Project title:	Optimising the *Macrolophus*-based *Tuta absoluta* IPM strategy: Phase 1 – Identification of species on UK nurseries.
Project number:	PC 302c
Project leader:	Dr Jennifer Hodgetts, The Food and Environment Research Agency (Fera)
Report:	Final report, March 2013
Previous report:	None
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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.

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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CONTENTS

GROWER SUMMARY

Headline

Commercial samples of *Macrolophus* from three suppliers and a residual nursery population dating from the mid-1990s were all identified as *Macrolophus pygmaeus* using molecular techniques.

Background

Macrolophus was first released into UK tomato crops as *M. caliginosus* (now = *M. melanotoma*) in 1995 to supplement the biological control of glasshouse whitefly. Within two growing seasons it became clear that the predators would also feed on tomato plants when invertebrate prey was limited. UK growers stopped releasing *Macrolophus* but populations survived between seasons and it became one of the most important pests of organic tomato crops. Research work from 2006 in HDC project PC 240 found a solution which allowed growers to obtain the predatory benefits of *Macrolophus* without suffering crop damage. Once pests had been controlled natural pyrethrins were used to reduce the population of *Macrolophus* before crop damage occurred. This resulted in renewed interest in the predator and some growers started to release it to supplement the control of tomato leaf miner (*Liriomyza bryoniae*). More recently, *Macrolophus* has formed the basis of an IPM strategy for the management of the new tomato pest, *Tuta absoluta*. As part of this IPM programme some growers have released *Macrolophus* at the start of each season while others have relied on the population already established on their nurseries. The *Macrolophus*-based IPM strategy has been successful in some situations but there have been inconsistencies which must be explained before these can be resolved.

During the last two decades, there has been considerable debate about the taxonomy of the *Macrolophus* complex of species. It was thought possible that the inconsistencies in the IPM results in UK tomato crops could be due to the presence of more than one species and the overall aim of the project was to study the genetic diversity of *Macrolophus* spp. and begin to consider the implications to the successful IPM of *Tuta absoluta*

As the first step to improving understanding of the use of *Macrolophus* it was important to compare the species of *Macrolophus* currently being sold by the main bio-control suppliers to those predator populations which become established in tomato crops each summer. In addition, the products were compared to a *Macrolophus* population which had been

established on a commercial nursery for 18 years without being supplemented with any further purchased material.

Summary

DNA sequences of four genes were analyzed from 21 separate adult *Macrolophus* specimens from seven separate sources / localities including three commercial bio-control agent suppliers and four commercial tomato growers. Three of the growers had released purchased *Macrolophus* during 2012 while the fourth had not released the predator since the mid-1990's.

Three genes (COI, ITS2 and D3) revealed identical DNA sequences across all the samples tested. One gene (CytB) revealed that one of the samples from a grower who released purchased material in 2012 had a slightly different DNA sequence to all other samples but they were still the same species. The CytB and COI DNA sequences were compared to publically available sequences of *M. melanotoma* and *M. pygmaeus*. This revealed that all samples tested were *M. pygmaeus*. Whilst this has demonstrated that overwintering and released populations of Macrolophus are the same species, any potential differences in the effectiveness of the two groups in terms of bio-control action cannot be inferred from this study. Therefore at present there are no recommended changes to the *Tuta absoluta* IPM strategy.

The results from the grower who had not released *Macrolophus* since the mid-1990's indicate that the material sold at that time was *M. pygmaeus* and not *M. caliginosus* (= *M. melanotoma*) as labelled. However, this was simply related to nomenclature and has probably not affected the IPM programmes used in the intervening period.

Financial Benefits

There is no immediate financial benefit to UK growers from this study. However, the information is a prerequisite to further fine tuning of the *Macrolophus*-based IPM strategy for *T. absoluta*.

Action Points

The results do not impact on growers at this stage but will influence further studies aimed at optimizing the overall IPM programme for tomato crops.

SCIENCE SECTION

Introduction

Taxonomy of Macrolophus spp.

As far as we know, plant bugs of the genus *Macrolophus* are naturally represented in the UK fauna by only two species; *M. rubi* Woodroffe and *M. pygmaeus* (Rambur). Both are widespread but not particularly common in the general environment with *M. rubi* usually associated with bramble (*Rubus* spp.) and *M. pygmaeus* with hedge woundwort (*Stachys sylvatica* L.), although both may be found on other plants.

The two species can be separated morphologically on the basis of two subtle characters; the relative length of the third antennal segment and the presence or absence of a dark mark at the apex of the clavus. Both of these characters are difficult to see in the field and, with the added complication of intra-specific variation, accurate species determination can be difficult.

To further complicate issues a third species *M. calignosus* Wagner was introduced into protected cultivation as a biological control agent. The separation of this species from both *M. rubi* and *M. pygmaeus* is also difficult, with some workers suggesting that *M. pygmaeus* and *M. calignosus* are in fact the same organism. *Macrolophus calignosus* has recently been renamed *M. melanotoma* and at present the two names are synonyms.

A brief history of the use of Macrolophus in UK tomatoes

Macrolophus spp. adults and nymphs are voracious predators. *Macrolophus caliginosus* was not indigenous to the UK and was first released under licence in 1995 to supplement the biological control of glasshouse whitefly. Within two growing seasons it became clear that the predators would also feed on the tomato plants when invertebrate prey was limited (Hayman & Jacobson, 1996). The damage to growing points and trusses could be extremely serious with losses in organic crops estimated to exceed £72k/ha per season (Starkey, unpublished data, 2004).

Although UK growers stopped releasing *M. caliginosus*, populations survived between seasons and it became one of the most important pests of organic tomatoes in the UK. A solution was found in 2006, which allowed UK growers to obtain the predatory benefits of *M. caliginosus* without suffering crop damage (Jacobson & Morley, 2006). This resulted in

renewed interest in the predator and some growers started to release it to supplement the control of *Liriomyza bryoniae* (Kaltenbach) larvae.

