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First occurrence of *Diaphorina citri* in East Africa, characterization of the *Ca*. Liberibacter species causing huanglongbing (HLB) in Tanzania, and potential further spread of *D. citri* and HLB in Africa and Europe

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Abstract Citrus surveys were conducted at high (>700 m), medium (300-600 m) and low (<200 m) altitudes in Tanzania in 2014/15. Adults and nymphs of *Trioza erytreae* (del Guercio) were abundant in the highlands and less abundant at medium altitudes. Unexpectedly, adults and nymphs of *Diaphorina citri* Kuwayama, the Asian citrus psyllid, were found and collected at medium altitudes, around Morogoro. No psyllids were observed at low altitudes. Severe huanglongbing symptoms and tree decline were evident at high altitudes, while mild and few symptoms were observed at intermediate and low altitudes, respectively. DNA was extracted from leaf and psyllid samples and subjected to conventional PCR (cPCR) with seven different primer sets and RT qPCR with two primer sets. cPCR bands were sequenced and subjected to phylogenetic analysis. *Candidatus* Liberibacter africanus (Laf) was detected in highland leaf and *T. erytreae* samples from high and medium altitudes by all methods. Sequences from leaves and psyllids were similar to those from South Africa. *Candidatus* Liberibacter asiaticus (Las) was detected by qPCR in medium altitude leaf samples, but cross-reaction with Laf was likely because presence of Las was not confirmed by cPCR and sequencing. Neither Laf nor Las were detected in *D. citri* samples. This is the first reported occurrence of *D. citri* in Africa. Predictions were made of the potential distribution of *D. citri* and Las in Africa and along the Mediterranean coast using the correlative models MAXENT and Multi-Model Framework. Additional surveys at medium and low altitudes and quarantine measures are recommended.

Keywords Asian citrus psyllid · Citrus greening · Emerging pathogen · Invasive species · Species distribution models · Vector

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Introduction

Citrus greening or huanglongbing (HLB) is considered one of the most damaging plant diseases worldwide, and it accounts for substantial economic losses in Asia, Africa and the Americas (Farnsworth et al. 2014; Bové 2006; da Graça et al. 2015). The disease consistently is associated with bacteria in the phloem of the host plants that are difficult to isolate and maintain in culture (Bové 2006; da Graça et al. 2015). The putative causative agents are *Candidatus* Liberibacter spp., which belong to the alpha proteobacteria. Three species of *Ca.* Liberibacter are known to colonize citrus trees: *Ca.* Liberibacter asiaticus (Las) in Asian countries, the Middle East, and the Americas (Shen et al. 2013b), *Ca*. L. africanus (Laf) in southern and eastern Africa, including Madagascar, and *Ca*. Liberibacter americanus (Lam) in Brazil and possibly in Texas (Bové 2006; Magomere et al. 2009; Nelson et al. 2013; Teixeira et al. 2008). Las was documented once in Africa, namely in Tigray, Ethiopia (Bové 2014; Saponari et al. 2010). None of the HLB-causing bacteria have been reported for Europe, Australia or New Zealand (Narouei-Khandan et al. 2015).

The three species of *Ca*. Liberibacter are transmitted by at least two psyllid vectors: Las and Lam by the Asian citrus psyllid, *Diaphorina citri* Kuwayama, and Laf by the African citrus psyllid, *Trioza erytreae* (del Guercio). In addition, Laf transmission by *D. citri* has been demonstrated under controlled conditions, and Las has been transmitted experimentally by *T. erytreae* (Bové 2006; Lallemand et al. 1986). *D. citri* has been documented as vector of Las in Asia, the Americas, the Arabian Peninsula, and several islands in the Pacific and Indian Oceans (Bové 2006; Halbert and Manjunath 2004; Shen et al. 2013b), while the vector of Laf, *T. erytreae*, mainly is limited to southern and eastern Africa, the Arabian Peninsula, and several islands in the Indian Ocean (Halbert and Manjunath 2004). The latter psyllid species recently was found in the Canary Islands, Madeira, northern Portugal and north-western Spain (Monzó et al. 2015; Pérez-Otero et al. 2015), but there are no reports of identified *Ca*. Liberibacter sp. in those areas. Neither *T. erytreae* nor Laf are heat tolerant, so they are restricted to higher elevations in Africa (da Graça et al. 2015). On the other hand, both *D. citri* and Las are heat tolerant, so they are found in the lowland tropics and subtropics. In Réunion Island and Mauritius, both vector species occur, with *T. erytreae* and Laf primarily in the highlands, and *D. citri* and Las in the lowlands. Thus, *D. citri* and Las might be limited to lower elevations (Aubert 1987; Bové 2006; Halbert and Manjunath 2004; Toorawa 1998).

All *Citrus* species and cultivars as well as some other plant genera in the family *Rutaceae*, are susceptible to infection by the three *Ca.* Liberibacter spp. (Halbert et al. 2012; Manjunath et al. 2008). The bacteria are transmitted from plant to plant by grafting, by the psyllid vectors, and experimentally by dodder. Adult psyllids feed and lay eggs on young shoots (flush) of their host plants (Patt and Sétamou 2010). Injection and acquisition of *Ca.* Liberibacter spp. by their psyllid vectors take place during feeding by nymphs and adults from phloem sap of the host plant (Chiyaka et al. 2012; Inoue et al. 2009; Lee et al. 2015). After pathogen acquisition by the nymphs, the bacteria multiply in the hemolymph and are transferred to the emerging adults (Chiyaka et al. 2012; Inoue et al. 2009).

The pathogen blocks the transport of sucrose to the roots, resulting in starch accumulation in the foliage and deterioration of the root system, various nutrient deficiency symptoms, and typical HLB symptoms: mottled leaves, yellow shoots, and lopsided, half-green, and sour fruits (Bové 2006; da Graça et al. 2015). Symptom expression is dependent on the age and nutritional status of the infected tree (Shen et al. 2013a), but similar symptoms are induced by the three species of *Ca.* Liberibacter. Severe losses have been caused by fruit drop, reduced fruit quality, tree death and tree removal. Moreover, production costs have increased due to more intensive fertilizer and insecticide applications, tree removal and replanting (Farnsworth et al. 2014).

The potential spread of *D. citri* into new areas has been studied with the aid of several different models (Gutierrez and Ponti 2013; Narouei-Khandan et al. 2015). The potential spread of the Asian form of HLB caused by Las was studied by Narouei-Khandan et al. (2015). In the latter study, two correlative modeling approaches, MaxEnt and the Multi-Model Framework, were used to predict the potential distribution of *D. citri* and Las from global long-term climate data. Both types of models predicted that central and southeastern Africa could be highly suitable for the Asian form of HLB and *D. citri* establishment if these species were introduced. Contrary to the situation in central Africa, citrus production is quite common in southeastern and southern Africa, and the chance of *D. citri* establishment could be high in countries such as Ethiopia, Kenya and Tanzania where citrus trees are grown in both high and low altitude areas. The African form of HLB is quite common at high and middle altitudes in these areas, and the chance of establishment of Las would depend on the competitive ability of Las versus Laf at these altitudes. Until now, *D. citri* has not been demonstrated to occur in Africa, even though Las was found in Ethiopia once (Bové 2014; Saponari et al. 2010). The potential presence of *D. citri* and Las could have a serious impact on citrus production in sub-Saharan Africa, because these organisms thrive at higher temperatures and lower elevations than *T. erytreae* and Laf, putting all citrus production areas in Africa at risk for one or the other species of *Ca*. Liberibacter.

Citrus is produced in most countries in Africa, except for the Sahara and Central Africa. In East Africa, Tanzania is the third ranking citrus producing country after Kenya and Madagascar, with a total production of about 45,084.00 mT annually (http://faostat3.fao.org/download/Q/QC/E). Commercial citrus orchards are located in Tanga, Coast region and Morogoro, mostly at altitudes below 600 m (Fig. 1 and S1). In addition, back-yard citrus trees grow throughout Tanzania at different altitudes up to 2000 m. Symptoms of HLB are widespread, especially in back-yards at medium and high altitudes (Evers and Grisoni 1991). The disease was discovered for the first time during a survey conducted between 1984 and 1985, based mainly on visual symptoms (Swai 1988). In 1991, HLB was provisionally confirmed using electron microscopy (Swai et al. 1992). The species of *Ca*. Liberibacter has not been identified in Tanzania thus far. Some countries in Africa have reported both species of *Ca*. Liberibacter, Las and Laf, including Ethiopia, Réunion and Mauritius (Bové 2014; Saponari et al. 2010). The climatic conditions in Tanzania would be suitable for *D. citri* and Las establishment (Narouei-Khandan et al. 2015). Although HLB was described in Tanzania over 20 years ago, there is no molecular evidence for the occurrence of either of the *Ca.* Liberibacter species and the occurrence of HLB vectors has not been differentiated according to altitude in Tanzania.

The objectives of this study were to: (i) identify potential psyllid vectors in citrus trees at different altitudes in Tanzania, (ii) identify the species of *Ca*. Liberibacter associated with citrus with HLB symptoms, (iii) determine the species of *Ca*. Liberibacter in different psyllid vectors in Tanzania, and (iv) predict the potential distribution of *D. citri* and Las in Africa and Europe using two correlative species distribution models.

