THE PANGAEOAN ORIGIN OF “CANDIDATUS LIBERIBACTER” SPECIES

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SUMMARY

Species of “Candidatus Liberiibacter”, all vectored by psyllids, are generally recognized as the cause of four serious plant diseases, Huanglongbing, Zebra Chip, Psyllid Yellows and Yellows Decline, which currently threaten and destroy the citrus, potato, tomato and carrot industries, respectively. These are relatively recent diseases in plant crops, thereby inferring a modern evolutionary trajectory, but no overall hypothesis on their origins has been presented. Plate tectonic movements provide an explanation for the disjunction between modern geographic range and phylogeny. Northern species, on Laurasia, represent both modern European and North American ranges. Southern species, on Gondwana, indicate a further speciation event as ancient India, conjoint with Madagascar, separated from Africa, left Madagascar on its own, and produced the single heat tolerant species while drifting across the equator to its modern position. Phylogeny, epidemiology and geography of the modern species point backwards to a single original speciation event from a free-living form, associated opportunistically with angiosperm and psyllid insect ancestors. The original heat-sensitive species lived in the equatorial but cool climate of the Central Pangaeon Mountains probably > 300 Ma. The obligately alternating insect/plant host lifestyle developed opportunistically with the evolution and spread of flowering plants and psyllids.

Key words: citrus, huanglongbing, potato, zebra chip, paleopathology.

There are six currently recognized “Candidatus Liberibacter” species, of which four produce significant symptoms in their crop hosts. Three are associated with Huanglongbing (HLB) of citrus, and one with (i) Zebra Chip (ZC) and Psyllid Yellows in solanaceous crops (primarily potatoes and tomatoes) and (ii) Yellows Decline in carrots. Recent reviews provide an in-depth understanding of the biology and epidemiology for both citrus (Bové, 2006; Da Graça, 2008; Gottwald, 2010; Wang and Trivedi, 2013) and potato (Munyaneza, 2012; Lin and Gudmestad, 2013) pathosystems.

HLB is the most serious disease affecting the modern citrus industry in Africa, Asia and the Americas. The very production of citrus in Florida is seriously endangered. ZC has resulted in many millions of dollars worth of damage to the North American New Zealand potato industries, first noted on a large scale affecting potatoes used for manufacturing crisps in Texas and Mexico (Munyaneza et al., 2007; Munyaneza, 2012), but more recently noted in crops in Oregon, Washington and Idaho (Crosslin et al., 2012a, 2012b) states representing over 50% of the USA potato production.

Three species result in the most significant crop damage, (i) “Ca. Liberibacter asiaticus” (Las) and (ii) “Ca. L. africanus” (Laf) in citrus [with “Ca. L. americanus” (Lam) (Teixeira et al., 2005) of lesser significance in Brazil, but most recently discovered in Texas] and (iii) “Ca. L. solanacearum” (Lso) in potatoes and carrots (Munyaneza, 2012; Munyaneza et al., 2010). The last two species, apparently asymptomatic in some plants at least, are “Ca. L. europaeus” (Leu) (Raddadi et al., 2010) and “Ca. L. crescens” (Lcr) (Leonard et al., 2012). In spite of the severity of HLB and ZC symptoms in crop plants, closely related plants are known to host the bacterium yet remain asymptomatic. Psyllids of various species are the only known insect vectors.

The position of the Laf subspecies “capensis” (LafCap) has not been included as the single sequence currently available (GenBank accession No. AF137368) contains a number of unresolved nucleotides that will impact its
position in the tree. This subspecies is known asymptotically or with mild symptoms from the rutaceous tree *Calodendrum capense* but has never been detected in commercial citrus (Garnier *et al.*, 2000; Phahladira *et al.*, 2012).

Laf, Las, Lam, Leu and Lso are obligately intracellular (except in the insect haemolymph), in a propagative, circulative and persistent manner, alternating between the plant and insect hosts. They enter the plant host as the psyllid feeds on phloem sap, and in turn enter the insect during feeding, making their way from the gut to haemolymph and then to the salivary glands. In the plant host, they are pleiomorphic and non-flagellar, moving with the phloem sap in the sieve tubes throughout the plant, including the roots. Las is currently the only species known to cope with relatively high temperatures (ca. 35°C), as heat-sensitivity seems to be the rule with the liberibacters (Bové *et al.*, 1974; Lopes *et al.*, 2009; Munyaneza *et al.*, 2012; Bové, 2013).

