Cooperative Research Centre for Viticulture

Australian Government
Grape and Wine Research and Development Corporation

Finalising and validating a diagnostic probe for the early detection of phylloxera



FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

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Abstract

Critical to the management of phylloxera in Australia is the early detection of new infestations, ensuring management options are implemented rapidly and minimising quarantine breakdown. There is an urgent need to develop a phylloxera-specific detection system, able to directly recognise the insect itself and not its associated symptoms on grapevines. Research presented in this study focuses on the early detection of phylloxera using DNA-based technology. Species-specific primers were developed for phylloxera and their specificity was confirmed after thorough screening using a wide range of vineyard organisms and aphid genera. Preliminary testing of the detection limits of the phylloxera-specific primers was conducted using field-sourced soil types spiked with a known number of phylloxera. The results indicate that the primers are both robust and sensitive enough to proceed to a thorough field testing stage aimed at comparing this technology to conventional ground-truthing methods.

Executive Summary

The present study focussed on the design and testing of phylloxera-specific DNA markers that could be developed into a diagnostic test for the routine detection of phylloxera from vineyard soil. A DNA-based approach is the only proposed phylloxera detection method based solely on the insect itself, rather than associated stress symptoms evident on the vine. DNA techniques have rapidly become the preferred tool for identification of many soil-borne pathogens including bacteria (*Mycobacterium* and *Legionella* (Sayler, 1990); soil borne fungi (*Gaeummannomyces graminis*, *Rhizoctonia solani* and *Fusarium* wilt of banana (Ophel-Keller et al. 1999; Pattemore et al. 2001); nematodes such as cereal cyst nematodes (Ophel-Keller et al. 1999) and root-knot nematodes (Quader et al. 2002). The most important benefits of adopting a DNA approach are increased sensitivity, accuracy and reduced labour inputs when compared to current spectral and ground-truthing methods. Phylloxera detection would therefore not rely on problems with visual detection of very tiny insects or galls, or evidence of weak spots on the vine, typically not seen until two years into a phylloxera infestation. The ability to directly quantify numbers of phylloxera in the soil would provide valuable information for quarantine and vine management decisions.

This research was undertaken at the Centre for Environmental Stress and Adaptation Research (CESAR), in collaboration with Kathy Ophel-Keller (South Australian Research and

Development Institute (SARDI), and Kevin Powell (Department of Primary Industries (DPI), Rutherglen).

Previous work by the CI successfully identified DNA regions that distinguished phylloxera from other vineyard organisms. Problems remained, however, with distinguishing phylloxera from all aphid genera common in Australia. Further work was required to accomplish this aim before a phylloxera-specific probe could be made commercially available. Additional regions needed to be isolated and screened. To complete this task, large numbers of the 6 major phylloxera genotypes were required. Field collections were undertaken so that populations of each genotype could be reared *in vitro*. Bulk genomic DNA extractions were then prepared for each genotype and used as standards for future screening.

Field collections of the major phylloxera genotypes were undertaken during late February and March 2005. Phylloxera populations were mass reared at PC2 quarantine facilities at DPI Rutherglen. Insect collections were shipped to the University of Melbourne where bulk extractions were undertaken.

11 gene regions were targeted for this study with the expectation that they would contain unique DNA sequence exclusive to phylloxera. Results of sequence analysis from the rDNA ITS2 gene region revealed highly variable regions of DNA sequence that contained DNA coding specific to phylloxera. Primer combinations were designed in these unique regions and extensively tested. These primers successfully amplified the six major phylloxera clones present in Australia. After extensive validation the ITS2 primers also proved to be specific to phylloxera as they failed to amplify all aphid genera tested. Preliminary testing of the detection limits of the phylloxera-specific primers in a soil-based environment was conducted using field-sourced soil types spiked with known number of phylloxera. The results indicated that the developed primers are both robust and sensitive enough to amplify down to a 1pg/ul concentration in soil. This result supports the further development of this DNA-based detection approach and field validation to compare the sensitivity and accuracy of this method to conventional ground-truthing methods.

Background

A combination of ground and aerial survey techniques are currently used in Australia to detect and monitor phylloxera infestations (Herbert et al., 2003). These methods rely on visual vine stress symptoms typically seen 2-3 years into a phylloxera infestation, making them unsuitable for early detection. In certain cases vines infested with less-virulent phylloxera strains do not exhibit foliar decline indicative of phylloxera.

While aerial surveys are suitable for screening large numbers of vineyards and pinpointing 'weak spots in the vineyard', the inherent problem lies with the follow-up ground level validation. Ground surveys can only be conducted in a limited 2-3 month period and are reliant on trained personnel observing the classic symptoms of phylloxera on the root system. Ground surveys do not survey whole vineyard blocks due high labour requirement.

Infestations therefore can remain undetected, especially in cases where galls are not yet present on the vine or phylloxera population levels are low. Furthermore a 2-3 year delay in the initial detection of phylloxera in vineyards can allow for widespread movement of the insect throughout the vineyard, imposing an even greater threat of spread to neighbouring vineyards and breakdown in quarantine.

In an effort to detect phylloxera after early infestation and to improve on the sensitivity and accuracy of phylloxera detection, DNA based detection formed a component of CRCV PhD Project 2.2.3. In conjunction with SARDI and Aventis, the development of a DNA-based diagnostic test for the detection of phylloxera in vineyard soils was commenced. DNA probes are powerful detection tools as they can detect single insects in large soil volumes.

The basis of this technique is the identification and utilisation of a DNA region that is specific to phylloxera. This is not a straightforward task because genetic information available on phylloxera is very limited and there is little understanding of the level of genetic similarity to other insects including aphids, the group that is most likely to be closely related to phylloxera. Initial research targeted genes that have been utilised in other insect studies. Three such regions were screened to determine the level of phylloxera specificity. These phylloxera regions were found to be genetically distinct from the same regions in all common vineyard organisms tested. However screening against aphid species common in Australia revealed almost 100% similarity at the DNA level between some aphids and phylloxera. While these

aphids are not normally found in vineyards, they may nevertheless occur in this environment sporadically.

Project Aims and Performance Targets:

The overall project aim was to identify unique phylloxera DNA sequence, design primers within this sequence and test its specificity using both commonly found vineyard organisms and closely-related aphid genera. The final component of this study was to test the sensitivity of the above primers in diluted field soils to determine its suitability for further development as a routine diagnostic test.

The project objectives and outputs are summarised as follows:

	Objectives	**Ouputs**
1	Isolate DNA regions containing unique phylloxera sequence.	11 gene regions were targeted, with one region (ITS2) identified as having DNA sequence most specific to phylloxera.
2	Test that above DNA regions are consistent amongst all phylloxera genotypes.	PCR testing confirmed that unique primers designed from this region amplified all major phylloxera genotypes.
3	Validate potential region(s) with commonly found vineyard organisms.	All vineyard organisms tested did not amplify using the phylloxera-specific primers.
4	Validate potential regions(s) with commonly found aphid species	All aphid genera tested did not amplify using the phylloxera-specific primers.
5	Conduct lab-based sensitivity tests in sterile soils.	This step was not conducted as the testing moved straight to field soils.
6	Conduct sensitivity lab-based sensitivity tests using field-collected soils.	High and low-risk field soils were spiked with known numbers of phylloxera to test sensitivity levels in soil. Recommendations on future work to complete the development of a diagnostic test to be given.

Method

Phylloxera Collection

Infested root material was collected from commercial *Vitis vinifera* vineyards from (a) King-Valley; (b) Nagambie; and (c) Rutherglen phylloxera-infested zones (PIZs) and transported under quarantine to the PC2 facilities at the Department of Primary Industries, Rutherglen. Adults were immediately removed from infested root material and placed into 100% ethanol for long-term storage. Eggs were transferred to excised root bioassays and the individual genotypes were mass reared as per the method outlined by Granett et al. (1987). Three phylloxera genotypes, confirmed as G1, G4 and G20 described using the method by Corrie et al. (2002) were sourced from the field collections. All insects were transported to the University of Melbourne for subsequent analysis.

Genomic DNA extractions

Bulk and single insect DNA extractions were performed using a chelating resin method outlined by Walsh *et al.* (1991). Briefly, individuals were placed in sterile 0.5ml tubes and air dried to remove excess ethanol. For single (S) insect extractions one adult was used and bulk (B) extractions used 20 adults per extraction. 5µl (S) or 20µl (B) of proteinase K (14mg/ml; Boehringer Mannheim) was pipetted onto the insect before finely grinding it with a plastic pestle. 100µl (S) or 500µl (B) of 5% Chelex® 100 (Biorad), pre-warmed approximately to 50°C was then added, followed by incubation at 55°C for 12 hours, then 95°C for five minutes. The tube was then centrifuged for 5 minutes at 14,000rpm to pellet the resin and stored at $^{-}$20°C until required. The aqueous phase containing the DNA was used as a template for all subsequent PCR reactions. To ensure representatives of all major phylloxera genotypes were included in this study, the following additional genotypes were included, kindly provided from the collections of Heidi Mackin and Angela Corrie: G2, G3, G7, G11, G12, G19; G29, G30, G38, G42, and G46.

Gene amplification derived from universal primers

A total of 11 different gene regions were targeted for this study for potential sequence variation across species: (1) ATP synthase subunit a; (2) ATP synthase subunit b; (3) Adenine nucleotide trasnsporter translocase; (4) Signal recognition particle 54; (5) Tata box binding protein; (6)

Lysidyl aminoaceyl transfer RNA synthase; (7) Zinc metalloproteins; (8) Cytochrome b; (9) rDNA internal-transcribed spacer (ITS) 2 region; (10) Cytochrome oxidase subnunit 1 (CO1) ; and (11) Dopa decarboxylase gene (DDC). Sources to the universal primers used for amplification of the gene regions numbered are listed as follows: gene regions (1 through to 7): Jarmon et al. 2002; (8): Simon et al. 1994; Figueroa et al. 1999; Bulman et al. 2005; and Raboudi et al. 2005; (9): Bulman et al. 2005; (10): Folmer et al. 1994; (11): Fang et al 1997.

For all gene regions, the PCR reaction mixture comprised of the following final reagent concentrations in a total reaction volume of 25 µls: 1.5mmM $MgCl_2$, 200 µM dNTPs (Fisher™), 1% bovine serum albumin (BSA) (Invitrogen™), 10 pmol appropriate primer pair, 2.5 µl 10 × PCR Buffer, 0.5 U Taq polymerase (Bioline™ Immolase) and 5µl DNA template solution (either S or B extraction). The PCR amplification conditions used were those outlined by the authors previously cited for each gene region. A 5 µl aliquot of the amplification product was visualised under UV light following electrophoresis through 1.8% agarose gels and ethidium bromide staining. Where multiple bands were present on the agarose gels, products were taken through a second electrophoresis step using a mini-acrylamide gel apparatus. Individual bands were cut from the acrylamide gel while visualised under UV light and then re-amplified as single PCR products for sequencing. Excess primers and nucleotides were removed from the remaining PCR products using a Qiagen™ clean up kit according to the manufacturer's instructions. One half of the eluted DNA product was sent to Macrogen (Seoul, Korea) for bidirectional sequencing.

Gene amplification targeting phylloxera-specific PCR products

Where a PCR product was obtained, sequence data from each gene region for the six major phylloxera genotypes (G1, G3, G4, G19, G20 and G30) were aligned using the BioEdit™ (Version 7.0.5). Consensus phylloxera sequence was compared with sequences in the Genbank Nucleic Acid database using the BLAST feature Version 2.2.05 (Altschul et al. 1997). Additional aphid species with homology above 95% in some instances were downloaded and aligned with phylloxera sequences using BioEdit™. Regions of low sequence homology were identified for phylloxera-specific primer design. Potential phylloxera-specific primers, typically of 17-22 basepairs (bp) length were designed to incorporate a minimum of two unique bases and to amplify a region containing a minimum of 30 phylloxera-specific base pairs. Potential primer sequences were tested for performance characteristics such as hairpin structure, potential self-dimer formation, and stability of 3′ termini by using the Primer 3

software package (S. Rozen and H.J. Skaletsky, Whitehead Institute of Biomedical Research, Cambridge, MA, USA).