Materials and methods

Surveys and sample collection in Tanzania

Intensive surveys were conducted between December 2014 to June 2015 in four regions of Tanzania namely Tanga, Coast, Morogoro and Kagera (Fig. 1 and Fig. S1). In this study, the Tanga and Coast regions represented low altitudes <300 m, Morogoro region represented both medium altitudes (300 m – 600 m) and higher altitudes (>700 m), and Kagera region represented higher altitudes above 1200 m. The surveys included both citrus orchards and backyard citrus trees. The locations were selected based on accessibility from paved and unpaved roads. A global positioning system, Garmin, GPSmap 62sc (GARMIN, USA), was used to obtain a reference to the locations' altitude, latitude and longitude. In orchards of <0.5 ha all trees (at distances of 3–4 m) were inspected, while in orchards of >0.5 ha trees were inspected by moving across the orchard in a W-pattern. All trees per household (up to 20) were inspected in backyards, and 20 trees were inspected in orchards >0.5 ha. At each location, the owners were interviewed briefly as to the age and pest and disease history of the trees. Thereafter, citrus trees were inspected for the presence of: (i) visual HLB symptoms characterized by leaves with blotchy mottle and/or chlorotic patterns similar to Zinc and Manganese deficiencies, small lopsided fruits, uneven coloration of the fruits, and shoot dieback (Fig. 2); (ii) living psyllid nymphs and/or adults on new flush (Fig. 3); and (iii) leaf galls induced by *T. erytreae* especially at medium and higher altitudes (Fig. 2d). Composite leaf samples (about 10 leaves per tree) were collected from all suspected symptomatic trees and stored in a cool box for transporting to the laboratories (Sokoine University of Agriculture (SUA) in Morogoro and the International Institute of Tropical Agriculture or IITA in Dar es Salaam), where they were preserved at −20 °C until DNA extraction. Psyllids (adults and nymphs) wherever observed, were collected and preserved in 70 % ethanol in Eppendorf tubes. The psyllid samples were labeled and sent to Florida Department of Agriculture and Consumer Services, Division of Plant Industry, (FDACS/DPI) for final identification.

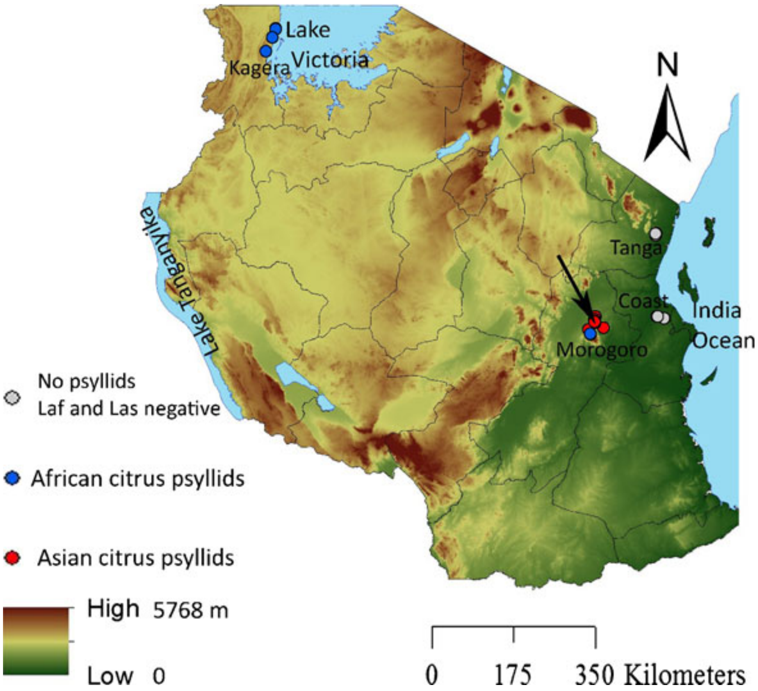

Fig. 1 Map of Tanzania showing locations where huanglongbing surveys were conducted. *White points*: locations where no psyllids were collected and all leaf samples tested negative for both *Candidatus* Liberibacter asiaticus and *Ca.* Liberibacter africanus; *blue points*: locations where African citrus psyllids (*Trioza erytreae* (del Guercio)) were collected, and both leaf and psyllid samples tested positive for *Ca.* Liberibacter africanus; *red points*: locations where Asian citrus psyllids (*Diaphorina citri* Kuwayama) were collected; *black arrow* indicates location where leaf samples tested positive for *Ca.*Liberibacter asiaticus by real time qPCR but negative by specific conventional PCR and sequencing

Psyllid identification

Preliminary identification of the different psyllids was carried out by the first author using a hand lens. The adult psyllids with a mottled brown or greyish-brown body, light brown head, probing at a steep angle were considered potential *D. citri* specimens, while black-headed psyllids with transparent wings that were probing at a less steep angle were considered *T. erytreae* (Fig. 3). The nymphs that were light yellow with red eyes and a white, waxy secretion were thought to be *Diaphorina* sp., while those with brown eyes and sometimes a pair of spots on the base of the abdomen in late stages were considered to be *T. erytreae* (Fig. 3c, d). *Trioza erytreae* nymphs typically were located in puckered galls on the leaves. Confirmation of 102 adults and 100 nymphs of *D. citri* and 68 adults and 5 nymphs of *T. erytreae* was provided by the third author at the FDACS/DPI in Gainesville, Florida (FDACS/DPI). Voucher specimens from most locations were deposited at the Florida State Collection of Arthropods (FSCA), and selected voucher specimens of *D. citri* also were deposited at the International Center for Insect Physiology and Ecology (ICIPE) in Kenya and the United States National Museum in Washington, DC.

DNA extraction from leaf samples

Total DNA of leaf samples was extracted using a DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, CA, USA) as per manufacturer's instructions. Briefly, the midribs were cut out from 5 to 10 leaves by using clean sterilized scissors. A total of 0.3 g of leaf midribs per tree (approximately 5–7 midribs) were finely chopped using sterile surgical blades, and then pulverized using a sterile mortar and pestle. Thereafter, the sample material was lysed, salt-precipitated, and centrifuged through the Qiagen DNeasy@ Plant Mini extraction kit columns (QIAGEN Inc., Valencia, CA, USA). Total DNA was purified with a silica-based membrane and elution buffer (AE buffer) (QIAGEN Inc., Valencia, CA, USA). The DNA extracts were stored at −20 °C for further analysis. The yield and purity of DNA samples were estimated by measuring the ratio of absorbance at 260 nm and 280 nm

Fig. 2 HLB-related symptoms; leaves with blotchy mottle **(a)**, leaves with chlorotic patterns similar to Zinc and Manganese deficiencies (**b** and **c**) and leaf galls induced by nymphs of African citrus psyllids (*Trioza erytreae* (del Guercio)) **(d)**

(260/280) using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware USA). Control citrus leaf samples that were positive for Las or negative for Las and Laf were kindly provided by Debra Jones (FDACS/DPI). The positive control sample for Laf was kindly provided by Prof Dr. Gerhard Pietersen, University of Pretoria, South Africa.

DNA extraction from psyllid samples

DNA from single psyllid nymphs or adults was extracted as described by Manjunath et al. (2008). Briefly, the psyllids were air-dried for 10 min, transferred to a 1.5-ml microfuge tube containing 300 μl of extraction buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA,

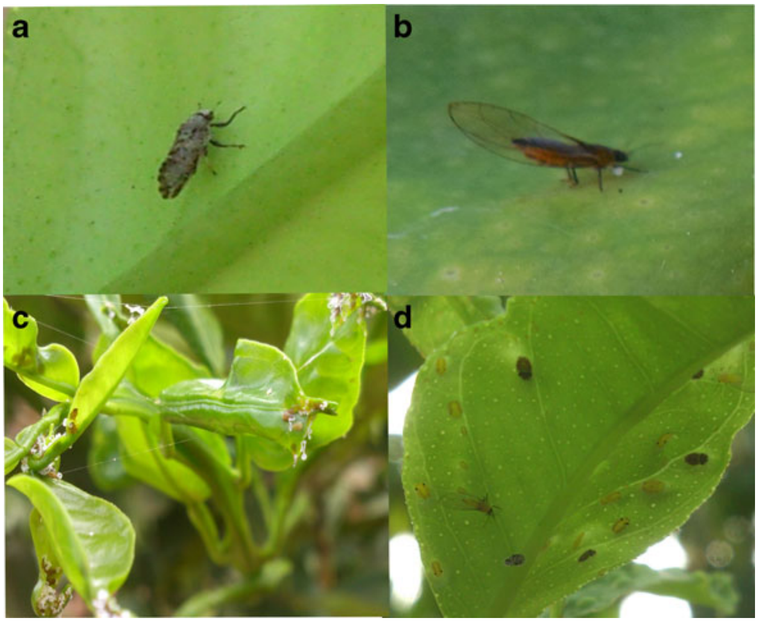

Fig. 3 Adults and nymphs of Asian citrus psyllids (*Diaphorina citri* Kuwayama) (**a** and **c**) and African citrus psyllids (*Trioza erytreae* (del Guercio)) (**b** and **d**) collected during huanglongbing surveys in Morogoro and Kagera regions, Tanzania, respectively

and 2 % SDS) and 20 units of Proteinase K (New England Biolabs), ground finely using a pellet pestle (Fisher Scientific Co.) connected to a cordless handheld motor. After incubating either at 50 °C for 3 h or 37 °C overnight, the nucleic acids were extracted using 300 μl of phenol-chloroform-isoamyl alcohol (25:24:1), followed by a second extraction using 300 μl of chloroform-isoamyl alcohol (24:1). The aqueous phase was ethanol precipitated and the resulting DNA pellet was dissolved in 20 to 50 μl of sterile water and stored at −20 °C. Steps were taken to avoid any possibility of contamination between samples. Psyllid DNA was used for conventional PCR amplification, as well as qPCR.

