Assessing mitochondrial DNA (CO1) barcodes for measuring diversity of *Schizaphis* spp. and abundance of *Aploneura lentisci*

A thesis submitted in partial fulfilment
of the requirements for the degree

of

Masters of Science in Biological Sciences

at

The University of Waikato

by

Colleen Podmore

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2015

Abstract

We examined the diversity of mitochondrial DNA (CO1) sequences in the New Zealand native aphids with a particular focus on the genus *Schizaphis* (syn. *Euschizaphis*). Previously, the genus was thought to consist of two species, each host-specific to endemic New Zealand plants (*Aciphylla* and *Dracophyllum*). These unnamed native taxa are reasonably common but have either a narrow distribution (*Aciphylla*-host) or low local abundances (*Dracophyllum*-host). There is also some uncertainty over the number of *Schizaphis* species present on the various host plants. Specimens from both host plants were collected from ten sites in the North (n=3) and South (n=8) Islands. A total of 29 new COI sequences were obtained. A further 189 sequences were obtained from the Barcode of Life Datasystems (n=187) and GenBank (n=2). Thirteen of the 15 known native aphids were analysed by Maximum Likelihood and their taxonomic classification confirmed, the majority belonging to the Aphidinae, but also representatives in other sub-families. Maximum Likelihood analyses and pairwise genetic distances confirmed taxonomic groupings of the Aphididae sub-family Aphidinae and within the Aphidinae the monophyly of the sub-tribe Rhopalosiphina which contains two genera: *Rhopalosiphum* spp.and *Schizaphis* spp. Two distinct, well supported clades within *Schizaphis* were clearly delineated according to the host plant. However, we also found two distinct clusters within the *Aciphylla*-host South Island individuals and four within the *Dracophyllum*-host individuals (North & South Islands), suggesting six potentially cryptic species. Based on these data, we suggest that the CO1 gene region is effective for identifying aphids and could assist in the on-going discovery and protection of these taxa in New Zealand. New information regarding the diversity of New Zealand native *Schizaphis* aphids is using COI gene sequences to reveal additional diversity. International databases such as the Barcode of Life Datasystems and GenBank will be particularly helpful in examining global relationships within the New Zealand native aphid taxon.

Root Aphids (*Aploneura lentisci*) are an introduced taxon, which live exclusively on the roots of Poaceae and can cause detrimental effects to New Zealand pastures, reducing the forage available for farmed livestock. In order to assess abundances of *A. lentisci* in soil samples, specific primers were first developed to target and amplify the mitochondrial gene region cytochrome *c* oxidase subunit 1 (CO1) gene region. Three DNA extraction protocols, the PowerSoil® DNA Isolation Kit , Extract-N-Amp™Tissue PCR and a phenol chloroform DNA extraction protocol were then assessed and real time quantitative polymerase chain reaction (qPCR) was used to measure the amount of amplicon produced, which was compared to a standard curve of known aphid numbers. The developed primers successfully amplified a 317 nucleotide fragment of the COI gene region and had limited cross reactivity with other aphid taxa tested. Of the three extraction methods, the Extract-N-Amp Tissue PCR Kit was the most effective. Adult root aphids were selected as representatives of infestations. DNA concentrations extracted using the Extract-N-Amp Kit were linear over the entire range of aphid numbers (R^2=0.98). Further dilutions were carried out: 10-fold, 100-fold and 1000-fold to test linearity and when combined produced a standard curve with a regression of 0.93 over a cycle threshold range of 18-33 cycles. DNA extractions from mixtures of 60 aphids in soil produced amplifiable aphid DNA. We conclude that this method will successfully measure root aphid abundances from soil samples using mitochondrial CO1 gene sequences and qPCR.

Acknowledgements

As I have examined the relationships of the most interesting and diverse of all insects: the aphids, my own relationships have come under scrutiny. They have not been found wanting and instead have withstood the test of time and pressure for which I am grateful.

This project would not have been possible without the support from my Supervisor, Ian Hogg. Since the first lecture I attended on molecular ecology and DNA Barcoding in particular, I was captivated. Only time will tell how this area of science will pan out, but so far so good! Thanks Ian for your Supervision of this project, words of encouragement and direction.

Simon Bulman collected the majority of the aphids. I'm really grateful for the time you took to travel to remote regions in the South Island and hunt out *Schizaphis*. Simon your enthusiasm is infectious. Jonathon Banks introduced me to qPCR and also edited this chapter, while Maria Tourna and Nigel Bell allowed access to their lab at AgResearch for carrying out qPCR experiments. Thanks also to Alison Popay for allowing me time to work on this project.

Thanks also to all my friends, colleagues and fellow students. So much advice and direction in how to do things, I'm really grateful. Cheryl Ward for assisting with Word formatting and EndNote. Gloria and Vicky in the office – always ready and willing to help. Stacey for instilling law and order in the PBRL laboratory and making us all better analysts. Chrissen Gemmil for helping with final editing, analysis of results and managing time constraints.

Special thanks to my family, husband Keith, and daughter Sandie. You guys kept me grounded and moving forward. Thanks for your patience and thanks Keith for coming on my aphid collecting expeditions and all your support!

Last but not least I thank my God who has sustained me and been my rock. I love your creatures – great and small!

Table of Contents

List of Figures

Chapter 2

Chapter 3

List of Tables

List of Appendices

Chapter 2

Chapter 3

Chapter One

Thesis Introduction

The identification and classification of animal life has traditionally been through the examination of morphological features. Experienced taxonomists have discovered, described and catalogued specimens of many diverse organisms which reside in museums around the world. While providing an invaluable natural history library, physical access by a range of researchers is often limited. In addition, there are now fewer experienced taxonomists available to do the work of identification. The use of molecular techniques focusing on specific gene regions has been suggested as an additional approach. Databases of DNA and RNA nucleotide sequences and protein related sequences such as GenBank (Benson *et al.*, 2012), have been established and now provide accessibility to molecular material through internet accessed websites, for example www.ncbi.nlm.nih.gov. In addition, search engines using the BLAST algorithm (Altschul *et al.*, 1990) make it possible to compare a fragment of DNA to others in the database and find similarities and differences.

More recently Hebert (2003a) and other researchers have established that the mitochondrial gene cytochrome c oxidase subunit 1 (CO1) can serve as a global bio-identification system for animals. Initially they showed that CO1 could accurately identify to phylum or order levels and also they correctly identified 200 species in the complex Lepidoptera order. Further work established that sequence divergences at CO1 regularly enable the discrimination of closely allied species in all animal phyla except the Cnidaria (Hebert *et al.*, 2003b). The 658 nucleotide region of the CO1 gene became known as the ‘barcode’ gene because it was sufficiently conserved within species to be reproducible, yet along this gene region there are sufficient differences to distinguish taxa (Savolainen *et al.*, 2005). Throughout the past decade these ‘barcodes of life’ have been amassed into a database or ‘library’ of barcodes, known by the acronym BOLD (Ratnasingham & Hebert, 2007), that can be freely accessed by anyone with an internet connection. In this way an unknown animal may be identified by comparison of extracted, amplified and sequenced DNA from the mitochondrial CO1 gene region of the

unknown animal to the same standardised sequence of an identified taxon held in the library. Identification may then be inferred depending on the percent similarity.

This thesis continues with two research chapters (Chapters 2 and 3) and ends with the general discussion (Chapter 4). In chapter 2 of this thesis, CO1 barcoding sequences are used to assess the diversity within and among populations of *Schizaphis* in New Zealand. A secondary goal was to determine genetic relationships among taxa and examine similarities with other New Zealand native and adventive aphids.
Also included in the chapter are CO1 sequences generated over the past 10 years by Plant & Food Researchers at Lincoln, New Zealand as well as those available on the global databases BOLD and GenBank. The research in Chapter 2 thus adds to the growing database of New Zealand aphid CO1 sequences that other researchers may access through internet based websites.

In Chapter 3 of this thesis, a quantitative PCR (qPCR) technique using the barcoding gene CO1 was investigated as a means of measuring root aphid abundance. This technique employed the same principles as end-point PCR but instead quantifies the number of copies of DNA as they are produced. An intercalating dye, Sybr, fluoresces when bound to double-stranded DNA, so as more DNA is produced, fluorescence increases. The original concentration of template can then be calculated from a standard curve constructed from known concentrations of DNA. The objects of this chapter were fourfold and that was to first investigate if primers could be developed to target root aphids, second what is the best method for extracting target DNA, third could a standard curve of aphid numbers versus cycle threshold (C_t) values be produced which is linear and finally would it be possible to detect and extract aphid DNA directly from soil and quantify them.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*(3), 403-410.

Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2012). GenBank. Nucleic Acids Research.

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003a). Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences, 270(1512), 313-321.

Hebert, P. D. N., Ratnasingham, S., & deWaard, J. R. (2003b). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society of London. Series B(Suppl), 270, S96-S99.

Ratnasingham, S., & Hebert, P. D. N. (2007). BOLD:The Barcode of Life Data System (www.barcodinglife.org). Molecular Ecology Notes, 7(3), 355-364.

Savolainen, V., Cowan, R. S., Vogler, A. P., Roderick, G. K., & Lane, R. (2005). Towards writing the encyclopaedia of life: an introduction to DNA Barcoding. Philosophical Transactions of the Royal Society B, 360, 1805-1811.

Chapter 2

Using mitochondrial DNA (CO1) sequences to assess diversity of New Zealand native aphids (Hemiptera: Aphididae)*

*To be published under the same title as: Podmore C, Hogg ID, Bulman, SR.

Introduction

Aphids are soft-bodied insects of between 1-2 mm in length that are parasitic on plants (Dixon, 1973) and there are an estimated 5000 species worldwide (Blackman & Eastop 2006). Most species are found in the temperate regions of the northern hemisphere (Blackman & Eastop, 2006), and of these, approximately 120 are also found in New Zealand (Teulon *et al.*, 2013). The majority are non-native and have been introduced in the past 200 years (Teulon *et al.*, 2002). Currently, it is considered that there are at least 15 species of native aphids in New Zealand, most belonging in the subfamily Aphidinae, two in the subfamily Neophyllaphidinae and one each in Taiwanaphidinae and Saltusaphidinae (Teulon*, et al.*, 2013).

New Zealand native aphids are relatively species-poor, compared to the adventive species. The latter also have more of an impact on New Zealand's horticulture and therefore attract more research attention. In addition, native species are often difficult to find, being found in small populations and being under pressure from natural enemies (ladybirds, lacewings, syrphids and spiders), from the loss of habitats, displacement by adventive aphids and by the impact of parasitoids, both naturally occurring and those introduced to control pest aphids. Having the ability to identify aphids without specialist training is an advantage in the study of their biology and any conservation efforts. COI sequences may be particularly helpful in this regard.

The genus *Schizaphis* (Aphidinae, Aphidini) was first described by Carl Börner in 1931 (Börner, 1931). Originally classified *as Rhopalosiphum* Koch, they were split into the new genus. The main difference between the genera being that the siphunculi, which are dorsal protrusions, are tapering in *Schizaphis* and media of the forewing is branched only once (Blackman & Eastop, 2006). Nevertheless, the two genera are very similar and taxonomically form the sub-tribe Rhopalosiphina which, along with the sub-tribe Aphidina, form part of the tribe Aphidini. The Aphidini and Macrosiphini are part of the sub-family Aphidinae, which is one of 25 sub-families (Aphididea, Hemiptera). According to Blackman (2006) there are

approximately 40 species of *Schizaphis* worldwide, with more than half being found in Europe and the remainder in the Middle East, Central Asia, East Asia, Africa and North America. There are at least two, known native *Schizaphis* species in New Zealand both of which are host-plant related. *Schizaphis* from the host plant *Aciphylla* spp*. (Apiaceae)* is described in Blackman & Eastop (2006) and the other on the host plant *Dracophyllum* spp*. (Ericaceae)* is undescribed in Blackman & Eastop (2006). A further species is also possible on *Dracophyllum* host shrubs as the siphunculi were noticed to be shorter on two of the populations previously collected (Teulon*, et al.*, 2013).

Mitochondrial cytochrome c oxidase subunit I (COI) DNA sequences (DNA barcodes) have been used to identify and to study species-level diversity in aphids worldwide (Foottit *et al.*, 2008; Foottit *et al.*, 2009; Coeur d'acier *et al.*, 2014). The diversity of native and non-native aphids in New Zealand were first examined using molecular methods by von Dohlen & Teulon, (2003) who used DNA sequences from mitochondrial tRNA leucine + cytochrome oxidase II and nuclear elongation factor-1α (EF1α) gene regions of the *Aphidini*, to reconstruct phylogenetic and biogeographic relationships. Kim & Lee (2008) used the same gene regions and their molecular data confirmed the taxonomic divisions (von Dohlen & Teulon 2003; Kim & Lee 2008,).

New Zealand aphids have also been previously sequenced at the CO1 gene region (Foottit unplubl. data) and are available on the Barcode of Life Datasystems (BOLD) database, under the project ‘Aphids of New Zealand’, 224 specimens representing 80 species are available. Another larger and closely related project represented 16 sub-families, and covered 134 genera and 335 species, and comprised 690 specimens examining sequence variation in the CO1 gene region and found that CO1 sequences are an effective tool for identification (Foottit*, et al.*, 2008).

Here, we refined this previous work with a focus on *Schizaphis* aphids found on *Aciphylla* and *Dracophyllum* host plants throughout New Zealand. These native aphid species were chosen for further investigation because they are widespread in the New Zealand montane environment (from the Central Plateau of the North Island south, and in targeted areas of the South Island). There are abundant habitats where it is likely that multiple aphid populations may be found and because these are in the sub-alpine region are not so affected by human activities. We tested the hypothesis that sequence variation within the CO1 gene can detect differences among populations of *Schizaphis* in New Zealand.

Methods and Materials

Sample collection

Schizaphis (Aphidinae, Aphidini) and other aphids were collected between October 2013 and January 2015 from sites throughout New Zealand (Table 1). Specimens were collected from *Dracophyllum* and *Aciphylla* host plants by shaking individual plants (*Dracophyllum*) *over* a white tray (approx 30cm x 23cm) or assessing individual plant leaves (*Aciphylla*). Specimens were confirmed as being aphids using a headband magnifier and then transferred to individually labelled vials with 100% ethanol. Specimens were kept cool in the field and stored at 4°C in the laboratory until required for molecular analyses. Complete collection details are provided in Appendix 2. Prior to DNA extraction, specimens were photographed and these are provided in Appendix 3. All collection data have been uploaded to the BOLD website under the project 'Soil Aphids of New Zealand' (NZAPH).

Genetic analyses

DNA was extracted from individual aphid legs or aphid bodies, using the Sigma Extract-N-Amp Tissue PCR Kit (Sigma Cat no. XNAT2-1KT) and following the manufacturer's instructions. The extraction solution was heated to 55°C for 10 mins. Following extraction, all solutions were stored at 4°C until needed for Polymerase chain reactions (PCR).

PCR's were carried out using an Eppendorf Mastercycler epgradient thermocycler. Negative and positive controls were run with samples: negative controls contained PCR grade milleQ water and positive controls contained known and previously amplified DNA to confirm that amplification had occurred. Reactions were carried out in 0.2mL microcentrifuge tubes in 20µl volumes containing 10µM each of the Folmer et al. (1994) primers HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') at a final concentration of 0.5µM, 6.2 µl PCR grade water, 9.4 µl of PCR master mix solution (i-Taq) (Intron Biotechnology Cat no. 25028) and BSA (Bovine serum albumin), to give a

final concentration of 0.2 mg/mL. The reaction mix was kept cool until 2 µl of the isolated DNA was added and the solution was briefly vortexed, spun down and immediately placed in the thermocycler for amplification.

Thermocycling conditions were as follows: initial denaturing at 94ºC for 5 minutes, followed by 36 cycles at 94ºC for 60 seconds, 52ºC for 90 seconds and 72 ºC for 60 seconds. Final elongation occurred at 72ºC for 5 minutes. PCR products were visualised by gel electrophoresis using a 1% agarose gel and 0.5x Tris-Borate-EDTA Buffer (TBE), Red Safe (Intron cat no. 21141) a commercially available nucleotide stain and a molecular ladder (Invitrogen Trackit, cat no. 10488-058) to verify the presence of a band at 600bp, indicating successful amplification before cleaning up the solution and sequencing.

PCR products were purified for sequencing by removing excess primer following the standard protocol for EXOSAP - Exonuclease I (EXO) and Shrimp Alkaline Phosphate (SAP) (Global Science & Tech Ltd. Cat no.GEHEE700 - 732 & 922). At the University of Waikato, DNA amplicons were sequenced in both directions using the same primers as the PCR (0.5mM concentration) on a capillary electrophoresis AB1 3130XL genetic analyser. At the Canadian Centre for DNA Barcoding, DNA was extracted using a Glass Fiber Plate protocol ((Ivanova *et al.*, 2006a; Ivanova *et al.*, 2006b), amplification using MLepF1/CLepFO1 and CLepFO1F/MEPTR1_t1 primers and the following thermocycling conditions: 96°C/120secs, then 30 cycles (96°C/30secs, 55°C/15secs, 60°C/4mins) followed by indefinite hold at 4°C (CCDB, 2014). PCR products were cleaned using a Sephadex® (Sigma-Aldrich G5080-500g) procedure before sequencing using the same primers as the PCR on an ABI 3730XL sequencer.

COI sequences were also obtained from four additional sources and included: 1) 73 unpublished aphid sequences from Plant & Food Research, Lincoln, New Zealand; 2) sequences from the ‘Aphids of New Zealand’ (RFNZ),

available on the Barcode of Life Datasystems (BOLD) website (www.boldsystems.org); 3). Sixteen *Rhopalosiphum* sequences, and two *Schizaphis* sequences from Foottit et al. (2008); and 4) two *Schizaphis* sequences from GenBank (Accession numbers AF220511.1, EU701901.1).

Data analyses

Forward and reverse sequences were assembled, primers removed and visually inspected using Geneious software version 6 (http://www.geneious.com, Kearse et al., 2012). The consensus sequence was attributed to genus and/or species levels using either the BOLD or GenBank BLASTn search engines. All newly generated sequences and trace files were deposited in the Barcode of Life Datasystems (BOLD) project "Soil Aphids of New Zealand" (NZAPH).

The complete dataset of newly and previously generated sequences (n= 232) was aligned using Muscle (Edgar, 2004) and trimmed to 407 nucleotides. Maximum Likelihood (ML) trees were produced using the General Time Reversible (GTR)+I+G model (Nei & Kumar, 2000). All analyses were carried out using MEGA v 6.06 software (Tamura *et al.*, 2013). Bootstrap support was calculated on ML trees using 1000 pseudoreplicates and relationship values of >95% were taken as statistically significant (Felsenstein, 1985).

Genetic distance analyses were also carried out separately on the *Schizaphis* sequences (Neighbour Joining pairwise distance analysis with the Tamura Nei model (Tamura & Nei, 1993) to analyse relationships within and between sites. Barcode Index Numbers (BIN), assigned by the BOLD database were used to cluster sequences 2% and less together as an indication of species-level divergences (Ratnasingham et al. 2013).

Table 1 *Schizaphis* approximate collection sites showing site name, New Zealand Province, latitude, longitude, elevation and host plant that specimens were collected from.(Map Fig. 1)

Site Code	Site Name	New Zealand Province	Lat.	Long.	Elevat-ion (m)	Host Plant
ND1	Pureora	Manawatu-Wanganui	-38.4957	175.5833	558	*Dracophyllum* sp.
ND2	Tukino	Manawatu-Wanganui	-39.3014	175.7419	1081	*Dracophyllum* sp
ND3	Egmont National Park	Taranaki	-39.2394	174.0355	1218	*Dracophyllum* sp
SD4	Lake Sylvester	Tasman	-41.1060	172.6349	1344	*Dracophyllum* sp
SD5	Cobb Valley	Tasman	-41.1313	172.6219	837	*Dracophyllum* sp
SD6	Arthur's Pass	Canterbury	-42.9096	171.5600	923	*Dracophyllum* sp
SD7	Mt. Lyford	Canterbury	-42.4662	173.1389	1232	*Dracophyllum* sp
SA8	St. James	Canterbury	-42.4415	172.7937	850	*Aciphylla* sp.
SA9	Korowai	Canterbury	-43.3396	171.6326	705	*Aciphylla* sp.
SA10	Porter's Pass	Canterbury	-43.2967	171.7420	939	*Aciphylla* sp.
SA11	Mt. Barrosa	Canterbury	-43.6380	171.2188	662	*Aciphylla* sp.

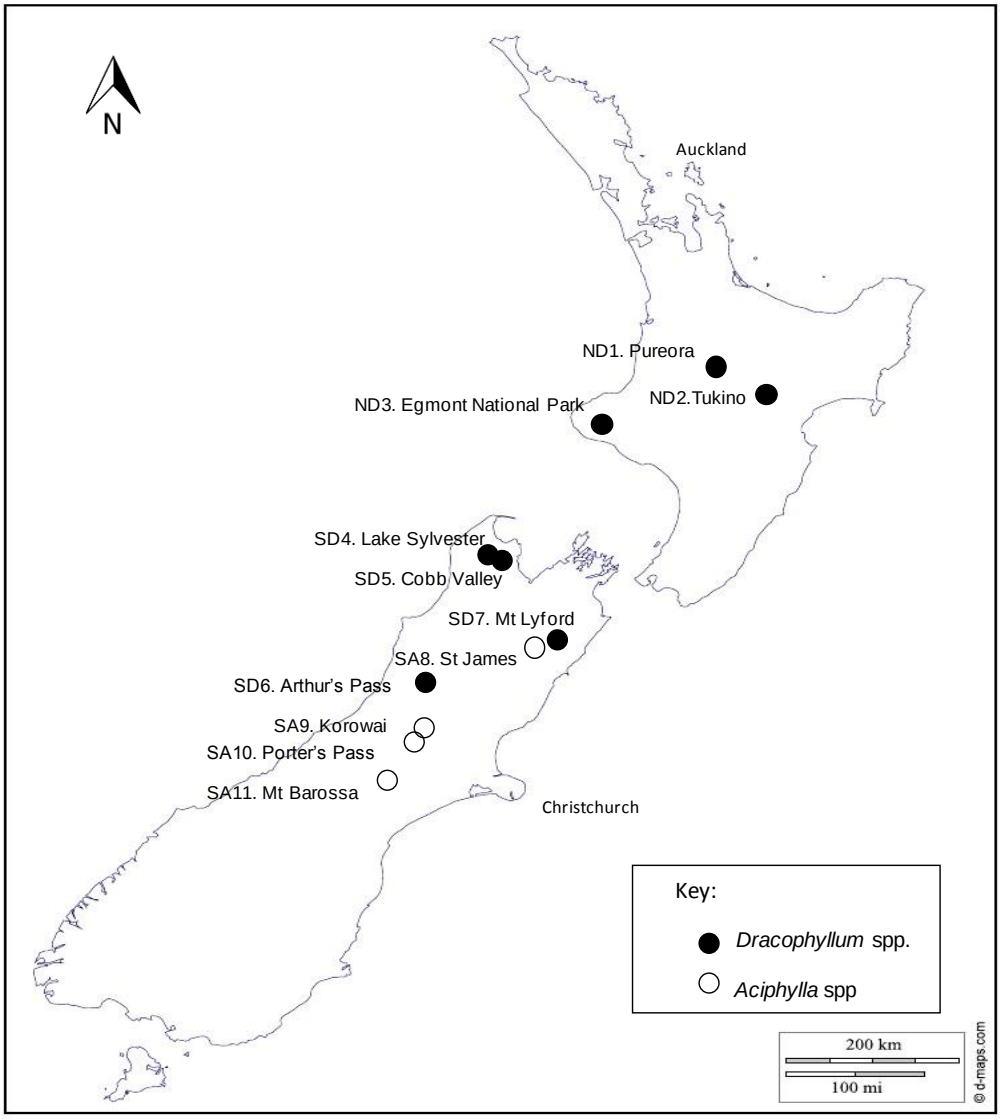


Figure 1 Map of New Zealand showing approximate *Schizaphis* collection sites. ND =North Island *Dracophyllum*. SD =South Island *Dracophyllum*. SA=South Island *Aciphylla*

Results

The Maximum Likelihood tree of the 232 CO1 sequences is shown in Figure 2. The tree with the highest log likelihood (-8641.04) is shown and the percentage of trees (bootstrap values) in which the associated taxa clustered, is shown next to the branches where these values are >75%. Nucleotide composition averaged over all taxa showed a strong A-T bias (A = 34.9%, T = 39.6%, C = 15.8%, G = 9.7

The *Aphidinae* CO1 sequences form a separate clade within the tree from the rest of the sub-families, supporting taxonomic divisions within the *Aphididae*, except for 5 species at Fig. 2E. The tree backbone is poorly supported but the terminal nodes are well supported. The *Aphidinae* (Fig. 2A) have been collapsed and are expanded in Fig. 3. Three New Zealand natives are found within small sub-families, *Thripsaphis foxtonensis (Saltusaphidinae)* (Fig. 2B), *Sensoriaphis nothofagi* (*Taiwanaphidinae*) is sister to *Phyllaphis fagi* (Fig. 2C) from the *Phyllaphidinae,* and *Neophyllaphis totarae (Neophyllaphidinae)* (Fig. 2D). The natives except for *S. nothofagi* are distinct and separate from the other sub-families also supporting traditional taxonomic groupings. Also included within this area of the tree are 2 species sequences belonging to members of the *Aphidina* (Fig. 2E)*: Aphis nerii,* and *Aphis gossypi.*

The *Macrosiphini* (Figure 3) are the largest tribe within the *Aphidinae* comprising 227 genera and 1813 species (89% & 73%). CO1 aphid sequences from the tribe make up 37% of the genera in this dataset. In this study, there are indicated at least four paraphyletic genera; *Brachycaudus* spp, (Fig. 3A), *Dysaphis* spp. (Fig.3B), *Myzus* spp. (Fig. 3C)*,* and *Acrythosiphon* spp (Fig. 3D*)* indicating unresolved cryptic species. Included for the first time is a sequence not found on the BOLD website, *Rhopalosiphoninus staphyleae* (Fig. 3E) Overall the *Macrosiphini* cluster into one group with generally good bootstrap support (>95%) at the terminal nodes. The aphid *Megoura stufkensi* (Fig. 3F) is the only putative native aphid within this group but there are questions around it being labelled as a

native, nevertheless it is unique in the world and has been found in only one location in New Zealand (Teulon 2013). The *Rhopalosiphina* sub-tribe form a distinct clade within the Aphidini, except for *Rhopalosiphum nymphaeae. Aphis craccivora (Aphidina) is also outside the main clade* (Fig 3). *Tuberculatus annulatus* of the sub-family Lachninae is also grouped within the Aphidini.

