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DNA characterization from gut content of larvae of *Megaselia scalaris* (Diptera, Phoridae)

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1. Introduction

Forensic entomology, a specialised tributary of forensic science, is the field where arthropod science interacts with the judicial system. The attitude of insects to colonise decomposing bodies is mainly used to estimate the post-mortem interval (PMI) of the corpse [1,2] but it can also provide answers to other relevant investigative questions such as the potential transfer of the cadaver from one location to another and also about the season of death in old/cold cases. In the last few decades DNA analyses, since its inception into criminal justice system, has acted as a multifaceted tool in crime scene investigation due to its power of discrimination. In forensic entomology, DNA analysis has been mainly used for insect species identification purposes [3,4]. Recently, such kind of analysis have also been applied for the identification of larval food source (Table 1). DNA, as evidence, is routinely collected from conventional sources (body fluids, soft tissues and bones) from a wide range of crime scenes. In the absence of such conventional sources, non-conventional sources such as fingerprints (touch DNA) [5], empty puparia [6] and the gut contents of larvae [7] can provide enough material (DNA) to identify the victim establishing a relation between the victim and the scene of crime.

Most of the studies of non-insect DNA extraction from immature stages of necrophagous insects focused on species with larvae and adults of relatively big size in the family Calliphoridae and Sarcophagidae and on few other species within the orders Phthiraptera and Coleoptera (Table 1).

Body decomposition is a temperature dependent process that can be altered by human activity especially when attempts are made to hide the decomposing body. In cases of indoor crime scenes and buried corpses, the access of large necrophagous species (Calliphoridae and Sarcophagidae) to the corpse is hindered [26,27]. In such context, species in the family Phoridae (genera *Conicera, Metopina, Thripleba, Diplonevra, Megaselia*, etc) are amongst the first, if not the only, colonizers of bodies. *Megaselia scalaris* Loew 1856, a cosmopolitan distributed species [28], has been reported from several indoor cases and buried bodies [1,27,29–31], but no studies about the extractability of DNA from their guts have been published.

In this paper we report the development of a comprehensive framework to extract non-insect DNA from the gut contents of III instars *M. scalaris* larvae fed on *Sus scrofa* Linneaus 1758 and *Bos taurus* Linneaus 1758 tissues. The DNA extracted was characterized using STR analysis.

2. Materials and methods

2.1. Sample preparation

Third instar larvae of *M. scalaris* feeding on pig tissue (*S. scrofa*) and bovine tissue (*B. taurus*) were obtained from the lines of fly population maintained at 25 °C in dark conditions at the University of Huddersfield. Before dissection, larvae were prepared using five different methods; (i) larvae were kept in hot water (> 80 °C) for 40 s [32], (ii) larvae were directly stored at −20 °C overnight [10], (iii) fresh larvae were stored in EtOH (98%) and then stored at −20 °C overnight [33], (iv) larvae were stored at −20 °C for 4 h and then added of EtOH (98%) and stored at −20 °C [33] overnight, (v) larvae were fixed in hot water (> 80 °C) for 40 s and then placed in EtOH (98%) and stored at −20 °C in the freezer before using it for the DNA extraction process [10]. After fixation, larvae were dissected under a Leica EZ4 stereomicroscope (Leica Microsystem GmbH, Germany) as described by Tuccia et al. [4]. The gut content was removed from the inner larval tissue with the help of sterile pins. The obtained gut content was weighed and transferred into 1.5 ml tube containing the lysis buffer as described in the following paragraph. Gut contents collected from 1, 3 and 5 larvae were analysed to define the minimum amount of material necessary for a successful identification.

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Table 1
Studies conducted on Human DNA extraction from necrophagous species commonly found inhabiting the decomposing corpse (A = adult, L = larvae, I = 1st instar, II = 2nd instar, III = 3rd instar, BM = Bloodmeal).