PCR detection of Ca. L. africanus and Ca. L. asiaticus in leaf extracts

DNA extracts from leaf samples were analyzed using PCR to detect the presence of Laf and/or Las genes. Primer pairs OA1/OI2C and OI1/OI2C were used for amplification of the 16S rDNA of Laf and Las, respectively (Jagoueix et al. 1996). Primers A2 /J5 were used for the amplification of Liberibacter β ribosomal protein gene of either Las or Laf (Hocquellet et al. 1999). Additionally, L10/L12 primers were used for amplification of the ribosomal protein rDNA of Laf (Magomere et al. 2009). All primers were ordered from Integrated DNA Technologies, BVBA (Leuven, Belgium). Finally, the specific Las primer pairs P25, P30, and P34 (Kogenaru et al. 2014) were used at the University of Florida for the sample from Mkuyuni, Morogoro, that had the lowest Ct value with HLBas and no reaction with HLBaf. The PCR reaction was performed in 25 μl of reaction mixture containing 1 μM of each of the primers, 1 μl DNA template, 9.5 μl H2O, and 12.5 μl of Dream Taq PCR master mix (2X) with the following composition: Dream Taq DNA polymerase optimized with 2X Dream Taq buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each and 4 mM MgCl2 (Thermo Fisher Scientific, Rochester, NY, USA). A SureCycler 8800 Thermal Cycler (Agilent Technologies, Santa Clara, CA, USA) was used for DNA amplification with the following program: 35 cycles each at 92 °C for 20 s (denaturation of template into single strands), 62 °C for 20 s (annealing) and 72 °C for 45 s (elongation). Following amplification, 8 ml aliquots of each reaction mixture were analyzed by electrophoresis on 1.2 % agarose gels, 90 V/2 h. The PCR results were based on the presence or absence of DNA bands on agarose gel (Jagoueix et al. 1996; Magomere et al. 2009).

Sequencing and phylogenetic analysis

The amplified products were sent to the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida (Gainesville, FL, USA) for Sanger sequencing (Sanger 3730xl, Applied Biosystems, ThermoFisher Scientific, Grand Island, NY, USA). All obtained sequences were submitted to the National Center of Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) (Table S1) and subjected to BLAST analysis. To investigate the phylogenetic relationship of the putative *Ca.* Liberibacter strains, the sequences were aligned along with other *Ca.* Liberibacter species sequences obtained from the NCBI database (Table S2) using MUSCLE, within MEGA 6 (Tamura et al. 2013). These included Laf strains from South Africa (Wellington 06–0354, Mpumalanga-UPCRI-06-0026, Rustenburg 06–0137, PTSAPSY, Nelspruit), the Kenyan Laf strain LM4, several subspecies of Laf from South Africa (*Ca.* Liberibacter africanus subsp. Zanthoxyli strain Ngele UPCRI 11–4561, subsp. Clausenae strain Port Alfred UPCRI 11–4361, subsp. Vepridis strain Knysna UPCRI 11–4038, subsp. Capensis strain ST. Francis Bay UPCRI 11–4270, subsp. Capensis strain Good Hope 10–2269), *Las* strain Ishi-1 from Japan and *Ca.* Liberibacter americanus from Brazil. Each alignment was subjected to jModeltest1.1 to select a nucleotide substitution model for phylogenetic analysis (Posada 2008). Phylogenetic trees based on 16S rDNA and L10/L12 intergenic rDNA were constructed using five strains from Tanzania. A concatenated alignment tree was generated from the alignment of 16S rDNA and L10/L12. MEGA 6 was used to construct Maximum Likelihood (ML) trees (Tamura et al. 2013).

Real-time qPCR for detection of Ca. L. africanus and Ca. L. asiaticus in leaf extracts

TaqMan-based real time qPCR assay for amplification of 16S rDNA of Laf and Las was carried out as described by Li et al. (2006). The primer pairs HLBas, HLBaf and HLBr, and a TaqMan probe HLBp were used for PCR amplification to target the 16S rRNA gene of Las and Laf respectively (Li et al. 2006). The citrus plant cytochrome oxidase (COX) gene was used as positive internal control. All primers and probes used were ordered from Integrated DNA Technologies, BVBA (Leuven, Belgium). The real-time qPCR amplifications were performed using a Mx3000P Real -Time PCR System (STRATAGENE) at IITA, Dar es Salaam, Tanzania, and CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA) at the University of Florida, USA. The reaction mixture was made to a final volume of 25 μl consisting of the following reagents: 12.5 μl of Dream Taq PCR master mix (2X) (Thermo Fisher Scientific, Rochester, NY, USA), 2 μL DNA template, 0.625 μL of 10 μM of each gene-specific primer pair HLBas, and HLBaf, 0.375 μL of 10 μM target probe HLBp, 0.75 μL of 10 μM of specific primer pair of COX gene and 0.375 μL of 10 μM COX probe (Li et al. 2006). Two-step thermal profiles consisted of 95 °C for 20 s, followed by 40 cycles of 1 s at 95 °C and 40 s at 58 °C, with plate reading at 58 °C for data acquisition. Each run contained one positive and one negative control sample. The positive and negative control samples for Las were kindly provided by Debra Jones (FDACS/DPI). The negative control samples were from citrus plants in a quarantine greenhouse at the Division of Plant Industry at Gainesville, FL. The positive control sample for Laf was kindly provided by Prof Dr. Gerhard Pietersen University of Pretoria, South Africa. Data analysis to determine cycle threshold (Ct) values were performed using the Stratagene software (STRATAGENE) and CFX Manager Software Version 2.0 (Bio-Rad Laboratories, Hercules, CA, USA). Samples with a Ct value of 34 or less were considered as positive for Laf or Las, depending on the primers used.

Real-time qPCR for detection of Ca. L. africanus and Ca. L. asiaticus in psyllids The DNA extracted from individual psyllids was amplified by conventional PCR with primers OI1/OI2c and OA1/OI2c as described previously (Manjunath et al. 2008). The remnants of the extracted DNA were analyzed by qPCR for detection of both *Ca*. Liberibacter species in An ABI ViiA7 machine (Manjunath et al. 2008) with slight modifications as described below. For detection of Laf, the Las forward primer HLBas was replaced with Laf specific forward primer HLBaf described by Li et al. (2006). PCR reactions were set up in fast qPCR plates and the fast qPCR reaction was carried out with an initial denaturation of 95 °C for 10 s followed by 40 cycles of 95 °C for 1 s and 60 °C 20 s. As positive control for Las dilutions of a plasmid with las 16 s (1167 bp insert) was used (http://www.ncbi.nlm.nih.gov/nuccore/EU130556.1). The LAF positive plant DNA that was provided by Dr. Gerhard Pietersen, University of Pretoria, South Africa, was also used as positive control for the DNA extracted from psyllids.

Modeling of the potential distribution of D. citri and Las

The current global distributions of *D. citri* and HLB-Las were gathered from online databases, literature review and personal communication with specialists (Narouei-Khandan et al. 2015). In total, 236 points were collected for HLB-Las and 330 points for *D. citri* (partially listed in Narouei-Khandan et al. 2015 who used 208 and 268 data points). The one and only report on the Asian form of HLB in Ethiopia was not included in order to check if that area would be predicted to be suitable for *D. citri* and Las establishment based on other global presence data. After spatially rarifying the locations where *D. citri* and/or Las were present (presence points) to avoid problems with clustered data, the remaining numbers of presence points were 300 for *D. citri* and 224 for HLB-Las. In the MaxEnt model the presence locations of *D. citri* and HLB-Las were compared with background points within circles with a radius of 500 km around the presence locations as described previously (Narouei-Khandan et al. 2015).

In the Multi-Model Framework or MMF, a large set of pseudo-absence points were generated in thousands of runs by the one-class support vector machines program (Narouei-Khandan et al. 2015). The generated pseudo-absence locations with similar environmental characteristics were clustered by K-means clustering, resulting in an equal number of clusters as the number of presence locations. The centroid of each pseudo-absence cluster was taken as absence point.

Long-term climate data including 19 temperature and rainfall variables were sourced from the Worldclim website (www.worldclim.org). The bioclim data resolution was in 10 arc minutes, or around 18.6 km (Narouei-Khandan et al. 2015). The Pearson correlation test was performed to avoid highly correlated environmental variables ($r > 0.8$). After removing highly correlated variables as described by Narouei-Khandan et al. (2015), 10 and 11 significant bioclimatic variables were selected for modeling *D. citri* and HLB-Las, respectively, using the random forest program in MMF (Narouei-Khandan et al. 2015). The same bioclimatic variables as listed in Narouei-Khandan et al. (2015) were used in the

MaxEnt and the MMF modeling procedures. The default features were used for both *D. citri* and HLB-Las (Narouei-Khandan et al. 2015). The prediction performance on the validation data set (25 % of the presence points) was evaluated by area under the curve or AUC for a plot of true positive predictions versus false positive predictions in MaxEnt. In MMF, the prediction performance was evaluated using ten different criteria, including AUC, and the support vector machines (SVM) model was selected based on all criteria (Narouei-Khandan et al. 2015). Model predictions were plotted globally, and output maps were generated in ASCII format that were exported into ArcMap 10.1 software. In addition, the global probabilities of HLB-Las and *D. citri* establishment derived from MaxEnt and MMF were converted to binary data in ArcGIS 10.1 using a threshold of 10 percentile presence probability in the training data set in MaxEnt and using a threshold of 0.5 in MMF (Narouei-Khandan et al. 2015). Maps of the binary data of the four data sets (*D. citri*-MaxEnt, *D. citri*-MMF, HLB-MaxEnt, and HLB-MMF) were overlaid using 'equal to frequency' in 'spatial analysis tools' in ArcGIS 10.1 to identify the areas where one, both or neither of the models agreed on the habitat suitability for both species, indicating hotspots of potential presence.