This study examined 13 of the approximately 15 New Zealand native aphids (Table 6). The majority of native aphids were part of the *Aphidina* sub-tribe (Figure 4). The native *Paradoxaphis aristoteliae* was well supported (97%) as being distinct from *Paradoxaphis plagianthi (96%)* (Fig. 4A)*. Aphis waiwera* (Fig. 4B) is a newly described native aphid. *Aphis hederae* and *A. fabae* (Fig. 4C) are very closely related morphologically and are likely to be *A. hederae* (S. Bulman pers comm).

Schizaphis spp. aphids were clustered together and distinct from the *Rhopalosiphum* spp. genera (Figure 5). The genera were collapsed here and expanded in Figure 6. *Schizaphis graminum* is shown as sister to *Rhopalosiphum maidis* (Fig. 5B), and is distinct from the *Schizaphis* clade (Fig. 5A). Genetic differences between some of these taxonomic groups are summarised in Table 2 *Schizaphis* are 14.3% different from sub-families outside Aphidinae

Schizaphis aphids formed a distinct clade (Figure 6), within the Rhopalosiphina sub-tribe, Terminal nodes are well-supported and most are greater than 95% bootstrap value, although the overall tree structure is weakly supported. The tree divides the New Zealand *Schizaphis* aphid sequences into distinct groups, which reflects both sites and host plants. Cobb Valley and Lake Sylvester locations in the South island (Figure 1) are next door to each other geographically, and this is reflected in the minimal differences between these sequences (SD4, SD5). Also closely related genetically and by physical location to the Cobb Valley and Lake Sylvester

aphids are the sequences from aphids collected at Arthur's Pass (SD6). The Egmont aphids in the North Island form a distinctly separate clade (ND3) as do the South Island, Mt Lyford taxa (SD7). The Tukino and Pureora aphids in the North island are sister taxa (ND1, ND2). The last two groups on the tree are *Aciphylla*-feeding aphids and interestingly have formed two distinct clades in the tree (SA 8-11). The second clade contains ten sequences which are identical - from 4 sites and 3 laboratories. Finally, all the New Zealand native *Schizaphis* are distinctly different to the three known *Schizaphis* species, *S. rotundiventris, S. scirpicola, S. graminum*, according to the CO1 barcode groupings, confirming the uniqueness of these New Zealand natives. The complete distance matrix may be viewed in Appendix 1.

Undescribed species were assigned a Barcode Index Number (BIN) when sequences were uploaded to the BOLD website. These are generated by the Refined Single Linkage (RESL) algorithm using uncorrected pair-wise (p-) distances. The *Schizaphis* aphid sequences were clustered to four BINs and the results are summarised in Table 4 Specifically, BIN AAH0489 corresponded to the Cobb Valley, Lake Sylvester and Arthur's Pass *Dracophyllum*-feeding aphids and BIN ACT4073 to the Mt. Lyford aphids. The *Aciphylla*-feeding aphids are assigned to BIN ACT4073. In the North Island Dracophyllum-feeding aphids at Egmont National park correspond to BIN ACT2294. The Tukino/Pureora sites are yet to be assigned. Analysis of pairwise distances within the *Schizaphis* aphids indicate some distinct differences and some similarities within *Schizaphis* site locations (Table 3). The distance matrix shows the *p*-distance value in the lower left quandrant and the Standard Error (SE) is in the upper right. The North Island Tukino and Pureora aphids are on average 8.9% divergent from all other sites. Egmont National Park aphids, the other North Island group, were distinctly different from the Tukino/Pureora aphids with a *p*-distance of 9%. However, they were only 2.3% and 2.5% different from the Arthur's Pass and Tasman aphids respectively. Furthermore, the Egmont National Park aphids were 4.8% distant from the Mt Lyford aphids in the South Island and on average 9.5%

distant from the Canterbury aphids. The Arthur's Pass and Cobb Valley/Lake Sylvester aphids differed by 1.3% and the Mt Lyford aphids were 8.9% different to the Tukino/Pureora aphids and on average 4.6% different from the Arthur’s Pass, Cobb valley/Lake Sylvester and Egmont aphids.

The South Island *Schizaphis* aphids collected from *Aciphylla* hosts clustered into two clades with a divergence of 6.6% and on average 12.4% divergence from all other aphids collected from *Dracophyllum* hosts.

Table 2 Distance matrix between members of *Aphidinae* and the other sub-families combined (rest) - from Neighbour Joining method using the Tamura Nei model showing *p*-distances in the lower left quadrant and Standard error (SE) in the upper right quadrant.

		1	2	3	4	5
1	Rhopalosiphum		0.012	0.013	0.011	0.013
2	Rest	0.124		0.012	0.014	0.010
3	Aphidina	0.112	0.131		0.014	0.012
4	Schizaphis	0.088	0.143	0.123		0.015
5	Macrosiphini	0.111	0.122	0.110	0.131	

Table 3 Distance matrix of *Schizaphis* site locations (*p*-distances) from Neighbour-Joining analysis using the Tamura-Nei model. Mitochondrial CO1 sequences have been grouped together according to the Maximum Likelihood tree clades (Fig.6). Genetic distance in the lower left quandrant. Upper right quadrant are Standard Error (SE) values.

Location			**Tukino/Pureora**	**Arthur's Pass**	**Cobb V/Lk Syl.**	**Egmont Nat. Park**	**Mt Lyford**	**Canterbury**	**Canterbury**
			1	2	3	4	5	6	7
ND1/ND2	**Tukino/Pureora**	1		0.016	0.015	0.016	0.015	0.015	0.017
SD6	**Arthur's Pass**	2	0.091		0.004	0.007	0.011	0.016	0.016
SD4/SD5	**Cobb Valley/Lake Sylvester**	3	0.087	0.013		0.007	0.010	0.015	0.016
ND3	**Egmont Nat. Park**	4	0.090	0.023	0.025		0.010	0.016	0.017
SD7	**Mt Lyford**	5	0.089	0.049	0.041	0.048		0.015	0.018
SA8-11	**Canterbury**	6	0.079	0.085	0.082	0.089	0.086		0.013
SA10-11	**Canterbury**	7	0.100	0.099	0.095	0.101	0.110	0.066	

Table 4 Barcode Index Numbers -BIN's, assigned to *Schizaphis* spp. sequences according to uncorrected p-distances.

Dracophyllum – host plant aphids

Site Code	Site Name	BOLD ID	BIN	Sequenced by:
ND1	Tukino	NZAPH034	Not assigned	University of Waikato
ND2	Pureora	NZAPH128 H	Not assigned	Plant & Food Nelson
ND3	Egmont Nat. Park	NZAPH058	ACT2294	University of Waikato
	Egmont Nat. Park	NZAPH059	ACT2294	University of Waikato
	Egmont Nat. Park	NZAPH060	ACT2294	University of Waikato
	Egmont Nat. Park	NZAPH062	ACT2294	University of Waikato
	Egmont Nat. Park	NZAPH061	ACT2294	University of Waikato
SD4	Lake Sylvester	NZAPH013	AAH0489	University of Waikato
	Lake Sylvester	NZAPH044	AAH0489	CCDB
	Lake Sylvester	NZAPH045	AAH0489	CCDB
SD5	Cobb Valley	NZAPH011	AAH0489	University of Waikato
	Cobb Valley	NZAPH012	AAH0489	University of Waikato
	Cobb Valley	NZAPH041	AAH0489	CCDB
	Cobb Valley	NZAPH042	AAH0489	CCDB
	Cobb Valley	NZAPH043	AAH0489	CCDB
	Cobb Valley	RFBAD386-08	AAH0489	CCDB
SD6	Arthur's Pass	NZAPH051	AAH0489	University of Waikato
	Arthur's Pass	RDBA480-06	AAH0489	CCDB
SD7	Mt Lyford	NZAPH054	ACT4073	University of Waikato
	Mt Lyford	NZAPH055	ACT4073	University of Waikato
	Mt Lyford	NZAPH056	ACT4073	University of Waikato

Aciphylla – host plant aphids

No.	Site Name	BOLD ID	BIN	Sequenced by:
SA8	St James	NZAPH50	AAH0490	University of Waikato
SA9	Korowai	NZAPH015	AAH0490	CCDB
	Korowai	NZAPH017	AAH0490	CCDB
SA10	Porter's Pass	NZAPH008	AAH0490	University of Waikato
	Porter's Pass	NZAPH037	AAH0490	CCDB
	Porter's Pass	RFBAD391-08	AAH0490	CCDB
	Porter's Pass	NZAPH129 H	Not assigned	Plant & Food Nelson
	Porter's Pass	NZAPH130 H	Not assigned	Plant & Food Nelson
SA11	Mt Barossa	NZAPH018	AAH0490	CCDB
	Mt Barossa	NZAPH019	Not assigned	CCDB
	Mt Barossa	NZAPH022	AAH0490	CCDB
	Mt Barossa	NZAPH023	AAH0490	CCDB
	Mt Barossa	NZAPH024	AAH0490	CCDB

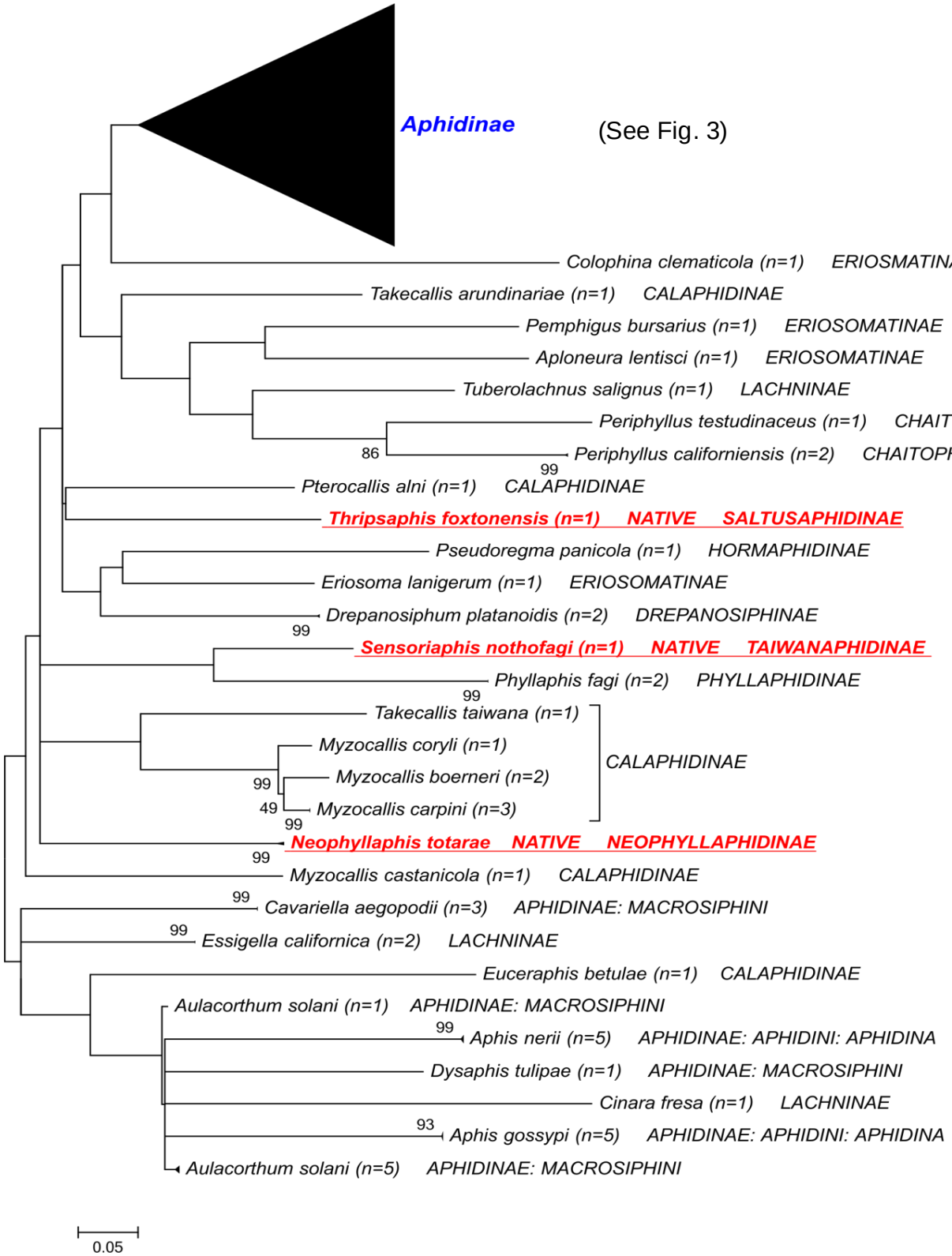


Figure 2 Maximum Likelihood tree of all aphid sequences. Aphidinae are collapsed and expanded in Fig.3. The GTR+G+I model was used to infer relationships based on mitochondrial CO1 gene sequences. Native aphids are underlined.

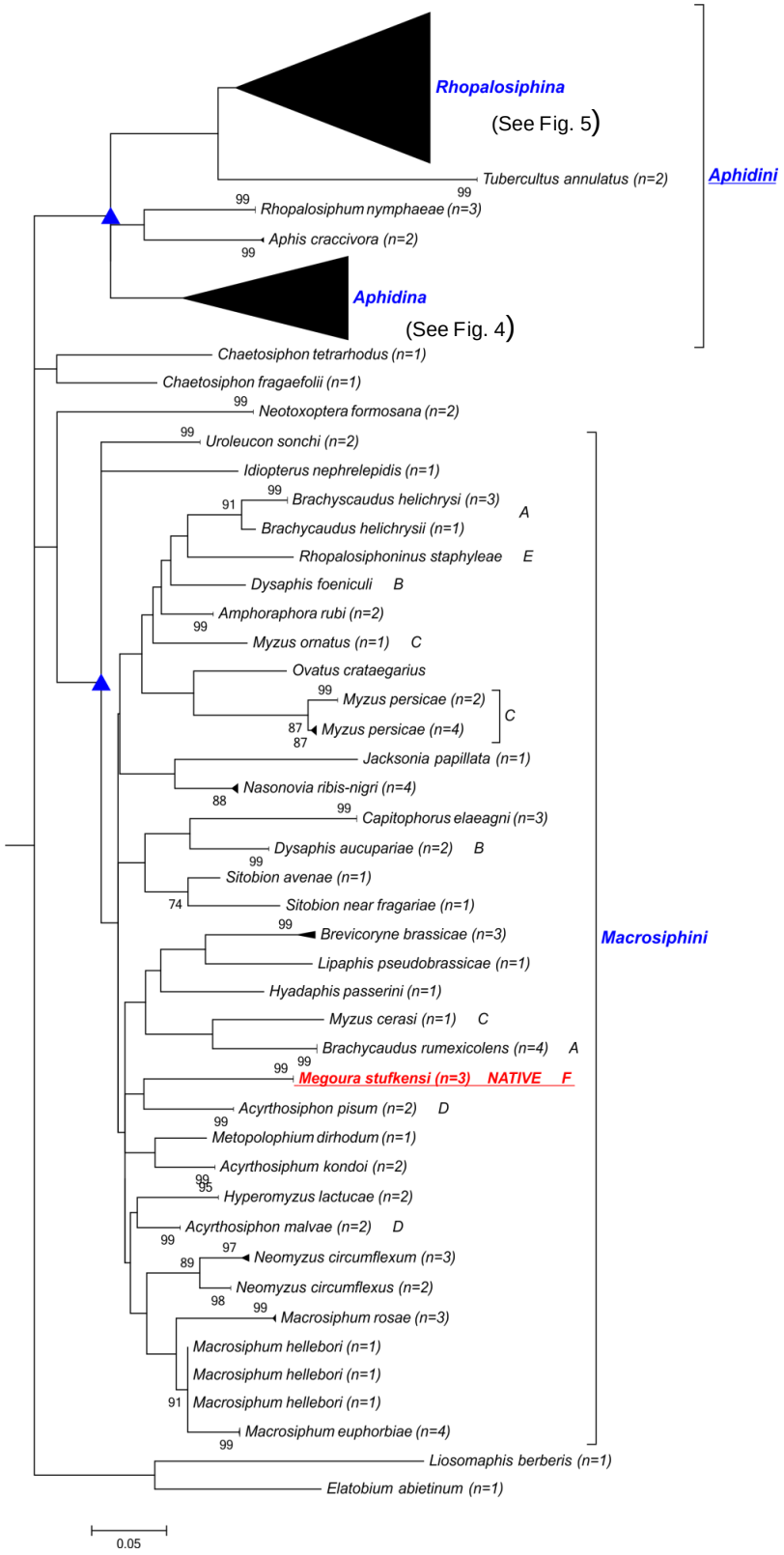


Figure 3 Maximum Likelihood tree for Aphidinae. Rhopalosiphina are expanded in Fig. 5. Aphidina are expanded in Fig. 4. The GTR+G+I model was used to infer relationships based on mitochondrial CO1 gene sequences. Native aphids are underlined.

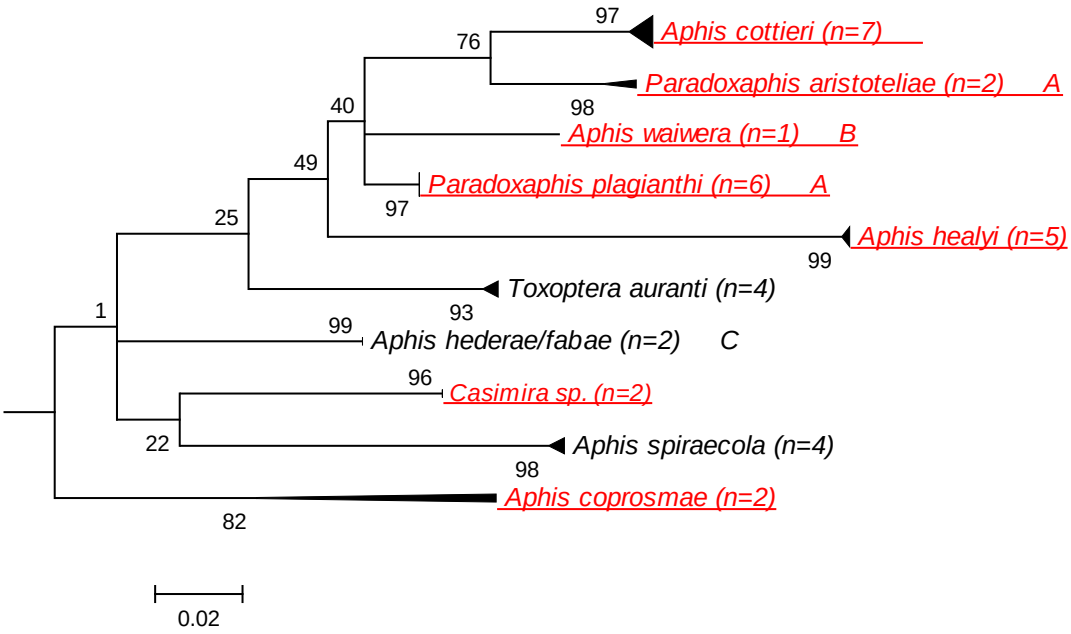


Figure 4 Maximum Likelihood tree for Aphidina showing the majority of natives belong to this tribe. The GTR+I+G model was used to infer relationships based on mitochondrial CO1 gene sequences. Native aphids are underlined.

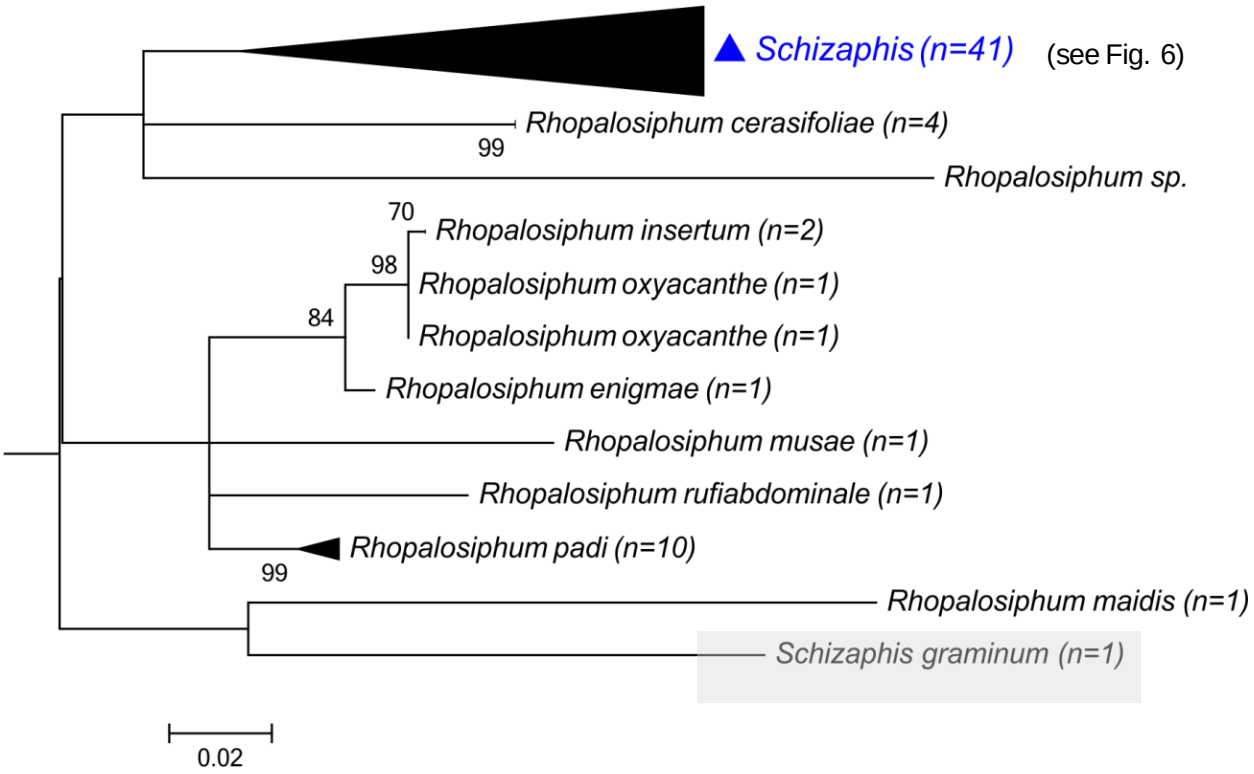


Figure 5 Maximum Likelihood tree for Rhopalosiphina. *Schizaphis* node is expanded in Figure 6. GTR +G+I model was used to infer relationships based on mitochondrial CO1 gene sequences.