Validation of potential phylloxera-specific primers

A testing procedure using a series of two PCR reactions was conducted on a number of commonly found vineyard and additional target organisms to determine the specificity of designed primers. The first PCR, using CO1 universal primers (Folmer et al 1994) tested viability and the second PCR tested designed primer specificity. Only DNA samples that gave a positive result with universal primers were used in the second PCR test. Where positive results were obtained for the second test, PCR products were purified and sequenced as above. Organisms tested were as follows:

Commonly-found vineyard insects and nematode species

These included: wasps (Order Hymenoptera), honey bees (*Aphis melifera*), fruit flies (*Drosophila melogaster*), thrips (Order Thysanoptera), caterpillars (Order Lepidoptera) and springtails (Order Collembola), all collected via the emergence trapping system from a number of vineyards in the Rutherglen and King Valley regions. Nematode species were kindly provided by Herdina (SARDI, Adelaide, South Australia) and comprised of the following species: stem nematode (*Ditylenchus dipsaci*), cereal cyst nematode (*Heterodera avenae*) and two root lesion nematodes (*Pratylenchus neglectus* and *Pratylenchus thornei*). Note that while not all the above nematode species are widely present in Australian vineyards, they were particularly targeted because they are soil dwelling parasites and, in the case of *P. neglectus* and *P. thornei*, cause root lesions on the grapevine root system.

Mite species

Bud mite (*Colomerus vitis*), blister mite (*Eriophyes vitis*) and rust mite (*Calepitrimerus vitis*) specimens, kindly provided by Melissa Carew (CESAR, The University of Melbourne), were tested. These three mite species are widely distributed throughout vineyards across Australia.

Aphid species

The aphid species used for validation were all kindly provided from the collections of Isabel Valenzuela (The University of Melbourne, Victoria). The 3 most common aphids present in Australian vineyards (Blackman and Eastop, 1941) were tested: cowpea aphid (*Aphis craccivora*), foxglove aphids (*Aulacorthum solani*) and cotton aphid (*Aphis gossipii*); as well as the following common aphid species: pea aphid (*Acyrtosiphon pisum*), spotted alfalfa aphid (*Therioaphis trifolii*), blackberry-cereal aphid (*Sitobion fragariae*), bird cherry-oat aphid (*Rhopalosiphum padi*), rice root aphid (*R. rufiabdominalis*), honeysuckle aphid (*Hyadaphis foeniculi*), gall-forming aphid (*Pemphigus* sp.), potato aphid (*Macrosiphum euphorbiae*), peach-potato aphid (*Myzus persicae*), grass and cereal aphid (*Metapolophium dilhodum*), ornate aphid (*M. ornatus*) and the rose aphid (*M. rosae*).

Sequence analysis

The programs Sequencer™ version 3.1.2 (Gene Codes Corporation) and BioEdit™ were used to check and align sequence data generated from phylloxera and aphid genera for which a PCR fragment was obtained using the ITS2 universal primers published in Bulman et al. (2005). Distance trees were constructed using the neighbour-joining method outlined by Saitou and Nei (1987) in the PAUP* program (Swofford, 1998). Confidence values for the branches were obtained using Rzhetski and Nei's (1993) bootstrapping method with 1,000 iterations.

Soil spiking

Two different soil types were selected for this study, classified as high (clay) and low (sand) phylloxera risk types. The high risk soils were obtained from the fibrous root zone of 15-year-old Cabernet sauvignon *V. vinifera* vines, located in the Yarra Valley, Victoria. Low risk soils originated from 2-year-old Shiraz *V. vinifera*, planted in a commercial vineyard in Drysdale, Bellarine Peninsula, Victoria. Both soils types were oven-dried at 50°C in bulk over night and then subsequently sub sampled into 200g amounts by weighing soils directly into individual sealable plastic bags. For each soil type, five replicates of the following phylloxera (G4) concentrations were prepared per 200g sub sample: 0 phylloxera/200g soil; 2 adults/200g soil; 5 adults/200g; 10 adults/200g soil; 20 adults/200g soil; 50 adults/200g soil; 100 adults/ 200g soil; and 200 adults/200g soil. After addition of phylloxera to the dilution series described above, all soils were oven-dried at 50°C for 50 hrs to ensure that the insect material was no longer viable (as per quarantine regulations and following a strict protocol for devitalisation of

infested soils for inter-state transport). Permits were then obtained from the relevant departments in Victoria and South Australia for transport of the soils under quarantine to a PC2 facility at SARDI, Urrbrae, South Australia.

Sensitivity testing of phylloxera-specific primers in a soil environment and soil DNA extraction

A bulk chelex extraction containing 100 G4 adults in 500 µls of chelex resin was quantified using the pico-green quantification reagent and standardised using a lambda standard into a DNA dilution series of 10ng/µl DNA; 5ng/µl DNA; 1ng/µl DNA; and 500pg/µl DNA. The dilution series was created in a DNA media that represented total DNA extracted from field soils located in South Australia. No phylloxera DNA was present in this soil media prior to the addition of the chelex DNA.

Total soil DNA (containing phylloxera DNA) was extracted from each of the soil dilutions at the Plant Research Centre, SARDI using a confidential extraction method. Soil DNA from the 100 adults/200g sub sample was first quantified using the pico-green method and diluted into the same serial dilution series as described for the chelex extraction dilution series as described above. i.e. 10ng, 5ng, 1ng and 500pg/ µl. A PCR reaction was then undertaken, using the ITS2 phylloxera-specific primers and two of the phylloxera spiked soils sub sample replicates (20 adults/200g and 200 adults/200g) against a known DNA dilution series prepared in phylloxera free soils (described previously) . The PCR reagents and final concentrations used for this step were the same as previously described, except BSA was replaced with a 5% Dimethyl sulfoxide (DMSO) additive. Amplification of an ITS2 product could be compared and quantified in both soils media.

Results and Discussion

Of the 11 gene regions selected for this study, 5 regions were successfully amplified using universal primers as shown in Table 1. Custom-made primers designed using unique bases located in the sequenced regions were constructed with the aim of producing primers that exclusively amplify phylloxera DNA (Table 1). Primers were tested to ensure that they consistently amplified all major phylloxera genotypes (Figure 1). This was true for all the phylloxera-designed primers except those in dopa carboxylase (DDC) gene region, which did not amplify consistently.

Extensive validation with vineyard organisms, mites and nematodes revealed that the designed primers to 4 gene regions [cytochrome oxidase subunit 1 (CO1), cytochrome b (cyt b), ATP synthase sub unit a (ATPSa) and the internal transcribed spacer unit 2 (ITS2)] were specific to phylloxera. Of these regions, however only primers designed from the ITS2 gene region did not amplify closely-related aphid genera. This result was not surprising since previous research by the CI has confirmed the extremely low (< 2%) genetic divergence between phylloxera and aphids and also other recorded instances of primers that can cross-amplify across different species of the Aphidoidea. All of the ITS2 primer combinations consistently amplified product, however the '61_F/68_F and 301_R primer' combinations were most visible as a clear single band on a 1.8% agarose gel.

Continued development of a diagnostic test for phylloxera therefore proceeded with the primers designed from the ITS2 region. A PCR gel illustrating the specific nature of the phylloxera-specific primers is shown in Figure 2. Sequence alignments comparing phylloxera and aphid sequence (Figure 3) illustrates the unique DNA bases to phylloxera. Regions selected for primer design are highlighted in red (Figure 3).

Neighbourhood-joining (NJ) analysis of the ITS2 gene regions was constructed (Figure 4) from aphid and phylloxera sequence generated using universal primers published in Bulman et al (2005). The NJ analysis was based on 48 informative characters and NJ tree includes 8 taxa. The NJ analysis clearly illustrates that the level of genetic divergence between phylloxera and aphid genera is such that these species can be distinguished from each other based on their ITS2 DNA composition.

Table 1: Custom-designed phylloxera primers for each gene region and their associated amplification conditions. Validation of results confirming their phylloxera specificity are also summarized.

Gene region	Designed primers	Amplification conditions	Primers phylloxera specific to: (a) vineyard orgs (VO) (b) aphid genera (AG)
Cytochrome Oxdase subunit 1 (CO1)	243_R: TGGGAAAGATATATCAGGAGC 432_R: AATTGATGAAATTCCTGCTAT 483_F: GGAGCAATTAATTTTATTTCTACC 586_F: TTATCTTTACCCGTATTAACATTA 663_F: CCATCAGGAGGAGGAGATCCAG	1 x 95°C (7 mins) 40 x (95°C, 30sec 56°C, 30sec 72°C, 1.0min) 1 x 72°C (7 mins) 4°C ∞	Yes to VO No to AG
CO1	30_F: ATCATAAAGACATTGGAACCC 32_F: CATAAAGACATTGGAACCCTT	As above	Yes to VO No to AG
Cytochrome B	CB_340_F: GCAAATCCAATAATTACACCAAC	As above except annealing temperature was 52°C	Yes to VO No to AG
ATP synthase sub unit a (ATPSa)	INT_57I_R: GGTGTCAATAGAAAAAGCAGTTTTAGAG EX3_R1: CTTCTGGTTGATGATCCTGTC EX3_R2: TGGTTGATGATGGTGTCAATAG EX2_F1: CAGCGWGAATTGATCATYGG EX2_F2: GATTGGAGACMGRCAGACTGG	As above except annealing temperature was 55°C	Yes to VO No to AG
Dopo Decarboxylase (DDC)	30_F: TGTGCTCGTATCCGTGGTG 443_R: AGGAATTGTTGGCTTAGGTAATG	1 x 95°C (7 mins) 40 x (95°C, 30sec 58°C, 30sec 72°C, 2.0min) 1 x 72°C (7 mins) 4°C ∞	PCR product did not consistently amplify phylloxera DNA.
Internal-Transcribed spacer (ITS) region 2	61_F: GTCGTATATCGGAAATTTGACG 68_F: ATCGGAAATTTGACGAGACC 276_F: GTCGGTTGAACGATCTCTCG 301_R: GCGATACGAGAGATCGTTCA 470_R: ATCTGAGGTCGGAACTCGTG	1 x 95°C (7 mins) 40 x (95°C, 30sec 54°C, 30sec 72°C, 1.0min) 1 x 72°C (7 mins) 4°C ∞	Yes to VO Yes to AG

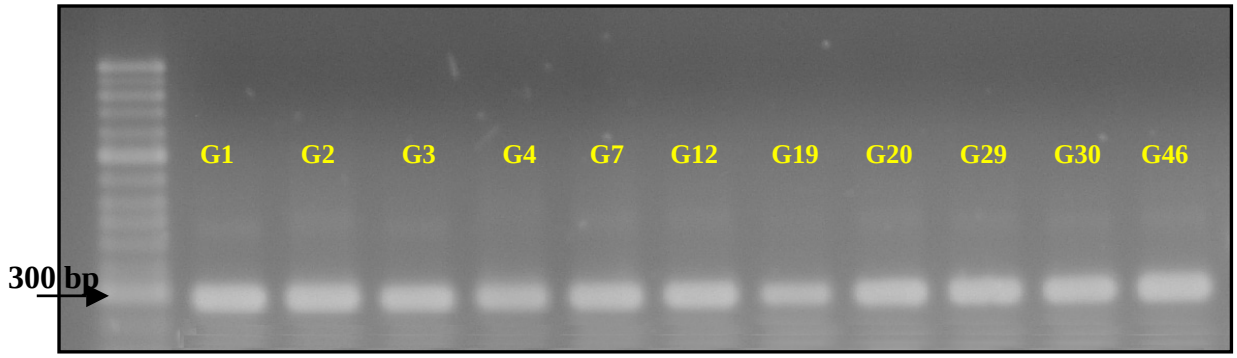


Figure 1: PCR agarose gel demonstrating ITS2 amplification across all major phylloxera genotypes.