The new Tuta absoluta control strategy

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) arrived in the UK in 2009 and it is now present and under official control on several sites across the country. The larvae cause extensive damage by mining in leaves and fruit. It is currently considered to be the most important pest of UK tomato crops.

A new season-long IPM strategy for the control of *T. absoluta* has been developed based on the predatory bug, *Macrolophus* spp. (Jacobson, 2011a and 2011b). The growing season has been divided into four distinct periods. The first period, from planting in December until early-mid spring, is the key period for establishing *Macrolophus* spp. Other methods are employed throughout this period to slow down *T. absoluta* population growth. These include exclusion, application of spinosad (applied under a Plant Health Order) through the irrigation system, deleafing, sticky floor treatments and mass trapping with pheromone and/or light traps. The second period is from mid- to late-spring. Despite the measures taken during the first period to delay *T. absoluta* population growth, it seems inevitable that at least one second line of defence (SLoD) treatment is required before the predatory bugs start to have a significant impact. The third period is from early summer through to early autumn. The predatory bugs should now be more numerous and suppress the *T. absoluta* population growth by feeding on eggs and larvae. However, careful monitoring is required to determine whether it becomes necessary to apply additional SLoD treatments. The fourth period is from early to late autumn when the main objective is to reduce the number of pests that survive to infest the following crop.

Although this strategy has been successful in some situations, there have been inconsistencies between nurseries and repeated application of pesticides have been needed. It is important to reduce this by optimising the use of *Macrolophus* spp.

The local populations of *Macrolophus* spp. are slow to establish in UK tomato crops and useful numbers are rarely found before May. In an attempt to speed up establishment of *Macrolophus* spp, many growers now release the predators at rates of up to 2/m^2 (cost about £1.6k/ha/season) at the start of the growing season. Despite this extra input, the *Macrolophus* spp, populations still do not reach useful levels before May. It is unclear whether the summer populations originate from those which overwinter locally or from the released biological control products. This must be clarified.

The overall aim of this project is to study the genetic diversity of *Macrolophus* spp. and begin to consider the implications to the successful IPM of *Tuta absoluta*.

The specific objectives are:

Objective 1: Collect a range of *Macrolophus* spp. specimens
Objective 2: Perform molecular analysis using DNA barcoding to establish the genetic relationship between collected samples

Background to molecular techniques

DNA barcoding employs the analysis of a short segment of an organisms DNA to identify what species it is - the segment of DNA used is called the 'DNA barcode'. Although the DNA of closely related species is generally quite similar there are distinctive differences in the sequences between species that provide a unique means of identification. Depending upon the species one or several DNA barcodes can be used.

DNA barcoding can be used for both identification and diagnostics, and is becoming a common technique for invertebrate identification. The technique can be applied to any species of interest and there are numerous world-wide DNA barcoding projects ongoing. This approach can be particularly useful for cryptic species that are difficult or impossible to differentiate using traditional morphological methods.

Although DNA barcoding is normally used to identify a species (by comparison of the DNA barcode to a database of known sequences) the technique can also be applied to studies aiming to differentiate species. Using this approach, barcodes from several genes are compared across the specimens of interest, and this can be used to establish if they are the same or different.

There are a range of genes which are common DNA barcodes. Some are mitochondrial and others nuclear genes; some are highly conserved, and others highly variable. By analysing a panel of genes with these different characteristics, DNA barcoding can be a very powerful tool. The implementation of DNA barcoding is a relatively simple lab procedure; DNA is extracted from individual insects, PCR performed, PCR amplicons subjected to DNA sequencing and then the resulting sequences analysed.

Once DNA barcoding has been undertaken, the information generated can be used in numerous ways. One use could be the development of a specific test for the detection of

the species required for an IPM strategy (such as TaqMan PCR for *M. caliginosus*) confirming the identity of the bio-control agent prior to release.

Materials and methods

Sample collection

Samples were collected by RJC Ltd from a range of sources including the commercial suppliers to the UK industry and a range of commercial growers as detailed in Table 1.

Table 1. Origin of samples used in the study

Sample origin	Acronym	Date Preserved	Sample type
Syngenta Bioline	Syngenta	02/02/2012	Commercial bio-control agent supplier
Koppert UK	Koppert	28/02/2012	Commercial bio-control agent supplier
BCP Certis	BCP	26/01/2012	Commercial bio-control agent supplier
Red Roofs, Cottingham, East Yorkshire	Durnford	28/08/2012	Commercial grower who only released in the mid-1990's and not since
Cornerways Phase 2, Wissington, Norfolk	Cornerways	14/02/2012	Commercial growers who released in 2012 growing season
VHB, Runcton, East Sussex	VHB Runcton	16/08/2012	Commercial growers who released in 2012 growing season
WSG, Arreton, Isle of Wight	WSG Arreton	30/08/2012	Commercial growers who released in 2012 growing season

Morphological Assessment

All the adult mirid bugs in the samples listed in Table 1 were examined using a Leica M205 C dissection microscope at up to 160X magnification.

Genetic Analysis

DNA extractions were performed on three individual adult specimens from the seven samples listed in Table 1 using the QIAGEN DNeasy Blood and Tissue kit, following the manufactures protocol for animal tissues.

Based on PCR primers used in the literature for previous studies of *Macrolophus* spp. combined with primers commonly used for insect DNA barcoding, primers for seven genes were selected (see Table 2). These were synthesized by Eurofins-MWG-Operon.

