Project Title:	**Development and validation of a molecular diagnostic test for strawberry tarsonemid mite**
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The results and conclusions in this report are based on an investigation conducted over a one-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

CONTENTS

GROWER SUMMARY

Headline

- Good progress is being made towards the development of a molecular diagnostic test for strawberry tarsonemid mite.

Background and expected deliverables

There has been a significant and threatening increase in the frequency and severity of attacks by tarsonemid mite (*Phytonemus (Tarsonemus) pallidus* ssp*. fragariae)* in UK strawberry production in the last few years and the problem has been particularly bad in 2010 and 2011 and threatens to get worse. Tarsonemid mite is usually introduced into plantations on infested planting material. 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. 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, such as 500-1,000 growing points, each from a separate randomly chosen plant. A highly sensitive test is needed to ensure the pest can be detected in such a sample. A decision will need to be taken as to the tolerance level (risk of the pest being present) in the sampled crop.

Following this project, a rapid, sensitive and reliable diagnostic test for strawberry tarsonemid mite will be made available to the suppliers of planting material to UK growers (and elsewhere if deemed appropriate) through FERA's diagnostic services. Sampling in the propagation field before runners are lifted would be desirable. The test will raise propagators' awareness of the problem and is likely to spur them into taking stronger action to ensure that their stocks are clean. The test could also be used by UK growers of fruiting crops, for early diagnosis of potential problems so effective evasive action can be implemented early.

The objectives of this work are:

1. To develop a molecular test for strawberry tarsonemid mite (Year 1)
2. To validate the test (Years 1 and 2)
3. To investigate specificity, sensitivity, reliability and determine optimum sample size (Years 1 and 2)

The development of a highly sensitive, rapid DNA-based molecular test for tarsonemid mite in strawberry plants is something that hasn't been attempted before and is a case of charting unknown territory, although the scientific team is armed with the methods that will successfully deliver the required result.

Summary of the project and main conclusions

The scientific team quickly established an efficient method of extracting the mites from samples of strawberry plants, based on an existing floatation method and this has provided much of the mite material needed and will be a key part of the final test.

At the same time, work in year 1 commenced to develop a pest specific test based on the COI gene. Previous research has shown the COI gene to be reliable for the identification of most invertebrate species. Thus far, for the mites used in this project, this has not been the case. The team has therefore switched to two other genes, namely 28S rRNA and ITS, that can also be used for species identification. The latest results support this change and encouraging results are being generated.

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 in some instances. A central aim of the project is to develop and validate a rapid, sensitive and reliable diagnostic test for strawberry tarsonemid mite that will be made available to the suppliers of planting material to UK growers (and elsewhere if deemed appropriate) through FERA's diagnostic services. The cost of similar tests is typically £40-60 per sample or less, depending on the numbers of samples being processed so the cost benefit ratio of a test is likely to be very large.

Action points for growers

- There are no action points at this early stage of the project

SCIENCE SECTION

Development and validation of a molecular diagnostic test for strawberry tarsonemid mite.

1. Objectives

This project aims to develop a highly sensitive, rapid DNA-based molecular test for tarsonemid mite in strawberry plants. The objectives are firstly development and secondly validation of the test. The project milestones are detailed in **Table 1**. At this point the project is at month 12 based on a contracted start date of 1 April 2011.

Table 1. Project milestones, tasks and target dates

Milestone Number	Task	Target date (month)
1.1	Sequence data for target species and other mite genera	6
1.2	Real-time (TaqMan) primers and probes developed	12
1.3	Primer specificity tested	15
1.4	Method of DNA extraction determined	15
2.1	Limit of detection determined	15
2.2	Sensitivity of DNA from dead mites determined	18
2.3	Diagnosis sensitivity validated	18
2.4	Sampling protocol/recommendations formulated	24

2. Methodology

2.1. Mite material

To provide specimens of mite species prevalent in the UK, EMR collected samples of strawberry plants from six separate strawberry plantations of varying ages and including both organic and non–organic crops.