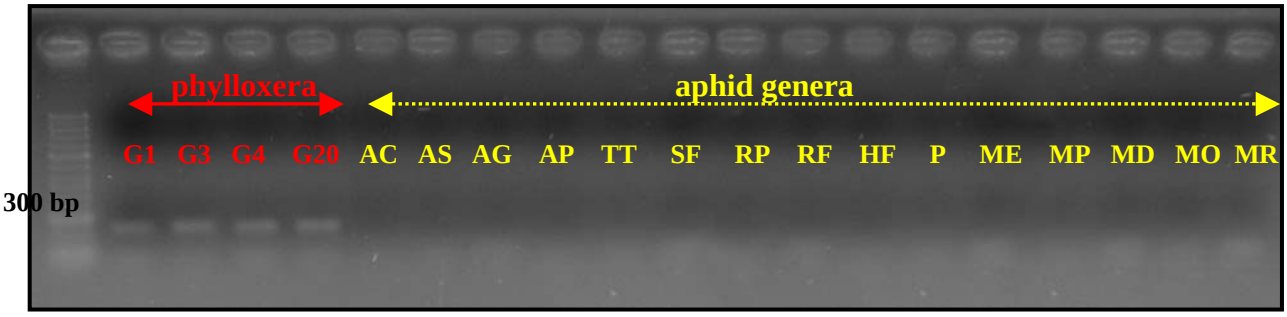


Figure 2: PCR agarose gel demonstrating positive ITS2 amplification using phylloxera DNA but negative amplification when aphid DNA is used from aphid genera. (Abbreviations: AC = *Aphis craccivora*; AS = *Aulacorthum solani*; AG = *Aphis gossipii;* AP = *Acyrtosiphon pisum*; TT = *Therioaphis trifolii*; SF = *Sitobion fragariae*; RP = *Rhopalosiphum padi*; RF = *R. rufiabdominalis*; HF = *Hyadaphis foeniculi*; P = *Pemphigus* sp.; ME = *Macrosiphum euphorbiae*; MP = *Myzus persicae*; MD = *Metapolophium dilhodum*; MO = *M. ornatus*; MR = *M. rosae*).

```
                          10         20         30         40         50
G1-ITS26F                 CAGTCGAACG CACATTGC?G CCTCGGTGAC CCGCGGGTCC CGGGCCACGC
G4-ITS26F                 .......... ........G. .......... .......... ..........
Rhopalosiphum_padi        .......... ........GC .......... .......... ..........
Aphis_gossypii            .......... ........G. .......... .......... ..........
Acyrthosiphon_pisum        .......... ........G. .......... .......... ..........
Myzus_persicae            .......... ........G. .......... .......... ..........
Metopolophium_dilhodum    .......... ........G. .......... .......... ..........
Aulacorthum_solani        .......... ........G. .......... .......... ..........

                          60         70         80         90         100
G1-ITS26F                 CTGTCTGAGG GTCGTATATC GGAAATTTGA CGAGACCGTT GGCGCCGCTC
G4-ITS26F                 .......... .......... .......... .......... ..........
Rhopalosiphum_padi        .......... ........C. ...T.C.C.G .C......A. .C-.....CG
Aphis_gossypii            .......... ........C. ...T.C.C.G .C......A. .C-.....CG
Acyrthosiphon_pisum       .......... ........C. ...T.C.C.G .C......A. .C-.....CG
Myzus_persicae            .......... ........C. ...T.C.C.G .C......A. .T-.....CG
Metopolophium_dilhodum    .......... ........C. ...T.C.C.G .C......A. .C-.....CG
Aulacorthum_solani        .......... ........C. ...T.C.C.G .C......A. .CA.....CG

                          110        120        130        140        150
G1-ITS26F                 GCTCGCCCGA GCGGACGCTC GTTTGGCGGT TCGACCGGCC TC--------
G4-ITS26F                 .......... .......... .......... .......... ..--------
#Rhopalosiphum_padi       ..AG..GTCC .AC.GG---- ---------- ---------- ----------
#Aphis_gossypii           ..A....G.C ...CG...G. CCGCCCGACC GTCGT.C--- ----------
#Acyrthosiphon_pisum      ..AG..GTCC .AC.G.A--- ---------- ---------- ----------
#Myzus_persicae           ..AG..GTCC .AC.G.C--- ---------- ---------- ----------
#Metopolophium_dilhodum   ..AG..GTCC .AC.G..GCG .CAGTC.TCC CG.GGACTGT C.GCC-----
#Aulacorthum_solani       ..AG..GTCC .ACT..CGCG .CGGC.G.CG GTCCT.CCGG GGACCGTTCG

                          160        170        180        190        200
G1-ITS26F                 ------CGCG CCGGTCGTCC GCTCAAGTAC GG---AAGGC AAACGTCCCG
G4-ITS26F                 ---------- .......... .......... ..-------- ..........
Rhopalosiphum_padi        --------A. A.C....G.. ....CG.CTG C.--CG..AT TGG..GTT..
Aphis_gossypii            --------.C .GTC.A.G.G ..GGCG.CGA C.GCCG.... G.C.AG.G..
Acyrthosiphon_pisum       --------.. ..C....G.. ....CG..GG T.--CG..AT TGG..GTT..
Myzus_persicae            --------A. ..C....G.. ....CG.CGG T.--CG..AT TGG..GTT..
Metopolophium_dilhodum    --------.. ..C....G.. ....CG..GG T.--CG..AT TGG..GTT..
Aulacorthum_solani        CCCGCC.... T.A....G.. ....CG.CGG C.TGCG..AT TGG..GTT..

                          210        220        230        240        250
G1-ITS26F                 GGTCCGCCAC CGCCGTCACG GCGAC----- ---------- GCGGCCCCGT
G4-ITS26F                 .......... .......... ..-------- ---------- ..........
Rhopalosiphum_padi        A-C.G.GG.A ..A..C.C.. C..T.TGCCT CGCGGCGACG ...A.GG..C
Aphis_gossypii            C-G.GCG.G. .--..CAC.. ...G.GCGAG ATTGGCGGCT CGAC.GG..C
Acyrthosiphon_pisum       A-C.G.GG.G A....CT-.. C--------- ---------- ------G..G
Myzus_persicae            A-C.G.GG.A .A.T.C.G.T C..------- ---------- ------G..G
Metopolophium_dilhodum    A-C.GTGG.A AA...CGCTC ..-------- ---------- ------G..G
Aulacorthum_solani        A-CGG.GG.A AA...CGCTC ...------- ---------- ------.G.G

                          260        270        280        290        300
G1-ITS26F                 CCGCCCGCCG CGCGCGCGTC GCCCGAGCCC TCGCGCTCGG GCTTCGTCCG
G4-ITS26F                 .......... .......... .......... .......... ..........
Rhopalosiphum_padi        ..------.T .CG.TCGTC. ....A..TTG .GA..GCAAA CAG..ACGG.
Aphis_gossypii            T.CAGG..GC .CG.TCGTC. ....A..TTG .GA..GCAAA CAG..ACGG.
Acyrthosiphon_pisum       ..------.T .CG.TCGTC. ....A..TTG .GA..GCAAA CAG..ACGG.
Myzus_persicae            .T------.T .CG.TCGTC. ....A..TTG .GA..GCAAA CAG..ACGG.
Metopolophium_dilhodum    ..------.T .CG.TCGTC. ....A..TTG .GA..GCAAA CAG..ACGG.
Aulacorthum_solani        ..------.T .C..TCGTC. ..T.A..TTG .GA..GCAAA CAG..ACGG.

                          310        320        330        340        350
G1-ITS26F                 CGGCCGGGTC GGTTGAACGA TCTCTCGTAT CGCCGCCGAC ACCGAGATGC
G4-ITS26F                 .......... .......... .......... .......... ..........
Rhopalosiphum_padi        TTCT..C.C. .-.AA....G -----.ACG. .C...T.CG. -...CCGC..
Aphis_gossypii            TTCT..C.C. .-.AA....G -----.ACG. .C...T.CG. -...CCGC..
Acyrthosiphon_pisum       TTCT..C... .-.CA....G -----.ACG. .C...T.CG. -...CCGC..
Myzus_persicae            TTCT..C... .-.CA....G -----.ACG. .C...T.CG. -...CCGC..
Metopolophium_dilhodum    TTCT..C... .-.CA....G -----.ACG. .C...T.CG. -...CCGC..
Aulacorthum_solani        TTCT..C... .-.CA....G -----.ACG. .C...T.CG. -...CAGC..
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                          360         370         380       390        400
G1-ITS26F                 GAACGGGGTC CCGCGTCCGC CGCGGCGG-- ---------- ----------
G4-ITS26F                 .......... .......... .........- ---------- ----------
Rhopalosiphum_padi        ..GA.C.CGG ...G...G.. T.A.AG---- ---------- ----------
Aphis_gossypii            ..GA.C.CGG ...G...G.. T.A.AGC.TT CGTCTCGACC GCGGTGTCGC
Acyrthosiphon_pisum       ..GA.C.CGG ...G.C.G.. T.A.AG---- ---------- ----------
Myzus_persicae            ..GA.C.CGG ...G...G.. T.A.AG---- ---------- ----------
Metopolophium_dilhodum    ..GA.C.CGG ...G...G.. T.A.AG---- ---------- ----------
Aulacorthum_solani        ..GA.C.C.G ...G...G.. T.A.AG---- ---------- ----------

                          410         420         430        440        450
G1-ITS26F                 -----TCTCG CGCG------ ---------- -------GAC --CGTTTCGG
G4-ITS26F                 -----..... ...------- ---------- -------... --........
Rhopalosiphum_padi        -----..CTA GA-------- ---------- -------... AC.TC.C...
Aphis_gossypii            GCGTG..CTC GA.ACGCGCG CGCGCCCGGC GGGACGC.G. GC.TC.C...
Acyrthosiphon_pisum       -----..CAC GA-------- ---------- -------... --.TC.C...
Myzus_persicae            -----..GTC GA-------- ---------- -------... --.TC.C...
Metopolophium_dilhodum    -----..CAC GA-------- ---------- -------... --.TC.C...
Aulacorthum_solani        -----..GAC GA-------- ---------- -------... --.TC.C...

                          460         470         480        490        500
G1-ITS26F                 GTTCCG---- GCCGCGGCGG CGGGCCGCGC GAAGTGTGTT ACACTTCTCG
G4-ITS26F                 .......... .......... .......... .......... ..........
Rhopalosiphum_padi        C.....AGAA ..G.AC.... AC..T...CG T.C.GAC.GA G.G.GGAC..
Aphis_gossypii            C.....AGAA ..G.AC.... ACC.TGT.CG ---.GAC.GA G.G.GGAC..
Acyrthosiphon_pisum       C.....AGAA ..G.AC.... GC..T.A.CG T.C.G.C.GA G.G.GGAC..
Myzus_persicae            C.....AGAA ..G.AC.... AC..T.A.CG T.C.G.C.GA G.G.GGAC..
Metopolophium_dilhodum    C.....AGAC ..G.AC.... AC..T.A.CG T.C.G.C.GA G.G.GGAC..
Aulacorthum_solani        C.....AGAA ..G.AC.... ACC.T.A.CG T.C.G.C.CA G.G.GGAC..

                          510         520         530        540         550
G1-ITS26F                 CGCGCCCGCC GTCGGATAAA T--------- -------ATA TAATATAAAT
G4-ITS26F                 .......... ......C... .--------- -------... ..........
Rhopalosiphum_padi        .A-.----..T .C..TG.T.. ---------- --GAAAA.A. -.CACA..CA
Aphis_gossypii            .A-.A..... .C..TGCG.G .CGGTTCGCC GACCGCACAC GC.C..T.TG
Acyrthosiphon_pisum       .A-.----..T .C..TG...T ---------- -------T.. AG.A.C...C
Myzus_persicae            .A-.----..T .C..TG.... ---------- -------CAT AG.A.-...A
Metopolophium_dilhodum    .A-.----..T .C..TG...T .TT------- --------.A. A..A.A..CA
Aulacorthum_solani        .A-.----..T .C..T.G.CG ---------- --------.A. A..A.A.T.C

                          560         570         580        590
G1-ITS26F                 TATACACGAG TTCCGACCTC AGATCAGGCA GGGACT
G4-ITS26F                 .......... .......... .......... ......
Rhopalosiphum_padi        C.CCG.TAC. .......... .........G ..ACTA
Aphis_gossypii            ......TAC. .......... .........G ..ACTA
Acyrthosiphon_pisum       A.CCG.TAC. .......... .........G ..ACTA
Myzus_persicae            ..CCG.TAC. .......... .........G ..ACTA
Metopolophium_dilhodum    ACCCG.TAC. .......... .........G ..ACTA
Aulacorthum_solani        A.CCG.T.C. .......... .........G ..ACTA
```

Figure 3: Partial 5.8S sequence, complete ITS2 sequence and partial 28S sequence that are consensus sequences representing G1 and G4 phylloxera genotypes aligned with closely related aphid genera. Primer regions are underlined and coloured in red.