Table 2. The target genes, primers and references used in the study

Target gene	Primer name	Sequence (5'-3')	Reference
Cytochrome B (CytB)	MacCal-F	CTTGATGCCTTTTATTGTGG	Machtelinckx *et al*., 2009
	MacCal-R	TGAATATGCACGGGGGTTAC	Machtelinckx *et al*., 2009
	MacPyg-F	ATGGCTATGAGGAGGGTTCTC	Machtelinckx *et al*., 2009
	MacPyg-R	TCTGGTTGAATATGGACTGGTG	Machtelinckx *et al*., 2009
Cytochrome oxidase I (COI)	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al*., 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al*., 1994
Cytochrome oxidase II (COII)	C2-J-3400F	ATTGGACATCAATGATATTGA	Simon *et al*., 1994
	CSL-372	TCCACAAATTTCTGCGCATTG	Glover *et al*., 2009
Cytochrome oxidase III (COIII)	C3-J-5014F	TTATTTATTKTWTCWGAAGT	Simon *et al*., 1994
	CSL-378	TCAACAAAGTGTCAGTATCATGC	Glover *et al*., 2009
D3 domain of 28S rRNA (D3)	D3A-F	GACCCGTCTTGAAACACGGA	Maraun *et al*., 2003
	D3B-R	TCGGAAGGAACCAGCTACTA	Maraun *et al*., 2003
Histone H3	H3NF	ATGGCTCGTACCAAGCAGAC	Colgan *et al*., 1999
	H3R	ATATCCTTRGGCATRATRGTGAC	Colgan *et al*., 1999
Internal transcribed spacer 2 (ITS2)	52R	GTTAGTTTCTTTTCCTCCCCT	Moritz *et al*., 2002
	P1	ATCACTCGGCTCGTGGATCG	Moritz *et al*., 2002

PCR reactions were performed for each gene using a proof-reading *Taq* Polymerase (BIOLINE BIO-X-ACT short) in 25 µl reactions using PCR parameters as described in the original reference. PCR products were visualized by agarose gel electrophoresis and samples suitable for DNA sequencing were subjected to QIAGEN QIAquick PCR purification kit before being submitted to Eurofins-MWG-Operon for DNA sequencing of both strands of DNA. DNA traces were manually edited and trimmed, consensus sequences prepared for each sample (where possible), and sequence alignments created in MEGA4. Phylogenetic trees were created using the neighbor-joining (NJ) algorithm to study the similarity of the DNA sequences.

Results

Literature Review

A mass of contradicting literature based primarily on morphological assessment was published in the 1990's leading to a state of confusion as to whether or not *M. melanotoma* (= *M. caliginosus*) and *M. pygmaeus* were one or two species. These primarily used the colouration of the first antennal segment and some morphological features to differentiate the two species.

Two defining pieces of literature were then published in the early 2000's both confirming that there are indeed two species, *M. melanotoma* (=*M. caliginosus*) and *M. pygmaeus*. Perdikis *et al*., 2003 performed hybridization experiments of populations of *M. melanotoma* and *M. pygmaeus* and applied two molecular techniques, studying the 16S rRNA gene by restriction fragment length polymorphism and random amplified polymorphic DNA PCR. All three approaches indicated the presence of two species. Martinez-Cascales *et al*., 2006 used a combination of morphological assessment and molecular data of the cytochrome B gene to confirm two distinct species.

However, these two papers still could not reach consensus as to whether the colouration of the first antennal segment should be used as a character to separate the species, with Perdikis stating this is a 'limitation' and that molecular markers are the 'appropriate method', and Martinez-Cascales stating that 'the shape of the black macula behind the eye may be used as a diagnostic character to differentiate' but with the caveat of 'with some degree of confidence'.

In 2012 a further paper was published where a very in-depth multivariate morphometric analysis was conducted (Castane *et al*., 2012) which could separate the males from the two species 'without error' and combined this with the development of new PCR primers for the cytochrome B gene which could discriminate the species by producing different sized PCR products for each. However to further muddy the water, in 2012 a paper by Gemeno *et al.,* stated that *M. pygmaeus* is 'morphologically undistinguishable' from *M. melanotoma*, yet one of the authors of this paper was Castane. This study used a third approach (separate to morphological and molecular studies) of chemical analysis of cuticular hydrocarbons which was able to discriminate the two species.

In summary, literature published after 2003 appears to reach a consensus that there are indeed two species. Molecular studies using a range of genes and approaches have confirmed this, as has cuticular hydrocarbon analysis. Separation based on morphological assessment is still an impractical means of separating *Macrolophus* to species level. This seems to remain a highly contentious issue to the present date, and may only be possible with a highly in-depth and skilled assessment.

Assessment of Publically Available Sequence

DNA sequences are typically deposited onto the National Centre for Biotechnology Information (NCBI) database by researchers globally. This is a freely accessible database and is the primary source of DNA sequences for researchers. However the main problem with the NCBI database is that it is not curated; meaning that it can contain DNA sequences which are mislabeled, contain sequencing errors or are attributed to the wrong species either because of samples being mixed up, or crucially, if the identification of the specimen from which DNA was taken from was initially incorrect. The key factor of relevance to this study is the initial morphological assessment and assignment of the specimen to species level. If this is not done correctly, then the DNA sequence can be very misleading. For this reason, it is considered prudent to use DNA sequences generated from multiple laboratories, to add robustness to the sequence, as this will likely mean that several entomologists will have classified specimens to the same species.

DNA sequences on NCBI categorized as Macrolophus were downloaded and sequence alignments and phylogenetic trees created to assess the clustering of sequences and the associated species name. For three genes (12S ribosomal RNA, cytochrome oxidase subunit I (COI) and cytochrome b (CytB)) sequences are available which are categorized as being from the two species of interest. The molecular study of *Macrolophus* species appears to be very fluidic and an area of interest at the present time. When the proposal for this project was prepared in July 2012, there were only a handful of sequences for a single gene available, a situation which has suddenly changed, with several research groups appearing to be actively studying these species again.

There are 18 sequences for the partial 12S ribosomal RNA gene, 12 for *M. pygmaeus* and 6 for *M. melanotoma*, all of which were added in December 2012. These were all generated by the same research group based at the Agricultural University of Athens in Greece, with the lead author Evangelou. The sequences are linked to a draft manuscript entitled '*Macrolophus* sp. and Biological Control: New Diagnostic Molecular Markers and Phylogenetic Relationships Based on Sequencing Data' which is at present unpublished.

