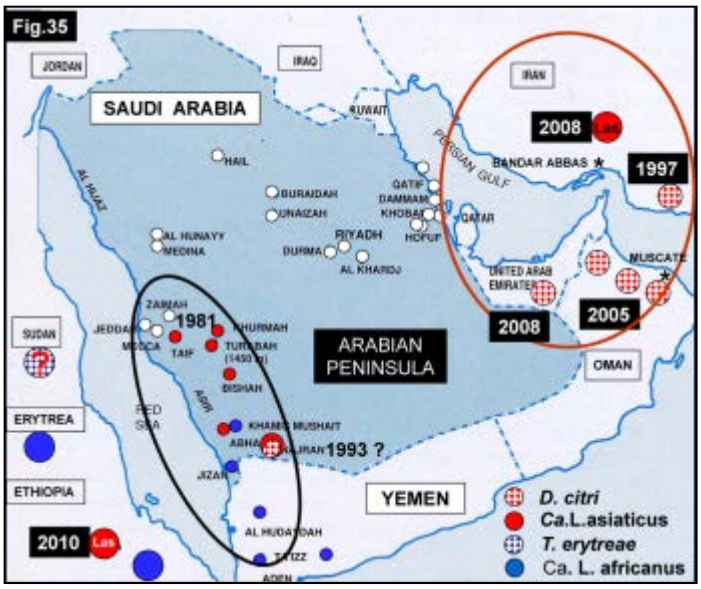
Identification of *D.citri* was based on the presence of adult psyllids and nymphs without bumps. *T. erytreae* was recognized by the adults and the typical, nymph-induced bumps on the upper leaf face.

Results have been reported in Bové and Garnier (1984), Bové (1986, 1995c, 1995e) and Aubert (1995).

27. Saudi Arabia (Fig. 35, 36, 37).

Citrus was grown essentially on the highland oases of the Southwestern Saudi Arabian plateau, from Taif (altitude: ~1700m) to Najran (~900m), and in the Kassim region, around Unaizah and Buraydah (~650m), northwest of Riyadh. During the 1980 surveys, HLB and citrus psyllids were discovered in the Mecca, Asir and Najran provinces, but not in the Kassim province.

27.1. *D. citri* and Asian HLB in Mecca and Northern Asir provinces.



During the November 1981 and April 1982 surveys, clear-cut HLB blotchy mottle symptoms and very high populations of *D. citri* were seen on small-fruited, acid lime (*Citrus aurantifolia*) trees in the following plateau oases of southwestern Saudi Arabia (names of areas, where the HLB bacterium was detected by TEM in samples from symptomatic trees, are underlined): Taif (altitude: 1700m), Khurmah (1400m) and Turabah (1400m) in Mecca province, Boua (1500m). and Bisha (1000m) in northern Asir province. HLB symptoms and *D. citri* were also found at altitudes below 1000m: at Zaimah (500m) and Wadi Nahman (500m) near Mecca. At even lower altitudes, *D. citri* was still present, but HLB symptoms were not clear: Usfan (150m), Khulays (200m), Haddat As Sham (200m). On the coastal area, no HLB symptoms and no psyllids were seen between Jeddah (~15m) and Jizan (~20m) at Al Lith, Al Ugdah, Muraibah, Ad Darb and Sabya (100m).

In the above areas, the only citrus species still grown at the time of the surveys was the small-fruited, acid lime. According to citrus growers, sweet orange and mandarin trees used to do well, but were wiped out in the 1970s. HLB was very probably the cause of this destruction since severe symptoms of HLB were still observed on diseased sweet orange and mandarin trees at Taif, Turabah and Bisha, and TEM showed the HLB bacterium to be present in these trees.

The small-fruited, acid lime, one of the best hosts of *D. citri*, proved to be less susceptible to HLB than sweet orange and mandarin. In the affected areas, lime trees showed leaf mottle and TEM detected the HLB bacterium, yet the trees reached relatively good sizes. An additional reason for good growth is that tristeza disease was not present. This is a remarkable situation, since in Africa and Asia, and now in America, HLB-affected trees are generally also infected with Citrus Tristeza virus (CTV) to which acid lime trees are very susceptible. The numerous, relatively well developed, tristeza-free, but HLB-infected lime trees, were vast reservoirs of the HLB bacterium. Thus very favorable conditions (good climate, large reservoir of inoculum, large populations of psyllid vectors, absence of citrus tristeza virus) existed for efficient transmission and spread of HLB.

In the above areas, high percentages of trees were affected. This was correlated with high populations of *D. citri*. The presence of *D. citri* was first observed in 1972 (FAO, 1972) and reported in 1974 (Wooler et al, 1974) on citrus in Djeddah, Wadi Kaulais, Mecca and Hadat Ash Shan. In certain areas such as Taif and Turabah, the psyllids had become a real pest and caused severe dieback, leaf dwarfing and curling.

The form of HLB present in the above areas was very probably the heat-tolerant Asian form for the following reasons (even though identification of the HLB bacterium as Las could never be achieved):

- The psyllid vector was *D. citri*. In nature, *D. citri* has never been shown to transmit African HLB, even though experimentally it can do so (Lallemand et al.,1986). *T. erytreae* eggs and nymphs cannot survive when the mean vapor saturation deficit (VSD) (Green and Catling, 1971) exceeds 35 millibars. In some of the above regions, such as Taif and Bisha, the VSD was well above this value from May to September.
- HLB was of the heat-tolerant form. Indeed, *D. citri* and Asian HLB were found in areas, such as Taif (1700m), with a mean monthly temperature in the summer months (June, July, August) as high as ~35°C. At ~38.5°C, some areas still carried *D. citri* and Asian HLB (Bisha, 1000m), but other areas did not. Finally, at ~42°C in the Medina region (500m) at Abiar Al-Mashy and Al Khelil areas, *D. citri* and Asian HLB were absent.
- Judging by the scale of destruction of sweet orange and mandarin trees in the 1970s, the disease must have been of the heat-tolerant Asian form, more severe than the heat-sensitive African form.

27.2. HLB, *D. citri* and *T. erytreae* in the Abha / Khamis Mushait region of Southern Asir province.

In the oasis of Brehim Khaibar in the Abha / Khamis Mushait area of southern Asir, at an altitude of 1500m, the two HLB vectors, *D. citri* and *T. erytreae*, occurred together in the same HLB-affected orchards. HLB symptoms were confirmed by TEM detection of the HLB bacterium. Bumps due to *T. erytreae* nymphs were present on the upper face of lime leaves, but no punctured nymph mummies were seen, indicating that *T. erytreae* was not parasitized or at least not to a great extend. *D. citri* nymphs however were parasitized and *Tamarixia radiata* was obtained in the emergence box.

Based on the severity of HLB symptoms, the disease was probably of the Asian form, widely distributed up North, throughout the oases of Asir and Mecca provinces, but the African form might also have been introduced by *T. erytreae* from neighboring Yemen. The mean monthly temperature in the summer months (June, July, August), ~30.9°C, allowed *T. erytreae* to be present and was probably also suitable for African HLB. In addition, the mean vapor saturation deficit (VSD) (Green and Catling, 1971), lethal for *T.*

erytreae eggs and nymphs when exceeding 35 millibars (mbar), was below this value every month of the year, including the summer months.