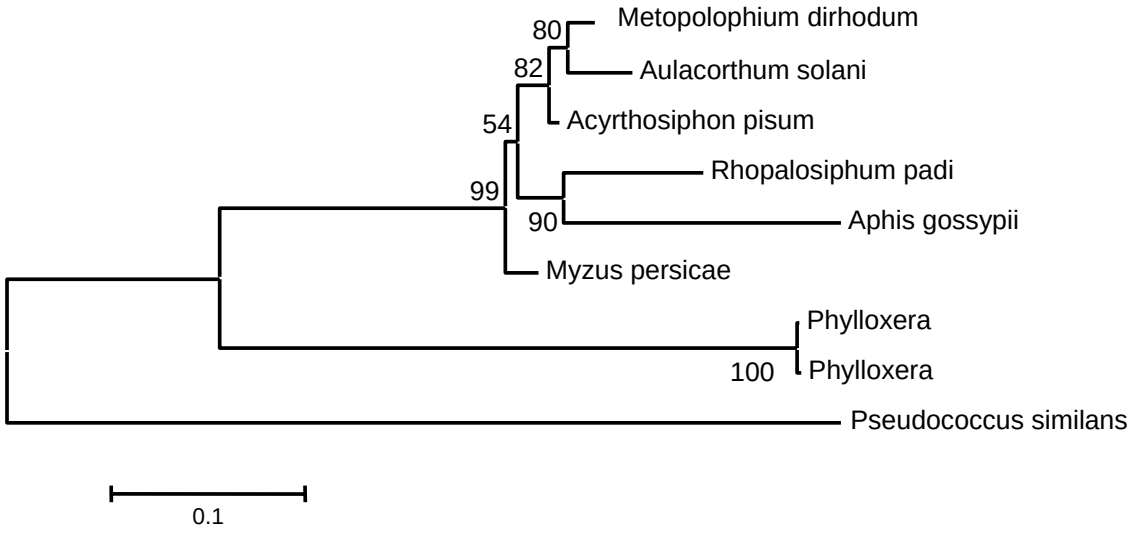


Figure 4: Consensus tree for neighbourhood-joining analysis of 834 base pair region of partial 5.8S sequence, complete ITS2 sequence and partial 28S sequence, comparing the sequence homology of G1 and G4 phylloxera sequences to aphid genera. *Pseudococcus similans* (mealybug) was obtained from Genbank (AF007265) and used as an outlier. Bootstrap values are shown. Scale bar represents 10% sequence divergence.

The results in Figure 5 (a) show that the phylloxera-specific primers developed from ITS2 sequence are able to amplify PCR products within the DNA concentration range of 100pg to 0.1pg in soil-based medium. Furthermore, PCR ITS2 products can be detected from spiked field soils containing 20 phylloxera adults/200g and 200 phylloxera adults/200g soil (Figure 5b). Due to time constraints, the remaining spiked soil dilutions could not be tested at this stage. Nonetheless the combined results so far indicate the robustness of the ITS2 primer set in soil-based environment, suggesting that they show potential to routinely detect phylloxera from infested soils.

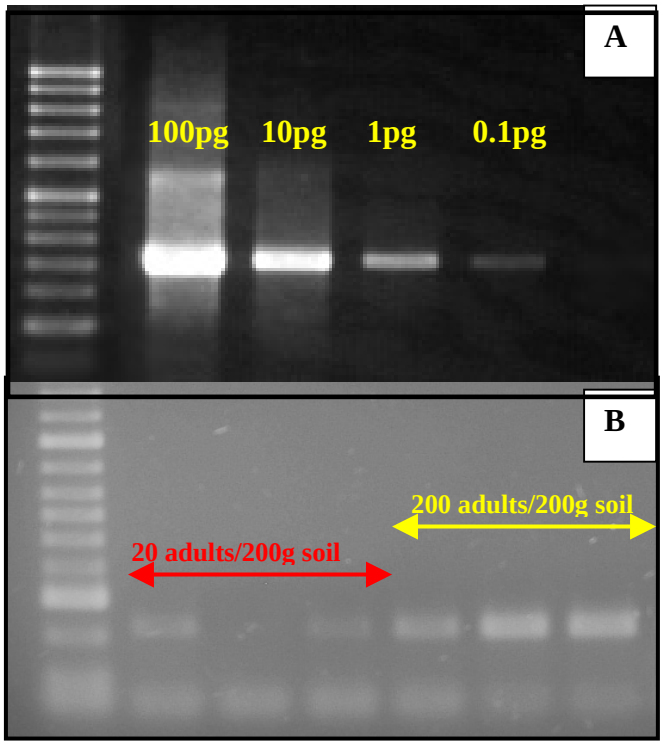


Figure 5: (a) Dilution series of phylloxera DNA in a soil DNA medium (free from phylloxera); (b) Gel result from soil spiking experiments that spiked high-risk field soils with known numbers of phylloxera. The 3 left bands represent soil DNA containing a phylloxera soil dilution of 20 adults in a 200g soil sub sample (The centre band did not amplify). The following 3 bands represent DNA yields from soils containing 200 adults in the same soil sub sample size. The PCR corresponding to 200 adults/200g soil DNA are the most visible.

A molecular diagnostic procedure designed to discriminate target organisms that can be utilised in quarantine laboratories should fulfil three criteria. Firstly, the diagnostic procedure should be reliable and validated against all possible cross reactions with similar taxa in the habitat under investigation; secondly, it should be a simple test comprised of a single PCR step thus allowing a high throughput of samples; and thirdly, it should be sufficiently sensitive to detect very low numbers of the target organism. Unfortunately, many existing diagnostics do not fulfil these criteria as they utilise a two-step molecular protocol, typically requiring post PCR restriction fragment analysis (for example, Fleming et a. 1998, Zheng et al. 2000, Ngugen et al. 2001) or duplex PCR reactions (Subbortin et al. 2001). While these 2-step diagnostic tests may be useful in a research laboratory as a confirmatory test for the presence of a particular pest species, they are impractical for use in a quarantine environment. These types of

methodologies are time-consuming relative to single-step PCR tests and, consequently more expensive in terms of both human and financial resources (Hübschen et al. 2004). Quantitative PCR assays could provide a simple testing procedure but there are currently few protocols available for plant pathogens as their utility is compromised by a failure to determine complete specificity (Mulholland et al. 1996) or sensitivity levels within their given environment (Blok et al. 2002).

A number of classical and real-time PCR protocols are available for the routine molecular detection of many bacterial (Louws and Couples, 2001), viral (Mumford and Barker, 1996; Klerks et al. 2001), fungal (Cullen et al. 2002; Lees et al. 2002; Li and Hartman, 2003; Destefano et al. 2004) and nematode pathogens (Amiri et al. 2002; Hübschen et al. 2004). However, there are few, if any, similar tests available for soil-dwelling insects such as phylloxera. A phylloxera-specific DNA probe would provide an improved method for the early detection of phylloxera because it would provide a rapid and highly accurate diagnostic test for the presence of this insect in vineyard soils.

With the purpose of designing a soil-based molecular test specific to phylloxera, the present study focused on the design and testing of species-specific primers designed from specific gene regions that have been widely used in arthropod research. Nuclear ribosomal RNA genes have been widely used for arthropod molecular systematics and are informative across a broad range of divergences in insects (Caterino, 2000). They have been widely used owing to their abundance within a genome and the relative ease of amplification and sequencing. Reviews on the utility of ribosomal gene data in phylogenetic analysis include Hillis and Dixon (1991), Simon et al. (1994) and Caterino et al. (2000). While nuclear ribosomal genes (i.e. 5.8S, 18S and 28S) themselves are highly conserved, significant genetic variation can be found both in discrete regions of the genes and in the length and sequence of the spacer regions such as the ITS1 and ITS2 spacer regions.

For the development of species-specific molecular identification, PCR primers and probe sequences must be designed to unique sequences and, therefore, nucleotide sequence information must be available from the target organism or it must be generated. Unique or characteristic DNA sequences need to be identified for the pathogen of interest. Furthermore, such primers need to be validated against all possible cross-reactions with similar taxa and other taxa present in the same habitat. As evidenced in this study, the detection of distantly related species using species-specific primers is possible due to high levels of sequence conservation seen in many gene families.

Phylloxera was successfully distinguished from every organism tested. Primers designed from sequence of the ITS2 gene did not amplify any DNA fragments from common vineyard organisms, mite, nematode, or aphid taxa. A high level of genetic divergence between phylloxera and aphid genera was found with the ITS2 gene region and this result was confirmed using NJ analysis. NJ analysis (using ITS2 sequence that positively amplified using universal primers) indicated that phylloxera and aphid sequences could be distinguished from each other evidenced by the high bootstrap values.

Outcomes/Conclusions

For the development of species-specific molecular identification, PCR primers and probe sequences must be designed to unique sequences and, therefore, nucleotide sequence information must be available from the target organism or it must be generated. Unique or characteristic DNA sequences need to be identified for the pathogen of interest. Furthermore, such primers need to be validated against all possible cross-reactions with similar taxa and other taxa present in the same habitat

In this study, species-specific PCR primers were successfully designed for phylloxera and successfully distinguished phylloxera from every organism tested. A high level of genetic divergence between phylloxera and aphid genera was found with the ITS2 gene region, indicating that routine tests using the DNA region would clearly distinguish phylloxera and aphid DNA. Results of the soil spiking experiments reveal that the ITS2 primers are capable of amplifying phylloxera DNA when immersed in soil-based environment, where soil inhibitors such as humic acids can be known to affect the performance of DNA primers in this media.

Therefore, this method shows a realistic potential for further development as an early detection tool for phylloxera. The benefits of adopting a DNA approach include increased sensitivity, accuracy and reduced labour inputs when compared to current spectral and ground-truthing methods. Furthermore, the ability to directly quantify numbers of phylloxera in the soil would provide valuable information on which quarantine and vine management decisions could be based.

The development of species-specific DNA tools for vineyard organisms means that information such as the identification, composition of pest, disease and beneficial and indicator organisms within a single vineyard can potentially be determined through universal sampling techniques and subsequent identification by a DNA service company.

Recommendations

Further development in the delivery of a diagnostic DNA test has already commenced. The PCR primers and ITS2 sequence obtained in this study have, under agreement, been given to SARDI for conversion to a high-throughput Taqman™ system. The financial costs of this conversion are being met by SARDI and CRCV. Conversion to a Taqman™ will provide a more sensitive, robust and faster approach to screening soils samples when compared to a standard PCR detection method.

This one-year research program was aimed at developing a tool for eventual use by agencies and growers for rapid and accurate assessment of the presence of phylloxera in vineyards. However the research led to the development of a species-specific probe rather than a commercial product that can be used by industry. Commercial adoption would require additional development and validation.

The next logical step involves extensive field evaluation of the DNA method for the detection and quantification of phylloxera infestations when compared to standard aerial and ground surveys. Field validation studies are crucial to determine the sensitivity levels in all soil types and to optimise sampling strategies.

This could be achieved by:

(1) Conducting DNA soil sampling alongside summer ground crews to directly compare the accuracy and sensitivity of both methods. Furthermore, DNA sampling could be conducted on a whole block basis to demonstrate its capabilities in quantifying and mapping variation in infestation levels. A greater understanding of the spatial distribution of phylloxera across individual blocks and infested zones in general will provide an excellent management tool and strengthen existing quarantine protocols.

(2) Sampling vines in a number of different soil types, to optimise the sampling strategies accordingly. This can be done in both phylloxera-infested and exclusion zones, i.e. in South Australia, and be incorporated with annual summer ground crew activities.

(3) Conduct extensive DNA sampling in phylloxera risk zones and around borders of infested zones to accurately access and map infestation levels. This assessment could be undertaken in both grafted and ungrafted vineyards, where correlations with vine performance can be simultaneously carried out.

Appendix 1: Communication

Papers:

K. Herbert, A. Hoffmann, K. Powell and K. Ophel- Keller (2006).
The Development of a PCR method for the rapid identification of phylloxera in vineyard soil. (manuscript).

K. Herbert, A. Hoffmann, A. Corrie, K. Powell and P. Umina (2006).
The use of DNA for pest management: the interaction between phylloxera clonal lineages and vine types. Acta Horticulturae (manuscript form currently).

K.S. Herbert, A.A. Hoffmann and K.S. Powell (2005).
Population Dynamics of Grape Phylloxera in ungrafted vineyards of south-eastern Australia. Journal of Economic Entomology. Accepted for publication January 2006.

Conference presentations:

K. Herbert, A. Hoffmann, K. Powell and K. Ophel- Keller
The Develoment of a PCR method for the rapid identification of phylloxera in vineyard soil. In: Abstracts of the Third International Phylloxera Symposium, October 7th, 2005. Fremantle, Perth, Australia. DPI (Eds: Trethowan, C. and K.S. Powell).

