DIAGNOSTIC STANDARD FOR HUANGLONGBING

DRAFT

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1. Introduction

Huanglongbing is a phloem-limited disease caused by a proteobacterium (*Candidatus* Liberibacter asiaticus) spread by the Asiatic citrus psyllid (*Diaphorina citri*). It the most serious impediment to citriculture in Asia as it can destroy orchards within 5 years of planting. It has prevented the establishment of viable citrus industries in tropical and subtropical Asia and as a consequence seriously affects the welfare of farmers and national economies. Huanglongbing's gradual movement eastward through Asia to Australia and Oceania is a potential threat to the biodiversity through the loss of citrus species and citrus relatives that are endemic to the region. It is a significant threat to the Australian citrus industry.

1.1. Transmission and spread of huanglongbing

Fourth and fifth instar nymphs and adults transmit L. asiaticus. *D. citri* requires an incubation period of about 21 days in which to transmit the pathogen, which it retains for life following a short access feeding (15-30 minutes) on a diseased plant (Capoor et al. 1974). It is unnecessary for adult psyllids arising from infectious nymphs to feed on diseased shoots in order to become vectors. Adult psyllids can transmit the disease in a minimum infection feeding of 15 minutes but percentage transmission with this duration of feeding is low. One hundred percent infection can occur when psyllids feed for 1 h or more. The pathogen multiplies in the body of the psyllid but there is no transovarial transmission (Capoor et al. 1974). Feeding on old leaves for long periods over winter makes adult *T. erytreae* highly infective on young flush in spring (Catling 1969). This may also apply to *D. citri*. The disease can be transmitted by grafting and marcotting, and these practices are a major source of inoculum in orchards throughout Southeast Asia. The bacterium is not seed transmissible (da Graca 1991).

1.3. Detection of huanglongbing

Detection of the disease is based on visual symptoms that can be confused with other disorders, particularly nutrient deficiencies. Where necessary, the presence of L. asiaticus can be confirmed by polymerase chain reaction (PCR) procedures that amplify rDNA fragments using huanglongbing-specific primers (see Villechanoux et al. 1992, Jagoueix et al. 1996, Ohtsu et al. 1998, Subandiyah et al. 2000). It is not practical to use PCR to detect the disease in citrus trees, or parts thereof, that do not exhibit symptoms.

1.4. Control of huanglongbing

Thermotherapy based on water-saturated hot air treatments can be used to eliminate the disease from budwood without loss of budwood viability. These treatments can be used with shoot-tip grafting to produce disease-free trees (da Graca 1991). Chemotherapy, based on the use of antibiotics such as tetracycline, has also been used to produce disease-free budwood and for successful control of the disease in the field (da Graca 1991). This technology is no longer a viable option in orchards due to cost, phytotoxicity, re-infection and residues (see for example da Graca 1991). Shoot-tip grafting is now the preferred for propagating disease-free seedlings as it can eliminate may other graft transmissible diseases (Aubert 1990b).

1.5. Description and general biology of the vector, *Diaphorina citri*

The brown, mottled adults are 3 to 4 mm long. The living insect is covered with whitish, waxy secretion, making it appear dusty. The adults leap when disturbed and may fly a short distance. They are usually found, sometimes in large numbers, on the lower sides of the leaves with their heads almost touching the leaf surface and the body raised almost to a 30º angle. The period of greatest activity of the psyllid corresponds with the periods of new growth of citrus. There are no galls or pits formed on the leaves as occurs with somepsyllid species; the nymphs are completely exposed. There are 5 nymphal instars ranging from 0.25 mm to 1.5 to 1.7 mm (first to fifth) that are generally yellowish orange. The eggs are approximately 0.3 mm long, elongate, almond-shaped, and thicker at the base. Fresh eggs are pale, but turn yellow and finally orange at hatching. They are laid on tips of growing shoots on and between unfurling leaves. Females may lay more than 800 eggs during their lives. There is no diapause. The duration of the life cycle in humid subtropical Florida requires 15 to 47 days, depending upon the season. Adults may live for several months and in Florida there are 9 to 10 generations a year; 16 have been observed in field cages (Mead 1997: http://creatures.ifas.ufl.edu/citrus/acpsyllid.htm).

1.6. Chemical control of *Diaphorina citri*

A range of insecticides, predominantly organophosphates and pyrethroids, has been used in management programs for *D. citri* (da Graca 1991). In Thailand, up to 52 sprays, each with up to 4 active ingredients, have been applied annually, predominantly for *D. citri*. Because control with sprays is aimed at nymphs and eggs on flush growth, the number and frequency of sprays is generally determined by the number of annual flushing cycles and the extent of flushing within cycles. Soil applications and trunk applications of systemic insecticides have also been practised (da Graca 1991). No sprays give 100% control, and applications of toxic pesticides, especially sprays, disrupt biological control of other pests, particularly scales and mites.

Biorational alternatives to the use of disruptive synthetic pesticides include horticultural and agricultural mineral oils (narrow and broad-range petroleum spray oils) (Beattie & Smith 1997) and plant-derived oils. Rae et al. (2000) showed that mineral oils can be used to control a range of citrus pests and diseases simultaneously, and that their effects on citrus production are well understood. They were also evaluated for control of *D. citri* (Rae et al. 1997). These results, and those of related studies on citrus leafminer (Beattie et al. 1995, Rae et al. 1996ab), formed the basis of oil spray strategies aimed at control of *D. citri* and citrus leafminer on immature flush growth and simultaneous control of other pests and diseases (Smith et al. 1997, Beattie et al. 1999). Simultaneous control of a range of pests was achieved with multiple low-concentration (0.25% to 0.5%) oil sprays in China (Rae et al. 2000), Vietnam (Nguyen et al. 2002) and in Sarawak (Leong et al 2002), where 0.3% to 0.5% sprays applied weekly or fortnightly controlled *D. citri* and other pests, as effectively as conventional pesticides, without causing phytotoxicity.