Family	Species	Stage	Analysis method	Reference
Insecta- Diptera				
Calliphoridae		LIII	STR analysis (Profiler Plus, 9 loci + amelogenin) and mt HV1 and HV2 analysis	[8]
		LIII	STR analysis (AmpF/STR® Identifiler®, 15 loci + amelogenin)	[9]
	Aldrichina graham	LIII	STR analysis (Identifiler®, 15 loci + amelogenin) and mt HV2 analysis	[10]
	Calliphora vicina	LIII	STR analysis (AmpF/STR Identifiler®, 15 loci + amelogenin;Y-filer PCR kit, 17 Y chromosome loci)	[11]
	Chrysomya albiceps	LIII	STR analysis (AmpF/STR Identifiler® Plus PCR, 15 loci + amelogenin; Argus-X-12 kit, 12 loci + amelogenin)	[12]
		LII	Quadruplex Y-STR analysis	[13]
		LI, LII, LIII	STR analysis 16 Y chromosome loci	[14]
	Protophormia terraenovae	LIII	STR analysis AmpFLSTR® Identifiler® Plus, 15 loci + amelogenin	[15]
Sarcophagidae	*Sarcophaga* sp.			
		LIII	STR analysis (Profiler Plus, 9 loci + amelogenin) and mt HV1 and HV2 analysis	[8]
		LIII	STR analysis (AmpF/STR® Identifiler®, 15 loci + amelogenin)	[9]
Muscidae	–	LIII	STR analysis (Profiler Plus, 9 loci + amelogenin) and mt HV1 and HV2 analysis	[8]
Culicidae	*Anopheles gambiae*	BM from female adult	VNTR analysis VNTR analysis Nuclear HLA-DQα and D1S80 loci analysis	[16–18]
	Anopheles stephensi	BM from female adult	mt-DNA cyt b RFLP analysis	[19]
	Aedes aegypti	BM from female adult	STR analysis (AmpF/STR® Identifiler® 15 loci + amelogenin) mt HV1 analysis	[20,21]
	Culex pipiens	BM from female adult	STR analysis (AmpF/STR® Identifiler® 15 loci + amelogenin)	[20]
Insecta- Phthiraptera				
Pediculidae	*Pthirus pubis*	Adult Excreta	AFLP analysis	[22]
	Pediculus humanus capitis	Adult Gut content	STR analysis (AmpF/STR1 NGM SElectTM PCR Amplification Kit, 15 loci + amelogenin)	[23]
Insecta- Coleoptera				
Nitidulidae	*Omosita* sp.	L	mt-DNA analysis	[24]
Arachnida- Acariforms				
Pyroglyphidae	*Dermatophagoides* sp.	A	STR analysis AmpF/STR® MiniFiler™ PCR Amplification Kit, 8 loci + amelogenin	[25]

2.2. DNA extraction and quantification

DNA extraction was performed using QIAamp® DNA investigator kit (Qiagen, Netherlands). The extractions were carried out according to the user manual procedures provided by the kit supplier. The reference samples from the food source were extracted with the same kit. DNA was eluted in 50 µl of elution ATE buffer and quantified using Invitrogen™Qubit® 3.0 (Life technologies, USA) via the dsDNA-High Sensitivity assay. DNA was then stored at −20 °C until further analysis.

2.3. DNA amplification and purification

Extracted DNA was amplified using species-specific cytochrome b (cyt b) primers (Table 2) resulting in fragment lengths of 149 bp and 116 bp for pig and bovine respectively. The PCR assay mix with a final volume of 20 µl comprised of 4 µl of 5× GoTaq flexi® Buffer (Promega, USA), 4 µl of 25 mM $MgCl_2$ (Promega, USA), 0.5 µl each of forward and reverse primer (10 pmol/µl) (IDT, USA), 0.5 µl of dNTPs (10 mM), 0.25 µl GoTaqG2 (5u/µl) polymerase (Promega, USA) and 6.25 µl of Ultrapure™ PCR grade water (Invitrogen, USA) along with 5 µl of DNA

Table 2
Primers used for specific PCR assay in this study. cyt b Pig [34], cyt b Bovine [35,36], 16S rRNA Pig [37] and 16S rRNA Bovine [37] were used to amplify specific targets of the DNA extracted from gut content of the larvae.

