A Survey for "*Candidatus* Liberibacter" species in South Africa confirms the presence of only "*Ca*. L. africanus" in commercial Citrus

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ABSTRACT

Greening disease of citrus is a serious disease known in South Africa since the late 1920's. In South Africa it is associated with infection by "*Candidatus* Liberibacter africanus" (Laf), a heat sensitive, phloem-limited, non-cultured alpha-proteobacterium. Huanglongbing (HLB), a similar, but more devastating disease which was described initially from China but which now occurs in several citrus producing countries, is associated with a different Liberibacter species, "*Ca.* L. asiaticus" (Las). A Laf sub-species, "*Ca.*" L. africanus ssp. capensis (LafC) has been found only in South Africa infecting an indigenous Rutaceous species, *Calondendrum capensis* (Cape Chestnut) in the Western Cape in 1995. The discovery of a new Liberibacter species in Brazil, "*Ca.* Liberibacter americanus" (Lam), and the spread of Las to a number of additional countries over the last few years prompted us to assess whether only Laf is present in commercial citrus orchards in South Africa. Samples displaying greening or similar symptoms were collected from 249 citrus trees from 57 orchards distributed throughout the greening affected citrus production areas of South Africa. Multiplex PCR was performed on DNA extracts to detect the known citrus Liberibacters. Amplicons were obtained from 197 samples. None of the samples yielded a 1027 bp amplicon indicative of Lam infection. The amplicons of 84 samples were sequenced and all were identical to the cognate Laf Nelspruit sequence in Genbank. No instance of Las or LafC sequence was found. Geographically representative samples which tested negative for Liberibacter also tested negative for phytoplasmas based on real-time PCR results. Based on the results of this survey it is concluded that to date only Laf is associated with citrus greening in commercial citrus in South Africa.

INTRODUCTION

A serious, debilitating, insect-transmissible citrus disease known in South Africa since the late 1920's as greening disease of citrus (29), is associated locally with "*Candidatus* Liberibacter africanus" (Laf), a heat sensitive, phloem-limited, non-cultured alpha-proteobacterium (17, 19). This is in contrast to Huanglongbing (HLB) (5) disease, initially described from China and which is now present in a number of other countries, and which is associated with infection by "*Ca.* L. asiaticus" (Las). Greening, therefore, refers to the Laf associated disease throughout the rest of this article. Greening is considered heat sensitive as the diseased plant appears to recover from symptoms at 32°C but not at 27°C (4). Laf was shown to be present in South Africa in the Rustenburg, Nelspruit, and Western Cape regions during 1998 (11). In addition, a second Liberibacter species, "*Ca.* L. africanus ssp. capensis" (LafC) was detected infecting an indigenous Rutaceous species, *Calondendrum capensis* (Cape Chestnut) in the Western Cape in 1998 (12). PCR tests on samples from South Africa confirmed the presence of Laf and the absence of the more destructive "*Ca* L. asiaticus" (Las) (16). However, since 2004, Las has spread to citrus orchards in, amongst other countries, Brazil (8), Cuba (26), the Dominican Republic (28) and Florida, USA, (13) where it was not previously found, and a new Liberibacter species, "*Ca.* L. americanus" (Lam) was found on citrus in Sao Paulo State, Brazil in 2004 (33). Lam is similar to Laf with regard to its heat sensitivity (25). A new Liberibacter species has also recently been discovered on solanaceous hosts in the USA and New Zealand (14, 23, 24). Recently, Liberibacter negative trees displaying HLB-like symptoms were found in Brazil (35) and in China (6) and were shown to be infected by phytoplasmas. HLB, caused by Las, is a far more debilitating disease than greening, with trees often dying within a few years whereas Laf most often will only affect a limited number of branches of a tree for many years (Van Vuuren, *per. comm.*). Furthermore, while greening's geographical distribution is limited by its heat-sensitive nature, HLB would affect citrus trees also in warmer citrus-production areas in South Africa currently greening unaffected. South Africa's citrus industry is primarily export based and while ranked as the 14th largest producer in the world by volume it is the third largest exporter of citrus. A premium is therefore placed on the production of good quality fruit and the introduction of Las would therefore severely affect the industry. As previous surveys in South Africa for greening were done prior to the advent of Liberibacter species-specific polymerase chain reaction (PCR)-based diagnostic tests, this study was conducted to assess whether Liberibacter species other than Laf may have been overlooked in past studies and may already occur in commercial citrus in South Africa.