The total invertebrate fauna was extracted using the methodology of a proven flotation extraction technique (Thind & Wallace, 1984). In summary, this involves chopping samples of leaf and crown material which are then dispersed into deionised water and mixed. Kerosene is then introduced into the base of this system and percolates upward to form a separate layer on top of the water. Because invertebrate cuticle is lipophilic the kerosene picks up and carries off any and all insects and mite material as it passes through the suspended plant material. The kerosene layer is then siphoned off through filter paper and the invertebrates are collected before being cleaned and preserved.

2.2. DNA extraction

DNA extraction was performed from individual mites to generate DNA for DNA bar-coding purposes. This was done with a minimum of three individuals from each species. Total DNA was extracted using a simple, rapid Chelex DNA extraction method.

2.3. DNA bar coding

Polymerase chain reaction (PCR) for a range of different genes was performed using various primers to generate PCR amplicons for sequencing. PCR was performed using BIO-X-ACT Taq polymerase (Bioline) with proof-reading capability, with a typical reaction shown in Table 2.

Table 2. The composition of a typical PCR reaction

Component	Per reaction µl	Final concentration
BIO-X-ACT Short mix (2x)	12.5 µl	1x
Forward primer (10µM)	1.0 µl	400nM
Reverse primer (10µM)	1.0 µl	400nM
Molecular grade water	9.5 µl (i.e. to 24 µl)	
DNA	1.0 µl	

PCR results were visually assessed by agarose gel electrophoresis. DNA sequencing was performed by Eurofins-MWG-Operon, and results processed using ABI sequence scanner v1.0 and phylogenetic analyses performed using MEGA 4.

3. Results

3.1. Mite material

An initial list of the mite taxa expected to be found in association with strawberry plants was drawn-up on the basis of previous records, to act as a guide to the taxa required for this study and with the intention of securing three distinct populations of each (**Table 3**).

Strawberry field samples from six separate strawberry plantations were provided by EMR starting from 9 June 2011 onward. The total invertebrate fauna was extracted (**Figure 1**) from five sub samples comprising of two crowns and five leaves and totalling 100g of material from each strawberry sample.

Table 3. Initial list of the mite species likely to be found in field crops of strawberries, with target species shown in red

Order	Family	Genus	Species	Authority	Availablility
Astigmata	Acaridae	*Tyrophagus*	*putrescentiae*	(Schrank)	In culture
		Tyrophagus	*palmarum*	Oudemans	In culture
	Winterschmidtiidae	*Calvolia*	*transversostriata*	(Oudemans)	
	Anoetidae	*Histiosoma*	*ferroniarum*	(Dufour)	
Mesostigmata	Phytoseiidae	*Amblyseius*	*andersoni*	Chant	
		Typhlodromus	*pyri*	Scheusten	
		Euseius	*finlandicus*	(Oudemans)	
		Phytoseiulus	*persimilis*	Athias-Henriot	
		Neoseiulus	*californicus*	McGregor	
	Parasitidae	*Parasitus*	spp.		
Prostigmata	Tarsonemidae	*Phytonemus*	*palidus* ssp. *fragariae*	(Zimmermann)	Yes
		Tarsonemus	spp.		
		Tarsonemus	*confusus*	Ewing	
		Tarsonemus	*setifer*	Ewing	
		Xenotarsonemus	*viridis*	Beer	
	Tetranychidae	*Tetranychus*	*urticae*	Koch	Yes
		Bryobia	*praetiosa*	Koch	Possibly
		Bryobia	*rubioculus*	(Schesten)	Possibly
		Panonychus	*ulmi*	(Koch)	
		Amphitetranychus	*viennensis*	(Zacher)	Yes
		Petrobia	*latens*	(Müller)	
Oribatidae	Humerobatidae	*Humerobates*	*rostrolamellatus*	Grandjean	

Figure 1. Total invertebrate fauna from one flotation extract

The invertebrate fauna composition from each sample was found to be largely the same, and from this material mites were removed, non-destructively identified by morphological means and prepared for molecular analysis. This process revealed the presence of mite taxa in additional to those expected from the initial literature search.

