Arthcollect 2019-F-323

Interlaboratory Test Performance Study

Final report

Interlaboratory Test Performance Study (TPS) for comparison of molecular tests identifying specimens of the Infraorder Coccomorpha: Coccidae & Pseudococcidae (soft scales and mealybugs). Organised as part of the Euphresco Arthcollect 2019-F-323 project.

Contents

1. Introduction

The scale insects and mealybugs of the infraorder Coccomorpha contain numerous pest species found on crop and ornamental plants worldwide. Although adult females can be identified morphologically to species, problems that are routinely encountered with classical taxonomy such as, distinguishing between very similar species and identifying eggs and juvenile specimens, remain. Normally DNA-based identification methods would be used in parallel alongside classical taxonomy however molecular identification of specimens from the infraorder Coccomorpha has also proven to be problematic (Park *et al.*, 2011[1]).

EPPO guidelines (EPPO, 2021[2]) recommend the use of the mitochondrial COI gene for molecular identification of arthropods, however amplification success rate and subsequent sequence generation can be lower for Coccomorpha using the recommended primers (Sethusa *et al.* (2014)[3], Malausa *et al.* (2009)[4]). Therefore, investigation into extraction methods and subsequent downstream analysis was carried out. Review of the literature reveals that using the CO1 gene in conjunction with nuclear genes; elongation factor 1α (EF-1α), Internal Transcribed Spacer 2 (ITS2) and large ribosomal subunit (28s), can increase the likelihood of a successful molecular identification (Park *et al.* (2011)[1], Sethusa *et al.* (2014)[3]).

This interlaboratory Test Performance Study (TPS) aimed to evaluate one DNA extraction method and effectiveness of two other barcoding primers alongside the LCO1490 & HCO2198 Folmer[5] primers for their usefulness in molecular identification of two different species of Coccomorpha: Coccidae & Pseudococcidae (soft scales and mealybugs). This TPS was organised through the Euphresco Arthcollect 2019-F-323 project, a significant part of which involved exchanging knowledge and experience of difficult to sequence arthropods such as Coccomorpha. Five partners from the Arthcollect 2019-F-323 project took part in this study, including SASA, UK who prepared the test samples. A sixth laboratory, separate from the Euphresco project, also participated.

2. Executive Summary

2.1. Aim

The interlaboratory Test Performance Study (TPS) was organised by SASA, UK. The main aim of the TPS was to compare molecular tests for identifying specimens of the Infraorder

[1] Park *et al.* (2011). DNA barcodes for two scale insect families, mealybugs (Hemiptera: Pseudococcidae) and armored scales (Hemiptera: Diaspididae). DOI: 10.1017/S0007485310000714

[2] EPPO (2021). DNA barcoding as an identification tool for a number of regulated pests. PM 7/129 (2) https://doi.org/10.1111/epp.12724

[3] Sethusa *et al.* (2014). DNA Barcode Efficacy for the Identification of Economically Important Scale Insects (Hemiptera: Coccoidea) in South Africa. https://doi.org/10.4001/003.022.0218.

[4] Malausa *et al.* (2009). DNA markers to disentangle complexes of cryptic taxa in mealybugs (Hemiptera: Pseudococcidae). DOI: 10.1111/j.1439-0418.2009.01495.x

[5] Folmer *et al.* (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. https://pubmed.ncbi.nlm.nih.gov/7881515/

Coccomorpha: Coccidae & Pseudococcidae (soft scales and mealybugs). Six laboratories across six different countries took part.

Objectives:

1. To evaluate the effectiveness of the DNeasy Blood & Tissue kit (QIAGEN) as a DNA extraction method of two species of Coccomorpha (using a non-destructive technique).
2. To test the effectiveness of three different barcoding primer sets for their usefulness in molecular identification (sequencing) of two species of Coccomorpha.
3. To determine if the voucher specimens were intact after the extraction process and identifiable morphologically [optional].

2.2. Key findings

Objective 1: All laboratories were able to easily acquire and use the DNeasy Blood & Tissue kit (QIAGEN). It was observed that the non-destructive method of piercing specimens was difficult, occasionally causing damage to the specimen (Pseudococcidae). Although some DNA concentrations were low, the QIAGEN kit was successful at extracting DNA from all specimens.

Objective 2: The 28s primer set had the highest sequencing success rate for Coccidae (soft scales) and Pseudococcidae (mealybugs), followed by the alternative COI primer set PCOF1/Lep-R. The Folmer COI primer set LCO1490/HCO2198 failed to produce a valid sequence for both Coccidae and Pseudococcidae samples.

Objective 3: Some laboratories observed that after the extraction process, the Coccidae (soft scales) and Pseudococcidae (mealybugs) voucher specimens were damaged and changed in appearance making them difficult to morphologically ID. Soft scale voucher specimens were noted desiccated, while mealybug voucher specimens appeared completely translucent. It is likely that piercing the specimen and incubating in lysis buffer overnight is damaging important morphological features used in species identification. Although this may prevent a species level identification, identification to genus was still possible in some cases.

2.3. Recommendations

For DNA extraction of Coccomorpha specimens, we recommend using the DNeasy Blood & Tissue kit (QIAGEN). For sequencing Coccomorpha species, we recommend using 28s primers alongside the alternative COI primer sets. Although a molecular identification to species level may not always be possible depending on data availability on public DNA databases, the sequences produced could assist in differentiating between morphologically similar species.

3. Materials and Methods

3.1. Test sample preparation and transport

Participants received four tubes labelled A, B, C and D (Table 1), prepared and dispatched by SASA, UK. Participants were informed of the contents of the tube e.g. Tube A: Coccidae (soft scales), however the species identification of the Coccomorpha samples was not revealed.

There was discussion between laboratories on the final species identity of the Coccidae soft scale specimens. Prior to dispatch, they were tentatively identified as a species within the *Pulvinaria* genus.

Tubes A and B had a minimum of three insect specimen replicates, with a few extra as spares. Insect specimens were preserved in ethanol. Tube D contained DNA in molecular grade water. All samples were transported in Eppendorf tubes.

Table 1. Test samples provided to participating laboratories

Tube/Sample	Contents (as disclosed to participants)	Type of sample	Species identification
A	Coccidae (soft scales)	Insect specimens in ethanol	Suspected *Pulvinaria* sp.
B	Pseudococcidae (mealybugs)	Insect specimens in ethanol	*Chryseococcus arecae* (Golden root mealybug)
C	PIC sample: *Philaenus spumarius*	Insect specimen in ethanol	*Philaenus spumarius* (Meadow spittlebug)
D	PAC sample: DNA of Pseudococcidae (mealybug)	DNA	*Pseudococcus longispinus* (Longtailed mealybug)

Tube C (*P. spumarius* insect specimen) was used as a positive isolation control (PIC) to verify the extraction method. Tube D (DNA of Pseudococcidae, aliquoted between participants) was used as a Positive Amplification Control (PAC) to monitor the efficiency of the amplification.

Samples were sent to participating laboratories via courier from SASA, UK. Upon receipt of samples, participants were asked to store Tube D containing DNA at -20°C and Tubes A, B and C containing insect samples at 4°C, until ready to proceed with the tests.

3.2. Extraction of DNA using DNeasy Blood & Tissue kit (QIAGEN)

Participating laboratories extracted 3 insect specimens from Tubes A and B, and 1 specimen from Tube C non-destructively with a piercing technique using the DNeasy Blood & Tissue kit (QIAGEN). Specimens were pierced once using a 0.1 mm pin or equivalent (e.g. stainless steel headless pin, size A2 or A3, Watkins & Doncaster) held in a pin/needle holder (e.g. universal needle holder, Watkins & Doncaster), from the dorsal through to the ventral side depending on orientation, until the pin tip emerged on the other side. Specimens were incubated in lysis buffer overnight. This technique is considered non-destructive as a voucher specimen can be retained after lysis. Laboratories followed the manufacturer's instructions with some modifications outlined in Appendix 1.

The quantity (DNA yield ng/µl) and quality (OD260/230) of DNA obtained from each extracted insect sample was recorded.

3.3. Comparison of primers

Laboratories tested and compared three different primer sets for DNA barcoding Coccomorpha (Table 2).

Table 2. Primer sets used in this study

Primer set number	Primers	Gene region	Reference
1	LCO1490/HCO2198	Cytochrome c oxidase subunit I (CO1) 'Folmer' region	Folmer *et al.* (1994)[6] and EPPO PM 7/129 (2)[7]
2	PC0F1/Lep-R	Cytochrome c oxidase subunit I (CO1) 'Folmer' region	Park *et al.* (2011)[8] Hajibabaei *et al.* (2006)[9]
3	28s-S3660/28s-a335	Large ribosomal subunit (28s)	Normark (2019)[10]

The PCR methods for all three primer sets are described in Appendices 2, 3 and 4. It was recommended the polymerase, MyFi™ DNA Polymerase (Meridian Bioscience) or other verified PCR master mixes containing a polymerase with proofreading activity were used.

In total, participants amplified the DNA extracted from tubes A and B (a total of 6 samples) and DNA from tube D (PAC) using all three primer sets (Table 3). The PIC, *P. spumarius*, was an extraction control for the QIAGEN DNeasy Blood & Tissue kit, the success of which was determined by the DNA concentration readings. While it was recommended for laboratories to also test the primer sets on the PIC, sequencing was optional.