These sequences show very large differences between *M. pygmaeus* and *M. melanotoma* (see Figure 1) and clearly split the specimens into two groups, with also quite substantial differences within each group, which the researchers have classified as haplotypes. As all the sequences of this gene are generated from one research group and the associated paper has not yet been published, it is not possible to critically assess this research, so this is not ideal as a genetic marker to study at this stage.

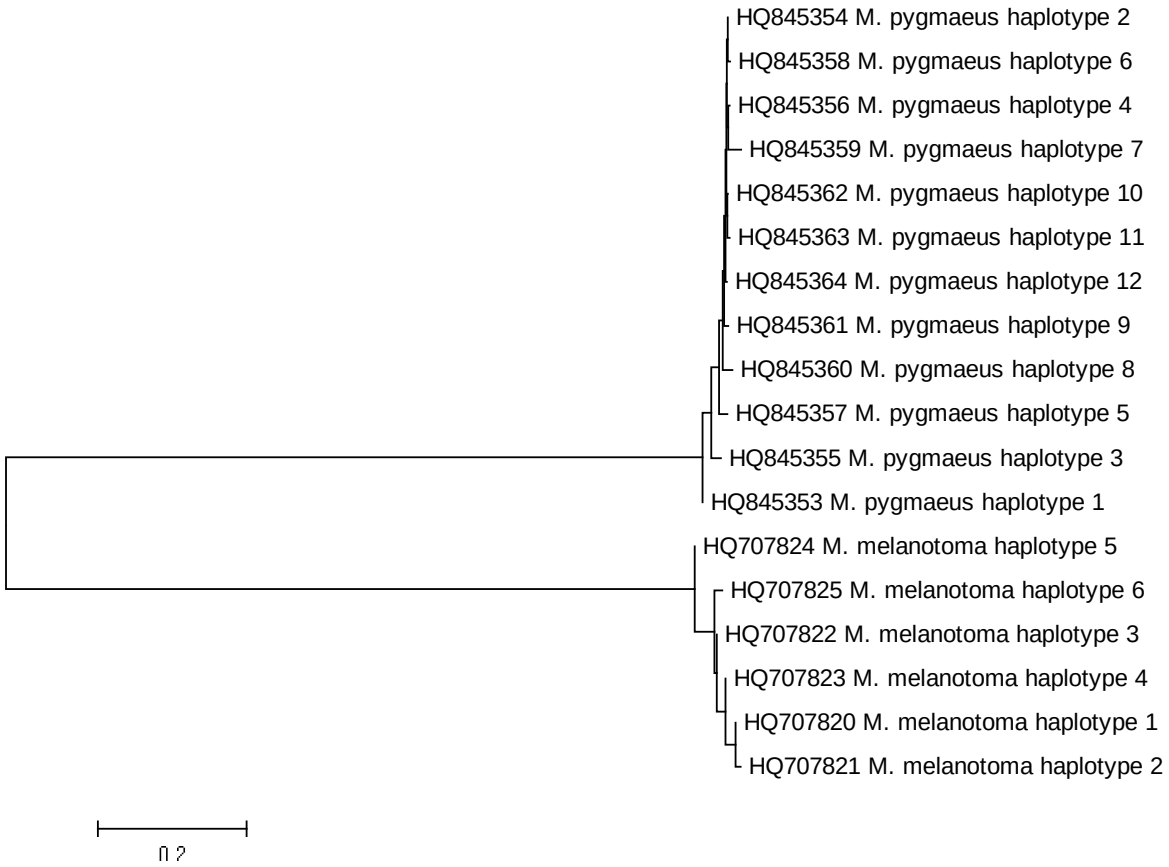

Figure 1. Phylogenetic tree of sequences for the 12S ribosomal RNA gene from NCBI

There are 29 sequences for the COI gene, identified as *M. pygmaeus*, *M. melanotoma* and *M. calignosus* on NCBI. There are sequences for both *M. pygmaeus* and *M. melanotoma* from the Evangelou group, but additionally (and importantly) there are sequences for each species from a different research group adding robustness to the data. A selection of these from each group were analyzed and revealed clustering of *M*. *pygmaeus* and *M. calignosus* together distinct from *M. melanotoma*, which splits into two distinct groups.