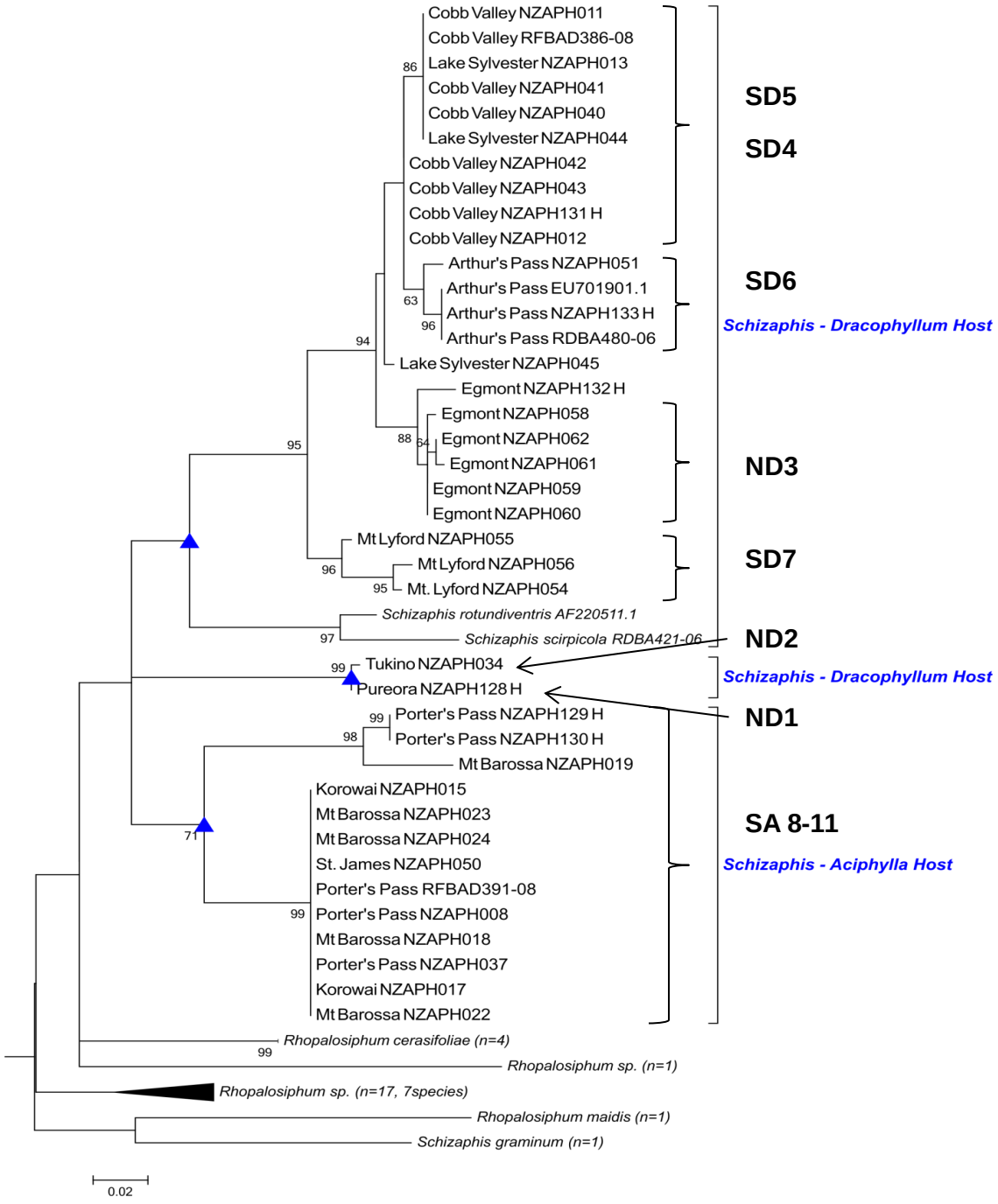


Figure 6 Maximum Likelihood tree for *Schizaphis* showing relationships based on the GTR+G+I model, using the mitochondrial CO1 gene sequences.

Discussion

Native aphids in New Zealand are found across four Aphididae sub-families. The majority are in the Aphidinae, but four other sub-families are also represented by New Zealand natives. Of the 15 known New Zealand natives, nine have been described while a further six await morphological description. In the interim, mitochondrial DNA sequences (DNA barcodes) collected as part of this study can provide an accurate method for documenting their occurrence and distribution.

The native *Schizaphis* spp. were collected from 11 sites throughout New Zealand. In the North Island, sites were revisited where *Schizaphis* had been previously collected. No aphids were found at Pureora National Park (ND1), just north of the Central Plateau, but a sequence had been generated from a previous collection. A single aphid was collected from the Central Plateau - Tukino site (ND2), east of Tongariro National Park. This area of New Zealand is dominated by the shrub *Dracophyllum subulatum* (Smale 1990), and is likely to be the host plant for the *Schizaphis* aphid sequences that have been included in this study.

D. subulatum is also common in the lower North Island and could yield more *Schizaphis* from these sites. Further to the west of the North Island at Egmont National Park (ND3), *Schizaphis* were collected at one site only and also included is a sequence from a previous collection. The main species of *Dracophyllum* at Egmont National Park is *Dracophyllum filifolium* (Clarkson, 1986), and this is likely to be the species that the aphids were collected from. In the South Island, *Dracophyllum longifolium* is distributed throughout and as far south as the sub-Antarctic Campbell Island (Allan, 1961), and is likely to be the host plant of the aphids collected from the South Island sites (SD4, 5, 6 & 7). Within the *Dracophyllum*-host, *Schizaphis*, there appear to be four cryptic species that are geographically separated. However, it is also possible that these cryptic species are separate species each found on different host species of *Dracophyllum*.

Previous studies in the *Aphididae* using the CO1 gene region have indicated that within-species variation is low (<0.2%), while sequence divergence among congeneric taxa averaged 7.25% (Foottit 2008). Within the *Dracophyllum*-host *Schizaphis,* genetic divergence ranges from 1.3% to 11.0%. Closely related are the Cobb Valley (SD5), Lake Sylvester (SD6) and Arthur's Pass (SD7) aphids (1.3%), suggesting the same species is present at these three sites. These sequences were clustered to a single Barcode Index Number (BIN), also suggesting that they are the same species. This group in the north-west corner of the South Island were genetically similar to those found in the North Island at Site ND3 (2.3% & 2.5% divergence). However, these individuals were assigned to a separate BIN suggesting the possibility of being separate species. The other North island group (ND1 & 2) is separated from all the other *Schizaphis* by an average *p*-distance of 8.9%. However these sequences have not been assigned to a BIN as the sequences were < 500 nucleotides long. Mt Lyford (SD7) is also different from the other South Island aphids by 4.6% and was also assigned a BIN separate from the other *Schizaphis.* Overall the *Aciphylla*-host aphids differ from the *Dracophyllum*-host aphids by 8.6% and the assignment to at least four BIN's also confirms this grouping within *Schizaphis.*

Within the *Aciphylla*-host, *Schizaphis,* there appear to be two cryptic species (6.6% sequence divergence). The majority of *Aciphylla*-host aphids were found within one cluster with bootstrap support of 100%. In addition, these sequences (10 sequences from 3 sites) were generated by three different laboratories based on at least two separate collections all of which have been assigned a single BIN. Three other sequences from the *Aciphylla*-host aphids formed a separate clade (SA10 &11) to the majority group (SA8, 9, 10 & 11) with 99% bootstrap support and 6.6% sequence divergence from the *Dracophyllum*-host individuals.

The complete COI sequence dataset covered a wide range of aphid sub-families. Of the 25 sub-families world-wide, 11 are represented here. Including a previous study (Foottit *et al.*, 2008) a total of 18 out of 26 sub-families have been sequenced at the COI gene locus. The dataset is listed in Appendix 2.

In order to test the genetic relationships between known *Schizaphis* species and the New Zealand natives, sequences from three other *Schizaphis* species were also included: 1) the common 'greenbug' *Schizaphis graminum*; 2)*, Schizaphis scirpicola*; and 3) *Schizaphis rotundiventris*. Genetic results using the CO1 marker indicate the New Zealand *Schizaphis* is a separate species to others in this genus, some of which are not currently found in New Zealand and also are clearly delineated from the genus *Rhoplaosiphum*. Sequences from sixteen extra *Rhopalosiphum* species, some of which are not currently found in New Zealand are also included to test the sub-tribal relationships.

This study has confirmed that DNA barcoding can detect species level differences within *Schizaphis*. In addition to the two known host-related *Schizaphis* species (*Dracophyllum*-host and *Aciphylla*-host), the CO1 sequences indicated that for the *Dracophyllum*-host aphids*,* there were possibly four cryptic species, two in the North Island and two in the South Island. Within the *Aciphylla*-host plants, there were possibly a further two cryptic species. This study has also contributed to the growing database of mitochondrial CO1 barcode sequences world-wide, which will aid in identification and enable further studies to be undertaken to examine Aphididae relationships and diversity both in New Zealand and globally.

Table 5 Sub-families within the Aphididae showing numbers of genera/species of those represented in two CO1 barcode sequence studies.

Sub-family[1]	genera/species in this study	genera/species in Foottit (2008)[2]	genera/species world-wide
Anoeciinae	No data	1/1	1/20
Aiceoniinae	No data	No data	1/14
Aphidinae	48/68	68/218	256/2483
Calaphidinae	4/8	18/22	62/358
Chaitophorinae	1/1	2/9	11/163
Drepanosiphinae	1/1	2/4	5/40
Eriosomatinae	4/4	16/35	48/301
Greenideinae	No data	1/2	16/150
Hormaphidinae	1/1	7/7	44/181
Israelaphidinae	No data	No data	1/4
Lachninae	5/5	8/18	19/346
Lizeriinae	No data	1/1	3/24
Macropodaphidinae	No data	No data	1/10
Mindarinae	No data	1/2	1/5
Neophyllaphidinae	1/1	1/1	1/15
Parachaitophorinae	No data	No data	2/2
Parastheniinae	No data	No data	No data
Phloeomyzinae	No data	No data	1/1-3
Phyllaphidinae	1/1	2/3	4/15
Pterastheniinae	No data	No data	2/4
Pterocommatinae	No data	No data	No data
Saltusaphidinae	1/1	3/3	12/55
Spicaphidinae	No data	No data	2/13
Taiwanaphidinae	1/1	No data	2/13
Tamaliinae	No data	1/4	1/4-5
Thelaxinae	No data	No data	4/18

[1] Classification follows that used by Remaudière, G., & Remaudière, M. (1997), with the revision of family names proposed by Nieto Nafría (1998).

[2] Barcoding the Aphididae (Foottit 2008)

Table 6 Summary of New Zealand native aphid sub-families, tribes, sub-tribes and status of species description or taxonomic authority

Genus/species	Authority:
Aphidinae: Aphidini: Rhopalosiphina	
Schizaphis (ex	undescribed
Schizaphis (ex	undescribed
Aphidinae, Aphidini, Aphidina	
Aphis ex*Veronica (* syn	undescribed
Aphis ex Clematis	undescribed
Aphis coprosmae	Laing ex Tillyard 1926
Casimira sp.	undescribed
Aphis healyi	Cottier 1953
Paradoxaphis plagianthi	Eastop 2001
Paradoxaphis	Sunde 1987
Aphis cottieri	Carver 2000
Aphis waiwera (new*)*	undescribed
Aphidinae, Macrosiphini	
Megoura stufkensi	Eastop 2011
Saltusaphidinae	
Thripsaphis foxtonensis	Cottier 1953
Taiwanaphidinae	
Sensoriaphis nothofagi	Cottier 1953
Neophyllaphidinae	
Neophyllaphis totarae	Cottier 1953

Acknowledgements

We would like to thank Simon Bulman from Plant & Food Research, Lincoln New Zealand for collecting the *Schizaphis* aphids and review of manuscripts. Thanks to the sequencing facility at the University of Waikato. Aphids were collected under DOC permit AK-32815-FLO

References

Allan, H. H. (1961). Flora of New Zealand. Vol. I. Indigenous Tracheophyta: Psilopsida, Lycopsida, Filicopsida, Gymnospermae, Dicotyledones. . Wellington: Government Printer.

Blackman, R. L., & Eastop, V. F. (2006). Aphids on the World's Herbaceous Plants and Shrubs. (2 vols). Chichester: Wiley.

Börner, C. (1931). Mitteilungen über Blattäuse (Communications on Aphids). Anzeiger für Schädlingskunde (Journal of Pest Science), 7, pg10.

Clarkson, B. D. (1986). Vegetation of Egmont National Park New Zealand National Parks Scientific Series Number 5. Wellington, New Zealand: Science Information Publishing Centre DSIR.

Coeur d'acier, A., Cruaud, A., Artige, E., Genson, G., Clamens, A.-L., Pierre, E., Hudaverdian, S., Simon, J.-C., Jousselin, E., & Rasplus, J.-Y. (2014). DNA Barcoding and the associated PhylAphidB@se website for the identification of European aphids (Insecta: Hemiptera: Aphididae). PLOS ONE, 9(6), 1-15.

Dixon, A. F. G. (1973). Biology of aphids: Edward Arnold Ltd.

Nei, M., & Kumar, S. (2000). Molecular Evolution and Phylogenetics. New York: Oxford University Press.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research, 32(5), 1792-1797.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution, 783-791.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit 1 from diverse invertebrates. Molecular Marine Biology and Biotechnology, 3(5), 294-299.

Foottit, R. G., Maw, H. E., & Pike, K. S. (2009). DNA Barcodes to explore diversity in aphids (Hemiptera aphididae and Adelgidae). Redia, XCII, 87-91.

Foottit, R. G., Maw, H. E., Von Dohlen, C. D., & Hebert, P. D. N. (2008). Species identification of aphids (Insecta:Hemiptera: Aphididae) through DNA barcodes. Molecular Ecology Resources, 8, 1189-1201.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software

platform for the organization and analysis of sequence data. Bioinformatics, 28(12), 1647-1649.

Kim, H., & Lee, S. (2008). A molecular phylogeny of the tribe Aphidini (Insecta: Hemiptera: Aphididae) based on the mitochondrial tRNA/COII, 12S/16S and the nuclear EF1α genes. Systematic

Nieto Nafría, J. M., Mier Durante, M. P., & Remaudière, G. (1998). Les noms des taxa du groupe-famille chez les Aphididae (Hemiptera). Revue Française d'Entomologie, 19, 77-92.

Remaudière, G., & Remaudière, M. (1997). Catalogue des Aphididae du monde- Catalogue of the world's Aphididae (Homoptera: Aphidoidea). France, French National Institute for Agricultural Research (INRA Editions).

Ratnasingham, S., & Hebert, P. (2013). A DNA-Based Registry for All Animal Species: The Barcode Index Number (BIN) System. PLoS ONE, 8(8), e66213.

Smale, M. C. (1990). Ecology of Dracophyllum subulatum-dominant heathland on frost flats at Rangitaiki and North Pureora, central North Island, New Zealand. New Zealand Journal of Botany, 28, 225-248.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution, 10, 512-516.

Tamura, K., Stecher, G., Peterson, D., Filipsk, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution. Molecular Biology and Evolution, 30, 2725-2729.

Teulon, D. A. J., & Stufkens, M. A. W. (2002). Biosecurity and Aphids in New Zealand. New Zealand Plant Protection, 55, 12-17.

Teulon, D. A. J., Stufkens, M. A. W., Drayton, G. M., Maw, H. E. L., Scott, I. A. W., Bulman, S. R., Carver, M., Von Dohlen, C. D., Eastop, V. F., & Foottit, R. G. (2013). Native aphids of New Zealand-diversity and host associations. Zootaxa, 3647(4), 201-217.

von Dohlen, C. D., & Teulon, D. A. J. (2003). Phylogeny and Historical Biogeography of New Zealand Indigenous Aphidini (Hemiptera, Aphididae):an Hypothesis. Annals of the Entomological Society of America, 96(2), 107-116.

Chapter 3

Using qPCR to determine root aphid (*Aploneura lentisci*) abundance*

*To be published under the same title as : Podmore C, Hogg ID, Banks JC, Tourna M.

Introduction

Root aphids (*Aploneura lentisci* Passerini), belonging to the sub-family *Eriosomatinae* (syn *Pemphiginae*) and the tribe *Fordini*, are known pests of pastures in New Zealand (Popay, 2004; Salmon *et al.*, 2008). As an introduced taxon to New Zealand they do not host alternate (loss of heteroecy) (Blackman & Eastop, 2006) as they do in other parts of the world, between the roots of *Poaceae* and the leaves of the Pistacia tree (*Pistacia lentiscis*) (Wertheim, 1954); Wool & Manheim, 1986), but instead live exclusively on the roots of *Poaceae* reproducing parthenogenically (anholocycly). The aphids are usually undetected because they are underground but they can be easily seen on the roots by the white, waxy exudate they produce. The root aphids can have long-term consequences for pasture growth, which reduces grazing available for livestock, especially when other stressors such as drought or overgrazing occur (Hume *et al.*, 2007; Thom *et al.*, 2013).

Previous research has indicated that the presence of fungal endophytes in pasture grasses can have a deterrent effect on root aphids (Jensen & Popay, 2007; Popay & Gerard, 2007). The results of pot-trials and pasture trials have produced grass cultivar and endophyte combinations that are resistant to root aphid infestations. Research is on-going as new endophytes are discovered (Stewart, 2005; Johnson *et al.*, 2013), and tested in pot-trials, which requires quantification of root aphid infestation to determine host plant resistance. In addition, the mechanisms of resistance to root aphids are not well understood (Popay *et al.*, 2012), another research area requiring root aphid quantification.

Current methods of quantifying root aphids involve floating the insects out of the soil with water in a bucket, decanting through two sieves, collecting the insects into a specimen container and manual counting using a stereomicroscope. This method works well and is low cost but is tedious and

time consuming when large numbers of samples are collected. For example, when testing 10 different cultivar/endophyte combinations requiring 15-20 replicates of each combination, a total of 200 samples may be generated for one trial, taking up to 15 minutes to count each sample and therefore up to 50 hours of intensive microscopic viewing.

Here, we investigated the use of the molecular technique, real-time quantitative PCR (qPCR) (Ginzinger, 2002) to quantify root aphids, using the barcode mitochondrial gene region CO1. Endpoint PCR produces an amplicon but it is difficult to then relate this back to the original amount of DNA. With qPCR, the amplification process is constantly monitored as the DNA is being copied and therefore when a certain threshold is reached (above the background levels), the amount of DNA in the original sample may be calculated from standard curves constructed from known concentrations of DNA. Agudelo *et al.* (2011), successfully employed qPCR to detect and identify nematodes directly from the soil, and others have used the technique to quantify nematodes (Galluzzi *et al.*, 2004; MacMillan *et al.*, 2006). Aphids have also been identified using qPCR and the CO1 gene and furthermore have adapted the technique to a portable system which can be used in the field for rapid identification (Naaum *et al.*, 2014). We targeted adult aphids in this study, as representatives of an infestation. Heavy infestations could contain between 50 to 100 aphids or more depending on the size of the pot and the age of the plant. An initial aspect of the study examined the average weight and length of adult aphids to determine a target size. We then determined whether qPCR could discriminate between low numbers of aphids and therefore the top-end of the curve was set at 64 aphids. Furthermore, three DNA extraction protocols were investigated to determine extraction efficacy and a cost benefit analysis was carried out to determine the best method for extracting aphid DNA directly from the soil. Our goal was to determine if the aphid-specific primers could successfully amplify mitochondrial DNA fragments of *A. lentisci* from a soil sample which could then be quantified by comparison to a standard curve.

Methods and Materials

Primer specificity

An *Aploneura lentisci* CO1 sequence was accessed from GenBank (Accession no. AY227083.1). Using the Primer 3 software (Ye *et al.*, 2012), primer pairs were designed with high specificity to the *A. lentisci* sequence (Table 1). The targeted 317 nucleotide region was preliminarily assessed by searching the amplicon sequence against the NCBI database using the Basic Local Alignment Search Tool (BLAST). We then tested for cross reactivity with some other common aphid species which were extracted and amplified; *Uroleucon sonchi, Metopolophium dirhodum and Rhopalosiphon padi* along with *A. lentisci*. Resulting PCR products were visualised by gel electrophoresis. Negative controls, using PCR grade water were included in all runs. Amplicons were sequenced to confirm identifications using the taxon identification function in the Barcode of Life Datasystems (BOLD) database (www.boldsystems.org)

Aphid collection and DNA extraction

Adult aphids were collected from the roots of potted grass plants that were carefully washed in a gentle stream of tap water into a bucket. The soil was allowed to settle and aphids floating on the surface of the water were decanted through one sieve (710 µm), to remove large debris, and collected in a second (210 µm) sieve (Appendix 6). The aphids were then washed onto an 18.5 cm fluted filter paper and were carefully removed with a fine paint brush with the aid of a stereomicroscope (40x magnification), according to size, shape and the presence of an anal plate indicating sexual maturity (Cottier, 1953). To minimise discrepancies caused by variations in individual aphid sizes, and to standardise the amount of DNA extracted, aphids were individually weighed on a 5-place balance and a sub-set was measured for length, using an eye-piece graticule at 40x magnification.

Three different DNA extraction protocols were investigated to determine their efficacy in extracting aphid DNA. The first was the PowerSoil® DNA Isolation Kit (Cat # 12888-100) (www.mobio.com), which uses an initial mechanical tissue disruption step and then various DNA purification steps. For the extraction protocol, aphids were randomly sorted into 3 replicates of 4 treatment groups, comprising 1; 4; 16; 64 aphids and then frozen at -20 °C before DNA extraction according to manufacturer protocol into a final volume of 100µL of PCR grade distilled water.

The second extraction protocol used the Extract-N-Amp™ Tissue PCR Kit (Cat # XNAT2) (www.sigma-aldrich.com) according to the manufacturer's instructions for 25 mg of animal tissue. For this protocol, sorted aphids were randomly allocated to 3 replicates of 4 treatment groups as above comprising 1, 4, 16, 64 aphids and then each treatment group was weighed to ensure a similar mass of aphid material was processed over three replicates. DNA was extracted according to the manufacturer's protocol, into a final volume of 450 µL. Tubes were then mixed by briefly vortexing and lightly centrifuged to settle any solids and 400 µL of supernatant was removed into a clean microcentrifuge tube and the liquid was held at -20 °C until required for further analysis.

The third extraction method was the Phenol Chloroform DNA extraction protocol for soil according to Griffiths (2000). Aphids were collected as above and randomly sorted into treatment groups, of 1, 4, 16, 64 aphids in triplicate. DNA was extracted into a final volume of 50 µL of PCR grade distilled water and held at -20 °C, until required for further analysis.

Total DNA concentration and purity were measured using a Nanodrop spectrophotometer (FisherScientific 2000c), in order to determine extraction efficacy of the three methods.

Quantitative PCR

Thermalcycler profile

All qPCR assays were carried out on an Eppendorf Mastercycler ep realplex. Following development of the aphid specific primers, the assay was adapted to a quantitative PCR (qPCR) format. Each reaction was conducted in 20 µL volumes containing 10 µL SensiFAST SYBR mix (Bioline Cat # 98002), 0.5 µM of each primer, 0.2 µM BSA (ThermoFisher) and 2 µL of template DNA.

Amplifications used an initial denaturation step of 95°C for 3 minutes, followed by 38 cycles of denaturation for 95°C (5 secs), annealing at 55°C (10 secs) and extension for 72°C (20 secs). This was increased to 40 cycles for all subsequent runs to ensure all values were captured. Fluorescence was read after each cycle and the default setting was used as a threshold. Any sample reaching a fluorescence value exceeding this threshold was considered positive, and the PCR cycle was the cycle threshold or 'Ct value'.

Melt curve (Tm) analysis was performed automatically after each qPCR run to check that the amplicons produced were for the correct sequence region and therefore species, by cooling to 60°C, then increasing to 95°C over 20 mins .(find out what steps and how long each step held for)

DNA from the treatments containing 1, 4, 16, 64 aphids was extracted using three extraction methods and then the DNA was amplified in the qPCR system and the cycle threshold (C_t) values determined. Lower concentrations of DNA template require more cycles than larger to attain a threshold reading. In this way, an unknown sample that has been analysed by qPCR will have a C_t value that can be used to calculate aphid numbers under the same conditions. Ideally, C_t values are inversely proportional to numbers of aphids and a standard curve should be log-linear.

Extraction comparison

DNA from the three extraction methods was analysed by qPCR according to the above protocol. Extract-N-Amp™ extracts of three replicates at four treatment groups were each diluted 10-fold, three times by serial dilution. The PowerSoil® and Phenol Chloroform extracts of three replicates and 4 treatment groups were serially diluted once to conserve reagents, and analysed by qPCR. DNA from the four aphid groups for each of the extraction protocols was diluted to test the linearity of the method. A composite was made by combining 50 μL of each replicate. Composites of the three extraction protocols were then analysed in duplicate by qPCR following the protocol out-lined above.

Aphids were added to a weighed amount of soil (10g) acquired locally from a commercial garden supply company, and the DNA was extracted using two extraction methods (The PowerSoil® and Extract-N-Amp™ kits) to determine: 1) if aphid DNA could be amplified from DNA extracted from the soil; and 2) which of the two DNA extraction methods was preferable for ease of use and accuracy. As the standard curve using the Phenol Chloroform procedure was not linear, this protocol was not investigated.

Four treatment groups were tested: 0; 5; 15; 60 aphids using the PowerSoil® Kit. The aphid/soil mixture was blended in a standard coffee bean grinder (Breville Coffee'n' Spice, model CG2B 37COBR) (2 x 10 sec bursts) until no aphids could be seen. Five replicates of 0.30 g (± 0.01 g), amounts of soil and aphid fragments were taken at random and placed into microcentrifuge tubes. The DNA was extracted according to the manufacturer's protocol, into 100 μL of PCR grade water and frozen until required for qPCR analysis.

For the Extract-N-Amp™ kit, soil samples containing 0 or 60 aphids were homogenised as per the PowerSoil® kit extractions. Duplicate 0.30 g (±0.01 g) amounts of aphid/soil were weighed into microcentrifuge tubes. DNA was extracted according to manufacturer's protocol. The final solution was briefly vortexed to mix, lightly centrifuged to settle any solids and 300 μL of supernatant pipetted into a clean microcentrifuge tube and stored at -20 °C until needed for qPCR.