The process of sorting and identification continues in order to increase the range of taxa and number of populations available for molecular analysis. At this point in time we have secured three separate populations of target species (*Phytonemus pallidus* ssp. *fragariae*) and several other taxa (Table 4).

Table 4: Amended list of mite species likely to be found in field crops of strawberries, included taxa located and secured from field strawberry samples and other sources (highlighted in green)

Order	Family	Genus	Species	Authority
Astigmata	Acaridae	*Tyrophagus*	*putrescentiae*	(Schrank)
		Tyrophagus	*palmarum*	Oudemans
		Tyrophagus	*similis*	
		Tyrophagus	*longior*	
	Winterschmidtiidae	*Calvolia*	*transversostriata*	(Oudemans)
	Anoetidae	*Histiosoma*	*ferroniarum*	(Dufour)
Mesostigmata	Ameroseiidae	*Ameroseius*	*corbiculus*	(Sowerby)
	Phytoseiidae	*Amblyseius*	*andersoni*	Chant
		Typhlodromus	*pyri*	Scheusten
		Euseius	*finlandicus*	(Oudemans)
		Phytoseiulus	*persimilis*	Athias-Henriot
		Neoseiulus	*californicus*	McGregor
	Parasitidae	*Parasitus*	spp.	
		Parasitus	*beta*	(Oudemans et Voigts)
Prostigmata	Tarsonemidae	*Phytonemus*	*palidus* ssp. *fragariae*	(Zimmermann)
		Steneotarsonemus	spp.	
		Tarsonemus	spp.	
		Tarsonemus	*confusus*	Ewing
		Tarsonemus	*setifer*	Ewing
		Xenotarsonemus	*viridis*	Beer
	Tetranychidae	*Tetranychus*	*urticae*	Koch
		Bryobia	*praetiosa*	Koch
		Bryobia	*rubioculus*	(Schesten)
		Panonychus	*ulmi*	(Koch)
		Amphitetranychus	*viennensis*	(Zacher)
		Petrobia	*latens*	(Müller)
	Tydeidae			
Oribatidae				
	Humerobatidae	*Humerobates*	*rostrolamellatus*	Grandjean
	Camisiidae	*Platynothrus*	*peltifer*	(Koch)
	Ceratozetidae	*Sphaerozetes*	*orbicularis*	(Koch)

3.2. DNA extraction

Analysis of the success of the selected DNA extraction method for single mites (not within a host background) was judged by whether DNA was extracted which could be amplified by PCR. DNA could be reliably extracted from the single mite species, regardless of the sample size.

3.3. DNA bar coding to identify target gene

3.3.1. Initial test panel for PCR testing

For selection of the best target gene for discrimination of *Phytonemus palidus* spp. *fragariae* a panel of mite samples was selected. This was comprised of a diverse range of mite species from three orders and four families to assess the range of genetic similarity or diversity (Table 5). Three adult individuals of each species (identities confirmed by morphological assessment) were randomly selected, and each given a unique designation within the Fera entomology DNA bar-coding database 'ENTOBAR'.

Table 5. Initial test panel for PCR

Collection Reference	Rep.	Order	Family	Genus	Species
ENTOBAR1245	1 of 3	Prostigmata	Tarsonemidae	*Phytonemus*	*pallidus fragariae*
ENTOBAR1246	2 of 3	Prostigmata	Tarsonemidae	*Phytonemus*	*pallidus fragariae*
ENTOBAR1247	3 of 3	Prostigmata	Tarsonemidae	*Phytonemus*	*pallidus fragariae*
ENTOBAR1248	1 of 3	Prostigmata	Tetranychidae	*Tetranychus*	*urticae*
ENTOBAR1249	2 of 3	Prostigmata	Teranychidae	*Tetranychus*	*urticae*
ENTOBAR1250	3 of 3	Prostigmata	Tetranychidae	*Tetranychus*	*urticae*
ENTOBAR1251	1 of 3	Mesostigmata	Phytoseiidae	*Neoseiulus*	*californicus*
ENTOBAR1252	2 of 3	Mesostigmata	Phytoseiidae	*Neoseiulus*	*californicus*
ENTOBAR1253	3 of 3	Mesostigmata	Phytoseiidae	*Neoseiulus*	*californicus*
ENTOBAR1254	1 of 3	Astigmata	Acaridae	*Tyrophagus*	*similis*
ENTOBAR1255	2 of 3	Astigmata	Acaridae	*Tyrophagus*	*similis*
ENTOBAR1256	3 of 3	Astigmata	Acaridae	*Tyrophagus*	*similis*
ENTOBAR1257	1 of 3	Prostigmata	Tydeidae		
ENTOBAR1258	2 of 3	Prostigmata	Tydeidae		
ENTOBAR1259	3 of 3	Prostigmata	Tydeidae		