27.3. HLB and *T. erytreae* in the Fayfa region of Jizan province.

East of Jizan, near the border with northern Yemen, in the Fayfa region (altitude 1200-1800m), many *T. erytreae* induced bumps were observed on sweet orange leaves. Very few punctured nymph mummies were seen, suggesting a low level of parasitism. *D. citri* was not observed. Symptoms of zinc deficiency were severe on many sweet orange trees carrying psyllid leaf-bumps and the HLB bacterium was detected in the leaves by TEM. In the absence of *D. citri*, but the presence of *T. erytreae,* it is very likely that the form of HLB is the African one, also present, South of the border, in North Yemen. HLB and both psyllid species were absent in nearby, coastal Jizan, 20m altitude and 38.2°C mean monthly temperature in the summer months (June, July, August).

27.4. An unclear situation in Old and New Najran, Najran province.

Further East, along the North Yemen border, in the Najran valley (~1200m), no evidence for the presence of HLB, *T. erytreae* or *D. citri* was found during the November 1981 and December 1983 surveys. However, in January 1984, the National Center for Horticultural Research and Development (NCHRD), established in 1982 with the support of FAO, observed *T. erytreae* bumps in three different orchards on small-fruited acid lime, lemon and mandarin leaves. Punctured nymph mummies indicated parasitism. The psyllid population seemed to be low and in a state of decline. The climatic conditions were probably not favorable enough for high populations of the African citrus psyllid to Thrive. Indeed, the mean monthly temperature from April to September was as high as 36.5°C. Also, the mean VSD was above 35mbar from April to September and reached ~59.6 mbar in the summer months (June July, August).

Najran valley has two areas: Old Najran to the West and New Najran to the East. Dr. A.A. Fudl-Allah, FAO Plant protection officer at the NCHRD, observed *D. citri*, for the first time in 1993, on citrus plants introduced from northern Asir into Old Najran, in spite of strong quarantine recommendations delivered to the growers as early as 1984. Some of the imported trees were also suspicious of HLB. Introduced originally within Old Najran, the Asian citrus psyllid was subsequently noticed in the western side of New Najran. *Tamarixia radiata* was found on two occasions and the percentage of parasitism of D. citri nymphs was estimated at 30% (Aubert, 1995).

Following the discovery of *D. citri* and trees suspicious of HLB, but without confirmation of HLB by TEM detection of the HLB bacterium or even DNA hybridization, already available at that time, an eradication campaign was launched in western New Najran. Trees from ten hectares of apparently contaminated citrus orchards and the plants from an “infected” nursery were uprooted and burned. In October 1985, B. Aubert on behalf of FAO, surveyed the area to check if the eradication had been carried out properly (Aubert, 1995). Thirty six adult *D. citri* psyllids from a small-fruited acid lime orchard as well as leaf-midrib and fruit samples from 18 suspicious trees (but without classic HLB symptoms) were collected and sent to INRA-Bordeaux for detection of the HLB liberibacters in psyllids by crush-blot DNA

hybridization with specific probes and in leaf midribs by PCR amplification of 16SrDNA. All psyllid samples and all plant samples gave negative reactions under conditions where negative and positive controls gave the expected results.

On the basis of these date, it is unclear whether HLB has ever been present in Najran, even though *T. erytreae* in 1984 and *D. citri* in 1993 have been observed.

27.5. HLB in Saudi Arabia: conclusions.

For reasons that were indicated above (27.1.), the major form of HLB present in the Mecca and Asir provinces was heat-tolerant Asian HLB with *D. citri* as the psyllid vector. In the Jizan province, close to North Yemen, heat-sensitive African HLB with *T. erytreae* as the psyllid vector was observed.

D. citri was first seen in Saudi Arabia in the Djeddah / Mecca region in 1972. When in November 1981, also in the Mecca region, HLB symptoms (confirmed by detection of the HLB bacterium by TEM) were first observed, mainly on small-fruited, acid lime trees, they were severe and widespread. In addition, many orange and mandarin trees had been wiped out in the 1970s. Thus, (i) HLB was already well established when it was first seen in 1981 in the Mecca area and (ii) the HLB liberibacter was most probably introduced into the Mecca region with infected *D. citri psyllids*, in the late1960s.

It is now well-known that often quarantine pests and disease agents are first seen in regions with international airports. It is likely that HLB has been introduced into the Mecca region through the Djeddah international airport, in which millions of pilgrims go through each year, many from countries where Asian HLB is established. Another possibility is the introduction of citrus material from Asian HLB affected countries. Saudi Arabia is known for having imported, over the years, citrus from Pakistan and southeastern Asia, without proper quarantine measures.

Starting in the Mecca region in the late 1960s, Asian HLB and *T. erytreae* have spread southwards (the region North of Mecca around Medina being too hot and dry) and have reached the Abha-Khamis Mushait region in southern Asir. There, the Asian HLB and *D. citri*, coming from tne North, have met with *T. erytreae* (and probably African HLB), coming from North Yemen, spreading northwards and already present in the Jizan province.

28. Yemen (Fig. 35, 37, 38).

The surveys in North Yemen were carried out in April 1982 and December 1983, and those in South Yemen in December 1983 and April 1987. In those years, North Yemen was still the Yemen

Arab Republic while South Yemen was called the People's Democratic Republic of Yemen. Yemeni unification took place on May 22, 1990, when North and South Yemen were reunited, forming the Republic of Yemen.

28.1. North Yemen.

Concern that HLB might be present arose from the fact that, in 1982, J. M. Bové discovered HLB, *D. citri* and *T. erytreae* in the southwestern part of Saudi Arabia and, in particular, African HLB with *T. erytreae* at Fayfa, Jizan province, close to the border with North Yemen. Indeed, the 1982 and 1983 surveys showed that HLB was also present in North Yemen in the following areas:

28.1.1. Regions where HLB and T. erytreae were present.

1. Ta'izz region (~1350m).

Advanced symptoms of HLB were observed in April 1982 on many trees of the citrus collection at the Agricultural research Station (Aussfera farm) near Ta'izz. This collection was established in 1974 with plant material from Italy, where HLB has never been seen. The trees from Italy were thus free of HLB, but became infected, once planted in the field. Typical *T. erytreae* bumps were present on leaves of sweet orange, lemon, sour orange and mandarin trees. The HLB bacterium was present in all leaves and fruit axes from symptomatic trees. Advice was given to uproot the heavily HLB-infected citrus collection. Unfortunately, rather than being eradicated, the the trees were instead severely pruned, as could be observed on a second visit to Aussfera farm in December 1983. This treatment resulted in the development of many young, tender shoots on which psyllids were happy to feed, thus enhancing transmission of the HLB bacterium. Evidence for this could be seen in a three-year-old sweet orange block, adjacent to the pruned collection, where several of the young trees were stunted, showed HLB symptoms and carried the HLB bacterium. Similarly, in a nearby young *C. aurantifolia* acid lime block, HLB blotchy-mottle symptoms and *T. erytreae* bumps affected many leaves, in which the HLB bacterium was detected.