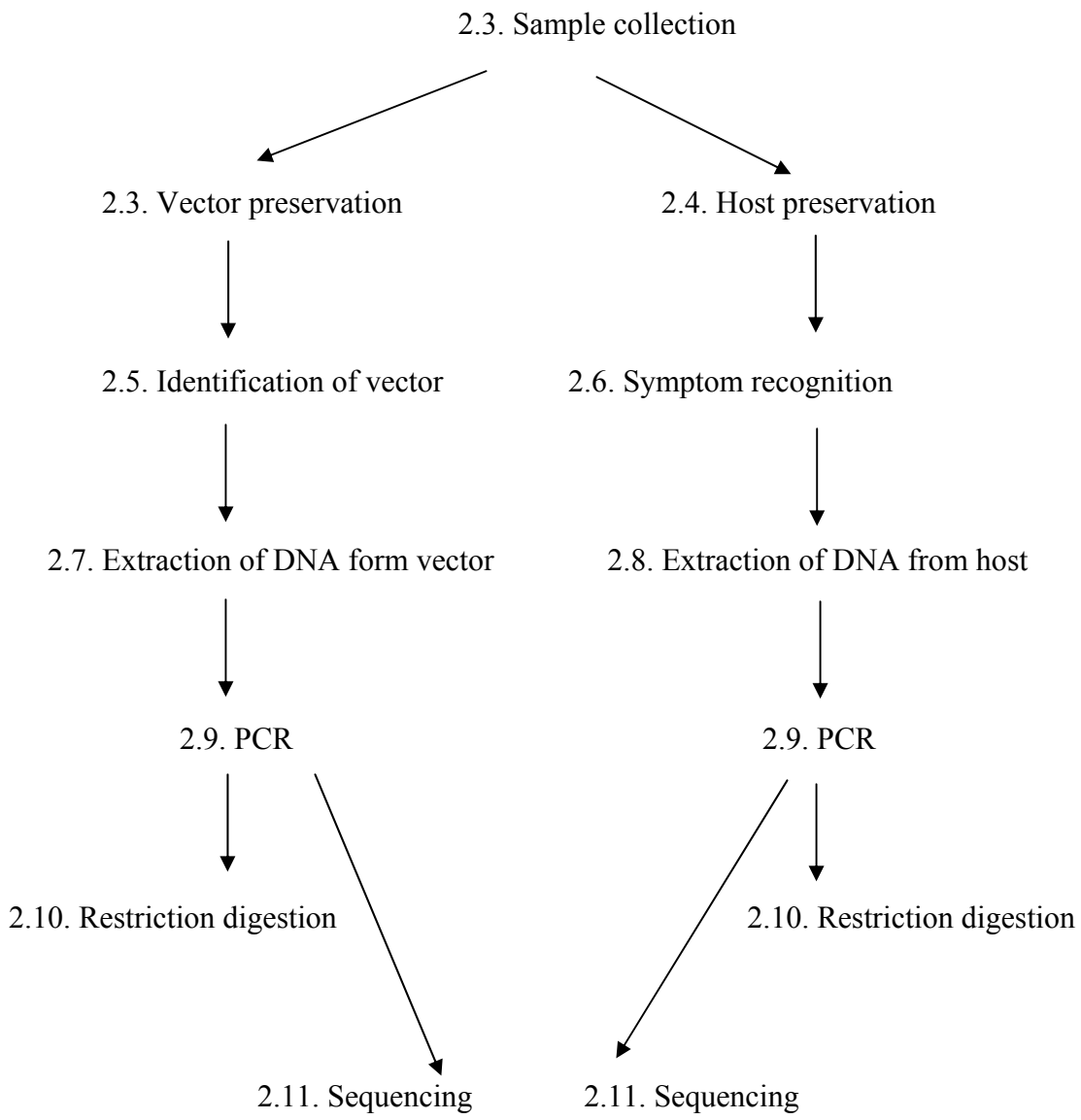
1.7. Variation in *Candidatus* Liberibacter

The causal organism of HLB is a phloem-limited proteobacterium assigned to the genus Canditatus Liberibacter (Jagoueix et al. 1994). To date, two species of this pathogen have been recognised: *Canditatus* Liberibacter africanus and *Canditatus* Liberibacter asiaticus (Villechanoux et al. 1993; Jagoueix et al. 1994; Planet et al. 1995). It is known that genetic variation exists within these species. A sub-species of *Canditatus* Liberibacter africanus,

Canditatus Liberibacter africanus sub sp. capensis, has been identified from differences in its reaction to monoclonal antibodies and from the sequencing of its 16S rDNA, intergenic 16S/23S rDNA and ribosomal protein genes (Garnier et al. 2000). Genetic variation among Asian strains of HLB has also been found (Villechanoux et al., 1992). It is likely that further genetic variation among strains of HLB will be detected. However, no studies have been published that show if variation in host range or virulence exists among strains of *L. africanus* or *L. asiaticus*.

2. Detection of Huanglongbing

2.1. Diagnostic flow chart



2.2. Sample collection

2.2.1. Sample collection within an orchard

When sampling material to determine the presence of huanglongbing from within an orchard, only collect material from trees that are showing symptoms. Gottwald's hierarchical block sampling layout (Appendix 1) can be used as a sampling aid and no more than 10% of trees need be sampled.

2.2.2. Sample collection from an individual tree

Trees should be sampled on 12 flushes, 3 from each cardinal (N, E, S, W) sector chosen randomly between knee and outstretched arm height depending on tree height.

2.3.3. Phenological description of the host plant

When collecting material, it may be useful to describe the phenological status of the host plant. The status of the flush should be categorised using the following descriptors:

- open buds ≤ 5 mm long
- immature flushes 6 mm to 10 mm long
- immature flushes > 10 mm long
- mature leaves

The number of immature flushes in each category with eggs present, and the number of immature flushes in each category with nymphs present (any stage, live or dead) should be recorded. The presence/absence of live adult psyllids that can be found on the undersides of mature leaves should only be recorded.

2.3. Preservation of vector

1. Collect adults using a pooter and nymphs using either fine forceps, a scalpel blade or paint brush and place them into a glass vial containing alcohol. The alcohol can be ethanol, methylated spirits or any local distilled spirit as long as the % of alcohol is more than 70%. 100% ethanol is preferred.
2. Give the vial an identification number and in the vial place a label with the following information written in pencil or Indian ink or other permanent ink that does not dissolve in alcohol (do not use a biro)
3. Keep a complete record of the sample:

- identification number
- date of collection
- country
- location – nearest major town
- GPS coordinates
- plant type and name (botanical name if possible, otherwise common name will do)
- person collecting sample

2.4. Identification of the vector

2.5.1. Morphology

Morphological identification will be based on existing descriptions (Hollis 1987) at nominated laboratories.

Australia:
Dr Paul De Barro, CSIRO Entomology, 120 Meiers Road, Indooroopilly, Queensland 4068

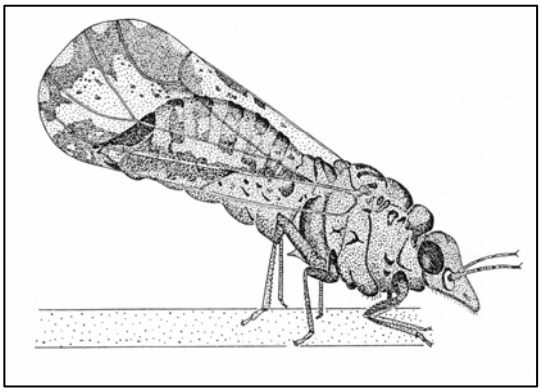
Other countries: ?

2.4.2. Suggestions for future research required for the identification of the vector

If the key developed by Hollis is not detailed enough for the identification of adults and nymphs, molecular tests will need to be produced. These will be PCR-based and will look for:

- sequence polymorphisms allowing diagnostic primers to be designed;
- size polymorphisms in amplified fragments;
- characteristic restriction enzyme recognition sites within fragments; or
- the determination of nucleotide sequence.

Regions suitable for the development of a diagnostic test include: CO1, ribosomal 18S and 28S D2 expansion regions and the ribosomal ITS1 region.

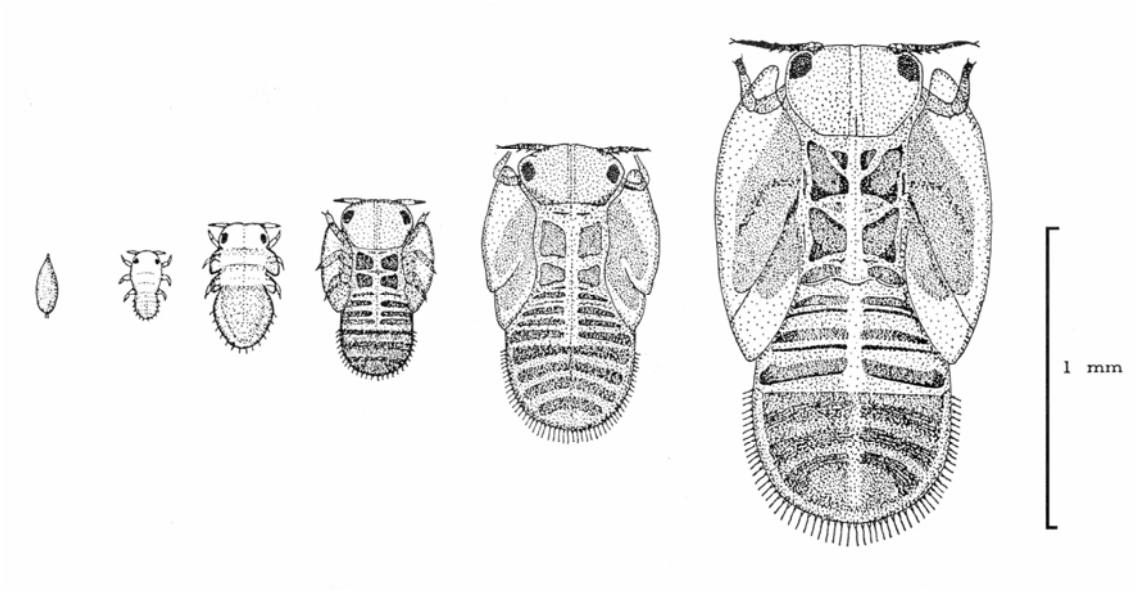


Adult psyllid (diagram taken from Aubert date?)

