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Analysis of Swine (*Sus scrofa*) DNA from Blowfly (*Chrysomya megacephala*) Larval Guts after Post-Feeding Periods by PCR Technique

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Abstract

This research aimed to investigate the DNA of food sources taken from blowfly (*Chrysomya megacephala*) larval guts, for application in forensic entomology. The muscle samples of domestic swine (*Sus scrofa*), decomposed in 2 conditions, in air and drowning, were used as case studies. Adult blowflies were allowed to lay eggs on the meat samples. Then, the third instar larvae were identified as *C. megacephala*, then transferred to non-meat food and collected every 6 h up to 36 h. Total DNA extraction was performed by the Phenol-Chloroform method, and used as DNA templates in a Polymerase Chain Reaction (PCR) test. The PCR amplified swine partial *cytochrome b* (*cyt b*) genes with 203 bp expected size. The results showed that the swine DNA from blowfly larvae grown on decayed tissue under air and drowning conditions could be detected by PCR after larvae post-feeding up to 18 and 36 h, respectively. The sequence analysis revealed that the forward and reverse strands undergo 94 and 98 % identities of the swine *cyt b* gene sequence retrieved from nucleotide databases. This study suggests that mitochondrial DNA of the food source from insect guts can be detected at limited times after feeding, combined with the condition of the decayed tissues.

Keywords: *Sus scrofa, cytochrome b*, blowfly, forensic entomology

Introduction

Forensic entomology is the analysis of insect evidence for forensic and legal purposes [1]. Entomological evidence can provide important information in a death investigation, especially, post-mortem interval (PMI) estimation and movement of the corpse. Since the age of fly larvae is relevant to PMI, this estimation is based on the age of the larvae developing and feeding on the victim [2]. This is useful in cases where larvae are directly collected from or near a corpse which shows any signs of decomposition. However, in certain circumstances, such as in the presence of live larvae at a crime scene without a dead body, the only alternative evidence which can be used for proving the identity of the victim is the food source left in the larvae guts [3]. Because of the higher copy number of mtDNA in cells, it can be effectively isolated and employed from aged and degraded tissues [4-6]. Human DNA could be successfully amplified and analyzed from several insect sources, according to the fact that, within the Dipteran larvae crops, the food is temporarily stored in the anterior portion into which proteolytic enzymes are not secreted [7]. Under this situation, human mitochondrial DNA (mtDNA) analysis is preferably chosen. Moreover, adult blood-feeding insects also serve to validate the reliability of human DNA typing methodologies, by using variable numbers of tandem repeats (VNTR) [8,9]. In 1999, Kreike and Kampfer [10] reported that human DNA in the mosquito blood meal can be qualitatively and quantitatively sufficient for DNA typing up to 26 h after ingestion. Determination of

vertebrate and human usually relies on mitochondrial D-loop in the hypervariable region; however, *cytochrome b* (*cyt b*) and *cytochrome oxydase* subunits *I* and *II* (*COI* and *COII*) are also loci of choices for vertebrate and insect identifications, respectively [11]. In a previous study, the periods in which it is feasible to detect DNA from the gut contents of *Aldrichina grahami* blowfly larvae ranged from a maximum of 24 h at 32 °C to 42 h at 16 °C [12]. Furthermore, how a corpse decomposes, and the factors that may alter the rate of decay, is extremely important for host DNA quality and quantity [13]. We investigated the effects of the conditions of decomposition (in air decayed and water drowning decayed tissues), and periods after post-feeding, for the possibility to detect host DNA in blowfly (*Chrysomya megacephala*) larval guts, based on PCR, and using swine muscle tissue as a case study.

Materials and methods

Blowfly larvae trapping, rearing and sampling

Swine muscle was bought from a local fresh market and divided into 4 portions of a hundred grams each, which were stored in conditions of (1) air decay, (2) water drowning decay, (3) at –20 °C as a reference, and (4) in a sealed box to verify no insect eggs contamination. The portions of (1) and (2) decomposed for 4 days before being used as traps to attract adult blowfly of genus *Chrysomya*. These decayed meats were separately placed in cases with 1 cm^2 mesh in a local forest in Khon Kaen University during March to June 2011. The experiment was performed 10 times, with a one week time interval. After allowing adult flies to lay eggs, larvae were continuously reared on the decayed meats, until they developed to the third instar larvae, in a laboratory at room temperature (30.66±1.78 °C). Then, larvae were morphologically identified by species [14] and moved to banana meal. Then, 3 larvae were randomly collected every 6 h (0, 6, 12, 18, 24, 30, and 36 h after meat feeding), and preserved in 70 % ethyl alcohol. The samples were kept at –20 °C until used for DNA extraction.