Primer	Primer name	Primer sequence	Fragment size (bp)	Annealing temperature (°C)	Reference
cyt b Pig	CytB pork F	ATGAAACATTGGAGTAGTCCTACTATTTACC	149	60	[34]
	CytB pork R	CTACGAGGTCTGTTCCGATATAAGG			[34]
16S rRNA Pig	16S SFI11 Pig F	CAACCTTGACTAGAGAGTAAAACC	138	58	[37]
	16S SFI11 Pig R	GGTATTGGGCTAGGAGTTTGTT			[37]
cyt b Bovine	CytB Bos F	CGGAGTAATCCTTCTGCTCACAGT	116	59	[35,36]
	CytB Bos R	GGATTGCTGATAGGTTGGTG			[35,36]
16S rRNA Bovine	SFI11-CowF	TATCTTGAACTAGACCTAGCCCAATG	131	58	[37]
	SFI11-Cow R	GGTACTTTCTCTATAGCGCCGTAC			[37]

Table 3
List of the loci analysed using the Animaltype Pig PCR Amplification Kit (Biotype® GmbH, Germany) and the Bovine Genotypes Panel 1.2 (Thermo Fisher Scientific, USA) respectively which include all the 12 STR loci recommended by ISAG for pork and bovine identification and parentage.

Animaltype Pig PCR amplification Kit (Biotype® GmbH)			Bovine genotypes panel 1.2 (Thermo Fisher Scientific)		
Locus name	Size range (bp)	Dye colour	Locus name	Size range (bp)	Dye colour
S0655	5–22	Blue	TGLA227 (D18S1)	63–115	Blue
SBH2	6–34	Blue	BM2113 (D2S26)	116–146	Blue
SBH4	47.3–66.1	Blue	TGLA53 (D16S3)	147–197	Blue
SBH18	9–23	Blue	ETH10 (D5S3)	198–234	Blue
SBH20	19–49	Green	SPS115 (D15)	240–270	Blue
SBH1	7–18	Green	TGLA126 (D20S1)	104–132	Green
SBH10	31–50	Green	TGLA122 (D21S6)	133–193	Green
SBH23 Y	–	Green	INRA23 (D3S10)	194–236	Green
SBH23 X	–	Green	BM1818 (D23S21)	248–276	Green
SBH19	10–16	Black	ETH3 (D19S2)	89–131	Black
SBH13	8–18	Black	ETH225 (D9S1)	132–166	Black
SBH22	18–28	Black	BM1824 (D1S34)	170–218	Black
387A12F	9–21	Black			

template. The C1000 Thermal cycler (Bio-Rad, USA) was used with the following amplification programme: initial denaturation step of 95 °C for 10 mins followed by 30 cycles each consisting of denaturation at 95 °C for 1 min, annealing at 60 °C or 59 °C for 1 min for pig and bovine respectively and extension at 72 °C for 1 min. A final elongation step of 72 °C for 10 min followed. The same protocol was repeated with species specific 16S rRNA primers (Table 2) with an annealing step at 58 °C. Positive and negative controls were used to test the quality of the PCR assay and to detect potential contaminants of the reaction.

PCR products were visualized on a 1.5% w/v agarose gel with a UV gel doc system (Syngene Bio Imaging System,UK) through Gene Snap (SynGene,UK) software. Positive samples were purified using QIAquick PCR Purification Kit (Qiagen, Netherlands) and sequenced by the external company Eurofins Genomics (Germany).