Adult psyllids

Adult psyllids and eggs

Psyllid eggs on a young shoot



Nymphal stages of *D. citri* (from Aubert date?)

2.4.3. Psyllid feeding damage

Apical notch

Twisting

Distorted flush

Mature leaf with cupped distortion

Mostly normal leaves

Lateral notching

Lateral distortion

Backward curling of leaf
caused by *Aphis spiraecol*

2.5. Preservation of plant host

Surface sterilise collected material with 70% alcohol or 1% household bleach.

Using a sterile blade, excise vascular material including midribs and/or petioles from leaves, peduncle/pedicel of fruits or flowers or the or columella/placental tissue of fruit. Chop tissue into approximately 5 mm lengths.

Wrap the excised material in paper tissue and place in calcium chloride granules (granule size approximately 7 mm diameter) in 25 mL plastic vials. Avoid using calcium chloride with a smaller granule size as the granules hydrate and fuse to form an impervious surface that inhibits air circulation and adequate desiccation.

Seal with Parafilm and keep refrigerated if possible.

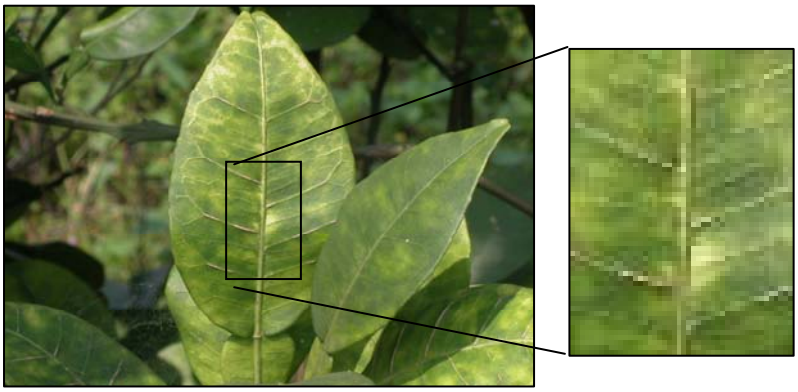
The following day replace the paper tissue and reseal in bottles containing calcium chloride. If there is excessive moisture present, repeat this process and if necessary replace calcium chloride.

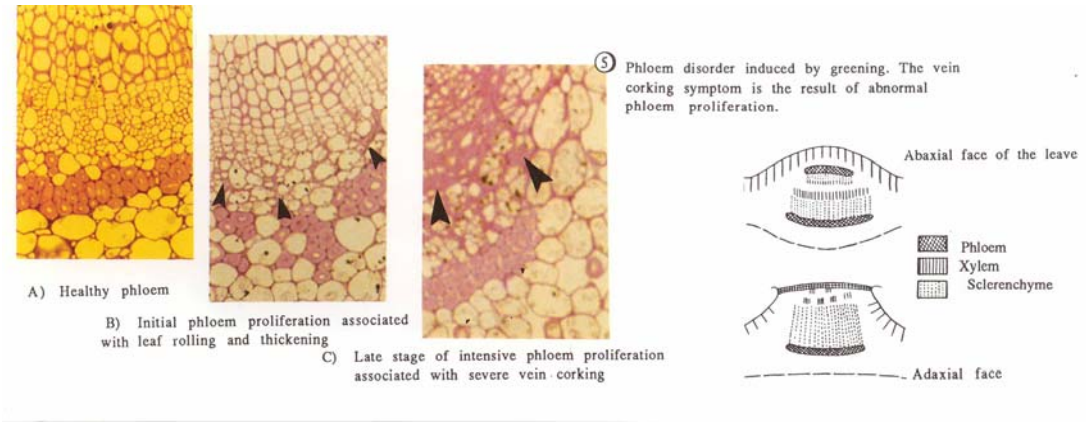
2.6. Symptom recognition

2.6.1. Stunting and die-back

2.6.2. Chlorosis

2.6.3. Vein corking





2.6.4. Microscopy

Liberibacter in the phloem of an orange (from Laflèche and Bové 1970)

2.7. Extraction of DNA from host (after Ahrens and Seemuller 1992)

The phytoplasma enrichment procedure of Ahrens and Seemuller (1992) works well for HLB and also permits the detection of phytoplasmas. It is suggested that trees are also tested for other citrus pathogens to determine multiple infections e.g., phytoplasmas.

2.7.1. Solutions

Ascorbic acid

0.062 g ascorbic acid in 10 mL double deionised water

Grinding Buffer

K_2HPO_4	16.7 g L^{-1}
KH_2PO_4	4.1 g L^{-1}
Sucrose	100 g L^{-1}
PVP-10	20 g L^{-1}
BSA	1.5 g L^{-1}

Make up as 2x concentration solution (i.e., quantities given above dissolved in 500 mL) in double deionised water. Store frozen in aliquots of 35 mL of 2x concentrate. Just before use, add 1 mL of the ascorbic acid to each 35 mL aliquot of 2x grinding buffer, adjust pH to 7.6 then make up solution to 70 mL with double deionised water. Keep on ice. This is sufficient to grind 4 specimens.

CTAB Buffer

CTAB	2 %
NaCl	1.4 M
Tris-HCl pH 8.0	100 mM
EDTA	20 mM
PVP-40	1 %

Check pH is 8.0 and store at room temperature. Just before use add 2 μL mL^{-1} β-mercaptoethanol (in fume cupboard). Make up a total volume equal to 1 mL per specimen. Aliquot CTAB buffer into 2 mL microfuge tubes and warm in 60°C water bath.

2.7.2. Method

1. Place 0.5–1 g of finely chopped, desiccated plant material in large pestle and mortar, add sufficient liquid nitrogen to freeze specimen, then grind thoroughly. If necessary add more liquid nitrogen and grind again.
2. Add 7 mL ice cold Grinding Buffer and continue grinding. Add a further 6 mL of Grinding Buffer and grind the material again.
3. Pour the contents of the pestle into precooled, 50 mL Oak Ridge tube and centrifuge at 1,100 x g for 5 mins at 4°C.
4. Pour supernatant into a second precooled Oak Ridge tube and centrifuge at 14,600 x g for 25 mins at 4°C.
5. Discard supernatant and drain briefly over a tissue.
6. Gently resuspend the pellet in 1 mL of prewarmed CTAB Buffer and transfer to a 2 mL microfuge tube.
7. Incubate at 60°C in water bath for 30 minutes. Mix by inverting the tubes several times during the incubation.
8. Spin tubes then add 0.8 mL chloroform:isoamyl alcohol (24:1) (in fume cupboard) and mix thoroughly but gently.
9. Centrifuge at 13,000 rpm for 5 minutes at room temperature.
10. Transfer aqueous phase to a fresh 2 mL microfuge tube and precipitate the DNA by adding an equal volume of ice cold (stored at –20°C) isopropanol and immediately mix thoroughly
11. Centrifuge at 13,000 rpm for 5 minutes at room temperature.
12. Discard supernatant and wash pellet twice in 0.5ml 70 % ice cold EtOH.
13. Allow pellet to dry.
14. Resuspend in 50 μL sterile PCR water or TE buffer.
15. Check DNA quality on a 1 % agarose gel made up in 0.5x TBE. Load 1–5 μl DNA solution + 1 μL Gel Loading Buffer in each well, and run at 80 V x 60 min or 120 V x 30 min. Post-stain in a 1mg L^{-1} ethidium bromide solution.