Hard copies of citrus production maps (orange, grapefruit, lemon and other citrus) for Africa and Europe was downloaded from the EPPO website (http://capra.eppo.org/maps.php). The maps were georeferenced and then digitized in ArcMap 10.1 to prepare a shapefile. The final shapefile which included a combined map of orange, grapefruit, lemon and other citrus was overlayed on *D. citri* and HLB-Las prediction maps (MaxEnt and MMF) to extract the probabilities of *D. citri* and HLB-Las occurrence in citrus production areas in Africa and Europe.

Results

HLB symptoms and psyllids

Most citrus trees encountered were orange trees on Rough lemon root stocks or on their own roots. The ages of the trees varied from 5 to 30 years. A total of 12 orchards and 70 households were visited during the surveys. Of all backyards and orchards inspected 56 % and 67 %, respectively, had trees that showed symptoms typical of HLB (Fig. 2). These symptoms were observed across all altitudes (Table 1). Most diseased trees were declining, especially at high altitudes (>700 m). A total of 47 composite leaf samples were collected from symptomatic trees: eight samples in Tanga, five samples in the Coast region, 26 samples in Morogoro and eight samples in Kagera (Table 1). Leaf galls induced by *T. erytreae* (Fig. 2d) were observed on the leaves at altitudes above 700 m. Sixty-eight adults and five nymphs of *T. erytreae* were collected from Kagera region at altitudes above 1100 m and from Morogoro region at altitudes above 700 m. At medium altitudes (400-600 m), 102 adults and 100 nymphs of *D. citri* were found in the vicinity of Morogoro (Table 1). No psyllid nymphs and/or adults were observed in Tanga and Coast regions.

Psyllid specimens collected from the Kagera and Morogoro regions at altitudes above 700 m were were confirmed to be *T. erytreae*, based on Hollis (1984). Nymphs in the Tanzanian collection had a brown band around the posterior of the abdomen, whereas the nymphs of South African specimens of *T. erytreae* in the FSCA have two prominent dark spots posterolaterally on the abdomen. Nevertheless, our specimens were considered to be *T. erytreae* because they matched our reference specimens in all other respects. Two other *Trioza* species were reported on citrus elsewhere (Halbert and Manjunath 2004). The adult specimens from Tanzania did not match *Trioza citroimpura* Yang and Li (1984) nor *Trioza litseae* Bordage (Hollis 1984), which has been reported from citrus but does not multiply on this crop (Halbert and Manjunath 2004). Other specimens observed in Tanzania were exactly like those in South Africa (Fig. 3d).

Psyllids collected from Morogoro region at altitudes below 600 m were all *D. citri*. Several other species of *Diaphorina* occur in Africa that have been reported on citrus and its close relatives. Most were ruled out because the pattern of markings on the wings was completely different. Several other species reported on citrus were ruled out because they do not actually colonize the crop (Halbert and Manjunath 2004). Of special interest was *Diaphorina auberti* Hollis, because it was described from citrus in the Comoro Islands, just offshore from Tanzania (Hollis 1987). *Diaphorina auberti* belongs to the *Diaphorina amoena* Capener species group, characterized by the hexagonal pattern of the spinules on the wings. The specimens from Tanzania did not have this feature, nor did they have the right

Table 1 Dates and locations where symptoms of huanglongbing and/or psyllids were observed in Tanzania in 2014 and 2015

Date of collection	Region	Location	Category	Altitude (m)	Host	symptoms	Leaf galls[1]	African citrus psyllids[2]		Asian citrus psyllids[2]	
								Nymphs	Adults	Nymphs	Adults
10 Sep. 2014	Morogoro	Langali	Backyard	1095	Lemon	Blotchy mottle[4]	+	+	−	−	−
10 Sep. 2014	Morogoro	Msikitini	Backyard	747	Orange	Blotchy mottle	+	+	−	−	−
10 Sep. 2014	Morogoro	Mgeta Kibaoni	Backyard	998	Orange	Blotchy mottle	+	+	−	−	−
10 Sep. 2014	Morogoro	Mgeta Kibaoni	Backyard	992	Orange	Chlorotic like Zinc deficiency	−	−	−	−	−
11 Sep. 2014	Morogoro	Mlali	Orchard	567	Orange	Chlorotic like Zinc deficiency	−	−	−	−	−
11 Sep. 2014	Morogoro	Mlali	Orchard	552	Orange	Blotchy mottle	−	−	−	−	−
11 Sep. 2014	Morogoro	Mzumbe	Backyard	531	Orange	Blotchy mottle	−	−	−	−	−
14 Dec 2014	Morogoro	Mkuyuni	Backyard	381	Orange	Blotchy mottle	−	−	−	−	−
14 Dec 2014	Morogoro	Mkuyuni	Mkuyuni	406	Mandarin	Chlorotic like Zinc deficiency	−	−	−	−	−
08 Jan. 2015	Morogoro	Mlali	Orchard	538	Orange	Blotchy mottle	−	−	−	−	−
08 Jan. 2015	Morogoro	Mzumbe	Backyard	515	Lemon	Blotchy mottle	−	−	−	−	−
09 Jan. 2015	Morogoro	SUA	Orchard	478	Orange	Blotchy mottle	−	−	−	+	+
09 Jan. 2015	Morogoro	Mafiga	Backyard	591	Mandarin	Blotchy mottle	−	−	−	+[3]	+
10 Jan. 2015	Morogoro	Mkuyuni	Backyard	400	Orange	Chlorotic like Zinc deficiency	−	−	−	−	+
10 Jan. 2015	Morogoro	Mkuyuni	Backyard	413	Orange	Chlorotic like Zinc deficiency	−	−	−	−	+
15 Jan. 2015	Morogoro	Mgeta Kibaoni	Backyard	1030	Orange	Blotchy mottle, Die back	+	+	−	−	−
15 Jan. 2015	Morogoro	Kihonda	Backyard	493	Orange	Blotchy mottle	−	−	−	+	+
15 Jan. 2015	Morogoro	Kihonda	Backyard	514	Lemon	Blotchy mottle	−	−	−		+
16 Jan. 2015	Morogoro	SUA	Orchard	479	Orange	Blotchy mottle	−	−	−	−	+
16 Jan. 2015	Morogoro	SUA	Orchard	476	Pumelo	Blotchy mottle	−	−	−	−	+
27 Apr 2015	Morogoro	Tumbaku	Backyard	472	Orange	Blotchy mottle	−	−	−	+	+
27 Apr 2015	Morogoro	Mafiga	Backyard	587	Orange	Blotchy mottle	−	−	−	+	+
28 Apr 2015	Morogoro	Kyegeya	Backyard	489	Orange	Blotchy mottle	−	−	−	+	+
28 Apr 2015	Morogoro	Kihonda	Backyard	514	Orange	Blotchy mottle, die back	−	−	−	+	+
16 Jun 2015	Morogoro	Mkuyuni	Backyard	400	Orange	Blotchy mottle	−	−	−	+	+
19 Jun 2015	Morogoro	Kyegeya	Backyard	480	Orange	Blotchy mottle	−	−	−	+	+
18 Jul 2015	Morogoro	Tumbaku	Backyard	468	Orange	Blotchy mottle	−	−	−	−	+
18 Jul 2015	Morogoro	Kyegeya	Backyard	481	Orange	Blotchy mottle	−	−	−	−	+
18 Jul 2015	Morogoro	Kyegeya	Backyard	481	Orange	Blotchy mottle	−	−	−	+	−
20 Dec 2014	Tanga	Kibanda	Backyard	185	Orange	Chlorotic like Zinc deficiency	−	−	−	−	−

Table 1 (continued)

