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The results and conclusions in this report are based on an investigation conducted over a two-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headlines

- A laboratory based molecular test that specifically identifies strawberry tarsonemid mite (*Phytonemus pallidus* ssp. *fragariae*) (Ppf) has been successfully developed.

- In conjunction with a mite extraction step this test can be used to screen strawberry foliage and crowns for the presence of both adult and immature Ppf and their eggs.

Background and expected deliverables

Management in UK fruiting plantations

UK growers currently use a combination of approaches to control the strawberry tarsonemid mite. (1) They source clean certified planting material but experience shows that the material from the main Dutch and Spanish suppliers often has low levels of infestation. (2) Plantations are inspected frequently in spring and early summer for signs of damage and infestation and infested plants are grubbed and destroyed. This approach rapidly becomes costly and uneconomic. (3) *Amblyseius* predatory mites are introduced to prevent or suppress outbreaks but this approach is only partially effective and cannot contain outbreaks in hot weather conditions. (4). Spray applications of abamectin (Dynamec) or tebufenpyrad (Masai) when damaging infestations start to develop, give partial control so delaying the spread or infestation and damage. The number of applications of abamectin (Dynamec) and tebufenpyrad (Masai) are limited to 3 and 1 respectively and, in any event, sprays used during flowering and fruiting on everbearers are undesirable.

Need for a rapid, sensitive and reliable diagnostic test

Ensuring that planting material is free from the pest is clearly the best way of controlling strawberry tarsonemid mite. Testing for the presence or absence of the pest currently relies on visual searching of samples of growing points for the presence of mites under a stereo microscope. It is very time consuming and laborious to search large samples in this way and there is a high risk that small numbers of mites will be missed. There is an opportunity to develop a highly sensitive, rapid DNA-based molecular test for the pest which will enable growers to ensure that planting material is free from tarsonemid mite, or at least to be more aware of the degree of risk. Note that the evidence suggests that tarsonemid mite is not ubiquitous and that it is not present in some propagation crops (e.g. UK planting material is normally free of the pest). The problem is more a question of sample size as of sensitivity of

the test. It will only be possible to sample a small sub-sample of the total number of plants in the propagation crop, e.g. 500-1000 growing points, each from a separate randomly chosen plant. A highly sensitivity test is needed to ensure the pest can be detected in such a sample; no such test is currently available and searches for sequence data upon which such a test would be developed are also not available, suggesting that no other groups outside of the UK are in the process of such a development. A decision will need to be taken as to the tolerance level (risk of the pest being present) in the sampled crop. The risk of one or more young leaves being infested in a sample of n leaves for a given tolerance can be estimated from probability statistics using the binomial distribution.

Summary of the project and main conclusions

Using conventional methods the identification of strawberry tarsonemid mite, *Phytonemus (Tarsonemus) pallidus* ssp. *Fragariae* (Ppf), requires the separation of mites from leaf material, slide mounting of the mites and identification based on the appearance of key morphological characteristics. The objective of this project was to develop a molecular approach (based on DNA detection) to identify Ppf with the aim of reducing the time and expertise required to perform the test, which it is hoped will reduce the cost of the analysis. A molecular marker that could be used to discriminate Ppf from other species of mite commonly found on strawberries was identified and an assay designed based on real-time PCR. The result is a species-specific and sensitive method that can be used for rapid identification of all life stages of Ppf. The method does not discriminate live and dead mites, although visual assessment of the sample prior to the PCR test does give an indication of viability. The complete test ensures the detection of an infestation level of 0.5% with 95% confidence. The test can be undertaken by scientists at Fera.

Financial benefits

Strawberry tarsonemid mite can cause devastating crop losses in highly valuable protected strawberry crops, with losses exceeding £10,000 per ha per season being incurred in some instances. Ensuring that planting material is free from the pest is clearly the best way of controlling strawberry tarsonemid mite and avoiding such potential losses. The development of a highly specific PCR test for Ppf will make the current screening method more effective as it will, for the first time, allow the non-adult stages of this pest to be identified thus increasing the chances of detecting infested stocks prior to planting.

Action points for growers

To achieve good and consistent results from Ppf screening tests, it is important that the following guidelines are observed by growers when collecting and submitting samples to the Fera laboratory:

- Sample the most actively growing parts of the plant i.e. crowns and small newly emergent or partially unfurled leaves as they are the most likely places to find Ppf when present.

- Each sample should be no more than 250g in weight and consist of approximately 600 leaves or pieces of crown material of a similar size.

- Do not include roots and avoid taking material from senescent or rotting leaves and crowns as well as excessive extraneous material such as soil which can interfere with the test.

- Samples should be clearly labelled and sent in sealed plastic bags by prior arrangement with the laboratory to avoid delays in processing the material, which may result in samples deteriorating and becoming unsuitable for screening.

SCIENCE SECTION

Introduction

The pest

The tarsonemid mite, *Phytonemus (Tarsonemus) pallidus* ssp. *Fragariae* (Ppf), sometimes called the strawberry mite, is a serious pest of strawberry. It feeds mainly on the upper surfaces of the young folded leaves of strawberry, mostly on the main vein, making their surfaces tough and crinkled as they expand. Sometimes the leaves turn brown and die and the whole plant usually becomes stunted. Mites also feed in the flowers and fruits, seriously affecting yield and quality, which can halt berry production. Damage is most severe in ever-bearing varieties and on plants grown under protection. June bearers can also be severely attacked.

Populations build up rapidly in warm conditions; the generation time is nine days at 25 ˚C. There has been a significant and threatening increase in the frequency and severity of attacks in UK strawberry production in the last few years. The problem was particularly bad in 2010 and threatens to get worse

Source of infestation

Tarsonemid mite is usually introduced into plantations on infested planting material. A large proportion of UK strawberry planting material is sourced from the Netherlands and Spain and industry feedback advises that a significant proportion of planting material from these sources is infested. Planting material from the UK does not appear to pose a significant risk currently.

Loss of suitable chemical actives and loss of methyl bromide fumigation of planting material are almost certainly the underlying causes of the increase in the incidence of the pest. Dutch propagators are now using elevated CO_2 levels in an attempt to disinfest planting material by asphyxiating the mites. Current experience is indicating that this treatment is at best only partially effective, which is not surprising as the mites are in diapause and presumably have a very low respiration rate in winter.

The problem is exacerbated because the Dutch pant health scheme has a low tolerance of tarsonemid mite, rather than the zero tolerance in the UK, which results in a higher probability of planting material, even if certified, being infested. In Spain hot water treatment of runners is being used to some extent. Hot water treatment is known

to be very effective, but is difficult to implement with adequate temperature control and heat penetration on a large scale. Hot water treatment also has an adverse effect on the subsequent vigour of plants.

Difficulty of chemical control

The difficulty of controlling strawberry tarsonemid mite is because most acaricides are contact-acting with no, or at best limited, translaminar activity. The mites are readily controlled when directly intercepted by an acaricide, but penetration into the young folded leaves, where the tarsonemid mites live and breed, is limited.

Furthermore, strawberry leaves are waxy and covered in hairs and as many products are not specifically formulated for the crop they have insufficient wetting properties. Work by EMR in HDC project SF 79 (report issued 2 Jan 2008) clearly demonstrated substantive improvements in the efficacy of abamectin when admixed with a silicone wetter. Nevertheless a high degree of efficacy is only likely to be achieved with a systemic acaricide

The project work has been broken down into two main phases:

- Phase one focused on the identification of a DNA sequence unique to Ppf and on which a test could be developed;

- Phase two focused on refining, validating and then developing a protocol for the practical application of the new test.

Materials and methods

Samples used in the study

To select and obtain an appropriate range of non-target mite species, samples of strawberry plants were provided by EMR during July and August of 2011. These had been collected from six separate plantations of varying ages and including both organic and non–organic crops.

Using a flotation extraction method (Thind, 2000) the total invertebrate fauna was extracted from 100g of strawberry leaves and crowns taken in five 20g subsamples (two of crowns and three of leaves) from each strawberry sample. Mites of relevance to the project were selected, slide mounted, examined using a Zeiss Universal Phase/DIC research microscope at up to 1,000x magnification and morphologically identified using

published keys and in comparison to verified voucher specimens.

Verified specimens of the taxa identified were also obtained from other sources, including the Fera living mite collection, Fera reference collection, field collected material, EMR and a UK supplier of biological control agents. A final list of the taxa to be used was developed (**Table 1**).