2.8. Extraction of DNA form vector (after Schneider et al (1999))

1. Place 10 insects (or less if 10 are not available) in a 2 mL microfuge tube with 1.0 mL of CTAB Buffer containing β-mercaptoethanol and a small amount (1 rice grain) of silicon carbide) and grind with a micropestle.
2. Incubate at 60°C in water bath for 0.5-2.0 h. Mix by inverting the tubes several times during the incubation.
3. Spin tubes briefly to collect the contents at the bottom, then add 0.8 mL chloroform:isoamyl alcohol (24:1) and mix thoroughly but gently.
4. Centrifuge at 13,000 rpm for 5 minutes at room temperature.
5. Transfer aqueous phase to a fresh 2 mL microfuge tube being careful to avoid any white material at the interface. Precipitate the DNA by adding an equal volume of ice cold (stored at –20°C) isopropanol and immediately mixing thoroughly.
6. Centrifuge at 13,000 rpm for 5 minutes at room temperature.
7. Discard supernatant and wash pellet twice in 0.5ml 70 % ice cold EtOH.
8. Allow pellet to dry.
9. Resuspend the pellet in 20 µL sterile molecular biology grade water or TE buffer.
10. Check DNA quality on a 1 % agarose gel made up in 0.5x TBE. Load 1–5 µl DNA solution + 1 µL Gel Loading Buffer in each well, and run at 80 V x 60 mins or 120 V x 30 mins. Post-stain in a 1mg/L ethidium bromide solution.

2.9. PCR

<u>2.9.1. Primers (after Jagoueix et al. (1996) and Subandiyah and Somowiyarjo (undated))</u>

The following primer pairs can be used for the amplification of the 16S ribosomal RNA region of L. asiaticus and L. africanus:

Forward primer: OI1 = 5’ GCG CGT ATG CAA TAC GAG CGG CA 3’
Reverse primer: OI2c = 5’GCC TCG CGA CTT CGC AAC CCA T 3’
Expected fragment size: 1,160 bp

Forward primer: Universal forward = 5’ GTG CCA GCA GCC GCG GTA ATA C 3’
Reverse primer: DC16SLibR = 5' CAC CTG TGT AAA GGT CTC CG 3’
Expected fragment size: 555 bp

The use of OI1/OI2c primers permits the separation of L. africanus from L. asiaticus through restriction digestion of the amplicon with *Xba*I. However, this primer pair can also amplify the sequences from *Wolbachia* spp. (Subandiyah and Somowiyarjo undated). OI1/DC16SlibR is specific for Liberibacter species but does not permit the use of restriction digestion to separate L. africanus from L. asiaticus

2.9.2. PCR reaction mixture (mastermix)

Mastermix – final concentration of reagents

Manufacturer's reaction buffer	1 x
BSA	200 μL mL^{-1}
$MgCl_2$	2 mM
Each dNTP	200 μM
Forward primer	500 nM
Reverse primer	500 nM
Taq	0.01U μL^{-1}
β-mercaptoethanol	10 mM

Typical mastermix (sufficient for 10 reactions)

Molecular biology grade water	149 μL
10 x manufacturer's reaction buffer	50 μL
BSA (2 mg mL^{-1})	50 μL
$MgCl_2$ (25 mM)	40 μL
dNTP mix (2 mM each dNTP)	50 μL
Forward primer (5 μM)	50 μL
Reverse primer (5 μM)	50 μL
Taq (5 units per μL)	1 μL
β-mercaptoethanol (100 mM)	50 μL
Total volume	490 μL

β mercaptoethanol
Mix 0.5 μL β-mercaptoethanol with 71 μL molecular biology grade water to give a 100 mM solution and briefly centrifuge to mix.

11. Aliquot 49 μL into thin walled PCR tubes.
12. PCR reactions are performed in duplicate
13. To pairs of tubes, add 1 μL of target DNA.
14. To one tube from each pair, add 1 μ L of DNA extracted from HLB-positive material to check for PCR inhibition
15. Mix well and spin briefly.
16. Add one drop of mineral oil to cover the reaction mixture if required.

A 1/10 dilution of DNA extracted from HLB-positive material can be used to spike one of each pair of tubes. This dilution should also be used as a positive control and water should be used as a negative control.

2.9.3. Thermolcycler program

For OI1/OI2c use:

Load tubes into thermocycler preset at 92°C for a 'hot start'

92°C for 40 s	35 cycles
72°C for 1.5 min	

For Universal forward/DC16SlibR use:

94°C for 10 min	1 cycle
94°C for 30 s	30 cycles
60°C for 30s	
72°C for 1.0 min	

2.9.4. Electrophoresis

Load 6 µL PCR product mixed with 2 µL Gel Loading Buffer in each well of a 1.0 % agarose gel made up in 0.5 x TBE. Use an appropriate marker (e.g., ϕX174). Run the gel in 0.5 x TBE at 80 V x 60 mins or 120 V x 30 mins. Post-stain in a 1mg L^{-1} ethidium bromide solution.

2.10. Restriction digestion (after Jagoueix et al. (1996))

A restriction enzyme digest, using *Xba*I, of the PCR product is then conducted to determine the Liberibacter species present.

Mastermix for *Xba*I (per reaction)

Molecular biology grade water	12.5 µL
10 x manufacturer's buffer	2.0 µL
*Xba*I	5.0 units
PCR product	5.0 µL
Total volume	20.0 µL

17. Incubate overnight at 37°C.
18. Add 6 µL Gel Lading Buffer to stop the digest.
19. Load 1 µL Gel Loading Buffer and 3 µL digested PCR product onto 4% agarose gel made with 0.5x TBE, run gel in 0.5x TBE with appropriate molecular weight markers at 90V for approximately 2 h.
20. Post-stain in ethidium bromide (1 mg L^{-1}) solution.

2.11. Sequencing of putative HLB fragments

The identity of putative HLB sequences can be compared with published sequence data from L. asiaticus (AB008366, AB038369 and L22532), L. africanus (L22533), and L. africanus subspecies capensis (AF137368). An alignment of these sequences including primer binding sites and the location of *Xba*I recognition sequences is given in Appendix 2.

3. Alternative hosts of *Diaphorina citri* and huanglongbing

3.1. Hosts of *Diaphorina citri*

The host range of *D. citri* is broader than that of huanglongbing. The preferred hosts are the Aurantieae [Citreae] species *Murraya paniculata* and *M. exotica* (possibly a cultivar of *M. paniculata*). *M. paniculata* comprises a number of distinctive forms found throughout Indomalesia to New Caledonia and Australia (Mabberley 1998). It is a common ornamental in Australia and has reached weed status in Queensland. Curry leaf, *Bergera* (formerly *Murraya*) *koenigii*, and most *Citrus* [Aurantieae] and *Clausena* [Clauseneae] species are good or common hosts. Other occasional or poor Aurantioideae hosts include species within the genera *Aegle*, *Atalantia*, *Limonia* (syn. *Feronia*), *Severinia*, *Swinglea* and *Triphasia* [Aurantieae], *Merrillia* [Clauseneae], *Toddalia* and *Vepris* [*Toddalieae*] (Khan & Borle 1989, Aubert 1990a, Ohtsu et al. 1995, Waterhouse 1998, Hung et al. 2001) but not all genera within the subfamily have been tested as hosts and it is likely that new hosts will be discovered (Aubert 1990a). Host records are summarised in Table 1. Note that *Citrus* now includes *Eremocitrus*, *Microcitrus*, and *Poncirus* and that the genus only comprises about 20 true species. Most common commercial varieties (e.g. grapefruit, oranges, lemons and limes) are hybrids but kaffir lime (*C. hystrix*), kumquat (*C. japonica*), true mandarins (*C. reticulata*), and pummelo (*C. maxima*) are valid species. Some 40% of valid species are native to Australasia. This suggests that the genus originated in Gondwana and continued to evolve in temperate, subtropical and tropical Asia and Australasia.