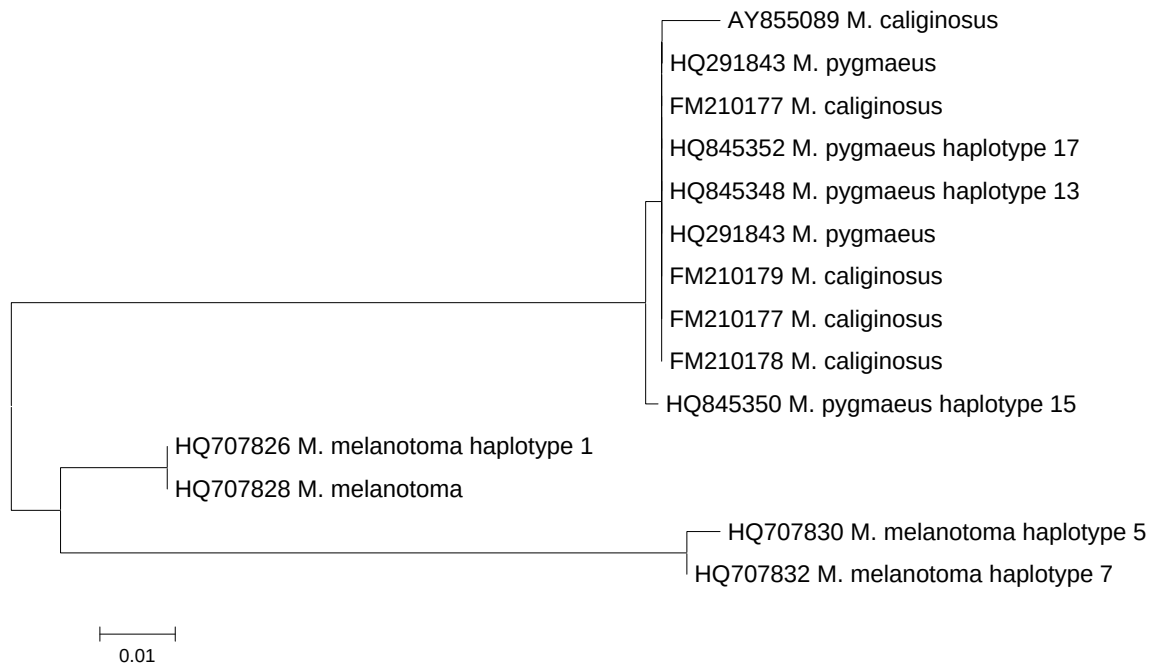

Figure 2. Phylogenetic tree of sequences for the cytochrome oxidase subunit I gene from NCBI

There are 99 sequences for the CytB gene, produced by two research groups, Martinez-Cascales and Machtelinckx. A sub-set of sequences were analysed (see Figure 3), revealing again a clear split into groups of *M. melanotoma* and *M. calignosus* together, separate to *M. pygmaeus* which themselves split into two groups.

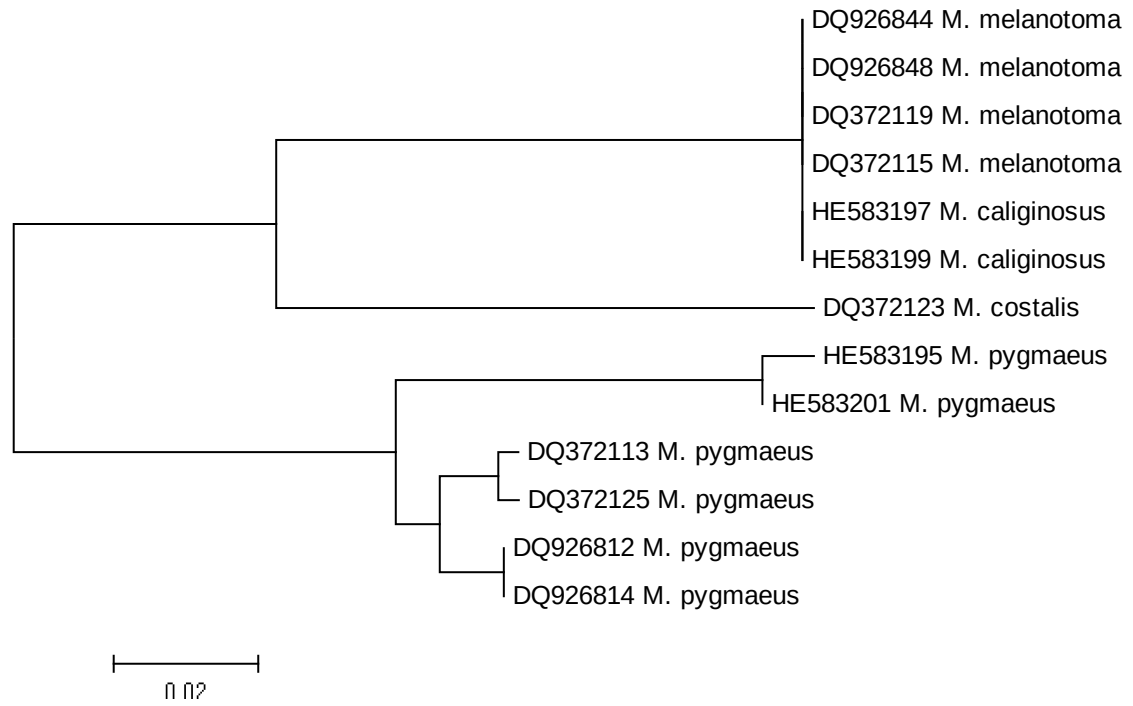

Figure 3. Phylogenetic tree of sequences for the cytochrome b gene from NCBI

In summary, the CytB and 12S genes separate *M. melanotoma* and *M. calignosus* from *M. pygmaeus*, yet the COI gene splits *M. calignosus* and *M. pygmaeus* from *M. melanotoma*. A possible explanation for the COI gene results is that the *M. calignosus* specimens were misidentified as *M. pygmaeus*, or that this may not be a suitable marker to differentiate the species.

Morphological Assessment

Assessment of all the adult specimens within the samples was undertaken, after which three typical specimens were selected for molecular analysis. Other than consistent sexual dimorphism there were no observable morphological differences between the specimens within or between the samples that would allow individuals to be identified to the species level with certainty, thus confirming the finding in previously published work.

Genetic Study of Specimens Obtained

PCR was performed on three individual adult specimens from the seven sample types detailed in Table 1 for seven genes to select a panel for sequence analysis. Two genes were not taken forward for sequence analysis; the histone H3 gene primers yielded multiple non-specific PCR products which were not suitable for DNA sequencing, and the cytochrome oxidase III failed to produce any PCR products with the samples.

PCR products for five genes (cytochrome B, cytochrome oxidase I, cytochrome oxidase II, D3 domain of 28S rRNA and internal transcribed spacer 2) were subjected to DNA sequencing. All of the samples with the cytochrome oxidase II gene generated DNA sequences which were not suitable for analysis so this gene could not be analysed. The remaining four genes all generated good sequences and were subjected to phylogenetic analysis.

For the ITS2 and D3 genes there are no publically available sequences for any macrolophus species so these were analysed just with an outlier, whilst for the cytochrome B and cytochrome oxidase I genes samples were compared to a selection of sequences from the NCBI database as well as the outlier. *Thrips palmi* Karny was selected to use as an outlier. Phylogenetic trees are shown for the D3 gene (Figure 4), the ITS2 gene (Figure 5), the COI gene (Figure 6) and the CytB gene (Figure 7).