Table 3. Sample ID and what testing was required for each sample.

				Primers to test		
Tube	Contents	Number of insects to extract	Extract using Qiagen kit	LCO1490 HCO2198	PCOF1 Lep-R	28s-S3660 28s-a335
A	Coccidae (soft scales)	3	Y	Y	Y	Y
B	Pseudococcidae (mealybugs)	3	Y	Y	Y	Y
C	PIC (Hemiptera, P. spumarius)	1	Y	Y	Y	Y
D	PAC (DNA of Pseudococcidae)	0	N	Y	Y	Y
	Y = Yes, N = No					

Laboratories recorded if the PCR was successful by gel electrophoresis. Successful PCR products were sequenced (Sanger sequencing) either in-house, or through an established sequencing laboratory supplier.

Participants analysed their own sequences using Geneious Prime. Sequencing success, consensus sequence quality (HQ%), and any molecular identification obtained using NCBI GenBank were recorded. As a guide, participants were referred to Appendix 7 of the "*DNA*

[6] Folmer *et al.* (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. https://pubmed.ncbi.nlm.nih.gov/7881515/

[7] EPPO (2021). DNA barcoding as an identification tool for a number of regulated pests. PM 7/129 (2) https://doi.org/10.1111/epp.12724

[8] Park *et al.* (2011). DNA barcodes for two scale insect families, mealybugs (Hemiptera: Pseudococcidae) and armored scales (Hemiptera: Diaspididae). DOI: 10.1017/S0007485310000714

[9] Hajibabaei *et al.* (2006). DNA barcodes distinguish species of tropical Lepidoptera. https://doi.org/10.1073/pnas.0510466103.

[10] Normark *et al.* (2019). Phylogeny and classification of armored scale insects (Hemiptera: Coccomorpha: Diaspididae). https://doi.org/10.11646/zootaxa.4616.1.1

barcoding as an identification tool for a number of regulated pests" (EPPO, 2021)[11] for the procedure for Sanger sequencing, consensus preparation and data analysis.

3.4. Analysing sequencing success and quality

Participants sent PCR and sequencing results, including a copy of consensus sequences to the organiser.

Due to small sample sizes, statistical analysis options were limited. Averages on the sequence success rate between each primer set were calculated to give approximations. For sequencing success, samples were recorded as having failed if they (i) failed to PCR correctly and therefore were not sequenced; (ii) failed to produce a sequence; or (iii) the consensus sequence had no NCBI GenBank match to the Order 'Hemiptera', as a minimum. Welch Two Sample t-tests were used to compare proportions of successfully sequenced samples between primer sets and between the two species of Coccomorpha.

Due to missing data in sequence quality (HQ%) (i.e. not all samples were sequenced due to failed PCR amplification), statistical analysis was not possible for analysing and comparing HQ%. Instead, when appropriate, sequences were aligned to ensure a close match between individual samples and between laboratories.

3.5. Observations of specimens after DNA extraction [optional]

As an optional part of the TPS, participants recorded the specimen appearance before and after overnight lysis (e.g. Good, Average, Poor) taking a photograph if practical. Morphological identification and other comments were also recorded.

4. Results

4.1. Test sample preparation and transport

All samples were delivered to participating laboratories. However, some were held up significantly due to courier and customs delays that resulted in lengthy liaising, additional administration work and costs for the recipients and sender. The delays had no obvious effects on results. Specimens were stored in ethanol in order to preserve DNA during transport. Laboratories observed similar nanodrop readings for the PAC sample, Table 4, indicating the DNA did not degrade during the lengthy transport.

4.2. Extraction of DNA using DNeasy Blood & Tissue kit (QIAGEN)

All laboratories successfully extracted the Coccomorpha specimens and extraction control using the DNeasy Blood & Tissue kit (QIAGEN).

[11] EPPO (2021). DNA barcoding as an identification tool for a number of regulated pests. PM 7/129 (2) https://doi.org/10.1111/epp.12724

Table 4. DNA concentration (ng/µL) of test samples from each laboratory.

Labs	DNA concentration (ng/µL)							
	Sample A Coccidae (soft scales) (*Pulvinaria* sp.)			Sample B Pseudococcidae (mealybugs) (C. *arecae*)			Sample C PIC (*P. spumarius*)	Sample D PAC (*P. longispinus*)
	A1	A2	A3	B1	B2	B3		
Lab 1	69.26	11.48	27.87	18.39	12.22	15.35	286.72	49.46
Lab 2	41.3	35.9	24.1	10.9	12.7	10.9	220.6	46.4
Lab 3	64.5	77.2	nd	3.7	7.2	nd	479	62.4
Lab 4	29.89	132.3	26.98	16.42	11.15	7.42	472.6	53.8
Lab 5	54.9	36.5	164.1	14.1	20.8	7.2	23.0	49.7
Lab 6	11.5	39.4	7.9	1.4	-1.9*	1.2	204.7	-

DNA concentrations were carried out on Nanodrop spectrophotometers. nd = DNA extraction not done. - = DNA concentration not tested. *Trace amounts lower than limit of detection for Nanodrop.

Table 5. DNA quality (A260/230) of test samples from each laboratory.

Labs	DNA quality (A260/230)							
	Sample A Coccidae (soft scales) (*Pulvinaria* sp.)			Sample B Pseudococcidae (mealybugs) (C. *arecae*)			Sample C PIC (*P. spumarius*)	Sample D PAC (*P. longispinus*)
	A1	A2	A3	B1	B2	B3		
Lab 1	2.18	3.24	2.79	2.49	2.37	2.82	2.3	1.05
Lab 2	-	-	-	-	-	-	-	-
Lab 3	2.04	2.16	nd	1.87	0.45	nd	2.1	1.46
Lab 4	1.3	1.81	1.34	1.05	0.82	0.49	1.66	1.43
Lab 5	1.38	1.85	0.86	4.21	0.99	1.24	0.7	1.45
Lab 6	1.8	2.0	2.2	1.3	0.8	5.1	2.1	-

nd = DNA extraction not done. - = DNA quality reading not recorded.

DNA concentrations varied between individual samples and laboratories (Table 4). DNA yields for Sample A (Coccidae, soft scales) ranged between 7.9 – 164.1 ng/µL; and between 1.4 – 20.8 ng/µL for Sample B (Pseudococcidae, mealybugs), excepting one reading of -1.9 ng/µL. Sample B2 for Lab 6 (-1.9 ng/µL) successfully amplified the target region in at least one primer set, indicating that DNA extraction was successful, despite having a very low DNA concentration. The lower limit of detection for Nanodrop spectrophotometers is 2.5 ng/µL, and lower concentrations than this can be difficult to produce accurate readings. Variation in DNA quantity could be explained by differences in specimen size and specimen quality pre-extraction. Further practise using the piercing technique could also aid in achieving higher DNA concentrations. Scale insect samples produced higher concentrations of DNA compared to mealybugs ($t = 3.8954$, $df = 16.722$, p-value = 0.001195), although this could be explained by specimen size difference between the two species.

DNA concentrations for the PIC, *P. spumarius*, were high for most laboratories (>200 ng/µL). Lab 5 removed and extracted one leg from the PIC specimen, rather than piercing and incubating the specimen whole, accounting for the lower DNA concentration (23.0 ng/µL), and potentially the lower A260/230 quality reading (Table 5). The *P. spumarius* specimens worked adequately as an extraction control for the DNeasy Blood & Tissue kit.

DNA quality readings varied between individual samples and laboratories (Table 5). In a pure sample, the A260/230 quality reading should be ~2.0. Some samples had A260/230 readings

lower than 1, indicating the presence of organic contaminants. On average, A260/230 quality readings were 1.92 and 1.85 for Coccidae and Pseudococcidae specimens, respectively. All samples extracted had success in sequencing in at least one primer set.

Some participants commented that the piercing of specimens was difficult. To avoid damaging important morphological features, it was advised that the mealybugs should be pierced between the mid and hind legs in the middle of the body (meta-thorax). Occasionally, this resulted in the specimen breaking or splitting.

4.3.1. Primer set 1: LCO1490 & HCO2198

PCR success

The Cytochrome c oxidase subunit I (CO1) 'Folmer' region primer set LCO1490 & HCO2198 produced mixed PCR results, with very few samples producing a clear, single band of correct size (Table 6). Most laboratories did not sequence Sample A and B Coccomorpha samples due to absent, faint or multiple bands. For Sample A (Coccidae) specimens, 2 samples appeared to PCR successfully (11.8%), i.e. single band of correct size. For Sample B (Pseudococcidae), 3 samples appeared to PCR successfully (17.6%), i.e. single band of correct size. All PIC samples produced strong single bands of the correct size, excluding one sample. Only 2 PAC samples produced single bands of the correct size.

Table 6. PCR result using primer set 1 (LCO1490 & HCO2198).

Labs	Consensus sequence quality (HQ%)							
	Sample A: Coccidae (soft scales) (*Pulvinaria* sp.)			Sample B: Pseudococcidae (mealybugs) (C. *arecae*)			Sample C PIC (*P. spumarius*)	Sample D PAC (*P. longispinus*)
	A1	A2	A3	B1	B2	B3	C	D
Lab 1	mb	mb	++	mb	mb	mb	++	mb
Lab 2	mb	++ mb	++ mb	++	+ mb	++ mb	++	++ mb
Lab 3	mb	mb	nd	very faint	very faint	nd	++	faint incorrect size
Lab 4	-	-	-	-	-	-	++	++
Lab 5	mb	mb	mb	+	+	-	++	++
Lab 6	+ mb	++ mb	++	-	-	-	++ mb	-

PCR results on gel electrophoresis: - = no band; + = faint band; ++ = strong band; mb = multiple bands or smear. nd = DNA extraction not done.