3.2. Hosts of huanglongbing

Within the Rutaceae, it is widely assumed that huanglongbing only affects species within the Aurantioideae, one of several subfamilies of the family Rutaceae. *Citrus*, *Severinia*, and *Clausena* are susceptible (Tirtawidjaja 1981, Aubert 1990a, Waterhouse 1998, Broadbent 1999). Garnier & Bové (1993) state that *M. paniculata* is not a host of the bacterium, but Aubert et al. (1985) and Li & Ke (2002) state that it can harbour the bacterium and recent work in Indonesia indicates that a form of *Murraya*, possibly *M. exotica*, is susceptible (Siti Subandiyah, Gadjah Mada University, pers. comm. February 2003) and Tirtawidjaja (1981) reported successful transmission of the disease by *D. citri* to *M. paniculata*. Tirtawidjaja (1981) also reported transmission by *D. citri* to *Swinglea glutinosa* and possibly *Pamburus missionis*and *Clausena indica*, but noted that the results needed to be confirmed in further studies. Hung et al. (2001) recently reported transmission to *Severinia buxifolia.*

Some non-rutaceous plants are also host of the disease. Pandey (1986) claimed that Madagascar periwinkle (*Catharanthus roseus* [Gentianales: Apocynaceae] could be infected by the citrus greening disease through wedge grafting and the vector *D. citri*. Huanglongbing has also been experimentally transmitted to periwinkle by dodder (*Cuscuta campestris* [Solanales: Cuscutaceae] (Garnier & Bové 1983, Ke et al. 1988) and by giant dodder (*C. reflexa*) from citrus to citrus (Raychaudhuri 1974). Dodder itself is a host for L. asiaticus, with titres higher than observed in citrus (Ghosh et al. 1977, Ke et al. 1988).

Some species and cultivars of citrus apparently vary in their tolerance or 'resistance' to huanglongbing. Nariani (1981) mentions that, of 20 'species' and cultivars tested for their reaction to huanglongbing, sweet lime (*C.* × *aurantiifolia*) was resistant, alone or as a rootstock for Mosambi orange (*C.* × *aurantium*), and Italian, Eureka and Lisbon lemons (*C.* × *limon*) were tolerant and showed mild symptoms. Koizumi et al. (1997) found rough lemon

(*C.* × *taitensis*), calamondin (*C.* × *microcarpa*), som-pan and ladu mandarin (*C.* × *reticulata*) trees to be tolerant, while large numbers of sweet orange, mandarin and tangelo trees were susceptible. Noto & Akihama (1991) state that no resistant rootstocks or cultivars are known but mention that pummelo and species or cultivars of certain mandarins are somewhat tolerant. However, pummelo can be severely affected by the disease, and successful selection for tolerance may be jeopardised by spontaneous mutations and hybridisation that occurs in *Citrus* and variable strains of L. asiaticus.

Tables 1 [Aurantieae (Citreae)], 2 [Clauseneae] and 3 [Toddalieae] list of species for possible evaluation as potential hosts of *Diaphorina citri* and *huanglongbing*. The tables also summarise current records and potential sources of plants. Host-testing records are indicative as tests and observations were undertaken under a range of conditions or not completed; blanks indicate no formal testing.

Table 1. *Aurantieae* (*Citreae*).

***Aurantieae* (*Citreae*) genera**	**Species**	**Host of *Diaphorina citri***			**Host of *huanglongbing***	**Potential sources of seeds or shoot-tips**							
		feeding	**oviposition**	**development**		**Australia**	**Indonesia**	**Papua New Guinea**	**Malaysia**	**Viet Nam**	**Philippines**	**Oceania**	**Elsewhere**
Aegle	***marmelos*** (bael, Indian bael fruit)	+				✓	✓		✓	✓	✓		India, Burma
Aeglopsis	***chevalieri***				Possibly								West Africa
Atalantia (outskirts)	***monophylla*** (Indian atalantia)	+					✓		✓	✓			India, Burma
	paniculata								✓				
	sp.	+	+	+			✓		✓				
Balsamocitrus	***dawei*** (Uganda powder-flask fruit)	+			Possibly								East Africa
Citrus*	***amblycarpa*** (possibly ×; sambal, djerook leemo, nasnaran mandarin, 'Celebes' papeda)						✓		✓				
	australis (Australian round lime, dooja)				✓	✓							
	australasica (*Australian finger-lime*)	++	++	++	✓	✓							
	garrawayae (Mount White lime, Garraway's Australian wild lime)				✓	✓		✓					
	glauca (Australian desert lime)				✓	✓							
	gracilis (Humpty Doo lime)				✓	✓							
	halimii (sultan lemon)				✓				✓				

	hystrix (***C. macroptera***: kaffir lime, limau purut, limau hantu)	++	++	++	✓		✓	✓	✓	✓	✓		Thailand
	ichangensis (Ichang papeda/lime)				✓								China, Burma, India
	inodora (Russell River lime, large-leaf Australian wild lime)				✓	✓							
	japonica (syn. ***Fortunella***; kumquat)	+	+	+	✓	✓				✓			China
	medica (citron)	++	++	++	✓	✓				✓			India
	maxima (pummelo)	++	++	++	✓	✓	✓		✓	✓	✓		
	reticulata (mandarin, tangerine, satsuma)	++	++	++	✓	✓			✓	✓	✓		'northern' China
	trifoliata (syn. ***Poncirus***)	+	+	—	✓	✓	✓		✓	✓			***'northern' China***
	warburgiana (Milne Bay lime, New Guinea wild lime)				✓			✓					
	wintersii (syn. ***Microcitrus papuana***; Brown River finger lime)				✓			✓					
	× ***aurantiifolia*** (lime)	+++	+++	+++	✓	✓			✓	✓	✓		
	× ***aurantium*** (sour, Valencia, and navel oranges; grapefruit; and king mandarin/orange tangors sometimes stated as ***C. nobilis***)	++	++	++	✓	✓			✓	✓	✓		
	× ***indica*** (Indian wild orange)												
	× ***insitorum*** (X *Citoncirus webberi*, citrange)												
	× ***limon*** (lemon)	++	++	++	✓	✓			✓	✓	✓		
	× ***macrophylla*** (Alemow)						✓				✓		
	× ***microcarpa*** (calamondin)	++	++	++	✓	✓			✓		✓		
	× ***suhuiensis*** (limau langkat)	++	++	++			✓		✓				
	× ***taitensis*** (= × ***jambhiri***, rough lemon)				✓	✓			✓				
Citropsis (C-4)	***gilletiana*** (Gillet's cherry-orange)								✓				African tropics

Clymenia (? monotypic; syn. ***Citrus***?)	***polyandra*** (clymenia)							✓			
Limonia (syn. ***Feronia***)	***acidissima*** (wood apple)	+	+	+	+		✓		✓		
Luvunga (C-1)	***crassifolia***						✓		✓		
	monophylla (syn. ***Paramignya trimera***)					✓	✓				
	sarmentosa						✓		✓		
	scandens (Indian liana lime)					✓				✓	India, Burma, Thailand
Merrillia	***caloxylon*** (monotypic; Malay lemon, ketengah, flowering merrillia)	+	—	—			✓		✓		Thailand
Murraya (C-2)	***paniculata*** (orange jessamine, mock orange)	+++	+++	+++	***Possibly***	✓	✓		✓		
	exotica (possibly cultivar of **M. paniculata**)	+			***Possibly***	✓	✓		✓		
Oxanthera	***aurantium*** (orange flower oxanthera)									✓	
	brevipes									✓	
	fragans (fragrant oxanthera)									✓	
	neocaledonica (false orange)									✓	
	sp.									✓	
	undulate (waxy-leaf oxanthera)									✓	
Pamburus	***missionis*** (pamburus)				+		✓			✓	India
Paramignya (= ***Luvunga***?)	***monophylla*** (?; Indian paramignya)					✓					