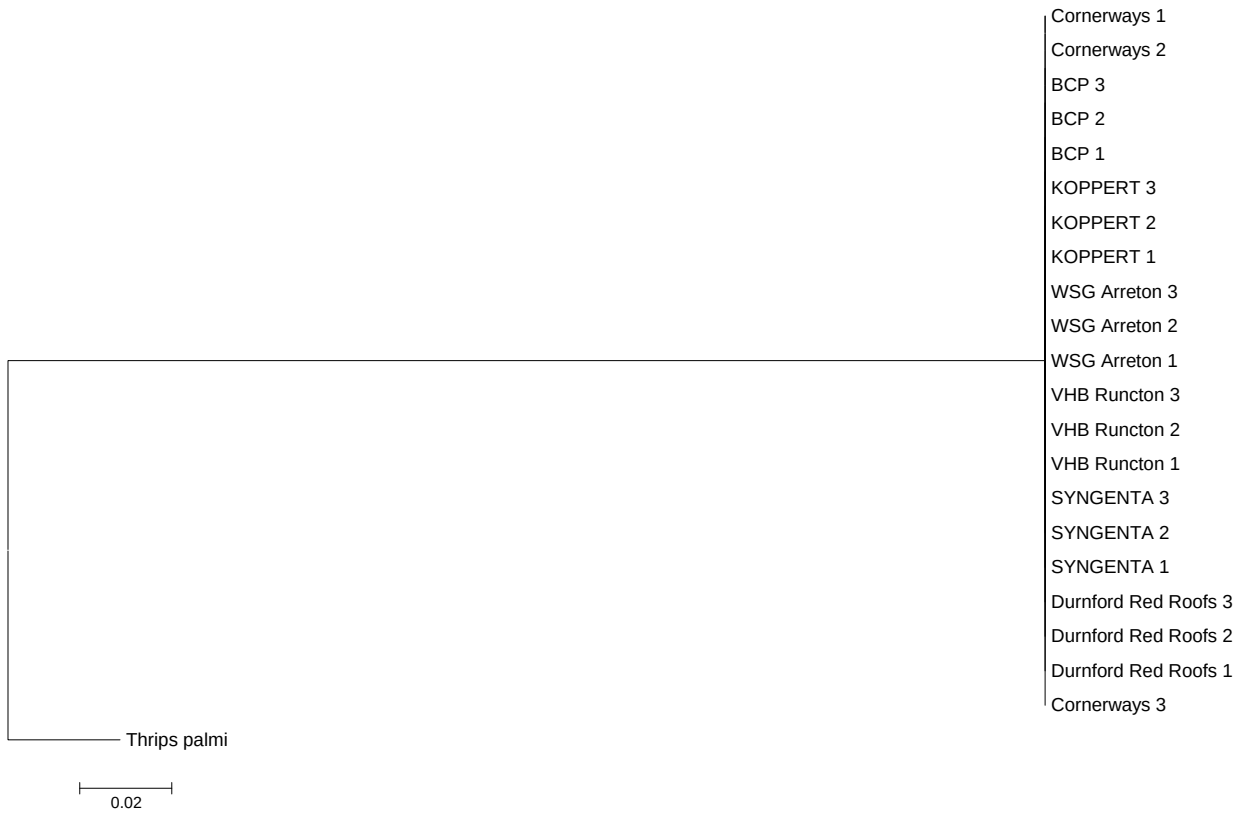

Figure 4. Phylogenetic tree of D3 gene sequences from the project samples

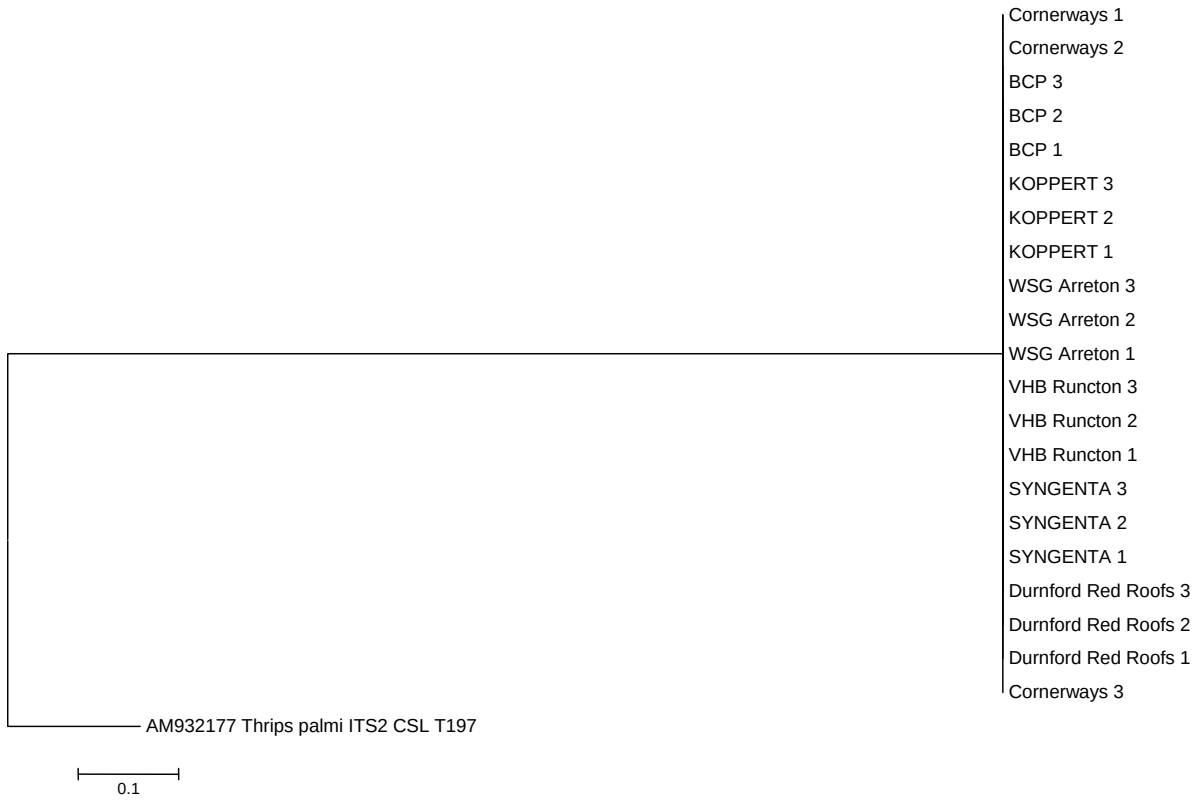