Sequencing success

Two laboratories attempted to sequence Samples A and B, however all samples tested failed to generate valid sequences (i.e. no sequencing product was produced; no contigs were found; or there was no close match to the Order 'Hemiptera' on NCBI as a minimum). The consensus sequence quality (HQ%) is recorded in Table 7. Samples that failed to produce valid sequences, or were not sequenced due to poor PCR results, are recorded as having failed.

Table 7. Consensus sequence quality (HQ%) using primer set 1 (LCO1490 & HCO2198).

Labs	Consensus sequence quality (HQ%)							
	Sample A: Coccidae (soft scales) (*Pulvinaria* sp.)			Sample B: Pseudococcidae (mealybugs) (C. *arecae*)			Sample C PIC (*P. spumarius*)	Sample D PAC (P. *longispinus*)
	A1	A2	A3	B1	B2	B3	C	D
Lab 1	Fail	Fail	Fail	Fail	Fail	Fail	25.9%	Fail
Lab 2	Fail	Fail	Fail	Fail	Fail	Fail	99.8%	97.8%
Lab 3	Fail	Fail	nd	Fail	Fail	nd	65.6%	Fail
Lab 4	Fail	Fail	Fail	Fail	Fail	Fail	ns (++)	99.7%
Lab 5	Fail	Fail	Fail	Fail	Fail	Fail	ns (++)	ns (++)
Lab 6	Fail	Fail	Fail	Fail	Fail	Fail	31.9%	Fail

Fail = sample either failed to PCR correctly, produce a sequence or had no NCBI GenBank match to the Order 'Hemiptera'. nd = DNA extraction not done. ns (++) = sample produced a strong band on gel, confirming PCR success, but was not sequenced.

The LCO1490 & HCO2198 primer set failed to sequence Sample A and B Coccomorpha specimens, including samples that appeared to PCR successfully. For some laboratories, the control samples C (*P. spumarius*) and D (*P. longispinus*) had poor sequence quality or failed to sequence. It was discovered that some *P. spumarius* specimens had been parasitised by the endoparasitoid big-eyed fly, *Verralia aucta* (a natural parasite of *P. spumarius*), explaining the sequences being of poorer quality or matching to *V. aucta* instead on NCBI GenBank. For those laboratories that did not sequence the PIC (this was optional), a strong band on the gel indicates primer success.

Since the LCO1490 & HCO2198 primers have shown to not work with the Sample B Pseudococcidae specimens, it is unsurprising that they had a low success rate for the PAC, also a Pseudococcidae. However, two laboratories were successful in producing sequences for the PAC using the COI 'Folmer' primers, suggesting that it may still be worth using these primers, depending on the species of Pseudococcidae. PCR cycler model and type of polymerase could also be affecting PCR outcome, although no obvious trends were found in this study. The results indicate that *Pseudococcus longispinus* was not an effective PAC for this primer set, and that future similar studies should use a species known to confidently amplify and sequence with the target primer set.

In summary, out of 34 individual samples tested (sample sets A and B) with the LCO1490 & HCO2198 primer set, no samples (0%) were successful in generating a sequence of high enough quality for molecular barcoding.

4.3.2. Primer set 2: PCOF1 & Lep-R

PCR success

The second COI primer set (PCOF1 & LepR) produced improved PCR results compared to LCO1490 & HCO2198 primers (Table 8). For Sample A (Coccidae) specimens, 14 samples appeared to PCR successfully (82.4%), i.e. single band of correct size. For Sample B (Pseudococcidae), 3 samples appeared to PCR successfully (17.6%), i.e. single band of

correct size. Only 2 PIC samples and 1 PAC sample produced single bands of the correct size.

Table 8. PCR result using primer set 2 (PCOF1 & Lep-R).

Labs	Consensus sequence quality (HQ%)							
	Sample A: Coccidae (soft scales) (*Pulvinaria* sp.)			Sample B: Pseudococcidae (mealybugs) (C. *arecae*)			Sample C PIC (*P. spumarius*)	Sample D PAC (*P. longispinus*)
	A1	A2	A3	B1	B2	B3	C	D
Lab 1	++	++	++	mb	mb	mb	mb	mb
Lab 2	++	++	++	+ mb	++ mb	++ mb	++	++ mb
Lab 3	++	++	nd	-	-	nd	+	faint incorrect size
Lab 4	++	++	++	++	++	+	mb	++
Lab 5	mb	mb	mb	mb	mb	mb	mb (target band strong)	-
Lab 6	++	++	++	-	-	-	-	-

PCR results on gel electrophoresis: - = no band; + = faint band; ++ = strong band; mb = multiple bands or smear. nd = DNA extraction not done.

Sequencing success

The consensus sequence quality (HQ%) for the second COI primer set (PCOF1 & Lep-R) is recorded in Table 9. Samples that failed to produce valid sequences, or were not sequenced due to poor PCR results, are recorded as having failed.

Table 9. Consensus sequence quality (HQ%) using primer set 2 (PCOF1 & Lep-R).

Labs	Consensus sequence quality (HQ%)							
	Sample A: Coccidae (soft scales) (*Pulvinaria* sp.)			Sample B: Pseudococcidae (mealybugs) (C. *arecae*)			Sample C PIC (*P. spumarius*)	Sample D PAC (*P. longispinus*)
	A1	A2	A3	B1	B2	B3	C	D
Lab 1	62.2%	*Rev	83.6%	79.4%	Fail	Fail	Fail	Fail
Lab 2	100%	99.8%	100%	*Rev	99.2%	98.5%	Fail	84%
Lab 3	95.2%	92.5%	nd	Fail	Fail	nd	98.9%	Fail
Lab 4	99.5%	99.7%	98.9%	98.8%	91.4%	Fail	Fail	97.2%
Lab 5	Fail	Fail	Fail	Fail	Fail	Fail	Fail	Fail
Lab 6	81.8%	*Rev	*Rev	Fail	Fail	Fail	Fail	Fail

Fail = sample either failed to PCR correctly, produce a sequence or had no close NCBI GenBank match to the Order 'Hemiptera'. nd = DNA extraction not done. *Rev = reverse sequence only.

Sample A: Coccidae (soft scales) sequencing success

The second COI primer set performed better for the Coccidae (soft scales) samples compared to the LCO1490 & HCO2198 primers (Table 9). However, there were differences in the sequencing success rates between laboratories. Laboratory 5 observed multiple bands and bands of incorrect sizes on the gel, and therefore did not submit their PCR products for sequencing.

Successful sequences for Coccidae specimens using PCOF1 & Lep-R primers were aligned, with 99.7% of nucleotides matching (Appendix 5). Samples with reverse sequences only were included in the alignment. The closest NCBI match was recorded as *Pulvinariella mesembryanthemi* (~92% match), indicating that the COI gene sequence for the Coccidae species used in this study is not currently in the NCBI GenBank database. The closest BOLD match was also *Pulvinariella mesembryanthemi* (~92% match).

In summary, out of 17 individual samples tested (Coccidae, Samples A) with the primer set PCOF1 & Lep-R, 14 samples (82.4%) were successful in generating a sequence of high enough quality for molecular barcoding.

Sample B: Pseudococcidae (mealybugs) sequencing success

The PCOF1 & Lep-R primer set was less successful for the Pseudococcidae (mealybugs) compared to Coccidae (soft scales) (Table 9).

Successful sequences for Pseudococcidae specimens using PCOF1 & Lep-R primers were aligned with 98.9% nucleotides matching (Appendix 6). Samples with reverse sequences only were included in the alignment. The closest NCBI match was recorded as Hemiptera sp. (~92% match), indicating that the COI gene sequence for the Pseudococcidae species used in this study is not currently in the NCBI GenBank database. The closest BOLD match was Pseudococcidae sp. (~93%).

In summary, out of 17 individual samples tested (Pseudococcidae, Samples B) with the primer set PCOF1 & Lep-R, 6 samples (35.3%) were successful in generating a sequence of high enough quality for molecular barcoding.

PIC and PAC sequencing success

Only one laboratory was successful in generating a sequence for the PIC extraction control, matching to *Verralia aucta* (endoparasitoid of *P. spumarius*). The alternative COI primer set performed worse for the PIC compared to the 'Folmer' region primer set, although it is unclear whether the primers were unsuccessful at binding to the correct COI region within *P. spumarius* or *V. aucta* DNA.

Only two laboratories were successful in generating valid sequences for the PAC. This result is again unsurprising due to the PCOF1 & LepR primers also performing poorly for the Sample B Pseudococcidae specimens. This highlights that *Pseudococcus longispinus* was not an effective PAC for this primer set.

4.3.3. Primer set 3: 28s-S3660 & 28s-a335

Primer success

The 28s primer set (28s-S3660 & 28s-a335) had the greatest PCR success rate for both Sample A (Coccidae) and Sample B (Pseudococcidae) specimens (Table 10). All samples, including controls PIC and PAC, showed 100% PCR success when observed on gel electrophoresis i.e. single band of correct size.