2.4. STR analysis

A 7500 Real Time PCR (Applied Biosystems, USA) was used to quantify the DNA based on SYBR-green (Life technologies, USA) assay using the primer reported in Table 2. A threshold value of 0.01 ng/μl was established prior to conduct the STR analysis. The Animaltype Pig PCR Amplification Kit (Biotype GmbH, Germany) [38] and Bovine Genotypes Panel 1.2 [39] (Thermo Fisher Scientific, USA) were used according to the manufacturer's instructions. The kits target 12 chromosomal loci including the amelogenin locus for sex determination (387A12F, S0655, SBH1, SBH2, SBH4, SBH10, SBH13, SBH18, SBH19, SBH20, SBH22, SBH23 X, Y) and 12 chromosomal loci respectively (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, BM1824 and BM1818) (Table 3). Capillary electrophoresis was performed with the 3130 Genetic Analyser (Applied Biosystem, USA); results were analysed using Genemapper V3.2 (Applied Biosystems, USA) with a threshold of 50 Relative Florescence Unit (RFU).

2.5. Statistical and bioinformatic analyses

Statistical analyses were performed using IBM SPSS Statistics, version 24 (IBM, USA). ANOVA test, with Tukey's post-hoc analysis, was used to compare the different amount of gut contents and DNA extracted between the different methods with a significance level of 0.05.

Researches for sequence identity on Genbank were performed using BLASTn [40].

3. Results

The wet and dry weight of 10 crops and the DNA amount extracted per crop are reported in Table 4. Wet weight of crops of larvae fed on *S. scrofa* tissue had a significant statistical variation amongst the different methods ($F = 369.000$, $df = 4$, $p = 0.000$), while wet weight of larvae fed on *B. taurus* tissue did not show any significant difference ($F = 2.925$, $df = 4$, $p = 0.77$). Similarly, for the dry weight, a significant difference was observed in the amount of gut content obtained by different preparation methods of larvae fed respectively on *S. scrofa* ($F = 10.157$, $df = 4$, p-value $= 0.000$) and *B. taurus* tissue ($F = 117.208$, $df = 4$, $p = 0.000$). The amount of DNA obtained, irrespective to the food source, showed significant difference amongst the different methods of larval fixation ($p < 0.05$). Direct fixation at −20 °C and fixation in EtOH and storage at −20 °C resulted in the maximum amount of DNA recovered from larvae fed respectively on *S. scrofa* and *B. taurus* tissues (Table 4).

DNA was successfully extracted from all the samples and the control and successfully amplified using universal primers targeting the mitochondrial cyt b gene specific to *S. scrofa* and *B. taurus*. The identity of the sequences was successfully confirmed upon comparison on GenBank database.

All the samples were successfully amplified via Real Time-PCR. Complete porcine and bovine STR profiles were obtained from the gut content of single crops fixed with EtOH only. Similar results were also obtained from gut content of larvae fed on *S. scrofa* tissue and fixed with freezing method.

The profiles matched with the standard profiles (*S. scrofa* and *B. taurus* tissue) at 50 RFU. On comparison, all the 12 loci were identical to one another (Table 5). This result was replicated with sets of 3 and 5 crops. On the contrary, only partial STR profiles were derived by using the other methods (Table 6).

4. Discussion

Successful non-insect DNA extraction from the gut contents of larvae of Calliphoridae and Sarcophagidae, which are generally the first

Table 4
Weight and Quantification of DNA of *M. scalaris* larvae. * represents the significance difference when methods 2 and 3 are compared with the other methods 1, 4 and 5 (- p value > 0.05; *p value < 0.05; ** p value < 0.01).