"Ca. Liberibacter" species are placed within Rhizobiaceae (α-proteobacteria) but phylogenetically fall further from the root than other species in this family (Duan *et al.*, 2009). This distance can be interpreted as a result of extremely early separation, or an event resulting in extreme selection pressure. Clearly, co-existence in psyllid cells with other bacteria has not influenced evolutionary drift since "Ca. Liberibacter" has far more homologous proteins with species within the Rhizobiaceae than the more distant Ricketsiaceae, in spite of opportunity for horizontal gene transfer (Duan *et al.*, 2009). So far, estimates of speciation dates using a molecular clock technique have only been conducted for the three HLB species. These place Laf/Las separation at 147 million years ago (Ma), and Lam separating from a common ancestor of Laf/Las at 309 Ma (Teixeira *et al.*, 2008).

A modern evolutionary trajectory has been inferred because of the description of recent and devastating diseases in crop plants. However, no hypothesis has been proposed covering the origin of the currently known species, nor to explain the obvious incongruities between phylogeny and modern geographic ranges. We propose an ancestral species associated with the Central Pangaean Mountains, with subsequent speciation events resulting in: (i) a Gondwanan species ancestral to Laf and Las, with speciation of Laf on eastern, Gondwanan Africa, and speciation of Las on the Indian plate, separated from Africa and Madagascar and drifting to its present day position in Asia; (ii) a species in southern Laurasia leading to Leu and Lam, and (iii) a northern Laurasian species ancestral to the Lso haplotypes. The origin of Lcr, the sixth and most recently described species, is as yet undetermined.

Literature review focusing on symptomatology, epidemiology and phylogeny, associated with continental geographic rearrangements as described by plate tectonics, was used to describe species associations, ancestral range reconstructions of hypothetical ancestral species and evolutionary trajectories in a novel manner.

The phylogenetic tree of "Ca. Liberibacter" species (Fig. 1) places the single culturable species, Lcr, at a distance to all the others. Of note is the closeness of Lam/Leu, Laf/Las and the Lso haplotypes. Placing currently known geographic zones of the "Ca. Liberibacter" species onto a world map reveals there could be a significant "back story" for these species (Fig. 2). In particular, there is a surprising lack of congruence between geographic and phylogenetic placements across the species and haplotypes. However, movement of these geographic regions as ascribed to plate tectonics (Kious and Tilling, 1996) suggests evolutionary ties congruent with phylogeny at about 120 and 300 Ma.

Four Lso haplotypes are recognised, two each in North America (in Solanaceae plants) and Europe (in Apiaceae plants) (Nelson *et al.*, 2011, 2013), illustrating the effect of tectonic movement on resulting geographic positions (Fig. 2). 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.

Laf and Las, while phylogenetically close are clearly geographically separate (Fig. 2). Prior to 2004, the only citrus liberibacter found in Africa was Laf, and the only liberibacter in Asia was Las (Bové, 2006; Garnier and Bové, 1996). The presence of Las and Lam in America after 2004 is the result of modern incursions not only of Las and Lam, but also of their psyllid host, *Diaphorina citri* (the Asian citrus psyllid, ACP). Here, we are addressing only the situation of Laf and Las before 2004.

Rafting of the Indian subcontinent from its position next to Africa/Madagascar, where Laf is still found, suggests the potential for a speciation event during the crossing of the equator, including the development of heat tolerance. Further, ACP, the most common psyllid vector of Las considered native to Asia, has a very close African relative, *Diaphorina punctulata*, that also favors rutaceous hosts (Halbert and Manjunath, 2004). This largely agrees with the previous suggestion of Beattie *et al.* (2008) that “Ca. Liberibacter” originated in Africa in association with plants within the Rutaceae and the African citrus psyllid, *Triozoa 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.*, 2005, 2008). Las has only been associated with ACP, and Laf only with the African citrus psyllid (Bové, 2006), indicating separation of these two pathosystems. We agree that neither Laf nor Las have had a long association with citrus species, and modern anthropogenic activities have spread them both widely within commercial citrus industries, the Arabian peninsula being the region where African and Asian HLB have met (Bové and Garnier, 1984; Bové, 2013).