MATERIALS AND METHODS

Collection of samples. Because of the heat sensitive nature of symptom expression by Laf and Lam, the survey was conducted during September 2006 as this time coincided with the visits of the French and Brazilian co-authors who are well versed in HLB, Lam induced

disease, and greening. Samples were collected from citrus trees displaying greening, HLB-like or greening-like symptoms from 57 orchards in 17 magisterial districts, representing all the major citrus-production regions of South Africa in which greening disease is known to occur (Figure 1). Orchards were selected based on their having a known history of greening spread. As the incidence of greening is generally low in many of the regions sampled (notable exceptions being Nelspruit and Rustenburg), samples were collected from trees found randomly by the collection team moving through the orchards without a predetermined pattern. Sample numbers at any given site were limited by the low incidence of symptomatic trees found. Where a high enough disease incidence occurred in a given orchard, larger numbers of samples from different symptomatic trees, up to a maximum of ten, were collected. A sample consisted of approximately 20 symptomatic leaves and budwood sticks from an individual tree and was collected from various branches on the tree that were displaying symptoms.

Extraction of DNA for PCR. Total DNA was extracted from 0.5 g of petiole or midrib from each field-collected sample using a standard CTAB (hexadecyltrimethylammonium bromide) procedure (9).

Liberibacter PCR amplification. A multiplex PCR for the simultaneous detection of Las, Laf and Lam was utilized which combined the previously published A2/J5 (16) and GB1/GB3 (34) primer sets directed at the the ß-operon of the ribosomal protein genes of Laf and Las, and the 16S rDNA of Lam respectively. The PCR was carried out in duplicate on DNA extracted from field-collected samples and was performed in a 25 µl reaction mixture consisting of 0.5 µM of each of the four primers, 200 µM of each of the four dNTP's, 67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.1% Tween-20 (NH4 reaction buffer Bioline, London, UK), 10 mM ß-mercaptoethanol, 10 µg Bovine serum albumin, 2 mM $MgCl_2$, 0.1% Triton X-100 and 2.5 U *Taq* polymerase (Biotaq, Bioline, London, UK). A 0.5 µl volume of DNA extract was used as template in the reaction. Amplification was conducted with an ABI Geneamp 2700 thermocycler (Applied Biosystems, Foster City, CA) and the conditions were 35 cycles of denaturation at 92°C for 20 s, annealing at 63°C for 20 s and primer extension at 72°C for 45 s. Amplicons were visualized on a UV transilluminator by ethidium bromide staining after electrophoresis at a constant voltage of 150V for 60 min in 2% agarose in sodium borate buffer (5 mM disodium borate decahydrate, adjusted to pH 8.5 with boric acid) gels. Total DNA extracts from known Las, Lam and Laf-infected plants were supplied by Dr. C. Saillard (INRA, Bordeaux) as positive controls in PCR.

Nucleotide sequencing. To obtain templates for sequencing, samples representative of each production site were amplified using the A2/J5 primer set (16). Reaction conditions were identical to those described for PCR for detection of Liberibacter except cycling conditions were conducted at an annealing temperature of 62°C for 20 s. Amplicons were

electrophoresed on a 1.0% agarose gel followed by ethidium-bromide staining. Desired bands were cut out and purified with a Wizard SV Gel and PCR clean up system (Promega, Madison, USA). The purified PCR products were subjected to cycle sequencing using the Big Dye® Terminator v3.1 Cycle Sequencing kit and Big Dye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturers specifications. The same forward and reverse primers used for the PCR amplification were used separately in cycle sequencing in order to sequence both sense and anti-sense strands. The extension products were purified by ethanol/sodium-acetate precipitation, and then sequenced at the core sequencing facility of the University of Pretoria on an ABI Prism 3100/3130 sequencer (Applied Biosystems, Foster City, CA).