K. Herbert, A. Hoffmann and K. Powell
Population Dynamics of Grape Phylloxera in ungrafted vineyards of south-eastern Australia. In: Abstracts of the Seventh International Symposium on Aphids October 2-7, 2005. Fremantle, Perth, Australia. CSIRO. (Eds. Edwards, O.).

Appendix 2: Intellectual Property

The information arising from this project includes the phylloxera DNA sequences and primers, crucial in the development of a species-specific test for phylloxera. Materials Transfer Agreements are in place between CRCV and SARDI to freely exchange this information for the further development of this test. The CRCV has indicated its intention to take out an inventors patent on the phylloxera specific DNA sequences.

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AAppendix 5: Publications

Herbert et al.: Changes in grape phylloxera abundance in ungrafted vineyards
Pest Management

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Changes in grape phylloxera abundance in ungrafted vineyards from south-eastern Australia.

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ABSTRACT

To examine seasonal changes in the abundance of grape phylloxera Daktulosphaira vitifoliae (Fitch), several sampling methods were tested at vineyards in Victoria, Australia. At a recently infested site, changes detected by root assessment, trunk trapping and emergence trapping were closely correlated, although the largest numbers of grape phylloxera were obtained using traps that collected phylloxera emerging from soil. This trapping technique was further used to investigate changes in grape phylloxera numbers across three different sites from south-east Australia as well as in three consecutive seasons at the same vineyard. Grape phylloxera numbers decreased as vines deteriorated, a single peak of emergence occurred in every summer. Size and timing of emergence peaks varied between sites and also between vine blocks within a site. The number of grape phylloxera trapped was correlated with degree days. Monitoring soil temperature may provide a way of timing control options against grape phylloxera, and identifying peak periods when phylloxera detection surveys should be completed or when grape phylloxera are at the highest risk of spreading among vineyards.

KEYWORDS Grape phylloxera, Daktulosphaira vitifoliae, population dynamics, degree days

INTRODUCTION

Grape phylloxera, *Daktulosphaira vitifoliae* (Fitch), is a highly destructive and worldwide insect pest of cultivated grapes, *Vitis vinifera* L. Shortly after its introduction to Australia in 1875 (Adcock 1902), quarantine areas known as 'Vine Disease Districts' were enforced to facilitate the eradication of grape phylloxera in certain grape-growing areas (Anon. 1878) and later to prevent the spread of infested grapevines (Anon. 1923). Today, grape phylloxera is present in a total of 2% of Australian vineyards, restricted to five declared Phylloxera Infested Zones (PIZs) in Victoria and two PIZs in New South Wales. With only three recorded incursions outside quarantine boundaries (now termed PIZs) in a 130-year history in Australia, quarantine has contributed to the containment of this insect to discrete regions.

Since their development, grafted resistant rootstocks have reduced the impact of phylloxera-related damage and are used by all grape-growing countries affected by this pest (Granett et al. 2001a). Of the major grape growing countries today, only Australia and Chile have large plantings of ungrafted vineyards. Grape phylloxera has not been reported to-date in Chile. The phylloxera 'free' status of many Australian viticulture regions as well as the relatively high costs of rootstocks and problems in their availability and management (Buchanan 1990) have seen many growers elect not to plant on phylloxera-resistant rootstocks, even though the spread of grape phylloxera to new regions outside PIZs has serious economic consequences (Ferris et al. 2002). It is estimated that more than 85% of Australian commercial vineyards are planted on ungrafted *V. vinifera* which are highly susceptible to grape phylloxera attack. Grape phylloxera containment in Australia relies primarily on the combination of detection of phylloxera infestations, and quarantine and to a lesser extent rootstock management to restrict the movement of phylloxera outside pre-existing quarantine boundaries.

The effectiveness of quarantine protocols can be assisted by the identification of periods in the viticulture season when grape phylloxera are most likely to spread between vineyards. In Australia, there are overlapping generations of the parthenogenetically reproducing radicicolae or root-dwelling life cycle, with only rare occurrences of the sexual leaf-galling gallicolae life cycle which are restricted to discrete vineyard regions (Powell, 2000; Corrie et al. 2002). The main mobile stages of grape phylloxera are the first instars (crawlers) and alates (Buchanan 1987, Powell et al. 2000, 2003a). The spread of grape phylloxera within and between vineyards is associated with the movement of the crawler stages, either naturally or through human activities, such as via vine material, equipment, tools and footwear. Studies of native grape phylloxera populations have demonstrated that they vary in their ability to infest different vine genotypes (Fergusson-Kolmes and Dennehy 1993, Hawthorne and Via 1994, Downie 1999), suggesting the potential for host adaptation within the species. Genetic variability within grape phylloxera populations has been demonstrated worldwide (reviewed in Granett et al, 2001a), notably in Australia by Corrie et al. (2002, 2003), who identified 85 different phylloxera genotypes from 28 of the 57 vineyards known to be infested (K. S. Powell, personal communication). The two most common and widespread genotypes in Australia are termed G1 and G4. Field based studies conducted to date have primarily focussed on the seasonal population dynamics of the two most common genotypes G1 and G4 (Powell et al. 2000 and Powell et al. 2003a) but as yet there is no model that predicts the population growth of grape phylloxera in the field or peak periods when crawlers are abundant and more likely to spread between vineyards.

The abundance of radicicole populations can vary dramatically throughout the growing season (Granett et al. 2001a; Powell et al. 2003b). Populations overwinter in small numbers as first instars or crawler life-stages, where a combination of temperature and daylength are believed to be the major factors contributing to their maturity into adults (Stevenson 1975, Granett and Timper 1987). As temperatures become favourable in the spring, exponential population increases occur on both fibrous (non-lignified) and storage (lignified) roots. In Australia, grape phylloxera numbers peak in mid- summer (Buchanan 1990, Powell et al. 2000) and then decline; instances of a secondary peak in population growth on grapevine roots through autumn have also been observed in Australian (Powell et al. 2000; 2003a) and Californian vineyards (Omer et al.1997). Extrinsic factors such as temperature, quality and quantity of root material and the activity of soil-inhabiting pathogens of grape phylloxera are all believed to have an important impact on population dynamics in the field (Omer et al. 1997). However there is only limited understanding of the environmental variables that might influence seasonal changes in grape phylloxera numbers.

Here we investigate changes in the abundance of grape phylloxera across three consecutive seasons in a commercial vineyard in the King Valley region in north-east Victoria, Australia. Different trapping methods were used to determine whether grape phylloxera life-stages vary in a predictable manner throughout the vine growth season and between seasons. Results were then compared to changes in grape phylloxera abundance from two other vineyards

containing genetically distinct grape phylloxera genotypes to test if there was variability in changes in phylloxera numbers that might be related to seasonal, regional and genotype-related effects. We also assessed whether environmental information can predict peaks in grape phylloxera numbers as a basis for the timing of management strategies such as the application of chemical insecticides.

MATERIAL AND METHODS

Sites. Three different monitoring methods were compared in a commercial vineyard 5 km east of Cheshunt, within the cool-climate King Valley PIZ, north-east Victoria. Grapevines were planted in 1990 and grape phylloxera was first detected in the vineyard in May 1997. A single phylloxera genotype characterised as G4 present (Corrie et al. 2002). Soil type, classified on the basis of texture and chemistry, a dystrophic brown kandosol (Isbell 1996), with the soil texture of a duplex nature comprising of a sandy clay loam A horizon overlying light to medium clay subsoils (Powell et al. 2003b).

To compare grape phylloxera numbers across sites, ungrafted *Vitis vinifera* L. cv. Cabernet Sauvignon (clone G9V3) located in the King Valley, Rutherglen and Upton regions were selected. The King Valley site was located 2 km south-west of the vines examined in the trapping comparison. Vines had been planted in 1996 and the G4 grape phylloxera genotype (Corrie et al. 2002) had first been identified from this block in 2001. The Rutherglen site was 5km west from the town centre, within the north-east PIZ. Vines had been planted in the spring of 1975 as an extension to an existing vineyard planted at the turn of the twentieth century. Grape phylloxera had first been detected at this site in the early 1900s. A mixed grape phylloxera population comprised of 14 different genotypes has been recorded from the affected block, reflecting the high level of genetic diversity of grape phylloxera in this region (Corrie et al. 2002). The two genotypes identified around the Cabernet Sauvignon vines selected for this study were G29 and G46, neither of which have been detected outside the Rutherglen and Milawa regions (Corrie et al. 2002). The soil type was classified as a mesotropic brown chromosol (Isbell 1996), with a soil texture of a sandy clay loam in the A horizon to a fine sand to light clay in the B horizon (Powell et al. 2003a).

The third vineyard studied was located 10 km northwest of the Upton township in the Upton PIZ, Central Victoria. Grape phylloxera genotype G1 was first detected on these 5-year old vines during April 2000. The soil-type is classified as a mixture of mottled-sodic red kurosols and acidic mesotrophic brown kandosols (Isbell 1996), with soil textures described as sandy loam A horizons over sandy to light clay subsoils (Powell et al. 2003a). A second ungrafted Cabernet variety, *V. vinifera* L. cv. Cabernet Franc clone C7115, was also monitored within the same vineyard block to compare varietal differences.

Comparison of monitoring techniques. The study was conducted during the 2000 -2001 grape vintage at the King Valley site. Nine adjacent rows (designated rows 1-9) of ungrafted *V. vinifera* L. cv. Sauvignon Blanc (clone FV5V10) vines were chosen for the study block. The row spacing was 3 m and the vine spacing was 1.8 m. Visual decline in vines attributed to grape phylloxera was more evident in the first five rows. A total of 12 vines were studied in the block, consisting of three vines from each of rows 2, 4, 6 and 8. The three vines selected for each row were the 18th, 26th and 34th vines positioned from the start of each row respectively.

First instars are the most mobile and abundant grape phylloxera dispersive stage (Powell et al. 2000) and move from below the ground onto the soil surface. First instars were collected with "emergence" traps, consisting of translucent plastic containers (4 litre Décor™), 22 cm ×13cm deep, open at one end and inverted onto the soil surface at a distance of 10cm from the sample vine trunk (Powell et al. 2000). Traps were fixed flush with the soil surface using metal tent pegs. On emergence from the soil, grape phylloxera trapped in condensation on the container sides. At fortnightly intervals commencing on the 26 October 2000 and ending on the 06 June 2001, insects were removed by rinsing the trap with 70% ethanol and collected in

plastic containers. Traps were then rinsed with tap water and replaced. Collected insects were counted using a low power binocular microscope.

Grape phylloxera movement (predominantly 1st instar) up and down the grapevine trunks was assessed using a trunk trapping technique involving the collection of grape phylloxera on tapes placed in two bands. The lower band collected grape phylloxera moving up the vine trunk from the soil surface whilst the upper band collected insects moving down the vine trunk from the vine canopy. A 10cm strip of grey duct tape was wrapped around each vine trunk, 20cm above the soil surface, and sealed at the top and bottom with a liquid sealant to prevent grape phylloxera moving into cracks in the vine bark. The duct tape formed a smooth surface on which the trunk traps could then be applied. Trunk traps consisted of two bands of white electrical tape wrapped around the trunks of sample vines at a distance of 25cm above the soil surface and 20mm apart. Tanglefoot™ was applied evenly to the centre of the electrical tape, at a width of 1 cm, using a paintbrush. Trunk traps were removed and replaced every two weeks. On removal, traps were stuck to A4 paper, covered with plastic food wrapping to prevent sample contamination, and insects were viewed and counted under a low power binocular microscope.

Root populations were monitored monthly from October 2000 to June 2001. Samples were not taken from vines selected for trap sampling (referred to as reference vines) to avoid disturbing grape phylloxera populations and to reduce root damage to these vines. Instead, samples were collected from the eight vines surrounding the reference vines. These were the vines to the left and right of the reference vine in the same row, and three vines each located in the buffer rows to the top and bottom of the reference vines. Vines selected for root sampling were labelled 1-8 (Fig. 1). The first vine to be sampled is shown in Fig. 1, marked with a number "1", and sampling proceeded in a clockwise order thereafter. Sampling commenced in October 2000. At the conclusion of the study in June 2001, a total of 9 samples had been taken, with the vine shown in position "1" (Fig. 1) sampled twice. Soil volume for root samples was comparable between vines, dates, and sites.