Severinia	***buxifolia*** (Chinese box-orange)	+			+			✓			China
	disticha (= ***Limonia***?; Philippine box-orange)						✓		✓		
Swinglea	***glutinosa*** (tabog)	+			+	✓	✓				
Triphasia (C-3)	***trifolia*** (limeberry, myrtle lime, triphasia)	+	+	+		✓	✓				

Table 2. *Clauseneae*

***Clauseneae* genera**	**Species**	**Host of *Diaphorina citri***			**Host of *huanglongbing***	**Potential sources of seeds or shoot-tips**							
		feeding	**oviposition**	**development**		**Australia**	**Indonesia**	**Papua New Guinea**	**Malaysia**	**Viet Nam**	**Philippines**	**Oceania**	**Elsewhere**
Bergera	***koenigii**** (***Murraya koenigii***; curry leaf)	+++	+++	+++		✓	✓		✓	✓	✓		
Clausena	***anisum-olens***** (anise)	+	+	+									
	brevistyla					✓							
	excavata	++	++	++			✓		✓	✓			
	harmandiana						✓						
	indica	+			Possibly		✓		✓				
	lansium (wampee)	++	++	++			✓		✓	✓			
	smyrelliana					✓							
	pinnata						✓						
Glycosmis	**pentaphylla**					✓	✓		✓	✓			
	trifoliata					✓							
Micromelum	***minutum***					✓	✓	✓	✓	✓	✓	✓	

* In Malaysia *Diaphorina citri* breeds well on *B. koenigii*.
** In the Philippines *Clausena anisumolens* is more attractive to adults than *Murraya paniculata*.

Table 3. *Toddalieae*

***Toddalieae* genera**	**Species**	**Host of *Diaphorina citri***			**Host of *huanglongbing***	**Potential sources of seeds or shoot-tips**							
		feeding	**oviposition**	**development**		**Australia**	**Indonesia**	**Papua New Guinea**	**Malaysia**	**Viet Nam**	**Philippines**	**Oceania**	**Elsewhere**
Acronychia	***laurifolia***						✓						
	trifoliata						✓						
Toddalia	***aculeata***						✓						
	asiatica	+	—	—									
Vepris	***lanceolata***	+	—	—									
	undulata						✓						

http://wwwx.ecoport.org/REFS/IPGRI/citrus.pdf
http://www.corse.inra.fr/sra/synonym.pdf
http://www.corse.inra.fr/sra/ecn2en.pdf
http://www.corse.inra.fr/sra/species.pdf
http://www.corse.inra.fr/sra/egspec2.pdf
http://www.corse.inra.fr/sra/html0.htm
http://www.inibap.org/publications/proceedings/managing-banana-citrus.pdf
http://biocontrol.ifas.ufl.edu/ctgysrch/ASP-hoy.htm

http://www.uga.edu/fruit/citrus.htm
http://rimba.um.edu.my/Flashback.html
http://apps3.fao.org/wiews/germplasm_species.jsp?species=C
http://www.ars-grin.gov/cgi-bin/npgs/html/site_holding.pl?RIV
http://www.winternet.com/~chuckg/dictionary/dictionary.23.html
http://www.innvista.com/health/foods/fruits/unusual.htm

http://www.museums.org.za/bio/plants/rutaceae/
http://www.life.uiuc.edu/plantbio/263/TROPFRUT.html
http://www.museums.org.za/bio/plants/rutaceae/citrus.htm
aggie-horticulture.tamu.edu/syllabi/422/ppt/class3.ppt
http://www.ipgri.cgiar.org/regions/apo/publications/tf_asia/chapter4.pdf
http://www.grcp.ucdavis.edu/publications/doc22/Ch5.pdf
http://www.ipgri.cgiar.org/regions/apo/publications/tfspecies/Chap-3.PDF
http://www.wcmc.org.uk/igcmc/s_sheets/meghalay/1565v.html
http://www.ipgri.cgiar.org/regions/apo/publications/tf_asia/chapter19.pdf
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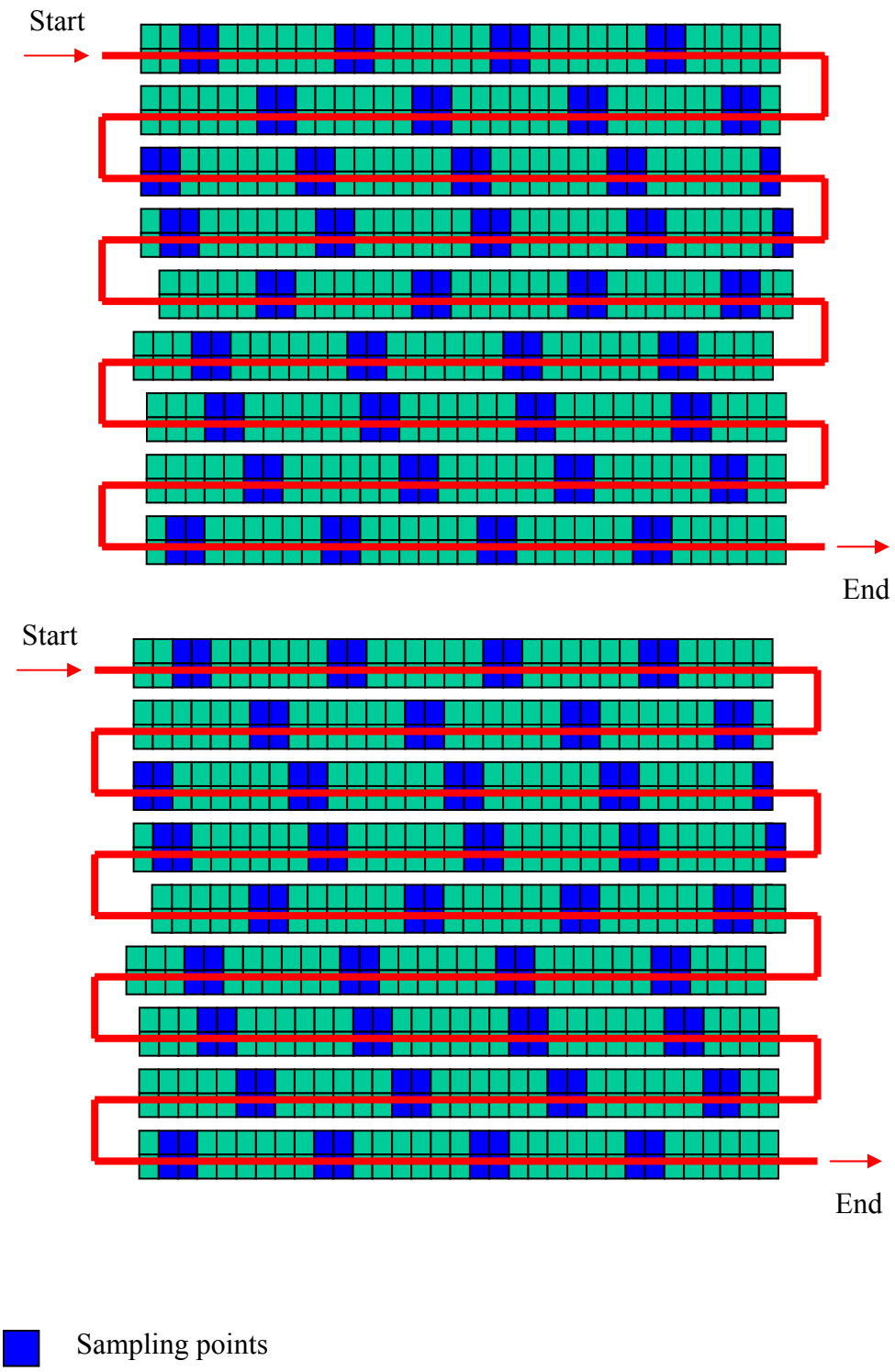
Appendix 1. Gottwald's hierarchical block sampling layout

Examples of hierarchical sampling method applied to a regular rectangular plot (1) and a plot with irregular edges (2).