Sequence analysis. Nucleotide sequence was analyzed using the DNAMAN software suite (Lynnon Biosoft, Quebec, Canada). Consensus sequences of the A2/J5 amplicons were prepared based on the sequence in both orientations. Nucleotide similarity searches of Genbank were conducted using the BLAST algorithm (1) of the National Centre for Biotechnology Information. Amplicon sequences were compared to cognate regions of the following Genbank accessions for Laf (LAU09675); LafC (AF248498), Lam (EF122254), Las (M94319), and a recently discovered liberibacter from tomato, *"Candidatus* Liberibacter solanacearum" (Lso) (EU834131) (23). Multiple alignments were prepared in DNAMAN and alignments were used to prepare phylogenetic trees using a maximum likelihood method with mutation rates estimated from the actual sequences (15, 32).

Graft propagation of Liberibacter from field samples. Budwood collected from field collected sources were used to establish the greening samples by side-grafting of two to three buds onto a single plant of sweet orange (*Citrus sinensis* cv. 'Bahianinha' navel) on Carrizo citrange *(Poncirus trifoliata* (L.) Raf.*)* X *Citrus sinensis* (L.) Osb.) rootstock. Inoculated plants could also serve as indicator plants. Plants were maintained in an insect-proof greenhouse under natural light conditions at temperatures between 24-28°C. The grafted plants were tested for Laf after one year using real-time PCR (22) using as template 1µl of the eluant from leaf petioles macerated in an alkaline buffer and spotted onto nitrocellulose membranes (2). Real-time PCR was performed using a Lightcycler® 1.5 (Roche, Mannheim, Germany) capillary-based thermocycler. Lightcycler® Taqman® Master kits were used along with the Laf-specific primers and probes and conditions described by Li et al (22).

Detection of phytoplasma with PCR. Of the 52 samples collected from symptomatic trees which yielded negative multiplex PCR results for all three Liberibacter species, 22 samples, one from each site, were tested for the presence of phytoplasmas. PCR was done using the Universal phytoplasma specific Taqman® probe and primer sequences previously published (7) using slightly modified conditions. Briefly, each sample was tested in duplicate in 20 µl glass capillaries in a Lightcycler® 1.5 apparatus (Roche, Mannheim, Germany) in a reagent

mixture of 0.5 µM of each of Phytop Forward and PhytoP Reverse primers, 0.25 µM of Phytop Probe in a 1x Taqman Universal PCR master mix (Roche, Mannheim, Germany). Cycling conditions consisted of 10 minutes at 95°C followed by 40 cycles of 92°C for 15 s, 60°C for 60 s and 72°C for 1 s. Single acquisition fluorescence readings were taken at the conclusion of each cycle and data processing and threshold cycle (Ct) calculations were done with the Lightcycler® instrument software. Positive controls included in all real-time tests were of Bois Noir phytoplasma obtained from Boudon-Padieu (INRA, France)

RESULTS

A total of 249 samples were collected from 57 groves in 17 magisterial districts (Table 1). The number of orchards monitored, type and number of samples collected per district are shown in Table 1. Samples included leaf material with one or more of the obvious greening symptoms, as agreed upon by most members of the survey team, as well as samples with more obscure symptoms which were collected primarily to ensure the detection of any Liberibacter associated with possible symptom variants of the disease. Graft transmission of Liberibacter to 'Bahianinha' navel sweet orange was very inefficient with only 12 recipients becoming infected. Real-time Laf-specific PCR performed on extracts from these trees yielded Ct values ranging from 24 to 35 one year post-inoculation. This low level of transmission likely was due to the fact that symptomatic branches did not necessarily have budwood of a physiological state adequate for efficient grafting, as poor bud take was experienced, with only 34 buds taking. No instances were obtained where graft inoculated trees displayed disease symptoms or contained Laf when budded with, field collected material which tested negative for Laf by multiplex PCR.