Root samples were collected by digging to expose the vine roots; between 2-5g fresh root material, comprising mostly storage (lignified) root and a small amount of fibrous (non-lignified) root, was removed. Root samples were then transported under quarantine conditions to a quarantine laboratory facility located at the Department of Primary Industries, Rutherglen, where they were washed using a sieve of 53μm aperture to collect grape phylloxera life-stages, with the filtrate transferred to a 125ml plastic screw-top container for storage. All grape phylloxera life-stages were counted using a low power binocular microscope. All root material was weighed, oven-dried at 50°C for 48h and reweighed to determine dry weight comparison across years. The emergence trapping technique was used to examine changes over three grape growing seasons because this technique captured more insects than the others tested (see Results section). Emergence trapping at the King Valley site was continued, using the same 12 reference vines described earlier, for the 2002 and 2003 grape-growing seasons, with traps being sampled every two weeks.

Variation in grape phylloxera emergence across different regions. Six vines were selected at random within a 100m × 100m area for each of the five vineyard blocks described above (three regions, two varieties sampled in two of the regions). Two emergence traps were placed either side of the vines, where weekly sampling was conducted from late December 2002 until mid-April 2003. At three sites (two trial blocks within the King Valley vineyard and one vineyard each at Upton and Rutherglen), soil temperature at a depth of 150mm was monitored at 15 minute intervals using Tinytag Ultra™ data loggers (Gemini Data Loggers (UK) Ltd.) fitted with a soil probe attachment. One data logger was present in each of the blocks with the exception of the Cabernet Franc block at Upton. At the King Valley sites soil temperature data were collected from the end of November until April in the following year. Temperature at the Upton site was only recorded when grape phylloxera were collected.

Analysis. For the monitoring method comparisons, insect numbers collected with emergence and trunk trap methods were summed over the twelve reference vines and plotted to examine

changes in abundance over time. For the root samples, insect life-stages per gram oven-dried (OD) root were plotted for each monthly sampling period. To compare emergence and trunk data with monthly root samples, fortnightly samples were summed to produce values for each month. To compare the numbers of grape phylloxera caught in different traps, correlation coefficients were computed. In addition, a simple sign test was used to test for consistent temporal changes in abundance between trapping methods. This test involved determining if numbers of grape phylloxera caught with a particular trapping method increased or decreased between sampling intervals. A comparison of the sign of the change between the different types of trapping methods was made. The number of consistent changes was compared with the expectation that inconsistent and consistent changes would occur equally using a Chi-square test.

For the comparison of crawler numbers across years, a univariate analysis of variance (ANOVA) was carried out on ln (x + 1) transformed numbers to test for effects of vine row and year on crawler number. To test if changes in phylloxera numbers over time were non-random (ie consisted of one or more peaks), a runs test was undertake (Sokal and Rohlf 1995). To compare the sites, crawler numbers were summed across the 12 emergence traps at each different site and plotted to compare peaks in first instar (or crawler) populations and differences in the total grape phylloxera genotype abundance. For the King Valley Cabernet Sauvignon site, the frequency of the alate adults and apterous crawlers was also compared. A simple sign test was used to examine for consistent temporal changes in grape phylloxera emergence between varieties grown in the same region and also to compare grape phylloxera emergence between the 3 regions. A runs test was also carried out to test if changes in phylloxera numbers over time deviated from a random pattern.

Degree day (°d) models were used to link grape phylloxera numbers to temperature. Models were calculated based on developmental studies conducted by Raspi et al. (1987) where the predetermined developmental zero was 8.7° and the thermal constant was 281.5 °d. The developmental zero was subtracted from mean daily temperature (based on hourly daily readings) to create °d units that were averaged across the sampling period and then plotted against summed ln (x + 1) emergence totals attained for the same sampling period. To determine if degree day data predicted grape phylloxera emergence numbers, linear regressions on the mean degree day values against ln (x + 1) grape phylloxera emergence at (a) the same sampling period and (b) degree day intervals moved earlier by one sampling period were carried out. Because emergence numbers at the Rutherglen site were extremely low, no attempt was made to link degree days to emergence numbers at this site.

RESULTS

Comparison of trapping techniques. Insect numbers collected with the three monitoring methods are shown in Fig. 2. First-instar and alate life-stages were collected in the emergence and trunk traps. The emergence trapping method (Fig. 2a) had by far the highest yields with summed numbers peaking to over 20,000 crawlers on 17 January, 2003. All traps indicated consistent changes in abundance, involving a steady increase of numbers caught from November and December, peaks in life-stages during January and February (apart from the 31st January sampling period) and a steady decline in all life-stages from March onwards.

For the trunk trapping method (Fig. 2b), higher numbers of crawlers were counted in the lower trap than the upper trap. Nonetheless, a significant correlation ($r = 0.921$, $N = 17$, $P < 0.001$) was found between the upper and lower trunk trap numbers.

For the root sampling method (Fig. 2c), numbers of eggs recovered from the roots peaked before numbers of 1st and then 2nd instars. The 3rd and 4th instars and adult life-stages (both alate and apterous) peaked at the same date (mid February). The 1st instar stage was by the far the most abundant stage recovered from the roots.

Direct comparison of the numbers and distribution of crawlers collected with the three trapping methods indicate similar patterns for all three trapping methods. This was particularly true for numbers recovered with the trunk and root sampling methods, which

showed the same signed changes between time intervals for all eight time periods ($X^2 = 8$, df = 1, $P < 0.01$). For the comparison of root versus emergence catches and trunk versus emergence catches, changes in abundance followed the same sign in seven of the eight comparisons ($X^2 = 4.5$; df = 1; $P < 0.05$). Grape phylloxera numbers therefore changed in the same way over a season regardless of the trapping method used.

Comparison across years. Emergence numbers in 2001, 2002 and 2003 are shown in Fig. 3. Comparisons of emergence data from successive vintages indicate a marked reduction in total insect numbers from season to season. The time point at which grape phylloxera crawler numbers peaked was similar for the 2001 and 2002 vintages, with an earlier peak evident for the 2003 vintage (Fig. 3). The 2002 emergence data showed a bimodal pattern, with a second peak occurring during March 2002, but this was not evident in other years. Above-ground changes in grapevines throughout the course of the study associated with phylloxera damage are clearly shown in Fig. 4.

Univariate ANOVAs were undertaken on data summed by rows and across the entire season to examine differences among rows and among seasons. This analysis indicated there was an overall significant difference in crawler emergence across the three seasons ($F = 30.83$; df= 2, 24; $P < 0.001$), and runs tests indicated that these changes over time were not random ($P < 0.01$ for all three years). Individual vine rows were also significantly different from each other ($F = 3.90$; df = 3, 24; $P < 0.05$), while there was no interaction between these factors ($F = 0.74$; df = 6, 24; $P = 0.62$) indicating that the same rows showed similar patterns from season to season. Above-ground symptoms of grape phylloxera damage were clearly seen across the whole vineyard block throughout the course of this study. Yield levels for this block decreased rapidly (data not presented), such that the 2003 vintage was not harvested due to uneconomic yields.

Grape phylloxera emergence across different regions. Peaks in crawler emergence differed in the three sites studied (Fig. 5). The numbers of crawlers collected across the vineyard regions varied in time and magnitude from peak levels of 14 insects at Rutherglen to over 16,000 crawlers detected on Cabernet Sauvignon vines in the King Valley site.

High numbers of winged alates were recovered from Cabernet Sauvignon vines in the King Valley which closely matched the distribution of apterous crawlers (Fig. 6). Where two varieties were studied in the same region, the pattern of emergence was similar between grapevine varieties (Figs. 5A and 5C). A significant association between the Cabernet Sauvignon and Cabernet Franc varieties was recorded at Upton ($X^2 = 15$; df = 1; $P < 0.001$), and also between the Cabernet Sauvignon and Sauvignon Blanc varieties in the King Valley ($X^2 = 5.4$; df = 1; $P = 0.02$). Runs tests indicated non-random changes in phylloxera numbers at the two King Valley sites ($P < 0.01$ for both varieties) but not at Rutherglen ($P = 0.27$ for Cabernet Sauvignon) and Upton ($P = 0.17$ for Cabernet Sauvignon and $P < 0.05$ for Cabernet Franc).

The results from the degree-day models (Fig. 7) indicate that soil temperature data could potentially be used to predict phylloxera emergence numbers for the two sites investigated. In the King Valley region, significant regressions were observed with degree-day values for both the actual and previous sample periods for the Cabernet Sauvignon ($r = 0.38$, $P < 0.01$ and $r = 0.66$, $P < 0.001$ respectively) and Sauvignon Blanc ($r = 0.33$, $P < 0.05$ and $r = 0.43$, $P < 0.01$ respectively) varieties. A significant regression for emergence numbers was also found for the Cabernet Sauvignon variety located in the Upton region when the sampling period was earlier than one interval ($r = 0.40$, $P < 0.01$) though not for the actual sampling period ($r = 0.01$, $P = 0.65$). Numbers of grape phylloxera emerging within the peak period therefore tended to be related to average degree days within an interval, particularly when measured in the week prior to the grape phylloxera samples being collected. For the King Valley where additional soil temperature data points were available, the degree day data indicate that grape phylloxera numbers only built up in traps when the degree days exceeded 10 per day, and then declined again as degree days fell below this point. For all three sample sites the number of

grape phylloxera detected in samples reached a peak after the average degree days within an interval had reached 13.

DISCUSSION

The different monitoring methods varied in their trapping efficiency, with emergence traps capturing more grape phylloxera than trunk traps and also being a more efficient and less destructive way of sampling for phylloxera than root sampling. These findings are consistent with Powell (2000), who found crawler numbers in the King Valley to be ten times higher in emergence traps compared to trunk traps. Buchanan (1990) and Powell et al. (2003b) have used emergence traps in two vineyards in the Nagambie and Upton PIZs respectively to show that first-instar crawlers, originating from radicicolae populations, were the most abundant dispersive stage of grape phylloxera. Crawler populations at the soil surface appear to be greater than those moving up (or down) the vine trunk. Nonetheless, all three trapping methods showed concordant seasonal changes. The high (< 90%) 1st instar mortalities found using the root sampling method suggests that the limiting factor for 1st instar to second instar survival may be an inability of 1st instars to initiate gall formation . Nonetheless, all three trapping methods showed concordant seasonal changes, suggesting that they are all suitable for assessing changes in grape phylloxera populations.

For all three field sites studied, changes in grape phylloxera numbers followed a similar trend. Low grape phylloxera populations occurred in early spring during the vegetative vine growth stage, rising exponentially during January to peak numbers seen in the mid-ripening stages, and dropping to relatively low levels during February/March before grape harvest. Peaks in all life-stages occurred during the vegetative and mid-ripening growth stages, rather than the post-harvest stage. Similar seasonal trends have been found in Canada (Stevenson 1964), the USA (Omer et al. 1997) and in other Australian vineyards (Buchanan 1990, Powell et al. 2000, 2003b). In field population studies in the USA by Omer et al. (1997), populations differed in abundance patterns across a season. In a Chardonnay vineyard at Stockton, large midsummer peaks were recorded during the vegetative/mid-ripening periods with a reduced peak post harvest. A contrasting pattern was found at an AXR#1 site in Ukiah where midsummer peaks were smaller than harvest peaks.

When comparing the King Valley emergence data over the three vintages, significant site and seasonal differences were evident. The most visually affected vines in Row 1 had the highest recorded grape phylloxera numbers when compared to the other three rows over the entire 3 year period of monitoring. This contrasts with field studies by Buchanan (1990), who recorded the highest number of grape phylloxera from emergence traps that were located in relatively vigorous vines at the edge of the grape phylloxera ‘weak spot’; these vines showed little visual evidence of infestation. A recent study by Powell (unpublished) shows that moderately vigorous vines had higher population numbers on roots at the same vineyard sites in King Valley and Upton.

Grape phylloxera development is influenced by temperature (Granett and Timper 1987, Belcari and Antonelli 1989, Connelly 1995, Turley et al. 1996). Laboratory experiments by Turley et al. (1996) showed that the temperature must exceed 18°C for grape phylloxera to establish feeding sites on tuberosities. Our results for degree days suggest that grape phylloxera development can be related to soil temperature, although numbers decline before temperatures fall below threshold levels. Regressions suggest that soil temperature recordings one week prior to grape phylloxera assessment are particularly useful in prediction. Grape phylloxera abundance will also be associated with other factors. The severity of grape phylloxera root infestations has been found by Nougaret and Lapham (1928) to be closely related to soil texture. In heavy clay soils with moderate temperatures, vine decline tends to be rapid compared to very hot or cold climates and in sandy soils (Nougaret and Lapham 1928, Stevenson 1963). A combination of extrinsic factors including temperature, quality and quantity of roots and the activity of soil inhabiting of pathogens of grape phylloxera are likely to account for variation in population dynamics (Omer et al. 1997).