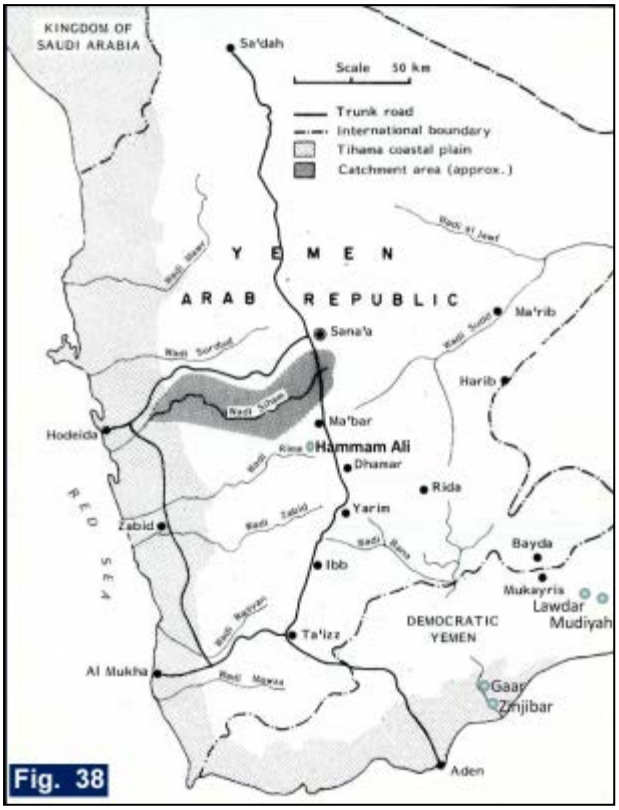


Fig. 38

In the Barakani area, South of Ta'izz, sweet orange and mandarin trees were known to have been dying during the 1970s. In 1979, one thousand declining sweet orange trees had been pulled out. During the December 1983 survey, it was most difficult to find sweet orange or mandarin trees in the area. However, many acid lime trees were still present, some of which were ~15-years-old. Practically all of them had clear-cut symptoms of HLB

with *T. erytreae*-induced bumps on leaves. TEM detected the HLB bacterium in all samples tested. A 20-years-old sweet lime tree also carried HLB. These observations showed that HLB was widely distributed in the Barakani area and that, most probably, the decline of sweet orange and mandarin trees in the 1970s was due to HLB, as such trees are more susceptible to HLB than acid lime trees. Ironically, the budwood for the sweet orange and mandarin trees that died or were pulled out in the 1970s, came from the Aussfera farm through the extension services. It is quite possible that HLB was introduced into the Barakani area with budwood from the Ta'izz station, where HLB was well established.

2. Hammam Ali region (~2300m).

In April 1982 in the Hammam Ali region, South of Sana'a, *T. erytreae*-induced bumps were seen on leaves of sweet orange, clementine and mandarin trees. Eggs and nymphs of the psyllid were abundant on young sweet orange leaves. HLB symptoms were seen on certain branches. The disease was probably of recent introduction.

3. Sana'a region (~2250m).

In December 1983 in the Wadi Dahr area, severe *T. erytreae* damage was observed on leaves of lemon, small-fruited acid lime and sweet orange trees. The HLB bacterium was detected by TEM in mottled sweet orange leaves.

4. Al Baida region (~2000m) (Fig. 38).

In December 1983, in the Dinahem area, severe *T. erytreae* damage was observed in an orchard of some hundred, 12-years-old Valencia sweet orange trees; HLB leaf- and fruit-symptoms occurred on "yellow" branches, as often seen with African HLB. TEM detected the HLB bacterium in leaf samples. The planting material came from Aden, free of HLB. No psyllid damage nor HLB symptoms could be seen in three nearby orchards of 3-years-old navel and Valencia late sweet orange trees from Egypt (free of HLB), suggesting that introduction of HLB and its psyllid vector was relatively recent.

In the Zahir area, severe HLB was seen on an 8-years-old sweet orange tree that was introduced from Ta'izz. TEM detected the HLB bacterium in the tree.

28.1.2. Surveyed regions in 1982 and 1983 where HLB and *T. erytreae* were absent.

1.Coastal plain or Tihama (altitude less than ~200m).

Garouba Usaid citrus project, Bait al Faqih region.

Jaraba UNDP citrus project, Bajil region.

Mauza farm, Wadi Safia region.

2. Marib (~1100m).

3. Harib (~1100m).

28.1.3. HLB in North Yemen: Conclusions.

The HLB present in North Yemen was most probably of the heat-sensitive African form for the following reasons. The psylld vector was *T. erytreae*; no evidence of *D. citri* was seen. HLB was only observed in the areas where *T. erytreae* was present. The psyllids were not observed in the coastal Tihama plain , as altitudes are below 200m, nor in the Marib-Harib region with altitudes around 1100m. The lowest

altitude, at which *T. erytreae* occurred, was 1350m in the Tai'zz region. In the other regions, the altitude reached 2000m! At these altitudes, the climat is cool enough and suits well the heat-sensitive African psyllid as well as the African HLB liberibacter.

The situation in the Barakani area, Tai'zz region, was very much reminiscent of what was happening in the southwestern part of Saudi Arabia (see 26.1.), where sweet orange and mandarin trees had also been wiped out in the 1970s, but the more tolerant acid lime trees survived. In both cases, the decline was most probably due to HLB, but with one important difference: the HLB involved in Saudi Arabia was the heat-tolerant Asian form of the disease, in North Yemen, it was the heat-sensitive African form.

It is most probable that HLB and *T. erytreae* entered Yemen over the narrow Bab al Mandab strait, at the southern end of the Red Sea, from neighboring Ethiopia where the African form of the disease and its African psyllid vector are known to be well established. There have been communications between Ethiopia and Yemen ever since immemorial times. The kingdom of queen Sheba (she is supposed to have paid a visit to King Solomon in Jerusalem, 10th century BC) is believed to have been composed of Ethiopia and Yemen, with Aksum (famous for the bath of the Queen of Sheba) as the capital in northern Ethiopia, and Marib (famous for the ancient dam which favored a flourishing agriculture) as the capital in Yemen.

The African citrus psyllid, moving northwards and after having spread through the highlands of Yemen, has eventually entered Saudi Arabia where it has already reached the Najran, Fayfa and Abha regions. The Abha-Khamis Mushait region harbored in the 1980s not only *T. erytreae*, but also *D. citri*, the Asian citrus psyllid, which, coming from the Mecca region (where it was first recorded), has moved down South, towards North Yemen. According to this scenario, *D. citri* (and Asian HLB !) should by now have reached northern Yemen…

28.2. South Yemen.

28.2.1. *T. erytreae*: present.

As seen above (28.1.1. 4), HLB and *T. erytreae* were observed in the Al Baida region of North Yemen in December 1983. At that time, North Yemen and South Yemen were still separate countries and it was not possible to cross the border and go from Al Baida (~2000m) in the North to nearby Mukairas (~2200m) in the South Fig. 38). Mukairas could however be reached by making a hudge detour via Aden and Lawdar. This possibility arose in April 1987, and the situation regarding HLB in Mukairas could be examined.

As expected, *T. erytreae* was present. Nymphs and young adults were seen on sour orange and small-fruited acid lime trees showing the typical bumps on the upper leaf face. In spite of the presence of the psyllid vector, HLB was not seen in the Mukairas region. True enough, there was very little citrus in the region. Citrus could only be seen in one farm, and even there the number of trees was very small. Yet on these few isolated trees, there was abundant psyllid multiplication.