Of the 249 samples collected and tested in the multiplex PCR, none yielded the 1027 bp amplicons expected from a Lam infected plant. Amplicons where obtained from 197 samples. The majority of these were the same size as the amplicon obtained with the Laf control. However, when high yields of amplicons were obtained electrophoresis conditions could not adequately differentiate the 669 bp band expected of Laf and the 703 bp band expected of Las (16). The differing yields of these amplicons, as indicated by staining and comparison to positive controls, suggest possible differences in Liberibacter amounts in sample tissues.

Eighty-four amplicons generated by multiplex PCR, selected mainly, but not exclusively, from those where the amplicon size could not be determined accurately due to the thickness of the band were subjected to cycle sequencing using the A2 and J5 primers to ensure sequence data of both orientations. Sequence analysis confirmed that all sequenced samples were infected with Laf only. Forty sequences were identical to each other and to the reference Laf Nelspruit Isolate (LAU09675). The lowest identity amongst all isolates was 98.7%. The sequence of thirteen geographically representative sources where submitted to Genbank.

These are; Rustenburg 06-0137 (GU120032); Brits 06-0204 (GU120033); Groblersdal 06-0224 (GU120034); Zebedeila 06-0234 (GU120035); Tzaneen 06-0241 (GU120036); Hoedspruit 06-0256 (GU120037); Nelspruit-2 06-0271 (GU120038); Whiteriver 06-0292 (GU120039); Swellendam 06-0307 (GU120040); Caledon 06-0330 (GU120041); Stellenbosch 06-0338 (GU120042); Paarl 06-0351 (GU120043) and Wellington 06-0354 (GU120044). A phylogenetic tree is presented in Figure 2 in which the A2/J5 amplified sequences of 13 samples from infected districts are compared to cognate sequences of known Liberibacter species.

None of the 22 samples which had displayed greening-like symptoms, but tested negative in the Liberibacter multiplex PCR, were positive in the phytoplasma real-time PCR test.

DISCUSSION

Only "*Candidatus*" Liberibacter africanus" (Laf) was detected amongst the commercial citrus samples during this survey. Therefore, it would appear that the other citrus Liberibacters are still absent from commercial citrus plantings in South Africa. This allays concerns that previous studies done on South African samples may have missed the presence of these other Liberibacters, especially Lam, due to the limitations of techniques available at the time and the relatively low number of samples previously tested for Las specifically. Symptoms of Laf and Lam differ from those of Las primarily by severity and temperature tolerance (5, 31, 25). Consequently it is possible that the symptoms induced by Laf in South Africa may mask the presence of another Liberibacter, especially Lam, for a significant period. In addition, the presence of a phytoplasma associated with citrus trees displaying greening-like symptoms in Brazil and China (6, 35) may indicate that the use of symptoms only to identify the disease may result in erroneous conclusions as to the causal organism. It was therefore essential that a survey be conducted in South Africa with techniques more sensitive and specific for the different Liberibacters.

Specific detection of Laf was first accomplished in the early 1990's with monoclonal antibodies (10, 20), and then later with DNA probes (30). A PCR specific for Laf (18), was used, along with DNA hybridization which was able to detect Laf and Las, to confirm the presence of only Laf in South Africa between 1993 and 1996 (19). During 1998, 82 samples from Nelspruit, Rustenburg, the Eastern and Western Cape were tested by Laf specific PCR's and resulted in the first detection of Laf in the Western Cape along with detection of "*Ca*. Liberibacter africanus spp. capensis" (LafC) infecting *Calodendrum capense* (11, 12).

A number of samples collected proved to be negative for Laf and phytoplasmas. This probably can be ascribed to the collection of samples from trees with greening or HLB-like symptoms which were due possibly to various nutrient deficiences, rather than due to Liberibacter infection. Although some samples possibly were collected unnecessarily, sample

collection based upon visual assessment is preferred to prevent possibly missing an unusual symptom potentially induced by a Liberibacter other than Laf.