3.3.2. Cytochrome c oxidase subunit 1 gene

PCR products were generated using standard published DNA bar-coding primers (C1-JF-1718 and C1-NR-2191) amplifying the cytochrome c oxidase subunit I gene (COI) (Figure 2). This yielded distinct specific PCR amplicons from at least one individual of each of the species tested, and these amplicons were then DNA sequenced.

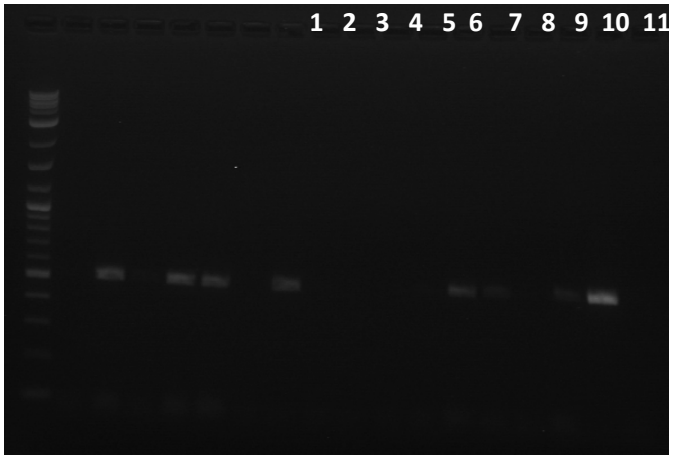

Figure 2. Results from COI PCR showing amplification products separated using agarose gel electrophoresis. Lanes; 1 = 2-log DNA ladder (NEB); 2 =ENTOBAR1245; 3 = ENTOBAR 1246; 4 = ENTOBAR 1247; 5 = ENTOBAR 1248; 6 = ENTOBAR 1249; 7 = ENTOBAR 1250; 8 = ENTOBAR 1251; 9 = ENTOBAR 1252; 10 = ENTOBAR 1253; 11 = ENTOBAR 1254; 12 = ENTOBAR 1255; 13 = ENTOBAR 1256; 14 = ENTOBAR 1257; 15 = ENTOBAR 1258; 16 = ENTOBAR 1259; 17 = positive control; 18 = negative (water) control

Analysis of the DNA trace files (Figure 3) revealed that for all samples and species a run of 10 ‘G’ bases caused a sequencing phenomenon known as polymerase slippage. This is a rare occurrence caused by the DNA sequencing being unable to process a long run of one single base, and causes the DNA sequence to degrade in quality so that it is unusable.