DNA extraction

Total DNA was extracted by the Phenol-Chloroform method, followed by ethanol precipitation [15]. Each preserved larva was washed with sterilized water, cut into small pieces, and ground with a plastic grinder in a 1.5 ml microcentrifuge tube containing 300 µl extraction buffer (10 mM Tris, 0.5 M NaCl, 10 mM EDTA, 2 % SDS, and 200 µg/ml Proteinase). Then, the lysate was incubated in a water bath at 37 °C overnight. A volume of 20 µL of 100 mg/ml RNaseA was added, mixed well, and incubated at 65 °C for 45 min. One volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the sample, and vortexed thoroughly, before being centrifuged at 13,500 rpm for 15 min. The aqueous layer was removed to a new microcentrifuge tube, and DNA was precipitated with 500 µl of cold absolute ethanol and washed with 70 % (v/v) ethanol. The DNA pellet was air-dried, and resuspended in 50 µl of TE buffer. DNA concentration was assessed using the absorbance at wavelength of 260 nm, and the DNA quality was estimated by a A260/280 ratio using a spectrophotometer (Shimadzu, Japan).

Amplification of swine partial mtDNA *cyt b* gene by PCR

The nucleotide sequence of the *Sus scrofa cytochrome b* (*cyt b*) gene (Accession number: AP003428) was retrieved from GenBank and used for primer design using Primer3 [16]. The primer pair targeting 203 base pairs (bp) of the partial *cyt b* gene was generated, which were PG2F (5'TCGCAGCCGTACATCT CATA3') and PG2R (5'TGCTGGGGTGTAGTTGTCTG3'). Each 20 µl PCR consisted of 1 unit of *Taq* polymerase master mix (Vivantis, Malaysia) (containing 0.05 U/µL of *Taq* polymerase, 4 mM $MgCl_2$, 0.4 mM of each dNTP, and 1x of buffer), 0.5 µM of each primer (PG2F and PG2R), and 25 ng of extracted DNA. PCR amplification reactions were set up using the following thermal cycle: an initial denaturation of 5 min at 95 °C, followed by 35 cycles at 95 °C for 45 sec, 58 °C for 45 sec, and 72 °C for 45 sec; and a final extension step at 72 °C for 5 min. PCR products were visualized by ethidium bromide stained 1.5 % agarose gel electrophoresis.

Sequencing and data analysis

A chosen 203 bp PCR amplicon was sequenced using a dye terminator technique performed by the Biomolecular Analysis Service Unit, Department of Biochemistry, Faculty of Medicine, Khon Kaen University. The nucleotide sequences of forward and reverse were aligned. The sequences used to search for similarities against the database using the BLAST nucleotide (blastn) alignment program, available on http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Results and discussion

Species identification

After adult blowflies laid eggs on decayed pork meat and developed to larvae, we identified the 3rd instar larvae from the morphological characteristics and found 2 species, which were *Chrysomya megacephala* (712 individuals = 94.9 %) and *Achoetandrus rufifacies* (38 individuals = 4.1 %) (**Figure 1**). The abundance of *C. megacephala* agreed with the previous report by Boonchu *et al.* [17] that *C. megacephala* represented the majority of all blowfly species because of its ability to survive in a wide range of ecological niches. Then, *C. megacephala* larvae were chosen for swine DNA analysis, as described in materials and methods.