Additional plant samples were used for DNA extraction / separation method development. These included:

1) Four Replicates of 300 strawberry leaves spiked with approximately 50 motile Ppf mites and four control replicates of 300 strawberry leaves supplied without Ppf supplied by EMR.
2) Additional sample of leaves (40 X 250g) supplied by EMR.
3) Eighty dormant strawberry plants var. Elsanta purchased from a commercial supplier.

Table 1. Mite taxa used in the study

Order: Acarina					
Suborder/Family	**Genus**	**Species**	**Authority**	**Habit**	**ENTOBAR***
Prostigmata					
Cheyletidae	*Cheyletus*	*eruditus*	(Schrank)	Predatory	1386-90
Tarsonemidae	*Phytonemus*	*pallidus* ssp. *fragariae*	(Zimmermann)	Phytophagous	1245-47
	Phytonemus	*pallidus* ssp. *fragariae*	(Zimmermann)	Phytophagous	1267-68
	Phytonemus	*pallidus* ssp. *fragariae*	(Zimmermann) EMR	Phytophagous	1367-83
	Polyphagotarsonem	*latus*	(Banks)	Phytophagous	1468-72
	Stenotarsonemus	*laticeps*	(Halbert)	Phytophagous	1418-27
	Tarsonemus	spp.	-	Phytophagous	1266
Tetranychidae	*Tetranychus*	*urticae*	Koch	Phytophagous	1248-50
	Bryobia	*praetiosa*	Koch	Phytophagous	1293-94
Tydeidae	-	-	-	Phytophagous	1257-59
Asigmata					
Acaridae	*Rhizoglyphus*	*robini*	Claparédè	Saprophytic	1392-96
	Tyrophagus	*putrescentiae*	(Schrank)	Fungivore/Phytophagous	1285-86
	Tyrophagous	*similis*	Volgin	Fungivore/Phytophagous	1254-56
Mesostigmata					
Parasitidae	*Parasitus*	*beta*	(Oudemans & Voigts)	Predatory	1287-88
Phytoseiidae	*Amblyseius*	*andersoni*	Chant	Mainly predatory	1568-77
	Amblyseius	*swirskii*	(Athias-Henriot)	Mainly predatory	1589-97
	Amblydromalus	*limonicus*	Garman & McGregor	Mainly predatory	1598-1607
	Euseius	*ovalis*	(Evans)	Mainly predatory	1638-47
	Neoseiulus	*californicus*	McGregor	Mainly predatory	1251-53
	Neoseiulus	*cucumeris*	(Oudemans)	Mainly predatory	1618-27
	Phytoseiulus	*persimilis*	Athias-Henriot	Mainly predatory	1648-57
	Typhlodromalus	*leilae*	(Schicha)	Mainly predatory	1578-87
	Typhlodromips	*montdoriensis*	(Schicha)	Mainly predatory	1608-17
Oribatidae					
Camisiidae	*Platynothrus*	*peltifer*	(Koch)	Saprophytic	1289-90
Ceratozetidae	*Sphaerozetes*	*orbicularis*	(Koch)	Saprophytic	1291-92
Order:	Unknown spider	As non-target	-	Predatory	1466-67
	Mixed invertebrates	As non-target	-	Various	1458-61

*ENTOBAR - A Fera database used to record and attribute unique reference number to all specimens used in molecular studies.

Sample DNA extraction methods

DNA was extracted from invertebrate samples using either a simple Chelex method (Boonham *et al*. 2002) or the QIAGEN DNeasy Blood and Tissue kit following the manufactures protocol for animal tissues. For non-target species samples were single adult specimens, whilst for the target species a range of samples sizes were used including 10, five and one adult(s), 10 immatures and 10, five, two or one egg(s).

Target sequence generation

To identify a region of DNA unique to *Phytonemus pallidus* ssp. *fragariae*, PCR and DNA sequencing was conducted of both the cytochrome oxidase I (COI) and the D3 fragment of the 28S rRNA (and flanking regions) using primers as detailed in **Table 2.** PCR was performed as detailed in the original reference (using a proof-reading DNA *Taq* polymerase
'BIO-X-ACT' from BIOLINE) and products visualised using agarose gel electrophoresis. The D3 primers were modified with the addition of a M13 primer tag to improve the quality of DNA sequencing reads. DNA sequencing of both strands was conducted by Eurofins-MWG- Operon.

Table 2. The target genes, primers and references used for DNA bar-coding of mite species.

Target gene	Primer name	Sequence (5'-3')	Reference
Cytochrome oxidase I (COI)	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al*., 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	
D3 domain of 28S rRNA (D3)	D3A-F	GACCCGTCTTGAAACACGGA	Maraun *et al*., 2003
	D3B-R	TCGGAAGGAACCAGCTACTA	
D3 domain of 28S rRNA (D3) [M13 modified]	D3A-M13rev(-29)	CAGGAAACAGCTATGACCGACCCGTCTTGAAACACGG	Modified from Maraun *et al*., 2003
	D3B-M13uni(-21)	TGTAAAACGACGGCCAGTTCGGAAGGAACCAGCTACTA	

Real-time assay development

DNA sequences were manually edited and trimmed using ABI sequence scanner v1 and consensus sequences were generated for each sample using MEGA 4. Sequence alignments were created using MEGA 4 and used for assay design. Real-

time PCR primes and probe were designed by eye using Primer Express V2 (Applied Biosystems) to ascertain primer/probe characteristics. Specific regions for primer/probe design were selected where the sequence of the target species was conserved but polymorphisms were identified to non-target species. Four primers were designed for testing, and one minor-groove binding

(MGB) probe (**Table 3**). All primers were synthesized by Eurofins MWG (Ebersberg, Germany) and the probe by Applied Biosystems with the reporter FAM.

Table 3. Primer and probe sequence for the real-time PCR assay specific to Ppf.

Primer/Prob	Sequence (5' - 3')
Ppf-F1	TTAGTATAGTTACGATGAACAGTTTAGTTATTTAAATT
Ppf-F2	TGTACAAGTTTAGTATAGTTACGATGAACAGTTTAG
Ppf-R1	CTACCGTATACGCTCTAATATGACTCGTA
Ppf-R2	CGGGTACTACCGTATACGCTCTAATA
Ppf-P	TTTAGCAGTAGCAATGTGATT (FAM-MGB)

Real-time PCR reactions were set up in 96-well reaction plates using TaqMan core reagent kits (Applied Biosystems), following the manufacturers protocols. All primers were used at a final concentration of 300 nM and the probe at a final concentration of 100 nM. Reactions were cycled using generic system conditions (2 min at 50°C and 10 min at 95°C, followed by 40 cycles, each consisting of 15 s at 95°C and 1 min at 60°C) on an ABI Prism 7900 instrument (Applied Biosystems). Results were analysed with Sequence Detection System V software in terms of the average cycle threshold (C_T) values (cycle that produces a positive PCR signal as determined by the algorithm built into the software). Plant samples were tested with the cytochrome oxidase (COX) gene real-time PCR assay as described in Weller *et al*., 2000.

DNA extraction development

Several approaches to achieving a DNA sample to test were examined. These included total DNA extractions from bulks of leaves (resulting in a DNA sample containing predominantly DNA from the host with low levels of mite DNA) as well as methods which separated the mites (along with other invertebrates and contaminating organic matter) from the leaves prior to DNA extraction from the 'invertebrate plug'.

The rational for this approach was two- fold; firstly it is known that extracting DNA from

strawberry leaves is difficult, due to large amounts of poly-phenolic and poly-sacharide material being present in the leaf which leads to contamination of the DNA with PCR inhibiting compounds. Secondly, due to the necessity to test large bulks of leaf material for potentially very small numbers of mites, the resulting DNA would contain very small amounts of mite DNA in a background of predominantly strawberry DNA. The problems of the former heavily compound the latter.

1) Total DNA extraction

Total DNA was extracted from duplicate samples of 300 strawberry leaves spiked with approximately 50 motile Ppf. DNA was extracted using a standard CTAB based magnetic bead extraction protocol to extract both plant and invertebrate DNA.

2) Methods to separate mites from samples

i) Flotation method

Extraction of invertebrates prior to molecular analysis using a published flotation method (Thind, 2000) was evaluated. In summary the methodology involves pre-treating samples to be extracted using various reagents (Appendix 1) and processes prior to suspension in an aqueous phase through which a quantity of kerosene is then passed. The invertebrate cuticle is lipophilic and adheres to the kerosene which then floats to the top of the aqueous phases, carrying with it any invertebrate that it has made contact with. The floating layer of kerosene is then drawn-off and filtered and the invertebrates collected.