Date of collection	Region	Location	Category	Altitude (m)	Host	symptoms	Leaf galls[1]	African citrus psyllids[2]		Asian citrus psyllids[2]	
								Nymphs	Adults	Nymphs	Adults
21 Dec 2014	Tanga	Kibanda	Backyard	168	Orange	Chlorotic like Zinc deficiency	–	–	–	–	–
21 Dec 2014	Tanga	Semngano	Orchard	241	Orange	Chlorotic like Zinc deficiency	–	–	–	–	–
12 Jan. 2015	Tanga	Semngano	Orchard	254	Orange	Blotchy mottle	–	–	–	–	–
13 Jan. 2015	Tanga	Semngano	Backyard	271	Orange	Chlorotic like Zinc deficiency	–	–	–	–	–
13 Jan. 2015	Tanga	Msangati	Backyard	155	Orange	Chlorotic like Zinc deficiency	–	–	–	–	–
14 Jan. 2015	Tanga	Msangati	Backyard	158	Orange	Chlorotic like Zinc deficiency	–	–	–	–	–
14 Jan. 2015	Tanga	Msangati	Backyard	152	Orange	Chlorotic like Zinc deficiency	–	–	–	–	–
06 Oct 2015	Kagera	Muhutwe	Backyard	1362	Orange	Blotchy mottle	+	+	–	–	–
06 Oct 2015	Kagera	Chudikye	Backyard	1290	Orange	Blotchy mottle, Die back	+	+	–	–	–
10 Oct 2014	Kagera	Maruku	Backyard	1293	Lemon	Blotchy mottle	+	+	–	–	–
15 Nov 2014	Kagera	Bumiro	Road side tree	1214	Lemon	Blotchy mottle	+	+	–	–	–
03 Jun 2015	Kagera	Maruku	Backyard	1294	Lemon	Blotchy mottle	+	+	+	–	–
04 Jun 2015	Kagera	Maruku	Backyard	1293	Orange	Blotchy mottle	+	+	+	–	–
05 Jun 2015	Kagera	Muhutwe	Backyard	1365	Orange	Blotchy mottle	+	+	+	–	–
10 Jun 2015	Kagera	Bumiro	Backyard	1221	Lemon	Blotchy mottle	+	+	+	–	–
10 Jul 2015	Kagera	Muhutwe	Backyard	1308	Orange	Blotchy mottle	+	–	+	–	–
23 Dec 2014	Coast	Kibaha	Orchard	122	Orange	Chlorotic like Zinc deficiency	–	–	–	–	–
23 Dec 2014	Coast	Visiga	Backyard	68	Orange	Chlorotic like Zinc deficiency	–	–	–	–	–
14 Apr 2015	Coast	Kibaha	Orchard	137	Orange	Chlorotic like Zinc deficiency	–	–	–	–	–
14 Apr 2015	Coast	Visiga	Orchard	64	Orange	Chlorotic like Zinc deficiency	–	–	–	–	–
14 Apr 2015	Coast	Visiga	Backyard	77	Orange	Chlorotic like Zinc deficiency	–	–	–	–	–

[1] Induced by *Trioza erytreae* (del Guercio)

[2] Psyllid samples from most locations were sent to Florida Department of Agriculture and Consumer Services, Division of Plant Industry (FDACS/DPI), Gainesville, FL, USA and confirmed as *Trioza erytreae* (del Guercio) or *Diaphorina citri* Kuwayama

[3] mummy only

[4] PCR and qPCR results are presented in Table 2; the samples can be matched by comparing dates and locations in the two tables

pattern of dark patches on the wings, nor the genal cone shape of *D. auberti*. Thus, *D. auberti* also was ruled out. To our knowledge, this is the first report of *D. citri* in mainland Africa. Voucher specimens of the collections from Tanzania were deposited in the FSCA. FSCA numbers for *D. citri* include E2015–6482-6487 and E2015–6493-6497. FSCA numbers for *T. erytreae* include E2015–6488-6492, and E2015–6498. Pinned specimens from E2015–6485, Tumbaku, 28-IV-2015, are deposited at the USNM and ICIPE. Slides from E2015–6482, Mgeta Kibaoni, 15-I-2015, are deposited at ICIPE.

Some of the nymphs from Kihonda, Tumbaku, and Mafiga showed evidence of parasitism, including hymenopterous pupae consistent with the family *Eulophidae* that includes *Tamarixia*, a genus of known psyllid parasites (personal communication, Dr. Kevin A. Williams, FDACS/DPI). No adult parasites were recovered, so it was not possible to determine the species at this time.

Detection of Candidatus Liberibacter species in leaf tissues with conventional PCR

When primers OI1/OI2c (known to amplify both Las and Laf) were used for PCR amplification of Liberibacter 16S rDNA, an amplified product of the expected size (1160 bp on a 1 % agarose gel) was observed from some leaves collected from Morogoro region at medium and high altitudes and Kagera region at high altitude (Fig. S2, lanes 4 (Mgeta Kibaoni), 9 (Kihonda), 13 (Mlali), 21(Maruku) and 22 (Bumiro)). No amplification was observed from any samples collected from low altitudes in Tanga, the Coast and Morogoro regions.

Similarly when the primers OA1/OI2c (known to amplify Laf only), A2/J5 (for Las and Laf), and L10/L12 (for Laf only) were used for PCR amplification, bands of 1160 bp, 703 bp, and 716 bp, respectively, were observed for DNA from leaves collected from medium and high altitudes in the Morogoro region (Mgeta Kibaoni, Kihonda and Mlali) and from high altitudes in the Kagera region (Maruku and Bumiro) (data not shown). Again, no amplification was observed for samples from low altitudes in Tanga, the Coast and Morogoro regions. No amplification was observed from healthy leaf samples and water with any of the primer sets (Fig S4). Taken together, the results from these primer sets indicate that Las was not found (Table 2). When three additional primer sets P25, P30 and P34 that were specific for Las were used for one sample from Morogoro region, no bands were detected on the gels.

Sequencing and phylogenetic analysis

Bands amplified with primer set OI1/OI2C with 16S rDNA from Mgeta Kibaoni, Kihonda, Maruku and Bumiro were sequenced, but only Laf was identified. Therefore, bands amplified with L10/L12 (for Laf only) from Mgeta Kibaoni, Kihonda, Mlali, Maruku and Bumiro were also sequenced. Phylogenetic analysis of 16S rDNA grouped the strains of *Ca.* Liberibacter africanus into three distinct clusters: one cluster with strains of *Ca.* Liberibacter africanus proper, including the Nelspruit and PTSAPSY strains from South Africa, one cluster of *Ca.* Liberibacter africanus subsp. Zanthoxyli, and one cluster with *Ca.* Liberibacter africanus subsp. Capensis and subsp. Vepridis with strains from South Africa (Fig. 4a). All three clusters were quite distinct from those of *Ca.* Liberibacter asiaticus and *Ca.* Liberibacter americanus. The Tanzanian strains M4 (Mgeta Kibaoni) and M9 (Kihonda) from Morogoro, and M21 (Maruku) and M22 (Bumiro) from Kagera appeared to be closely related to *Ca.* Liberibacter africanus strains Nelspruit and PTSAPSY (Fig. 4a). In a phylogenetic tree based on the L10/L12 rDNA gene (laf only), all Tanzanian strains clustered together with other Laf strains from South Africa like PTSAPSY, Nelspruit, Wellington 06–0354, and Rustenburg 06–0137 (Fig. 4b). They differed slightly from strain LM4 from Kenya (Fig. 4b). In a concatenated tree of 16S rRNA and L10/L12 genes, the same three clusters described above for 16S rRNA were distinguished. All strains from Tanzania were again closely related to the South African Laf strains PTSAPSY and Nelspruit (Fig. S3).

Detection of Candidatus Liberibacter species in leaf tissues with RT-qPCR

In RT-qPCR tests, DNA samples from leaves collected from Kagera and Morogoro regions at altitudes above 700 m, were all positive for Laf with Ct values ranging from 22.84–28.40 (Table 2); these results were consistent with HLB symptoms observed. However, not all these samples produced positive results with conventional PCR with the primer sets OA1/OI2C, A2/J5, and

Table 2 Dates and locations of collection of citrus leaf samples with huanglongbing symptoms in Tanzania and the results of conventional PCR and RT-qPCR with primers for *Ca*. Liberibacter africanus (Laf) and *Ca*. Liberibacter asiaticus (Las)

Date of Collection	Region	Location	Host	Altitude	PCR Primer pairs				RT– qPCR[2]	Ct value	RT- qPCR[2]	Ct value
					OI1/OI2c[1]	OA1/OI2c[1]	A2/J5	L10/L12[1]	Laf	Laf	Las	Las
10 Sep. 2014	Morogoro	Langali	Lemon[3]	1095	–	–	–	+	+	27.84	X[4]	X
10 Sep. 2014	Morogoro	Msikitini	Orange	747	–	–	–	–	+	24.79	X	X
10 Sep. 2014	Morogoro	Mgeta Kibaoni[5]	Orange	1030	+	+	+	+	+	23.64	X	X
10 Sep. 2014	Morogoro	Mgeta Kibaoni[5]	Orange	992	–	–	–	–	+	28.40	X	X
10 Sep. 2014	Morogoro	Mgeta Kibaoni[6]	Orange	998	+	+	+	+	+	25.52	X	X
14 Dec 2014	Morogoro	Mkuyuni[7]	Orange	381	–	–	–	–	–	40	+	33.88
14 Dec 2014	Morogoro	Mkuyuni[5]	Mandarin	406	–	–	–	–	–	40	–	40
11 Sep. 2014	Morogoro	Mlali[6]	Orange	552	+	+	+	+	+	23.15	+	32.04
11 Sep. 2014	Morogoro	Mlali	Orange	538	–	–	–	–	+	26.13	–	36.14
11 Sep. 2014	Morogoro	Mzumbe	Orange	531	–	–	–	–	+	25.14	+	33.81
10 Jan. 2015	Morogoro	Mkuyuni[5]	Orange	413	–	–	–	–	–	40	–	39.67
10 Jan. 2015	Morogoro	Mkuyuni[7]	Orange	400	–	–	–	–	–	40	+	25.77
16 Dec 2014	Morogoro	SUA	Orange	479	–	–	–	–	+	25.7	+	33.89
16 Dec 2014	Morogoro	SUA[5]	Pumelo	476	–	–	–	–	–	34.55	–	39.81
15 Dec 2014	Morogoro	Kihonda	Orange	498	–	–	–	–	+	27.4	+	25.26
28 Apr 2015	Morogoro	Kihonda[6]	Orange	514	+	+	+	+	+	24.45	+	25.9
15 Dec 2014	Morogoro	Kihonda	Lemon	514	–	–	–	–	+	27.29	+	24.85
14 Jan. 2015	Tanga	Msangati	Orange	152	–	–	–	–	–	40	–	40
21 Dec 2014	Tanga	Semngano	Orange	241	–	–	–	–	–	40	–	37.65
12 Jan. 2015	Tanga	Semgano[5]	Orange	254	–	–	–	–	–	40	–	40
20 Dec 2014	Tanga	Kibanda	Orange	185	–	–	–	–	–	40	–	38.01
14Jan 2015	Tanga	Msangati	Orange	158	–	–	–	–	–	40	–	40
21 Dec 2014	Tanga	Kibanda	Orange	168	–	–	–	–	–	40	–	40
13 Jan. 2015	Tanga	Semgano	Orange	271	–	–	–	–	–	40	–	39.11
13 Jan. 2015	Tanga	Msangati[5]	Orange	155	–	–	–	–	–	40	–	40
08 Jan. 2015	Morogoro	Mlali	Orange	538	–	–	–	–	+	24.13	–	36.12
08 Jan. 2015	Morogoro	Mzumbe	Orange	515	–	–	–	–	+	27.25	+	33.83
09 Jan. 2015	Morogoro	SUA[7]	Orange	479	–	–	–	–	+	30.02	+	32.82
09 Jan. 2015	Morogoro	Mafiga[5]	Mandarin	591	–	–	–	–	–	38.13	–	36.79