28.2.2. Surveyed regions in 1983 and/or 1987 where HLB and *T. erytreae* were not seen.

1. Lawdar-Mudia (~1000m), on the road to Mukairas, is a major citrus region, but *T. erytreae* and HLB were not seen in the many orchards visited in 1983 and 1987, probably because at the altitude of only ~1000m, in comparison with Al Baida at ~2000m, the climatic conditions are too hot and dry. The same is true for the following regions:

2. Say'un and Tarim (~700m) in the Hadramawt province.

3. Al Musaymir (~800m), on the road from Aden to Ta'izz, half way between the two cities.

4. Zinjibar (~20m) and Gaar (~70m) in the Tihama.

28.3. Yemen: Conclusions.

In Yemen, *T. erytreae* and severe HLB, most probably of the heat-sensitive African form, are present in what used to be North Yemen at elevations of ~1350m and higher. In former South Yemen, the climate is probably too hot and dry for heat-sensitive African HLB and *T. erytreae* to become established. Only the psyllid has been observed in the Mukayras area, but where, at ~2200m, the conditions are adequate for HLB, and the disease might come in from nearby Al Baida (~2000m). The elevation of ~1000m at Lawdar-Mudia might be low enough to protect this major citrus growing area from HLB.

29. Sultanate of Oman, United Arab Emirates (Fig. 33, 39).

The Sultanate of Oman is known particularly well for witches' broom disease of lime (WBDL). The disease was first observed in the sultanate in 1986 (Bové, 1986b) and is caused by *Candidatus* Phytoplasma aurantifolia (Garnier et al., 1991a; Bové, 1995a; Zreik et al., 1995). WBDL was seen in the United Arab Emirates (UAE) in 1989 (Garnier et al., 1991a) and in Iran by 1997 (Bové et al., 2000a). The disease affects most severely acid lime (*C. aurantifolia*) and citron (*C. medica*). Sweet lime (*C. limettioides* Tan., *C. limetta* Risso) is also susceptible. The same presumed leafhopper vector, *Hishimonus phycitis*, was identified in Oman, the UAE and Iran, respectively in 1991, 1993 and 1997 (Bové et al., 1993b; Bové et al., 2000a) and shown to be indeed the vector in 2007 in Iran (Salehi et al., 2007).

In Iran, *D. citri* was first seen in late 1997, while searching for *H. phycitis* at Chah Bahar on the coast and, inland, at Gasre Gand (southern Sistan/Baluchistan), not far from the Pakistan border (Bové et al., 2000a). Las-associated HLB was reported in 2009 (Faghihi et al., 2009). By 2011, *D. citri*

and Las-associated HLB were widely distributed in citrus growing regions of Sistan/Baluchistan and Hormozgan provinces of southern Iran (Salehi et al., 2012) .

In Oman, *D. citri* was reported in June 2008 (Al-Zadjali et al, 2008), but was seen at Barka (Al Batinah coastal region) already in September 2005, eight years after it was first seen in Iran, suggesting that it might have been introduced into Oman from Iran, WBDL having most probably been introduced, inversely, from Oman.into Iran. Today, the psyllid is found all over the coastal Batinah region as well inland in the Nizwa region.

D. citri was reported from the UAE in 2008 (Burckhardt, 2008). Introduction of the psyllid from Oman into the UEA is likely.

It is to be feared that, soon, Asian HLB will be reported from Oman and the UAE.

30. Arabian Peninsula: Conclusion (Fig. 41, 42).

HLB in the Arabian Peninsula has been studied in the 1980s, from 1981 to 1987. At that time, there were no techniques yet to identify the liberibacter involved in HLB: Laf, the African liberibacter, or Las, the Asian HLB liberibacter. TEM was able to detect the liberibacter in HLB affected trees, but could not make the difference between Laf or Las. However, the Asian HLB pathosystem, associated with Las, and the African HLB pathosystem, associated with Laf, could be distinguished on the basis of their distribution according to climate and altitude.

Fig. 41

It was shown in Part I that, in the African HLB zone, from South Africa to Ethiopia, only one pathosystem occurred: heat-sensitive African HLB with *T. erytreae* as the psyllid vector. Similarly, as Part II illustrates, only one pathosystem was present in the Asian HLB zone, from New Guinea Island to Iran: heat-tolerant Asian HLB with *D. citri* as the psyllid vector. Part III has shown that the Arabian Peninsula, located in between the African HLB zone (to the West) and the Asian HLB zone (to the East), carries both pathosystems: (i) the heat-

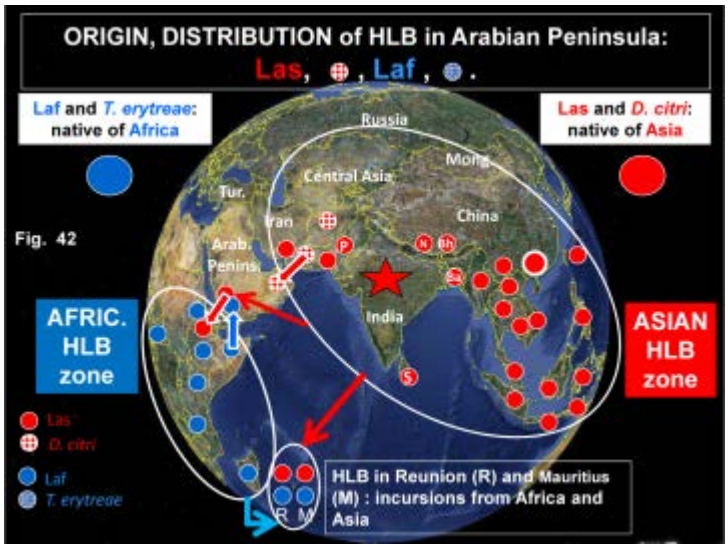


Fig. 42

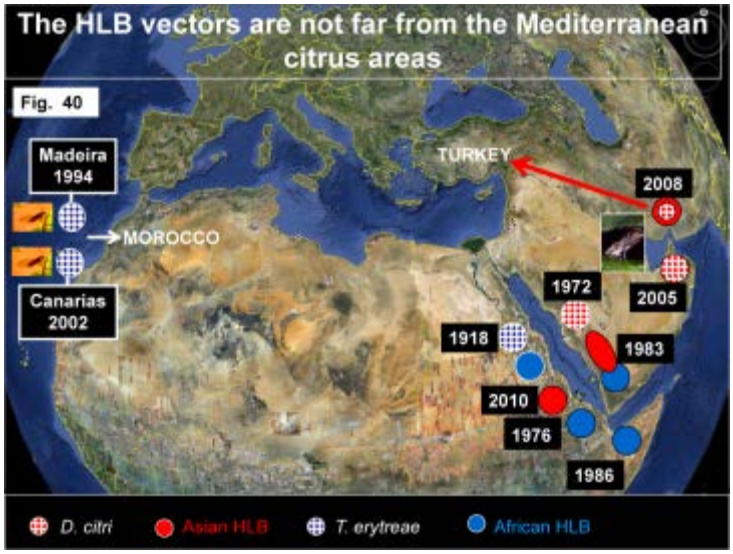
sensitive African HLB pathosystem at altitudes above ~1300m in western Yemen and spreading northwards, and (ii) the heat-tolerant Asian HLB pathosystem in southwestern Saudi Arabia, not only at altitudes above ~1300m but also at lower altitudes, and spreading southwards. The two pathosystems have probably met in southern Saudi Arabia in the Abha / Khamis Mushait region, where both *T. erytreae* and *D. citri* psyllid species occurred in the presence of severe HLB.