Lam and Leu are phylogenetically close (Fig. 1) yet the modern descriptions put them distant geographically (Fig. 2). Leu is reasonably wide-spread within its Rosaceae
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., 2005): the known heat-tolerant, Asian liberibacter, Las, and a new, heat-sensitive species, Lam, both transmitted by the adventive ACP, first reported in Brazil in 1942. Lam was by far the dominant species, infecting commercial citrus and some ornamental rutaceous species. Over the years, the proportion of trees newly infected with Lam decreased, while the proportion of Las-infected trees increased greatly, and today the dominant species is Las. Out of Brazil, Lam has been reported only from one single citrus tree in China in 2008 (Lou et al., 2008), but this report has not been confirmed. However, with no native plant or insect hosts having been described, we do not see Lam as having originated in Brazil; it is most probably a recent incursion into South America. 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 end of the Central Pangaean Mountains (Fig. 2). If so, after continental drift of the North American plate, 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, the recent, March 2013, report of Lam from a psyllid sample collected in a residential property near Mission, southern Texas, is noteworthy, even though so far only a solitary case (John da Graça, cited by Hawkes, 2013). This is only the second reliable report of Lam and, more precisely, from a region in reasonable proximity with the Ouachitas in Arkansas and Oklahoma.
and the Appalachians in Georgia and Alabama. Since 1998 when ACP was first reported in Florida, the psyllid has invaded all southern states, from Florida to Texas. It might have picked up Lam in a, so far unknown, plant host 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 region, might have retarded its detection.

Phylogeny, epidemiology and geography of the modern species point backwards to a single original speciation event from a free-living form, associated opportunistically with angiosperm and psyllid insect ancestors. The original heat-sensitive species lived in the equatorial but cool climate of the Central Pangaean Mountains. The fairly generic “yellows” and “decline” nature of plant symptomatology suggests a physiological rather than a virulent pathology. More species are likely to be discovered, and the introduction of species into more plant crop/psyllid vector combinations is inevitable. Solanaceae and Rutaceae crops are already severely affected; significant Eucalyptus and Acacia forestry investments are likely highly susceptible as they host many psyllid species. Of these, the most problematic is likely to arise if the more heat tolerant species spreads to more crops.

**Phylogeny.** Molecular clock dating systems are potentially fickle unless a good proxy is available to set the clock, or other events can confirm the resultant dating. Estimates of speciation events at 147 and 309 Ma (Teixeira et al., 2008) appear not to have resulted in any particular change in assuming a modern evolutionary trajectory, but these dates fit the phylogenetic tree structure extraordinarily well, as well as providing a clue to the related geographic alignments associated with the species described here.

LafCap is undoubtedly a member of the Laf/Las clade evolving on the Gondwanan continent, the precise position is marred by limited gene information useful for phylogenetic study. Future studies will be required to provide further evidence of the relationship between LafCap, Laf and Las.

Lcr is clearly separated phylogenetically, reflected also as being the only cultured species, as well as having a significantly larger genome at 1.5 Mbp (Leonard et al., 2012) compared to 1.23 Mbp of Las (Duan et al., 2009), 1.26 Mbp of Lso (Lin et al., 2011) or 1.18 Mbp of Lam (Lin et al, 2013).

**Epidemiology.** If “Ca. Liberibacter” species evolved to essentially their modern form prior to 300 Ma, they pre-date by some considerable period the approximately 100-120 Ma date for the spread of angiosperms (Wang et al., 2007), which is also approximately coincident with the evolution of the Psylloidea (Hodkinson, 1980). However, ancestral forms are being discovered from somewhat earlier periods, with the Sternorrhyncha developing from about 250 Ma (Ouvrard et al., 2010) and angiosperms from the early Jurassic period (150-200 Ma) (Wang et al., 2007). Psyllids are thought to have evolved on conifers and even now they frequently use them as over-wintering hosts (Hodkinson, 1980), although no conifers have yet been reported to host “Ca. Liberibacter”. Precisely when and how these bacteria became associated with and dependant on their insect hosts to provide entry to their plant hosts is currently unknown.

In one line of thoughts, “Ca. Liberibacter” species have been suggested to have developed their alternating plant/insect lifestyle from an earlier plant pathogenic lifestyle, possibly derived from the same mechanism that allowed other species within the Rhizobiaceae to develop intimacy with plants (Wang and Trivedi, 2013). However, bacteria that interact directly with their plant host cells in this
manner have mobile and unstable genetic elements (Batut et al., 2004), not present in “Ca. Liberibacter”. Further, “Ca. Liberibacter” requires the insect to insert it into the plant host (lack of virulence) and examples of bacterial plant pathogens that have subsequently infected their insect vectors are generally non-circulative in the insect (Nadarasah and Stavrínides, 2011).