Table 2 (continued)

Date of Collection	Region	Location	Host	Altitude	PCR Primer pairs				RT– qPCR[2]	Ct value	RT- qPCR[2]	Ct value
15 Nov 2014	Kagera	Bumiro[6]	Lemon	1214	+	+	+	+	+	22.84	X	X
10 Oct 2014	Kagera	Maruku[6]	Lemon	1293	+	+	+	+	+	25.61	X	X
06 Oct 2015	Kagera	Chudikye[5]	Orange	1290	–	–	–	+	+	24.28	X	X
06 Oct 2015	Kagera	Muhutwe[5]	Orange	1362	–	–	–	+	+	25.58	X	X
27 Apr 2015	Morogoro	Tumbaku	Orange	472	–	–	–	–	–	39.98	+	31.3
28 Apr 2015	Morogoro	Kyegeya	Orange	489	–	–	–	–	–	40	+	30.01
27 Apr 2015	Morogoro	Mafiga	Orange	587	–	–	–	–	–	40	+	32.78
19 Jun 2015	Morogoro	Kyegeya	Orange	480	Y[8]	Y	Y	Y	–	40	–	35.35
16 Jun 2015	Morogoro	Mkuyuni	Orange	400	Y	Y	Y	Y	–	38.20	–	35.43
05 Jun 2015	Kagera	Muhutwe	Orange	1365	Y	Y	Y	Y	+	25.47	+	26.61
03 Jun 2015	Kagera	Maruku	Lemon	1294	Y	Y	Y	Y	+	26.01	+	29.47
04 Jun 2015	Kagera	Maruku	Orange	1293	Y	Y	Y	Y	+	25.79	+	26.53
10 Jun 2015	Kagera	Bumiro	Lemon	1221	Y	Y	Y	Y	+	28.40	+	28.06
23 Dec 2014	Coast	Kibaha	Orange	122	–	–	–	–	–	40	–	38.07
23 Dec 2014	Coast	Visiga	Orange	68	–	–	–	–	–	40	–	35.91
14 Apr 2015	Coast	Kibaha	Orange	137	–	–	–	–	–	40	–	37.58
14 Apr 2015	Coast	Visiga	Orange	64	–	–	–	–	–	40	–	40
14 Apr 2015	Coast	Visiga[5]	Orange	77	–	–	–	–	–	39.06	–	40

[1] PCR with primers OA1/OI2C, OI1/OI2C and L10/L12 conducted at Sokoine University of Agriculture (SUA) Tanzania and repeated at University of Florida, Gainesville, FL, USA

[2] RT-qPCR conducted at International Institute of Tropical Agriculture (IITA) and repeated at University of Florida, Gainesville, FL, USA

[3] Symptoms are presented in Table 1; the samples can be matched by comparing dates and locations in the two tables

[4] X indicates tha RT-qPCR was not performed because samples collected from high altitudes were initially not tested for Las

[5] Samples not sent to University of Florida, Gainesville, FL, USA

[6] Samples were sequenced and used to make phylogenic trees; the sequences were submitted to NCBI data base (Table S1)

[7] Samples tested with primer sets P25, P30 and P34 specific for Las, at University of Florida, Gainesville, FL, USA

[8] Y indicates that only RT-qPCR was performed on these leaf samples

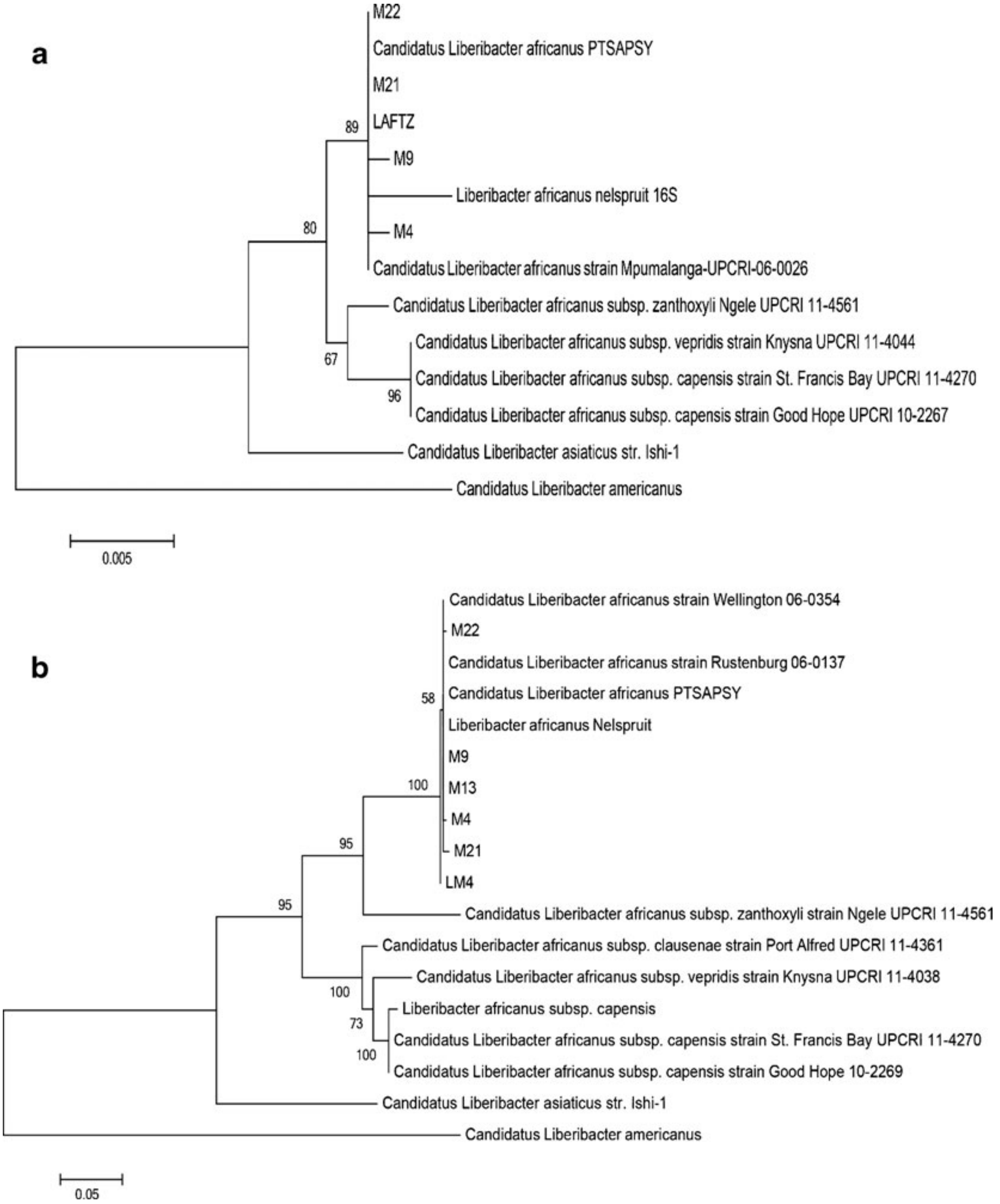

Fig. 4 Phylogenetic trees based on sequences obtained from16S rDNA (OI1/OI2C primers) **(a)** and ribosomal protein rDNA (L10/L12 primers) **(b)** isolated from citrus leaves collected in Tanzania (M4 from Mgeta Kibaone, M9 from Kihonda, M13 from Mlali, M21 from Maruku and M22 from Bumiro in Tanzania), one isolate from *T. erytreae* (LAFTZ) collected in Maruku, one Kenyan isolate (LM4) and 12 entries from the National Center of Biotechnology Information (NCBI) database

L10/L12 (Table 2). Besides Laf, Las seemed to be detected in leaf samples collected from medium altitudes (300 - 600 m) at Mlali, Kihonda, SUA, and Mazumbe, with Ct values ranging from 23.15–30.02 for Laf and from 24.85–33.89 for Las (Table 2; Fig. S4). Leaf samples collected from medium altitudes (300 m – 600 m) in Kyegeya, Mkuyuni, and Mafiga in 2015 were negative for Laf and positive for Las with Ct values ranging from 25.77–33.88 (Table 2; Fig. S4). However, positive qPCR results for Laf and Las in samples collected in Kagera (high altitudes) strongly suggest cross-reaction of Laf to the primer HLBas.

