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Running title: DNA barcoding for mealybug vectors of *Cacao Swollen Shoot Virus*

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Abstract

BACKGROUND: Mealybugs (Hemiptera: Coccoidea: Pseudococcidae) are key vectors of badnaviruses, including *Cacao Swollen Shoot Virus* (CSSV) the most damaging virus affecting cacao (*Theobroma cacao* L.). The effectiveness of mealybugs as virus vectors is species dependent and it is therefore vital that CSSV resistance breeding programmes in cacao incorporate accurate mealybug identification. In this work the efficacy of a CO1-based DNA barcoding approach to species identification was evaluated by screening a range of mealybugs collected from cacao in seven countries.

RESULTS: Morphologically similar adult females were characterised by scanning electron microscopy and then, following DNA extraction, were screened with CO1 barcoding markers. A high degree of CO1 sequence homology was observed for all 11 individual haplotypes including those accessions from distinct geographical regions. This has allowed for the design of a High Resolution Melt (HRM) assay capable of rapid identification of the commonly encountered mealybug pests of cacao.

CONCLUSIONS: HRM Analysis (HRMA) readily differentiated between mealybug pests of cacao that can not necessarily be identified by conventional morphological analysis. This new approach, therefore, has potential to facilitate breeding for resistance to CSSV and other mealybug transmitted diseases.

Keywords: mealybugs; Pseudococcidae; *Theobroma cacao*; *Cacao Swollen Shoot Virus*; DNA barcoding; High Resolution Melt

1 INTRODUCTION

There are at least 61 species of mealybugs (Hemiptera: Coccoidea: Pseudococcidae) found on *T. cacao*, of which 19 have been reported in West Africa[1] and to date 16 of those are thought to act as vectors of *Cacao Swollen Shoot Virus* (CSSV), the most damaging virus affecting the crop in that region.[2] The effectiveness of mealybugs as virus vectors is species dependent and varies according to their favoured feeding sites on the cacao plant and with respect to the age of the plant. Differences in cacao infection rates, for example, have been observed between the mealybug vectors *Formicococcus njalensis* (Laing) and *Ferrisia virgata* (Cockerell) with distinct stylet dimensions and frequency of phloem penetration being proposed as the cause.[3] *F. njalensis* and *Planococcus citri* (Risso) are thought to be the most important viral vectors on cacao as they are generally the predominant mealybugs on the crop in the Afrotropical region. Persistence of the virus within the vectors reportedly differed between the two species with *F. njalensis* showing a gradual decline in infectivity up to 18 h post acquisition feeding while *P. citri* transmission rates over a similar period were constant.[4] However, of these two species, while *P. citri* is usually present in lower numbers in West Africa,[5,6] its greater mobility and ability to infest new cacao trees make it potentially the more important virus vector.[7] It is vital therefore that CSSV resistance breeding programmes incorporate accurate identification of the mealybug species with which candidate cacao plants are inoculated. This presents a challenge as mealybugs are morphologically cryptic with some species characterised only relatively recently and regular instances of misidentification occurring in the literature e.g. [8,9]

Morphological keys for mealybugs require a high degree of expertise depending as they do upon characterisation of microscopic structures that are, *in vivo*, often obscured by filamentous wax exudates (using scanning electron microscopy Cox and Pearce[10] were able to distinguish three species of mealybugs based on their wax exudates though this was not proposed as a practical means of identification). The keys cannot be definitive as some species are known to exhibit misleading phenotypic plasticity [11] and, with few exceptions (e.g. [12]), morphological keys for mealybugs describe only adult females leaving the peripatetic and therefore more pathogenically important juveniles[5] largely anonymous.

In this work we describe the use of a mitochondrial cytochrome c oxidase 1 (CO1)-based DNA barcoding approach to mealybug identification that circumvents many of these problems. CO1 markers have been shown to be effective for the separation of haplotypes within the mealybug genus *Planococcus*[13] and we have found that, using PCR primers newly designed from conserved hemipteran CO1 sequences, it has proved possible to distinguish all mealybug species so far sampled from cacao plants. The simple test is effective at the level of a single egg and scanning electron microscopy has allowed correlation of morphology of individual mealybugs with their CO1 sequences, confirming the robustness of the procedure. In order to make a DNA barcoding approach a practical tool to support *in situ* breeding for improved pest resistance of tropical crops such as cacao we have also tested High Resolution Melt Analysis (HRMA) for CO1-based mealybug identification. HRMA is a well-established technique for gene scanning based on monitoring the melting behaviour of whole amplicons after a common PCR amplification,[14] and as it does not require any processing, reagent addition or separations after PCR, it provides a cheaper, more rapid means of species characterisation compared to DNA sequencing. This study seeks to establish the efficacy of HRMA as a means of haplotype discrimination for mealybugs found on cacao.