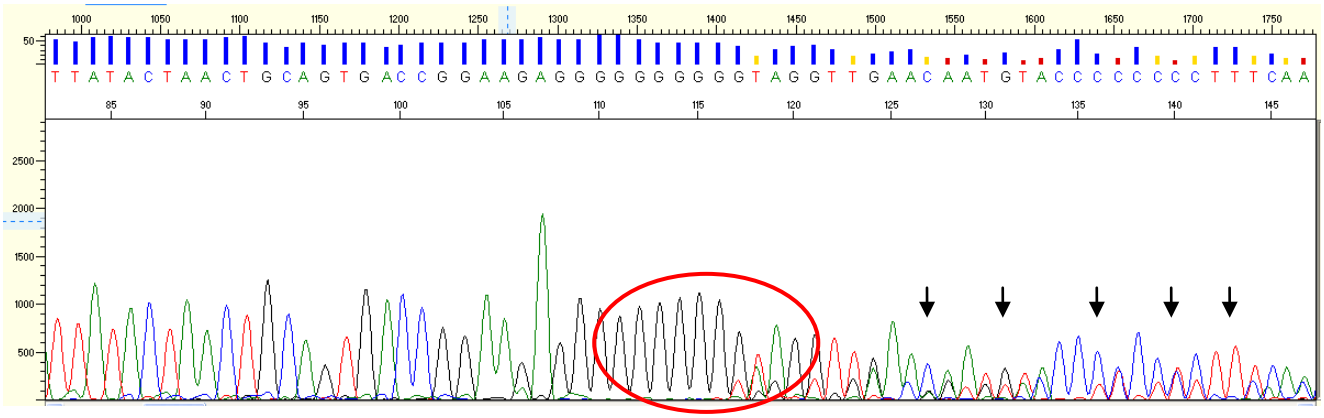

Figure 3. Example trace file of ENTOBAR1251 sequenced with primer C1-JF-1718 with the polymerase slippage site circled. Note sequence before the site has single defined peaks, and after the site there are peaks over-laying each other (some example of this are marked with arrowheads)

Regardless of this sequencing issue, useable sequence was generated at either side of the site for all species, and an alignment of these created to assess the genetic diversity across the species (Figure 4). This revealed that for all five species the COI gene was identical. This is a highly surprising finding as this is the gold-standard gene for identification of a wide

range of invertebrates. This means that this gene will not be a suitable target to allow species specific detection of *Phytonemus palidus* spp. *fragariae*.

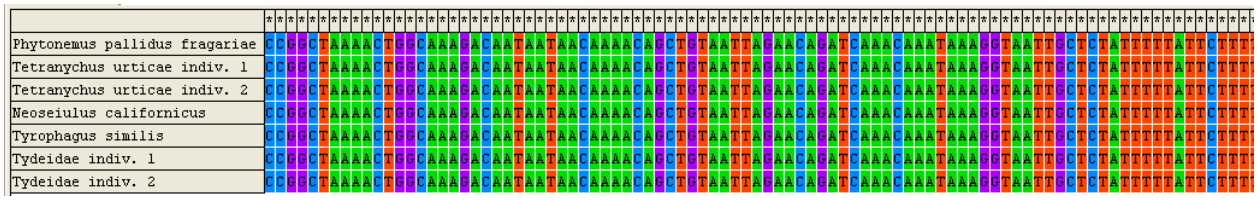

Figure 4. Alignment of partial COI gene sequence for five mite species

3.3.3. Internal transcribed spacer gene

Primers designed to amplify the internal transcribed spacer (ITS) of several mite species from different genera have been published by Navajas *et al* (1999). These have been shown to amplify several of the non-target species in this project, and the ITS is commonly used in molecular taxonomy. For this reason PCR with these primers (Mite-ITS-F and Mite-ITS-R) were performed on the initial test panel (**Table 5**). This yielded PCR amplicons for four samples from three species (**Figure 5**), although two were not suitable for DNA sequencing due to the low concentration of the PCR product.