Table 3 Summary of RT qPCR results for psyllid (*Trioza erytreae* (del Guercio) *and Diaphorina citri* Kuwayama) samples collected during citrus huanglongbing surveys in Morogoro and Kagera regions, Tanzania, in 2015

Location	Month	Latitude	Longitude	Altitude (m)	Psyllid species	Adults	Nymphs	Sent for PCR	Las+[1]	Las Ct range	Laf+[1]	Laf Ct range
SUA	Jan	−6.644	37.663	479	*D. citri*	2	–	0 adults	–	–	–	–
Mafiga	Jan	−6.968	37.539	591	*D. citri*	23	–	15 adults	0	40	0	40
Tumbaku	Apr	−6.781	37.660	472	*D. citri*	26	2	10 adults	0	40	0	40
Kihonda	Mar	−6.812	37.650	514	*D. citri*	9	18	4 adults +10 Nymphs	0	40	0	40
Tumbaku	Apr	−6.825	37.655	511	*D. citri*	1	20	10 nymphs	0	40	0	40
Maruku	Jun	−1.419	31.779	1293	*T. erytreae*	27	–	18 adults	3	30.1–33.0	14	25.6–33.8
Maruku	Jun	−1.419	31.779	1293	*T. erytreae*	6	–	4 adults	0	40	1	31
Muhutwe	Jun	−1.585	31.719	1362	*T. erytreae*	13	5	10 adults	0	40	3	31.5–33.1
Bumiro	Jun	−1.841	37.597	1214	*T. erytreae*	9	–	6 adults	0	40	3	32.1–32.9
Bumiro	Jun	−1.841	31.597	1214	*T. erytreae*	5	–	2 adults	0	40	1	33.8
Mkuyuni	Jun	−6.934	31.817	400	*D. citri*	19	–	14 adults	0	40	0	40
Kyegeya	Jun	−6.727	37.668	489	*D. citri*	4	–	2 adults	0	40	0	40
Tumbaku	Jul	−6.813	37.650	468	*D. citri*	10	–	8 adults	0	40	0	40
Kyegeya	Jul	−6.722	37.680	481	*D. citri*	8	–	6 adults	0	40	0	40
Kyegeya	Jul	−6.726	37.673	481	*D. citri*		~60	40 nymphs	0	40	0	40
Muhutwe	Jul	−1.584	31.723	1308	*T. erytreae*	8		6 adults	1	32.3	2	28.7–32.7
								4 + control	4	15.1–23.1	4	19.4–26.2
								4 -control	0	>40	0	>40

[1] Number of specimens that tested positive for Las or Laf (with Ct value below 34.0) in RT qPCR tests

Detection of Candidatus Liberibacter species in psyllids

Twenty four adult psyllid samples (*T. erytreae*) collected from Maruku and Muhutwe in Kagera region tested positive for Laf in qPCR tests, with Ct values ranging from 25.6–33.8 (Table 3). Also, four adult psyllids collected from the same areas tested positive for Las with Ct values ranging from 30.1–33.0 (Table 3). All Asian citrus psyllid samples (*D. citri*) collected from Morogoro region tested negative for both Las and Laf. One DNA sample from *T. erytreae* collected at Maruku in Kagera region, amplified by HLBas/HLBr and named LAFTZ, was sequenced, and the sequences were similar to those of the leaf samples from Tanzania and South Africa (Fig. 4a).

Potential spread of D. citri and Las in Africa and Europe

Both MaxEnt and Multi-Model showed a good fit to the training data of *D. citri* and HLB-Las. For *D. citri*, the AUC of the test data in MaxEnt and MMF was 0.76 and 0.98 respectively. Both models predicted that most of the citrus growing areas in Africa and some in Europe are suitable for *D. citri* establishment (Fig.3). However, some citrus production locations in Congo, Central African Republic and Southern Sudan were predicted as unsuitable by MaxEnt (Fig. 5a) while SVM in MMF predicted high probability of *D. citri* establishment at these locations (Fig. 5b). Some other citrus production areas were predicted unsuitable by both models such as Central parts of South Africa, Egypt, Central Spain, Northern Italy, Turkey (except for the coast), the Balkan and the Greek inland (Fig 3). Additionally, some areas with interior Mediterranean climate in Algeria, Tunisia and Morocco were predicted unsuitable for *D. citri* establishment by both models. The consensus map (overlayed on citrus production areas) showed the hot-spots where both modelling approaches agreed on the climate suitability for both *D. citri* and HLB-Las establishment (Fig. S5).

Discussion

In addition to the native African citrus psyllid *T. erytreae*, the Asian citrus psyllid *D. citri* was found

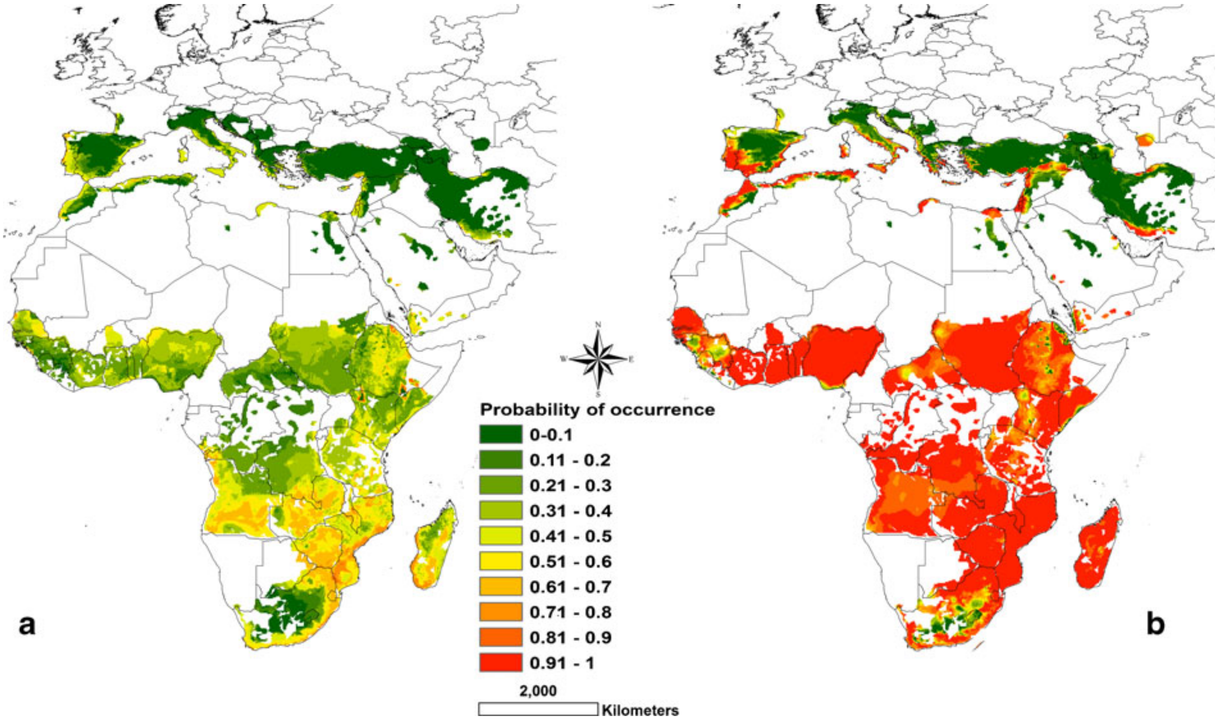

Fig 5 The potential climate suitability for *Diaphorina citri* Kuwayama in citrus growing areas in Africa and Europe predicted by the MaxEnt model **(a)** and the support vector machines (SVM) model in Multi-Model Framework (MMF) **(b)**. MAXENT is a correlative presence only model that is generally conservative in its predictions. MMF includes 9 correlative models with presence and pseudo-absence locations; the SVM model was selected as the most accurate model in validation tests. Both models used global 50-year climate data to predict potential establishment of *D. citri* from the current global occurrence locations, and pseudo-absence locations in SVM

for the first time in Tanzania. *Diaphorina citri* was found at medium altitudes (300-600 m) but not at lower or higher altitudes. Several other species of *Diaphorina* have been reported on citrus and its close relatives in Africa, but these were ruled out based on biology and morphology (Halbert and Manjunath 2004; Hollis 1987). To our knowledge, this is the first occurrence of *D. citri* in mainland Africa, although both *D. citri* and *T. erytreae* occur in Mauritius and Réunion (Bové 2014). As expected from prior observations (Evers and Grisoni 1991), *T. erytreae* and damage from this psyllid (leaf galls) were found at high altitudes (>700 m), but *T. erytreae* was not observed at medium altitudes (500-600 m).