Alternatively, the bacterium first infected an insect host and subsequently developed the ability to live and multiply within the new plant host. An example is “Ca. Arsenophonus” that is both vertically transmitted within the insect host as well as being a plant pathogen (Bressan et al., 2011). Spiroplasma citri and S. kuskelli, respectively the agents of citrus stubborn and corn stunt diseases, are similarly injected into the plant phloem system by their insect host, in which they reproduce (Bové et al., 2003; Regassa and Gasparich, 2006), as are species of “Ca. Phytoplasma” (Gundersen et al., 1996). “Ca. Liberibacter” epidemiology is therefore more similar to the plant pathogenic “Ca. Arsenophonus”, Spiroplasma, and “Ca. Phytoplasma” than to phylogenetically closer Rhizobiaceae.

Plant symptomatology is primarily a generic yellows and decline, often readily confused with general nutrient deficiencies. Marked starch accumulation, followed by yellowing of the leaves, could indicate a disruption of phloem transport with physiological cascade effects. Photomicrographs of infected cells suggest quite high numbers of bacteria develop within a single phloem sieve element, thus contributing to hydraulic resistance within the phloem system in the same way that organelles do (Jensen et al., 2012), suggesting a mechanism of phloem transport disruption without virulence. Variation in degree of symptom development, or even lack of symptoms, is marked across the “Ca. Liberibacter” species, with marked symptom development in species highly unlikely to have any evolutionary history with the bacteria, particularly the modern crop plants of citrus, potato and tomato.

Within the psyllid hosts, there is growing evidence for a lack of specificity of bacteria with psyllid species. For example, Las and Laf can both be vectored by either Asian or African citrus psyllids (Bové, 2006). Lso is vectored by three different Triozid psyllids across its geographic range (Munyaneza et al., 2007; Alfaro-Fernández et al., 2012; Munyaneza et al., 2010). Leu is vectored by many species of Cacopsylla (Camerota et al., 2012) or Ancylostina psyllids (Thompson et al., 2013). This emphasises the caution to be taken during experimentation, since it is quite plausible that a new crop/psyllid cycle could be inadvertently unleashed and also many of the recent disease patterns could represent exactly such a new crop/psyllid combination derived from currently unknown natural plant/insect hosts.

Geography. Plate tectonics and phylogenies provide a new viewpoint towards a common geographic and genetic origin. The Central Pangaean Mountains, set close to the equator, provides the optimal position for a heat sensitive ancestral species radiating into both hemispheres. The plant host of LfCap is a forest tree species, suggesting how heat sensitive bacteria could spread across otherwise hot regions by hopping between “islands” of cooler vegetation. Similarly, in regions too hot for Lf and its heat-sensitive African psyllid vector, the only citrus trees showing symptoms of HLB are in the shade of large windbreaks.

As noted earlier, the geographic position for Lam suggests a southern Laurasian ancestry. The potential for it to have been moved from North America (for example near the Ouachita mountains of Arkansas and Oklahoma) to Brazil seems even more plausible now that: (i) Lam has been detected in Texas in psyllids and (ii) Las is the only currently known species from Asia and has a good, plausible Gondwanan ancestry. Lcr is reported from Puerto Rico but is presumably adventive there since the phylogeny suggests Lcr to be older even than Leu/Lam, yet the geology of Puerto Rico is considerably more modern (Mitchell, 1954). The large phylogenetic gap between Lcr and the other liberibacters strongly suggests that there could be other species not yet discovered, as well as species closer to the other Rhizobiaceae.

In conclusion, an ancient and extremely conserved genome, combined with the already wide range of plant and psyllid species able to host “Ca. Liberibacter”, suggests that all psyllids and most angiosperm phloem cells should be considered suitable hosts. In this respect, it is relevant to note that Las affects not only citrus, its major host, but also tobacco (Garnier and Bové, 1993; Munyaneza et al., 2013) and tomato (Duan et al., 2008), the latter being one of the major hosts of Lso. It would not be surprising to find that, inversely, Lso can also affect citrus. Psyllids are common pests in many angiosperm crops, including vegetables and fruits as well as in significant forestry industries based on Acacia and Eucalyptus species, which host a broad range of psyllids. The experience of these emerging diseases in the citrus and potato industries presents a warning that a psyllid, feeding briefly outside its normal plant host range, could introduce a devastating disease to another crop.

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REFERENCES