Appendix 2. Alignment of sequenced portions of the 16S ribosomal RNA gene

AB008366 = Liberibacter sp. gene for 16S rRNA, partial sequence Subandiyah et al. (Unpublished 1997).
AB038369 = Liberibacter endosymbiont of *Diaphorina citri.* Subandiyah et al. (2000).
L22532 = Liberibacter asiaticus 16S ribosomal RNA gene, partial sequence (Jagoueix et al. 1994).
L22533 = Liberibacter africanus 16S ribosomal RNA gene, partial sequence (Jagoueix et al. 1994).
AF137368 = Liberibacter africanus subsp. capensis 16S ribosomal RNA gene, partial sequence (Garnier et al. 2000).

```
                                                        OI1
                                                   ____________
           1                                                  50
AB008366   .......... .......... .......... .......... ........aa
AB038369   .......... .......... .......... .......... ..........
L22532     aacgaacnct ggcggcaggc ctaacacatg caagtcgagc gcgtatgcaa
L22533     aacgaaccct ggcggcaggc ctaacacatg caagtcgagc gcgtatttta
AF137368   .......... .......... .........a attcgattgc gcgtatcgaa

           ___________
           51                                                100
AB008366   tacgagcggc agacgggtga gtaacgcgta ggaatctacc tttttct.ac
AB038369   .......... .gacgggtga gtaacgcgta ggaatctacc tttttct.ac
L22532     tacgagcggc agacgggtga gtaacgcgta ggaatctacc tttttctanc
L22533     tacgagcggc agacgggtga gtaacgcgta ggaatctacc tttttct.ac
AF137368   tacgacgggc agacgggtga gtaacgcgta ggaatctacc tttttct.ac

           101                                               150
AB008366   gggataacgc atggaaacgt gtgctaatac cgtatacgcc ctattggggg
AB038369   gggataacgc atggaaacgt gtgctaatac cgtatacgcc ctattggggg
L22532     gggataacgc atggaaacgt gtgctaatac cgtatacgcc ctattggggg
L22533     gggataacgc atggaaacgt gtgctaatac cgtatacgcc ctattggggg
AF137368   gggataacgc ntggaaacgt gtgctaatac cgtatacgcc ctatt..ggg

           151                                               200
AB008366   aaagatttta ttggagagag atgagcctgc gttggattag ctagttggta
AB038369   aaagatttta ttggagagag atgagcctgc gttggattag ctagttggta
L22532     aaagatttta ttggagagag atgagcctgc gttggattag ctagttggta
L22533     aaagatttta ttggagagag atgagcctgc gttggattag ctagttggta
AF137368   aaagatttta ttggagagag atgagcctgc tgtggattag ctagttggta

           201                                               250
AB008366   gggtaagagc ctaccaaggc tacgatctat agctggtctg agaggacgat
AB038369   gggtaagagc ctaccaaggc tacgatctat agctggtctg agaggacgat
L22532     gggtaagagc ctaccaaggc tacgatctat agctggtctg agaggacgat
L22533     gggtaaaggc ctaccaaggc tacgatctat agctggtctg agaggacgat
AF137368   gggtaaaggc ctaccaaggc tacgatctat agctggtctg agaggacgat
```

```
          251                                                300
AB008366  cagccacact gggactgaga cacggcccag actcctacgg gaggcagcag
AB038369  cagccacact gggactgaga cacggcccag actcctacgg gaggcagcag
L22532    cagccacact gggactgaga cacggcccag actcctacgg gaggcagcag
L22533    cagccacact gggactgaga cacggcccag actcctacgg gaggcagcag
AF137368  cagccacact gggactgaga cacgccagac tctac..... ggagcagcag

          301                                                350
AB008366  tggggaatat tggacaatgg gggcaaccct gatccagcca tgccgcgtga
AB038369  tggggaatat tggacaatgg gggcaaccct gatccagcca tgccgcntga
L22532    tggggaatat tggacaatgg gggcaaccct gatccagcca tgccgcgtga
L22533    tggggaatat tggacaatgg gggcaacccg aatccagcca tgccgcgtga
AF137368  tggggaatat tggacaatgg gggcaa.cct gatccagcca tgccgcgtga

          351                                                400
AB008366  gtgaagaagg cctt..aggg ttgtaaagct ctttcgccgg agaagataat
AB038369  gtgaagaagg cctt..aggg ttgtaaagct ctttcgccgg agaagataat
L22532    gtgaagaagg cctt..aggg ttgtaaagct ctttcgccgg agaagataat
L22533    gtgaagaagg cctt..aggg ttgtaaagct cttnnnccgg agaagataat
AF137368  gtgaagaagg cctt..aggg ttgtanagct ctttcgccgg agaagataat
```