The emergence trapping technique was the easiest method to use with regards to sample collection in the field and quantification of grape phylloxera number in the laboratory. Current grape phylloxera detection methods are based on root assessments for visual evidence of nodosities and tuberosities. Root assessments are very labour intensive and visual evidence of nodosities and tuberosities on grape roots may be difficult to confirm, and absent in the case of tuberosities (Herbert et al. 2003). They can also cause damage to the vine root system. Emergence traps may be a non-destructive alternative to root sampling in some situations due to their simple and effective application, as well as lower labour requirement. The optimal time period to sample for grape phylloxera, independent of the sampling method used, was typically found to occur between mid-January and mid-February for the three field sites studied; insect numbers peaked in all three sites within this 4-5 week period.

Soil temperature measurements can be used to assist in predicting peaks. The degree day and emergence data indicate that peaks of emergence only occur after the mean weekly degree day values have reached 10 degrees or greater above the threshold temperature of 8.7°C. When these values are recorded, the period of peak emergence is likely to be approached, but ideally sampling for grape phylloxera and potential treatments should be applied soon after values greater than 10 degree days are determined. Although these patterns need to be validated in other regions and vineyards, they provide a starting point for developing guidelines for routine phylloxera sampling. Such temperature-based monitoring will need to be applied on a block by block basis, given that aspect, soil conditions, irrigation practices and other factors will influence local soil temperature.

Grape phylloxera populations on the root system showed a synchronised pattern of life-stage development. Even though the phylloxera life cycle is complicated with overlapping generations, peaks in egg numbers were detected during December, followed by peaks in 1st instar number in mid-January, shortly followed by peaks in the 2nd instar life-stage in late January. The numbers of alates were low. Field studies in Australia in the Nagambie region (Buchanan 1990) also indicate alate numbers to be much lower than 1st instar numbers, and in comparable field studies in New Zealand by King et al (1981) similar trends in the ratio of these two life-stages were obtained. The physiological mechanism by which the alate form is triggered is not known (Granett et al. 2001b), however it may be stimulated by overcrowding (Granett et al 2001b), host plant quality (Dixon 1973) or high soil moisture (Maillet 1957). First instars were the most abundant life stage detected, as has already been well documented in Australia (Buchanan 1990, Powell et al. 2000) and overseas (Stevenson 1964, Granett et al. 2001a, Omer et al. 1997, 2002). This life-stage therefore poses the greatest threat to quarantine. Due to the very high crawler numbers detected on the canopy floor, this can cause a substantial risk of transfer from infested to uninfested vineyards via machinery, vine material or footwear and clothing of vineyard personnel.

In summary, a combination of environmental, physiological and genetic factors are likely to influence seasonal and regional changes in grape phylloxera numbers. Grape phylloxera numbers were found to be variable within the same region and across seasons, however overall seasonal trends in grape phylloxera numbers were consistent between two varieties in the same/nearby vineyard(s). Predicting peaks in grape phylloxera populations for timing of chemical insecticide application may be possible using soil temperature and emergence traps given that these traps can collect large numbers of grape phylloxera with low effort and that numbers in these traps reflect activity of grape phylloxera on roots and moving onto the soil surface and up the grapevine trunk.

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FIGURE LEGENDS

Fig. 1. Sampling procedure employed at a commercial vineyard in the King Valley throughout the 2001 vintage. Triangles represent individual vines located within the buffer or sample rows. Monthly root sampling was conducted around the 'reference' vines (R) within the sample rows. Reference vines were used for emergence and trunk trapping. Root sampling was not conducted on these vines to avoid root damage and to ensure that the grape phylloxera populations were not disturbed. Root sampling commenced at position 1 (as indicated above) and proceeded in a clockwise direction over subsequent monthly intervals.

Fig. 2. Mean number of grape phylloxera (genotype G4) life-stage numbers found with (A) emergence traps, (B) trunk traps and (C) oven-dried (O.D.) root sampling methods at a commercial vineyard in the King Valley, North-East Victoria, for the 2001 vintage.

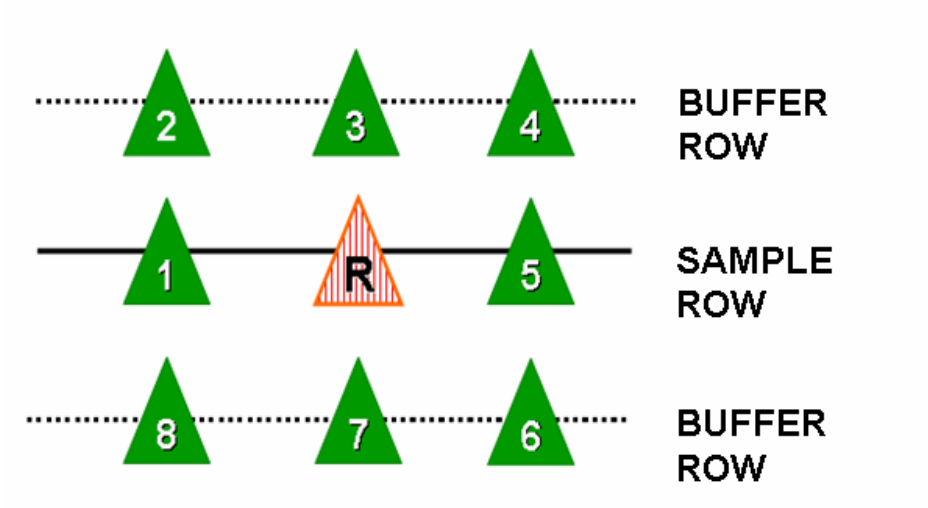
Fig. 3. Mean of grape phylloxera crawlers (genotype G4) found per emergence trap located across four rows of grapevines in a commercial vineyard in the King Valley for three consecutive seasons (2001-2003).

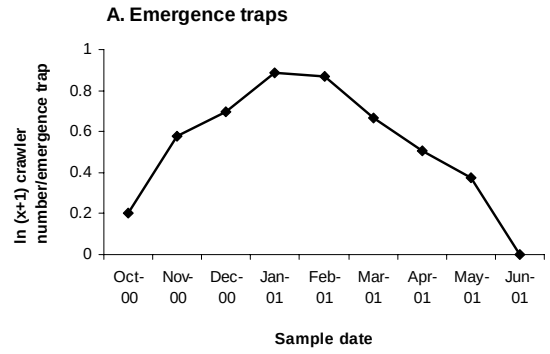
Fig. 4. Visual changes associated with phylloxera damage on ungrafted Sauvignon Blanc grapevines at the commencement of the trial in 2000 to the conclusion of the trial in 2003.

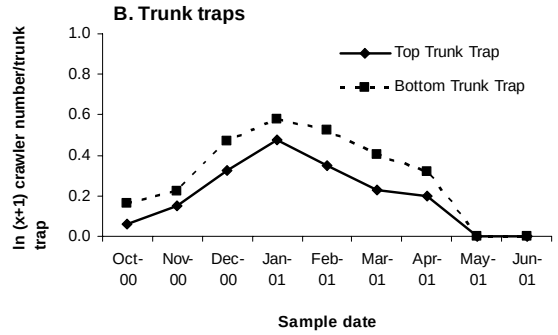
Fig. 5. Crawler emergence at three vineyards across north-east Victoria: (A) Upton with G1 genotype; (B) Rutherglen with a mixed G29 and G46 grape phylloxera population; and (C) King Valley with G4 genotype

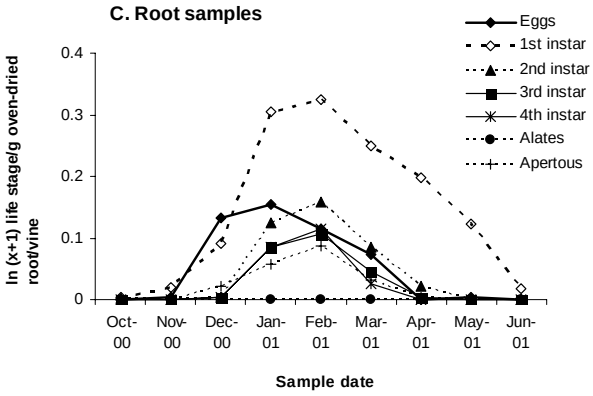
Fig. 6. Comparison of G4 apterous crawler and alate numbers at the Cabernet Sauvignon site in the King Valley throughout the 2003 growing season.

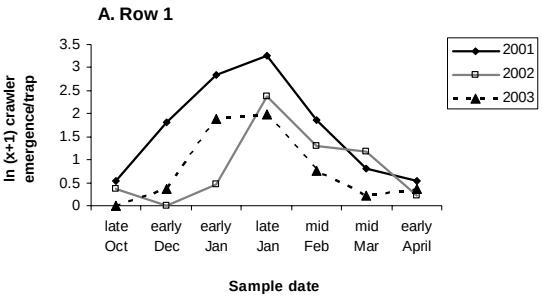
Fig. 7. Predicted values of degree days (°d), as computed from Raspi et al. (1987), based on soil temperature data (depth 150mm), against summed emergence totals categorised as: (A) Upton, Cabernet Sauvignon ; (B) King Valley, Sauvignon Blanc; (C) King Valley, Cabernet Sauvignon.

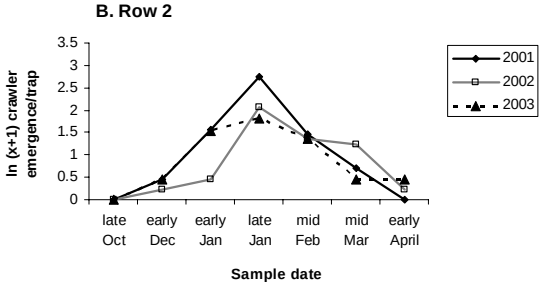


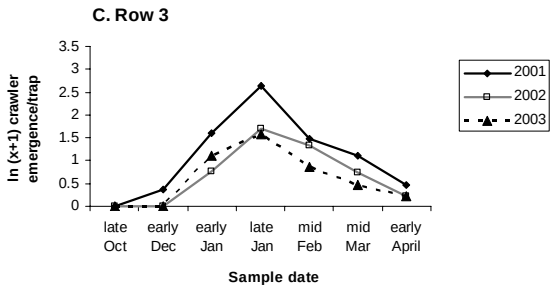

A. Emergence traps
ln (x+1) crawler number/emergence trap
1
0.8
0.6
0.4
0.2
0
Oct-00
Nov-00
Dec-00
Jan-01
Feb-01
Mar-01
Apr-01
May-01
Jun-01
Sample date

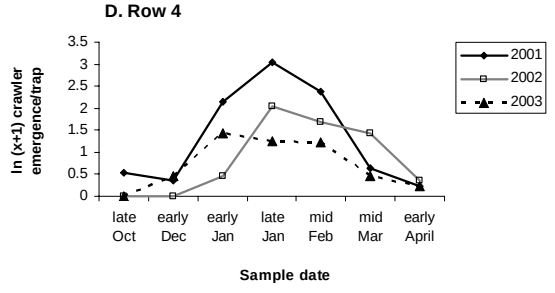

B. Trunk traps
ln (x+1) crawler number/trunk trap
1.0
0.8
0.6
0.4
0.2
0.0
Top Trunk Trap
Bottom Trunk Trap
Oct-00
Nov-00
Dec-00
Jan-01
Feb-01
Mar-01
Apr-01
May-01
Jun-01
Sample date


C. Root samples
ln (x+1) life stage/g oven-dried root/vine
0.4
0.3
0.2
0.1
0
Eggs
1st instar
2nd instar
3rd instar
4th instar
Alates
Apertous
Oct-00
Nov-00
Dec-00
Jan-01
Feb-01
Mar-01
Apr-01
May-01
Jun-01
Sample date


A. Row 1
ln (x+1) crawler emergence/trap
3.5
3
2.5
2
1.5
1
0.5
0
2001
2002
2003
late Oct
early Dec
early Jan
late Jan
mid Feb
mid Mar
early April
Sample date