Table 10. PCR result using primer set 3 (28s-S3660 & 28s-a335).

Labs	Consensus sequence quality (HQ%)							
	Sample A: Coccidae (soft scales) (*Pulvinaria* sp.)			Sample B: Pseudococcidae (mealybugs) (C. *arecae*)			Sample C PIC (*P. spumarius*)	Sample D PAC (*P. longispinus*)
	A1	A2	A3	B1	B2	B3	C	D
Lab 1	++	++	++	++	++	++	++	++
Lab 2	++	++	++	++	++	++	++	++
Lab 3	++	++	nd	++	++	nd	++	++
Lab 4	+	++	++	++	++	++	++	++
Lab 5	++	++	++	++	+	++	++	++
Lab 6	++	++	++	++	++	++	++	++

PCR results on gel electrophoresis: + = faint band; ++ = strong band. nd = DNA extraction not done.

Sequencing success

The consensus sequence quality (HQ%) for the third primer set (28s-S3660 & 28s-a335) is recorded in Table 11. Samples that failed to produce valid sequences are recorded as having failed.

Table 11. Consensus sequence quality (HQ%) using primer set 3 (28s-S3660 & 28s-a335).

Labs	Consensus sequence quality (HQ%)							
	Sample A Coccidae (soft scales) (*Pulvinaria* sp.)			Sample B Pseudococcidae (mealybugs) (C. *arecae*)			Sample C PIC (*P. spumarius*)	Sample D PAC (*P. longispinus*)
	A1	A2	A3	B1	B2	B3	C	D
Lab 1	55.2%	81.4%	85.1%	82.6%	82.4%	84.9%	66.1%	*Rev
Lab 2	100%	*For	100%	100%	100%	100%	100%	*Rev
Lab 3	97.2%	94.8%	nd	96.6%	96.1%	nd	83.8%	93.8%
Lab 4	Fail	94%	93.5%	97.2%	94.9%	94.9%	ns (++)	Fail
Lab 5	82.9%	79.4%	81.3%	93.8%	87.8%	88.8%	82.6%	ns (++)
Lab 6	94.2%	96.3%	93.7%	95.4%	94.1%	93.3%	95%	*Rev

Fail = sample failed to produce a sequence or had no NCBI GenBank match to the Order 'Hemiptera'. nd = DNA extraction not done. ns (++) = sample produced a strong band on gel, confirming PCR success, but was not sequenced. *For = forward sequence only. *Rev = reverse sequence only.

Sample A: Coccidae (soft scales)

The third primer set, 28s-S3660 & 28s-a335, had the highest sequencing success rate for Coccidae (soft scales) (Table 11).

Successful sequences for Coccidae samples using the 28s region primers were aligned with 99.5% of nucleotides matching (Appendix 7). Samples with forward sequences only were included in the alignment. The closest NCBI match was recorded as *Pulvinariella*/*Pulvinaria mesembryanthemi* (~97-99% match). It is important to note that the sequences matched to only one 28s *P. mesembryanthemi* sequence available on NCBI GenBank. Caution should therefore be taken on accepting this ID.

In summary, out of 17 individual samples tested (Coccidae, Samples A) with the 28s primer set, 16 (94.1%) were successful in generating a sequence of high enough quality for molecular barcoding.

Sample B: Pseudococcidae (mealybugs)

The 28s primer set had the highest sequencing success for Pseudococcidae (mealybugs) (Table 11).

Successful sequences for Pseudococcidae samples using the 28s region primers were aligned, with 99.1% of nucleotides matching (Appendix 8). The closest NCBI match was recorded as *Nipaecoccus viridis* (~95% match), which is within the same Pseudococcidae family as *Chryseococcus arecae*. There are currently no *C. arecae* 28s gene sequences on NCBI GenBank.

In summary, out of 17 individual samples tested (Pseudococcidae, Samples B) with the 28s primer set, 17 (100%) were successful in generating a sequence of high enough quality for molecular barcoding.

PIC and PAC sequencing success

Four out of five laboratories that sequenced the PAC with the 28s primers were successful in producing a sequence matching to *Pseudococcus longispinus*. Lab 5 did not sequence the PAC however the strong band on the gel indicated primer success. Three laboratories produced reverse sequences only for the PAC, however the HQ quality was still high enough to confirm a species ID.

All laboratories that sequenced the extraction control with the 28s primers were successful in producing a sequence matching to *Philaenus spumarius*, indicating that this primer set could have a greater affinity to 28s *P. spumarius* DNA over 28s *V. aucta* DNA.

4.3.4. Summary of primer set results

For sequencing success, samples were recorded as having failed if they (i) failed to PCR correctly and therefore were not sequenced; (ii) failed to sequence; or (iii) the consensus sequence had no NCBI GenBank match to the Order ‘Hemiptera’, as a minimum.

On average, primer set 3 (28s-S3660/28s-a335), had a higher sequencing success rate for both Coccidae (100%) and Pseudococcidae (94.1%), followed by primer set 2 (PCOF1/Lep-R), Table 12 and Figure 1. 0% of samples produced valid sequences with Primer set 1 (LCO1490/HCO2198).

Table 12. Average % of samples that successfully sequenced between all laboratories.

Primer sets	Average % of samples that produced successful sequences	
	Sample A (Coccidae, soft scales)	Sample B (Pseudococcidae, mealybugs)
Primer set 1 (LCO1490/HCO2198)	0%	0%
Primer set 2 (PCOF1/Lep-R)	82.4%	35.2%
Primer set 3 (28s-S3660/28s-a335)	94.1%	100%

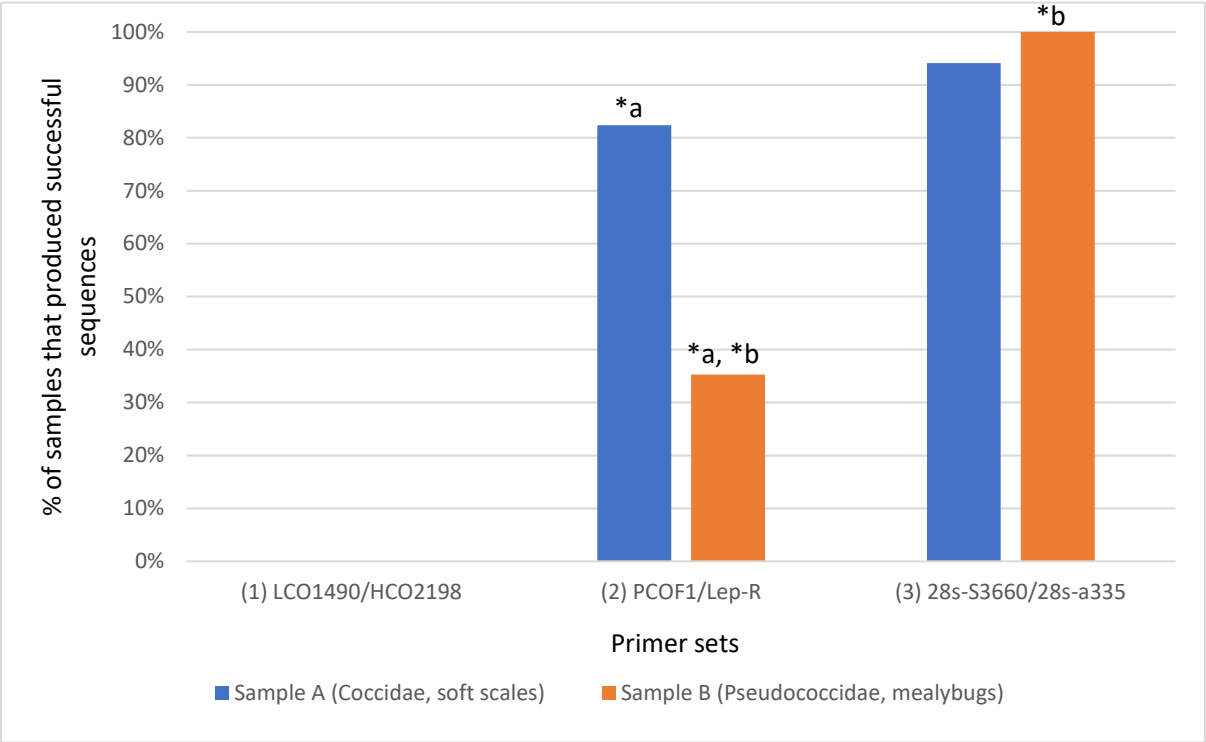


Figure 1. The percentage (%) of Coccidae and Pseudococcidae samples that produced successful sequences using three different primer sets. Samples that failed to sequence or had no match to the Order 'Hemiptera' on NCBI GenBank were recorded as failed. *a indicates a significant difference in sequencing success between Sample A Coccidae and Sample B Pseudococcidae for primer set (2). *b indicates a significant difference in sequencing success between primer set (2) and primer set (3) for Sample B Pseudococcidae.

There is a significant difference between Coccidae and Pseudococcidae sequencing success for Primer set 2 (t = 3.0792, df = 30.494, p-value = 0.004367), with Coccidae samples showing greater sequencing success (Figure 1, *a). There is also a significant difference in the sequencing success between primer set 2 and 3 for Pseudococcidae samples, with primer set 3 generating significantly more sequences (t = 5.416, df = 16, p-value = 5.713e-05), (Figure 1, *b). There was no significant difference in sequencing success for Coccidae (soft scales) samples between primer sets 2 and 3 (t = 1.0505, df = 26.646, p-value = 0.3029).