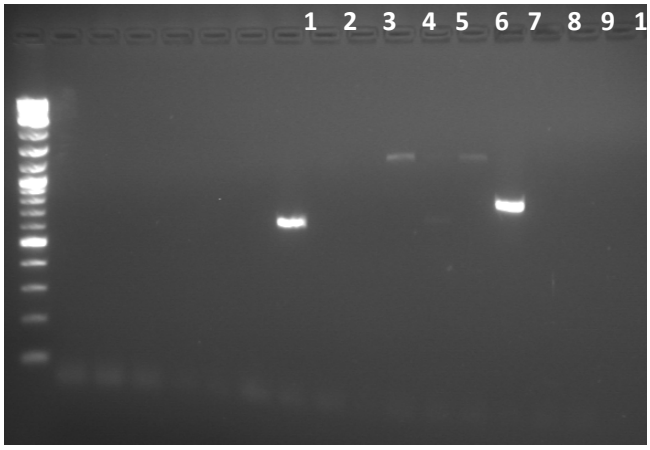

Figure 5. Results from ITS PCR showing amplification products separated using agarose gel electrophoresis. Lanes; 1 = 2-log DNA ladder (NEB); 2 =ENTOBAR1245; 3 = ENTOBAR 1246; 4 = ENTOBAR 1247; 5 = ENTOBAR 1248; 6 = ENTOBAR 1249; 7 = ENTOBAR 1250; 8 = ENTOBAR 1251; 9 = ENTOBAR 1252; 10 = ENTOBAR 1253; 11 = ENTOBAR 1254; 12 = ENTOBAR 1255; 13 = ENTOBAR 1256; 14 = ENTOBAR 1257; 15 = ENTOBAR 1258; 16 = ENTOBAR 1259; 17 = negative (water) control

Analysis of the ITS sequences revealed no sequencing issues and high levels of difference between the two species from two orders, suggesting that this target gene may have potential to differentiate between the mite species of interest. Efforts are now underway to generate ITS sequences from a wider range of species.

Figure 6. Alignment of partial ITS gene sequence for two mite species

3.3.4. 28S ribosomal RNA gene

The 28S rRNA is another gene commonly used for molecular identification and differentiation of invertebrate species and Fera have designed primers for routine DNA bar-coding of certain mite species with this gene (primer sequences unpublished). PCRs were performed on the initial test panel (**Table 5**) of five diverse species (**Figure 7**).

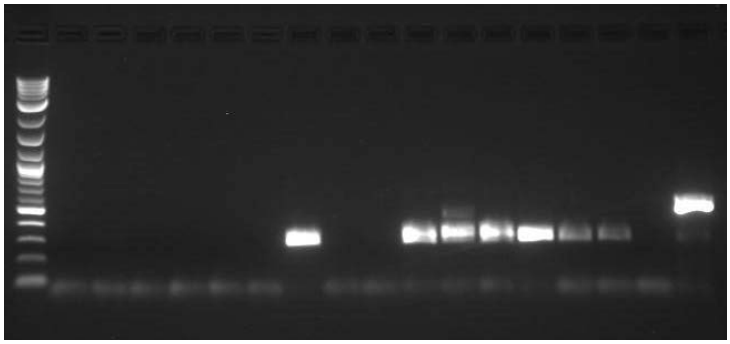

Figure 7. Results from 28S rRNA PCR showing amplification products separated using agarose gel electrophoresis. Lanes; 1 = 2-log DNA ladder (NEB); 2 =ENTOBAR1245; 3 = ENTOBAR 1246; 4 = ENTOBAR 1247; 5 = ENTOBAR 1248; 6 = ENTOBAR 1249; 7 = ENTOBAR 1250; 8 = ENTOBAR 1251; 9 = ENTOBAR 1252; 10 = ENTOBAR 1253; 11 = ENTOBAR 1254; 12 = ENTOBAR 1255; 13 = ENTOBAR 1256; 14 = ENTOBAR 1257; 15 = ENTOBAR 1258; 16 = ENTOBAR 1259; 17 = negative (water) control; 18 = positive control

Sequencing of these products yielded useable sequences from six individuals of three species. Alignments of the sequences (Figure 8) showed regions of similarity and diversity between the species. This revealed unique bases within all of the species, suggesting that this target may be able to differentiate the target and non-target species in a diagnostic assay. Efforts are now underway to generate 28S rRNA sequences for a broader range of mite species.