Universal forward

```
          401                                                450
AB008366  gacggtattc ggagaagaag ccccggctaa cttcgtgcca gcagccgcgg
AB038369  gacggtattc ggagaagaag ccccggctaa cttcgtgcca gcagccgcgg
L22532    gacggtattc ggagaagaag ccccggctaa cttcgtgcca gcagccgcgg
L22533    gacggtattc ggagaagaag ccccggctaa cttcgtgcca gcagccgcgg
AF137368  gacggtattc ggag.agaag ccccggctaa cttcgtgcca gcagccgc.g

          451                                                500
AB008366  taatacgaag ggggcgagcg ttgttcggaa taactgggcg taaagggcgc
AB038369  taatacgaag ggggcgagcg ttgttcggaa taactgggcg taaagggcgc
L22532    taatacgaag ggggcgagcg ttgttcggaa taactgggcg taaagggcgc
L22533    taatacgaag ggggcgagcg ttgttcggaa taactgggcg taaagggcgc
AF137368  taatacgaag ggggcgagcg ttgttcggaa taactgggcg taaagggcgc

          501                                                550
AB008366  gtaggcgggc ...gattaag ttagaggtga aatcccaggg ctcaaccttg
AB038369  gtnggcgggc ...gattaag ttagaggtga aatcccaggg ctcaaccttg
L22532    gtaggc.ggc ...gattaag ttagaggtga aatccca.gg ctcaaccttg
L22533    gtaggc.ggc ...gattaag ttagaggtga aatccca.gg ctcaaccttg
AF137368  gtaggc..gt ...gattaag ttagaggtga a...tccagg ctcaaccttg

          551                     XbaI                       600
AB008366  gaactgcctt taatactggt tgtctagag. tttagga.ga ggtgagtgga
AB038369  gaactgcctt taatactggt tgtctagag. tttagga.ga ggtgagtgga
L22532    gaactgcctt taatactggt tgtctagagc tttagga.ga ggtgagtgga
L22533    gaactgcctt taatactgat tgtctagag. ttcagga.ga ggtgagtgga
AF137368  gaactgcc.t taatactggt tatctagag. ttcagga.ga ggtgagtgga
```

```
          601                                                650
AB008366  attccgagtg tagaggtgaa attcgtagat attcggagga acaccggtgg
AB038369  attccgagtg tagaggtgaa attcgtagat attcggagga acaccggtgg
L22532    attccgagtg tagaggtgaa attcgtagat attcggagga acaccggtgg
L22533    attccgagtg tagaggtgaa attcgtagat attcggagga acaccggtgg
AF137368  attccgagtg tagaggtgaa attcgtagat attcggagga acaccggtgg

          651                                                700
AB008366  cgaaggcggc tcactggcct gatactgacg ctgaggcgcg aaagcgtggg
AB038369  cgaaggcggc tcactggcct gatactgacg ctgaggcgcg aaagcgtggg
L22532    cgaaggcggc tcactggcct gatactgacg ctgaggcgcg aaagcgtggg
L22533    cgaaggcggc tcactggcct gatactgacg ctgaggcgcg aaagcgtggg
AF137368  cgaaggc.gc tcactggcct gatactga.g ctgaggc.cg aaagcgtggg

          701                                                750
AB008366  gagcaaacag gattagatac cctggtagtc cacgccgtaa acgatgagtg
AB038369  gagcaaacag gattagatac cctggtagtc cacgccgtaa acgatgagtg
L22532    gagcaaacag gattagatac cctggtagtc cacgccgtaa acgatgagtg
L22533    gagcaaacag gattagatac cctggtagtc cacgctgtaa acgatgagtg
AF137368  gagcaaacag gattagatac cctggtagtc cacgctgtaa acgatgagtg

          751                                                800
AB008366  ctagctgttg ggtggtttac cattcagtgg cgcagctaac gcattaagca
AB038369  ctagctgttg ggtggtttac cattcagtgg cgcagctaac gcattaagca
L22532    ctagctgttg ggtggtttac cattcagtgg cncacgtaac gcattaagca
L22533    ctagctgttg ggtggtttac cattcagtgg cgcacgtaac gcattaagca
AF137368  ctagctgttg ggtggtttac cattcagtgg cgc.nnnaac gcattaagca


          801                                                850
AB008366  ctccgcctgg ggagtacggt cgcaagatta aaactcaaag gaattgacgg
AB038369  ctccgcctgg ggagtacggt cgcaagatta aaactcaaag gaattgacgg
L22532    ctccncctgg ggagtacggt cgcaagatta aaactcaaag gaattgacgg
L22533    ctccgcctgg ggagtacggt cgcaagatta aaactcaaag gaattgncgg
AF137368  ctccgcctgg ggagtacggt cgcaagatta aaactcaaag gaattgacgg

          851                                                900
AB008366  gggcccg... .cacaagcgg tggagcatgt ggtttaattc gatgcaacgc
AB038369  gggcccg... .cacaagcgg tggagcatgt ggtttaattc gatgcaacgc
L22532    gggcccg... .cacaagc.g tggagcatgt ggtttaattc gatgcaacgc
L22533    gggcccg... .cacaagc.g tggagcatgt ggtttaattc gatgcaacgc
AF137368  ggg..ng... .cacaagcgg tggagcatgt ggtttaattc gatgcaacgc

          901                                                950
AB008366  gcagaacctt accagccctt gacatgtata ggacgatatc agagatggta
AB038369  gcagaacctt accagccctt gacatgtata ggacgatatc agagatggta
L22532    gcagaacctt accagccctt gacatgtata ggacgatatc agagatggta
L22533    gcagaacctt accagccctt gacatatgtt ggacgatatc agagatgata
AF137368  gcagaccctt accagctctt gaca..tatg agacgatatc agagatgata
```

DC16SlibR/GOrev

```
           951                                                1000
AB008366   ttttcttttc ggagaccttt acacaggtgc tgcatggctg tcgtcagctc
AB038369   ttttcttttc ggagaccttt acacaggtgc tgcatggctg tcgtcagctc
L22532     ttttcttttc ggagaccttt acacaggtgc tgcatggctg tcgtcagctc
L22533     ttttcttttc ggagactttc atacaggtgc tgcatggctg tcgtcagctc
AF137368   ttttcttttc .gagactttc atacaggtgc tgcatggctg tcgtcagctc

           1001                                               1050
AB008366   gtgtcgtgag atgttgggtt aagtcccgca acgagcgcaa cccctgcctc
AB038369   gtgtcgtgag atgttgggtt aagtcccgca acgagcgcaa cccctgcctc
L22532     gtgtcgtgag atgttgggtt aagtcccgca acgagcgcaa cccctgcctc
L22533     gtgtcgtgag atgttgggtt aagtcccgca acgagcgcaa cccctacctc
AF137368   gtgtcgtgag atgttgggtt aagt.ccgca acgagcgcaa cccctacctc

           1051                        XbaI                   1100
AB008366   tagttgccat caagtttagg tttttaccta gatgttgggt actttatagg
AB038369   tagttgccat caagtttagg tttttaccta gatgttgggt actttatagg
L22532     tagttgccat caagtttagg tttttaccta gatgttgggn actttatagg
L22533     tagttgccat caagttta.g atttta**tcta ga**tgttgggt actttatagg
AF137368   tagttgccat caagttta.g atttta**tcta ga**tgttgggt actttatagg

           1101                                               1150
AB008366   gactgccggt gataagccgg aggaaggtgg ggatgac... ...gtcaagt
AB038369   gactgccggt gataagccgg aggaaggtgg ggatgac... ...gtcaagt
L22532     gactgccggt gataagccgg aggaaggtgg ggatgan... ...gtcaagt
L22533     gactgccggt gataagccgg aggaaggtgg ggatgac... ...gtcaagt
AF137368   gactgccggt gataagccgg aggaaggtgg ggatgac... ...gtcaagt

           1151                                               1200
AB008366   cctcatggcc cttatgggct gggctacaca cgtgctacaa tggtgg....
AB038369   cctcatggcc cttatgggct gggctacaca cgtgctacaa tggtggttac
L22532     cctcatgcgc cttatgggct gggctacaca cgtgctacaa tggtggttac
L22533     cctcatgcgc cttatgggct gggctacaca cgtgctacaa tggtggttac
AF137368   cctcat.cgc gttatgggct gggctacaca cgtgctacaa tggtggttac
```

OI2c

```
           1201                                               1250
AB008366   .......... .......... .......... .......... ..........
AB038369   a......... .......... .......... .......... ..........
L22532     aatgggttgc gaagtcgcga ggcggagcta atccccaaaa gccatctcag
L22533     aatgggttgc gaagtcgcga ggcggagcta atccccaaag tccatctcag
AF137368   aatgggttgc gaagtcgcga ggcaatcact agtgaattcg cggccgcctg

           1251                                               1300
AB008366   .......... .......... .......... .......... ..........
AB038369   .......... .......... .......... .......... ..........
L22532     ttcggattgc actctgcaac tcgagtgcat gaagtcggaa tcgctagtaa
L22533     ttcngattgc actctgcaac tcgagtgcat gaagttggaa tcgctagtaa
AF137368   cagg...... .......... .......... .......... ..........
```

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          1301                                                1350
AB008366  .......... .......... .......... .......... ..........
AB038369  .......... .......... .......... .......... ..........
L22532    tcgcggatca gcatgcnncg gtgaatacgt tctcgggcct tgtacacacc
L22533    tcgcggatca gcatgccgcg gtgaatacgt tctcgggcct tgtacacacc
AF137368  .......... .......... .......... .......... ..........

          1351                                                1400
AB008366  .......... .......... .......... .......... ..........
AB038369  .......... .......... .......... .......... ..........
L22532    gcccgtcaca ccatgggagt tggttttgcc tgaagacggt gt.ntaaccg
L22533    gcccgtcaca ccatgggagt tggttttgcc tgaagacggt gtgctaaccg
AF137368  .......... .......... .......... .......... ..........

          1401                                                1450
AB008366  .......... .......... .......... .......... ..........
AB038369  .......... .......... .......... .......... ..........
L22532    caaggaggca gccggccacg gtagggtcag cgactggggt gaagtcgtaa
L22533    caagggggca gccggccacg gtaaggtcag cgactggggt gaagtcgtaa
AF137368  .......... .......... .......... .......... ..........

          1451                    1476
AB008366  .......... .......... ......
AB038369  .......... .......... ......
L22532    caaggtagcc gtagggga.. ......
L22533    caaggtagcc gtaggggaac ctgcgg
AF137368  .......... .......... ......
```