100g of material taken from duplicate samples of 300 strawberry leaves provided by EMR and spiked with approximately 50 motile Ppf were extracted using this method to initially assess its suitability. DNA extraction was performed on the flotation plug using a simple Chelex method. Modifications to the method were made to eliminate some reagents (such as concentrated acid and Methylene blue dye) and a heating step to attempt to mitigate / prevent damage to the DNA within the organisms extracted.

ii) Washing method

The current standard method used to screen samples of strawberry plants for the presence of Ppf is a previously unpublished procedure developed within Fera and involves a three stage process. The procedure starts with a visual examination of the sample using a binocular microscope at magnifications of at least X 80 followed by a

washing and filtration step and a final examination of the filtrate produced, again using a binocular microscope at magnifications of at least X 80.

Normally 250g of strawberry leaf or crown material is extracted in five 50g replicates, each being chopped into small pieces before being washed in 250ml of 10% ethanol solution in a 500ml beaker. For the purposes of this study the sample replicates were scaled down to 20g to be directly comparable to the flotation extraction method that is currently set-up for, and works most efficiently on, 20g sub- samples of material.

Once added to separate flasks of ethanol solution each test replicate is allowed to stand for 30 minutes before being stirred vigorously for 10 seconds by hand immediately prior to being filtered through a Whatman 551 90mm diameter black filter paper using a Buchner funnel/vacuum flask aspirated with a hand pump. For the purposes of this project the mite recovery rate achievable from strawberry leaves and crowns using this extraction method was assessed.

This assessment was done by taking three 100g samples of strawberry leaves and the three 100g samples of strawberry crowns and divided each into five replicates of 20g. Each replicate was spiked with either one, five or 10 adult Ppf prior to washing and filtration. Controls consisting of five 20g replicates each of leaves and crowns to which no Ppf had been added were also extracted. All the extracts produced were then examined visually for the presence of Ppf and any tarsonemids found were slide mounted and checked. This washing method was later modified further by replacing the black filter paper with white filter paper of the same dimensions.

DNA extraction was performed on generated filter papers using either a simple Chelex method or the DNeasy Blood and Tissue kit (QIAGEN) following the manufactures protocol for animal tissues. Depending upon the size of the filter paper for processing, the Blood and Tissue kit method was modified to scale up the lysis step appropriately. To enable this, the filter paper was cut into small pieces and placed into sample tubes of an appropriate size in order to allow the buffer used to adequately cover the sample.

Sensitivity to DNA from dead mites

The original intention was to base this study on mites killed following a standard application of abamectin (Dynamec) but this was not possible. As an alternative twenty-five preserved adult Ppf (from the EMR Stock culture) were dipped into a 0.5ml/l solution

of abamectin (Dymanec) for 30 minutes and then placed separately into open 0.5ml Eppendorf tubes and stored in a laboratory incubator at 23°C under a 12h light/dark regime. At 1, 3, 5, 7 and
14 day intervals, five replicates were removed, sealed and frozen at • 20°C. All of the replicates were then subjected to DNeasy Blood and Tissue kit (QIAGEN) DNA extraction and tested on the same day.

Results

Species included in the study

An initial list of the mite taxa expected to be found in association with strawberry plants was developed from previous records. This was refined using survey data generated from flotation extractions of the total invertebrate fauna (**Figure 1**) taken from the six samples of strawberry plants first supplied by EMR. The final list was thus a truly representative panel of the non-target mite taxa.

Figure 1. Total invertebrate fauna from one flotation extract.

The invertebrate fauna composition from each sample extracted was found to be largely the same, including representatives from all invertebrate classes (see Figure 1). From this material mites were removed, slide mounted and identified on the basis of the presence of morphological features. The initial intention was to use the same specimen for developing the molecular test, but this proved impossible to achieve as the processes needed to prepare the specimens sufficiently to be identified as slide mounts denatured the DNA and rendered them unsuitable for that purpose. However, 25 of the most commonly occurring species were identified and selected by this method, representing the four main terrestrial mite suborders (Astigmata, Mesostigmata, Oribatida and Prostigmata) and including three

other members of the family Tarsonemidae i.e. in the same family and therefore more closely related than the other mite taxa to Ppf (**Table 1.**). With the exception of Ppf which is host specific to strawberry, the other mite taxa detected can be found widely across a range of plants and many are commonly introduced as biological control agents. Verified alcohol preserved material of each of the taxa identified were obtained from various sources, including three populations of Ppf and prepared for molecular analysis.

Generation of sequence for target and other mite species

Initially sequences for the standard DNA bar-coding gene cytochrome c oxidase subunit I gene (COI) (which is used for species identification of many insect species) were generated from a range of target and non-target species. This gene proved unsuitable for differentiation of Ppf and non-target species being studied within this project. The reasons for this are two-fold; firstly a rare DNA sequencing phenomenon called polymerase slippage was identified within the mite species being studied; this means that this region of the gene is not ideal for diagnostic methods (**Figure 2**) because it makes the resolution of sequence distal to this region impossible. Secondly, DNA sequence of the COI gene was revealed to be identical across the five species selected for the initial test panel (**Figure 3**). These five species were selected for their diversity, being from three orders and five families, and should therefore be as diverse as possible. This is an ideal panel for method development as it allows the best specific gene fragments for the target species to be identified, and minimizes the risk of any diagnostic tool cross-reacting with non-target species.

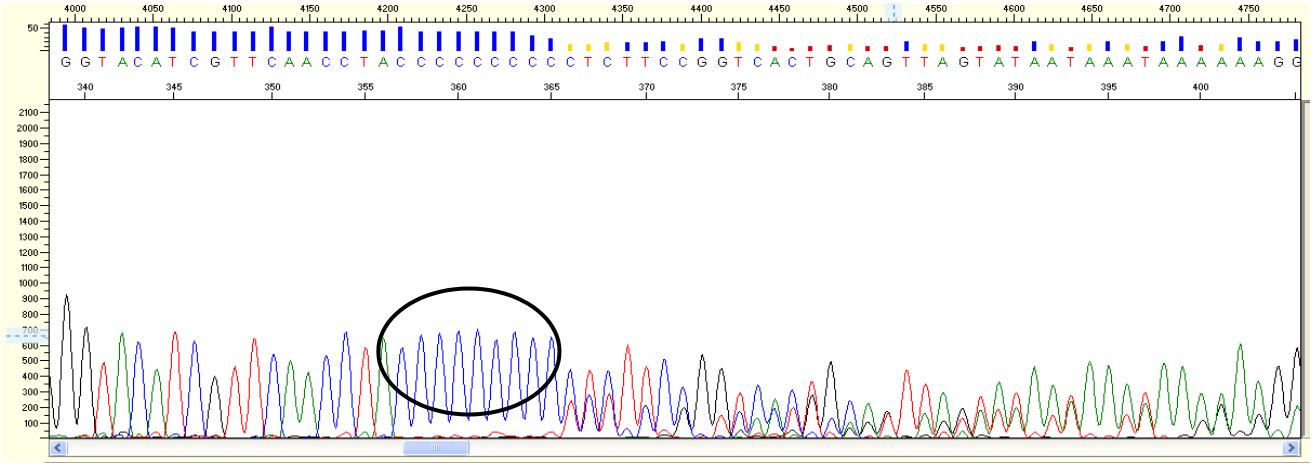

Figure 2. An example DNA sequencing trace from a family Tydeidae specimen, with the polymerase slippage site, a run of 10 'C' nucleotides, circled. Note single peaks to the left where sequence can be resolved and overlapping peaks to the right where it cannot.

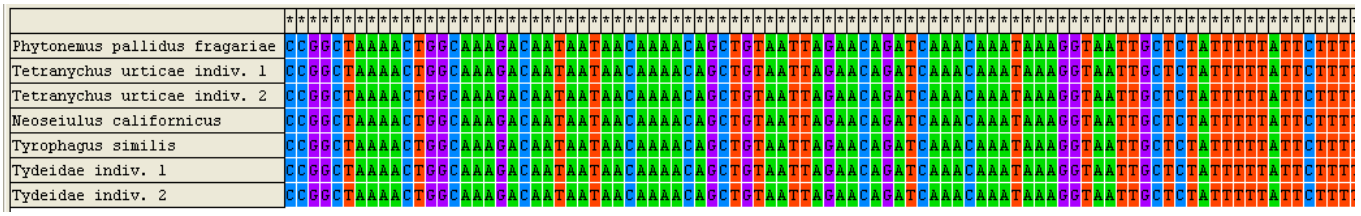

Figure 3. Partial alignment of the COI gene from five mite species (the initial test panel) showing the sequence is identical.