Larval food source	*Sus scrofa* tissue			*Bos taurus* tissue	
Method for fixing the larvae	Wet weight of 10 crops (mg)	Dry weight of 10 crops (mg)	Extracted DNA per crop (ng/μl)	Wet weight of 10 crops (mg)	Dry weight of 10 crops (mg)	Extracted DNA per crop (ng/μl)
Hot water (> 80 °C), 40 s [32]	5.02 ± 0.21	0.79 ± 0.09	0.93 ± 0.03	2.63 ± 0.15	0.97 ± 0.06	1.89 ± 0.06
At −20 °C overnight [10]	2.46 ± 0.10**--	0.58 ± 0.16--**	3.67 ± 0.05**-**-**	2.10 ± 0.10	0.43 ± 0.06**--**	2.80 ± 0.07**-**-**
EtOH (98%) at −20 °C overnight [33]	3.43 ± 0.19**-**-**	0.59 ± 0.09**--**	2.55 ± 0.06**-**--	2.73 ± 0.58	1.37 ± 0.06--**-**	3.36 ± 0.14**-**-**
−20 °C for 4 h and then in EtOH (98%) at −20 °C overnight [33]	2.37 ± 0.06	0.93 ± 0.06	2.32 ± 0.04	2.37 ± 0.06	0.93 ± 0.06	2.82 ± 0.04
Hot water (> 80 °C) for 40 s [32], EtOH (98%) at −20 °C [10]	2.20 ± 0.10	0.57 ± 0.06	1.02 ± 0.02	2.20 ± 0.10	0.57 ± 0.06	1.51 ± 0.05

Table 5
Allele calling at 50 RFU results of the positive samples in comparison to the allele calling of DNA extracted from *Sus scrofa* and *Bos taurus* tissue that was fed to the larvae while breeding (standard). All the above samples were obtained from gut content of larvae which were fixed by directly placing the larvae at EtOH and then storing at −20 °C. These results were also replicated on the larvae fixed at −20 °C feed on *Sus scrofa* tissue.

Sus scrofa Animaltype Biotype STR kit			*Bos tauras* Bovine panel 1.2 STR kit		
Locus tested	Allele calling at 50 RFU		Locus tested	Allele calling at 50 RFU	
	Standard	Sample		Standard	Sample
387A12F	14.1, 15.1	14.1, 15.1	TGLA227	79, 97	79, 97
S0655	13	13	BM2113	135, 139	135, 139
SBH1	13, 14	13, 14	TGLA53	158, 166	158, 166
SBH2	6, 26	6, 26	ETH10	219, 221	221
SBH4	56, 57	56, 57	SPS115	252, 258	252, 258
SBH10	49	49	TGLA126	123	123
SBH13	11, 14	11, 14	TGLA122	151	151
SBH18	12	12	INRA23	200, 216	200, 216
SBH19	14, 15	14, 15	BM1818	270	270
SBH20	23, 37	23, 37	ETH3	117, 127	117, 127
SBH22	23	23	ETH225	140, 150	140, 150
SBH23 Y	Y	Y	BM1824	178, 180	178, 180
SBH23 X	X	X			

colonizer of the exposed bodies, have been repeatedly demonstrated in the literature [7,11]. In cases of buried corpse and indoor crime scene, *M. scalaris* is found to be one of the primary colonizers due to its small size and its ability to crawl in tight spaces thus making it an important fly of forensic interest [28,30,31,41]. The size of larvae belonging to *M. scalaris* is remarkable smaller compared to the previous mentioned taxa (Calliphoridae and Sarcophagidae): 1–2 vs 9–10 mm [41,42] (Fig. 1). Despite the reduced size of the larvae and the low amount of gut content (Table 3) we demonstrated that DNA extraction was still possible.

During the larvae dissection, liquefaction was observed in the larvae fixed with hot water which made the separation of the host tissue from the gut content difficult. This problem was overcome when dissection was performed on larvae fixed with freezing and only EtOH methods. Despite these fixing methods have been proven to be better in terms of ease of dissection and in the amount of DNA yield per crop (Table 2), the preservation of some morphological features useful for PMI estimation (e.g. length) [43] is not guaranteed. So, if possible, we strongly recommend collecting enough specimens in order to avoid the risk to lack of sufficient material to perform both the analyses as above mentioned if requested by the Court.