Table 1 Primer pairs used for *Aploneura lentisci* (Aphididae) amplification

Name	**Sequence (5’ -> 3’)**	**Length (as bp)**
*Aploneura lentisci*_CO1_F	TGGTCTATGATCTGGAATAATTGGG	25
*Aploneura lentisci*_CO1_R	TTGTTCATCCTGTTCCTGTACCA	23
Product length		317

Results

Primer specificity

The amplified sequences provided no matches to any known CO1 sequences on GenBank or BOLD except *Aploneura lentisci*. (%similarity and e value GenBank) Primer specificity was not completely supported by amplification of a DNA fragment of the expected size using endpoint PCR and comparing amplicon size to other aphid species (Figure 1). There was some cross-reactivity with *Metopolophium dirhodum* (Figure1, lane 4), amplicon sequence matched 99.29% on BOLD to *M. dirhodum*. There was no match for the amplicon in Figure1, lane 3, *Uroleucon sonchi*, and minimal cross-reactivity to *Rhopalosiphum padi* (Figure1, lane 5). *A. lentisci* amplicon identities were confirmed (99.29% similarity)

DNA extraction

Individual aphids varied in weight from 0.11 mg to 1.13 mg. The median weight was 0.44 mg and aphids were selected with weights of one standard deviation on either side of the mean to give a range from 0.2 mg to 0.7 mg. The upper range was then extended to 0.8 mg to increase the numbers of aphids available for analysis. The length of the aphids varied from 1.1 mm to 2.4 mm (Table 2). Overall the mean weight per aphid was 0.56 mg for the Extract-N-Amp™ Tissue PCR Kit and 0.49 mg for the Phenol-Chloroform extraction protocol. (Appendix 4)

DNA concentration means (n=3), using the PowerSoil® extraction kit were 4.1 ng/µL ± 0.8 for 1 aphid, 8.5 ng/µL ± 2.8 for 4 aphids, 32.9 ng/µL ± 7.7 for 16 aphids and 36.2 ng/µL ± 25.8 for 64 aphids (Figure 3). There was a linear correlation between aphid numbers and DNA concentrations for up to 16 aphids (R^2 - 0.998), (Figure 4)

DNA concentration means for the Extract-N-Amp™ Kit were 320 ng/µl ± 7.6 for 1 aphid, 356 ng/µL ± 8.8 for 4 aphids, 448ng/µL ± 48.1 for 16 aphids and 663 ng/µL ± 86.3 for 64 aphids. There was a linear correlation between DNA

concentration extracted using the Extract-N-Amp kit and aphid numbers, with a correlation coefficient of 0.98 (Figure 5).

DNA concentrations ranged from 320 to 663 ng/µL.

DNA concentration means (n=3) for the Phenol Chloroform method were 10ng/µL ± 6.4 for 1 aphid, 45.2 ng/µL ± 7.6 for 4 aphids, 85 ng/µL ± 15.7 for 16 aphids and 55.8 ng/µL ± 54.4 for 64 aphids. The correlation between aphid numbers and the concentration of DNA extracted using the Phenol Chloroform method was linear up to 16 aphids (R^2 = 0.91), (Figure 7). However, the extraction from 64 aphids did not produce a proportional increase in DNA concentration. DNA concentrations ranged from 6 to116 ng/µL.

Quantitative PCR

Correlation between C_t and manually sorted aphids

The correlation between the number of aphids and Cycle threshold (C_t) using the PowerSoil® DNA Isolation Kit was linear up to the 16 aphids, for undiluted extract and extract diluted 10-fold. However, as there was slightly lower DNA concentrations in the 64 aphid group compared with the 16 aphid group, this resulted in higher C_t values (Figure 8). The correlation between aphid numbers and Ct values was higher when the 64 aphid group was removed (R2= 0.98), (Figure 9).

DNA concentrations extracted using the Extract-N-Amp™ Kit were linear over the entire range of aphid numbers (Figure 10). In addition, as this protocol produced the most linear DNA extraction results, further dilutions were carried out on the extracts to test the linearity of 10, 100 and 1000-fold dilutions of DNA (Figure 10). The results were then combined to produce a standard curve with a regression of 0.93 over a range of $\log_{10}$ 0.001 aphids to $\log_{10}$ 64 aphids, and C_t values of 18-33 cycles (Figure 11).

The replicate extracts were pooled together into one composite of each of the groups of aphids and analysed in duplicate to test the reproducibility of the analysis (Figure 12). The regression was 0.99 and the C_t range was 17 to 22 cycles. Examples of the qPCR fluorescent signals are shown in Figure 10. The distinct C_t values of each group can be seen as they cross the threshold (red line). Also included are the melt curve analysis which occur automatically at the end of each run. Values of 8 amplicons with a mean of 73.33°C ± 0.13.

The Phenol Chloroform extraction method did not produce a linear relationship between number of aphids and C_t (Figure 14).

The reproducibility of the three extraction protocols was tested for linearity and compared by diluting one of the aphid groups serially up to 1:1000 (Figure 15). Melt temperatures were consistent at 75.4°C ± 0.5°C and a CV of 0.6%, and indicates that the primers were amplifying the same CO1 gene

region in all three extraction methods. In addition, for each ten-fold dilution there was a corresponding number of cycles (usually 3,3 if the amplification is working with optimal efficiency and log linearity) (Table 3). The PowerSoil® Kit DNA extraction qPCR was the most consistent over the dilution range, increasing by 3.79, 3.62, 3.81 cycles every 10-fold dilution. The Extract-N-Amp™ qPCR increased by 2.88, 3.42, 6 cycles and the Phenol Chloroform qPCR by 3.72, 3.43, 1.88 cycles.

Correlation between C_t and aphids in soil

PowerSoil®

The qPCR analysis results for the PowerSoil aphid DNA extractions of 0, 5, 15 and 60 aphids indicate that only the 60 aphid extractions were successfully extracted and amplified (Table 4)). Mean Ct value was 25.31 ± 0.53. Mean Melt temperatures of the amplicons were 75.48°C ± 0.23.

Five of the soil/aphid subsamples with no aphids were measured for C_t value (Table 4), two had C_t > 40 (i.e. no amplification detected) and three had a C_t between 32.28 - 35.95. The amplicons were visualised by gel electrophoresis and the band of one of them was subsequently sequenced and returned a 100% match to *A. lentisci*. Ten-fold dilutions of the extracts produced a similar result (Figure 1). Changes in C_t value over each dilution was 4.07, 2.11 cycles.

Overall, qPCR was able to amplify aphid DNA extracted from an aphid/soil mixture using the PowerSoil Kit to extract DNA.

Extract-N-Amp™ DNA extractions from mixtures of 0 and 60 aphids with soil produced amplifiable aphid DNA (Table 6). Duplicate extractions of the 60 aphids in soil had a mean C_t 33.35 ± 1.6.

Table 2 Adult aphid (*Aploneura lentisci*) weights and body lengths used in PowerSoil® kit DNA extraction

Aphid weight (mg)		Aphid length (mm	
N	350	N	96
Mean	0.44	Mean	1.6
Std dev.	0.24	Min	1.1
Range (1sd)	0.19-0.68	Max	2.4

Table 3 Cycle Thresholds (C_t) and melt temperatures obtained using three extraction protocols at 4 dilutions.

Extraction Protocol	Dilution	C_t (Sybr)	Change in C_t	Tm (°C)
PowerSoil®	1	15.25		75.1
PowerSoil®	0.1	19.04	3.79	75.1
PowerSoil®	0.01	22.66	3.62	75.1
PowerSoil®	0.001	26.47	3.81	75.2
Extract-N-Amp™	1	21.03		75.1
Extract-N-Amp™	0.1	23.91	2.88	75.2
Extract-N-Amp™	0.01	27.33	3.42	75.3
Extract-N-Amp™	0.001	33.33	6	75.4
Phenol Chloroform	1	21.67		75.2
Phenol Chloroform	0.1	25.39	3.72	75.3
Phenol Chloroform	0.01	28.82	3.43	75.3
Phenol Chloroform	0.001	30.7	1.88	75.4

Table 4 qPCR results of aphid DNA extracted from soil by the PowerSoil® DNA Isolation Kit

No. of aphids	Rep	C_t (SYBR)	Tm (°C)
0	1	>40	no result
0	2	35.95	no result
0	3	32.28	76.1
0	4	>40	no result
0	5	34.48	76.8
5	1	33.65	76.5
5	2	>40	no result
5	3	34.54	76.5
5	4	32.10	76.0
5	5	20.54	no result
15	1	33.50	76.5
15	2	32.10	76.1
15	3	>40	no result
15	4	>40	no result
15	5	34.37	76.5
60	1	25.00	75.2
60	2	24.47	75.3
60	3	25.64	75.7
60	4	25.99	75.7
60	5	25.45	75.5

Table 5 Effects of dilution on qPCR results of aphid DNA extracted from soil using the PowerSoil® DNA Isolation Kit

No. of aphids	Dilution	C_t (SYBR)	Tm (°C)
0	0.1	>40	no result
0	0.01	>40	no result
0	0.001	>40	no result
5	0.1	>40	no result
5	0.01	>40	no result
5	0.001	>40	no result
15	0.1	>40	no result
15	0.01	>40	no result
15	0.001	>40	no result
60	0.1	28.08	76.0
60	0.01	32.15	76.2
60	0.001	34.26	77.1

Table 6 qPCR results of aphid DNA extracted from soil using the Extract-N-Amp™ kit

No. of aphids	Dilution	C_t (Sybr)	Tm (°C)
0	1	>40	No result
0	1	>40	No result
0	0.1	33.12	76.4
0	0.01	>40	No result
60	1	32.23	75.3
60	1	34.47	75.3
60	0.1	38.95	No result
60	0.01	>40	No result

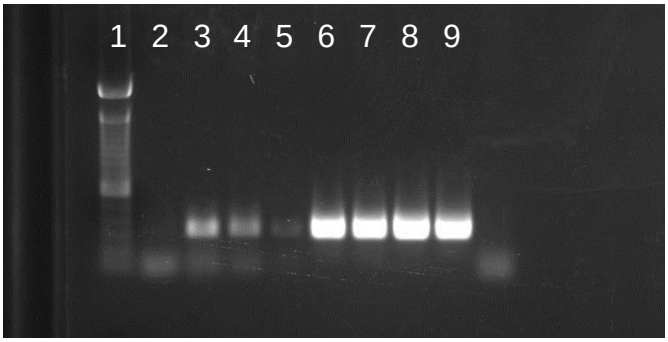


Figure 1 Electrophoresis gel showing cross-reactivity of *Aploneura lentisci*-specific primers Lane1 molecular ladder Lane 2. negative Lane 3. *Uroleucon sonchi* Lane 4 *Metopolophium dirhodum* Lane 5 *Rhopalosiphum padi* Lanes 6 - 9 *Aploneura lentisci* Lane 10 negative

- >0025_044-1_(CO_1R_CO_1F) *Aploneura lentisci* amplicon
- TCTTCTTTAAGAATATTAATTCGACTTGAATTAAGACAAATTAAT
  TCTATTATTAATAATAGTCAATTATATAATGTAATTGTTACAATTC
  ATGCCTTTATTATAATTTTTTTTATAACTATACCTATTGTAATTGG
  AGGTTTTGGTAATTGATTGATTCCTATAATAATAGGTTGCCCTG
  ATATATCTTTTCCTCGACTTAATAATATTAGATTTTGATTGTTAC
  CTCCTTCATTAATAATAATAATCTCAAGATTTTTAATTAATAA

Figure 2 Sequence of nucleotide amplified by *Aploneura lentisci*-specific primers

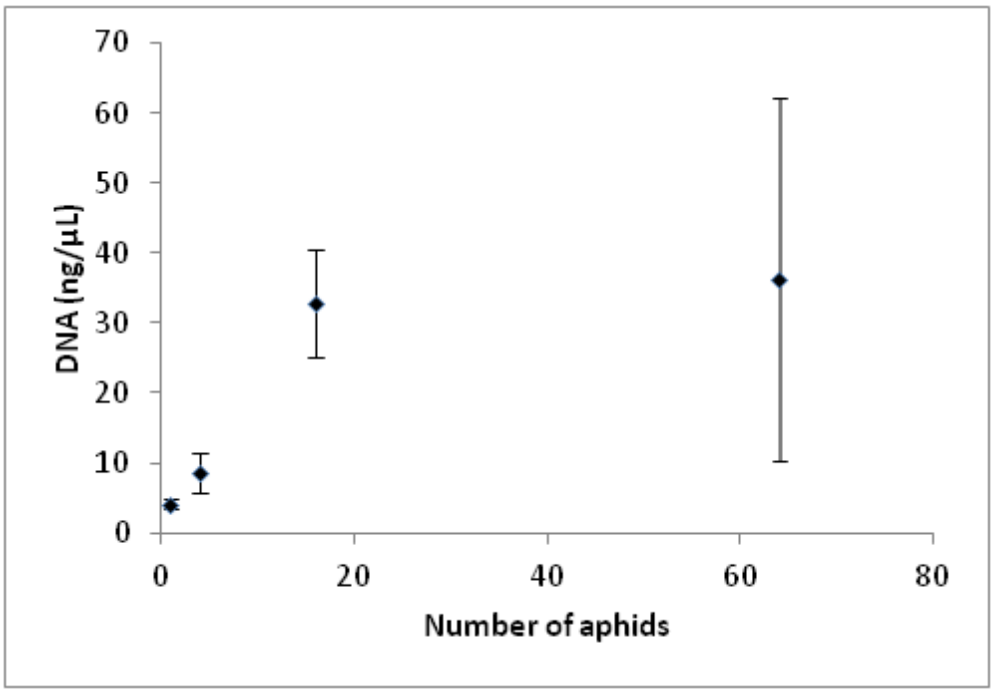


Figure 3 Relationship between aphid numbers 1,4,16,64 (*Aploneura lentisci*) and concentration of DNA (nglµL) extracted using the PowerSoil® DNA Isolation Kit. Error bars =1 sd (n=3).

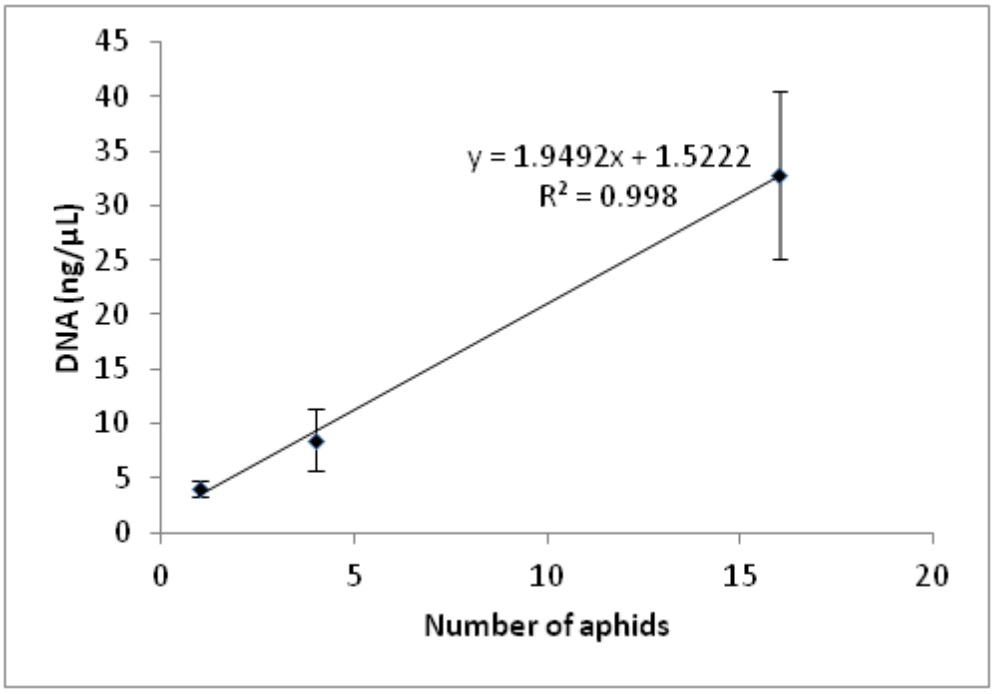


Figure 4 Relationship between aphid numbers 1,4,16 (*Aploneura lentisci*) and concentration of DNA (ng/µL) extracted using the PowerSoil® DNA Isolation Kit . Error bars = 1 sd (n=3).

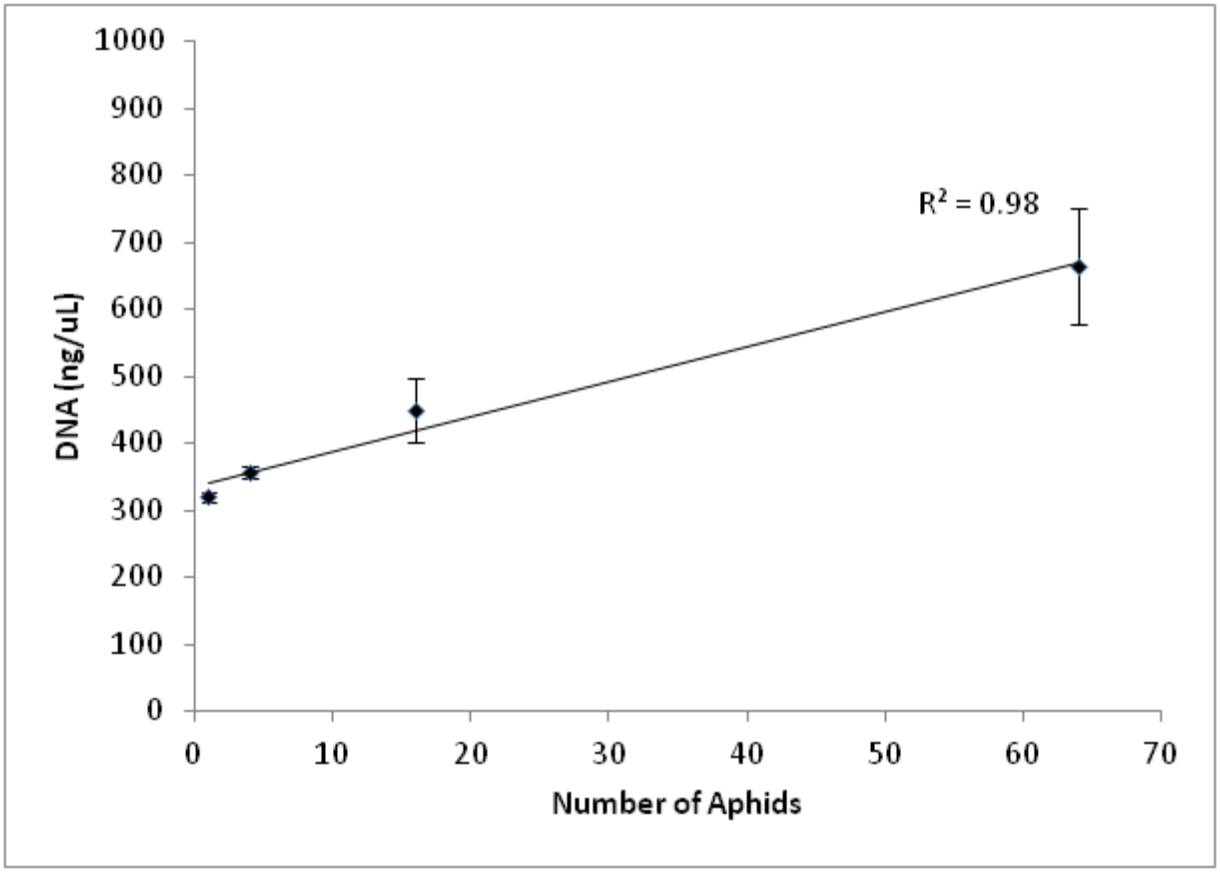


Figure 5 Relationship between number of aphids 1, 4, 16, 64 (*Aploneura lentisci*) and concentration of DNA (ng/µL) extracted using the Extract-N-Amp™ kit. Error bars=1 sd (n-3).

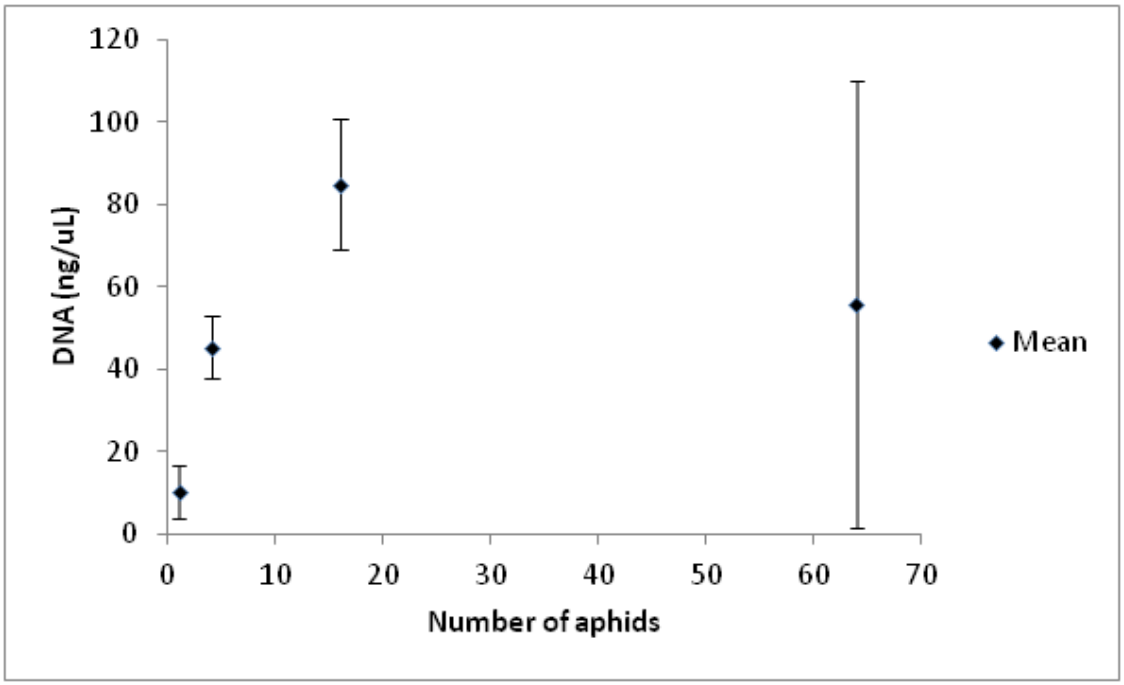


Figure 6 Relationship between number of aphids (*Aploneura lentisci*) and concentration of DNA (ng/µL) extracted using the Phenol Chloroform extraction protocol according to Griffiths *et al* 2000. Error bars= 1sd (n=3)

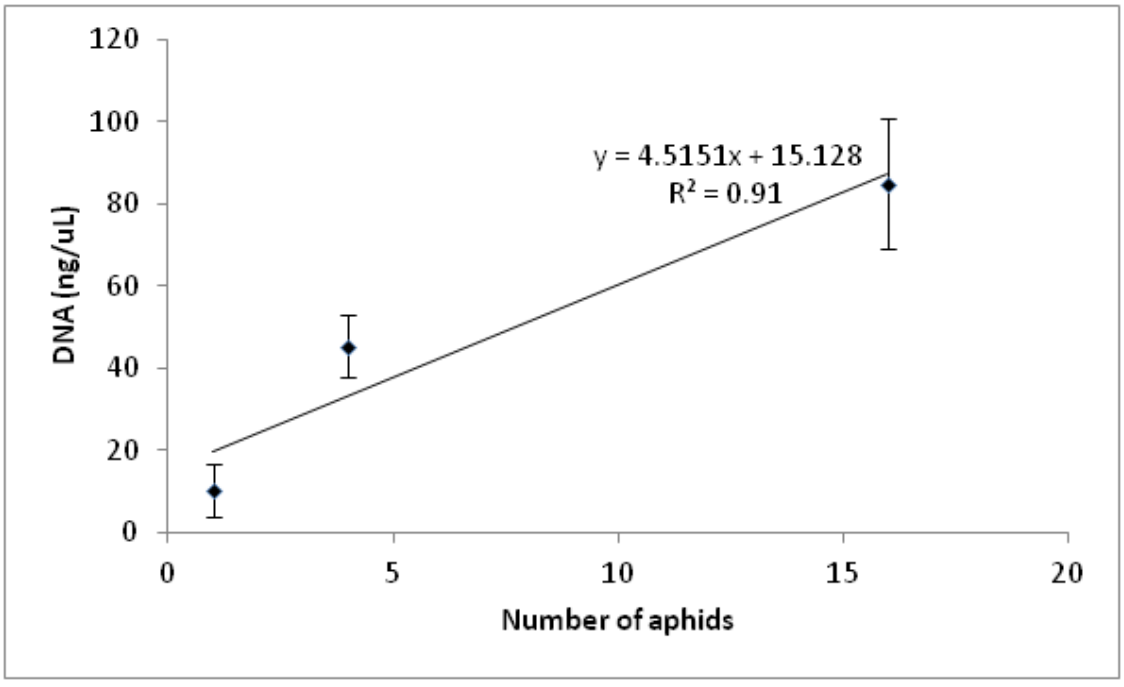


Figure 7 Relationship between number of aphids (*Aploneura lentisci*) and concentration of DNA (ng/µL) extracted using the Phenol Chloroform extraction protocol according to Griffiths *et al* 2000. Error bars= 1 sd (n=3).