It is proposed that Asian HLB might have been introduced from the Asian HLB zone into the Jeddah / Mecca / Taif region with infected *D. citri* psyllids.

African HLB and *T. erytreae* have probably been introduced into Yemen from Ethiopia, at the northern end of the African HLB zone.

In 2010, citrus trees infected with Las, the Asian HLB liberibacter, have been detected for the first time in the African HLB zone: in Ethiopia. It is likely that Las has been introduced into Ethiopia across the Red Sea from southwestern Saudi Arabia. This might be the first intrusion of the Asian zone into the African zone.

D. citri was found in Oman in 2005 and in the UAE, in 2008. It might have originated in Iran where it was recorded several years earlier, in 1997, close to the border with Pakistan, a country known to have carried the psyllid, at least since the 1920s. So, Oman gave WBDL to Iran; in return, Iran gave *D. citri* (and perhaps tomorrow also HLB) to Oman...



Finally, as shown on Fig. 40, the Mediterranean region, still free of HLB and the two citrus psyllids, is threatened to the West by *T. erytreae* (in Madeira and Canarias islands), and to the East by *D. citri* and Asian HLB (in Iran, Saudi Arabia, Oman, and the Emirates) and *T. erytreae* and African HLB (in Ethiopia)…

Part IV:
Gondwanan Origin of African HLB and Asian HLB, Laurasian Origin of Leu, Lam and Lso.

31. Incongruities between geographic and phylogenetic placements of Laf and Las (Fig. 43, 44).

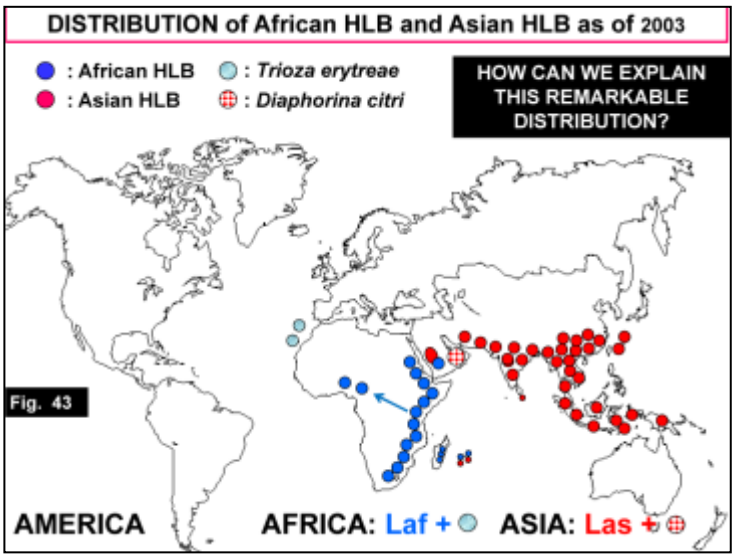


Fig. 43

As seen in the three previous parts, the African HLB zone is clearly separate from the Asian HLB zone. The African HLB zone extends from South Africa to Ethiopia, while the Asian HLB zone goes from Iran to New Guinea island. The Arabian Peninsula is in between the two zones. The presence of African and Asian HLB in the Peninsula is seen as the result of HLB incursions, respectively from the African HLB zone and the Asian HLB zone. The liberibacter present in the African HLB zone is only Laf and in the Asian zone, only Las: the two citrus HLB liberibacters occur in two geographically very different and clearly distinct zones. Yet, the two liberibacter species, laf and Las, are phylogenetically very close. On phylogenetic trees, they always cluster together: Las always with Laf, Laf always with Las, never with other liberibacters, such as *Ca*. L. americanus (Lam) (Teixeira et al., 2005d), *Ca*. L. europaeus (Leu) (Raddadi et al., 2010), *Ca*. L. solanacearum (Lso) (Liefting et al., 2009) or *Ca*. L. crescens (Lcr) (Leonard et al., 2012). For instance, Lam clusters with Leu, not with Laf or Las. There is thus a disjunction and a surprising lack of congruence between geographic and phylogenetic placements of Laf and Las. Also, why is Las heat-tolerant, knowing that Laf and all the other liberibacters are heat-sensitive?

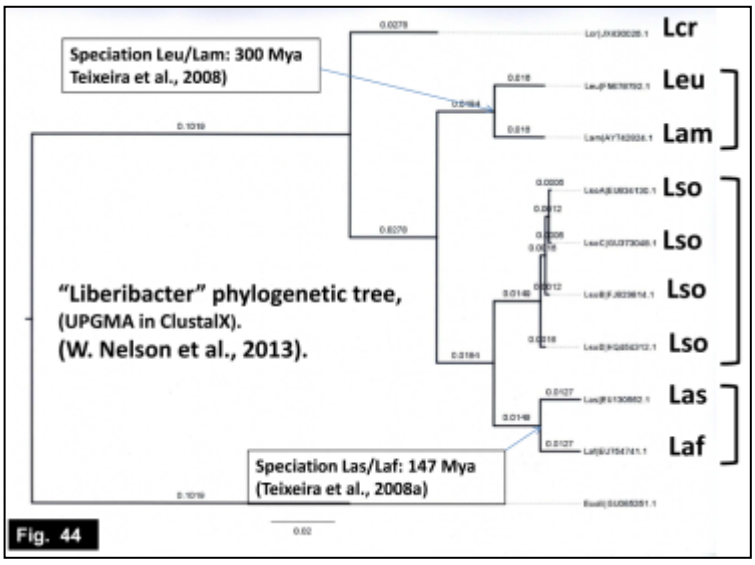
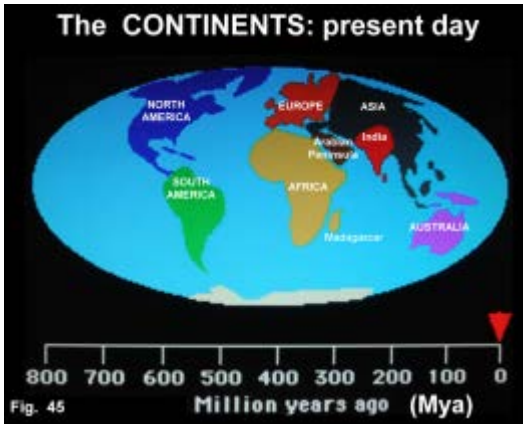