Following this discovery, a larger number of genes were analysed to identify a region suitable for discrimination of Ppf from other closely related mites. This included evaluating two regions of 28S ribosomal RNA, two regions of the internal transcribed spacer (ITS), two regions of elongation factor (EF) and heat shock protein 82 (HSP82) genes. The primer sets for most of these targets failed to give consistent amplification with all species within the core panel of target and non-target mites, presumably due to sequence polymorphisms in the primer regions. This prevented the generation of DNA sequences for assay design for all targets for all species. However PCR primers which amplify the D3 fragment of the 28S rRNA (and flanking regions) yielded PCR products from a wide range of species and DNA sequencing of this region revealed substantial differences between the target and non- target species (**Figure 4**), thus indicating that this would likely be a suitable DNA marker for development of a species specific assay.

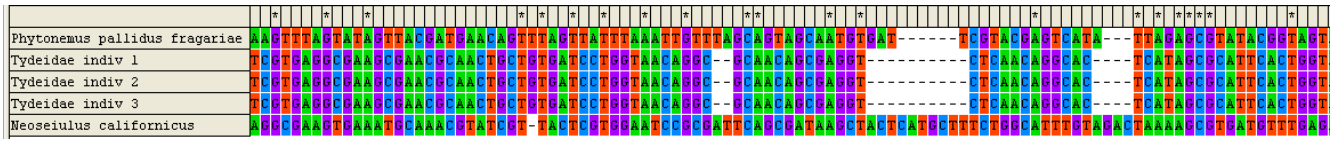

Figure 4: Partial alignment of the D3 fragment of the 28S rRNA and flanking region from three mite species showing sequence variability.

Develop and assessment of real-time PCR assay

Four primers (two forward and two reverse) were designed within the D3 fragment along with a single probe, which in combination gave four putative species specific assays. All four versions of the assay (all primer combinations) were tested with all samples both target and non-target (see **Table 4**). All of the target samples, regardless of sample size, were positive and none of the non-target species cross reacted. The presence of amplifiable DNA was confirmed in all DNA extracts by performing PCR using conserved primers for either the COI or D3 genes. Therefore any negative results can be taken as genuine. The primer combination Ppf-F2 and Ppf-R1 was taken forward as the best assay, since this

combination consistently generated the lowest C_T and high ΔR_N value. This assay was used for all remaining experiments.

Table 4. Real-time PCR data for all four assay versions for all samples tested. A C_T value of 40 indicates the sample is negative; values are presented as an average from duplicate wells (NT indicates not tested).

Sample	Species	n.	C_T Value			
			F1/	F1/	F2/	F2/
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	5	33.	33.	30.	N
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	5	31.	32.	29.	N
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	5	30.	27.	29.	N
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	5	36.	37.	32.	N
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	5	40.	40.	37.	N
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	5	36.	31.	29.	30.
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	1	37.	32.	30.	31.
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	1	35.	32.	30.	31.
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	1	35.	30.	29.	29.
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	1	37.	35.	30.	34.
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	1	32.	30.	29.	29.
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	1	29.	27.	26.	26.
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	1	27.	NT	NT	N
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	1	30.	27.	26.	26.
ENTOBAR13	*Phytonemus pallidus fragariae* EMR Stock culture	1	32.	28.	26.	26.
ENTOBAR12	*Phytonemus pallidus fragariae* Norfolk July	1	33.	33.	33.	35.
ENTOBAR12	*Phytonemus pallidus fragariae* Norfolk Nov.	5	34.	36.	37.	40.
ENTOBAR12	*Phytonemus pallidus fragariae* Norfolk Nov.	1	31.	32.	40.	36.
ENTOBAR15	*Amblydromalus limonicus*	1	40.	40.	40.	40.
ENTOBAR16	*Amblydromalus limonicus*	1	40.	40.	40.	40.
ENTOBAR15	*Amblyseius andersoni*	1	40.	40.	40.	40.
ENTOBAR15	*Amblyseius andersoni*	1	40.	40.	40.	40.
ENTOBAR15	*Amblyseius swirskii*	1	40.	40.	40.	40.
ENTOBAR15	*Amblyseius swirskii*	1	40.	40.	40.	40.
ENTOBAR15	*Amblyseius swirskii*	1	40.	40.	40.	40.
ENTOBAR12	*Bryobia praetiosa*	1	40.	40.	40.	N
ENTOBAR13	*Cheyletus eruditus*	1	40.	40.	40.	40.
ENTOBAR13	*Cheyletus eruditus*	1	40.	40.	40.	40.
ENTOBAR13	*Cheyletus eruditus*	1	40.	40.	40.	40.
ENTOBAR13	*Cheyletus eruditus*	1	40.	40.	40.	40.
ENTOBAR13	*Cheyletus eruditus*	1	40.	40.	40.	40.
ENTOBAR16	*Euseius ovalis*	1	40.	40.	40.	40.
ENTOBAR16	*Euseius ovalis*	1	40.	40.	40.	40.
ENTOBAR12	*Neoseiulus californicus*	1	40.	40.	40.	40.
ENTOBAR12	*Neoseiulus californicus*	1	40.	40.	40.	40.
ENTOBAR16	*Neoseiulus californicus*	1	40.	40.	40.	40.
ENTOBAR16	*Neoseiulus californicus*	1	40.	40.	40.	40.
ENTOBAR16	*Neoseiulus cucumeris*	1	40.	40.	40.	40.
ENTOBAR12	*Parasitus beta*	1	40.	40.	40.	N
ENTOBAR16	*Phytoseiulus persimilis*	1	40.	40.	40.	40.
ENTOBAR16	*Phytoseiulus persimilis*	1	40.	40.	40.	40.
ENTOBAR12	*Platynothrus peltifer*	1	40.	40.	40.	40.

ENTOBAR12	*Platynothrus peltifer*	1	40.	40.	40.	40.
ENTOBAR14	*Polyphagotarsonemus latus*	5	40.	40.	40.	40.
ENTOBAR14	*Polyphagotarsonemus latus*	5	40.	40.	40.	40.
ENTOBAR14	*Polyphagotarsonemus latus*	5	40.	40.	40.	40.
ENTOBAR14	*Polyphagotarsonemus latus*	5	40.	40.	40.	40.
ENTOBAR14	*Polyphagotarsonemus latus*	5	40.	40.	40.	40.
ENTOBAR13	*Rhizoglyphus robini*	1	40.	40.	40.	40.
ENTOBAR13	*Rhizoglyphus robini*	1	39.	40.	40.	40.
ENTOBAR13	*Rhizoglyphus robini*	1	40.	40.	40.	40.
ENTOBAR13	*Rhizoglyphus robini*	1	40.	40.	40.	40.
ENTOBAR13	*Rhizoglyphus robini*	1	40.	40.	40.	40.
ENTOBAR12	*Sphaerozetes orbicularis*	5	40.	40.	40.	40.
ENTOBAR12	*Sphaerozetes orbicularis*	5	40.	40.	40.	N
ENTOBAR14	*Steneotarsonemus laticeps*	5	40.	40.	40.	40.
ENTOBAR14	*Steneotarsonemus laticeps*	5	40.	40.	40.	40.
ENTOBAR14	*Steneotarsonemus laticeps*	5	40.	40.	40.	40.
ENTOBAR14	*Steneotarsonemus laticeps*	5	40.	40.	40.	40.
ENTOBAR14	*Steneotarsonemus laticeps*	5	40.	40.	40.	40.
ENTOBAR14	*Steneotarsonemus laticeps*	5	40.	40.	40.	40.
ENTOBAR14	*Steneotarsonemus laticeps*	5	40.	40.	40.	40.
ENTOBAR14	*Steneotarsonemus laticeps*	5	40.	40.	40.	40.
ENTOBAR14	*Steneotarsonemus laticeps*	5	40.	40.	40.	40.
ENTOBAR14	*Steneotarsonemus laticeps*	5	40.	40.	40.	40.
ENTOBAR12	*Tarsonemus sp.*	1	40.	40.	40.	40.
ENTOBAR12	*Tetranychus urticae*	1	40.	40.	40.	40.
ENTOBAR12	*Tetranychus urticae*	1	40.	40.	40.	40.
ENTOBAR12	Tydeidae	1	40.	40.	40.	40.
ENTOBAR12	Tydeidae	1	40.	40.	40.	40.
ENTOBAR15	*Typhlodromalus leilae*	1	40.	40.	40.	40.
ENTOBAR15	*Typhlodromalus leilae*	1	40.	40.	40.	40.
ENTOBAR16	*Typhlodromips montdoriensis*	1	40.	40.	40.	40.
ENTOBAR16	*Typhlodromips montdoriensis*	1	40.	40.	40.	40.
ENTOBAR16	*Typhlodromips montdoriensis*	1	40.	40.	40.	40.
ENTOBAR12	*Tyrophagus putrescentiae*	1	40.	40.	40.	N
ENTOBAR12	*Tyrophagus putrescentiae*	1	40.	40.	40.	N
ENTOBAR12	*Tyrophagus similis*	1	40.	40.	40.	40.
ENTOBAR12	*Tyrophagus similis*	1	40.	40.	40.	40.
ENTOBAR14	Unknown SPIDER	1	40.	40.	40.	40.
ENTOBAR14	Unknown SPIDER	1	40.	40.	40.	40.