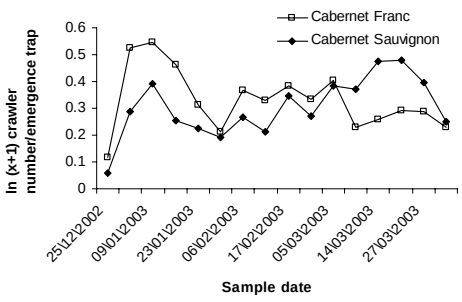

B. Row 2
ln (x+1) crawler emergence/trap
3.5
3
2.5
2
1.5
1
0.5
0
2001
2002
2003
late Oct
early Dec
early Jan
late Jan
mid Feb
mid Mar
early April
Sample date


C. Row 3
ln (x+1) crawler emergence/trap
3.5
3
2.5
2
1.5
1
0.5
0
2001
2002
2003
late Oct
early Dec
early Jan
late Jan
mid Feb
mid Mar
early April
Sample date

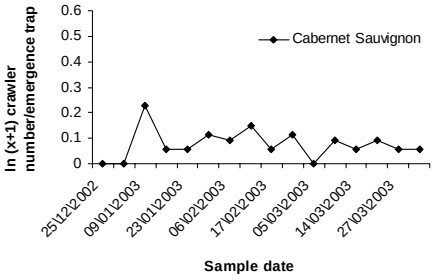

D. Row 4
ln (x+1) crawler emergence/trap
3.5
3
2.5
2
1.5
1
0.5
0
2001
2002
2003
late Oct
early Dec
early Jan
late Jan
mid Feb
mid Mar
early April
Sample date



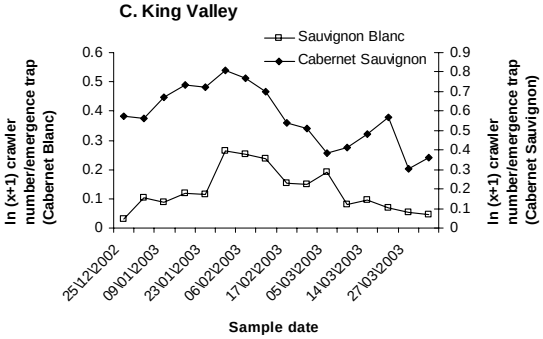
A. Upton

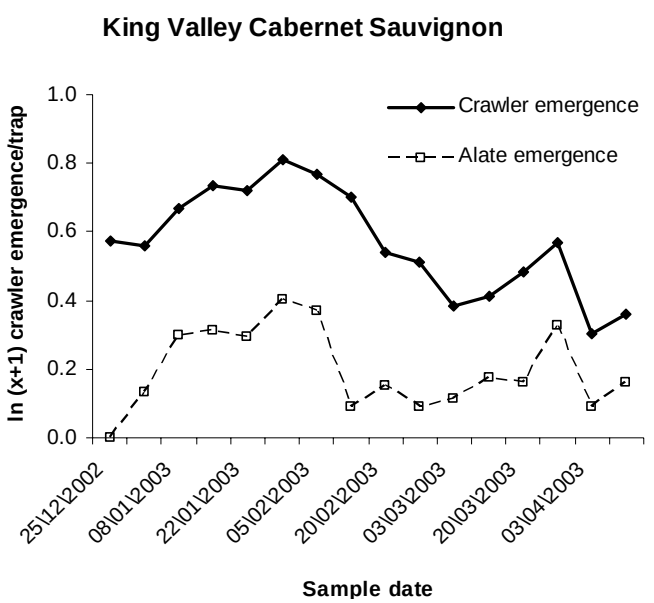


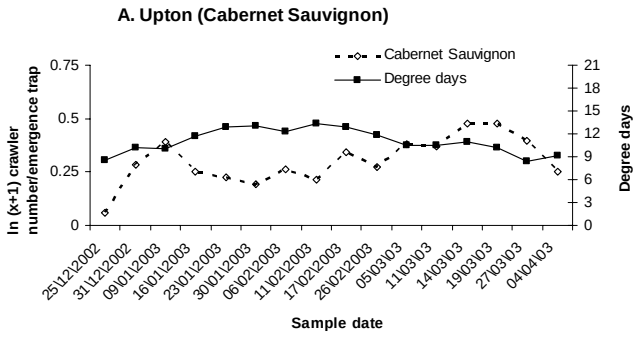
B. Rutherglen

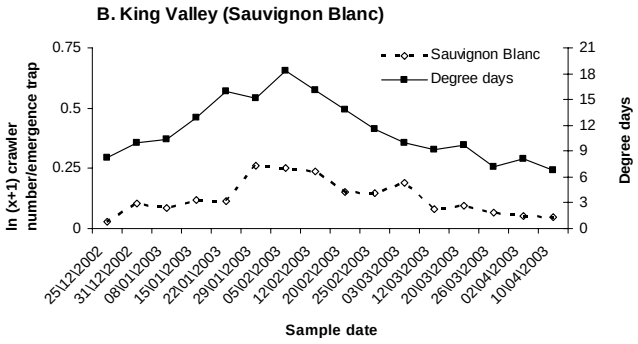


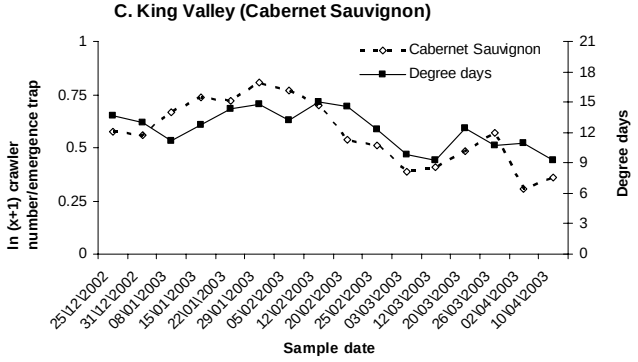
C. King Valley




King Valley Cabernet Sauvignon
ln (x+1) crawler emergence/trap
Crawler emergence
Alate emergence
1.0
0.8
0.6
0.4
0.2
0.0
25\12\2002
08\01\2003
22\01\2003
05\02\2003
20\02\2003
03\03\2003
20\03\2003
03\04\2003
Sample date


A. Upton (Cabernet Sauvignon)
ln (x+1) crawler number/emergence trap
Cabernet Sauvignon
Degree days
Degree days
Sample date


B. King Valley (Sauvignon Blanc)
ln (x+1) crawler number/emergence trap
Sauvignon Blanc
Degree days
Degree days
Sample date


C. King Valley (Cabernet Sauvignon)
ln (x+1) crawler number/emergence trap
Cabernet Sauvignon
Degree days
Degree days
Sample date

Appendix 6: Budget reconciliation

PROJECT BUDGET (cash component only)

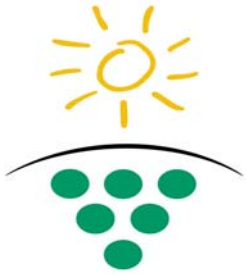
	2004/2005	2005/2006
Salary	$26,344	$26,344
On-costs	$7,376	$7,376
Operating	$7,500	$7,500
Equipment	nil	nil
Total	$41,220	$41,220

CRCV Attachment 1: Project Summary & Impact Form

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Milestone	Year 1	Progress	Revised Date for Achievement of Milestone
Perform field collections and DNA extractions of major phylloxera genotypes.	--	Achieved	
Isolate potential DNA regions likely to contain high genetic variability enabling the design of species-specific primers	--	Achieved	
Design primers and that test that these amplify DNA regions consistent amongst all phylloxera genotypes.	--	Achieved	
Validate potential region(s) with commonly found vineyard organisms.	---	Achieved	
Validate potential regions(s) with commonly found aphid species	----	Achieved	To be completed by 31st December 2005
Conduct lab-based sensitivity tests in sterile soils.	------	Achieved	
Conduct sensitivity lab-based sensitivity tests using field-collected soils.	-------	Achieved	

CRCV Attachment 2: CRCV Annual Report Requirements



COOPERATIVE RESEARCH CENTRE *for* VITICULTURE

Project Leaders are required to submit reports to Program Managers for compilation for the information of the Executive Management Group, the CRCV Board and the Australian Government's CRC Program

The format and reporting dates have been developed to satisfy the requirements of the CRC Program and the CRCV. This form seeks to collect information from Project Leaders for the CRCV's 05/06 Annual Report.

Project Title	**Finalising and Validation a Diagnostic Tool for the Early Detection of Phylloxera**
Period Report Covers	**1st February 2005 to 31st March 2006**
CRCV Project Code Number	**2.2.3a**
Organisation	**Cooperative Research Centre for Viticulture**

Intellectual Property

The information arising from this project includes the phylloxera DNA sequences and primers, crucial in the development of a specific-specific test for phylloxera. Materials Transfer Agreements are in place between CRCV and SARDI to freely exchange this information for the further development of this test.

Publications and Technical Communications (2005/2006 only)

K.S. Herbert, A.A. Hoffmann and K.S. Powell (2005).
Population Dynamics of Grape Phylloxera in ungrafted vineyards of south-eastern Australia. Journal of Economic Entomology. Accepted for publication January 2006.

K. Herbert, A. Hoffmann, K. Powell and K. Ophel- Keller (2006).
The Development of a PCR method for the rapid identification of phylloxera in vineyard soil. Acta Horticulturae (in prep.).

K. Herbert, A. Hoffmann, A. Corrie, K. Powell and P. Umina (2006).
The use of DNA for pest management: the interaction between phylloxera clonal lineages and vine types. Acta Horticulturae (in prep.).

Research Conference, Seminars, Workshop Presentations and Technical Reports (non-refereed)
(2005/2006 only)

Addresses:

K. Herbert, A. Hoffmann K. Powell and K. Ophel- Keller
The Development of a PCR method for the rapid identification of phylloxera in vineyard soil. In: Abstracts of the Third International Phylloxera Symposium, October 7th, 2005. Fremantle, Perth, Australia. DPI (Eds: Trethowan, C. and K.S. Powell).

K. Herbert, A. Hoffmann and K. Powell
Population Dynamics of Grape Phylloxera in ungrafted vineyards of south-eastern Australia. In: Abstracts of the Seventh International Symposium on Aphids October 2-7, 2005. Fremantle, Perth, Australia. CSIRO. (Eds. Edwards, O.).

Posters
None

Technical Reports and Industry Journal articles (non-refereed)
None

Leaflets/Brochures/Technical Guides
None

Other Communications
Seminar of research progress given at the Bi-Annual CESAR TalkFest.
June, 2005

Public Presentations – 2005/2006 only

Presentations None		
Event	**Date**	**Number of Attendees**

Public Relations and Communication 2005/2006 only
None

Industry Contribution to the Project ***2005/2006 only***	
Vineyard area (ha) made available by collaborating growers	5 hectares
Vineyard staff (no. by category) made available by collaborating growers: (Insert grower name)	
Manager (s)	N/A
Technical staff	N/A
Field staff	N/A
Materials (by item):	N/A
Vineyard chemicals	N/A
Vineyard products	N/A
Laboratory apparatus	N/A
Consumables	N/A
Cash support ($).	N/A
Personal in-kind participation associated with review or advisory activities (persons x hr p.a.)	N/A

Contributions of Project Staff to Viticultural Education and Training 2005/2006 only
(Show time (hours) spent by project staff in an average working week in education and training programs.

Name	Undergraduate Lectures	Postgraduate Supervision	Industry Training*

*include seminars, workshops, demonstrations; show annual figure if more applicable.

Grants and Awards – 2005/2006 only **(List awards, honours or other special recognition to the project and/or team members)**	
Award	**Recipient(s)**
Finalist in the DPI Science Awards 2006	Collaborate Phylloxera Research Team

Project Team Meetings – 2005/2006 only
(Complete details under headings shown)

Date	**Location**	**Purpose**	**Participants**
01/03/05		Project Meeting & Update	K. Herbert & A. Hoffmann
01/05/05		Project Meeting & Update	K. Herbert & A. Hoffmann
01/07/05		Project Meeting & Update	K. Herbert & A. Hoffmann
01/09/05		Project Meeting & Update	K. Herbert & A. Hoffmann
01/11/05		Project Meeting & Update	K. Herbert & A. Hoffmann
01/12/05		Project Meeting & Update	K. Herbert & A. Hoffmann
01/02/06		Project Meeting & Update	K. Herbert & A. Hoffmann
01/03/06		Project Meeting & Update	K. Herbert & A. Hoffmann