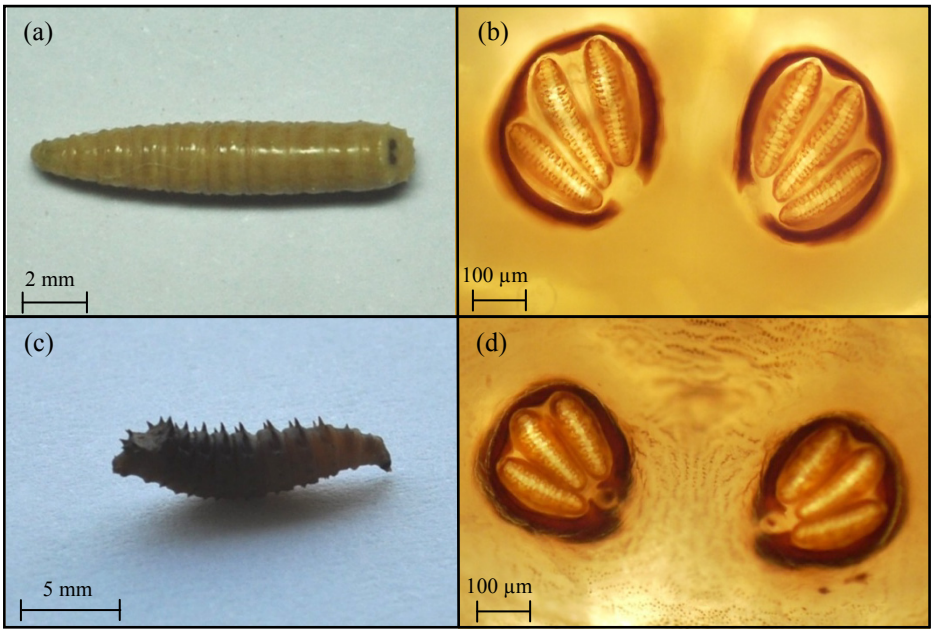

Figure 1 Morphological characteristics of *Chrysomya megacephala* and *Achoetandrus rufifacies*; (a) surface body, (b) posterior spiracle of *C. megacephala* 3rd instar larva, (c) surface body, and (d) posterior spiracle of *A. rufifacies* third instar larva.

DNA isolation and swine mtDNA *cyt b* gene detection

The total DNA concentrations obtained from the pork meat (as references) and *C. megacephala* ranged from 990 to 8,979 µg/µl. A stock dilution of 25 ng/µl was prepared for PCR reaction. We were consistently able to amplify a fragment of approximately 203 bp from each DNA sample extracted from the fresh and decayed pork meats. The detection of swine *cyt b* fragments by PCR from the DNA obtained from blowfly larvae grown on decayed pork meats varied greatly, depending on the time after meat-feeding. Swine *cyt b* gene could be successfully amplified from the total DNA of larvae fed on air decayed meat at 0 h, 6 h, 12 h, and 18 h after meal (**Figure 2**). Meanwhile, DNA extracted from larvae

grown on water drowning decayed meat at periods of 0, 6, 12, 18, 24, 30, and 36 h could be used to amplify swine *cyt b* gene fragments (**Figure 3**). These PCR products showed consistency with the same maximum periods of the DNA detection in all 3 replicates. The results suggested that the rate of decay from water drowning decayed tissue was slower than air decayed tissue; therefore, the DNA can be preserved for a longer period as well. The results agree with the fact that the decomposition rate of animal tissue is ordinarily decreased by being submerged in water. Air exposure conveys microorganisms in tissue, and proper moisture promotes the growth of microorganisms, speeding degradation of the meat tissue [18].

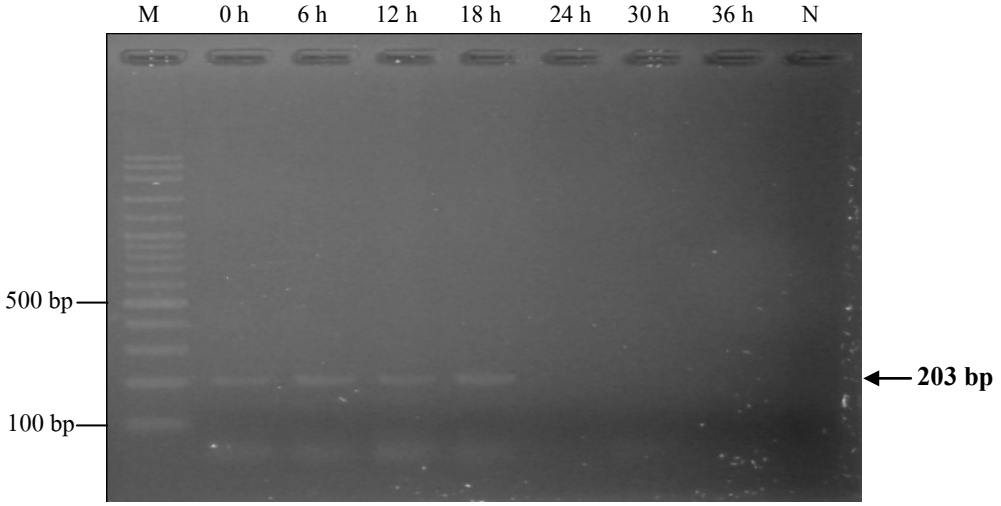

Figure 2 Agarose gel electrophoresis showing swine-specific partial *cyt b* PCR products amplified from larval guts after being fed on air decayed meat at varying post-feeding periods (0 - 36 h). M= 100 bp DNA ladder, and N = negative control.