2 EXPERIMENTAL METHODS

2.1 Sample collection

Mealybugs were collected from stems, foliage and pods of cacao trees in Brazil, Costa Rica, Indonesia, the Philippines and Trinidad. In addition mealybugs were collected from cocoa producing areas in Côte d'Ivoire and Ghana, the countries most extensively affected by CSSV. Samples were sent to the University of Reading and either processed immediately for Environmental Scanning Electron Microscopy (ESEM) or stored at -80°C.

2.2 Morphological analysis

Mealybugs were treated for wax removal using a simplified version of the methodology of Banks and Williams,[15] by soaking them overnight in the detergent Decon 90. Individual mealybugs were mounted on aluminium stubs using double-sided adhesive tape and examined in an environmental scanning electron microscope (Quanta 600F, FEI, Amsterdam, Netherlands) operating in the low vacuum mode using its secondary electron detector. Following morphological assessment[16,17] individual samples were coded and stored at -80°C for subsequent DNA extraction.

2.3 DNA extraction, amplification and sequencing

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and quantified with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). PCRs were performed in a total volume of 25 µl: 12.5 µl of BioMix (Bioline, London, UK), 1.25 µl of each 2 µM primer, 2 µl of diluted DNA (between 2 and 30 ng) and 8 µl of water. Primers were designed from published pseudococcid sequences to amplify a 379 bp partial region of the CO1 gene (MFCO1 5'ATATCTCAAATTATAAATCAAGAA3'; MRCO1 5'ATTACACCTATAGATAAAACATAATG3'). PCR conditions were: initial denaturation at 94°C for 4 min, followed by 40 cycles of (i) denaturation at 94°C for 30 s, (ii) annealing at 56°C for 30 s, (iii) elongation at 72°C for 60 s and a final extension period at 72°C for 5 min. PCR products were separated by electrophoresis in 1% agarose gels to check their quality. PCR products were Sanger sequenced on both strands by Source BioScience Ltd (Oxford, UK). Consensus sequences were produced and alignments were manually edited with Geneious 5.5.6 (Biomatters Ltd, Auckland, New Zealand).

2.4 HRMA

A new set of CO1 primers was designed (HRM3F 5'AATTTCCATTGGAATTTTAGG3'; HRM3R 5'TTCCATTTAAAGTTATTATTCA3') yielding a 158 bp fragment also diagnostic for all sequences generated in the present work but better suited for HRMA. All PCR amplifications and HRMA were performed on a Rotorgene 6000 (software version 1.7, Qiagen, UK). For all reactions 10 ng DNA was included in a 20 µl PCR mixture prepared from 2X Sensimix (Quantace Ltd, London, UK) and containing both primers (final concentration 200 nM) and 1 µl of Evagreen (Quantace Ltd). PCR amplifications were performed using initial denaturation at 95°C for 10 min then five cycles of (i) 60 s at 95°C, (ii) 90 s at 45°C and (iii) 90 s at 72°C and then 30 cycles of (i) 60 s at 95°C, (ii) 90 s at 50°C and (iii) 60 s at 72°C.

HRMA was performed both on pure PCR products and mixtures comprising an individual PCR product combined ('spiked') with a reference sample amplified separately and exhibiting CO1 haplotype 2 (H2). This product mixing was performed in order to produce artificial heteroduplexes which should enhance the differentiation in melting curves. HRMs were performed by combining 20 µl of the PCR amplicon with 20 µl of PCR products from template H2 with an HRM procedure comprising: a first step at 95°C for 2 min, a hold at 50°C for 2 min and then a melting step of 59°C for 90 s followed by a graduated increase of 0.1°C with a 2s hold at each step up to 75°C. Fluorescence levels were acquired at the end of each step and a melting curve of the PCR product was obtained from the recorded values. The melting curves were normalized by calculation of the 'line of best fit' between normalization regions before and after the major fluorescence decrease.

2.5 Statistical analysis

The HRMA was assessed for statistically significant identification of specific CO1 sequence haplotypes. The significance of observed differences between treatments in melt phase midpoint temperature (Tm) and altered curve shape was calculated using Rotor-Gene ScreenClust HRM software (version 1.10.1.2, Qiagen, UK). After normalization of the melting curve, a residual plot was created by subtracting the differentiated curves from a median of all of the curves. Principal Components (PCs) could then be determined based on the residual plots.

The ScreenClust software calculates the optimal number of clusters and allocates each sample to the most appropriate cluster (indicating which nucleotide sequence differences generate significantly different HRM curves), provides the probability that each sample belongs to the assigned cluster(s) and shows the typicality of samples within its allocated cluster. To optimise the discriminatory power, a preliminary analysis was run by fixing the maximum number of possible clusters to 11 distinct haplotypes (supervised mode) and then compared to the optimal number of clusters generated automatically by the software (unsupervised mode). Only groups of samples separated by both types of analysis and with probabilities >0.999 and typicalities >0.05 were deemed statistically different.