4.4. Observations of specimens after DNA extraction [optional]

Sample A: Coccidae (soft scales)

The Coccidae specimens were observed to stay intact after overnight lysis. Participants described the post appearance as: poor; brittle; desiccated; colour faded; and likely difficult to be morphologically identified. Photographed examples of the scale insects prior to and after extraction are displayed in Figure 2.

Two participating laboratories attempted to morphologically identify the specimens after the extraction process. The quality of specimens after the extraction (and subsequent slide mounting) did not allow for a species level identification, with *Pulvinaria* sp. being the closest identification, confirming the original tentative identification. One specimen was identified as *Pulvinariella mesembryanthemi*; although another laboratory noted that the marginal setae appeared different to a typical *P. mesembryanthemi*.

Whilst the appearance of the scale insects was poorer after extraction, it was still possible in some cases to obtain a genus level ID. It is important to highlight that a species level identity for this scale insect was difficult to determine prior to extraction, suggesting that the physical impact on the specimens may not have been too severe.

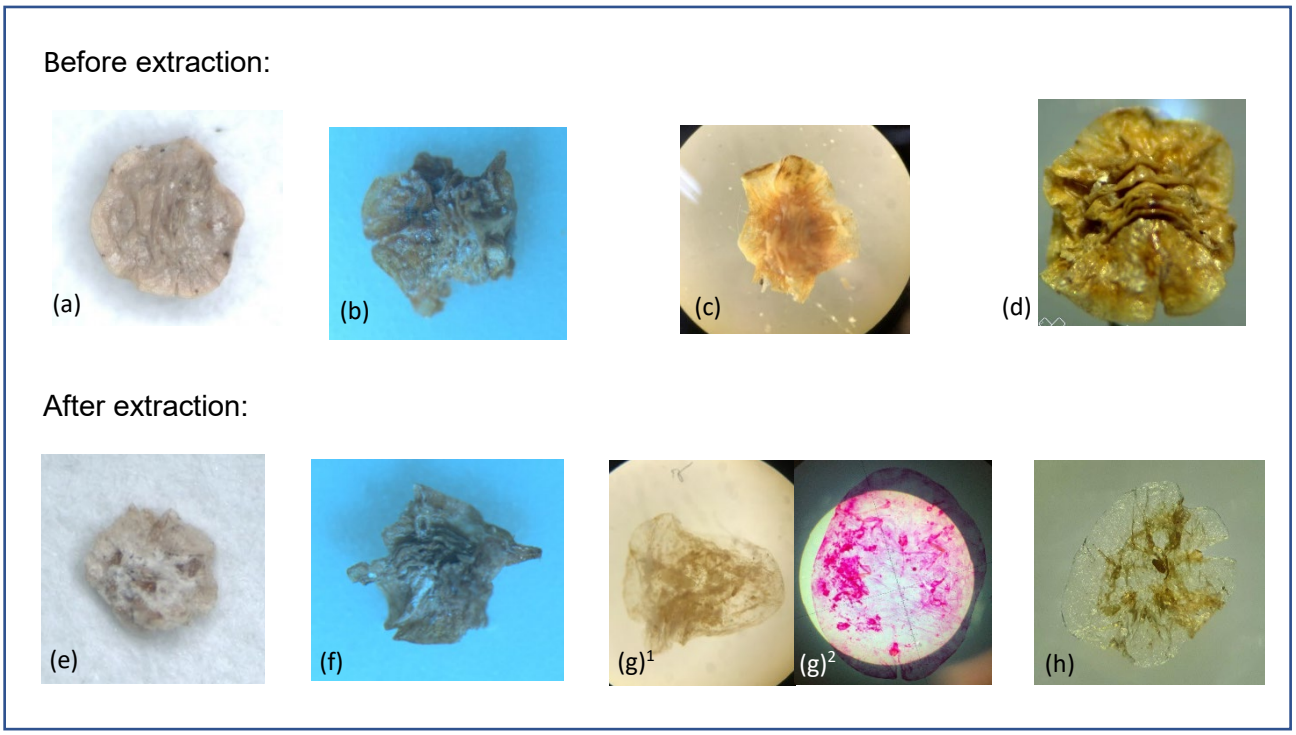


Figure 2. Sample A Coccidae specimens (soft scales) before and after extraction. Lab 1 = (a) and (e); Lab 4 = (b) and (f); Lab 5 = (c), $(g)^1$ and $(g)^2$; Lab 6 = (d) and (h). Images provided courtesy of participants of the TPS.

Sample B: Pseudococcidae (mealybugs)

Some participants observed that the *Pseudococcidae* specimens separated or split when pierced and during the overnight lysis. Specimens after extraction were described as translucent and 'sac-like', making it difficult to manipulate under a microscope. Most participants concluded that it would not be possible to identify to species level morphologically. Photographed examples of the mealybugs prior to and after extraction are displayed in Figure 3.

One laboratory was successful at mounting and photographing the specimens after the extraction to attempt morphological ID. However, their ID examination concluded it was a *Chorizococcus* sp. suggesting that some of the key features of *Chryseococcus arecae* were lost or damaged during the extraction process. It is also important to note that the striking and indicative golden colour of *Chryseococcus arecae* (golden root mealybug) is lost when stored in ethanol.

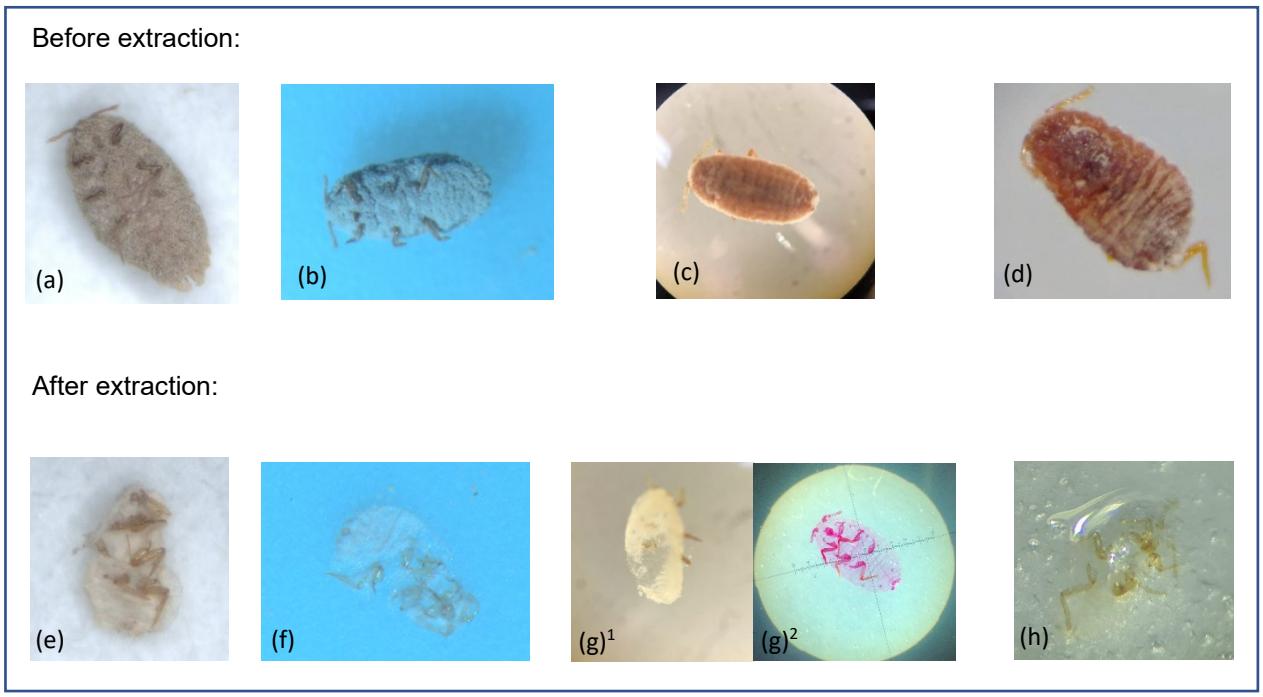


Figure 3. Sample B Pseudococcidae specimens (mealybugs) before and after extraction. Lab 1 = (a) and (e); Lab 4 = (b) and (f); Lab 5 = (c), (g)[1] and (g)[2]; Lab 6 = (d) and (h). Images provided courtesy of participants of the TPS.

5. Discussion

Extraction of DNA using the DNeasy Blood & Tissue kit (QIAGEN)

All participating laboratories were able to easily acquire and use the DNeasy Blood & Tissue kit from QIAGEN. All laboratories successfully extracted all Coccidae (soft scales) and Pseudococcidae (mealybugs) specimens using the non-destructive method of piercing the

specimen and incubating it in lysis buffer overnight, a technique adopted from Sjölund (2017)[12]. The benefits of a non-destructive extraction method enable a voucher specimen (exoskeleton) to be retained and therefore re-examined if the DNA sequence analysis conflicts the morphological identity (Sjölund, 2016[13]). Not crushing or grinding the specimen can also reduce unwanted carryover of tissue debris into the spin columns (authors' observations).