Determination of most appropriate DNA extraction method

Total DNA extraction

The total DNA extracts of strawberry leaves spiked with Ppf were assessed using the real-time PCR assays for the plant COX gene (as an internal positive control) and all four versions of the Ppf assay. All of the samples were negative, indicating that DNA extractions were unsuccessful from large bulks of strawberry leaves.

Methods to separate mites from samples

Flotation

Flotation samples were processed using the standard methodology and then the plugs examined and several invertebrate samples retrieved. These were subjected to PCR of the COI gene to assess if the DNA was damaged during the flotation process. Additionally, each sample was subjected to PCR combined with DNA of a known positive sample to check that the DNA extract supported PCR. Of the five samples tested, only one gave a PCR product, yet all were able to support PCR, possibly indicating that the DNA had been damaged by the sample processing (see **Figure 5**). Further leaf samples spiked with Ppf were subjected to flotation and DNA extracted from the plugs using the Chelex method. When these samples were screen using the COX assay for plant DNA and Ppf assay for
mite DNA all of the samples were again negative for all targets (see **Table 5**).

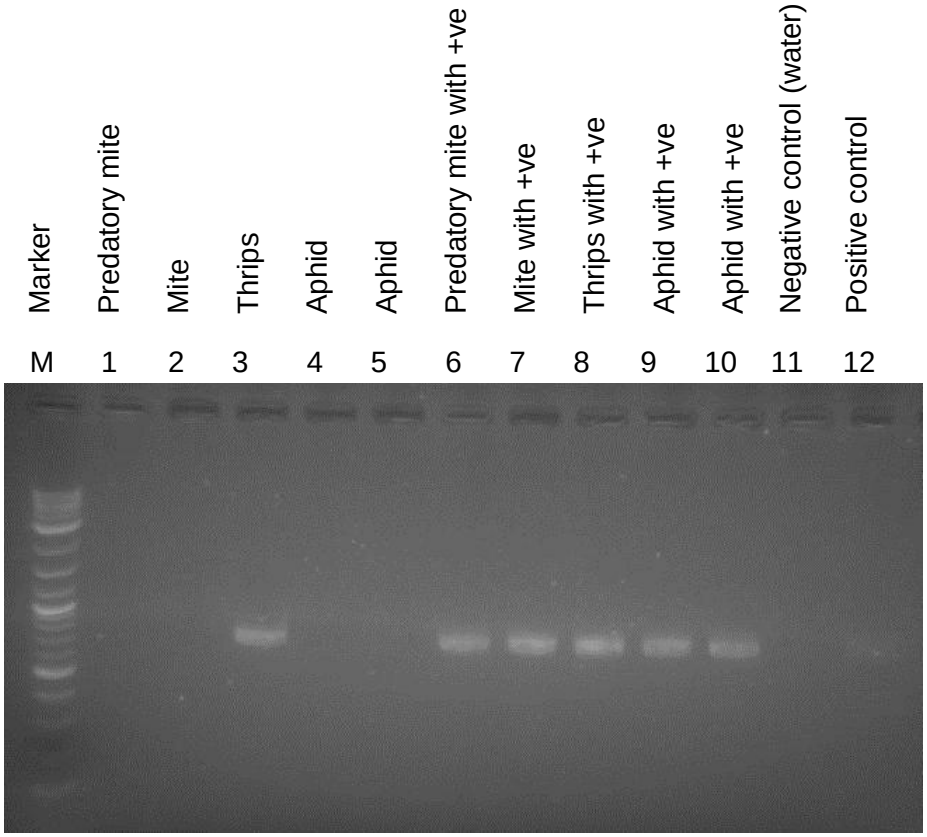

Figure 5. PCR of the COI gene of single invertebrates from flotation, visualised by agarose gel electrophoresis.

Table 5. Real-time PCR results of DNA extracts of leaf samples spiked with Ppf and subjected to flotation extraction, tested for COX (plant DNA) and Ppf (target mite). A C_T value of 40 indicates the sample is negative; values are presented as an average from duplicate wells.

Sample	Ppf	COX
Sample 1 (ENTOBAR 1503)	40.0	40.0
Sample 2 (ENTOBAR 1504)	40.0	40.0
Negative control (water)	40.0	40.0
Positive control	25.6	19.0

To investigate if the problems with testing were due to the flotation process or the DNA extraction from plugs of invertebrates, the most appropriate method of DNA extraction of the invertebrate plugs was evaluated. A range of simulated plugs (not passing through the flotation method) were created using various invertebrate materials, and half were then spiked with Ppf. The simulated plugs were subjected to DNA extraction, using either the Chelex or QIAGEN DNA extraction methods, and assessed by COI PCR, to confirm the DNA extractions were successful, and real-time PCR to detect the Ppf mite spiked into the samples. Invertebrate DNA could be detected in all of the samples extracted by both methods and Ppf mite could be detected in all of the spiked samples, with marginally lower C_T values from the Chelex extracts (see **Table 6**). The results suggest that the DNA within the invertebrates was being degraded during the flotation method, such that the samples were inconsistent in amplification after flotation, which would likely lead to false negative results if deployed further.

Table 6. Real-time PCR for Ppf on simulated plugs after DNA extracted by either Chelex or QIAGEN methods. A C_T value of 40 indicates the sample is negative; values are presented as an average from duplicate wells

Sample	Description	Ppf
ENTOBAR1458	Chelex non-spiked	40
ENTOBAR1459	Chelex non-spiked	40
ENTOBAR1462	Chelex spiked	28.8
ENTOBAR1463	Chelex spiked	27.5
ENTOBAR1460	Qiagen non-spiked	40
ENTOBAR1461	Qiagen non-spiked	40
ENTOBAR1464	Qiagen spiked	34.9
ENTOBAR1465	Qiagen spiked	40

Washing

An established extraction process involving ethanol based separation of the invertebrate fraction from leaves or crowns was used. Before this method was deployed it was formally reassessed on a small scale for samples of two types, strawberry leaves and strawberry crowns. Mite recovery rates of 60-62% for leaves and 60-70% for crowns were achieved (see **Appendix 2**).

In addition to Ppf a small number of *Tarsonemus* spp. were also extracted that could be easily mistaken for Ppf by an untrained observer in the absence of a suitable molecular test as the diagnostic characters are subtle (see **Figure 6**). As this method yields the invertebrate fraction on filter paper, an initial experiment was conducted to assess the ability of the Chelex and QIAGEN DNA extraction methods to extract mite DNA from a background of filter paper.

Single adult mites were placed on $1cm^2$ of Whatman No. 1 white filter paper and subjected to Chelex extraction (combined with bead beating with glass beads to homogenise the paper) or QIAGEN blood and tissue extraction with an initial overnight lysis step. Five replicate samples were processed with each method and screened with the Ppf real-time PCR assay. Using QIAGEN DNA extraction three out of five replicates yielded good C_T values (C_T ~ 30-32), and with Chelex three out of five replicates yielded poorer C_T values (C_T ~ 33-36).

Whilst both methods successfully extracted mite DNA, the QIAGEN method gave better (lower) C_T values for the target mite, more consistent extractions and is easily scale-able to larger sample sizes so this approach was taken forward for further assessment.