Both Laf and Las seemingly were detected by qPCR of DNA extracted from adult *T. erytreae* samples collected from higher altitudes in Kagera region. Only four (8.7 %) out of 46 adult *T. erytreae* samples tested by qPCR were Las positive, while 24 (52.2 %) of the 46 adult *T. erytreae* samples were Laf positive. The Las positive reactions could have been cross-reactions to the primers used, as cross-reactions have been observed more frequently in recent years (Li et al. 2006; J. Rascoe, personal communitation). Also, the sequences obtained from one sample were similar to those of Laf although both OI1/OI2c and OA1/OI2c were used in the prior cPCR reactions. It is therefore impossible at this time to conclude that Las may have occurred in *T. erytreae* from Kagera region. Both *D. citri* and *T. erytreae* have been shown to experimentally transmit Laf and Las (Lallemand et al. 1986), but from field studies and surveys *D. citri* appeared to be the only vector of Las and *T. erytreae* of Laf (Bové 2006; Bové 2014). Laf and Las were absent from the *D. citri* samples collected from medium altitudes in Morogoro region, but the titers may have been too low for detection (Manjunath et al. 2008; Razi et al. 2014; Teixeira et al. 2008). It is important to test the psyllids in Tanzania more extensively for the presence of Las, because positive insects can be found months to years prior to the development of disease symptoms induced by Las (Manjunath et al. 2008; Shen et al. 2013b).

Likewise, both Laf and Las were detected by RT qPCR in DNA extracts from symptomatic leaf samples collected from medium and higher altitudes in Morogoro and Kagera regions. Laf first was detected provisionally in Tanzania by electron microscopy and grafting in the early 1990's (Swai et al. 1992; Evers and Grisoni 1991), but molecular characterization of Laf is reported first here. The DNA sequences identified in samples from Tanzania were very similar to the South African Laf strains PTSAPSY and Nelspruit. A recent report showed that Kenyan strains of *Ca. Liberibacter* also were related closely to the South African Laf strains (Magomere et al. 2009), indicating that Laf strains similar to those of South Africa are distributed widely in East Africa.

The positive reactions to the HLBas primers used in RT qPCR probably were due to cross-reaction with Laf, although there were 3 Las-positive and Laf-negative samples in Morogoro. Las could not be detected by sequencing of 16S rDNA from leaf samples collected in Tanzania. Thus, at this time it is not possible to say that Las is present or not present in Tanzania. More surveys, using more specific primers and protocols for Las detection (Bertolini et al. 2014; Coy et al. 2014; Keremane et al. 2015; Kogenaru et al. 2014; Orce et al. 2015) are needed to prevent potential spread into other parts of Tanzania and Africa.

Predictions from Maxent and Multi-model framework indicate that East Africa and part of South-central Africa, as well as some areas in West Africa, have a highly conducive climate for the establishment of *D. citri* and Las. Similarly, the climate along the Mediterranean coast, including Southern Europe, is suitable for these species, as was predicted earlier (Gutierrez and Ponti 2013). Climate suitability for *D. citri* and HLB-Las is primarily determined by precipitation (Narouei-Khandan et al. 2015), which is quite high in South-central Africa. More arid environments, such as inland areas of South Africa and Spain, are less suitable possibly due to the absence of frequent flushing of citrus trees (Lee et al. 2015; Narouei-Khandan et al. 2015; Patt and Sétamou 2010). In addition to rainfall, a minimum temperature below 4 °C in the coldest quarter is not conducive for *D. citri* survival (Narouei-Khandan et al. 2015). In warm seasons, a mean temperature higher than 33 °C can limit *D. citri* presence because eggs and young nymphs are sensitive to high temperatures and desiccation (Liu and Tsai 2000). Optimal and limiting temperature conditions for HLB-Las are dependent on rainfall. The combination of dryness and high temperatures seems to form a stress factor either on the pathogen itself or on its vector or both (Narouei-Khandan et al. 2015). It is well-known that the Asian form of HLB is most severe in warm and wet climates (Bové 2014), which are common in large parts of South-central and West Africa as well as parts of East Africa, where HLB-Las is still unknown. Las was detected once in Tigray (Saponari et al. 2010), which is only slightly suitable for this pathogen according to our model. North-western Portugal and Spain have a moderately warm and humid climate and are highly suitable for *D. citri* and Las establishment. *T. erytreae* has been observed in these areas, but a *Ca*. Liberibacter species has not been detected in citrus as yet (Monzó et al. 2015; Pérez-Otero et al. 2015).

Potential invasion of Africa and Europe by *D. citri* and Las not only would depend on climate suitability, but also on competition between *D. citri* and *T. erytreae* and between Las and Laf at medium altitudes. *Trioza erytreae* and Laf are well established in Southern and Eastern Africa, and it is unknown which of the two combinations (African or Asian) would be more competitive. In Latin America, Las outcompeted Lam in a relatively short period (da Graça et al. 2015; Lin et al. 2013; Lopes et al. 2009), but the relative competitive ability of Las and Laf is unknown. Our research indicates that Las and Laf as well as both psyllid species ultimately may occur in the same groves in Tanzania, similar to the situation in Réunion and Mauritius islands (Bové 2006), although some segregation by altitude has been noted on those islands and in the Arabian peninsula (Aubert 1987; Bové 2006; Toorawa 1998). In addition to competition from *T. erytreae* and Laf, the spread of *D. citri* and Las could be limited by parasitism. Some *D. citri* nymphs from Tanzania showed evidence of parasitism, although no parasites were recovered. *Diaphorina citri* may have arrived in the African mainland accompanied by one or more of its known parasites, or it may have acquired local parasites associated with other species of *Diaphorina* (Halbert and Manjunath 2004) that occur in Africa (Hollis 1984). In other citrus producing areas, parasites have not been effective at controlling HLB (Halbert and Manjunath 2004), with the possible exception of Réunion Island (Aubert 1987).

The arrival of *D. citri* in the African mainland has serious implications for African citriculture. Formerly, HLB in Africa was restricted to highland cooler areas where *T. erytreae* and Laf could survive. *Diaphorina citri* can colonize the lowland tropics and subtropics, putting citrus in these areas at risk, should Las be introduced. The insects and the pathogens can be transported over long distances in strong winds, on fruit and nursery stock in trailers (Halbert et al. 2012). Once HLB is established, citriculture can become economically non-viable in a few years due to poor fruit quality, leaf and fruit drop, tree decline and death (Farnsworth et al. 2014; Halbert and Manjunath 2004).

Thus, strict control measures are needed urgently. First of all, an extensive survey needs to be conducted at medium and low altitudes to delineate the area that needs to be put under quarantine. Although quarantine measures are needed to prevent further spread, the indiscriminate application of insecticides to attempt eradication of *D. citri* is not recommended (Ichinose et al. 2010; Shen et al. 2013a; Tiwari et al. 2011). Also, removal of Las infected trees has not been effective in most regions with a mosaic of small orchards (including in Florida and Brazil) due to the long incubation period relative to the latent period, and the inability to reduce psyllid populations to zero (Bassanezi et al. 2013a, b; Belasque et al. 2010; Ichinose et al. 2010; Lee et al. 2015; Sisterson and Stenger 2013). Therefore, tree removal will not be practical or effective in the small orchards and backyard plantings that are common in Africa.

Much of the long distance movement of vectors and pathogens can be accounted for by movement of infected and infested plants (Halbert et al. 2012; da Graça et al. 2015). In keeping with this, the establishment of insect-proof nurseries with disease-free and psyllid-free trees is the most important measure to be taken. In Tanzania, citrus nurseries are located in Morogoro, where *D. citri* was detected during our survey. Citrus nurseries may need to be relocated to drier and warmer lowland areas where the probability of HLB development is limited as long as the psyllids are kept out. It is important to keep the entire nursery operation in protected conditions, and to treat all plants with soil-applied systemic insecticides. Transmission of the HLB pathogens is most efficient in vector colonies (Lee et al. 2015), so preventing colonization in nursery settings is crucial. Finally, movement of citrus planting materials needs to be regulated, so that they cannot be moved out of infested areas.

In conclusion, *D. citri* probably still is restricted to a limited area at medium altitudes in Tanzania, but spread of this species to other parts of Africa and Europe is likely unless action is undertaken to prevent its movement in and outside of Tanzania. So far, Las was reported only in Tigray province of Ethiopia, although *D. citri* was not found (Saponari et al. 2010). Thus far, we do not know much about the occurrence of *D. citri* and possibly Las in Tanzania. Our results of the prediction models indicate that the coast of East Africa is highly suitable for both *D. citri* and Las establishment. Quarantine measures and the establishment of nurseries for clean planting materials are very important to curb spread of *D. citri* and HLB. Roguing of infected trees in large orchards (about 10 ha in Tanzania) could contribute to controlling HLB in the beginning of the epidemic, provided that regional control is coordinated (Sisterson and Stenger 2013). Psyllid control by insecticides is likely not effective if applications are not coordinated regionally (da Graça et al. 2015), and is likely not possible in the small-scale production situation of Tanzania. Moreover, intensive spraying can lead to insecticide resistance if strict resistance management is not followed (Tiwari et al. 2011). In the long run, the best solution may be HLB resistance. There is some variation in sensitivity of citrus root stocks (Boava et al. 2015), and breeding for resistance is ongoing (Stover and McCollum 2011), but current citrus cultivars are susceptible to all forms of HLB, and other HLB management measures are inadequate in small-scale citrus production areas worldwide.

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