Figure 5. Phylogenetic tree of ITS2 gene sequences from the project samples

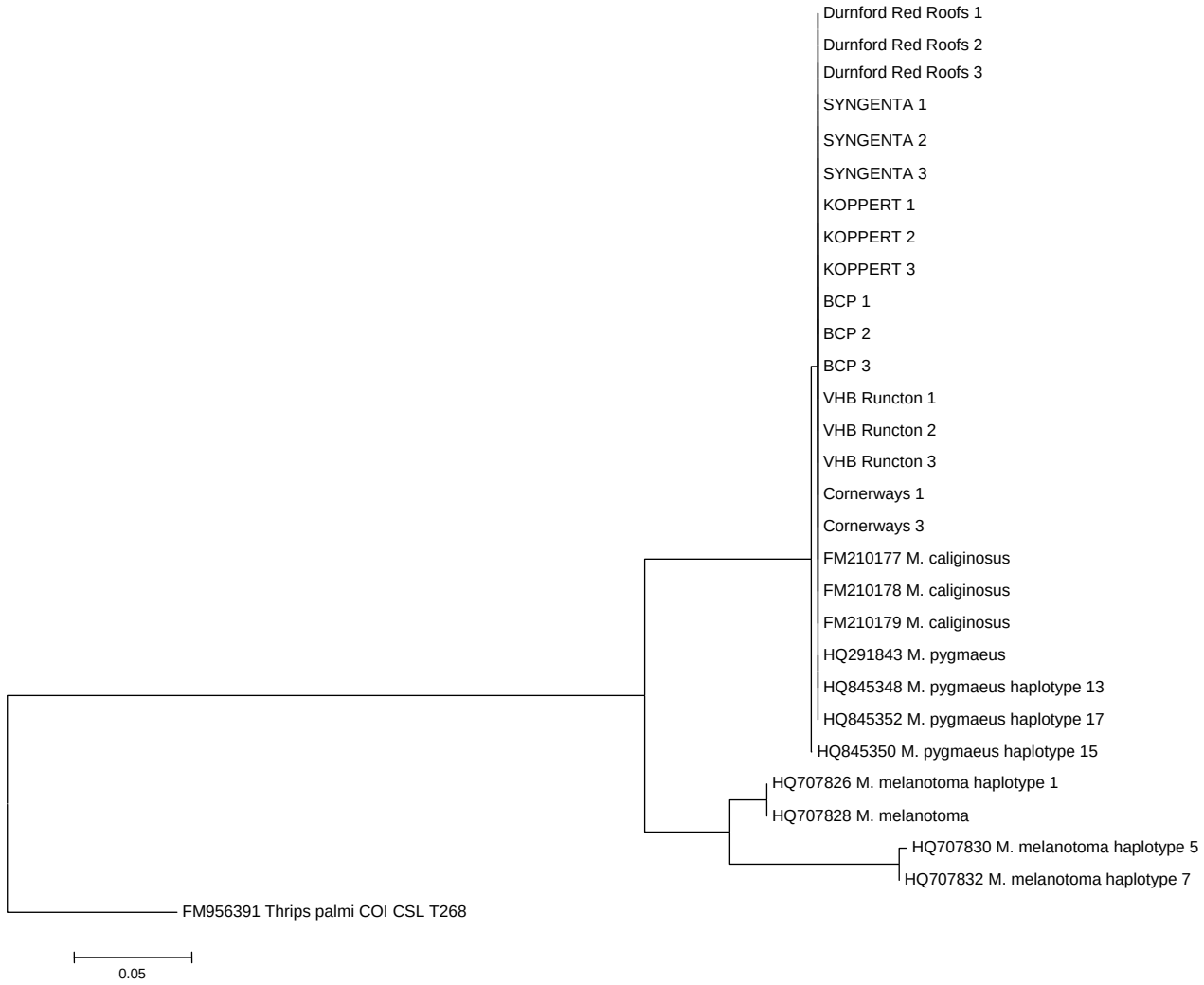

Figure 6. Phylogenetic tree of COI gene sequences from NCBI and the project samples