3 RESULTS

3.1 Morphological analysis

During preparation for ESEM analysis the mealybug samples were positioned sideways on to the aluminium stubs (Fig. 1). This orientation meant that by movement of the specimen stage observations could be made of diagnostic structures on both the dorsal and ventral surfaces of individual samples (an option not available with specimens mounted for light microscopy). Detergent washing was invariably required beforehand to remove wax meal (Figs 1 a and b) that otherwise obscured diagnostic structures such as the pores and setae of the cerarii (Figs 1 c and d). Features such as antennae segment number, which are helpful species indicators in adults, are variable in samples that prove to be late instar juveniles and so had to be used with caution (Figs 1 e and f). Unlike the preparation of mealybug specimens for morphological assessment via conventional light microscopy, the ESEM preparation and imaging left the samples intact and readily available for DNA extraction and subsequent PCR analysis. DNA yield and CO1 sequence comparisons between extractions from fresh and post-microscopy samples indicated that any DNA degradation caused by the ESEM did not undermine the DNA barcoding process (data not shown). While all groups were examined by ESEM, some did not yield images that were sufficiently informative for a definitive description to be made. In these cases the 'morphological assessment' category in Table 1 indicates only the likely genus (haplotypes H5, H6, and H10) or that identification of the haplotype was inconclusive (H8, H9 and H11).

3.2 CO1 sequence analysis

Sequences were obtained from all mealybug samples analysed which included all developmental stages from eggs to adults (Table 1). Sequence analysis of the CO1 region from 64 samples revealed 11 unique haplotypes. Where possible, species prediction had been made based on morphological assessment and DNA sequence searches on NCBI looking for closest possible matches. As shown in Table 1 morphological and molecular characterisation allowed for the categorisation of three haplotypes to the species level and one to the genus level. The remaining seven haplotypes were either unresolved morphologically or differ in their morphological and molecular characterisation. It is important to note that the latter category includes two haplotypes which have been morphologically identified as *Formicococcus njalensis*, a species for which there is no current CO1 sequence available on NCBI (accounting for the similarity of only 93%, Table 1). This is therefore the first report of a CO1 partial sequence for *F. njalensis*. Five of these 11 haplotypes were found at West African sites.

3.3 HRMA

Twenty seven nucleotides out of the 115 bp section of the CO1 region sequenced in this study were polymorphic and enabled the characterization of all 11 haplotypes (see Table 2). The level of sequence polymorphism between haplotypes varied from one to 14 Single Nucleotide Polymorphisms (SNPs). The HRMA was performed on 34 selected mealybugs representative of the 11 haplotypes detected (Table 1) each spiked with the same CO1 PCR product from the reference sample H2 (KM378731). As expected for an identical sequence haplotype, HRM curves of replicate samples from H2 spiked and non-spiked samples showed no differences in profile (Fig. 2). Furthermore, Sample Clustering Analysis (SCA) showed high probabilities (P) and typicalities (T) of the H2 samples exposed or not to spiking belonging to the same haplotype cluster 6 ($P>0.999$; $T>0.05$) (Fig. 3, Table 3). In contrast, the analysis by heteroduplexing with H2 of the 10 remaining haplotype sequences described in this study generated significantly different melting patterns from H2 (Fig. 2). Furthermore, these samples clustered according to their haplotype and in distinct locations from H2 (Fig. 3). Analysis also showed highly significant P and T values for all samples belonging to their own assigned haplotype cluster ($P>0.999$; $T>0.05$) (Table 3).

4 DISCUSSION

An attribute of the approach employed in the present study was the DNA barcoding of individual specimens that had first been morphologically assessed via ESEM. Mealybugs can be found on cacao as single species populations but not exclusively so.[6] This may in part account for instances of apparent mislabelling among mealybug CO1 sequences that have been submitted previously. For instance, comparison of 36 published sequences of CO1 regions identified as *P. citri* (see online material) revealed that, while 35 of them do not differ by more than 1.8% (12/657 bp), a single sequence (AF483206) differed by 12.8% (84/657 bp). This higher level of sequence difference observed in AF483206 strongly suggests species misidentification.

To establish an efficient PCR based diagnostic protocol it is vital that the primers used in the analysis are effective for all potential CSSV vector species. Published universal primers LC01490-HC02198,[18] were initially tested on 24 mealybug samples originating from various developmental stages but only two of these samples generated discrete, reliable PCR products. Therefore in order to maximise the effectiveness of this approach the primers used in the present study were designed from all published pseudococcid CO1 sequences then available i.e. January 2012. These new primers have been 100% reliable for all mealybug species collected from cacao and were effective with all developmental stages from eggs to adults for both males and females.

While HRM can be performed on any size fragment, trials have shown that optimum resolution is usually achieved with fragments of less than 200 bp.[19,20] A dedicated HRM primer pair was designed from the initial CO1 sequences obtained in this project and proved to be as effective as the original MFCO1/MRCO1 combination. Therefore, while it will have to be tested, we anticipate that any species from the Pseudococcidae could be assessed following the methodology described in this paper.