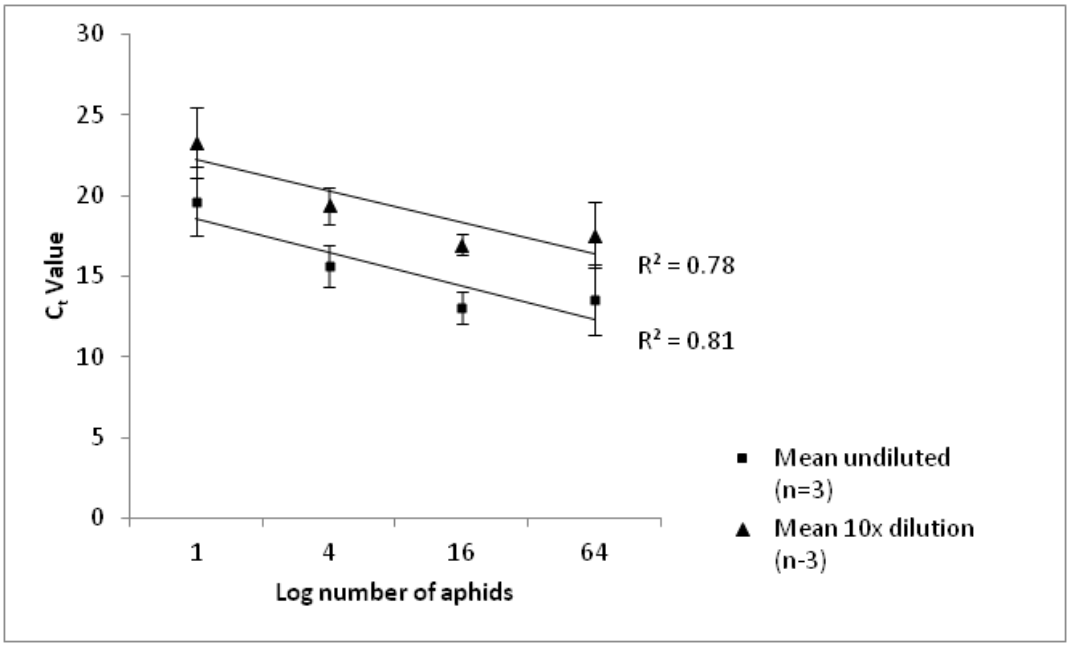


Figure 8 Relationship between qPCR results of undiluted DNA extracts and 10-fold diluted extracts and C_t values using the PowerSoil® DNA Isolation Kit for aphid number range 1 to 64. Error bars =1sd.

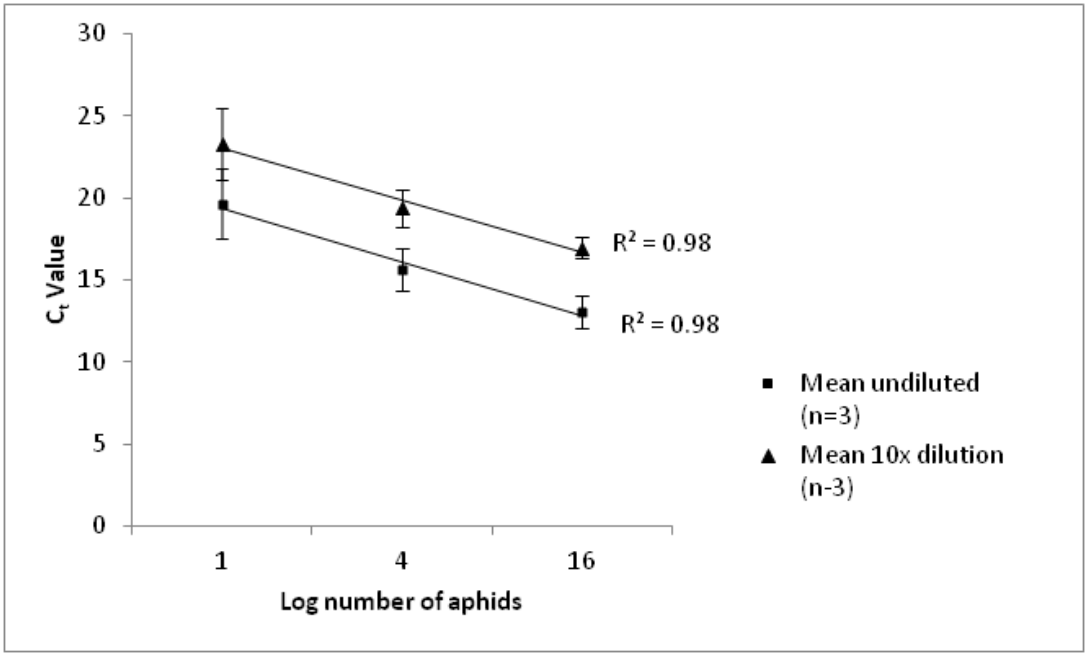


Figure 9 Relationship between qPCR results of undiluted DNA extracts and 10-fold diluted extracts and C_t values using the PowerSoil® DNA Isolation Kit. for aphid number range from 1 to 16 aphids. Error bars =1sd.

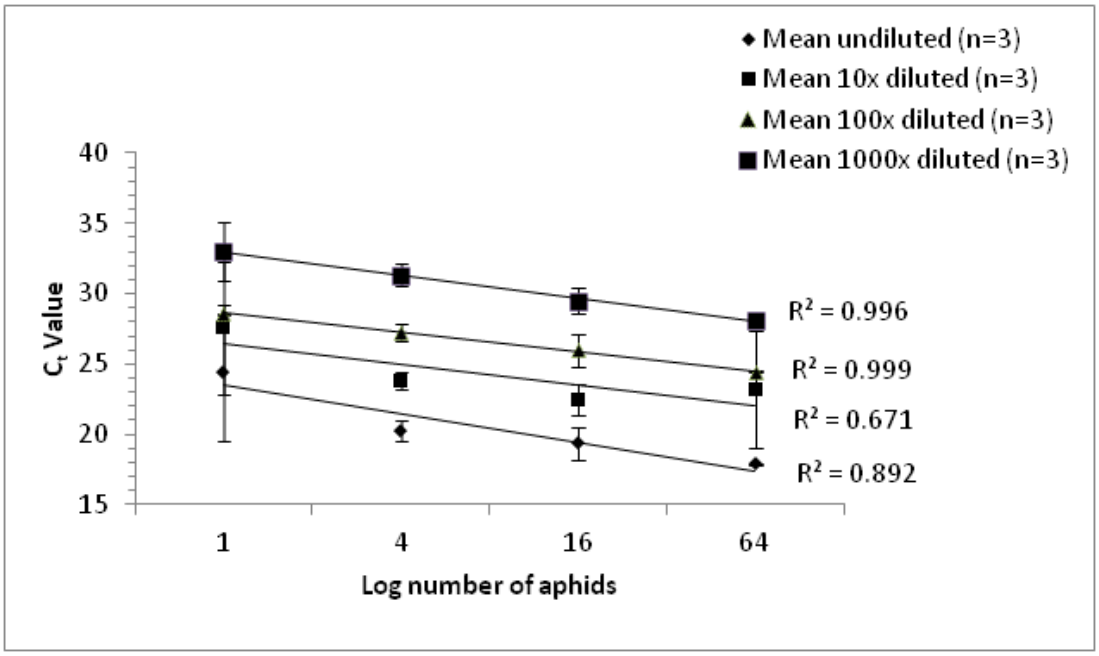


Figure 10 Relationship between qPCR results of undiluted and diluted DNA extracts and C_t values using the Extract-N-Amp™ Kit. Error bars = 1 sd.

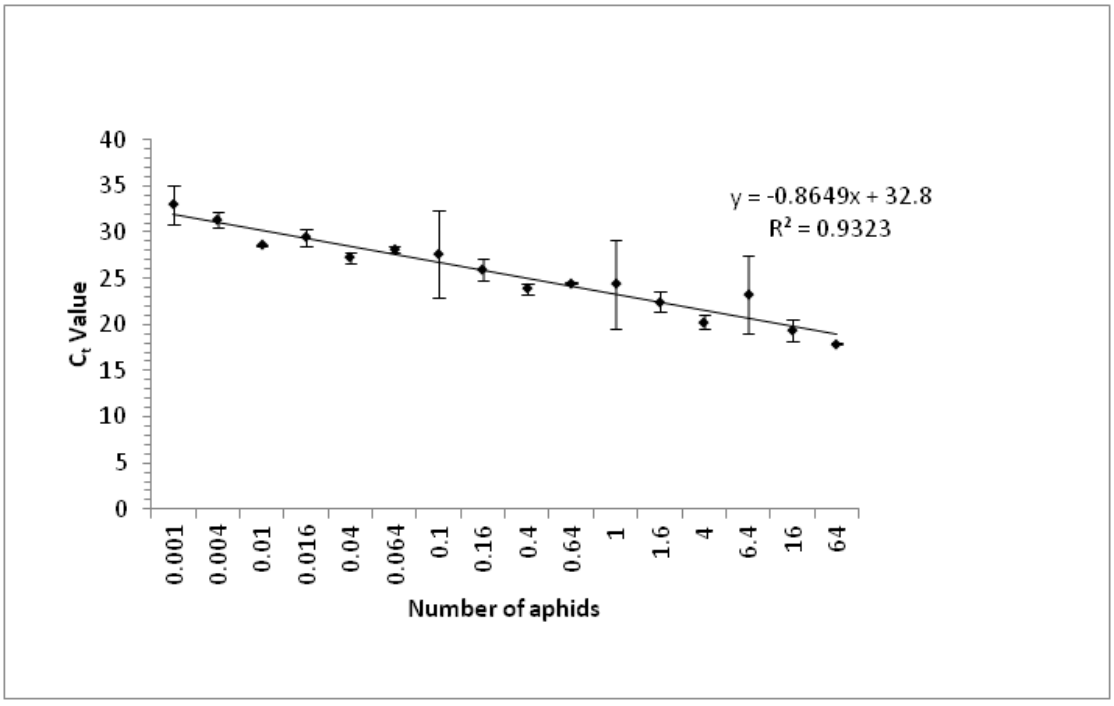


Figure 11 Quantification of undiluted and diluted Extract-N-Amp™ DNA extracts from aphids. Error bars = 1 sd (n=3).

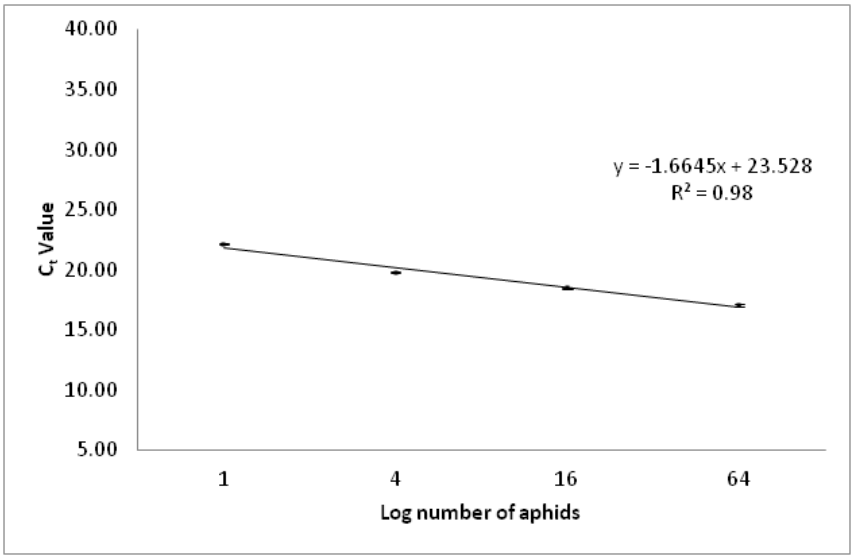


Figure 12 Standard Curve of Extract-N-Amp DNA extracts. Composite of 3 replicates were analyzed in duplicate.

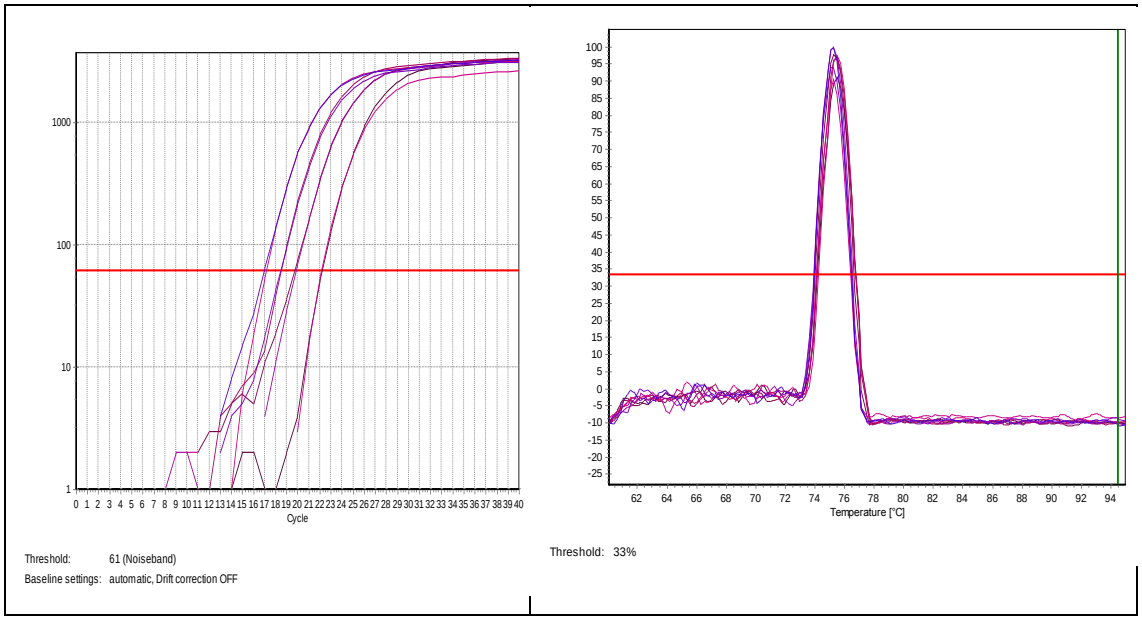


Figure 13 Graph of qPCR amplification in real-time (left) for the duplicate Extract-N-Amp™ DNA extractions and (right) melt curves.

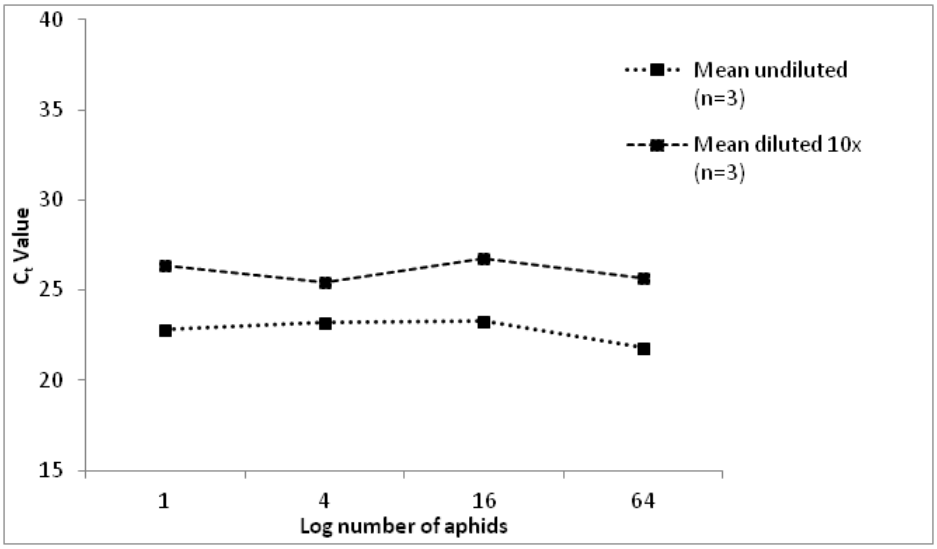


Figure 14 Relationship between number of aphids (*Aploneura lentisci*) and concentration of DNA (ng/µL) extracted using the Phenol Chloroform extraction protocol according to Griffiths *et al* 2000. Error bars =1sd

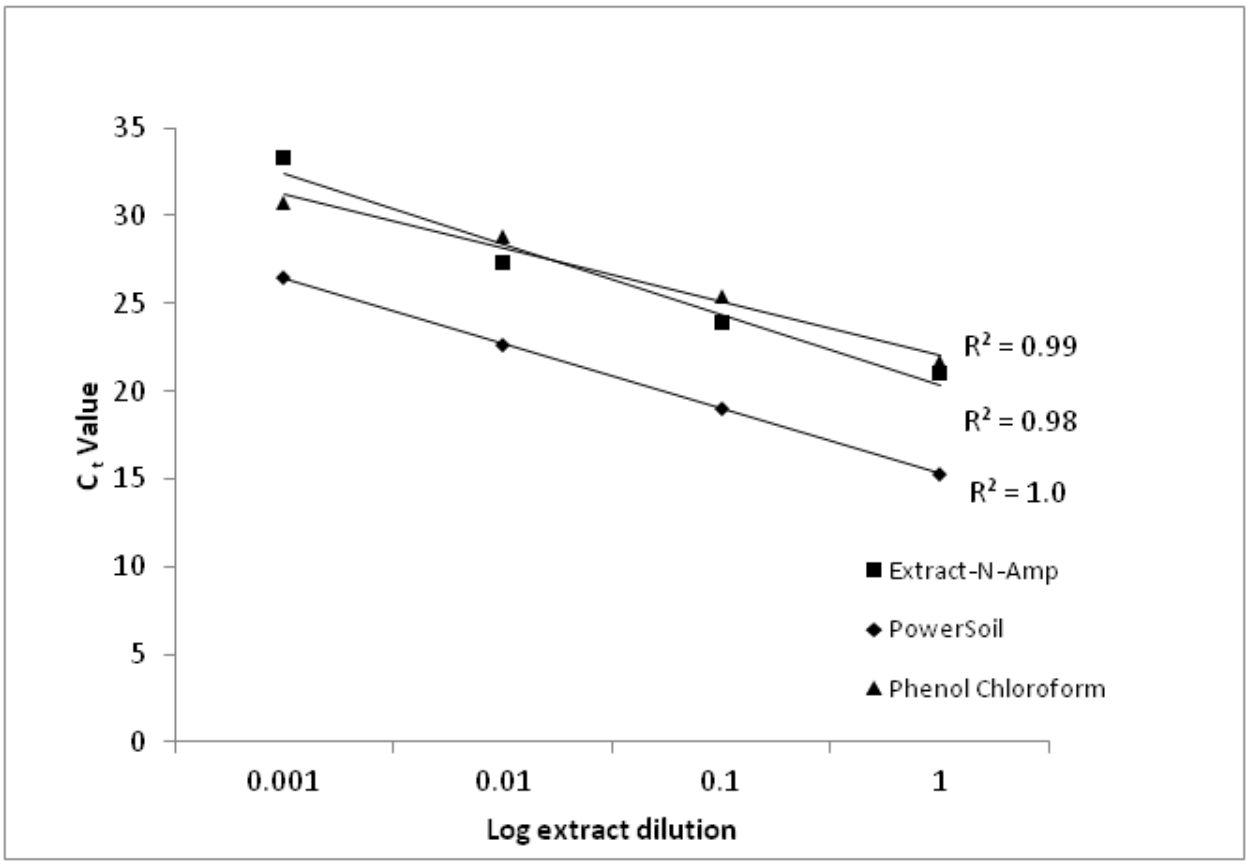


Figure 15 Three DNA extracts of the four aphid group, diluted and analysed by qPCR to show linearity of dilutions.

Discussion

Primer specificity

The amplified CO1 region of 317 nucleotides was longer than the 100 nucleotides recommended for qPCR, to ensure the success of amplification. The primers had some cross reactivity with other aphid species. However, in a New Zealand context, experience indicates the dominant aphid in screening roots infested with root aphids will be *A. lentisci* and occasionally a few *R.padi*, hence cross-reactivity is unlikely to provide a confounding influence for the purpose of this application.

Effect of body size or life stage

Adult aphids are easily identified by their elliptical body shapes, absence of siphunculi, and short antennae, even without magnification. They produce a characteristic white flocculent material on roots which is easy to spot and identify as *A.lentisci*. As they age, they vary in size and their length is variable as they have imbricated body segments (Cottier, 1953) which can 'telescope' (overlap). In addition, nymphs or immature morphs of varying sizes are present alongside the adults. This study focussed on establishing specific primers and qPCR protocols, DNA extraction protocols and extraction of DNA from soil and the aphid body size variability was overcome by selecting adults as representative of the colony

Efficacy of DNA extraction methods and qPCR results

The initial step of the PowerSoil® DNA Isolation Kit procedure provides complete disruption of tissue and release of DNA. However, the kit is designed as a qualitative tool and therefore residual amounts of DNA are left behind at each step as supernatants are pipetted off, leaving enough liquid behind to avoid contamination with the pellet. To measure the loss, the supernatant left-over in the initial powerbead tube was decanted/poured off to see how much DNA was left behind and the procedure was followed according to the manufacture instructions, proportioning out reagents

according to amount of liquid at each step. A reasonable amount of DNA was further recovered (results not shown) and may account for the non-linear relationship between aphid numbers and DNA yield. This aspect would need to be taken into account for this procedure.

The correlation between log number of aphids and Cycle threshold (C_t) using the PowerSoil® extraction protocol was linear up to the 16 aphid group. However, it did not produce a proportional increase in DNA concentration for the 64 aphid treatment group and indicates that there may be too much DNA for this procedure. An alternative would be to proportionately increase all the volumes used in the kit, effectively 'scaling up' at each step to cope with the extra DNA.

The C_t values (Table 4) of five replicate soil aliquots weighed from the 10 g sample with 60 aphids is an indication of the effectiveness of the coffee grinder to homogenise the aphids and soil. The 10 g of soil is a small volume for pot-trials but nevertheless could be incorporated into a protocol for large numbers of samples. Seeds of trial cultivars planted into 10 g volume trays are able to establish and grow successfully (C. Podmore unpubl. data) for up to 6 months before needing to be transplanted. Visual inspection and qPCR confirmation of low, medium, high aphid infestations could then be carried out quickly and effectively using the qPCR protocol developed here. In our protocol only 0.3 g of soil was removed from a 10 g soil sample giving a reduction of 1 in 33.3. Methods for dealing with larger amounts of soil, or methods of extracting aphids from the soil prior to the DNA extraction step could be investigated in future studies.

The presence of *A. lentisci* DNA in three of the controls (no aphids added to soil) could have been either because aphid DNA was already present in the soil or because contamination occurred during the extraction process. Similarly, there was a large range for the aphid DNA concentrations measured by in the five replicates of five aphids and 25 aphids added to soil

that could be due to the concentrations being at the lower end of the method's sensitivity.

There was a linear correlation between aphid numbers and C_t value for DNA extracted using the Extract-N-Amp™ Kit. The dilution of extracted DNA up to 1 in 1000 effectively increased the range of the correlation from 18 to 33 cycles (figure 3.6). C_t values of ≤ 29 are strong reactions, C_t =30-37 are moderate and anything ≥38-40 are considered weak and may even be the result of reaction artefacts such as primer dimerization or contamination. Therefore the results from this study are within an ideal range of qPCR cycles. However the mean C_t value for the DNA extracted from the soil is at the extreme range of the method. Increasing aphid numbers 10-fold may overcome this.

The results using the Phenol Chloroform DNA extraction method indicated a linear correlation from 1 to 16 aphids. However, this method did not show a linear correlation between aphid numbers and C_t values. It may be possible to look more closely at the method and improve this result. It may be that high numbers of aphids are required for a correlation

A cost benefit analysis looking at the three extraction methods is summarised in Table 7 Both the Extract-N-Amp and PowerSoil kits cost approximately $7 per test. However, the Extract-N-Amp™ reagents may be available in bulk which would reduce this price. The Extract-N-Amp™ Kit was the quickest and easiest to use and could return a result on the same day, depending on batch sizes. The PowerSoil® kit required a reasonably experienced operator. In addition, 12 samples took three hours to extract and may not be efficient in terms of throughput and labour intensity. The reagents for this kit are safe to use unlike those in the Phenol Chloroform extraction method.

Conclusion

The method developed here has been effective in amplifying *Aploneura lentisci* mitochondrial CO1 DNA from the soil. The Extract-N-Amp™ Kit provided an efficient means of extracting DNA from aphids that resulted in a linear correlation between aphid numbers and DNA concentration measured by spectrophotometer or by qPCR. The number of aphids (1 to 64) added to 10 g of soil in this study was too few to achieve a result in the optimal working range of the standard curve given the dilution effect of the soil. A 10-fold increase in aphid numbers would be necessary to overcome this. Nevertheless, with more development of the extraction system, the barcode gene is effective for the quantification of *Aploneura lentisci* in soil and would improve current methods of aphid quantification.

Table 7 Comparison of three DNA extraction methods of aphids (*A.lentisci*)

PowerSoil	**Extract-N-Amp**	**Phenol Chlorofrom**
Expensive	Expensive but may be reduced	Low cost
Labour intensive	Quick turnaround	Labour intensive
Safe materials	Safe materials	Hazardous materials
Non-linear result	Linear result	Non-linear results

Acknowledgements

Thanks to Jonathan Banks for explaining in plain English how qPCR works and for welcoming me to Cawthron. Thanks for editing the chapter.

Thanks to Maria Tourna who allowed me to work in her Lab and helped me formulate a plan. Thanks also to Alison Popay for allowing me the time to complete my research even though it put pressure on an already busy lab.

Thanks to my colleagues Jo and Louise for your encouragements over the course of this study.

References

Agudelo, P., Lewis, S. A., & Fortnum, B. A. (2011). Validation of a real-time polymerase chain reaction assay for the identification of *Meloidogyne arenaria*. *Plant Disease, 95*(7), 835-838.

Blackman, R. L., & Eastop, V. F. (2006). *Aphids on the World's Herbaceous Plants and Shrubs. (2 vols)*. Chichester: Wiley.

Cottier, W. (1953). Aphids of New Zealand Vol. Bulletin 106. Wellington: NZ Department of Scientific and Industrial Research.

Galluzzi, L., Penna, A., Bertozzini, E., Vila, M., Garces, E., & Magnani, M. (2004). Development of a real-time PCR assay for rapid detection and quantification of *Alexandrium minutum (*a Dinoflagellate). *Applied and Environmental Microbiology, 70*(2), 1199-1206.