The DNA concentrations varied across laboratories and individual samples. The soft scales did produce higher concentrations of DNA compared to the mealybugs, although as previously suggested, this could be explained by specimen size difference between the two species. The *P. spumarius* specimens worked adequately as an extraction control.

Some participants found the piercing technique difficult and required practise. It was also brought to the group's attention that for mealybugs, it was important to aim the piercing between the mid and hind legs in the middle of the body (in the meta-thorax), so as to avoid damaging the circulus, a key characteristic of some genera. It is imperative that if adopting this non-destructive piercing technique with the aim to store voucher specimens, the location of piercing should be carefully considered.

Primer comparison

It was clear that primer set 3 (28s-S3660 & 28s-a335, Normark (2019)[14]), within the large ribosomal subunit (28s) gene, performed better and produced the highest quality sequences for Coccomorpha, with a 94.1% and 100% success rate for Coccidae and Pseudococcidae specimens, respectively. There was a solid alignment of successful sequences between samples and laboratories, with 99.5% identical sites for Coccidae sequences and 99.1% identical sites for Pseudococcidae sequences.

The mitochondrial COI Folmer primers, LCO1490 & HCO2198, as described and recommended for the molecular identification of arthropods in the EPPO guidelines (EPPO, 2021[15]), failed to barcode Coccomorpha samples used in this TPS. Many laboratories observed PCR amplification failure using gel electrophoresis e.g. multiple bands; no bands or bands with incorrect product sizes. Some sequence products were produced, however the sequences were either of very poor quality, had no matches on NCBI or had false matches to the wrong insect Order. For analysis purposes, these were interpreted as having failed. These results were somewhat expected. The participating laboratories have shared and discussed their own issues and experiences with attempting to sequence Coccomorpha using the COI 'Folmer' primers, and these difficulties have been documented by others previously (Sethusa

[12] Sjölund J. (2017) Non-destructive DNA extraction from Psyllids. https://www.ponteproject.eu/protocols-calsol/non-destructive-dna-extraction-psyllids/

[13] Sjölund, M. J., Ouvrard, D., Kenyon, D. & Highet, F. Developing an RT-PCR assay for the identification of psyllid species. Proc. Crop Prot. North. Britain 279–282 (2016).

[14] Normark *et al.* (2019). Phylogeny and classification of armored scale insects (Hemiptera: Coccomorpha: Diaspididae). https://doi.org/10.11646/zootaxa.4616.1.1

[15] EPPO (2021). DNA barcoding as an identification tool for a number of regulated pests. PM 7/129 (2) https://doi.org/10.1111/epp.12724

et al. (2014)[16], Malausa *et al.* (2009)[17], Park *et al.* (2011)[18]). Interestingly, two laboratories were successful in producing sequences for the Pseudococcidae positive amplification control (*P. longispinus*) using the COI 'Folmer' primers, suggesting that it may still be worthwhile using these barcoding primers, depending on the species of Coccomorpha. PCR cycler model and type of polymerase could also be affecting PCR outcome, although no obvious trends were found in this study. Further investigation into comparing PCR cycler models and polymerases could prove useful. The alternative COI primer set PCOF1 & Lep-R (Park *et al.* (2011)[17], Hajibabaei *et al.* (2006)[19]) had greater success, with 82.4% of Coccidae samples and 35.2% and Pseudococcidae samples producing valid sequences.

Participants that sequenced the extraction control (*P. spumarius*) and the positive amplification control (*P. longispinus*), as discussed above, did not always achieve PCR or sequencing success. Some *P. spumarius* specimens had unknowingly been parasitised by the endoparasitoid big-eyed fly, *Verralia aucta* (a natural parasite of *P. spumarius*), resulting in poorer quality sequences or matching to *V. aucta* instead on NCBI GenBank. Only two laboratories were successful in producing sequences for *P. longispinus* using the COI 'Folmer' and alternative COI primer sets, highlighting that this was not an effective PAC for these primer sets. For future similar studies, a positive amplification control that is known to be effectively amplified by each primer set should be used.

Coccidae 28s sequences matched to *Pulvinariella mesembryanthemi* (~97-99% match) on NCBI, however it is important to note that the sequences matched to only one 28s *P. mesembryanthemi* sequence on NCBI. Furthermore, the Coccidae COI sequences had a lower (~92% match) to *P. mesembryanthemi*. The soft scales used in this TPS were collected from Scotland, and it is unlikely that *P. mesembryanthemi* would survive in the Scottish climate. It is likely that the scale insects used in this TPS are a closely related *Pulvinaria* species that do not currently have 28s or COI barcodes on NCBI. The closest NCBI match for Pseudococcidae 28s sequences was *Nipaecoccus viridis* (~95% match), which is within the same Pseudococcidae family as *Chryseococcus arecae*. There are currently no *C. arecae* 28s or COI gene sequences on NCBI.

Scarcity of sequences for Coccomorpha species and groups on NCBI GenBank combined with potential database inconsistencies from studies that do not keep voucher specimens is a limitation of molecular taxonomy (Amouroux *et al*. 2017[20]); and highlights the importance of using morphological taxonomy in conjunction with molecular barcoding. Using other

[16] Sethusa *et al.* (2014). DNA Barcode Efficacy for the Identification of Economically Important Scale Insects (Hemiptera: Coccoidea) in South Africa. https://doi.org/10.4001/003.022.0218.
[17] Malausa *et al.* (2009). DNA markers to disentangle complexes of cryptic taxa in mealybugs (Hemiptera: Pseudococcidae). DOI: 10.1111/j.1439-0418.2009.01495.x
[18] Park *et al.* (2011). DNA barcodes for two scale insect families, mealybugs (Hemiptera: Pseudococcidae) and armored scales (Hemiptera: Diaspididae). DOI: 10.1017/S0007485310000714
[19] Hajibabaei *et al.* (2006). DNA barcodes distinguish species of tropical Lepidoptera. https://doi.org/10.1073/pnas.0510466103.
[20] Amouroux, P., Crochard, D., Germain, JF. et al. Genetic diversity of armored scales (Hemiptera: Diaspididae) and soft scales (Hemiptera: Coccidae) in Chile. Sci Rep 7, 2014 (2017). https://doi.org/10.1038/s41598-017-01997-6

databases with reliable sequences generated from vouchered material e.g. EPPO-Q-bank is also advised.

Without repeating the tests with additional Coccidae and Pseudococcidae species, it is difficult to confirm from this study that the 28s primer set and alternative COI primer set are effective primers for barcoding Coccomorpha, as there was no confident species level match for either species used in this TPS. The 28s primer results are however promising. We have confirmed that there is a strong alignment in the sequences produced from each laboratory, demonstrating that the primers work well and have amplified the correct region in all cases. Furthermore, the closest matches on NCBI and BOLD were within the same Coccidae and Pseudococcidae Family, indicating that the sequences produced are valid. It is instead likely that the species in this TPS have not yet been added to NCBI GenBank. Whilst the limitations of public DNA databases may mean that a species level match cannot always be made, the sequences produced by using primers like the 28s set are of high quality and could be used alongside COI primers to help differentiate between morphologically similar species.

Observations of specimens after DNA extraction

Piercing and incubating Coccomorpha in lysis buffer overnight can damage and alter the appearance of specimens, making it difficult to morphologically ID. Soft scale voucher specimens were noted desiccated, while mealybug voucher specimens became translucent and difficult to manipulate under a microscope. In some cases, a genus level identification was still possible. Incubating Coccomorpha specimens without piercing was not tested within this study but could be worth further investigation as a comparison study.

6. Conclusions and recommendations

The DNeasy Blood & Tissue kit (QIAGEN) extraction kit was successful at extracting DNA from all Coccomorpha specimens tested. Piercing the specimen as opposed to crushing achieved sufficient DNA yields for downstream molecular analyses e.g. sequencing, with the added benefit of retaining a voucher specimen. Voucher specimens could be damaged in the extraction process so extra care should be taken on the location of piercing, if adopting this technique. While the standard ‘Folmer’ region COI primers failed to PCR specimens, the alternative COI primer set (PC0F1/Lep-R) had more success and are worth considering for Coccomorpha COI barcoding, particularly for Coccidae (soft scales). The 28s primers generated a 100% sequencing success rate for Pseudococcidae (mealybugs) specimens tested, and a 94.1% sequencing success rate for Coccidae (soft scales) specimens tested. The findings from this TPS support recommendations to use 28s primers for generating high quality sequences for genetic analyses and barcoding of Coccomorpha species, alongside alternative COI primers. We encourage laboratories to continue to submit 28s sequences for Coccomorpha species to public DNA databases as well as COI, which will greatly aid in identifications of immature or morphologically ambiguous species.

Appendix 1 - DNA extraction method

DNeasy Blood & Tissue kit (Qiagen, Cat. No. / ID: 69504) - as per PM 7/129 (2)[21] with minor modifications.