Screening mealybugs collected from multiple cacao sites across three continents gave rise to 11 haplotypes, five of which were apparent among the West African samples. These haplotypes were identified morphologically as belonging to the species *P. citri*, *Pseudococcus longispinus* (Targioni Tozzetti) and *F. njalensis*. This is a subset of the 19 mealybug species reported to be present on West African cacao[1] and represents a first step towards the establishment of a comprehensive set of reliable, morphologically established exemplars. This surveying work is on-going but, in keeping with diversity studies of invertebrate fauna on West African cacao,[5-7] *F. njalensis* and *P. citri* (both known vectors of CSSV[21,22]) continue to be the most commonly encountered mealybug species.

HRMA allows for indirect sequence assessment and can be performed quickly without the need for a sequencing service making the technique of particular value in developing countries. HRMA has been effective in large scale studies involving the rapid haplotyping of invertebrate vectors of crop disease and their results have been shown to be robust according to subsequent DNA sequencing.[23] The approach is also being increasingly utilised where analysis of large populations necessitates a cost effective means of haplotype identification.[24]

When the identity of the vector mealybug species to be employed in a virus transmission trial is established, it can be used as a permanent reference sample. With such an exemplar available, stock mealybug lines maintained for resistance breeding work can be readily tested to verify their integrity. Using a spiking approach for HRMA of single mealybugs, a reference species sequence employed as a probe will reveal any sequence variation through melt profile changes due to heteroduplex product formation. Indeed, our results show that HRMA of a number of different haplotypes can be separated in distinct groups corresponding to either haplotype H2-like sequence (identical sequence and no heteroduplex formed) or non H2 haplotype (distinct sequences leading to the formation of a heteroduplex). While sequencing-free CO1-based screening has previously been used to identify mealybugs from a set of seven specific alternatives,[25] that multiplex PCR approach was dependent upon one dimensional separation of products on electrophoretic gels and so lacked the resolving power associated with three dimensional principal component analysis used in the present study. It is this enhanced capacity for haplotype discrimination that would make HRMA well suited to the detection of invasive arthropod species. It has also been demonstrated that HRMA is effective for the detection of mixed populations with the presence of cancerous cells still identifiable when mixed with wild type samples at dilutions as low as three per cent:[19] such a sample pooling approach could further enhance the utility of HRMA for the rapid assessment of species purity in virus vector populations.

Our results indicate that hemipteran haplotypes can be distinguished using HRMA in the same way that the technique has been utilized in the surveillance of a range of higher and lower organisms.[26,27] This HRMA approach uses a universal primer pair and is effective for species separation but also for the detection of novel haplotypes regardless of the number of SNPs. For this reason the technique will be appropriate for distinguishing mealybug species without a requirement for the use of multiple primer pairs, as distinct from multiplex PCR approaches (e.g .[25,28]). Thus, in the context of quarantine systems, an HRM approach such as this could be utilised to rapidly identify potential pathogen vectors present on internationally exchanged germplasm. DNA barcoding allied to a morphological characterisation of reference exemplars would be required to fully implement such a screening system and the present work constitutes the foundation for such an approach. HRMA's capacity to facilitate identification from damaged samples and from developmental stages for which taxonomic keys are not available will be of particular value in quarantine scenarios.

This work utilised a CO1-based DNA barcoding methodology for mealybug haplotype characterisation because of that gene's established utility for species identification applications. However, HRMA has also been successfully applied using microsatellites as DNA markers allowing resolution below the species level[29] and this raises the possibility of more detailed assessment of geographical origin of CSSV vectors via such an SSR approach.

5 CONCLUSIONS

HRMA allied to morphological characterisation of mealybug exemplars has immediate utility for the support of CSSV resistance screening in cacao. Once exemplar haplotypes have been established for all species found on West African cacao, reference DNA will be made available so that only HRMA will be necessary at remote sites for the identification of potential CSSV vectors. The approach makes dependence on access to DNA sequencing superfluous and its sensitivity means that samples can be characterised regardless of developmental stage thereby also benefitting quarantine applications.

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References

1 ScaleNet. [Online]. www.sel.barc.usda.gov/SCALENET/scalenet.htm [Accessed 10 November 2014].

2 Roivainen O, Mealybugs In Vectors of Plant Pathogens, KF Harris and K Maramorosch (eds) Academic Press, New York pp. 15-38 (1980).

3 Entwistle PF and Longworth, JF, The relationship between cacao viruses and their vectors: the feeding behaviour of three mealybug (Homoptera: Pseudococcidae) species. *Ann Appl Biol* **52**: 387-391 (1963).

4 Posnette AF and Robertson NF, Virus diseases of cacao in West Africa. VI Vector investigations. *Ann Appl Biol* **37**: 363-377 (1950).