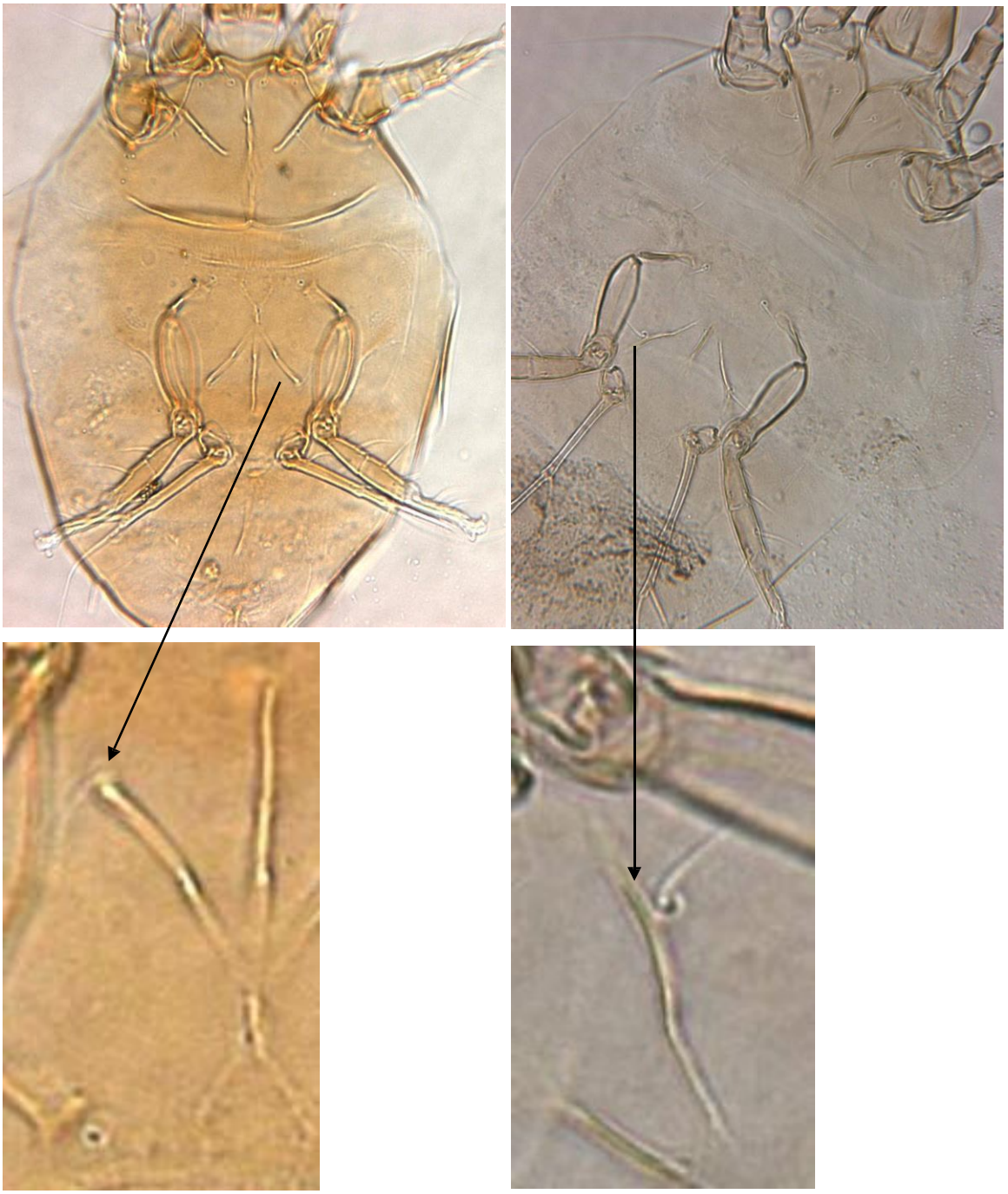

Figure 6. Left *Tarsonemus* sp. right *Phytonemus pallidus* ssp. *fragariae* and insert the position of a seta (arrowed) a character key to separating the two genera.

To further challenge this method samples were prepared simulating a real sample, where strawberry leaves were subjected to the separation, applied to the filter paper and then 1cm^2 excised and single adult mites spiked onto each paper square. Five replicate samples were prepared, using both white (No. 1) and black (No. 551) Whatman filter paper. DNA was extracted with the QIAGEN method and tested with the Ppf real-time PCR assay.

All replicates on white filter paper were positive, with C_T values ranging from 28-36, whilst all samples from the black filter paper were negative (indicating the black filter paper inhibited either the DNA extraction or PCR).

After successfully demonstrating that a single mite on 1cm^2 of white filter paper with strawberry leaf extract could be detected, the same experiment as conducted using bare root strawberry crowns cv. Elsanta (commercially sourced), that were visually check for the presence of Ppf prior to use. Five replicates were spiked with Ppf and five remained un-spiked to confirm that the crowns were not infected with Ppf. Initial PCR of the samples with the real-time PCR assays were negative. It was confirmed that the DNA extractions supported PCR by testing each sample combined with a known positive.

As the failure of the PCR was thought to be due to PCR inhibitors in the DNA extracts, three approaches to overcoming this were tested. The samples were tested using core reagents with double the concentration of *Taq* polymerase, with the addition of 0.04% bovine serum albumin (BSA) or using Applied Biosystems Environmental Master-mix (see **Table 7**). Whilst both the environmental master-mix and addition of BSA overcame the PCR inhibition, the BSA gave an improved C_T value and maintained the ΔR_N so this was chosen as the most appropriate approach. Optimisation of the concentration of BSA added to the reaction at 0.04%, 0.2%, 0.4% and 0.6% had no effect on the C_T value, so 0.2% BSA was used for all remaining PCR testing.

Table 7. Assessment of approaches to overcome PCR inhibition of crown separation samples. A C_T value of 40 indicates the sample is negative; values are presented as an average from duplicate wells.

Sample		**C_T value**		
		Double *Taq*	**Environmental**	**0.04 %**
ENTOBAR172	Flotation prep - extraneous material only (x 5 repeats)	40.0	40.0	40.0
ENTOBAR172		40.0	40.0	40.0
ENTOBAR172		40.0	40.0	40.0
ENTOBAR172		40.0	40.0	40.0
ENTOBAR172		40.0	40.0	40.0
ENTOBAR172	1x Ppf adult (extraction	32.4	34.4	32.5
ENTOBAR173		40.0	33.9	31.6
ENTOBAR173	Flotation prep - extraneous material spiked with 1 x Adult (x 5 repeats)	40.0	40.0	40.0
ENTOBAR173		40.0	32.1	29.9
ENTOBAR173		40.0	35.2	32.5
ENTOBAR173		40.0	35.6	33.3
ENTOBAR173	1x Ppf adult (extraction	31.3	33.7	30.9

With the addition of BSA to the real-time PCR reaction, a single adult mite on 1cm^2 of filter paper could be readily detected. This approach was then scaled up to a sample size more realistic of a routine diagnostic test. Small dormant strawberry crowns (10 crowns = approx 20g of material) were subjected to the separation process and applied to a 9cm Whatman filter paper. The paper was divided into four quarters, and each spiked with a different number of adult Ppf using the range two, four, eight and 16. These were subjected to the QIAGEN DNA extraction process with a large scale lysis step and each lysate applied to two extraction columns (samples labelled A and B), followed by assessment with real-time PCR. All of the sample sizes, including two adult mites per quarter filter paper were readily detected by the assay (see **Table 8**), giving a preliminary indication of the limit of detection of the extraction process.

Table 8. Real-time PCR using the Ppf assay of crown separation samples on large filter papers. A C_T value of 40 indicates the sample is negative; values are presented as an average from duplicate wells.

Sample		C_T value
ENTOBAR1781-A	2 adult mites on 1/4 9cm filter paper with crown extract	32.7
ENTOBAR1781-B		30.9
ENTOBAR1782-A	4 adult mites on 1/4 9cm filter paper with crown extract	36.2
ENTOBAR1782-B		32.3
ENTOBAR1783-A	8 adult mites on 1/4 9cm filter paper with crown extract	29.9
ENTOBAR1783-B		29.9
ENTOBAR1784-A	16 adult mites on 1/4 9cm filter paper with crown extract	31.5
ENTOBAR1784-B		29.6

Determine limit of detection

Early evaluation of the real-time PCR assay demonstrated it could readily detect single adults, so to further gauge the limit of detection, QIAGEN extractions were performed on a range of sample sizes of Ppf eggs (see **Table 9**). Whilst not all replicates of each sample size could be detected, it was possible to readily detect Ppf eggs, with detection success rate increasing the greater the number of eggs present. Combined with the earlier sensitivity experiments indicating that a single adult is reliably detected (whether extracted by Chelex or QIAGEN), this indicates that the assay has a good sensitivity.