A complete STR profile with a perfect match with the control was obtained from the DNA extracted from gut content of one larva fed on *S. scrofa* and *B. taurus* tissues. This is concurrent to studies previously conducted on larvae of species of bigger size by Wells et al. [7], Zehner et al. [8], Di Luise et al. [11] etc. (Table 1) despite some unsuccessful results as reported in Campobasso et al. [44]. The improvement of the DNA extraction/amplification techniques but also the fixation of the specimens when in the feeding phase and their preservation and storage can be listed as the reasons of our positive results.

5. Conclusion

This study expands the source of DNA extraction aiding criminal investigations by successfully extracting and typing non-insect DNA from the gut content of the larvae of *M. scalaris*, a species commonly found in indoor and burial cases. Moreover, this study identifies 2 preservation techniques (preservation by freezing at −20 °C and preservation in EtOH (98%)) as optimal for this kind of analysis as they not only aid the process of dissection but do not interfere with the molecular analysis. Based on our results, obtained on small larvae, these two

Table 6
Percentage of full (FP) or partial (PP) profile and percentage of loci detected in samples fixed with different methods.

Larval food source	*Sus scrofa* animaltype biotype STR kit (12 loci)			*Bos taurus* Bovine panel 1.2 STR kit (12 loci)		
Method for Fixing the larvae	1 crop	3 crops	5 crops	1 crop	3 crops	5 crops
Hot water (> 80 °C), 40 s [32]	FP: 0%	FP: 0%	FP: 0%	FP: 0%	FP: 0%	FP: 0%
	PP: 0%	PP: 100%, 16.6% alleles detected	PP: 100%, 16.6% alleles detected	PP: 0%	PP: 100%, 8.3% alleles detected	PP: 100%, 24.9% alleles detected
At − 20 °C overnight [10]	FP: 100%	FP: 80%	FP: 90%	FP: 0%	FP: 0%	FP: 0%
	PP: 0%	PP: 20%, 83.3% alleles detected	PP: 10%, 83.3% alleles detected	PP: 100%, 91.6% alleles detected	PP: 100%, 83.3% alleles detected	PP: 100%, 83.3% alleles detected
EtOH (98%) at − 20 °C overnight [33]	FP: 70%	FP: 80%	FP: 90%	FP: 100%	FP: 100%	FP: 90%
	PP: 20%, 83.3% alleles detected PP: 10%, 75% alleles detected	PP: 20%, 91.7% alleles detected	PP: 10%, 83.3% alleles detected	PP: 0%	PP: 0%	PP: 10%, 91.7% alleles detected
− 20 °C for 4 h and then in EtOH (98%) at − 20 °C overnight [33]	FP: 0%	FP: 0%	FP: 0%	FP: 0%	FP: 0%	FP: 0%
	PP: 100%, 33.2% alleles detected	PP: 100%, 41.6% alleles detected	PP: 100%, 91.7% alleles detected	PP: 100%, 16.6% alleles detected	PP: 100%, 66.7% alleles detected	PP: 80%, 83.3% alleles detected PP: 20%, 58.3% alleles detected
Hot water (> 80 °C) for 40 s [32], EtOH (98%) at − 20 °C [10]	FP: 0%	FP: 0%	FP: 0%	FP: 0%	FP: 0%	FP: 0%
	PP: 0%	PP: 0%	PP: 100%, 24.9% alleles detected	PP: 0%	PP: 100%, 8.3% alleles detected	PP: 100%, 33.2% alleles detected

FP: full profile; PP: partial profile.

Fig.1. *M. scalaris* (Diptera: Phoridae) (A) and *C. vomitoria* (Diptera: Calliphoridae) (B) larvae. Scale bar: 1 mm.

methods can be applied in general in all the cases where the victim's DNA identification is requested from entomological samples.

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