Fig. 44

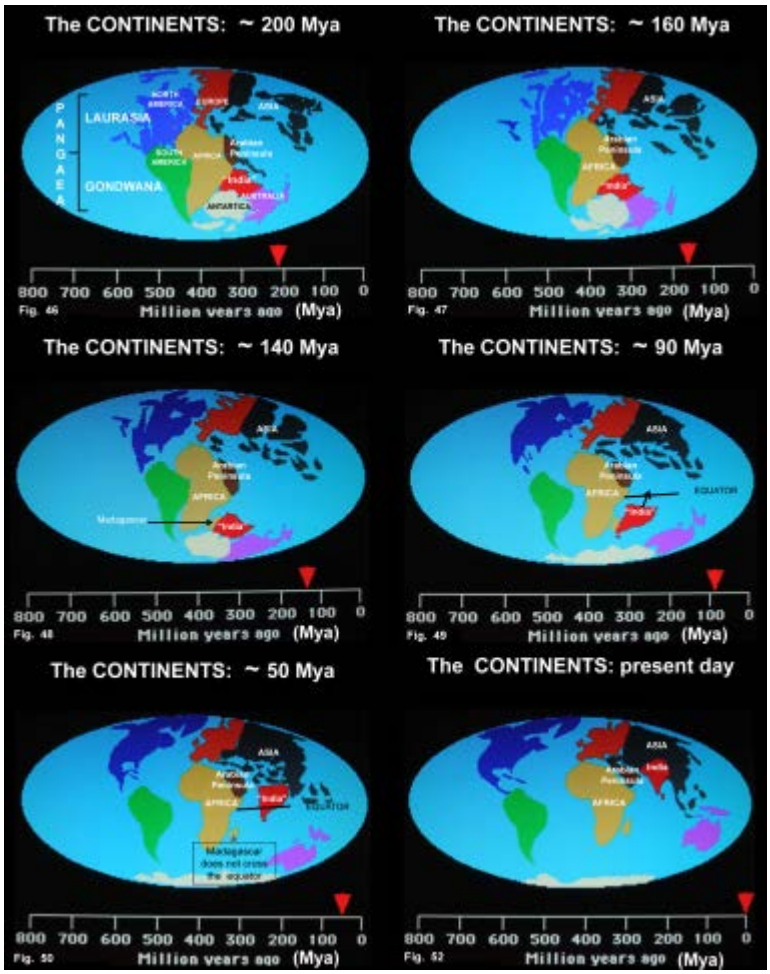
Fig. 51

32. Continental drift, Pangaea, Gondwana and Laurasia (Fig. 45 to 52).

Nelson and colleagues, in their hypothesis on the Pangaean origin of the liberibacters, have recently proposed answers to these incongruities and questions (Nelson et al., 2013). Their



explanation takes into account Plate Tectonic Movements and Continental Drift of ancient continents (Kious, W. & Tilling, R. 1996). The supercontinent “Pangaea” (from Ancient Greek *pan* meaning "entire", and *Gaia* meaning "Mother Earth") was composed of “Laurasia” in its northern hemisphere part and “Gondwana” in its southern hemisphere part. Laurasia was formed of the landmasses to become North America, Europe and Asia, and Gondwana was made of those to become South America, Africa, the Indian subcontinent, Australia and Antartica. Pangaea was one large landmass in which nearly all of the Earth's continents were connected. Cathaysia, which was made up of northern and southern China, was not a part of the larger Pangea landmass. Pangea began forming about 300 million years ago (Mya), was fully together by 270 Mya and began to separate around 200 Mya. During the formation of Pangaea, collision between two minor supercontinents gave rise to the “Central Pangaean Mountains” (Fig. 53). The Appalachian and Ouachita Mountains of North America are Remnants of this massive mountain range.



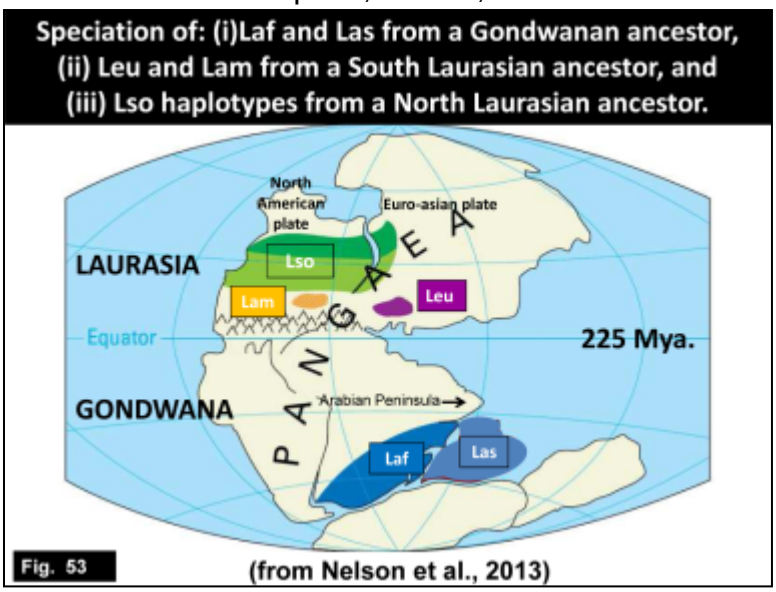
33. Speciation of an ancestral Pangaean species into one Gondwanan and two Laurasian ancestors (Fig. 53).

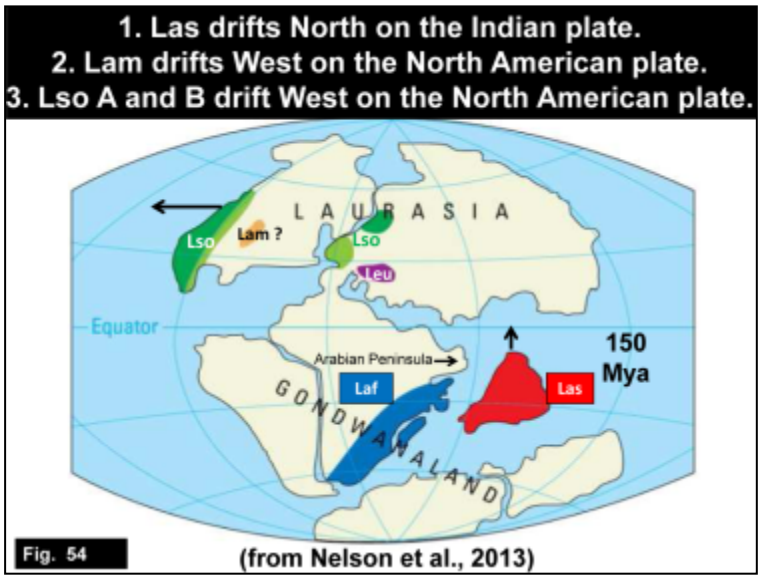
According to Nelson et al. (2013), the very ancestor of the liberibacters is seen as a heat sensitive, free-living form, associated opportunistically with angiosperm and psyllid insect ancestors, and living in the equatorial but cool climate of the Central Pangaean Mountains. Speciation of the ancestral species results in: (i) a Gondwanan ancestor species on eastern Gondwanan Africa, ancestral to Laf and Las, (ii) a southern Laurasian ancestor species leading to Leu and Lam, and (iii) a northern

Laurasian ancestor species ancestral to the Lso haplotypes. The origin of Lcr, the sixth and most recently described species, is as yet undetermined.

34. Speciation of the Gondwanan ancestor into Laf and Las (Fig. 53, 54, 55).

The Gondwanan ancestor of Laf and Las is seen as colonizing the East coast of Africa as well as Madagascar and the Indian plate, "India", but not the Arabian Peninsula plate (Fig.54). Speciation of this ancestor into Laf and Las occurs on dislocation and fractionation of Gondwana, isolating the African liberibacter (Laf) lineage within East Africa and Madagascar, and the Asian liberibacter (Las) lineage within "India". Around 150 Mya, the island of Madagascar, squeezed within Gondwana between Africa and "India", split away from Africa, while being still conjoint to "India". It broke off from "India" and was on its own by ~88 Mya (Fig. 51). It stayed close to Africa. On the contrary, "India" drifted northwards (Fig. 54) and collided with the Eurasian plate, a collision which resulted in the formation of the Himalayan chain. On its movement to its present position, "India" had to cross the hot equator region, with heat conditions for a speciation event leading to heat-tolerance of Las. Indeed, Las is the only liberibacter whose speciation had an equatorial event and it is also the only one to have acquired heat tolerance, all other liberibacters, including Laf, being heat-sensitive. In the above scenario, speciation of Laf and Las occurs with the dislocation of Gondwana, 150 Mya. From gene sequence comparisons, it has been calculated that speciation of laf and Las occurred ~147 Mya (Teixeira et al., 2008a). The two independent figures fit remarkably well.