Nucleic acid extraction and purification

1. Tissue material (whole insect) of all life stages of a single scale or mealybug is used as input for DNA extraction.
2. DNA is extracted using the DNeasy Blood & Tissue kit (Qiagen) according to the "Purification of Total DNA from Animal Tissues" protocol.
3. When tissue material is stored in ethanol, all the ethanol should be removed prior to DNA extraction. To achieve this, the insects can be transferred for a few minutes to a dry filter paper and may be further dried in a SpeedVac centrifuge to facilitate evaporation of the solvent.
4. Pierce the specimen using a 0.1 mm pin or equivalent (*e.g.* stainless steel headless pin, size A2 or A3, Watkins & Doncaster) held in a pin/needle holder (*e.g.* universal needle holder, Watkins & Doncaster). For mealy bugs aim to pierce between mid and hind legs in the middle of the body (in the meta-thorax). See protocol[22] and video[23] for more details on how to pierce insect specimens.
5. Once pierced, each specimen should be incubated overnight in 180 µl lysis buffer and 20 µl proteinase K at 56°C in a slow shaking heat block (300 rpm). Include a tube with no specimen to act as a Negative Isolation Control (NIC) to monitor for contamination during DNA extraction.
6. Continue with the rest of the extraction the following day, as per the manufacturer's instructions.
7. Please note, when transferring the liquid to spin column, take care to leave the specimen behind. The insect can then be stored in ethanol until required. Continue with the rest of the extraction as per manufacturer's instructions.
8. When working with small amounts of tissue material (*e.g.* less than 10 mg) DNA is eluted in 50 µl of elution buffer (provided).
9. No DNA clean-up is required after DNA extraction.
10. The extracted DNA should either be used immediately or stored at -20°C until used.

[21] EPPO (2021). DNA barcoding as an identification tool for a number of regulated pests. PM 7/129 (2) https://doi.org/10.1111/epp.12724

[22] https://www.ponteproject.eu/protocols-calsol/non-destructive-dna-extraction-psyllids/

[23] Videos preparing insects for non-destructive extraction: https://www.youtube.com/channel/UC-wM5CV-89CBB3BoQtLozlw

Appendix 2 - PCR method: Primer set 1

Mitochondrial cytochrome c oxidase subunit I (COI)

Primer	Primer sequence (5'–3' orientation)	Reference
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al* (1994)[24]
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	

Master mixes are prepared according to the table below:

Basic PCR (MyFi™)*	Amount per reaction (µl)	Final concentration
Master mix 2x	10µl	X1
Primer 10µM (F & R)	0.4µl of each	0.2µM
Water	7.2µl	
Template (DNA)	2µl	5-40ng
Total	20µl	

*Or other verified PCR master mixes containing a polymerase with proofreading activity

Thermocycler profile:

Temp.		No. of cycles
94°C	5min	
94°C	30sec	x5
44°C	30sec	
72°C	1min	
94°C	30sec	x35
51°C	1min	
72°C	1min	
72°C	10min	

Expected product: 709 bp

Controls

For a reliable test result to be obtained, the following external controls should be included in the PCR:

i. The negative isolation control (NIC) from the DNA extraction,
ii. Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: include a tube with no added template, instead add 2 µL of molecular-grade water that was used to prepare the reaction mix.
iii. Positive amplification control (PAC) to monitor the efficiency of the amplification.

[24] Folmer *et al.* (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. https://pubmed.ncbi.nlm.nih.gov/7881515/

Appendix 3 - PCR method: Primer set 2

Mitochondrial cytochrome c oxidase subunit I (COI)

Primer	Primer sequence (5'–3' orientation)	Reference
PcoF1	CCTTCAACTAATCATAAAAATATYAG	Park *et al.* (2011)[25]
Lep-R	TAAACTTCTGGATGTCCAAAAAATCA	Hajibabaei *et al.* (2006)[26]

Basic PCR (MyFi™)*	Amount per reaction (µl)	Final concentration
Master mix 2x	10µl	X1
Primer 10µM (F & R)	0.4µl of each	0.2µM
Water	7.2µl	
Template (DNA)	2µl	5-40ng
Total	20µl	

*Or other verified PCR master mixes containing a polymerase with proofreading activity

Thermocycler profile:

Temp.		No. of cycles
94°C	5min	
94°C	30sec	x5
44°C	30sec	
72°C	1min	
94°C	30sec	x35
51°C	1min	
72°C	1min	
72°C	10min	

Expected product 709 bp

Controls

For a reliable test result to be obtained, the following external controls should be included in the PCR:

iv. The negative isolation control (NIC) from the DNA extraction,

v. Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: include a tube with no added template, instead add 2 µL of molecular-grade water that was used to prepare the reaction mix.

vi. Positive amplification control (PAC) to monitor the efficiency of the amplification.

[25] Park *et al.* (2011). DNA barcodes for two scale insect families, mealybugs (Hemiptera: Pseudococcidae) and armored scales (Hemiptera: Diaspididae). DOI: 10.1017/S0007485310000714

[26] Hajibabaei *et al.* (2006). DNA barcodes distinguish species of tropical Lepidoptera. https://doi.org/10.1073/pnas.0510466103.

Appendix 4 - PCR method: Primer set 3

Large ribosomal subunit (28s D2&D3 expansion region)

Primer	Primer sequence (5'–3' orientation)	Reference
28s_S3660	GAGAGTTMAASAGTACGTGAAAC	Normark (2019)[27]
28s_a335	TCGGARGGAACCAGCTACTA	

Basic PCR (MyFi™)	Amount per reaction (µl)	Final concentration
Master mix 2x	10µl	X1
Primer 10µM (F & R)	0.4µl of each	0.2µM
Water	7.2µl	
Template (DNA)	2µl	5-40ng
Total	20µl	

*Or other verified PCR master mixes containing a polymerase with proofreading activity

Thermocycler profile:

Temp.			No. of cycles
95°C		5min	
95°C		30sec	x33
58-48°C	-1° per 3 cycles	1min	
72°C		1min	
95°C		30sec	x11
48°C		1min	
72°C		1min	
72°C		10min	

Expected product 750bp

Controls

For a reliable test result to be obtained, the following external controls should be included in the PCR:

vii. The negative isolation control (NIC) from the DNA extraction,

viii. Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: include a tube with no added template, instead add 2 µL of molecular-grade water that was used to prepare the reaction mix.

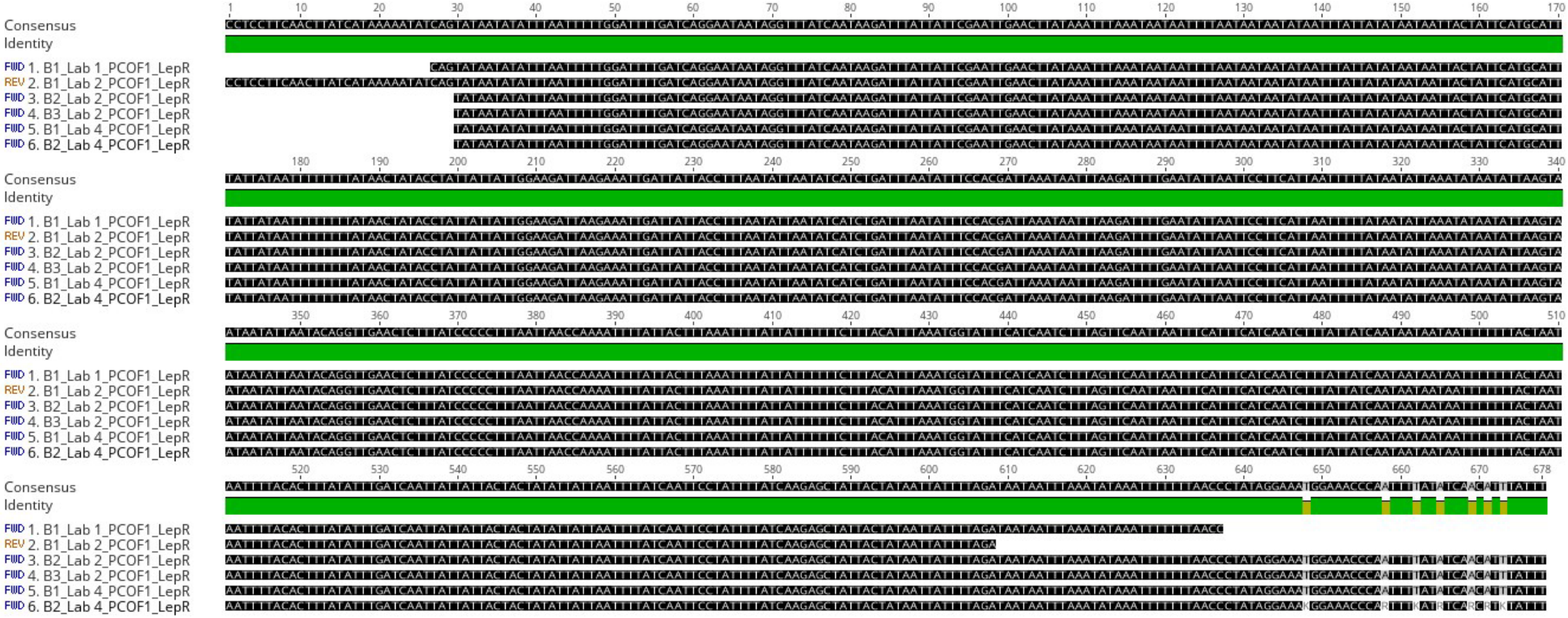
ix. Positive amplification control (PAC) to monitor the efficiency of the amplification.