Table 9. Real-time PCR of Ppf egg DNA extracts. A C_T value of 40 indicates the sample is negative; values are presented as an average from duplicate wells.

Sample		C_T value
ENTOBAR1785	1 egg	40.0
ENTOBAR1786		40.0
ENTOBAR1787		40.0
ENTOBAR1788		29.8
ENTOBAR1789		40.0
ENTOBAR1790		40.0
ENTOBAR1791	2 eggs	29.7
ENTOBAR1792		33.3
ENTOBAR1793		33.7
ENTOBAR1794		40.0
ENTOBAR1795		38.6
ENTOBAR1796	5 eggs	40.0
ENTOBAR1797		40.0
ENTOBAR1798		33.9
ENTOBAR1799		33.2
ENTOBAR1800		40.0
ENTOBAR1801	10 eggs	36.4
ENTOBAR1802		33.0
ENTOBAR1803		29.1
ENTOBAR1804		31.8

Sensitivity to DNA from dead mites

Mites were exposed to a 0.5ml/l solution of abamectin (Dymanec) for 30 minutes and then aged for a range of time points prior to DNA extraction and testing for the presence of amplifiable target mite DNA. One, three and five days post mortem 80-100% of the mites could still be detected and for seven and 14 days post mortem 40-60% of the mites could still be detected (see **Table 10**). Because a significant number of dead mites could still be detected at least 14 days after death, in common with other DNA tests there is a risk that field samples would give positive results with the DNA test where the mites were all dead.

Table 10. Real-time PCR with the Ppf assay on adult Ppf mites exposed to Abamectin and aged prior to DNA extraction. A C_T value of 40 indicates the sample is negative; values are presented as an average from duplicate wells.

Sample	Number of days after exposure	C_T value
ENTOBAR1761		39.8
ENTOBAR1762		35.1
ENTOBAR1763	Day 1	37.0
ENTOBAR1764		30.9
ENTOBAR1765		39.7
ENTOBAR1766		33.8
ENTOBAR1767		38.3
ENTOBAR1768	Day 3	40.0
ENTOBAR1769		29.9
ENTOBAR1770		40.0
ENTOBAR1771		39.9
ENTOBAR1772		40.0
ENTOBAR1773	Day 5	30.8
ENTOBAR1774		31.1
ENTOBAR1775		29.7
ENTOBAR1776		30.9
ENTOBAR1777		33.1
ENTOBAR1778	Day 7	40.0
ENTOBAR1779		40.0
ENTOBAR1780		40.0
ENTOBAR1805		35.6
ENTOBAR1806		30.5
ENTOBAR1807	Day 14	40.0
ENTOBAR1808		40.0
ENTOBAR1809		29.9

Discussion

The objective of this research was to develop a method that would enable strawberry leaf material to be tested using a real-time PCR specific for *Phytonemus pallidus* ssp. *fragariae* (Ppf).

For the first time a species specific DNA sequence was identified for Ppf and a molecular test developed that will, without error, identify this species from single adults and in some instances single eggs. The development of this molecular test is a big step forward as it allows this pest to be identified without the need to slide mount adult specimens for confirmatory morphological identification by a specialist and also allows eggs to be positively identified for the first time.

The preparation of the samples for testing by either the extraction of mites or the extraction of total DNA (including the mite DNA) from strawberry leaves followed by testing using the species specific real-time PCR, proved to be a considerable challenge. Total DNA extraction from large bulk strawberry leaf samples generated samples contaminated with compounds inhibitory to PCR to a degree that detection of small numbers of Ppf was not possible. Despite this the programme of work also included an evaluation of two current extraction methods for their suitability as a means of removing and concentrating any potential targets from a sample prior to molecular testing to avoid such inhibitory effects.

The first of these was a flotation technique based on the use of kerosene. This method was developed and optimised for use in extracting and quantifying the numbers of invertebrates found in 20g samples of food or feedstuffs and had been demonstrated to be up to 98% efficient at extracting mites, but had not previously been used to extract invertebrates from plant material or for the purpose of securing samples for DNA analysis. When applied to samples of strawberry leaves this method proved to be very effective at extracting invertebrates of all classes including mites. This was borne out by a trial where it was estimated that invertebrate recovery rates of 90% could be expected for 20g samples however an attempt to scale-up samples to a more useful size of 50g reduced the recovery rate to 60-75%. Nevertheless this technique was successfully used as a survey tool in the initial stages of the project to help determine the most appropriate list of non-target mite species needed for the development of the molecular test.

One unforeseen problem with this technique was that the DNA extracted from the separated invertebrates could not be amplified using PCR methods. It is suspected that some of the chemical reagents and processes such as heating used in preparing and

extracting the samples had damaged the DNA of the specimens. Some of these techniques included concentrated hydrochloric acid, which functions as a wetting agent, and Methylene blue dye, which is added to help visualize any mites extracted on the filter papers produced. Despite eliminating these from the separation step the samples still proved recalcitrant to DNA amplification using PCR, indicating that the damage was probably caused by one or more of the reagents that could not be removed. It is for example known that the naphthalene and its derivatives, one of the main components of kerosene can cause DNA fragmentation. As a result this extraction method and further experiments planned using were abandoned.

The second extraction method, a basic washing technique using a 10% ethanol solution, is part of the current screening procedure used to check strawberry samples for the presence of Ppf. The efficacy of this method had been informally assessed many times in the past, giving mite recovery rates of 60-70%. For the purposes of this project it was formally reassessed on a small scale on samples of two types: strawberry leaves and strawberry crowns. To allow the mite recovery rates achieved using this method to be directly compared with those of the flotation method the sample size was scaled down from the normal 50g sample to 20g. This had no apparent effect on the recovery rates, which were found to be 60-62% for strawberry leaves and 60-70% from strawberry crowns respectively.

It has been shown statistically (see **Appendix 3**) that the number of units (either leaves or crowns) that need to be examined in order to detect either a 0.5% and or 0.1% level of Ppf infestation with 95% confidence, assuming that the sampling regime is 100% efficient, would be approximately 600 and 3,000 respectively and to detect the same levels of infestation with a 99% level of confidence the sample size would have to be increased again to approximately 900 and 4,600 respectively.

The washing method has been shown to be the only viable extraction process available at the present time, but is at best 70% efficient. If sole reliance was placed on this as a means of detecting an infestation in a sample prior to a confirmatory test the sample sizes required to detect either a 0.5 or 0.1% level of infestation at the 95 or 99% confidence interval would range from 800-4,000 to 1,200-6,000 units respectively and clearly samples of this size would be impractical both in terms of collection and processing. However the current screening method includes an initial visual screen as a first step that does detect most infestations prior to an extraction by washing. The washing step will ensure that most (up to 70%) of the mites that may have been missed visually will be picked-up and will also pick-up mite eggs that usually are not easily seen on the surfaces of plant material. The efficiency of the visual screen combined with washing does however need to

be properly evaluated and quantified, but will be in excess of 70% efficient. It would therefore be prudent to keep the initial visual screen as part of the screening protocol until a sufficiently efficient mite extraction process can be developed that allows its exclusion. The current sample size recommended for a screen for Ppf is 250g taken from the most actively growing parts of the plant i.e. the crowns and small newly emergent or not fully unfurled leaves where Ppf is more likely to be found, rather than older tougher or senescent foliage. The number of leaves within a 250g sample would be dependent on the individual leaf sizes, but it was found that a fully unfurled leaf 5cm in length typically weighed between 0.5 and 0.4 g, thus a 250g sample consisting entirely of leaves of this size would contain between 500 and 625 leaves. Obviously smaller leaf sizes would increase the total leaf number. Based on these figures, and that an initial visual screen together with extraction by washing will detect most mites, a sample of 250g would be a sufficient to ensure the detection of an infestation level of 0.5% with 95% confidence.

Work on the detection of adult mites on the filter papers used when samples were extracted by washing showed that it was possible to detect eight mites per filter paper (two per quarter), and given further experimental development it may be possible to achieve detection for much lower numbers of mites and even eggs per filter paper. The extraction process is relatively time consuming and would require scaling up (five filter papers per sample) to process large sample sizes. It is however a relatively quick and easy process to visually screen filter papers for the presence of mites and eggs, which can then be picked-off and identified using the new molecular test. The additional advantage of adopting this approach is that the live and dead mites can be differentiated visually i.e. live or freshly dead mites are usually turgid, white or pale yellow and shiny in appearance whereas dead mites are often flaccid or completely flattened, often a dark amber colour and dull in appearance, whereas the new molecular test could not differentiate between live and dead mites.