Ginzinger, D. G. (2002). Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Experimental Hematology, 30*(6), 503-512.

Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G., & Bailey, M. J. (2000). Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition. *Appl. Environ. Microbiology, 66*(12), 5488-5491.

Hume, D. E., Ryan, D. L., Cooper, B. M., & Popay, A. J. (2007). Agronomic performance of AR37-infected ryegrass in northern New Zealand. *Proceedings of the New Zealand Grassland Association, 69*, 201-205.

Jensen, J. G., & Popay, A. J. (Compiler) (2007). Reductions in root aphid populations by non-toxic endophyte strains in tall fescue. New Zealand Grassland Association:Endophyte Symposium.

Johnson, L., de Bonth, A. C. M., Briggs, L. R., Caradus, J. R., Finch, S. C., Fleetwood, D., Fletcher, L. R., Hume, D. E., Johnson, R. D., Popay, A. J., Tapper, B. A., Simpson, W. A., Voisey, C. R., & Card, S. D. (2013). The exploitation of epichloae endophytes for agricultural benefit. *Fungal Diversity 60*, 171-188.

MacMillan, K., Blok, V., Young, I., Crawford, J., & Wilson, M. J. (2006). Quantification of the slug parasitic nematode Phasmarhabditis hermaphrodita from soil samples using real time qPCR *International Journal for Parasitology 36*, 1453-1461.

Naaum, A. M., Foottit, R. G., Maw, H. E. L., & Hanner, R. (2014). Real-time PCR for identification of the soybean aphid, Aphis glycines Matsumura *Journal of Applied Entomology*.

Popay, A. J. (2004). *Aspects of the root ecology of Neotyphodium Endophytes in Lolium perenne.* thesis, Waikato.

Popay, A. J., & Gerard, P. J. (2007). Cultivar and endophyte effects on a root aphid, *Aploneura lentisci,* in perennial ryegrass. *New Zealand Plant Protection, 60*, 223-227.

Popay, A. J., Cotching, B., Moorhead, A., & Ferguson, C. M. (2012). AR37 endophyte effects on porina and root aphid populations and ryegrass damage in the field. *Proceedings of the New Zealand Grassland Association, 74*, 165-170.

Salmon, R. W., Popay, A. J., Pennell, C. G. L., Walmsley, W. H., & Moorhead, A. J. (Compiler) (2008). Root Aphid (*Aploneura lentisci)* : A pest of pastures in Australia and New Zealand. Proceedings of the 49th Annual Conference of the Grasslands Society of Southern Australia inc. Bairnsdale, Victoria.

Stewart, A. (2005). The commercial use of endophyte in turf ryegrass in New Zealand. *International Turfgrass Society Research Journal, 10*, 641-644.

Thom, E. R., Popay, A. J., Waugh, C. D., & Minnee, E. M. K. (2013). Impact of novel endophytes in perennial ryegrass on herbage production and insect pests from pastures under dairy cow grazing in northern New Zealand. *Grass and Forage Science, 69*, 191-204.

Wertheim, G. (1954). Studies on the biology and ecology of the gall-producing aphids of the tribe fordini (homoptera: aphidoidea) in Israel. *Transactions of the Royal Entomological Society of London, 105*(5), 79-96.

Wool, D., & Manheim, O. (1986). Population ecology of the gall-forming aphid, *Aploneura lentisci* (Pass.) in Israel. *Researches on Population Ecology, 28*(1), 151-162.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics, 13*, Article 134.

Chapter 4

Thesis Summary and Conclusions

This thesis has demonstrated two different applications of the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene, for taxon identification and also for assessing species' abundance. In the first research chapter (Chapter 2), I used COI gene sequences to measure the diversity of New Zealand native aphids, specifically *Schizaphis*. In all cases, COI sequences were successfully able to discriminate between known species as well as highlight the possibility of previously unknown, cryptic species. Specifically, I was able to demonstrate that there were at least five separate groups/species, either related to geographical location or host plant species. All of the sequences generated as part of this work have been made publically available through the Barcode of Life Datasystems (BOLD) database (Ratnasingham & Hebert, 2007) This will enable other researchers to access this resource and progress knowledge and understanding of this taxon and build on previous work (von Dohlen & Teulon, 2003; Foottit *et al.*, 2009; Teulon *et al.*, 2013). Future work could focus on collecting more aphids from across New Zealand and also collecting and vouchering host plants. In addition, the host plants, *Dracophyllum* are also found in 'Gondwanan' distributions on the East coast of Australia, Lord Howe Island and New Caledonia (Venter, 2009; Wagstaff *et al.*, 2010) and it would be interesting to investigate the possibility of *Schizaphis* being present on these plants. Obtaining COI sequences for other *Schizaphis* species world-wide may also yield some insight into the New Zealand species provenance (Teulon*, et al.*, 2013). This study did not examine morphological features that may exist between the genetically diverse *Schizaphis* groups and this would be an important aspect of future work. Previous collections (Teulon*, et al.*, 2013) have included alate specimens according to taxonomic identification, which could be now confirmed by barcoding. In the current collection only apterous adults and some nymphs were collected and this raises the question of: what is the reproductive cycle of *Schizaphis* in New Zealand? Many introduced aphid species which are heterocyclic, loose this aspect and only reproduce asexually (anholocycly) (Blackman & Eastop, 2006). If this is the case with *Schizaphis*, it would be interesting to study how the genetic diversity within

New Zealand has occurred without the gene recombination that occurs through a sexual reproductive cycle; otherwise geographical segregation over time has occurred. Given that COI is a mitochondrial gene and therefore maternally inherited, the use of other, nuclear markers would be necessary to address such issues. Another interesting aspect of the aphid collections was the number of weevils found on the North island *Dracophyllum.* These were prevalent across all the North Island sites and I analysed a single specimen which was barcoded at the COI gene locus. However, this individual did not match any CO1 sequences currently on the BOLD database. However, specimens were also sent to Landcare Research in Auckland and identified as a flower weevil (*Peristoreus maorinus*) by Dr Richard Leschen who also states that this species is a common New Zealand native which feeds in the flowers of the plant An interesting study would be to determine if this weevil competes with *Schizaphis* on the host plants.

In Chapter 3, I was able to demonstrate the application of CO1 in measuring the abundance of root aphids (*Aploneura lentisci)*, known pests of pastures (Salmon *et al.*, 2008), through the application of quantitative real-time PCR. These aphids are pests of agriculture and in heavy infestations can destroy pastures (Hume *et al.*, 2007). Fungal endophytes present in plants can have a deterrent effect on the aphids and therefore on-going investigation requires the quantification of aphids to produce robust naturally resistant plant cultivars (Stewart, 2005; Johnson *et al.*, 2013). While easy to identify on the roots of plants, a new methodology based on molecular methods would be beneficial to screen and process larger numbers of plants. For this work, I first needed to develop specific primers to amplify a region of the CO1 gene specific to *A. lentisci*. The primers amplified a 317 nucleotide region which is quite a large region within the 658 nucleotide barcode region. Some limited cross-reactivity occurred with other aphids where the nucleotide sequences were in common. However, this was unlikely to be a confounding factor for the purposes of evaluating root aphids in New Zealand. Three quite different approaches to DNA extraction were then investigated. I used the PowerSoil Kit method that has been designed to remove DNA from soil samples and

has various ‘cleanup’ stages. The Phenol Chloroform protocol was very similar but the main disadvantage of this method was the toxic chemicals used. The final method examined was the Extract-N-Amp which employs an enzymatic extraction and proved to be very effective. However, in terms of a quick turn-around the Extract-N-Amp protocol was the quickest, once reagents had been added to the soil, a short incubation time of 10-15 mins at 55°C was all that was needed and once the DNA was extracted, in both the kits, the process of amplification was relatively straightforward. Any improvements in the process should focus on the extraction of DNA. Future work could look at various other methodologies of extracting aphid DNA from larger quantities of soil or removing aphids from the soil before extracting DNA. I found a linear correlation between aphid numbers and DNA concentration and also was able to directly extract and sequence *A. lentisci* DNA from soil, meaning that this application could effectively determine the abundance of aphids accurately and efficiently in the same day. In addition, other technology which utilises hand-held devices could be utilised for even faster turn-around times.

Molecular methods have rapidly replaced conventional ways of examining the flora and fauna in the natural world. The CO1 gene region has been highlighted in the past decade or so by many researchers around the world, since the pioneering work of Hebert et al.(2003a) at the University of Guelph in Canada. They have since set up infrastructure to deal with the massive amounts of information being generated that need to be stored and regularly accessed (Hajibabaei *et al.*, 2005). Computer technology, the world-wide web and the publication of research are able therefore to enhance the advances made by researchers throughout the world as information sharing is simplified. The possibility of barcoding the world’s biota is a distinct reality. It is a privilege to be part of the global community investigating the natural world to understand this complex, diverse and seemingly well-designed planet.

References

Blackman, R. L., & Eastop, V. F. (2006). *Aphids on the World's Herbaceous Plants and Shrubs. (2 vols)*. Chichester: Wiley.

Foottit, R. G., Maw, H. E., & Pike, K. S. (2009). DNA Barcodes to explore diversity in aphids (Hemiptera aphididae and Adelgidae). *Redia, XCII*, 87-91.

Hajibabaei, M., deWaard, J. R., Ivanova, N. V., Ratnasingham, S., Dooh, R. T., Kirk, S. L., Mackie, P. M., & Hebert, P. D. N. (2005). Critical factors for assembling a high volume of DNA barcodes. *Philosophical Transactions of the Royal Society B, 360*, 1959-1967.

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003a). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 270*(1512), 313-321.

Hume, D. E., Ryan, D. L., Cooper, B. M., & Popay, A. J. (2007). Agronomic performance of AR37-infected ryegrass in northern New Zealand. *Proceedings of the New Zealand Grassland Association, 69*, 201-205.

Johnson, L., de Bonth, A. C. M., Briggs, L. R., Caradus, J. R., Finch, S. C., Fleetwood, D., Fletcher, L. R., Hume, D. E., Johnson, R. D., Popay, A. J., Tapper, B. A., Simpson, W. A., Voisey, C. R., & Card, S. D. (2013). The exploitation of epichloae endophytes for agricultural benefit. *Fungal Diversity 60*, 171-188.

Ratnasingham, S., & Hebert, P. D. N. (2007). BOLD:The Barcode of Life Data System (www.barcodinglife.org). *Molecular Ecology Notes, 7*(3), 355-364.

Salmon, R. W., Popay, A. J., Pennell, C. G. L., Walmsley, W. H., & Moorhead, A. J. (Compiler) (2008). Root Aphid (*Aploneura lentisci)* : A pest of pastures in Australia and New Zealand. Proceedings of the 49th Annual Conference of the Grasslands Society of Southern Australia inc. Bairnsdale, Victoria.

Stewart, A. (2005). The commercial use of endophyte in turf ryegrass in New Zealand. *International Turfgrass Society Research Journal, 10*, 641-644.

Teulon, D. A. J., Stufkens, M. A. W., Drayton, G. M., Maw, H. E. L., Scott, I. A. W., Bulman, S. R., Carver, M., Von Dohlen, C. D., Eastop, V. F., & Foottit, R. G. (2013). Native aphids of New Zealand-diversity and host associations. *Zootaxa, 3647*(4), 201-217.

Venter, S. (2009). *A Taxonomic Revision of the genus Dracophyllum Labill.(Ericaceae).* thesis, Victoria University, Wellington.

von Dohlen, C. D., & Teulon, D. A. J. (2003). Phylogeny and Historical Biogeography of new Zealand Indigenous Aphidini (Hemiptera, Aphididae):an Hypothesis. *Annals of the Entomological Society of America, 96*(2), 107-116.

Wagstaff, S. J., Dawson, M. I., Venter, S., Munzinger, J., Crayn, D. M., Steane, D. A., & Lemson, K. L. (2010). Origin, diversification, and classification of the Australasian genus *Dracophyllum* (Richeeae, Ericaceae). *Annals of the Missouri Botanical Garden, 97*(2), 235-258.

Appendicies

Appendix 1 *Schizaphis* Distance matrix (Neighbour Joining - Tamura Nei model)

	Site Code	Schizaphis Location	BOLD ID	Rhopalosiphum enigmae	Tukino	Pureora	Schizaphis graminum	Arthur's Pass	Arthur's Pass	Arthurs Pass	Arthurs Pass	Lake Sylvester	Cobb Valley	Cobb Valley	Cobb Valley	Cobb Valley	Lake Sylvester	Cobb Valley	Cobb Valley	Lake Sylvester	Cobb Valley	Cobb Valley	Egmont	Egmont	Egmont	Egmont	Egmont	Egmont	Mt Lyford	Mt Lyford	Mt Lyford	Schizaphis rotundiventris	Schizaphis scirpicola	Korowai	Mt Barossa	Mt Barossa	St James	Porter's Pass	Porters Pass	Mt Barossa	Porter's Pass	Korowai	Mt Barossa	Mt Barossa - 1	Porter's Pass - 1	Porter's Pass - 1
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
1		Rhopalosiphum enigmae	RDBA160-05																																											
2	ND2	Tukino	NZAPH034	0.080																																										
3	ND1	Pureora	NZAPH128	0.077	0.002																																								
4		Schizaphis graminum	RDBA262-05	0.062	0.091	0.088																																							
5	SD6	Arthur's Pass	NZAPH133	0.091	0.094	0.091	0.104																																						
6	SD7	Arthur's Pass	EU701901.1	0.088	0.092	0.089	0.101	0.000																																					
7	SD8	Arthur's Pass	RDBA480-06	0.088	0.092	0.089	0.101	0.000	0.000																																				
8	SD9	Arthurs Pass	NZAPH051	0.084	0.091	0.089	0.101	0.010	0.007	0.007																																			
9	SD4	Lake Sylvester	NZAPH045	0.079	0.088	0.085	0.098	0.018	0.015	0.015	0.018																																		
10	SD5	Cobb Valley	NZAPH042	0.076	0.085	0.083	0.095	0.010	0.007	0.007	0.010	0.007																																	
11	SD5	Cobb Valley	NZAPH043	0.076	0.085	0.083	0.095	0.010	0.007	0.007	0.010	0.007	0.000																																
12	SD5	Cobb Valley	NZAPH131	0.076	0.085	0.083	0.095	0.010	0.007	0.007	0.010	0.007	0.000	0.000																															
13	SD5	Cobb Valley	NZAPH012	0.076	0.085	0.083	0.095	0.010	0.007	0.007	0.010	0.007	0.000	0.000	0.000																														
14	SD4	Lake Sylvester	NZAPH044	0.079	0.091	0.088	0.100	0.015	0.013	0.013	0.015	0.012	0.005	0.005	0.005	0.005																													
15	SD5	Cobb Valley	NZAPH040	0.079	0.091	0.088	0.100	0.015	0.013	0.013	0.015	0.012	0.005	0.005	0.005	0.005	0.000																												
16	SD5	Cobb Valley	NZAPH041	0.079	0.091	0.088	0.100	0.015	0.013	0.013	0.015	0.012	0.005	0.005	0.005	0.005	0.000	0.000																												
17	SD4	Lake Sylvester	NZAPH013	0.079	0.091	0.088	0.100	0.015	0.013	0.013	0.015	0.012	0.005	0.005	0.005	0.005	0.000	0.000	0.000																												
18	SD5	Cobb Valley	NZAPH011	0.079	0.091	0.088	0.100	0.015	0.013	0.013	0.015	0.012	0.005	0.005	0.005	0.005	0.000	0.000	0.000	0.000																												
19	SD5	Cobb Valley	RFBAD386-08	0.079	0.091	0.088	0.100	0.015	0.013	0.013	0.015	0.012	0.005	0.005	0.005	0.005	0.000	0.000	0.000	0.000	0.000																												
20	ND3	Egmont	NZAPH132	0.086	0.099	0.096	0.111	0.028	0.025	0.025	0.033	0.025	0.028	0.028	0.028	0.028	0.033	0.033	0.033	0.033	0.033	0.033																											
21	ND3	Egmont	NZAPH062	0.084	0.088	0.086	0.098	0.023	0.020	0.020	0.023	0.020	0.023	0.023	0.023	0.023	0.028	0.028	0.028	0.028	0.028	0.028	0.015																										
22	ND3	Egmont	NZAPH061	0.084	0.091	0.088	0.101	0.025	0.023	0.023	0.025	0.023	0.025	0.025	0.025	0.025	0.030	0.030	0.030	0.030	0.030	0.030	0.018	0.002																									
23	ND3	Egmont	NZAPH059	0.087	0.091	0.088	0.101	0.020	0.018	0.018	0.025	0.018	0.020	0.020	0.020	0.020	0.025	0.025	0.025	0.025	0.025	0.025	0.012	0.002	0.005																								
24	ND3	Egmont	NZAPH060	0.087	0.091	0.088	0.101	0.020	0.018	0.018	0.025	0.018	0.020	0.020	0.020	0.020	0.025	0.025	0.025	0.025	0.025	0.025	0.012	0.002	0.005	0.000																							
25	ND3	Egmont	NZAPH058	0.084	0.088	0.086	0.098	0.018	0.015	0.015	0.023	0.015	0.018	0.018	0.018	0.018	0.023	0.023	0.023	0.023	0.023	0.023	0.015	0.005	0.007	0.002	0.002																						
26	SD7	Mt Lyford	NZAPH055	0.065	0.082	0.079	0.091	0.044	0.042	0.042	0.039	0.030	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.033	0.046	0.036	0.038	0.038	0.038	0.036																				
27	SD7	Mt Lyford	NZAPH056	0.079	0.096	0.093	0.099	0.057	0.055	0.055	0.052	0.046	0.046	0.046	0.046	0.046	0.046	0.046	0.046	0.046	0.046	0.046	0.060	0.049	0.052	0.052	0.052	0.049	0.020																			
28	SD7	Mt Lyford	NZAPH054	0.068	0.094	0.091	0.091	0.055	0.052	0.052	0.050	0.044	0.044	0.044	0.044	0.044	0.044	0.044	0.044	0.044	0.044	0.044	0.060	0.049	0.052	0.052	0.052	0.049	0.018	0.007																	
29		Schizaphis rotundiventris	AF220511.1	0.088	0.107	0.104	0.098	0.079	0.077	0.077	0.080	0.076	0.074	0.074	0.074	0.074	0.076	0.076	0.076	0.076	0.076	0.076	0.087	0.071	0.073	0.073	0.073	0.071	0.060	0.068	0.065																
30		Schizaphis scirpicola	RDBA421-06	0.096	0.104	0.101	0.113	0.082	0.080	0.080	0.083	0.079	0.077	0.077	0.077	0.077	0.079	0.079	0.079	0.079	0.079	0.079	0.090	0.077	0.079	0.079	0.079	0.077	0.078	0.080	0.077	0.039															
31	SA9	Korowai	NZAPH015	0.080	0.080	0.077	0.090	0.086	0.084	0.084	0.087	0.083	0.081	0.081	0.081	0.081	0.083	0.083	0.083	0.083	0.083	0.083	0.095	0.087	0.090	0.090	0.090	0.087	0.078	0.089	0.090	0.066	0.081														
32	SA11	Mt Barossa	NZAPH023	0.080	0.080	0.077	0.090	0.086	0.084	0.084	0.087	0.083	0.081	0.081	0.081	0.081	0.083	0.083	0.083	0.083	0.083	0.083	0.095	0.087	0.090	0.090	0.090	0.087	0.078	0.089	0.090	0.066	0.081	0.000												
33	SA11	Mt Barossa	NZAPH024	0.080	0.080	0.077	0.090	0.086	0.084	0.084	0.087	0.083	0.081	0.081	0.081	0.081	0.083	0.083	0.083	0.083	0.083	0.083	0.095	0.087	0.090	0.090	0.090	0.087	0.078	0.089	0.090	0.066	0.081	0.000	0.000											
34	SA8	St James	NZAPH050	0.080	0.080	0.077	0.090	0.086	0.084	0.084	0.087	0.083	0.081	0.081	0.081	0.081	0.083	0.083	0.083	0.083	0.083	0.083	0.095	0.087	0.090	0.090	0.090	0.087	0.078	0.089	0.090	0.066	0.081	0.000	0.000	0.000										
35	SA10	Porter's Pass	RFBAD391-08	0.080	0.080	0.077	0.090	0.086	0.084	0.084	0.087	0.083	0.081	0.081	0.081	0.081	0.083	0.083	0.083	0.083	0.083	0.083	0.095	0.087	0.090	0.090	0.090	0.087	0.078	0.089	0.090	0.066	0.081	0.000	0.000	0.000	0.000									
36	SA10	Porters Pass	NZAPH008	0.080	0.080	0.077	0.090	0.086	0.084	0.084	0.087	0.083	0.081	0.081	0.081	0.081	0.083	0.083	0.083	0.083	0.083	0.083	0.095	0.087	0.090	0.090	0.090	0.087	0.078	0.089	0.090	0.066	0.081	0.000	0.000	0.000	0.000	0.000							
37	SA11	Mt Barossa	NZAPH018	0.080	0.080	0.077	0.090	0.086	0.084	0.084	0.087	0.083	0.081	0.081	0.081	0.081	0.083	0.083	0.083	0.083	0.083	0.083	0.095	0.087	0.090	0.090	0.090	0.087	0.078	0.089	0.090	0.066	0.081	0.000	0.000	0.000	0.000	0.000	0.000						
38	SA10	Porter's Pass	NZAPH037	0.080	0.080	0.077	0.090	0.086	0.084	0.084	0.087	0.083	0.081	0.081	0.081	0.081	0.083	0.083	0.083	0.083	0.083	0.083	0.095	0.087	0.090	0.090	0.090	0.087	0.078	0.089	0.090	0.066	0.081	0.000	0.000	0.000	0.000	0.000	0.000	0.000					
39	SA9	Korowai	NZAPH017	0.080	0.080	0.077	0.090	0.086	0.084	0.084	0.087	0.083	0.081	0.081	0.081	0.081	0.083	0.083	0.083	0.083	0.083	0.083	0.095	0.087	0.090	0.090	0.090	0.087	0.078	0.089	0.090	0.066	0.081	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000				
40	SA11	Mt Barossa	NZAPH022	0.080	0.080	0.077	0.090	0.086	0.084	0.084	0.087	0.083	0.081	0.081	0.081	0.081	0.083	0.083	0.083	0.083	0.083	0.083	0.095	0.087	0.090	0.090	0.090	0.087	0.078	0.089	0.090	0.066	0.081	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000			
41	SA11	Mt Barossa - 1	NZAPH019	0.097	0.111	0.108	0.130	0.110	0.107	0.107	0.110	0.107	0.104	0.104	0.104	0.104	0.107	0.107	0.107	0.107	0.107	0.107	0.117	0.104	0.107	0.106	0.106	0.104	0.105	0.116	0.117	0.092	0.107	0.072	0.072	0.072	0.072	0.072	0.072	0.072	0.072	0.072	0.072		
42	SA10	Porter's Pass - 1	NZAPH129	0.091	0.098	0.095	0.121	0.094	0.092	0.092	0.095	0.091	0.089	0.089	0.089	0.089	0.092	0.092	0.092	0.092	0.092	0.092	0.105	0.095	0.098	0.098	0.098	0.095	0.102	0.113	0.114	0.095	0.111	0.063	0.063	0.063	0.063	0.063	0.063	0.063	0.063	0.063	0.063	0.033	
43	SA10	Porter's Pass - 1	NZAPH130	0.091	0.098	0.095	0.121	0.094	0.092	0.092	0.095	0.091	0.089	0.089	0.089	0.089	0.092	0.092	0.092	0.092	0.092	0.092	0.105	0.095	0.098	0.098	0.098	0.095	0.102	0.113	0.114	0.095	0.111	0.063	0.063	0.063	0.063	0.063	0.063	0.063	0.063	0.063	0.063	0.033	0.000