The above scenario largely agrees with the previous suggestion of Beattie *et al.* (2005, 2008) that "*Ca*. Liberibacter" originated in Africa in association with plants within the Rutaceae and the African citrus psyllid, *Trioza erytreae*, but disagrees with their later thoughts that

Laf was subsequently taken to Asia with modern trade and changed there to Las (Beattie et al., 2008). As suggested here, Las is native to "India", speciation being underway and heat-tolerance being initiated while the Indian plate was drifting across the equator to its present day position. Furthermore, *D. citri*, the natural psyllid vector of Las, is also thought to be native to "India", especially since it has a very close African relative, *Diaphorina punctulata*, that also favors rutaceous hosts (Halbert & Manjunath, 2004). In other words, Las and *D. citri* are native of Gondwanan "India" in the same way than Laf and *T. erytreae* are native of Gondwanan East Africa. Eventually, Las and *D. citri* ended up in the Indian subcontinent, the region where, most likely, Asian HLB showed up for the first time in citrus. From there on, Asian HLB has spread in all directions to other citrus growing countries in Asia, Iran being the last one to have been affected, in 2008, in western Asia (Faghigi et al., 2009), and Papua-New Guinea in eastern Asia, in the early 2000s (Weinert et al, 2004). The presence of Las and *D. citri* in South, Central and North America is also the result of incursions.

35. "Arabian Peninsula – Ethiopia", "Reunion – Mauritius" versus Madagascar.

As indicated above, the continental plate of the future Arabian Peninsula is seen as being excluded from the Gondwanan zone in which the speciation of Laf and Las occurred (East Africa, Madagascar, "India"). This has been made apparent on figures 46 to 52. African HLB, Asian HLB and their psyllid vectors are not native to the Peninsula, but rather the result of recent incursions from the African HLB zone into Yemen, and from the Asian HLB zone into Saudi Arabia. Similarly, the presence of Las in Ethiopia, as reported in 2010 (Saponari et al., 2010), is also an incursion, possibly from Saudi Arabia (Fig. 42).

In addition to the Arabian Peninsula and Ethiopia, two other regions carry both African and Asian HLB, including the two psyllid vectors: Reunion and Mauritius islands in the Indian Ocean, East of Madagascar. While Madagascar has Gondwanan origin and was detached from "India" by ~88 Mya, Reunion and Mauritius emerged from the Indian Ocean as volcanoes much later, respectively ~5 Mya and ~8 Mya (Fig. 42). They are not part of the Gondwanan zone where speciation of Laf and Las took place. The presence of the two HLB pathosystems is, here also, the results of recent incursions.

Madagascar has Gondwanan origin and is included in the African zone where speciation of Laf took place. The island did not move up North to cross the equator and stayed not far away from the African Continent, in an environment probably not much different from that of the African East coast (Fig. 54). It would however be interesting to check whether Laf in Madagascar has haplotype differences with Laf in Africa.

36. Sri Lanka.

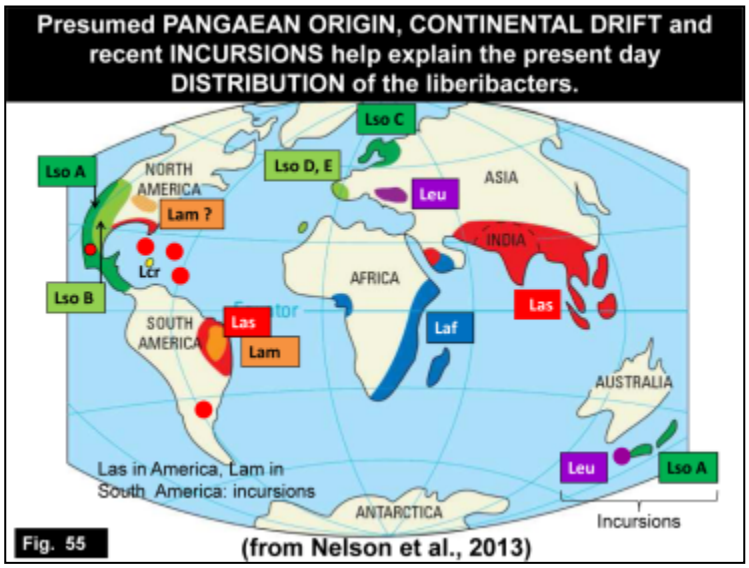
Sri Lanka is an interesting place!. The island was part of Gondwana, in very close juxtaposition with southeast Madagascar and the southern tip of India (Dissanayake and Chandrajith, 1999). Contrary to Madagascar, which broke off from

the Indian plate and the stayed near the eastern coast of Africa, Sri Lanka remained part of the Indian plate and moved with it up North. After collision of the Indian plate with the Asian plate and formation of the Himalaya some 45 Mya, Sri Lanka separated from "India" and was an island some 20 Mya. However, during subsequent periods there have been land connections and severances several times. There might have been four such occurrences, the last one having taken place as recently as 25,000 or even 10,000 years ago.

D. citri was mentioned in 1961(Ahmad, 1961) and the first evidence for the presence of Las-associated HLB in Sri Lanka was in 1993 (Garnier and Bové, 1996) (see 18.4.). However, the psyllid vector and the disease might have been present much before 1961 and 1995, respectively. The presence of Las in the island might be the result of modern incursions, but Sri Lanka, as part of the Indian plate, might also have been part of the zone where speciation of Las occurred. For the latter hypothesis, It would be important to know the early rutaceous plant population. The amount of diversity between Indian Las and Sri Lanka Las would also help making the decision.

37. Speciation of southern Laurasian ancestor into Leu and Lam (Fig. 44, 53, 54, 55).

Lam and Leu are phylogenetically close (Fig. 44) but geographically far apart. Leu is reasonably wide-spread within its Rosaceae plant and *Cacopsylla* psyllid hosts in Europe (Camerota *et al.*, 2012), suggesting Europe (originally South Laurasia) as the native region. As to Lam, when HLB was identified for the first time in Brazil in 2004, two liberibacters were found to be involved (Teixeira *et al*, 2005b): the known heat-tolerant, Asian liberibacter, Las, and a new, heat-sensitive species, *Candidatus* Liberibacter americanus (Lam) (Teixeira et al., 2005d; Lopes et al., 2009), both transmitted by the adventive Asian citrus psyllid, *D. citri*, (Yamamoto et al., 2006) and first reported in Brazil in 1942.



Up to now, Lam was restricted to São Paulo and Minas Gerais states, Brazil. Recently, 330 symptomatic and symptomless leaf samples were collected from HLB orchards in the following provinces of China: Guangdong, Guangxi, Fujian, Jiangxi, Zhejiang, Hunan, Yuannan and Guizhou. While, on the basis of gene sequences, *Ca*. L. asiaticus was detected in 96 samples, *Ca*. L. americanus was found in only one sample, a sample from Hunan (Lou *et al*., 2008). However, there has never been a follow up of this report, because the presumptive Lam-infected tree was eradicated without samples having been kept.