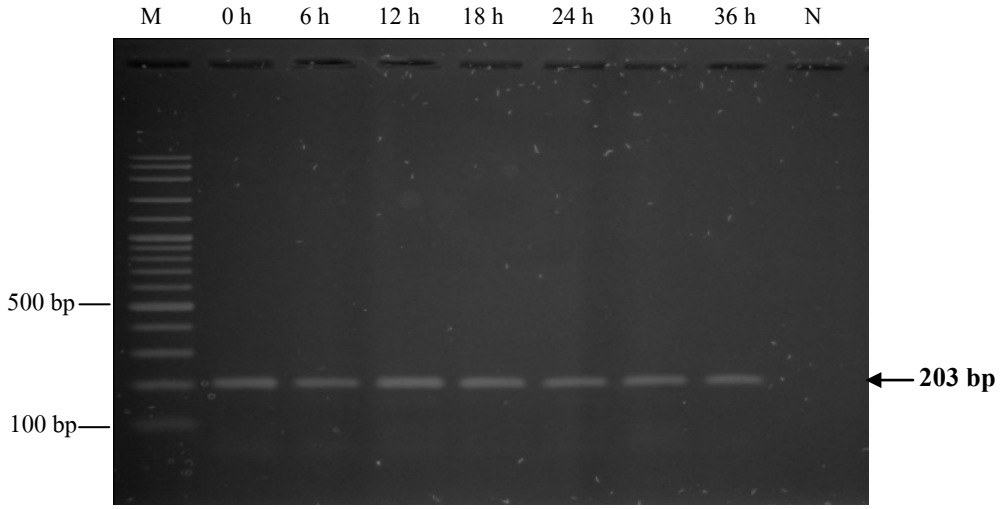

Figure 3 Agarose gel electrophoresis showing swine-specific partial *cyt b* PCR products amplified from larval guts after being fed on water drowning decayed meat at varying post-feeding periods (0 - 36 h). M = 100 bp DNA ladder, and N = negative control.

Sequences analysis and comparison

PCR product sizes of 203 bp of the partial *cyt b* genes were sequenced to confirm that the amplicons were swine DNA. The lengths of the obtained forward and reverse strand sequence lengths were 175 bp and 172 bp, respectively. The difference between the forward and reverse sequences was generated from gaps and a non-identified nucleotide, which were however replaced with correct ones. The sequences were then used to search in nucleotide databases via the BLASTN program on the NCBI. The most similar sequence retrieved was the *Sus scrofa cyt b* gene (Accession number: KR049170), with 100 % identities (**Figure 4**). This alignment result confirmed that the PCR products from DNA templates extracted from blowfly larval guts belonged to swine, i.e., the food source.

Sus scrofa isolate C3 breed Yimeng-Black cytochrome b (CYTB) gene, complete cds; mitochondrial

Sequence ID: gb|KR049170.1| Length: 1140 Number of Matches: 1

Range 1: 609 to 778 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
315 bits(170)	2e-82	170/170(100%)	0/170(0%)	Plus/Plus

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Query  3    CGGATCCAACAACCCTACCGGAATCTCATCAGACATAGACAAAATTCCATTTCACCCATA  62
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  609  CGGATCCAACAACCCTACCGGAATCTCATCAGACATAGACAAAATTCCATTTCACCCATA  668

Query  63   CTACACTATTAAAGACATTCTAGGGGCCTTATTTATAATACTAATCCTACTAATCCTTGT  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  669  CTACACTATTAAAGACATTCTAGGGGCCTTATTTATAATACTAATCCTACTAATCCTTGT  728

Query  123  ACTATTCTCACCAGACCTACTAGGAGACCCAGACAACTACACCCCAGCAA  172
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  729  ACTATTCTCACCAGACCTACTAGGAGACCCAGACAACTACACCCCAGCAA  778
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Figure 4 The retrieval results from the BLASTN program, using nucleotide sequences (Query) from PCR product identical to the *Sus scrofa cyt b* gene (Sbjct) with 100 % identities.

Conclusions

In this present study, swine tissue was used as a case study, instead of real human corpse tissue, to investigate the possibility to detect host DNA from blowfly larval guts under 2 conditions of decomposition, air decayed and water drowning decayed tissues. The results showed that 2 species of blowfly were collected, which were *C. megacephala* (94.9 %) and *A. rufifacies* (4.1 %). The high-yielded and qualified DNA could be extracted from *C. megacephala* larval gut grown on swine tissue, before removal to non-meat food. The sources of obtained DNA were from larvae themselves, from swine tissue and, probably, from banana. However, the PCR specifically amplified swine mtDNA *cyt b* gene with 203 bp amplicons. The amplicons were sequenced and aligned against the nucleotide database BLASTN to confirm that they were partial fragments of the *cyt b* gene. Moreover, we found that, under water drowning decayed conditions, DNA could be detected within a longer period (36 h after meat feeding) than under air decayed conditions (18 h after meat feeding). These results are useful for further consideration of estimation of PMI by forensic entomology.

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