The results of this project have clearly highlighted the problems with DNA extraction from large bulks of material, especially difficult to extract material such as strawberry leaves, which are notorious for their high concentration of inhibitors of PCR such as polysaccharides or polyphenolic compounds. Whilst this highlights potential future research directions, the project has generated a robust, economic though relatively time-consuming protocol that can be used to identify Ppf in samples. This has now been augmented with a new molecular test that provides another means of detecting Ppf without the need for specialist morphological identification and the associated time and resourcing issues this traditional method entails.

Conclusions

- A DNA sequence unique to the target organism Ppf was identified.
- A specific real-time PCR test based on the DNA sequence unique to Ppf was developed and will identify all life stages of Ppf.
- The test will give positive results for live Ppf and well as for most dead Ppf for at least 14 days post mortem.
- A flotation extraction method based on the use of kerosene and other reagents was highly efficient for isolating mites, but was found not to be suitable due to the damaging effects that it had on DNA.
- Extraction using a basic alcohol washing procedure was effective but was less efficient at extracting mites.
- Samples should be no more than 250g in weight and collected following the guidelines outline in the 'Action Points' section of the grower summary.
- The initial visual inspection of a sample for Ppf should be kept as part of the screening process and together with extraction by washing should ensure the detection of an infestation level of 0.5% with 95% confidence.
- The new PCR test will be integrated into the current screening protocol, removing the need for specialist morphological identification skills.
- Other possible directions for future research have been identified including: work to overcoming the problem of PCR inhibition in total DNA extractions (i.e. including host plant material); development of a more efficient extraction and work to developing a molecular means of differentiating between live and dead mites.

Knowledge and Technology Transfer

April 2011 - Year 1 Report - Project SF124 Development and validation of a molecular diagnostic test for strawberry tarsonemid mite.

November 2012 - Project SF124 poster provided for the BGG Autumn Growers' Meeting and technical Conference - *Development and Validation of a Molecular Diagnostic Test for Strawberry Tarsonemid Mite*.

November 2012 - Project SF 124 oral presentation presented by Dr. M. Fountain (EMR) at the BGG Autumn Growers' Meeting and technical Conference.

March 2012. Project risk report - SF124 - Development and validation of a molecular diagnostic test for strawberry tarsonemid mite.

July 2012. Project risk report - SF124 - Development and validation of a molecular diagnostic test for strawberry tarsonemid mite.

September 2012. Project risk report - SF124 - Development and validation of a molecular diagnostic test for strawberry tarsonemid.

November 2012. Project risk report - SF124 - Development and validation of a molecular diagnostic test for strawberry tarsonemid.

Glossary

Aspirate(d) – Suction applied for the removal of a liquid or gas.

Kerosene - A thin, clear liquid formed from hydrocarbons with a density of 0.78–0.81 g/cm^3 obtained from the fractional distillation of petroleum and consisting mainly of - dodecane, alkyl benzenes, and naphthalene and its derivatives.

Lipids - A group of naturally occurring molecules that include fats, waxes, sterols, fat- soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, triglycerides, phospholipids, and others.

Lipophilic - Having a strong affinity for lipids

Saprophytic - Feeding on dead or decaying organic matter.

References

Boonham, N. Smith, P. Walsh, K. Tame, J. Morris, J. Spence, N. Bennison, J. Barker, I. (2002). The detection of tomato spotted wilt virus (TSWV) in individual thrips vectors using real time fluorescent RT-PCR (TaqMan). Journal of Virological Methods, 1010:37–48.

Grogan, H. (1997). Examination of the efficacy of two novel fungicides against *Dactylium dendroides*. Horticultural Development Company Annual report for project M 22.

Thind, B. B. (2000). Determination of low levels of mite and insect contaminants in food and feedstuffs by a modified flotation method. Journal of AOAC International 83.1: 13-119.

Weller, S.A. Elphinstone, J.G. Smith, N.C. Boonham, N. and Stead. D.E. 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Applied and Environmental Microbiology, 66:2853–2858.

Appendix 1 - Reagents and apparatus used in flotation extraction protocol

Reagents
Deodorised kerosene (s.g. 0.786 - 0.795) Industrial Methylated Spirits (IMS)
50% aqueous alcohol (specific gravity 0.925) (50% IMS)
Methylene blue solution (2g of Methylene blue dissolved in one litre of 50% IMS) Hydrochloric acid (12M, 35% technical)
Saturated sodium chloride solution
Glycerol

All reagents should be of recognised analytical grade (e.g. AnalaR). Where water is referred to demineralised water should be used. All reagents must be kept in closed containers.

Apparatus
Flotation flask
1 way disc valve
3 piece Hartley funnel located in the 1000 ml filtration flask by means of a bung
Filtration flasks: 500 ml and 1000 ml. 1000 ml attached to vacuum pump via the 500 ml flask that serves as an oil trap
Rotary vacuum pump
Geared motor with attached paddle
Kerosene reservoir
600 ml Pyrex beaker
Spatula
Filter paper (Whatman no. 41 110 mm) ruled into 10 mm squares
TrayAluminium,foil
Soft paint brush
Glass slides (67 x 67 mm)
Petri dish lid (150 mm diameter)
Measuring cylinders (2 x 200 ml, 2 x 25 ml) for use with separate reagents

Wash bottles for separate reagents
Balance accurate to 0.001 g
Vacuum desiccator (255 mm diameter) Microwave oven
Fume cabinet
Stereoscopic microscope with x10 – x90 magnification zoom lens
Red narrow nibbed felt tip pen
Grid for use with dust samples
Glass Microscope slides
Glass cover slips (10-13 mm diameter)
Heinz medium (available from Mite Taxonomy) Microscope slide labels
Bung marked with a large black spot
Fine mounted needle
Stereoscopic microscope
Drying oven
Mite Identification forms
Teepol
Cloths and bottle brushes
100 ml measuring cylinder
Parafilm
Wide bore 10 ml pipette
Laboratory tissues

Appendix 2 – Data for Ppf recovery rates on strawberry plant material

Data for Ppf recovery rates from 20g samples of leaves and crowns spiked with different numbers of mites.

Leaves	**Replicate**					**No.**	**% Ppf Recovere**
	1	**2**	**3**	**4**	**5**		
No. Ppf *spiked*							
I	1	1	0	1	0	3	60
Control	0	0	0	1*	0	0	
5	5 (1*)	4	0	3	3	15	60
Control	0	0	1*	0	0	0	
10	6	8	6	8	5	31	62
Control	0	0	0	0	0	0	

Data for Ppf recovery rates from 20g samples of leaves and crown spike with different numbers of mites.

Crowns	**Replicate**					**No.**	**% Ppf Recovere**
	1	**2**	**3**	**4**	**5**		
No. Ppf *spiked*							
I	1	0	0	1	1	3	60
Control	0	0	0	0	0	0	
5	3	5	5	2	2	17	68
Control	1*	0	0	0	0	0	
10	7	9	4	8	7	35	70
Control	0	0	1*	1*	0	0	

**Tarsonemus* spp.

Appendix 3 - Sampling statistics for large lots (Binomial or Poisson based sampling)

When the lot size is sufficiently large and mixed the likelihood of finding an infested unit is approximated by simple binomial statistics. The hypergeometric distribution is appropriate for small lots.

Sample sizes for 95% and 99% confidence at varying levels of detection and efficacies of detection (lot size large and mixed, binomial distribution)

Percentage efficacy of	P = 95% (confidence level) % level of detection					P = 99% (confidence level) % level of detection				
	5	**2**	**1**	**0.5**	**0.1**	**5**	**2**	**1**	**0.5**	**0.1**
100	59	14	29	59	299	90	22	45	91	460
99	60	15	30	60	302	91	23	46	92	465
95	62	15	31	63	315	95	24	48	96	484
90	66	16	33	66	332	10	25	51	102	511
85	69	17	35	70	352	10	26	54	108	541
80	74	18	37	74	374	11	28	57	114	575
75	79	19	39	79	399	12	30	61	122	613
50	11	29	59	119	599	18	45	91	184	920
25	23	59	119	239	1198	36	91	184	368	184
10	59	149	299	599	2995	91	230	460	920	460