Our finding that only Laf occurs in the commercial citrus samples examined in this study in South Africa is important. The recent spread of Las to Brazil (8, 33), Florida, USA (13) and Cuba (26) and the discovery of a new species, Lam, found on citrus in Sao Paulo State, Brazil in 2004 (33), were all preceded by the introduction of the Las and Lam natural vector *Diaphorina citri*, an insect not yet reported in South Africa.. However, both *D. citri* and *Trioza erytreae*, the natural vector of Laf in South Africa, have been shown to at least experimentally transmit both Laf and Las (21, 27), and the introduction of Las to South Africa may not require the introduction of *D. citri* also to spread. The absence of LafC in any of the citrus samples is however interesting, as both occur in at least the Western Cape, where 25% of the samples were collected. It may be that the epidemiology of the Laf and LafC do not overlap. This is the subject of an additional study.

Very low nucleotide sequence variability in the genome region amplified from the Laf sources was found, with 40 of the 87 sequenced amplicons yielding identical sequences, and the remaining samples diverging by a maximum of only 1.3% of the nucleotides. As the amplified region is part of the 50S ribosomal subunit protein gene, this high level of sequence conservation is expected. It is possible too that part of the differences amongst the samples is due to amplification and sequencing errors and that with cloning and sequencing of a number of individual clones the variation may be even less. However, serological variation amongst South African Laf populations has been demonstrated in the past, with the 10H8 and MG8 monoclonal antibodies produced against Laf differentially binding to different Laf sources (20). Studies to determine the variation that exists amongst Laf sources should target a more variable gene, for example the outer membrane protein gene (*omp*), which has been demonstrated to vary with different isolates of Las (3).

Future studies to ensure that the Liberibacter *status quo* is being maintained in South Africa and that Liberibacter spp. reported from other crops (14, 23, 24) don't occur here (eg. those from the Solanaceous crops) will require that a multiplex PCR, based on specific primers resulting in amplicons with greater size differences, will need to be developed In the current study it was necessary to sequence the amplicons in order to identify the different Liberibacter species.

The aim of the survey was to confirm the validity of previous conclusions from studies using more limited detection techniques that Laf alone is associated with greening in commercial citrus in South Africa where greening is generally well controlled and the incidence is low. As Laf remains the only Liberibacter found it is essential that the South African

Phytosanitary authorities and the citrus industry remain vigilant against the unintentional introduction of Las and Lam. Finding a newly introduced Liberibacter in South Africa, where greening symptoms are common will be much more difficult than in countries where the disease symptoms would be unique. Having absolute certainty that neither Las or Lam occurs in South Africa would require that practically all citrus trees showing any HLB or greening-like symptoms would have to be tested, including large numbers of backyard trees. Should Lam, which also induces a heat sensitive disease, be introduced to South Africa it is likely to remain undetected for a long time as its symptoms would be attributed to Laf infection. The introduction of Las possibly may be observed locally as a more severe disease epidemic than greening, or as an expansion of disease to warmer production areas that are currently greening free. Producers are urged to report such an observation through numerous awareness campaigns by the local citrus industry. Should Las or Lam be introduced, delineating surveys would probably have to be based on specific laboratory tests rather than on symptoms as these would be confused with those induced by Laf. This would make containment and localized eradication extremely difficult if not impossible. Furthermore, due to the successful management of Laf-induced greening in South Africa and the associated complacency regarding this disease, it would be difficult in the period directly after introduction to convince producers to eradicate trees newly infected with either Las or Lam, especially the relatively mildly symptomtic Lam infected trees. Nevertheless, in view of the crises that the introduction of HLB would cause in the South African citrus industry, and the need to act as quickly as possible after its introduction, growers are encouraged to submit samples with greening-like diseases for laboratory tests. This is especially so where such symptoms appear more severe than usual or are from citrus trees in the warmer citrus production areas.

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LEGENDS OF FIGURES

Figure 1. Map of South Africa illustrating sites where samples were collected and showing number of samples collected per site. Main towns within South Africa are indicated by unfilled squares and filled circles with numbers indicate the number of plant samples collected at the sites named in Table 1.