5 Strickland AH, The entomology of swollen shoot of cacao. *Bull Entomol Res* **41:** 725-748 (1951).

6 Campbell CAM, The assessment of mealybugs (Pseudococcidae) and other Homoptera on mature cocoa trees in Ghana. *Bull Entomol Res* **73**: 137-151. (1983)

7 Bigger M, The relative abundance of the mealybug vectors (Hemiptera: Coccidae and Pseudococcidae) of cocoa swollen shoot disease in Ghana. *Bull Entomol Res* **71**: 435-448 (1981).

8 Cox JM, The mealybug genus *Planococcus* (Homoptera: Pseudococcidae), *Bulletin of the British Museum (Natural History), Entomology* **58**: 1-78, (1989).

9 Kaydan MB and Gullan PJ, A taxonomic revision of the mealybug genus *Ferrisia* Fullaway (Hemiptera: Pseudococcidae), with descriptions of eight new species and a new genus. *Zootaxa* **3543**: 1-65 (2012).

10 Cox JM and Pearce MJ, Wax produced by dermal pores in three species of mealybug (Homoptera : Pseudococcidae). *Int J Insect Morphol Embryol* **12**: 235-248 (1983).

11 Cox J, An experimental study of morphological variation in mealybugs (Homoptera: Coccoidea: Pseudococcidae). *Systematic Entomology* **8**: 361–382 (1983).

12 Gullan PJ, Identification of the immature instars of mealybugs (Hemiptera: Pseudococcidae) found on citrus in Australia. *Australian Journal of Entomology* **39**: 160–166 (2000).

13 Rung A, Scheffer SJ, Evans G and Miller D, Molecular identification of two closely related species of mealybugs of the genus *Planococcus* (Homoptera: Pseudococcidae). *Ann. Entol Soc Am* **101**: 525-532 (2008).

14 Gundry CN, Vandersteen JG, Reed GH, Pryor RJ, Chen J and Wittwer CT, Amplicon melting analysis with labelled primers: a closed-tube method for differentiating homozygotes and heterozygotes. *Clin Chem* **49**: 396-406 (2003).

15 Banks HJ and Williams DJ, Use of the surfactant, Decon 90, in the preparation of coccids and other insects for microscopy *J Austr Ent Soc* **11**: 347-348 (1972).

16 Williams DJ Mealybugs of Southern Asia. Southdene, Kuala Lumpur, Malaysia, (2004).

17 Williams DJ and Granara de Willink MC, Mealybugs of Central and South America. CABI, London, England, (1992).

18 Hebert PDN, Cywinska A, Ball SL and deWaard JR, Biological identifications through DNA barcodes. *Pro R Soc London Ser B* **270**: 313–321 (2003).

19 Krypuy M, Newnham GM, Thomas DM, Conron M and Dobrovic A, High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: *KRAS*

codon 12 and 13 mutations in non-small cell lung cancer. *BMC Cancer* **6**: 295 doi:10.1186/1471-2407-6-295 (2006).

20 Salim B, Bakheit MA and Sugimoto C, Rapid detection and identification of *Theileria equi* and *Babesia caballi* by high-resolution melting (HRM) analysis. *Parasitol Res* **112**: 3883-3886 (2013).

21 Box HE, Insect transmission of the swollen-shoot virus. *Nature* **155**: 608-609 (1945).

22 Posnette AF and Strickland AH, Virus diseases of cacao in West Africa. III Technique of insect transmission. *Ann Appl Biol* **35**: 53-63 (1948).

23 Kylie D, Swisher KD, Munyaneza JE and Crosslin JM, High resolution melting analysis of the cytochrome oxidase I gene identifies three haplotypes of the potato psyllid in the United States. *Environ Entomol* **41**: 1019-1028 (2012).

24 Lin S-Y, Su Y-N, Hung C-C, Tsay W, Chiou S-S, Chang C-T**,** Ho H-N and Lee C-N Mutation spectrum of 122 hemophilia A families from Taiwanese population by LD-PCR, DHPLC, multiplex PCR and evaluating the clinical application of HRM. *BMC Med Genet* **9**: 53 doi:10.1186/1471-2350-9-53 (2008).

25 Daane KM, Middleton MC, Sforza R, Cooper M, Walton VM, Walsh DB, Zaviezo T and Almeida RPP, Development of a Multiplex PCR for Identification of Vineyard Mealybugs. *Environ Entomol* **40**: 1595-1603 (2012).

26 Dobrowolski SF, Gray J, Miller T and Sears M, Identifying sequence variants in the human mitochondrial genome using high-resolution melt (HRM) profiling. *Human Mutat* **30**: 891-898 (2009).

27 Adaszek L and Winiarczyk S, Application of the SYBR Green real-time HRM PCR technique in the differentiation of the *Babesia canis canis* protozoa isolated in the areas of eastern Poland. *Parasitol Res* **106**: 1253-1256 (2010).