Appendix 2 List of species

Genus/Species	Sample ID	Site Name	Province/Country	Host Plant	Collector	BOLD ID*	Source
Aphidinae: Aphidini: Aphidina							
Aphis coprosmae	Aph_cop_SRB004	Banks Peninsula	Canterbury/NZ	*Coprosma rubra*	S. Bulman	NZAPH068	Plant & Food Research
Aphis coprosmae	CP055	Pureora	Manawatu-Wanganui/NZ	*Hebe* sp.		NZAPH035	Soil Aphids of NZ
Aphis cottieri	CNC#HEM056596	Kaitorete Spit	Canterbury/NZ	*unknown*	G. Drayton	RFBAC097-07	Aphids of NZ
Aphis cottieri	CNC#HEM056672	Lake Forsyth	Canterbury/NZ	*unknown*	unknown	RFBAC134-07	Aphids of NZ
Aphis cottieri	A_cotKS_NA1701_SRB005	Kaitorete Spit	Canterbury/NZ	*Meuhlenbeckia complexa*	G. Drayton	NZAPH069	Plant & Food Research
Aphis cottieri	A_cotKS_NA1702_SRB006	Kaitorete Spit	Canterbury/NZ	*Meuhlenbeckia complexa*	G. Drayton	NZAPH070	Plant & Food Research
Aphis cottieri	A_cotKS_NA1802_SRB007	Kaitorete Spit	Canterbury/NZ	*Meuhlenbeckia complexa*	G. Drayton	NZAPH071	Plant & Food Research
Aphis cottieri	Acott_Tumbledown_69_SRB008	Banks Peninsula	Canterbury/NZ	*Meuhlenbeckia complexa*	S. Bulman	NZAPH072	Plant & Food Research
Aphis cottieri	Acott_Magnet_69_SRB009	Banks Peninsula	Canterbury/NZ	*Meuhlenbeckia complexa*	S. Bulman	NZAPH073	Plant & Food Research
Aphis cottieri	Acott_Lwairewa_SRB010	Banks Peninsula	Canterbury/NZ	*Meuhlenbeckia complexa*	S. Bulman	NZAPH074	Plant & Food Research
Aphis craccivora	CNC#HEM072065	Little Shoal Bay	Auckland/NZ	*Vicia sativa*	NA. Martin	RFNZ045-12	Aphids of NZ
Aphis craccivora	CP006	AgResearch	Waikato/NZ	Graminae		NZAPH025	Soil Aphids of NZ
Aphis fabae	Aphis_fivefinger(assembly)_SRB011	Banks Peninsula	Canterbury/NZ	*Pseudopanax arboreus*	S. Bulman	NZAPH075	Plant & Food Research
Aphis gossypii	CNC#HEM056598	Le Bons Bay	Canterbury/NZ	*unknown*	S. Bulman	RFBAC099-07	Aphids of NZ
Aphis gossypii	CNC#HEM061667	Glen Eden	Auckland/NZ	succulent	R. Henderson	RFBAD423-08	Aphids of NZ
Aphis gossypii	CNC#HEM061670	Preston Ave	Auckland/NZ	*Cucurbita pepo*	OR. Green	RFBAD426-08	Aphids of NZ
Aphis gossypii	CNC#HEM064124	Rangitoto Is	Auckland/NZ	*Hebe stricta*	NA. Martin	RFBAE154-09	Aphids of NZ
Aphis gossypii	CNC#HEM072066.1	Mt Wellington	Auckland/NZ	*Kalanchoe blossfeldiana*	NA. Martin	RFNZ049-12	Aphids of NZ
Aphis healyi	CNC#HEM064100	Palmer Rd	West Coast/NZ	*Carmichaelia odorata*	C. Till	RFBAE141-09	Aphids of NZ
Aphis healyi	A_heaBGRR_NA1501_SRB012	Blue Grey River	West Coast/NZ	*Carmichaelia* sp.	M. Stufkens	NZAPH076	Plant & Food Research
Aphis healyi	A_heaBGRR_NA1502_SRB018	Blue Grey River	West Coast/NZ	*Carmichaelia* sp.	M. Stufkens	NZAPH077	Plant & Food Research
Aphis healyi	A_heaHT_NA1001_SRB014	Hihitahi, Nth Island	Manawatu-Wanganui/NZ	*Carmichaelia* sp.	M. Stufkens	NZAPH078	Plant & Food Research
Aphis healyi	A_heaHT_NA1002_SRB015	Hihitahi, Nth Island	Manawatu-Wanganui/NZ	*Carmichaelia* sp.	M. Stufkens	NZAPH079	Plant & Food Research
Aphis hederae	CNC#HEM061674	Pleasant Rd	Auckland/NZ	*Hedera helix*	R. Henderson	RFBAE430-08	Aphids of NZ
Aphis nerii	CNC#HEM056602	Fern Dr	Canterbury/NZ	*unknown*	A. Inder	RFBAC102-07	Aphids of NZ
Aphis nerii	CNC#HEM061659	Preston Ave	Auckland/NZ	*Asclepias* sp. *(swan plant)*	OR. Green	RFBAD415-08	Aphids of NZ
Aphis nerii	Aph_ner_SRB016	Verona	Italy	*unknown*	S. Bulman	NZAPH080	Plant & Food Research
Aphis nerii	A_ nerii_140523_2_SRB017	Christchurch	Canterbury/NZ	*Asclepias* sp. *(swan plant)*	S. Bulman	NZAPH081	Plant & Food Research
Aphis nerii	CP029	Pureora	Manawatu-Wanganui/NZ	*Pittosporum* sp.		NZAPH030	Soil Aphids of NZ
Aphis spiraecola	CNC#HEM061664	Smiths Bush East	Auckland/NZ	*Parsonia hetero*	NA. Martin	RFBAD420-08	Aphids of NZ
Aphis spiraecola	CNC#HEM061665	Tamaki Campus	Auckland/NZ	*Pittisporum teunifolium*	RE. Beever	RFBAD421-08	Aphids of NZ
Aphis spiraecola	CNC#HEM072024	Manurewa	Auckland/NZ	*Schleffera digitata*	NA. Martin	RFNZ055-12	Aphids of NZ
Aphis spiraecola	Aph_spir_SRB018	Christchurch	Canterbury/NZ	*unknown*	S. Bulman	NZAPH082	Plant & Food Research
Aphis waiwera	Aphis_waiwera_69_cns_SRB052	Lake Wairewa, Little River	Canterbury/NZ	*Clematis foetida*	S. Bulman	NZAPH116	Plant & Food Research
Casimira sp.	C_Ozot_NA0501_SRB074	Catlins	Southland/Otago/NZ	*Ozothamnus* sp.	M. Stufkens	NZAPH138	Plant & Food Research
Casimira sp.	C_Ozot_NA0502_SRB075	Catlins	Southland/Otago/NZ	*Ozothamnus* sp.	M. Stufkens	NZAPH139	Plant & Food Research
Paradoxaphis aristoteliae	CNC#HEM064095	Dolamore Park	Southland/NZ	*Aristotelia serrata*	D. Teulon	RFBAE135-09	Aphids of NZ
Paradoxaphis aristoteliae	P_ arist_SRB058	Korowai Reserve	Canterbury/NZ	*Aristotelia fruticosa*	S. Bulman	NZAPH122	Plant & Food Research
Paradoxaphis plagianthi	P_plaChBotGdns_NA0901_SRB053	Christchurch Bot. Gdns	Canterbury/NZ	*Plagianthus regius*	G. Drayton	NZAPH117	Plant & Food Research
Paradoxaphis plagianthi	P_plaChBotGdns_NA0902_SRB054	Christchurch Bot. Gdns	Canterbury/NZ	*Plagianthus regius*	G. Drayton	NZAPH118	Plant & Food Research
Paradoxaphis plagianthi	P_plaDeansBush_NA0401_SRB055	Christchurch Riccarton Bush	Canterbury/NZ	*Plagianthus regius*	G. Drayton	NZAPH119	Plant & Food Research
Paradoxaphis plagianthi	P_plaDeansBush_NA0402_SRB056	Christchurch Riccarton Bush	Canterbury/NZ	*Plagianthus regius*	G. Drayton	NZAPH120	Plant & Food Research
Paradoxaphis plagianthi	Paradoxaphis_ex_wineberry(ass'bly)_SRB057	Banks Peninsula	Canterbury/NZ	wineberry	S. Bulman	NZAPH121	Plant & Food Research

Genus/Species	Sample ID	Site Name	Province/Country	Host Plant	Collector	BOLD ID*	Source
Aphidinae: Aphidini: Rhopalosiphina							
Paradoxaphis plagianthi	CP035	Kaituna	Canterbury/NZ	*Plagianthus regius*		NZAPH007	Soil Aphids of NZ
Toxoptera aurantii	CNC#HEM061657	Dingle Del Reserve	Auckland/NZ	*Myrsine australis*	R. Henderson	RFBAD413-08	Aphids of NZ
Toxoptera aurantii	CNC#HEM061672	Dingle Del Reserve	Auckland/NZ	*Myrsine australis*	R. Henderson	RFBAD428-08	Aphids of NZ
Toxoptera aurantii	CNC#HEM072046	Auckland Domain	Auckland/NZ	*Metrosideros robusta*	NA. Martin	RFNZ065-12	Aphids of NZ
Toxoptera aurantii	Toxoptera_auranti_Camelia_69_2_SRB072	13A Hackthorne Rd	Canterbury/NZ	*Camellia* sp.	S. Bulman	NZAPH136	Plant & Food Research
Rhopalosiphum cerasifoliae	CNC#HEM010262		Saschatchewan/Canada	*Prunus virginiana*	E.Maw	RDBA698-06	Barcoding the Aphididae
Rhopalosiphum cerasifoliae	CNC#HEM007432		Ontario/Canada	*Prunus virginiana*	E.Maw	RDBA699-06	Barcoding the Aphididae
Rhopalosiphum cerasifoliae	CNC#HEM032973		British Columbia/Canada	*Prunus virginiana*	E.Maw	RDBA710-06	Barcoding the Aphididae
Rhopalosiphum cerasifoliae	CNC#HEM007545		New Brunswick/Canada	*Prunus virginiana*	E. Maw	RDBA697-06	Barcoding the Aphididae
Rhopalosiphum enigmae	CNC#HEM049321		Tennessee/US	*Typha latifolia*	E.Maw	RDBA160-05	Barcoding the Aphididae
Rhopalosiphum insertum	CP067a	Chateau Tongariro	Manawatu-Wanganui/NZ	*Dracophyllum* sp.	C.Podmore	NZAPH057	Soil Aphids of NZ
Rhopalosiphum maidis	CNC#HEM054017.2		Ontario/Canada	*Zea mays*	E.Maw	RDBA375-05	Barcoding the Aphididae
Rhopalosiphum musae	CNC#HEM064117	Waiatarua Res	Auckland/NZ	*Juncus gregiflorus*	NA. Martin	RFBAE148-09	Aphids of NZ
Rhopalosiphum nr. insertum	Rho_nr.ins_SRB060	Lincoln	Canterbury/NZ	Apple	M. Stufkens	NZAPH124	Plant & Food Research
Rhopalosiphum nymphaeae	CNC#HEM056626	Oderings Nursery	Canterbury/NZ	*unknown*	J. Fletcher	RFBAC126-07	Aphids of NZ
Rhopalosiphum nymphaeae	CNC#HEM051877		Hawaii/US	*Nymphaea alba*	R. Millar	RDBA278-05	Barcoding the Aphididae
Rhopalosiphum nymphaeae	Rho_nym_SRB061	Christchurch	Canterbury/NZ	Water lily	M. Stufkens	NZAPH125	Plant & Food Research
Rhopalosiphum oxyacanthae	CNC#HEM007472		Ontario/Canada	*Crataegus mollis*	E. Maw	RDBA079-05	Barcoding the Aphididae
Rhopalosiphum oxyacanthae	CNC#HEM007427		Ontario/Canada	*Crataugus* sp.	E. Maw	RDBA458-06	Barcoding the Aphididae
Rhopalosiphum padi	CNC#HEM056627	Pye Rd	Canterbury/NZ	*unknown*	D. James	RFBAC127-07	Aphids of NZ
Rhopalosiphum padi	CNC#HEM061654	Landcare	Canterbury/NZ	*unknown*	unknown	RFBAD410-08	Aphids of NZ
Rhopalosiphum padi	CNC#HEM072051.1	Mt Wellington	Auckland/NZ	*Hesperantha pearsonii*	NA. Martin	RFNZ013-12	Aphids of NZ
Rhopalosiphum padi	CNC#HEM007396		Ontario/Canada	*Prunus virginiana*	E. Maw	RDBA470-06	Barcoding the Aphididae
Rhopalosiphum padi	CNC#HEM055880		Washington/US	*Musa* sp.	K.S Pike	RDBA034-06	Barcoding the Aphididae
Rhopalosiphum padi	CNC#HEM025924		Ontario/Canada	*Prunus nigra*	E. Maw	RDBA090-05	Barcoding the Aphididae
Rhopalosiphum padi	Rho_pad2_SRB062	Lincoln	Canterbury/NZ	wheat	M. Stufkens	NZAPH126	Plant & Food Research
Rhopalosiphum padi	Rho_pad1_SRB063	Lincoln	Canterbury/NZ	wheat	M. Stufkens	NZAPH127	Plant & Food Research
Rhopalosiphum padi	CP015	AgResearch	Waikato/NZ	Graminae	C.Podmore	NZAPH028	Soil Aphids of NZ
Rhopalosiphum padi	CP030	Pureora	Manawatu-Wanganui/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH032	Soil Aphids of NZ
Rhopalosiphum rufiabdominale	CNC#HEM053450		Ontario/Canada	*Lycopersicon esculentum*	G.Zilahi-Balogh	RDBA148-05	Barcoding the Aphididae
Rhopalosiphum sp.	CNC#HEM062938.1	Mt Benger	Otago/NZ	*unknown*	B. Barrett	RFBAE001-09	Aphids of NZ
Schizaphis graminum	CNC#HEM040487		Ontario/Canada	*Hordeum jubatum*	E. Maw	RDBA262-05	Barcoding the Aphididae
Schizaphis rotundiventris	AF220511.1					GenBank	GenBank
Schizaphis scirpicola	CNC#HEM012135		Ontario/Canada	*Scirpus atrocinctus*	E. Maw	RDBA421-06	Barcoding the Aphididae
Schizaphis sp.	CNC#HEM059961	Cobb Valley	Tasman/NZ	*Dracophyllum sp.*	D. Teulon	RFBAD386-0	Aphids of NZ
Schizaphis sp.	CNC#HEM061642	Porter's Pass	Canterbury/NZ	*Aciphylla sp.*	C. Till	RFBAD391-08	Aphids of NZ
Schizaphis sp.	E_DracPureora_NA1302_SRB064	Pureora	Manawatu-Wanganui/NZ	*Dracophyllum* sp.	M. Stufkens	NZAPH128	Plant & Food Research
Schizaphis sp.	E_AciPorPass_NA2401_SRB065	Porter's Pass	Canterbury/NZ	*Aciphylla* sp.	M. Stufkens	NZAPH129	Plant & Food Research
Schizaphis sp.	E_AciPorPass_NA2402_SRB066	Porter's Pass	Canterbury/NZ	*Aciphylla* sp.	M. Stufkens	NZAPH130	Plant & Food Research
Schizaphis sp.	CobbValleyEusCO1_cns_SRB067	Cobb Valley	Tasman/NZ	*Dracophyllum* sp.	S. Bulman	NZAPH131	Plant & Food Research
Schizaphis sp.	E_DracEgmont_NA1202_SRB068	Egmont National Park	Taranaki/NZ	*Dracophyllum* sp.	M. Stufkens	NZAPH132	Plant & Food Research
Schizaphis sp.	E_DracArtPass_NA1602_SRB069	Arthur's Pass	Canterbury/NZ	*Dracophyllum* sp.	M. Stufkens	NZAPH133	Plant & Food Research
Schizaphis sp.	CP036	Porter's Pass	Canterbury/NZ	*Aciphylla* sp.	S.Bulman	NZAPH008	Soil Aphids of NZ
Schizaphis sp.	CP039	Cobb Valley	Tasman/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH011	Soil Aphids of NZ

Genus/Species	Sample ID	Site Name	Province/Country	Host Plant	Collector	BOLD ID*	Source
Aphidinae:Aphidini:Rhopalosiphina							
Schizaphis sp.	CP040	Cobb Valley	Tasman/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH012	Soil Aphids of NZ
Schizaphis sp.	CP041a	Lk. Sylvester	Tasman/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH013	Soil Aphids of NZ
Schizaphis sp.	CP057a	Korowai	Canterbury/NZ	*Aciphylla* sp.	S.Bulman	NZAPH015	Soil Aphids of NZ
Schizaphis sp.	CP057c	Korowai	Canterbury/NZ	*Aciphylla* sp.	S.Bulman	NZAPH017	Soil Aphids of NZ
Schizaphis sp.	CP058a	Mt. Barossa	Canterbury/NZ	*Aciphylla* sp.	S.Bulman	NZAPH018	Soil Aphids of NZ
Schizaphis sp.	CP058b	Mt. Barossa	Canterbury/NZ	*Aciphylla* sp.	S.Bulman	NZAPH019	Soil Aphids of NZ
Schizaphis sp.	CP059a	Mt. Barossa	Canterbury/NZ	*Aciphylla* sp.	S.Bulman	NZAPH022	Soil Aphids of NZ
Schizaphis sp.	CP059b	Mt. Barossa	Canterbury/NZ	*Aciphylla* sp.	S.Bulman	NZAPH023	Soil Aphids of NZ
Schizaphis sp.	CP059c	Mt. Barossa	Canterbury/NZ	*Aciphylla* sp.	S.Bulman	NZAPH024	Soil Aphids of NZ
Schizaphis sp.	CP054	Tukino	Manawatu-Wanganui/NZ	*Dracophyllum* sp.	C.Podmore	NZAPH034	Soil Aphids of NZ
Schizaphis sp.	CP036b	Porter's Pass	Canterbury/NZ	*Aciphylla* sp.	S.Bulman	NZAPH037	Soil Aphids of NZ
Schizaphis sp.	CP039b	Cobb Valley	Tasman/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH040	Soil Aphids of NZ
Schizaphis sp.	CP039c	Cobb Valley	Tasman/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH041	Soil Aphids of NZ
Schizaphis sp.	CP040b	Cobb Valley	Tasman/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH042	Soil Aphids of NZ
Schizaphis sp.	CP040c	Cobb Valley	Tasman/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH043	Soil Aphids of NZ
Schizaphis sp.	CP041b	Lk. Sylvester	Tasman/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH044	Soil Aphids of NZ
Schizaphis sp.	CP041c	Lk. Sylvester	Tasman/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH045	Soil Aphids of NZ
Schizaphis sp.	CP062	St. James	Canterbury/NZ	*Aciphylla* sp.	S.Bulman	NZAPH050	Soil Aphids of NZ
Schizaphis sp.	CP063	Arthur's Pass	Canterbury/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH051	Soil Aphids of NZ
Schizaphis sp.	CP065b	Mt. Lyford	Canterbury/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH054	Soil Aphids of NZ
Schizaphis sp.	CP065c	Mt. Lyford	Canterbury/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH055	Soil Aphids of NZ
Schizaphis sp.	CP065d	Mt. Lyford	Canterbury/NZ	*Dracophyllum* sp.	S.Bulman	NZAPH056	Soil Aphids of NZ
Schizaphis sp.	CP069	Egmont National Park	Taranaki/NZ	*Dracophyllum* sp.	C.Podmore	NZAPH058	Soil Aphids of NZ
Schizaphis sp.	CP071	Egmont National Park	Taranaki/NZ	*Dracophyllum* sp.	C.Podmore	NZAPH059	Soil Aphids of NZ
Schizaphis sp.	CP072	Egmont National Park	Taranaki/NZ	*Dracophyllum* sp.	C.Podmore	NZAPH060	Soil Aphids of NZ
Schizaphis sp.	CP073a	Egmont National Park	Taranaki/NZ	*Dracophyllum* sp.	C.Podmore	NZAPH061	Soil Aphids of NZ
Schizaphis sp.	CP073b	Egmont National Park	Taranaki/NZ	*Dracophyllum* sp.	C.Podmore	NZAPH062	Soil Aphids of NZ
Schizaphis sp. A rgf-2008	CNC#HEM050100	Arthur's Pass	Canterbury/NZ	*Dracophyllum* sp.	D. Teulon	RDBA480-06	Barcoding the Aphididae
Schizaphis sp. *A. RGF-2008*	EU701901.1	ex RDBA480-06	Canterbury/NZ	*Dracophyllum* sp.	D. Teulon	GenBank	GenBank
Aphidinae:Macrosiphini							
Acyrthosiphon kondai	CNC#HEM056616	Kaitorete Spit	Canterbury/NZ	*unknown*	G. Drayton	RFBAC116-07	Aphids of NZ
Acyrthosiphon kondai	Acy_kon2_SRB001	Lincoln	Canterbury/NZ	*unknown*	M. Stufkens	NZAPH065	Plant & Food Research
Acyrthosiphon kondai	Acy_kon2_SRB002	Lincoln	Canterbury/NZ	*unknown*	M. Stufkens	NZAPH065	Plant & Food Research
Acyrthosiphon malvae	CNC#HEM056619	Spreydon	Canterbury/NZ	*unknown*	W. Nelson	RFBAC119-07	Aphids of NZ
Acyrthosiphon malvae	Acy_mal_SRB002	Lincoln	Canterbury/NZ	*unknown*	M. Stufkens	NZAPH066	Plant & Food Research
Acyrthosiphon pisum	CNC#HEM059953	Lincoln Uni	Canterbury/NZ	Broadbean	C. Till	RFNBAD378-08	Aphids of NZ
Acyrthosiphon pisum	Acy_pis_SRB003	Lincoln	Canterbury/NZ	pea	M. Stufkens	NZAPH067	Plant & Food Research
Amphoraphora rubi	CNC#HEM072020.1	Waitakere Ranges	Auckland/NZ	*Rubus australis*	NA. Martin	RFNZ033-12	Aphids of NZ
Amphoraphora rubi	CNC#HEM072020.2	Waitakere Ranges	Auckland/NZ	*Rubus australis*	NA. Martin	RFNZ034-12	Aphids of NZ
Amphoraphora rubi	Arubraxxxx_SRB019	Christchurch	Canterbury/NZ	blackberry	M. Stufkens	NZAPH083	Plant & Food Research
Aulacorthum solani	CNC#HEM056621	Torvill & Dean Lane	Canterbury/NZ	*unknown*	D. Teulon	RFBAC121-07	Aphids of NZ
Aulacorthum solani	CNC#HEM064126	Lakeside Park	Northland/NZ	*Microtis unifolia*	NA. Martin	RFBAE156-09	Aphids of NZ
Aulacorthum solani	CNC#HEM072014	Mt Albert	Auckland/NZ	*Coprosma repens*	NA. Martin	RFNZ031-12	Aphids of NZ
Aulacorthum solani	CNC#HEM072054	Tahuna Torea Nature Res	Auckland/NZ	*Salix fragilis*	NA. Martin	RFNZ056-12	Aphids of NZ