Figure 2. A phylogenetic tree of nucleotide sequences amplified by A2/J5 primers from representative South African samples from the indicated districts and the cognate sequences of known Liberibacters. Reference isolates and their Genbank Accession numbers are: Laf = "*Candidatus* Liberibacter africanus" (LAU09675); LafC= "*Candidatus* Liberibacter africanus spp. capensis" (AF248498); Lam= "*Candidatus* Liberibacter americanus" (EF122254); Las="*Candidatus* Liberibacter asiaticus" (M94319); and Lso="*Candidatus* Liberibacter solanacearum" (EU834131). Geographically representative South African local and Genbank Accession numbers are as follows: Rustenburg 06-0137 (GU120032); Brits 06-0204 (GU120033); Groblersdal 06-0224 (GU120034); Zebedeila 06-0234 (GU120035); Tzaneen 06-0241 (GU120036); Hoedspruit 06-0256 (GU120037); Nelspruit-2 06-0271 (GU120038); Whiteriver 06-0292 (GU120039); Swellendam 06-0307 (GU120040); Caledon 06-0330 (GU120041); Stellenbosch 06-0338 (GU120042); Paarl 06-0351 (GU120043) and Wellington 06-0354 (GU120044). Scale bar refers to branch length.

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Table 1. Results of sampling for "*Ca*. Liberibacter spp." in citrus samples from various locations in South Africa.

District	Scion	No. of Orchards Sampled/ District/Scion	No. of Samples/District/Scion	No. of infected samples
Brits	Citrus reticulata Blanco cv. Nova	1	2	2
	C. sinensis Osbeck cv. Valencia	2	12	10
Caledon	C.limon (L.) Burm f. cv. Eureka	1	6	6
	C. reticulata Blanco cv. Nules	1	5	4
Groblersdal	C. sinensis Osbeck cv. Robyn Navel	1	1	0
	C. sinensis Osbeck cv. Washington Navel	3	9	4
Hoedspruit	C. paradisi Macf. cv. Marsh	1	1	1
	C. sinensis Osbeck cv. Delta Valencia	1	6	6
	C. sinensis Osbeck cv. Du Roi Valencia	1	1	0
	C. sinensis Osbeck cv. Valencia	2	9	9
Malelane	C. sinensis Osbeck cv. Valencia	5	7	0
Melmouth	C. sinensis Osbeck cv. Valencia	2	7	0
	C. sinensis Osbeck cv. Navel	1	1	0
	C. sinensis Osbeck cv. Valencia	1	1	0
Montagu	Citrus reticulata Blanco cv. Afurer	1	12	8
Nelspruit	C.limon (L.) Burm f. cv. Eureka	1	1	1
	C.limon (L.) Burm f. cv. Limonera	1	2	1
	C. sinensis Osbeck cv. Delta Valencia	1	10	10
	C. sinensis Osbeck cv. Midnight Valencia	1	5	4
	C. sinensis Osbeck cv. Valencia	1	10	9
Ngonini, Swaziland	C. sinensis Osbeck cv. Delta Valencia	1	6	6
Paarl	C.limon (L.) Burm f. cv. Eureka	1	8	4
	C. reticulata Blanco cv. Satsuma	1	2	2
Rustenburg	C.limon (L.) Burm f. cv. Eureka	1	6	6
	C.limon (L.) Burm f. cv. Fino	1	1	0
	C. reticulata Blanco X C. paradisi Macf.	1	6	5
	C. reticulata Blanco cv. Clemantine	1	1	0
	C. sinensis Osbeck cv. Delta Valencia	1	5	5
	C. sinensis Osbeck cv. Midnight Valencia	1	2	2
	C. sinensis Osbeck, cv. Not recorded	1	5	2
	C. sinensis Osbeck cv. Palmer Navel	2	20	19
	C. sinensis Osbeck cv. Robyn Navel	1	6	6
	C. sinensis Osbeck cv. Valencia	5	18	18
Stellenbosch	C.limon (L.) Burm f. cv. Eureka	1	8	8
Swellendam	C. sinensis Osbeck cv. Navelina Navel	1	10	9
Tzaneen	C. sinensis Osbeck cv. Delta Valencia	1	1	1
	C. sinensis Osbeck cv. Midnight Valencia	3	10	10
Wellington	C.limon (L.) Burm f. cv. Eureka	1	11	4
Whiteriver	C.limon (L.) Burm f. cv. Eureka	1	6	6
Zebediela	C. sinensis Osbeck cv. Delta Valencia	1	2	2
	C. sinensis Osbeck cv. Palmer Navel	1	7	7
Totals		57	249	197