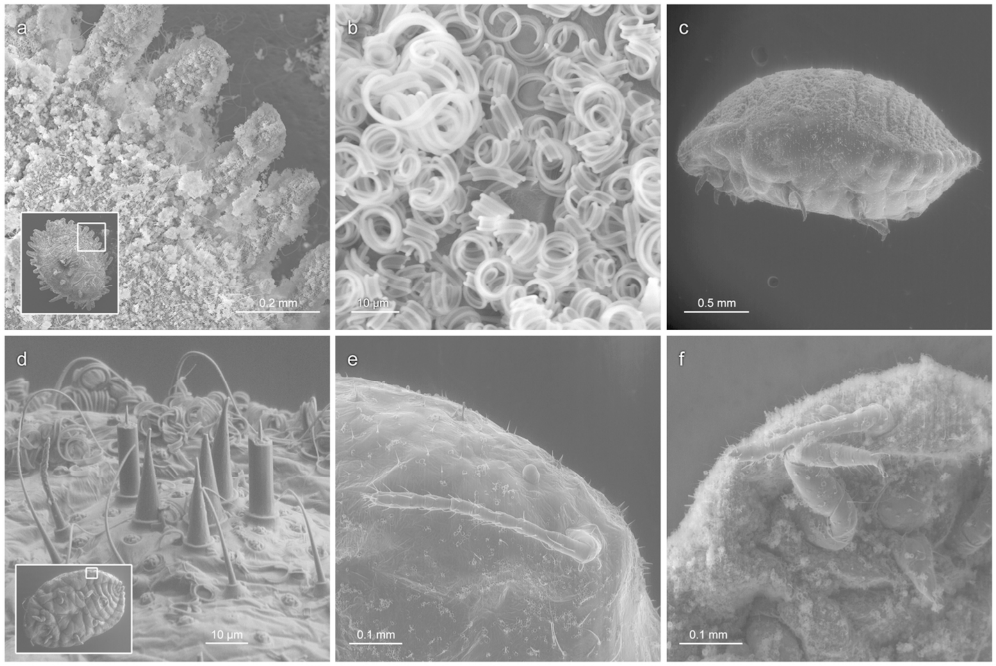
28 Hosseini R and Hajizadeh J, Molecular identification of three of the most important mealybug species (Hemiptera: Sternorrhyncha: Coccoidea: Pseudococcidae) on ornamental plants in Guilan province, Iran. *Zootaxa* **3009**: 46-54 (2011).

29 Mackay JF, Wright CD and Bonfiglioli RG, A new approach to varietal identification in plants by microsatellite high resolution melting analysis: application to the verification of grapevine and olive cultivars. *Plant Methods* **4**: 8 doi:10.1186/1746-4811-4-8 (2008).

Figure 1. Morphological analysis using environmental scanning electron microscopy of mealybugs collected from cacao: (a) lateral filaments covering cerarii (inset showing position of image on whole mealybug); (b) detail of wax meal exuded from trilocular pores on ventral surface of unwashed sample (c-e Decon 90-washed samples); (c) (Côte d'Ivoire) 18 of the 18 possible pairs of cerarii visible excluding all genera except *Phenacoccus, Planococcus* and *Formicococcus*; (d) (Côte d'Ivoire) 13th cerarius with four conical setae excluding genus *Planococcus* (inset showing position of image); (e) (Brazil) eight-segmented antenna and pre-ocular cerarius suggests genus *Planococcus*; (f) (Côte d'Ivoire) claw lacking denticle on juvenile excludes *Phenacoccus madeirensis* while the seven segmented antenna implicates *F. njalensis*.

Figure 2. Typical High Resolution Melt (HRM) curves generated by Rotor-Gene 6000 software for 11 haplotypes of mealybugs collected from cacao detected according to partial CO1 sequences generated using the HRM3 PCR assay. Each haplotype curve was produced as an average of 4 to 8 replicates. HRM analysis was performed on all samples spiked with the reference sample Haplotype 2. All haplotypes exhibit a distinct melt curve. Reference curve H2 (black) masks H2 spiked with H2 (grey).

Figure 3. Cluster plot generated by ScreenClust HRM software showing the differentiation of 68 mealybug accessions according to partial CO1 sequences generated using the HRM3 PCR assay. HRM analysis was performed on all samples spiked with the reference H2 and the non spiked reference sample. Circled in red are the reference samples H2 (black) clustering with test samples H2 (grey). The remaining samples all group according to their specific haplotype. Cluster plot produced according to Principal Components 2 and 3.