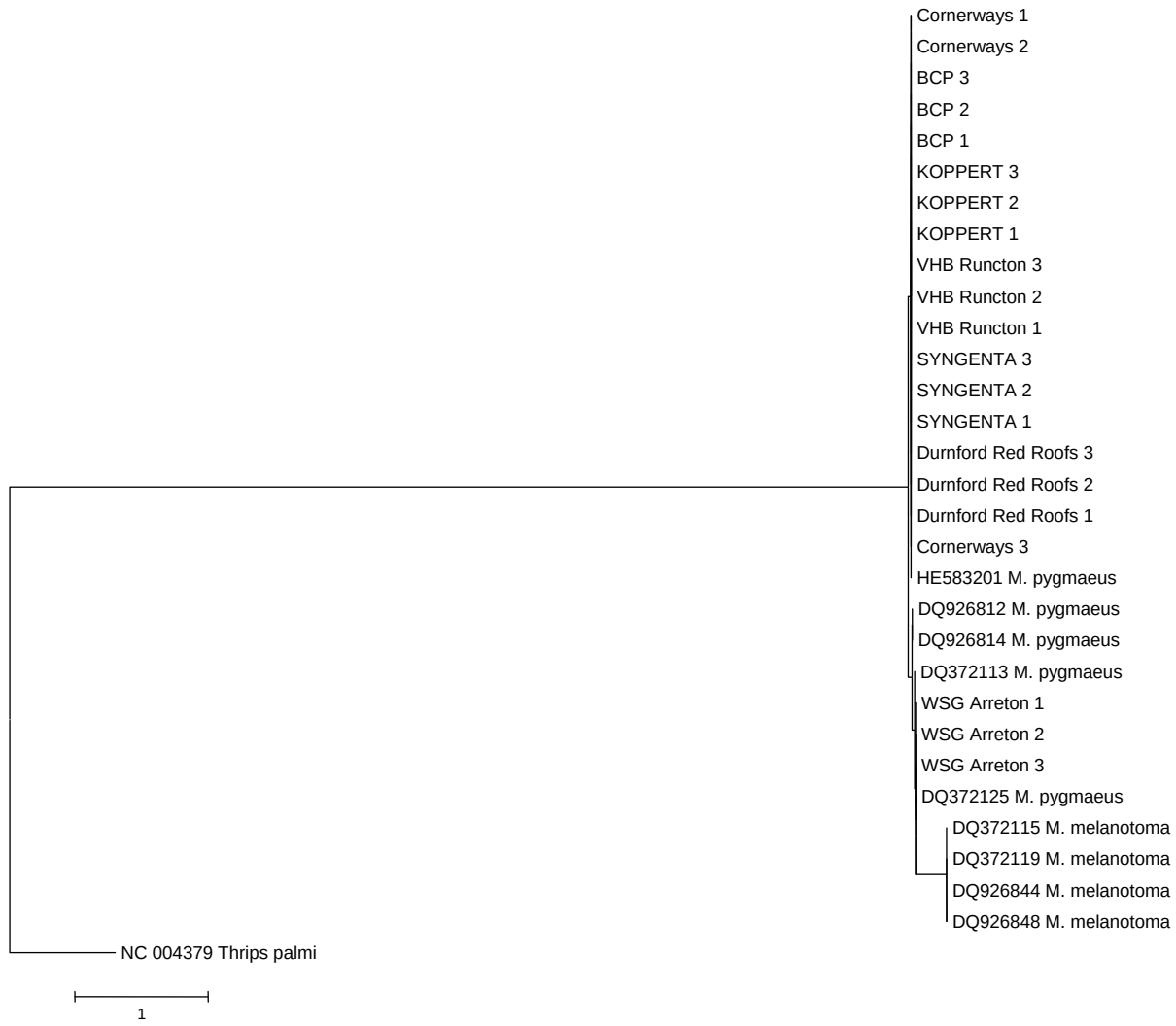

Figure 7. Phylogenetic tree of CytB gene sequences from NCBI and the project samples

Analysis of the D3 (Figure 3) and ITS2 (Figure 4) genes reveal that all the samples studied have identical DNA sequence for these regions. As there are no named sequences available from the two species available, the sequences were searched against the NCBI database which revealed no significant similarity or high percentage similarity matches to other sequences within the database. Whilst we cannot assign the samples to a species from these genes, we can confirm that all of the samples are the same species.

Four samples were subjected to PCR and sequencing of the COI gene twice, but on both occasions failed to generate DNA sequence which could be used (samples Cornerways 2, and WSG 1, 2 and 3). Analysis of the COI gene (Figure 6) for the samples again revealed that the samples have identical sequences, and cluster with sequences from NCBI labeled as *M. pygmaeus* (and the likely mis-identified *M. calignosus*) whilst distinct from the *M.*

melanotoma sequences. This suggests that the samples tested are *M. pygmaeus* and again are all the same species.

Analysis of the CytB gene (Figure 7) reveals differences within all of the samples studied. The *M. pygmaeus* sequences from NCBI split into 2 clusters which are separate to *M. melanotoma*. All of the samples analyzed cluster within the *M. pygmaeus* sequence clusters, however the WSG samples cluster to one group, and all of the other samples to the other group.

Discussion

The key aim of this study was to analyze the populations of *Macrolophus* currently present at commercial tomato growers and compare these to those currently available from bio-control suppliers. This would hopefully establish if these were all the same species and then help in the understanding of the implementation of IPM schemes. By analyzing a panel of genes the relationship between a range of samples could be determined.

If any differences between current bio-control supplies and natural populations were present, these would have been expected to be evident in the Red Roofs samples, as this grower had not released purchased *Macrolophus* since the mid 1990's. This was not found to be the case. And further, of all the samples studied, only very small differences were found within one of four genes studied in samples from a grower who released in the 2012 growing season. On this basis the WSG sample appeared to be indicative of a slightly different population of *M. pygmaeus*. Comparisons of IPM success with this population compared to others may help to elucidate if population differences of *M. pygmaeus* (at the strain level) are contributing to the observed inconsistencies in IPM control, given that this study has shown the species involved are the same and not the root cause.

Conclusions

- Three genes (COI, ITS2 and D3) revealed identical DNA sequences across all the samples tested.
- One gene (CytB) revealed the WSG samples to have slightly different DNA sequence to all other samples but were still the same species.
- The CytB and COI DNA sequences were compared to publically available sequences of *M. melanotoma* and *M. pygmaeus*. This revealed that all samples tested were *M. pygmaeus*.
- Variation was seen within the CytB gene, however, all samples were shown to be *M. pygmaeus.*
- The results from the grower who had not released *Macrolophus* since the mid-1990's indicate that the material sold at that time was *M. pygmaeus* and not *M. caliginosus* (= *M. melanotoma*) as labelled.

Knowledge and Technology Transfer

Progress has been shared at the Tomato Growers' Association Technical Committee meetings throughout the course of the project.

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