With no native plant or insect hosts having been described, it is unlikely that Lam has originated in Brazil; it is most probably a recent incursion into South America and so is *D. citri*. Also, since Lam and Leu are phylogenetically close, a common South Laurasian source can be expected for both: Leu from the European part of Laurasia and Lam from the western, North American part (Central Pangaean Mountains) (Fig. 53). If so, after continental drift of the North American plate (Fig. 54), Lam would be found in the North American Appalachian and Ouachita mountains in a region with adequate climate for heat sensitive Lam and rutaceous hosts. In this respect, an April 8 announcement of Lam in samples of *D. citri* psyllids collected in a residential property near Mission, southern Texas, is noteworthy (John Da Graça, 2013). Apparently, an isolate of Lam would also have been obtained from citrus leaves. Without confirmation of the presence of Lam in China, the Texas announcement seems to be the first reliable report of Lam outside of Brazil and, more precisely, from a region in reasonable proximity with the Ouachitas in Arkansas and Oklahoma, and the Appalachians in Georgia and Alabama (Fig. 55). Since 1998, when *D. citri* was first reported in Florida, the psyllid has invaded all southern states, from Florida to Texas. It might have picked up Lam in a plant host, so far unknown, and moved it to southern Texas through alternating citrus/psyllid hosts. Similar to the situation in Brazil, competition with heat-tolerant Las, the major liberibacter in the greater southern USA region, might have retarded its detection.

38. Speciation of northern Laurasian ancestor into Lso haplotypes (Fig.53, 54, 55).

Speciation of the northern Laurasian ancestor species has led to the Lso haplotypes. Five Lso haplotypes are recognized, two in North America (in *Solanaceae* plants) and three in Europe (in *Apiaceae* plants) (Nelson et al*.,* 2011; 2013b), illustrating remarkably well the effect of tectonic movement on resulting geographic positions. This indicates an extraordinarily conserved species, with only haplotype-level divergence since the breakup of Laurasia 150-200 Ma. The native range of each haplotype is probably not much different to the currently known regions of crop disease, although in each case the symptomatic crops are not the native plant host.

FINAL REMARKS ON HLB DISTRIBUTION.

Prior to 2003, only two HLB liberibacters, laf and las, were known and the disease was restricted to: (i) an African zone, with heat-sensitive Laf and *T. erytreae (*African HLB), (ii) an Asian zone with heat-tolerant Las and *D. citri* (Asian HLB), and (iii), in between the African zone and the Asian zone, the Arabian Peninsula zone with both African HLB (essentially in Yemen) and Asian HLB (in Saudi Arabia).

After 2003, a third liberibacter, Lam, was discovered in Brazil in 2004 and Asian HLB was reported for the first time in America, namely in Florida in 2005. By 2013, the disease was present in (i) North America (USA, with, in particular, Texas

and California in addition to Florida, and Mexico), (ii) Central America and Caribbean islands, and (iii) South America with Brazil and now Argentina. In America, the disease involves Las and *D. citri.* In São Paulo State, Brazil, when HLB was discovered in 2004, most of the affected trees (95%) were infected with Lam. Only a minority of trees was infected with Las. By 2013, the situation had reversed; only a small proportion of the newly infected trees carry Lam; most of the newly infected trees carry Las.

Understanding the present day distribution of the citrus liberibacters (Laf, Las, Lam) as well as the non-citrus liberibacters (Lso, Leu) greatly benefits from their presumed Pangaean origin and, thus, their association with continents drifting to their present day positions after dislocation of Pangaea, Gondwana and Laurasia. For instance, the recent discovery of Lso haplotypes in northern Europe (Scandinavian countries) and southern Europe (France, Spain: Valencia, Canarias) came after the "potato" liberibacter had been discovered first in North America (western USA, Mexico) and Central America. This distribution finds a satisfactory explanation by (i) assuming the Lso ancestor to be present in Laurasia on both the North American plate and the euro-asian plate (Fig. 53), and (ii) separation of the two plates and movement of the North American plate (Fig. 54) to its present day postion (Fig. 55). Similarly, the speciation of Laf on Gondwanan Africa and Madagascar (Fig. 53), and the speciation of Las on the "India "plate drifting up North through the equator to join with Asia (Fig. 54) result in two totally distinct HLB zones: the African heat-sensitive zone with Laf and *T. erytreae*, and the Asian heat-tolerant zone with Las and *D. citri.* As to Leu, its present day distribution in Central Europe suggests a South Laurasian origin within the European part of the Euro-Asian plate (Fig. 53). Because of its close phylogenetic relation with leu, lam is expected to have an origin similar to that of Leu: a South Laurasian origin, but from the North American plate (Fig. 53). Drift of the North American plate to its present day position would bring Lam to the southern Appalachian and Ouachita mountains (Fig. 54, 55). This hypothesis was formulated early February 2013 at the Orlando HLB symposium at a time when Lam had never been reported from North America. Therefore, the report of Lam in Texas in March 2013 came as an unexpected surprise. Whether the presence of Lam in Texas confirms the South Laurasian hypothesis or whether it is only circumstantial evidence and the result of an incursion from São Paulo State, remains to be seen.

In summary, there are zones to which the liberibacters are native: (i) the African HLB zone with Laf, (ii) the Asian HLB zone with Las, (iii) the European Leu zone, (iv) the North and Central American Lso zones, (v) the North and South European Lso zones, and (vi) perhaps a North American Lam zone. In these zones and in modern times, the liberibacters have spread, with their psyllid vectors, from an initial area to new areas more or less concomitant with the development and extension of the susceptible crops. For instance, in the Asian HLB zone, Las has probably spread (with *D. citri*) from an initial area on the Indian subcontinent to further areas. For instance, Nepal became infected through importation of infected plant material from India in the early 1960s and began to show symptoms by the mid

1960s. Iran to the West and Papua New Guinea to the East have shown symptoms only in 2008 and 2000, respectively.

The present day distribution of the liberibacters shows that they are present even in areas, to which they cannot be native: (i) Las in America, (ii) Lam in South America, (iii) Lso and Leu in New Zealand, (iv) Laf and Las and their vectors in Reunion and Mauritius, two very "recent" islands, only a few millions-years-old, which did not exist when the speciation of Laf and Las started, ~150 Mya, (vi) Laf and Las and their vectors in the Arabian Peninsula, whose continental plate was probably not involved in the speciation of Laf and Las, (vii) Las in Ethiopia only since 2010. In these "non-native" areas, the presence of the liberibacters and their vectors are the result of incursions.

Techniques have become available to study more deeply the diversity of the various isolates of a given species. For instance, the single Lso species occurs as two haplotypes in North America and three haplotypes in Europe. Similarly, there are indications that the diversity of Laf and Lafcap (the "capensis" subspecies of Laf from the rutaceous tree, *Calodendrum capense*), are at the haplotype level (W. R. Nelson, personal communication). Diversity between isolates of Las from various countries of Asia, and America has already been demonstrated. In 2010, Las has been identified in northern Ethiopia, for the first time in Africa. Does it originate in nearby Saudi Arabia? The origin and distribution of the liberibacters worldwide greatly benefit from these diversity studies.

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