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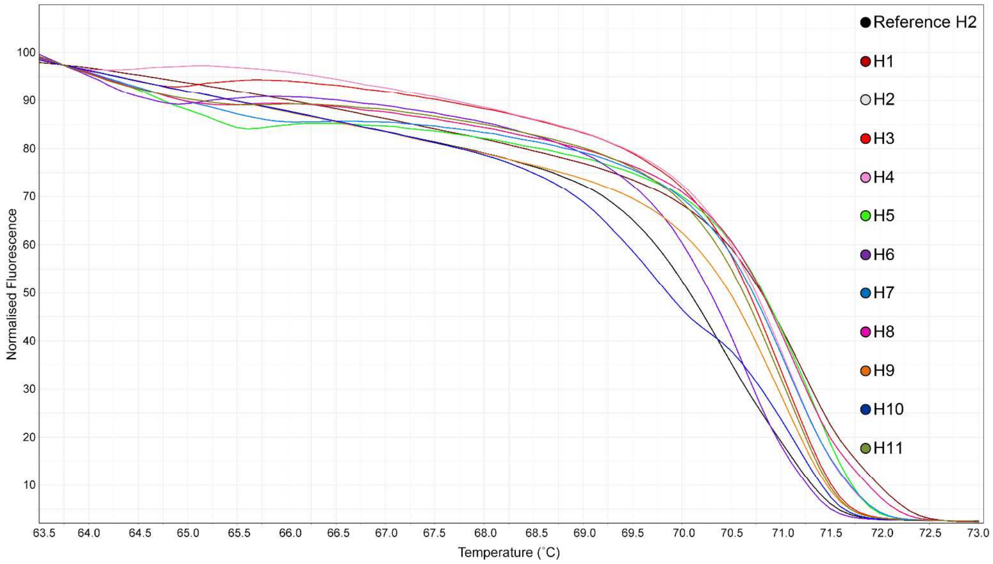

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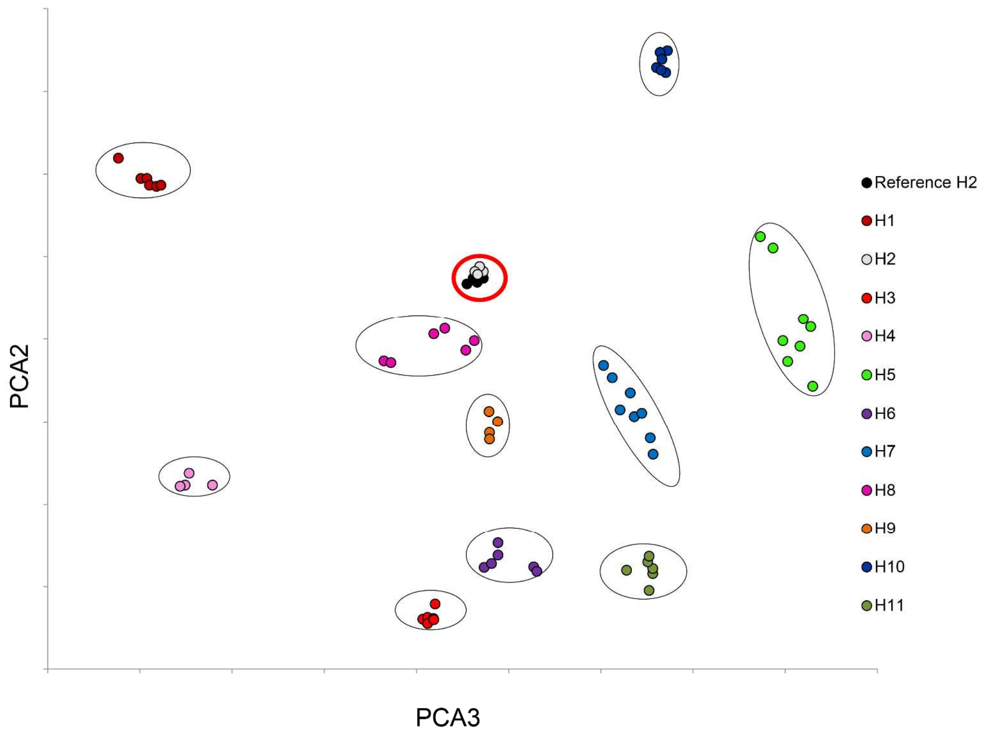

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Table 1. Characterisation of 11 CO1 haplotypes of mealybugs collected from cacao growing areas. Sequence ID corresponds to each haplotype submitted to Genbank. Morphological assessment was as specific as ESEM images allowed for these samples. The NCBI closest match was determined using BLAST search.