FIGURE 1

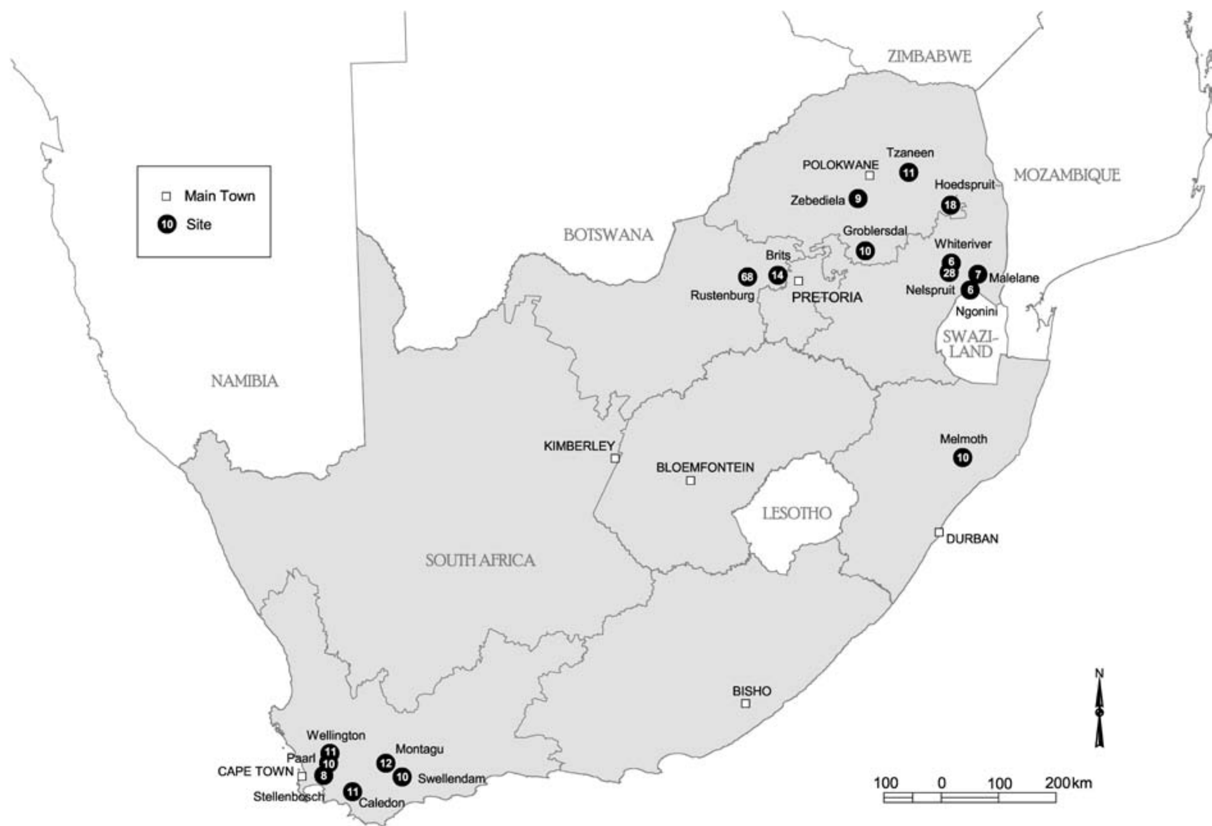

FIGURE 2