[27] Normark *et al.* (2019). Phylogeny and classification of armored scale insects (Hemiptera: Coccomorpha: Diaspididae). https://doi.org/10.11646/zootaxa.4616.1.1

Appendix 5 - PCOF1 & Lep-R primer sequence alignments for successful Sample A: Coccidae (soft scale) sequences

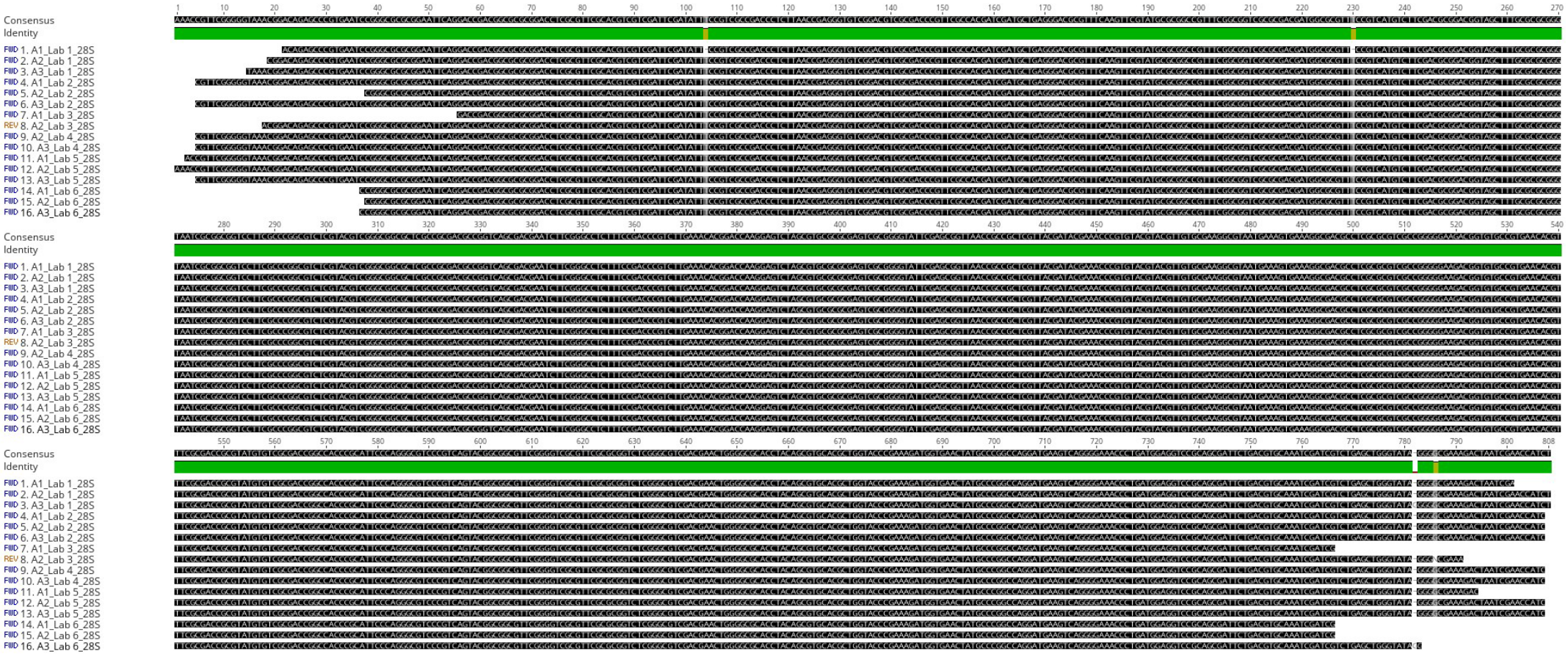
14 individual sequences, extracted and analysed by 5 different laboratories (Lab 1, Lab 2, Lab 3, Lab 4 and Lab 6) were aligned.
Nucleotide Statistics: Length (mean): 638 bp; Sequences: 14; Identical Sites: 654 (99.7%); Pairwise Identity: 99.96%
Geneious version 2022.0 created by Biomatters. Available from https://www.geneious.com

Appendix 6 - PCOF1 & Lep-R primer sequence alignments for successful Sample B: Pseudococcidae (mealybug) sequences



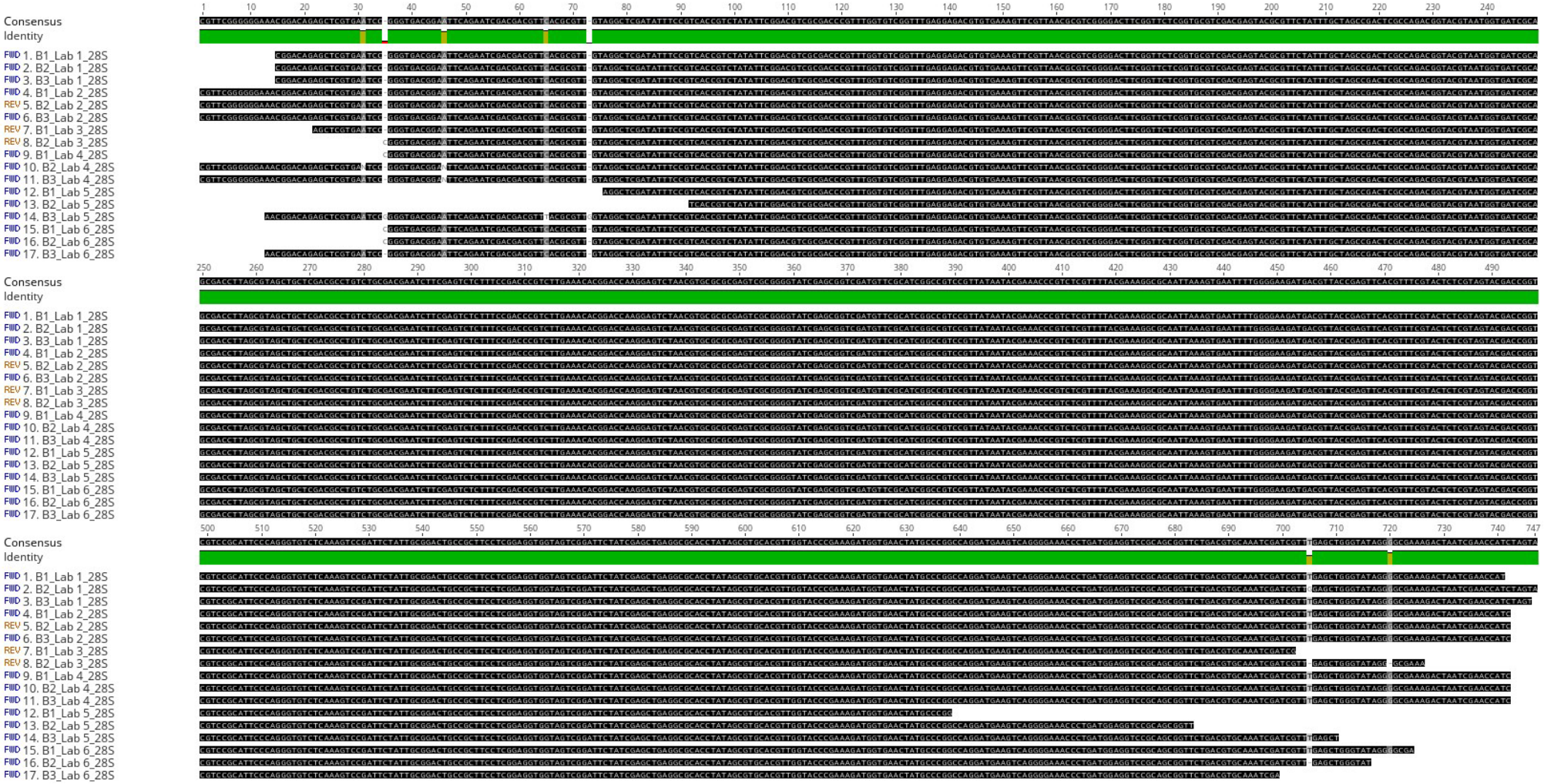
6 individual sequences, extracted and analysed by 3 different laboratories (Lab 1, Lab 2 and Lab 4) were aligned.
Nucleotide Statistics: Length (mean): 636 bp; Sequences: 6; Identical Sites: 645 (98.9%); Pairwise Identity: 99.9%
Geneious version 2022.0 created by Biomatters. Available from https://www.geneious.com

Appendix 7 - 28s primer sequence alignments for successful Sample A: Coccidae (soft scale) sequences



16 individual sequences, extracted and analysed by 6 different laboratories (Lab 1, Lab 2, Lab 3, Lab 4, Lab 5 and Lab 6) were aligned.
Nucleotide Statistics: Length (mean): 777 bp; Sequences: 16; Identical Sites: 802 (99.5%); Pairwise Identity: 99.9%
Geneious version 2022.0 created by Biomatters. Available from https://www.geneious.com

Appendix 8 - 28s primer sequence alignments for successful Sample B: Pseudococcidae (mealybug) sequences



17 individual sequences, extracted and analysed by 6 different laboratories (Lab 1, Lab 2, Lab 3, Lab 4, Lab 5 and Lab 6) were aligned.
Nucleotide Statistics: Length (mean): 698 bp; Sequences: 17; Identical Sites: 739 (99.1%); Pairwise Identity: 99.8%
Geneious version 2022.0 created by Biomatters. Available from https://www.geneious.com

7. Acknowledgements

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