Haplotype ID	n	Dvt. stage	Sequence ID	Country of origin	Morphological assessment	NCBI closest match		
						Species	GenBank ID	% similarity
H1	5	*(a,b)*	KM378730	Ghana	*Pseudococcus longispinus*	*Pseudococcus longispinus*	JN112804	97
H2	6	*(a,c)*	KM378731	Ghana	*Planococcus citri*	*Planococcus citri*	EU250572	98
H3	4	*(a)*	KM378732	Ghana	*Formicococcus njalensis*	*Planococcus sp.*	EU250534	93
H4	2	*(a)*	KM378733	Ghana	*Formicococcus njalensis*	*Planococcus sp.*	EU250534	93
H5	5	*(a)*	KM378734	Côte d'Ivoire	*Formicococcus sp.*	*Planococcus sp.*	EU250534	93
H6	14	*(a,b)*	KM378735	Indonesia & Philippines	*Dysmicoccus sp.*	*Dysmicoccus neobrevipes*	EU267213	99
H7	12	*(a)*	KM378736	Indonesia	*Planococcus lilacinus*	*Planococcus lilacinus*	GQ906767	96
H8	4	*(a)*	KM378737	Philippines	inconclusive	*Planococcus lilacinus*	GQ906767	96
H9	7	*(a,c)*	KM378738	Costa Rica	inconclusive	*Planococcus citri*	AB439517	100
H10	2	*(a,b)*	KM378739	Trinidad	*Planococcus sp.*	*Planococcus minor*	EU250518	100
H11	3	*(a)*	KM378740	Brazil	inconclusive	*Ferrisia virgata*	GQ906765	94

n = number of individuals sequenced; developmental stage = *(a)* adult, *(b)* juvenile and *(c)* egg.

Table 2. Sequence variation detected across all 11 mealybug haplotypes using HRM3 assay. Single Nucleotide Position of polymorphisms are indicated with reference to the CO1 partial sequence obtained for haplotype 2 (KM378731) used for heteroduplex spiking.

		Position of nucleotide polymorphism																										
Haplotype	Genbank acc. no.	28	37	40	43	46	58	61	64	70	76	79	83	85	88	95	96	97	100	103	115	118	121	124	127	136	139	142
2	KM378731	T	T	A	T	T	T	C	A	T	T	A	T	A	T	T	C	A	T	A	T	T	T	A	A	C	T	A
1	KM378730	A	C	.	A	.	A	T	.	.	.	.	C	C	C	.	.	.	A	.	A	.	A	T	T	T	.	.
3	KM378732	.	A	.	.	.	.	T	.	.	C	.	.	.	.	.	.	T	C	T	.	.	.	C	C	T	.	T
4	KM378733	.	A	.	.	.	.	T	G	.	C	.	.	.	.	.	.	T	C	T	.	.	.	C	C	T	.	T
5	KM378734	A	.	.	C	.	.	T	.	.	C	.	.	.	.	.	.	T	.	.	.	C	.	.	.	T	.	T
6	KM378735	A	.	.	.	.	C	T	.	.	.	T	.	.	.	A	T	.	.	.	.	.	C	.	.	T	.	T
7	KM378736	.	.	.	.	.	.	T	.	C	C	T	.	.	.	.	.	.	.	.	.	C	.	T	T	T	C	.
8	KM378737	.	.	G	.	.	.	T	.	C	C	T	.	.	.	.	.	.	.	.	.	C	.	T	T	T	C	.
9	KM378738	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10	KM378739	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
11	KM378740	.	A	.	A	C	.	T	.	.	.	.	A	.	.	.	.	.	A	T	A	.	.	.	.	T	.	.

Dots indicate nucleotide match with the sequence of haplotype 2.

Table 3. ScreenClust HRM analysis cluster, typicality and probability results of 11 mealybug CO1 haplotypes.

Haplotype	Cluster *(a)*	Typicality *(b)*	Posterior probabilities *(c)*										
			Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7	Cluster 8	Cluster 9	Cluster 10	Cluster 11
H1	Cluster 2	0.27 - 0.88	0	1	0	0	0	0	0	0	0	0	0
H2	Cluster 6	0.29-0.88	0	0	0	0	0	1*	0	0	0	0	0
H3	Cluster 7	0.29-0.85	0	0	0	0	0	0	1	0	0	0	0
H4	Cluster 9	0.52	0	0	0	0	0	0	0	0	1	0	0
H5	Cluster 3	0.18-0.96	0	0	1	0	0	0	0	0	0	0	0
H6	Cluster 1	0.33-0.72	1	0	0	0	0	0	0	0	0	0	0
H7	Cluster 4	0.19-0.97	0	0	0	1	0	0	0	0	0	0	0
H8	Cluster 11	0.38-0.60	0	0	0	0	0	0	0	0	0	0	1
H9	Cluster 5	0.52	0	0	0	0	1	0	0	0	0	0	0
H10	Cluster 8	0.32-0.66	0	0	0	0	0	0	0	1	0	0	0
H11	Cluster 10	0.26-0.89	0	0	0	0	0	0	0	0	0	1	0

(a) The genotype result for a sample.

(b) Typicality measures how well a sample falls within the cluster for which it has been classified.

(c) Probability of each sample fitting into a particular cluster is given as a value from 0 to 1. The sum of all probability values for a single sample is 1.

Each sample is called into the cluster with the highest probability. Samples with a probability of less than 0.7 of belonging to a particular cluster should be treated with caution.

* indicates the result for the screen of both test and reference sample H2