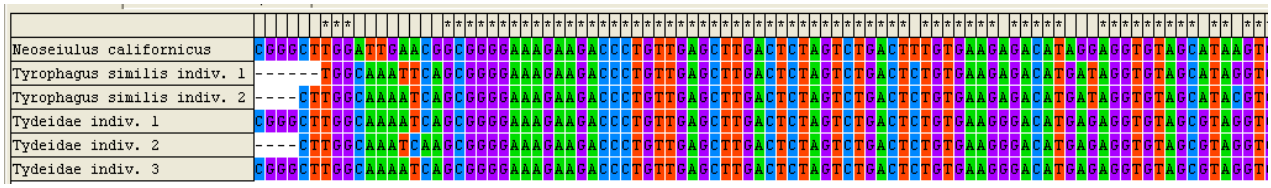

Figure 8. Alignment of partial 28S rRNA gene sequence for three mite species, with arrows indicating examples of bases conserved across all species (left hand), unique to one species (centre) and varying in all three species (right hand)

4. Conclusions and future work

Analysis of the gold standard gene for most invertebrate species identification, the COI gene, revealed that this is identical across several mite species of interest within this project. Therefore DNA sequences have been generated for two alternate genes, the 28S rRNA and ITS, which can be used for species identification. To date, sequences have been generated for several non-target species from different orders and this has shown both of these genes to be variable across different mite species. The focus of the project is now generating additional sequences from a wider range of target and non-target species for these genes, and this data can then be used to design a species specific TaqMan assay. The methods for separation of mites from plant material have proven to be quite effective.

Given the challenges faced with the molecular method development side of the project, it is proposed that some of the project milestone dates are modified as detailed in **Table 6**. This will not affect the project final end date, not the overall project budget.

Table 6. Proposed modifications to project milestones

Milestone Number	Task	Contract target date (month)	Proposed new target date (month)
1.1	Sequence data for target species and other mite genera	6	15
1.2	Real-time (TaqMan) primers and probes developed	12	15
1.3	Primer specificity tested	15	18
1.4	Method of DNA extraction determined	15	18
2.1	Limit of detection determined	15	18
2.2	Sensitivity of DNA from dead mites determined	18	21
2.3	Diagnosis sensitivity validated	18	21
2.4	Sampling protocol/recommendations formulated	24	24

Reviews of primary literature have suggested that the challenges faced in this project have been experienced by many others within the field, as demonstrated in Figure 9, showing the lack of published information for the target family.

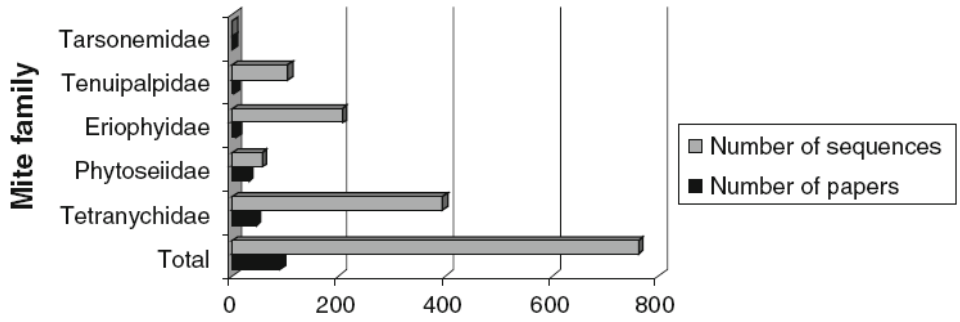

Fig. 1 Contribution of DNA data to the study of several plant mite families, estimated in number of published papers (CAB 1990–2009 database) and number of DNA sequences submitted to GenBank (on March 28th, 2009)

Figure 9. Figure from Navajas and Navia, 2010 paper, demonstrating the lack of published information (sequences or primary literature) on the family Tarsonemidae

The results of the latest testing suggest that two potential genes have now been identified that enable specific detection of the target species, allowing the project to progress. The change to the anticipated target gene does not in any way affect the methods by which the test is completed, nor the cost of the final test.

5. References

Navajas, M. Lagnel, J. Fauvel, G. and De Moraes, G. 1999. Sequence variation of ribosomal Internal Transcribed Spacers (ITS) in commercially important Phytoseiidae mites. *Experimental and Applied Acarology,* 23: 851–859.

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