Genus/Species	Sample ID	Site Name	Province/Country	Host Plant	Collector	BOLD ID*	Source
Aphidinae:Macrosiphini							
Aulacorthum solani	Aul_solNZ_SRB020	Lincoln	Canterbury/NZ	potato	M. Stufkens	NZAPH084	Plant & Food Research
Brachycaudus helichrysii	CNC#HEM056607	Greenpark	Canterbury/NZ	*unknown*	M. Marshall	RFBAC107-07	Aphids of NZ
Brachycaudus helichrysii	CNC#HEM059957	Tautuka Outdoor Centre	Otago/NZ	*Ozothamum*	D. Teulon	RFBAD382-08	Aphids of NZ
Brachycaudus helichrysii	Bra_hel_SRB021	Christchurch	Canterbury/NZ	*Helichrysum* sp.	S. Bulman	NZAPH085	Plant & Food Research
Brachycaudus helichrysii	CP064	Mt. Lyford	Canterbury/NZ	*Dracophyllum* sp.	S. Bulman	NZAPH052	Soil Aphids of NZ
Brachycaudus rumexicolens	CNC#HEM059864	Plant & Food, Lincoln	Canterbury/NZ	*Rumex*	C. Fletcher	RFBAD208-08	Aphids of NZ
Brachycaudus rumexicolens	Bpers_Ghgreen_69_cns_SRB022	Banks Peninsula	Canterbury/NZ	*unknown*	S. Bulman	NZAPH086	Plant & Food Research
Brachycaudus rumexicolens	Bpersicae_CO1_cns_SRB023	Banks Peninsula	Canterbury/NZ	*unknown*	S. Bulman	NZAPH087	Plant & Food Research
Brachycaudus rumexicolens	Kaik_Brachy_mtd69_241108_cns_SRB024	Kaikoura	Canterbury/NZ	*Mueblenbeckia* sp.	S. Bulman	NZAPH088	Plant & Food Research
Brevicoryne brassicae	CNC#HEM056623	Royal Park Dr	Auckland/NZ	*unknown*	J. Kaiser	RFBAC123-07	Aphids of NZ
Brevicoryne brassicae	CNC#HEM061661	Preston Ave	Auckland/NZ	*Brassica oleraceae*	OR Green	RFBAD417-08	Aphids of NZ
Brevicoryne brassicae	Bre_bra_SRB025	Lincoln	Canterbury/NZ	Broccoli	S. Bulman	NZAPH089	Plant & Food Research
Capitophorus eleaegni	CNC#HEM061641	Plant & Food, Lincoln	Canterbury/NZ	*Carduinae*	C. Till	RFBAD390-08	Aphids of NZ
Capitophorus eleaegni	CNC#HEM072031	Howick	Auckland/NZ	*Cynara cardunculus*	NA. Martin	RFNZ032-12	Aphids of NZ
Capitophorus eleaegni	Capitophorus_SRB026	Plant & Food Res, Lincoln	Canterbury/NZ	*Elaeagnus* spp	S. Bulman	NZAPH090	Plant & Food Research
Cavariella aegopodii	CNC#HEM056600	Millstream Dr	Canterbury/NZ	*unknown*	R. van Toor	RFBAC101-07	Aphids of NZ
Cavariella aegopodii	CP037	Mt. Somers	Canterbury/NZ	*Aciphylla* sp.	S. Bulman	NZAPH009	Soil Aphids of NZ
Cavariella aegopodii	CP038	Mt. Somers	Canterbury/NZ	*Aciphylla* sp.	S. Bulman	NZAPH010	Soil Aphids of NZ
Chaetosiphon fragaefolii	CNC#HEM056620	Torvill & Dean Lane	Canterbury/NZ	*unknown*	D. Teulon	RFBAC120-07	Aphids of NZ
Chaetosiphon tetrarhodus	Cha_tet_SRB027	New Regent St, Christchurch	Canterbury/NZ	Rose	S. Bulman	NZAPH091	Plant & Food Research
Dysaphis aucupariae	CNC#HEM061640	Ward's Rd	Canterbury/NZ	plantain	C. Till	RFBAD389-08	Aphids of NZ
Dysaphis aucupariae	Dys_auc_SRB028	SHW75 corner, Halswell River	Canterbury/NZ	Plantain	S. Bulman	NZAPH092	Plant & Food Research
Dysaphis foeniculi	Dys_foe_SRB029	13A Hackthorne Rd	Canterbury/NZ	*Aciphylla* sp.	S. Bulman	NZAPH093	Plant & Food Research
Dysaphis tulipae	Dya_tul_SRB030	Plant & Food Res, Lincoln	Canterbury/NZ	Bearded iris	S. Bulman	NZAPH094	Plant & Food Research
Elatobium abietinum	Ela_abi_SRB031	Lincoln	Canterbury/NZ	Pine tree	M. Stufkens	NZAPH095	Plant & Food Research
Hyadaphis passerini	CNC#HEM056625	Anderson Ave	Canterbury/NZ	*unknown*	C. Wilde	RFBAC125-07	Aphids of NZ
Hyperomyzus lactucae	CNC#HEM056614	Torvill & Dean Lane	Canterbury/NZ	*unknown*	D. Teulon	RFBAC114-07	Aphids of NZ
Hyperomyzus lactucae	Hyp_lac_SRB033	Lincoln	Canterbury/NZ	Sow thistle	M. Stufkens	NZAPH097	Plant & Food Research
Idiopterus nephrelepidis	CNC#HEM056629	Aniseed Valley	Tasman/NZ	*unknown*	I. Scott	RFBAC128-07	Aphids of NZ
Jacksonia papillata	nr_Jacksonia_CO1_SRB034	Jack's Pass, Hanmer	Canterbury/NZ	*unknown*	S. Bulman	NZAPH098	Plant & Food Research
Liosomaphis berberis	Liosomaphis_SRB035	Wright's Bush, Little River	Canterbury/NZ	*unknown*	S. Bulman	NZAPH099	Plant & Food Research
Lipaphis pseudobrassicae	CNC#HEM056594	Boundary Rd	Canterbury/NZ	Pak choi	unknown	RFBAC095-07	Aphids of NZ
Macrosiphum euphorbiae	CNC#HEM056613	Locharburn Res	Otago/NZ	*unknown*	M. Marshall	RFBAC113-07	Aphids of NZ
Macrosiphum euphorbiae	CNC#HEM064123	The Tunnel	Auckland/NZ	*Meuhlenbeckia complexa*	NA. Martin	RFBAD153-09	Aphids of NZ
Macrosiphum euphorbiae	CNC#HEM061328	Waiau St	Canterbury/NZ	*unknown*	D. Teulon	RFBAE100-07	Aphids of NZ
Macrosiphum euphorbiae	Mac_eupNZ_SRB036	Lincoln	Canterbury/NZ	Sow thistle	S. Bulman	NZAPH100	Plant & Food Research
Macrosiphum hellebori	CNC#HEM056595	Yaldhurst	Canterbury/NZ	*Hellebore orientalis*	J. Lyall	RFBAD096-07	Aphids of NZ
Macrosiphum hellebori	CNC#HEM059857	Christchurch Bot. Gdns	Canterbury/NZ	*Hellebore orientalis*	C. Till	RFBAD201-08	Aphids of NZ
Macrosiphum hellebori	Mac_hel_SRB037	Plant & Food Res, Lincoln	Canterbury/NZ	*Helleborus* sp.	S. Bulman	NZAPH101	Plant & Food Research
Macrosiphum rosae	CNC#HEM056622	Estuary Rd	Canterbury/NZ	*unknown*	H. Wilson	RFBAC122-07	Aphids of NZ
Macrosiphum rosae	Mac_ros_SRB038	Christchurch	Canterbury/NZ	Rose	S. Bulman	NZAPH102	Plant & Food Research
Macrosiphum rosae	Mac_rosNZ_SRB039	Christchurch	Canterbury/NZ	Rose	S. Bulman	NZAPH103	Plant & Food Research
Megoura stufkensi	Sp_nov_NA0101_SRB040	Kaitorete Spit	Canterbury/NZ	*Meuhlenbeckia complexa*	M. Stufkens	NZAPH104	Plant & Food Research
Megoura stufkensi	Sp_nov_NA0102_SRB041	Kaitorete Spit	Canterbury/NZ	*Meuhlenbeckia complexa*	M. Stufkens	NZAPH105	Plant & Food Research

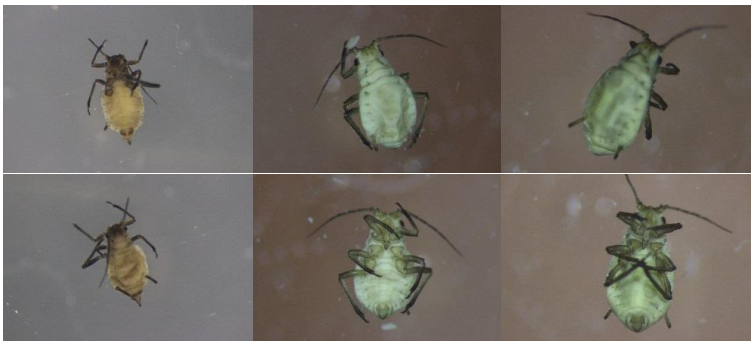
Genus/Species	Sample ID	Site Name	Province/Country	Host Plant	Collector	BOLD ID*	Source
Aphidinae:Macrosiphini							
Megoura stufkensi	Megoura101_69_SRB042	Kaitorete Spit	Canterbury/NZ	*Meuhlenbeckia complexa*	M. Stufkens	NZAPH106	Plant & Food Research
Metopolophium dirhodum	CNC#HEM056624	Opawa	Canterbury/NZ	*unknown*	R. Toonen	RFBAC124-07	Aphids of NZ
Metopolophium dirhodum	Met_dir_SRB043	Lincoln	Canterbury/NZ	wheat	M. Stufkens	NZAPH107	Plant & Food Research
Myzus cerasi	CNC#HEM056608	Waianakarua	Otago/NZ	*unknown*	M. Marshall	RFBAC108-07	Aphids of NZ
Myzus ornatus	Myz_orn_SRB045	Lincoln	Canterbury/NZ	*unknown*	M. Stufkens	NZAPH109	Plant & Food Research
Myzus persicae	CNC#HEM061655	Preston Ave	Auckland/NZ	*Impatiens*	OR Green	RFBAD411-08	Aphids of NZ
Myzus persicae	CNC#HEM072013.1	Tahuna Torea Nature Res	Auckland/NZ	*Myoporum laetum*	NA. Martin	RFNZ005-12	Aphids of NZ
Myzus persicae	CNC#HEM072030	Waiheke Is	Auckland/NZ	*Myoporum laetum*	NA. Martin	RFNZ037-12	Aphids of NZ
Myzus persicae	CNC#HEM072021	Piha	Auckland/NZ	*Tetragonia implexicoma*	NA. Martin	RFNZ044-12	Aphids of NZ
Myzus persicae	Myz_per_SRB046	Lincoln	Canterbury/NZ	potato	M. Stufkens	NZAPH110	Plant & Food Research
Myzus persicae	Mpers_Copros_LR_May08_69_cns_SRB047	Banks Peninsula	Canterbury/NZ	*Coprosma* sp.	S. Bulman	NZAPH111	Plant & Food Research
Nasonovia ribis-nigri	CNC#HEM056604	Cronin Rd	Auckland/NZ	*unknown*	P. Workman	RFBAC104-07	Aphids of NZ
Nasonovia ribis-nigri	Nas_ribNZ_SRB048	Lincoln	Canterbury/NZ	Lettuce	M. Stufkens	NZAPH112	Plant & Food Research
Nasonovia ribis-nigri	CP065	Mt. Lyford	Canterbury/NZ	*Dracophyllum* sp.	S. Bulman	NZAPH053	Soil Aphids of NZ
Nasonovia ribis-nigri	CP074	Stratford Plateau	Taranaki/NZ	*Dracophyllum* sp.	C.Podmore	NZAPH063	Soil Aphids of NZ
Neomyzus circumflexus	CNC#HEM056632	Aniseed Valley	Tasman/NZ	*unknown*	I. Scott	RFBAC131-07	Aphids of NZ
Neomyzus circumflexus	CNC#HEM064120	Albert Park	Auckland/NZ	*Metrosideras robusta*	NA. Martin	RFBAE151-09	Aphids of NZ
Neomyzus circumflexus	CNC#HEM072012.1	Tahuna Torea Nature Res	Auckland/NZ	*Melicytus ramiflorus*	NA. Martin	RFNZ009-12	Aphids of NZ
Neomyzus circumflexus	Aul_cirUK_SRB049_SRB049	Lincoln	Canterbury/NZ	*unknown*	M. Stufkens	NZAPH113	Plant & Food Research
Neomyzus circumflexus	N_circumflexus_CO1_SRB050	Banks Peninsula	Canterbury/NZ	*Mahoe*	S. Bulman	NZAPH114	Plant & Food Research
Neotoxoptera formosana	CNC#HEM056630	Cashmere	Canterbury/NZ	*unknown*	R. Falloon	RFBAC129-07	Aphids of NZ
Neotoxoptera formosana	Neo_tox_SRB051	Trent St, Christchurch	Canterbury/NZ	Onion	S. Bulman	NZAPH115	Plant & Food Research
Ovatus crataegarius	CNC#HEM064104	Wilson's Rd	Canterbury/NZ	*Lamiaceae*	D. Teulon	RFBAE129-09	Aphids of NZ
Rhopalosiphoninus staphyeae	Rstaphyleae_69_100802_SRB052	13A Hackthorne Rd	Canterbury/NZ	*unknown*	S. Bulman	NZAPH123	Plant & Food Research
Sitobion avenae	Sit_mis_SRB070	Lincoln	Canterbury/NZ	Wheat or grass sp.	M. Stufkens	NZAPH134	Plant & Food Research
Sitobion nr. fragariae	Sit_nr_fra_SRB071	Lincoln	Canterbury/NZ	Wheat or grass sp.	M. Stufkens	NZAPH135	Plant & Food Research
Uroleucon sonchi	CNC#HEM056603	Cronin Rd	Auckland/NZ	*unknown*	P, Workman	RFBAC103-07	Aphids of NZ
Uroleucon sonchi	Uro_son_SRB073	unknown	Canterbury/NZ	Sow Thistle	M. Stufkens	NZAPH137	Plant & Food Research
Calaphidinae:Calaphidini							
Euceraphis betulae	CNC#HEM072016	Mt Wellington	Auckland/NZ	*Betula pendula*	NA. Martin	RFNZ029-12	Aphids of NZ
Calaphidinae:Panaphidini							
Myzocallis boerneri	CNC#HEM056612	Waianakarua	Otago/NZ	*unknown*	M. Marshall	RFBAC112-07	Aphids of NZ
Myzocallis carpini	CNC#HEM059859	Christchurch Bot. Gdns	Canterbury/NZ	*Ulmus glabra*	C. Till	RFBAD203-08	Aphids of NZ
Myzocallis carpini	CNC#HEM059958	Christchurch Bot. Gdns	Canterbury/NZ	*Carpinus betulae*	D. Teulon	RFBAD383-08	Aphids of NZ
Myzocallis carpini	CNC#HEM072034.1	Dunedin	Otago/NZ	*Carpinus betulae*	NA. Martin	RFNZ051-12	Aphids of NZ
Myzocallis castanicola	CNC#HEM059863	Lincoln University	Canterbury/NZ	*Quercus* sp.	C. Till	RFBAD207-08	Aphids of NZ
Myzocallis coryli	CNC#HEM056609	Waianakarua	Otago/NZ	*unknown*	M. Marshall	RFBAC109-07	Aphids of NZ
Pterocallis alni	CNC#HEM056631	Christchurch Bot. Gdns	Canterbury/NZ	*unknown*	B. Barrett	RFBAC130-07	Aphids of NZ
Takecallis arundinariae	CNC#HEM061669	Glen Eden	Auckland/NZ	*Phyllostachys bambusoides*	R. Henderson	RFBAD425-08	Aphids of NZ
Takecallis taiwana	CNC#HEM064093	unknown	Auckland/NZ	Poaceae	R. Henderson	RFBAE137-09	Aphids of NZ
Chaitophorinae:Chaitophorini							
Periphyllus californiensis	CNC#HEM056605	Warren Cres	Canterbury/NZ	*unknown*	E. Parr	RFBAC105-07	Aphids of NZ
Periphyllus californiensis	CNC#HEM059860	Christchurch Bot. Gdns	Canterbury/NZ	*Acer palmatum*	C. Till	RFBAD204-08	Aphids of NZ

Genus/Species	Sample ID	Site Name	Province/Country	Host Plant	Collector	BOLD ID*	Source
Drepanosipinae							
Drepanosiphum platanoidis	CNC#HEM056597	Kaitorete Spit	Canterbury/NZ	*unknown*	G. Drayton	RFBAC098-07	Aphids of NZ
Drepanosiphum platanoidis	CNC#HEM072018.1	Cornwall Park	Auckland/NZ	*Acer platanoides*	NA. Martin	RFNZ016-12	Aphids of NZ
Eriosomatinae:Eriosomatini							
Colophina clematicola	CNC#HEM064109.1	Ngaio St	Canterbury/NZ	*Clematis var Monaco*	C.Till	RFBAE124-09	Aphids of NZ
Eriosoma lanigerum	CNC#HEM059861	Massey University	Manawatu-Wanganui/NZ	*Pyrocanthus*	D. Teulon	RFBAD205-08	Aphids of NZ
Eriosomatinae:Fordini							
Aploneura lentisci	CP056	AgResearch	Waikato/NZ	*Graminae*	C. Podmore	NZAPH036	Soil Aphids of NZ
Eriosomatinae:Pemphigini							
Pemphigus bursarius	CNC#HEM064110	Plant & Food, Lincoln	Canterbury/NZ	*Populus*	C.Till	RFBAE122-09	Aphids of NZ
Hormaphidinae:Cerataphidini							
Pseudoregma panicola	CNC#HEM061675	Dingle Del Reserve	Auckland/NZ	*Oplismenus hirtellus*	NA. Martin	RFBAD431-08	Aphids of NZ
Lachninae:Eulachnini							
Essigella californica	CNC#HEM059952	Plant & Food, Lincoln	Canterbury/NZ	*Pinus radiata*	C.Till	RFBAD394-08	Aphids of NZ
Lachninae:Lachnini							
Cinara fresa	CNC#HEM059951	Riverlaw Terrace	Canterbury/NZ	*Thuja* sp.	D. Teulon	RFBAD377-08	Aphids of NZ
Periphyllus testudinaceus	CNC#HEM064107	Plant & Food, Lincoln	Canterbury/NZ	*Acer* sp.	C. Till	RFBAE126-09	Aphids of NZ
Tuberculatus annulatus	CNC#HEM056611	Waianakarua	Otago/NZ	*unknown*	M. Marshall	RFBAC111-07	Aphids of NZ
Tuberculatus annulatus	CNC#HEM059862	Lincoln Uni	Canterbury/NZ	*Quercus* sp.	C. Till	RFBAD206-08	Aphids of NZ
Tuberolachnus salignus	CP043	Hamilton River Walk	Waikato/NZ	*Saliceae*	N. Binks	NZAPH049	Soil Aphids of NZ
Neophyllaphidinae							
Neophyllaphis totarae	CNC#HEM059867	Art Gallery, Christchurch	Canterbury/NZ	*Podacarpa totara*	D. Teulon	RFBAD211-08	Aphids of NZ
Neophyllaphis totarae	CP032	Mangorei Rd Track	Taranaki/NZ	*Dracophyllum* sp.	C. Podmore	NZAPH033	Soil Aphids of NZ
Phyllaphidinae							
Phyllaphis fagi	CNC#HEM056610	Waianakarua	Otago/NZ	*unknown*	M. Marshall	RFBAC110-07	Aphids of NZ
Phyllaphis fagi	CNC#HEM059858	Christchurch Bot. Gdns	Canterbury/NZ	*Fagus sylvatica*	C. Till	RFBAD202-08	Aphids of NZ
Saltusaphidinae							
Thripsaphis foxtonensis	CNC#HEM059964	Papatowai Highway	Otago/NZ	*Carex* sp.	C. Fletcher	RFBAD388-08	Aphids of NZ
Taiwanaphidinae							
Sensoriaphis nothofagi	CNC#HEM056617	Mt Albert Research Centre	Auckland/NZ	*unknown*	D. Teulon	RFBAD117-07	Aphids of NZ

Appendix 3 *Schizaphis* photos

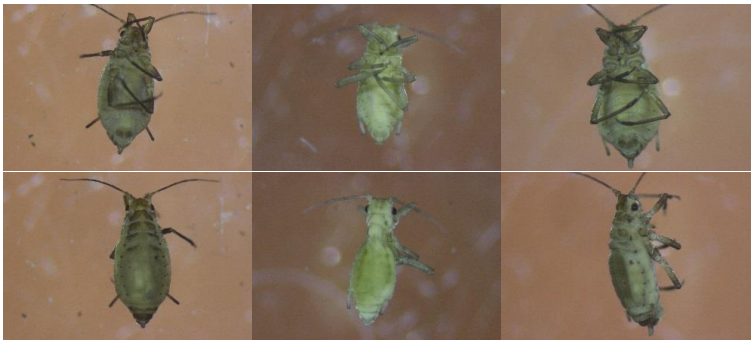
ND2 Tukino

ND3 Egmont National Park



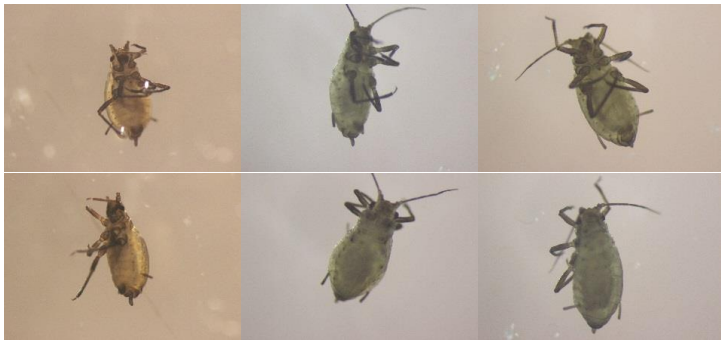
NZAPH034 NZAPH058 NZAPH059

ND3 Egmont National Park



NZAPH060 NZAPH061 NZAPH062

SD4 Lake Sylvester



NZAPH013 NZAPH044 NZAPH045

SD5 Cobb Valley

NZAPH011 40x NZAPH012 40x NZAPH0 40x

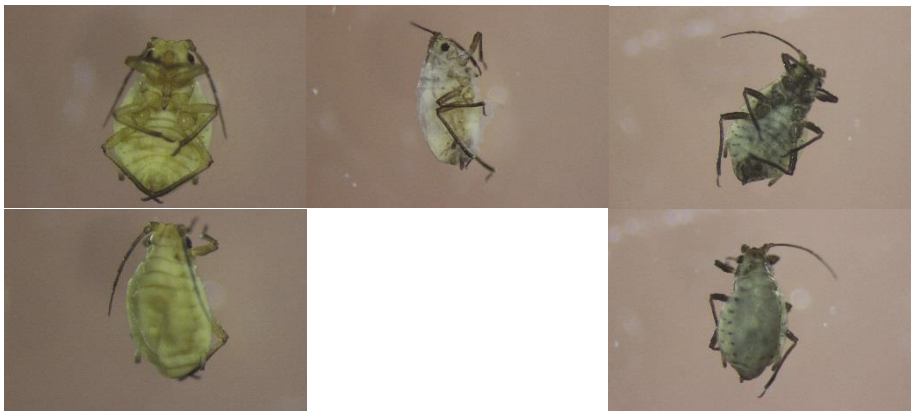
SD5 Cobb Valley

NZAPH041 60x NZAPH042 50x NZAPH043 50x

SD6 Arthur s Pass

NZAPH051 50x

SD7 Mt Lyford



NZAPH054 70x

NZAPH055 50x

NZAPH056 50x

SA8 St James

SA9 Korowai

NZAPH050 50x

NZAPH015 50x

SA10 Porter s Pass

NZAPH008 70x

NZAPH037 40x

Appendix 4: Phenol Chloroform DNA extraction protocol

DNA extraction protocol for soil (Griffiths et al., 2000)

Protocol:

- Weigh 0.5 g soil in 2-ml Lysing Matrix tubes E (containing a mixture of ceramic and silica beads)
- Add 0.5ml of CTAB Buffer (see recipe below)
- Add 0.5 ml of Phenol/Chloroform/Isoamyl alcohol (25:24:1)
- Place tubes in Fast prep machine (30 sec at speed 4)
- Cool tubes on ice (1-2 min)
- Centrifuge top speed for 10 min
- Transfer supernatant in new tube and add equal volume (≈ 0,5 ml) of Chloroform/Isoamyl alcohol (24:1)
- Centrifuge top speed for 10 min
- Transfer supernatant in new tube and add 2x volume of PEG (see recipe below), mix by hand
- Incubate either at room temperature for 2 hours or overnight at $4^{o}C$
- Centrifuge top speed at $4^{o}C$ for at least 30 min
- Remove supernatant and wash with 70% cold ethanol
- Centrifuge at top speed for 10 min
- Dry the pellet and re-suspend in 50 µl of dH_2O or TE buffer pH 7.5

DNA extraction protocol for soil (Griffiths et al., 2000)

Reagents:

5% CTAB/Phosphate Buffer (120mM, pH 8)

For this buffer you have to mix 1:1 **solutions A and B**

Solution A: 10% CTAB in 0.7M NaCl

For 100 ml: NaCl: 4.09 g

CTAB: 10 g

Up to 100 ml with dH2O

Solution B: 240 mM Phosphate buffer pH 8

For 100 ml: 1M K_2HPO_4: 22.56 ml

1M KH_2PO_4: 1.44 ml

dH2O Up to 100 ml

Mix the two solutions, treat with 0.1% DEPC over night and autoclave

PEG solution

1.6 M NaCl

30% PEG 6000 or 8000 (whatever you have)

For 200 ml you add:

18.7 g NaCl

60 g PEG

Treat with 0.1% DEPC over night and autoclave

Appendix 5: *Aploneura lentisci* weights

Table 1 PowerSoil® extraction aphid weights

	1	4	16	64
rep 1	0.39	1.38	6.21	24.93
rep 2	0.48	1.82	7.56	26.74
rep 3	0.40	1.66	6.15	26.11
total mean wt/trt	**0.42**	**1.62**	**6.64**	**25.93**
mean wt/trt	**0.42**	**0.41**	**0.42**	**0.41**

Table 2 Total aphid weights for each treatment group used for Extract-N-Amp™ extraction

	1	4	16	64
Rep 4	1.56	2.27	8.55	25.81
Rep 5	0.52	2.32	8.45	30.10
Rep 6	0.26	1.48	6.64	29.76
total mean wt/trt	**0.78**	**2.02**	**7.88**	**28.56**
mean wt/trt	**0.78**	**0.51**	**0.49**	**0.45**

Table 3 Phenol Chloroform extraction aphid weights

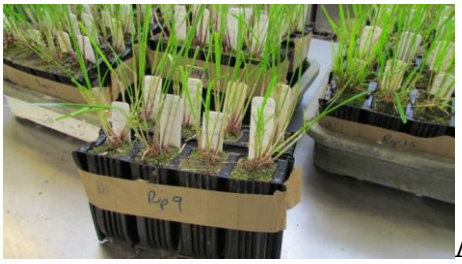
	1	4	16	64
Rep 7	0.32	1.73	5.61	27.43
Rep 8	0.70	2.19	7.36	29.21
Rep 9	0.64	2.65	8.00	26.55
total mean wt/trt	**0.55**	**2.19**	**6.99**	**27.73**
mean wt/trt	**0.55**	**0.55**	**0.44**	**0.43**

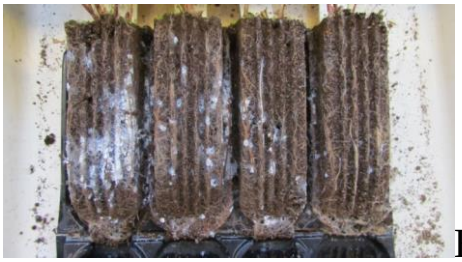
Table 4 Summary of *Aploneura lentisci* individual mean and overall weight variation

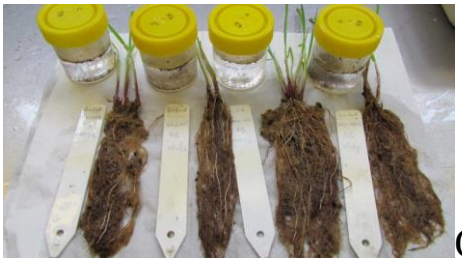
Extraction Protocol	Treatment Groups - Number of aphids 1	4	16	64	mean	sd	%CV
PowerSoil®	0.42	0.41	0.42	0.41	0.41	0.01	2.1
Extract-N-Amp™	0.78	0.51	0.49	0.45	0.56	0.15	27.2
Phenol Chloroform	0.55	0.55	0.44	0.43	0.49	0.07	13.5
	overall mean	0.49					
	overall sd	0.1					
	overall %CV	22					

Appendix 6 *Aploneura lentisci* recovery from roots of *Poaceae*

Plants are grown in root trainers (A) in a sand:soil (2:1) mix. Once fully grown and infested with aphids-the white material on the surface of the roots (B), the roots are washed and the aphids transferred to 70mL specimen containers and refrigerated before being counted. The red circle in D indicates a root aphid. E shows the two sieves used in the procedure.


A


B


C


D


E